

Steven L.Gersen
Martha B.Keagle
Editors

The Principles of Clinical Cytogenetics

Third Edition



Springer

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Preface

In April 2011, the website *The DNA Exchange* ran a story about the origin of our convention of referring to the short and long arms of chromosomes as “p” and “q.” Several possible explanations for how this usage came into being were presented in a somewhat whimsical manner.

Did we really go with p from the French *petite* and q because it alphabetically follows p? Was there really a “French vs. English” argument? Was it supposed to be p and g (from the French *grande*) but changed due to a typesetting error? Was Hardy-Weinberg equilibrium ($p+q=1$) invoked?

This prompted a flurry of comments over the Listserv used by cytogeneticists. Ultimately, several participants of the 1966 “Chicago Conference” weighed in, and Dr. Kurt Hirschhorn, who chaired the session at that conference, confirmed that the decision to go with p and q resulted from a combination of (sometimes spirited) debate, compromise (p really is for *petite*), logic, and, yes, agreement that $p+q=1$.

This is all great fun. But the story in *The DNA Exchange* also spawned other comments. It opened with:

Karyotypes are sooooo 20th century. Time was when a ripe crop of G-banded chromosomes promised a fruitful harvest of genetic secrets. But nowadays a Giemsa-stained karyotype seems like a quaint low resolution black and white TV set – those cute little D & G groups even have rabbit-ear antennas – compared with the bright, sexy colors of FISH, the fine oligonucleotide detail of microarrays, and the dense volumes of data generated by high throughput DNA sequencing.

Some cytogeneticists took offense at this.

People have been predicting the demise of cytogenetics for decades; this tended to happen each time new technology, such as DNA analysis or fluorescence *in situ* hybridization, became available. And yet we are still here.

Interestingly, this idea was significant as the previous edition of this book went to press in 2005 due to the increasingly important role of many FISH assays. In the preface to that edition, we discussed that while some classically trained cytogeneticists were concerned that FISH was going to put them out of work, Dorothy Warburton had predicted, years earlier, that FISH would actually provide the cytogenetics lab with an even more important diagnostic and prognostic role. She was of course correct.

Now we have microarrays. This edition of our book has a chapter dedicated to this technology, and several authors also deal with it in their individual chapters. The term “cytogenomics” (chromosome analysis using molecular techniques) is working its way into our lexicon.

Once again, there is talk, if not concern, that arrays could mean the unemployment line for cytogeneticists and, if not arrays, then perhaps next-generation sequencing. And once again, Dorothy put things into perspective:

The way I look at it is that cytogenetics is not about a technique, but a field of knowledge. We may change the way we look at chromosomes, but the questions and problems remain the same. A technique is only as good as our ability to interpret what we see in a way that helps families, and having molecular training does not provide the experience necessary to do this. We would never have known about bal-

anced translocations without looking at chromosomes, but now we have a way to tell if they are really balanced or not. I also believe that we will never be able to stop using chromosome preparations to interpret what we see on arrays. We have many examples where confirming array data has revealed unexpected kinds of rearrangements, as well as mosaicism. These are things that have much more significance for counseling than a simple call of a dup or del. I don't believe sequencing will change this.

I was first advised to find another field in 1969 (right before banding). So far I still have a job, although what I look at day to day has changed a great deal. "Classical" is pretty much a synonym for "in the past," so yes, classical cytogenetics may no longer be practiced. However, what is here is exciting and challenging and requires every technique in our playbook.

This third edition of *The Principles of Clinical Cytogenetics* was prompted by significant advances in the field since the last edition of this book was published. So while it is true that the way we look at chromosomes will likely continue to evolve, we do not expect to stop looking at them any time soon.

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Part I

Basic Concepts and Background

History of Clinical Cytogenetics

Steven L. Gersen

The beginning of human cytogenetics is generally attributed to Walther Flemming, an Austrian cytologist and professor of anatomy, who published the first illustrations of human chromosomes in 1882. Flemming also referred to the stainable portion of the nucleus as *chromatin* and first used the term *mitosis* [1]. In 1888, Waldeyer introduced the word *chromosome*, from the Greek words for “colored body,” and several prominent scientists of the day began to formulate the idea that determinants of heredity were carried on chromosomes [2]. After the “rediscovery” of Mendelian inheritance in 1900, Sutton (and, independently at around the same time, Boveri) formally developed a “chromosome theory of inheritance” [3, 4]. Sutton combined the disciplines of cytology and genetics when he referred to the study of chromosomes as *cytogenetics*.

Due in part to improvements in optical lenses, stains, and tissue manipulation techniques during the late nineteenth and early twentieth centuries, the study of cytogenetics continued, with an emphasis placed by some on determining the correct number of chromosomes, as well as the sex chromosome configuration, in humans. Several reports appeared, with differing estimates of these. For example, in 1912, von Winiwarter concluded that men have 47 chromosomes and women 48 [5]. Then, in 1923, T. S. Painter studied (meiotic) chromosomes derived from the testicles of several men who had been incarcerated, castrated, and ultimately hanged in the Texas State Insane Asylum. Based on this work, Painter definitively reported the human diploid chromosome number to be 48 (double the 24 bivalents he saw), even though, 2 years earlier, he had preliminarily reported that some of his better samples produced a diploid number of 46 [6]. At this time, Painter also proposed the X and Y sex chromosome mechanism in man. One year later, Levitsky formulated the

term *karyotype* to refer to the ordered arrangement of chromosomes [7].

Despite continued technical improvements, there was clearly some difficulty in properly visualizing or discriminating between individual chromosomes. Even though Painter’s number of 48 human chromosomes was reported somewhat conservatively, it was increasingly treated as fact with the passage of time and was “confirmed” several times over the next few decades. For example, in 1952, T. C. Hsu reported that, rather than depending upon histologic sections, examination of chromosomes could be facilitated if one studied cells grown with tissue culture techniques published by Fisher [8]. Hsu then demonstrated the value of this method by using it to examine human embryonic cell cultures, from which he produced both mitotic metaphase drawings and an idiogram of all 48 human chromosomes [9]!

As with other significant discoveries, correcting this inaccuracy required an unplanned event—a laboratory error. Its origin can be found in the addendum that appears at the end of Hsu’s paper:

It was found after this article had been sent to press that the well-spread metaphases were the result of an accident. Instead of being washed in isotonic saline, the cultures had been washed in hypotonic solution before fixation [9].

The hypotonic solution caused water to enter the cells via osmosis, which swelled the cell membranes and separated the chromosomes, making them easier to visualize. This accident was the key that unlocked the future of human cytogenetics. Within one year, Hsu, realizing the potential of this fortuitous event, reported a “hypotonic shock” procedure [10]. By 1955, Ford and Hamerton had modified this technique and had also worked out a method for pretreating cells grown in culture with colchicine so as to destroy the mitotic spindle apparatus and thus accumulate dividing cells in metaphase [11]. Joe Hin Tjio, an American-born Indonesian, learned about these procedures and worked with Hamerton and Ford to further improve upon them.

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In November of 1955, Tjio was invited to Lund, Sweden, to work on human embryonic lung fibroblast cultures in the laboratory of his colleague, Albert Levan, a Spaniard who had learned the colchicine and hypotonic method in Hsu's laboratory at the Sloan-Kettering Institute in New York. Tjio and Levan optimized the colchicine/hypotonic method for these cells and in January of 1956 (after carefully reviewing images from decades of previously reported work) diplomatically reported that the human diploid chromosome number appeared to be 46, not 48 [12]. They referenced anecdotal data from a colleague who had been studying liver mitoses from aborted human embryos in the Spring of 1955 but temporarily abandoned the research "because the workers were unable to find all the 48 human chromosomes in their material; as a matter of fact, the number 46 was repeatedly counted in their slides." Tjio and Levan concluded their paper:

...we do not wish to generalize our present findings into a statement that the chromosome number of man is $2n=46$, but it is hard to avoid the conclusion that this would be the most natural explanation of our observations [12].

What was dogma for over 30 years had been overturned in one now classic paper. Ford and Hamerton soon confirmed Tjio and Levan's finding [13]. The era of clinical cytogenetics was at hand. It would take three more years to arrive, however, and it would begin with the identification of four chromosomal syndromes.

The concept that an abnormality involving the chromosomes could have a phenotypic effect was not original. In 1932, Waardenburg made the suggestion that Down syndrome could perhaps be the result of a chromosomal aberration, but the science of the time could neither prove nor disprove his idea; this would take almost three decades [14]. In 1958, Lejeune studied the chromosomes of fibroblast cultures from patients with Down syndrome and in 1959, described an extra chromosome in each of these cells [15].

The trisomy was reported to involve one of the smallest pairs of chromosomes and would eventually be referred to as trisomy 21. Lejeune had proved Waardenburg's hypothesis by reporting the first example of a chromosomal syndrome in man, and in December of 1962, he received one of the first Joseph Kennedy Jr. Foundation International Awards for his work (Fig. 1.1).

Three more chromosomal syndromes, all believed to involve the sex chromosomes, were also described in 1959. Ford reported that females with Turner syndrome have 45 chromosomes, apparently with a single X chromosome and no Y, and Jacobs and Strong demonstrated that men with Klinefelter syndrome have 47 chromosomes, with the additional chromosome belonging to the group that contained the X chromosome [16, 17]. A female with sexual dysfunction was also shown by Jacobs to have 47 chromosomes and was believed to have an XXX sex chromosome complement [18].

The sex chromosome designation of these syndromes was supported by (and helped explain) a phenomenon that had been observed 10 years earlier. In 1949, Murray Barr was studying fatigue in repeatedly stimulated neural cells of the cat [19]. Barr observed a small stained body on the periphery of some interphase nuclei, and his records were detailed enough for him to realize that this was present only in the nuclei of female cats. This object, referred to as sex chromatin (now known as X chromatin or the Barr body), is actually the inactivated X chromosome present in nucleated cells of all normal female mammals but absent in normal males. The observation that the Turner syndrome, Klinefelter syndrome, and putative XXX patients had zero, one, and two Barr bodies, respectively, elucidated the mechanism of sex determination in humans, confirming for the first time that it is the presence or absence of the Y chromosome that determines maleness, not merely the number of X chromosomes present, as in *Drosophila*. In 1961, the single active X



Fig. 1.1 Jérôme Lejeune receives a Joseph P. Kennedy Jr. Foundation International Award for demonstrating that Down syndrome results from an extra chromosome (Photo courtesy of the John F. Kennedy Library, Boston, MA)

chromosome mechanism of X-dosage compensation in mammals was developed by Mary Lyon and has been since known as the Lyon hypothesis [20].

It was not long after Lejeune's report of the chromosomal basis of Down syndrome that other autosomal abnormalities were discovered. In the April 9, 1960, edition of *The Lancet*, Patau et al. described two similar infants with an extra "D-group" chromosome who had multiple anomalies quite different from those seen in Down syndrome [21]. In the same journal, Edwards et al. described "a new trisomic syndrome" in an infant girl with yet another constellation of phenotypic abnormalities and a different autosomal trisomy [22]. The former became known as Patau syndrome or "D trisomy" and the latter as Edwards syndrome or "E trisomy." Patau paper incredibly contains a typographical error and announces that the extra chromosome "belongs to the E group," and Edwards reported that "the patient was ... trisomic for the no. 17 chromosome," but we now know these syndromes to be trisomies 13 and 18, respectively.

Also in 1960, Nowell and Hungerford reported the presence of a small chromosome in patients with chronic myelogenous leukemia. Using the proposed nomenclature method at the time, this was designated Philadelphia chromosome 1 (Ph¹), and it demonstrated, for the first time, an association between chromosomes and cancer [23–25] (Fig. 1.2). Still referred to as the "Philadelphia chromosome" for historical purposes, this phenomenon was eventually relegated to nothing more than a curiosity during the 1960s, as the concept of a clinical association between chromosomes and cancer fell out of favor.

In 1963 and 1964, Lejeune et al. reported that three infants with the *cri du chat* ("cat cry") syndrome of phenotypic anomalies, which includes severe mental retardation and a characteristic kitten-like mewing cry, had a deletion of the short arm of a B-group chromosome, designated as chromosome 5 [26, 27]. Within two years, Jacobs et al. described

"aggressive behavior, mental subnormality and the XYY male," and the chromosomal instabilities associated with Bloom syndrome and Fanconi anemia were reported [28–30].

Additional technical advancements had facilitated the routine study of patient karyotypes. In 1960, Peter Nowell observed that the kidney bean extract phytohemagglutinin, used to separate red and white blood cells, stimulated lymphocytes to divide. He introduced its use as a mitogen, permitting a peripheral blood sample to be used for chromosome analysis [31]. This eliminated the need for bone marrow aspiration, which had previously been the best way to obtain a sufficient number of spontaneously dividing cells. It was now feasible to produce mitotic cells suitable for chromosome analysis from virtually any patient.

Yet, within nine years of the discovery of the number of chromosomes in humans, only three autosomal trisomies, four sex chromosome aneuploidies, a structural abnormality (a deletion), an acquired chromosomal abnormality associated with cancer, and two chromosome breakage disorders had been described as recognizable "chromosomal syndromes." A new clinical laboratory discipline had been created; was it destined to be restricted to the diagnosis of a few abnormalities?

This seemed likely. Even though certain pairs were distinguishable by size and centromere position, individual chromosomes could not be identified, and as a result, patient-specific chromosome abnormalities could be observed but not defined. Furthermore, the existence of certain abnormalities, such as inversions involving a single chromosome arm (so-called *paracentric inversions*) could be hypothesized, but not proven, because they could not be visualized. Indeed, it seemed that without a way to definitively identify each chromosome (and more importantly, regions of each chromosome), this new field of medicine would be limited in scope to the study of a few disorders.

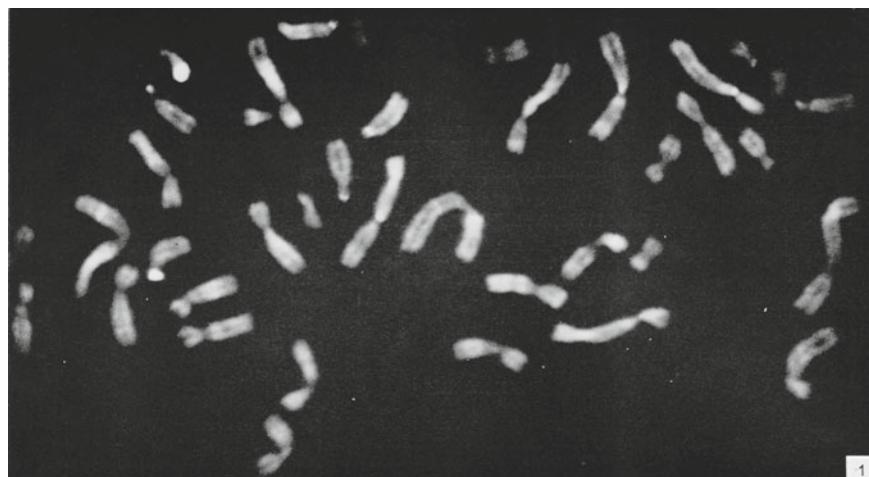


Fig. 1.2 The first photograph of a Q-banded cell published by Caspersson in 1970. The figure was originally labeled "Quinacrine mustard treated human metaphase chromosomes (male) from leukocyte culture. Fluorescence microscope $\times 2,000$ " (Reprinted with permission from Caspersson et al. [33], Elsevier)

For three more years, clinical cytogenetics was so relegated. Then, in 1968, Torbjörn Caspersson observed that when plant chromosomes were stained with fluorescent quinacrine compounds, they did not fluoresce uniformly but rather produced a series of bright and dull areas across the length of each chromosome. Furthermore, each pair fluoresced with a different pattern, so that previously indistinguishable chromosomes could now be recognized [32].

Caspersson then turned his attention from plants to the study of human chromosomes. He hypothesized that the quinacrine derivative quinacrine mustard (QM) would preferentially bind to guanine residues and that C-G rich regions of chromosomes should therefore produce brighter “striations,” as he initially referred to them, while A-T rich regions would be dull. Although it ultimately turned out that it is the A-T rich regions that fluoresce brightly and that ordinary quinacrine dihydrochloride works as well as QM, by 1971, Caspersson had successfully produced and reported a unique “banding” pattern for each human chromosome pair [33, 34]. See Fig. 1.3.

For the first time, each human chromosome could be positively identified. The method, however, was cumbersome. It required a relatively expensive fluorescence microscope



Fig. 1.3 One of the first photomicrographs of a metaphase spread from a patient with chronic myelogenous leukemia, indicating the Philadelphia chromosome. Reported a decade before routine chromosome banding, the authors (correctly) interpreted the abnormal chromosome to represent the next-to-smallest human chromosome and reported it as being a chromosome 21: “Note the Ph¹ chromosome (arrow). To right are shown, from bottom to top, 21, Ph¹, 22, 22, and Y. The Ph¹ chromosome is apparently a 21 which has lost approximately one half of its long arm.” However, although chromosome banding demonstrated that the chromosome involved in Down syndrome is actually the smallest human chromosome, the term “trisomy 21” was already too common to be changed, and so the numbering of the two smallest human chromosomes was reversed. The Philadelphia chromosome is therefore described as being derived from chromosome 22 (Figure courtesy of Alice Hungerford and reprinted with permission from Nowell and Hungerford [25])

and a room that could be darkened, and the fluorescence tended to fade or “quench” after a few minutes, making real-time microscopic analysis difficult.

These difficulties were overcome a year later, when Drets and Shaw described a method of producing similar chromosomal banding patterns using an alkali and saline pretreatment followed by staining with Giemsa, a compound developed for identification, in blood smears, of the protozoan that causes malaria [35]. Even though some of the chromosome designations proposed by Drets and Shaw have been changed (essentially in favor of those advocated by Caspersson), this method, and successive variations of it, facilitated widespread application of clinical cytogenetic techniques. While the availability of individuals with the appropriate training and expertise limited the number and capacity of laboratories that could perform these procedures (in some ways still true today), the technology itself was now within the grasp of any facility.

What followed was a cascade of defined chromosomal abnormalities and syndromes: aneuploidies, deletions, microdeletions, translocations, inversions (including the paracentric variety), insertions, mosaicism, and a seemingly infinite number of patient- and family-specific rearrangements.

In 1973, Janet Rowley demonstrated that the “Philadelphia chromosome” was actually the result of a translocation involving chromosomes 9 and 22, and in that same year, she also described an (8;21) translocation in AML [36, 37]. The association between chromosomes and cancer could no longer be ignored. The decades that followed saw an ever-increasing collection of rearrangements and other cytogenetic anomalies associated with neoplasia. These were eventually cataloged by Felix Mitelman in what has become an ongoing project of incredible dedication; the first volume was published in 1983, and the most recent version is an online database with close to 60,000 entries [38, 39].

Thanks to the host of research applications made possible by the precise identification of smaller and smaller regions of the karyotype, genes began to be mapped to chromosomes at a furious pace. The probes that resulted from such research have given rise to the discipline of molecular cytogenetics, which utilizes the techniques of fluorescence *in situ* hybridization (FISH). In recent years, this exciting development and the many innovative procedures derived from it have created even more interest in the human karyotype. A perfect example involves the union of information gleaned from the Human Genome Project with molecular techniques such as comparative genomic hybridization (GCH) or single nucleotide polymorphism (SNP) analysis. Combining these using computer and droplet technologies has given rise to the chromosome microarray, which is already becoming the next step in the evolution of clinical cytogenetics.

In the summer of 2006, geneticists from around the world met in Bethesda, Maryland, to celebrate “50 Years of 46



Fig. 1.4 In July 2006, geneticists from around the world met in Bethesda, Maryland, to celebrate “50 Years of 46 Human Chromosomes: Progress in Cytogenetics”

Human Chromosomes: Progress in Cytogenetics” (Fig. 1.4), and in 2010, we gathered in Philadelphia for a “Philadelphia Chromosome Symposium: Past, Present, and Future—The 50th Anniversary of the Discovery of the Philadelphia Chromosome.” This group had the honor of being addressed by Dr. Peter Nowell, Dr. Janet Rowley, Dr. Felix Mitelman, and Mrs. Alice Hungerford, wife of the late Dr. David Hungerford.

More than one million cytogenetic and molecular cytogenetic analyses are now performed annually in more than 400 laboratories worldwide, and this testing is now often the standard of care [40, 41]. Pregnant women over the age of 35, or those with certain serum-screening results, are routinely offered prenatal cytogenetic analysis, and many also have prenatal ploidy analysis via FISH. For children with phenotypic and/or mental difficulties and for couples experiencing reproductive problems, cytogenetics has become a routine part of their clinical workup. FISH has permitted us to visualize changes that are too subtle to be detected with standard chromosome analysis, and chromosome microarrays provide even greater resolution. Cytogenetics and FISH also provide information vital to the diagnosis, prognosis, therapy, and monitoring of treatment for a variety of cancers, and cancer arrays are gaining utility as well.

It was really not so long ago that humans had 48 chromosomes. One has to wonder whether any of the pioneers of this field could have predicted the modern widespread clinical use of chromosome analysis, in all its forms. But perhaps it is even more exciting to wonder what lies ahead.

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Martha B. Keagle

Introduction

The molecule deoxyribonucleic acid (DNA) is the raw material of inheritance and ultimately influences all aspects of the structure and functioning of the human body. A single molecule of DNA, along with associated proteins, comprises a chromosome. Chromosomes are located in the nuclei of all human cells (with the exception of mature red blood cells), and each human cell contains 23 different pairs of chromosomes.

Genes are functional units of genetic information that reside on each of the 23 pairs of chromosomes. These units are linear sequences of nitrogenous bases that code for protein molecules necessary for the proper functioning of the body. The genetic information contained within the chromosomes is copied and distributed to newly created cells during cell division. The structure of DNA provides the answer to how it is precisely copied with each cell division and to how proteins are synthesized.

DNA Structure

James Watson and Francis Crick elucidated the molecular structure of DNA in 1953 using X-ray diffraction data collected by Rosalind Franklin and Maurice Wilkins, and model building techniques advocated by Linus Pauling [1, 2]. Watson and Crick proposed the double helix: a twisted, spiral ladder structure consisting of two long chains wound around each other and held together by hydrogen bonds. DNA is composed of repeating units—the nucleotides. Each nucleotide consists of a deoxyribose sugar, a phosphate group, and one of four nitrogen-containing bases: adenine (A), guanine (G), cytosine (C), or thymine (T). Adenine and

guanine are purines with a double-ring structure, whereas cytosine and thymine are smaller pyrimidine molecules with a single ring structure. Two nitrogenous bases positioned side by side on the inside of the double helix form one rung of the molecular ladder. The sugar and phosphate groups form the backbone or outer structure of the helix. The fifth (5') carbon of one deoxyribose molecule and the third (3') carbon of the next deoxyribose are joined by a covalent phosphate linkage. This gives each strand of the helix a chemical orientation with the two strands running opposite or antiparallel to one another.

Biochemical analyses performed by Erwin Chargaff showed that the nitrogenous bases of DNA were not present in equal proportions and that the proportion of these bases varied from one species to another [3]. Chargaff noted, however, that concentrations of guanine and cytosine were always equal, as were the concentrations of adenine and thymine. This finding became known as Chargaff's rule. Watson and Crick postulated that in order to fulfill Chargaff's rule and to maintain a uniform shape to the DNA molecule, there must be a specific complementary pairing of the bases: adenine must always pair with thymine, and guanine must always pair with cytosine. Each strand of DNA, therefore, contains a nucleotide sequence that is complementary to its partner. The linkage of these complementary nitrogenous base pairs holds the antiparallel strands of DNA together. Two hydrogen bonds link the adenine and thymine pairs, whereas three hydrogen bonds link the guanine and cytosine pairs (Fig. 2.1). The complementarity of DNA strands is what allows the molecule to replicate faithfully. The sequence of bases is critical for DNA function because genetic information is determined by the order of the bases along the DNA molecule.

DNA Synthesis

The synthesis of a new molecule of DNA is called replication. This process requires many enzymes and cofactors. The first step of the process involves breakage of the hydrogen

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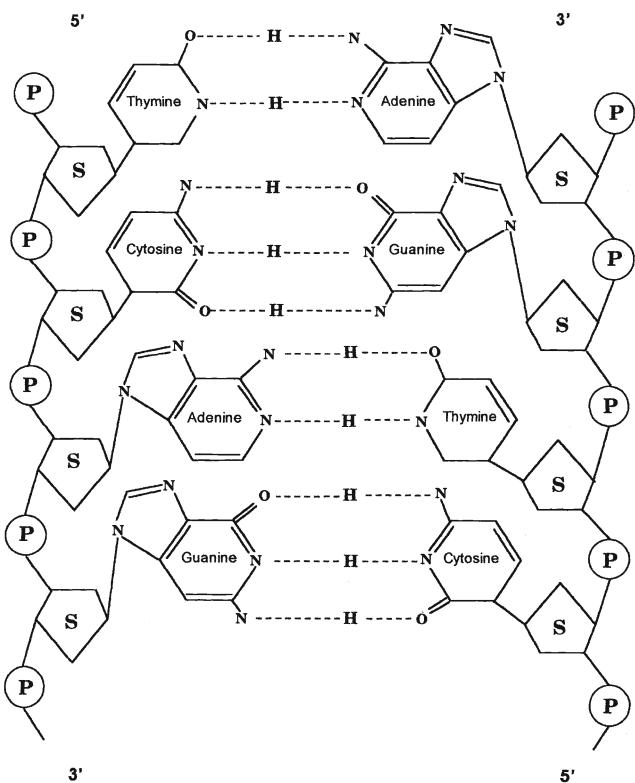


Fig. 2.1 DNA structure. Schematic representation of a DNA double helix unwound to show the complementarity of bases and the antiparallel structure of the phosphate (P) and sugar (S) backbone strands

bonds that hold the DNA strands together. DNA helicases and single-strand binding proteins work to separate the strands and keep the DNA exposed at many points along the length of the helix during replication. The area of DNA at the active region of separation is a Y-shaped structure referred to as a replication fork. These replication forks originate at structures called replication bubbles, which, in turn, are at DNA sequences called replication origins. The molecular sequence of the replication origins has not been completely characterized. Replication takes place on both strands, but nucleotides can only be added to the 3' end of an existing strand. The separated strands of DNA serve as templates for production of complementary strands of DNA following Chargaff's rules of base pairing.

The process of DNA synthesis differs for the two strands of DNA because of its antiparallel structure. Replication is straightforward on the leading strand. The enzyme DNA polymerase I facilitates the addition of complementary nucleotides to the 3' end of a newly forming strand of DNA. In order to add further nucleotides, DNA polymerase I requires the 3'-hydroxyl end of a base-paired strand.

DNA synthesis on the lagging strand is accomplished by the formation of small segments of nucleotides called Okazaki fragments [4]. After separation of the strands, the

enzyme DNA primase uses ribonucleotides to form a ribonucleic acid primer.

The structure of ribonucleic acid (RNA) is similar to that of DNA, except that each nucleotide in RNA has a ribose sugar instead of deoxyribose and the pyrimidine thymine is replaced by another pyrimidine, uracil (U). RNA also differs from DNA in that it is a single-stranded molecule. This RNA primer is at the beginning of each Okazaki segment to be copied, provides a 3'-hydroxyl group, and is important for the efficiency of the replication process. The ribonucleic acid primer then attracts DNA polymerase I. DNA polymerase I brings in the nucleotides and also removes the RNA primer and any mismatches that occur during the process. Okazaki fragments are later joined by the enzyme DNA ligase. The process of replication is semiconservative because the net result is creation of two identical DNA molecules, each consisting of a parent DNA strand and a newly synthesized DNA strand. The new DNA molecule grows as hydrogen bonds form between the complementary bases (Fig. 2.2).

Protein Synthesis

The genetic information of DNA is stored as a code; a linear sequence of nitrogenous bases in triplets. These triplets code for specific amino acids that are subsequently linked together to form protein molecules. The process of protein synthesis involves several types of ribonucleic acid.

The first step in protein synthesis is transcription. During this process, DNA is copied into a complementary piece of messenger RNA (mRNA). Transcription is controlled by the enzyme RNA polymerase, which functions to link ribonucleotides together in a sequence complementary to the DNA template strand. The attachment of RNA polymerase to a promoter region, a specific sequence of bases that varies from gene to gene, starts transcription. RNA polymerase moves off the template strand at a termination sequence to complete the synthesis of an mRNA molecule (Fig. 2.3).

Messenger RNA is modified at this point by the removal of introns—segments of DNA that do not code for an mRNA product. In addition, some nucleotides are removed from the 3' end of the molecule, and a string of adenine nucleotides are added. This poly(A) tail helps in the transport of mRNA molecules to the cytoplasm. Another modification is the addition of a cap to the 5' end of the mRNA, which serves to aid in attachment of the mRNA to the ribosome during translation. These alterations to mRNA are referred to as mRNA processing (Fig. 2.4). At this point, mRNA, carrying the information necessary to synthesize a specific protein, is transferred from the nucleus into the cytoplasm of the cell, where it then associates with ribosomes. Ribosomes, composed of ribosomal RNA (rRNA) and protein, are the site of

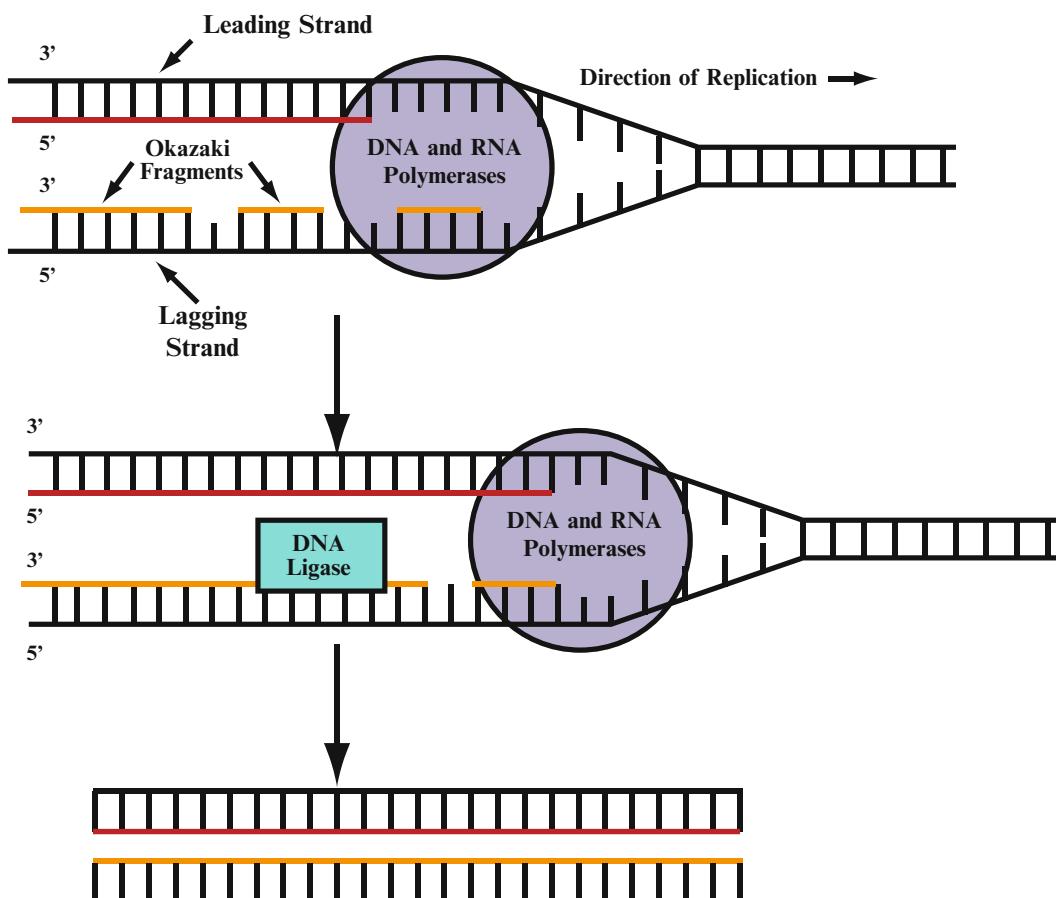


Fig. 2.2 Semiconservative replication. Complementary nucleotides are added directly to the 3' end of the leading strand, whereas the lagging strand is copied by the formation of Okazaki fragments

protein synthesis. Ribosomes consist of two subunits that come together with mRNA to read the coded instructions on the mRNA molecule.

The next step in protein synthesis is translation. A chain of amino acids is synthesized during translation by using the newly transcribed mRNA molecule as a template, with the help of a third ribonucleic acid, transfer RNA (tRNA). Leder and Nirenberg and Khorana determined that three nitrogen bases on an mRNA molecule constitute a codon [5, 6]. With four nitrogenous bases, there are 64 possible three-base codons. Sixty-one of these code for specific amino acids, and the other three are “stop” codons that signal the termination of protein synthesis. There are only 20 amino acids, but 61 codons. Therefore, most amino acids are coded for by more than one mRNA codon. This redundancy in the genetic code is referred to as degeneracy.

Transfer RNA molecules contain “anticodons”—nucleotide triplets that are complementary to the codons on mRNA. Each tRNA molecule has attached to it the specific amino acid for which it codes.

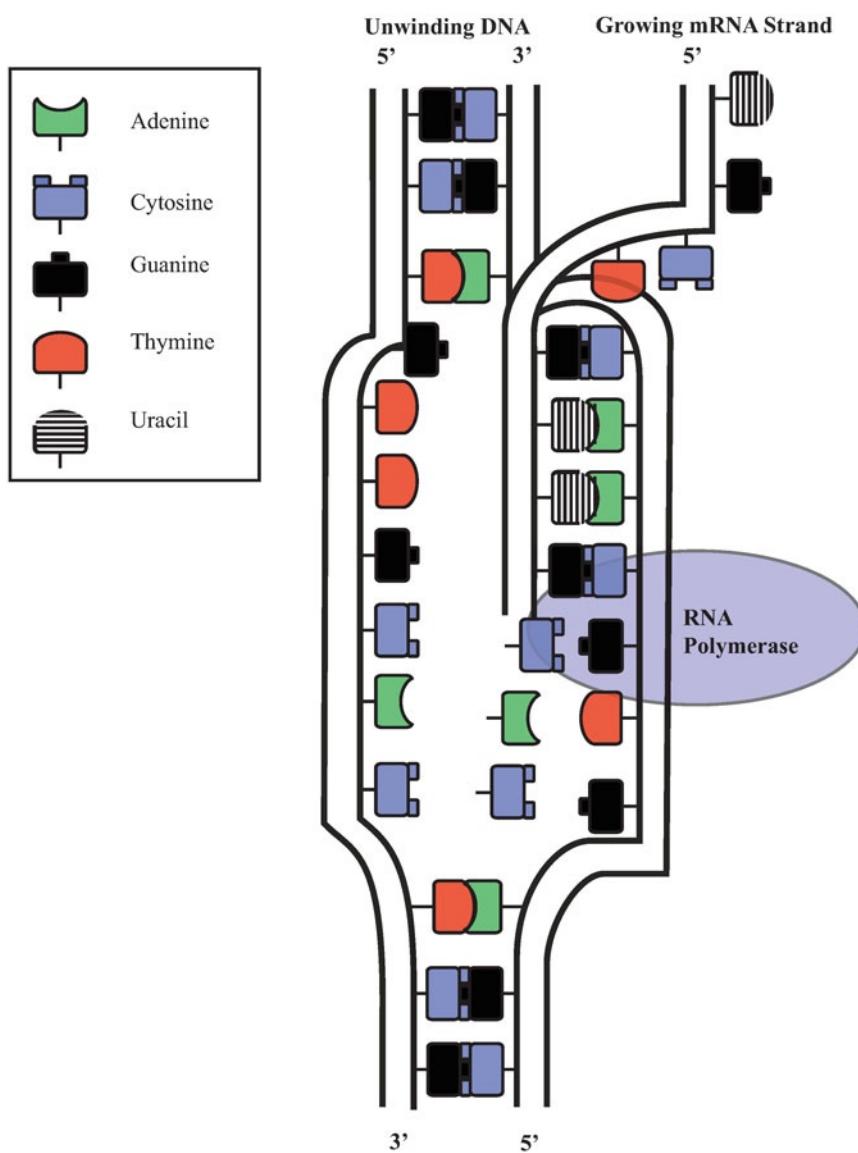
Ribosomes read mRNA one codon at a time. Transfer RNA molecules transfer the specific amino acids to the synthesizing protein chain (Fig. 2.5). The amino acids are joined to this chain by peptide bonds. This process is continued until a stop codon is reached. The new protein molecule is then released into the cell milieu and the ribosomes split apart (Fig. 2.6).

DNA Organization

Human chromatin consists of a single continuous molecule of DNA complexed with histone and nonhistone proteins. The DNA in a single human diploid cell, if stretched out, would be approximately 2 m in length and therefore must be condensed considerably to fit within the cell nucleus [7]. There are several levels of DNA organization that allow for this.

The DNA helix itself is the first level of condensation. Next, two molecules of each of the histones H2A, H2B, H3, and H4 form a protein core: the octamer. The DNA double

Fig. 2.3 Transcription. A DNA molecule is copied into mRNA with the help of RNA polymerase



helix winds twice around the octamer to form a 10-nm nucleosome, the basic structural unit of chromatin. Adjacent nucleosomes are pulled together by a linker segment of the histone H1. Repeated, this gives the chromatin the appearance of “beads on a string.” Nucleosomes are further coiled into a 30-nm solenoid, with each turn of the solenoid containing about six nucleosomes. The solenoids are packed into DNA looped domains attached to a nonhistone protein matrix. Attachment points of each loop are fixed along the DNA. The looped domains coil further to give rise to highly compacted units, the chromosomes, which are visible with the light microscope only during cell division. Chromosomes reach their greatest extent of condensation during mitotic metaphase (Fig. 2.7).

Chromosome Structure

A chromosome consists of two sister chromatids, each of which is comprised of a contracted and compacted double helix of DNA. The centromere, telomere, and nucleolar organizer regions are functionally differentiated areas of the chromosomes (Fig. 2.8).

The Centromere

The centromere is a constriction visible on metaphase chromosomes where the two sister chromatids are joined together. The centromere is essential to the survival of a chromosome

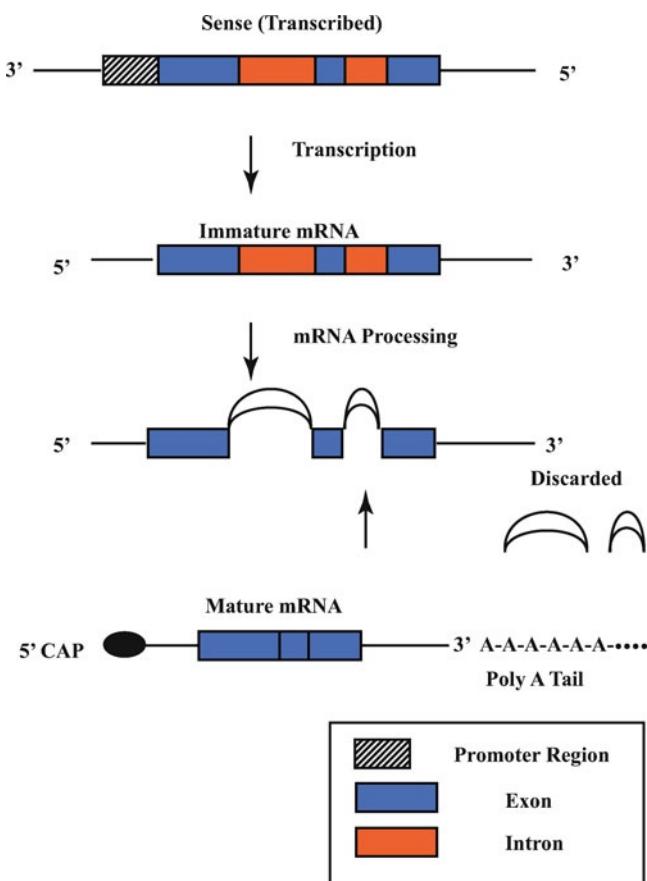


Fig. 2.4 Messenger RNA processing. The transcribed strand of DNA is modified to produce a mature mRNA transcript

during cell division. Interaction with the mitotic spindle during cell division occurs at the centromeric region. Mitotic spindle fibers are the functional elements that separate the sister chromatids during cell division.

Human chromosomes are classified based on the position of the centromere on the chromosome. The centromere is located near the middle in metacentric chromosomes, near one end in acrocentric chromosomes, and between the middle and end in submetacentric chromosomes. The kinetochore apparatus is a complex structure consisting of proteins that function at the molecular level to attach the chromosomes to the spindle fibers during cell division. Although the kinetochore is located in the region of the centromere, it should not be confused with the centromere. The latter is the DNA at the site of the spindle-fiber attachment.

The Nucleolar Organizer Regions

The satellite stalks of human acrocentric chromosomes contain the nucleolar organizer regions (NORs), so-called because this is where nucleoli form in interphase cells. NORs are also the site of ribosomal RNA genes and production of

rRNA. In humans, there are theoretically ten nucleolar organizer regions, although all may not be active during any given cell cycle.

The Telomeres

The telomeres are the physical ends of chromosomes. Telomeres act as protective caps to chromosome ends, preventing end-to-end fusion of chromosomes and DNA degradation resulting after chromosome breakage. Nonhistone proteins complex with telomeric DNA to protect the ends of chromosomes from nucleases located within the cell [9]. The telomeric region also plays a role in synapsis during meiosis. Chromosome pairing appears to be initiated in the subtelomeric regions [10].

Telomeres contain tandem repeats of the nitrogenous base sequence TTAGGG over 3–20 kb at the chromosome ends [11]. At the very tip of the chromosome, the two strands do not end at the same point, resulting in a short G-rich tail that is single stranded. Because of this, DNA synthesis breaks down at the telomeres and telomeres replicate differently than other types of linear DNA. The enzyme telomerase synthesizes new copies of the telomere TTAGGG repeat using an RNA template that is a component of the telomerase enzyme. Telomerase also counteracts the progressive shortening of chromosomes that results from many cycles of normal DNA replication. Telomere length gradually decreases with the aging process and with increased numbers of cell divisions in culture. The progressive shortening of human telomeres appears to be a tumor-suppressor mechanism [12]. The maintenance of telomeric DNA permits the binding of telomeric proteins that form the protective cap at chromosome ends and regulate telomere length [12]. Cells that have defective or unstable telomerase will exhibit shortening of chromosomes, leading to chromosome instability and cell death.

Types of DNA

DNA is classified into three general categories: unique sequence, highly repetitive sequence DNA (>105 copies), and middle repetitive sequence DNA (102–104 copies). Unique sequence or single-copy DNA is the most common class of DNA, comprising about 75% of the human genome [13]. This DNA consists of nucleotide sequences that are represented only once in a haploid set. Genes that code for proteins are single-copy DNA. Repetitive or repeated sequence DNA makes up the remaining 25% of the genome and is classified according to the number of repeats and whether the repeats are tandem or interspersed among unique sequence DNA [13].

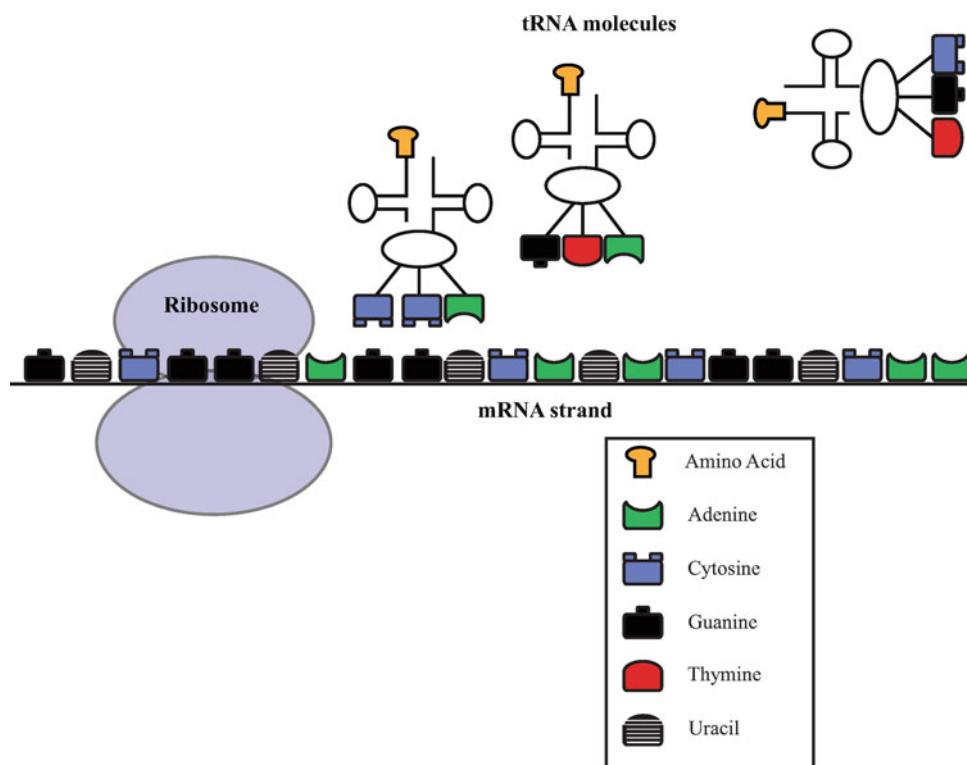


Fig. 2.5 Translation. Transfer RNA molecules bring in specific amino acids according to the triplet codon instructions of mRNA that are read at the ribosomes

Repetitive, tandemly arranged DNA was first discovered with a cesium chloride density gradient. Repetitive, tandem sequences were visualized as separate bands in the gradient. This DNA was termed satellite DNA [14]. Satellite DNA is categorized, based on the length of sequences that make up the tandem array and the total length of the array, as α (alpha)-satellite, minisatellite, and microsatellite DNA.

Alpha-satellite DNA is a repeat of a 171-base pair sequence organized in a tandem array of up to a million base pairs or more in total length. Alpha-satellite DNA is generally not transcribed and is located in the heterochromatin associated with the centromeres of chromosomes (see later). The size and number of repeats of satellite DNA is chromosome specific [15]. Although α -satellite DNA is associated with centromeres, its role in centromere function has not been determined. A centromeric protein, CENP-B, has been shown to bind to a 17-base pair portion of some α -satellite DNA, but the functional significance of this has not been determined [16].

Minisatellites have repeats that are 20–70 base pairs in length, with a total length of a few thousand base pairs. Microsatellites have repeat units of two, three, or four base pairs, and the total length is usually less than a few hundred base pairs. Minisatellites and microsatellites vary in length among individuals and, as such, are useful markers for gene mapping and identity testing.

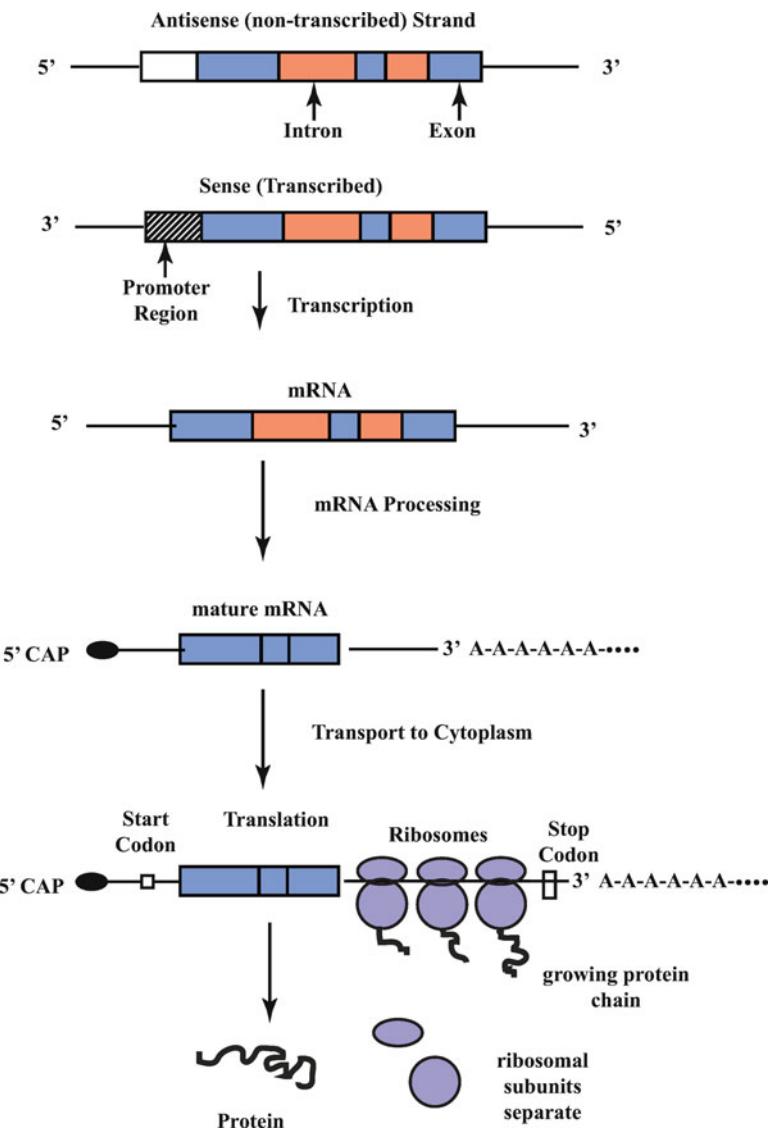
The genes for 18S and 28S ribosomal RNAs are middle repetitive sequences. Several hundred copies of these genes are tandemly arranged on the short arms of the acrocentric chromosomes.

Dispersed repetitive DNA is classified as either short or long. The terms SINEs (short interspersed elements) and LINEs (long interspersed elements) were introduced by Singer [17]. SINEs range in size from 90 to 500 base pairs. One class of SINEs is the Alu sequence. Many Alu sequences are transcribed and are present in nuclear pre-mRNA and in some noncoding regions of mRNA. Alu sequences have high G-C content and are found predominantly in the Giemsa-light bands of chromosomes [18]. LINEs can be as large as 7,000 bases. The predominant member of the LINE family is a sequence called L1. L1 sequences have high A-T content and are predominantly found in the Giemsa-dark bands of chromosomes [17]. See Chaps. 3 and 4.

Chromatin

There are two fundamental types of chromatin in eukaryotic cells: euchromatin and heterochromatin. Euchromatin is loosely organized, extended, and uncoiled. This chromatin contains active, early replicating genes, and stains lightly with GTG-banding techniques (see Chap. 4).

Fig. 2.6 Overview of protein synthesis. DNA is transcribed to mRNA, which is modified to mature transcript and then transferred to the cytoplasm of the cell. The codons are read at the ribosomes and translated with the help of tRNA. The chain of amino acids produced during translation is joined by peptide bonds to form a protein molecule



There are two special types of heterochromatin that warrant special mention: facultative heterochromatin and constitutive heterochromatin. Both are genetically inactive, late replicating during the synthesis (S) phase of mitosis, and are highly contracted.

Constitutive Heterochromatin

Constitutive heterochromatin consists of simple repeats of nitrogenous bases that are generally located around the centromeres of all chromosomes and at the distal end of the Y chromosome. There are no transcribed genes located in constitutive heterochromatin, which explains the fact that variations in constitutive heterochromatic chromosome regions apparently have no effect on the phenotype. Chromosomes 1, 9, 16, and Y have variably sized constitutive heterochromatic regions.

The heterochromatic regions of these chromosomes stain differentially with various special staining techniques, revealing that the DNA structure of these regions is not the same as the structure of the euchromatic regions on the same chromosomes. The only established function of constitutive heterochromatin is the regulation of crossing-over—the exchange of genes from one sister chromatid to the other during cell division [19].

Facultative Heterochromatin

One X chromosome of every female cell is randomly inactivated. The inactivated X is condensed during interphase and replicates late during the synthesis stage of the cell cycle. It is termed facultative heterochromatin. Because these regions are inactivated, it has been proposed that facultative heterochromatin regulates gene function [20].

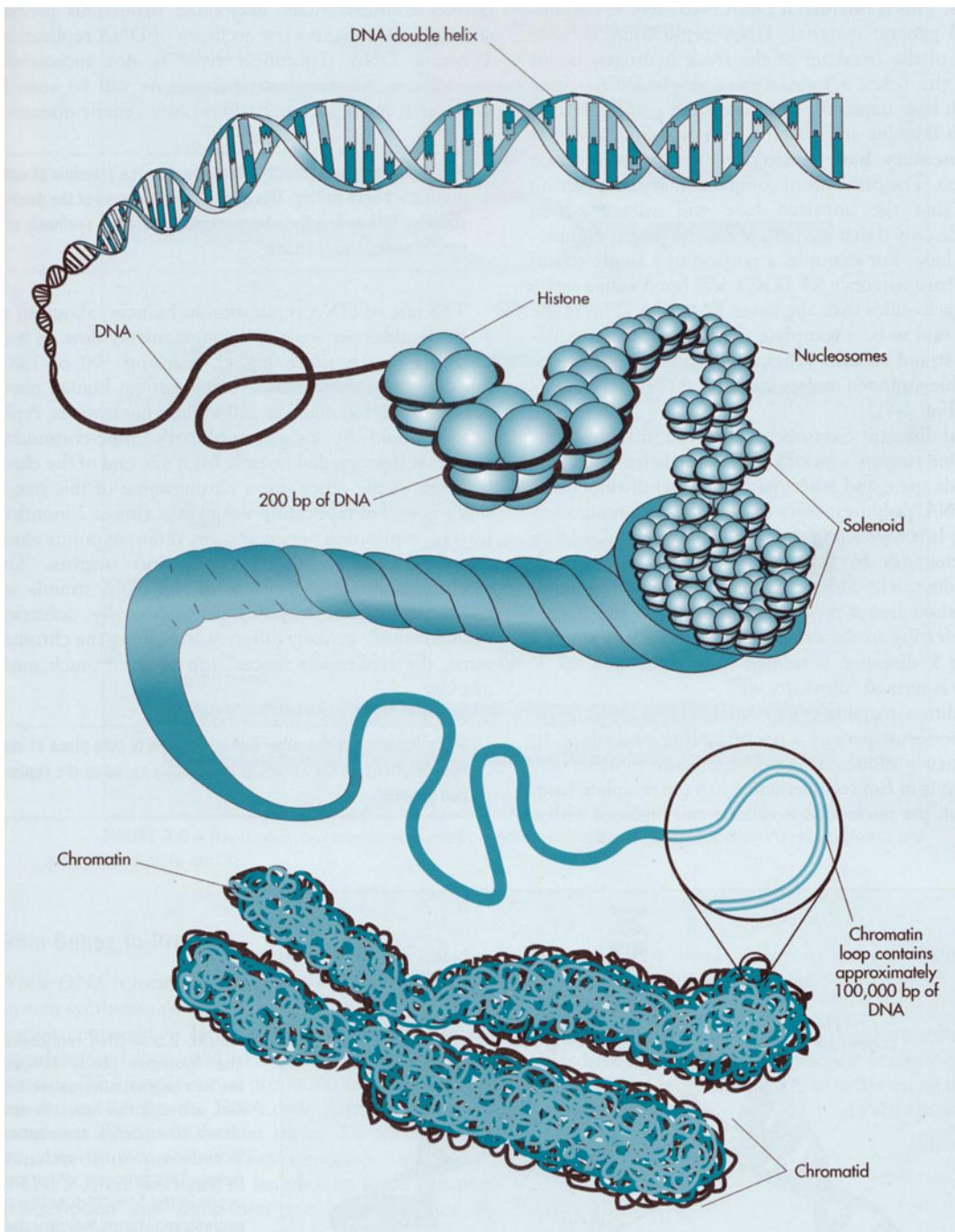


Fig. 2.7 The levels of DNA organization (Reprinted with permission from Jorde et al. [8])

Cell Division

An understanding of cell division is basic to an understanding of cytogenetics. Dividing cells are needed in order to study chromosomes using traditional cytogenetic techniques, and many cytogenetic abnormalities result from errors in cell division.

There are two types of cell division: mitosis and meiosis. Mitosis is the division of somatic cells, whereas meiosis is a special type of division that occurs only in gametic cells.

The Cell Cycle

The average mammalian cell cycle lasts about 17–18 h and is the transition of a cell from one interphase through cell division and back to interphase [21]. The cell cycle is divided into four major stages. The first three stages, gap 1 (G₁), synthesis (S), and gap 2 (G₂), comprise interphase. The fourth and final stage of the cell cycle is mitosis (M) (Fig. 2.9).

The first stage, G₁, is the longest and typically lasts about 9 h [21]. Chromosomes exist as single chromatids during this

Fig. 2.8 The functional and structural components of metaphase chromosomes

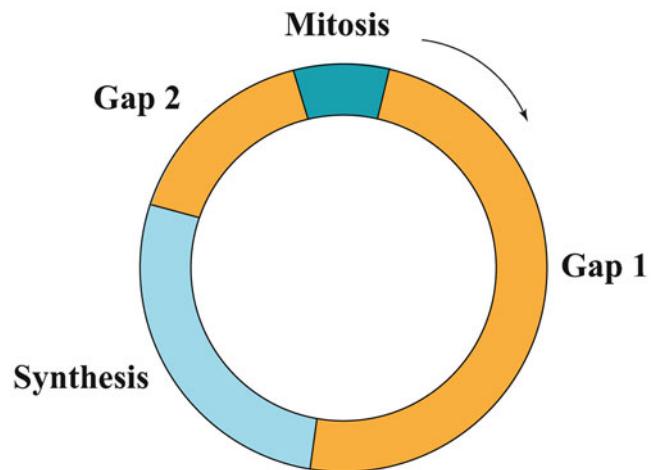
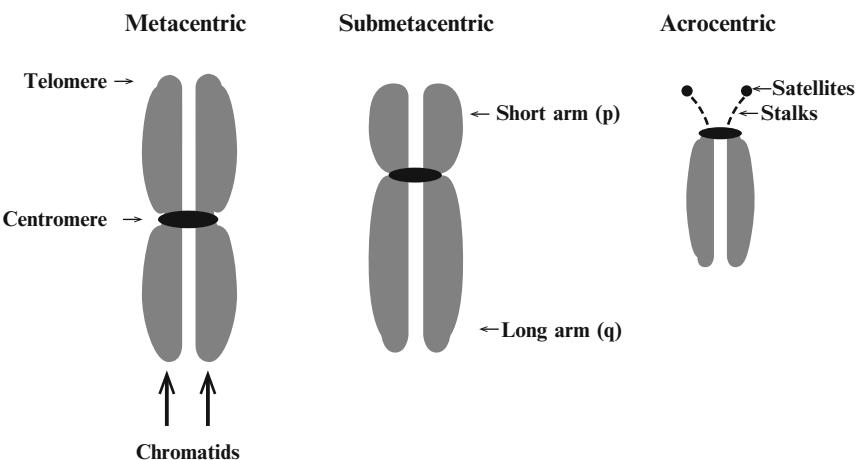


Fig. 2.9 The cell cycle: gap 1, synthesis, gap 2, and mitosis

stage. Cells are metabolically active during G₁, and this is when protein synthesis takes place. A cell might be permanently arrested at this stage if it does not undergo further division. This arrested phase is referred to as gap zero (G₀).

Gap 1 is followed by the synthesis phase, which lasts about 5 h in mammalian cells [21]. This is when DNA synthesis occurs. The DNA replicates itself, and the chromosomes then consist of two identical sister chromatids.

Some DNA replicates early in S phase, and some replicates later. Early replicating DNA contains a higher portion of active genes than late-replicating DNA. By standard G-banding techniques, the light-staining bands usually replicate early, whereas the dark-staining bands and the inactive X chromosome in females replicate late in the S phase.

Gap 2 lasts about 3 h [21]. During this phase, the cell prepares to undergo cell division. The completion of G₂ represents the end of interphase.

The final step in the cell cycle is mitosis. This stage lasts only 1–2 h in most mammalian cells. Mitosis is the process by which cells reproduce themselves, creating two daughter cells that are genetically identical to one another and to the original parent cell. Mitosis is itself divided into stages (Fig. 2.10).

Mitosis

Prophase

Chromosomes are at their greatest elongation and are not visible as discrete structures under the light microscope during interphase. During prophase, chromosomes begin to coil, become more condensed, and begin to become visible as discrete structures. Nucleoli are visible early in prophase but disappear as the stage progresses.

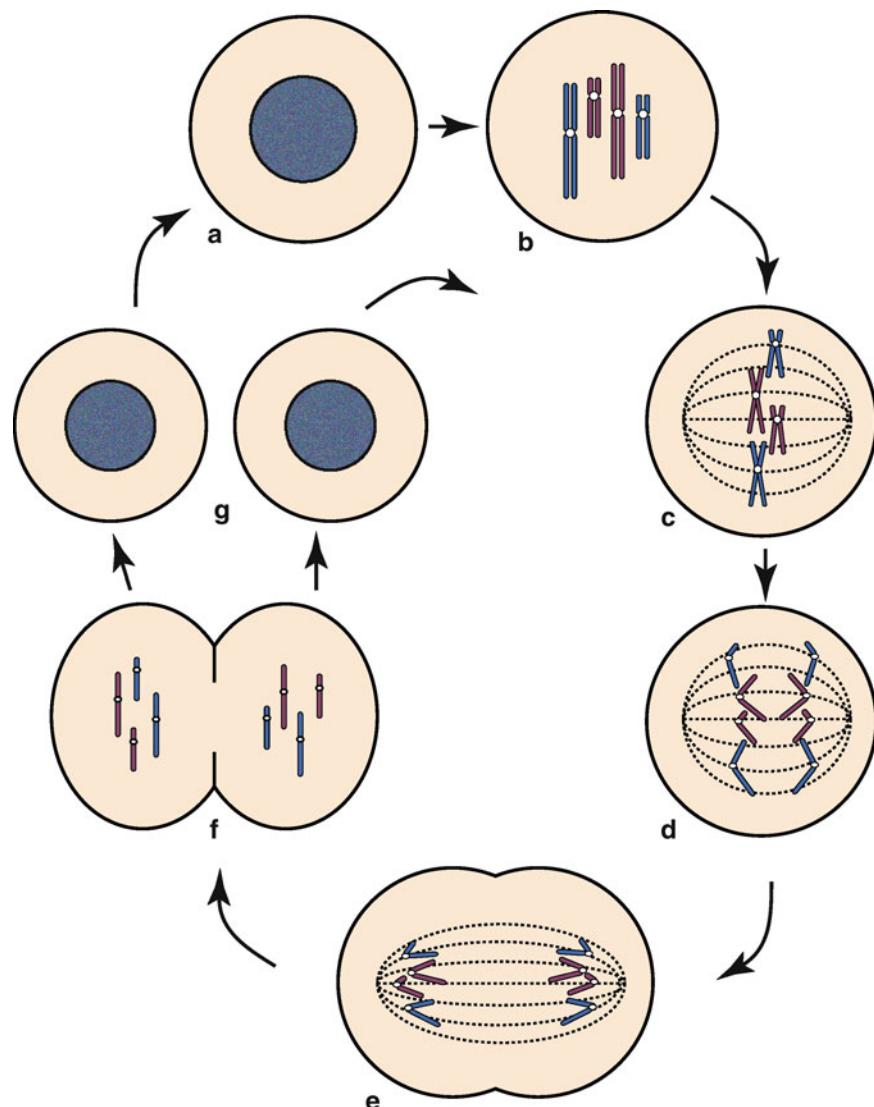
Prometaphase

Prometaphase is a short period between prophase and metaphase during which the nuclear membrane disappears and the spindle fibers begin to appear. Chromosomes attach to the spindle fibers at their kinetochores.

Metaphase

During metaphase, the mitotic spindle is completed, the centrioles divide and move to opposite poles, and the chromosomes line up on the equatorial plate. Chromosomes reach their maximum state of contraction during this phase. It is metaphase chromosomes that are traditionally studied in cytogenetics.

Fig. 2.10 Mitosis. Schematic representation of two pairs of chromosomes undergoing cell division: (a) interphase, (b) prophase, (c) metaphase, (d) anaphase, (e) telophase, (f) cytokinesis, and (g) interphase of the next cell cycle



Anaphase

Centromeres divide longitudinally and the chromatids separate during this stage. Sister chromatids migrate to opposite poles as anaphase progresses.

Telophase

The final stage of mitosis is telophase. The chromosomes uncoil and become indistinguishable again, the nucleoli reform, and the nuclear membrane is reconstructed. Telophase is usually followed by cytokinesis, or cytoplasmic division. Barring errors in DNA synthesis or cell division, the products

of mitosis are two genetically identical daughter cells, each of which contains the complete set of genetic material that was present in the parent cell. The two daughter cells enter interphase, and the cycle is repeated.

Meiosis

Meiosis takes place only in the ovaries and testes. A process involving one duplication of the DNA and two cell divisions (meiosis I and meiosis II) reduces the number of chromosomes from the diploid number ($2n=46$) to the haploid number ($n=23$). Each gamete produced contains only one copy of each chromosome. Fertilization restores the diploid number in the zygote.

Meiosis I

Meiosis I is comprised of several substages: prophase I, metaphase I, anaphase I, and telophase I (Fig. 2.11).

Prophase I

Prophase I is a complex stage that is further subdivided as follows.

Leptotene

In leptotene, there are 46 chromosomes, each comprised of two chromatids. The chromosomes begin to condense but are not yet visible by light microscopy. Once leptotene takes place, the cell is committed to meiosis.

Zygotene

Zygotene follows leptotene. Homologous chromosomes, which in zygotene appear as long thread-like structures, pair locus for locus. This pairing is called synapsis. A tripartite structure, the synaptonemal complex, can be seen with electron microscopy. The synaptonemal complex is necessary for the phenomenon of crossing-over that will take place later in prophase I.

Synapsis of the X and Y chromosomes in males occurs only at the pseudoautosomal regions. These regions are located at the distal short arms and are the only segments of the X and Y chromosomes containing homologous loci. The nonhomologous portions of these chromosomes condense to form the sex vesicle.

Pachytene

Synapsis is complete during pachytene. Chromosomes continue to condense and now appear as thicker threads. The paired homologs form structures called bivalents, sometimes referred to as tetrads because they are composed of four chromatids.

The phenomenon of crossing over takes place during pachytene. Homologous or like segments of DNA are exchanged between nonsister chromatids of the bivalents. The result of crossing over is a reshuffling or recombination of genetic material between homologs, creating new combinations of genes in the daughter cells.

Diplotene

In diplotene, chromosomes continue to shorten and thicken, and the homologous chromosomes begin to repel each other. This repulsion continues until the homologous chromosomes are held together only at points where crossing-over took place. These points are referred to as chiasmata. In males, the sex vesicle disappears, and the X and Y chromosomes associate end to end.

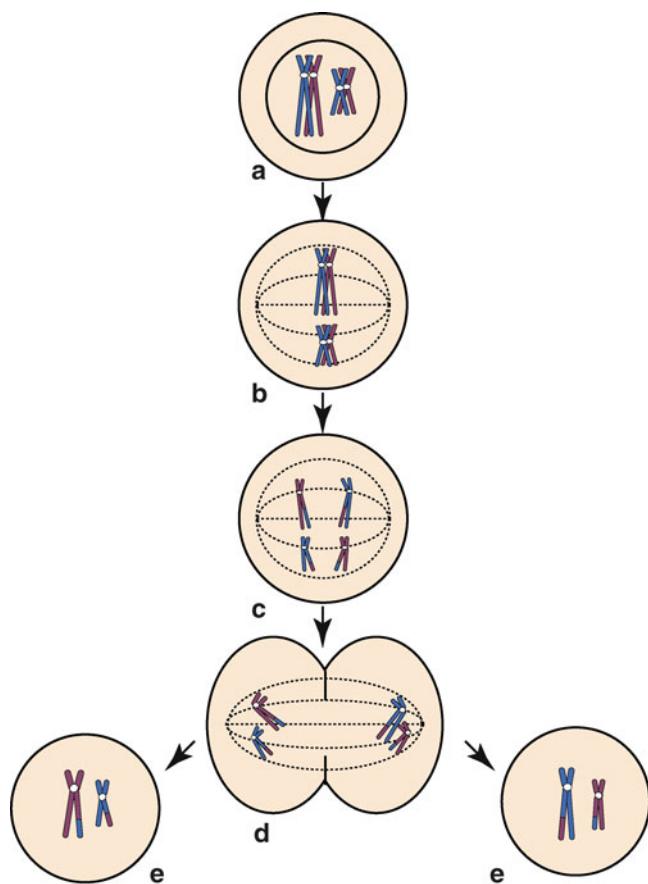


Fig. 2.11 Schematic representation of two chromosome pairs undergoing meiosis I: (a) prophase I, (b) metaphase I, (c) anaphase I, (d) telophase I, and (e) products of meiosis I

Diakinesis

Chromosomes reach their greatest contraction during this last stage of prophase.

Metaphase I

Metaphase I is characterized by disappearance of the nuclear membrane and formation of the meiotic spindle. The bivalents line up on the equatorial plate with their centromeres randomly oriented toward opposite poles.

Anaphase I

During anaphase I, the centromeres of each bivalent separate and migrate to opposite poles.

Telophase I

In telophase, the two haploid sets of chromosomes reach opposite poles, and the cytoplasm divides. The result is two cells containing 23 chromosomes, each comprised of two chromatids.

Meiosis II

The cells move directly from telophase I to metaphase II with no intervening interphase or prophase. Meiosis II proceeds much like mitotic cell division except that each cell contains only 23 chromosomes (Fig. 2.12).

The 23 chromosomes line up on the equatorial plate in metaphase II, the chromatids separate and move to opposite poles in anaphase II, and cytokinesis occurs in telophase II. The net result is four cells, each of which contains 23 chromosomes, each consisting of a single chromatid. Owing to the effects crossing-over and random assortment of homologs, each of the new cells differs genetically from one another and from the original cell.

Spermatogenesis and Oögenesis

The steps of spermatogenesis and oögenesis are the same in human males and females; however, the timing is very different (Fig. 2.13).

Spermatogenesis

Spermatogenesis takes place in the seminiferous tubules of the male testes. The process is continuous and each meiotic cycle of a primary spermatocyte results in the formation of four nonidentical spermatozoa. Spermatogenesis begins with sexual maturity and occurs throughout the postpubertal life of a man.

The spermatogonia contain 46 chromosomes. Through mitotic cell division, they give rise to primary spermatocytes. The primary spermatocytes enter meiosis I and give rise to the secondary spermatocytes, which contain 23 chromosomes, each consisting of two chromatids. The secondary spermatocytes undergo meiosis II and give rise to spermatids. Spermatids contain 23 chromosomes, each consisting of a single chromatid. The spermatids differentiate to become spermatozoa, or mature sperm.

Oögenesis

Oögenesis in human females begins in prenatal life. Ova develop from oögonia within the follicles in the ovarian cortex. At about the third month of fetal development, the oögonia, through mitotic cell division, begin to develop into diploid primary oöcytes. Meiosis I continues to diplotene, where it is arrested until sometime in the postpubertal reproductive life of a woman. This suspended diplotene is referred to as dictyotene.

Subsequent to puberty, several follicles begin to mature with each menstrual cycle. Meiosis I rapidly proceeds with an uneven distribution of the cytoplasm in cytokinesis of meiosis I, resulting in a secondary oöcyte containing most of the cytoplasm, and a first polar body. The secondary oöcyte,

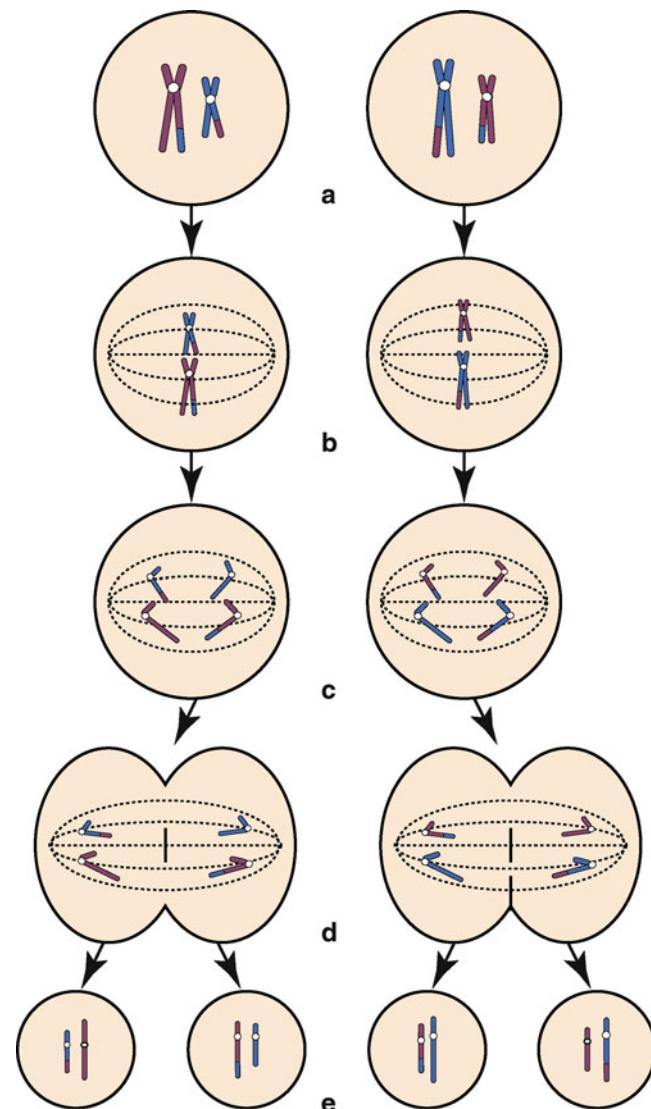


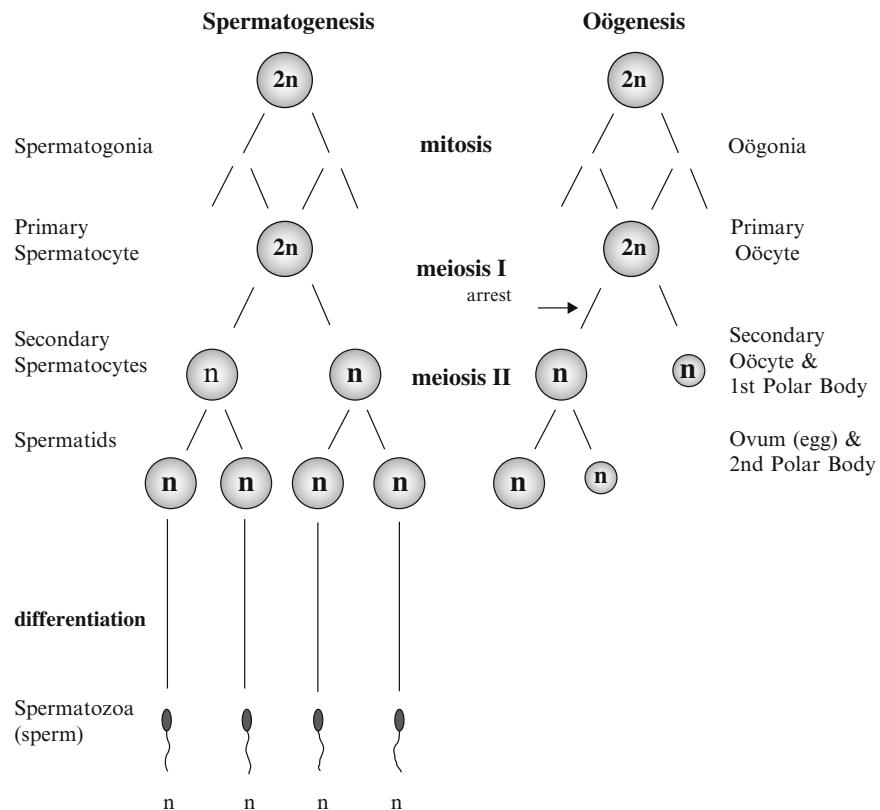
Fig. 2.12 Schematic representation of two chromosome pairs undergoing meiosis II: (a) products of meiosis I, (b) metaphase II, (c) anaphase II, (d) telophase II, and (e) products of meiosis

which has been ovulated, begins meiosis II. Meiosis II continues only if fertilization takes place. The completion of meiosis II results in a haploid ovum and a second polar body. The first polar body might undergo meiosis II, or it might degenerate. Only one of the potential four gametes produced each menstrual cycle is theoretically viable.

Fertilization

The chromosomes of the egg and sperm produced in meiosis II are each surrounded by a nuclear membrane within the cytoplasm of the ovum and are referred to as pronuclei. The male and female pronuclei fuse to form the diploid nucleus of the zygote, and the first mitotic division begins.

Fig. 2.13 Spermatogenesis and oogenesis. The events of spermatogenesis and oogenesis are the same, but the timing and net results are different. Oogenesis begins prenatally and is arrested in meiosis I until the postpubertal life of a woman; spermatogenesis begins with the sexual maturity of the male and is continuous. Each cycle of spermatogenesis results in four functional gametes, while each cycle of oogenesis results in a single egg.



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Human Chromosome Nomenclature: An Overview and Definition of Terms

Marilyn L. Slovak, Aaron Theisen, and Lisa G. Shaffer

Introduction

Science is about generating, interpreting, and communicating information. The need to establish a common language or communication tool to describe human chromosomes and chromosomal aberrations associated with human disease became apparent in the mid-1950s soon after Tjio and Levan reported that the correct chromosome number in humans was 46 [1]. Variations in chromosome number and structure were quickly associated with multiple congenital anomalies, intellectual disabilities, and cancer. To effectively describe chromosomal changes in a systematic manner, a group of 17 forward-thinking investigators who had previously published human karyotypes teamed up in Denver, Colorado, in 1960 to create the foundation of the celebrated communication tool known today as *An International System for Human Cytogenetic Nomenclature* or ISCN. ISCN is an abbreviated symbolic writing method used to describe genetic changes by copy number (dosage) and position (locus). This international language allows cytogeneticists to describe the results of cytogenetic-based assays, communicate across cultures and languages, create databases, publish scientific activities, and foster collaborations worldwide. To keep the nomenclature current with the latest technological advances without losing the foundation on which it was built, the ISCN recommendations are updated periodically by an elected standing committee with global representation; ten conferences have been held since its first printing in 1960. The latest version of

ISCN [2] is one of several “dog-eared” books within an arm’s reach of every practicing clinical cytogeneticist.

For those unfamiliar with the language of cytogenetics, the deceptively simple act of communication can be a barrier to understanding or becoming involved in the field. The goal of this chapter is to present the general concepts of the ISCN and offer guidance on writing abnormal cytogenetic results by providing nomenclature examples. It is not intended to replace the latest edition of the ISCN. To get started, an easy-reference glossary of the most commonly used ISCN terms may be found at the end of this chapter. After mastering the *lingua franca* of cytogeneticists offered in this primer, ISCN [2] should be consulted to sharpen human nomenclature skills and extend proficiency to include chromosome breakage and meiotic chromosome nomenclature (Chaps. 10 and 12, respectively, in ISCN [2]).

Visualizing Human Chromosomes

Human cells have 23 pairs of chromosomes (22 pairs of autosomes and one pair of sex chromosomes) for a total of 46 chromosomes per cell (Fig. 3.1). The autosomes are assigned a number (1–22) based on size (with one exception; chromosome 22 is slightly larger than chromosome 21). The sex chromosomes are noted by the letters X and Y. The female sex chromosome complement is XX and the male complement is XY. Chromosomes are divided into long and short arms, separated by a centromere, or primary constriction. A chromosome may be metacentric, with its centromere in the middle; submetacentric, with the centromere closer to one end of the chromosome; or acrocentric, in which the centromere is near one end of the chromosome and the short arm is essentially comprised of repetitive DNA that constitutes the satellites and nucleolar organizing regions. Chromosomes 1 and 3 are examples of metacentric chromosomes, chromosomes 4 and 5 are large submetacentric chromosomes, and chromosomes 13–15 are considered medium sized acrocentric chromosomes.

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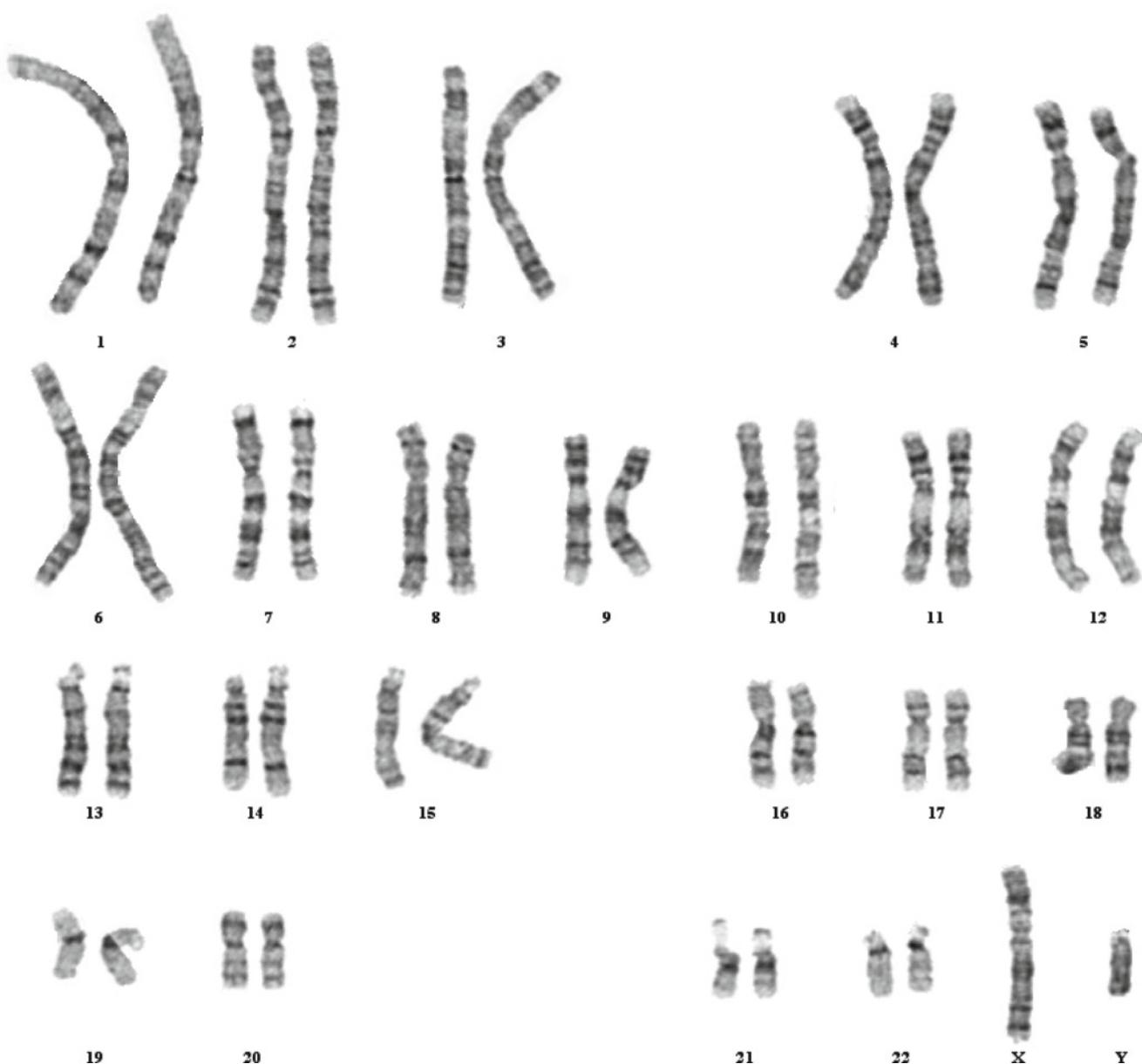


Fig. 3.1 G-banded normal male karyotype illustrating the characteristic size, centromere position, and G-banding banding pattern for each human chromosome pair

Chromosome Banding and Identification

Launched in the early 1970s, banding methods allow for the identification of chromosomes not only by length and centromere position, but also by their unique banding properties. Figure 3.1 illustrates the characteristic size, centromere position, and G-banding banding pattern for each human chromosome pair. The commonly used G-, Q-, and R-banding techniques show bands distributed along the entire chromosome, whereas the C-, T-, or NOR-banding techniques are used to identify specific chromosome structures that are

heritable features (Table 3.1; see also Chap. 4). To identify each chromosome in the human karyotype, it is important to be familiar with the characteristic morphological features or landmarks of each chromosome, such as the telomeres that cap the ends of the chromosomes, the centromere or “primary constriction” that divides a chromosome into two arms, and certain defined bands (Fig. 3.2). The symbols p and q are used to designate the short and long arms, respectively. Convention places the short arm or “p” (from the French *petite*) arm at the top in diagrammatic representations and the long or “q” arm at the bottom. Characteristic

Table 3.1 Commonly used banding and staining techniques in human cytogenetics

Commonly used banding and staining techniques in human cytogenetics	
Q	<i>Q-banding</i> —a fluorescent stain (quinacrine dihydrochloride) produces specific banding patterns for each pair of homologous chromosomes similar to G-banding, excellent for identifying centromeric regions of chromosomes 3, 4, and 13, some acrocentric chromosomes and the Y chromosome. AT-rich (gene poor) regions fluoresce brightly with Q-banding
QF	Q-bands by fluorescence
QFQ	Q-bands by fluorescence using quinacrine
QFH	Q-bands by fluorescence using Hoechst 33258 dye
G	<i>G-banding</i> —Giemsa stain produces specific banding patterns for each pair of homologous chromosomes similar to Q-banding. The chromosomes are treated with trypsin to partially digest the chromosome prior to being stained. AT-rich (gene poor) regions stain darkly with G-banding
GT	G-bands by trypsin
GTG	G-bands by trypsin using Giemsa
GTL	G-bands by trypsin using Leishman stain
GTW	G-bands by trypsin using Wright stain
C	<i>C-banding</i> —after barium hydroxide treatment, Giemsa stain is used to stain constitutive heterochromatin close to the centromeres and on the long arm of the Y chromosome. C-banding is used to identify dicentric chromosomes and variations of constitutive heterochromatin
CB	C-bands by barium hydroxide
CBG	C-bands by barium hydroxide using Giemsa
R	<i>R-banding</i> —a staining method in which chromosomes are heated in a phosphate buffer and then stained to produce a banding pattern that is the reverse of that produced with G-banding
RF	R-bands by fluorescence
RFA	R-bands by fluorescence using acridine orange
RH	R-bands by heating
RHG	R-bands by heating using Giemsa
RB	R-bands by 5-bromo-2-deoxyuridine (BrdU)
RBG	R-bands by BrdU using Giemsa
RBA	R-bands by BrdU using acridine orange
DAPI	<i>4',6-diamidino-2-phenylindole (DAPI) staining</i> —permits characterization of AT-rich (DAPI+) or AT-poor (DAPI-) heterochromatic regions, especially when counterstained with chromomycin A3, which preferentially binds to GC-rich DNA
DA-DAPI	DAPI-bands by Distamycin A and DAPI
NOR	<i>Nucleolus organizing region staining</i> —a staining method utilizing silver nitrate, which preferentially accumulates in the NORs located on the stalks of the acrocentric chromosomes that contain active ribosomal RNA genes
T	<i>T-banding</i> —a Giemsa staining technique that stains the telomeres (ends) and the centromeres of chromosomes

regions and bands within a given chromosome are observed when banding techniques are used. Chromosome regions refer to those areas lying between two distinct landmarks and are divided into bands. For example, the long arm of chromosome 7 has three regions: 7q1, 7q2, and 7q3 (Fig. 3.2). These regions are further subdivided into bands. A band is defined as a part of the chromosome that is clearly distinguishable from its adjacent segments based on its staining properties.

As a general rule, a chromosome band contains ~5–10 megabases (Mb) of DNA. “High-resolution” cytogenetic techniques (see Chap. 4) produce elongated chromosomes that allow further refinement of karyotypic aberrations by subdividing bands into smaller sub-bands. Banding resolution and patterns may vary depending on the banding method employed (Table 3.1), so it is important to state the level of banding resolution and banding method employed on the final report when describing a cytogenetic result.

The gene content of chromosome bands is also variable and, in general, reflects functionality.

Giemsa or G-banding is the most common banding method employed in North American cytogenetics laboratories. G-dark (positive) bands are AT rich, gene poor, and late replicating. The early replicating G-light (negative) bands are GC rich, gene rich, and late replicating. Reverse or R-banding shows this banding pattern in reverse (i.e., reversal of light and dark G-bands). However, the numbering of the bands is identical with both banding methods. Additional banding/staining methods are used to detect specific chromosome regions or abnormalities. For example, centromeric and pericentromeric DNA are comprised of alpha-satellite and various other families of repetitive satellite DNA, which are easily visualized using constitutive heterochromatin (C-banding) methods. C-banding is particularly useful when identifying the morphologically variable heterochromatin regions of the Y chromosome and chromosomes 1, 9, and 16.

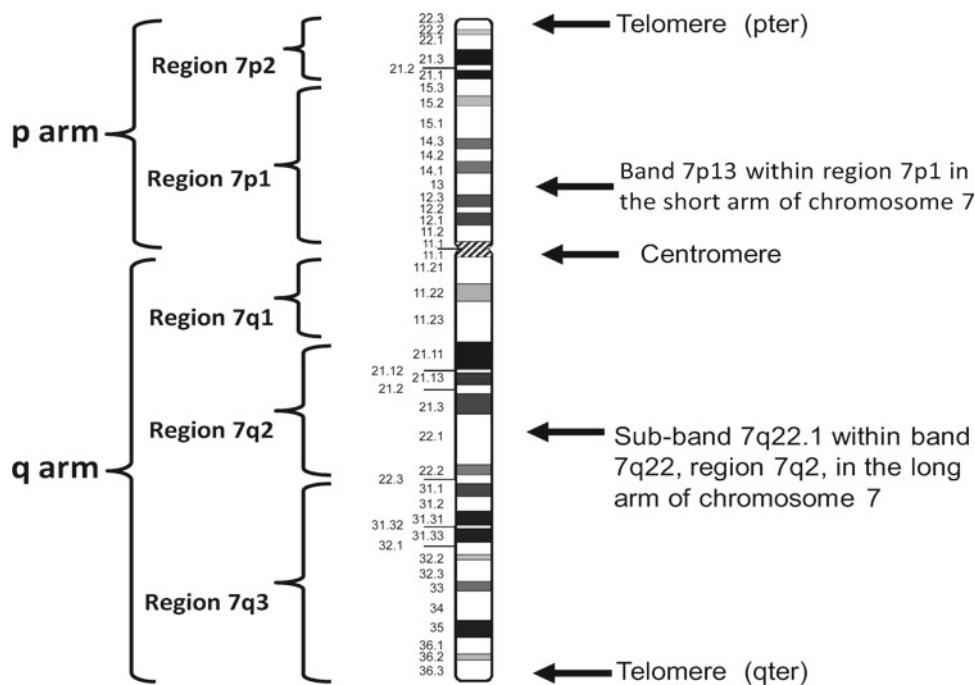


Fig. 3.2 Characteristic morphological features of a human chromosome. Chromosome 7 is used in this example. Chromosomes have major landmarks, including the centromere or primary constriction, certain bands, and the telomeres that cap both ends of the chromosomes.

The centromere divides the chromosomes into the short or “p” and long or “q” arms. Each arm is divided into 1–4 regions. Each band within a region is numbered centromere to telomere. Bands may be subdivided into sub-bands

The short arms of the acrocentric chromosomes house the ribosomal RNA gene clusters in the nucleolar organizing regions (NORs), which form the nucleolus of the cell. NORs are detected by silver-based NOR staining. Finally, telomeres are comprised of $(TTAGGG)_n$ mini-satellite repeats that stain darkly with T-banding. Technical details of the various chromosome banding methods may be found in Chap. 4.

Figure 3.3 shows the idiograms or diagrammatic representations of the G-banding patterns for normal human chromosomes 1 and 13 at five successive levels of resolution. The centromere itself is designated as “10,” with the part adjacent to the short arm as “p10” and the part adjacent to the long arm as “q10.” The bands and regions are numbered outward from the centromere to the telomeres. Four distinct chromosome units—the chromosome number, the chromosome arm, the region number, and the band number within a region—are needed to describe a precise location within a specific chromosome. For example, 7q34 refers to chromosome 7, long arm, region 3, band 4 (Fig. 3.2). This is referred to as “seven q three four,” *NOT* “seven q thirty-four.” If “high-resolution” banding is used, the band may be further subdivided using a decimal point after the band designation. Having a copy of the human chromosome idiograms and a reference set of well-banded karyotypes representing the banding methods and banding level of resolution routinely employed by the laboratory is helpful.

Karyotype Descriptions

Karyotype descriptions convey the total number of chromosomes, the sex chromosome complement, and a description of any chromosome abnormalities present. The correct use of punctuation in the nomenclature string brings structure and meaning to the description. Table 3.2 provides a quick reference guide of the conventional cytogenetic ISCN punctuation symbols and their meaning.

The description of any human karyotype begins with two basic components separated by a comma; the total number of chromosomes is listed first, followed by the sex chromosome complement. Thus, a normal male karyotype is written as 46,XY, and a normal female karyotype is designated as 46,XX.

There are a few additional basic rules to describing chromosome aberrations:

- As with normal karyotypes, chromosome number (or chromosome range, see later in chapter) is listed first, followed by the sex chromosome complement and any aberrations. Commas separate chromosome number, the sex chromosome complement, and each abnormality from one another within the nomenclature string. An exception exists when a triplet abbreviation is needed before the chromosome number (e.g., mos for mosaic, see later). In these circumstances, a space must be used after any abbreviation and before the chromosome number (Table 3.3).

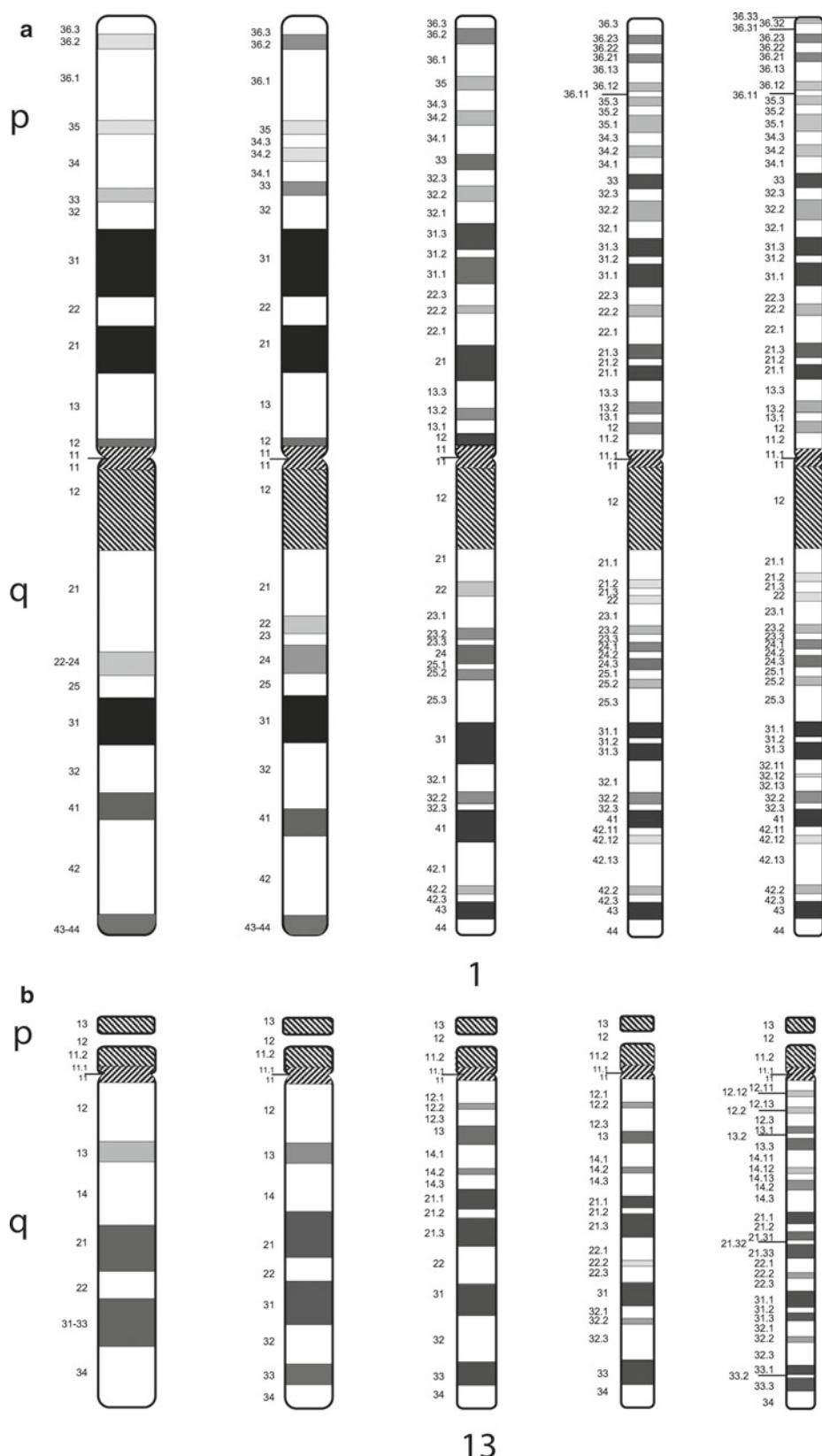


Fig. 3.3 Idiograms or diagrammatic representations of the G-banding patterns for normal human chromosomes (a) 1 and (b) 13 at five successive levels of resolution. From left to right, the chromosomes represent a haploid karyotype of 300-, 400-, 550-, 700-, and 850-band level. The

dark bands represent the G-positive and bright Q-bands, with the exception of the variable regions. R-bands will have the reverse banding pattern, but the numbering of the bands remains unchanged (Reproduced from ISCN [2] with permission from Nicole L. Chia)

Table 3.2 ISCN punctuation and significance

Description	Symbol	Significance
Approximate sign	~	Denotes intervals and expresses uncertainty about breakpoints, number of chromosomes, fragments, or markers; denotes chromosome range when the exact number cannot be determined
Arrow	→	Denotes from → to in the detailed system
Bracket, angle	<>	Denotes ploidy level
Bracket, square	[]	Denotes number of cells in a cell line/clone
Colon, single	:	Break in the detailed system
Colon, double	::	Break and reunion in the detailed system
Comma	,	Separates chromosome, numbers, sex chromosomes, and chromosome aberrations
Decimal point	.	Denotes sub-bands
Equal sign	=	Number of chiasmata
Letter “c”	c	Indicates a constitutional abnormality in a cancer karyotype. Always placed immediately after the constitutional abnormality
Minus sign	-	Loss
Multiplication sign	×	Multiple copies of rearranged chromosomes or number of copies of a chromosomal region
Parentheses	()	Surround structurally altered chromosomes and breakpoints
Period	.	Separates various banding/staining techniques
Plus sign	+	Gain
Question mark	?	Questionable identification of a chromosome or chromosome structure
Semicolon	;	Separates altered chromosomes and breakpoints in structural rearrangements involving more than one chromosome
Slant line, single	/	Separates cell lines/clones
Slant line double	//	Separates chimeric cell lines/clones
Underlining, single	—	Used to distinguish homologous chromosomes

Table 3.3 Reporting mosaicism, chimerism, and chimerism secondary to bone marrow stem cell transplantation

Example	Interpretation or rule used
mos 47,XY,+18/46,XY	When present, a normal cell line is listed last
mos 45,X[20]/47,XXX[10]/46,XX[20]	When several cell lines are present, size matters: the largest is presented first, then the second largest, etc.; normal cell lines are listed last, when present
chi 46,XY[25]/46,XX[10]	Largest clone is presented first in chimeras
45,X[20]/46,X,i(X)(p10)[20]	In the event of equivalent clone size, numerical abnormalities are reported before structural ones
47,XX,+8[15]/47,XX,+21[15]	In the event of equivalent clone size with numerical abnormalities, the cell lines are listed from lowest to highest autosome number
47,XXX[15]/47,XX,+21[15]	Clones with sex abnormalities are always reported first
46,XX[4]/46,XY[16]	Four cells from the female recipient were detected along with 16 cells from the male donor
46,XX,t(8;21)(q22;q22)[5]/46,XY[15]	Five female recipient cells showed an (8;21) translocation, along with 15 normal male donor cells
//46,XY[20]	All 20 cells analyzed were derived from the male donor
46,XX[20]//	All 20 cells analyzed were derived from the female recipient (host)

2. Sex chromosome abnormalities are listed before any autosomal aberrations, and X chromosome abnormalities are presented before those involving the Y chromosome.
3. Autosomal abnormalities follow any sex chromosome aberration and are listed in numerical order irrespective of aberration type. Multiple structural changes of homologous chromosomes are listed in alphabetical order according to their abbreviated term (e.g., a deletion would be written before an insertion).
4. If a chromosome has both numerical and structural aberrations, numerical aberrations are listed first followed by structural aberrations; for example, trisomy 8 is listed before a translocation involving chromosomes 8 and 14.
5. Letters or triplets are used to specify structurally altered chromosomes (see Table 3.4).
6. Parentheses are used to identify chromosomes involved in a specific aberration. The first set of parentheses identifies which chromosome or chromosomes are involved. The second set of parentheses denotes the exact chromosome band of the aberration for each of the chromosomes listed in the first set of parentheses. In both sets of parentheses, semicolons are used to separate multiple chromosomes or bands.
7. If the aberration involves a sex chromosome, it is always listed first; otherwise, the autosome with the lowest number is specified first. However, if an aberration involves a

Table 3.4 Short and detailed ISCN for common cytogenetic aberrations

Aberration type	Description
add	Additional material of unknown origin attached to a chromosome region or band
Short	add(1)(q21)
Detailed	add(1)(pter→q21::?)
Description	Material of unknown original attached to the long arm of chromosome 1 at band 1q21. Chromosome 1 material distal to band q21 is lost
del	Deletion or loss of chromosome material. May be either terminal or interstitial
Interstitial	
Short	del(7)(q22q31)
Detailed	del(7)(pter→q22::q31→qter)
Description	Interstitial deletion with breakage and reunion (::) of bands 7q22 and 7q31. The segment lying between those two bands is deleted
Terminal	
Short	del(7)(q22)
Detailed	del(7)(pter→q22::)
Description	Terminal deletion with a break in band 7q22. Segment distal to 7q22 is deleted
der	Derivative or structurally rearranged chromosome with an intact centromere
Short	der(5)inv(5)(p13q13)del(5)(q31q33)
Detailed	der(5)(pter→p13::q13→cen→p13::q13→q31::q33→qter)
Description	Derivative chromosome 5 with a pericentric inversion (breakage and reunion of bands 5p13 and 5q13 and a 180° rotation of the segment) with an interstitial deletion with breakage and reunion (::) of band 5q31 and 5q33. The segment lying between the latter two bands is deleted
dic	Dicentric chromosome has two centromeres but counted as one chromosome. There is no need to indicate that one normal chromosome is missing.
Short	45,XY,dic(13;14)(q14;q24)
Detailed	45,XY,dic(13;14)(13pter→13q14::14q24→14pter)
Description	Dicentric chromosome with breaks and reunion at bands 13q14 and 14q24. The missing chromosomes 13 and 14 are not indicated because they are replaced by the dicentric. The karyotype has one normal chromosome 13, one normal chromosome 14, and the dic(13;14). The resulting net imbalance is loss of the segments distal to 13q14 and 14q24.
dup	Duplication of genetic material is present. Band order indicates whether this is direct or inverted
Short	dup(1)(q21q32)
Detailed	dup(1)(pter→q32::q21→qter)
Description	Direct duplication of the segment between bands 1q21 and 1q32
Short	dup(1)(q32q21) or dup(1)(q32q21)
Detailed	dup(pter→q32::q32→q21::q32→qter)
Description	Inverted duplication of the segment 1q21 to 1q32. The detailed system clarifies the location of the duplicated segment
hsr	Intrachromosomal homogeneously staining region indicating gene amplification
Short	hsr(8)(q24.1)
Detailed	hsr(8)(pter→q24.1::hsr::q24.1→qter)
Description	Homogeneously staining region in band 8q24.1
ins	Insertion of material from one site into another site. Band order indicates whether this is direct or inverted. May involve one or more chromosomes.
Short	ins(5)(p13q31q15)
Detailed	ins(5)(pter→p13::q15→q31::p13→q15::q31→qter)
Description	Inverted insertion of the long arm 5q15 to 5q31 segment into the chromosome 5 short arm at 5q13. Band orientation within the segment is reversed with respect to the centromere, that is, 5q15 is more distal to the centromere than 5q31
inv	Inversion of a chromosome segment: breakpoints may be on either side of the centromere (pericentric) or within the same chromosome arm (paracentric)
Short	inv(9)(p13q21)
Detailed	inv(9)(pter→p13::q21→p13::q21→qter)
Description	Pericentric inversion with breakage and reunion at bands 9p13 and 9q21
i	Isochromosome, a mirror image of chromosome from its centromere
Short	i(17)(q10)
Detailed	i(17)(pter→q10::q10→qter)
Description	Isochromosome for the entire long arm of one chromosome 17. The centromeric band q10 indicates an isochromosome of the long arm. If band p10 was listed, the isochromosome would be comprised of the short arm. The shorter designation of i(17q) may be used in the text but never in the nomenclature string

(continued)

Table 3.4 (continued)

Aberration type	Description
r	Ring chromosome
Short	r(6)(p23q25)
Detailed	r(6)(::p23→q25::)
Description	Ring chromosome with breakage and reunion at bands 6p23 and 6q25. The segments distal to the two breakpoints are deleted
rec	Recombinant chromosome due to meiotic crossing-over. This term is only used when the parental karyotype is known (include “mat” or “pat”); otherwise, use “der”
Short	rec(6)dup(6p)inv(6)(p22.2q25.2)pat
Detailed	rec(6)(pter→q25.2::p22.2→pter)pat
Unknown rec	der(6)(pter→q25.2::p22.2→pter)
t	Balanced translocation involving two or more chromosomes. Also use “t” to describe balanced whole-arm translocations. See text for reporting Robertsonian translocations.
Short	t(8;9;22)(p21;q34.1;q11.2)
Detailed	t(8;9;22)(22qter→22q11.2::8p21→8qter;9pter→9q34.1::8p21→8pter;22pter→22q11.2::9q34.1→9qter)
Description	3-way balanced translocation where the segment distal to 8p21 is translocated to chromosome 9 at band 9q34.1, the segment distal to 9q34.1 is translocated to chromosome 22 at band 22q11.2, and the segment distal to 22q11.2 is translocated to chromosome 8 at band 8p21
trc	Tricentric chromosome is counted as one chromosome (note chromosome count). The chromosome with the lowest number is specified first followed by the order of appearance within this chromosome
Short	44,XY,trc(5;11;8)(q31;q13p15;q22)
Detailed	44,XY,trc(5;11;8)(5pter→q31::11q13→11p15::8q22→8pter)
Description	A tricentric chromosome where band 5q31 is fused with 11q13 and 11p15 is fused with 8q22
trp	Tripllication of chromosome material. Orientation of the triplicated segment is only obvious using the detailed system
Short	46,XX,trp(1)(q31q21)
Detailed	46,XX,trp(1)(pter→q31::q31→q21::q21→qter)
Description	Inverted triplication of the 1q21 and 1q31 segments

- three-break rearrangement, such as observed in “[Insertions](#)” (see later), the receptor chromosome is specified before the donor chromosome (also see “[Translocations](#)” later).
8. A semicolon is used between chromosomes and breakpoints within sets of parentheses if two or more chromosomes have been altered in a rearrangement. No semicolon is used in the second set of parentheses for any rearrangement that involves a single chromosome.
 9. A break suspected at the interface of two bands should be assigned the higher band number or the number more distal to (farther from) the centromere.
 10. Different clones or cell lines are separated by a single slant line (/).
 11. Square brackets [] are placed after the karyotype string to designate the number of cells of each cell line or clone. In constitutional studies, the size of the cell lines determines the order of presentation in the karyotype. In cancer studies, the use of square brackets is critical because multiple clones indicating clonal evolution of disease may be observed at varying levels, and various therapies may eliminate or lessen one subclone but give another subclone a growth advantage.

The following are examples using these basic guidelines; refer also to each specific section below for additional information:

46,XX,inv(3)(q21q26.2)

This is a female with a balanced paracentric inversion (an inversion involving a single chromosome arm) of the long arm of chromosome 3. One break occurred at band 3q21 and the other at 3q26.2. The chromosome segment between those breakpoints is present but inverted 180°. There are no spaces after any commas in the description, and there is no semicolon in the second set of parentheses because the aberration affects a single chromosome. When normal chromosomes are replaced by structurally altered chromosomes, there is no need to record the normal chromosome as missing. In this case, the nomenclature implies that one morphologically normal chromosome 3 and one inv(3q) are present in this XX karyotype with 46 chromosomes. See also “[Inversions](#)” later.

46,Y,t(X;9)(p22.3;q22)

This is a male karyotype showing a balanced translocation between the X chromosome and chromosome 9. The breakpoints for this translocation are Xp22.3 and 9q22, respectively. The chromosomal segments distal to these breakpoints have been exchanged. Note that the normal sex chromosome in this example is written before the X chromosome aberration. Semicolons are present in both sets of parentheses because two chromosomes are involved.

46,XX,ins(5;2)(q13;q22q32)

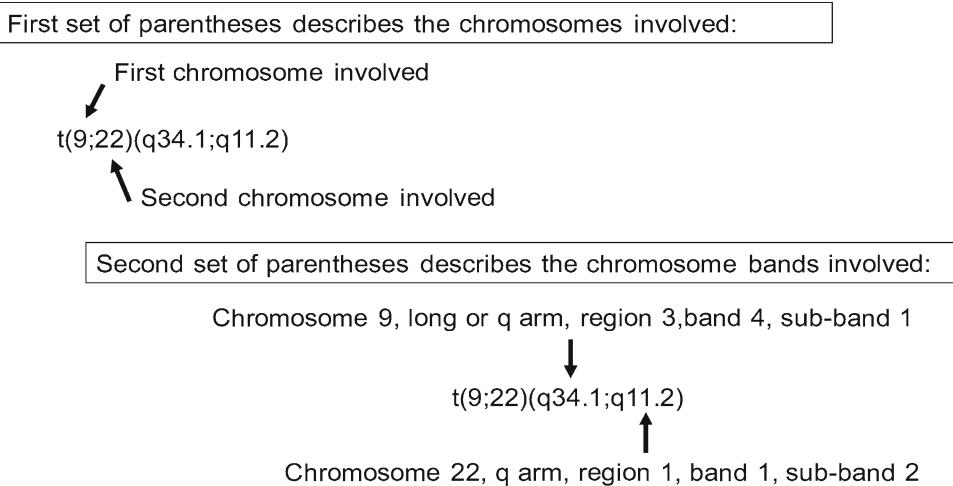


Fig. 3.4 Diagrammatic dissection of translocation nomenclature. A translocation is indicated by the letter “t” followed by two sets of parentheses. The first set of parentheses will describe the chromosomes involved in the translocation. In this example, chromosomes 9 and 22 are involved. If a sex chromosome was involved, it would be listed before any autosomal aberration with X chromosome abnormalities presented before those involving the Y chromosome.

Autosome abnormalities are listed in numerical order. The second set of parentheses denotes the exact chromosome band of the aberration for each of the chromosomes listed in the first set of parentheses. The breakpoints involved in this translocation involve bands 9q34.1 and 22q11.2. Semicolons are used to identify the different chromosomes and their corresponding breakpoints.

This is a female karyotype in which material from the long arm of chromosome 2 between bands q22 and q32 (donor chromosome) is inserted into the long arm of chromosome 5 at band q13 (receptor chromosome). This is a direct insertion because the original orientation of the inserted segment has been maintained in its new position; that is, band 2q22 remains more proximal, or closer, to the centromere than band 2q31. If the insertion were inverted in the receptor chromosome 5, the ISCN would be written as ins(5;2)(q13;q32q22), indicating that band 2q22 is now more distal to the centromere than band 2q32.

46,X,t(X;18)(q22;p11.2) - or - 46,Y,t(X;18)(q22;p11.2)

These denote the same aberration, a translocation involving an X chromosome and chromosome 18, in either a female or male, respectively. The normal sex chromosome is listed before the rearranged one.

46,t(X;18)(q22;p11.2),t(Y;13)(q11.2;q12)

If both the X and Y chromosome are involved in aberrations, the abnormality involving the X chromosome is listed before that of the Y chromosome.

mos 45,X[25]/46,XX[15]

This describes a mosaic karyotype with one cell line showing a single X chromosome in 25 cells and a second cell line with a normal female (XX) sex chromosome complement in 15 cells. Note the use of a space between mos and

the chromosome number and the (optional) use of square brackets to indicate the number of cells in each cell line. See also “[Mosaicism and Chimerism](#)” later.

47,XX,+8,t(8;14)(q24.1;q32)

This is a female with two different aberrations involving chromosome 8. The numerical aberration is listed before the structural aberration.

46,XY,t(9;22)(q34.1;q11.2)[16]/47,XY,+8[4]

This male patient has two clones; these are separated by a slant line (/). The first clone of 16 cells shows a translocation between the long arms of chromosomes 9 and 22, with breaks at bands 9q34.1 and 22q11.2, respectively. The segments distal to the breakpoints have been exchanged (Fig. 3.4). The second clone of four cells shows gain of chromosome 8 as the sole clonal aberration. The abnormalities observed in the first clone are not seen in the second clone and vice versa. This situation is seen in neoplasia; see “[Describing Cancer Karyotypes](#)” later.

Constitutional and acquired (neoplastic) karyotypes may show a tremendous range of structural abnormalities. ISCN allows for both an abbreviated or short system as well as a detailed system of nomenclature. Whenever possible, use of the short system is strongly encouraged; all examples to this point are written in this way. Using the short system, the chromosome number, sex chromosome complement, type of rearrangement, the chromosome(s) involved, and the breakpoints

are indicated. However, complex rearrangements, especially structural aberrations with multiple gains and losses or involving multiple chromosomes, may necessitate the use of a detailed system that describes the involved chromosomes from end to end. The detailed system is particularly useful when describing complex acquired aberrations in malignant disorders.

The rules used in the short system are retained in the detailed system with one exception. Instead of writing the breakpoints within the last parentheses, an abbreviated description of the band composition of the rearranged chromosome(s) starting from the end of the short arm (pter) and proceeding to the end of the long arm (qter) is specified, that is, the bands are identified in the order in which they occur in the derivative chromosome. In the detailed system, a single colon denotes a break, a double colon denotes breakage and reunion, and an arrow indicates “from→to.” If there are doubts as to whether to use the short or detailed system, first write the short system and determine if the aberration(s) can be accurately drawn as described in the nomenclature string. If the abnormalities cannot be correctly visualized using the short system, the detailed system should be used. The detailed system was devised to be flexible; therefore, if only one of several chromosome aberrations requires the use of the detailed system, it is acceptable to combine the short and detailed systems to describe the karyotype. Table 3.4 lists the most common structural aberrations found in human karyotypes with interpretation of the findings and examples of how to write them in both the short and detailed systems.

Numerical Abnormalities and Ploidy

Gains and losses of whole chromosomes in the karyotype string are usually denoted by the use of either a plus (+) or minus (−) sign before the aberrant chromosome; for example, 47,XY,+21. The exception is the sex chromosomes in constitutional studies, where sex chromosome gains and losses are indicated by listing the chromosome(s) present (e.g., 45,X or 47,XXY) without use of plus or minus signs. Acquired sex chromosome aberrations are written with plus and minus signs (see “[Describing Cancer Karyotypes](#)” later).

Ploidy refers to the number of sets of chromosomes present. Thus, diploid refers to the normal situation of two sets of each chromosome (e.g., 46,XX or 46,XY). A haploid, triploid, or tetraploid karyotype is evident from the chromosome number; for example, 23,X, 69,XXY, or 92,XXYY, respectively. If additional chromosome changes are evident, these are expressed in relation to the appropriate ploidy level. The

ploidy levels most commonly used in human karyotyping, most often in acquired diseases, are:

- *Near-haploid* (1n), which describes chromosome counts up to 34 chromosomes; numerical abnormalities are expressed in relation to 23 chromosomes.
- *Near-diploid* (2n), which describes counts with 35–57 chromosomes; numerical abnormalities being expressed in relation to 46 chromosomes.
- *Near-triploid* (3n), which describes karyotypes with 58–80 chromosomes; numerical aberrations are expressed in relation to 69 chromosomes.
- *Near-tetraploid* (4n), which describes karyotypes with 81–103 chromosomes; numerical changes are expressed in relation to 92 chromosomes.

25,X,+4,+10

This represents a near-haploid karyotype with two copies of chromosomes 4 and 10 and single copies of all other chromosomes.

70,XXY,+13

This describes a near-triploid karyotype with four copies of chromosome 13 and three copies of all other chromosomes.

94,XXYY,−2,−5,+8,+8,+21,+21

This represents a near-tetraploid karyotype with three copies of chromosomes 2 and 5, five copies of chromosomes 8 and 21, and four copies of all other chromosomes.

For more complex ploidy changes, please refer to ISCN [2].

At times, the biology of the study or the chromosome number will vary between two ploidy levels. Because precise communication of the karyotypic data is key, these cases may be written with the ploidy level in angle brackets “<>” immediately after the chromosome number and before the sex chromosome complement. For example, high hyperdiploidy, a favorable finding in pediatric acute lymphoblastic leukemia (ALL), may be written relative to 2n ploidy even though it represents a near-triploid clone; for example, 59<2n>,XX,+X,+4,+5,+6,+10,+10,+14,+14,+17,+17,+18,+18,+21.

Endoreduplication (end) is a special form of duplication of the genome without mitosis, giving rise to four-stranded chromosomes at prophase and metaphase. Endoreduplication should be written as end 46,XY. Note the space after the triplet and before the chromosome number.

Structural Chromosome Abnormalities

Abbreviations are used to specify structural abnormalities (see Table 3.4) and precede the chromosome(s) involved in the aberration in the nomenclature string.

Deletions (del)

Deletions result in loss of a chromosome segment. *Terminal deletions* are caused by a single break with loss of the segment distal to the break. *Interstitial deletions* result from two breaks in a chromosome, loss of the intervening segment, and reunion of the breakpoints.

del(5)(p15.3)

This describes a terminal deletion of the short arm of chromosome 5. All chromosomal material distal to band p15.3 is missing.

del(20)(q11.2q13.3)

This represents an interstitial deletion of the long arm of chromosome 20. The material between bands q11.2 and q13.3 is deleted. Note that no semicolon separates the breakpoints, as this abnormality involves a single chromosome.

Additional examples are presented in Table 3.4. “High-resolution” banding (see Chap. 4) allows for the detection of deletions within a single chromosome band. Such deletions should be written denoting that two breaks have occurred in a single band, for example, del(4)(q12q12).

Ring Chromosomes (r)

Ring chromosomes, or rings, are donut-shaped structures that may involve one or more chromosomes. When a single chromosome is involved, a semicolon is not used between the band designations (see additional example in Table 3.4):

46,XX,r(7)(p22q36)

This describes a ring derived from chromosome 7. Breaks have occurred at bands p22 and q36, and the ends of the segment between the breakpoints have rejoined. The acentric (without a centromere) segments distal to the breakpoints have been lost.

When two chromosomes are involved and a monocentric (one centromere) ring chromosome and an acentric segment results, “der” should be used (see “[Derivative \(der\) and Recombinant \(rec\) Chromosomes](#)” later).

46,XY,der(18;?)(p11.2q22;?)

This indicates a ring derived from the segment between the breakpoints p11.2 and q22 of chromosome 18 and an acentric fragment of unknown origin.

If the origin of a ring chromosome is not known, it is listed after all known aberrations but before other markers:

51,XX,+8,+13,+r1,+r2,+mar

This indicates that two distinctly different clonally occurring rings and a marker chromosome are present.

If multiple rings are present but it is not known if any of the rings are identical, the rings are denoted by a plus sign and the number of rings identified; for example, the presence of three rings is described as +3r.

Inversions (inv)

In an inversion, a chromosomal segment breaks, reorients 180°, and reinserts itself. If an inversion involves the centromere, with one break in each chromosome arm, it is said to be pericentric. A paracentric inversion is isolated to one chromosome arm and does not involve the centromere.

46,XX,inv(16)(p13.1q22)

This is a pericentric inversion of chromosome 16. A break has occurred in the short arm at band 16p13.1 and the long arm at band 16q22. The chromosome segment between these bands is present but inverted. This aberration is commonly observed in acute myelomonocytic leukemia with eosinophilia (see also Chap. 15).

46,XY,inv(3)(q21q26.2)

This is a paracentric inversion involving bands q21 and q26.2 in the long arm of chromosome 3. This rearrangement is also seen in acute myeloid leukemia (see also Chap. 15).

For additional examples, see Table 3.4.

Duplications (dup)

The orientation of duplications is either *direct* or *inverted* and is indicated by the order of the bands with respect to the centromere in the karyotype designation. The band closest to the centromere is written first in the short system; only the detailed system can pinpoint the exact location of the duplicated segment.

46,XY,dup(1)(q21q42)

This is a direct duplication of the segment between bands 1q21 and 1q42 in the long arm of chromosome 1.

46,XX,dup(13)(q34q21)

This is an inverted duplication of the segment between bands 13q21 and 13q34 in the long arm of chromosome 13.

For additional examples, see Table 3.4.

Insertions (ins)

As the name implies, an insertion involves the movement of a segment of intrachromosomal material from one chromosomal location into another. The recipient can be another chromosome or a different part of the chromosome of origin. The orientation of the inserted segment may be direct, retained in its original orientation, or inverted. In inverted insertions, the “normal” orientation of the bands will be reversed with respect to the centromere.

46,XY,ins(2)(q13p11.2p14)

This is an example of an insertion within a chromosome, a direct insertion of the short arm segment between bands 2p11.2 and 2p14 into the long arm at band 2q31.

46,XX,ins(12;?)(q13;?)

This is an example of an insertion between two chromosomes. Material has been inserted into chromosome 12 at band 12q13; however, the origin of the inserted material is not known.

46,XY,ins(19;11)(p13.1;q23q13)

This is an example of an inverted insertion between two chromosomes. The long arm segment between bands 11q13 and 11q23 is present but is inverted and inserted into the short arm of chromosome 19 at band 19p13.1 Note that the 11q13 band of the inserted segment is more distal to the centromere, indicating that the inverted segment is inverted compared to its normal orientation.

For additional examples, see Table 3.4.

Translocations (t)

A translocation is an abnormality resulting from an exchange of genetic material between two chromosomes. Translocations may be balanced or unbalanced (the latter resulting in derivative chromosomes and loss or gain of material). See “Derivative (der) and Recombinant (rec) Chromosomes” later.

46,XY,t(12;14)(q13;q32)

This is a translocation involving two chromosomes. Breaks have occurred at bands 12q13 and 14q32. The segments distal to the two breakpoints are present but exchanged with no apparent loss of genetic material.

46,XX,t(9;22;11)(q34.1;q11.2;q13)

This is a 3-way translocation. Breaks have occurred in three chromosomes at bands 9q34.1, 22q11.2, and 11q13.

The material distal to 9q34.1 is translocated to chromosome 22 at band 22q11.2, the material distal to 22q11.2 is translocated to chromosome 11 at band 11q13, and the material distal to 11q13 has been translocation to chromosome 9 at band 9q34.1. This 3-way translocation appears to be balanced at the cytogenetic level.

For additional examples, see Table 3.4

Derivative (der) and Recombinant (rec) Chromosomes

Derivative chromosomes are structurally abnormal chromosomes that can be generated in three ways: more than one rearrangement within a single chromosome, one rearrangement involving two or more chromosomes, including rearrangements between chromosome homologues, or more than one rearrangement involving two or more chromosomes. All three possible scenarios may result in an unbalanced karyotype. The term “der” refers to a chromosome that has an intact centromere.

Derivative Chromosomes Generated by More than One Rearrangement Within a Single Chromosome

46,XY,der(7)del(7)(p11.2)del(7)(q11.2)

This describes a male karyotype with a chromosome 7 centromere showing deletions in both the short and long arms, namely, deletion of the material distal to bands 7p11.2 and 7q11.2. This karyotype may appear as monosomy 7 with a small centric fragment. Fluorescence *in situ* hybridization (FISH) studies may have identified the centric fragment as a chromosome 7 centromere using a FISH probe (see Chap. 17).

Derivative Chromosome Generated by One Rearrangement Involving Two or More Chromosomes Whole-Arm Translocations

45,XX,der(1;7)(q10;p10)

This describes a female karyotype with an unbalanced whole-arm translocation (see also previous section “Translocations (t)”). The derivative chromosome is comprised of the long arm of chromosome 1 and the short arm of chromosome 7. The derivative chromosome has replaced one normal chromosome 1 and one normal chromosome 7, resulting in a chromosome count of 45. The two missing normal chromosomes are not specified. The resulting net imbalance is monosomy 1p and monosomy 7q.

46,XX,+1,der(1;7)(q10;p10)

This describes a female karyotype with two normal chromosomes 1 and a derivative chromosome comprised of the long arm of chromosome 1 and the short arm of chromosome 7. The derivative chromosome has replaced one normal chromosome 1 and one normal chromosome 7, but +1 in this karyotype changes the net imbalance to trisomy 1q and monosomy 7q.

Other Derivative Chromosomes Generated by One Rearrangement Involving Two or More Chromosomes

46,XX,der(1)t(1;2)(p34.1;q31)

This describes a female karyotype with a derivative chromosome 1. The unbalanced translocation results in loss of 1p34.1→pter and gain of 2q31→qter.

47,XY,t(9;22)(q34.1;q11.2),+der(22)t(9;22)

This describes a male karyotype with the standard (9;22) “Philadelphia chromosome” translocation and duplication of the Philadelphia (Ph) chromosome—the der(22). Note that once the breakpoints are written in the description, there is no need to repeat the breakpoints again for each additional copy of the derivative chromosome. Also, the Ph chromosome abbreviation may be used in the text of the report, but only der(22)t(9;22) should be used in the ISCN description.

Derivative Chromosome Generated by More than One Rearrangement Involving Two or More Chromosomes

The derivative chromosome is specified in parentheses, followed by all aberrations involved in its generation. These aberrations are listed according to the breakpoints of the derivative chromosome from pter to qter and should not be separated by a comma.

Short system: 46,XX,der(1)t(1;2)(p34.1;q31)dup(1)(q21q32)

or detailed system:

46,XX,der(1)(2qter→2q31::1p34.1→1q32::1q21→1qter)

Both the short and detailed systems describe a female karyotype with a derivative chromosome 1 resulting from an unbalanced (1;2) translocation with a 1p24.1 breakpoint and a duplication of the segment between 1q21 and 1q32.

The arrows in the detailed system describe the derivative chromosome 1 from pter to qter.

In some instances, the centromere of the derivative chromosome is not known, but other parts of the chromosome are clearly recognizable. These abnormal chromosomes should be designated as der(?) and placed after all identified aberrations. Other unidentified ring chromosomes, markers, and double minutes (see Chap. 15) are listed behind the der(?), in that order.

52,XY,....+der(?)t(??;5)(?;q15),+r,+mar,6~15dmin

This represents a hyperdiploid clone with multiple aberrations (not listed in this example) including a derivative chromosome resulting from an unbalanced translocation between chromosome 5 and a chromosome of unknown origin; a ring chromosome, the chromosomal origin of which is unknown; one marker, and six to 15 double minutes.

Recombinant Chromosomes

Recombinant chromosomes are structurally rearranged chromosomes with a new segmental composition resulting from meiotic crossing-over (see Chap. 9); thus, this term should never be used to describe acquired (cancer) aberrations. Recombinants usually originate from heterozygotes carrying inversions or insertions, and the term always refers to the chromosome that has an intact centromere. The triplet “rec” should be used when the parental karyotypes are known and a parental inversion is identified (see Table 3.4). If parental karyotypes are unknown in a suspected recombinant, the abnormal chromosome should be designated as a derivative chromosome (der).

Isochromosomes (i)

An isochromosome is an abnormal chromosome with two identical arms due to duplication of one arm and loss of the other arm (mirror image of a chromosome from its centromere).

46,XY,i(6)(p10)

An isochromosome for the short arm of chromosome 6 has replaced one copy of chromosome 6.

46,X,i(X)(q10)

This is a female with one normal X chromosome and one isochromosome for the long arm of the X chromosome. This karyotype is a frequent finding in patients with Turner syndrome (see also Chap. 10).

For additional examples, see Table 3.4.

Dicentric (dic), Isodicentric (idic), and Pseudodicentric (psu dic) Chromosomes

These are structurally altered chromosomes with two centromeres. The term “der” may be used instead of “dic” but the combination of “der dic” is not appropriate. In the karyotype description, both dicentric and isodicentric chromosomes are counted as one chromosome without the need to indicate the missing normal chromosome(s).

45,XY,dic(14;14)(q11.2;q32)

This represents a dicentric chromosome formed by breakage and reunion at bands 14q11.2 and 14q32 on the two homologous chromosomes 14. However, if a dicentric chromosome is proven to originate through breakage and reunion of sister chromatids, it may be designated as dic(14)(q11.2q32). To avoid ambiguity, it is always good practice to describe complex dicentric chromosomes as derivative chromosomes.

47,XY,+idic(14)(q13)

47,XY,+idic(14)(pter→q13::q13→pter)

This karyotype exhibits two normal chromosomes 14 and an isodicentric chromosome 14 consisting of two copies of the short arm, centromere, and proximal long arm. The detailed nomenclature clearly describes the abnormal chromosome.

At times, it is clear that only one centromere of the dicentric is active and the other is inactive (visualized by a decondensed region of the inactive centromere in the majority of mitotic cells analyzed). These structurally altered chromosomes are commonly referred to as pseudodicentric chromosomes (psu dic), and the presumed active centromere should be written first in the karyotype string.

45,XX,psu dic(15;14)(q11.2;q11.2)

This is an abnormal karyotype with a pseudodicentric chromosome comprised of the short arms, centromeres, and proximal long arms of chromosomes 14 and 15 replacing one normal copy each of chromosomes 14 and 15. The nomenclature implies that the centromere of chromosome 15 is active.

Isoderivative Chromosomes (ider)

An isoderivative chromosome designates an isochromosome formation for one of the arms of a derivative chromosome. The breakpoints are assigned to the centromeric bands

(p10 or q10) depending on which arm is present in the isoderivative chromosome.

46,XY,ider(22)(q10)t(9;22)(q34.1;q11.2)

This is an isoderivative chromosome comprised of the long arm of the “Philadelphia chromosome.” It is one of the most common isoderivative chromosomes seen in cancer cytogenetics (see “[Describing Cancer Karyotypes](#)” later).

Robertsonian Translocations (rob)

Robertsonian translocations are a special type of translocation in humans involving the acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22). Typically, the participating chromosomes break in their short arms and give the appearance that the long arms fuse to form a single chromosome with a single centromere. If the location of the breakpoints is unproven, “rob” may be used. Because the short arms of acrocentric chromosomes contain repetitive ribosomal gene clusters, loss of these arms due to this type of translocation has no phenotypic consequences. A karyotype with a single Robertsonian translocation by definition will have a 45 chromosome count. If the “rob” is proven to be a dicentric chromosome with breakpoints of p11.2 or q11.2, the abbreviation “dic” should be used. Either “rob” or “der” adequately describes these whole-arm translocations in a constitutional karyotype; however, for acquired Robertsonian translocations in cancer, “rob” should not be used.

45,XY,der(14;21)(q10;q10)

- or -

45,XY,rob(14;21)(q10;q10)

This describes a male karyotype with one normal chromosome 14, one normal chromosome 21, and a der(14;21) or whole-arm translocation involving the long arms of chromosomes 14 and 21. The resulting net imbalance is loss of the short arms of chromosomes 14 and 21, which is of no clinical consequence.

46,XY,der(14;21)(q10;q10),+21

- or -

46,XY,rob(14;21)(q10;q10),+21

These are similar to the previous example but with gain of chromosome 21. This male karyotype has one normal chromosome 14, two normal chromosomes 21, and the der(14;21). The resulting net imbalance is inconsequential loss of the short arms of chromosomes 14 and 21 and trisomy for the

long arm of chromosome 21, resulting in Down syndrome in a live-born individual.

46,XY,+21,der(21;21)(q10;q10)

- or -

46,XY,+21,rob(21;21)(q10;q10)

These denote a male karyotype with a derivative chromosome comprised of both copies of the long arms of chromosome 21. One additional normal chromosome 21 is also present, denoted with a plus sign. The net imbalance is referred to as translocation trisomy 21.

45,XY,dic(14;21)(p11.2;p11.2)

- or -

45,XY,dic(14;21)(14qter→14p11.2::21p11.2→21qter)

These describe a translocation proven to be a dicentric, with breakage and reunion at 14p11.2 and 21p11.2. In this case, the triplet “dic” should be used in both the short or detailed version, and the dicentric chromosome is counted as single chromosome. The resulting net clinically inconsequential “imbalance” of this karyotype is loss of the segments distal to 14p11.2 and 21p11.2.

Balanced Rearrangements Involving Three or More Chromosomes

As with any science, the general principles create a strong foundation, but karyotype complexity sometimes requires modifications or exceptions to the guidelines. As noted for balanced translocation nomenclature involving two chromosomes, a sex chromosome or the autosome with the lowest number is always specified first, with subsequent abnormalities listed in numerical order regardless of type. However, when three or more chromosomes are involved in a balanced rearrangement, the next chromosome listed is the one that received a segment from the first chromosome, and the chromosome specified last is the one that donates material to the first chromosome listed.

46,Y,t(X;22;2)(q22.1;q22.2;p21)

This represents an XY karyotype with the segment of the X chromosome distal to Xq22.1 translocated to chromosome 22 at band 22q22.2, the segment distal to chromosome 22q22.2 translocated to chromosome 2 at band 2p21, and the segment distal to chromosome 2p21 translocated to the X chromosome at band Xq22.1. Note that the segment from the last chromosome listed in the first set of parentheses has been translocated to the first chromosome listed.

Neocentromeres (neo)

Neocentromeres are fully functional centromeres that are present in non-centromeric regions. They lack α -satellite DNA. A derivative chromosome with a neocentromere may be described using either the triplet “neo” or “der,” both of which are equally acceptable:

47,XY,+der(18)(qter→q21.1:)

- or -

47,XY,+neo(18)(qter→q21.1:)

For example, a derivative chromosome containing a neocentromere within the segment 18q21 through 18qter, which normally lacks a centromere, is written with the short system. However, to adequately describe the location of a neocentromere, the detailed system is required:

47,XY,+der(18)(qter→q21.1→neo→q21.1:)

Additional Material of Unknown Origin (add)

The triplet “add” is used to describe material of unknown origin attached to a chromosome region or band. The material may have come from the same chromosome or another chromosome, and no known mechanism is implied (see additional example in Table 3.4). Despite the implication that such material is always additional, it often actually replaces part of a chromosome arm.

46,XX,add(5)(q13)

Material of indeterminate origin is present on chromosome 5 at band q13, replacing the material distal to this band. Note that the nomenclature does not describe the size of this piece of chromosomal material.

Marker Chromosomes (mar)

Marker chromosomes are structurally abnormal chromosomes in which no part can be unambiguously identified by conventional banding techniques. A plus sign is always used when describing the presence of a marker in the karyotype, and marker chromosomes are usually listed last in the nomenclature string. If multiple markers can be distinguished as distinct from one another, they should be written as +mar1, +mar2; otherwise, +2mar should be used. If any part of

the abnormal chromosomal marker can be recognized, even if the origin of the centromere is unknown, the correct designation of the abnormal chromosome is “der” and not “mar,”—e.g., +der(?)t(?)7(?)q11.2.

49,XY,+8,+2mar

An additional copy of chromosome 8 is present, as are two marker chromosomes.

Multiple Copies of Rearranged Chromosomes

When multiple copies of a rearranged chromosome are present, the *multiplication sign* (\times) is placed directly after the aberration. Note that a multiplication sign should never be used to describe multiple copies (gains) of a normal chromosome.

46,XX,del(5)(q13q31) \times 2

This indicates that two copies of a deletion involving 5q were found.

52,XY.....+mar1 \times 3

This indicates that three copies of marker 1 was a clonal finding.

Chromosome Breakage

Cultured cells from patients with chromosomal breakage syndromes, such as ataxia telangiectasia, Bloom syndrome, Fanconi anemia, and xeroderma pigmentosum may exhibit spontaneous or elevated levels of chromosomal breakage following clastogenic exposure. Examples of abbreviations used here are chrg (chromosome gap), chrb (chromosome break), and chte (chromatid exchange).

Fragile Sites (fra)

Chromosomal fragile sites are inherited in a codominant Mendelian fashion and are commonly considered to be normal variants with no phenotypic consequences. However, they may result in chromosome abnormalities such as deletions, multi-radial figures, or acentric fragments. Fragile sites have been known to be associated with a specific disease or phenotype, such as the fragile X syndrome (see Chap. 19). Regardless of their biological consequences, fragile sites are denoted by the triplet “fra,” for example, fra(X)(q27.3).

Incomplete Karyotypes (inc)

Every attempt should be made to describe all aberrations in an abnormal cell or clone. However, when this is not possible (such as when chromosome morphology is poor), the triplet “inc” is placed at the end of the nomenclature string, after the description of the identifiable abnormalities:

46,XY,del(7)(q22),inc[10]

Ten cells were examined; for cancer karyotypes, this is indicated using square brackets (see “[Describing Cancer Karyotypes](#)” later). The number of chromosomes is determined, as is the presence of a deletion involving the long arm of chromosome 7. The triplet “inc” indicates that other abnormalities are also present but cannot be described.

Parental Inheritance

When parental inheritance is known, the triplet “mat” for maternally inherited or “pat” for paternally inherited should be used, immediately following the designation of the abnormality. If multiple different aberrations are inherited from the parents, the parental origin should be designated for each individual aberration even if both aberrations came from the same parent. If the parental chromosomes are normal with respect to the abnormality, the abnormality may be designed as “dn” for *de novo*.

46,XX,t(8;9)(q13;p13)mat,inv(13)(q14q32)mat

Both aberrations were inherited from the mother.

46,XX,t(8;9)(q13;p13)mat,inv(13)(q14q32)dn

The translocation was inherited from the mother and the inversion arose *de novo*.

Additional Symbols

Additional punctuation signs and symbols within the nomenclature string clarify gains, losses, and variable heteromorphisms (chromosome morphology) or uncertain chromosome features.

Plus (+) and Minus (-) Signs

Plus and minus signs are used in conjunction with other symbols such as “h” and “s,” to distinguish normal variation in length from additions or deletions of other origins; however, they should not be used after the chromosome arm in the nomenclature string. For example, 16qh+ describes an

increase in length of the heterochromatin on the long arm of chromosome 16, but the use of 16q+ in an ISCN nomenclature string is inappropriate. This should be written as add(16)(q?), indicating that material of unknown origin has been added to the long arm of chromosome 16, but the exact location of the added material is unknown. Wyandt and Tonk have compiled an excellent resource for the range of variation representing human chromosomal heteromorphisms [3], and a comprehensive guide for describing these variations may be found in ISCN [2].

Questionable Karyotypic Results

Uncertainty within a chromosome is denoted by using a question mark (?), the approximate sign (~), or the term “or” in the nomenclature string.

A question mark (?) can be for uncertainty in a chromosome band or a chromosome structure; the symbol must be placed immediately in front of the uncertain band or structure that is questioned. For example, if a translocation is suspected in a karyotype but additional testing is required for confirmation, the question mark should go in front of the “t” and not behind it, for example, 46,XX,?t(15;17)(q22;q12). If additional testing proves that the translocation is present, the report should be revised (i.e., issue an addendum stating that new information allowed for ISCN refinement) to include the new confirmed data with revision of the nomenclature string. If the banding quality is particularly poor, a question mark may replace a chromosome, region, or band designation; for example, 46,XY,del(1)(q?) indicates a long arm deletion of chromosome 1 in which neither the region nor band can be identified.

The approximate sign (~) is most useful when describing uncertain breakpoints or boundaries of a chromosome segment such as t(2;5)(p21~23;q33~35), but it may also be used to describe a range of chromosomes when the specific chromosome modal number is not known (38~48,XX...) or when the number of markers within a karyotype appears to be variable as in 48~53,XY,+2~7mar[12].

The word “or” with a space on either side is used in human chromosome nomenclature to indicate an alternative interpretation of an aberration, based on the banding level. For example, 46,XY,add(19)(p13.1 or q13.1) denotes a karyotype in which additional material of unknown origin is attached to chromosome 19, but the banding is too poor to show with certainty whether the material is attached to the short or long arm.

The term “or” is also used to describe two possible breakpoint interpretations of a translocation. In the example of t(7;10)(q22;q24) or t(7;10)(q32;q22), the two alternative interpretations give rise to identical-looking derivative chromosomes. If the breakpoints could be either one band or another, use “or,” but if the breakpoints are less certain and a

variety of combinations within the uncertain region is possible, use “~.”

Uniparental Disomy (UPD)

Uniparental disomy (UPD) is a condition in which both homologous chromosomes are derived from the one parent. In most cases, UPD is detected by polymorphic markers such as microsatellite polymorphisms, but in some instances, UPD may be identified cytogenetically or through single nucleotide polymorphism (SNP) microarray analysis (see Chap. 20).

46,XX,upd(15)pat

This is a female karyotype indicating UPD for a paternally derived chromosome 15.

Mosaicism and Chimerism

Mosaicism is the presence of two or more populations of cells with different genotypes originating from the same zygote. To differentiate mosaicism from a chimerism, which has two or more different populations of genetically distinct cells or cell lines originating from different zygotes, the triplet “mos” or “chi” may be used, for example, mos 45,X/47,XXX/46,XX versus chi 46,XX/46,XY. When writing these karyotype descriptions, the triplet is only needed for the initial karyotype description within the report, and a space must be used after the triplet abbreviation and before the chromosome number. A normal diploid cell line, when present, is always listed last.

Constitutional chimerism in humans is rare, but chimerism secondary to bone marrow stem cell transplantation (SCT) is a common finding in patients who have had such a transplant. In these cases, the recipient or host cell lines (clones) are listed first, followed by the donor cell line(s). A double slant line (//) is used to clearly separate the chimeric recipient//donor cell populations. Table 3.3 provides examples on how to write and report karyotypes with mosaicism/chimerism with respect to clone size and type of aberration.

46,XX,t(9;22)(q34;q11.2)[15]//46,XY[5]

Fifteen of the twenty metaphase cells examined exhibit a (9;22) translocation and represent the female recipient. The remaining five cells represent her male bone marrow donor.

//46,XX[20]

All 20 metaphase cells examined from this male patient are of (female) donor origin.

Describing Cancer Karyotypes

Acquired abnormalities associated with neoplastic disorders imply the presence of both normal (constitutional) and abnormal (neoplastic) cells in the same patient and frequently in a single specimen. Terms used exclusively in neoplasia nomenclature include clone, mainline, stemline, sideline, composite karyotype, and unrelated clones.

Clone

A clone is a cell population derived from a single progenitor cell. As in the constitutional setting where cell line is the preferred term, a clone constitutes at least two mitotic cells with the same chromosome gain or structural aberration, or at least three cells with the same chromosome loss. The requirement of three cells for the identification of clonal chromosome loss is due to the fact that chromosome loss may occur during the slide-making process.

The *modal number* (mn) is the most common chromosome number in a tumor cell population and may be expressed as a range.

Stemline, Sideline, and Mainline

The *stemline* (sl) is the most basic clone of a tumor cell population and is always written first in the nomenclature string.

Based on the well-founded assumption that clones with more abnormalities tend to have evolved from those with fewer abnormalities, clone order in oncology samples reflects order of increasing complexity to describe the presence of clonal evolution of disease or genetic instability. Square brackets are used to enumerate clone size.

Additional related clonal aberrations (subclones) are referred to as *sidelines* (sdl). Sidelines follow the stemline in the nomenclature string in order of increasing complexity (note the difference from constitutional cell line order). If multiple sidelines are present in a tumor karyotype, they may be numbered sdl1, sdl2, sdl3, etc., and used within the nomenclature string to refer back to that part of the stemline or previous sideline that is also present in the new subclone. The term “*idem*” (Latin for “the same”) may also be used to represent a subclone. However, when used in a nomenclature string, “*idem*” refers back only to the stemline clonal aberrations. The purpose for these terms is to reduce redundancy when multiple clones are present in a tumor karyotype.

The *mainline* (ml) is the largest clone in a tumor, but this abbreviation is rarely used in the nomenclature string because it is implied by the number of cells in brackets. The mainline may or may not be the stemline.

Examples of using cancer nomenclature follow:

46,XY,t(8;14)(q24.1;q32)[20]/
47,XY,t(8;14)(q24.1;q32),+der(14)t(8;14)[6]

46,XY,t(8;14)(q24.1;q32)[20]/
47,idem,der(14)t(8;14)[6]

46,XY,t(8;14)(q24.1;q32)[20]/
47,sl,der(14)t(8;14)[6]

These three nomenclature strings describe the same karyotype: a clone (stemline) with 46 chromosomes and an (8;14) translocation in 20 cells, with a subclone of six cells with 47 chromosomes showing the t(8;14) and gain of a derivative chromosome 14 resulting from the t(8;14). The subclone denotes clonal evolution of the stemline. The terms “*idem*” and “*sl*” are alternative ways to describe the same result—in this case the t(8;14) in the subclone. The terms “*sl*” and “*idem*” should never be intermixed when describing a single tumor sample. Note that the stemline is the mainline in this example.

46,XY,t(8;14)(q24.1;q32)[12]/45,sl,-X[18]/46,sdl1,+8[5]/
47,sdl2,+der(14)t(8;14)[6]

46,XY,t(8;14)(q24.1;q32)[12]/45,idem,-X[18]/
46,idem,-X,+8[5]/47,idem,-X,+8,der(14)t(8;14)[6]

These two examples show a male karyotype with t(8;14) as the sole abnormality in the stemline. Three related subclones or sidelines are present. Note that the sidelines are listed in order of increasing complexity. In this example, the mainline (18 cells) is sideline 1 with 45 chromosomes and two clonal aberrations: t(8;14) and loss of one chromosome X. Sideline 2 (5 cells) shows t(8;14), -X, and gain of chromosome 8. Sideline 3 (6 cells) shows t(8;14), -X, +8, and gain of the derivative chromosome 14. Note that the use of “*sdl*” in the karyotype string indicates that all clonal aberrations found in that subclone are present in the new subclone in addition to the new aberrations. The term “*idem*” refers back to the stemline only. If using “*idem*,” the additional clonal aberrations found in each subclone relative to the stemline must be restated.

46,XY,t(8;14)(q24.1;q32)[14]/92,sl×2[4]/93,sdl1,+8[6]

46,XY,t(8;14)(q24.1;q32)[14]/92,idem×2[4]/93,idem×2,+8[6]

In these two examples, the clone with t(8;14) is the stemline. Two additional subclones were detected: one subclone

(sideline 1) is a doubling product ($\times 2$) of the stemline. The second subclone (sideline 2) is a near-tetraploid subclone of sd1 with gain of chromosome 8 (sideline 2). Note that complexity, not size, determines clone order.

Composite Karyotype (cp)

A composite karyotype may be created in samples showing vast tumor heterogeneity, a common finding in solid tumors, acute leukemias, and aggressive lymphomas. A composite karyotype lists all clonally occurring abnormalities. In such cases, the nomenclature string usually begins with a chromosome range, the sex chromosome component, and clonal aberrations per ISCN standard guidelines, and is closed with the total number of cells in which the clonal aberrations were observed in square brackets:

45~50,XX,+X,add(1)(p34.1),+8,i(9)(q10),add(13)(q32),
t(14;18)(q32;q21.3),+2~10mar[cp24]

This female composite karyotype has from 45–50 chromosomes in 24 cells denoted by “cp” in the square brackets and the chromosome number written as a range. A composite karyotype contains all clonally occurring abnormalities observed in a tumor. Each of the abnormalities listed in this example has been seen in at least two cells, but there may be no cells with all abnormalities. The chromosome range present in cancers, particularly solid tumors, may reflect incompletely analyzed or over/under-spread metaphase cells, or too few analyzable mitotic cells. When reporting neoplastic cases, every effort should be made to describe subclones so that clonal evolution is made evident. Nevertheless, it is often practical, if not necessary, to describe observed abnormalities as a single composite clone.

Unrelated Clones

Unrelated clones are clones found within a single tumor that fail to show “relatedness” by conventional cytogenetics. This term does not rule out the possibility of a common molecular mutation. If a normal diploid clone is also present, it is listed last.

47,XX,+8[7]/46,XX,t(9;22)(q34.1;q11.2)[4]/46,XX[9]

This is an example of a female tumor karyotype with unrelated clones. Gain of chromosome 8 was observed in seven cells. The (9;22) translocation was observed as an unrelated clone in four cells. Nine cells showed a normal female karyotype. Note that the number of cells that constitute each clone is given in square brackets, and the normal diploid clone is listed last.

When describing cancer karyotypes, several ISCN recommendations are critical.

Acquired sex chromosome numerical abnormalities

Acquired sex chromosome numerical abnormalities are expressed with plus and minus signs. A tumor karyotype with loss of one X chromosome is written as 45,X,-X. Similarly, a tumor with loss of the Y chromosome is written as 45,X,-Y. Gains of sex chromosomes in cancer karyotypes are written with plus sign:

48,XY,+X,+Y

This denotes a male tumor karyotype with one additional X and one additional Y chromosome.

Presence of a constitutional chromosome aberration

If a known constitutional chromosome aberration is present in a cancer karyotype, the letter “c” should be used in the karyotype.

46,XXYc,-X

This is a male with Klinefelter syndrome (47,XXY, see Chap. 10) who has an acquired loss of one X chromosome in his tumor karyotype.

48,XXYc,+X

This indicates gain of one X chromosome in the previous patient.

49,XY,+8,+21c,+21[20]

This indicates a tumor from a patient with constitutional trisomy 21 (Down syndrome) and acquired gain of chromosomes 8 and 21.

Clone size

The number of cells that constitutes a clone is given in square brackets [] after the description of that clone. Moreover, to provide an estimate of tumor load or effectiveness of treatment, the number of cells that constitute a clone must be given in square brackets after the karyotype, even if the karyotype appears to be a normal. Normal karyotypes are always written last. Cancer karyotyping standards require a total of 20 mitotic cells that are analyzed band for band (see Chap. 4).

46,XX[20]

This indicates the presence of a normal karyotype, and the cell count in brackets indicates that the study was performed to rule out an acquired aberration.

46,XY,t(8;14)(q24.1;q32)[20]

This describes a male karyotype with t(8;14) detected in all twenty cells examined.

Non-clonal aberrations

Non-clonal or single cell aberrations should not be reported in the nomenclature string, with two exceptions:

If a single abnormal metaphase cell showed an abnormality found to be clonal by a second genetic testing method (e.g., FISH), the karyotype should be reported:

46,XY,del(13)(q12q12)[1]/46,XY[19].nuc ish
(D13S319 \times 1,LAMP1 \times 2)[25/200]

A single cell with an interstitial deletion of chromosome 13 was observed but is reported since 25 cells with such a deletion were also detected with FISH using a D13S319 probe, which maps to 13q13.3 (see “[Molecular Cytogenetic Techniques](#)” later).

If at follow-up (post treatment), a patient shows the same clonal aberration in a single cell detected at disease onset, the cell should be reported in the karyotype:

46,XY,t(9;22)(q34.1;q11.2)[1]/46,XY[19]

A single cell with a (9;22) translocation was observed in a patient in whom this rearrangement was observed previously.

Gene amplification

Homogeneously staining regions (hsr) and *double minutes* (dmin) are two different cytological forms of amplified gene sequences. The term “hsr” refers to uniformly staining regions within a chromosome arm, even though the staining is not always homogeneous. These hsrs can range in size, and the nomenclature does not indicate how large an hsr is.

46,XX,hsr(8)(q24.1)

A homogeneously staining region is present in band q24.1 of chromosome 8.

Double minutes are extrachromosomal amplified gene sequences that appear as two small cojoined chromosomal fragments in the nuclear matrix. A single version of a double minute is known as a minute or min. When writing the nomenclature string, double minutes are not included in the chromosome count because they do not possess centromeres,

and a plus (+) sign should never be placed in front of the symbol “dmin.” It is also common to report a range of double minutes since they do not segregate equally with each division:

46,XY,t(8;14)(q24.1;q32),10~28dmin

In addition to an (8;14) translocation, cells with as few as ten and as many as 28 double minutes were observed.

Molecular Cytogenetic Techniques

Molecular cytogenetic assays such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and array-based comparative genomic hybridization (aCGH) are valuable adjuncts to conventional chromosomal banding techniques (see Chaps. 17 and 21). These hybridization-based assays use labeled complementary DNA probes to localize a specific DNA sequence (or demonstrate the lack thereof) in a patient or tissue sample. Table 3.5 has a partial list of abbreviations and symbols pertaining to molecular cytogenetic techniques. For a complete list, please refer to ISCN [2].

Fluorescence *In Situ* Hybridization (FISH)

The fluorescence *in situ* hybridization assay is a targeted approach to determine whether a specific DNA sequence, as visualized with a probe, is present within chromosomes on microscope slides. The DNA, which may be in the nucleus (nuc ish) in nondividing or interphase cells or in metaphase chromosomes (ish), is fixed on a slide and denatured in place (*in situ*) to expose the two strands of DNA and allow a denatured-labeled probe to hybridize to the chromosomal DNA. The location of the hybridization signal is visualized by fluorescence microscopy and pinpoints the location of the DNA segment to which the probe is hybridized (see Chap. 17).

Metaphase FISH

To describe abnormal metaphase FISH results, the following sequence is used: the triplet “ish” followed by a space, the triplet denoting the abnormality, then, in separate parentheses, the chromosome, the band designation for the breakpoint(s), and the locus or loci from which clones were used. Whenever possible, the clone name should be used. If the clone name is not available, the locus designation or D-number should be used [4]. If the locus designation is not available, then the gene name, using HUGO nomenclature [5], may be substituted. Locus designations are immediately followed by “ \times 2” to indicate a normal

Table 3.5 Common symbols and abbreviations used in molecular cytogenetics

Symbol/Abbreviation	Interpretation
minus sign (-)	Loss of a probe signal; single minus sign represents one copy loss, double minus sign indicates two copy or homozygous deletion
plus sign (+)	Signal is present on a specific chromosome; two plus signs indicate gain of signal
multiplication sign (\times)	Precedes the number of signals observed
period (.)	Separates cytogenetic observations from results of <i>in situ</i> hybridization
semicolon (;)	Separate probes on different derivative chromosomes
aCGH	Array-based comparative genomic hybridization
arr	Microarray or results from microarray testing
amp	Amplified gene sequences
cgh	Comparative genomic hybridization
con	Signals are connected
htz	Heterozygous
hmz	Homozygous
ish	Refers to <i>in situ</i> hybridization result. ish usually refers to metaphase FISH
nuc ish	Refers to an interphase <i>in situ</i> hybridization result. nuc indicates nuclear or non-mitotic cells
sep	Signals that are separated. Used to denote separated signals using break-apart probe set (implies translocation)
subtel	Subtelomeric

hybridization pattern to an autosome or pseudoautosomal region and “ $\times 1$ ” for normal hybridization to X or Y probes in a male.

A minus sign is used to indicate that one copy (-) or both copies (--) of a probe are absent. When multiple probes are used, those that are present are indicated by a plus sign. Additional plus signs indicate duplication of a locus. Locus designations should be separated by commas.

Normal *in situ* hybridization results are designated with the symbol “ish” followed by the chromosome, region, band, or sub-band designation of the locus or loci tested, followed in parentheses by the locus or loci tested, multiplication or plus/minus signs, and the number of signals detected. When conventional cytogenetics is not performed, the nomenclature string begins with “ish”:

ish 22q11.2(HIRA $\times 2$)

This indicates a normal hybridization result for the DiGeorge syndrome locus HIRA.

ish.del(7)(q11.23q11.23)(ELN-)

This indicates a microdeletion associated with Williams syndrome identified by ish with a probe for the elastin gene.

ish.del(2)(q13q13)(NPHP1--)

This indicates a homozygous deletion for NPHP1, the gene associated with nephronophthisis.

If conventional cytogenetics was also performed, the karyotype results are written prior to the *in situ* hybridization

findings, with a period separating the two results. The triplet “ish” indicates the beginning of the FISH results:

46,XY.ish del(22)(q11.2q11.2)(HIRA-)

This describes a male with a normal karyotype by cytogenetics with a microdeletion within the DiGeorge syndrome critical region on chromosome 22 identified by metaphase FISH using a probe for HIRA.

46,XY.ish del(22)(q11.2q11.2)(HIRA-,N25-,D22Z1+)

This describes a male with a normal karyotype who has a deletion of the DiGeorge syndrome critical region identified via ish using probes for the HIRA, N25, and D22Z1 loci. HIRA and N25 are deleted, whereas D22Z1 is retained.

Subtelomere FISH (subtel)

Use of FISH probes for the 41 unique human subtelomeric regions is a diagnostic tool used in many cytogenetics laboratories, although this is becoming less common due to the increasing utilization of microarrays (see later and see also Chap. 18).

When describing normal results for subtelomeric FISH, the short form is preferred:

ish subtel(41 $\times 2$)

This indicates a normal subtelomere FISH result using probes to the 41 unique subtelomeric regions.

Abnormal results following subtelomeric FISH are written the same way as standard *in situ* hybridization

(see previous); however, instead of using distal bands and probe names, the presence or absence of a signal may be designated by a plus or minus sign, following the symbol for the appropriate short or long arm (pter or qter), respectively:

ish del(8)(qter-)

This indicates a terminal deletion of 8qter identified by subtelomere FISH.

ish der(8)t(8;10)(qter-;pter+)

This indicates an unbalanced translocation between the distal long arm of chromosome 8 and the distal short arm of chromosome 10, resulting in loss of the 8q subtelomeric region and gain of the 10p subtelomeric region. Alternately, distal band designations and probe names may be used:

ish der(8)t(8;10)(q24.3-,p15.3+)
(RP11-6515-,RP11-581022+)

Interphase FISH (Nuclear *In Situ* Hybridization, nuc ish)

When writing a karyotype description using results obtained from non-mitotic or interphase cells (nuc ish), the number of signals and their relative positions are indicated. Because chromosomal bands cannot be visualized in the nucleus of interphase cells, the band location is not necessary in the nomenclature string.

When single probes are used, the probe name, followed by a multiplication sign and the number of signals seen, is given within parentheses. If multiple probes are used in the same hybridization experiment, they are placed within parentheses and separated by a comma. The number of signals seen is placed outside the parentheses if equal for both probes; otherwise, the number of signals follows each probe within the parentheses. The description of multiple probes follows the same general principles as basic karyotype description; multiple probes on the same chromosome are listed pter to qter, and multiple probes on different chromosomes are listed in the same order as with cytogenetic nomenclature—probes for the sex chromosomes are listed first, followed by probes for autosomes in ascending numerical order:

nuc ish(DSCR×2)

Two copies of DSCR were detected.

nuc ish(RB1,D18Z1,DSCR)×2

Two copies each of RB1, D18Z1, and DSCR were detected.

nuc ish(RB1×3),(D18Z1,DSCR)×2

Three copies of RB1 and two copies each of D18Z1 and DSCR were detected.

Many cytogenetics laboratories use one or multiple probe FISH strategies for acquired chromosomal abnormalities (single fusion, single-fusion with extra signals, dual-fusion, and break-apart) to interrogate a specific or targeted regions of interest for a disease subtype (see Chap. 17). Each type of probe can be reported with ISCN.

Dual-Fusion Probes

nuc ish(ABL1,BCR)×2[200]

This is a negative result using the dual-fusion BCR-ABL1 fusion probe set. The result is from single hybridization, and each probe produced two signals in 200 interphase cells.

nuc ish(ABL1,BCR)×3(ABL1 con BCR×2)[100/200]

This is an abnormal (positive) result using the dual-fusion BCR-ABL1 fusion probe set. The result is from a single hybridization. One hundred cells show three signals for each probe because one probe for each locus is split to form two BCR-ABL1 fusion signals (connected or “con”) on both the der(9) and der(22), as indicated in the second set of parentheses (ABL1 con BCR×2). There are also two signals localized to the normal chromosomes 9 and 22, for a total of three signals for each probe. The abnormal or BCR-ABL1 positive cells were found in 100 of the 200 interphase cells scored. Normal results do not need to be reported because it is understood that the remaining 100 cells scored in this study produced a normal FISH signal pattern.

Break-Apart FISH Probes

Short system : nuc ish(MLL×2)[200]

Detailed system :

nuc ish(5'MLL,3'MLL)×2(5'MLL con 3'MLL×2)[200]

Break-apart probes are made up of two probes that are in close proximity to one another. The two examples above depict negative results for MLL. The detailed form indicates that the two probes associated with the 5' and the 3' part of the MLL gene, respectively, are connected (con) and therefore represent a normal signal pattern. The short form does not convey the normal location of probes, that is, 5' versus 3' to the breakpoint site, but a negative result is clearly evident.

nuc ish(MLL×2)(5'MLL sep 3'MLL×1)[150/200]

This indicates the 5' and 3' MLL signals are separated (sep), presumably as the result of a translocation in 150 of the 200 interphase cells scored. If conventional cytogenetics studies were not performed previously or are negative, metaphase FISH studies would be needed to identify the translocation partner.

Oncology FISH Probe Panels

The following are examples of probe panels for chronic lymphocytic leukemia (CLL) where the ATM and TP53 probes are hybridized in one experiment and probes for chromosomes 12 and 13 are hybridized in a second experiment:

nuc ish(ATM×1,TP53×2)[75/200],
(D12Z3×2,D13S319×2,LAMP×2)[75/200]

This abnormal result indicates that an ATM deletion and gain of chromosome 12 were detected by FISH in 75 of the 200 interphase cells scored. Note that the probes co-hybridized together are reported together.

nuc ish(ATM,TP53×2)[200],
(D12Z3×2,D13S319×2,LAMP×2)[120/200]

This FISH study is positive for a biallelic 13q deletion. In this case, the ATM and TP53 probes are hybridized together, and they are normal, so the ×2 can be placed outside the parentheses. Compare this example to the previous one.

nuc ish(MYB,ATM,D12Z3,D13S319,LAMP1,TP53)×2[200]

This describes a normal hybridization pattern in 200 interphase cells for all probes scored. When the results are normal and the number of cells counted are identical for each hybridization, the ×2 (normal diploid result) may be written outside the parentheses. However, since not all probes are hybridized together and more than 200 cells must be examined in total, this can also be described as

nuc ish(MYB×2)[200],(ATM,TP53)×2[200],
(D12Z3,D13S319,LAMP)×2[200]

Gene Amplification Detected by FISH

nuc ish(D17Z1×2,ERBB2×15~20)[52/60]

This indicates that 15–20 copies of ERBB2 (HER2) were detected in 52 of 60 interphase cell scored. Only two copies of the chromosome 17 centromere probe were observed in the same cells. This result is positive for an ERBB2 gene amplification.

nuc ish(D17Z1,ERBB2)×2[60]

This indicates a normal (diploid) result indicating no evidence of ERBB2 amplification in 60 cells.

nuc ish amp(MYC)[100]

This indicates that 100 cells showed amplification of MYC, but the number of amplified copies cannot reliably be determined.

Chimerism Studies in Sex-Mismatched Bone Marrow Stem Cell Transplants

Interphase FISH may be used to quantify the number of donor and recipient cells after bone marrow stem cell transplantation. As used in conventional cytogenetics, the double slash (//) denotes a chimeric state.

nuc ish(DXZ1×2)[150]//(DXZ1,DYZ3)×1[200]

This FISH result describes 150 recipient (host) female cells and 200 male donor cells seen using probes specific for the X and Y chromosomes. Note that the recipient (host) cell line is listed before the double slant line, followed by the donor cell line.

Comparative Genomic Hybridization (cgH)

Comparative genomic hybridization (CGH) is a technique in which genomic DNA from test and reference (control) samples are differentially labeled and competitively hybridized to metaphase chromosomes. CGH detects relative DNA copy-number gains or losses. Alterations detected by CGH may be written as follows:

ish cgh del(12)(q24.3qter)

A deletion of the long arm of chromosome 12 is identified with CGH.

If FISH is used to confirm the CGH results, the nomenclature may be rewritten to incorporate the new findings if they clarify the CGH results.

Microarray Analysis (arr)

Array-based comparative genomic hybridization (aCGH) has become an important technique for interrogating the entire genome for unbalanced chromosomal abnormalities. Thousands of probes from throughout the genome are fixed on a solid support and interrogated in a single assay. The clones used as targets include cosmids, fosmids, synthetic oligonucleotides, or bacterial artificial chromosomes (BACs). Unlike aCGH-based microarrays, which perform a direct comparison between a control sample and a test sample,

single nucleotide polymorphism (SNP)-based arrays determine relative copy number for a region quantitatively within a single genome in comparison to known SNP reference controls. See Chap. 18.

To accommodate varying platforms, microarray nomenclature specifies nucleotide positions rather than probe types. Additional information, such as clone name or accession number, gene name, GDB D-number, and type of cloned DNA, can be included in the final report.

arr(1–22,X)×2
arr(1–22)×2,(XY)×1

These represent normal female and male microarray analysis results. The autosomes are listed first, followed by the sex chromosomes.

If the results are normal using only probes targeted to a specific chromosome or region, the results are written as follows:

arr(7)×2

Microarray analysis using a microarray comprised of clones specific to chromosome 7 shows a normal (diploid) DNA copy number.

For abnormal results, only the relevant regions affected are described. For multiple abnormalities, sex chromosome aberrations are listed first, followed by the autosomes in ascending chromosome number. Only the band designations of the aberrant clones are listed. Unlike chromosome nomenclature, which is written from the centromere out to the telomeres, aberrant nucleotides should be listed from pter to qter for each chromosome to be consistent with the formatting of the public genome databases. Multiple nucleotide positions should be listed separated by a comma, or a dash may be placed between two nucleotide positions to indicate an aberration of the intervening sequence. The parental origin of the abnormality may follow the copy number (×1 mat, ×3 pat, etc.). There is a space between the copy number and the inheritance abbreviation (dn, mat, pat), but a space is not used if the inheritance abbreviation follows a parenthesis in the detailed system:

arr 4q32.2q35.1(163,146,681–183,022,312)×1 dn
arr 4q32.2q35.1(163,002,425×2,163,146,681–183,022,312×1, 184,332,231×2)dn
arr 17p11.2(16,512,256–20,405,113)×3 dn

The specific genome build (human genome assembly [6]) may now be added to the array nomenclature if desired:

arr[hg18]6q25.3(157,052,244–157,341,934)×1

Oncology Microarrays

Similar to constitutional arrays, only the abnormal aberrations are listed in the ISCN. Two examples follow.

Microarray analysis of a chronic lymphocytic leukemia sample shows two microdeletions, a 216 kb deletion within band 11q22.3 and a ~1.53 Mb deletion within the 13q14.2q14.3 region:

arr 11q22.3(108,526,054–108,726,070)×1,
13q14.2q14.3(50,331,912–51,863,519)×1

Microarray analysis of a patient with a myelodysplastic syndrome showing an ~86.2 Mb deletion of the long arm of chromosome 5, a 42.8 Mb copy-neutral loss of heterozygosity (CN-LOH) 7q segment and loss of chromosome 17:

arr 5q12.3q32(63,329,099–149,510,083)×1,
7q31.2q36.3(116,250,176–159,119,707)×2 hmqz,
17p13.3q25.3(0–81,195,210)×1

ISCN is a dynamic document that is periodically updated. The ISCN Committee met and released a 2013 update [7]. New terminology was added to accommodate the description of complex oncology microarrays and evolving targeted technologies. Four revisions are worth mentioning here:

1. For arrays, the genome build should now be specified in the string:
arr[hg19]4q32.2q35.19163,146,681–183,022,321)×1
2. The triplet “cth” for chromothripsis was added to describe multiple alternating copy number changes (normal, gain, and/or loss) along the length of a chromosome or chromosomal segment in neoplastic samples. For example, the following chromosome 13 aberrations can be described using the detailed form:

arr[hg19]13q12.12q12.2(24,226,609–28,672,052)×4,
13q12.3(29,136,283–30,993,921)×1,13q12.3
(31,377,869–31,803,043)×2,13q13.1q13.2(32,518,057–
34,768,219)×1,13q13.2(4,771,543–35,030,739)×3,
13q13.2q13.3(35,435,048–35,879,773)×1,13q13.3
(36,509,727–36,857,806)×2,13q13.3q14.1(38,167,497–
47,219,875)×3,13q14.13q14.3(47,219,983–53,057,363)×1

This may be shortened to:

arr[hg19]13q12.12q14.3(24,226,609–53,057,363)cth

3. For complex rearrangements seen in high-grade tumor samples, the use of “cx” for complex rearrangements by

array may be used for complex cases that cannot be further characterized.

arr(1-22,X)cx

4. A new triplet code has been added for those laboratories that use targeted region-specific assays or “rsa.” The rsa triplet should be used when describing results from MLPA, QF-PCR, or bead-based methods. Five examples follow:

rsa(13,18,21,X)×2

This is a normal female.

rsa(13,18,21)×2,(X,Y)×1

This is a normal male.

46,XX,rsa 22q11.2(HIRA)×2

Two copies of HIRA were identified after a region-specific assay.

46,XY,rsa 8p23.1(GATA4)×1

One copy of GATA4 was identified after a region-specific assay.

rsa(13)×3

Trisomy 13.

Summary

ISCN recommendations provide a strong foundation for communicating consistent and accurate results among cytogeneticists and to physicians worldwide. Valuable teaching tools can be created by supplementing a current copy of ISCN with illustrations of rare or less frequent cytogenetic abnormalities and complex FISH patterns seen in the laboratory. Despite the fact that ISCN guidelines are merely recommendations, they provide “proof of principle” of how standardized concepts keep communication flowing globally, providing a means for genetic databases to be compared and contrasted to facilitate the application of personalized medicine.

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Glossary of commonly used ISCN terms

Term (ISCN abbreviation)	Definition
Acentric fragment (a)	A segment of a chromosome that lacks a centromere
Additional material of unknown origin (add)	Indicates additional material of unknown origin has been added to a chromosome region or band. The “add” triplet does not imply any particular mechanism, and the additional material may actually replace part of a chromosome arm.
Allele	A sequence variant of a gene
Band	A part of a chromosome that is distinguishable from its adjacent segments by appearing darker or lighter with one or more banding techniques
Centromere (cen)	The primary constriction that divides the chromosome into the short arm (p arm) and the long arm (q arm); the region of a chromosome that contains the kinetochore, a microtubule organizing center (MTOC) responsible for attachment of the sister chromatids to the spindle apparatus at mitosis
Chiasma or chiasmata (xma)	The point where two homologous non-sister chromatids exchange genetic material during meiosis (sister chromatids also form chiasmata between each other, but because their genetic material is identical, it does not cause any change in the resulting daughter cells). The chiasmata become visible during the diplotene stage of prophase I.
Chimera (chi)	Cell lines originating from different zygotes. A chimera is formed by the merging of two nonidentical twins in early blastocyst phase or acquired through allogeneic hematopoietic stem cell transplantation.
Chromatid (cht)	One of the two identical copies of a replicated chromosome
Chromatid gap (chtg)	A nonstaining region of a single chromatid in which there is minimal misalignment of the chromatid
Chromosome (chr)	An organized structure of DNA-bound proteins that houses many genes and regulatory elements. Each chromosome is made up of DNA tightly coiled many times around histones that support its structure.
Chromosome paint	Fluorescent probes stretching over the entire length of a specific chromosome. These probes consist of libraries of DNA sequences derived from flow-sorted chromosomes.
Clone	A cell population derived from a single progenitor cell. A clone comprises two mitotic cells with the same gain or structural aberration or three cells with the same chromosome loss.
Comparative genomic hybridization (cgħ)	A molecular cytogenetic method for the detection of copy number changes (gains/losses/chromosomal imbalances) in a patient’s DNA
Composite karyotype (cp)	A karyotype containing all clonally occurring abnormalities in a tumor
Constitutional anomaly (c)	An abnormality present at conception. The letter “c” in a nomenclature string refers to a constitutional abnormality that is present in a tumor karyotype.
Deletion (del)	A mutation that results in the loss of nucleotides from a DNA sequence or a chromosome
<i>De novo</i> (dn)	Genetic mutation that neither parent possesses or transmits to their offspring
Derivative chromosome (der)	A structurally rearranged chromosome generated either by a rearrangement involving two or more chromosomes or by multiple aberrations within a single chromosome. The term always refers to a chromosome that has an intact centromere.
Dicentric (dic)	An aberrant chromosome with two centromeres that forms when two chromosome segments (from different chromosomes or from the two chromatids of a single chromosome), each with a centromere, fuse, with loss of the resulting acentric fragments
Double minute (dmin)	Extrachromosomal DNA associated with gene amplification and a selective growth advantage in human tumors
Duplication (dup)	A segment of a chromosome that is present more than once on that chromosome
Endoreduplication (end)	Duplication of the genome without mitosis, giving rise to four-stranded chromosomes at prophase and metaphase
Exchange (e)	Refers to either chromatid (chte) or chromosome (chre) exchanges. Exchange is the result of two or more chromatid or chromosome lesions and subsequent rearrangement of chromatid or chromosome material.
Fission (fis)	Centric fission refers to breakage through the centromere resulting in two derivative chromosomes
Fluorescence <i>in situ</i> hybridization (FISH)	A technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes using fluorescent probes that bind with high sequence similarity to the part of the chromosome being interrogated
Fragile site (fra)	Heritable chromosomal sites that exhibit gaps or constrictions on chromosomes when exposed to partial replication stress. Fragile sites are classified as either “common” or “rare,” depending on their frequency.
Heterochromatin, Constitutive	Highly condensed, repetitive DNA found in the centromeres and telomeres that are transcriptionally silent.
Heterozygous (htz)	Diploid cell or organism that contains two different variants of a given gene, chromosome, or chromosome region/arm
Homozygous (hmz)	Diploid cell or organism that contains two identical copies of a given gene, chromosome, or chromosome region/arm
Homogeneously staining region (hsr)	Intrachromosomal segments of various length and uniform staining intensity after G-banding that house amplified genes
Idem	Latin for the same. Refers to the stemline or most basic acquired aberrations in a subclone of a tumor population. The terms idem and sl may be used interchangeably if only one additional subclone is present in a tumor population.
Idiogram	A diagrammatic representation of a karyotype
Incomplete karyotype (inc)	The karyotype present is incomplete, usually because of poor chromosome quality. The term inc is placed at the end of the nomenclature string, after the description of the identifiable abnormalities.

Glossary of commonly used ISCN terms

Term (ISCN abbreviation)	Definition
Insertion (ins)	A chromosomal segment is displaced (two breaks) and relocated into a different chromosomal region (necessitating a third break). The orientation of the inserted segment may be retained in its original orientation or inverted. An insertion may involve different chromosomes or may be intrachromosomal
Inversion (inv)	A chromosomal segment created by two breaks is rotated 180° and reinserted into the same chromosome
Isochromosome (i)	An abnormal chromosome with two identical arms
Isoderivative chromosome (ider)	Designates an isochromosome formation from one of the arms of a derivative chromosome
Isodicentric chromosome (idic)	Designates an isochromosome with two centromeres
Karyogram	A systematic array (picture or figure) of chromosomes
Karyotype	The chromosomal complement of an individual (if constitutional) or tissue (if acquired) or cell line
Landmark	A cytological feature of a chromosome that aids in the identification of that specific chromosome, for example, the centromere, p arm, q arm, telomere, or certain defined bands
Mainline (ml)	A quantitative term referring to the most frequent chromosome constitution of a tumor cell population
Marker chromosome (mar)	A structurally abnormal chromosome in which no part can be unambiguously identified by conventional banding techniques
Maternal origin (mat)	Derived from the mother
Microarray (arr)	An ordered array of microscopic elements on a planar substrate that allows the specific binding of genes or gene products
Modal number (mn)	The most frequent chromosome number in a tumor cell population. The modal number may be expressed as a range.
Mosaic (mos)	Two or more cell lines are present
Neocentromere (neo)	A functional centromere in a novel (non-centromeric) location. May lack specific classes of deoxyribonucleic acid (α -satellite DNA) that are usually present in a centromere.
Nucleolar organizing regions (NOR)	A part of the acrocentric short arm that contains tandem copies of ribosomal or rRNA genes in large, clusters (~40 copies per gene), present on the stalks of the short arms of chromosomes 13, 14, 15, 21, and 22. NORs are detected with silver staining.
Paternal origin (pat)	Derived from the father
Premature centromere division (pcd)	Premature centromere division represents a loss of control over the sequential separation and segregation of chromosome centromeres because the chromosomes are not attached at the centromere; for example, pcd with chromatid puffing in areas of constitutive heterochromatin is found in Robert syndrome.
Premature chromosome condensation (pcc)	Results when an interphase cell fuses with a mitotic cell, causing the interphase cell to produce condensed chromosomes prematurely; for example, pcc may be achieved following cell fusion mediated either by fusogenic viruses or by polyethylene glycol.
Quadriradial (qr)	An interchange figure with four chromosome arms
Ring chromosome (r)	Results when a chromosome breaks in two places and the ends of the chromosome arms fuse together to form a circular structure
Reciprocal (rcp)	An exchange of material (translocation) between two nonhomologous chromosomes
Recombinant chromosome (rec)	A structurally rearranged chromosome with a new segmental composition resulting from meiotic crossing-over involving a displaced segment and its normally located counterpart in certain types of structural (inversion or insertion) heterozygotes
Region	An area of a chromosome lying between two adjacent landmarks. Regions are numbered outward from the centromere on both chromosome arms
Robertsonian translocation (rob)	A translocation involving two acrocentric chromosomes that fuse near the centromere with resulting (inconsequential) loss of the short arms
Sister chromatid exchange (sce)	An interchange of homologous segments between two chromatids of one chromosome
Stemline (sl)	A term referring to the most basic clone of a tumor cell population. The stemline is always listed first in the nomenclature string.
Subtelomeric region (subtel)	The chromosomal region just proximal to the telomere (end of the chromosome) comprised of highly polymorphic repetitive DNA sequences that are typically situated adjacent to gene-rich areas
Telomere (tel)	A region of repetitive DNA at the end of a chromosome that protects it from deterioration. In humans, the telomeres are comprised of a repeating string of TTAGGG, between 5 and 20 kilobases in length, and stain darkly by T-banding.
Telomeric association (tas)	Fusion of chromosomes by their telomeres, which predisposes a cell to genetic instability
Translocation (t)	A chromosome abnormality caused by an exchange of genetic material between two chromosomes. Translocations may be balanced or unbalanced (resulting in loss or gain of material and derivative chromosomes)
Tiradial (tr)	An interchange figure with three chromosome arms
Uniparental disomy (upd)	The condition of having both homologs, a chromosome region, or gene from only one parent

Part II

Examining and Analyzing Chromosomes

Basic Cytogenetics Laboratory Procedures

Martha B. Keagle and Steven L. Gersen

Introduction

The study of chromosomes using traditional cytogenetic techniques requires cells that are actively dividing. Chromosomes are individually distinguishable under the light microscope only during cell division and are best examined during metaphase. Metaphase chromosomes can be obtained from specimens that contain spontaneously dividing cells or ones that are cultured and chemically induced to divide *in vitro*.

Specimens that contain spontaneously proliferating cells include bone marrow, lymph nodes, solid tumors tissue biopsies, amniotic fluids, and chorionic villi. If there are not enough naturally dividing cells for a chromosome analysis, these specimen types may also be cultured in the laboratory without the aid of mitotic stimulants. Peripheral blood lymphocytes usually require the addition of a mitotic stimulant. The choice of specimen for chromosome analysis depends on clinical indications and whether the diagnosis is prenatal or postnatal.

The individual details of culture initiation, maintenance, and cell harvest vary somewhat for the different sample types; however, the general steps and requirements are similar. These are summarized below.

Overview of cell culture and harvest

Culture initiation →	Culture maintenance → Cell harvest
• Living cells	• Sterility
• Sterility	• Optimal temperature
• Proper growth medium	• Optimal pH
• ± Mitotic stimulant	• Optimal humidity
• Microbial inhibitors	• Optimal time interval
	• Arrest division
	• Swell cells
	• Fix cells
	• Prepare slide
	• Stain/band

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The most critical requirement is that *living cells* capable of cell division be received by the laboratory. The manner in which the sample is collected and subsequently handled will greatly influence whether or not the cells will grow and divide, and the quality of the resulting metaphases. Specimen containers must be sterile and must be labeled with the patient's name and a second identifier. The laboratory may reject specimens that are improperly labeled or unlabeled.

Specimen Collection and Handling

Sample Requirements

Peripheral Blood Specimens

Peripheral blood samples should be collected in sterile syringes or vacuum tubes containing preservative-free sodium heparin. Vacuum tubes should be discarded if outdated. Peripheral blood cultures can be initiated several days after the blood is drawn; however, for best results, blood samples should be set up within 24 h of collection. Temperature extremes must be avoided if samples are transported or stored. Specimens should be kept at *room temperature* or refrigerated above 4°C until they can be processed. Culture medium is sometimes added to small blood samples, as these have a tendency to dry up, especially if collected in large containers.

A repeat sample should be requested if these requirements are not met (e.g., the sample is received clotted, on ice, more than 24 h old). It is not always practicable or possible to obtain a new sample, and in such cases, the laboratory should attempt to salvage the original specimen. There may be enough viable cells for a cytogenetic analysis, though the number and quality of cells may be compromised.

Bone Marrow Aspirates

The collection requirements for bone marrow samples are essentially the same as for peripheral blood. Bone marrow aspirates should be collected in sterile syringes or vacuum

tubes containing preservative-free sodium heparin and transported at room temperature. The first few milliliters of the bone marrow tap contain the highest proportion of cells and are the best sample for the cytogenetics laboratory. Blood dilutes the bone marrow sample in later taps and reduces the number of actively dividing cells present in the sample. The success of bone marrow culture is dependent on the number of actively dividing cells. Bone marrow specimens should be processed without delay upon receipt to avoid cell death.

Amniotic Fluid Specimens

Amniocentesis can be performed from as early as 10 weeks of gestation until term (see Chap. 12). Fifteen to thirty milliliter of amniotic fluid should be obtained under sterile conditions and collected in a sterile container approved for cell culture. For amniocenteses performed earlier than 15 weeks, 1 mL of fluid is generally drawn for each week of gestation. The first few milliliters of an amniotic tap are the most likely to be contaminated with maternal cells and should not be submitted to the cytogenetics laboratory. Samples should be transported at room temperature. Temperature extremes and long transport times should be avoided.

The amniocentesis procedure has an inherent, albeit small, risk of miscarriage and should not be repeated unless absolutely necessary. Every effort to salvage samples improperly collected or handled should be made to diminish the need for a repeat procedure.

Solid Tissue Biopsies

Solid tissue sources include skin biopsies, chorionic villi, products of conception, lymph node and solid tumor biopsies, and tissue from stillbirths. Products of conception and stillbirths (and in most cases, tumor biopsies) are one-of-a-kind specimens that cannot be recollected, and repeat collection of chorionic villi increases the risk of miscarriage, although subsequent amniocentesis is an option here. Microbial contamination is a common problem for many types of solid tissue samples. Unlike amniotic fluid, blood, bone marrow, and chorionic villi, some solid tissue specimens are not sterile prior to collection. In addition, viable cells may be few or even nonexistent. These factors threaten the integrity of the sample and pose problems for the laboratory.

Small samples should be collected and transported in sterile culture vessels containing growth or tissue culture medium (not formalin). Sterile saline is not optimal for this purpose but should be used if no other option is available. If distance and timing permit the laboratory to receive and process the sample at once, it may be delivered with no liquid added at all. Larger samples may be sent to the laboratory *in toto* for dissection. Solid tissue samples may be transported and stored on ice until culture is established. Storing tissue specimens on ice slows the action of enzymes that degrade the tissue and slows microbial growth in the event of contamination.

Culture Initiation

Growth Media

All specimens for chromosome preparation are grown and maintained in an aqueous growth medium. Some media are formulated for specific cell types (e.g., AmnioMAX™, Chang Medium®, or Amniochrome™ for amniocytes, giant cell tumor-conditioned medium for malignancies, PANDIS for breast tumors), while others are appropriate for a broad spectrum of cell types (e.g., RPMI 1640, MEM). All culture media are balanced salt solutions with a variety of additives including salts, glucose, and a buffering system to maintain the proper pH. Phenol red is often used as a pH indicator in many media. If the medium becomes too acidic, it will turn yellow, while medium that is too basic becomes pink or purple.

Commercial media are available either in powder forms that must be rehydrated, or as ready-to-use aqueous solutions. Both complete and incomplete media are commercially available, but most commercial media are incomplete. Incomplete media do not contain all of the nutrients and additives necessary for cell growth. Incomplete culture medium must be supplemented with one or more additives before being used for cell culture:

L-Glutamine

L-Glutamine is an amino acid essential for cell growth. L-Glutamine is unstable and breaks down on storage to D-glutamine, a form that cannot be used by cells. L-Glutamine must therefore be stored frozen to retain its stability, and it is optimal to add it to the culture medium just prior to use. There are some commercially available complete media that contain L-glutamine.

Serum

Serum is essential for good cell growth. Too little does not allow for maximum cell growth, but too much can have a detrimental effect. Fetal bovine serum (FBS) is preferred; culture medium is generally supplemented with 10–30% FBS.

Antibiotics

Microbial inhibitors are added to culture media to retard the growth of microorganisms. This is a stopgap measure at best, and should never be relied upon to compensate for sloppy technique. Good sterile technique is always the best defense against contamination.

Penicillin/streptomycin, kanamycin, and gentamicin are bacterial inhibitors commonly used in tissue culture. Fungicides routinely used include nystatin and amphotericin B. Fungicides can adversely affect cell growth and generally

are only used when the potential for contamination outweighs this potentially negative effect.

Bacterial contamination of cultures imparts a cloudy appearance to the culture medium. Fungal contamination presents to the unaided eye as “woolly” masses in the medium, or when observed under an inverted microscope, as branching hyphae. Mycoplasma and viral contamination can be hard to detect and treat. Mycoplasma should be suspected if the background level of chromosome breaks and rearrangements is higher than usual.

Mitotic Stimulants (Mitogens)

Some cells, particularly mature lymphocytes, do not spontaneously undergo cell division and must be stimulated to divide by the addition of an appropriate mitogen to the cell culture.

Phytohemagglutinin (PHA) is an extract of red kidney beans that stimulates division primarily of T-lymphocytes. Cell division starts 48 h after the addition of PHA, with additional waves of division at 24-h intervals. The culture period for blood specimens is based on this knowledge. For routine peripheral blood cultures, 72 h is usually optimal. Blood specimens from newborns may require a shorter culture period. T-cell mitogens may also be indicated for bone marrow samples when some chronic lymphoproliferative disorders (depending upon the immunophenotype), or well-differentiated T-cell disorders are suspected.

Some hematopoietic studies require stimulation of B-lymphocytes, and B-cell mitogens are indicated for bone marrow samples when some chronic lymphoproliferative disorders (depending upon the immunophenotype) or mature B-cell disorders are suspected. There are a number of B-cell mitogens available, including Epstein–Barr virus, LPS (lipopolysaccharide from *E. coli*), protein A, TPA (12-O-tetradecanoylphorbol-13-acetate), and pokeweed. A cocktail including PHA and interleukin-2 (IL2) has proven successful as a lymphoid mitogen for bone marrow samples. The synthetic oligonucleotide DSP-30 has been shown to improve detection of abnormalities in patients with CLL, often together with IL2, and may be useful for other B-cell neoplasms as well [1–3].

Growth Factors

A variety of additional growth factors are commercially available and are used by some laboratories to achieve optimal cell growth for different sample types. These include giant cell tumor extract (GCT) for bone marrow culture and specially formulated amniotic fluid culture media.

Culture Vessels

Choice of culture vessel depends in part on the growth needs of the sample and in part on the individual preference of the

laboratory. Blood and bone marrow samples consist of single free-floating cells. For such suspension cultures, sterile centrifuge tubes or tissue culture flasks (T-flasks) may be used. The cells from samples such as amniotic fluid, chorionic villi, skin biopsies, and other solid tissues need to attach to a surface to grow. Such samples may be grown in T-flasks or with an *in situ* method.

Flask Method

Cells are grown on the inner surface of T-flasks until adequate numbers of dividing cells are present. Cell growth is monitored using an inverted microscope. To remove the cells from the surface of the culture flask where they have been growing, the cultures are treated with an enzyme such as trypsin. This enzymatic treatment releases the individual cells into the fluid environment and permits their collection, harvest, or subculture, as needed.

In Situ Method

Amniotic fluid, CVS, and other tissue samples can be grown directly on coverslips in small petri dishes, in “flaskettes,” or in slide chambers. Growth of these cultures is also monitored with an inverted microscope. They are harvested as “primary” cultures (those that have not been sub-cultured) when adequate numbers of dividing cells are present, and cells do not have to be enzymatically removed prior to harvest. The cells can therefore be analyzed as they grew *in situ*.

Advantages of the *In Situ* Method

Over the Flask Method

The primary advantage of using the *in situ* method is that it provides information about the colony of origin of a cell. This is important when deciding whether an abnormality seen in some but not all cells represents true mosaicism (constitutional mosaicism) or an artifact of tissue culture (pseudomosaicism). True mosaicism is said to be present when there are multiple colonies from more than one culture with the same chromosomal abnormality. Pseudomosaicism is suggested if a single colony with all or some cells exhibiting a chromosomal abnormality is found. In such cases, all available colonies should be studied to rule out the possibility of true mosaicism. If only a single colony with a potentially viable abnormality is found, it may result in an equivocal diagnosis. Low-level mosaicism cannot be completely ruled out in such cases. Clinical correlation may help clarify the picture. A repeat amniocentesis may confirm the presence of true mosaicism but cannot, of course, eliminate the results of the first study.

No inference can be made about the origin of cells when using the flask method, since cells from all colonies are mixed together after they are released from the growing surface. It is impossible to tell if multiple cells exhibiting the same chromosomal abnormality arose from one or multiple

colonies. Thus, two or more cells exhibiting the same structural abnormality or having the same extra chromosome or three or more cells lacking the same chromosome must be treated as potential true mosaics if the flask method is used. However, it should be noted that the presence of multiple abnormal colonies in the same *in situ* culture might also represent artifact. Guidelines for interpretation of mosaicism are available for both methods.

Another advantage of the *in situ* method is that there is usually a shorter turnaround time (TAT), since only primary cultures are harvested. Flask cultures are often sub-cultured, adding days to the culture time.

Preparation of Specimens for Culture

Amniotic fluid specimens, whole blood, and bone marrow samples arrive in the laboratory as single cells in a fluid environment. Whole blood or bone marrow can be added directly to the culture medium, or the white blood cells can be separated from the other blood elements and used to inoculate the culture medium. Separation of the white blood cells is easily accomplished by centrifuging the sample or allowing it to rest undisturbed until the blood settles into three distinct layers. The lowest layer consists of the heavier red blood cells, the top layer consists of plasma, and the narrow middle layer—the buffy coat—consists of the desired white blood cells. The buffy coat can be removed and used to establish the suspension culture.

Amniotic fluid contains a variety of cells that arise from the fetal skin, urinary and gastrointestinal tracts, and the amnion. These are collectively referred to as amniocytes. Most of the cells in an amniotic fluid sample are dead or dying and are not suitable for cytogenetic analysis. Amniotic fluids are centrifuged at low speed (800–1,000 rpm) to retrieve the small number of viable cells. The cell pellet is then used to establish the cultures. The supernatant may be used for a variety of biochemical tests including α -fetoprotein (AFP) and acetylcholinesterase (AChE) assays for open fetal defects.

Solid tissue samples received in the cytogenetics laboratory are usually too large to culture directly and must be disaggregated before use. To obtain single cells, the sample must be finely minced using sterile scissors or scalpels, or alternately, cell dispersion can be achieved by enzymatic digestion of the sample using collagenase and/or trypsin.

Culture Maintenance

After cultures have been initiated, they are allowed to grow under specific conditions of temperature, humidity, and pH until adequate numbers of dividing cells are present. The

optimal temperature for human cell growth is 37°C, and it is essential that incubators be maintained at this temperature. Cultures are maintained either “open” or “closed” systems, depending upon the type of incubator used.

Open systems are those that allow the free exchange of gases between the atmosphere inside the culture vessel and the surrounding environment of the incubator. To facilitate the exchange of gases, the tops or caps of tissue culture vessels are loosely applied. A CO₂ incubator is required for open systems to maintain the 5% CO₂ level necessary to sustain the ideal pH of 7.2–7.4. A humidity level of 97% should be maintained to prevent cell death due to cultures drying out. This can be accomplished by placing pans of sterile water in the bottom of the incubator. A major disadvantage of open systems is that they are susceptible to microbial contamination, especially fungi, due to the moist warm surfaces in the incubator. An open system is required for samples grown on coverslips using the *in situ* method.

Closed systems are those in which the culture vessels are tightly capped to prevent exchange of gases. Humidification is self-maintained, and CO₂ incubators are not required. Commercial media are buffered to the appropriate pH necessary to sustain short-term cultures such as those from blood and bone marrow samples. Long-term cultures from amniotic fluid and solid tissue specimens require the use of additional buffering systems to maintain the proper pH over the longer culture period. Microbial contamination is not as great a risk with closed systems.

In the final analysis, the decision to use an open or closed system, or a combination of both, involves the type of sample being processed and the preference of the laboratory.

Culture Maintenance and Growth Interval

Once the culture requirements are met, the cells must be allowed time to grow and divide. The time in culture varies depending upon the cell type involved.

Peripheral blood cultures require little maintenance once the growth requirements have been met. The culture vessels are placed in an incubator for a specified period of time, usually 72 h.

Likewise, bone marrow cultures need little attention once the culture has been initiated. Bone marrow contains actively dividing cells and therefore can be harvested directly, without any time in culture, or a 24- to 48-h culture time may be used to increase the mitotic index. Longer culture periods are generally not advised since the abnormal cancerous cells may be lost over time or be diluted out by normal precursor cells that may be present. A short growth period usually provides a more accurate reflection of makeup of the tumor; however, there are exceptions, as some tumor cells are slow growing, and some mitogens require longer culture times.

Amniotic fluid and solid tissue specimens require longer culture periods and do not grow at predictable rates. Cell growth is monitored periodically until there are sufficient numbers of dividing cells present, indicating that the culture is ready for harvest. An inverted phase-contrast microscope is used to visualize the mitotic cells that appear as small, refractile spheres. *In situ* amniotic fluid cultures are generally harvested at 6–10 days, sometimes earlier. For amniotic fluid and solid tissue specimens grown using the flask method, the culture interval may be 2 weeks or more.

Amniotic fluid and solid tissue specimens cultured with either the *in situ* or flask method become depleted of required nutrients and additives during the culture period. Depleted medium must be removed and replenished with fresh medium. This process is called “feeding” the culture and is done on a regular basis throughout the culture maintenance period dependent upon the number of cells growing, the length of time in culture, and the protocol of the laboratory. Exhausted medium becomes acidic and will appear yellow if the medium contains a pH indicator such as phenol red.

Cell Harvest

After the cell cultures have grown for the appropriate period of time and there is a sufficient number of dividing cells, the cells are harvested. Harvest is the procedure of collecting the dividing cells at metaphase, their subsequent hypotonic treatment and fixation, and the placement of the chromosomes on glass slides so they may be stained and microscopically examined. The basic steps of cell harvest are the same for all specimen types, with minor variation. An example is shown in Fig. 4.1.

Mitotic Inhibitor

A mitotic inhibitor must be used to obtain adequate numbers of cells in metaphase. Colcemid, an analog of colchicine, is used in most cytogenetics laboratories. Colcemid binds to the protein tubulin, obstructing formation of the spindle fibers or destroying those already present. This prevents separation of the sister chromatids in anaphase, thus collecting the cells in metaphase. Exposure time to colcemid is a trade-off between quantity and quality. A longer exposure results in more metaphases being collected, but they will be shorter because chromosomes condense as they progress through metaphase. Longer chromosomes are generally preferred for cytogenetic studies. Exposure time to colcemid varies by specimen type.

Hypotonic Solution

A hypotonic solution is added to the cells after exposure to colcemid. The hypotonic solution has a lower salt concentration than the cell cytoplasm, allowing water to move into the cell by osmosis. This swells the cells and is critical for adequate spreading of the chromosomes on the microscope slide. Timing is crucial, as too long an exposure will cause the cells to burst. Too short an exposure to hypotonic solution will not swell the cells sufficiently, which results in poor spreading of the chromosomes.

There are a variety of acceptable hypotonic solutions including 0.075 M potassium chloride (KCl), 0.8% sodium citrate, dilute balanced salt solutions, dilute serum, and mixtures of KCl and sodium citrate. Morphology of the

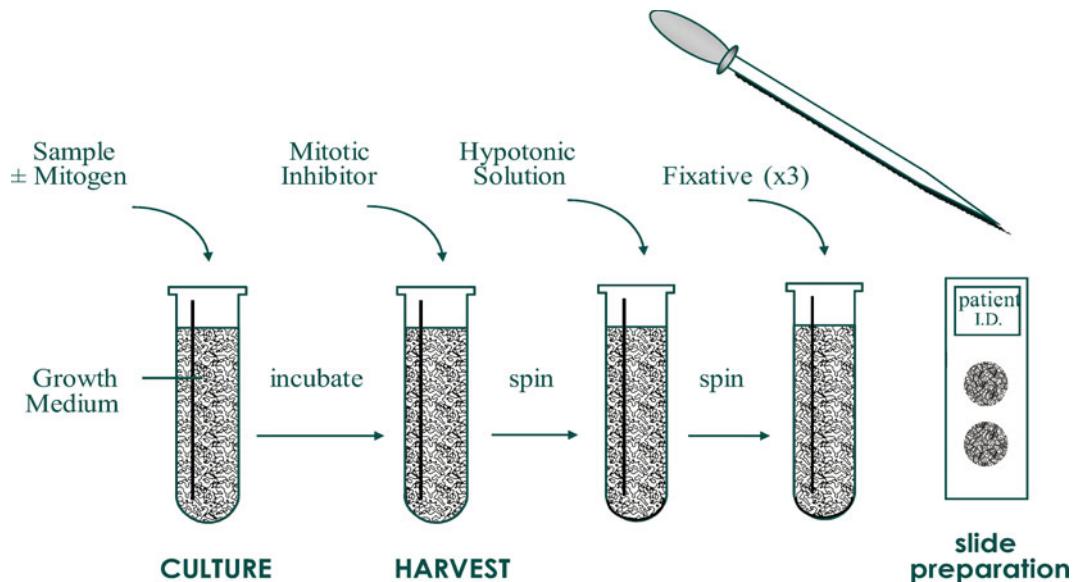


Fig. 4.1 Overview of peripheral blood cell culture and harvest for chromosome analysis. This procedure, with minor variations, is utilized for all specimen types

chromosomes is affected by the hypotonic solution used. The choice of hypotonic solution is based on specimen type and laboratory protocol.

Fixative

A solution of three parts absolute methanol to one part glacial acetic acid is used to stop the action of the hypotonic solution and to fix the cells in the swollen state. This fixative also lyses any red blood cells present in the sample. The fixative must be prepared fresh before use since it readily absorbs water from the atmosphere, which adversely affects chromosome quality and staining.

Slide Preparation

The final step of the harvest procedure is slide preparation. A well-prepared slide has sufficient numbers of metaphases that are not crowded on the slide, metaphases that are well spread with minimal overlapping of the chromosomes, and no visible cytoplasm.

Fixed cells from suspension cultures are dropped onto glass slides to allow for subsequent staining and analysis. When the swollen, fragile cells hit the glass slide, the fixative spreads across the slide and begins to evaporate. The surface tension of the fixative exerts a downward pressure on the cells, and the cells become squashed between the slide and the meniscus of the fixative. As the fixative evaporates, the cell membranes are stretched further and further, and the cells become flatter and flatter, taking up more surface area on the slide. The longer evaporation takes, the more spread the cells and chromosomes become. The rate of slide drying is therefore of major importance in producing good-quality metaphase spreads. Variables that hasten evaporation (like heat and dryness), will decrease spreading, while those that slow evaporation, (like cold temperature and increased humidity) will enhance spreading.

A number of variables affect the rate of evaporation of fixative from the slide, and thus influence the spreading of chromosomes, and the overall quality of the slide preparation. Ambient temperature and humidity; the length of the hypotonic treatment; the height from which the cells are dropped; the use of wet versus dry slides; the use of cold versus room temperature versus warm slides; the use of steam, airflow, or flaming the slides; and the angle at which the slide and/or pipette is held all affect spreading of chromosomes. Test slides should be made and checked under a phase-contrast microscope for metaphase quality and adjustments made if they are not optimal. The concentration of the cell suspension can also be adjusted if the cells are too dense or too dilute on the test slide. Every technolo-

gist must have an arsenal of techniques to effectively deal with these variables.

Some labs use slide drying chambers that control airflow, humidity, and temperature to standardize several of the important variables in slide preparation.

Fixed cells from *in situ* cultures are not dropped because they are already attached to a coverslip or other solid surface. The coverslips are dried under conditions that favor optimal chromosome spreading (see Chap. 7) and are checked with a phase-contrast microscope for metaphase quality and number.

After slides are prepared, they are aged overnight at 60°C or 1 h at 90°C to enhance chromosome banding. There are also techniques that allow chromosomes to be “aged” by brief exposure to ultraviolet (UV) light.

Chromosome Staining and Banding

Prior to the 1970s, human chromosomes were “solid” stained using orcein or other stains with an affinity for chromatin. The chromosomes were classified according to their overall length, centromere position, and ratio of the short arm to long arm. Solid stains provided limited information. Simple aneuploidies could be recognized, but structural aberrations were difficult to characterize and, in some cases, impossible to detect. In addition, it was not possible to specifically identify individual chromosomes. See Chap. 1.

A large number of banding and staining techniques have since been developed. These can be divided into two broad categories: those that produce specific alternating bands along the length of each entire chromosome, and those that stain only a specific region of some or all chromosomes.

Methods that produce specific alternating bands along the length of the chromosomes create unique patterns for each individual chromosome pair. This property allows for the positive identification of the individual chromosome pairs and permits characterization of structural abnormalities. These banding techniques answer many questions by facilitating the numerical and structural examination of the entire karyotype.

Those techniques that selectively stain specific regions of chromosomes are used in special circumstances when a particular piece of information cannot be answered using a routine banding method. These special stains are typically utilized to obtain such specific data.

Techniques That Create Bands Along the Length of the Chromosomes

An important measurement associated with these methods is the level of banding resolution obtained. As chromosomes condense during mitosis, sub-bands begin to merge into larger landmarks along the chromosome. Obviously, as this



Fig. 4.2 G-banding (Giemsma banding). Note the light and dark bands along the length of each chromosome (Image provided by Alma Ganezer)

progresses, the ability to visualize subtle abnormalities is reduced. Chromosomes with a greater number of visible bands and sub-bands (higher resolution) are therefore more desirable. Laboratories accomplish this in two ways: by optimizing the banding and staining procedures themselves so that a maximum number of sharp, crisp bands is produced, and by choosing (and in some cases manipulating cultures to produce) cells with longer, less-condensed chromosomes.

Cytogenetic nomenclature (see Chap. 3) utilizes approximations of the *number of bands present per haploid set of chromosomes*, estimates of the number of light and dark bands one would arrive at by counting these in one of each chromosome (the definition of a haploid set). Minimum estimates usually begin at approximately 300 bands. Well-banded, moderately high-resolution metaphases are usually in the 500- to 550-band range, while prometaphase cells can achieve resolutions of 850 or more bands.

G-Banding (Giemsma Banding)

G-banding is the most widely used routine banding method in the USA. GTG banding (G bands produced with trypsin and Giemsma) is one of several G-band techniques. With this method, prepared and “aged” slides are treated with the enzyme trypsin and then stained with Giemsma. This produces a series of light and dark bands that allow for the positive identification of each chromosome (Fig. 4.2). The dark bands are A-T-rich, late-replicating, heterochromatic regions of the chromosomes, while the light bands are C-G-rich, early-replicating, euchromatic regions. The G-light bands are biologically more significant because they represent the active regions of the chromosomes, while the G-dark bands contain relatively few active genes. There are also G-banding techniques



Fig. 4.3 Q-banding. The fluorescence banding pattern is essentially the same as with G-banding. Note, however, the bright fluorescence on the long arm of the Y chromosome (arrow)

that actually utilize stains other than Giemsma, such as Wright’s and Leishman’s stains.

Q-Banding (Quinacrine Banding)

Q-banding is a fluorescent technique and was the first banding method developed for human chromosomes (see Chap. 1). Certain fluorochromes, such as quinacrine dihydrochloride, will bind to DNA and produce distinct banding patterns of bright and dull fluorescence when excited with the proper wavelength of light. Because adjacent A-T pairs are necessary to create binding sites, the brightly fluorescing regions are A-T rich. The Q-banding pattern is similar to the G-banding pattern with some notable exceptions. In particular, the large polymorphic pericentromeric regions of chromosomes 1 and 16, and the distal long arm of the Y fluoresce brightly; the distal long arm of the Y chromosome is the most fluorescent site in the human genome. There are also Q-band polymorphic regions at the centromeres of chromosomes 3 and 4 that cannot be appreciated with G-banding. Q-banding is therefore useful to confirm the presence of Y material or when studying the cited polymorphic regions. See Fig. 4.3.

Most fluorescent stains are not permanent and require the use of expensive fluorescence microscopes and a darkened room. Q-banding is therefore not conducive to routine work in most laboratories and has essentially been supplanted by fluorescence *in situ* hybridization (FISH) technology. However, for an example of the clinical application of Q-banding, see Chap. 20, Fig. 20.3.

R-Banding (Reverse Banding)

R-banding techniques produce a banding pattern that is the opposite or reverse of the G-banding and Q-banding patterns.

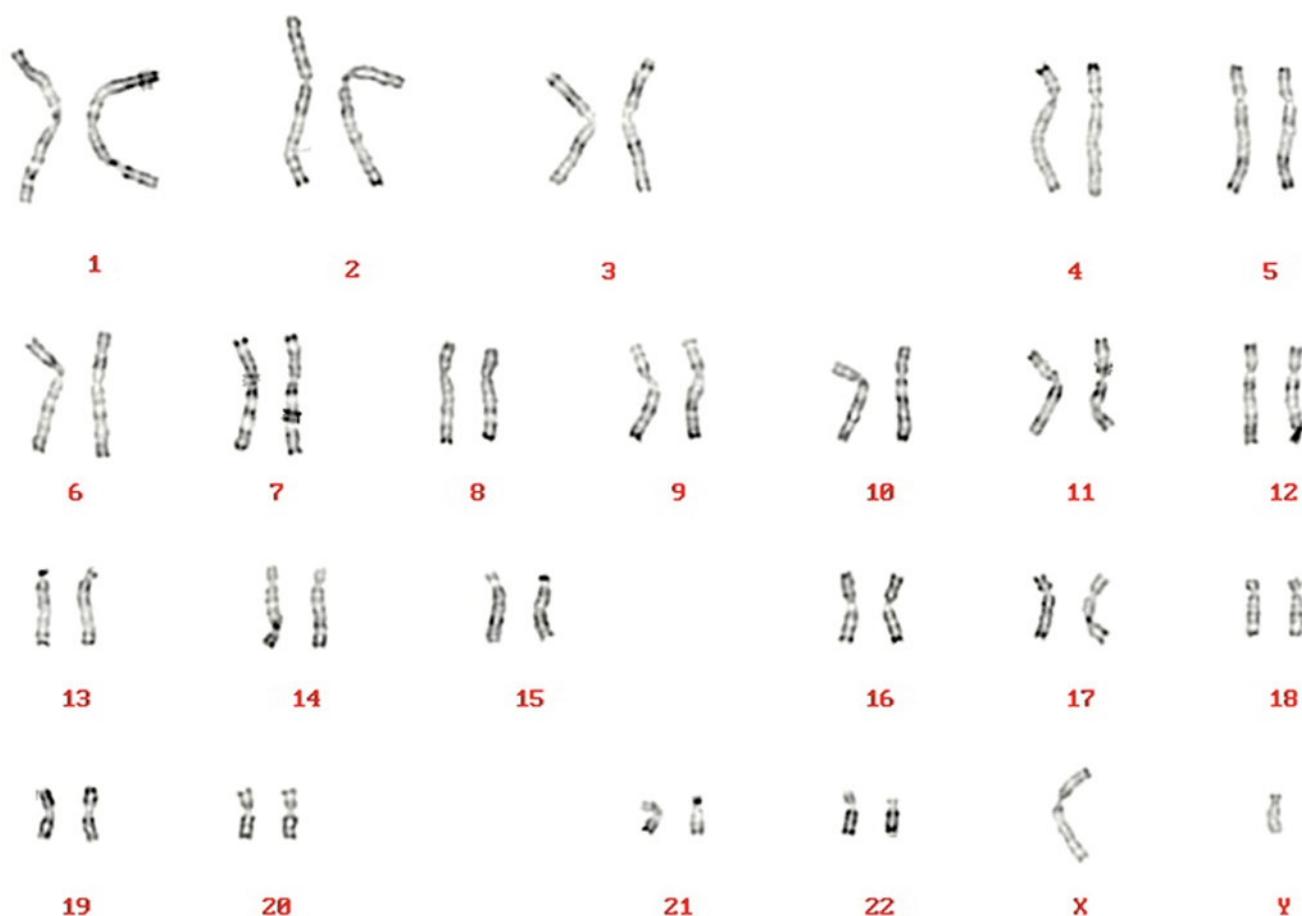


Fig. 4.4 R-banding (reverse banding). The *light* and *dark* bands are the opposite of those obtained with G-banding. R-banding can also be performed with fluorescent staining (Image courtesy of Dr. Sylvie Szapiro-Tapia)

There are fluorescent and non-fluorescent methods. The C-G-rich, euchromatic regions stain darkly or fluoresce brightly, while the A-T-rich heterochromatic regions stain lightly or fluoresce dully. The euchromatic, R-band-positive regions are the more genetically active regions of the chromosomes. Many human chromosomes have euchromatic terminal ends that can be difficult to visualize with standard G-band techniques, since the pale telomeres may fade into the background. R-banding is a useful technique for the evaluation of these telomeres. R-banding is typically used as an additional procedure in many countries but is the standard method for routine banding in France (Fig. 4.4).

Techniques That Stain Selective Chromosome Regions

C-Banding (Constitutive Heterochromatin Banding)

C-banding techniques selectively stain the constitutive heterochromatin around the centromeres, the areas of inherited polymorphisms present on chromosomes 1, 9, 16, and the distal

long arm of the Y chromosome. C-band-positive areas contain highly repetitive, late-replicating sequences of α -satellite DNA. The function of constitutive heterochromatin is not understood, but it is stable and highly conserved evolutionarily.

With CBG banding (C-bands by barium hydroxide, using Giemsa), the DNA is selectively depurinated and denatured by barium hydroxide, and the fragments are washed away by incubation in a warm salt solution. Constitutive heterochromatin resists degradation and is therefore the only material left to bind with the Giemsa stain. The result is pale, almost ghost-like chromosomes with darkly stained areas around the centromeres, at the pericentromeric polymorphic regions of chromosomes 1, 9, and 16, and at the distal Y long arm (Fig. 4.5). C-banding is useful for determining the presence of dicentric and pseudodicentric chromosomes, and also for studying marker chromosomes and polymorphic variants.

T-Banding (Telomere Banding)

T-banding is an offshoot of R-banding that results in only the terminal ends or telomeres of the chromosomes being stained. A more harsh treatment of the chromosomes diminishes

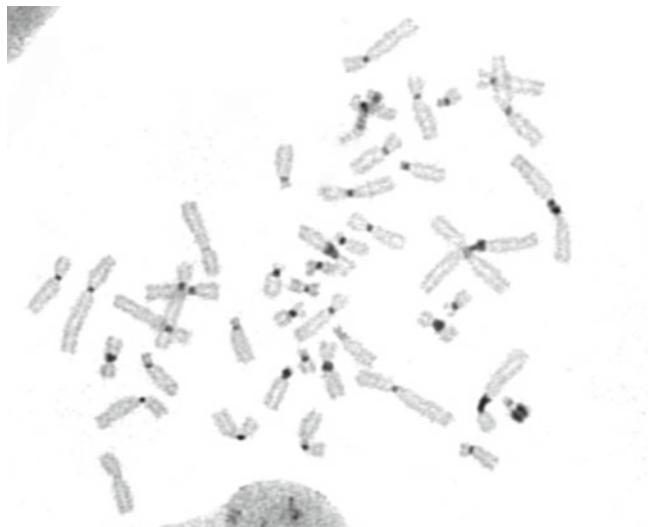


Fig. 4.5 C-banding. This technique stains the constitutive heterochromatin found in each chromosome (hence the term C-banding) and is useful for clarification of polymorphisms. Note the *large* heterochromatic regions in some of the chromosomes (Image provided by Alma Ganezer)

staining except at the heat-resistant telomeres. There are fluorescent and non-fluorescent T-banding techniques.

Cd Staining (Centromeric Dot or Kinetochore Staining)

This technique produces a pair of dots at each centromere, one on each chromatid. These are believed to represent the kinetochores or the chromatin associated with them. The dots are specific to the centromeric region and are not the same as C-bands. Only active or functional centromeres will stain with Cd staining, in contrast to C-banding that will stain inactive as well as active centromeric regions. Cd staining can be used to differentiate functional from nonfunctional centromeres and to study Robertsonian translocations (centromere to centromere translocations of acrocentric chromosomes), ring chromosomes, and marker chromosomes.

G-11 Banding (Giemsa at pH 11)

This technique specifically stains the pericentromeric regions of all chromosomes, the heterochromatin regions of chromosomes 1, 9, 16, and the distal Yq, and the satellites of the acrocentric chromosomes. An alkaline treatment of the chromosomes causes loss of the Giemsa binding sites. Optimal results are achieved at pH of 11.6. At this high alkaline pH, only the azure component of Giemsa binds with the majority of the chromosomes, staining them light blue. The eosin component of Giemsa binds specifically to the heteromorphic regions cited previously, staining them magenta. G-11 banding is used for delineating these heterochromatin polymorphisms.

G-11 banding also has research applications. It is used to differentiate between human and rodent chromosomes in



Fig. 4.6 NOR staining (silver staining). This procedure identifies active nucleolar organizer regions, found on the stalks of acrocentric chromosomes. Silver nitrate produces *dark* staining in these areas. Arrow indicates an abnormal chromosome with satellites at the ends of both arms (Image provided by Alma Ganezer)

hybrid cells. The human chromosomes stain pale blue, while the rodent chromosomes stain magenta.

NOR Staining (Silver Staining for Nucleolar Organizer Regions)

This technique selectively stains the nucleolar organizer regions (NORs) located on the satellite stalks of the acrocentric chromosomes. These regions contain the genes for ribosomal RNA and can be stained with silver nitrate. Theoretically, there are ten NORs per cell, one for each acrocentric chromosome. However, not all will usually stain at any one time because the silver stains the activity, not presence, of rRNA genes. NOR staining is useful for the identification of marker chromosomes and rearrangements or polymorphisms involving the acrocentric chromosomes. See Fig. 4.6.

DAPI/DA Staining (4,6-Diamino-2-Phenole-Indole/Distamycin A)

This stain combines DAPI, a fluorescent dye, with distamycin A, a non-fluorescent antibiotic. Both form stable bonds preferentially to similar, but not identical, A-T-rich, double-stranded regions of DNA. Used together, DAPI/DA fluoresces certain A-T-rich areas of constitutive heterochromatin in the

C-band regions of chromosomes 1, 9, and 16, the distal Yq, and the short arm of chromosome 15. Prior to the development of fluorescence *in situ* hybridization techniques, this was the only stain that differentiated between satellite regions of any of the acrocentric chromosomes.

DAPI/DA is used to identify rearrangements of chromosome 15; to confirm variations in the polymorphic regions of chromosomes 1, 9, and 16 and distal Yq; and to study marker chromosomes with satellites.

Fluorescence *In Situ* Hybridization (FISH)

The development of fluorescence *in situ* hybridization technology represents an important advancement in cytogenetics. FISH is a marriage of classical cytogenetics and molecular technologies and has a large number of applications (see Chap. 17). While many laboratories still utilize traditional special stains in select circumstances, FISH techniques have replaced special stains in many laboratories.

Chromosome Elongation Studies

Chromosomes are routinely examined during metaphase, when they are at their most contracted state. While this is often sufficient for chromosomal analysis, small structural abnormalities may not be detected in chromosomes of metaphase length. In such cases, longer, less-contracted prophase or prometaphase chromosomes are needed. Historically, these were referred to as *high-resolution* cytogenetic studies, but with the advent of arrays (see Chap. 18), they are no longer truly high resolution, so the term has gone into disfavor. To achieve longer chromosomes, the cells can be synchronized and harvested earlier in the cell cycle, or chemical elongation techniques can be used to prevent condensation of the chromosomes.

Cell Synchronization Techniques

Randomly dividing cells can be synchronized with knowledge of the average timing of the stages of the human cell cycle. The cells are blocked and then released at the appropriate time so that a large percentage of cells accumulate in prophase or prometaphase at the time of harvest. There are several protocols for generating such synchronization.

One method involves the addition of FUdR (5-fluorodeoxyuridine) to peripheral blood cultures prior to harvest. FUdR is an inhibitor of thymidylate synthetase, which plays an important role in the folic acid pathway. Folic acid is required for incorporation of thymidine during DNA synthesis. The addition of FUdR blocks cell division at the G1/S border. After 17 h, the accumulated cells are released from the block by the addition of a high level of thymidine.

The peak prometaphase index occurs 5–6 h later, and this is when the harvest is performed.

To achieve cell synchrony, (+) amethopterin or methotrexate (MTX) can also be used, and BrdU (5-bromodeoxyuridine), an analog of thymidine, can be used to release the block.

Chemical Elongation

Ethidium bromide (EB) can be added to cultures prior to harvest to achieve longer chromosomes. Ethidium bromide acts by intercalating between the bases of DNA, thus preventing or slowing its contraction. This results in the collection of long, if not truly prometaphase, chromosomes. The procedure is technically very simple and is used routinely on blood and bone marrow cultures.

The major drawback to using EB is that it is highly mutagenic. Extreme care must therefore be taken when utilizing this reagent.

Newer, less toxic reagents that produce similar results have recently become available, including Chromosome Resolution Additive (CRA).

In previous decades, before the introduction of molecular analysis for fragile X syndrome (see Chap. 19), the diagnosis of this disorder was made in the cytogenetics lab, using special culture conditions. Among these was the inclusion of FUdR, described previously. Laboratories observed that one by-product of this procedure was longer chromosomes. Although the exact mechanism is not known, the addition of FUdR to blood cultures 24 h prior to harvest does in fact seem to produce chromosomes of greater length, and this technique is used in several labs. One consideration, however, is that this can facilitate the expression of folate-sensitive fragile sites (see Chap. 14).

Some laboratories employ an amniotic fluid harvest technique that includes overnight exposure to colcemid. Many have also found that the addition of BrdU to these cultures also increases chromosome length, probably by replacing thymidine with a larger base, thereby reducing chromosome condensation.

Culture Failure

All culture failures must be investigated. The circumstances of the failure should be recorded as a part of an ongoing quality assurance program (see Chap. 6). A record of failure rates for each specimen type in the laboratory must be kept as a baseline so that deviations from the norm can be detected. It is important to isolate the reason(s) for a culture failure so that steps can be taken to prevent future similar failures. Some culture failure is unavoidable, but adherence to strict standards and rigorous investigation of all failures should keep this number to a minimum.

There are many possible origins of culture failure. It can be due to improper specimen collection or transport, improper laboratory technique, or the condition of the sample. There are general sources of failure that apply to all sample types and specific ones that pertain to one or more of the sample types.

Errors in sample collection and handling include failure to submit an adequate amount of sample, collection under non-sterile conditions resulting in microbial contamination, use of an inappropriate collection vessel or medium, failure to use an anticoagulant, use of an inappropriate or expired anticoagulant, delay in transport, and improper storage before and/or during transport of the sample.

In the laboratory, errors can occur at any step from culture initiation to staining. Failure to follow proper protocol can cause loss of a culture. This is one reason for establishing multiple cultures for all samples and harvesting them at different times. Faulty media, sera, or other reagents can also result in culture failure. It is therefore important to test all new lots of media and sera for sterility and ability to support cell growth before using these on patient samples. It is also important to maintain a log of lot numbers of all reagents used and the date each was put into use to help identify the source of any problem. During the culture period, improper temperature, CO₂ level, or pH of the culture can have deleterious results. The temperatures and CO₂ levels of all incubators must therefore be monitored and recorded at least daily, and samples should be split and grown in separate incubators in the event an incubator malfunctions. In general, all equipment used in the laboratory must be monitored at regular intervals and maintained to prevent malfunction.

Lack of viable cells or unsuitable cell type can compromise amniotic fluid samples. Samples from patients with advanced gestational age (20 weeks or greater) may consist primarily of mature nondividing cells or dead cells. Some samples consist principally of epithelial cells, which typically produce few metaphases of poorer quality than the desired fibroblasts.

Amniotic fluid samples are usually clear yellow in appearance. A brown fluid indicates prior bleeding into the amniotic cavity, which may suggest fetal death or threatened miscarriage. In such samples, there may be few if any viable cells present. Bloody taps containing large numbers of red blood cells can be problematic. The physical presence of large numbers of red blood cells can prevent the amniocytes from settling on and attaching to the growth surface of the culture vessel. In addition, the red cells utilize nutrients in the culture medium, thereby competing with the amniocytes.

Patient factors can influence the success of peripheral blood and bone marrow samples. Disease conditions, immunosuppression, and use of other drugs can affect both the number of lymphocytes present and their response to mitotic

stimulants. The laboratory is not always made aware of these confounding factors. Bone marrow samples that have been contaminated with blood may not have adequate numbers of spontaneously dividing cells present. For this reason, it is important that the cytogenetics laboratory receive the first few milliliters of the bone marrow tap. Bone marrow samples are notorious for producing poor-quality metaphases. There are sometimes adequate numbers of metaphases, but the chromosomes are so short and so poorly spread that analysis is difficult or impossible. In addition, metaphases of poor quality often represent an abnormal clone.

The failure rate of solid tissues may be quite high and is often due to the samples themselves. In the case of products of conception or stillbirths, the sample may not contain viable cells, or the wrong tissue type may have been collected. Additionally, microbial contamination is a frequent contributing factor, since many solid tissue samples are not sterile prior to collection.

Preservation of Cells

Cells do not survive indefinitely in tissue culture. After a period of time, they become senescent and eventually die. At times, a sample may need to be saved for future testing, to look at retrospectively, or because it is unusual or interesting and might be of some value in the future. In such cases, the cells need to be kept alive and capable of division long term or indefinitely.

Cultured cells can be kept alive by cryopreservation, the storage of cell in liquid nitrogen. The freezing process is critical to cell survival. Rapid freezing will cause cell death due to formation of ice crystals within the cells. Improper freezing can also denature proteins, alter the pH, and upset electrolyte concentrations. The cells must be cooled slowly so that water is lost before the cells freeze. The addition of 10% glycerol or dimethyl sulfoxide (DMSO) to the storage medium lowers the freezing points and aids in this process. One-milliliter aliquots of the sample in storage medium are placed in cryogenic freezing tubes. The samples are then slowly frozen under controlled conditions at a rate of 1°C per minute to a temperature of -40°C. The sample can then be rapidly frozen to about -80°C. Alternately, the samples may be placed in a -70°C freezer for 1–4 h. After this initial freezing has been accomplished, the cells are stored in the liquid phase at about -190°C.

Thawing of the sample is also critical. Rapid thawing is necessary to prevent the formation of ice crystals.

B-lymphocytes can be transformed so that they will proliferate indefinitely in tissue culture by exposing them to Epstein–Barr virus (EBV). These immortalized lymphoblastoid cell lines do not become senescent and can therefore be maintained indefinitely in culture.

Chromosome Analysis

Selection of the correct specimen for chromosome analysis and additional tests is not always straightforward, and the submission of an inappropriate sample to the laboratory can create frustration for both patient and clinician.

This was not always as complex an issue as it is today. In the 1970s, prenatal diagnosis involved an amniotic fluid specimen, often obtained at exactly 17 weeks of gestation, for chromosome analysis and alpha-fetoprotein testing. Other tests were available but rare. The cytogenetic contribution to hematology/oncology essentially involved whether a bone marrow specimen was “positive or negative” for the “Philadelphia chromosome.” Constitutional chromosome analysis from peripheral blood implied that the patient had to be an adult or a child.

Today’s prenatal caregivers and their patients must choose between traditional amniocentesis, early amniocentesis, chorionic villus sampling, or, sometimes, percutaneous umbilical blood sampling. A decision must be made concerning whether ploidy analysis via FISH is warranted, and acetylcholinesterase is often a factor in the diagnosis of certain open fetal lesions, but AFP and AChE cannot be performed on all sample types. Many disorders can be also diagnosed by biochemical or molecular methods, and ethical dilemmas surround the potential to diagnose, prenatally, late-onset disorders such as Huntington’s disease. Screening for increased risk or predisposition to developing certain cancers or other diseases has created new moral and ethical pitfalls. Each collection method may ultimately affect the number of cells available for chromosome analysis, and all of these issues can play a role in the timing and choice of sampling procedure.

Today, the cytogenetics laboratory provides indispensable information for the diagnosis, prognosis, or monitoring of patients with a wide variety of hematological disorders and other neoplasms, using not only bone marrow but also, in some cases, blood, lymph node biopsies, or tumor tissue or aspirates. Treatment decisions often rest on the results of a chromosome analysis, but some tissue types are only appropriate under certain conditions, and an incorrect selection here can delay a vital diagnosis.

A blood sample today could be from a patient with leukemia or from a fetus rather than a child or an adult suspected of having a constitutional chromosome abnormality. These must all be handled differently, and the information they provide is unique in each circumstance.

Procedure

After all of the appropriate laboratory manipulations and staining procedures have been performed, there are several steps involved in the clinical analysis of chromosomes. These

begin with the microscope, where selection of appropriate metaphases begins the process. Although technologists are trained to recognize well-spread, high-quality cells under low-power magnification, they must also remember to examine some poor-quality metaphases when analyzing hematological samples, as these often represent abnormal clones.

Under high power, the chromosome morphology and banding resolution are evaluated. If these are appropriate, the number of chromosomes is counted, and the sex chromosome constitution is typically determined. The microscope stage coordinates of each metaphase are recorded, and in many laboratories, an “identifier” of the cell is also noted. This is typically the position of one or more chromosomes at some reference point(s) and serves to verify that the correct metaphase has been found should there be a need to relocate a cell. Any other characteristics of the metaphase being examined, such as a chromosome abnormality or quality of the banding and chromosome morphology, are also noted.

In the United States, certifying agencies such as the College of American Pathologists (CAP) require that a minimum number of metaphases be examined for each type of specimen, barring technical or clinical issues that can sometimes prevent this (see Chap. 6). There are also requirements for a more detailed analysis (typically band-by-band) of a certain number of cells, as well as standards for the number of metaphases from which karyograms are prepared. Regulations notwithstanding, it is clearly a good laboratory practice to analyze every chromosome completely in several cells and even more important to check *all* chromosomes in certain situations, such as when analyzing cancer specimens. Depending upon the results obtained and/or initial diagnosis, additional cells may be examined in order to correctly identify all cell lines present (see Chap. 6 for additional discussion of guidelines and standards).

Once the appropriate number of mitotic cells has been examined and analyzed, a representative sample must be selected for imaging and ultimate preparation of karyograms. Today, computer imaging and automated production of karyograms have virtually replaced traditional photography and manual arrangement of chromosomes (see Chap. 7). Many laboratories also image additional cells to be included as references in the patient chart. Ultimately, summary information (patient karyotype, banding resolution, number of cells examined, analyzed, imaged, etc.) is recorded in the patient’s file and is used in the clinical report (see Chap. 6).

The final steps of the process typically involve a clerical review of all relevant clinical, technical, and clerical data, examination of the patient’s chart and karyograms by the laboratory director (often preceded by the supervisor and/or other senior laboratory personnel), and generation of the formal clinical report. In addition to the appropriate physician and patient demographic information, this should include the number of metaphases that were examined microscopically,

the banding resolution obtained for the specimen, the number of cells that were analyzed in detail, the number of karyograms prepared, the patient's karyotype, and the clinical interpretation of the results, including, where appropriate, recommendations for additional testing and/or genetic counseling.

Summary

The purpose of this chapter is to provide a general overview of the many steps involved from receipt of a sample in the cytogenetics laboratory to the generation of a patient report and to impress upon the reader the labor-intensive nature of this work. While the basic procedure is always the same, there are culturing and processing variations that are sample type-dependent, choices of methodology that are diagnosis-dependent, and microscopic analysis decisions that are results-dependent. All of these in turn depend upon individuals with the appropriate expertise and dedication to patient care.

Due to the nature of this chapter, individual citations were not always practical. In addition to the references cited below and the authors' personal experience, the following were used as supplemental sources of information:

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The Essentials of Light Microscopy

5

Nathan S. Claxton and Stephen T. Ross

Introduction

Light microscopy is an indispensable tool in the cytogenetics laboratory, both for routine analysis and for techniques such as fluorescence *in situ* hybridization (FISH). Modern tools, such as digital imaging and advances in image analysis, have aided the microscopist in drawing conclusions from microscope images. A clear understanding of optical systems and the trade-offs involved in imaging is essential to maximizing the quality of such images. This chapter covers the basic principles and applications of the light microscope, including brightfield and contrasting techniques, and an introduction to the fluorescence microscope.

Brightfield Microscopy

The modern compound microscope is the most important diagnostic tool in the cytogenetics laboratory and is designed to enhance the observable detail of specimens by magnifying images, resolving structures, and applying various contrasting techniques. In transmitted light brightfield microscopy, specimens are typically mounted on glass slides and light is passed through them to illuminate and resolve structures not visible to the naked eye. While some specimens may be viewable in their natural state, optical dyes and stains are often used to add contrast to typically transparent and colorless cellular features.

The Microscope Optical Train and Conjugate Planes

A knowledge of conjugate focal planes helps in the understanding of how the various components of the microscope work in concert to produce the best possible images. There are two sets of conjugate focal planes, typically referred to as the “image” plane and “aperture” plane. When aligned properly, the specimen, field diaphragm, and detector (eye or camera) are focused together so that a sharp image of the specimen is projected onto the detector. The “aperture” components (the light source, condenser aperture, and back focal plane of the objective) are also focused to each other in a reciprocal manner relative to the image plane. Light rays focused in one set of conjugate planes are nearly parallel in the reciprocal set of conjugate planes. The microscope cutaway in Fig. 5.1 shows conjugate planes in a modern upright microscope.

Köhler Illumination

Proper centering and focusing of the condenser is necessary for the full and even illumination that is essential for good resolution and high-quality imaging. Köhler illumination, developed in the late nineteenth century by August Köhler, completely defocuses the inherently uneven light source (such as an incandescent filament) in the image plane resulting in a very even field of illumination, while focusing the light source in the aperture plane for optimal brightness and resolution. Setting Köhler illumination requires a lamp with a collector lens to focus light at the front aperture of a focusable and centerable condenser. A simple method for setting basic Köhler illumination follows:

1. Using a low-magnification objective, place a specimen on the stage and bring it into focus. Remove the specimen.
2. Close down the field aperture, typically located at the base of the microscope, so that the shutter blades are visible in the field of view.
3. Rack the condenser up or down until the field aperture blades come into sharp focus and center it in the field of view.

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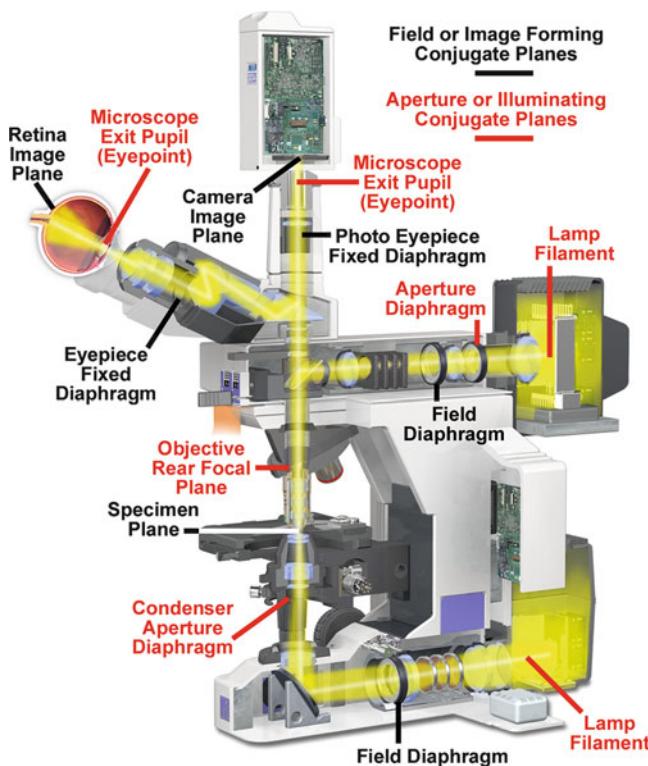


Fig. 5.1 Conjugate planes in the optical microscope (cutaway). In this cutaway of a modern microscope, “image” planes are marked in black while “aperture” planes are marked in red, denoting the two conjugate sets of light planes (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

4. Reopen the field diaphragm to the field of view, whether eye or digital detector.

This aligns the illuminating components into precise physical locations resulting in optimal illumination. Köhler illumination is also the starting point for the proper operation of various advanced contrasting techniques and should be performed each time the microscope is used and for individual objectives. Figure 5.2 shows the conjugate planes of a microscope aligned for Köhler illumination in both the illuminating and image-forming light paths.

Transmitted Light Source

The transmitted light source for brightfield microscopy is usually located in an external housing or the microscope base and is most commonly an incandescent tungsten-halogen bulb. Some newer microscopes have integrated light-emitting diodes (LEDs). The housing reflects as much light as possible toward the collector lens, which then directs light into the microscope condenser. In some cases, the light source is manually centered and focused, but most modern housings automatically center the bulb. Halogen bulbs produce heat and often require some amount of ventilation, while LEDs operate at much cooler temperatures and have extended life.

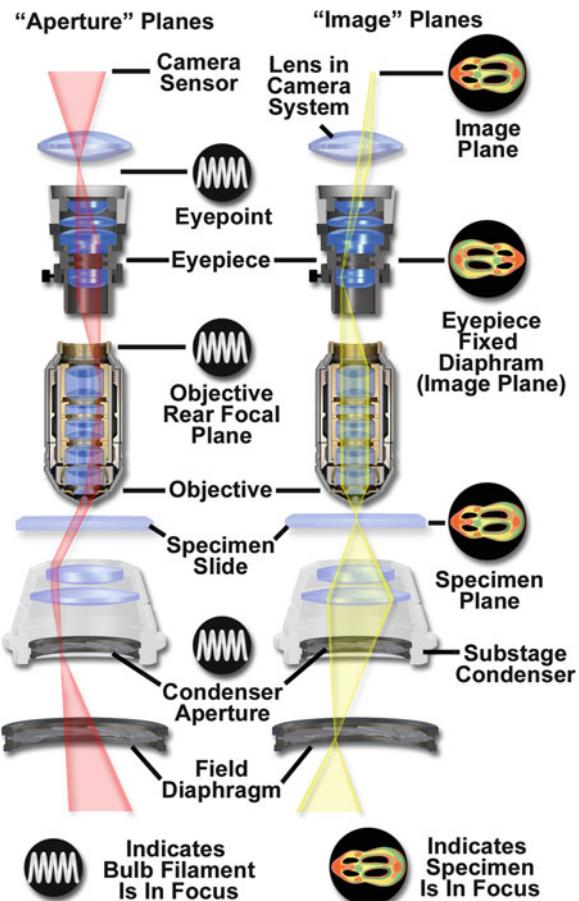


Fig. 5.2 Conjugate planes in the optical microscope (light paths). The ray-trace shown in red illustrates the path taken by light focused at or originating from an aperture plane. The ray-trace shown in yellow illustrates the path taken by light focused at or originating from an image plane (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

An adjustable rheostat, located on the microscope body or the external power supply, regulates the voltage delivered to the bulb and adjusts the intensity of the light. With incandescent bulbs, such voltage adjustment also changes the color temperature, and this can cause major changes to the hue or color property of the image, particularly when combined with digital imaging. Neutral density filters (NDs) can be used to control the light intensity without changing the color balance by attenuating light evenly across the entire spectrum. While digital cameras have greater ability than film to adjust for changes in color temperature by automatically “white balancing,” which digitally shifts the hue of colors in relation to each other, the use of NDs to adjust light intensity reduces this need. Unlike incandescent bulbs, LEDs have a constant color temperature regardless of voltage adjustment.

Halogen bulbs emit a continuous spectrum of light that extends from about 300 to 1,400 nm. The collector lens for

the lamp typically blocks ultraviolet (UV) light, while a separate infrared (IR) filter may be used to block IR light that can cause eye strain and high background on digital images. LEDs tuned for white light do not emit UV or IR light, thus reducing eye strain for the operator and eliminating the need for extra UV- or IR-blocking filters. A neutral color-balancing filter, typically NCB11, is often placed in the light path to adjust the color temperature of the incandescent light nearer to that of daylight, while LEDs are often pre-tuned to this color balance as a manufacturing specification without the need for an additional filter.

Other filters may be used to increase the visual contrast in cytogenetics specimens. For instance, contrast in G-banded chromosomes can be improved using a simple green glass filter that absorbs light of all colors except green. Depending on the correction level of the microscope optics, performance may be improved with monochromatic green light. In this case, a more efficient green interference filter is better at producing monochromatic green light for the best imaging conditions. Interference filters reject unwanted wavelengths by reflecting and causing destructive interference. A green interference filter (often labeled GIF) can be differentiated visually from a green glass filter by its unique reflective property, which often produces an orange or yellow tint when viewed at an angle. Interference filters, including those used for fluorescence, have very thin-layered coatings on their surfaces, and great care should be taken when cleaning them.

Field Diaphragm, Condenser, and Aperture

Diaphragm

The field diaphragm, typically located after the light source and its associated filters, is an adjustable iris-type diaphragm that defines the total area of illumination. It should be opened just past the field of view, whether to the eye or camera sensor, to fully illuminate the specimen while reducing stray light. Proper Köhler illumination will focus the image of the field diaphragm in the specimen plane.

The microscope condenser gathers and focuses light from the source and passes it through the specimen, providing full and even illumination. The condenser assembly contains an adjustable diaphragm in its front focal plane known as the aperture diaphragm and may also house various light conditioners used in advanced contrasting techniques.

The maximum angle of incidence for light rays in the cone of light that the condenser can deliver is determined by the numerical aperture (NA) of the condenser. This optical property of lenses ultimately determines the resolving power of the optical system, which is the limit of its ability to separate fine details. While the objective lens may be the most prominent component that affects magnification and resolution, the effective NA (and thus resolving power) of the objective collecting transmitted light cannot exceed the NA of the

condenser that delivers that light. For optimal resolution, the NA of the condenser should closely match or exceed the NA of objective lens.

The simplest common condenser is known as an Abbe condenser named for its inventor, Ernst Abbe. While Abbe condensers are available with a variety of NAs, they do not have significant correction for optical aberrations. The Abbe condenser can be used for basic inspection of routine brightfield samples but may not be suitable for critical or high-detail investigations, such as in cytogenetics.

Aplanatic condensers are corrected for spherical aberration, which is an optical imperfection characteristic of lenses with curved surfaces in which light rays passing near the lens periphery focus to a different point than rays traveling through the center of the lens, leading to a reduction in sharpness. Correction for this aberration may be accomplished for individual wavelengths of light. The performance of aplanatic condensers is best using green light, and this is assumed in the optical design since aplanatic condensers are not corrected for chromatic aberrations.

Chromatic aberration in microscopy generally refers to axial chromatic aberration, in which light of differing wavelengths does not focus to the same point. Achromatic condensers are corrected for axial chromatic aberrations to bring blue and red light to the same focus as green light, but they are not corrected for spherical aberration. Aplanatic-achromatic condensers are corrected for both spherical and chromatic aberrations.

The effective NA of the condenser can be adjusted by opening or closing the aperture diaphragm, thus increasing or reducing the angle of light entering the specimen and objective. Since higher NA relates to higher resolving power, it may seem counterintuitive to purposely reduce the NA of the condenser by restricting the diaphragm. However, as the aperture diaphragm is closed down and the NA is reduced, visual contrast and depth of field are both increased even as ultimate resolving power is decreased. Visual contrast lost by opening the aperture can often be restored by processing digital images. A clear understanding of this interplay between contrast and resolution helps the user navigate challenging samples and extract the maximum amount of information.

Objective Lenses

The objective lens is the major determinant of magnification and resolution and is perhaps the most significant single component of the optical train with regard to observational capability. Most manufacturers offer a wide selection of objectives with various magnifications and NAs and with varying optical design considerations for aberration correction and application. Objectives typically have markings on

the barrel, indicating magnification, NA, aberration correction, working distance, immersion medium, and coverslip correction.

Resolution vs. Magnification

The two main functions of objectives are to resolve detail and magnify the image. It is a common misconception that magnification determines resolution when, in fact, the resolving power of a lens is a function of numerical aperture. The ability to resolve detail is directly tied to the numerical aperture of the objective such that higher NAs translate to higher resolving power, while magnification makes those resolved details easier to observe. Magnification increases size but not resolution.

As waves of light encounter very small objects, particularly of a size approaching the wavelength of the light, they are bent around the object. This property of light is described as diffraction. It can also refer to the way light waves spread as they pass through small apertures. As light is passed through a small object of interest on a microscope, such as a chromosome, the light that holds the information about that object may propagate at very steep angles relative to the optical axis. NA describes an objective's ability to collect this highly diffracted light. The more highly diffracted the light that is collected, the better that object can be resolved by the lens. Since NA describes light-collecting ability, it also indicates relative brightness of the image produced, which is of major importance in light-challenged applications such as fluorescence.

The method of observation (visual vs. imaging system) should be considered when selecting magnification. For visual inspection, magnification selection is dependent on whether relevant resolved details can be observed by the human eye. For digital imaging, the required level of magnification needed to accurately record resolved details depends on the physical pixel size of the detector. In general, higher NAs call for smaller pixels, which are usually a consequence of a greater number of pixels on the detector, but this need is mitigated as magnification increases. The Nyquist sampling theorem states that to accurately record and reconstruct a continuous analog signal using discrete units (pixels in this case), the sampling rate, or frequency of the digital data point in time or space, must be at least two times the smallest observable signal.

A common rule of thumb for digital cameras is that effective pixel size in the specimen plane should be 2.5 times the maximum resolution of the objective. For example, to determine the resolution of an objective with a 1.3 NA, assuming long green light of 589 nm, the Rayleigh criterion for resolution ($0.61\lambda/NA$) gives a result of ~ 276 nm for resolvable detail. Magnifying this result by 100 \times results in a resolved size at the detector of 27.6 μ m. In order to sample

2.5 times per 27.6 μ m, the physical pixel size of the detector must be smaller than $27.6/2.5$ or ~ 11 μ m. However, if the magnification of the objective were only 40 \times , the resolved size at the detector is 276 nm \times 40 or 11.04 μ m, which would require a pixel size smaller than $11.04/2.5$ or ~ 4.4 μ m.

Objective Types

Another major consideration in objective selection is the level of correction for optical aberrations. Common types of objectives include achromat, plan, fluorite, and apochromat. While the specific details of correction naming conventions are not completely standard across all manufacturers in the industry, these serve as a good general description of the type of optical corrections engineered in the lens.

Plan lenses are corrected for flatness of field so that the periphery of the field of view lies in the same focal plane as the center. Non-plan lenses may exhibit field curvature in which the edges of the image are out of focus, while the center is in focus. Flatness of field is very important for digital imaging, though in practice, even non-plan lenses may be flat in the field of view of the camera sensor, which is often smaller than the full visual field of view. The plan designation is often combined with other corrections, such as plan achromat, plan fluorite, or plan apochromat.

Modern achromat objectives are corrected for spherical aberration in a single wavelength (typically green light) and axial chromatic correction in two colors (typically blue and red). If color information is not necessary, an achromat objective will perform best with monochromatic green light due to the correction for spherical aberration in this wavelength range.

Fluorite objectives (also called FL, fluor, fluar or semi-apo), whose name is derived from the calcium fluoride crystal called fluorite or fluorspar from which the lenses were originally manufactured, are corrected for spherical aberration in at least two colors and chromatic aberration in at least two colors.

Apochromat objectives (often abbreviated apo) are the most highly corrected lenses and are corrected for spherical aberration in at least three colors as well as chromatic aberrations in at least two colors. Many modern apo objectives exceed these specifications by correcting spherical and chromatic aberration in four or more colors.

Immersion Objectives and Oils

While the speed of light in a vacuum is a constant, the speed of light as it travels through a medium is defined by its refractive index (RI or n). This is expressed as a ratio of the speed of light in a vacuum over the speed of light in the medium. Thus, the refractive index of air is very nearly 1, while the refractive index for crown glass, a common optical lens material, is about 1.5.

As light passes from a medium of one refractive index to a medium of differing refractive index at an angle, the light will change speed and direction. If light passes from a higher refractive index to a lower one, the light is bent toward the interface. In microscopy, this means that if light exiting a specimen and coverglass ($n \approx 1.5$) at a high angle passes through air ($n \approx 1.0$), it will be bent further away from the objective and thus may not be collected, resulting in a loss of resolving power. However, by filling the space between the glass coverslip and the objective with oil having a refractive index similar to glass, this loss of light and thus information can be avoided.

Refractive index also directly relates to numerical aperture in that the effective NA of an objective cannot be greater than the refractive index of the medium through which light passes. This means that for a “dry lens,” the theoretical limit of NA is 1.0 (in practice, often 0.90 or 0.95). Thus, these lenses, usually available in magnifications of 40 \times to 100 \times , provide relatively good resolving power without the use of oil.

An NA higher than 1.0 may be needed to observe the finest details. For an objective to achieve this higher NA, an immersion medium with a refractive index higher than that of air must be used. For standard oil immersion objectives, the theoretical NA limit is about 1.5 (in practice, often 1.4–1.49). The same is true in transmitted light microscopy for the delivery of light to the sample by the condenser. In order to achieve the same high NAs, the condenser must be oiled so that light does not pass through air between the condenser front element and the glass slide.

Since oils may have varying optical or chemical properties, the use of the particular type of oil specified by the objective manufacturer for that lens is recommended. Oils of different brands should not be mixed without consulting the manufacturer. As immersion oils dry, they can become very gummy or sticky and sometimes degrade optical coatings or cements. Therefore, oil objectives should always be wiped clean with optical tissue after use.

Coverglass Correction and Correction Collars

If a coverglass (coverslip) is used, the optical design of the objective must take into consideration the thickness of this glass. Most standard objectives are corrected for a 0.17-mm-thick coverslip, and this property is marked on the outer casing of the objective.

In coverglass parlance, a #1.5 coverglass has a target thickness of 0.17 mm. Manufacturing tolerances of standard coverslips generally allow that a #1.5 coverslip may vary in thickness from 0.16 to 0.19 mm. This small variation can induce spherical aberration that, when using high NA

objectives to observe fine detail, will significantly degrade resolution. To correct for this, some objectives are fitted with correction collars that adjust internal lens spacing as they are rotated. Similarly, some objectives have correction collars to correct for other optical path length differences such as the thickness of a plastic culture dish or when imaging deep into a thick sample.

Other objectives are designed to be used without a cover-glass. These are marked as “NCG” (no coverglass) on the outer barrel of the objective.

Eyepieces

The eyepieces of the microscope further increase the magnification of the image and project it to a point where it can be comfortably viewed. Magnification is typically listed on the eyepiece along with another important specification known as the field number, which defines the field of view. To determine the field of view, the field number as listed in millimeters is divided by the objective magnification (and any intermediate magnification). Eyepieces are also available in high-eyepoint versions to allow the use of eyeglasses or a more comfortable viewing position or with individual diopters to correct for focus without the need for corrective lenses. Additionally, various markers such as crosshairs, pointers, or measuring reticles can be positioned in the field plane of the eyepiece so that they appear in focus with the specimen.

Beam Splitter

Microscopes that have digital imaging capabilities include a beam splitter to direct light either toward the eyepieces or the detector. The beam splitter may send 100% of the available light in either direction or in some cases will split some percentage of the light to both the eyepieces and detector at the same time for simultaneous visual inspection and imaging.

The Microscope Stage and Coordinate Location

The microscope stage provides a flat, level surface for the microscope slide and a means of affixing the slide to the stage. Controls on a mechanical stage allow the microscope slide to be moved in x- and y-axes. Mechanical stages usually have a coordinate grid on each axis to precisely identify the location of an object on the slide. The microscope stage can also be moved in an up-down manner (z-axis) by using the coarse- and fine-focus controls.

Coordinate Location

Recording accurate coordinates is essential for documentation of cytogenetic findings. In most instances, notation of the x and y coordinates are used for this purpose.

Vernier Grids and England Finders®

When a metaphase is to be relocated at a microscope other than that used for the original analysis, a system of coordinate conversion between the two microscopes needs to be employed.

Microscopes of the same manufacturer and model can often have their stages aligned so that the coordinates of one scope can be used at another. Vernier grids or England Finders® allow for easy conversion of coordinates between similar microscopes whose stages cannot be aligned or when the microscopes are made by different manufacturers. This technique provides a printed grid whose value is read at one microscope and then simply relocated at the second.

Microscope Slides, Coverslips, and Mounting Media

The microscope slides, coverslips, and mounting media play a significant role in the contrast and resolution of an image. Microscope slides and coverslips should be made from high-quality glass to allow light to pass with the least generation of optical aberrations. A microscope slide with a thickness of 1.0 mm is well suited for cytogenetics microscopy. Coverslip thickness can be 0.17–0.18 mm, depending upon the recommendation of the microscope manufacturer. It is important to note that high numerical aperture lenses have a very low tolerance to variance of slide, mounting medium, and cover-slip thickness (± 0.05 mm for NAs greater than 0.7). Images that cannot be brought into good Köhler illumination are often a sign of a specimen whose thickness has exceeded the capacity of the microscope lenses.

Brightfield Contrasting Techniques

In brightfield microscopy, samples that are thin or transparent are often stained to enhance visual contrast. There are times, however, when it is desirable to observe samples without staining, such as when monitoring living cells in culture or checking the quality of chromosome spreading prior to staining. There is almost no detectable visual contrast when light passes through cells and subcellular structures, since there is little to no absorption, but the speed of light does change as it passes through them resulting in a phase shift. The human eye and digital cameras only detect changes in intensity (amplitude) of light and cannot easily detect phase shifts. By converting these very small phase shifts into large

changes in amplitude, visual contrast can be enhanced using phase contrast microscopy. Through a rather different mechanism, another technique known as differential interference contrast (DIC) converts optical path length gradients into amplitude changes observed as visual contrast.

Phase Contrast

There are two major obstacles that must be overcome in phase contrast microscopy. First, the specimen information is too dim compared to the background, and second, small phase shifts must be converted to intensity differences. Light that interacts with the specimen is diffracted, while light that passes through without interacting, termed zero-order light, contains no specimen information but adds overall brightness to the resultant image (background). The first step is to reduce the intensity of zero-order non-diffracted light. Since this zero-order light is much higher in amplitude than higher orders of diffracted light, attenuating its intensity helps in the visualization of the higher-order diffracted light.

To accomplish this, illuminating light is focused into an annulus in the condenser front focal plane resulting in a ring of parallel illuminating light exiting the condenser front element. Figure 5.3 shows the placement of the components and the light path in phase contrast microscopy. Any light that is not diffracted by the specimen, and thus contains no information, will enter the objective as parallel rays, meaning it will be focused at the back aperture of the objective. By placing a ring-shaped phase plate in the back aperture of the objective, this non-diffracted zero-order light (surround light, shown in yellow in Fig. 5.3) can be blocked, typically by 60–90%. Light that is diffracted by the specimen will be defocused at the back aperture of the objective and will be largely unaffected by the phase plate (shown in red in Fig. 5.3).

As light is diffracted by the specimen, a phase shift is also introduced that is typically a retardation of approximately $\frac{1}{4}$ wavelength. In positive phase contrast, the phase plate in the objective also advances the phase of light passing through it by approximately $\frac{1}{4}$ wavelength, resulting in a total phase difference of $\frac{1}{2}$ wavelength between diffracted and zero-order light. The diffracted light then destructively interferes with any remaining zero-order light to produce intensity variations that are observed as visual contrast, where phase objects appear darker than the background. In negative phase contrast, the zero-order light is retarded with respect to diffracted light, leading to phase objects appearing brighter than the background. See Fig. 5.4 for an example of the images created using the two variations of phase contrast.

In practical application, the phase ring in the objective and the condenser annulus must be aligned so that they overlap along the optical axis. For proper phase contrast operation, it is essential to start with Köhler illumination. Since the size of the phase ring in the objective varies with NA and

Fig. 5.3 Light path in a phase contrast system. After the illumination light passes through the condenser annulus and specimen, any non-diffracted zero-order light, termed the surround light and marked in yellow, is largely blocked and phase-shifted by the phase ring in the objective. This light represents mostly background signal with no information. Light that is diffracted by the specimen, marked in red, remains mostly unaffected by the objective phase ring (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

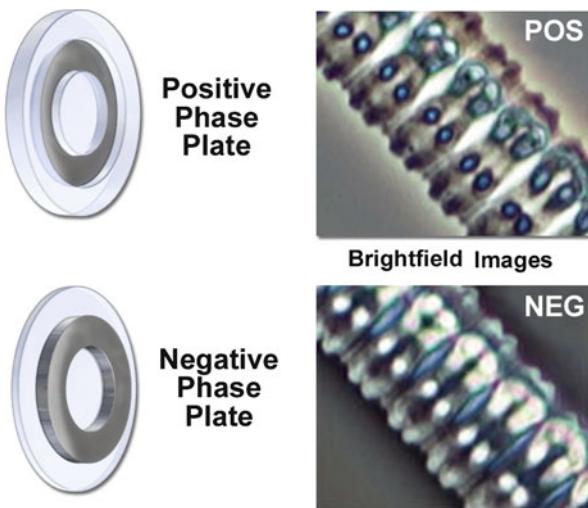
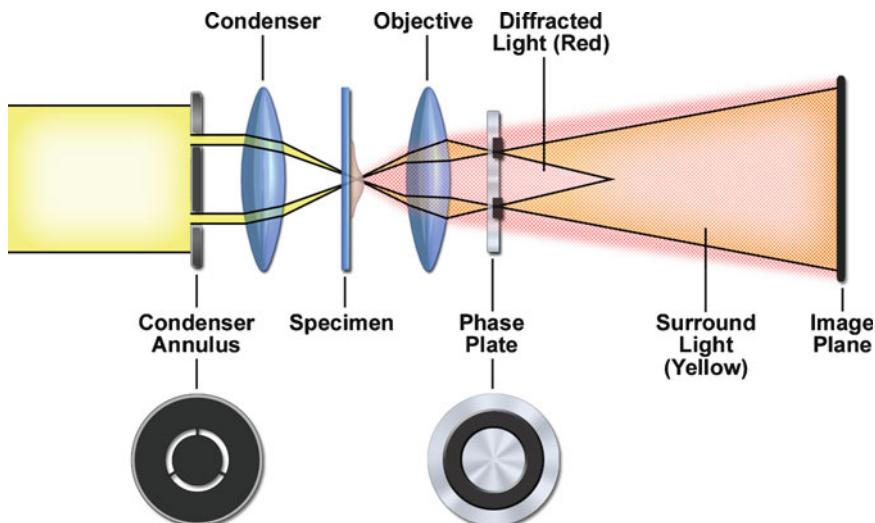


Fig. 5.4 Positive and negative phase contrast systems. Representative images created by positive and negative phase contrast systems (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

magnification, a properly sized condenser annulus must be selected. The objective will be marked on the outer barrel with an indication of the proper condenser annulus, such as Ph1, in which case a condenser annulus also marked Ph1 should be used. To align the fixed phase ring to the adjustable condenser annulus, the aperture plane must be visualized using an eyepiece telescope or Bertrand lens built into the eyepiece tube. The objective phase ring will appear as a dark circle and should overlap the illuminating ring from the condenser annulus. When the phase contrast microscope is properly aligned, very small changes in phase can be detected

allowing living cells or unstained chromosomes to be easily observed.

Since the phase ring is typically deposited on a glass plate in the objective, specific phase contrast objectives must be used. In some specialized systems, the phase ring is located in a conjugate aperture plane outside the objective so that phase objectives are not needed, but this is not typical.

Differential Interference Contrast

In differential interference contrast (DIC), constructive and destructive interference between light rays that traverse slightly different optical path lengths creates visual contrast in the image. DIC allows for detailed visualization of transparent specimens with several advantages over phase contrast, including the absence of the halo artifact sometimes associated with phase contrast, as well as the ability to create high-quality images even through relatively thick specimens.

Since plane-polarized light is required for this technique, a polarizer is placed between the light source and condenser. The plane-polarized light, which is vibrating in only one direction, passes through a birefringent prism in the condenser known as a Nomarski-modified Wollaston prism. This splits the beam into two beams that are vibrating perpendicular to each other (often termed the ordinary and extraordinary wavefronts; see Fig. 5.5). The two beams travel slightly different optical path lengths induced by specimen refractive index and thickness, and are recombined by a second prism behind the objective. As a result of phase shifts in the beams with different optical path lengths, constructive and destructive interference create light and dark areas particularly along the edges of optical path length gradients. A second polarizer (termed the analyzer) is in a crossed orientation to the first and blocks unmodified background

Wavefronts In a Nomarski DIC System

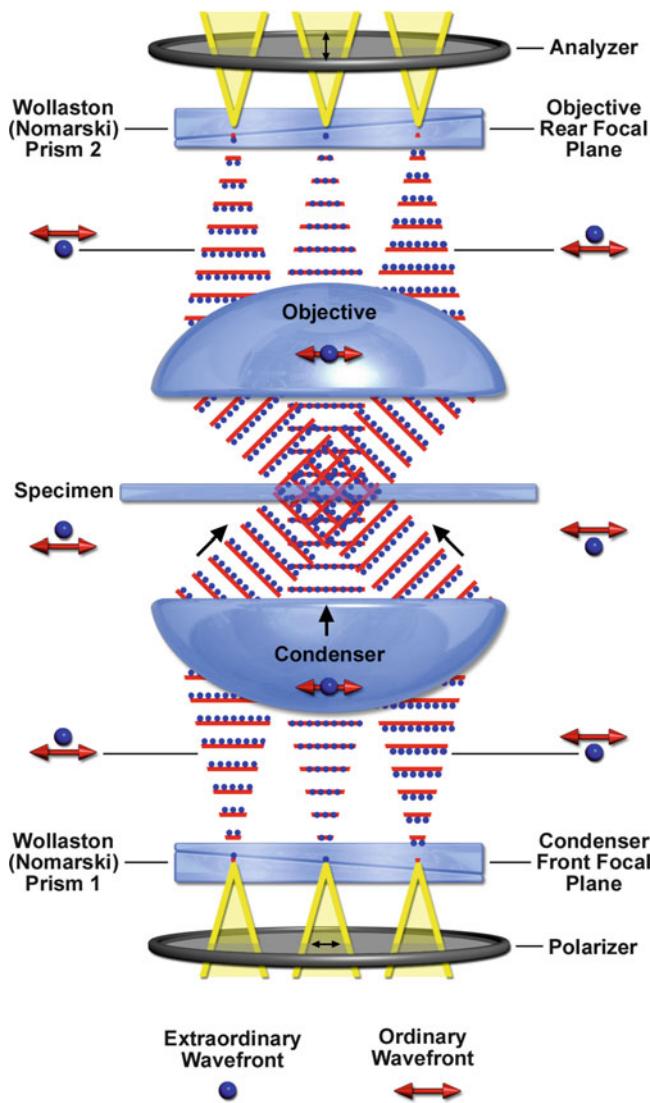


Fig. 5.5 Wavefronts in a Nomarski DIC system. Ordinary wavefronts (red arrows) showing vibration across the page are shown together with extraordinary wavefronts, represented by blue circles that are essentially arrows going into and out of the plane of the page to show perpendicular vibration to ordinary wavefronts. In the center optical axis of the system, both wavefronts propagate together. On the sides of the optical axis, one wavefront becomes advanced or retarded relative to the other, represented by either the blue trailing the red or the red trailing the blue (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

light, darkening the field, while passing elliptically polarized light that represents the specimen information. The result is a shadow-mask effect that appears as a seemingly three-dimensional pseudo-relief, although it is not indicative of an actual topographical structure.

Following Köhler illumination, the polarizer and analyzer should be crossed for maximum extinction, which is observed

as they are rotated relative to each other as a minimum of intensity. Since the required shear angle, or distance between the two beams following splitting or “shearing” by the Wollaston prism, may vary according to objective magnification, the condenser prism should be matched to the objective (check marking on the objective barrel). There are several methods that may be employed to control the level of contrast, including physically moving the objective prism relative to the optical axis (introduction of bias retardation) or using a rotating polarizer attached to a fixed quarter-wavelength retardation plate (termed de Séarnmont compensation).

For the best DIC imaging, strain-free microscope optics that are manufactured without material stresses, which could create strain-induced birefringence, should be used to avoid artifacts that can be caused by polarized light. In addition, imaging through birefringent or strained materials like the extruded plastic used for culture dishes can also produce poor results.

Epifluorescence Microscopy

The basic principle of fluorescence microscopy is that light of a particular wavelength can be efficiently absorbed by a fluorescent dye (also termed a fluorophore or fluorochrome) and emitted at a longer wavelength. The absorbed energy from incident photons raises the fluorophore molecule to an excited state. As the fluorophore returns to the ground state, this energy is emitted as a photon. The emitted photon is usually of longer wavelength than the originally absorbed photon. This change in wavelength is described as the Stokes shift.

Epifluorescence capabilities can often be added to modular brightfield microscopes with the addition of an epifluorescence illumination system. Originally used in the cytogenetics laboratory to observe Q-banded chromosomes (see Chap. 4 and Fig. 4.3), epifluorescence microscopes are now utilized in a number of molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH), and multiplex FISH (M-FISH) or spectral karyotyping (SKY). See also Chap. 17.

The Fluorescence Microscope

Unlike transmitted light brightfield microscopy, epi-illumination microscopes deliver and collect light from the same side of the sample. A light delivery path is introduced behind the objective lens, which focuses light onto the sample and collects light that returns. This illuminator may include aperture and field diaphragms for the epifluorescent light path, as well as various filters. Epifluorescence techniques typically

require relatively intense light that is filtered from white light to a specific bandwidth.

Fluorescence Light Sources

While halogen bulbs can be used, arc lamps such as mercury vapor, metal halide, or xenon are the most common light sources for epifluorescence due to their higher intensity in various wavelengths optimal for widely used fluorophores. These lamps produce an electric arc between two electrodes in a gas-filled envelope and operate under high pressure and temperature for efficiency and brightness. This requires ample ventilation. They are often contained in a housing at the rear of the microscope that may also include bulb alignment controls, adjustable reflectors, and an adjustable collector lens.

Many modern microscopes forgo the adjustable attached housing in favor of remote housing that uses a pre-centered bulb and delivers light to the illuminator through a liquid-filled light guide and collimating light adapter. These light sources eliminate the need for manual alignment of the bulb while integrating the power supply and often use extended-life bulbs. These self-contained boxes may also integrate an iris and neutral density or other filters.

Light-emitting diodes (LEDs) are gaining popularity as light sources for fluorescence work and enjoying the advantages of very long lifetimes, low heat output, and the ability to switch on and off very quickly, potentially eliminating the need for a physical shutter. However, since they are tuned by the manufacturer to a specific wavelength range with some bandwidth, they are not as flexible as a white-light source if the desired wavelength range changes.

The intensity of arc lamps is not generally directly controllable by voltage adjustment, so neutral density filters become important for attenuating brightness. Infrared filters are also often employed since even though excitation light is defined to a spectral band by an excitation filter (see “[Fluorescence Filters](#)”, next section), IR light may inadvertently pass through these interference filters. Sometimes called “heat filters,” the IR filter helps reduce background signal on a digital detector that is caused by detector sensitivity to IR light and helps to extend the life of multicoated fluorescence bandpass filters.

Fluorescence Filters

Through the use of filters, the fluorescent property of fluorophores and the resulting Stokes shift are exploited to allow for efficient illumination of the sample and collection of the emitted longer wavelength light, while blocking the collection of any returning illumination light, resulting in high specificity and signal-to-noise ratio. In this case, the “signal” is the emitted light, while the “noise” might be excitation or stray light, as well as autofluorescence, which is the property of some tissues and materials to fluoresce without

Light Path of a Fluorescence Filter Cube

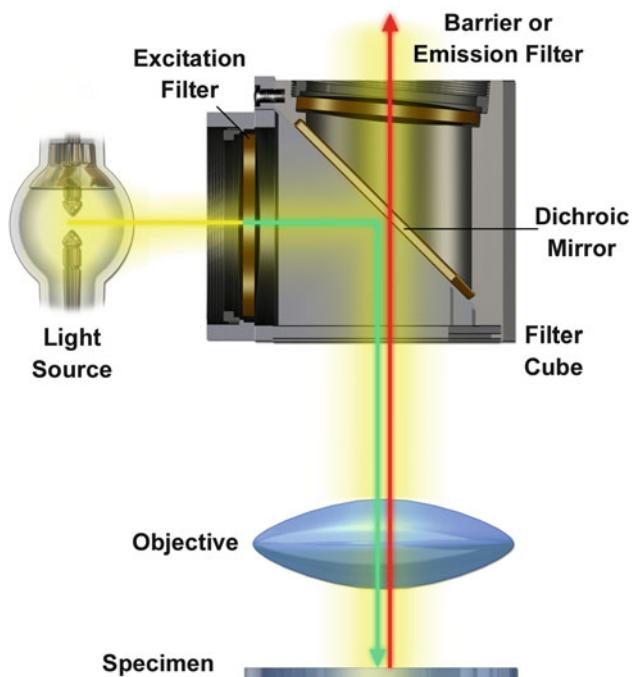


Fig. 5.6 Light path of a fluorescence filter cube. The “cube” contains three optical elements: an excitation filter, a dichroic mirror, and an emission filter. Excitation light is first filtered by the excitation filter and is reflected toward the sample by the dichroic mirror. Returning light is collected by the objective and sent back toward the dichroic mirror. Longer wavelength light produced by fluorescence passes through while any back-reflected excitation light is blocked. Light passing through the dichroic mirror is then filtered by the emission filter (Figure used by permission of Michael W. Davidson of the National High Magnetic Field Laboratory and MicroscopyU.com)

any added fluorophores, producing nonspecific emission. The typical fluorescence filter set consists of three filters: the excitation filter, the dichroic mirror, and the emission or barrier filter, in a single housing commonly referred to as a “cube” (illustrated in Fig. 5.6).

The full-spectrum light from the source passes through the excitation filter, which defines the spectral range that is efficiently absorbed by a particular fluorophore. The light is then directed toward the back of the objective by reflecting off a dichroic mirror placed at a 45° angle to the optical axis. A standard dichroic mirror is reflective to wavelengths below a certain cutoff and transmissive to wavelengths longer than the cutoff range. The objective focuses excitation light onto the specimen where fluorophores are illuminated and emit light of longer wavelength as defined by the Stokes shift. This longer wavelength light is collected by the objective and passes through the dichroic mirror and then through the emission filter. Any scattered or reflected excitation light does not pass through the dichroic mirror due to its reflective property to the excitation wavelength. The emission filter

defines a spectral band specific for the emission of the target fluorophore while blocking background autofluorescence of other wavelengths and light from any other fluorophores and secondarily blocks any excitation light that may have leaked through the dichroic mirror.

Since excitation and emission bandwidths are discretely defined, careful planning can allow multiplexing of multiple fluorophores on a single sample. Multiband filters can allow for simultaneous viewing of multiplexed fluorophores, which can also be recorded by a color digital camera. However, for reasons of efficiency and specificity, individual fluorescent colors are usually captured sequentially as “channels” by a monochromatic digital camera using single bandpass filter cubes. These channels can then be “pseudocolored” and overlaid in analysis software to produce a multispectral image.

Fluorescence Considerations

Since the amount of light emitted by fluorophores is relatively dim compared to brightfield microscopy, high NA optics are greatly preferred for fluorescence microscopy. When performing multispectral fluorescence, chromatic correction may also be of great importance to ensure that different fluorophores focus to the same plane. Phase contrast objectives are not ideal for dim fluorescence, as the phase plate will result in a significant reduction of brightness.

Special considerations may need to be made depending on the wavelengths used for fluorescence microscopy. In the case of UV or IR excitation and emission, objectives specifically made to transmit those wavelength ranges may be needed. Beam splitters used with fluorescence should direct 100% of the available light to the detector.

Background signal can greatly obscure the information of interest. To reduce this, low-autofluorescence oils should be used with oil immersion objectives. The transmitted condenser and light source do not play a role in epifluorescence and should be defocused and blocked. Stray room light can also contribute to background, so critical fluorescence imaging is often performed in a darkened room.

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Quality Control and Quality Assurance

Martha B. Keagle

Introduction

Upon receiving news that results of a chromosome analysis are abnormal (and even sometimes that they are normal), a patient will frequently ask: "How do I know that the lab didn't make a mistake? How do I know that the sample they reported on was really mine? How can I be certain that this is all correct?" Most would be surprised to learn of the myriad of checks and balances that exist in clinical cytogenetics laboratories. Based on the consensus of professionals and on common sense, The American College of Medical Genetics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories are the basis for oversight by regulatory agencies and are intended to prevent clinical and clerical errors [1]. These comprise the area of laboratory medicine known as quality assurance and quality control (QA/QC). They are supplemented by both total quality management (TQM) and complete quality improvement (CQI) programs that seek to minimize errors when the laboratory interfaces with referring physicians and their patients.

The nature of clinical cytogenetics is such that it includes both quantitative and qualitative components of tests. Some aspects are generic to practices in laboratories of any kind, while others are specific to cytogenetics laboratory tests.

A proper QA/QC program requires that policies for validation of protocols and reagents, training and credentials of individuals performing cytogenetic analysis, sample identification, safety for laboratory staff, and other compliance issues must all be in place. Laboratories are inspected periodically by various state and national entities, and most have institutional and internal regulations and guidelines as well.

There are many steps that occur between obtaining a specimen for chromosome analysis and the generation of a final clinical report. After collection of the specimen itself, accessioning, culturing, harvesting, slide preparation and staining (probe hybridization for fluorescence *in situ* hybridization [FISH]), microscopic analysis, electronic imaging, karyogram production, creation of a final report, and actual reporting of results are the path that specimens follow as they progress into and out of the cytogenetics laboratory. During this process, many variables can subject a specimen or data to a variety of conditions that must be managed for a proper diagnosis to ultimately be reached.

Central to any QA/QC program is the laboratory's standard operating procedure (SOP) manual. This often formidable document contains the policies and procedures that must be followed in order for the laboratory to perform chromosome analysis. It includes requirements of physical space and mechanical systems, specimen requirements and collection procedures, transport requirements, personnel experience and credentials, and safety and protection for personnel. It includes sections on training and compliance with the various regulatory agencies that monitor and inspect laboratories, and, finally, it may contain a section pertaining to quality assurance and quality control. The majority of these issues pertain to the analytic component of testing.

With the rapid growth of knowledge and expansion of genetic testing, the laboratory has become increasingly involved in ensuring that the pre-analytic and post-analytic aspects of testing are also designed to ensure the appropriate use of tests and their results. These commonly include issues of analytical test validation, documentation of clinical validity, interpretation of test results, and educational materials that allow the laboratories' clients to interface with it. These aspects are commonly encompassed in a complete quality improvement program.

Entire books could be written that address each of these issues in detail; entire chapters could be devoted to labels alone! Such detail is beyond the scope of this book, however. This chapter will provide an overview of the ways in which

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laboratories deal with many of these steps in order to ensure proper patient care.

Pre-analytical Testing Components

Before a test specimen arrives in the laboratory, there are a number of things that must be done correctly to ensure that an accurate and useful test result is provided. Laboratories often develop and provide materials to their clients to guide them in understanding when to test, what to test, and how to order tests. Often considered outside of the day-to-day functioning of the laboratory, these are important to ensuring safe and effective testing.

Test Validation

Prior to initiating testing, there should be evidence of clinical validation of the test. This may be done by the laboratory developing the test or may be apparent from the scientific literature and merely documented. With the advent of the 1992 modifications to the 1988 Clinical Laboratory Improvement Amendments (“CLIA ‘88”) regulations, laboratories are required to validate all tests being introduced into service whether they were newly developed or long used in other laboratories [2]. Further, all new tests must be reevaluated every 6 months. Approaches to validation vary for quantitative versus qualitative tests. Classical concepts such as sensitivity (the ability to detect a target when it is present) and specificity (the ability to not detect a target when it is not present) are common measures of analytical validity for quantitative tests. These are most often applied to FISH (particularly when interphase based) and microarray tests, (see Chaps. 17 and 18) but also are important when mosaicism is under consideration. Requirements for validation may vary with the regulatory status of a product. When a test is approved by the US Food and Drug Administration (FDA), the laboratory is expected to demonstrate that the test operates within the performance characteristics described by the manufacturer. When tests are not FDA approved or have been modified, the laboratory is expected to demonstrate their validity independently. For the more qualitative classical chromosome analysis, laboratories commonly validate their ability to process particular specimen types, to perform particular tests, or to detect a particular abnormality by testing samples from individuals with those abnormalities.

Specimen Submission

Specimens are almost always collected by individuals who rely upon the laboratory to provide a requisition form and

instructions for specimen collection and transport. Thus, quality assurance and quality control begins by interactions with the health-care providers who will collect and submit specimens for chromosome analysis.

Collection Protocol

A collection protocol from the cytogenetics laboratory is of critical importance, as it establishes the collection guidelines for individuals who are not intimately familiar with the operating procedures of the laboratory. A collection protocol should include:

- Ideal volume of specimen for collection.
- Suitable transport containers, anticoagulants, or media.
- Transport temperature and the maximum permissible transport time to ensure optimum specimen growth.
- Confirmation of the identification of the patient from whom the specimen was collected.
- Specimen container labeling and requisition form requirements.
- Laboratory hours, phone numbers, contact individuals, and after-hours procedures.

Once established, it is important to keep copies of this protocol anywhere a specimen might be collected, including a hospital’s general laboratory, departmental clinics and operating room suites, and outpatient clinics and referring physician’s offices. It is also a good idea to routinely discuss collection protocols with the appropriate individuals, especially those who do not frequently submit samples to the laboratory. Regular interaction helps promote a complete understanding of collection requirements, as well as general expectations for samples submitted for cytogenetic analysis. It also provides an opportunity to discuss questions, concerns, or suggested improvements of collection or submission procedures.

Specimen Labeling and Requisition Forms

Accurate specimen identification is one of the most important policies to implement. Specimen labels should include at least two sources of identification, such as patient name, date of birth, or a unique patient-specific number, for proper identification in the event of a labeling error.

The requisition form is equally important, as it supplies the laboratory with the patient and clinical data associated with the specimen. When Medicare is to be billed for laboratory tests and the physician believes that a portion of the laboratory charges may not be covered, the requisition (or an accompanying document) must also include an advanced beneficiary notification (ABN), which informs the patient that he or she will be billed should Medicare deny payment. Certain states or other regulatory agencies also require that informed patient consent be part of, or accompany, the requisition form.

For obvious reasons, it is desirable to have a properly completed requisition (paper or electronic) accompany each

specimen submitted to the laboratory, but it is also important for the laboratory to develop a policy for dealing with specimens that are not accompanied by a requisition, or for requisitions that have not been filled out completely. Of special importance are those requests for chromosome and/or FISH analysis that are made verbally with the laboratory. In these instances, it is important for the laboratory to obtain written or electronic authorization for the study. The provision of sufficient clinical information to ensure that appropriate tests and analyses have been requested is a valuable cross-check.

Rejection Criteria

It is very important for individuals to clearly understand the minimum requirements for submission of a specimen for chromosome analysis, FISH, or arrays, and what circumstances would prevent a laboratory from performing analysis. The collection protocol and requisition forms should clearly state these requirements. Although extremely rare, circumstances can arise that prevent a laboratory from accepting a specimen for analysis.

In the event of a problem with a sample, the laboratory should make immediate contact with the individual submitting the specimen, either to obtain clarification of the specimen identity or to discuss potential difficulties in obtaining a result. In most instances, both parties will elect to proceed, knowing that the success of the analysis may be impacted. In some instances, the problems are insurmountable, and a repeat sample is needed. When this occurs, it is a requirement for the lab to carefully document the reason for rejection or failure, as well as disposition of the specimen in the patient report and appropriate log.

Analytical Testing Components

The analytical phase of testing includes the actual processing and analysis of the specimen. Although specimen accessioning are often considered pre-analytical, it is included here because labeling and tracking of specimens through a test is among the most common causes of error in clinical laboratory testing. This phase usually ends when a laboratory test result is apparent.

Specimen Accessioning

Once a specimen has been received, an accession process is used to log it into the laboratory and to prepare it for analysis. During this time an accession and/or laboratory number is assigned to a specimen, relevant patient and clinical data are entered into a logbook and/or database, and the culture and analysis requirements for the studies requested are identified.

Assessing the Condition of the Specimen and Requisition

After receipt of the specimens in the laboratory, the individual responsible for accessioning specimens must check the sample and requisition for the appropriate labels, transport reagents (medium, anticoagulants, etc.), specimen condition (color, clotted, adequate sample size, transport temperature, etc.), and date of collection. When a problem is detected, the individual should follow the laboratory procedure for informing the “submitter” of the specimen and take appropriate actions. Problems with the specimen and action taken might also be documented.

Accession Numbers and Patient Database

It is important to assign a unique identifier to each specimen as it enters the laboratory, distinguishing it from other specimens, as well as from a patient's previous studies. The lab number, patient data, and clinical information are then often transferred into a logbook or electronic database, creating a patient record that can be tracked and cross-referenced against previous and/or future studies. In addition, other data can be entered into a database record as a study progresses, allowing the laboratory to track:

- Culture conditions
- Results
- Turn around times (TATs)
- Dates of specimen receipt, processing, and report
- Individual(s) issuing reports
- Cytogenetic results versus the findings of patients with similar histories or abnormalities (interpretation of results)
- Culture failures, labeling errors, transcription errors, mis-diagnoses, and actions taken
- Incidence of submission problems

Electronic databases need to be managed within the laboratory to ensure the accuracy of the data as well as patient confidentiality.

Once a specimen has been logged into the cytogenetics laboratory, it must be prepared for cell culture. This may include notification of appropriate individuals of its receipt, creation of culture records and container labels, and creation of a patient folder or file for paper records. If the sample is not set up immediately, it needs to be stored under appropriate conditions.

There should be a system for identifying specimens that require special handling such as an accelerated study, a preliminary report, or a completion by a certain date to meet anticipated turn around times. These requirements should be clearly indicated on all appropriate forms and/or computer fields, and all individuals involved with the study should be notified.

Specimen Labels

The accuracy of any laboratory result requires correct specimen labeling. After the initial accessioning process, a number

of items need to be labeled, including a culture worksheet; culture flasks, tubes, or Petri dishes; microscope slides; a microscope analysis worksheet; metaphase prints; karyograms; FISH images; and reports. The laboratory labeling policy should allow patient identification to be cross-checked in the event of a labeling error.

Specimen Culture, Harvesting, Slide Preparation, and Staining

All equipment and supplies used for culture and harvesting of cells, preparation of slides, and banding and staining of chromosomes should be monitored in order to provide high-quality analyses.

Cell Culture

Whenever possible, duplicate or independently established cultures should be created for all samples, and these should be placed in separate incubators, each equipped with its own power, CO₂ source (if utilized), and emergency alarm. A backup procedure must also be created that ensures that cultures will be maintained in the event of a power (emergency generator) or CO₂ (automatic gas tank supply change) failure.

Precautions to prevent contamination should be taken when a specimen is added to culture medium, a culture is transferred between containers, or reagents are added to a specimen culture. Working with specimens within the area of a laboratory designated for biological hazardous materials and using sterile technique in laminar flow hoods will greatly reduce the risk of bacterial contamination of the specimen and exposure of staff to biohazards. In addition, using latex gloves, cleaning work surfaces with alcohol before and after use, and exposing container openings, pipettes, or other measuring devices to a flame will reduce the likelihood of contamination.

Working with one specimen at a time and disposing of all used pipettes or containers that come into contact with a specimen (before moving onto the next) will greatly reduce the likelihood of cross contamination or improper identification. It is also important to note that the transfer of reagents into a culture should be performed using a fresh pipette when there is any risk of contact with a specimen or specimen aerosol.

Culture Protocols

Cell and tissue culture begins with a protocol that outlines tested and reproducible steps to produce cells and metaphase chromosomes for analysis. The quality control of new reagent lots and changes in established protocols should be completed prior to their use with patient specimens. For critical reagents that may be of variable quality from manufacturer

to manufacturer or from lot to lot (such as serum), prepurchase testing of multiple lots can ensure that the highest-quality reagent is available to the laboratory. The methods of QC testing should be appropriate to the reagent and method being tested and may include parallel testing of the current validated reagents/devices against the new lots of reagents/devices using nonclinical control specimens or reference materials. It is also important to track the history of protocol modifications, allowing a comparison of past culture techniques and successes. The format of a culture protocol should comply with the requirements of the agency used for laboratory accreditation.

Equipment Maintenance

Consistency and reliability of laboratory procedures cannot be accomplished without well-maintained equipment, and there are many regulations that reflect this.

Refrigerators, freezers, and water baths should be closely monitored daily for temperature and cleaned following regular schedules. Centrifuges should be monitored for accurate speed semiannually. Laminar flow hoods should be cleaned before and after use and be equipped with an antibacterial light or cover to prevent contamination during periods of nonuse. Biological safety cabinets also should be checked and certified annually for airflow and bacterial contamination, and pH meters should be cleaned and calibrated regularly. Balances should be kept clean of laboratory reagents and calibrated regularly to ensure proper weight measurements. Ovens need to be monitored daily for temperature. Trays for slide preparation and storage should be kept clean to reduce chemical contamination of staining reagents.

Incubator temperature and gas (CO₂) concentration should be monitored continuously and documented daily. Incubators should be on a regular cleaning schedule and, as discussed earlier, should also be equipped with separate power and gas sources, as well as emergency alarms. Incubator gas and power supplies should also have a backup in the event of a failure, and the laboratory should maintain an emergency plan in the event of complete incubator failure. Records of equipment monitoring and maintenance should be documented in an equipment log.

Automated harvesting procedures are used by many cytogenetics laboratories as a way of increasing laboratory productivity and improving consistency (see Chap. 7). However, automation does not imply “carefree.” Laboratories that utilize such technology must strictly follow the manufacturer’s recommended operational guidelines and closely monitor the equipment for acceptable performance. A procedure for the use of automated equipment that details the procedural steps for operation, appropriate reagents, calibration and cleaning requirements, and preventive maintenance must be prepared. It is also important for individuals operating the

equipment to receive proper training before using it on clinical specimens.

Harvesting, Slide Making, and Staining

The transition from cell/tissue culture to microscopically analyzable chromosomes is achieved by harvesting the dividing cells (which involves mitotic arrest, osmotic swelling of cell membranes, and fixation), spreading of the chromosomes on microscope slides, and staining the chromosomes with one of various methods which produce an appropriate banding pattern (see Chap. 4). Each of these steps must be optimized to facilitate correct diagnoses.

Protocols

After cells have been successfully cultured, the techniques of harvesting, slide making, and banding/staining will determine the ultimate quality of the metaphase chromosomes available for analysis. Following validated protocols is very important for these procedures, but frequent modifications may be required to address changing laboratory conditions. It is important to note that these procedures can be especially sensitive to individual technique, particularly fixation and slide making, and that mastery of these skills requires individuals to observe and document minor variations in procedure or laboratory conditions that improve or detract from chromosome morphology.

New protocols, procedural changes, introduction of new reagents, reagent concentrations, microscope slides, etc. must be validated under controlled conditions. The method of validation should be one that is appropriate for the reagent or technique being tested and may include parallel testing of current versus new, testing on nonclinical control specimens, or direct analysis using reference materials. It is also important to track the history of harvesting, slide preparation, and staining protocol modifications in order to allow a comparison of past techniques to present successes. Documentation of proactive and reactive factors from these procedures is important to ensure quality metaphase chromosomes, as well as to identify and track problems that reduce specimen quality.

Slide Preparation

The chromosomes present in harvested metaphases must be spread apart so that they can be microscopically analyzed. They must lie flat so that staining is uniform and all chromosomes are in a single plane of focus, and they must be aged (literally or artificially) in order for most banding and staining procedures to work properly.

Even when all else has gone well with the tissue culture and harvesting procedures, poor slide preparation can result in scarce, poorly spread, or improperly aged metaphase spreads for staining and microscope analysis. The following variables should be considered:

- Harvesting method (centrifuge tubes vs. *in situ* processing) (see Chap. 4)
- The humidity and temperature of the laboratory or drying chamber utilized (see Chap. 7)
- The number of fixations and the method of fixing the specimen
- The slide temperature
- Wet or dry slides? How much water?
- The angle of the slide during specimen application
- The method of applying the specimen
- The method of drying the slide
- The slide-aging technique

Each of these factors can significantly contribute to the success of slide preparation. As these can be variable from day to day and between individuals, close observation and documentation of technique may allow the highest proficiency of these skills.

Banding and Staining

While slide preparation and aging are important factors contributing to the lab's ability to successfully stain a specimen, adjustments to solution concentrations, the time slides are left in the staining solution, etc., can also influence successful staining of cytogenetic samples. Careful preparation of reagents and documentation of adjustments made to staining procedures help the laboratory personnel to refine their techniques.

The shelf life and storage conditions of banding and staining reagents are important considerations and should also be documented in a staining log. As reagents arrive in the laboratory, lot numbers should be recorded and compared with previous lots used. Reagent containers should be labeled with the reagent name, quantity, concentration, storage requirements, date received, and expiration date. Reagents that require refrigeration should have minimum and maximum permissible temperatures documented, and these should not be exceeded. Existing supplies of reagents should be rotated so that they are depleted before new supplies are used.

Although good specimen staining is critical for optimal microscope analysis, it is also necessary to consider the microscope on which a specimen will be analyzed and the staining requirements of the recording medium. When a laboratory has a variety of microscopes, each may have a light source, contrast or interference filters, objectives, or other lenses that produce images with a unique set of visual characteristics. Additional variables, such as excitation and barrier filters, are introduced with the use fluorescence microscopy, and features such as the numerical aperture of lenses or bulb intensity may be critical (see Chap. 5). Individual preference is also an important factor in identifying a staining intensity that is well suited for microscope analysis.

When accessing the quality of banding in G-banded images, it is important to identify staining intensities that produce:

- Chromosome pale ends that contrast well against background areas
- A wide range in mid-gray intensity
- Dark bands in close proximity that appear as distinct bands

Comparing the requirements of the individual performing the microscope analysis against the requirements of the recording media and documentation of ideal conditions in a staining log will help laboratories gain control of the many variables of a staining procedure.

Specimen Analysis

Any chromosome analysis begins by identifying the specific requirements for the specimen type being examined. Following this, the basic steps are: the microscope analysis itself (location of metaphase spreads suitable for analysis, counting the chromosomes and determining the sex chromosome complement, and analysis of the band pattern of the individual chromosomes), imaging of the metaphase spreads, preparation of karyograms, and documentation and reporting of results. The procedure begins with a protocol that must be accessible and thoroughly understood by all individuals performing chromosome analysis. An analogous process is required for FISH studies.

Analysis Protocols

An analysis protocol must identify the general requirements for each specimen type. The protocol should identify normal parameters and normal variants and should distinguish between true abnormality and artifact. The number of cells from which chromosomes are to be counted and the sex chromosome complement identified and analyzed in detail (band-for-band) must be clearly stated, including whether each type of examination is to occur at the microscope, on an image, or via a karyogram. A protocol should set standards for the selection of suitable metaphase spreads, as well as the number of cultures (and colonies, when applicable) from which cells should be examined. When an abnormality is detected, the appropriate steps to take should be specified. Other things, such as an appropriate banding resolution level, maximum allowable number of overlapping chromosomes, random chromosome loss, and dealing with metaphases in close proximity, might also be included.

A protocol should identify the procedures used to document each metaphase, as well as the data to be recorded on a microscope analysis worksheet, requirements for imaging, the number of cells to create karyograms from, the number of individuals who should take part in performing the analysis, and the individual who should verify the results. Finally,

a protocol should establish the policies for the storage of microscope slides and retention of images, both during analysis and once analysis has been completed.

Personnel Requirements

The experience level, credentials, and workload of each technologist are all important considerations, and the laboratory must be appropriately staffed to allow for complete, accurate, and timely results of all samples received. When possible, it is often recommended to split the analysis of a specimen between two individuals in some way, increasing the potential for detection of a subtle abnormality.

Establishing goals for individuals or groups to meet, such as turn around time and the number of cases to be completed in a week, is an important aspect of effective laboratory management. The quality of analysis should not, however, be sacrificed in the attainment of these goals, and performance monitors should include frequent statistical analysis of failure rates and percentage of abnormal cases.

Microscopy

A significant part of quality microscopy lies in the training an individual receives on the components of a microscope and their proper use. Any protocol for microscopy should therefore include training of personnel in the use of microscopes, quality checks to identify equipment in need of service or adjustment, and identification of individuals in need of additional training.

The selection of microscopes for analysis and documentation of results (image production) is also a very important consideration. It is not unusual for a laboratory to have microscopes of various quality grades, and users need to understand the limiting factors of any given scope. “Newer” does not necessarily imply “better,” and many “veteran” microscopes produce excellent images. It is often the resolution of the objective (lens), not extraneous accessories, that is the key to image clarity. Also, good images are more likely to come from well-prepared microscope slides. Controlling the slide preparation process and using a microscope with the appropriate lenses and features will promote quality cytogenetic analysis and image documentation. For additional details on microscopy, see Chap. 5.

General Analysis Requirements

Analysis requirements have evolved as a mix of “conventional wisdom” and statistically validated needs for specific types of studies. Professional organizations have developed consensus-based standards for different types of analyses (e.g., The American College of Medical Genetics (ACMG) 2009/revised 2010 [1]), and regulatory bodies have typically used these as guides when specifying minimum requirements for each sample type processed for chromosome analysis. Individual laboratory protocols and individual

state requirements frequently augment these. It should be noted that minimum requirements are just that; the standard of care frequently requires more rigid guidelines. It must also be remembered that most listed standards apply to chromosomally normal samples. Once an abnormality has been discovered, it is important to confirm its presence or absence in each cell examined and to identify additional procedures that may be necessary for correct diagnosis. It is also important to realize that a patient's clinical indications may dictate that analytical resolution should be higher than the stated minimums.

The following are some general guidelines for constitutional chromosome studies for different specimen types:

PHA-Stimulated Blood (Non-neoplastic Disorders)

At least two cultures should be established for each sample. The chromosome count and sex chromosome complement should be determined for at least 20 cells. If a mosaic sex chromosome abnormality is suspected and confirmed in the 20-cell analysis, no additional counts are required; however, if not confirmed in the standard 20-cell evaluation, a minimum of ten additional metaphases should be examined. At least five metaphase cells should be completely analyzed, and at least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each. A minimum band resolution of 550 should be the goal for constitutional studies, but greater resolution may be required for focused studies of specific chromosome pairs.

Although guidelines for the diagnosis of fragile X syndrome via cytogenetic analysis did at one time exist, current standard of care now involves analysis via molecular methods (see Chap. 19).

Amniotic Fluid, *In Situ* Method

The chromosome count and sex chromosome complement should be determined for one cell from each of at least 15 colonies. As many colonies as possible should be examined when a true mosaic condition is detected or, in some cases, to clarify pseudomosaicism. Cells must originate from at least two independent cultures (from more than one sample syringe or tube, when possible). At least five metaphases from independent colonies should be completely analyzed, and at least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each. The band resolution should be as high as possible and should not be less than 450.

Amniotic Fluid, Flask Method

The chromosome count and sex chromosome complement should be determined for at least 20 metaphases from at least two independent cultures as described earlier. Other requirements are the same as for the *in situ* method.

Chorionic Villus Samples

Many laboratories examine cells from both "direct" (uncultured) and cultured preparations, but in clinical use, uncultured preparations should only be used if a culture technique of 48 h or more is also used. Band resolution should be as high as possible.

For cultured preparations, the chromosome count and sex chromosome complement should be determined for at least 20 cells, distributed as widely as possible from at least two independent cultures. At least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each.

For combined direct/cultured preparations the chromosome count and sex chromosome complement should be determined for a minimum of 20 cells, at least 10 of which are from the cultured preparations. Additional cells should be examined when mosaicism is detected, particularly when there are discrepancies between the direct and cultured preparations, which are often an indication of confined placental mosaicism (see Chap. 11).

At least five metaphases (four from cultured material, if possible) should be completely analyzed, and at least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each. A resolution of at least 450 bands should be obtained if possible.

Percutaneous Umbilical Blood Sampling (PUBS)

A minimum of two cultures from fetal blood should be established if there is adequate sample, and harvests at 48 and 72 h are recommended. At least five metaphase cells should be completely analyzed, and at least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each. The fetal origin of the sample should be confirmed.

Solid Tissue (Non-neoplastic Studies)

At least two cultures should be established for each sample. The chromosome count and sex chromosome complement should be determined for at least 20 cells from at least two independent cultures. If a mosaic sex chromosome abnormality is suspected and confirmed in the 20-metaphase analysis, no additional counts are required; however, if not confirmed in the standard 20-cell evaluation, a minimum of ten additional metaphases should be examined. At least five metaphases should be completely analyzed, and at least two karyograms should be prepared. If more than one cell line is present, at least one karyogram must be prepared from each.

The following are some general guidelines for analysis of acquired chromosome abnormalities for different specimen types:

Bone Marrow and Unstimulated Peripheral Blood

Bone marrow is usually the sample of choice for the study of premalignant and malignant hematologic conditions. If an adequate bone marrow aspirate cannot be obtained, an unstimulated peripheral blood study may yield satisfactory results if the circulating blast count is greater than 10–20%.

Guidelines vary by the type of study, and the reader is referred to the ACMG Standards and Guidelines (ACMG 2009/revised 2010) for more specific information [1]. In general, thorough, for initial diagnostic studies, examination of 20 consecutive cells from unstimulated cultures is recommended, when possible. Metaphase selection should *not* be based on good chromosome morphology. All 20 cells should be fully analyzed. Attempts should be made to count and identify structural abnormalities in cells skipped because of poor morphology.

If one abnormal clone is present, two karyograms should be created. If more than one related abnormal clone is present, two karyograms from each stemline and one from each sideline should be prepared. If unrelated clones are present, two karyograms from each stemline and one from each pertinent sideline should be generated. If only normal cells are present, two karyograms should be made. If both normal and abnormal cells are present, only one karyogram from the normal cell line is required.

For follow-up studies of patients with previous G-band studies who have not received an allogeneic hematopoietic cell transplant, 20 cells should be analyzed. If all cells are normal, additional cells may be evaluated by G-banding or FISH for a specific abnormality.

For follow-up studies of patients who have had a hematopoietic cell transplant for whom donor versus recipient cells can be distinguished by sex chromosome complement or cytogenetic polymorphisms, 20 cells should be analyzed (note: FISH or molecular methods are more sensitive than G-banding for determining engraftment status and should be used in preference to G-banding in such cases).

If only donor cells are present, two karyograms should be prepared.

If donor and recipient cells are present, all recipient cells of the 20 should be analyzed fully for previously identified clonal abnormalities and for any newly acquired abnormalities. Analysis or scoring of additional recipient cells may be indicated, depending upon the number of recipient cells in the initial 20 cells. For the recipient cells, two karyograms of the stemline and one of each sideline, if present, should be prepared. For donor cells that have been previously documented, one metaphase image should be captured; if donor cells have not been previously documented, two karyograms should be generated.

If only recipient cells are present, 20 cells should be analyzed fully for previously identified clonal abnormalities and for any newly acquired abnormalities. Two karyograms of

the stemline and one of each sideline present, if any, should be prepared.

For follow-up studies of patients who have had a hematopoietic cell transplant for whom donor and recipient cells cannot be differentiated by G-banding, 20 cells should be analyzed fully for previously identified clonal abnormalities and for any newly acquired abnormalities. Two karyograms of the stemline and one of each sideline should be produced.

Lymph Nodes

The number of cultures established should be based on the apparent cellularity of the sample, but if lymphoma is part of the differential diagnosis, the cultures should include at least one unstimulated, 24-h suspension culture. Selection of cells for analysis should *not* be based on good chromosome morphology. Twenty metaphase cells should be analyzed whenever possible. Since lymphomas can have very complex karyotypes, and thus can be very labor intensive, some laboratories may choose to perform an abbreviated study (usually at least ten cells) when the abnormal clone has been characterized. Documentation guidelines are the same as for neoplastic blood and bone marrow specimens. If there is a confirmed diagnosis of lymphoma and only normal metaphases are seen on an analysis of 20 cells, additional analysis/scoring by G-banding or FISH for specific lymphoma-related chromosomal abnormalities may be appropriate.

Solid Tumors

Cytogenetic evaluation of solid tumors can be performed to establish a diagnosis, to assess prognosis, or for patient management. Since cytogenetic abnormalities in pediatric tumors are often disease-specific and may have prognostic value, cytogenetic evaluation is recommended whenever sufficient tissue is available. FISH analysis may also be used as a primary or secondary method of evaluation when a rapid diagnosis is needed. Cytogenetic analysis of adult tumors should be based on diagnostic and prognostic value.

Culture methods should be chosen based on the type of tumor submitted. Non-small cell round tumors grow better as attached cell cultures (flask or *in situ*), and small cell round tumors (SCRT) are usually best grown in suspension culture, but SCRTs will also grow in monolayer; thus, if sufficient material is available, both types of culture should be established.

Analysis of 20 metaphase cells or sufficient numbers to characterize all abnormal clones and subclones should be performed. If there are only normal cells, or if there is one abnormal clone, two karyograms should be made. If there is more than one related abnormal clone, two karyograms of the stemline and one of each sideline should be prepared. If unrelated clones are present, two karyograms from each

stemline and one from each related sideline should be made. If both normal and abnormal cells are present, one karyogram should be prepared from the normal cells, plus karyograms from the abnormal clones as previously described.

Supplemental FISH studies can be used to provide rapid results to aid in making the differential diagnosis, for planning therapy, for appraising prognosis, or for ruling out cryptic aberrations in solid tumors. FISH can also be used longitudinally once a baseline FISH pattern has been established in the initial study. FISH is also useful when there are inadequate cells for G-band analysis in cases where there is a suspected diagnosis for which a recurring abnormality is known, or when conventional cytogenetics studies are normal.

The following are some general guidelines for metaphase FISH (including repeat sequence and whole chromosome probes):

Applications for metaphase FISH include evaluation/identification of marker chromosomes and material of unknown origin attached to or within a chromosome, clarification and characterization of structurally rearranged chromosomes, detection of losses or gains of chromosome segments (including microdeletions), and detection of multiple cell lines.

Metaphase FISH is considered adjunctive to conventional cytogenetics in most circumstances; it only provides information about the probe(s) in question and does not replace a full cytogenetic evaluation.

Regulatory requirements for different metaphase FISH probes vary based on the origin of the probes and their FDA approval status (refer to the ACMG Standards and Guidelines for specific information [1]).

Probe localization and analytic validation, including sensitivity and specificity, must be established for all metaphase FISH probes (refer to the ACMG Standards and Guidelines for further details [1]). In a laboratory that has attained 98% sensitivity during its internal test validation, a minimum of five cells should be evaluated when characterizing non-mosaic marker chromosomes or unknown chromosomal regions within or attached to a chromosome. For non-mosaic microdeletions, a minimum of 10 cells should be evaluated. If any discordant cells are found, additional cells from a second slide should be examined.

Since the probes in whole chromosome cocktails are not always uniformly distributed along the entire length of chromosomes, caution must be used when interpreting results in target regions of small size. Care must also be taken when interpreting negative results of repeated sequence probes studies since, infrequently, some individuals have small numbers of the target sequence. Additionally, results of metaphase FISH for confirmation of microdeletions in which the probe is not specifically for the gene in question need to be interpreted carefully.

Metaphase FISH results should be confirmed by at least two persons with experience evaluating FISH samples; one may be the laboratory director. At least two images should be maintained by the laboratory.

The following are some general guidelines for interphase/nuclear fluorescence in situ hybridization:

Applications for interphase FISH include detection of numerical abnormalities, duplications, deletions, chromosomal rearrangements, constitutional sex chromosome complement, mosaicism (with proper caution), and gene amplification. The specificity and limitations of the probes used must be considered when interpreting results.

Interphase FISH only provides information about the probe(s) in question and does not replace a full cytogenetic evaluation. If the suspected abnormality can also be detected with conventional cytogenetics, confirmatory chromosomal analysis should be performed, although the interphase FISH result may be issued as a preliminary finding. If the interphase FISH study is for disease monitoring, adjunctive conventional cytogenetics may not be necessary.

Regulatory requirements for different interphase FISH probes vary based on the origin of the probes and their FDA approval status (refer to the ACMG Standards and Guidelines for specific information [1]).

Probe localization and analytic validation, including sensitivity and specificity, must be established for all interphase FISH probes (refer to the ACMG Standards and Guidelines for further details [1]). Establishment of databases and reportable reference ranges and biannual calibrations are required.

Whole chromosome probes and probes for whole chromosome arms are not appropriate for interphase FISH analysis. When multiple probes are used concurrently, different fluorochromes should be used to enable their differentiation. Care must be taken when interpreting results of repeated sequence probes studies since, infrequently, some individuals have small numbers of the sequence target. Normal results of interphase FISH for detection of microdeletions or micro-duplications in which the probe is not specifically for the gene in question need to be interpreted carefully, and a disclaimer should be included in the written report.

A minimum of 50 nuclei should be scored (split between two independent readers); many laboratories routinely examine 200 or more nuclei for each probe, especially for oncology diagnoses. Additional nuclei may need to be scored if there is significant discrepancy between the two readers, if a result does not meet the laboratory's established reporting ranges, or if mosaicism for a constitutional abnormality is suspected.

Interphase FISH results should be confirmed by at least two persons with experience evaluating FISH samples; one may be the laboratory director. At least two images should be maintained by the laboratory.

The following are some general guidelines for multi-target FISH tests:

FISH tests developed to analyze several chromosome loci in a single test format provide information only on the specific probe loci used and are not a substitute for complete karyotypic analysis. Abnormalities detected by multi-target FISH should be confirmed by another method when possible (G-banding, locus-specific FISH, etc.).

Regulatory requirements for different interphase FISH probes vary based on the origin of the probes and their FDA approval status (refer to the ACMG Standards and Guidelines for specific information [1]).

Probes used in multi-target FISH tests must be validated for localization, sensitivity, and specificity. Established databases and reportable reference ranges and biannual calibrations are required for multi-target probes that include interphase FISH probes.

The following are some general guidelines for microarray analysis (constitutional studies):

CGH- and SNP-based arrays can be used to detect copy number gains and losses resulting from aneuploidies and unbalanced structural chromosomal abnormalities. They will not detect balanced structural rearrangements, some ploidy changes, or single gene abnormalities. CGH-based arrays will not detect uniparental disomy, but this can be detected with SNP-based arrays. Additionally, array CGH may not detect low-level mosaicism for aneuploidies and unbalanced structural rearrangements.

Arrays can be used as adjuncts to conventional cytogenetics and targeted FISH or as a primary diagnostic test.

There are various types of arrays that can be used in the clinical laboratory (FDA approved, for investigational use only, for research use only, and “home brews”), some of which can be purchased commercially, and others that are developed within the laboratory itself. Each type may require different levels of validation depending upon their intended use (refer to the ACMG Standards and Guidelines for a more complete discussion [1]). The laboratory must also demonstrate expertise in array performance and analysis.

The laboratory should have a laboratory procedure manual that includes written protocols for DNA extraction, labeling, and quantification; obtaining adequate DNA quality and quantity; proper fragmentation; and fluorescent labeling. These should be documented in each patient record. The laboratory should also have protocols for confirmation of abnormal or ambiguous array findings (G-band karyotyping, FISH, PCR, etc.).

Both male and female control samples should be established, and the laboratory should have guidelines indicating how they are to be used in same-sex and opposite-sex comparisons. Controls usually have normal karyotypes, but there are circumstances when it is appropriate to use controls with

a particular karyotypic abnormality. The laboratory should also have a written protocol to determine whether any regions covered by the array represent known regions of copy number variants. The finding of abnormal copy number in such regions should be characterized by another method (FISH, parental studies, quantitative PCR, etc.).

Follow-up studies (FISH, chromosome analysis, arrays, etc.) of biological parents or other family members may be indicated when an abnormality is detected by arrays to rule out balanced rearrangements, inherited duplications or deletions, or for interpretation of findings of uncertain clinical significance.

Analysis Worksheets

Laboratories routinely use some form of worksheet to document microscopic analysis data. This is the technologist’s working document but becomes part of the patient’s permanent laboratory chart, and as such serves as an additional clinical and clerical cross-check.

The analysis worksheet typically includes patient data (patient name, laboratory accession, and case numbers), indication for study, and specimen type. The identification of each slide examined should be verified, and previous studies might be noted. The technologist(s) performing the analysis and the date of analysis should be recorded. The microscope being used is often indicated, and microscopic coordinates are recorded for each metaphase examined, along with other data (slide number, culture of origin, banding method, and identifiers for relocating the cell). The number of chromosomes and the sex chromosome complement is typically noted, along with other relevant data such as quality of banding, abnormalities, polymorphisms, chromosome breakage, whether the cell was analyzed and/or imaged, which cells should be considered for karyotyping, etc. Finally, a summary of the results, including the patient’s karyotype, can be included, along with documentation that the entire case has been reviewed for clerical accuracy prior to release of the final report.

Imaging Systems and Karyogram Production

During or upon completion of the microscopic analysis, selected metaphases must be imaged and karyograms must be created and printed.

Historically, photomicroscopy was used to capture images of metaphases, darkroom techniques were used to make photographic prints, and the chromosomes were cut out with scissors and manually arranged on the karyogram form.

Today, electronic tools are used to record microscope images and create karyograms. Saved images document the findings and allow the chromosomes to be reanalyzed as necessary. Understanding how to operate, optimize, and maintain the materials and equipment used in the imaging process

is necessary in order to achieve optimum results from any sample.

Computer-driven imaging systems are essentially the digital equivalent of photography. Instead of photographing a metaphase, it is electronically captured in digital form, and instead of developing film and using filters to produce prints with the appropriate contrast and background, the image is electronically enhanced to achieve a similar appearance, and a printer provides a hard copy if needed. Finally, images are stored not as photographic negatives, but as digital files on optical disks, DVD-R, or other digital storage media. Many laboratories store images on a server. An understanding of the theory and hardware, and appropriate training, is requisite to utilizing an imaging system properly and efficiently (see also Chap. 7).

Karyogram Production

The final laboratory manipulation required for chromosome analysis is typically generation of the ordered arrangement of chromosomes known as a *karyogram* (while the term *karyotype* was historically used to refer to both the nomenclature describing the chromosomal complement *and* the systematized array of the chromosomes, in 2005 the ISCN committee recommended that the term *karyogram* be used for the systematized array of chromosomes and that the word *karyotype* be restricted to describing the nomenclature of the chromosome complement. See also Chap. 3).

If there was ever a perfect example of the value of training in laboratory medicine, it is this process. A bright individual with a modest comprehension of the theory behind cytogenetics and essentially normal pattern recognition and motor skills can be taught the normal human chromosome identification well enough to perform this task in about a week. Yet, the comment most often made by visitors to a cytogenetics lab is typically: “These chromosomes all look alike. How do you tell them apart? I’d never be able to do that.” In reality, all that is required is a sufficient number of images for repeated attempts, plus sufficient patience on the part of the individuals doing the training. By making attempt after attempt (and receiving the appropriate corrections each time), the novice eventually begins to recognize certain pairs and then eventually all pairs. Mastery of the subtleties sufficient to perform actual microscopic analysis, of course, requires much more training, but in many laboratories, technicians, lab aides, interns, or students are often employed to generate karyograms. When such adjunct personnel are used, a good rule to follow is that no such individual be permitted to create karyograms for an entire case without supervision or review by a trained technologist.

Karyogram production is one method laboratories can use to divide analyses between two or more technologists. A guideline that specifies that the technologist(s) who per-

formed the microscopic analysis cannot prepare or review the karyograms for that patient assures that multiple individuals see every case (an exception to this rule can be made for abnormal cases, particularly for oncology samples, where the technologist who performed the analysis has a better feel for the abnormalities that are present and the risk of missing an abnormal karyotype is no longer a concern). Preparation of karyograms by appropriately trained individuals who are not technologists as described earlier also accomplishes this. When review by the laboratory supervisor or another senior individual followed by final review by the laboratory director is added, a well-designed protocol can ensure that at least four or more trained “pairs of eyes” examine chromosomes from every patient, increasing the likelihood of detecting a subtle abnormality or clerical error.

A special consideration in this area involves the use of the computerized imaging system to prepare patient karyograms. Pattern-recognition software has improved to the point that many sophisticated systems can now arrange the chromosomes with little or no human input (see Chap. 7). This, of course, creates a quality concern. Laboratories using such tools can deal with the issue by putting in place protocols that require appropriate review of all computer-generated karyograms. When properly monitored, such systems can increase laboratory efficiency by markedly reducing the time required for karyogram production.

Retention of Case Materials

Slides used in diagnosis and stained by a permanent method (e.g., G-, C-, or R-banding; NOR staining) must be retained by the laboratory for at least 3 years. Retention of slides stained using fluorescent methods is at the discretion of the laboratory director.

Residual original patient samples, such as cell pellets, should be kept until sufficient metaphase preparations have been made to complete the analysis. Processed patient samples should be retained until the final report is signed, and many laboratories keep them for longer periods of time. Some laboratories do not discard cell pellets from abnormal cases.

Post-analytical Testing Components

Laboratory Reports

Reporting the results of chromosome analysis and/or FISH can have a direct impact on the diagnosis and treatment of a patient; thus, it is important to establish a reporting procedure that:

- Summarizes the findings of the laboratory
- Cross-checks the findings against the various specimen labels for labeling errors
- Interprets the test results, where appropriate
- Establishes a reporting process to outside individuals so that the data, the individual issuing the report, the individual receiving the data, and the report date are properly documented

Preliminary Reports

Although potentially risky, preliminary results are sometimes released by a laboratory before the full chromosome analysis has been completed. Preliminary reports are often issued verbally once enough data has been collected to formulate a likely indication of the final result, or once the data already available is clinically critical and must be communicated to a physician (this might also pertain to FISH analysis that has been completed while results of chromosome analysis are still pending, in which case a preliminary report could be in writing). Once verified, it is important to follow an established procedure for reporting preliminary results. Individuals reporting the data should be qualified to interpret the preliminary findings and to give an indication of the possible outcome once a complete study has been conducted. It is important for this individual to document the microscope analysis data, patient and cytogenetic data reported, date of the report, and individual receiving preliminary data. It is also vital to impress upon the person receiving the report what may change once the study is completed.

Final Reports

The final report summarizes and interprets the results of the study. Some states and regulatory agencies also require a statement describing the limitations of chromosome and/or FISH analyses, and many laboratories choose to include such statements, whether required or not. A protocol for the creation of final reports should include a checklist to insure that all appropriate procedures have been completed and that all data is clerically correct. The final report should include the following:

- Case identification (including patient name, date of collection and receipt by the laboratory, laboratory accession number, sample source, name of physician or authorized agent who ordered the test)
- Reason for study
- Number of cells in which chromosomes were counted, analyzed, and karyogrammed
- Culture methods when significant to the cytogenetic findings
- Banding method, band resolution, and ISCN description of analyzed cells

- Indication of additional work performed (e.g., to resolve possible mosaicism), correlation to previous studies, controls used, etc.
- Interpretation of results written for a non-geneticist physician (including clinical correlation, discussion of abnormal results, recommendations for additional genetic studies or genetic counseling)
- Information about any preliminary reports
- Signature of qualified laboratory director
- Date of final report

Once completed, final reports can be generated electronically or on paper. If a preliminary report was provided, any variations from it should be stated. Once the final report has been completed, a record should be kept of the individuals to whom a report was issued, as well as the date(s) of issue. In most instances, a report is typed or printed electronically by a computer program and filed in a patient folder. Patient folders are retained in the laboratory or filed in an outside facility. Whether stored within a laboratory or at an off-site facility, it is important to have access that allows prompt data retrieval.

Quality Assurance

Laboratories can experience a variety of difficulties with the samples themselves. Some of these are inevitable and therefore are not preventable (insufficient volume, wrong sample type, no living or dividing cells present, etc.), while others may be due to improper collection or transport of the specimen before it reaches the laboratory, incorrect labeling, or errors in handling and processing in the laboratory itself. Any of these can result in an incorrect diagnosis, or in failure to reach one at all. It is therefore very important for improvement of overall laboratory quality to investigate and document all problems that arise, thereby determining ways to prevent similar occurrences in the future.

It is also important to monitor specific types of laboratory test outcomes in order to judge a laboratory's performance. This is most commonly done when a laboratory can expect a particular distribution of outcomes. In studies of products of conception (POCs), for example, review of distribution of results can alert laboratory personnel of potential problems with tissues provided and dissected for study (e.g., if the male: female ratio is not close to 1). In leukemia and cancer testing, there may be subsets of cases for which there is an expectation of study success and abnormality detection rates. For instance, among patients entered in national cancer cooperative group studies, there is usually a group-wide expectation based on prior performance of laboratories in the group.

Specimen Failures

The inability of a laboratory to provide a diagnostic result is typically due to one of two basic reasons: cells from the sample did not grow in culture, and therefore no mitotic cells were produced, or a problem occurred in one of the many post-culture steps, rendering the processed material useless. The purpose of this section is not to convince the reader that problems are inevitable, but rather to impress upon the reader the amount of care and attention to detail that is required, and the critical role quality assurance plays in the cytogenetics laboratory.

Culture Failure

As described in Chap. 4, the basic procedure for producing chromosomes for analysis from any tissue type requires living cells that can somehow be coaxed into active division. Without mitosis, there can be no chromosomes to process and examine.

There are several possible reasons for cell culture failure:

- *The sample did not contain any living cells.* In some cases, this is clinically not surprising; it is frequently the case with products of conception obtained after a fetal demise, or in necrotic or aplastic bone marrow samples. At other times, the cause can be deduced (such as a delay in sample transport or exposure of the specimen to extremes of temperature during transport when an outside reference lab is used). In other instances, however, no explanation is readily available. In these cases, the entire path the specimen followed between the point of collection and delivery to the laboratory is suspect and must be investigated.
- *An inappropriate specimen is submitted to the laboratory.* This may involve a peripheral blood with no circulating blasts having been collected instead of bone marrow (without blasts, there are no spontaneously dividing cells present and the unstimulated cultures used for many hematopoietic disorders will not produce metaphases). It might be due to the wrong collection tube being used, or to products of conception being placed in formalin and then sent to the lab. The specimen and the way it is collected must match the intended application of chromosome analysis.
- *An insufficient specimen is submitted to the laboratory.* Specimen descriptions such as “2 mL of extremely bloody amniotic fluid” or “0.5 mL of watery bone marrow” frequently accompany cultures that fail. However, *all such samples should be submitted to the laboratory*, which will do everything it can to generate a result, no matter how unlikely this may seem.
- *The laboratory suffers a catastrophic equipment failure.* With proper precautions in place, this is unlikely. Specimens should be divided, and multiple cultures should be initiated and placed in separate incubators,

whenever possible. There should also be appropriate backup power, redundant CO₂ and alarm/warning systems in place, and all major equipment should be on a preventative maintenance schedule. Nevertheless, unusual hardware problems do occur.

- *Reagent failure.* There are rare but unfortunate examples of supplies that are supposedly quality-controlled by the manufacturer being released (unknowingly) for purchase by laboratories without actually meeting the appropriate criteria. Improperly cleaned water storage tanks have poisoned entire lots of culture medium, and syringes made with natural rubber stoppers have periodically resulted in amniotic cell death on contact. Again, with proper precautions in place (testing all supplies before use and dividing all cultures between two lots of everything), these risks can be minimized.
- *Human error.* While also unlikely, it is always possible for a technologist to inadvertently prepare culture medium incorrectly, forget to add the appropriate mitogen, or utilize equipment improperly.

Every culture failure must be documented and the cause investigated to the extent possible. The laboratory should keep records of these, along with periodic measurements of culture failures *for each specimen type*, as a way of detecting an increasing trend before it becomes a serious problem.

Post-culturing Errors

There are few things as frustrating to those working in a cytogenetics laboratory as having seemingly good cell cultures or routine blood cultures produce no usable metaphases. While these are admittedly rare events, they do occur and, as with all culture failures, must be fully investigated and documented. Some examples are:

- *Harvesting errors.* As outlined in Chap. 4, there are a variety of steps in the harvest procedure, and each provides the potential for error. If Colcemid® is not added, an insufficient number of mitotic cells can be the result. If fixative is added before the hypotonic solution (unfortunately an easy thing to do but a mistake a good technologist makes only once), cells will not swell, and chromosome separation is impossible. If a centrifugation step is omitted, all cells except those that have settled due to gravity will be removed via aspiration or pour-off. Other errors, such as adding the wrong hypotonic solution, making any of the reagents incorrectly, or using incorrect timing, can also render a harvest unusable. Finally, a catastrophic event that results in the loss of all material (e.g., spillage or breakage of a rack of tubes) will, of course, result in loss of usable material.
- *Problems with robotic harvesters.* As described in Chap. 7, many types of cultures are now amenable to harvesting on a robotic device. Although the motivation for using such a machine is often to free up technologist time for

other vital functions, it is not a good practice merely to load the cultures onto the harvester, press the start button, and walk away. Solution bottles must be filled with the proper reagents, lines must be free of clogs, and the computer program must be functioning correctly. All of these must be verified before a technologist leaves the machine alone, and it is a good laboratory policy to make periodic checks up until the cultures are in fixative.

- *Slide-making and drying errors.* It has often been said that clinical cytogenetics is part art and part science. Producing high-quality metaphases during the slide-making process is one example. This procedure is described in Chap. 4 and also discussed in Chap. 7; suffice it to say that if not done properly, the laboratory's ability to correctly analyze a patient's chromosomes can be compromised.
- *Banding/staining errors.* Banding and staining are examples of the art of cytogenetics. Correct "aging" (actual or artificial via baking slides in an oven) and timing of each step in this process is critical to producing well-banded chromosomes (see Chap. 4), and a failure to interpret results and adjust parameters accordingly can ruin even the best of preparations.
- *Miscellaneous accidents or human error.* Although each of the basic post-culture steps has been covered, there are still unusual things that can occur at any point in the process, from wiping the wrong side of a slide to breaking it completely.

Labeling Errors

The result of a labeling error can range from an incorrect laboratory number appearing on a report to the misdiagnosis of a specimen. Collection containers, requisition forms, computer databases, culture flasks or dishes, culture worksheets, microscope slides, and microscope analysis worksheets are all places where specimen labeling errors can occur. Regardless of the outcome, labeling errors lead to improper identification of or assign incorrect/inaccurate information to a specimen and are therefore a significant concern of any laboratory. Processing specimens one at a time using controlled, standardized procedures serves to greatly reduce the likelihood of labeling errors. Nevertheless, it is important to remember that people make mistakes, and the laboratory must therefore implement a system that cross-checks the accuracy of the labels assigned to a patient, as well as the data collected from a cytogenetic study. Each step that creates the possibility for misidentification should have a cross-check built into it, and some form of overall clerical review of a patient chart is frequently carried out before results are released.

Misdiagnosis

While perfection is always a goal in medicine, realistically it is never achieved. Every laboratory discipline strives to eliminate all mistakes, but given the fact that human beings are involved, all lab areas have "acceptable" error limits. A small cytogenetics laboratory processing 2,000 samples per year that achieves a 99.97% accuracy rate (far in excess of the performance of the typical excellent pathology lab) will make six diagnostic errors in a 10-year period.

Misdiagnosis in the cytogenetics laboratory can occur in three ways: as the result of incorrect specimen labeling (described earlier), by incorrect interpretation of a chromosome abnormality, or by failing to identify abnormality that is present. Despite the many "pairs of eyes" that typically see each specimen in most laboratories, as described previously in this chapter, some errors occasionally still manage to get all the way through such a system undetected.

The consequences of an incorrect interpretation of a chromosome abnormality can range from negligible to serious. Because of the less than optimal chromosome morphology often produced by bone marrow aspirates or solid tumors, or to the complex abnormalities frequently present in such samples, it is often difficult, if not impossible, to correctly identify every chromosomal change that may be present. It is not uncommon for a laboratory to receive serial bone marrow aspirates from a patient only to discover that, due to improved resolution in the current sample, an abnormality can be more accurately characterized and that either a previous interpretation was not quite correct, or an abnormality initially described as uninterpretable can now be described. This is typically of little clinical consequence and can easily be addressed in the current clinical report. On the other hand, misidentification of, or failure to detect, a disease-specific rearrangement can lead to incorrect therapy and potentially disastrous results.

Incorrect identification of a constitutional chromosome abnormality is less common than it once was, since most such changes can be confirmed or further characterized via FISH or microarrays (see Chaps. 17 and 18). Many structural rearrangements are family-specific, but predicting the phenotype likely to result from an unbalanced aberration is never an exact science. Nationwide proficiency tests often result in numerous similar but different interpretations of the abnormalities presented in any given challenge, demonstrating that "getting it right" can be subjective in the field of cytogenetics.

Failure to identify a chromosome abnormality that is, in fact, present can be a serious issue if the inaccuracy is ultimately discovered, but such is not always the case. As discussed earlier, an abnormality may be detected in one bone marrow aspirate but not in a prior one, particularly if there is a difference in quality between the two. If the same labora-

tory receives both specimens, this can be detected and interpreted correctly, and while it may not make a therapeutic difference, it is possible that the referring physician(s) may comment that the patient's treatment would have been different had the abnormality been detected earlier. However, it is not uncommon today for different labs to be used for serial studies, and in the scenario presented earlier, the initial diagnostic failure might never be revealed.

Perhaps the most serious example of a missed diagnosis is an unbalanced chromosome abnormality that is not detected in a prenatal sample. Failure to identify a balanced rearrangement could have consequences for the extended family, usually by resulting in the failure to identify other family members who are at risk for carrying it (see Chaps. 9 and 21) but (fortunately) rarely has an impact on the current pregnancy. Failure to identify an unbalanced abnormality, however, will very often result in the birth of an abnormal child, and should the parents believe that they would have interrupted the pregnancy had the abnormality been identified prenatally, a lawsuit can result. The outcome of such cases often depends on whether the laboratory's methods, quality systems, and results measure up to what is considered to be the standard of care (i.e., everything covered in this chapter) and whether or not the abnormality "should have been detected." The latter often involves presenting uninformed professionals with the karyograms to determine whether or not they can identify the abnormality (a biased process, since these individuals obviously must know that something is wrong) and soliciting their opinions as "expert witnesses" concerning whether or not the laboratory should have caught the abnormality, or whether it was too subtle to detect.

Regardless of the nature of the error that is detected, it is important to determine the cause of the problem and to put into place the necessary changes to minimize recurrence.

The Laboratory Quality Assurance Program

An organized process of review, communications, and staff education is required to realize the benefits of a laboratory system that tracks and monitors its functions, performance, and problems. While at times this may involve subsets of the laboratory personnel, it is often part of the ongoing training and continuing education program that should be available to the entire staff.

Oversight

In addition to the numerous steps already described, cytogenetics labs, like all other clinical laboratories, are subject to many external guidelines, inspections, and tests that are designed to ensure and improve quality. These vary from

country to country and even from state to state in the USA. Federally, the Food and Drug Administration (FDA) regulates manufacturers of devices, some reagents, some software, and testing kits sold to laboratories. Though the FDA has suggested that the regulation of laboratories is within the purview of its federal mandate because the laboratories make some of their own reagents, there is no precedent for their involvement at this level. The majority of direct laboratory oversight is focused on laboratory practices including personnel requirements, general quality control and assurance, and quality control and assurance specific to the area of practice. Clinical cytogenetics is among the areas with specialty-specific requirements under the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) regulations (CFR§493.1276) [2].

Accreditation, Inspections, and External Proficiency Testing

Under CLIA '88, every laboratory performing moderate- to high-complexity testing (i.e., every cytogenetics laboratory in the USA) must enroll in US Department of Health and Human Services-approved external inspection and testing programs [2]. Almost all clinical laboratories in the USA do so under the auspices of the CLIA-deemed program of the College of American Pathologists (CAP) [3]. This accrediting organization inspects laboratories and provides the American College of Medical Genetics and Genomics (ACMG)/CAP proficiency testing survey program, according to CLIA requirements, several times a year [3]. A lab's ability to perform and be reimbursed for testing depends upon successful participation in each aspect of this process, and repeated failure can lead to loss of accreditation. As of this time, no areas of genetic testing have mandated performance requirements for these proficiency testing programs.

CAP sends a team, typically from another laboratory, to inspect each facility every other year; during off years, the laboratory must conduct and report the results of a self inspection. Proficiency testing and interlaboratory comparison programs vary according to specialty; in cytogenetics, the proficiency tests generally consist of four unknown cases in the form of banded metaphase preparations and sufficient clinical information for the lab to make a diagnosis. A fifth unknown case, in the form of a peripheral blood sample, is also frequently submitted, but there are obvious logistical and medical challenges of this procedure; there are enough cytogenetics laboratories in the USA that care must be taken not to exsanguinate the individual (typically a carrier of a rearrangement) who has volunteered to be the test subject! Depending on the probe type, proficiency testing for FISH can involve slides to be hybridized, images to be interpreted, or both.

State requirements can be quite variable. Several require participation in the CAP programs. One of the more rigorous

programs is administered by the New York State Department of Health, which conducts its own inspections and proficiency tests of all labs in the USA that process specimens from New York State residents [4]. This body also has its own certification process for clinical laboratory directors.

Laboratory Staff Qualifications

Many US states require, either formally or informally, that the individual who signs chromosome analysis reports (typically the director of the cytogenetics lab) be board-certified in clinical cytogenetics by the American Board of Medical Genetics (ABMG), a body that is recognized by both the American Board of Medical Specialties and the American Medical Association. It is similarly approved by the US Department of Health and Human Services under the CLIA '88 regulations [2] as among the boards required of laboratory directors. Such certification is awarded to a doctor (M.D. or Ph.D.) who passes a comprehensive examination in general genetics as well as a specialty exam (in this case in clinical cytogenetics). Both exams must be passed for an individual to be board-certified. Diplomates certified during or after 1993, when maintenance of certification became a requirement, must recertify after 10 years. Those certified prior to 1993 are not required to recertify but are encouraged to do so.

Many technologists, supervisors, and even directors in clinical cytogenetics labs in the USA learned to perform chromosome analysis on-the-job, and such experience was all that was needed in order to find employment. While many cytogenetic technologists are still on-the-job trained, a baccalaureate degree is now required, and postbaccalaureate certificate and degree programs in cytogenetics exist in several colleges, universities, and laboratories in the USA.

Qualified individuals can become certified as technologists in cytogenetics by passing the national certification examination in cytogenetics given by the American Society for Clinical Pathology (ASCP) Board of Certification (BOC) [5]. Candidates who pass the examination, which includes both didactic and practical components, can use the credential CG(ASCP)^{CM}. Initial certification is valid for 3 years. Recertification via the Certification Maintenance Program (CMP) requires 36 units (hours) of continuing education every 3 years and is a requirement of continued certification.

Formal cytogenetics education programs can attain accreditation by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS) by meeting specified standards that include minimum qualifications of the program director, didactic and clinical faculty, and curricular requirements that include didactic and clinical components [6]. Programs seeking NAACLS accreditation are required to complete a comprehensive self-study and site visit. Programs are reaccredited periodically by similar requirements.

Graduates of NAACLS-accredited programs are eligible to sit for the American Society for Clinical Pathology Board of Certification (ASCP BOC) examination in cytogenetics immediately upon graduation from the program using the education route. On-the-job-trained technologists can qualify to take the ASCP BOC cytogenetics examination through other routes that require combinations of educational achievement and work experience in a cytogenetics laboratory.

State Licensure

Credentialing requirements vary by state, and some states have their own licensing requirements for cytogenetics laboratories and personnel. Laboratories in those states and/or laboratories receiving specimens from residents of those states, and personnel working in them, must abide by the state requirements.

International QA/QC

There are cytogenetics laboratories worldwide, and a comprehensive discussion of the quality control/quality assurance and personnel issues in other countries is not feasible here. Those interested are encouraged to contact professional cytogenetics and clinical laboratory organizations in the countries of interest. However, as a close neighbor, a few words about personnel requirements in cytogenetics laboratories in Canada are included.

Canada

In Canada, technologists working in cytogenetics and molecular laboratories are required to have earned a Bachelor of Science (B.Sc.) degree and to have graduated from a post-degree diploma program that includes extensive clinical laboratory experience. There are only two such programs in Canada: one at the British Columbia Institute of Technology (BCIT) in Burnaby, British Columbia, and the other at the Michener Institute in Toronto, Ontario.

The Canadian Society of Medical Laboratory Science (CSMLS) is the credentialing/certification organization in Canada [7]. In all provinces except Ontario, candidates take a combined cytogenetics/FISH/molecular examination, and successful candidates receive the credential MLT (clinical genetics). In Ontario, instead of a combined credential, technologists can become certified in cytogenetic/FISH or molecular technology. There are no longer on-the-job routes for certification in Canada. Canadian certification does not have a requirement of continuing education, thought it is usually encouraged by employers.

There is no uniformity in the credentials needed to be a cytogenetics laboratory director in Canada, although many are certified by the Canadian College of Medical Genetics (CCMG) [8]. Candidates for this certification must have successfully completed a CCMG-accredited training program.

Related Topics

This chapter has covered most issues involved in the generation of clinical results in the cytogenetics laboratory. However, no such work would be complete without making mention of the ancillary QA/QC that must also be dealt with on an ongoing basis:

- *Safety.* In past decades, laboratory design and protocols put the specimen first and the technologist second. Mouth pipetting was common, even with potentially toxic reagents (e.g., Giemsa stain is frequently dissolved in methanol). Gloves were not used, and “medical waste” was any garbage can that had come in contact with a specimen. Cytogenetics labs often reeked of acetic acid (used in fixing samples; see Chap. 4), and laminar flow hoods (“sterile hoods”) were constructed with no separation between the specimen and the technologist and utilized a back-to-front horizontal flow of filtered air. The sample was protected from microbial contamination as air blew over it directly into the technologist’s face! The reader is reminded that hepatitis existed long before the human immunodeficiency virus (HIV).

Hoods in use today (“biological safety cabinets”) feature split vertical airflow and protective glass windows. Pipetting devices are typically required, and in the USA, material safety data sheets (MSDS) for every reagent used in the laboratory must be available to all employees. Acceptable concentrations of all volatile reagents are maintained via ventilation systems and are monitored, and universal precautions govern every process that involves contact with patient samples. Most laboratory inspections include a safety component. All laboratories should have general and specific laboratory, chemical, biological, and, if needed, radiation safety programs. Many also include ergonomics (including, but not limited to, correct hand, arm, and body position for working at a microscope, hood, or computer) as part of their safety program.

- *Reference laboratories.* Not every cytogenetics lab performs every type of test on every type of sample. Some specimens require additional non-cytogenetic testing. Some laboratories experience backlogs or other similar difficulties, which require that some samples be sent to another lab to enable them to “catch up.” For these reasons, proper record keeping and other regulations exist to ensure proper handling and timely reporting of results for such specimens. Reports must clearly indicate where the testing took place.
- *Ethics policies.* While most laboratorians who perform prenatal testing consider themselves to be “pro-choice” regarding a patient’s right to make informed decisions, many feel compelled to contribute only clinically relevant results. Prenatal analysis for gender identification/selec-

tion is an example of a nonclinical indication for study, and specimens submitted solely for this purpose are therefore refused by some laboratories. Because of the obvious difficulties faced by all involved with such issues, a written policy, created by an internal ethics committee, can be extraordinarily helpful and is recommended.

Laboratories can also be faced with (often unique) ethical dilemmas on a case-by-case basis; these require thought and discussion to allow the laboratory to address them in what it deems to be the most appropriate manner.

- *Health Insurance Portability and Accountability Act of 1996 (HIPAA).* The HIPAA privacy rules created new requirements for health-care providers to protect the privacy and security of individually identifiable health information. This is defined as information that is created or received by a health-care provider that relates to the past, present, or future physical or mental health or condition of an individual; the provision of care to an individual; or past, present, or future payment for the provision of health care to an individual or information that identifies an individual. They went fully into effect on April 14, 2003.

The requirement to comply is triggered when the medical geneticist of the institution at which he or she practices electronically transmits health information for billing or other purposes. Once required to comply, it applies to all information including that in nonelectronic form. There are three main areas of requirement. These include the monitoring and control of the uses and disclosures of protected health information (PHI), providing patients with certain rights with respect to their PHI, and establishing and implementing certain administrative policies and procedures to ensure maintenance of privacy. Not all rules apply equally to clinicians and laboratories. For instance, because the laboratory is considered to have an indirect treatment relationship with the patient, it is considered exempt from the consent requirements that require distribution of a Notice of Privacy Practices and from obtaining the acknowledgment. Clinicians may extend their protection under HIPPA to a third party, such as a laboratory, by entering into a business associate agreement. If the laboratory is only analyzing specimens, such an agreement should not be necessary. However, part of the American Recovery and Reinvestment Act of 2009 known as the Health Information Technology for Economic and Clinical Health Act (HITECH) has a subtitle that deals with privacy and security issues associated with electronic transmission of PHI and extends those aspects of HIPAA (including updated civil and criminal penalties) to business associates (in this case, a laboratory) of entities covered by these provisions (i.e., clinicians).

There is a wide range of information that may be considered “individually identifiable,” including names, Social Security numbers, geographic subdivisions smaller

than a state, etc. Care must also be taken in the use of photographs.

The HIPAA privacy rules and offshoots like HITECH are likely to evolve as their intent is interpreted over time. They set the floor for the protection of an individual's information. About half of the states have enacted more specific genetic information privacy statutes. Consultation with local or institutional compliance officers for specific needs is recommended.

- *Compliance training.* Many labs, particularly those in commercial settings, are subjected to an increasing number of restrictions designed to prevent "kickbacks" or other potentially fraudulent finance-related practices. While the average technologist is unlikely to be faced with decisions that may involve such regulations, training in this area is becoming a common precaution.

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Instrumentation in the Cytogenetics Laboratory

Steven L. Gersen

Introduction

All laboratory procedures were essentially manual at one time. Primitive centrifuges were hand operated, and the earliest microscopes (examples of “new technology” in their own time) utilized operator-positioned mirrors to gather sunlight or candlelight before the discovery of electricity and the invention of the electric light bulb. Today, however, the typical clinical laboratory is dominated by technology, computers, and automated instrumentation. These have improved laboratory practice in three basic ways:

- Automation of tasks, which can free up technologist time, thereby improving efficiency and reducing costs
- An increase in the speed, accuracy, and reproducibility at which tasks can be performed
- Performance of tasks that cannot be accomplished manually

Previous editions of this book made a point of emphasizing the manual nature of chromosome analysis and drew a distinction between cytogenetics and other aspects of clinical laboratory medicine when it came to automation. In certain ways, this has not changed. Chromosome analysis still requires a great deal of labor; cultures must be initiated, chorionic villus samples and products of conception require technologist time and manipulation at a dissecting microscope, and even where technology exists, human interaction with that technology is often required; whether through a microscope or on a computer screen, visual examination of chromosomes by human beings is necessary. Nevertheless, technology has advanced considerably in recent years, and many steps in the process of preparing and examining chromosomes have now been automated to some degree.

Such instrumentation can assist with specimen processing and chromosome analysis and falls into several basic categories:

robotic harvesters, environmentally controlled drying chambers, and computerized imaging systems, which can also include automation of certain microscopy steps. There have also been devices developed to eliminate some of the manual steps involved in performing chromosome banding and staining and fluorescence *in situ* hybridization (FISH) studies, and instrumentation and computing power are required for microarray analysis. It should be pointed out that some cytogenetics laboratories use many if not all of these types of devices, while most use one or a few.

Robotic Harvesters

As described in Chap. 4, harvesting of mitotic cells for cytogenetic analysis involves exposing the cells to a series of reagents that separate the chromosomes, fix them, and prepare them for the banding and staining process. This traditionally involves pelleting the cells by centrifugation between steps, in order to aspirate one reagent and add another. However, the *in situ* method of culture and harvest of amniotic fluid (and other) specimens requires that the cells remain undisturbed in the vessel in which they were cultured. Reagents are therefore removed and added without the need to collect the cells in a tube that can be centrifuged. Thanks to improvements in technology both processes lend themselves to automation.

Webster defines a robot as “...an automatic apparatus or device that performs functions ordinarily ascribed to human beings....” In this context, those functions are aspiration of the growth medium from the centrifuge tube or culture dish, addition of a hypotonic solution, and, after an appropriate incubation time, removal of the hypotonic solution and addition of several changes of fixative, each with its own duration. For suspension cultures, a centrifugation step is also necessary before each aspiration. What is required, then, is a device that can aspirate and dispense liquids (and, for suspension cultures, perform the necessary centrifugation), monitor the timing of each step, and control these steps

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Fig. 7.1 Multiprep robotic harvester. This device was designed specifically for cytogenetics laboratories, with enhancements such as automatic fixative mixing, integral fume extraction, multiple dispensing, and aspiration probes to reduce the risk that blockage will ruin a harvest, and onboard programming, which eliminates the need for an external computer, reducing bench space requirements (Photo courtesy of Genial Genetic Solutions)

correctly regardless of the number of cultures or tubes being processed at any one time, i.e., some form of computer control that can be “told” how many specimens there are and where on the device they are.

The initial approach to automation of certain *in situ* harvesting steps was modification of existing computer-controlled horizontal liquid handling devices that utilized a probe that moved along its X and Y axes. This is no longer necessary; laboratories can now choose from among several instruments designed specifically for harvesting specimens for chromosome analysis

For *in situ* cultures, liquid handling devices have been replaced by those built specifically for cytogenetics laboratories; one such instrument is programmed by the user directly, without the need for an external computer. This, along with the machine’s vertical design, dramatically reduces its footprint, conserving valuable bench space (Fig. 7.1).



Fig. 7.2 Hanabi-PII metaphase chromosome harvester (Photo courtesy of Transgenomic, Inc.)

Robotic processors are also available for the blood/bone marrow harvest procedure. Because these devices must be capable of both liquid handling and centrifugation, they require a substantial amount of space and are designed to be freestanding rather than benchtop units (Fig. 7.2).

Drying Chambers/Slide Makers

Again, as described in Chap. 4, the typical end product of the cytogenetic harvest is a centrifuge tube with fixed cells, both mitotic and nonmitotic. Spreading of chromosomes is achieved by placing one or more drops of this suspension on a number of microscope slides and is controlled by the height from which the suspension is dropped, the temperature and condition of the slide, and any number of manipulations while the slide is drying (including the ambient conditions in the laboratory). Results are monitored with phase contrast microscopy, and any slide that is not satisfactory can be discarded and replaced; trained individuals can determine

the adjustments necessary to improve drying and spreading. Provided that such adjustments are made properly and quickly, running out of cell suspension is generally not a problem.

This is not always true, however, particularly with small volume bone marrow aspirates, and it is never the case with *in situ* culture and harvesting, typically utilized for prenatal diagnosis. Most cytogenetics laboratories initiate four to six *in situ* cultures from each amniotic fluid or CVS sample (see Chap. 12), depending on the condition of the specimen upon receipt. Regulations and good clinical sense require that cells from at least two of these are examined, and in many cases, three or more cultures are required. When one considers that at least one culture or some other form of backup should be retained against an unexpected need for additional testing, it becomes evident that every culture dish must produce useable metaphases. The concept of discarding one and trying again, possible in many cases when making slides from cell suspensions, does not apply. Further complication is introduced by the fact that the physical force generated by dropping the cells onto a glass slide is not available when *in situ* processing is used, and so spreading of chromosomes is accomplished solely by the manner in which the cultures are dried.

As the 3:1 absolute methanol:glacial acetic acid fixative used in cytogenetics laboratories dries, it “pulls” the cell membrane across the slide or coverslip with it, allowing the chromosomes of mitotic cells to separate. If this process is viewed with a phase contrast microscope, the metaphases appear to open much like a flower blossom. Clearly, the ambient temperature and humidity, as well as air flow over the cells (and possibly, as suggested by some studies, the barometric pressure), all affect the rate of drying and the ultimate quality of the chromosome preparation [1, 2]. When utilizing *in situ* processing, controlling these parameters is the only way to control chromosome spreading.

In fact, of greatest importance is not merely controlling conditions, but maintaining them with a high degree of consistency. With each change in any one parameter, drying and spreading of chromosomes changes; once the correct combination is achieved, it is of paramount importance that it be maintained throughout the entire harvest. This is true regardless of the specimen type or harvest method.

There have probably been almost as many solutions to this situation as there are cytogenetics laboratories. Some constructed enclosed chambers in which air flow, humidity, and temperature could be varied, although these were typically prone to failure whenever the air-conditioning broke down since it is easy to warm the air inside the chamber but almost impossible to cool it. Some labs designed climate-controlled rooms; these frequently functioned well, but the drawbacks here were the need to maintain conditions while properly venting out fixative fumes (an engineering challenge, but certainly possible) and the potential to expose the technologist to uncomfortable if not unhealthy conditions. Such rooms were also costly to build.

The availability of specialized equipment has all but eliminated the need for such homegrown solutions.



Fig. 7.3 Benchtop drying chamber. Initially developed for the culture of insect cells (which are grown at room temperature, and so the incubator must be capable of cooling as well as heating), this chamber has been modified to control humidity as well, and fans have been installed to allow for control of airflow over coverslips or slides (Photo courtesy of Percival Scientific, Inc.)

Several companies have developed self-contained chambers specifically for the purpose of drying *in situ* cultures; an example is shown in Fig. 7.3. The advantages to this type of hardware are its ability to maintain conditions, quick recovery time after opening the chamber to insert or remove dishes, and potential for external venting if necessary. The disadvantage is the necessity to remove the fixative prior to placing the dishes in the chamber, creating the potential for drying to begin under noncontrolled conditions if there is any delay in getting the dishes into the chamber.

A variation on this theme is shown in Fig. 7.4. Here, the entire drying process, including aspiration of fixative, can take place inside a freestanding chamber. The technologist sits at the unit and manipulates the processing with a glove-box approach. The advantage here is that the drying process takes place under controlled conditions from the instant the fixative is removed; there is no need to rush to get dishes or slides into the chamber. The drawback to this concept is the large size of the unit, and a somewhat more cumbersome and limiting setup; removing one or more cultures for examination (an absolute requirement) can be more intrusive to the workflow.

A benchtop device that combines advanced computer control capability for precise control and monitoring of conditions with the ability to perform aspiration inside the chamber is also available (Fig. 7.5).

These condition-controlled chambers are gaining in popularity in cytogenetics laboratories, and some use them not only for *in situ* processing but for routine slide making as well due to the consistency they provide. For this reason,



Fig. 7.4 Floor model drying chamber (Photo courtesy of Thermotron Industries)



Fig. 7.6 Hanabi metaphase spreader. Temperature, humidity, and airflow are set and rapidly stabilized so when chromosome preparations are placed on microscope slides, they are dried in a consistent and reproducible manner. Multiple slides can be created simultaneously (Photo courtesy of Transgenomic, Inc.)



Fig. 7.5 Monalisa® ambient conditions chamber. Exact specifications for multiple programs, to accommodate different tissue types, are software-controlled via a laptop computer for ease of operation (Photo courtesy of elja, Inc.)



Fig. 7.7 Hanabi-PIV automated slide maker (Photo courtesy of Transgenomic, Inc.)

devices designed specifically for slide making are now also available (Fig. 7.6), and one of these actually automates the entire slide-making process (Fig. 7.7).

Instrumentation for FISH

While fluorescence *in situ* hybridization (FISH, see Chap. 17) represents one of the most exciting and clinically significant developments in cytogenetics, most of the steps involved in preparing samples for analysis are unremarkable and often repetitive and therefore lend themselves to automation. When one considers the FISH sample volume that many cytogenetics laboratories receive, any device that can reduce the labor component of the process becomes indispensable.

The entire FISH process can be performed automatically (Fig. 7.8), but laboratories also have the option of utilizing instruments that are specifically designed for different aspects of the procedure.

Pretreatment

For many applications of FISH, the only thing one must do to prepare a sample for analysis is make one or more additional slides or, in some cases, destain a slide that has already been examined so as to be able to interpret the results of hybridization to already-analyzed metaphases. However, some applications of the technology (e.g., *ERBB2* analysis for breast cancer or FISH for bladder cancer recurrence; see

Chap. 17) utilize specimen types that are not processed for chromosome analysis, such as slides cut from paraffin blocks or made from bladder wash/urine samples. Such sample types require deparaffinization or other pretreatment before any FISH procedure can be performed. While not difficult or complicated, these procedures are repetitive and time consuming. Fortunately for the laboratory, devices that automate such steps are available (Fig. 7.9). These devices also offer the laboratory the flexibility of performing other FISH pre-treatment procedures, and they can even be programmed to perform certain routine cytogenetic or cytological procedures, making them more cost efficient for certain institutions. This can be significant, as these instruments are not inexpensive.

Hybridization

As with any DNA hybridization procedure, FISH requires a series of heating and cooling steps to facilitate denaturation and renaturation/hybridization of probe and target DNA. Analogous to the thermocyclers utilized for the polymerase chain reaction (PCR) in the molecular genetics laboratory, devices are available that permit a technologist to add FISH probes to a sample slide, close the cover, initiate a preprogrammed series of temperature changes, and walk away. These instruments can handle a modest number of slides at one time, and store several user-defined programs for analytical flexibility. Newer models include fluid handling capabilities so that various pretreatment steps can also be performed prior to hybridization (Fig. 7.10a, b).

The drawback to these devices is that large volume or frequent use of probes that require different programming necessitate the purchase of more than one unit. Some have, however, come down in price in recent years.



Fig. 7.8 Xmatrix™ automated FISH processing system (Photo courtesy of Abbott Molecular, Inc.)



Fig. 7.9 VP 2000 Processor. This device automates various laboratory protocols, such as a pretreatment or deparaffinization step prior to performing a FISH assay (Photo courtesy of Abbott Molecular, Inc.)

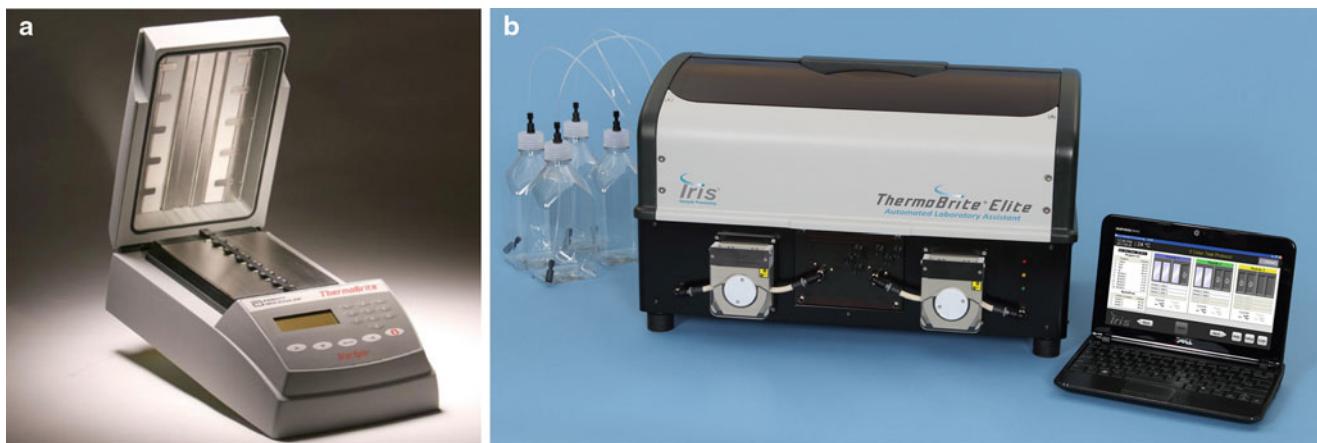


Fig. 7.10 (a) Thermobrite StatSpin® programmable temperature controlled slide processing system. Up to 12 slides can be placed in the device, which can be programmed to heat and cool as required for various FISH protocols (Photo courtesy of Abbott Molecular,

Inc.). (b) Thermobrite® Elite Automated Laboratory Assistant. This device adds an automated fluidic system to facilitate pretreatment of different specimen types (Photo courtesy of Iris Sample Processing)

Enrichment

Eliminating the need for actively dividing cells notwithstanding, FISH assays can still suffer from difficulties in detecting abnormal cell populations if these are present in small numbers. Techniques to enrich the population of target cells prior to performing FISH are gaining popularity and are expected soon to become standard of care for certain neoplasms (see also Chap. 17). One approach to enrichment is to chemically couple magnetic beads with an antibody to a specific cell surface marker. Target cells can then be magnetically separated from a specimen prior to a FISH assay. Instruments to automate this process are now available; an example is shown in Fig. 7.11.

Automated Imaging Systems

Introduction

The original method of imaging chromosomes was photomicrography. A photograph of metaphase chromosomes was taken, the film was developed and photographs were printed in a darkroom, and the chromosomes were cut out and arranged to form a karyogram. Though it was a standard technique for many years, this process increased the already time-consuming nature of clinical cytogenetics, and it has for all intents and purposes been replaced by digital imaging.

Automated imaging systems dramatically reduce the time it takes to produce a karyogram and therefore can be seen as one of the most important developments in automation of the cytogenetics laboratory. Furthermore, the growth in fluorescent techniques such as multicolor fluorescence *in*



Fig. 7.11 RoboSep instrument for automating the magnetic enrichment of a target cell population prior to performing FISH or other procedures (Photo courtesy of Stemcell Technologies, Inc.)

situ hybridization (FISH), interphase FISH, and comparative genomic hybridization (CGH) can also be attributed to automated imaging (see Chap. 17).

Although the primary application of an imaging system in many cytogenetics laboratories used to be the production of karyograms, the use of automated imaging systems for FISH rapidly gained popularity, and some laboratories perform more FISH imaging than chromosome imaging. Automated metaphase finding and fluorescent spot counting also represent important applications for imaging systems in cytogenetics.

Benefits

There are clear advantages to eliminating the need to rely on photography. Laboratories no longer need to dedicate valuable space to creating and maintaining a darkroom, staff no longer need be exposed to the chemicals and fumes involved, and there is no longer a need to arrange and pay for proper disposal and reclamation of silver. Nevertheless, a reduction in the time it takes to complete an analysis is unquestionably the major benefit of an automated imaging system. Laboratories can also save operator time by automating metaphase scanning, karyotyping, and FISH applications, in some cases resulting in a faster turnaround time and higher throughput of cases. Reduction in labor also translates to reduced costs.

Another big advantage of digital images is easy and compact storage. With current compression technologies and digital storage devices, this is much easier and less space consuming than it was with photographs and negatives. In addition, photographs could deteriorate over time, making them harder to reexamine if necessary.

Automated imaging systems can also provide consistency, especially when performing interphase FISH assays. Whereas manual spot counting can be highly subjective, an automated system will use predefined parameters for spot counting and, using those parameters, will produce consistent results.

Sharing of data can be important in a clinical lab setting and is clearly facilitated by the use of digital images; with the current use of the Internet and electronic mail, digital images are more easily shared for consultation and discussion. However, with data sharing via the Internet comes the need for compression and, more pressing, the need for patient record

security. Partly to address this need for patient record security, the U.S. Congress passed the Health Insurance Portability and Accountability Act (HIPAA) in 1996 and subsequently the Health Information Technology for Economic and Clinical Health Act (HITECH) as part of the American Recovery and Reinvestment Act of 2009 [3, 4] (see also Chap. 6).

While traditional photographic techniques offered some degree of contrast and other image adjustment, automated imaging systems further offer easy image enhancements, visualization techniques, and quantification, potentially providing additional information.

Limitations

Despite image enhancement features, the quality of the final image is ultimately dependent on the quality of the original microscope image (see Chap. 5). An image may be improved through background elimination, contrast and color enhancement, or even longer exposure times, but all these will not make up for a poor image due to poor microscope configuration or suboptimal slide preparation (see Chap. 4).

Imaging System Components

In general, an imaging system for cytogenetics comprises a microscope with a camera adapter, a camera, and a computer and software (Fig. 7.12). A printer and a method of archiving images are also required.

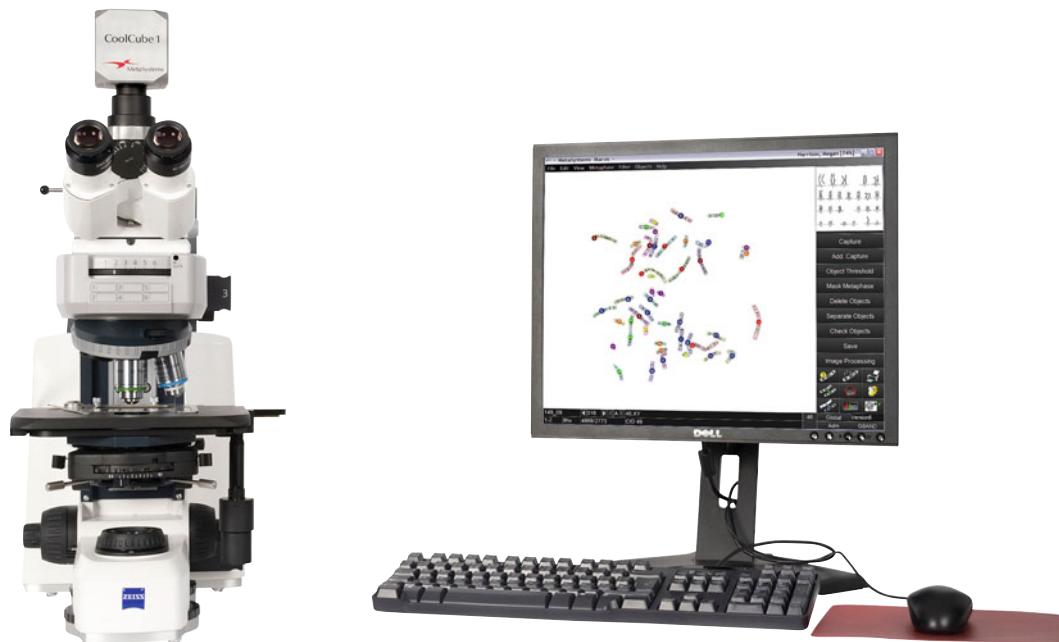


Fig. 7.12 Ikaros automated chromosome imaging system (Photo courtesy of MetaSystems Group, Inc.)

Microscope with Camera Adapter

A detailed discussion of microscopes and microscopy can be found in Chap. 5. As the name already implies, the camera adapter (known as a C-mount) is the device designed to attach a camera to a microscope. This adapter also permits the microscope image to be projected onto the photosensitive area of the camera, and ensures that the digitally captured image is scaled correctly to match the microscope objective. It is important to be certain that the captured image reflects the size and proportion of the image viewed through the microscope. This can be accomplished through the use of an additional lens that will enlarge or reduce the size of the image captured. For example, a 63 \times objective would be paired with a 1 \times camera adapter, which introduces no additional magnification or reduction. However, a 100 \times lens would require a .7 \times camera adapter to reduce the size of the image to fit correctly on the camera.

Camera

Though a wide range of different camera options are available (analog, digital, cooled, uncooled, monochrome, color), the most commonly used camera on automated imaging systems for the cytogenetic laboratory is a black-and-white, uncooled CCD (charge coupled device) camera.

Computer

Though both PC- and Macintosh-based systems have been available, the recent trend has been a move toward PC-based imaging systems. The computer(s) can be networked, allowing the actual analysis of the images to be performed off-line and to facilitate data sharing. Network storage of digital images has become more prevalent, allowing laboratories to share data and improve efficiencies, workflow, and turnaround times. Additionally, network storage of images has removed the need to store and maintain data on individual computers.

Software

The software for automated imaging systems for cytogenetics consists of at least two parts: acquisition or capture and the actual analysis. These can be two distinct steps or can be seamlessly integrated into one application. The acquisition step drives the camera in order to take a digital picture (capture an image). It also includes image enhancement features such as contrast adjustment, background subtraction, shading correction, and image sharpening. After image capture and enhancement, the user can analyze the image using the analysis applications of the software. Though there are many commercial packages available for image analysis, cytogenetics software, especially developed to address the specific requirements of a cytogenetics laboratory, includes several important features that are not available in conventional image analysis packages. Some of these features include the automatic generation of karyograms and the automated scoring of interphase FISH slides.

Printer

Although the trend seems to be moving toward the paperless laboratory, hard copy prints can still be used for diagnostic and/or archival purposes. In addition to the high-resolution black-and-white images of karyograms prepared by cytogenetics laboratories, a printer used for FISH applications must be capable of reproducing the range of colors generated by modern FISH software. Laser printers are widely used for these purposes.

Archival Device

As mentioned earlier, there is a need (often imposed by regulation) for long-term archiving of patient data. With the use of automated imaging systems, the data is in digital form and is easy to store. Currently, DVDs and optical discs can be used as solid media, while many labs choose to store data directly on a server, often as part of a network as indicated earlier.

Applications of Automated Imaging

Preparation of Karyograms

One main application of automated imaging systems is karyotyping. This involves separating and classifying chromosomes based on length, position of the centromere, and banding pattern (See Chap. 3). These automated systems should provide ease of use, speed, image quality, and accuracy.

Imaging systems have improved significantly over the years. Early systems required the user to “cut out” the chromosomes using a trackball or mouse, and place them into a karyogram. In semiautomated systems, the system could “cut out” the chromosomes, but the user had to arrange them into a karyogram. Today’s fully automated imaging system will capture the image, separate or “cut out” the metaphase chromosomes, classify them, and arrange them into a karyogram (Fig. 7.13). However, some metaphases contain several

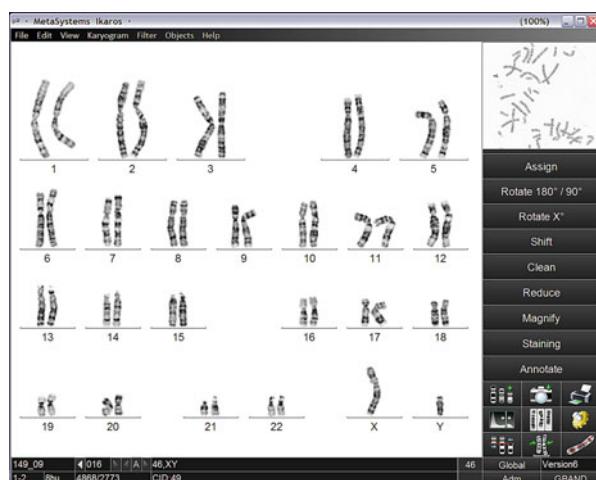


Fig. 7.13 An example of karyotyping software. The original metaphase is in the upper right (Photo courtesy of MetaSystems Group, Inc.)

overlapping chromosomes, and the user may still need to intervene and manually separate these using the mouse. Also, abnormal chromosomes are often not identified by imaging software, requiring user interaction for creation of an accurate karyogram.

A fully automated karyotyping system can also be used in conjunction with metaphase finding capability. This means that the system will automatically scan the slide in search of good metaphase spreads that can be used for analysis and creation of karyograms.

Scanning and Metaphase Finding

Finding metaphases acceptable for analysis is an integral part of cytogenetics. In many samples, good quality metaphases are abundant. However, in some specimens, such as in cancer cytogenetics, cells are often of poor quality and metaphase spreads acceptable for analysis are few and hard to find. A system that will automatically scan a slide for metaphase spreads can greatly reduce the time spent by a technologist on these samples looking for those metaphases.

The microscope in a metaphase finding system is outfitted with a motorized stage and focus drive for automated focusing. While automatically scanning one slide saves the user time, it does not make much sense to continuously have to change slides for scanning. To increase the throughput of the system, many suppliers add a stage or even slide loader to the system that holds multiple slides (Fig. 7.14). Based on several parameters, the system images metaphase spreads at high power and presents them to the user for review and analysis (Fig. 7.15).

Key factors for a metaphase finding system are the ability to recognize appropriate metaphases or cells, accuracy of

relocation to a metaphase of interest, speed of scanning, and sensitivity (the percentage of metaphase cells found by the system). Newer technologies allow the metaphases finders to automatically select the best metaphases after search, apply oil to the slide, and capture these metaphases under high magnification.

Software features important for metaphase finding include:

- Definition of classification parameters to ensure optimum scan results. The user can define the parameters that are utilized by the system.
- The ability to quickly relocate to a metaphase or rare cell for review.
- A sort function to organize metaphases or cells after scanning based on specific parameters and quality preferences.

A metaphase scanning system can be set up to continuously scan slides for metaphases or rare events while technologists are analyzing the detected metaphases or cells on remote review stations. Some laboratories find that this streamlines their workflow, while others find these systems to be neutral in terms of time gained. Laboratory volume and workflow will determine whether use of such a system makes sense.

Due to the general nature of the scanning system, it can also be used in other applications that require scanning for particular cells, such as FISH spot counting for detection of tumor cells in body fluids.

Fluorescent Spot Counting

FISH technology is based on fluorescently labeled probes that hybridize to unique DNA sequences along the chromosomes and can be performed on either metaphase preparations or interphase cells. One application is fluorescent spot



Fig. 7.14 An automated slide scanning system, which can be used with both brightfield and fluorescence microscopy on the same tray. The tray loader allows up to 81 slides to be scanned without interruption, but slide trays can also be loaded and unloaded while the system is operating (Photo courtesy of Applied Spectral Imaging, Inc.).

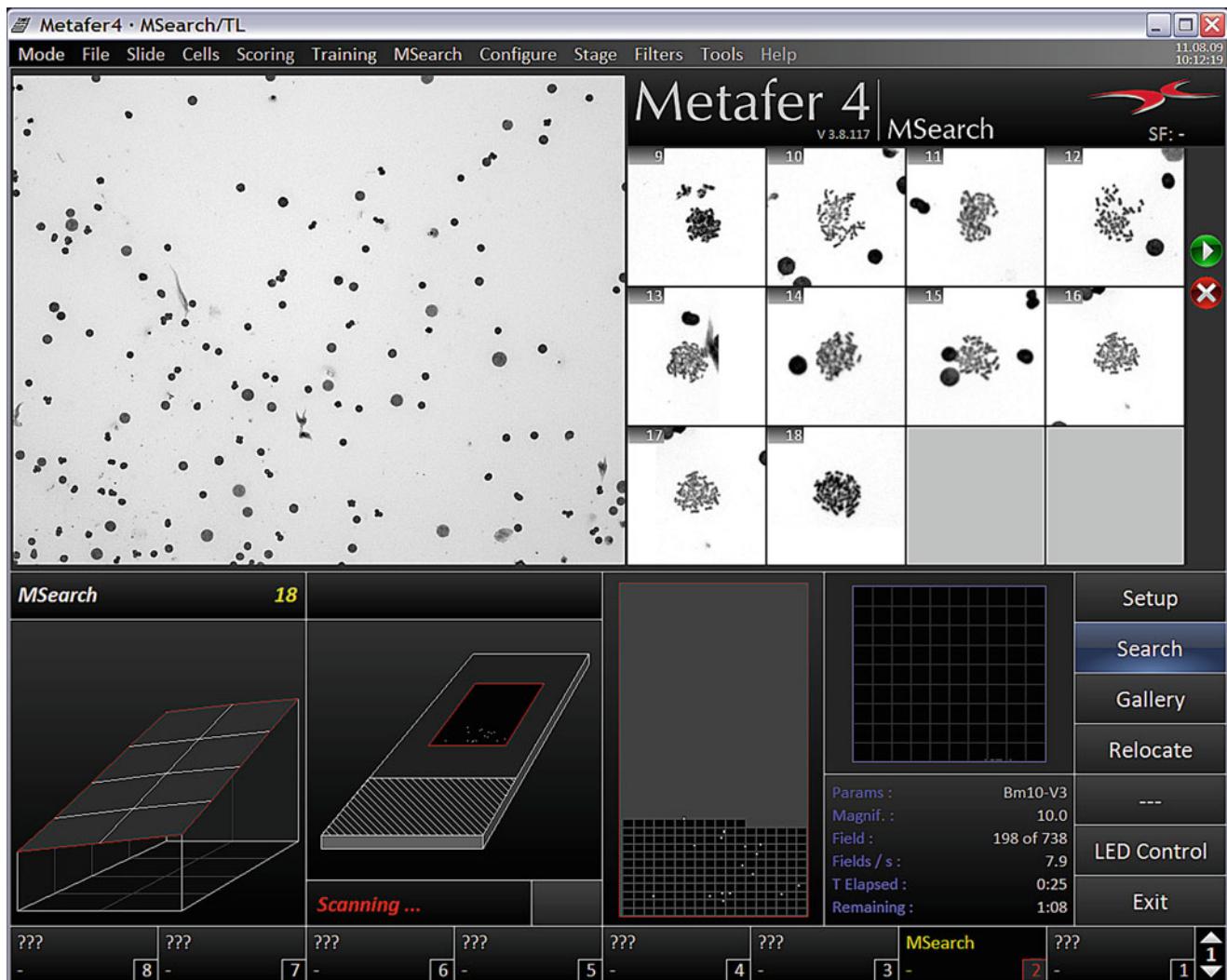


Fig. 7.15 Software interface of a metaphase finding system, showing thumbnails of the metaphase spreads located by the system (Photo courtesy of MetaSystems Group, Inc.)

counting and examination used for translocation or copy number analysis performed on interphase cells (Fig. 7.16).

Generally, an imaging system for FISH needs to be able to capture low light level images in multiple wavelengths, quantify the number of each fluorescent signal, and estimate the intensity ratio of the different signals.

Since interphase cells are three-dimensional (3-D) structures, the fluorescent signals in interphase FISH can be present in different focal planes. This means that to be able to see all signals, the user will need to focus on the different planes (the Z-axis), making the presence of a motorized focus drive on an automated system imperative. The automated focusing allows for resolution of the multiple signals across a large focal depth. Images from different focal planes are captured, processed, and compiled into one pseudo 3-D image that shows all signals in focus. This 3-D image capture is often referred to as Z-stack.

To visualize the different fluorochromes, the system uses different bandpass filters and a single, epi-illuminating light source (see Chap. 5, Fig. 5.6). An image is acquired for each fluorescent label used in the protocol, and the computer combines those into a color image. If the system is not equipped with an automated microscope with motorized filter block changing, a motorized filter wheel that will hold the different filters is highly desirable.

The microscope focus, camera, and filter wheel can be automatically controlled and synchronized by Z-stack software for multiplane, multicolor fluorescence image capture. Images in different focal planes are acquired and combined in a focused, color image to ensure that faint signals that would otherwise be omitted are incorporated in subsequent analyses.

FISH analysis is also amenable to automated software. To ensure consistent scoring and analysis of interphase FISH, such software should include:

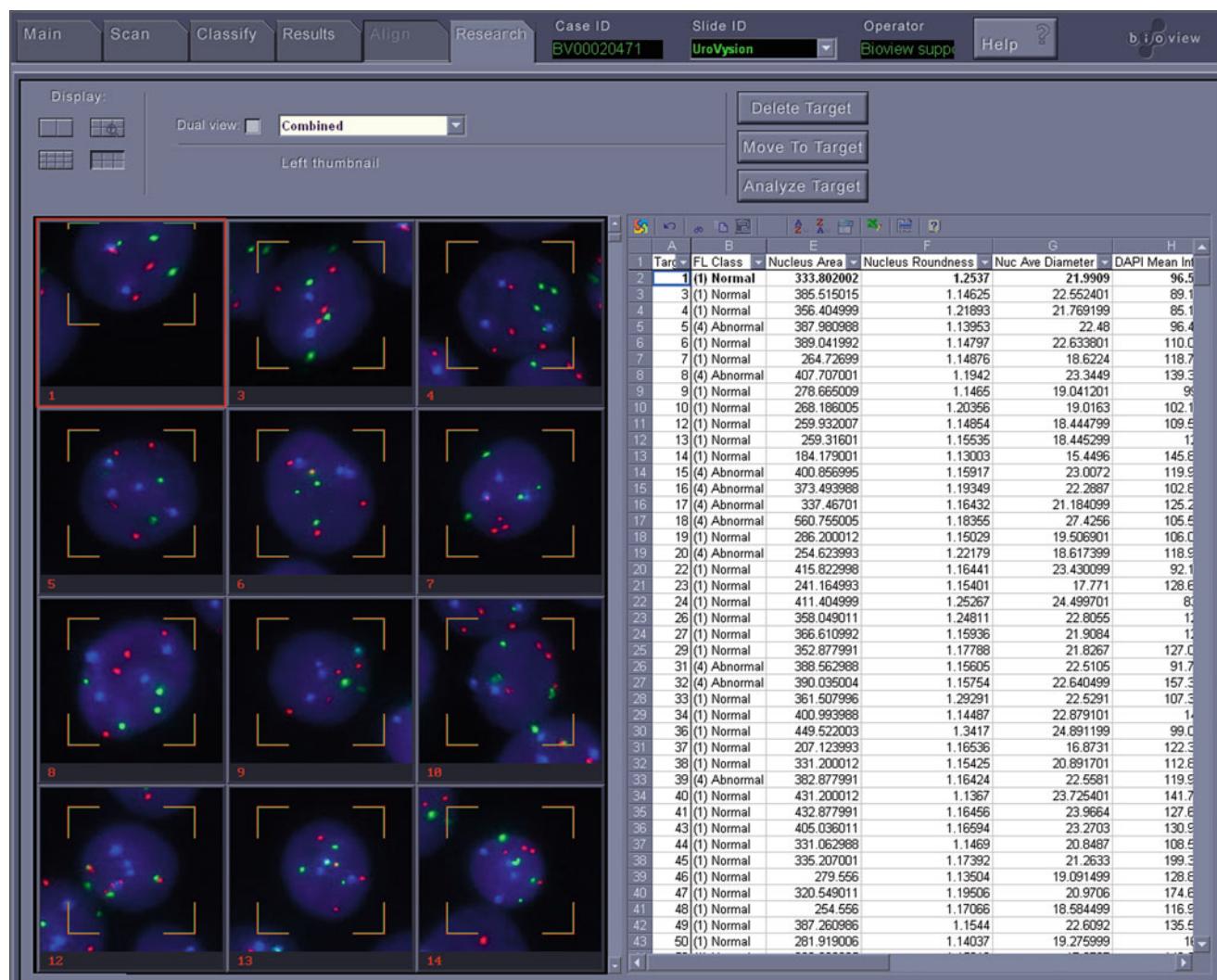


Fig. 7.16 Example of a software interface for spot counting of interphase FISH, showing thumbnails of cells and spots located by the system. This application is using the Abbott Molecular UroVysis® kit for

the detection of chromosomal abnormalities associated with the recurrence and progression of bladder cancer (Photo courtesy of Bioview, Inc.)

- Trainable classifiers to determine which cells to score, so users can “teach” the system to work with their own results and standards.
- User-definable parameters to determine the scoring rules. Such parameters include spot size and spot separation distances (measured three-dimensionally), fusion detection, and the number of cells to score.
- The ability to reprocess the images under different scoring rules without having to rescan the slide.
- The ability to sort the scored cells in user-definable categories, allowing for easier analysis of complex signal patterns.
- A reporting function that presents the results for review. Reports should be customizable to reflect the user’s preferred data layout and should include images of scored cells and different representations of the results, such as bar charts and scatter plots.

Software that facilitates analysis of multiple (sequential) fluorescence assays by relocating previously analyzed cells is also available (Fig. 7.17).

M-FISH

M-FISH, also referred to as multicolor FISH, multiplex FISH, or one company’s proprietary name of spectral karyotyping (SKY), can be viewed as fluorescent multicolor karyotyping and is mainly used for the detection and classification of interchromosomal aberrations (see Chap. 17). In this form of FISH, probes labeled with a combination of different fluors are hybridized with the chromosomes in a metaphase spread. Currently, five different fluorochromes are used. The five different fluors give $31 (2^n - 1)$ color combinations, enough to uniquely identify the 24 different chromosomes in the human genome. It has been suggested that the resolution

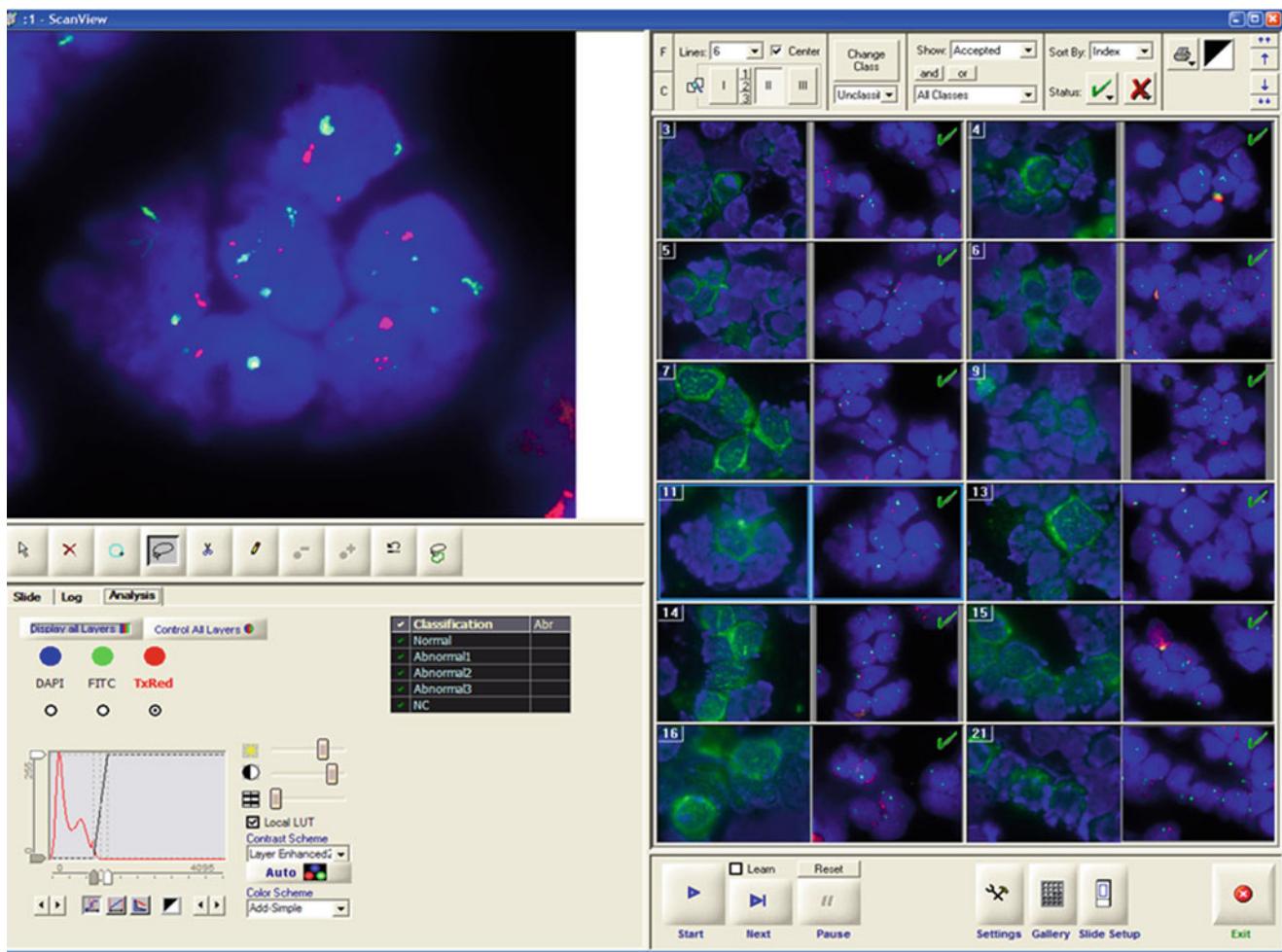


Fig. 7.17 ASI MultiStain software for relocation to previously found cells after restaining with different markers, enabling cross correlation of findings from the same slide after multiple staining. After the slide is washed following initial staining, the cells can be restained and quickly

relocated. This process can be repeated up to three times. Afterward, all images of the same, differently stained cells can be viewed side by side in the gallery (Image courtesy of Applied Spectral Imaging, Inc.)

of using a 5-fluorochrome set could result in some small aberrations being missed and that this problem can be eliminated by increasing the number of fluors to seven [5]. However, this concept has not been introduced into common laboratory practice.

From a hardware perspective, the requirements of an automated system for M-FISH are similar to the requirements of an automated system for interphase FISH: the system should include the fluorescent, epi-illuminating light source and a filter wheel containing the appropriate filters. In addition, the system could include a metaphase finding capability as well as motorized focusing.

The software for M-FISH incorporates:

- Sophisticated algorithms that analyze the images to determine the fluor combination a chromosome is labeled with and then assign a pseudo-color to each fluor combination. These pseudo-colors should be user changeable to improve visualization of rearranged chromosomes.

- Karyotyping capabilities so that the colored chromosomes can be arranged in a karyogram (Fig. 7.18; see also Chap. 17, Fig. 17.18).
- Individual pseudo-color display of any single chromosome to facilitate visualization of chromosomal aberrations.

Comparative Genomic Hybridization (CGH) and Chromosome Microarray

Whereas M-FISH is a useful technique to determine interchromosomal rearrangements, comparative genomic hybridization (CGH) provides information concerning losses or gains of DNA within a chromosome (see Chap. 17). In CGH, the probes are generated from two different sources: one from genetically normal cells and the other from the patient sample. The two different probe sets are labeled with different fluors. These two pools of probes are then hybridized to a slide that contains normal metaphases and will compete for hybridization to the corresponding loci. The ratio of the patient DNA to



Fig. 7.18 M-FISH capture and analysis. The software analyzes the signals produced by the various combinations of fluors, produces a false color for each chromosome, and arranges the chromosomes into a karyogram. See text for details (Photo courtesy of MetaSystems Group, Inc.)

normal DNA will indicate whether the patient DNA is normal (the ratio is 1:1) or whether there is an addition or deletion of DNA in any given region. When there is an addition, the ratio will increase; when there is a deletion, the ratio will decrease.

Chromosomal CGH requires the use of a high-quality and quantitative FISH imaging system with dedicated software that will:

- Accurately measure and average the ratio of the two fluors over multiple metaphases. This requires sophisticated algorithms.
- Correct the measurements for unequal chromosome length.
- Plot the ratios along the chromosome length for ease of interpretation, highlighting the areas of statistically significant differences (see Chap. 17, Fig. 17.17).

For microarrays, specific DNA targets are “printed” onto a microscope slide and CGH is performed *in situ* on the slide (see Chap. 18). A scanner reads the slide and sends the data to a computer for analysis (Fig. 7.19a, b).

Multipurpose Instruments

Some of the instruments described in this chapter are capable of performing more than one function in the cytogenetics laboratory. Given the direction that instrumentation and electronics are going in today’s world, such cross functionality will ultimately become more common. Devices designed to

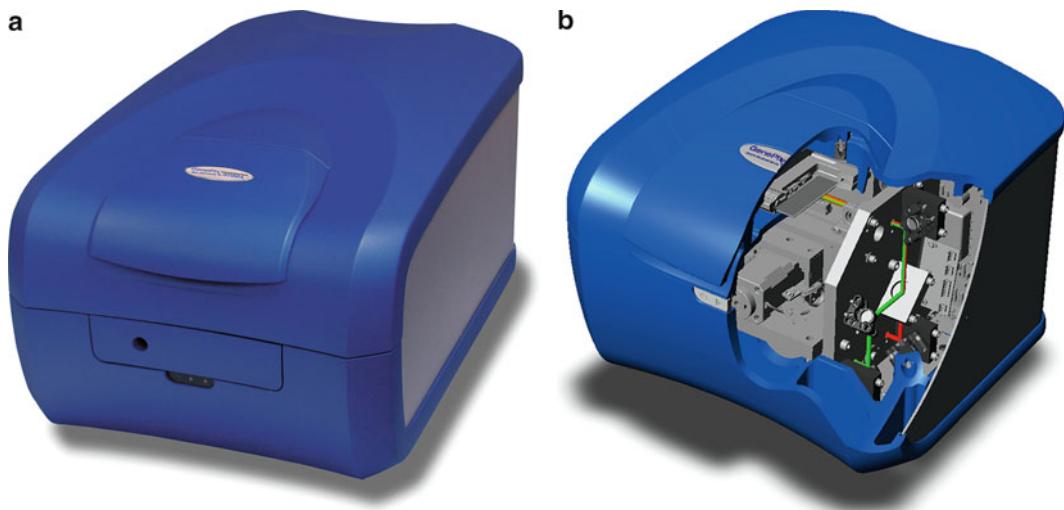
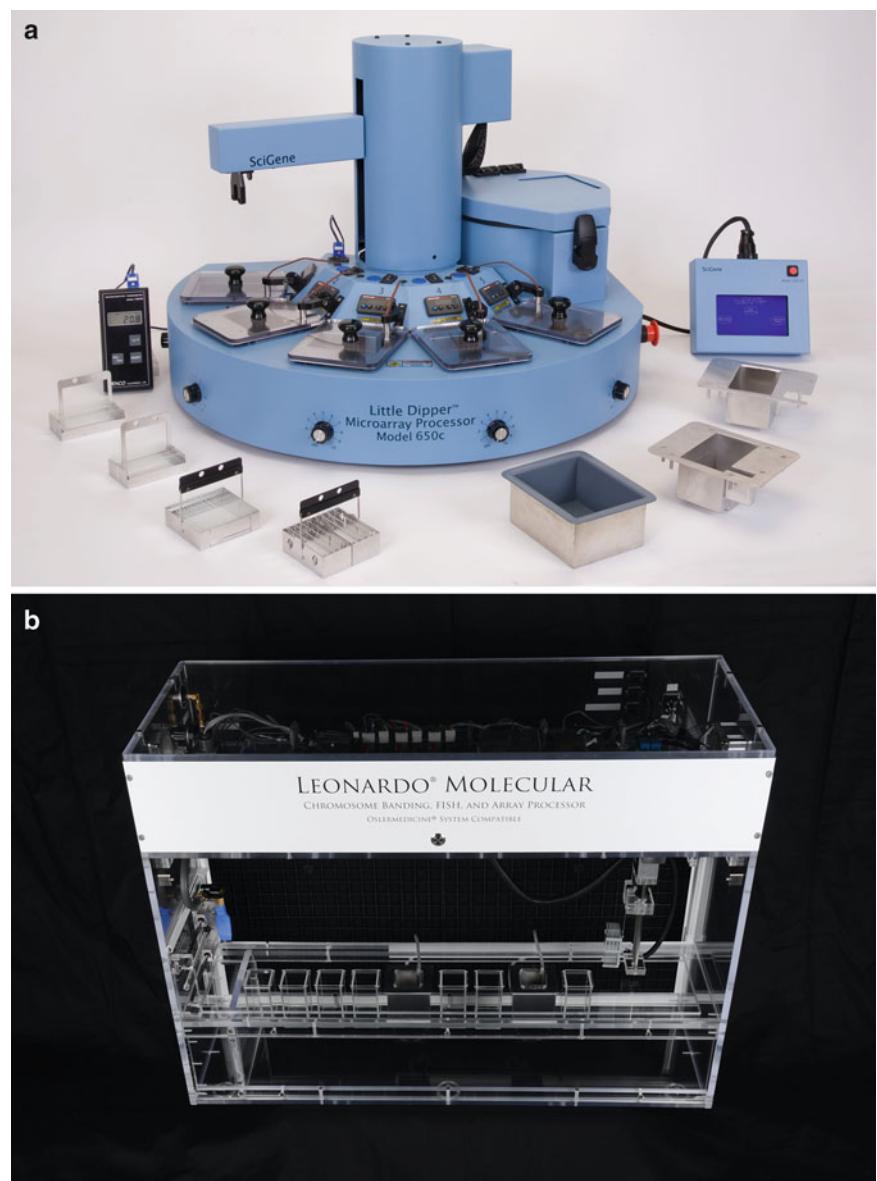


Fig. 7.19 GenePix microarray scanner. (a) This benchtop unit features an 8-position emission filter wheel to allow the user flexibility in choosing fluorescent dyes. (b) The image on the right shows a schematic of the light path through the scanner (Photos courtesy of Axon Instruments, Inc.)

Fig. 7.20 Multipurpose devices designed to process chromosome banding, FISH, and microarrays. (a) SciGene Little Dipper. Wash time, agitation, buffer temperatures, and drying are controlled, and the device uses an integrated centrifuge (Photo courtesy of SciGene). (b) elja Leonardo® Molecular Processor. Laptop software allows the user to modify every aspect of the procedure for accuracy and reliability (Photo courtesy of elja, Inc.)



be used for some of the steps involved in chromosome banding, FISH analysis, and microarray studies are already available (Fig. 7.20a, b).

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Part III

Clinical Cytogenetics

Autosomal Aneuploidy

Jin-Chen C. Wang

Introduction

The term *aneuploidy* refers to cytogenetic abnormalities in which all or part of one or more chromosomes is duplicated or deleted. Autosomal aneuploidy refers to all such abnormalities that do not involve the sex chromosomes. These can be either numerical (the topic of this chapter) or structural, the vast majority being trisomies, and may be present only in some cells (mosaic aneuploidy) or in all cells (nonmosaic). The incidence of autosomal aneuploidy in newborns is estimated to be 0.2% [1]. Many autosomal aneuploidies are incompatible with fetal survival and therefore have much higher incidences (approximately 27–30%) in spontaneous abortuses [2–4]. These are discussed in this chapter and covered in detail in Chap. 12.

Cytogenetic studies of human oocytes and sperm reveal that the overall frequency of abnormalities is approximately 15–20 and 10%, respectively [5–7]. More than 90% of the abnormalities observed in oocytes and less than 50% of those seen in sperm are numerical. Since structural abnormalities are difficult to detect, the observation that the abnormalities identified in oocytes are mostly numerical could be a result of ascertainment bias [8]. Studies using fluorescence *in situ* hybridization (FISH) or primed *in situ* labeling (PRINS), which do not require the presence of dividing cells, have shown that the frequency of autosomal aneuploidy in human sperm is relatively uniform for all chromosomes studied (chromosomes 3, 7, 8, 9, 10, 11, 13, 16, 17, 21), with a range of 0.26–0.34% [9–11]. On the other hand, one study using FISH reported a higher frequency of aneuploidy for chromosome 21 (0.29%) than for other chromosomes studied (0.08–0.19% for chromosomes 1, 2, 4, 9, 12, 15, 16, 18, 20) [12]. It is therefore possible that meiotic nondisjunction is random for

all autosomes, with the possible exception of chromosome 21. It has also been reported that the frequency of aneuploidy varies among different individuals and can increase over a 5-year period in the same individual [13].

Trisomies for all autosomes have been reported in spontaneous abortuses, including trisomy 1, which has been reported at least one clinically recognized pregnancy at 8–9 weeks of gestation and in a clinically recognized *in vitro* fertilization (IVF) pregnancy at 6 weeks of gestation [3, 14, 15, 16]. However, no fetal pole ever developed in either case. Unlike in sperm, the observed frequency of each trisomy varies greatly in spontaneous abortuses or liveborns. For example, trisomy 16 accounts for approximately 30% of all autosomal trisomies in abortuses [3]. In liveborns, the only trisomies that have not been reported in either mosaic or nonmosaic form are those involving chromosomes 1 and 11, although trisomies other than 13, 18, and 21 are rare. Autosomal monosomies, on the other hand, are extremely rare in both liveborns and recognized abortuses.

The supposition that, with the probable exception of trisomy 21, the frequencies of trisomy for each chromosome might be similar at the time of conception but differ greatly among abortuses and liveborns can be explained by the devastating effect of chromosomal imbalance. Many autosomal aneuploidies are so deleterious that they are lethal in the preembryonic stage and thus result in unrecognized and, therefore, unstudied in spontaneous abortions. The lethality of a particular autosomal aneuploidy correlates with the gene content of the chromosome involved [14]. Aneuploidies for “gene-rich” chromosomes are less likely to survive. Trisomies 13, 18, and 21, which involve chromosomes that are “less gene-rich,” are therefore relatively “mild” and fetuses can survive to term.

This chapter addresses only those autosomal aneuploidies, both trisomies and monosomies, that have been observed in liveborns. Polyploidy, or changes in the number of *complete sets* of chromosomes, are also included, as are aneuploidies that are the result of supernumerary “marker” chromosomes.

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Mechanism and Etiology

Errors in meiosis (nondisjunction) result in gametes that contain abnormal numbers of chromosomes and, following fertilization, produce aneuploid conceptuses. Using DNA markers, the parental origin of the additional chromosome in autosomal aneuploidies has been studied for trisomies 2, 7, 13, 14, 15, 16, 18, 21, and 22 [2, 17–32]. These studies suggest that most trisomies are of maternal origin but that the proportion varies among different chromosomes and that (with the exception of chromosomes 7, 13, and 18) nondisjunction in maternal meiosis stage I accounts for the majority of cases (Table 8.1). A more recent study on nondisjunction of chromosome 13 in a large number of cases reported an equal number of maternal meiosis I (MI) and meiosis II (MII) errors [33].

The association between autosomal aneuploidy and maternal age has long been recognized. In 1933, Penrose demonstrated that maternal age was the key factor for the birth of Down syndrome children [34]. Why aneuploidy is maternal age dependent, and what constitutes the mechanism and etiology of chromosomal nondisjunction have been topics of much research, as summarized later in this chapter.

Nondisjunction can occur during either meiosis I (MI) or meiosis II (MII). In MI, homologous chromosomes pair and form bivalents (see Chap. 2). Malsegregation of homologous chromosomes can occur in one of two ways. The first involves

nondisjunction of the bivalent chromosomes with both homologs going to the same pole (Fig. 8.1d,e). The second type of error involves premature separation of the sister chromatids of one homolog of a chromosome pair. Subsequent improper distribution of one of the separated chromatids results in its segregation with the other homolog of the chromosome pair [35] (Fig. 8.2d,e). In MII, sister chromatids separate. Malsegregation occurs when both chromatids go to the same pole (Fig. 8.3g,h). It has been shown that error involving premature sister chromatid separation, especially in the smaller chromosomes, is in fact a more common mechanism leading to aneuploidy than malsegregation of the whole chromosome [6, 36, 37].

Earlier cytogenetic studies of oocytes, performed mostly on unfertilized or uncleaved specimens obtained from *in vitro* fertilization programs, have provided conflicting results regarding whether the frequency of gamete aneuploidy actually increases with maternal age [38–41]. A FISH study of human oocytes using corresponding polar bodies as internal controls demonstrated that nondisjunction of bivalent chromosomes (MI error) does in fact increase with maternal age, and a study using multiplex FISH on fresh, noninseminated oocytes also indicated an increase in premature separation of the sister chromatids in MI with increasing maternal age [42, 43]. More recent data based on studies of large numbers of oocytes further provided evidence for a direct correlation between advanced maternal age and increased aneuploidy frequency [6, 44].

Table 8.1 Parental and meiotic/mitotic origin of autosomal trisomies determined by molecular studies (number of cases)

Trisomy	Maternal					Paternal					References
	MI	MII	MI or MII	Mitotic	Total ^a	MI	MII	MI or MII	Mitotic	Total ^a	
2	4	1	6	1	13	5				5	[30]
7	2	3	1	6	12				2	2	
13	4		17		21	1	1	1		3	[24]
	34	33	3	3			5	1	3		[33]
14	3	4	2		9		2			2	[24]
15	21	3		3	27				5	5	[16] (UPD study)
	10				10				2	2	[18] (UPD study)
	17	2	10		29		4	1		5	[24, 30]
16	56		6		62					0	[25]
18	11	17			56		1			6	[28]
					17					5	[21]
	16	≥35		3	61				2	2	[26]
	10	17		1	28	2			1	3	[31]
21	9	1			22					3	[17]
					91					6	[18]
	128	38			188	2	7			9	[19]
							7	15		8	36
	174	58	79		311	9	15	8		32	[23] (paternal study only)
	62				81		10			13	[24]
	67	22			97	4	4			10	[27]
22	20	1	15		37			1		1	[24, 30]

^aTotal numbers may not add up because not all origins of error can be determined

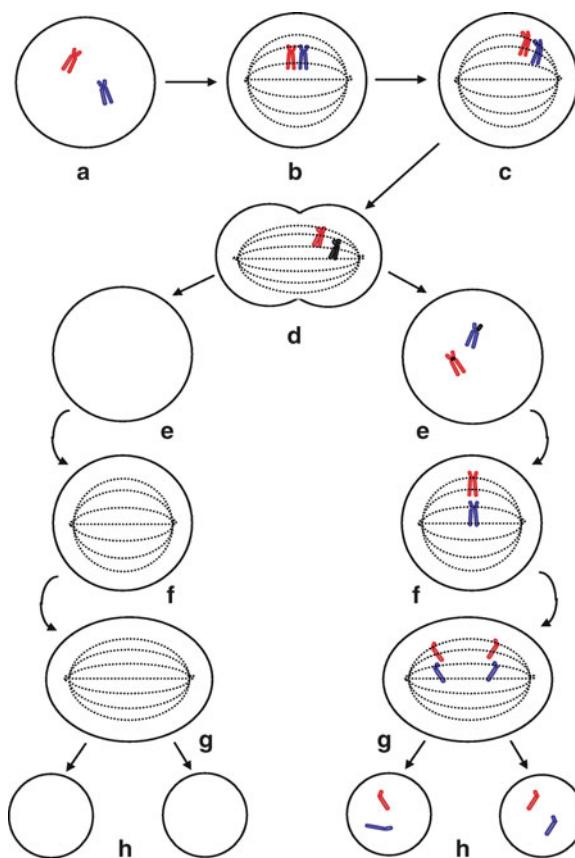


Fig. 8.1 Schematic representation of meiosis I nondisjunction. (a) Prophase I. (b) Metaphase I. (c) Anaphase I. (d) Telophase I, with both homologs of one chromosome pair segregating together. (e) Products of meiosis I. (f) Metaphase II. (g) Anaphase II. (h) Meiotic products—two gametes lack one chromosome and two gametes contain two copies of one chromosome

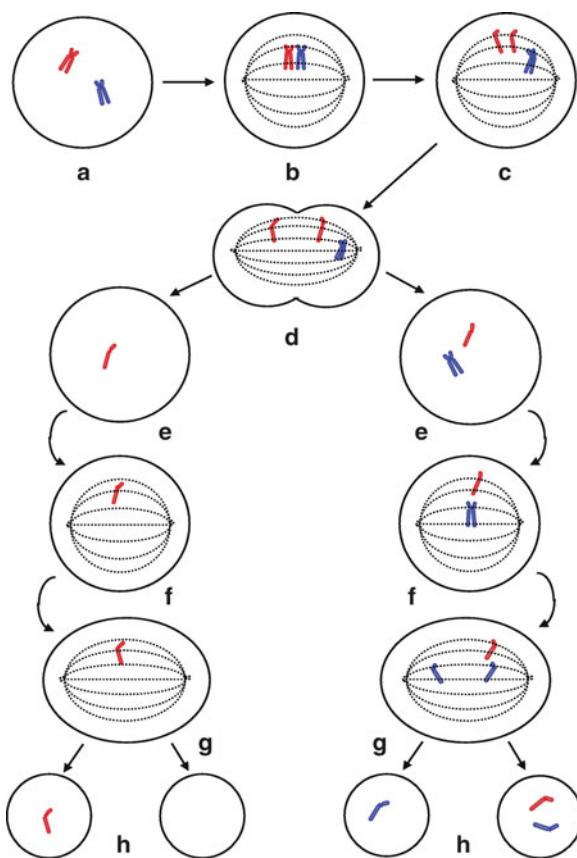


Fig. 8.2 Schematic representation of meiosis I error resulting from premature sister chromatid separation. (a) Prophase I. (b) Metaphase I. (c) Anaphase I, with premature separation of centromere of one chromosome. (d) Telophase I, with one prematurely separated chromatid segregating with its homologous chromosome. (e) Products of meiosis I. (f) Metaphase II. (g) Anaphase II. (h) Meiotic products—two gametes with a normal chromosome complement, one gamete lacking one chromosome, and one gamete containing two copies of one chromosome

Different mechanisms have been proposed to account for the observation of the correlation between maternal age and aneuploidy frequency. One example is the “production line” hypothesis [45, 46]. This hypothesis proposes that oocytes mature in adult life in the same order as the corresponding oogonia entered meiosis in fetal life. Oogonia that enter meiosis later in development may be more defective in the formation of chiasmata and thus more likely to undergo nondisjunction. One direct cytological support for this hypothesis was provided by a study that examined the frequency of unpaired homologs in MI pachytene and diplotene in oocytes obtained from abortuses at 13–24 weeks and 32–41 weeks of gestation [47]. Of the six chromosomes studied (chromosomes X, 7, 13, 16, 18, and 21), the rate of pairing failures in early specimens (0–1.2%) was significantly lower than that in later specimens (1.3–5.5%). No corroborating data are available. It remains an interesting question whether the oocytes first committed to meiosis in fetal life

are the first to ovulate in adult life. Another example is the “limited oocyte pool” model [48]. At the antral stage of each menstrual cycle, multiple follicles at various stages of development are present. When stimulated with high levels of plasma follicle stimulating hormone (FSH), only one follicle, presumably the one at the most optimal stage, will complete MI and eventually achieve ovulation. The number of follicles in the antral stage decreases with increasing maternal age. When the number of these follicles is low, it is more likely that an oocyte that is not at the optimal stage will be selected for ovulation. If such “less optimal” oocytes are more likely to undergo MI nondisjunction, then the ovulated oocytes of older women will have higher rates of aneuploidy. More recent data, however, does not appear to support this hypothesis [49, 50].

One probable factor that predisposes gametes to nondisjunction is aberrant recombination [51] (see Chap. 2). Data on recombination patterns are available for trisomies

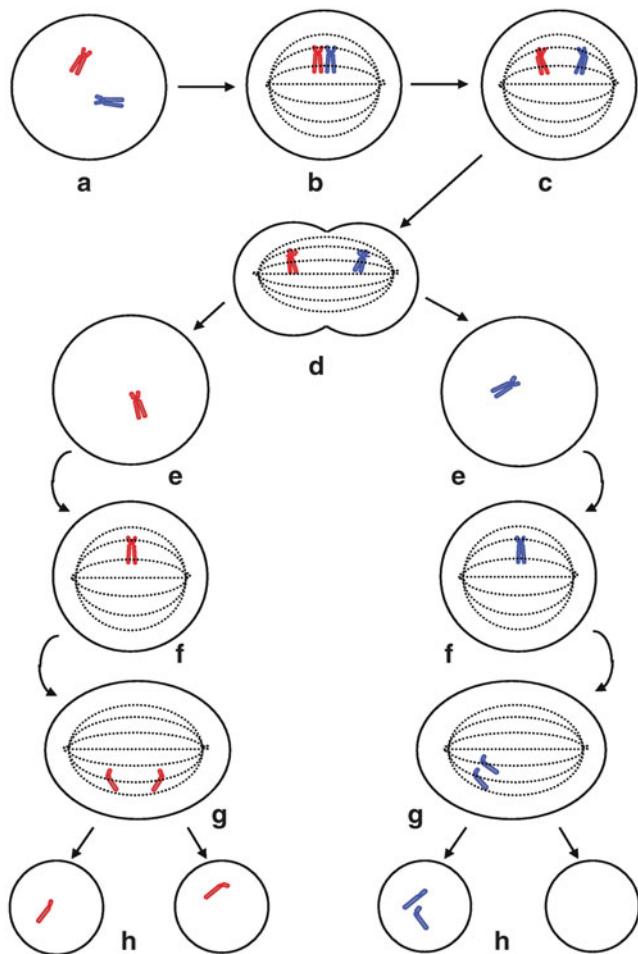


Fig. 8.3 Schematic representation of meiosis II nondisjunction. (a) Prophase I. (b) Metaphase I. (c) Anaphase I. (d) Telophase I. (e) Products of meiosis I. (f) Metaphase II. (g) Anaphase II, with both sister chromatids segregating together. (h) Meiotic products—two gametes with a normal chromosome complement, one gamete lacking one chromosome

15, 16, 18, and 21. Studies of chromosome 15 nondisjunction in uniparental disomy (see Chap. 20) revealed that there was a mild reduction in recombination in association with maternal nondisjunction, with an excess of cases that have zero or one crossover and a deficiency of cases that have multiple crossovers [20]. In contrast, in a study of trisomy 18, approximately one-third [5/16] of maternal MI nondisjunctions were associated with a complete absence of recombination, whereas the remaining MI and all MII nondisjunctions appeared to have normal rates of recombination [26]. Studies of trisomy 16 and trisomy 21 reported similar findings between the two. In trisomy 16, it was shown that recombination was reduced, but not absent, and that distribution of recombination was altered, with rare crossovers in the proximal regions of the chromosome [25]. A recent study performed on oocytes from young egg

donors after hormone-induced superovulation demonstrated that 2.5% of chromosome 16 bivalents had no crossovers and a high percentage (19.8%) had only a single recombination [37]. In trisomy 21, there was an overall reduction in recombination with an increase in both zero and one crossover in maternal MI nondisjunction [52]. Lamb et al. showed that in maternal MI nondisjunction for chromosome 21, the average number of recombination events was decreased, with approximately 35–45% of cases having no crossovers [53]. When at least one crossover was present, it occurred largely at distal 21q. This study, together with one on trisomy 16, suggests that, at least for trisomies 16 and 21, distal chiasmata are less efficient in preventing nondisjunction in MI [25]. In contrast, in maternal MII nondisjunction, the number of recombination events appeared to be increased, especially in proximal 21q. These proximal recombinations may cause an “entanglement” effect. Entanglement of the two homologs can cause the bivalent to move to the same pole at MI, and then at MII the two homologs finally separate, resulting in two disomic gametes each having two chromatids with identical centromeres. Alternatively, the entanglement may disrupt sister chromatid cohesion resulting in premature separation of the sister chromatids at MI. If the two separated sister chromatids travel to the same pole at MI and again at MII, an apparent MII nondisjunction would be observed. Thus, these data suggest that all nondisjunction events may be initiated during MI. The observation that for chromosome 21, MI error is associated with distal recombination while MII error is associated with proximal recombination has been independently confirmed recently in a study of a population in India [54]. Lamb et al. showed that the alteration in recombination pattern was not maternal age dependent. They proposed a “two hit” model and hypothesized that certain recombination configurations are less likely to be processed properly in older women [53, 55]. This could result from, for example, an age-dependent loss of spindle forming ability, thus explaining their observation for trisomy 21 that although an altered recombination pattern is not maternal age dependent, meiotic disturbance is age dependent [56]. The same argument was used by Hassold et al. to explain their findings with trisomy 16 [51].

It has been proposed that the cellular mechanism assuring correct segregation of chromosomes into daughter cells is provided by a four-protein complex (SMC1, SMC3, SCC1/RAD21, SCC3/SA/STAG) that together form a ring-like structure known as the cohesin complex [57]. The cohesin complex acts as “chromosome glue” and thus mediates cohesion of the two sister chromatids during cell division. Additional proteins are needed for the establishment and maintenance of cohesion. Loss of cohesion of both arms, telomere, and centromere during the metaphase/anaphase transition is also tightly controlled by various proteins including a specific protease separin/separase.

It has also been suggested, at least for chromosome 21, that chiasmata and recombination in oocyte are as efficient as in spermatocyte [58]. The less effective meiotic checkpoint mechanism in oocytes allowing aneuploid oocytes to progress through meiosis appeared to be the basis for the observation that the majority of trisomy 21 conceptions are of maternal origin. On the other hand, evidence against a defective spindle assembly checkpoint being the cause of aneuploidy associated with advanced maternal age has also been reported [59].

The possibility of the presence of a genetic predisposition to nondisjunction has also been proposed. One study involving consanguineous families in Kuwait showed that the relative risk for the occurrence of Down syndrome was approximately four times greater for closely related parents (first cousins, first cousins once removed, second cousins) than for unrelated parents [60]. As consanguinity is usually perpetuated in certain families or sections of the population, these results were taken as evidence for the existence of an autosomal recessive gene that facilitates meiotic nondisjunction in homozygous parents. Thus, in a subgroup of trisomy 21 patients, nondisjunction may be genetically determined. In a study of trisomy recurrence based on North American data, a significantly increased risk for recurrent of a different trisomy was observed [61]. This supports the hypothesis of possible genetic predisposition to nondisjunction.

While maternal age and altered recombination remain the only well-established risk factors for nondisjunction, our understanding of the underlying mechanism of this observation is still not complete. It is possible that more than one mechanism, including possibly environmental and hormonal factors, contributes to the observed maternal age effect [62].

Nondisjunction occurring at mitosis, on the other hand, will result in mosaicism, usually with both normal and abnormal cell lines.

Discussion of autosomal aneuploidies in this chapter will be limited largely to those observed in liveborns only.

Autosomal Trisomies

Trisomy 21

Incidence

Trisomy 21 [47,XX or XY,+21] (Fig. 8.4) was the first chromosome abnormality described in man by Lejeune et al. in 1969 [63]. The phenotype was delineated by John Langdon Down (1828–1896) in 1866 and is referred to today as Down syndrome [64]. It is the most common single known cause of mental retardation. The frequency in the general population is approximately 1 in 700. Down syndrome is more frequent in males, with a male-to-female ratio of 1.2:1. A recent study using multicolor FISH showed that among sperm disomic

for chromosome 21, significantly more were Y-bearing than X-bearing [65]. This finding was consistent with earlier reports showing an excess of males among trisomy 21 conceptuses that resulted from paternal meiotic errors [23]. This preferential segregation of the extra chromosome 21 with the Y chromosome contributes to a small extent to the observed sex ratio in trisomy 21 patients. Other mechanisms, such as *in utero* selection against female trisomy 21 fetuses, must also exist.

Trisomy 21 accounts for approximately 95% of all cases of Down syndrome. Mosaicism and Robertsonian translocations (see Chap. 9) comprise the remaining 5%. As described previously, the incidence of trisomy 21 in newborns is closely associated with maternal age (Table 8.2).

Phenotype

The clinical phenotype of Down syndrome has been well described [68, 69]. Briefly, there is a characteristic craniofacial appearance with upward-slanting palpebral fissures, epicanthal folds, flat nasal bridge, small mouth, thick lips, protruding tongue, flat occiput, and small and overfolded ears. Hands and feet are small and may demonstrate clinodactyly, hypoplasia of the midphalanx of the fifth finger, single palmar crease (Fig. 8.5), and a wide space between the first and second toes. Hypotonia and small stature are common, and mental retardation is almost invariable. Cardiac anomalies are present in 40–50% of patients, most commonly endocardial cushion defects, ventricular septal defects (VSD), patent ductus arteriosus (PDA), and atrial septal defects (ASD). Other observed major malformations include duodenal atresia, annular pancreas, megacolon, cataracts, and choanal atresia. In addition, a 10- to 20-fold increase in the risk for leukemia, most commonly acute megakaryoblastic leukemia, has been observed in Down syndrome patients of all ages, with a bimodal age of onset in the newborn period and again at 3–6 years [70]. Moreover, a transient abnormal myelopoiesis (TAM) with clinical and morphologic findings indistinguishable from acute myeloid leukemia occurs in approximately 10% of Down syndrome newborns [71]. Spontaneous remission within the first 3 months of life is observed in most patients; occasional relapse and life-threatening disease have also been noted. Of interest is the observation of the presence of a trisomy 21 clone in association with TAM in 15 phenotypically normal children, at least 4 of whom were determined to be constitutional mosaics for Down syndrome [72].

Overall, the clinical phenotype is typically milder in mosaic Down syndrome patients, but there is no clear correlation between the percentage of trisomy 21 cells and the severity of clinical presentation. This can be as severe in mosaic patients as in nonmosaic trisomy 21 individuals.

Delineation of the regions of chromosome 21 responsible for the Down syndrome phenotype has been attempted using molecular methods to study patients with partial trisomy 21

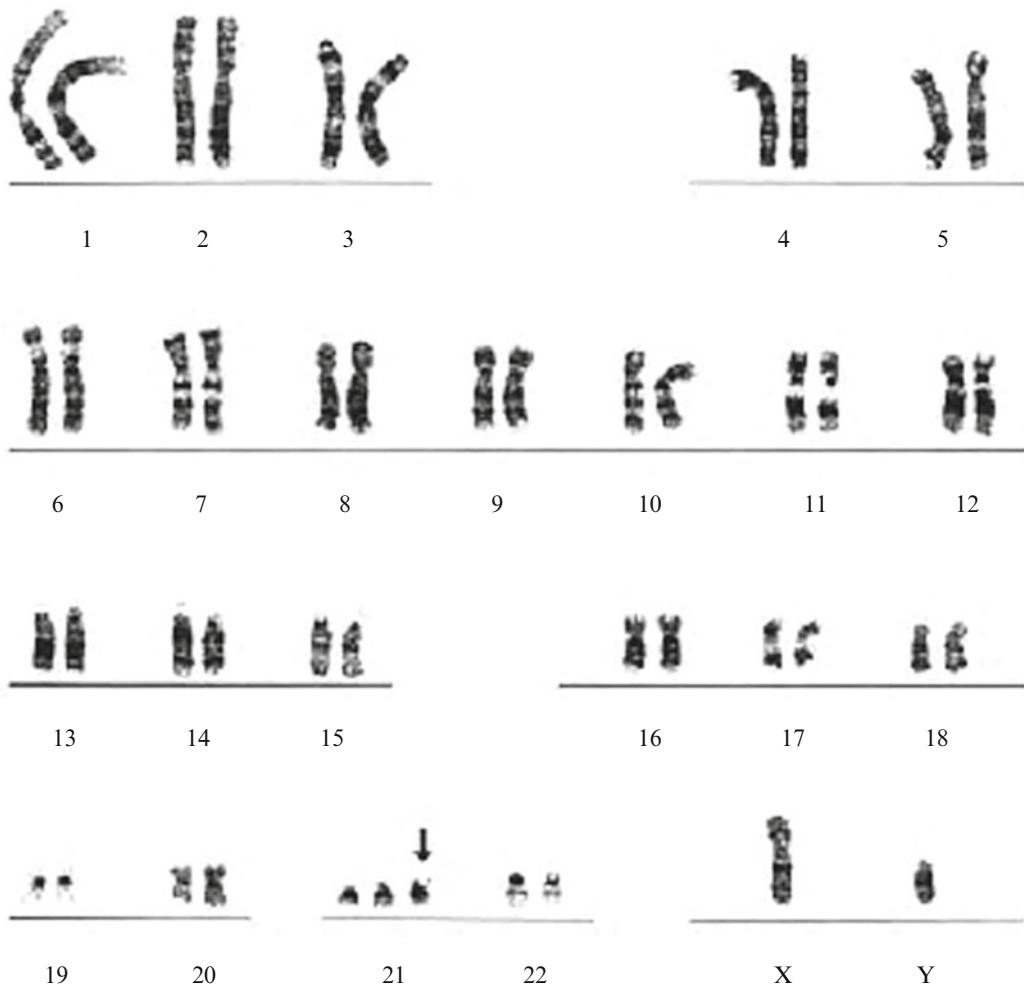


Fig. 8.4 Trisomy 21 Down syndrome male karyogram (47,XY,+21)

Table 8.2 Maternal age-specific risks for trisomy 21 at birth

Maternal age (years)	Incidence at birth (1 in)	Maternal age (years)	Incidence at birth (1 in)
15	1,580	33	570
16	1,570	34	470
17	1,565	35	385
18	1,555	36	307
19	1,540	37	242
20	1,530	38	189
21	1,510	39	146
22	1,480	40	112
23	1,450	41	85
24	1,400	42	65
25	1,350	43	49
26	1,290	44	37
27	1,210	45	28
28	1,120	46	21
29	1,020	47	15
30	910	48	11
31	800	49	8
32	680	50	6

Modified from Cuckle et al. [66]. Data were based on eight pooled studies. Restriction of analysis to two studies with the most complete ascertainment yielded higher rates [67]

who present clinically with various features of the syndrome [73–80]. Studies by Korenberg et al. in a panel of cell lines derived from 16 partial trisomy 21 individuals suggest that, instead of a single critical region, many chromosome 21 regions are responsible for various Down syndrome features [79]. The study was expanded to include a total of 30 subjects carrying rare segmental trisomies of various regions of chromosome 21. By using current genomic technologies including high-density isothermal oligonucleotide DNA tiling arrays, a high-resolution genetic map of Down syndrome phenotype was constructed corresponding to discrete regions of 1.8–16.3 Mb likely to be involved in the development of eight Down syndrome phenotypes: acute megakaryocytic leukemia, transient myeloproliferative disorder, Hirschsprung disease, duodenal stenosis, imperforate anus, severe mental retardation, Down syndrome-Alzheimer disease, and Down syndrome-specific congenital heart disease [81]. The map also provided evidence against both the existence of a single Down syndrome consensus region and the previous supposition that a synergistic role of *DSCR1*, *DYRK1A*, and/or *APP* was sufficient for many of the Down syndrome phenotypes.



Fig. 8.5 The hand of a Down syndrome child showing small hand, clinodactyly, only one crease in the fifth finger, and single palmar crease

The additional copy of chromosome 21 is proposed to result in the increased expression of many of the genes encoded by this chromosome. The knowledge of which of the genes, when present in three copies, leads to each of the different Down syndrome-associated phenotype, together with research using Down syndrome mouse models, may provide insight into possible pharmacological approach to improving some of the symptoms [82].

Recurrence

Various estimates of the recurrent risk for trisomy 21 have been reported. The overall empirical recurrence risk is about 1% in women under 30 years of age and includes trisomies other than 21. For women over 30, the recurrence risk may not be significantly different from the age-specific risk [83]. A more recent study reported 5,960 women with a previous trisomy 13, 18, or 21 pregnancy; 75 of the 3,713 subsequent pregnancies were trisomic [84]. The relative risk of a subsequent trisomy 21 compared to the expected number of trisomies based on maternal age-related risk alone was 2.2. The risk of a different trisomy subsequent to trisomy 21 might also be increased (relative risk 1.4). The increase in risk was greater for women under age 35 at the first trisomic pregnancy. A similar increase in the rate of

trisomy pregnancy following an initial trisomy pregnancy was reported in a study of trisomy recurrence based on North American data [61].

One study of 13 families with two trisomy 21 children showed that three had a parent who was mosaic for trisomy 21 (by cytogenetic studies), and two had a parent who was potentially mosaic (by DNA polymorphism analysis) [85]. In a family with three trisomy 21 children, Harris et al. reported that the mother was mosaic for trisomy 21 in lymphocytes and skin fibroblasts [86]. In another single case report involving a family with four trisomy 21 children, the mother was found to have a trisomy 21 cell line in an ovarian biopsy specimen [87]. In a study compiling data from 80 families with either maternal (61 families) or paternal (19 families) gonadal mosaicism for trisomy 21, a total of 142 Down syndrome offspring were reported [88]. Among these offspring, mosaicism was observed in 12 families and the proportion of mosaics among affected female offspring (14%) was significantly higher compared to that among affected male offspring (0%). Based on these observations, it was proposed that female-specific trisomy rescue might be a mechanism of formation of both gonadal mosaicism and somatic mosaicism. Gonadal mosaicism in one parent is an important cause of recurrent trisomy 21 and should be looked for in families with more than one affected child.

The recurrence risk for mosaic trisomy 21 that results from mitotic nondisjunction should, in general, not be increased. However, several studies investigating the mechanism and origin of mosaic trisomy 21 have shown that in a relatively high proportion of cases (probably over 50%), the mosaicism results from the loss of one chromosome 21 during an early mitotic division in a zygote with trisomy 21 [89, 90]. In such cases, the recurrence risk for nondisjunction will be the same as for nonmosaic trisomy 21.

Trisomy 18

Incidence

Trisomy 18 [47,XX or XY,+18] was first described by Edwards et al. in 1960 [91]. The incidence is 1 in 6,000–8,000 births. It is more frequent in females, with a male-to-female ratio of 1:3–4. The risk for trisomy 18 also increases with maternal age.

Phenotype

The most common features of trisomy 18 include mental and growth deficiencies, neonatal hypotonicity followed by hypertonicity, craniofacial dysmorphisms (prominent occiput, narrow bifrontal diameter, short palpebral fissures, small mouth, narrow palate, low-set malformed ears, micrognathia) (Fig. 8.6), clenched hands with a tendency for the second finger to overlap the third and the fifth finger to overlap the



Fig. 8.6 Profile of a trisomy 18 child showing prominent occiput, low-set malformed ear, and micrognathia



Fig. 8.7 Trisomy 13 stillborn with midline cleft lip and holoprosencephaly

fourth, short dorsiflexed hallux, hypoplastic nails, rocker bottom feet, short sternum, hernias, single umbilical artery, small pelvis, cryptorchidism, hirsutism, and cardiac anomalies (mainly ventricular septal defect [VSD], atrial septal defect [ASD], and patent ductus arteriosus [PDA]). Studies show that median survival averages approximately 5 days, with 1-week survival at 35–45%; one later study indicated a median survival of 14.5 days [92–96]. Fewer than 10% of patients survive beyond the first year of life. A few patients over 10 years of age, all females with one exception, have been described; however, the presence of a normal cell line in these patients was not always searched for [97–99].

Mosaic trisomy 18 patients have, in general, milder phenotypes. At least six mosaic trisomy 18 patients, again all females, with normal intelligence and long-term survival have been reported [100–105].

Two molecular studies, performed on a total of 10 patients with partial trisomy 18, suggest that the region proximal to band 18q12 does not contribute to the syndrome, while two critical regions, one proximal (18q12.1→q21.2) and one distal (18q22.3→qter), may work in cooperation to produce the typical trisomy 18 phenotype [106, 107]. In addition, severe mental retardation in these patients may be associated with trisomy of the region 18q12.3→q21.1.

Recurrence

Single case reports of trisomy 18 in sibs (e.g., [105]), and of trisomy 18 and a different trisomy in sibs or in prior or

subsequent abortuses (e.g., [108–110]) are recorded. In the same studies referenced for trisomy 21, an increased risk of trisomy 18 subsequent to a previous pregnancy with trisomy 18 was observed [61, 84]. The relative risk was 1.7–3.8. Again, the increase in risk was greater for women under age 35 at the first trisomic pregnancy. Given the low baseline age-related risk, the absolute risk of recurrence is nonetheless quite low.

Trisomy 13

Incidence

Trisomy 13 [47,XX or XY,+13] was first described by Patau et al. in 1960 [111]. The incidence is estimated to be 1 in 12,000 births. It is seen slightly more in females than in males. Again, the risk for trisomy 13 increases with maternal age.

Phenotype

The most prominent features of trisomy 13 include the holoprosencephaly spectrum (Fig. 8.7), scalp defects, microcephaly with sloping forehead, large fontanels, capillary hemangioma (usually on the forehead), microphthalmia, cleft lip, cleft palate, abnormal helices, flexion of the fingers, polydactyly, hernias, single umbilical artery, cryptorchidism, bicornuate uterus, cardiac abnormalities in 80% of patients (mostly VSD, PDA, and ASD), polycystic kidneys, increased

polymorphonuclear projections of neutrophils, and persistence of fetal hemoglobin. Prognosis is extremely poor, with a median survival of 2.5–7 days and a 6-month survival of 5% [94, 96]. Severe mental deficiencies, failure to thrive and seizures are seen in those who survive. Mosaic trisomy 13 patients are, again, in general less severely affected; however, the degree is very variable and can be as severe as in nonmosaic trisomy 13 individuals.

Development of a karyotype-phenotype correlation by studying partial trisomies for different segments of chromosome 13 has also been attempted [112, 113]. These studies were based on cytogenetic methods and suggested that the proximal segment (13pter→q14) contributes little to the trisomy 13 phenotype, while the distal segment (all or part of 13q14→qter) is responsible for the complete trisomy 13 features. A prenatally diagnosed pure partial trisomy 13 involving 13q14→qter with breakpoints delineated by array comparative genomic hybridization (aCGH; see Chap. 18) analysis was reported recently. The fetus had agenesis of the corpus callosum and the diaphragm, severe pulmonary hypoplasia, and generalized hydrops [114].

Recurrence

An increased risk of trisomy 13 subsequent to a previous pregnancy with trisomy 13 was noted in the same references as recorded for trisomy 18 above [61, 84]. The relative risk was 3.8–8.6. The relative risk for a subsequent different trisomy was 1.9 [61]. The absolute risk remains very low.

Trisomy 8

Trisomy 8 [47,XX or XY,+8] was first reported by Grouchy et al. in 1971 [115]. It is rare, with an unknown incidence. More than 100 cases have been reported in the literature, most of them mosaics [47,+8/46] [116–121]. The male-to-female ratio is 2–3:1.

Growth and the degree of mental deficiency are variable. Mild to severe retardation is seen, while a proportion of patients have normal IQs. Craniofacial dysmorphism (Fig. 8.8) includes prominent forehead, deep-set eyes, strabismus, broad nasal bridge, upturned nares, long upper lip, thick and everted lower lip, high arched or cleft palate, micrognathia and large dysplastic ears with prominent anti-helices. Skeletal abnormalities include a long, thin trunk, hemivertebrae, spina bifida, kyphoscoliosis, hip dysplasia, multiple joint contractures, camptodactyly, dysplastic nails, and absent or dysplastic patella. The presence of deep palmar and plantar furrows is characteristic. Renal and ureteral anomalies and congenital heart defects are common. A case with extremely elevated maternal serum alpha-fetoprotein noted prenatally without open defect was recorded [121]. A few cases of hematological malignancy have been reported



Fig. 8.8 An infant with mosaic trisomy 8. Note prominent forehead, strabismus, broad nasal bridge, upturned nares, long upper lip, and everted lower lip

in mosaic trisomy 8 patients [122, 123]. This is of particular interest because trisomy 8 is a frequently acquired cytogenetic abnormality in myeloid neoplasms (see Chap. 15). When studied, the abnormal cells in these patients appeared to have developed from the trisomic cell population. The significance of this is not clear, but the possibility remains that constitutional trisomy 8 may predispose individuals to myeloid neoplasia.

There is no direct correlation between the proportion of the trisomy 8 cells and the severity of the phenotype. The percentage of trisomic cells is usually greater in skin fibroblasts than in blood lymphocytes. In addition, the proportion in lymphocytes usually decreases with time.

The risk for recurrence is not known.

Trisomy 9

The first cases of trisomy 9 in either nonmosaic [47,XX or XY,+9] or mosaic [47,+9/46] form were reported in 1973 [124, 125]. More than 40 cases of liveborns or term stillbirths with trisomy 9 have been reported. Most were mosaics [126–130]. The male-to-female ratio is close to 1:1.

Clinical features include craniofacial anomalies (high narrow forehead, short upward-slanting palpebral fissures, deep-set eyes, microphthalmia, low-set malformed auricles, bulbous

nose, prominent upper lip, micrognathia), skeletal malformations (abnormal position/function of various joints, bone dysplasia, narrow chest, 13 ribs), overlapping fingers, hypoplastic external genitalia, and cryptorchidism. Cardiac anomalies are seen in more than 60% of cases, most frequently VSD. Renal malformations are present in 40% of patients. A case of mosaic trisomy 9 with holoprosencephaly and another case with XX sex reversal were reported [131, 132]. The majority of patients die in the early postnatal period. With rare exceptions, all survivors have severe mental deficiency. Mosaic patients tend to survive longer, but the proportion of trisomy 9 cells does not predict the severity of the condition or the length of survival. It is possible that a normal cell line could be present in some tissues in apparently nonmosaic patients.

The mean maternal age of women bearing trisomy 9 offspring was reported to be significantly increased over that of the general population [127]. This suggests that the occurrence of trisomy 9 may also be associated with advanced maternal age. The risk for recurrence is not known.

Trisomy 16

Trisomy 16 is the most frequently observed autosomal aneuploidy in spontaneous abortuses (see Chap. 13). Full trisomy 16 is almost always lethal during early embryonic or fetal development, although a single case of a stillborn at 35 weeks gestation has been recorded [133].

Mosaic trisomy 16 fetuses, however, may occasionally survive to term. More than ten such cases have been reported [134–141]. Intrauterine growth restriction is nearly invariable. An elevated maternal serum hCG or alpha-fetoprotein level during pregnancy was noted in more than 50% of cases. Congenital cardiac defects (mainly VSD or ASD) were present in 60% of patients. Other clinical findings included postnatal growth retardation, mild developmental/speech delay, craniofacial asymmetry, ptosis, flat broad nasal bridge, low-set dysplastic ears, hypoplastic nipples, umbilical hernia, deep sacral dimple, scoliosis, nail hypoplasia, and single transverse palmar crease. One patient had normal growth and development at 11 months of age [141]. Approximately 50% of the patients died within the first year of life. Long-term follow-up is not available; however, survival to more than 5 years has been observed (Hajianpour and Wang, personal observation).

The risk for recurrence is probably negligible.

Trisomy 20

Although mosaic trisomy 20 is one of the most frequent autosomal aneuploidies detected prenatally, its occurrence in liveborns is very rare [142]. The majority of prenatally diagnosed

cases are not cytogenetically confirmed in postnatal life. It appears that in conceptuses capable of surviving to the second trimester, trisomy 20 cells are largely confined to extraembryonic tissues. Liveborns with documented mosaic trisomy 20 have been reported and most were phenotypically normal at birth [143–150]. In cases with long-term follow-up, hypopigmentation, mild psychomotor delay, and facial dysmorphism have been observed in some cases. The possibility of a more consistent phenotype associated with mosaic trisomy 20 has been recently suggested, including spinal abnormalities (spinal stenosis, vertebral fusion, kyphosis), hypotonia, lifelong constipation, sloped shoulders, and significant learning disabilities [150]. No case of liveborn nonmosaic trisomy 20 has been recorded.

Phenotypic abnormalities in abortuses with cytogenetically confirmed mosaic trisomy 20 include microcephaly, facial dysmorphism, cardiac defects, and urinary tract anomalies (megapelvis, kinky ureters, double fused kidney) [151].

Trisomy 20 cells have been found in various fetal tissues including kidney, lung, esophagus, small bowel, rectum, thigh, rib, fascia, and skin [142, 151, 152]. Postnatally, they have been detected in cultured foreskin fibroblasts and urine sediments [143–148]. The detection of trisomy 20 cells in newborn cord blood has been reported in one case, but subsequent study of peripheral blood at 4 months of age produced only cytogenetically normal cells [145]. There are no other reports of trisomy 20 cells in postnatal blood cultures.

The risk for recurrence is probably negligible.

Trisomy 22

Trisomy 22 was first reported in 1971 [153]. Since then, more than 20 liveborns have been reported in the literature [154–161]. Although most cases were apparently nonmosaic full trisomies, the presence of an undetected, normal cell line confined to certain tissues cannot be excluded, as pointed out by Robinson and Kalousek [162].

The most consistent phenotypic abnormalities include intrauterine growth restriction, low-set ears (frequently associated with microtia of varying degrees plus tags/pits), and midfacial hypoplasia. Other frequently seen abnormalities are microcephaly, hypertelorism with epicanthal folds, cleft palate, micrognathia, webbed neck, hypoplastic nails, anal atresia/stenosis, and hypoplastic genitalia. Cardiac defects, complex in some cases, are seen in 80% of patients. Renal hypoplasia/dysplasia is also common. Skin hypopigmentation (hypomelanosis of Ito) is usually present in mosaic cases. Intestinal malrotation and Hirschsprung disease were recently reported in a prenatally diagnosed mosaic trisomy 22 infant with normal development [160]. A 4-year-old girl with confirmed trisomy 22 mosaicism in skin had normal cognitive, behavioral, and physical development [161].

Prolonged survival to over 20 years has been observed in mosaic patients.

Most nonmosaic patients die in the first months of life. The longest survival reported is 3 years [163]. That patient had severe growth and developmental delay and died a few days before his third birthday.

Trisomy 22 cells can be detected in both blood lymphocytes and skin fibroblasts. The risk for recurrence is unknown and probably negligible.

Other Rare Autosomal Trisomies

As noted in the introduction, mosaic or nonmosaic autosomal trisomies for chromosomes other than 1 and 11 have been reported in liveborns. Trisomies are detected much more frequently in spontaneous abortuses or in prenatal diagnostic specimens, following which elective terminations are often performed. Thus, the occurrence of such trisomies in liveborns is extremely rare and only isolated case reports are available. The risks for recurrence for these rare trisomies are probably negligible. The following discussion will include cytogenetically confirmed postnatal cases only.

At least two cases of liveborn mosaic trisomy 2 have been reported [164, 165]. In one case, the mosaicism was detected in amniocytes and confirmed postnatally in liver biopsy fibroblasts (4 of 100 cells) but not in blood, skin fibroblasts, or ascites fluid cells. At 16 months of age, the child had hypotonia, microcephaly, and growth and developmental delay. In the second case, mental retardation, multiple congenital anomalies, and dysmorphic findings similar to Pallister-Killian syndrome were observed. Another case of possible mosaic trisomy 2, detected at amniocentesis and observed in a single cell of a foreskin fibroblast culture following the birth of a dysmorphic child, was reported in an abstract [166].

Three cases of mosaic trisomy 3 have been reported; one of these, a severely mentally retarded woman, was alive at age 32 [14, 167, 168]. Clinical features in the three cases vary, except all had prominent forehead, ear, and eye anomalies.

At least two cases of postnatally confirmed mosaic trisomy 4 have been reported [169, 170]. In both cases, the trisomic cells were detected in prenatal amniocytes and confirmed postnatally in skin fibroblasts, but not in blood lymphocytes. One of the cases also had low-level mosaicism for trisomy 6 with clinical features of prenatal growth restriction, right facial hypoplasia, dysplastic and posteriorly rotated right ear, high vaulted palate, retrognathia, aplasia of the right thumb, hypoplasia of the fingernails, deep sacral dimple, and patchy skin hypopigmentation of the right leg [170]. Long-term follow-up was available on the other case [171]. The patient had right hand and ear anomalies. At age 14, she had delayed puberty with no menarche, asymmetrical breast development, and low normal intelligence.

One case of postnatally confirmed mosaic trisomy 5 has been reported [172]. The trisomic cells were detected in prenatal amniocytes and confirmed postnatally in skin fibroblasts, but not in blood lymphocytes. The patient had multiple dysmorphic features and congenital anomalies, including eversion of the diaphragm and ventricular septal defect.

At least two cases of mosaic trisomy 6 have recently been reported. The first patient was born at 25 weeks of gestation. Clinical features included heart defects (ASD and peripheral pulmonary stenosis), large ears, cleft right hand, cutaneous syndactyly, overlapping toes of irregular shape and length, and epidermal nevi. Growth was considerably delayed, but development was relatively normal at age 2½. Trisomy 6 cells were detected in skin fibroblasts but not in blood [173]. Mosaic trisomy 6 was prenatally diagnosed in fetal urine in the second case. The infant was born at term with normal growth parameter, heart defect, and malformations of hands and feet [174].

At least seven cases of cytogenetically documented mosaic trisomy 7 in skin fibroblasts have been recorded [175–178]. All patients were phenotypically abnormal. Common features included growth and developmental delay, skin pigmentary dysplasia with hypo- and hyperpigmentation, facial or body asymmetry, and facial dysmorphism. One mentally retarded male was 18 years old at time of report. A few cases of liveborn mosaic trisomy 10 have been reported [179–181]. One patient was mosaic for trisomy 10 and monosomy X in skin fibroblasts, whereas only monosomy X cells were present in blood. This infant died at 7 weeks of age from heart failure. Another patient was mosaic for trisomy 10 and had maternal uniparental disomy for chromosome 10 in the diploid cell line [181]. The common clinical phenotype included growth failure, craniofacial dysmorphism (prominent forehead, hypertelorism, upslanted palpebral fissures, blepharophimosis, dysplastic large ears, retrognathia), long slender trunk, deep palmar and plantar fissures, cardiac defects, and short survival.

At least seven cases of cytogenetically confirmed trisomy 12 have been reported in liveborns; all were mosaics [110–113, 115–187]. The earliest reported case was that of an infertile man. A more recent case was a girl with pituitary malformation associated with growth retardation responding to growth hormone therapy. The patient also had a polycystic right ovary. Phenotypic presentation was variable among patients and included facial dysmorphism, scoliosis, ASD, PDA, dysplastic pulmonary and tricuspid valves, short stature, and mental retardation. Trisomy 12 cells have been found in lymphocytes, skin fibroblasts, urine sediments, and internal organs including liver, spleen, adrenal, ovary, and thymus.

More than 20 cases of mosaic trisomy 14 have been reported in liveborns [188–190]. The most consistent phenotypic abnormalities were growth and mental retardation, broad nose, low-set dysplastic ears, micrognathia, congenital

heart defects, and micropenis/cryptorchidism in males. One prenatally diagnosed patient had alobar holoprosencephaly and died at 36 days of age [189]. Survival varied from days to more than 29 years. Trisomy 14 cells were detected in both lymphocytes and fibroblasts, with a generally higher percentage in lymphocytes. There was no clear correlation between the proportion of trisomic cells and the severity of the phenotype. In patients with body asymmetry, trisomic cells were usually limited to the atrophic side.

At least ten cases of liveborn trisomy 15 have been recorded, two of them were reportedly nonmosaics [191–197]. In some cases, the trisomy 15 cell line was present only in skin fibroblasts and not in peripheral blood lymphocytes. The concurrent finding of maternal uniparental disomy 15 (see Chap. 20) in the normal cell line was reported in two of the cases [192, 194]. These cases appeared to have the most severe phenotype. Phenotypic abnormalities include hypotonia, various craniofacial dysmorphisms, minor skeletal anomalies, congenital heart defects, and short survival. One patient with longer survival had short stature, mild mental retardation, hemihypotrophy, atrial septal defect, bilateral branchial cleft fistulas, and abnormal skin pigmentation [195].

At least four cases of confirmed mosaic trisomy 17 have been reported [198–200]. The trisomic cells were not seen in lymphocytes but were found in high percentage in skin fibroblasts. One patient, age 8½ years at the time of reporting, had mental and growth retardation, microcephaly, minor dysmorphism, seizures, hearing loss, attention deficit hyperactivity disorder, and autistic behavior. Peripheral motor and sensory neuropathy, hypoplastic cerebellar vermis, zonular cataract, and body asymmetry have also been reported.

At least two cases of mosaic trisomy 19 were in the literature, one of them was a stillborn male and the other died on day 13. Clinical features were varied and included facial dysmorphism with no report of major malformation [201, 202].

Autosomal Monosomies

As noted in the introduction, autosomal monosomies are extremely rare in either liveborns or abortuses, reflecting the severity of the genetic imbalance resulting from the loss of an entire chromosome. The only monosomies that have been reported are monosomy 21 and mosaic monosomy 22.

Monosomy 21

Mosaic monosomy 21 was reported in four liveborns in the early literature [203–206]. The most prominent features included intrauterine growth restriction, postnatal growth and mental retardation, hypertonia, facial dysmorphism with downward slanting palpebral fissures, large low-set ears, and micrognathia. A more recent report described pathological

findings of an electively terminated 20-week female fetus after mosaic monosomy 21 was diagnosed by repeated amniocenteses [207]. The facial abnormalities previously described were present in this abortus. In addition, a complex cardiac malformation, malrotation of the bowel, uterus didelphys, small dysplastic ovaries, and focal cystic dysplasia of the lung were noted.

Approximately ten cases of apparently nonmosaic monosomy 21 have been reported in liveborns [208–211]. Some of these cases have subsequently been shown to represent partial monosomy 21 resulting from an undetected subtle translocation [212–214] with part of chromosome 21 material attached to a derivative chromosome, explaining the observation that mosaic monosomy 21 is less commonly observed than apparently nonmosaic monosomy 21 and indicating that complete monosomy 21 is almost always incompatible with life. The phenotypic features were similar to those observed in the mosaics and included intrauterine growth restriction, postnatal growth and mental deficiencies, microcephaly, hypertelorism with downward slanting palpebral fissures, large low-set ears, prominent nose, cleft lip/palate, micrognathia, cardiac anomalies, and abnormal muscle tone. Most patients died before 2 years of age. A case of full nonmosaic monosomy 21 confirmed by fluorescence *in situ* hybridization analysis was reported in a liveborn who died shortly after birth [211]. The phenotype of this infant included severe intrauterine growth restriction, microcephaly, semilobar holoprosencephaly, hypotonia, bilateral microphthalmia, facial dysmorphism, agenesis of the external auditory meatus, redundant skin in the neck, narrow chest, cryptorchidism, hypospadias, micropenis, camptodactyly, congenital heart disease, and agenesis of the right kidney.

Monosomy 22

At least four cases of mosaic monosomy 22 in liveborns have been reported [215–218]. All four were male. One was a 34-week premature infant with gastroschisis who died from intracranial hemorrhage shortly after birth. No dysmorphic features were noted, and autopsy was not performed [217]. Two patients had growth and developmental deficiencies, microcephaly, and mild facial dysmorphism. The fourth patient was a 30-week premature infant with facial features of DiGeorge syndrome, hypertonicity, limited extension of major joints, and flexion contractures of all fingers.

Polypliody

Polypliodies are numerical chromosome abnormalities with changes in the number of complete sets of chromosomes. They are usually incompatible with fetal survival and are extremely rare in liveborns.

Triploidy

The chromosome number in triploidy is $3n=69$ (Fig. 8.9). It is estimated to occur in approximately 1% of all human conceptions and is found in 17–18% of all chromosomally abnormal abortuses [219, 220]. Only very rarely do triploid conceptuses survive to term. Two distinct phenotypes have been recognized [221]. One type presents as a relatively well-grown fetus with or without microcephaly, and an abnormally large and cystic placenta usually classified as a partial hydatidiform mole. The parental origin of the extra haploid set of chromosomes in such cases is determined to be paternal (diandry) by analysis of cytogenetic heteromorphisms or DNA polymorphisms [221, 222, 180]. Diandry results from the fertilization of a normal ovum with either two sperm (dispermy) or a sperm that has a diploid chromosome complement resulting from a failure of meiotic division. The other type is characterized by severe intrauterine growth restriction with relative macrocephaly and a small and noncystic placenta. The extra haploid set of chromo-

somes in such cases is maternal (digyny) [221–224]. Digyny can result from a failure of the first maternal meiotic division, generating a diploid egg, or from retention of the second polar body. While the occurrence of triploidy does not appear to be associated with maternal age, digyny may play a major role in the generation of triploidy in the advanced maternal age group [220]. Early cytogenetic studies indicated that the majority of triploid conceptuses were diandric partial moles [222, 225]. Later studies based on DNA polymorphisms have suggested that a maternal contribution to triploidy may occur more frequently than was previously realized [223, 226]. Yet in a more recent study of 87 informative cases of triploid spontaneous abortuses at 5–18 weeks of gestation, Zaragoza et al. showed that approximately two-thirds are androgenetic in origin and that many, but not all, androgenetic triploids developed a partial molar phenotype [227]. The sex chromosome complement in triploidy is either XXX or XYY, with XYY occurring only rarely. For example, the reported numbers of XXX:XYY:XYY cases in two studies performed on spontaneous abortuses were 82:92:2 and

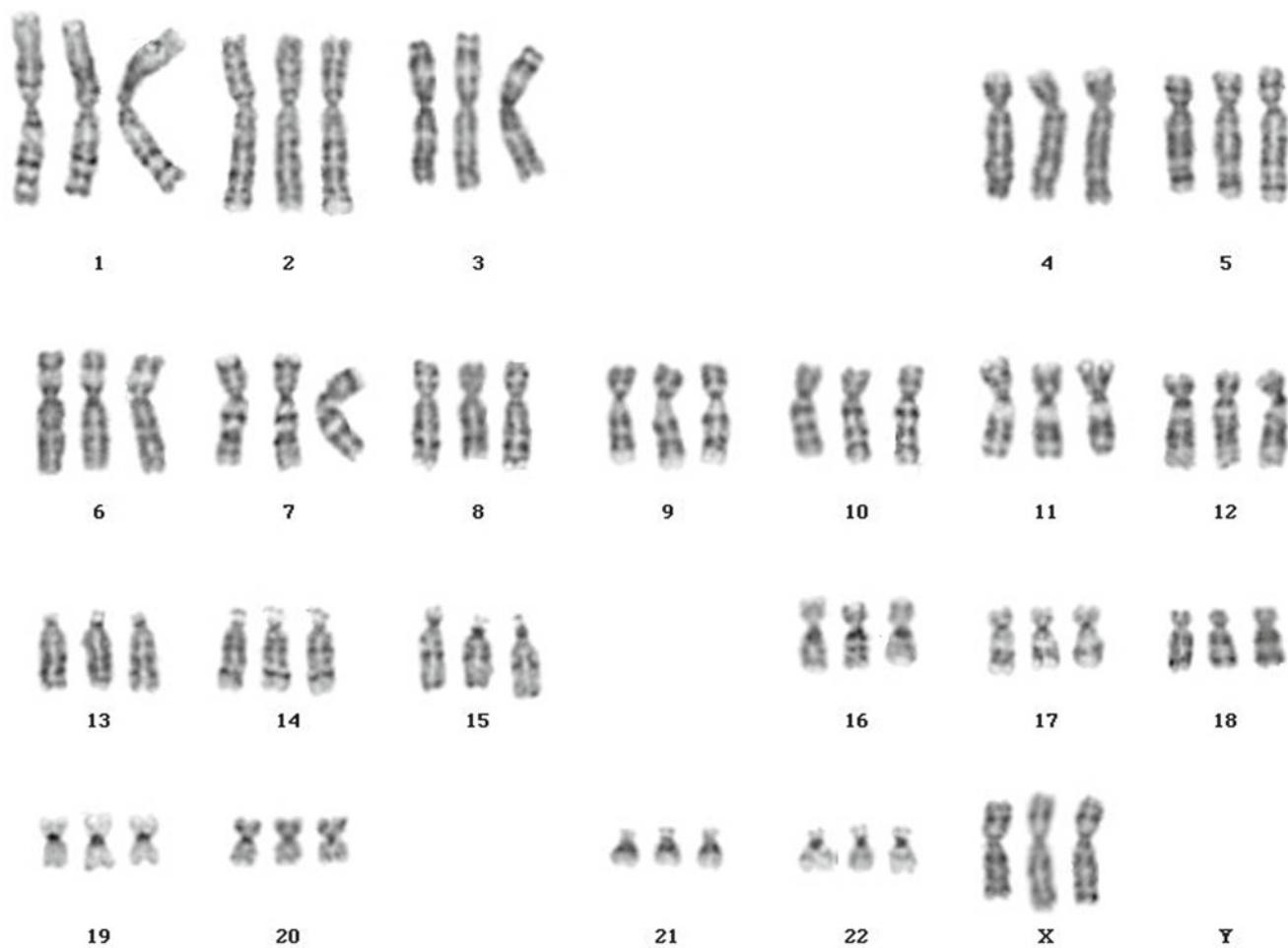


Fig. 8.9 Karyogram of a triploid fetus (69,XXX)

26:36:1, and in one study performed on amniotic fluid cells this ratio was 6:8:0 [3, 177, 228]. It has been suggested that 69,XYY triploid conceptuses are incompatible with significant embryonic development [3].

The observation that the phenotype of triploidy depends on the parental origin of the extra set of chromosomes is an example of genomic imprinting, or the differential expression of paternally and maternally derived genetic material [229, 230]. It correlates well with observations obtained from mouse embryo studies using nuclear transplantation techniques, which demonstrated that maternal and paternal genomes function differently and are both required for normal development [231–233]. See Chap. 20.

More than 50 cases of apparently nonmosaic triploidy, either 69,XXX or XXY, have been reported in liveborns. Most patients died shortly after birth. Eight patients with survival longer than 2 months have been reported, with the longest being 10½ months [234, 235]. The origin of the extra set of chromosomes was determined by cytogenetic polymorphisms or human leukocyte antigen (HLA) to be maternal in three cases and paternal in one case [236]. One study based on DNA polymorphism in an infant who survived for 46 days indicated a maternal meiosis II failure as the origin of the triploid [236]. These findings suggest that in general digynic triploids survive longer than diandric triploids. The most frequent phenotypic abnormalities include intrauterine growth restriction, hypotonia, craniofacial anomalies (macro/hydrocephalus, low-set dysplastic ears, broad nasal bridge), syndactyly, malformation of the extremities, adrenal hypoplasia, cardiac defects, and brain anomalies.

Mosaic triploidy (diploid/triploid mixoploidy) has been reported in approximately 20 patients. Triploid cells were found in both lymphocytes and fibroblasts, although in a number of cases the triploid cell line was limited to fibroblasts [237]. Patients with such mixoploidy are less severely affected than nonmosaics, and survival beyond 10 years has been observed. Usual clinical features include intrauterine growth restriction, psychomotor retardation, asymmetric growth, broad nasal bridge, syndactyly, genital anomalies, and irregular skin pigmentation [238]. Truncal obesity was seen in some patients [239]. A recent case of a 46,XX/69,XXY diploid/triploid mixoploid 8-year-old girl with normal female genital and ovarian development despite normal expression of SRY expression was reported [240].

Mitotic nondisjunction cannot readily explain the occurrence of diploid and triploid cell lines in the same individual. One possible mechanism is double fertilization of an ovum by two sperm; one sperm nucleus fuses with the ovum nucleus producing the diploid line, followed by a second sperm fertilizing one of the early blastomeres producing the triploid line. Cytogenetic evidence for such a mechanism has been reported in at least one case [241]. Another proposed mechanism supported by molecular evidence is delayed incorporation of the

second polar body into one of the early blastomeres. The triploid cell line in this case is digynic [242].

Tetraploidy

The chromosome number in tetraploidy is $4n=92$. It is rarer than triploidy in spontaneous abortuses, seen in approximately 6–7% of such specimens with chromosome abnormalities [219, 220]. Tetraploid conceptuses usually abort spontaneously early in gestation and only rarely do they survive to term. A probable origin of tetraploidy is chromosome duplication in the zygote resulting from a failure of cytoplasmic division during the first division. Other theoretically possible mechanisms require the occurrence of two independent, rare events and are thus highly unlikely.

At least nine apparently nonmosaic tetraploid liveborns have been reported [243, 244]. The sex chromosome complement was either XXXX or XXYY. No 92,XYYY or XXXY conceptuses have been reported. The most frequent abnormalities were growth and developmental delay, hypotonia, craniofacial anomalies (short palpebral fissures, low-set malformed ears, high arched/cleft palate, micrognathia), and contracture/structural abnormalities of the limbs, hands and feet. Cardiac defects were present in four cases. Urinary tract abnormalities, such as hypoplastic kidneys, have also been recorded. Most patients died before 1 year of age. One girl had survived to 22 months at the time of report [245].

Mosaic tetraploidy (diploid/tetraploid mixoploidy) has been reported in at least 12 liveborns [246, 247]. This can occur as a result of postzygotic nondisjunction with failure of cytoplasmic division in a diploid conceptus. Tetraploid cells were seen in peripheral blood lymphocytes, skin fibroblasts, and bone marrow cells. In one severely malformed patient who died at 2 days of age, tetraploid cells were found in 95% of bone marrow cells [248]. In two females, aged 11 and 21 years, with severe intellectual handicaps and skin pigmentary dysplasia, tetraploid cells, were found only in skin fibroblasts [247]. In lymphocytes, the proportion of tetraploid cells decreases with age [249]. Overall, clinical features are similar to, but less severe than, those in nonmosaic tetraploidy patients. In addition to the longer survivals already mentioned, survivals to 6 years at the time of reporting have also been recorded [247, 250].

Partial Autosomal Aneuploidies

Partial duplication/deletion as a result of structural rearrangement is discussed in Chap. 9. Only those partial autosomal aneuploidies that result from the presence of a supernumerary chromosome and have been detected in postnatal specimens will be presented in this chapter.

Tetrasomy 5p

Tetrasomy 5p [47,XX or XY,+i(5)(p10)] resulting from the presence of a supernumerary isochromosome for the entire short arm of chromosome 5 is rare and has been reported in only five liveborns, all of whom were mosaics with both normal and abnormal cell lines [251, 252]. The abnormal cell line has been found in lymphocytes, skin fibroblasts, and chondrocytes. The phenotype appears to be similar to that of trisomy 5p. This includes hypotonia, seizures/abnormal electroencephalogram (EEG), psychomotor retardation, macrocephaly, facial dysmorphism, and respiratory difficulties. Skin hyperpigmentation was observed in two patients. Survival was variable; the most recent case reported was a 35-year-old male with a normal phenotype [252]. One patient died at 6 months of age, and another was 5 years old at the time of reporting.

Tetrasomy 8p

Tetrasomy 8p [47,XX or XY,+i(8)(p10)] usually results from the presence of a supernumerary isochromosome for the entire short arm of chromosome 8. All except one of the cases reported were mosaics, with both normal and abnormal cell lines. The abnormal cell line was found in lymphocytes and skin fibroblasts. In some cases, the origin of the abnormal isochromosome was confirmed by molecular cytogenetic (FISH) studies [253–255]. At least 12 cases have been reported [255–257]. A few patients died before the first year of life, but survival beyond 5 years was not uncommon. Weight and head circumference were normal at birth. The most frequently observed phenotypic features include mental retardation, speech and motor delay, dilatation of cerebral ventricles, mild facial dysmorphism (depressed nasal bridge, short nose, upturned nares, low-set and posteriorly rotated ears), and vertebral abnormalities. Agenesis of the corpus callosum was noted in six patients and cardiac defects in five. Deep palmar and plantar creases have also been reported. The phenotype resembles, to some degree, that of mosaic trisomy 8. A single apparently nonmosaic case was recorded with isochromosome 8p present in all blood lymphocytes while prenatal amniocytes showed a normal karyotype [257]. The girl had congenital ventricular septal defect, agenesis of corpus callosum, and facial, ear and bone anomalies.

Tetrasomy 9p

Tetrasomy 9p [47,XX or XY,+i(9)(p10)], resulting from the presence of a supernumerary isochromosome, has been reported in more than 20 liveborns [258–262]. The isochromosome consists of either the entire short arm of chromosome 9 as previously described, the entire short arm and part

of the heterochromatic region of the long arm, or the entire short arm and part of the long arm extending to the euchromatic region. No consistent phenotypic differences have been observed among the three types. Both mosaic and apparently nonmosaic patients have been reported. The tetrasomy 9p cells were seen in both lymphocytes and skin fibroblasts. In contrast to tetrasomy 12p (described later), the 9p isochromosomes were present only in lymphocytes in five patients and in fibroblasts at a much lower percentage than in lymphocytes in two others [258, 259, 263, 264, 265, 266]. The mechanism for this observed tissue-limited mosaicism for different chromosomes is not clear.

Survival is variable, ranging from a few hours to beyond 10 years. The most frequent phenotypic abnormalities include low birth weight, growth and developmental delay, craniofacial anomalies (microphthalmia, low-set malformed ears, bulbous tip of the nose, cleft lip/palate, micrognathia), short neck, skeletal anomalies, joint contracture, nail hypoplasia, and urogenital anomalies. Cardiac defects are present in more than 50% of patients. Diaphragmatic hernia was reported in an apparently nonmosaic patient [262].

Overall, nonmosaic patients are more severely affected. One patient, who had the i(9p) present in 75% of lymphocytes but not in skin fibroblasts, had only mild developmental delay and minor anomalies [258].

Tetrasomy 12p

Tetrasomy 12p (Pallister-Killian syndrome) results from the presence of a supernumerary isochromosome for the entire short arm of chromosome 12 [i(12)(p10) or i(12p)] (Fig. 8.10). The syndrome was first described in 1977 by Pallister et al. in two adults, a 37-year-old man and a 19-year-old woman [267]. In 1981, Killian and Teschler-Nicola reported a 3-year-old girl with similar clinical manifestations [268]. Subsequently, many cases have been reported, and many more have been observed but not reported in the literature [269, 270]. All cases were mosaics, with a normal cell line in addition to cells containing i(12p). Maternal age for reported cases has been shown to be significantly higher than that for the general population [271]. This observation has been taken to suggest that the isochromosome arises from a meiotic error and that the normal cell line results from subsequent loss of the i(12p) from some cells. In 6 of 7 cases studied by molecular analysis, the meiotic error was determined to be maternal [272, 273]. Tissue specificity and both the *in vivo* and *in vitro* age dependencies of the i(12p) have been well demonstrated [274]. The i(12p) is found in a high percentage of skin fibroblasts and amniocytes but is rarely seen in blood lymphocytes. The percentage of cells containing the isochromosome also decreases with age. The presence of tetrasomy 12p in 100% of bone marrow cells has been

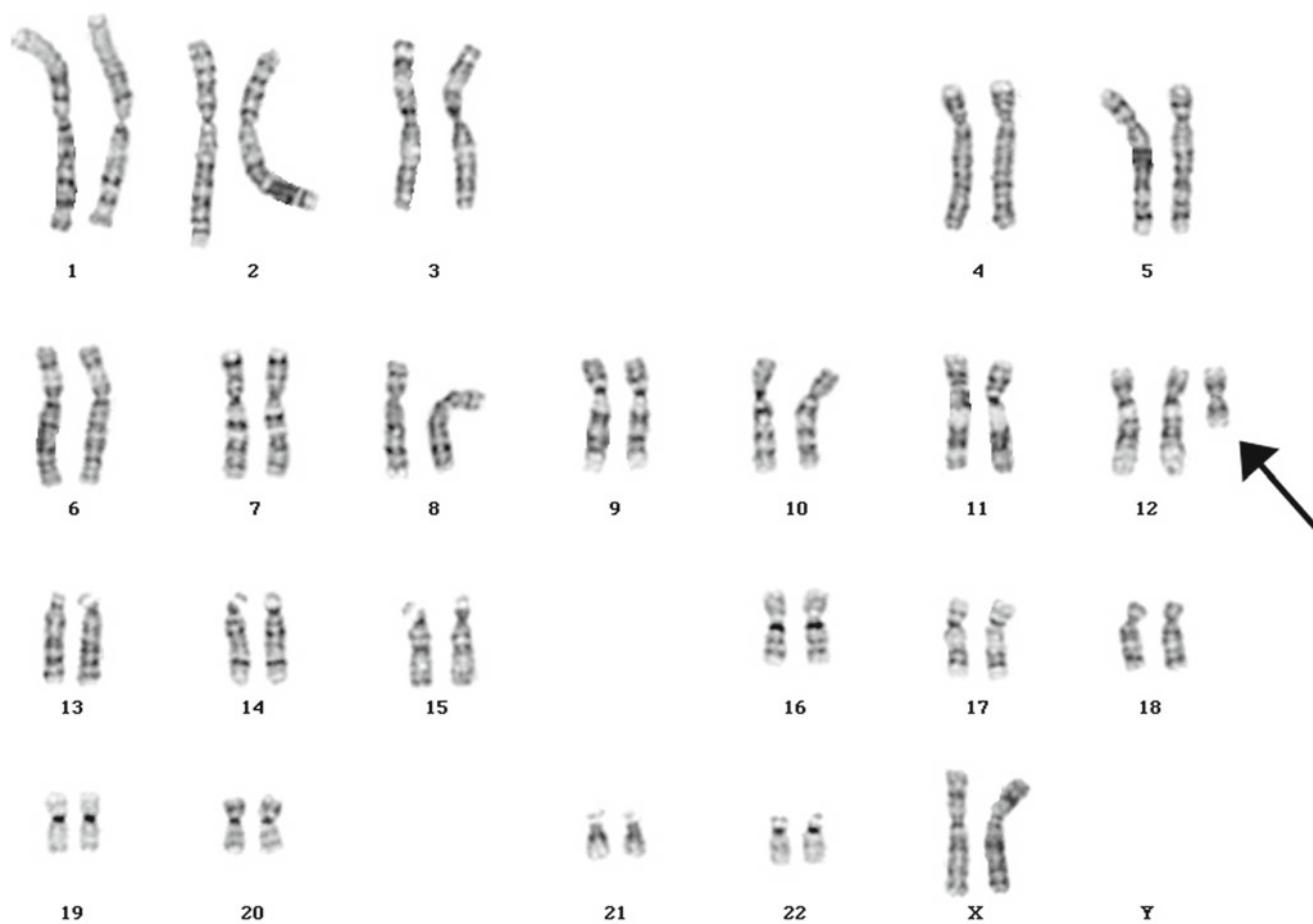


Fig. 8.10 Tetrasomy 12p female karyogram

reported in at least two newborn infants and in only 6% of marrow cells in a 3½-year-old child [275–277]. In lymphocytes it has been found in fetal blood, but has never been seen beyond childhood [274, 278]. In a case reported by Ward et al., the i(12p) was present in 10% of lymphocytes initially but was not seen in these cells when the patient was 2 months old [275]. The isochromosome is more stable in skin fibroblasts and can be found in adults, usually at lower percentage than in younger patients. When fibroblast cultures were examined, the percentage of cells containing the isochromosome decreased with increasing numbers of cell passages [272, 274–276, 279]. One study using FISH showed that in lymphocytes, the i(12p) was present in a significantly higher proportion of interphase nuclei than in metaphase cells [280]. With the availability of array CGH (see Chap. 18), gain of 12p has been detected in total genomic DNA from blood specimens [281]. These indicate that lymphocytes containing i(12p) may fail to divide upon phytohemagglutinin (PHA) stimulation. These observations suggest that tissue-limited mosaicism in Pallister-Killian syndrome may

result from differential selection against cells containing i(12p) in different tissues and that this selection can occur both *in vivo* and *in vitro*.

Many patients die shortly after birth, but survival to adulthood is possible. Clinically, a distinct pattern of anomalies is observed in these patients. Growth parameters at birth are usually normal. Profound hypotonia is present in the newborn period, while contractures develop later in life. Sparse scalp hair, especially bitemporally, is observed in infancy, with coarsening of facial features over time. Craniofacial dysmorphism includes prominent forehead, large malformed ears, hypertelorism, epicanthal folds, broad flat nasal bridge, short nose, upturned nares, long philtrum, thin upper lip, and high arched palate. Most patients have a generalized pigmentary dysplasia with areas of hyper- and hypopigmentation. Other abnormalities include short neck, macroglossia, micrognathia progressing to prognathia, accessory nipples, umbilical and inguinal hernias, urogenital abnormalities, and congenital heart defects. Severe mental retardation and seizure are seen in those who survive.

All cases are sporadic. The recurrence risk is probably negligible.

Tetrasomy 18p

Tetrasomy 18p [47,XX or XY,+i(18)(p10)] results from the presence of a supernumerary isochromosome for the entire short arm of chromosome 18. The syndrome was first described by Froland et al. in 1963, although identification of the marker as an i(18p) was not made until after the introduction of banding techniques in 1970 [282]. Confirmation of the origin of the marker has been possible in recent years by FISH studies. Of interest is the finding of a loss of approximately 80% of chromosome 18 alpha-satellite DNA in the i(18p) in one case [283].

At least 60 cases have been reported [284–287]. Most are nonmosaics. The i(18p) is usually readily detectable in lymphocytes. Its presence in amniocytes and cultured chorionic villus cells has also been reported [283, 288].

The most frequent clinical features include low birth weight, microcephaly, feeding problems, various degrees of psychomotor retardation, spasticity, seizures, craniofacial characteristics (oval shaped face, arched eyebrows, strabismus, low-set dysplastic ears, small pinched nose, small triangular mouth, high arched palate, micrognathia), narrow shoulders and thorax, small iliac wings, scoliosis, camptodactyly, and simian creases. Cardiac defects including ASD, VSD, and PDA have been observed in some cases. Urogenital anomalies including horseshoe kidneys, double ureter, and cryptorchidism have occasionally been seen. One case with aggressive behavior was reported in a 41-year-old male who also had dysmorphic features, marked obesity, and profound mental retardation [289].

It is not clear whether patients with tetrasomy 18p are born to mothers of increased age. Most of the reported cases are sporadic. The presence of i(18p) in maternal lymphocytes has been reported in at least three families. In two families, the mothers had an abnormal chromosome 18 with deletion of the short arm and a supernumerary i(18p), and thus were trisomic for 18p. The offspring inherited the normal chromosome 18 and the i(18p), and were, therefore, tetrasomic for 18p [290, 291]. In the third family, the mother had low-level mosaicism for a supernumerary i(18p) and was mildly affected clinically. The child apparently had nonmosaic tetrasomy 18p and had the full clinical presentation of the syndrome [292]. In another report, the presence of an i(18p) in two maternal half siblings was observed. No i(18p) was found in the mother's lymphocytes or fibroblasts, raising the possibility of gonadal mosaicism [287]. The recurrence risk in such families will be high.

Other Partial Autosomal Aneuploidies

Supernumerary Marker Chromosomes

In addition to the tetrasomies described previously, partial autosomal aneuploidies can result from the presence of small supernumerary marker chromosomes of cytogenetically indeterminate origin. The frequency of such markers is approximately 0.7 per 1,000 in newborns and 0.8–1.5 per 1,000 in prenatal specimens [293–296]. Since their cytogenetic origins are not initially known, these markers may or may not represent autosomal aneuploidy. Identification of such markers is now typically achieved using FISH or array CGH and is covered in Chap. 17.

These supernumerary markers are often classified as satellited or nonsatellited and are frequently present in mosaic form. They are a heterogeneous group and the clinical significance of a marker depends on its origin and characteristics. Markers that contain only heterochromatin and/or the short arms of acrocentric chromosomes are typically of no phenotypic consequence. On the other hand, markers that contain euchromatin are generally not benign and can result in phenotypic abnormalities. Among these are the dicentric bisatellited markers that contain variable amounts of long arm euchromatin of an acrocentric chromosome.

Markers derived from all autosomes have been reported [297–300]. The most common marker is the so-called inverted duplication of chromosome 15, “inv dup(15)”. This is an archaic misnomer that dates from an incorrect assessment of the mechanism of formation of such chromosomes and represents a heterogeneous group of small markers consisting of two copies of the short arm of chromosome 15, with or without variable amounts of long arm material. These are correctly identified as isochromosomes or isodicentric chromosomes and account for approximately 40% of all marker chromosomes [299, 301]. The amount of long arm euchromatin present in the marker dictates its phenotypic significance. A direct correlation has been observed between the presence of the Prader-Willi/Angelman syndrome regions (located at 15q11.2) on the marker and mental retardation or developmental delay [302–304]. Of particular interest is the observation of a few patients with this type of marker who present clinically with Prader-Willi syndrome or Angelman syndrome [303, 305–309]. Molecular studies performed on some of these patients indicate that the abnormal phenotype results not from the presence of the marker, but from either uniparental disomy of the two normal chromosomes 15 or a deletion of 15q11.2-q13 on one of the apparently cytogenetically normal 15s [303, 308, 309].

Another type of marker chromosome that results in a clinically recognizable multiple congenital anomaly syndrome is the supernumerary bisatellited dicentric marker derived from chromosome 22. This marker contains two copies of a small

segment of proximal long arm euchromatin (22q11.2), thus resulting in tetrasomy for 22q11.2. Clinically, these patients usually present with cat-eye syndrome [310–312]. Characteristic features include craniofacial anomalies (vertical coloboma of the iris, which gives the syndrome its name; coloboma of the choroid or optic nerve; preauricular skin tags/pits; down-slanting palpebral fissures) and anal atresia with rectovestibular fistula. Cardiac defects are present in more than one-third of cases. Renal malformations include unilateral agenesis, unilateral or bilateral hypoplasia or dysplasia. Other less frequent findings include microphthalmia, microtia, atresia of the external auditory canal, biliary atresia, and malrotation of the gut. Intelligence is usually low normal to mildly deficient.

Other types of supernumerary markers, such as ring chromosomes derived from chromosome 22 resulting in either trisomy or tetrasomy for 22q11.2, can also cause various features of the cat-eye syndrome. The critical region of this syndrome has been shown to lie within a 2.1-Mb DNA segment defined distally by locus D22S57 and containing the *ATP6E* (the E subunit of vacuolar H-ATPase) gene [313].

Clinically definable entities have not been observed for other markers, as each is typically unique. However, this may change as data concerning the composition of marker chromosomes accumulates through the use of FISH, array CGH, and other molecular technologies.

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Structural Chromosome Rearrangements

Kathleen Kaiser-Rogers and Kathleen W. Rao

Introduction

The subject of structural chromosome rearrangements is an immense one, to which entire catalogs have been devoted. Indeed, there are theoretically an almost infinite number of ways in which chromosomes can reconfigure themselves from the familiar, normal, 23-pair arrangement. While structural rearrangements are often thought of in terms of chromosome pathology, some rearrangements are fairly innocuous. In fact, a few such benign rearrangements (such as certain pericentric inversions of chromosome 9) are seen frequently enough to be considered polymorphic variants of no clinical significance.

This chapter will discuss and provide examples of the ways in which chromosome rearrangements can occur, and will begin with an overview of general concepts that relate to all structural rearrangements and their association with human pathology. Each category of structural rearrangement will then be dealt with as a unique entity in the second half of the chapter.

Mechanism of Formation

The exchange of genetic material between sister chromatids and homologous chromosomes is a normal occurrence in

somatic and germ cells. These types of exchanges ensure mixing of the gene pool and appear to be obligatory for normal cell division. It is only when exchanges occur between nonallelic chromosomal regions that structural rearrangements result. Since chromosome breakage can theoretically occur anywhere within the human genome and the involved chromosome(s) can recombine in innumerable ways, the number of potential rearrangements that can result is immense. In practice, however, there appear to be particular areas of the genome that are more susceptible to breakage and rearrangement than others because of their underlying architecture. The presence of a DNA sequence that is repeated elsewhere in the genome, susceptible to the formation of double-stranded breaks, and/or capable of forming a particular secondary DNA structure, all appear to influence the likelihood that a particular chromosome region is involved in a structural rearrangement [1–7].

Numerous studies have now shown that many recurring and some sporadic rearrangements occur secondary to recombination between nonallelic regions of homology. While these regions of homology sometimes represent high-copy-number repeats such as Alu or satellite DNA sequences, the majority appear to involve low-copy repeats (LCRs). There are now many examples in the literature of recurring duplications, deletions, inversions, translocations, isochromosomes, and marker chromosomes that form secondary to LCR-mediated nonallelic homologous recombination (NAHR). The LCRs that serve as substrates for these recombination events typically range in size from 10 to 500 kilobase pairs (kb) and share ≥95% sequence identity. Although distributed throughout the genome, LCRs may appear preferentially within pericentromeric chromosomal regions. The ultimate size and types of rearrangements that result from these nonallelic homologous recombination events appear to reflect the location, size, and orientation of the involved LCRs, as well as the number of crossover events that occur between them.

Direct LCRs (those with the same orientation) located on the same chromosome can mediate both duplications and deletions, as shown in Fig. 9.1. When a single, nonallelic,

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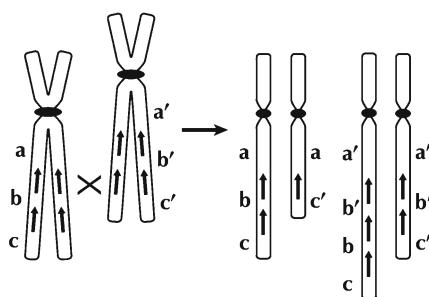
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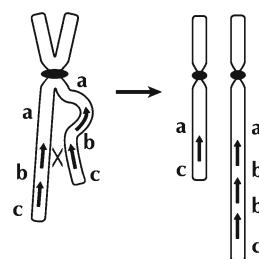
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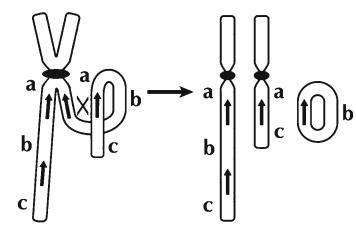
a Direct Repeats & Homologues Recombine



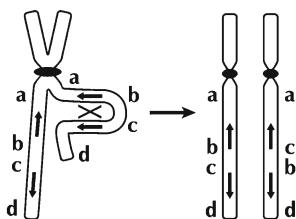
b Direct Repeats & Sister Chromatids Recombine



c Direct Repeats with Intrachromatid Recombination



d Inverted Repeats with Intrachromatid Recombination



e Direct Repeats & Nonhomologous Chromosomes Recombine

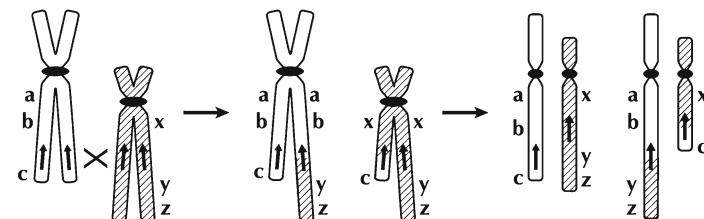


Fig. 9.1 Chromosome rearrangements can be produced by nonallelic homologous recombination between shared sequences or repeats of identical (direct repeats) or opposite (inverted repeats) orientation. Recombination between direct, nonallelic repeats on homologous chromosomes (**a**) or sister chromatids (**b**) can produce complementary duplications and deletions. Recombination between direct repeats located at different sites within a single chromatid can produce both

deletions and acentric ring chromosomes (**c**). If instead recombination occurs between inverted repeats within a single chromatid, a chromosome inversion is produced (**d**). Translocations and other more complex rearrangements can occur secondary to recombination events between shared sequences that are located on different chromosomes (**e**). Shared sequences or repeats are designated by arrows and lower case letters represent unique sequences

homologous recombination event involving homologous chromosomes (interchromosomal) or sister chromatids (intrachromosomal) is mediated by direct LCRs, complementary duplications and deletions occur (Fig. 9.1a, b). Only deletions are predicted to occur, however, if nonallelic homologous recombination involving direct LCRs occurs within a single chromatid (intrachromatid; Fig. 9.1c). As shown in Fig. 9.1d, inversions can form secondary to intrachromatid recombination events within a pair of nonallelic homologous inverted LCRs. Nonallelic recombination events involving LCRs located on completely different chromosomes would be expected to produce translocations (Fig. 9.1e) as well as other more complex rearrangements [8].

The size of the inversions, duplications, and deletions produced by the recombination events described earlier is dependent upon the length and proximity of the LCRs mediating the rearrangement. In general, the larger the rearranged region, the larger the LCR that mediates the recombination event. Single gene rearrangements occur when the recombining homologous sequences flank or are within a single gene. These rearrangements are submicroscopic, require molecular techniques for their identification, and typically

result in Mendelian genetic disorders such as Hunter syndrome, hemophilia A, familial juvenile nephronophthisis, and others [6, 9]. In contrast to single gene rearrangements, recombination events that utilize nonallelic homologous sequences that are separated by large regions of the genome (typically 1.5–5 Mb) or are located on different chromosomes altogether can produce cytogenetically visible rearrangements involving multiple genes. Included within this group are many of the recognized microdeletion and microduplication syndromes, as well as several recurring rearrangements such as the (4;8) translocation with breakpoints at 4p16 and 8p23 and the inverted duplicated chromosomes derived from chromosomes 15 and 22 (see respective sections later).

While LCRs appear to serve as the recombination substrates for many chromosomal rearrangements, high-copy-number repeats such as Alu or satellite DNA sequences also play a role. At least 32 cases of single gene disorders and 16 cases of cancer have been attributed to intrachromosomal Alu-mediated recombination events [10]. While much less common, interchromosomal Alu-Alu recombination events also appear to occur. This is evidenced by a report of

an XX male who carried an XY translocation mediated by Alu repeats [11]. Additionally, interchromosomal nonallelic recombination events mediated by high-copy satellite DNA sequences and/or other adjacent repetitive sequences located within the short arms of the acrocentric chromosomes are hypothesized to be responsible for the formation of at least some of the Robertsonian translocations (see section “[Robertsonian Translocations](#)”, later). Recombination events mediated by satellite DNA sequences are also thought to be responsible for the recurring inversions involving the heterochromatic region within the proximal long arm of chromosome 9 [12].

That DNA architecture may create “hot spots” for chromosome rearrangements has been supported by studies addressing the recurring (11;22) translocation (see section “[Reciprocal Autosomal Translocations](#)”, later). The breakpoints involved in this translocation are not associated with regions of homology but rather with unstable AT-rich palindromic sequences (DNA sequences that contain two inverted regions complementary to each other) susceptible to double-stranded breaks that are repaired by a process referred to as nonhomologous end joining (NHEJ). These palindromic sequences are predicted to form imperfect hairpin- or cruciform-shaped secondary structures susceptible to nucleases that produce double-stranded breaks, which are then ligated to form the resulting translocation. Short, typically 2–5 base pair regions of homology at, or in close proximity to, the double-stranded breaks are often, but not always, used to promote ligation.

Despite the fact that the breakpoints of a number of translocations have now been examined, palindromic sequences have rarely been identified. Among the few that have been identified, with one exception, the same AT-rich palindromic sequence within the proximal short arm of chromosome 22 employed in the recurring (11;22) translocation has been utilized [13]. Two reported cases involve independent (17;22) translocations mediated by a palindrome within intron 31 of the neurofibromatosis I (*NFI*) gene, at least five cases involve a recurring (8;22) translocation with nearly identical breakpoints within an AT-rich palindrome at 8q24.13, while the others involved palindromic sequences at 1p21.1 and 4q35.1 [13–18]. Most rearrangements formed secondary to NHEJ therefore do not appear to utilize palindromic sequences. Instead, they tend to occur within other areas of the genome that are predicted to form cleavage-sensitive chromatin structures vulnerable to the formation of double-stranded breaks such as topoisomerase II cleavage sites, DNase I-sensitive sites, scaffold attachment regions, or expanded trinucleotide repeat regions [1, 19]. Additionally, although the most thoroughly studied example of a rearrangement formed secondary to NHEJ is the recurring (11;22) translocation described earlier, most rearrangements that utilize this mechanism are nonrecurring. Examples of nonrecurring rearrangements

suspected of forming primarily by NHEJ include the 1p36 and 11p11.2 deletions associated with 1p36 deletion and Potocki-Shaffer syndrome, respectively, the 10q24 duplications associated with split hand-split foot malformation [20], and others [7].

Recently, a third mechanism, fork stalling and template switching (FoSTeS), has been proposed to explain the formation of multiple complex nonrecurrent structural chromosome rearrangements that do not appear to have formed secondary to either NAHR or NHEJ [21, 22]. The complex rearrangements associated with FoSTeS contain duplications and/or deletions that are interspersed with nonduplicated or nontriplicated segments that are believed to form secondary to a promiscuous lagging replication strand that may not only have moved discontinuously within its own replication fork but may also have invaded other replication forks. This abnormal replication process is thought to be initiated by fork stalling secondary to particular DNA structures and/or protein-DNA complexes. Once stalled, the lagging DNA strand disengages from its original template and, using short regions of homology, reinitiates replication elsewhere within the same chromosome, the homologous chromosome, or a nonhomologous chromosome in close proximity. Whether the same or a different chromosome has been invaded, the location of strand invasion is upstream or downstream relative to the original replication fork, and whether the leading or lagging strand of the new replication fork has been invaded will dictate whether an interstitial or interchromosomal rearrangement has formed and whether genetic material has been duplicated in a direct or inverted orientation or has been completely deleted. The number of serial replication fork disengagements and invasions that occur prior to reestablishing normal replication on the original strand will dictate the complexity of the resulting rearrangement. Many of the *PLP1* gene duplications associated with Pelizaeus-Merzbacher disease, as well as the nonrecurrent 17p11.2 duplications associated with Potocki-Lupski, are believed to occur secondary to FoSTes [23].

In contrast to the maternal bias noted for numerical chromosome abnormalities, approximately 75% of structural chromosome rearrangements appear to be paternally derived [24, 25]. Exactly why the male bias for *de novo* structural rearrangements exists is currently unknown. It has been suggested, however, that the lifelong mitotic proliferation of spermatogonial cells, compared to the finite number of mitotic divisions responsible for oögonial cell production in the female embryo, may promote the accumulation of mutations. Additionally, studies on mouse and drosophila suggest that male gametogenesis may be more sensitive to mutagens than oogenesis [26]. It is interesting to note, however, that although structural rearrangements as a group are more commonly paternal in origin, there are some exceptions to this rule. For example, approximately 90% of *de novo* nonhomologous

Robertsonian translocations and 80% of terminal chromosome 1 short arm deletions are maternal in origin [27, 28]. Several supernumerary isochromosomes and inverted duplicated chromosomes also appear to form primarily during maternal gametogenesis [29–32]. No parental bias has been noted for several other types of rearrangements including the interstitial microdeletions associated with DiGeorge and Williams syndrome [33, 34]. Although the differences noted in male versus female gametogenesis are thought to affect our respective predispositions to producing specific types of *de novo* rearrangements, other factors, such as the effect of imprinting on fetal survival, have also been proposed to play a role (see Chap. 20).

In theory, chromosome breakage, rearrangement, and reunion can occur during meiosis or mitosis. Meiotic errors, since they occur prior to conception, would be expected to be present in every cell of the resulting pregnancy. Postconception mitotic errors, in contrast, would be predicted to produce a mosaic pregnancy containing both normal and abnormal cells. Interestingly, with the exception of mitotically unstable chromosomes such as rings or dicentrics, structural chromosome rearrangements are rarely seen in mosaic form. While this observation suggests that many structural rearrangements may be formed during meiosis, ascertainment bias likely plays a role as well. Since mosaic individuals typically have milder phenotypes than comparable nonmosaics, they are less likely to be ascertained and karyotyped. This would be especially true of individuals carrying mosaic balanced rearrangements. Additionally, mosaicism is difficult to detect, particularly when it is limited to a specific tissue or group of tissues, is present at a low level, and/or involves a subtle structural change.

Differentiating Between Balanced and Unbalanced Structural Rearrangements

Structural rearrangements are often divided into two general categories, balanced and unbalanced. Balanced rearrangements contain no net loss or gain of genetic information and the individuals who carry them are generally phenotypically normal. In contrast, additional and/or missing genetic material is present in individuals who carry unbalanced rearrangements. Just as modifications in the amount of the various ingredients added to any recipe cause changes in the final product, deviation from the normal disomic genetic complement results in a clinically affected individual.

While it is easy to define balanced and unbalanced rearrangements, distinguishing between a truly balanced and an unbalanced rearrangement using traditional cytogenetic techniques is often impossible. The maximum level of resolution obtained using standard microscopy of G-banded pro-metaphase chromosomes is reported to be 3–5 megabases or

$3\text{--}5 \times 10^6$ base pairs. This number will vary, however, depending on the quality of the chromosome preparations and the skill of the cytogeneticist examining the karyogram(s). The ability to resolve or identify a rearrangement will also be influenced by the degree to which the banding pattern, overall size, and centromere location of an involved chromosome is altered. Obviously, the more apparent the change, the more likely it is to be detected. A number of molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH), 24-color karyotyping, and chromosome microarray analysis are currently being used to detect submicroscopic or otherwise cryptic rearrangements that cannot be detected using traditional cytogenetics (see Chaps. 17 and 18). Recent studies suggest that chromosome imbalances can be found in an additional 10–14% of patients using chromosome microarray analysis [35–37]. Additionally, chromosome microarray analysis has demonstrated that as many as 40% of phenotypically abnormal individuals with apparently balanced simple chromosome rearrangements detected by traditional karyotyping actually contain submicroscopic gains and/or losses at or near one or more rearrangement breakpoints, while others have been found to have cryptic imbalances unrelated to their identified balanced rearrangements [38–41].

Associated Risks

Once a structural chromosome rearrangement is detected, regardless of whether it is balanced or unbalanced, the subsequent steps to take depend on the type of specimen that was analyzed. For prenatal samples or those obtained from a child, parental karyotypes or molecular cytogenetic studies should be obtained whenever possible to assess whether the rearrangement has been inherited or represents a *de novo* mutation. If neither parent is found to be a carrier of the rearrangement, the most likely scenario is that it represents a *de novo* abnormality rather than an inherited one. Non-paternity should be considered. Since the possibility of gonadal mosaicism can never be excluded, this family would be given a very low risk of having another child with the same structural abnormality. Prenatal testing would also be offered for all future pregnancies.

In contrast to the very low recurrence risk quoted to a couple with a child or pregnancy carrying a *de novo* rearrangement, the risk of chromosomally abnormal conceptions for an adult who carries a balanced structural rearrangement is much higher. In fact, for some familial rearrangements, the risk can approach 50%, and for very rare carriers of a homologous Robertsonian translocation, the risk for an abnormal conception is 100%. It is therefore imperative that these families are identified so that they can be given accurate genetic counseling regarding their reproductive risks and options. In situations where a familial rearrangement is

identified, it must be remembered that it is not just the immediate family but distant relatives as well who may be at risk for having children with unbalanced karyotypes and associated mental and/or physical abnormalities. By systematically karyotyping the appropriate individuals in each generation, all those with elevated reproductive risks can be identified and appropriately counseled regarding their risks and options. Although there has been some debate regarding the appropriateness of karyotyping the phenotypically normal minors of balanced carriers, 50% of whom would be expected to be balanced carriers themselves, there is a consensus that these children should be referred for appropriate genetic counseling when they reach reproductive age.

The situation becomes a bit more complex when chromosome analysis of a bone marrow or tumor specimen results in an apparently balanced rearrangement, not associated with any particular neoplasm, in all cells examined. In these cases, it is imperative to ascertain whether such a rearrangement represents a patient-specific acquired change (which can then be monitored during treatment, remission, relapse, or any change in disease aggression) or a constitutional abnormality present from birth. The reasons for this are twofold. First of all, from the point of view of the physician treating the patient, the presence of any acquired cytogenetic change is significant (see Chaps. 15 and 16). Equally as important in the long term, however, is establishing whether the chromosome rearrangement is familial and therefore has potential reproductive consequences for the extended family. While the potential familial reproductive issues are understandably not the primary concern of oncology patients nor their physicians, given the critical nature of their disease and the fact that cancer patients are often well beyond childbearing age themselves, this is an issue that should not be overlooked. Genetic counseling is covered in detail in Chap. 21.

De Novo Rearrangements

Every chromosome rearrangement was at one time a new or *de novo* rearrangement that carried the risks associated with an undefined entity. Children who carry unbalanced rearrangements, regardless of whether they represent new mutations or an unbalanced form of a familial rearrangement, almost inevitably demonstrate an abnormal phenotype. An imbalance is an imbalance regardless of how it arose.

In contrast, accurate predictions regarding the phenotype of a child or fetus that carries an apparently balanced *de novo* chromosome rearrangement are more difficult to make. In this situation, it is not known what has occurred at the molecular level within the rearrangement, and there are no family members with the rearrangement from whom inferences can be made. The risk for an abnormal phenotype is therefore

always higher for an individual with an apparently balanced *de novo* rearrangement than for an individual who has inherited a similar rearrangement from a normal parent. Obviously, these individuals also carry a significantly higher risk for phenotypic abnormalities than their chromosomally normal counterparts. Several population studies have shown, for example, that the incidence of *de novo* apparently balanced rearrangements among the mentally retarded is approximately seven times that reported in newborns [42]. Apparently balanced *de novo* rearrangements detected at amniocentesis have also been associated with a risk for congenital abnormalities that is two- to threefold that observed within the general population [1].

A number of different mechanisms are thought to be responsible for the abnormal phenotypes observed in children with apparently balanced *de novo* rearrangements. One possibility is that the translocation is not truly balanced. As discussed earlier, structural rearrangements that appear balanced at the microscopic level may actually contain large duplications and/or deletions at the molecular level. Another possibility is that the rearrangement is “balanced,” but a break has occurred within a critical gene or its surrounding regulatory sequences such that the gene product or its expression is altered. This scenario has been demonstrated in several patients with Duchenne muscular dystrophy, for example [43]. A position effect, in which the expression of a specific gene or group of genes is altered when the chromosome segment containing them is moved to a different location, could also result in an abnormal phenotype. Such an effect has been demonstrated in several X;autosome translocation chromosomes in which inactivation seems to spread from the inactive X chromosome into neighboring autosomal segments. This phenomenon has been documented in drosophila and plants as well. Finally, the possibility that an individual’s abnormal phenotype may be completely unrelated to his or her rearrangement must always be examined. Other non-chromosomal genetic disorders, prenatal exposures, birth trauma, non-paternity, etc., must all be considered.

Familial Rearrangements

Balanced structural rearrangements may pass through multiple generations of a family without detection. When these families are ascertained, it is usually due to the presence of infertility, multiple spontaneous pregnancy losses, and/or clinically abnormal family members (Fig. 9.2). Meiotic events that result in cytogenetically unbalanced conceptions can explain the presence of all three occurrences within these families.

During normal meiosis, homologous chromosomes pair utilizing a mechanism of formation thought to depend, at least in part, upon interactions between their shared

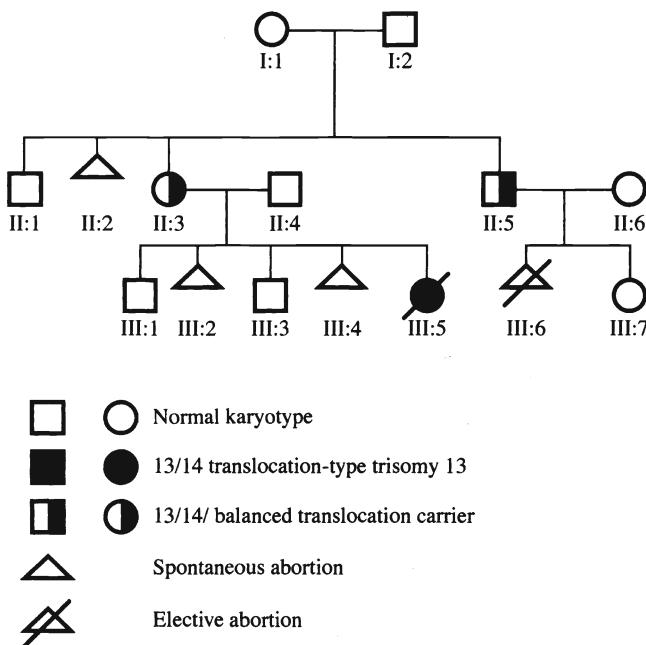


Fig. 9.2 A pedigree of a family in which a balanced Robertsonian (13;14) translocation is segregating. Multiple spontaneous abortions (see individuals II-2, III-2, and III-4), abnormal children (III-5), and infertility are frequently observed in families segregating a balanced rearrangement

sequences. Under normal circumstances, all 23 pairs of homologous chromosomes align themselves to form 23 paired linear structures or bivalents that later separate and migrate to independent daughter cells (see Chap. 2). In cells carrying structurally rearranged chromosomes, pairing cannot occur in a simple linear fashion. Instead, complex pairing configurations are formed in an attempt to maximize pairing between homologous regions that now differ with regard to their chromosomal location and/or orientation (see the later sections “Deletions,” “Duplications,” “Inversions,” “Reciprocal Autosomal Translocations,” and “Insertions”). Chromosome malsegregation and/or particular recombination events within these complex configurations can then lead to unbalanced conceptions, many of which never implant or are spontaneously lost during gestation.

Cytogeneticists are frequently asked to make predictions regarding a balanced carrier’s risk of producing an abnormal liveborn child. While this is a legitimate question, it is in practice very difficult to answer accurately. One source of difficulty is the fact that, with very few exceptions, each family’s rearrangement is unique. Therefore, unless a family is large and accurate information regarding the reproductive history and phenotype of each family member is available, typically no empiric data are available from which to obtain risk values. A second source of difficulty one encounters in assessing the reproductive risks associated with a particular balanced rearrangement is the breadth and complexity of the variables involved.

One important factor that is considered when assessing the reproductive risks of a carrier parent is the extent of imbalance demonstrated by the potential segregants. In general, the smaller the imbalance, the less severe the phenotype, and the more likely the survival. An additional rule of thumb is that the presence of excess genetic material is less deleterious than the absence of genetic material. Another variable to be considered is the quality of the genetic information involved. Some chromosomes, such as 16 and 19, are infrequently involved in unbalanced structural rearrangements. Presumably, this occurs because of the importance of maintaining a critical dosage for a gene or group of genes on these chromosomes. Conversely, imbalances involving other chromosomes such as 13, 18, 21, X, and Y appear to be more easily tolerated. In fact, a complete trisomy involving any of these chromosomes is survivable.

Each family’s reproductive history can also provide important clues regarding the most likely outcome for an unbalanced pregnancy. As might be expected, those families or individuals who have had a liveborn child or children with congenital abnormalities, especially when an unbalanced form of the familial rearrangement has been documented, are at highest risk for having unbalanced offspring. In families or individuals in whom multiple spontaneous abortions and/or infertility are noted, the risk for liveborn unbalanced offspring would be expected to be lower. In these families, it is assumed that the unbalanced conceptions are being lost very early as unrecognized pregnancies (infertility) or later during gestation. Interestingly, the sex of the carrier parent, in some cases, also influences the risk of having unbalanced offspring. In situations where a sex bias does exist, the female carrier invariably possesses the higher risk. Why male carriers appear to produce fewer unbalanced offspring than their female counterparts is not known. Perhaps fewer unbalanced segregants form during spermatogenesis relative to oogenesis, and/or the selective pressure against unbalanced gametes is greater in the male, and/or imprinting effects may cause the unbalanced embryos of male carriers to be less viable than those of their female counterparts. Male infertility may also play role [26, 44] (see Chap. 11).

On rare occasions, an abnormal phenotype is observed in an apparently balanced carrier of a familial rearrangement. While some of these cases may simply represent coincidental events, other possible explanations exist as well. Very rarely, abnormal offspring resulting from uniparental disomy, or the inheritance of both homologous chromosomes from a single parent, has been documented in the offspring of balanced translocation carriers [45] (see Chap. 20). Incomplete transmission of a partially cryptic rearrangement has also been observed in the abnormal offspring of a phenotypically normal carrier parent. Wagstaff and Herman, for example, describe a family in which an apparently balanced (3;9) translocation was thought to be segregating [46]. After

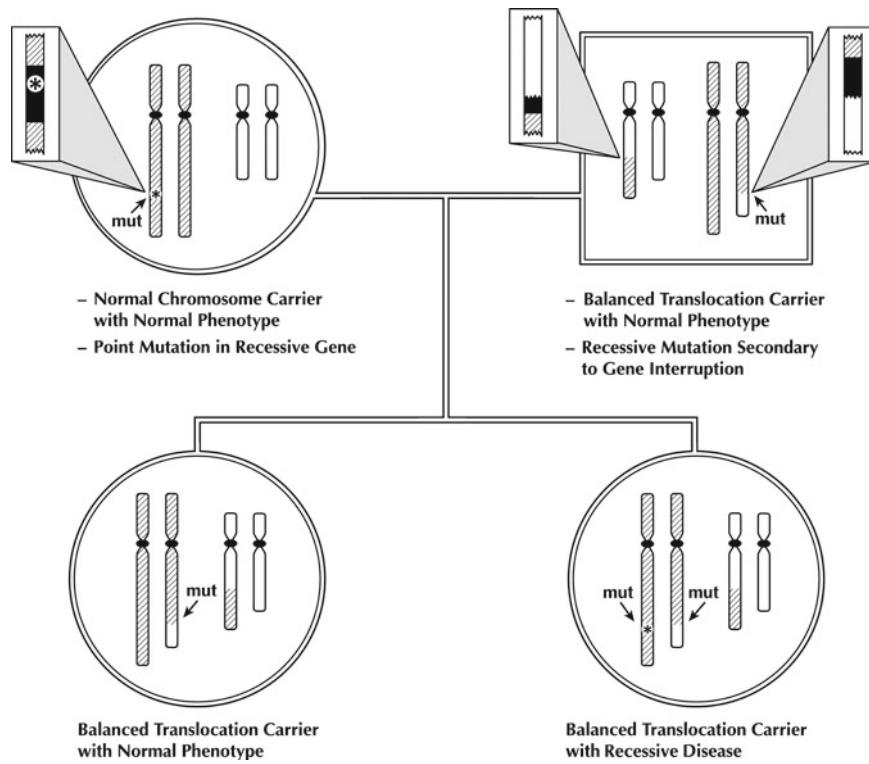


Fig. 9.3 In the example here, the mother (top left) carries a recessive point mutation (asterisk) within a gene (black box) located on one chromosome homolog (hatched). The father (top right) carries a mutation in the same gene secondary to interruption via a translocation event. Because the second homolog in each parent contains a normal allele, both parents are phenotypically normal. This is also true for their first child (bottom left) who inherited the balanced

translocation from her father and the normal hatched chromosome from her mother. Although their second child (bottom right) is also a balanced translocation carrier, she has inherited two mutated copies of the gene and therefore manifests the recessive disease. The allele she inherited from her mother contains a point mutation, while the comparable paternally inherited allele has been interrupted secondary to a translocation

the birth of two phenotypically abnormal offspring with apparently balanced karyotypes, molecular analysis demonstrated that the father's apparently balanced (3;9) translocation was actually a more complex rearrangement involving a cryptic insertion of chromosome 9 material into chromosome 8. Abnormal segregation of this complex rearrangement led to a cryptic deletion of chromosome 9 material in one sibling and a duplication of the same material in the other.

Phenotypic discrepancies between child and parent may also be explained by the presence of a recessive allele that is inherited from a chromosomally normal parent. While the parent is phenotypically normal due to the presence of a complementary normal allele on the homologous chromosome, the abnormal allele can be expressed in the offspring, who has no normal allele. The affected child inherits two mutant alleles; one mutant allele is inherited secondary to the balanced chromosome rearrangement, while the other is inherited from the cytogenetically normal parent (Fig. 9.3). In a slight variation of this theme, the second inherited hit or mutation in the affected child is nonallelic but presumably

functions within a biological pathway identical to or related to the first hit. The idea is that each parent carries a single mutation that is benign or causes only mild clinical manifestations of disease. When both mutations are inherited together, however, the involved pathway(s) is sufficiently altered to cause disease. Alternatively, one predisposing mutation could be inherited, while the other occurs *de novo*.

The two-hit scenario described earlier has also recently been invoked to provide one possible explanation for the variable expressivity and decreased penetrance observed in association with many microdeletions and microduplications. This is especially true of some of the smaller copy number changes that are currently being identified by chromosome microarray analysis [47, 48].

Deletions

Autosomal deletions that can be detected by traditional, “high-resolution,” or molecular cytogenetic methods produce monosomies that are generally associated with significant

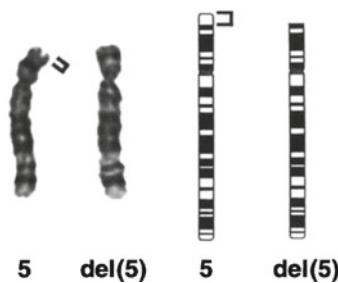


Fig. 9.4 A terminal deletion involving the distal short arm of chromosome 5 [del(5)(p15.3)]. Patients with similar deletions are said to have *cri du chat* or cat cry syndrome because of the characteristic catlike cry present in many during infancy

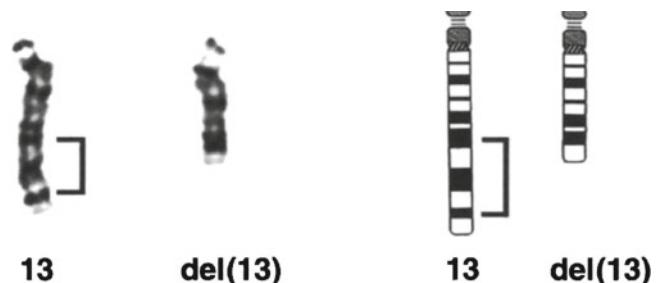


Fig. 9.5 An interstitial deletion involving the long arm of chromosome 13 [del(13)(q21.3q33)]

pathology. Some exceptions, however, do exist. Loss of the short arm material from acrocentric chromosomes during the formation of Robertsonian translocations, for example, has no impact on phenotype. Similarly, the striking size variation of heterochromatic regions in normal individuals suggests that loss of some, if not all, of this material is insignificant. There have even been reports of “benign” deletions in regions that are considered euchromatic. Gardner and Sutherland catalog deletions of this type in bands 2p11.2-p12 and 2q13-q14.1; 3p25.3-pter; 5p14; 8p23.1-pter and 8q24.13-q24.22; 9p21.2-p22.1; 11p12; 13q21; 16q21 and 16q13-q22; and 18p11.2-pter [49]. Close examination of these regions reveals that many are relatively gene poor and therefore less likely to contain a dosage-sensitive gene. For example, the 11 Mb of genomic material within the 5p14 band and the 7 Mb of genomic material within the 11p12 band contain only eight and nine genes, respectively.

Among deletions of pathological significance, classic cytogenetic deletions that can be detected by routine methodology tend to be larger and associated with major malformations. Generally, large deletions have a more significant impact on phenotype and survival than smaller ones. The nature of the deleted material, however, also plays an important role in determining whether a specific deletion is viable. Thus, deletions of large segments of the short arms of chromosomes 4 and 5, and of the entire short arm of chromosome 18, are recurrent abnormalities among infants with major malformations, while deletions of similar size involving the short arms of chromosomes 17 and 19 are rarely, if ever, seen in liveborns [50].

Classic deletions have traditionally been described as either terminal (Fig. 9.4) or interstitial (Fig. 9.5) based on chromosome banding patterns. A deletion is considered “terminal” if there is no discernable material beyond the site of initial breakage. Conversely, interstitial deletions have a proximal breakpoint, missing material, and a more distal breakpoint beyond which the chromosome continues with a

normal banding pattern to its terminus. All stable chromosomes have telomeres comprised of the human consensus telomere sequence $(TTAGGG)_n$. Chromosomes with apparent terminal deletions are no exception and are assumed to have acquired “new” telomeres following the deletion event.

Several mechanisms for acquiring or retaining a telomere have now been documented among chromosome deletions. One mechanism referred to as telomere healing involves the addition of a new $(TTAGGG)_n$ sequence at or near the deletion breakpoint [51–53]. In these cases, a telomerase recognition site in the vicinity of the deletion breakpoint is bound by the enzyme telomerase, which synthesizes a completely new telomere. These therefore represent true terminal deletions. Other chromosomes with apparent terminal deletions have been shown to actually represent derivative chromosomes that have acquired their subtelomeric and telomeric regions from another chromosome secondary to a translocation event. These translocation or “telomere capture” events are hypothesized to occur secondary to homologous recombination mediated by regions of shared homology that exist within the deleted chromosome and the subtelomeric region of a separate chromosome [54, 55]. Still other deletions appear to be terminal by traditional cytogenetic analysis but have been shown by molecular analytic analysis to be interstitial. It is estimated that 7–25% of apparent terminal deletions fall into this category [56–58]. Because a chromosome with an interstitial deletion retains its original telomere, there is no reason to synthesize or acquire a new one.

The use of “high-resolution” banding and molecular cytogenetic techniques has led to the identification of another class of cytogenetic abnormality variously referred to as chromosomal microdeletions, contiguous gene syndromes, and segmental aneusomy syndromes (SAS). These abnormalities are mostly very small interstitial deletions, often at or below the resolution of microscopic

analysis, that recur with appreciable frequency and are associated with distinct clinical phenotypes. The term, “microdeletion,” is descriptive but fails to include the minority category of “microduplications” (e.g., CMT1A; see also section “[Duplications](#),” later) and the variable etiologies for some of the disorders. The term “contiguous gene syndromes” was introduced in 1986 to describe the involvement of multiple contiguous genes in the production of a clinical phenotype [59]. While this terminology remains appropriate for some of the disorders in this new category, others are actually single gene disorders, or the result of imprinting defects or uniparental disomy (see Chap. 20). In an effort to more accurately characterize the pathogenesis of these disorders, the term segmental aneupomy syndrome was proposed to imply that the phenotype is the result of “inappropriate dosage for a critical gene(s) within a genomic segment” [60].

Williams syndrome is one example of an SAS that results from a small deletion [61]. These patients typically carry a ~1.5 Mb deletion within the proximal long arm of chromosome 7 that encompasses approximately 30 different known and predicted genes. At least some of these genes appear to be responsible for the cardiovascular abnormalities, growth and developmental delays, infantile hypercalcemia, and dysmorphic facial features that are associated with Williams syndrome. Deletion of the elastin gene (*ELN*), for example, has been implicated in the cardiovascular abnormalities. This gene is also presumed to play a causative role in some of the other features associated with this syndrome including renal artery stenosis, hypertension, hoarse voice, premature sagging of the skin, and perhaps some of the facial features. Similarly, loss of LIM-kinase 1 (*LIMK1*), a novel kinase expressed in the brain, is predicted to explain some of the cognitive abnormalities in these patients. Presumably, some, or all, of the remaining genes identified within the common Williams syndrome deletion also contribute to the physical features associated with this contiguous gene syndrome.

Molecular studies of the Williams syndrome deletions have revealed the presence of flanking low-copy repeat (LCR) sequences at the common breakpoint sites. These LCR sequences appear to provide recombination sites for unequal meiotic and mitotic exchange events that produce the recurring Williams syndrome deletions [62–64]. In some cases, these unequal exchange events seem to be promoted by the presence of heterozygosity for a submicroscopic paracentric inversion that spans the same low-copy repeat sequences that mediate the common 1.5 Mb Williams syndrome deletion [65–68]. It is estimated that the risk of having a child with a Williams syndrome deletion is approximately fivefold higher for an individual who is heterozygous for this inversion when compared to an indi-

vidual who does not carry it. However, despite their elevated relative risk, it is important to note their absolute risk remains low at approximately 1 in 1,750. Several studies have now demonstrated that, at least in some cases, these inversions increase the probability of a rearrangement by producing better LCR substrates for recombination. In the case of the recurring 15q13.3, 16p12.1, and 17q21.31 microdeletions, for example, the associated inversion polymorphism increases the probability of an unequal exchange by changing the directional relationship between the mediating LCRs [48, 69, 70]. Without the inversion polymorphism, the LCRs are in an inverted orientation and unequal exchange is not promoted. With the inversion, however, the LCRs are placed in a direct orientation and as such are ideally suited for the NAHR events between homologous chromosomes and sister chromatids that are primarily responsible for these deletions (Fig. 9.1a, b). In other cases, the inversion may improve upon the involved LCR substrates by increasing their length. It is also possible that the inversion loop that forms to maximize homologous chromosome pairing in the heterozygous parent renders the paired chromosomes more susceptible to unequal crossing-over (see section “[Inversions](#)” and Fig. 9.12 later).

As noted for Williams syndrome, flanking LCR sequences have also been found at the deletion sites of several other SASs. Recombination events localized to these LCR sequences appear to account for the size consistency and the frequency of the deletions associated with these disorders as well. A partial listing of classic cytogenetic deletion or SASs can be found in Table 9.1. Given the recent widespread use of chromosome microarray technology in many research and clinical cytogenetics laboratories, the number of microdeletion and microduplication syndromes being identified and characterized is growing at a rapid pace. For an extensive list of chromosome abnormalities that includes some of the more recently identified syndromes and their associated phenotypes, see the Websites for Wellcome Trust Sanger Institute “DECIPHER,” the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA), and Unique – The Rare Chromosome Disorder Support Group [71–73].

In contrast to the size consistency and recurrent use of specific LCR sequences documented among many of the interstitial SAS deletions, other deletions appear to have multiple independent breakpoints and vary considerably in size. This size variability has been noted in association with multiple deletions including those that involve the short arms of chromosomes 1, 4, and 5 [28, 74, 75]. Nonhomologous end joining (NHEJ) and fork stalling template switching (FoSTeS), rather than nonallelic homologous recombination (NAHR), are believed to be responsible for these nonrecuring rearrangements [7, 76, 77].

Table 9.1 Some recurring deletion syndromes

Deletion syndrome	Deleted region	Key clinical features
Monosomy 1p36	1p36 ^a	Mental retardation, growth delay, hypotonia, early puberty, deafness, eye problems, cardiomyopathy, seizures and/or abnormal EEGs, enlarged anterior fontanel, deep-set eyes, flat nasal bridge, orofacial clefting or palatal abnormalities, pointed chin, ear abnormalities
Wolf-Hirschhorn	4p ^a	Mental and growth retardation, microcephaly, hypertelorism, broad nasal bridge, downturned mouth, cleft lip and/or palate, micrognathia, cryptorchidism, hypospadias
Cri du chat	5p ^a	Mental and growth retardation, cat-like cry in infancy, microcephaly, round face, hypertelorism, down-slanting palpebral fissures
Sotos	5q35 ^b	Cardinal features include intellectual disability, overgrowth, and characteristic long, thin facies with a broad forehead, sparse frontoparietal hair, and down-slanted palpebral fissures. Macrocephaly, advanced bone age, behavior problems, hypotonia, feeding problems, renal anomalies, scoliosis, and seizures are also seen
Williams	7q11.23 ^b	Mental retardation, short stature, supravalvular aortic stenosis, hypercalcemia, friendly disposition, hoarse voice, periorbital fullness, stellate pattern in the iris, anteverted nares, long philtrum, full lips
Potocki-Shaffer (DEFECT 11 syndrome for <u>deletion</u> , <u>enlarged</u> foramina, <u>exostoses</u> , cranial <u>dysostosis</u> , retardation)	11p11.2 ^a	Mental retardation, biparental foramina, brachycephaly, turricephaly, multiple exostoses, micropenis, and minor facial dysmorphism including a high forehead, small upturned nose with broad tip, downturned mouth
Jacobsen	11q24.1–11qter ^a	Mental and growth retardation, trigonocephaly, strabismus, cardiac defects, digit anomalies, thrombocytopenia
Langer-Giedion (trichorhinophalangeal syndrome type II)	8q24.11–8q24.13 ^a	Mental and growth retardation, multiple exostoses, cone-shaped epiphyses, fine scalp hair, bulbous nose, prominent ears, simple but prominent philtrum, loose redundant skin in infancy
Angelman	Maternal 15q11.2–15q13.1 deletion complementary to the 15q11.2–15q13.1 microduplication syndrome	Mental and growth retardation, frequent laughter, ataxia and jerky arm movements, seizures, maxillary hypoplasia, deep-set eyes, large mouth with protruding tongue, widely spaced teeth, prognathia
Prader-Willi	Paternal 15q11.2–15q13.1	Mental and growth retardation, hypotonia and feeding problems in infancy, later obesity associated with hyperphagia, narrow bifrontal diameter, almond-shaped eyes, small hands and feet, hypogonadism, skin picking
15q13.3 Microdeletion	15q13.3 ^b	Developmental delay with mild to moderate learning disability, autism spectrum disorder, schizophrenia, epilepsy, seizures, digit anomalies, and facial features that include hypertelorism, short philtrum, and a thick, everted upper lip. Extensive phenotypic variability and incomplete penetrance have been reported
Rubinstein-Taybi	16p13.3 ^b	Mental retardation, postnatal growth retardation, hypotonia, broad thumbs and toes, cryptorchidism, abnormal facies with downward-slanting palpebral fissures; heavy, highly arched eyebrows; long eyelashes; prominent and/or beaked nose; hypoplastic maxilla with narrow palate
Miller-Dieker	17p13.3 ^a	Mental and growth retardation, lissencephaly, microcephaly, bitemporal depression, long philtrum, thin upper lip, mild micrognathia, ear dysplasia, anteverted nostrils
Hereditary neuropathy with liability to pressure palsies (HNPP)	17p11.2 ^b Deletion complementary to the CMT1A syndrome duplication	Asymmetric recurrent palsies precipitated by focal pressure beginning in the second or third decade of life and electrophysiologic findings of prolonged sensory motor nerve conduction
Smith-Magenis	17p11.2 ^a	Mental retardation, behavioral problems, hyperactivity, sleep disturbance, decreased pain sensitivity, short stature, brachycephaly, midface hypoplasia, prognathism, fingertip pads, hoarse voice
17q21.3 Microdeletion	17q21.3 ^b Deletion complementary to the 17q21.2 microduplication syndrome	Mental retardation/developmental delay, delayed speech, friendly disposition, hypotonia, normal growth, epilepsy, heart anomalies, renal/urologic anomalies, abnormal hair color or texture, and typical facies with high broad forehead, ptosis, blepharophimosis, up-slanting palpebral fissures, epicanthal folds, a tubular- or pear-shaped nose, prominent ears
Alagille	20p12.2 ^b	Cholestasis, peripheral pulmonic stenosis, vertebral arch defects, posterior embryotoxin, abnormal facies including deep-set eyes, broad forehead, long straight nose, prominent chin, small low-set or malformed ears

(continued)

Table 9.1 (continued)

Deletion syndrome	Deleted region	Key clinical features
DiGeorge/velo-cardio-facial (Shprintzen)	22q11.2 ^a Deletion complementary to proximal 22q11.2 microduplication syndrome	Learning disabilities, short stature, overt or submucous cleft palate, velopharyngeal incompetence, prominent nose with squared nasal root and narrow alar base, conotruncal cardiac defects, and psychiatric disorders in some
Phelan-Mcdermid	22q13.3 ^a	Moderate to severe developmental delay, severe expressive speech delay, behavior disturbance, increased tolerance to pain, hypotonia, normal to accelerated growth, dysplastic toenails, large hands, and minor dysmorphic features including dolichocephaly, ptosis, abnormal ears, pointed chin
Kallmann ^c	Xp22.3 ^b	Hypogonadotropic hypogonadism, eunuchoid habitus, anosmia or hyposmia, bimanual synkinesis
Ichthyosis (X-linked) ^c	Xp22.3 ^b	Hypertrophic ichthyosis, corneal opacities without impairment of vision

^aDeletion is frequently visible or ^btypically not visible using traditional cytogenetics

^cThis has been seen in association with several other X-linked disorders when it occurs as part of a contiguous gene syndrome

Duplications

The term “duplication” as applied to chromosome abnormalities implies the presence of an extra copy of a genomic segment resulting in a partial trisomy. A duplication can take many forms. It can be present in an individual as a “pure duplication,” uncomplicated by other imbalances (Fig. 9.6), or in combination with a deletion or some other rearrangement. Examples of some types of rearrangements that involve duplications include isochromosomes, dicentrics, derivatives, recombinants, rings, and markers. The origins and behavior of these abnormal chromosomes are discussed elsewhere in this chapter.

Tandem duplications represent a contiguous doubling of a chromosomal segment. The extra material can be oriented in the same direction as the original (a direct duplication) or in opposition (an inverted duplication). Most cytogenetically detectable tandem duplications in humans appear to be direct [78].

Autosomal duplications produce partial trisomies and associated phenotypic abnormalities. As mentioned in the introduction, the phenotypes associated with duplications are typically less severe than those associated with comparable deletions. Relatively few duplications, however, have occurred with sufficient frequency or been associated with such a strikingly characteristic phenotype that they have been recognized as defined clinical syndromes (Table 9.2). A few cases of distal 3q duplication have been reported in patients with features similar to Cornelia de Lange syndrome. However, these patients also have additional abnormalities not usually associated with the syndrome [50]. Paternally derived duplications of distal 11p have also been associated, in some cases, with Beckwith-Wiedemann syndrome [79]. More intriguing, and perhaps more significant, is the emerging recognition of recurring duplications that involve the same genomic segments that are associated with some of the

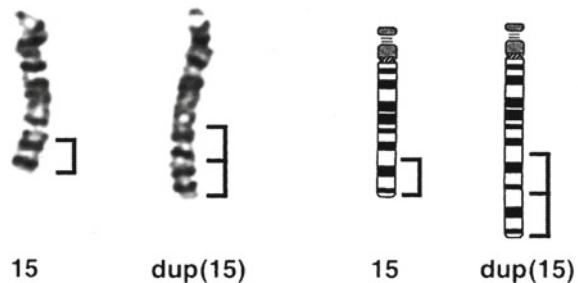


Fig. 9.6 A duplication involving the distal long arm of chromosome 15 [dup(15)(q24q26.3)]. This duplication was initially observed in the bone marrow of a patient with mental retardation and leukemia. By obtaining a peripheral blood karyotype, it was possible to demonstrate that the duplication was constitutional and apparently unrelated to the leukemia

established microdeletion syndromes. These complementary microduplication/microdeletion syndromes are thought to represent the reciprocal products of recurring unequal exchange events that are mediated by flanking homologous low-copy repeat (LCR) sequences. The causative unequal exchange events can occur following misalignment of either sister chromatids or homologs as shown in Fig. 9.1.

One of the first complementary microduplication syndromes identified involves the Prader-Willi and Angelman syndrome (PWS/AS) region within the proximal long arm of chromosome 15 (Fig. 9.7). These duplications are mediated by the same LCRs and encompass the same loci that are deleted in PWS/AS. The clinical significance of these particular duplications was initially difficult to assess because many affected patients had phenotypically normal relatives with the same apparent duplication. Molecular studies have now provided us with an explanation for the apparent absence of a genotype-phenotype correlation in these families. With few exceptions, the clinically affected individual(s) within these families demonstrate mental retardation, decreased motor coordination, autism spectrum disorder, and mild to no dysmorphic features and carry a maternally derived

Table 9.2 Some recurring duplication/triplication syndromes

Duplication/triplication syndrome	Duplicated/triplicated region	Key clinical features
Duplication 3q	?3q26.3	A Cornelia de Lange-like phenotype that includes mental retardation postnatal growth retardation, long philtrum, palate anomalies, anteverted nares, clinodactyly, talipes, renal and cardiac abnormalities
7q11.23	7q11.23 ^b	Cognitive abilities range from normal to moderate mental retardation, but all have speech delay. Other features include autism, hypotonia, heart defects, diaphragmatic hernia, cryptorchidism, and dysmorphic facial features including a short philtrum, thin lips, and straight eyebrows
Microduplication	Duplication complementary to the 17q21.2 microdeletion syndrome	Macrosomia, macroglossia, organomegaly, omphalocele, ear creases, hypoglycemia, tumor susceptibility. Beckwith-Wiedemann patients with cytogenetic duplications are more likely to have learning difficulties
Beckwith-Wiedemann	11 p15.5 ^b (Paternal)	Wiedemann patients with cytogenetic duplications are more likely to have learning difficulties
Pallister-Killian	Mosaic tetrasomy 12p usually secondary to an extra metacentric isochromosome 15q11.2-15q13.1 ^a	Mental retardation, streaks of hyper- and hypopigmentation, sparse anterior scalp hair, sparse eyebrows and eyelashes, prominent forehead, protruding lower lip, coarsening of face with age
Proximal 15q11.2 microduplication	Complementary to Prader-Willi/ Angelman syndrome deletion region	Mild to severe intellectual impairment particularly with regard to language, autism spectrum disorders, decreased motor coordination, hypotonia, reduced deep tendon reflexes, joint laxity, mild or no dysmorphic features. Phenotype typically associated with maternal (but not paternal) duplication
Pseudodicentric 15 ("inverted duplicated 15")	Tetrasomy 15pter-15q13 due to the presence of an extra pseudodicentric chromosome	Mental and growth retardation, autism, behavioral disturbance, seizures, low posterior hairline, epicanthal folds, low-set ears, strabismus
17p13.3 Duplication	17p13.3	The smaller pseudodicentric 15 chromosomes may not cause phenotypic abnormalities
	Most are unique nonrecurrent duplications that overlap with but do not correspond to the recurring Miller-Dieker syndrome deletion region	Mental retardation and/or learning difficulties, speech difficulties, autism, hypotonia, subtle hand and foot malformations, lack of severe congenital anomalies, and normal to increased growth parameters. Facial features include prominent forehead and pointed chin
Charcot-Marie-Tooth 1A (CMT1A)	17p11.2 ^b Duplication complementary to the HNPP syndrome deletion	Abnormal nerve conduction velocities, distal muscle weakness, muscle atrophy, and sensory loss. Symptoms begin between ages 5 and 25 and progress slowly
Potocki-Lupski	17p11.2 ^a	Mild to borderline mental retardation, behavioral problems, hypotonia, failure to thrive, cardiac anomalies, and variable dysmorphic features that include triangular face, frontal bossing, microcephaly, hypertelorism, wide nasal bridge, epicanthal folds, and a flat philtrum
17q21.3 Duplication	17q21.3 ^b	Intellectual disability, autism spectrum disorders, and variable dysmorphic features
Proximal 22q11.2 microduplication	Duplication complementary to the 17q21.3 microdeletion syndrome	
	22q11.2 ^a	Currently no clearly established phenotype recognized. Some patients noted to have features that overlap with DiGeorge syndrome including mental retardation and developmental delay, abnormalities of the palate, conotruncal heart defects, absent thymus, and corresponding T-cell deficiency. Phenotype variable and ranges from mild to severe
Cat eye	Tetrasomy 22q11.2 (occasionally trisomy) usually secondary to an extra pseudodicentric or ring chromosome	Usually mild mental retardation, coloboma of the iris, down-slanting palpebral fissures, preauricular tags and/or fistulas, anal atresia

^aDuplication is frequently visible or typically not visible using traditional cytogenetics



Fig. 9.7 A duplication involving the proximal long arm of chromosome 15 [dup(15)(q11.2q13.1)] that appears to be complementary to the common deletions that are observed in Prader-Willi syndrome and Angelman syndrome patients. Duplication of the Prader-Willi/Angelman syndrome region was confirmed using fluorescence *in situ* hybridization (*not shown*.)

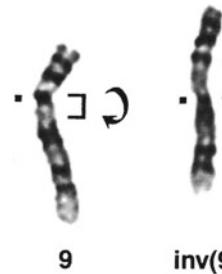
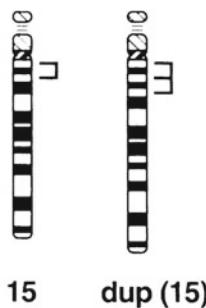


Fig. 9.8 This benign inversion of chromosome 9 [inv(9)(p11q13)] represents a pericentric inversion with breakpoints in both chromosome arms. The material between the two breakpoints has been inverted, the block of heterochromatin that normally sits in the long arm has been shifted to the short arm, and the banding pattern has been subtly changed. Because the breakpoints have not occurred symmetrically with respect to the centromere, the short arm-to-long arm ratio of the inverted chromosome has been altered as well

duplication. In contrast, the duplicated chromosome in the normal relatives of these patients is typically paternally inherited. These data suggest that imprinting within the PWS/AS region is responsible not only for the phenotypic differences we observe with maternal versus paternal deletions but also for the presence or absence of a clinical phenotype in patients with a duplication (see Chap. 20).

In addition to the PWS/AS region, complementary microdeletion-microduplication syndromes have also been documented for the Williams, Smith-Magenis, 17q21.31, and DiGeorge syndrome critical regions, as well as for the hereditary neuropathy with liability to pressure palsies (HNPP)/Charcot-Marie-Tooth type I region (Table 9.2). More recently, chromosome microarray analysis has resulted in the identification of additional previously unrecognized complementary recurring microdeletions and duplications involving 1q21.1, 3q29, 15q13.3, 16p11.2, 16p13.11, 17q12, distal 22q11.2, and multiple other regions [48]. For an extensive list of chromosome abnormalities that includes some of the more recently identified microduplications and microdeletion syndromes and their associated phenotypes, see the Websites listed earlier in the section “Deletions” [71–73].

Inversions

Inversions are intrachromosomal rearrangements formed when a chromosome breaks in two places and the material between the two breakpoints reverses orientation. Inversions can be of two types: pericentric or paracentric. In pericentric inversions, the breakpoints lie on either side of the centromere and formation of the inversion often changes the chromosome arm ratio (centromere position) and alters the banding pattern of the chromosome (Figs. 9.8 and 9.9). Paracentric inversions, on the other hand, have

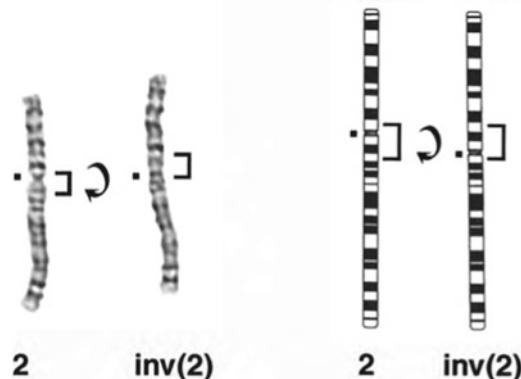


Fig. 9.9 Although this recurring pericentric inversion [inv(2)(p11q13)] is considered to be benign, individuals who carry this inversion might have a slightly increased risk for miscarriages

both breakpoints on the same side of the centromere, or within a single chromosome arm (see Chap. 10, Fig. 10.6). In paracentric inversions, the centromere position does not change, and the only clue to their presence is an alteration in the chromosome banding pattern. Prior to the development of banding techniques, the existence of paracentric inversions was theorized but could not be proven.

In those studies in which parents of a proband with a unique inversion have been karyotyped, the inversion is found in a parent as often as 85–90% of the time [80–82]. Most inversions of both types therefore appear to be inherited. Additionally, among the handful of apparent recurring inversions studied thus far, most have not been formed from multiple independent events but instead have been inherited from a single common distant ancestor. Examples include inv(3)(p25q21), inv(5)(p13q13), inv(8)(p23q22), inv(10)(p11.2q21.2), and inv(11)(q21q23). The recurrent variant inv(2)(p12q13), however, appears to be one of the few

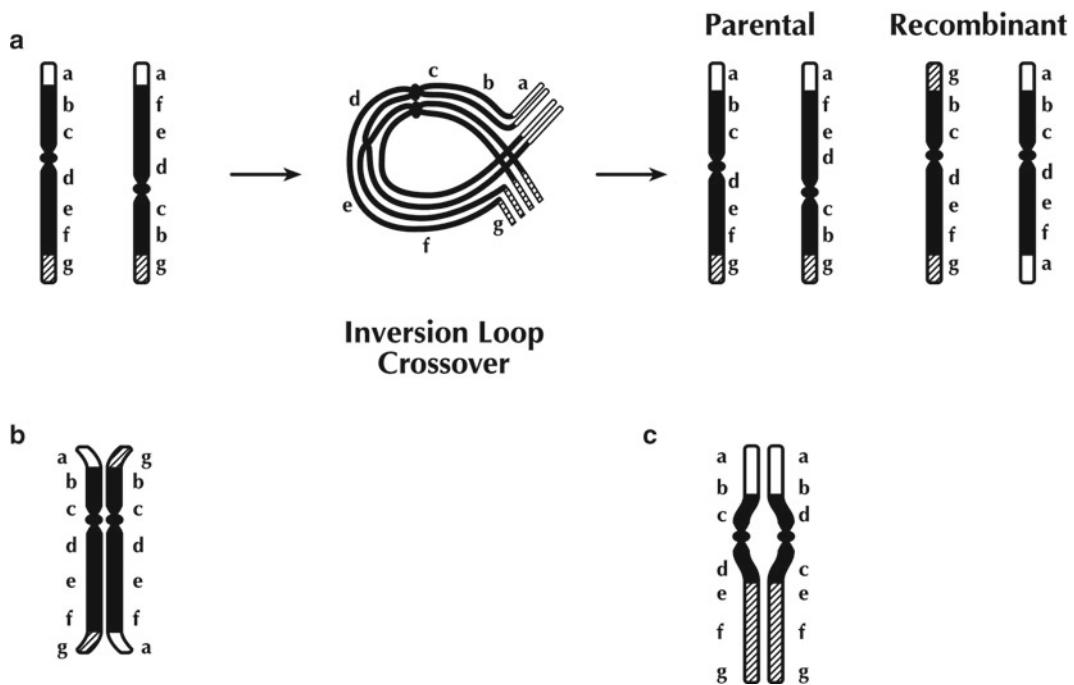


Fig. 9.10 Several models for meiotic pairing in a pericentric inversion heterozygote. (a) An inversion loop containing a single crossover and the resulting parental and recombinant chromosomes. Note that only

the material that is distal to the inversion breakpoints has been duplicated/deleted in each recombinant chromosome. (b, c) Alternate models for pairing during which only partial pairing or synapsis occurs

exceptions (Fig. 9.9). This inversion has occurred in the human genome on multiple occasions, and its recurring formation appears to occur secondary to nonallelic homologous recombination (NAHR) [83, 84].

Pericentric Inversions

As discussed earlier, both recurring and unique pericentric inversions have been reported in man. Some recurring inversions are considered normal variants. In some of these variant inversions, a block of heterochromatin normally situated in the proximal long arm of the chromosome is inverted into the short arm of the chromosome. Such inversions are found in chromosomes 1, 9, and 16. A second group of apparently benign recurring inversions, which have breakpoints very near the centromere in both the long and short arms, are found in chromosomes 2, 3 and 10, and in the Y chromosome. These variant forms have been observed in a large number of families and appear to segregate without deleterious effect. One group of investigators, however, has reported an increased risk for miscarriage among carriers of a pericentric inversion of chromosome 2 [inv(2)(p11q13), Fig. 9.9] [85]. Other inversions have been observed in many families but are not without consequence. Of particular note is the inversion of chromosome 8 with breakpoints at p23 and q22, which has been seen in families of Mexican-American descent [86].

Unique inversions are those observed in a single individual or family. The clinical significance of these inversions must therefore be determined on a case-by-case basis; as described later, some inversions can impart substantial reproductive risk, depending on the chromosome segment involved.

Excluding the variant inversions discussed previously, the frequency of pericentric inversions in the human population has been estimated at 0.12–0.7% [49].

Meiotic Behavior and Risks for Carriers of Pericentric Inversions

In order to understand the reproductive risks of an inversion carrier (heterozygote), the meiotic behavior of inverted chromosomes must first be considered. In meiosis, homologous chromosomes pair in close association. During this pairing phase, genetic information is exchanged between homologs through a process known as crossing-over or recombination (see Chap. 2). Crossing-over appears to be a necessary step for orderly chromosome segregation and is the mechanism that ensures human genetic individuality. A chromosome pair that consists of one normal chromosome and one chromosome with an inversion cannot achieve the intimate pairing of homologous regions necessary for normal meiosis through simple linear alignment. The classic model for pairing in an inversion heterozygote is the inversion or reverse loop demonstrated in Fig. 9.10a. In this model, the inverted segment forms a loop that can then pair with homologous

regions on the normal chromosome. The noninverted portions of the chromosome (the chromosome segments distal to the inversion breakpoints) pair linearly with homologous regions on the normal chromosome. An odd number of crossovers between the same two chromatids within the inversion loop will result in the production of recombinant chromosomes, while an even number of crossovers between the same two chromatids within the inversion loop should result in the production of normal or balanced chromosomes.

Two types of recombinant chromosomes are formed when crossing-over occurs between the inversion breakpoints. One recombinant will contain a duplication of the material distal to the breakpoint on the short arm and a deletion of the material distal to the breakpoint in the long arm. The second recombinant is complementary to the first and contains a short arm deletion and a long arm duplication (Figs. 9.10 and 9.11). Both recombinants are known as duplication-deficiency chromosomes.

Alternate models for pairing in an inversion heterozygote are seen in Fig. 9.10b, c. In inversions with very small inverted segments (breakpoints are close to the centromere and the distal segments are large), the noninverted segments of both chromosomes may pair in linear fashion, with asynapsis or failure to pair in the small inverted segment. In this model, crossing-over can only take place in the noninverted segments of the chromosomes, and thus abnormal recombinant chromosomes are not formed. In the opposite situation, where the inverted segment is very large relative to the size of the entire chromosome and the distal segments are small, pairing may occur only between the inversion breakpoints, and the distal material will remain unpaired. In this situation, a crossover between the inversion breakpoints would produce recombinant chromosomes in a manner similar to the reverse loop model discussed previously. Crossing-over could not take place in the segments distal to the inversion breakpoints since those regions do not pair.

Careful examination of the recombinant chromosomes produced when crossing-over takes place between the breakpoints in a pericentric inversion reveals that the genetic imbalance always involves the material distal to the inversion breakpoints. Thus, large inversions have small distal segments and produce recombinant chromosomes with small duplications and deficiencies, while small inversions have large distal segments and produce recombinant chromosomes with large duplications and deficiencies. In general, then, large inversions are associated with a greater risk of producing abnormal liveborn offspring since the recombinant chromosomes associated with them carry small duplications and deficiencies that have a greater probability of being compatible with survival. Furthermore, the larger the inversion, the greater the likelihood that a recombination event within the inversion loop will occur and

form recombinant chromosomes. The opposite is true of small inversions with large distal segments, which are usually associated with a very low risk of liveborn abnormal offspring.

In addition to the size of the inverted segment, other factors must be considered when determining the reproductive risk associated with any given pericentric inversion. Since monosomies are generally more lethal than trisomies, an inversion that produces a recombinant with a very small monosomy may be associated with a relatively high risk of abnormal offspring.

The nature of the genetic material in the inverted chromosomes can also be important. For instance, both trisomy and partial monosomy of chromosomes 13, 18, and 21 are seen in liveborn infants with birth defects and mental retardation. Once the duplications and deficiencies associated with the recombinants from a particular inversion are identified, review of the medical literature for evidence that these duplications and/or deficiencies are compatible with survival can aid in predicting the magnitude of the risk associated with that particular inversion.

Another clue to the level of risk associated with a given inversion is the manner in which the inversion was ascertained. If a balanced inversion is ascertained fortuitously, for instance, during a prenatal chromosome study because of advanced maternal age, the risk associated with such an inversion is probably very low. On the other hand, an inversion that is ascertained through the birth of an infant with anomalies secondary to the presence of a recombinant chromosome is associated with a much higher risk since the important question of whether the recombinant offspring is viable has already been answered. Careful examination of the family history in both types of ascertainment can provide additional important information in assessing risk.

Gardner and Sutherland reviewed several studies that contain data about the risks associated with pericentric inversions and estimated the risk for an inversion heterozygote to have an abnormal child secondary to a recombinant chromosome [49]. This risk was estimated to be 5–10% in families ascertained through an abnormal child and approximately 1% for families ascertained for any other reason. For families segregating very small inversions, the risk of having a liveborn recombinant child may be close to zero. In cases of recurring inversions, additional information about the risks can be gained from studying the literature. In the case of the inversion (8)(p23q22) mentioned earlier, for example, enough recombinant offspring have been observed to derive an empiric risk of 6% for a heterozygote to have a liveborn recombinant child [87]. Large inversions with distal segments that have been seen in liveborn children as monosomies or trisomies may be associated with high risk regardless of their mode of ascertainment in a particular family.

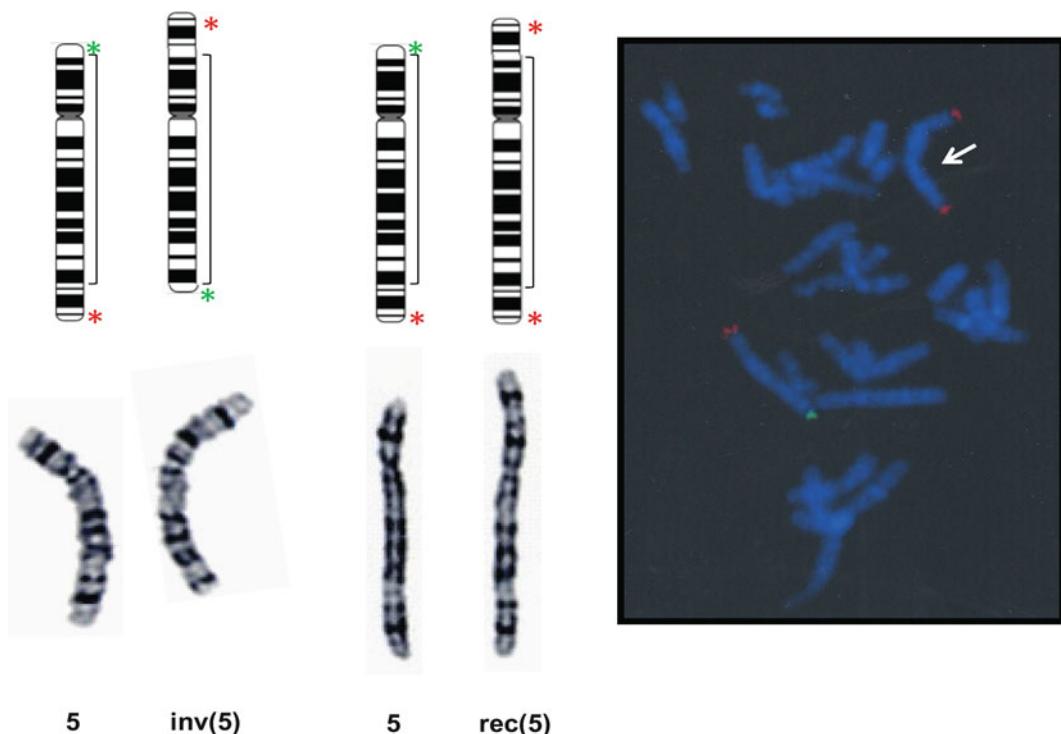


Fig. 9.11 *Left:* a normal chromosome 5 and a chromosome 5 with a large pericentric inversion, with breakpoints at p15.33 and q33.1, observed in a parent (* 5p terminal material, * 5q terminal material). *Middle:* a normal chromosome 5 and a recombinant chromosome 5 [rec(5)dup(5q)inv(5)(p15.33q33.1)] resulting from recombination within the inversion loop of the parental inversion carrier. This duplication-deficiency chromosome is missing the short arm material

that lies distal to the short arm inversion breakpoint and has two copies of the long arm material positioned distal to the long arm inversion breakpoint. *Right:* a metaphase hybridized with subtelomere probes for the short arm (green) and long arm (red) of chromosome 5. Note that the recombinant or duplication-deficiency chromosome (arrowed) has two red long arm subtelomere probe signals and no green short arm subtelomere probe signal

Paracentric Inversions

The presence of paracentric inversions in the human population was only appreciated after the advent of chromosome banding, and they are still reported less frequently than pericentric inversions. Their incidence has been estimated at 0.09–0.49 per thousand [80]. While various cytogenetically visible recurrent paracentric inversions have been reported in a variety of chromosomes, recent data suggests that most do not represent multiple independent mutation events but are instead identical by descent [83]. One of the most frequently cited is the recurring inv(11)(q21q23), which is believed to have arisen as a single mutation in the Netherlands and is now observed in a large number of families in the Netherlands as well as in Canadian Hutterites [88, 89]. With the aid of fluorescence *in situ* hybridization (FISH) and other molecular techniques, a number of recurring submicroscopic inversions within the human genome are being identified.

Several studies have now demonstrated that some recurring submicroscopic inversions confer susceptibility to other rearrangements. For example, a submicroscopic inversion

polymorphism that spans the same low-copy repeat sequences that mediate the recurring Williams syndrome deletion has been observed in the transmitting parent of some Williams syndrome parents [65–68]. The presence of a submicroscopic inversion polymorphism has also been associated with several other recurring microdeletions including Sotos, Angelman, the 15q13.3, 16p12.1, and 17q21.3 microdeletion syndromes, as well as several other types of disease-associated structural rearrangements. For example, submicroscopic inversions that span the olfactory receptor gene clusters at both 4p16 and 8p23, and mediate the recurring (4;8) translocation, have been seen in all nine of the transmitting parents examined [8, 90]. The same 8p inversion polymorphism associated with this (4;8) translocation has also been observed in each of the nine mothers who transmitted a recurring inverted duplicated 8p or supernumerary derivative 8p chromosome to their offspring [91]. Recombination between the homologous *PRKX* and *PRKY* genes located on the short arm of the X and Y chromosome, respectively, results in a translocation involving the material distal to the involved breakpoints and the formation of both XX males

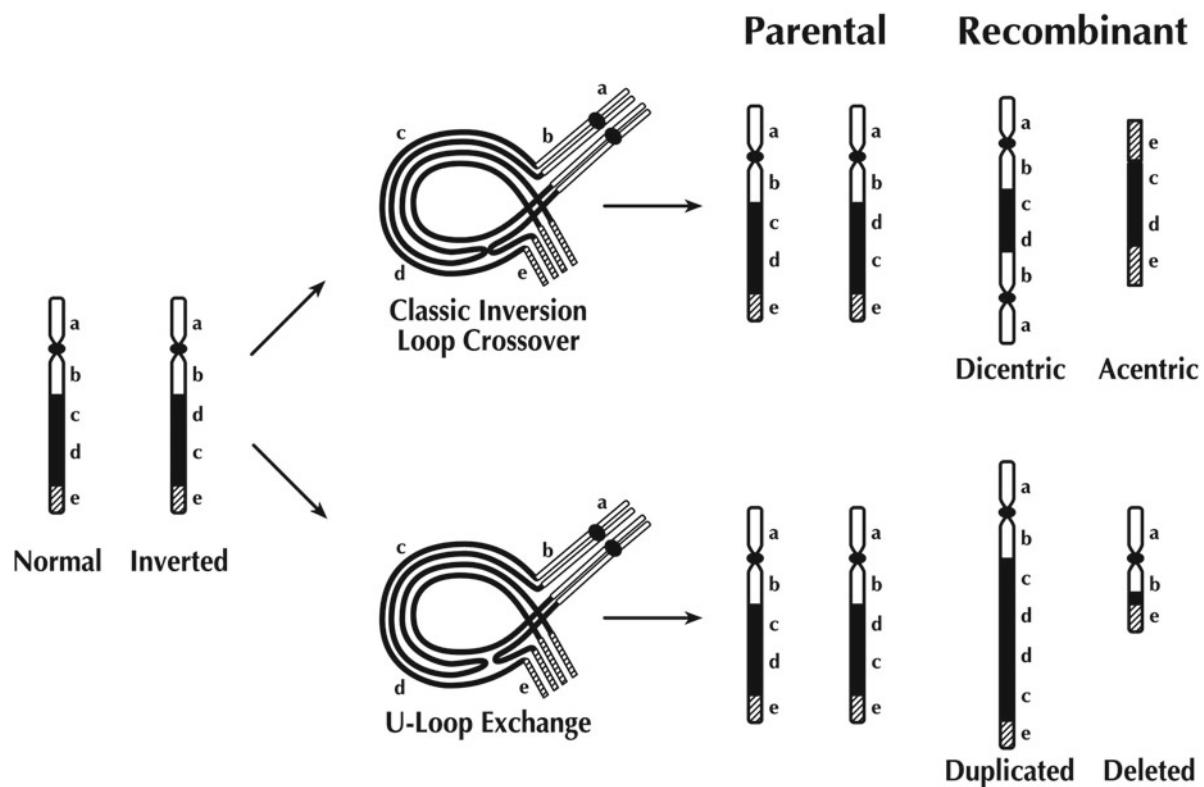


Fig. 9.12 The type of recombinant chromosome produced depends on which mechanism of chromosome exchange occurs within the paracentric inversion loop. A classic crossover within the inversion loop results

in the formation of an acentric and a dicentric recombinant chromosome (*top*), whereas a U-type exchange produces only monocentric chromosomes (*bottom*)

and XY females. This particular recombination event also appears to occur preferentially in association with a Yp inversion polymorphism [92].

The manner in which small paracentric inversion polymorphisms promote the formation of some chromosome rearrangements is not currently known in most cases. It has been demonstrated, however, that in the case of the recurring 15q13.3, 16p12.1, and 17q21.31 microdeletions the inversion produces an improved substrate for nonallelic homologous recombination (NAHR). In both cases, the inversion converts a pair of inverted LCRs into a pair of direct LCRs that are ideally suited to mediate the causative unequal exchange event [48, 69, 70].

Meiotic Behavior and Risk for Carriers of Paracentric Inversions

As with pericentric inversions, the classic solution to the problem of homologous pairing in paracentric inversions is the reverse loop. In this case, however, the centromeres are found in the segment distal to the inversion loop. On a theoretical basis, an odd number of crossovers within the inversion loop of a paracentric inversion should produce one dicentric and one acentric recombinant chromosome

(Fig. 9.12). The dicentric recombinant is genetically unstable because each of the two centromeres could potentially orient toward opposite poles of the dividing cell. The material between the two centromeres would remain stretched between the poles of the two reorganizing daughter nuclei or break. Thus, with each cell division, the dicentric recombinant chromosome has a new opportunity to contribute a different and possibly lethal genetic imbalance to a new generation of cells. The acentric fragment, on the other hand, has no ability to attach to a spindle since it lacks a centromere. Consequently, at cell division, it can be passively included in the daughter nuclei or be lost. Dicentric and acentric recombinant chromosomes are almost always lethal and are rarely found in liveborns (see sections “[Acentric Chromosomes](#)” and “[Dicentric Chromosomes](#),” later).

Although dicentric and acentric recombinants are very rarely seen, there have been several reports of monocentric recombinants among the children of paracentric inversion carriers. Pettenati et al., for example, identified 17 recombinant chromosomes among 446 inversions [80]. While two of these recombinant chromosomes were dicentric, each of the remaining 15 was monocentric with duplications and/or deletions. A variety of mechanisms have now been proposed

for the formation of these abnormal monocentric chromosomes, including breakage of dicentric recombinants, unequal crossing-over, and abnormal U-loop exchanges similar to the one diagrammed in Fig. 9.12. All of these mechanisms involve abnormal processes of one type or another.

There is currently a fair amount of controversy surrounding the question of risk for liveborn children with abnormalities secondary to the presence of a familial balanced paracentric inversion. Much of this controversy may be based on our inability, in some cases, to distinguish between a paracentric inversion and an intrachromosomal insertion using G-banded chromosomes [93]. Indeed, if the distance between the original site of the inserted segment and the new point of insertion is small, the resulting insertion is more likely to be interpreted as an inversion rather than a relatively rare insertion. By inadvertently combining data from intrachromosomal insertion carriers, whose risk for a recombinant offspring can approach 50%, with that from true paracentric inversion carriers, some studies may have overestimated the reproductive risks of paracentric inversion carriers. Generation of an accurate empiric risk estimate has been further complicated by ascertainment bias. Some express concern that not all of the associations of abnormal phenotypes with apparently balanced inherited paracentric inversions can be explained by the presence of misidentified intrachromosomal insertions, ascertainment bias, or coincidence [80, 94]. Others believe that familial paracentric inversions are relatively innocuous and carry a small risk for abnormal offspring; Gardner and Sutherland estimate that the risk “lies in the range of 0.1–0.5%” [49, 93, 95, 96]. Clearly, many questions remain to be answered concerning the clinical significance of apparently balanced inherited paracentric inversions.

Dicentric Chromosomes

Any chromosome exchange in which the involved donor and recipient chromosome segments each contain a centromere will result in the formation of a chromosome with two centromeres. These chromosomes are referred to as dicentrics. The most common dicentric chromosomes are those that are derived from a Robertsonian translocation event (see section “[Robertsonian Translocations](#),” later). Recombination within a paracentric inversion loop is also a well-documented method by which a dicentric chromosome can form (see section “[Inversions](#),” earlier).

The presence of two active centromeres in a single chromosome has the potential to wreak havoc during cell division. Normal segregation can only occur when the spindle apparatus from a single pole binds both centromeres of the dicentric chromosome. If instead, spindles from both poles independently bind only one of each of the two centrom-

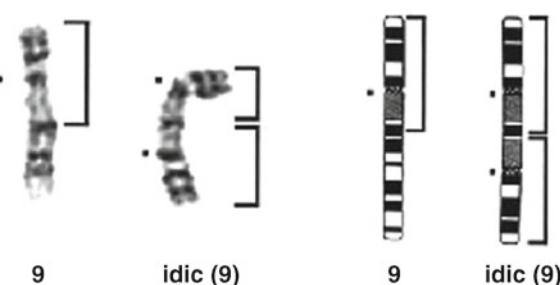


Fig. 9.13 A pseudoisodicentric chromosome involving the entire short arm and a portion of the long arm of chromosome 9. It appears to have one constricted active centromere (*upper dot*) and one unconstricted inactive centromere (*lower dot*). This chromosome was found in each of the cells of a phenotypically abnormal infant with the following karyotype: 47,XY,+psu idic(9)(q21.1)

eres, the chromosome will be simultaneously pulled in two opposing directions. As a result of this bipolar pulling, the chromosome may continue to straddle both daughter cells in a state of limbo until it is ultimately excluded from both. Alternatively, the chromosome may break, allowing some portion to migrate to each daughter cell. Regardless of which of these takes place, changes in the genetic content of the resulting sister cells will occur and mosaicism can result. Interestingly, not all dicentric chromosomes demonstrate mitotic instability. Some of these stable dicentric chromosomes appear to have closely spaced centromeres that function as a single large centromere [97–99]. The presence of one active and one inactive centromere is also frequently observed among stable dicentric chromosomes. These “pseudodicentric” chromosomes contain two copies of the centromeric heterochromatin, but only the centromere with the primary constriction appears to bind the appropriate centromere proteins required for activity [97, 100]. An example of a pseudoisodicentric chromosome 9 is shown in Fig. 9.13.

“Acentric” Chromosomes

Because the centromere is essential for chromosomal attachment to the spindle and proper segregation, chromosomes lacking this critical component are rapidly lost. Therefore, although single cells with acentric chromosomes or fragments are occasionally observed, individuals with constitutional karyotypes that include a true acentric chromosome are never seen. More than 90 chromosomes with atypical centromeres have, however, been reported in the literature [101, 102]. With the exception of chromosome 19, these atypical centromeres or neocentromeres have now been reported in association with each of the 24 human chromosomes. The majority of these chromosomes are small supernumerary chromosomes composed of two mirror image

copies of the terminal end of a single chromosome. However, neocentromeres have also been identified in ring chromosomes, in linear chromosomes with deletions, and very rarely in structurally normal chromosomes that appear to also contain their original intact centromere in an inactive form. While typically associated with unbalanced karyotypes, neocentromeres have been observed as part of a balanced rearrangement in a handful of cases. Approximately 82% of cases demonstrate a partial tetrasomy, while trisomies and more rarely monosomies are also seen. Mosaicism is frequently observed.

Like traditional centromeres, neocentromeres are denoted by the presence of a primary constriction, and with the exception of the centromere protein B (CENP-B), they bind the same centromeric proteins. Interestingly, however, neocentromeres are located in noncentromeric regions, they do not react to stains specific for centromeric heterochromatin, nor do they hybridize to centromere-specific fluorescence *in situ* hybridization probes. These staining differences suggested early on that the composition of these neocentromeres differed from that of a traditional centromere. Subsequent mapping and sequencing studies have since confirmed this. Current data suggests that neocentromeres do not contain DNA sequences that we typically associate with centromeres, such as alpha satellite DNA. In fact, the DNA sequence within such an atypical centromere does not appear to be altered relative to the homologous region of the parental chromosome from which it was derived [103]. These data, in addition to DNA modeling studies and the observation that duplicons are frequently present at the sites of neocentromere formation, suggest that it is the conformation or secondary structure formed by the DNA, rather than the DNA sequence itself, that enables a chromosomal region to function as a neocentromere. Neocentromere development may also be promoted by repair-initiated epigenetic changes that occur during formation of the chromosome rearrangements. Some speculate that neocentromeres may represent ancient centromere sequences that have been reactivated as a consequence of chromosome rearrangement [104, 105].

Isochromosomes

An isochromosome consists of two copies of the same chromosome arm joined through a single centromere in such a way that the arms form mirror images of one another. Individuals with 46 chromosomes, one of which is an isochromosome, are monosomic for the genes within the lost arm and trisomic for all genes present on the isochromosome. Tetrasomy for the involved chromosome segment is present when an isochromosome is present as a supernumerary chromosome. In general, the smaller the isochromosome, the smaller the imbalance and the more likely the survival of

the fetus or child that carries the isochromosome. It is therefore not surprising that, with few exceptions, the most frequently reported autosomal isochromosomes tend to involve chromosomes with small arms. Some of the more common chromosome arms involved in isochromosome formation include 5p, 8p, 9p, 12p, 18p, and 18q. The relatively large isochromosome involving the long arm of the X chromosome shown in Chap. 10, Fig. 10.4, is the most common structural abnormality found in Turner syndrome patients.

Over the years, a number of theories have been proposed to explain the mechanism of isochromosome formation [31, 32, 97, 106–109]. One of the more popular proposals has been that isochromosome formation is the result of centromere misdivision (Fig. 9.14). Instead of splitting longitudinally to separate the two sister chromatids, the centromere was hypothesized to undergo a transverse split that separated the two arms from one another. However, molecular studies suggest that the breakage and reunion events required to form many isochromosomes occur predominantly within the area adjacent to the centromere rather than within the centromere itself [97, 110–115]. The resulting chromosome, which appears monocentric at the cytogenetic level, would actually have two closely spaced centromeres and would more appropriately be called an isodicentric chromosome. Other theories that invoke exchanges between homologous chromosomes have also been challenged as common mechanisms of isochromosome formation. Molecular evidence indicating that at least some isochromosomes are formed from genetically identical arms, rather than homologous arms, suggests that one predominant mechanism of isochromosome formation may rely on sister chromatid exchange [109, 110, 116–119]. Breakage and reunion involving the pericentromeric regions of sister chromatids, an event sometimes referred to as a sister chromatid U-type exchange, may therefore represent an important mechanism of isochromosome formation. Nonallelic homologous recombination events mediated by low-copy repeat sequences appear to be responsible for many of these U-type exchanges. Additional molecular studies suggest that most isochromosomes are maternal in origin and that nondisjunction occurs prior to isochromosome formation in the majority of cases involving supernumerary chromosomes [30–32, 120, 121]. Most of these nondisjunction events appear to be meiotic rather than mitotic in origin.

From existing data, it is clear that multiple mechanisms of isochromosome formation are likely to exist. Precisely which mechanism is found to predominate may largely depend on the chromosomal origin of the isochromosome, whether the chromosome is present in a disomic karyotype or represents an extra or supernumerary chromosome, and whether formation occurs during meiosis or mitosis. Clearly, additional studies are needed to establish a more complete understanding of isochromosome formation.

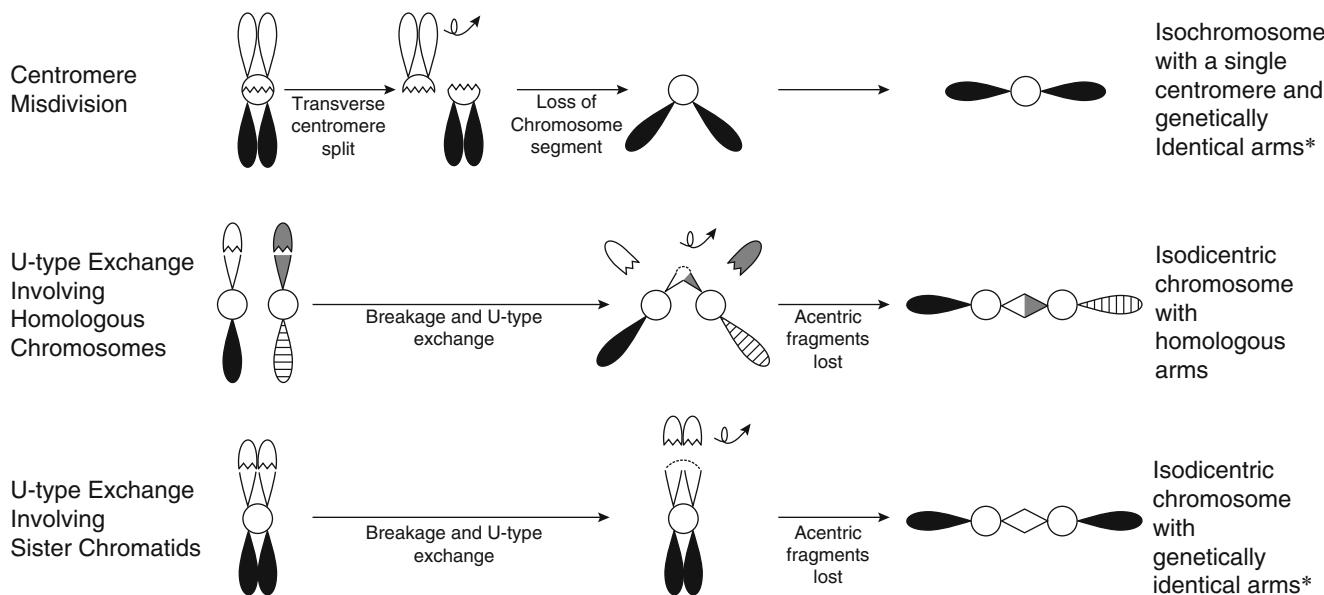


Fig. 9.14 Some of the mechanisms proposed for isochromosome formation. *Because recombination occurs during normal meiotic cell division, the arms of an isochromosome formed during meiosis would be identical only for markers close to the centromere

Ring Chromosomes

Autosomal ring chromosomes are rare and usually arise *de novo* (Fig. 9.15). Reported frequencies range from 1 in 27,225 to 1 in 62,279 in consecutive newborn and prenatal diagnosis studies [81]. Rings have been reported for all chromosome pairs, although those involving chromosomes 13 and 18 are among the most common [122]. When ring chromosomes replace a normal homolog in a karyotype, they often represent a partial monosomy for both long and short arm material. When rings are present as supernumerary chromosomes, partial trisomies result. In rare cases, a ring chromosome is found as part of a balanced chromosome complement [102, 123].

Rings are traditionally thought to form as a result of breakage in both arms of a chromosome, with subsequent fusion of the ends and loss of the distal segments. Molecular studies, however, have suggested that additional mechanisms exist. In a 1991 study, Callen et al. characterized ten small supernumerary rings using FISH [124]. They found that some of the rings were missing specific satellite DNA sequences from one side of the centromere, suggesting that these rings originated from a “transverse misdivision of the centromere” combined with a U-type exchange on one of the chromosome arms. In other studies, investigators have demonstrated that some rings form by telomere fusion, with no detectable loss of genetic material [125]. A number of ring chromosomes that are composed of discontinuous sequences have also been reported in the literature, suggesting still other

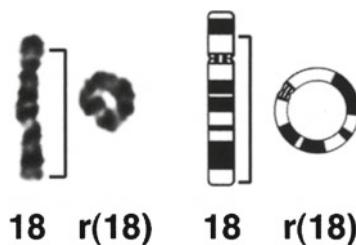


Fig. 9.15 A ring chromosome 18 [r(18)(p11.2q23)]. This ring chromosome is the result of fusion between two broken arms. The chromosome material distal to the breakpoints in each arm has been lost because it lacks a centromere

mechanisms of ring formation. Some of these discontinuous ring chromosomes are believed to represent the “breakdown” products of larger rings, while others appear to be derived from a structurally abnormal chromosome rather than a normal one [126–129].

An additional class of ring chromosome that lacks a traditional centromere has also been identified [101, 102]. In contrast to the traditional ring chromosomes described earlier, which are formed following breakage in both arms or within the centromere and one arm, these rings form following fusion of the ends of a chromosome fragment that lacks centromeric DNA. In the absence of a traditional centromere, a new centromere (neocentromere) forms from previously noncentromeric DNA within the resulting ring chromosome (see section “Acentric Chromosomes,” earlier).

One of the more striking characteristics of ring chromosomes is their instability. This instability is thought to result

from sister chromatid exchanges that occur in the ring chromosome before cell division. Such exchanges are normal events that, because of the unique structure of the ring chromosome, lead to the formation of double-sized dicentric rings and interlocking rings. Rings with even larger numbers of centromeres are also occasionally seen. The centromeres of these multicentric and interlocking rings can orient toward opposite poles during cell division. This can lead to breakage of the ring at anaphase, with subsequent generation of new ring structures. Alternatively, the entire ring chromosome can be lost. This active process of creating new cells with altered genetic material is termed “dynamic mosaicism” [49, 122]. Not all ring chromosomes exhibit instability. Although the relationship between ring size and stability is not entirely clear, in most cases, smaller rings appear to be more stable than large rings [49].

In addition to mosaicism, the genetic content and breakpoints of the rings will also have a significant impact on the patient’s phenotype. An individual with one normal chromosome homolog and one partially deleted ring chromosome will have clinical findings associated with a partial monosomy. The specific phenotype of the individual will depend on both the amount and the nature of the deleted material. Similarly, for a patient with a supernumerary ring chromosome and therefore a partial trisomy, the size of the ring, its genetic content, and the proportion of cells that contain the ring will all influence phenotype.

Another phenomenon that has the potential to impact the phenotype of individuals with ring chromosomes is uniparental disomy (UPD, see Chap. 20). Petersen et al., for example, described a patient with mosaicism for a normal cell line and a cell line in which one normal copy of chromosome 21 was replaced by a ring [130]. Uniparental isodisomy for chromosome 21 was present in the normal cell line. The authors suggested the isodisomy developed when the normal 21 was duplicated in a cell that had lost the ring (“monosomy rescue”). Rothlisberger et al. have reported a single case of mosaicism involving a cell line with a supernumerary ring derived from chromosome 1 and a normal cell line with maternal uniparental heterodisomy for chromosome 1 [131]. The presence of uniparental heterodisomy (rather than isodisomy as described earlier) suggests that both of the abnormal cell lines in this patient may have arisen secondary to a trisomy rescue event (see Chap. 20). Presumably, the original zygote had three copies of chromosome 1: one paternal chromosome 1 and two different maternal chromosomes 1. Conversion of the paternal chromosome 1 into a small ring would then produce a cell line with a survivable partial trisomy 1 rather than a lethal complete trisomy. Subsequent loss of the ring chromosome would then ultimately produce a disomic cell with the expected two copies of chromosome 1 and uniparental maternal heterodisomy for chromosome 1. Given that current data suggests there are no maternally

imprinted genes on chromosome 1 that influence phenotype, the resulting disomic cell line would be expected to demonstrate normal viability and perhaps a selective growth advantage compared to the cell line with partial trisomy 1.

One recurring phenotype seen in ring chromosome heterozygotes is the “ring syndrome,” originally proposed by Cote et al. in 1981 [132]. These patients have 46 chromosomes, one of which is a ring chromosome with no detectable clinically significant deletion. The ring is derived from one of the larger chromosomes in the karyotype, and the larger the chromosome, the more severe the phenotype. Typically, these patients have severe growth retardation without major malformations. Minor anomalies and mild to moderate mental retardation are often part of the picture. The ring syndrome is believed to result from instability of the ring chromosome. The larger chromosomes are thought to be more unstable than the smaller ones because they present more opportunities for sister chromatid exchange. The breakage that occurs during cell division generates new ring structures, most of which represent a more serious genetic imbalance than the previous forms and are thus less viable. This results in increased cell death and contributes to growth failure and the disturbance of developmental pathways [133]. Kosztolanyi has proposed that this phenomenon may also contribute to the severity of the phenotype in patients who have ring chromosomes with obvious deletions [133].

A 1991 literature review discovered 32 reported cases in which a ring chromosome was inherited from a carrier parent. The authors concluded that no more than 1% of ring chromosomes are inherited, and in 90% of these cases, the mother is the carrier parent. Among the 32 patients with inherited rings, half had a phenotype similar to the carrier parent, while approximately one third were more severely affected [49, 134].

In addition to the risks associated with ring instability, carriers of ring chromosomes may also be at risk for having children with other abnormalities involving the chromosome from which their ring is derived. There are at least three reports of carriers of a ring chromosome 21 who had offspring with trisomy 21 secondary to a translocation or tandem duplication of chromosome 21 [134].

Reciprocal Autosomal Translocations

Reciprocal translocations represent one of the most common structural rearrangements observed in man. Estimates of the population frequency range from 1/673 to 1/1,000 [1, 135]. A reciprocal translocation forms when two different chromosomes exchange segments. In the example shown in Fig. 9.16, a balanced translocation involving chromosomes 1 and 9 has occurred. The distal short arm of chromosome 1 has replaced the distal short arm material on chromosome 9, and vice versa.

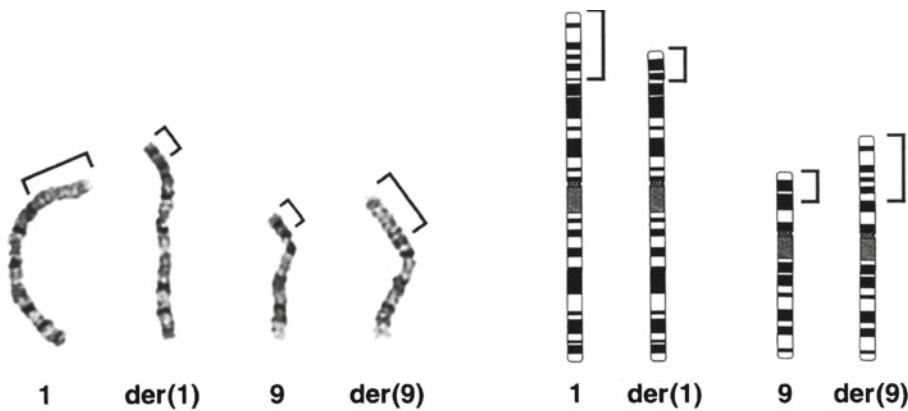


Fig. 9.16 A balanced reciprocal translocation involving the short arm of chromosomes 1 and 9 [$t(1;9)(p32.3;p21)$]. The translocated segments of each chromosome have been bracketed

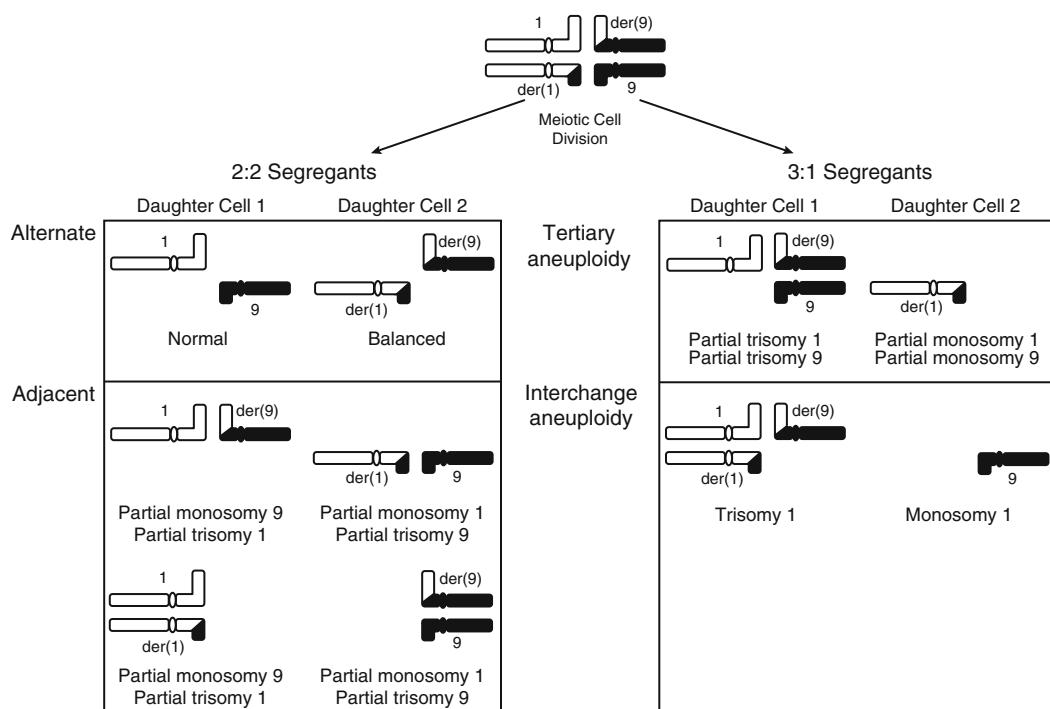


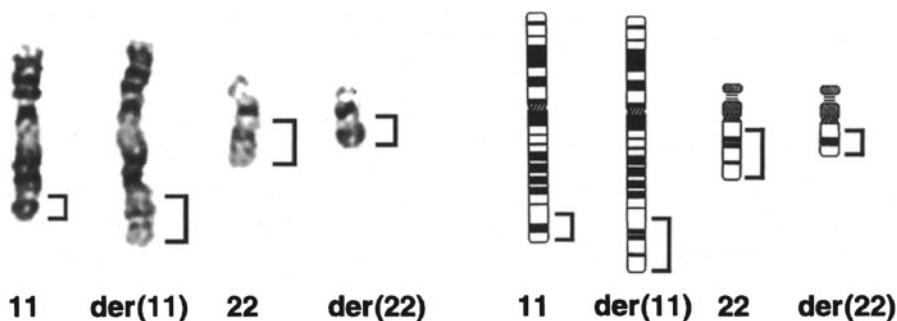
Fig. 9.17 The expected meiotic pairing configuration for the (1;9) translocation described in Fig. 9.16. Each of the 2:2 and 3:1 segregants typically produced during meiotic cell division are shown

The individual who carries this balanced translocation is clinically normal. His rearrangement was identified when his wife had prenatal karyotyping because of advanced maternal age and a fetus with the same (1;9) translocation was found.

Although individuals who carry truly balanced reciprocal translocations are themselves clinically normal, they do have an increased risk for having children with unbalanced karyotypes secondary to meiotic malsegregation of their translocation. As discussed in the introduction to this chapter and in Chap. 2, during normal meiotic prophase, all 23 sets of homologous chromosomes couple to produce 23

paired linear structures or bivalents that later separate and migrate to independent daughter cells. In a cell with a reciprocal translocation, 21 rather than 23 bivalents are formed. The remaining two derivative chromosomes involved in the reciprocal translocation and their normal homologs form a single pairing structure called a quadrivalent. The expected quadrivalent for the reciprocal (1;9) translocation described earlier is diagrammed in Fig. 9.17. Notice that the four chromosomes within the quadrivalent have arranged themselves such that pairing between homologous regions is maximized.

Fig. 9.18 A balanced reciprocal translocation involving the long arm of chromosomes 11 and 22 [$t(11;22)(q23.3;q11.2)$]. This is the first recurring constitutional translocation reported in multiple, unrelated families



Segregation of the chromosomes within a quadrivalent can occur in multiple ways, most of which will result in chromosomally unbalanced gametes. Only a 2:2 segregation, during which the two alternate chromosomes within the quadrivalent travel together to the same daughter cell, yields chromosomally balanced gametes. In theory, 50% of the resulting gametes would carry a normal chromosome complement, while the other 50% would be balanced translocation carriers. Each of the remaining segregation patterns for a reciprocal translocation produces unbalanced gametes. A 2:2 segregation, during which two chromosomes with adjacent rather than alternate centromeres migrate to the same daughter cell, produces gametes with partial trisomies and monosomies. Both 3:1 and 4:0 segregations also occur, resulting in trisomies and monosomies. Studies examining the sperm obtained from balanced reciprocal translocation carriers suggest that approximately equal numbers of alternate and adjacent segregants are generally formed and that these two groups represent the most common types of segregants. The remaining 3:1 and 4:0 segregants appear to be much rarer. Female translocation carriers are capable of producing the same types of unbalanced 2:2, 3:1, and 4:0 segregants that have been documented in male carriers, and like their male counterparts, they produce approximately equal numbers of alternate and adjacent segregants and relatively few 4:0 segregants. In contrast, however, the 3:1 segregants that are rarely observed in sperm are a relatively common finding in oöcytes [136–138].

In addition to being inherited, reciprocal translocations can also occur as new or *de novo* mutations. As discussed in the introduction to this chapter, the risk for an abnormal outcome associated with a *de novo* apparently balanced rearrangement is always greater than that associated with an equivalent rearrangement that has been inherited from a normal parent. The actual risk associated with a *de novo* apparently “balanced” translocation has been reported to be approximately 6–9% [1]. This is 2–3 times the overall rate of congenital abnormalities observed in the population.

The (11;22) Translocation

The (11;22) translocation, with breakpoints within bands 11q23.3 and 22q11.2, is unique because it represents the first recognized recurring constitutional reciprocal translocation in man (Fig. 9.18). More recently, evidence for two additional recurring translocations, a (4;8) translocation with breakpoints at 4p16 and 8p23.1 and an (8;22) translocation with breakpoints at 8q24.1 and 22q11.2, have been reported (see later).

More than 100 apparently unrelated families with this (11;22) translocation have been reported to date. For many years, it was not known whether the ostensible reoccurrence of this translocation was best explained by the efficient transmission of a single ancient unique translocation through multiple generations or by multiple independent translocation events between two susceptible regions. However, it is now known that the latter is the case. Mapping studies involving many different unrelated families have demonstrated that the translocation breakpoints cluster within long AT-rich palindromic sequences and both the size and symmetry of these sequences influence the *de novo* translocation frequency (a palindrome is a DNA sequence that contains two inverted regions that are complementary to each other) [139–142]. In each case, the breakpoints are localized to the tip of the imperfect hairpin or cruciform structures that are predicted to form. Palindromic sequences have also been implicated in the formation of several other, mostly nonrecurring, translocations (see section “Mechanisms of Formation,” earlier). Although precisely how these structures promote this translocation is unknown, it has been suggested that they are substrates for hairpin-specific nucleases. Once nicked, these structures become susceptible to other nucleases that produce double-stranded breaks and further erosion of the palindromic DNA surrounding the initial nick site. Ultimately, the two double-stranded ends are joined utilizing a repair process referred to as nonhomologous end joining (NHEJ), to produce the recurring (11;22) translocation. Interestingly, all eight of the *de novo* (11;22) translocations examined thus far

have been paternal in origin, suggesting the translocation mechanism employed may be spermatogenesis specific [141].

The presence of multiple families with the same (11;22) translocation has made it possible to obtain good empiric data concerning viable segregants, expected phenotypes, and the various risks associated with this rearrangement. It is known, for example, that a carrier's empiric risk for having a liveborn child with an unbalanced karyotype is 2–10% and that the unbalanced, liveborn offspring of (11;22) translocation carriers inevitably have 47 chromosomes: 46 normal chromosomes plus an extra or supernumerary chromosome representing the derivative chromosome 22 [143, 144]. These individuals are therefore trisomic for the distal long arm of chromosome 11 and the proximal long arm of chromosome 22. Severe mental retardation, congenital heart disease, microcephaly, malformed ears with preauricular skin tags and/or pits, a high-arched or cleft palate, micrognathia, anal stenosis or atresia, kidney anomalies, and genital abnormalities in males are common features shared by these unbalanced (11;22) segregants. In 2004, the condition was named Emanuel syndrome, to honor the work of Dr. Beverly Emanuel.

Balanced carriers of the (11;22) translocation are phenotypically normal, with one possible exception. There is a single, unconfirmed report in the literature indicating that female carriers may have a predisposition to breast cancer [145]. While cytogenetically the breakpoints involved in this translocation appear to be identical to those identified in the acquired chromosome rearrangements seen in Ewing sarcoma, peripheral neuroepithelioma, and Askin tumor, molecular studies have shown that they differ [146–148] (see also Chap. 16). The gene(s) and mechanisms responsible for the development of these neoplasms therefore have provided no clues regarding the etiology of breast cancer development in these patients.

The (4;8) Translocation

At least 18 unrelated families with similar (4;8) translocations, or a chromosome derived from this translocation, have been reported in the literature [8, 149, 150]. In each case, the breakpoints involved appear to correspond to bands 4p16 and 8p23. Most of these families have been ascertained secondary to the birth of a clinically abnormal child with the derivative chromosome 4 but not the complementary abnormal chromosome 8. These children are monosomic for distal chromosome 4 short arm material and trisomic for a small amount of distal chromosome 8 short arm material. Despite the presence of 8p trisomy, these patients are clinically indistinguishable from Wolf-Hirschhorn patients with pure 4p deletions (Table 9.1) [149]. Both groups of patients

demonstrate mental retardation, poor growth, hypotonia, heart defects, and an abnormal facies including hypertelorism, prominent forehead, broad nasal bridge, large down-turned mouth, cleft lip and/or palate, micrognathia, and dysplastic ears.

In contrast to the frequent reports of chromosomally unbalanced children who inherited the abnormal chromosome 4, only one chromosomally unbalanced child who inherited the abnormal chromosome 8 has been reported. This child was reported to be less dysmorphic and have milder mental retardation than her third cousin and other unrelated individuals reported in the literature who inherited the derivative chromosome 4 [151].

It has been demonstrated that this particular (4;8) translocation is a recurring one, mediated by nonallelic homologous recombination between olfactory receptor gene clusters located on both chromosomes [8]. Among the six individuals whose translocation breakpoints were examined, the 8p23 breakpoint was confined to a single olfactory receptor cluster, while the 4p16 breakpoint was distributed between two different clusters. Interestingly, the translocation was of maternal origin in all five of the *de novo* cases examined, and each transmitting mother was heterozygous for a submicroscopic inversion at both 4p16 and 8p23. The 4p16 inversion seen in each of these mothers spanned the two olfactory receptor clusters that serve as breakpoint sites for the (4;8) translocation. Similarly, the 8p23 inversion spanned the distal olfactory receptor cluster involved in the (4;8) translocation and a more proximally placed cluster. Presumably, both of these submicroscopic inversions, like the (4;8) translocation, were also mediated by these clusters. Of note, heterozygosity for the same 8p23 inversion polymorphism was also found in the transmitting parents of patients who carry several other types of recurring chromosome 8 rearrangements that are mediated by the same 8p23 olfactory receptor gene clusters implicated in the (4;8) translocation [91]. Heterozygosity for a submicroscopic inversion has also been reported in association with several other chromosome rearrangements (see section “[Paracentric Inversions](#),” earlier).

The (8;22) Translocation

At least 12 families with a recurring (8;22) translocation involving breakpoints at 8q24.1 and 22q11.21 have been identified [13]. Molecular characterization of the translocation breakpoints involved in the five families studied thus far indicates this rearrangement is mediated by AT-rich palindromic sequences. Interestingly, this translocation utilizes the same palindrome at 22q11.2 that has been associated with the recurring (11;22) translocation as well as several other nonrecurring rearrangements (see sections “[Mechanism of Formation](#)” and “[The \(11;22\) Translocation](#),” earlier).

Also, as with the recurring (11;22) translocation, all viable cytogenetically unbalanced offspring of t(8;22) are the result of a 3:1 meiotic segregation event. These individuals carry 46 normal chromosomes in addition to a supernumerary derivative chromosome 22 and are therefore trisomic for proximal chromosome 22 and distal chromosome 8 long arm material. The phenotype associated with this imbalance is variable but is most often associated with a normal birth weight and normal subsequent growth, mild developmental delays and/or mild mental retardation, prominent ears with preauricular pits, and clinodactyly. While the balanced carriers are typically clinically normal, two of the six individuals reported have autoimmune disorders.

Robertsonian Translocations

A Robertsonian translocation occurs when the long arms of any two acrocentric chromosomes join to produce a single metacentric or submetacentric chromosome [49, 50, 56, 62, 63] (Figs. 9.19 and 9.20). Although these translocations may in fact be reciprocal, the small complementary chromosome composed of short arm material is only occasionally seen, presumably because it is typically acentric and therefore lacks the stability conferred by a centromere [152]. Balanced carriers of Robertsonian translocations therefore typically have 45 chromosomes rather than the usual 46. The only notable genetic material within the short arm region of each of these chromosomes is a nucleolar organizer region composed of multiple copies of the ribosomal RNA genes. Because this is redundant information, loss of this material from the two chromosomes involved in the translocation is therefore not clinically significant. It has been suggested that the close association of these nucleolar organizer regions within the cell nucleus may promote the formation of Robertsonian translocations.

Since Robertsonian translocations were first described by W. R. B. Robertson in 1916, it has come to be recognized that they are among the most common balanced structural rearrangements in the human population [153]. Numerous studies examining both spontaneous abortions and liveborn individuals indicate a frequency of approximately 1/1,000 [154–156]. Although pairwise association of the five human acrocentric chromosomes can form 15 different Robertsonian translocations, these rearrangements do not occur with equal frequency, and their mechanisms of formation appear to differ.

Nonhomologous Robertsonian Translocations

Approximately 95% of all Robertsonian translocations are formed between two nonhomologous or different

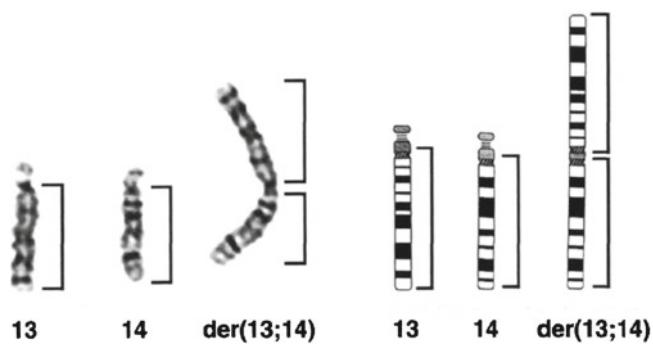


Fig. 9.19 This (13;14) translocation is the most common Robertsonian translocation observed in man [der(13;14)(q10;q10), sometimes described as der(13;14)(p11.2;p11.2); see Chap. 3]

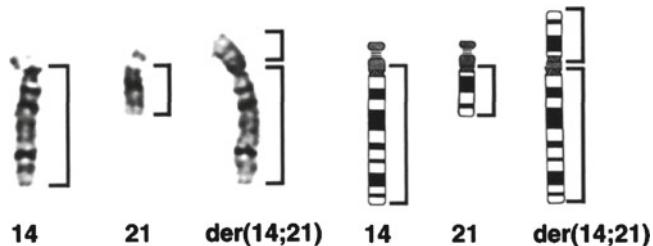


Fig. 9.20 Although less common than the (13;14) translocation, the Robertsonian (14;21) translocation is more clinically significant because the affected offspring of such a carrier are more likely to survive to birth. Their unbalanced offspring will inevitably have three copies of chromosome 21 long arm material or Down syndrome, a chromosome abnormality that is more compatible with survival than trisomy 13 [der(14;21)(q10;q10)]

chromosomes. Among this group, the (13;14) and (14;21) translocations are the most common and constitute approximately 75 and 10% of all nonhomologous Robertsonian translocations, respectively [49] (Figs. 9.19 and 9.20). Molecular studies performed to explore the origins of these rearrangements suggest that they occur predominantly during oogenesis [27, 157].

Despite the monocentric appearance of many of these chromosomes, most are in fact dicentric [158–160]. The majority of these chromosomes therefore appear to form as a result of short arm fusion rather than centromere fusion or a combination of both. A single pair of short arm breakpoint regions has been observed in most (13;14) and (14;21) translocations, while multiple short arm breakpoint regions are utilized during formation of each of the remaining types of Robertsonian translocations [158, 161–163]. Precisely where the breakpoint occurs within the short arm therefore seems to be dependent upon the type of Robertsonian translocation being formed and perhaps the mechanism responsible for the rearrangement. While the mechanisms responsible for Robertsonian translocation formation are not currently known, recombination involving repetitive satellite III DNA

sequences and/or other repetitive DNA sequences located within the short arms of the acrocentric chromosomes has been proposed. Nonrandom suppression of one centromere appears to provide mitotic stability to some of the dicentric Robertsonian chromosomes [100, 164]. In other cases, both centromeres appear to be active. It is believed that because of their close proximity, both centromeres are able to function as one in these dicentric chromosomes [165]. It should be noted that current nomenclature (see Chap. 3) still calls for the description of all Robertsonian translocations as monocentric and that this is used in most laboratories.

Homologous Robertsonian Translocations

In contrast to nonhomologous Robertsonian translocations, *de novo* whole arm exchanges involving homologous or like chromosome pairs are very rare. They appear to be predominantly monocentric and several of them have been shown to form postmeiotically [110, 160, 119, 166, 167]. While historically all such rearrangements were collectively called homologous Robertsonian translocations, recent molecular studies have shown that approximately 90% of the chromosomes within this category may actually be isochromosomes composed of identical rather than unique homologous arms [110, 111, 118, 168]. Molecular studies exploring the parental origin of *de novo* homologous Robertsonian translocations suggest that no parental bias exists. Equal numbers of maternally and paternally derived isochromosomes have been reported, and true homologous Robertsonian translocations in balanced carriers appear to be composed of both a maternal and a paternal homolog.

Reproductive Risks for Carriers of Robertsonian Translocations

Carriers of Robertsonian translocations are at risk for miscarriages and for offspring with mental retardation and birth defects associated with aneuploidy and, rarely, uniparental disomy or the inheritance of both copies of a chromosome pair from a single parent (see Chap. 20). The relative risk for each of these outcomes is a function of the sex of the heterozygous parent and/or the particular acrocentric chromosomes involved. In theory, all chromosome segregations within the carrier parent of a homologous Robertsonian translocation and all malsegregations within nonhomologous Robertsonian carriers produce monosomic or trisomic conceptions. Since all potential monosomies and most of the potential trisomies are lethal during the first trimester, miscarriage is not uncommon. Only those Robertsonian translocation chromosomes containing chromosomes 21 or 13 are associated with an increased risk for having liveborn trisomic

offspring. Trisomy 22 occurring secondary to a Robertsonian translocation may also represent a rare possibility. Because their risk for aneuploidy is greater than that of the general population, it is recommended that all Robertsonian translocation carriers be offered prenatal testing (see Chap. 12).

Occasionally, abnormal offspring with uniparental disomy (UPD) have also been observed among the children of balanced Robertsonian translocation carriers [45]. UPD has been reported in association with both *de novo* and familial, homologous and nonhomologous, translocations. Currently, the risk for UPD in a fetus with a balanced nonhomologous Robertsonian translocation is estimated to be 0.6%, while that for a fetus with a balanced homologous Robertsonian translocation is predicted to be approximately 66% [169]. Among liveborn offspring with congenital anomalies who carry a balanced nonhomologous or homologous Robertsonian translocation, the risk for UPD has been reported to be 4 and 100% (2/2 homologous Robertsonian cases studied), respectively [170]. The higher incidence of UPD noted in association with the balanced homologous Robertsonian translocations parallels the observation that most of these translocations actually represent true isochromosomes. Because both arms of a true isochromosome are derived from a single chromosome, by definition, uniparental isodisomy should be present in these balanced Robertsonian translocation carriers. Whether the risk for UPD varies depending on whether the translocation is familial or *de novo* is not currently known.

Postzygotic correction of a trisomy through chromosome loss (trisomy rescue) is thought to represent the most likely mechanism for UPD, although monosomy correction and gamete complementation may occur as well [169–171]. Current data indicate that uniparental disomy is most concerning when Robertsonian translocations containing chromosomes 14 or 15 are involved since both chromosomes appear to have imprinted regions. Maternal and paternal UPD for chromosome 15 result in Prader-Willi syndrome and Angelman syndrome, respectively [172, 173]. Clinically abnormal offspring have also been documented in association with paternal and maternal UPD for chromosome 14 [174–178]. A single reported case of maternal UPD in a normal individual has created uncertainty regarding the association between maternal UPD 14 and phenotype [175]. Because UPD involving chromosomes 14 and 15 is associated with an adverse outcome, it has been proposed by the American College of Medical Genetics and Genomics that prenatal UPD testing be offered when a fetus carrying a balanced Robertsonian translocation involving one or both of these chromosomes is ascertained.

While an abnormal phenotype is not likely to be directly associated with UPD for chromosomes 13, 21, and 22, residual disomy/trisomy mosaicism and recessive disease resulting from reduction to homozygosity through isodisomy may influence the phenotype of all uniparental disomy offspring [171]. These etiologies for disease should be

remembered when dealing with any fetus that carries a balanced Robertsonian translocation involving these chromosomes, especially if the fetus is clinically abnormal (see Chap. 20).

As discussed in the introduction, for some types of rearrangements, the risk for unbalanced offspring appears to be significantly higher for a female carrier than a male carrier. This appears to be the case for nonhomologous Robertsonian translocations involving chromosome 21. In female carriers of these translocations, an unbalanced karyotype is detected in 13–17% of second trimester pregnancies [49, 179]. For male carriers, the same risk appears to be less than 2%. Precisely why male carriers appear to produce fewer unbalanced offspring than their female counterparts is not known. Current data, however, suggests that female Robertsonian translocation carriers produce greater numbers of unbalanced gametes than their male counterparts [180, 181].

Jumping Translocations

The term “jumping translocation” refers to dynamic or changing translocations that are rarely observed in constitutional karyotypes. It is used most often to describe a type of mosaicism in which a specific donor chromosome segment is translocated to two or more different recipient sites over the course of multiple mitotic cell divisions [182–184]. To date, at least 50 cases have been described, with the majority involving at least one acrocentric chromosome [182, 183]. Jewett et al., for example, have described an individual with four different cell lines in which long arm material of chromosome 15 was translocated to five different sites [184]. Within the child’s main cell line, the chromosome 15 long arm segment was transferred to the distal long arm of chromosome 8 and the distal short arm of chromosome 7. In additional cell lines, this same segment was transferred to the long arm of chromosome 12, the short arm of chromosome 6, or the short arm of chromosome 8.

In other rare situations, families are described in which translocations involving a common donor chromosome segment but a different recipient chromosome are observed in parent and child [185, 186]. Tomkins et al., for example, describe a mother and daughter with different, apparently balanced translocations involving the same short arm segment of chromosome 11 [185]. The mother carried an (11;22) translocation, while the daughter carried a similar (11;15) translocation. In families like this, chromosome “jumping” appears to occur during gametogenesis rather than during mitosis as described earlier.

The breakpoints observed in jumping translocations frequently involve regions known to contain repetitive DNA sequences such as telomeres, centromeres, and nucleolar organizers [184, 186–188]. The location of breaks within

these repetitive regions and the suspicion that evolutionary chromosome rearrangements have distributed inactive forms of these sequences throughout the genome suggest that recombination between homologous sequences may play a role. Evidence also exists for the involvement of fragile sites and viral integration sites in the formation of some jumping translocations [182]. For now, however, the mechanism by which jumping translocations occur is unknown.

Insertions

Insertions are complex three-break rearrangements that involve the excision of a portion of a chromosome from one site (two breaks) and its insertion into another site (one break). The orientation of the chromosomal material that has been moved can remain the same in relation to the centromere (a direct insertion) or be reversed (an inverted insertion). When the material is inserted into a different chromosome, the insertion is considered interchromosomal, while with intrachromosomal insertions, material excised from one portion of a chromosome is reinserted into another portion of the same chromosome. An example of an interchromosomal insertion involving chromosomes 5 and 11 is shown in Fig. 9.21.

While the incidence of insertions detectable by traditional karyotyping is estimated to be approximately 1 in 80,000 to 1 in 10,000, not surprisingly, more recent studies using microarray analysis in conjunction with fluorescence *in situ* hybridization (FISH) suggest the incidence is likely to be higher; Kang et al. report an incidence of 1 in 500, while Neil et al. report an incidence of 1 in 3,400 to 5,200 [189–192]. While the large cytogenetically detectable insertions typically result in abnormal phenotypes when inherited in an



Fig. 9.21 Insertion. A portion of chromosome 11 short arm material has been inserted into the proximal long arm of chromosome 5 to produce an apparently balanced, inverted, interchromosomal insertion [ins(5;11)(q13.1;p15.3p13)]. The individual who carries this insertion was ascertained following the birth of a cytogenetically unbalanced child who inherited the derivative 5 but not the complementary derivative 11 (Courtesy of Dr. Frank S. Grass, Department of Pediatrics, Carolinas Medical Center)

unbalanced form, many of the submicroscopic insertions appear to cause few, if any, phenotypic consequences. In one study by Kang et al., a parent was found to carry the same chromosomal imbalance as the proband in 69% of the cases involving inherited submicroscopic insertions [189]. Regardless of the size of an insertion, however, because the risk for an abnormal pregnancy outcome can approach 50% for a carrier, it is important to identify these individuals.

Intrachromosomal Insertions

Intrachromosomal insertions can occur within a single chromosome arm or between chromosome arms. Direct within-arm insertions have occasionally been mistaken for paracentric inversions [93, 193, 194].

During meiotic pairing, the inserted segment and its complementary region on the normal chromosome may loop out allowing synapsis, or pairing, of the rest of the chromosome (Fig. 9.22). A single crossover in the paired interstitial segments of such a bivalent would result in the formation of recombinant chromosomes that are either duplicated or deleted for the inserted segment. The theoretical risk for the formation of such recombinant chromosomes could approach 50% for each meiosis, depending on the size of the interstitial segment. The risk for having a liveborn child with an unbalanced karyotype will depend, to some extent, on the viability of the duplications and deletions produced.

Alternatively, in the case of large inserted segments, complete pairing between the homolog with the insertion and its normal counterpart can be achieved through the formation of

double-loop structures during meiosis. Crossing-over or recombination in these fully synapsed chromosomes can result in the generation of chromosomes with duplications, deletions, or both. Madan and Menko, in their review of 27 cases, observed an overall 15% risk for each pregnancy that a carrier of an intrachromosomal insertion will have a live-born child with an unbalanced karyotype [195]. This risk may differ greatly for individual insertions depending on the size of the inserted segment and the viability of the partial trisomies and monosomies produced by the abnormal recombinant chromosomes.

Interchromosomal Insertions

Interchromosomal insertions involve the movement of material from one chromosome to another. As discussed earlier, the inserted segment can be either direct or inverted relative to its original position in the chromosome. Approximately 85% are inherited, usually from a carrier mother, and no fertility differences were noted between the two sexes [189, 192].

For relatively small inserted segments, it seems most likely that the homologs involved in the rearrangement will pair independently [196]. The inserted segment and its homologous region on the normal chromosome can loop out, allowing full pairing of the uninvolved segments of the bivalents (Fig. 9.22). Independent 2:2 segregation of the homologs in these two bivalents can result in the formation of four gamete types, two of which have a normal or balanced chromosome complement and two of which have an unbalanced

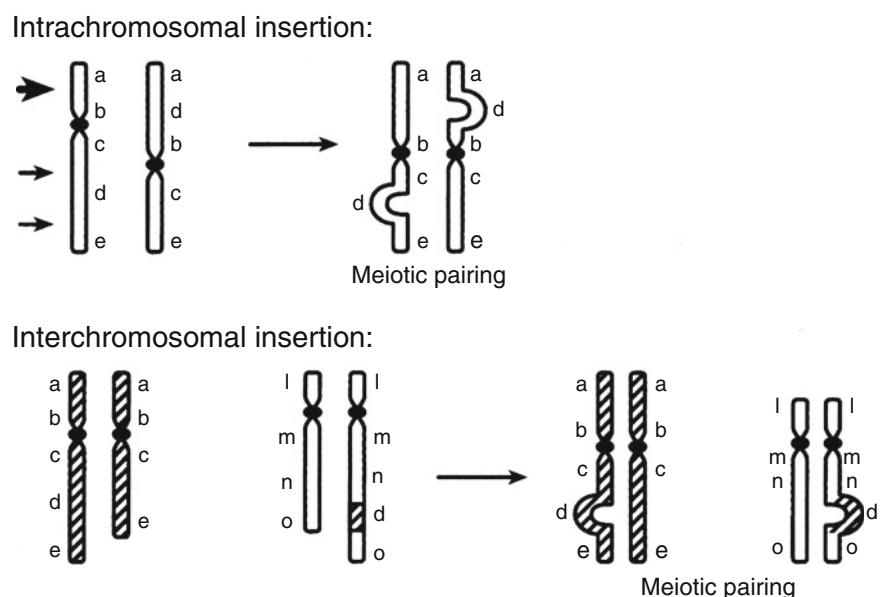


Fig. 9.22 Models for meiotic pairing during which partial pairing is observed between the insertion chromosome and its homolog

complement, one duplicated and one deleted for the inserted segment. The theoretical risk, in this situation, would be 50% for producing a conceptus with an unbalanced karyotype. The risk for having a liveborn abnormal child would depend on the viability of the partial trisomy or partial monosomy of the inserted segment involved.

In the case of very long inserted segments, a quadrivalent containing an insertion loop may be formed, allowing complete pairing of the chromosomes involved in the rearrangement [197]. If no crossover occurs within the insertion loop, the consequences are the same as described earlier for non-paired bivalents. If a crossover occurs within the insertion loop, however, recombinant chromosomes that would lead to the production of gametes with duplications and deletions may be formed. Once again, the risk for having a liveborn abnormal child will depend on the viability of the partial trisomies and monosomies produced.

Regardless of whether complete pairing is achieved between the chromosomes involved in an interchromosomal insertion or whether recombination takes place, compared to carriers of other chromosome rearrangements, an insertion carrier's risk of having an abnormal liveborn child is among the highest. VanHemel and Eussen reviewed the data from a number of individual case reports and found the average risk for having an abnormal child to be approximately 32% for a male carrier and 36% for a female [192]. The theoretical risk, as mentioned earlier, approaches 50%.

Complex Chromosome Rearrangements

Although the definition of what constitutes a complex chromosome rearrangement (CCR) appears to vary somewhat, a rearrangement involving two or more chromosomes and at least three breakpoints is generally considered to be complex [198]. The more complex the rearrangement, the greater the number of chromosome breaks and the higher the probability that an essential gene has been interrupted or that genetic material has been lost or gained during its formation. It is therefore not surprising that CCRs are only rarely seen in constitutional karyotypes.

The majority of reported constitutional CCRs represent *de novo* events that appear to have occurred during spermatogenesis. Because many *de novo*, apparently balanced CCRs have been shown to include cryptic imbalances, a high-resolution genome-wide chromosome microarray analysis may be warranted for such patients, especially if they are phenotypically abnormal [38]. In contrast to the male preponderance of *de novo* CCRs, the less frequently reported familial CCRs appear to be transmitted predominately through females, in keeping with the observation that chromosome rearrangements are more readily tolerated in female meiosis than male meiosis. This female transmission

prevalence may also reflect the presence of male sterility associated with some CCRs.

As one might suspect, meiotic pairing and segregation can become quite complex in a CCR carrier. In theory, the more complex the rearrangement, the more elaborate the chromosome contortions required to optimize pairing between the rearranged chromosomes and their homologs. Similarly, the greater the number of involved chromosomes, the greater the potential number of unbalanced gametes. It is therefore somewhat surprising that a balanced CCR carrier's empiric risk for an unbalanced liveborn child does not appear to differ significantly from that of a comparable simple balanced reciprocal translocation carrier. The risk for miscarriage among these carriers does, however, appear to be somewhat higher, suggesting that early loss of unbalanced pregnancies may partially explain this observation [179, 199–202]. Selection against grossly unbalanced gametes at fertilization could also play a role. As discussed in the introduction to this chapter, the actual reproductive risks for any CCR carrier will vary depending upon the precise rearrangement involved as well as many other variables. However, the empiric risk for a liveborn child with an unbalanced chromosome complement and phenotypic abnormalities is estimated to be approximately 18% for a CCR carrier [202].

Variant Chromosomes

There are a number of structural chromosome rearrangements that have no apparent clinical consequences for the patients that carry them [203–208]. The chromosomes that carry these rearrangements are referred to as normal heteromorphic or polymorphic variants.

Changes in the C-band-positive heterochromatic DNA found in the distal long arm of the Y chromosome and within the pericentromeric region of every chromosome are responsible for some of the most common chromosome variants that we see. Because C-band-positive heterochromatin represents DNA that has been permanently inactivated, it is not surprising that alterations in the size, position, and/or orientation of this material would be benign. Among the most common chromosomal variants observed in humans is a pericentric inversion of chromosome 9 (Fig. 9.8 and section “[Pericentric Inversions](#),” earlier). While the heterochromatic C-band-positive material typically sits within the proximal long arm of chromosome 9, when inverted, it becomes situated within the proximal short arm. Inverted or not, the size of this heterochromatic material is also quite variable. Some chromosomes 9 have little to no pericentromeric heterochromatic material, while in others this region can be quite large; the largest ones are comparable in length to the long arm of chromosome 17.

The short arms of the acrocentric chromosomes (13, 14, 15, 21, and 22) represent another major region of variability within the human genome. Both the proximal short arm and distal satellite region of these short arms are composed of repetitive satellite DNA that is devoid of coding sequences. As described earlier with respect to C-band-positive heterochromatin, because these sequences do not contain DNA that is expressed, changes in the size, orientation, and position of this acrocentric short arm material is clinically benign. In contrast to the proximal and distal regions of the acrocentric short arms, the stalk region sandwiched between encodes ribosomal RNA. Typically, many copies of these ribosomal RNA genes are located within the stalk region of each of the five pairs of acrocentric chromosomes. This region of the genome is therefore highly redundant, and the presence of missing or extra copies of this sequence is of no phenotypic consequence. Translocation of this region to another chromosome, provided that critical genes have not been deleted or interrupted secondary to the rearrangement, also appears to have no clinical consequences. Multiple examples of *de novo* and familial normal variant chromosomes with terminal translocations and interstitial insertions of these ribosomal RNA sequences have been documented in the literature.

In addition to the common C-band heterochromatic and acrocentric short arm variants described earlier, numerous other variant chromosomes also exist in the human karyotype. Some of these actually appear to involve duplications and deletions of apparent euchromatic (expressed) DNA. Because no phenotype is associated with an altered copy number of these sequences, it is assumed that the genes within them are not dosage sensitive. As one might suspect, these variants appear to be rarer than those described earlier, and they can cause a great deal of consternation when they are observed in a karyotype.

Unless a variant chromosome is very common, most cytogeneticists would agree that the variation should be reported and follow-up familial studies should be offered in an attempt to document the same variation in at least one other normal family member. If the variant chromosome is a rare one, particularly if it is one that appears to represent duplication or deletion of euchromatic material, attempts may be made to document the variant chromosome in multiple normal family members and to further characterize the variant chromosome using molecular techniques such as fluorescence *in situ* hybridization (FISH, see Chap. 17). This more extensive workup ensures that the rearrangement has been correctly interpreted and that the presence of imprinting or a more complex rearrangement with reproductive consequences for the family has not been overlooked.

In addition to the variant chromosomes discussed earlier that have been detected using traditional karyotyping, numerous previously unrecognized submicroscopic copy number

variants have recently been identified by chromosome microarray testing. While many of the identified copy number variants are recognized as benign changes in the genome, the clinical significance of others remains unknown. The variants that fall into this latter category are referred to as variants of unclear significance (VUS) and constitute a significant portion of the copy number changes detected by chromosome microarray analysis. As described earlier for cytogenetically visible variants, parental studies to establish whether the copy number change in question was inherited from a clinically normal parent or instead represents a *de novo* mutation can be helpful for establishing clinical significance. However, because variable expressivity and incomplete penetrance have been reported in association with multiple copy number changes identified by microarray testing, the presence of the same mutation in a parent is not considered definitive proof of clinical irrelevance [48]. These copy number changes typically remain VUS until large studies are completed to ascertain the incidence of the copy number change in question within a well-vetted normal population and the extent of clinical variability within the affected population. See also Chap. 18.

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Sex Chromosomes, Sex Chromosome Disorders, and Disorders of Sex Development

Cynthia M. Powell

Introduction

It can be argued that the sex chromosomes are the most important pair of chromosomes given their role in determining gender and, therefore, allowing for reproduction and procreation. Considered together, sex chromosome aneuploidies are the most common chromosome abnormalities seen in live-born infants, children, and adults. Physicians in many specialties including pediatrics, obstetrics and gynecology, endocrinology, internal medicine, and surgery commonly encounter individuals with sex chromosome disorders. There has been a great deal of misinformation in the past regarding outcomes and developmental profiles of these patients, leading to bias and discrimination. This chapter attempts to provide a summary of information regarding the sex chromosomes, sex chromosome disorders, and disorders of sex development.

The X and Y Chromosomes

Role in Sexual Differentiation

Genetic sex is established at the time of fertilization and is dependent on whether an X- or Y-bearing sperm fertilizes the X-bearing egg. The type of gonads that develop (gonadal sex) is determined by the sex chromosome complement (XX or XY) and sex-determining genes. Before the seventh week of embryonic life, the gonads of both sexes appear identical [1]. Normally, under the influence of the Y chromosome, the immature gonad becomes a testis. In the absence of the Y chromosome and with two normal X chromosomes, the gonad differentiates into an ovary. The term phenotypic sex refers to the appearance of the external genitalia and in

some disorders may not correspond to the genetic or gonadal sex (see section “[Disorders of Sex Development with “Normal” Sex Chromosomes](#)”).

Pseudoautosomal Regions

The distal region of the short arms of the X and Y chromosomes contains highly similar DNA sequences. During normal meiosis in the male, crossing-over occurs between these regions. Because this resembles the crossing-over that occurs between autosomes, these regions have been termed pseudo-autosomal or PAR1 (Fig. 10.1). There is also a region of homology (PAR2) at the distal ends of Xq and Yq, which has been observed to associate during male meiosis, with proven recombination events [2]. PAR1 is 2.6 Mb and contains at least 24 genes, whereas PAR2 is only 320 kb and has 4 genes [3].

All genes within PAR1 escape X inactivation in women. One of the genes in this region, *SHOX*, requires two functional copies, without which there will be short stature with or without features of Leri-Weill dyschondrosteosis. If no functional copy of *SHOX* is present, a more severe skeletal dysplasia, Langer mesomelic dysplasia, results. Families segregating deletions or mutations of *SHOX* can have unusual inheritance patterns due to the very high recombination frequency in PAR1. Males may inherit the deleted or mutated gene from their mother but pass it to their sons due to recombination. This has important implications for genetic counseling [4].

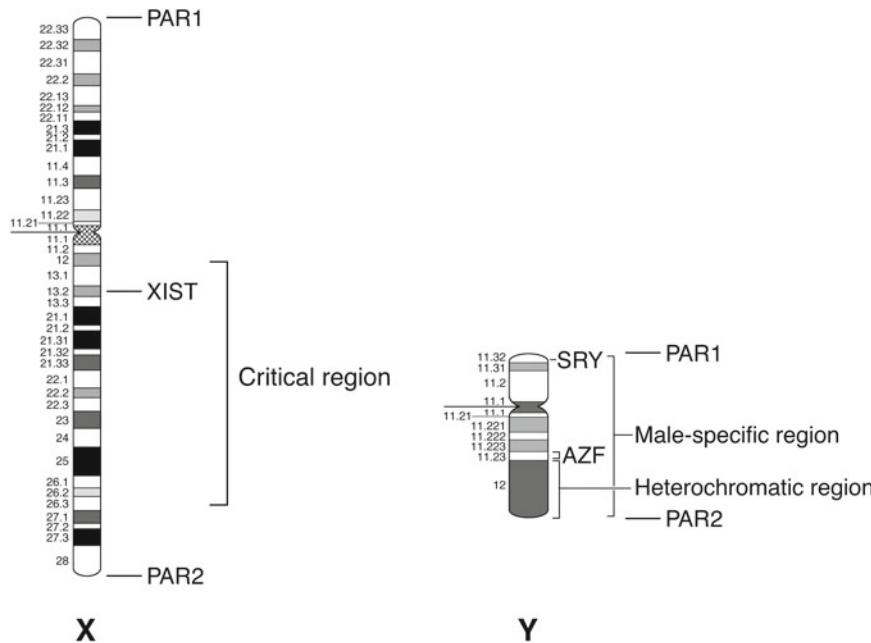
X-Chromosome Inactivation

There are thousands of genes on the X chromosome but relatively few on the Y chromosome. The explanation for the fact that males survive quite nicely with only one X chromosome while females have two involves a concept called “dosage compensation,” and is termed the Lyon hypothesis after its proponent, Dr. Mary Lyon [5].

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Fig. 10.1 Idiograms of the X and Y chromosomes showing the pseudoautosomal regions (PAR1 and PAR2), the locations of the X-inactive-specific transcript (*XIST*) gene, the critical region on Xq, the sex-determining region Y (*SRY*), the azoospermia factor region (AZF), the heterochromatic region of the Y, and the male-specific region located between PAR1 and PAR2



In somatic cells in females, only one X chromosome is active. X inactivation occurs early in embryonic life, beginning about 3 days after fertilization, and is completed by the end of the first week of development. The inactivation is random between the two X chromosomes. Either the maternal or paternal X can be inactivated, and after one X has become inactive, all the daughter cells from that original cell have the same inactive X. In female germ cells, the inactive X chromosome is reactivated as the cells enter meiosis, and in male germ cells, the single X chromosome becomes inactive.

The inactive X has properties characteristic of heterochromatin, with late DNA replication in the S phase of the cell cycle and remaining condensed during interphase. Histone proteins associated with the inactive X are underacetylated, and the cytosines in the CpG islands are methylated [6]. A gene that controls X inactivation is *XIST* (X-inactive-specific transcript) and is located at the X-inactivation center (XIC) at band Xq13 (Fig. 10.1). Only the inactive X expresses this gene. *XIST* codes for Xist RNA, which appears to coat or paint the inactive X chromosome and is responsible for inactivation [7]. About 15% of genes on the X-chromosome escape inactivation and remain active to some degree on both X chromosomes in females [8]. An additional 10% of genes show variable patterns of inactivation and are expressed to different extents from some inactive X chromosomes [9]. Many more genes on Xp escape inactivation as compared to Xq [6]. These genes are clustered and primarily map to the distal portion of the short arm [9]. In individuals with extra or missing X chromosomes, a single X remains active. However, in triploids, there are usually two active X chromosomes, suggesting a counting

mechanism protecting a single X-chromosome inactivation for every two autosome sets [6]. Genes retaining Y homology tend to be expressed on the inactive X [9]. Although all genes in PAR1 escape X-chromosome inactivation (XCI), some in PAR2 do not escape XCI but achieve dosage compensation through inactivation on both the inactive X and the Y [10].

Early evidence for the existence of the inactive X was the observation of the Barr body, named for the Canadian cytologist Murray Barr [11]. This is a dark-staining chromatin body, present in one copy in normal females, which is the condensed, inactive X chromosome. Normal males have no Barr body. Initially, a buccal smear was obtained from patients to look for Barr bodies. Because of improved methods for looking at sex chromosomes and the inaccuracy of the buccal smear technique, it is now considered an obsolete test. The sex chromatin body in polymorphonuclear leukocytes takes the form of the “drumstick,” seen attached to the nucleus in approximately 2% of these cells in XX women but not in XY men [12].

Techniques for detecting the inactive X have been based on the fact that it is late replicating. The most commonly used cytogenetic method involves the use of bromodeoxyuridine (BrdU) [13]. Newer methods for detecting the inactive X involve molecular techniques often using differential methylation analysis [14–18].

Despite the fact that *XIST* was identified in 1990, there remains an incomplete understanding of the cis- and trans-acting elements that control X-chromosome inactivation and determine how some genes escape it as well as how *XIST* is only able to associate with the chromosome from which it is transcribed [19].

Skewing of X-chromosome inactivation (defined as greater than 80% of cells with one of the two chromosomes inactivated) may occur through *XIST* promoter mutations or mutation of other regulatory elements within the XIC, defined as primary skewing. Secondary skewing is post-X-chromosome inactivation selection due to slower proliferation of cells expressing a mutant allele. Evidence supporting this is a study by Amos-Landgraf in which 4.9% of cord blood samples but 14.2% of adult peripheral blood samples showed skewing of XCI [20].

The Y Chromosome

The Y chromosome is made up of several different regions. These include the pseudoautosomal regions at the distal short and long arm, PAR1 and PAR2, the heterochromatic region on the long arm, and the recently sequenced male-specific region of the Y (MSY) located between PAR1 and PAR2, with 78 protein-coding genes that encode 27 distinct proteins (Fig. 10.1). Twelve of the MSY genes are ubiquitously expressed in many organs throughout the body, while 11 MSY genes are expressed predominantly in the testes [21].

The testis-determining factor (TDF) that leads to differentiation of the indifferent gonads into testes is located on the short arm of the Y chromosome. TDF was mapped by molecular analysis of sex-reversed patients (chromosomally female but phenotypically male and vice versa), and the gene *SRY* (sex-determining region Y) was identified in 1990 [22]. It is located on the short arm of the Y at band p11.3 in the MSY region (Fig. 10.1). Deletions and mutations in this gene have been found in some 46,XY females (see [Disorders of Gonadal \(Testicular\) Development](#)).

The Y chromosome has a highly variable heterochromatic region on its long arm. The length of this region is usually constant from one generation to the next. A gene controlling spermatogenesis, termed the azoospermia factor (*AZF*), was first proposed by Tiepolo and Zuffardi in 1976 and mapped to the distal part of the euchromatic Yq11 region (Yq11.23) [23] (Fig. 10.1). In studies of men with azoospermia or severe oligospermia, deletions in different intervals of Yq11 have been found, and three nonoverlapping regions or azoospermia factors (*AZFa*, *AZFb*, and *AZFc*, from proximal to distal Yq) have been defined as spermatogenesis loci [24] (see also Chap. 11).

A locus for susceptibility to gonadoblastoma (*GBY*) has been proposed on the Y chromosome based on the high incidence of gonadoblastoma in females with 45,X/46,XY mosaicism or XY gonadal dysgenesis [25]. Deletion mapping has localized this putative gene to a region near the centromere but has raised the possibility of multiple *GBY* loci

dispersed on the Y chromosome [26, 27]. One of the most likely candidate genes in this region is the testis-specific protein Y-encoded (*TSPY*) gene. This gene functions normally in male germ cell proliferation and differentiation but is ectopically expressed in early and late stages of gonadoblastoma [28, 29].

Numerical Abnormalities of the Sex Chromosomes

Introduction

Numerical abnormalities of the sex chromosomes are one of the most common types of chromosomal aneuploidy, with a frequency of 1 in 500 live births. This might be the result of the fact that abnormalities of sex chromosomes have less severe clinical abnormalities and are more compatible with life as compared to autosomal disorders. Reasons for this include inactivation of all additional X chromosomes and the small number of genes on the Y chromosome.

Sex chromosome disorders are more commonly diagnosed prenatally than autosomal aneuploidies, and genetic counseling for these conditions is often more complex and challenging than that for an autosomal abnormality. In the past, many individuals with sex chromosome disorders would have gone through life undetected, as they do not have physical or developmental problems that would have warranted a chromosome study [30]. Women undergoing amniocentesis and chorionic villus sampling (see Chap. 12) should be informed about the possibility of detecting a sex chromosome disorder, and when a fetal sex chromosome abnormality is detected prenatally, information should be provided to the patient by a clinical geneticist or pediatric endocrinologist when possible [30]. Cytogenetics labs reporting results to physicians and genetic counselors discussing results with patients should provide up-to-date and accurate information about these conditions. It is important for couples faced with having a child with a sex chromosome disorder to see the potential problems for developmental delay in the context of any chromosomally normal child having a risk of developmental delay (by definition, a 5% chance) [30].

There appears to have been a trend toward a higher rate of pregnancy continuation in more recent years [31]. This might be at least in part from results of long-term studies of individuals with sex chromosome disorders revealing a better prognosis than previously reported. [32]. Average percentages of pregnancy terminations for sex chromosomal aneuploidies reported range from 10 to 88% depending on the population, type of aneuploidy, maternal age, presence of fetal abnormalities on ultrasound, and the medical professional providing information [33–39].

Turner Syndrome

45,X (and its variants) occurs in approximately 1 in 2,500 live-born females but is one of the most common chromosome abnormalities in spontaneous abortions and is estimated to occur in 1–2% of all conceptuses. The syndrome was first described in 1938, and a report that it is caused by a single X chromosome appeared in 1959 [40, 41]. The older medical literature sometimes referred to the Turner syndrome karyotype as 45,XO. *This terminology is incorrect and should not be used; there is no O chromosome.*

Ninety-nine percent of 45,X conceptuses result in spontaneous loss, usually by 28 weeks (Fig. 10.2). These fetuses usually have ultrasound abnormalities including a cystic hygroma and hydrops. Although 45,X is quite lethal in the fetus, those that survive to term have relatively minor problems. The reasons for this are not known, although it has been speculated that all conceptions that survive have some degree of undetected mosaicism for a normal cell line [42]. Prenatally diagnosed cases with mosaicism for a 45,X cell line and a cell line with a second structurally normal sex chromosome usually result in the birth of a child with a normal phenotype [43, 44].



Fig. 10.2 A 45,X fetus with large cystic hygroma and hydrops

Origin of the X Chromosome in Turner Syndrome

In approximately 75% of patients with 45,X, the X chromosome is maternal in origin [37, 45, 46]. There is no parental age effect [46]. Although phenotypic differences have not been found between Turner patients with a maternal or paternal X chromosome, there may be some cognitive differences particularly in memory function [47]. This has been theorized to be on the basis of an imprinted X-linked locus; however, no human imprinted X-linked genes have yet been identified [19, 48].

Phenotype

Clinical features of Turner syndrome in newborns may include decreased mean birth weight (average weight 2,800 g), posteriorly rotated ears, neck webbing (Fig. 10.3a), and edema of hands and feet (Fig. 10.3c, d), although more than half are phenotypically normal [49]. Congenital heart defects, especially coarctation of the aorta, and structural renal anomalies are common and should be checked for. Most older children and adults with Turner syndrome have short stature and ovarian failure, and variable dysmorphic features including down-slanting eyes, posteriorly rotated ears, low posterior hairline, webbed neck (Fig. 10.3a), a broad chest, short fourth metacarpals (Fig. 10.3b), and cubitus valgus [49]. Adults with Turner syndrome have a four- to fivefold increased rate of premature mortality, mainly due to complications of congenital heart disease [50]. There is a risk of aortic dilatation leading to dissection. Regular follow-up throughout life with a cardiologist and cardiac magnetic resonance imaging is recommended [49]. Without hormonal supplementation, there is usually lack of secondary sex characteristics. The gonads are generally streaks of fibrous tissue. Although germ cells normally form in the 45,X embryo, there is accelerated oocyte loss by 15 weeks of gestation [51]. Standard treatment includes use of growth hormone and estrogen. It is recommended that these patients be followed by endocrinologists familiar with Turner syndrome. There are several published guidelines for health supervision for children and adults with Turner syndrome [49, 52–55].

The *SHOX* gene, located in the distal part of the pseudo-autosomal region on Xp, escapes X inactivation. Haploinsufficiency for *SHOX* causes short stature and Turner skeletal features [56–58]. A gene determining lymphedema has been proposed at Xp11.4 [59].

Some degree of spontaneous puberty occurs in 10–30% of girls with Turner syndrome, but fewer reach complete puberty with menarche, and most require estrogen treatment beginning at age 12 for normal secondary sex characteristic development, and throughout adulthood to prevent osteoporosis [49]. Spontaneous pregnancy may occur in 2–5% [49, 54].

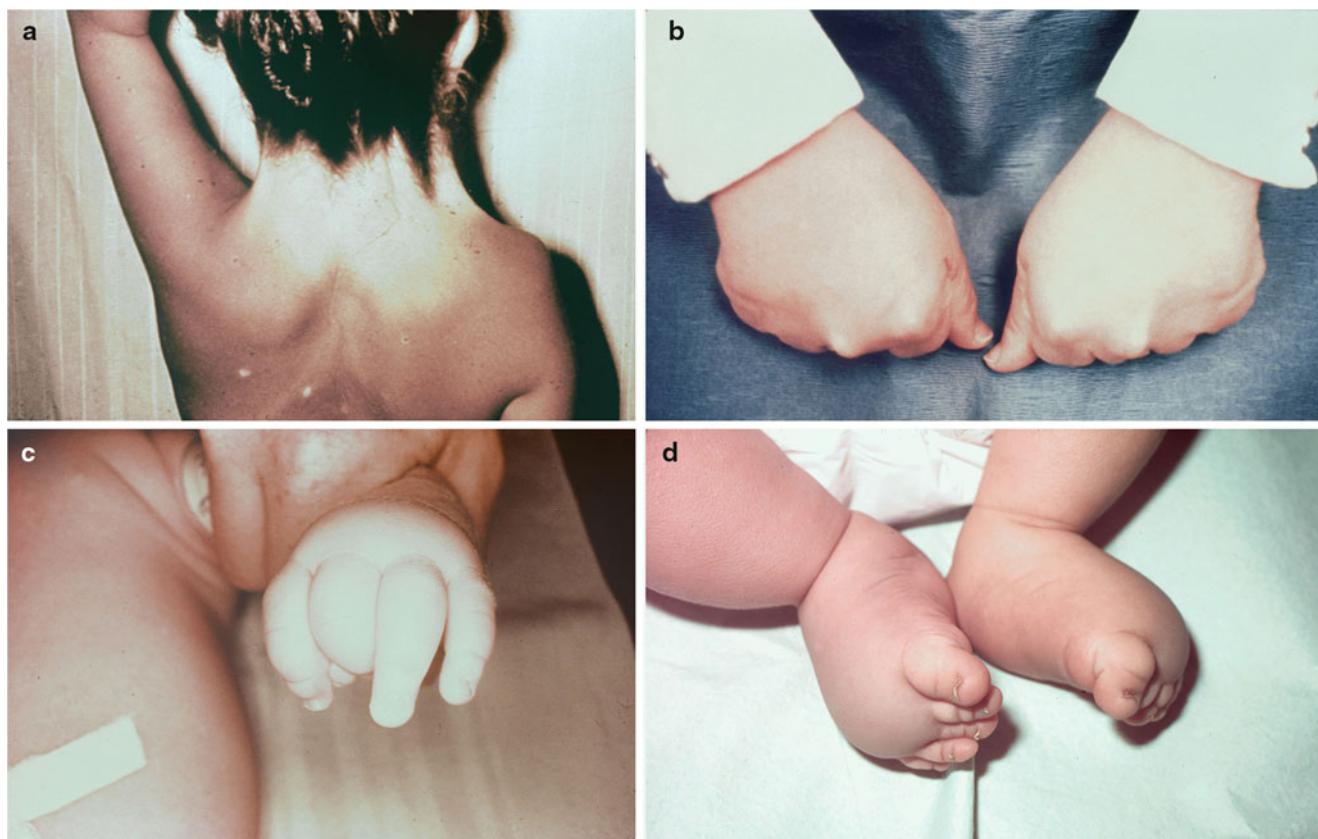


Fig. 10.3 Child with Turner syndrome and low posterior hairline and webbed neck (a) and short fourth metacarpals (b). Infant with Turner syndrome and lymphedema of the hand (c) and feet (d)

Development

Intelligence in individuals with Turner syndrome is average to above average, although there is an increased risk of a nonverbal learning disorder and behavioral problems. Infants can have feeding problems and developmental delay. Problems with visual-spatial skills, working memory, executive functions, and social skills can occur. Verbal IQ scores are typically higher than performance IQ [55]. With appropriate therapeutic and educational intervention, women with Turner syndrome can do well academically and socially.

Turner Syndrome Variants

Approximately half of all individuals with Turner syndrome have a 45,X karyotype. The remainder exhibit mosaicism and/or structural abnormalities of the X chromosome. In a study of cytogenetic and cryptic mosaicism in 211 patients with Turner syndrome, Jacobs et al. reported pure 45,X in 46%, 47% had a structurally abnormal sex chromosome (41% with an abnormal X and 6% with an abnormal Y), and 7% had a 46,XX or 47,XXX cell line [60]. Two patients were found to have cryptic X mosaicism, and none had cryptic Y mosaicism.

Mosaicism

Mosaicism for 45,X and another cell line is found in 15–20% of patients with Turner syndrome with lymphocyte chromosome analysis. A 46,XX cell line may modify the phenotypic features of the syndrome. As mentioned earlier, in order to explain why 99% of 45,X conceptions terminate in miscarriage, it has been proposed that most surviving 45,X fetuses have some degree of mosaicism. In a study of both lymphocytes and fibroblast cultures in 87 patients with Turner syndrome, mosaicism was found in 66.7% [61]. In a patient with several typical features of Turner syndrome but normal lymphocyte chromosome analysis, analysis of another tissue such as skin for fibroblast culture or buccal cell analysis using an X-chromosome probe and fluorescence *in situ* hybridization (FISH; see also Chap. 17) should be considered [61, 62].

In prenatal diagnosis, when multiple cells from a single culture are identified with a 45,X karyotype, a moderate work-up is warranted with examination of an additional 20 cells from cultures other than the one with the initial finding or 12 colonies from coverslips other than the one with the abnormality [62, 63]. Markers identified prenatally should

have their origin determined using X and Y centromere FISH. When a small ring or marker chromosome is determined to be derived from the X chromosome, FISH with a probe for *XIST* should be performed. Lack of the *XIST* locus in a ring X chromosome may be associated with a more severe phenotype that includes developmental disability (see section “[Ring X](#)”).

Mosaicism with a Y Chromosome

Patients with 45,X/46,XY mosaicism may have external genitalia ranging from normal male to ambiguous to female with features of Turner syndrome. The Y chromosome is often structurally abnormal. One study of 92 prenatally diagnosed cases found that 95% had normal male genitalia. Abnormal genitalia included hypospadias, micropenis, and abnormal scrotum. In those fetuses for which pathologic studies were possible, 27% had abnormal gonadal histology, classified as dysgenetic gonads. The percentage of mosaicism found in amniotic fluid samples was a poor predictor of the phenotype [44]. Another study of 42 cases of 45,X/46,XY mosaicism diagnosed prenatally found phenotypically normal male offspring in 90%, with 10% having questionably abnormal phenotypes, including three cases with mixed gonadal dysgenesis [64]. In another study, three of 27 patients with mosaic 45,X/46,XY diagnosed postnatally had mixed gonadal dysgenesis (a streak gonad on one side and testis on the other) and normal plasma testosterone levels. The streak gonads were removed in these patients but the testes were not, and all three had normal puberty. Mild intellectual disability (mental retardation) and autism were seen in four and two patients, respectively, in this series, although there might have been biased ascertainment [65]. Abnormal gonadal development including gonadal dysgenesis, infertility, low testosterone level, and azoospermia can occur in patients with 45,X/46,XY and an apparently normal external male phenotype [65]. Fertility cannot be evaluated until puberty, but infertility is common.

Using fluorescence *in situ* hybridization (FISH) analysis, Robinson et al. looked at the structure of the Y chromosomes present in 14 cases of Turner syndrome with at least one cell line with an abnormal Y chromosome [66]. Ten patients had a pseudodicentric Yp chromosome, two had an isodicentric Yq, one a pseudodicentric Yq, and one had a derivative Y chromosome (see Chap. 9). Results suggested that the majority of Turner syndrome patients with structurally abnormal Y chromosomes contain two copies of most of the euchromatic Y material (see the section “[Structural Abnormalities if the Y Chromosome](#),” later). In a study of 211 patients with Turner syndrome, Jacobs et al. found a clinically significant structural abnormality of the Y chromosome in 13 patients (6%) [60]. One hundred cells were examined from each patient. No patients were found to have 45,X/46,XY mosaicism with a structurally normal Y.

The frequency of occult Y mosaicism in Turner syndrome has varied widely depending on the study and type of analysis used. One study using polymerase chain reaction (PCR) and Southern blot analysis found that 40% of 45,X patients had SRY sequences [67]. Most patient samples produced only a faint signal, indicating a low percentage of cells with Y-chromosome material (or contamination). A more recent study using PCR and Southern blot analysis found evidence of Y mosaicism in only 3.4% of patients with Turner syndrome who were cytogenetically nonmosaic [68]. In a population study in Denmark of 114 females with phenotypic Turner syndrome, 12.2% of patients had Y chromosome material by PCR analysis. Fifty percent of these patients did not have evidence of Y detected by karyotype analysis [69]. Nishi et al. studied 122 patients with Turner syndrome and compared results of nested PCR in these patients with those of 100 women with no known chromosome abnormality [70]. First-round PCR identified Y sequences in four patients (3%); all were also found to have a marker chromosome with cytogenetic analysis. Fourteen percent of DNA samples from 100 “normal” women showed positive amplification after nested PCR. The authors hypothesized contamination with PCR amplification products. The possibility of microchimerism in these women secondary to having had a male conception or a male twin has also been raised [71]. Jacobs et al. found no occult mosaicism for Y in 178 patients with Turner syndrome using Y-specific PCR primers [60]. Thirteen patients had Y-chromosome material detected by routine cytogenetic analysis of 100 cells. Only two of the thirteen patients had fewer than 10% of cells with a Y chromosome: one had 7% and one had 8%. It is likely that these would have been detected with standard analysis of 30 cells [60].

These studies suggest that the presence of cryptic mosaicism involving the Y chromosome is rare and might involve less than 1% of patients. Nested PCR appears to overestimate the frequency of Y sequences in patients with Turner syndrome and should be avoided to prevent false-positive results, which lead to unnecessary surgical treatment in these patients [70]. At this time, PCR analysis for Y sequences in patients with Turner syndrome does not appear to be warranted unless a marker chromosome is found. In such instances, identification of the marker with FISH or molecular analysis is critical due to the risk of gonadoblastoma. Most marker Y chromosomes in patients with Turner syndrome should be detected with standard G-band analysis of 30 cells. The use of FISH analysis to look for Y-chromosome mosaicism in 45,X patients has been recommended [52, 62]. If a 30-cell analysis reveals an apparently nonmosaic 45,X karyotype in patients without virilizing features, FISH analysis using X and Y probes should be performed to identify low-level sex chromosome mosaicism to prevent a potential life-threatening cancer [62, 72]. Identification of mosaicism for a marker, ring, or isochromosome derived from the X

chromosome eliminates the need to pursue additional investigation for Y-chromosome material. All ring and marker chromosomes should be further analyzed with FISH to determine their origin.

If a patient with Turner syndrome has evidence of virilization with clitoromegaly or other masculinizing features, mosaicism for a Y-containing cell line is likely. FISH with X and Y centromere probes should be performed on a minimum of 200 cells to detect low-level mosaicism. If lymphocyte analysis is normal, study of a second tissue should be considered [62].

The risk of gonadoblastoma is increased in phenotypic females with 45,X/46,XY mosaicism [73]. Some but not all patients with Turner syndrome and a Y cell line will have masculinization. In one study, gonadoblastoma was found in one of ten patients with Y-chromosome material who had ovariectomy performed [69]. This patient had a 45,X/46,XY karyotype. None of the patients with only PCR-detected Y-chromosome material developed gonadoblastoma. In another study, 10 of 171 patients with Turner syndrome without clitoromegaly or other evidence of masculinization had Y-chromosome mosaicism with conventional cytogenetic analysis of at least 50 metaphase cells. Four additional patients were identified using PCR for *SRY* and the DYZ3 region, two of whom subsequently had marker chromosomes identified cytogenetically. Of the 12 who underwent gonadectomy, gonadoblastoma was identified in 4 (33.3%), with the youngest patient aged 2.8 years. The percentage of Y-bearing cells in the blood or gonadal tissue did not correlate with gonadoblastoma incidence. Combining their data with previous studies, there was a 26.9% risk of gonadoblastoma in patients with 45,X and Y-chromosome material. In those with gonadoblastoma, the risk of malignancy was 14% [72]. As the risk of malignant degeneration of gonadoblastoma cannot be predicted, surgical removal of gonadal tissue is generally indicated for patients with Turner syndrome and Y-chromosome material. The appropriate timing for this surgery should be discussed with a pediatric endocrinologist. For those patients with a male phenotype and external testes, the risk of neoplasm is not as high, but frequent physical and ultrasound examinations are recommended [74].

Isochromosome X

An isochromosome X [i(X)(q10)] (see Fig. 10.4), consisting of two copies of the long arm (missing all or most of the short arm), is seen in 18% of patients, either as a single cell line or, more commonly, in mosaicism with a 45,X cell line. Although most i(Xq) chromosomes look monocentric, molecular evidence suggests that many are actually dicentric and result from breakage within the proximal short arm of a single X chromosome with subsequent reunion of the sister chromatids [60, 75]. The i(Xq)s are equally likely to be maternal or paternal [60]. The error occurs during male or

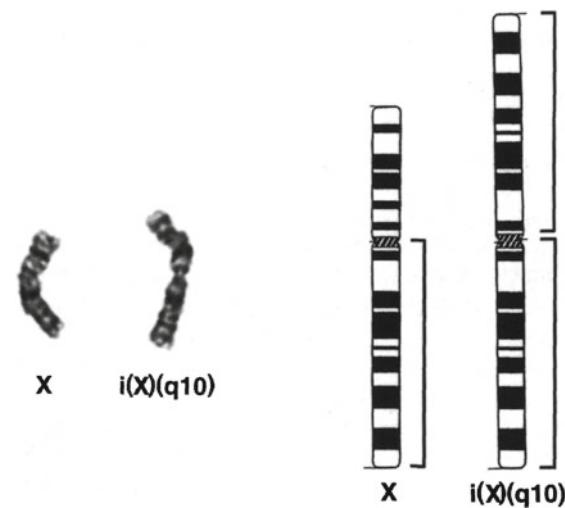


Fig. 10.4 Normal X chromosome and isochromosome Xq: 46,X,i(X)(q10) in a patient with Turner syndrome. Brackets indicate region of duplication on Xq

female gametogenesis. When the error occurs postzygotically, it is likely to be at the first postzygotic division because 45,X/46,X,i(X)(q10)/46,XX mosaics are extremely rare [60].

These patients are phenotypically indistinguishable from those with a pure 45,X karyotype although there have been reports of an increased risk of autoimmune problems in patients with an isochromosome X [76]. In a study of 145 women with Turner syndrome, 83% with an i(Xq) had positive thyroid autoantibodies compared with 33% of women with other karyotypes. The women with an i(Xq) chromosome were also more likely to become hypothyroid (37.5%) and require thyroxine compared to the 45,X women (14%) [77].

Ring X

A subset of patients with Turner syndrome, estimated at approximately 15%, have one normal X chromosome and a ring X chromosome, most often as a mosaic cell line [60]. In a study of patients with a 45,X/46,X,r(X) karyotype in whom the parental origin of the ring could be determined, the ring was derived from the opposite parent from the normal X. In one patient, there was uniparental disomy (UPD) for the X chromosome (see Chap. 20). In two-thirds of patients, the normal X was maternal in origin, and in one-third of patients, it was paternal, as it is in 45,X patients [78]. The size of the ring varies from minute to nearly the size of a normal X. An important first step in characterizing a ring is to perform FISH (see Chap. 17) with a painting probe for the X to confirm that it is derived from the X and not Y [79] (see the section “Mosaicism with a Y Chromosome”).

In general, ring X patients lack many of the classic somatic features of Turner syndrome [80]. Those with ring X often do not have lymphedema and, therefore, are less often ascertained at birth compared to 45,X individuals [81].

Although some have typical features of Turner syndrome, others have a severe phenotype with developmental disability, facial dysmorphism, and congenital malformations. Some patients with a ring X and severe phenotype have features similar to those of patients with Kabuki syndrome, a multiple malformation syndrome due to mutations in the *MLL2* gene on chromosome 12 [80–83]. El Abd et al. reported a characteristic behavioral profile in five patients with a small active ring X that included autistic-like features, obsessive compulsive behavior, and social and communication problems [84].

Studies have suggested that the more severely affected patients have smaller rings that are deleted for *XIST*. It has been hypothesized that the lack of *XIST* causes the ring to fail to inactivate, thus causing functional disomy for genes present on the ring, resulting in phenotypic abnormalities [85–87]. Larger rings have *XIST* present and are preferentially inactivated. However, Turner et al. reported that in seven females with 45,X/46,r(X) and a *XIST*-negative ring, only one had a severe phenotype and this was explained by the absence of *XIST* expression, a large amount of Xp material in the ring, and, possibly, the concomitant maternal uniparental isodisomy [88] (see Chap. 20). The remaining six patients had physical phenotypes consistent with Turner syndrome. The size of the ring X chromosome lacking *XIST* correlates with the degree of clinical severity [81, 87–89]. Those with extremely small rings have been found to have cognitive functioning similar to those with 45,X. There could be particular gene sequences that, when functionally disomic, result in the severe physical phenotype.

Other factors that could contribute to the phenotype in patients with small ring X chromosomes are the tissue-limited distribution of the ring X cell line or ring formation from an inactive X after the establishment of X inactivation. In patients with an inactivated ring X chromosome, having a larger proportion of cells with the ring was associated with lower verbal and nonverbal IQ scores [88, 89]. Migeon et al. reported two patients with inactive ring X chromosomes, mental retardation, and a severe phenotype [90]. Cultured fibroblasts from these patients showed a second ring in a small percentage of cells. The authors hypothesized that the severe phenotype with an inactive X chromosome is the result of the presence of a second ring X that was active in some tissues during embryogenesis.

The prognosis for patients with small ring X chromosomes might be better than previously proposed [87]. However, a ring X chromosome appears to be associated with a substantially increased risk of significant learning difficulties, requiring special educational provision, compared to 45,X [89]. It might not be possible to accurately predict, prenatally, the phenotype that will be associated with the ring X chromosome after birth. Although a relatively large, active ring X (*XIST* not expressed) is more likely to be associated with

severe phenotypic abnormalities, demonstration of an inactive ring X is not necessarily reassuring [90]. The etiology of the abnormal phenotype in ring X is complex and cannot be based solely on the inactivation status of the ring. Size and gene content, extent of X inactivation, parental origin, and timing of ring formation and of cell selection likely play a role in the broad phenotypic variability [79].

45,X/47,XXX Mosaicism

Approximately 2% of patients with Turner syndrome have a 45,X/47,XXX mosaic karyotype [60, 91]. A study of seven girls with this type of mosaicism aged 6.1–20.4 years found that three of seven did not require growth hormone, five of six girls older than 10 years had spontaneous puberty, and four of five girls older than 12 years had spontaneous menarche with regular menstrual cycles without medication. No renal or cardiac anomalies and no cognitive or behavioral problems were found in this small group of patients. In general, patients with Turner syndrome caused by 45,X/47,XXX mosaicism are more mildly affected clinically with regard to phenotype and ovarian function [91].

Marker Chromosomes in Patients with Turner Syndrome

It is important to identify the origin of a marker chromosome in a patient with Turner syndrome, because of the risk of gonadoblastoma if it is made up of Y material (see the section “Mosaicism with a Y chromosome” earlier) or the increased risk of phenotypic and developmental abnormalities if the marker is of autosomal origin. This can be done either with FISH or molecular techniques.

47,XXX

Trisomy X or triple X is the most frequent sex chromosome disorder present at birth in females, occurring in 1 in approximately 1,000 live female births [92]. It was first described in 1959 by Jacobs et al. [93]. Unfortunately, the term originally used for this cytogenetic abnormality was “superfemale,” which gives a misconception of the syndrome and is no longer in use. Due to variation in phenotype, with some individuals very mildly affected, only approximately 10% of cases are ascertained clinically [94].

Origin

Most 47,XXX conceptions result from maternal nondisjunction at meiosis I, and so there is an association with increased maternal age. Two of the X chromosomes are inactivated, and abnormalities could result from three active X chromosomes early in embryonic development, prior to X inactivation and/or from genes on the X chromosome that escape inactivation.

Phenotype

In contrast to the result of a 45,X karyotype, there is not a recognizable syndrome in females with an additional X chromosome. The majority are physically normal, although there is a slight increase in the frequency of minor anomalies. The mean birth weight is at the 40th percentile, the mean birth length is at the 70th percentile, and the mean birth head circumference is at the 30th percentile [95]. In general, as adults, these women have moderately tall stature, with an average height of 172 cm (5 ft and 7.7 in.). Pubertal development is normal, and most have normal fertility, although a small number have ovarian dysfunction and premature ovarian insufficiency sometimes associated with autoimmune thyroid disease [96, 101, 97]. Renal and ovarian abnormalities and congenital heart defects have been reported in small numbers of patients. There appears to be an increased risk of seizures [94]. Most have good health, although one study found that 25% had recurrent nonorganic abdominal pain as teenagers [98].

There is a small but slightly increased risk of chromosomally abnormal offspring of 47,XXX women [99, 100]. Although they do not, remarkably, appear to be at significantly increased risk of having XXX or XXY children, prenatal diagnosis should be offered for all pregnancies.

Development

Usually, 47,XXX females have normal intelligence, but most have lower IQs than their siblings. There is a great deal of variability and many women have normal intelligence and are well adjusted. Precise predictions regarding an individual child's prognosis are not possible, but there does appear to be a risk for mild to moderate developmental problems in the areas of motor, speech and language, and learning [95, 101, 102]. Verbal IQ tends to be lower than performance IQ, with many having problems with expressive language.

Many studies of 47,XXX females have ascertainment bias; however, in a group of 11 females with 47,XXX ascertained at birth by unbiased screening of all newborns who were then followed into adulthood, most had serious patterns of dysfunction [102]. Most showed early delays in motor, language, and cognitive development and were described as shy, withdrawn, and immature, with poor coordination [95]. The full-scale IQ was 26 points lower than in normal sibling controls; average IQ was in the 85–90 range, and subjects were at the 24th percentile in academic achievement scores, but mental retardation was rare. Nine of the eleven needed special education in high school either full time or part time, and less than half completed high school, but two achieved "A"s and "B"s, and one excelled in math. Most did not participate in extracurricular activities. They were described as socially immature. All had heterosexual

orientation. Compared to individuals with other types of sex chromosome disorders, 47,XXX females seemed to have the most psychological problems, including depression and, occasionally, psychoses. However, one woman attended college, and many were able to overcome psychological problems and become independent, hold jobs, and marry. Stability of the home environment was somewhat related to outcome but not to such an extent as is seen in other sex chromosome disorders [102]. In adulthood most of these women were employed full time (unskilled or semiskilled jobs), had married, and had children [103]. In another long-term study, 47,XXX women were found to have more work, leisure, and relationship problems as compared to a control group [104]. They were found to have poorer psychosocial adaptation and more psychiatric impairment as compared to their female siblings. However, most were self-sufficient and functioned reasonably well as adults. Severe psychopathology and antisocial behavior were rare, and there was a larger variability in behavioral phenotype than originally appreciated [104]. In another longitudinal study of 16 girls with 47,XXX ascertained at birth through a cytogenetic survey of consecutive newborns, 50% had speech delay and IQ scores averaged 85.3 verbal (range 67–109) and 88.3 performance (range 67–110). Higher IQ scores were positively correlated with the level of parental education. All attended regular schools, but most required extra help in math and reading. Behavior problems required psychiatric referral in 25% of the girls [105].

In a study of five girls ranging in age from 7 to 14 years with 47,XXX diagnosed prenatally, only one had motor and language delays and learning problems; the others had normal IQs (range 90–128) and were doing well in school [106]. Another longitudinal study by Linden and Bender of 17 47,XXX females, ages 7–18, initially diagnosed prenatally, found that eleven needed academic assistance and seven required speech therapy. Many were shy in younger years but became more outgoing as teenagers, and three had no developmental problems. Their IQs ranged from 73 to 128, with a mean of 103. The girl with a 73 IQ was from a family with a history of learning problems [107]. Reasons for the difference between the two groups could be the higher socioeconomic status and greater stability of the prenatally diagnosed group.

Experts in this field advocate for anticipatory guidance for these patients, emphasizing the child's normal development but remaining prepared to identify and provide early intervention when developmental problems occur. Appropriate speech, occupational and physical therapy, educational assistance, and psychiatric treatment should be instituted as soon as a need is identified [104]. There have been several recent reviews of 47,XXX [94, 105, 106].

Variants with Additional X Chromosomes

48,XXXX

As compared to 47,XXX, there is almost always mild to moderate intellectual disability (mental retardation) in individuals with 48,XXXX or tetrasomy X syndrome, with an average IQ of 60, ranging from less than 30 to 75 [110]. One individual was reported to have a normal IQ [111]. Phenotypic features include mild hypertelorism, epicanthal folds, micrognathia, and midface hypoplasia [112]. Tall stature is common, with an average height of 169 cm (approx. 5 ft and 6 in.) [110]. Skeletal anomalies include radioulnar synostosis and fifth finger clinodactyly. Incomplete development of secondary sex characteristics could occur with scant axillary and pubic hair and small breasts, and some patients have gonadal dysgenesis [113]. Speech and behavioral problems are common. Fertility is reduced, and primary ovarian failure has been reported, although some have had chromosomally normal offspring [114].

49,XXXXX

Nondisjunction in successive meiotic divisions is the probable mechanism for pentasomy X syndrome, and molecular studies have shown that, at least in some cases, the extra X chromosomes are all maternally derived [115, 116].

Phenotypic features seen in penta-X females include intrauterine growth restriction, short stature, microcephaly, upslanting palpebral fissures, low hairline, and coarse, Down syndrome-like facial features. Congenital heart defects, renal anomalies, hip dysplasia, and joint subluxation have been reported [117–120]. One individual with penta-X and hyper-IgE syndrome has been reported [121]. An infant with laryngomalacia and extreme neonatal hypotonia has been reported with mosaicism for tetrasomy X and pentasomy X [122]. Most have moderate developmental disability (IQ range 20–75, average IQ 50) and are described as shy and cooperative [110, 120]. There have been no reports of pregnancy in women with this chromosomal aneuploidy [110].

47,XXY (Klinefelter Syndrome)

Klinefelter syndrome (KS) was the first sex chromosome disorder to be described and its cytogenetic cause identified, and is the most common cause of hypogonadism and infertility in males [120, 123–125]. It is found in approximately 1 in 575–1,000 newborn males, although only 25% are ever diagnosed [30, 92, 126].

Origin

In one study, the extra chromosome arose at paternal meiosis I in 53% of patients, 34% at maternal meiosis I, 9% at maternal meiosis II, and 3% from postzygotic errors. There is an

association with increased maternal age in those with maternal meiosis I errors [127]. A recent study found the additional X chromosome to be paternal in approximately 25% of cases, and these were associated with increased paternal age, although other studies have not supported this finding [128, 129].

Maternal nondisjunction during meiosis I leads to uniparental heterodisomy for the X chromosome, while an error during meiosis II results in uniparental isodisomy (see Chap. 20). If the father contributes the X, there are two different X chromosomes with different parental origins. So far, studies have found contradicting results regarding differences in phenotype and development between KS males with a paternal versus a maternal additional X [130]. No phenotypic differences between heterodisomy and isodisomy for the X in KS have yet been found. The repeat length and inactivation status of the androgen receptor at Xq11.2-q12 has also been studied as to its correlation with the KS phenotype, but so far there have been no definitive findings [130].

Phenotype

47,XXY males have taller than average stature, with mean height at the 75th percentile, and might have a eunuchoid build with long limbs and pear-shaped hips, although there is a great deal of phenotypic variability [98]. Head circumference during infancy is usually average but, beginning at age 4 years, tends to be below the mean for age, although generally within normal limits [131]. Testicular and penile size is usually small during childhood, although prepubertal phenotype is often unremarkable. Gynecomastia occurs in up to 50% of 47,XXY males during adolescence. Most enter puberty normally, although there is usually inadequate testosterone production and most require testosterone supplementation. Measurement of serum testosterone level in male infants with Klinefelter syndrome at 6 weeks of age can help predict the amount of natural testosterone production these patients will have.

Testes are small in adulthood, and there is hypergonadotropic hypogonadism. Almost all have infertility with absent spermatogenesis, tubular hyalinization, and Leydig cell hyperplasia. Most are born with spermatogonia, but during early puberty, the spermatogonia are thought to undergo massive apoptosis along with damage to the germinal epithelium [132]. Many men with KS are diagnosed in adulthood, with a chief complaint of infertility, but based on a population study, as many as 74% might never be diagnosed [30]. There have been more than 200 reports of pregnancies resulting from intracytoplasmic sperm injection (ICSI) from nonmosaic 47,XXY patients [130]. In one study of 38 pregnancies, there were 34 karyotypically normal neonates, two karyotypically normal pregnancy losses, one healthy unkaryotyped neonate, and one 47,XXY prenatally diagnosed fetus [133] (see also Chap. 12).

Development

Boys with Klinefelter syndrome have been reported to have decreased muscle tone during infancy, delayed speech and language skills, and an increased incidence of reading disability and dyslexia [134]. IQs are lower than in controls and compared to siblings, with the average between 85 and 90, although there is a wide range [98]. Verbal IQ is usually lower than performance IQ. The majority require special help in school, especially in the areas of reading and spelling. They are often described as awkward with mild neuro-motor deficits, shy, immature, restrained, reserved, and lacking confidence [98]. A group of 13 males with Klinefelter syndrome, diagnosed as newborns and followed into adulthood, were said to have struggled through adolescence with limited academic success but were able to function independently in adulthood [102]. Most needed at least some special education help in school; nine completed high school, and four went to college. All were heterosexual. Some had psychological problems, including conduct disorder and depression, and difficulties with psychosocial adjustment. A stable and supportive family environment was found to correlate with better outcome [102]. Another long-term study of this group found that as adults, ten of eleven were employed full time and eight had married [103].

In a group of five boys ranging in age from 7 to 14 years who had been prenatally diagnosed with 47,XXY karyotypes, there were fewer language and motor deficits in childhood as compared to the postnatally diagnosed group, and all were doing well in school. IQs ranged from 90 to 131. The reason for the better outcome might be the result of environmental and other genetic factors [108]. In a long-term study of 14 prenatally diagnosed boys with Klinefelter syndrome followed to 7–18 years, ten had average school performance, eight required educational assistance, three had attention deficit disorder, and four had speech problems. In general, they were healthy and had pleasant personalities [109]. A study of 50 boys with KS diagnosed prenatally and through clinical ascertainment found problems with complex language processing, impaired attention, and motor function [134]. An excellent review of the developmental phenotype in KS is by Boada et al. [135].

In summary, individuals with Klinefelter syndrome can have productive and fulfilling lives but often require special assistance in school and could have social and behavioral problems. Early evaluation and intervention are strongly recommended because the prognosis can be improved significantly with appropriate therapeutic intervention [136].

Variants with Additional X or Y Chromosomes

48,XXYY

This is the most common variant of Klinefelter syndrome [110]. There is overlap between this condition and both

Klinefelter and XYY syndrome (see the section 47,XYY). The incidence is estimated at 1 in 50,000 male births [137]. Men are tall statured, with adult height above 6 ft, a eunuchoid body habitus, and long thin legs. There is hypergonadotropic hypogonadism, small testes, and sparse body hair. Gynecomastia occurs frequently [110].

Most 48,XXYY patients have mild intellectual disability, although IQs ranging from 60 to 111 have been reported. Speech and motor delays and hypotonia are seen in 75%, with average age at ambulation of 18 months [138]. Developmental outcomes described range from milder language-based learning disability (reading disability, dyslexia) to mild mental retardation in 30% of patients. Overall adaptive functioning is often more impaired than would be expected based on IQ scores [139]. Psychosocial and behavior problems are generally more severe than in 47,XXY individuals, although patients without significant behavior problems have been reported [110, 140]. Four patients with 48,XXYY observed over a 10-year period had psychiatric disorders, including aggressiveness, self-injury, and mental retardation [141].

48,XXYY is not associated with advanced parental age. Nondisjunction in both the first and second male meiotic divisions leading to an XYY sperm has been hypothesized [142].

48,XXX

This is a rare condition, with more abnormal features and developmental disability as compared to 47,XXY. It was first described by Barr in 1959 [143]. Stature is normal to tall, and dysmorphic features include epicanthal folds, hypertelorism, protruding lips, prominent mandible, radioulnar synostosis, and fifth finger clinodactyly. There is hypergonadotropic hypogonadism and hypoplastic penis in 25% of patients, and gynecomastia is common. Testosterone therapy has been shown to be beneficial. Affected individuals are infertile.

Males with this condition have mild to moderate intellectual disability, with most in the 40–60 IQ range, although an individual with an IQ of 79 has been reported [110]. Most have speech delay, slow motor development, and poor coordination. Behavior is immature, with personality traits described as passive, pleasant, placid, and cooperative [110]. A 10–15 point decrease in IQ for each additional X chromosome has been reported, although there is still wide variability in outcomes [144].

49,XXXXY

49,XXXXY has an approximate incidence of 1 in 85,000 male births, and more than 100 cases have been described in the literature since this karyotype was first reported by Fraccaro et al. in 1960 [145]. It results from nondisjunction of the X in maternal meiosis I and II [146].

Common features include low birth weight, short stature in some patients, craniofacial features consisting of round face in infancy, and coarsening of features in older age, with

hypertelorism, epicanthal folds and prognathism, and a short, broad neck [110, 142]. Congenital heart defects are found in 15–20%, with patent ductus arteriosus the most common defect described, but atrial ventricular septal defects and tetralogy of Fallot are also reported [110, 147, 148]. Skeletal anomalies include radioulnar synostosis, genu valgus, pes cavus, and fifth finger clinodactyly. Muscular hypotonia and hyperextensible joints are often present. There is hypergonadotropic hypogonadism [110]. Genitalia are hypoplastic with short penile length, small testicular volume, and cryptorchidism [110, 139]. Because of the distinctive phenotype, some authors have suggested classifying this condition separately from Klinefelter syndrome [139, 148].

Mild to moderate intellectual disability (mental retardation) is seen in most patients, although reported IQs range from 20 to 78. Language development is most severely impaired with some patients having speech aphasia [148]. Behavior has been described as timid, shy, pleasant, anxious, and irritable [110]. Testosterone replacement therapy has been found to be beneficial in some patients, with improvement in attention and behavior [149]. Recent studies have suggested that there may be less significant cognitive impairment in these individuals than previously thought [150]. In 13 children aged 2–7 years, the mean nonverbal IQ was 87.1 [139].

49,XXXYY

Only five cases of live-born males with this sex chromosome disorder have been described. Physical features include tall stature, dysmorphic facial features, gynecomastia, and hypogonadism. All have had moderate to severe mental retardation, with passive but occasionally aggressive behavior and temper tantrums. One patient had autistic-like behavior [110].

47,XY,i(X)(q10)

There have been reports in the literature of 19 individuals with Klinefelter syndrome with an isochromosome Xq or i(Xq) [151]. The phenotype is very similar to typical Klinefelter syndrome with azoospermia, hypogonadism, and gynecomastia, but height is in the normal range, presumably due to a normal copy number of the pseudoautosomal region on Xp/Yp, including the *SHOX* gene. Using microdissection, Hockner et al. demonstrated the i(Xq) chromosome observed in their patient was an isodicentric, maternally derived, true isochromosome and not the result of an Xq:Xq translocation [151]. These findings are consistent with the proposed mechanism of origin for most of the i(Xq) chromosomes observed in Turner syndrome females (see section “**Isochromosome X**” above).

Origin of Extra Chromosomes

The extra chromosomes in polysomy X syndromes most likely arise from sequential nondisjunction events during either maternal or paternal gametogenesis. Studies using

polymorphic microsatellite DNA markers have shown a maternal origin of extra X chromosomes in 30 cases of 49,XXXXY and 49,XXXXXX [115, 116, 157, 159–162, 165–168]. There does not appear to be a maternal age effect as is seen for 47,XXX and 47,XXY. Two cases of 48,XXYY have been shown to arise from paternal nondisjunction [116, 162].

For cases of 48,XXXY studied with Xg blood groups or other polymorphic markers, two are maternal and five are paternal in origin [45, 116, 146, 159–164]. Nondisjunction at first and second meiotic divisions is proposed versus fertilization of an ovum by an XY sperm followed by postzygotic nondisjunction, because mosaicism has not been detected in these patients [116].

47,XYY

Origin

One in 800–1,000 males has an extra Y chromosome [92]. This arises through nondisjunction at paternal meiosis II.

Phenotype

Males with 47,XYY tend to have normal birth length and weight, but when older, most are above the 75th percentile in height. Minor anomalies are found in 20% of patients, but the rate of major malformations is not increased. Most infants are normal in appearance [152]. Pubertal development is usually normal, although onset of puberty in one group of patients studied was approximately 6 months later than average for males with no sex chromosome disorder. Patients often have severe facial acne, are described as tall and thin, and have good general health. Sexual orientation is typically heterosexual. Individuals are described as somewhat awkward and have minor neuromotor deficits [98]. Most have normal fertility and are able to father children. It has been estimated that only 12% of men with XYY are ever diagnosed. Half of those identified were karyotyped because of developmental delay and/or behavior problems [30].

Development

Intelligence is normal, although there is an increased incidence of learning disabilities. There have been two groups of patients with 47,XYY studied long term: one diagnosed in a newborn screening program and the second diagnosed prenatally. The latter group of patients comes from families with an above-average socioeconomic status. The first group had an IQ range of 93–109, and all required part-time special education. The second group had an IQ range of 109–147, and all were reported to be getting “A”s and “B”s in school. IQ is usually somewhat lower than in siblings [98, 108]. Most older boys attend college or have jobs after high school. Longer follow-up of the boys in the second group found that five of 14 required extra assistance in school for academic

difficulties and two were in special education programs for the learning disabled [109]. Overall, school performance in this group was above average. IQ scores were available for six of the boys and ranged from 100 to 147. Most were involved with sports, although five were described as poorly coordinated. Two had serious emotional problems. Five had no academic or behavior problems. In general, there was wide variability of development and adaptation [109].

Delays in speech and language development occur in some boys. An increased rate of autism spectrum disorder in boys with XYY has been reported [153]. In a systematic literature review, Leggett et al. found that although males with XYY had lower IQ scores than expected for their social background, they were not impaired in relation to general population norms. Although some have problems with reading, others report reading as a relative strength, and some are noted to have proficiency in science and mathematics [105].

Hyperactive behavior, distractibility, temper tantrums, and a low frustration tolerance are reported in some boys in late childhood and early adolescence. Aggressiveness is not common in older boys. Although early studies raised the possibility of an increase in criminal behavior in these individuals, recent studies have shown that although there is a higher percentage of males with 47,XYY in prisons than in the general population, there was not an increase in violent behavior in these individuals [154]. Differences in rates of incarceration may be due to the lower intelligence of some XYY males. A study of a newborn cohort of XYY males followed into adulthood reported a higher proportion with antisocial behavior than their peers, but there was no statistical difference when diagnostic criteria for antisocial behavior disorder was used and compared with a group of unaffected males [105, 155].

The condition is clearly variable. Most blend into the population as normal individuals. Better outcomes seem to be associated with a supportive, stable environment.

Variants with Additional Y Chromosomes

(See also the section “**48,XXYY**” presented earlier).

48,XYYY

There is no consistent phenotype for males with two additional Y chromosomes. Mild mental retardation to low normal intelligence and sterility has been described [156, 157].

49,XYYYY

Five nonmosaic cases of 49,XYYYY have been reported. Facial features include hypertelorism, low-set ears, and micrognathia. Skeletal abnormalities include clinodactyly, radioulnar synostosis, scoliosis, and spina bifida occulta. Mental retardation and speech delay, along with impulsive and

aggressive behavior, were reported [110, 158–162]. In a patient followed from age 4 months to 26 years, adult height was at the 80th percentile, walking began at 2 years, and IQ at age 7 years was 75, but he was described as having mild to moderate mental retardation as an adult with impulsivity, behavioral disturbance, and difficulties with social interaction [162].

Sex Chromosome Aneuploidy and Age

Increased aneuploidy with advancing age was first reported by Jacobs et al. in 1961 [169]. This was subsequently found to be the result of the loss of the X chromosome in females and of the Y in males [170]. Premature centromere division in the X chromosome and loss through anaphase lag and formation of a micronucleus are one proposed mechanism in females [171, 172]. This is supported by the finding that hyperdiploidy for the X is much less common than monosomy, which would not be expected if nondisjunction was the mechanism [173]. It is usually the inactive X chromosome that is missing in monosomy X cells [174]. Another proposed mechanism is telomere shortening, as telomeres play a key role in chromosome segregation [175]. X-chromosome aneuploidy is not observed in bone marrow preparations from older women but is seen in phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes. Although some early studies suggested an increase in sex chromosome aneuploidy in women with a history of reproductive loss, recent studies have shown that this is probably not true and that it is purely a phenomenon of aging [173, 176].

In a prospective study of 11 women from 83 to 100 years of age, Jarvik et al. found a fourfold increase in X-chromosome loss after 5 years, compared with the initial level [177]. Galloway and Buckton found a tenfold increase in X-chromosome aneuploidy in women aged 25–35 years compared with those between 65 and 75 years [170]. Between 30 and 55 years, the rate of hypodiploid cells was 3–5% in females, increasing to 8% at age 70 years. This holds true for the loss of any chromosome, but the most common was loss of an X chromosome. It should be noted that there is variability of sex chromosome loss between individuals of the same age, so that what is “normal” aneuploidy at a specific age is impossible to predict. This makes it difficult to interpret the clinical implications of X-chromosome loss seen in an older woman who can also have features of Turner syndrome such as ovarian failure and/or infertility. Because the age-related loss is limited to peripheral blood lymphocytes, analysis of other tissues such as skin fibroblasts or buccal cells can be helpful in clarifying these situations. Russell et al. studied metaphase cells from cultured peripheral blood lymphocytes in 655 females ranging in age from birth to 80 years and found that below age 16 years there is 0.07% X-chromosome loss and above age 65 years there is 7.3% X-chromosome

loss. The authors provide their data as a reference graph to illustrate normal percent X-chromosome loss based on number of cells counted (ranging from 30 to 60 cells) [178].

Age-associated loss of the Y chromosome in men is found more often in bone marrow than in peripheral blood, and it approaches the rate generally seen in peripheral blood in women [173]. Studies of bone marrow preparations have shown that Y-chromosome loss was restricted to males over age 40–50 years, with a frequency of 8–10% of cells [179, 180]. For men of varying ages with no hematological malignancies, 7.7% had Y-chromosome loss; it was rare below age 50 years but was found in 20% of men above 80 years of age. The percentage of Y-negative cells ranged from 10 to 100% [180].

Most studies comparing age-related sex chromosome aneuploidy were done on metaphase preparations and are, therefore, at risk for preparation aneuploidy. Guttenbach et al. performed an *in situ* study of lymphocyte interphase nuclei to look at sex chromosome loss and aging [181]. In males, the rate was 0.05% up to age 15 years, 0.24% in 16- to 20-year-olds, and then steadily increased to 1.34% at age 76–80 years. The mean value of monosomy X cells in females was 1.58% in 0- to 5-year-olds and increased to 4–5% in women over 65 years. Only women over 51 years of age showed a distinct age correlation. This study also found no difference in aneuploidy rates between cultured and non-cultured cells [181]. Bukvic et al. performed analysis of sex chromosome aneuploidy in interphase cells of 16 centenarians and found loss of Y signal in 10% of cells in males compared to 1.6% of cells in younger control men and loss of X signal in 22% of females compared to 1.7% of cells in young women [182].

These findings should be considered when analyzing peripheral blood chromosomes in older females and bone marrow from older men, in order to avoid misinterpretation of normal age-related aneuploidy as clinically significant mosaicism or acquired changes.

Structural Abnormalities of the X Chromosome

In addition to the isochromosome Xq commonly found in patients with Turner syndrome, the X chromosome can be involved in translocations, both balanced and unbalanced, and can also have deletions and duplications (see Chap. 9).

Structural abnormalities of the X in males are generally associated with more severe phenotypic manifestations than in females. This is partly explained by preferential inactivation of the structurally abnormal X in cases of duplications or deletions or in unbalanced X;autosome translocations in females. In cases of balanced X;autosome translocations, there is usually preferential inactivation of the normal X chromosome. Theories explaining this are discussed in the

following paragraphs. “High-resolution” chromosome analysis should be performed on females manifesting X-linked disorders to look for a structural X abnormality. Chromosome microarray can also be helpful to look for X-chromosome imbalance (see Chap. 18).

The molecular X-inactivation pattern seems to correlate with phenotype in women with structural abnormalities of the X chromosome. Completely nonrandom X inactivation of the abnormal X is generally associated with a normal phenotype, whereas those with skewed or random inactivation patterns usually have nonspecific intellectual disability (mental retardation) and/or congenital abnormalities. The X-inactivation status of women with structurally abnormal X chromosomes and an abnormal phenotype should be assayed as part of a routine clinical workup. The phenotype could be correlated with differences in X-inactivation ratios [183]. There have been very few reports on the use of prenatal X-inactivation studies in amniotic fluid or CVS [184] (see also Chap. 12). Studies comparing prenatal and postnatal analysis of X inactivation and their correlation with phenotypic and developmental outcomes are needed before these could be used to give prognostic information in female fetuses with X-chromosome abnormalities.

X;Autosome Translocations

Balanced Translocations

In females, balanced X;autosome translocations can be divided into four phenotypic categories: normal phenotype with or without history of recurrent miscarriage, gonadal dysfunction with primary amenorrhea or premature ovarian failure (POF), a known X-linked disorder, or congenital abnormalities and/or developmental delay [185]. The reasons for the variable phenotypes are complex and not fully understood, making genetic counseling in cases of prenatal detection of these translocations very difficult.

Translocations involving the X chromosome and an autosome often lead to primary or secondary ovarian failure and sometimes Turner syndrome-like features if the translocation occurs within the critical region of Xq13-q26 [186–188]. There are several different hypotheses concerning the cause of gonadal dysfunction in these cases, including disruption of POF-related genes, a position effect resulting from local alteration of chromatin caused by the translocation, and incomplete pairing of X chromosomes at pachytene [186–190]. In cases in which the derivative X chromosome is inactive, inactivation will spread from the translocated X segment to the attached autosomal material, where it will inactivate genes. The other X-chromosome segment will remain active. There is incorrect dosage of both autosomal and X-linked genes in these cells, with functional autosomal monosomy for the derived (X)t(X;autosome) chromosome that contained

the X-inactivation center and functional X-chromosome disomy for the portion of the X chromosome translocated onto the active (autosomal) reciprocal translocation product [191]. There is strong selection against such cells. In general, the normal X is preferentially inactivated in approximately 75% of such patients [191, 192]. When the translocation disrupts a gene located on the X chromosome, a female with such a translocation could manifest a disease condition [193, 194]. Any mutated genes on the derivative X chromosome will be fully expressed, as they would be in a male [5]. Several X-linked genes have been mapped in this way.

A “critical region” determining normal ovarian function has been hypothesized at Xq13-Xq26 [188, 195]. The majority of females with balanced translocations with breakpoints in this region usually have premature ovarian failure (secondary amenorrhea associated with elevated gonadotropin levels before the age of 40 years). Although there have been several candidate genes for POF identified in this region, molecular characterization of the translocation breakpoints of women with balanced translocations involving the critical region has often shown no gene disruption [196, 197]. This supports the hypothesis that a position effect secondary to chromatin alteration or pairing abnormalities at meiosis causes ovarian dysfunction.

It has been thought that the majority of females with balanced X;autosome translocations with breakpoints above the X-inactivation center at Xq13 are phenotypically normal [198, 199]. Schmidt and DuSart found that most X;autosome translocation patients with phenotypic abnormalities or developmental delay had breakpoints clustered in the subtelomeric bands Xp22 and Xq28 [191]. This was thought to be the result of persistence of cells with inactivation of the translocated X in these patients. However, in a study by Waters et al. that reported 104 cases ascertained from cytogenetics laboratories in the United Kingdom, female

X;autosome translocation carriers had a significantly higher number of abnormalities, including developmental delay and learning problems, than would be expected from literature review [185]. Those with congenital anomalies and/or developmental delay showed random X-chromosome breakpoint distribution. *De novo* translocations were significantly more likely to be associated with an abnormal outcome (18 of 19 cases), suggesting that *de novo* status versus breakpoint location is the most important risk factor in predicting phenotypic outcome [185].

Some studies have indicated that in those patients with phenotypic and/or developmental abnormalities, the translocated X was late replicating (inactive) [200]. However, Waters et al. did not find an association with aberrant late replication and abnormal phenotype. Eight of their patients showed a deviation from the expected pattern of consistent early replication of the derived X chromosome and late replication of the normal X chromosome. Five of these patients were phenotypically normal [185].

Because of variability in X inactivation from one person to another with the same X;autosome translocation, it is possible for a phenotypically normal mother to have a daughter with phenotypic abnormalities and intellectual disability (mental retardation) even though both carry the same such rearrangement. This can be because of skewed (nonrandom) X inactivation in the former and random X inactivation in the latter, leading to functional X disomy and functional autosomal monosomy in some cells. This is estimated to occur in approximately 25% of females with X;autosome translocations [193].

A fertile woman with a balanced X;autosome translocation is at risk for having offspring with an unbalanced rearrangement (Fig. 10.5). There is also the risk that even balanced offspring could be abnormal as a result of random or skewed inactivation of the abnormal X in a female child

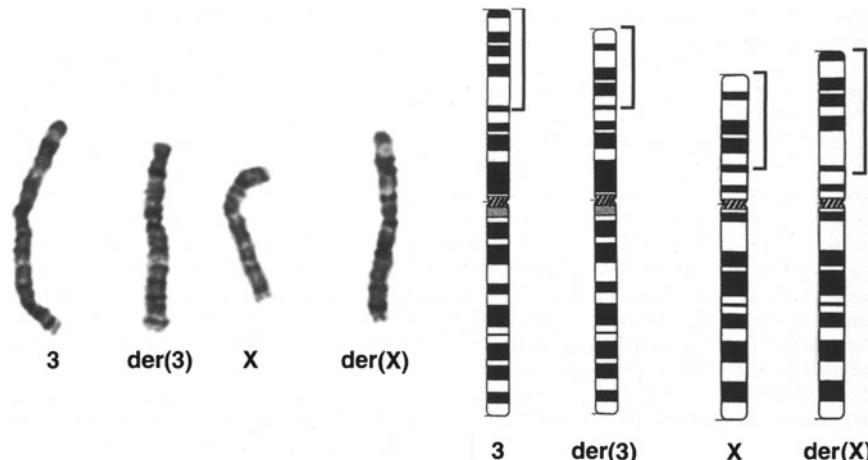


Fig. 10.5 Balanced reciprocal translocation between the short arms of chromosomes X and 3: 46,X,t(X;3)(p11.3;p21.2). Brackets indicate regions involved in translocation on the derivative chromosomes. The patient was a 30-year-old clinically normal, fertile female who had a

daughter with an unbalanced translocation consisting of a normal X, the derivative X, and two normal chromosomes 3 (partial monosomy Xp and partial trisomy 3p)

or by disruption of a functional gene on the X in a male. The risk for a female with a balanced X;autosome translocation to have a live-born child with a structural and/or functional aneuploidy has been estimated at 20–40% [192]. Phenotypic abnormalities can range from mild effects to severe intellectual disability (mental retardation) and birth defects.

Males with balanced X;autosome translocations are usually phenotypically normal but almost all are infertile [201] (see also Chap. 11). There have been reports of severe genital abnormalities in males with such translocations and of multiple congenital anomalies in a man with an apparently balanced (X;6) translocation inherited from his mother [202, 203]. As noted earlier, there is also a risk of an X-linked recessive disorder because of disruption of a gene by the translocation.

Further characterization of breakpoint regions in X-chromosome rearrangements has revealed that although some interrupt specific genes, others are in gene-poor regions. X-linked gene interruption is not a common finding in patients with POF and X;autosome translocations. Two critical regions on Xq associated with POF have been proposed. Critical region I at Xq21 has a low meiotic recombination frequency, is gene poor, is high in repetitive sequences, and is close to the X-inactivation center [204]. Translocations in this region could cause POF as a result of a position effect of autosomal genes important in ovarian function or as a result of meiotic pairing abnormalities [205, 206]. Deletions in this region rarely cause POF. Critical region II, at Xq23-q28, has different properties. Translocations involving this region could cause POF because of a position effect of X-linked genes. Since deletions in this region are frequently associated with POF, haploinsufficiency for missing or interrupted genes in this region could be the cause.

Unbalanced Translocations

In females with unbalanced X;autosome translocations, the abnormal X is generally inactive if the X-inactivation center is present, probably secondary to selection against cells with an autosomal imbalance and functional X disomy. If the X-inactivation center is not present in the translocated segment, phenotypic abnormalities usually result from such imbalances and can include intellectual disability (mental retardation) and multiple congenital anomalies [207]. There have also been patients described who have unbalanced X;autosome translocations but no phenotypic abnormalities and only mild behavioral problems [208].

Earlier studies relied on replication timing to investigate inactivation in X;autosome translocations. The translocated autosomal material can become delayed in its replication timing, and this has been used to examine the extent of the spread of X inactivation in such cases. It has recently been demonstrated that late replication is a poor correlate of the

spread of gene silencing [209]. The spreading of late replication is often incomplete and might skip some autosomal bands and affect others [210]. This suggests that autosomal chromatin does not transmit or maintain the inactivation signal as efficiently as the X chromosome [209].

Sharp et al. reported a family with both a balanced and unbalanced (X;10) translocation segregation [209]. A female with the unbalanced form was phenotypically normal except for secondary amenorrhea. Although the derivative X chromosome was late replicating, the late-replicating region extended only to the X;autosome boundary and did not appear to spread into the translocated segment of 10q. However, transcriptional analysis showed that the translocated segment of 10q was mostly inactive, consistent with the phenotype of the patient. There have been several other reports of patients with X;autosome translocations with mild phenotypes in which no spreading of late replication into the attached autosome was observed [211, 212]. This suggests that silencing of autosomal genes by X inactivation can occur without apparent delay in the replication timing of the surrounding chromatin. The use of replication-timing studies to evaluate the extent of spread of inactivation in X;autosome translocations can be misleading and should not be used to make predictions of phenotype [209]. In a study of five cases with X;autosome translocations, there appears to be some correlation between the pattern of gene silencing and clinical phenotype [15]. However, use of such techniques for prognostic purposes on a clinical basis awaits further studies. Cytogenetic features such as depletion of histone acetylation and H3 lysine 4 dimethylation provide more reliable indicators of the extent of spread of X inactivation than replication-timing studies [15].

Prenatal detection of an unbalanced X;autosome translocation presents a difficult genetic counseling problem (see Chap. 21). Although there have been reports of affected females having only secondary amenorrhea or mild developmental delay, many have had a more severe phenotype with mental retardation and birth defects [15, 200, 209, 213].

In males with unbalanced X;autosome translocations, there is *in utero* lethality or, if they survive, multiple congenital anomalies and intellectual disability (mental retardation) [214].

Functional disomy for the distal long arm of the X caused by unbalanced X;autosome translocations involving Xq28 appears to cause a distinctive phenotype [215] (see also the section on “[Xq Duplications](#)” later).

Deletions of Xp

Males with deletions of the short arm of the X show contiguous gene syndromes characterized by different combinations of phenotypes, depending upon the location and length

of the deletion [216]. X-linked ichthyosis, Kallmann syndrome (anosmia and hypogonadism), intellectual disability (mental retardation), and chondrodysplasia punctata (skeletal dysplasia) are seen in males with deletions involving distal Xp. Deletions in Xp21 cause a contiguous gene syndrome of Duchenne muscular dystrophy, retinitis pigmentosa, adrenal hypoplasia, mental retardation, and glyceral kinase deficiency [217]. Larger Xp deletions in males are lethal.

In females, there is usually preferential inactivation of the structurally abnormal X when the deletion is in or proximal to Xp22.1. In those with breakpoints in Xp22.3, the normal and abnormal X can be active in various proportions of cells [218]. Females with Xp deletions do not usually manifest any of the recessive disorders due to presence of a normal X chromosome, although almost all have short stature and some have phenotypic features of Turner syndrome. Short stature in these patients is likely the result of haploinsufficiency for the *SHOX* gene, within the pseudoautosomal region on Xp [56, 219]. Turner syndrome features could include variable skeletal anomalies associated with *SHOX* deletion and soft tissue anomalies such as nuchal webbing and low posterior hairline reported in some patients with Xp11.1 terminal deletions possibly related to a proposed lymphedema critical region in Xp11.4 [43, 59].

Females with terminal deletions at Xp11.1 usually have complete ovarian failure, although in a series reported by Ogata et al., almost 50% of those with deletions in this region had spontaneous puberty and one had fertility [220]. Females with terminal deletions originating at Xp21 are more likely to show premature rather than complete ovarian failure, although they may have normal fertility [220, 221]. The phenotypes associated with Xp deletions can vary, even within the same family [222]. This is most likely due to variable X inactivation and modifying genes.

Studies have shown that most *de novo* Xp deletions originate on the paternal chromosome [218]. Uniparental disomy (UPD; see also Chap. 20) for the deleted and nondeleted X chromosomes was not found in a study of 25 females with Xp deletions [218].

Interstitial deletions of Xp in females can have intrafamilial phenotypic variability, even when the involved genes escape X inactivation [223]. The cause of this variability is not known.

Deletions of Xq

Large Xq deletions in males are not compatible with survival. Smaller deletions are associated with severe phenotypes [224].

Deletions of the long arm of the X lead to variable phenotypic outcomes in females. Forty-three percent of women with Xq deletions have short stature [225]. In a study by Geerkens et al., it was found that women with breakpoints in

Xq13 to Xq25 had both average and short stature, suggesting variable inactivation of growth genes in Xp or proximal Xq [226]. Deletions in various regions of the long arm are sometimes associated with gonadal dysgenesis or POF. Females with terminal deletions originating at Xq13 are more likely to have complete ovarian failure, whereas those with deletions at Xq24 might have POF [221]. In a series of women with Xq deletions ranging from Xq13.3 to Xq27 reported by Maraschio et al., seven of eight patients had secondary amenorrhea. One woman with deletion at Xq27 had fertility and menopause at 43 years [227]. Clinical features of Turner syndrome are less common in Xq as compared to Xp deletions but more common in patients with deletions proximal to Xq25 [227, 228]. The likelihood of an abnormal phenotype in a female with an Xq deletion is low, although primary or secondary ovarian failure is likely. Recent studies using molecular techniques to characterize breakpoints in Xq deletion cases have reported that normal fertility is more likely if the deletion is in critical region I (Xq21) but most women with deletions in critical region II (Xq23-Xq28) have ovarian failure [205]. Women with Xq deletions and fertility should be advised about their 50% risk of passing the abnormal X to male offspring, with likely miscarriage or severe phenotype depending on the size and location of the deletion.

Xp Duplications

Duplications of Xp involving bands p21.2–21.3, plus a Y chromosome, have been reported in patients who were phenotypic females, suggesting a sex-determining gene locus on Xp [229, 230]. These patients also had mental retardation and multiple anomalies. This area of the X has been termed the dosage-sensitive sex reversal (DSS) region (see the section “*46,XY Disorders of Sex Development*” later). Dosage-sensitive sex reversal is the result of duplication of the *DAX1* gene, which, when deleted or mutated, leads to congenital adrenal hypoplasia [231]. Males with duplications involving more distal Xp have been reported with mental retardation and autism but without sex reversal [232].

Both normal and abnormal phenotypes, and normal fertility as well as amenorrhea, have been reported in females with Xp duplications and one normal X chromosome [229, 230, 233]. The abnormal phenotype—including Turner syndrome features, short stature, seizures, and amenorrhea, but normal intelligence—was seen in a female with complete inactivation of the duplicated X, suggesting that random inactivation was not the cause [233]. An interstitial duplication at Xp11.1-p21.2 was reported in a female with macrocephaly, cleft lip, hypertelorism, and other dysmorphic features who died at age 2 months. There was random X-chromosome replication pattern in this patient [234]. In a review of 52 females with partial X duplications involving Xp or Xq,

Matsuo et al. found that random or skewed but not completely selective X inactivation occurred in nine of 45 patients examined for X-inactivation pattern, independent of the size or location of the duplicated segments [235]. For Xp duplications, four of six patients with random or skewed X inactivation had an apparently normal phenotype, and three of 12 patients with selective inactivation of the duplicated chromosome had clinical abnormalities [235].

A dicentric inverted duplication of most of the short arm of the X [dic dup(X)(qter→p22.3::p22.3→cen)] has been reported in a mother and daughter with short stature, mental retardation, and dysmorphic features. The mother had the duplicated X as the inactive X in all cells, but the daughter had the duplicated X active in 11% of lymphocytes [236].

Females with duplications of Xp including the *SHOX* gene region have been reported with tall and normal stature [237, 238].

With the increasing use of chromosomal microarray technology (see Chap. 18), recurrent microduplications associated with variable phenotypes are being delineated and characterized. A recurrent microduplication of Xp11.22-p11.23 has been reported in association with intellectual disability, speech delay, and electroencephalogram (EEG) abnormalities in both males and females [239]. The size of the duplication ranges from 0.8 to 9.2 Mb. Other clinical features include a hoarse or nasal voice, early puberty, and obesity. X-inactivation analysis of affected females showed selective inactivation of the normal X chromosome in six of nine subjects and random inactivation in the other three. The degree of X inactivation did not correlate with clinical phenotypic severity within families. A female with a *de novo* duplication of Xp11.22-p11.4 has been reported with intellectual disability and structural brain anomalies. X-inactivation studies in peripheral blood lymphocytes showed that the duplicated X was active in the majority of cells [240]. This is a gene-rich region, with numerous genes associated with X-linked intellectual disability, and all genes in this region typically undergo X inactivation, which may account for the abnormal phenotypes of both sexes even with relatively small duplications [9].

Duplication of the region from Xp21-p22 may also be of clinical significance and related to developmental delay and intellectual disability in both males and females along with variable dysmorphic features, including genital anomalies and seizures in a male [241].

An interstitial microduplication of Xp22.31, ranging in size from 149 kb to 1.74 Mb, and including the steroid sulfatase gene has been found with a frequency of 0.15% in a healthy control population and 0.37% in a cohort of individuals with abnormal phenotypes that included developmental delay, intellectual disability, autism, dysmorphic features, and/or multiple congenital anomalies. The most frequent clinical features observed in the abnormal group

were intellectual disability, hypotonia, failure to thrive/feeding difficulties, and seizures. Eight phenotypically normal females tested had random or preferential inactivation of the duplicated X as compared to those with an abnormal phenotype in whom the duplicated X was preferentially active in two of seven, preferentially inactive in one, and there was random X inactivation in four [242]. Whether microduplication of Xp22.31 is clinically significant and related to intellectual disability or is a benign copy number variant remains unclear.

Xq Duplications

Males with duplications of the long arm of the X usually have significant intellectual disability (mental retardation) and birth defects due to functional disomy of the duplicated regions. Most females with Xq duplications have normal phenotypes and are ascertained after the birth of an abnormal male child. However, there have been females with phenotypic abnormalities including short stature, microcephaly, developmental delay/mental retardation, and gonadal dysgenesis reported. Reasons for this variability may be the size or location of the duplicated segment, random or nonskewed X inactivation, duplication of dosage-sensitive genes and genes that normally escape inactivation, incomplete inactivation of a portion of the duplicated segment, or an imprinting effect [243, 244] (see also Chap. 20).

In a review of Xq duplications, phenotypically normal females had smaller and more proximal duplicated Xq segments compared to the Xq duplications in females with clinical abnormalities [243]. In a review of Xq duplications, Zhang et al. also reported that the duplicated segments in individuals with abnormal phenotypes were more frequently located in proximal Xq [244]. A review by Matsuo et al. showed that normal phenotypes are more commonly associated with smaller and more proximal duplications of Xq, and abnormal phenotypes tend to have larger and more distal duplications, but that there is a great deal of overlap [235].

Goodman et al. reported three families with duplication of Xq27-qter on the short arm of the X [245]. Affected males had mental retardation and minor anomalies. The abnormal chromosomes were inherited from the mothers, who were phenotypically normal. Replication studies in two of the mothers showed the abnormal X to be late replicating. However, most phenotypically abnormal females have also been reported to preferentially inactivate the abnormal X chromosome [246]. Therefore, both normal and abnormal phenotypes can be seen even when there is preferential inactivation of the abnormal X. One of three patients with random or skewed X inactivation had an abnormal phenotype, and 9 of 22 cases with selective inactivation of the duplicated X had an abnormal phenotype [235]. The reason for the vari-

able phenotypes but similar inactivation patterns could be the result of differential patterns of inactivation along the chromosome. The activation status of the material present in excess copy number might be what differentiates females with normal phenotypes from those with abnormal phenotypes. The functional disomy of genes might affect the phenotype [246].

Replication studies cannot distinguish phenotypically normal and abnormal females with Xq duplications [233, 243]. Correlations of X-inactivation pattern and phenotype in patients with small duplications should be interpreted carefully [183].

Recurrent Xq duplications identified through chromosome microarray have been reported for Xq25 and associated with hemihyperplasia and digital anomalies and also for Xq27 involving the *SOX3* gene causing pituitary failure in males [247, 248]. Xq28 duplications involving *MECP2* cause the Lubs X-linked mental retardation syndrome (MRXSL) in males [249, 250]. A different condition involving copy number gains at Xq28 has also been reported that involves not *MECP2* but *FLNA* and *GDI1*, with severity correlating with number of additional copy numbers and causing non-syndromic intellectual disability [251]. Sanlaville et al. have reviewed distal Xq duplications [252].

Inversions of the X Chromosome

Paracentric Inversions

Paracentric inversions of the X chromosome (Fig. 10.6) are relatively rare. There has been a wide range of phenotypes described. In general, when long-arm paracentric inversions involve the critical region at Xq13–26, females have some degree of ovarian dysfunction [253]. When the inversion is outside the critical region, normal phenotype and fertility have been reported, although there are exceptions to this [254, 255]. There has also been variability in mental function in females, with some having mental retardation and others

with normal intelligence, even in the same family [256]. Males can be phenotypically normal or have mental retardation [255, 257]. Fertility in males is also variable [258, 259].

Pericentric Inversions

Most females with pericentric inversions of the X have normal phenotypes and fertility [260–264]. However, pericentric inversions of the X have been reported in females with gonadal dysgenesis and with mental retardation [261]. Keitges et al. reported dizygotic twins with the same pericentric X inversion [inv(X)(p11;q22)] [261]. One twin was phenotypically normal with normal intelligence and menses and had random X inactivation. The other was mildly mentally retarded and had psychiatric problems, irregular menses, minor anomalies, and selective inactivation of the inverted X. Proposed explanations for these findings include different normal Xs, a nondetectable deletion or duplication in the abnormal twin, or chance. This also raises the likelihood that the replication pattern of the inverted X is a better predictor of fertility than the breakpoints. Interestingly, females with random X inactivation are more likely to have normal fertility than those with skewed inactivation of the inverted X [261]. Offspring of females with pericentric inversions are at risk for inheriting a recombinant chromosome with associated phenotypic abnormalities [260, 263–265].

Most males with inherited pericentric inversions of the X have a normal phenotype and fertility [260, 265, 266]. However, X-linked disorders have been found to segregate with pericentric inversions of the X, presumably by disruption or deletion of a gene by the inversion [267–270].

Analysis of X-chromosome inactivation in women with apparently balanced pericentric inversions might determine whether an imbalance is present at the molecular level. Random inactivation is usually associated with a balanced inversion, whereas skewed inactivation is more likely associated with an unbalanced inversion [266, 268]. Inactivation status of the mother might provide helpful information in cases of prenatal detection of a male fetus with a maternally inherited inversion [271]. Chromosome microarray is also helpful (see Chap. 18).

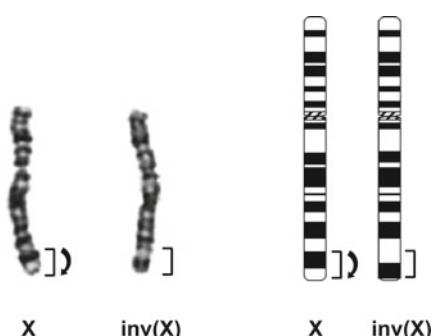


Fig. 10.6 Distal paracentric inversion of Xq: inv(X)(q26q28) in a woman with normal phenotype and fertility. Brackets indicate region involved in inversion

Isodicentric X Chromosomes

Isodicentric Xp Chromosomes

Isodicentric X chromosomes are formed by the fusion of two X chromosomes [272]. The phenotypic effects are variable and dependent on whether the chromosomes are fused at long or short arms, as well as on the location and extent of the deletion. The isochromosomes that are composed of two copies of the entire short arm [*i*(Xp)] are rare and have only been reported in females; they are believed to be nonviable in male

conceptions [273]. These i(Xp) chromosomes are typically pseudodicentric with one active and one inactive centromere, and the *XIST* locus is retained in at least one of the deleted long arms [273, 274]. Most of these females have gonadal dysgenesis that could be explained by the mosaicism for a 45,X cell line that is often present, loss of critical regions on Xq, and/or meiotic pairing abnormalities. Emotional and behavioral problems and intellectual disability have been reported in these patients [273]. Stature ranges from tall to short. Tall stature could be related to the presence of three copies of *SHOX* in those patients with long arms joined.

Isodicentric Xq Chromosomes

Isodicentric X chromosomes consisting of two copies of the long arm joined at the proximal short arms with two centromeres are the most common structural abnormality of the X chromosome seen in Turner syndrome. Although most look monocentric in a G-banded karyotype, they are actually dicentric with one active centromere. The correct nomenclature (in the absence of direct evidence of a dicentric chromosome) is 46,X,i(X)(q10), with the understanding that in many instances it would be more accurate to use 46,X,idic(X)(p11.2).

Females with isodicentric X chromosomes joined at their short arms exhibit short or normal stature, gonadal dysgenesis, and, occasionally, Turner syndrome features [275]. Explanations for the phenotype of short stature when the short arms are joined include deletion of the distal short arm at the region of *SHOX* and/or as a result of a 45,X cell line. There are some males with Klinefelter syndrome who have one normal X and one isochromosome X [276] (see also the previous section on “[47,XXY \(Klinefelter Syndrome\)](#)”).

Mechanisms to explain formation of terminal rearrangements between homologous chromosomes include the following:

1. Breakage and deletion of a single chromosome followed by rejoining of sister chromatids
2. Breakage and deletion of two homologous chromosomes at the same breakpoints followed by interchromosomal reunion
3. Terminal fusion without chromatin loss between sister chromatids and homologous chromosomes [277]

The isodicentric X is almost always late replicating, suggesting nonrandom inactivation of the derivative X. The second centromere is usually nonfunctional, making it a pseudodicentric chromosome [278] (see also the subsections “[Turner Syndrome](#),” “[Isochromosome X](#)” earlier). Studies have shown that those that are functionally dicentric tend to have anaphase lag and, therefore, are more likely to be found in association with a 45,X cell line. Those that are functionally monocentric tend to segregate normally in mitosis [278, 279]. The occurrence of a 45,X cell line also correlates with the distance between the centromeres, presumably due to less mitotic stability of the isodicentric chromosome [75]. Most

are thought to be derived from exchanges between sister chromatids. Analysis of breakpoints in cell lines with idic(X) (p11.2) has found that they lie within large inverted repeat sequences associated with low copy repeats or highly repetitive elements on Xp11.2, suggesting that the principal mechanism in their formation involves nonallelic homologous recombination. The Xp11.2 region appears to be susceptible to rearrangements leading to isochromosome formation and other rearrangements [280].

Structural Abnormalities of the Y Chromosome

Structural abnormalities of the Y chromosome that lead to deletion of the proximal long arm might be associated with azoospermia, infertility, and short stature. Marker chromosomes derived from Y chromosomes are important to detect due to the risk of gonadoblastoma in females with Turner syndrome. FISH probes and chromosome microarray have improved the ability to identify marker Y chromosomes.

Translocations Involving the Y Chromosome

The Y chromosome can be involved in translocations with any other chromosome (another Y, an X, or an autosome).

(X;Y) Translocations

Although the short and long arms of the Y chromosome are now divided into subbands, several references and the original cases cited in them designate “Yp11” or “Yq11.” In order to avoid misrepresentation of the authors’ original work, this nomenclature will be retained here.

Hsu reviewed 51 reported cases of (X;Y) translocations, 47 with a derivative X and four with a derivative Y [281]. The (X;Y) translocations with a derivative X were divided into seven types, with the most common types involving translocation of a portion of Yq11→Yqter onto Xp22.

Patients with type 1, in which there is a normal Y chromosome and a derivative X with a portion of Yq translocated to Xp [46,Y,der(X)t(X;Y)(Xqter→Xp22::Yq11→Yqter)], were phenotypic males. For those with reported heights (14 of 15 reported), all were short, presumably as a result of nullisomy for *SHOX* on Xp22.3. Eleven cases with information available on skin condition showed evidence of ichthyosis, presumably the result of nullisomy for the steroid sulfatase gene on Xp22. All 12 of the patients for whom information was provided on intelligence were mentally retarded. Minor facial anomalies, including flat nasal bridge and hypertelorism, were also reported. Four patients had short limbs compatible with the diagnosis of chondrodysplasia punctata, presumed secondary to nullisomy for the X-linked chondrodysplasia punctata gene on Xp (*ARSE* or

CDPX). In two adult males, azoospermia and small testes were reported. The size of the Xp deletion varies, and phenotypes reflect which genes on Xp are missing. Short stature is a consistent finding; hypogonadism with infertility is common. Patients can have short stature with or without Leri-Weill dyschondrosteosis (as a result of *SHOX* deletion), chondrodysplasia punctata (*ARSE* deletion), mental retardation (presumed MRX locus deletion), ichthyosis (*STS* deficiency), and hypogonadotropic hypogonadism in combination with anosmia (Kallmann syndrome) when the deletion is large and encompasses all of the genes in this region [282].

With probes for the *STS* and Kallman syndrome regions on Xp, it is now possible to use FISH (see Chap. 17) or chromosome microarray (see Chap. 18) to delineate the extent of deletions of Xp22. This will be important in helping to predict phenotype, especially in prenatally diagnosed cases.

Type 2 patients had a translocation of Yq11→Yqter onto Xp22, with one normal X chromosome and a derivative X: 46,X,der(X)t(X;Y)(Xqter→Xp22::Yq11→Yqter) (see Fig. 10.7). Most of these women were ascertained through sons with a type 1 translocation. All 25 reported cases were phenotypic females, and 17 of 22 with height information were short. Most had proven fertility or reportedly had normal ovaries [281]. Most have normal intelligence, but mild mental retardation has been reported [283].

Type 3 patients had one normal X chromosome and a second sex chromosome that was dicentric, consisting of major portions of both X and Y: 46,X,dic(X;Y)

(Xqter→Xp22::Yp11→Yqter). All three patients reported were phenotypic males and had short stature and hypogonadism or azoospermia [281].

Type 4 patients had a portion of Yq translocated to band p11 of the second X chromosome. Of one type 4 case reported, the patient was a phenotypic female, with short stature, streak gonads, and secondary amenorrhea [281].

Types 5 and 6 patients had varying amounts of Yq material translocated to Xq22; of two patients described, both had streak gonads [220].

Type 7 has a dicentric chromosome: 46,X,dic(X;Y) (Xpter→Xq22::Yp11→Yqter), and the one case reported was a phenotypic female with streak gonads, normal stature, and secondary amenorrhea [281].

Four cases of (X;Y) translocations with a derivative Y were reported, which Hsu classified into four types. All involved a portion of Xp22 (three cases) or Xq28 (one case) translocated to Yq11, and all patients had normal stature, hypogonadism with hypoplastic male external genitalia or ambiguous genitalia, mental retardation, and various dysmorphic features [281, 284].

One case has been reported of a 45,X male with an (X;Y) translocation, in which distal Yp was translocated to Xp: 45,der(X)t(X;Y)(Xqter→Xp22.3::Yp11→Ypter). The patient had short stature, a short broad neck, broad chest, wide-spaced nipples, short metacarpals and slight cubitus valgus, normal male external genitalia but small testes, and normal intelligence [285].

It should be noted that the presence or absence of a 45,X cell line in addition to one with an (X;Y) translocation can be of significance concerning the development of external genitalia. When a 45,X cell line is present, there is an increased likelihood of a female phenotype with features of Turner syndrome [281].

(Xp;Yp) translocations involving the testis-determining factor can be found in XX males or, rarely, XY females with sex reversal (see Disorder of Sex Development with “Normal” sex chromosomes later). These translocations are usually not seen with cytogenetic analysis and require molecular probes for diagnosis [216].

There has been a case described of translocation of Yp sequences including the *SRY* gene onto the long arm of the X in a patient who had both ovarian and ovotesticular gonadal tissue [286].

The finding of different phenotypes of XX males and patients with both ovarian and testicular tissue or ovotestes who carry the same translocation has been explained by a different pattern of inactivation of the Y-bearing X chromosomes [287]. Inactivation on the X chromosome spreading into a translocated Yp fragment is the proposed mechanism for an intersex phenotype in some 46,XX (*SRY*+) individuals [288].

Because most males with (X;Y) translocations will inherit the translocation from their mothers, it is important to advise

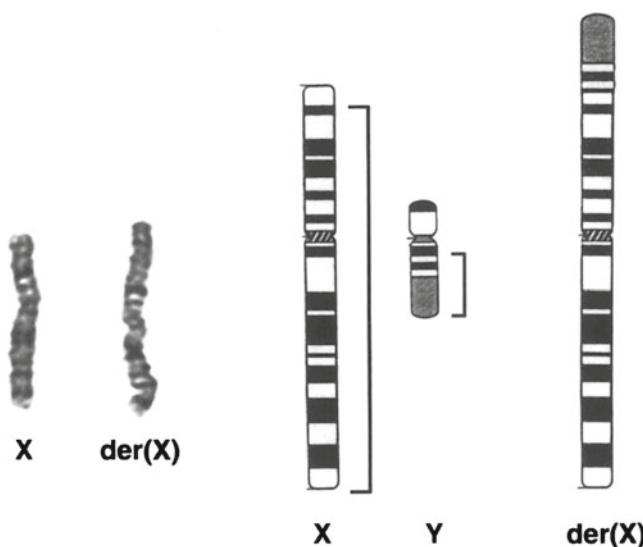


Fig. 10.7 Derivative X chromosome consisting of a small terminal Xp deletion and translocation of Yq:46,X,der(X)t(X;Y)(p22.3;q11.2) mat. This was seen in a 5-year-old girl with short stature who had inherited the chromosome from her mother, who also had short stature but was otherwise normal. Brackets indicate regions on X and Y making up the derivative X

women with such translocations of the risk for more severe manifestations in their male offspring who inherits the derivative X.

Y;Autosome Translocations

Y;autosome translocations are estimated to occur with a frequency of 1 in 2,000 in the general population [289]. In a review of more than 130 cases of Y;autosome translocations, Hsu reported that the most common involved translocation of the fluorescent heterochromatic region of Yq to the short arm of a “D group” (13–15) or “G group” (21 and 22) chromosome [281]. Most of these are familial, and an otherwise normal 46,XX or 46,XY karyotype with this translocation is associated with a normal phenotype. Chromosomes 15 and 22 are most commonly involved: t(Yq12;15p) and t(Yq12;22p). When the translocation is familial, it is unlikely to have any phenotypic effects, and fertility is not affected. When the diagnosis is made prenatally in a 46,XX,der(“D group” chromosome) or der(“G group” chromosome) fetus and the translocation can only be found in male relatives, the possibility of the presence of Yp material in the derivative chromosome cannot be ruled out [281]. There would be a significant risk of masculinization or sex reversal in the female. Molecular studies using Yp probes are indicated in such situations.

Translocations have been reported involving all autosomes except 11 and 20. Twenty-nine of fifty cases that did not involve a “D group” or “G group” chromosome involved a reciprocal translocation, of which 27 were associated with a male phenotype and 2 with a female phenotype. Eighty percent of the adult males had azoospermia/oligospermia or infertility, although there was bias of ascertainment, making the true risk of infertility in males with a balanced Y;autosome translocation unknown. Four of the patients were infants or boys with mental retardation and/or multiple congenital abnormalities. The two patients with female phenotypes had gonadal dysgenesis and streak gonads. A small Yp deletion or 45,X mosaicism could not be ruled out in these patients.

Hsu also reviewed 21 cases with unbalanced Y;autosome translocations, of which 13 had a male phenotype [281]. Two of five adult males had azoospermia or hypogonadism; the other three were phenotypically normal and fertile. Eight were infants or children with abnormalities secondary to autosomal aneuploidy. Six patients were phenotypic females, five with gonadal dysgenesis, and one with Turner syndrome features; three had developed gonadoblastoma.

Males with 45,X and Y;autosome translocations involving all of Yp or a portion of distal Yp might have azoospermia or infertility, although some have normal fertility [281]. The presence of Yp in a Y;autosome translocation explains the male sex determination.

In most cases, when the breakpoint in the Y chromosome is in Yq12, the heterochromatic region of the Y, there is normal fertility. When the breakpoint involves the distal Yq11.2 euchromatic region at the azoospermia factor locus, there is usually infertility. Exceptions to this have been reported [290]. Studying meiotic configurations in a patient with a Y;autosome translocation at Yq12 and infertility, the authors found pairing abnormalities involving the compartment of the sex vesicle (the condensed sex chromosomes). Possible causes of degeneration of spermatocytes after the pachytene stage and thus infertility in such patients include extensive asynapsis, spreading of X inactivation to the autosomal segments partially included in the sex vesicle, autosomal genes involved in the different rearrangements, and the modifying factors of the genetic background [290] (see also Chap. 12).

Yp Deletions

Individuals with deletions of the short arm of the Y involving band p11.3, the location of *SRY*, are usually phenotypic females. Most have streak gonads with Turner syndrome features, especially lymphedema, but normal stature [281]. These individuals are at risk for gonadoblastoma (see previous subsection). This is in contrast to females with 46,XY “pure” gonadal dysgenesis who do not have features of Turner syndrome (see section “[Disorders of Sex Development with “Normal” Sex Chromosomes](#)”).

Males with cryptic deletions of Yp involving the pseudoautosomal region (PAR1) usually have short stature with Madelung deformity and other features of Leri-Weill dyschondrosteosis secondary to haploinsufficiency of *SHOX*. These deletions are often associated with Yp translocations [291–293].

Yq Deletions

Deletions involving the heterochromatic portion of Yq are compatible with normal genital development and sexual differentiation (see the section “[Y-Chromosome Polymorphisms](#)” later). Larger deletions involving the euchromatic portion of Yq could cause azoospermia [294]. When detected prenatally or in a young patient, the father should be tested to see whether the deleted Y is an inherited or a *de novo* abnormality.

Hsu reviewed 52 cases of Yq deletions. In all cases Yq12 was deleted, but for most the cytogenetic findings did not provide specific information about the breakpoint on Yq11. Forty-eight were phenotypic males, and most were infertile with azoospermia or oligospermia [281]. Based on patients with Yq deletions, the azoospermia factor (AZF) was identified (see section “[The Y Chromosome](#)” earlier). Males with these

deletions could have short or normal stature. No patients had gonadoblastoma. Of the three patients who were phenotypic females, two had streak or dysgenetic gonads, and two had normal stature. One patient had ambiguous external genitalia with left testis and right streak gonad, normal stature, and Turner syndrome features. Mosaicism for a 45,X cell line could not be ruled out [281].

Interstitial microdeletions in the euchromatic portion of the Y chromosome occur in 10–15% of men with azoospermia and severe oligozoospermia [295, 296]. AZFa in proximal Yq (Yq11.21) contains two genes (*USP9Y* and *DDX3Y*) whose absence or mutation causes spermatogenic failure [297–299]. Complete absence of AZFa is associated with complete absence of germ cells. AZFb (Yq11.23) contains seven Y genes (*CDY2A*, *EIF1AY*, *PRY*, *RBMY1*, *KDM5D*, *TTTY5*, and *TTTY6*). AZFb absence is associated with a meiotic maturation arrest; that is, spermatogonia and spermatocytes are present in the patients' testis tubules in normal amounts, but postmeiotic germ cells are completely absent. AZFc (Yq11.23) contains seven genes (*BPY2*, *CDY1*, *CSPG4LY*, *DAZ*, *GOLGA2LY*, *TTY3*, and *TTY4*). AZFc deletions are the most common genetic abnormality in men with azoospermia or severe oligozoospermia. They are associated with variable testicular pathology and occasionally are inherited, although most are *de novo* in origin [300–302, 306]. Polymerase chain reaction (PCR) techniques are needed to identify various deleted regions (see also Chap. 12).

Short stature in males with Yq deletions might be the result of the loss of the proposed *GCY* (growth control gene[s] on the Y chromosome) locus near the pericentromeric region of Yq [303, 304]. No gene has yet been identified in this region. Using FISH analysis, Kirsch et al. demonstrated 45,X cell lines in metaphase preparations from all patients with terminal Yq deletions, suggesting that at least in some patients, short stature could be explained by mosaicism for a 45,X cell line [304].

Y Isochromosomes

In most cases of isochromosome for Yp or Yq, the abnormal chromosome is dicentric and present in mosaic fashion, usually with a 45,X cell line (see the section “[Turner Syndrome Variants](#)” earlier). The dicentric Y is among the most commonly detected structural abnormalities of the Y chromosome (Fig. 10.8) [281]. Most dicentric Ys have the breakpoint in the long arm, with duplication of the proximal long arm and entire short arm, while some have the break in the short arm with the proximal short arm and entire long arm duplicated. Most isodicentric Y chromosomes are thought to arise through homologous recombination between opposing arms of palindromes on Y sister chromatids [305].

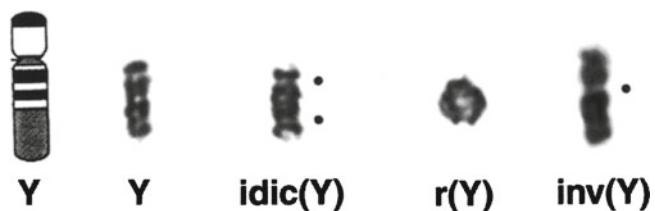


Fig. 10.8 (Left to right) Normal Y; isodicentric Y consisting of two copies of the short arm, centromere, and proximal long arm (q11.2); ring Y, and pericentric inversion of the Y: inv(Y)(p11q11). Dots indicate location of centromeres

The presence of a 45,X cell line in addition to any cell line with an isochromosome Y or isodicentric Y and the degree of mosaicism in different tissues leads to variable phenotypic manifestations, ranging from phenotypic male with azoospermia to ambiguous genitalia to phenotypic females with typical or atypical Turner syndrome features [281]. Many reported patients have short stature. Gonadoblastoma has been reported in females with a dicentric Y cell line. Males often have hypospadias. Azoospermia is common in phenotypic males with an isodicentric Y. Again, this has been proposed to be due to loss of an AZF gene [281, 307, 308]. Mental retardation has been reported in a few patients, and schizophrenia in two patients, although there is a bias of ascertainment in postnatally diagnosed cases and there are very few reports of prenatally diagnosed cases [248, 308, 309, 310, 314]. In a report of outcomes of nine cases ascertained prenatally with nonmosaic 46,X,idic(Yp) or with mosaicism for a 45,X cell line, one had ambiguous genitalia, two of the pregnancies were terminated but the fetuses had normal male genitalia, and six had normal male genitalia at birth and at follow-up examinations. The patient with ambiguous genitalia also had short stature, but in five cases carried to term and followed postnatally with available height information, stature was in the normal range [311]. Another study reported that the likelihood of female anatomic development increases proportionately to the intercentromeric distance in idic(Yp) chromosomes, presumably due to increased instability of the isodicentric chromosome with higher likelihood of a 45,X cell line [305].

Infertility in males with isochromosome Yp (despite having two copies of *SRY*) might arise through lack of distal Yq genes that play critical roles in spermatogenesis, from meiotic pairing disruption as a result of duplication of the pseudoautosomal region of Yp and loss of the pseudoautosomal region on Yq and/or from mitotic instability of the isochromosome and resulting 45,X mosaicism [305].

In a report of five phenotypic females with isodicentric Y and a 45,X cell line who had removal of their gonads, three had microscopic gonadoblastoma, one had evidence of a previous gonadoblastoma that according to the authors had probably “burned out,” and one had a microdysgerminoma

(malignant tumor) [312]. Gonadal tumors have been reported in patients with isodicentric Y chromosomes with breakpoints in both the short and long arms. This could be explained by the presence of multiple gonadoblastoma loci (GBY) on both arms of the Y that may correspond to the *TSPY* gene that has clusters in several locations on both arms [312]. This gene has recently been reported in gonadoblastoma tissue as well as in surrounding normal tissue in five of six gonadoblastoma specimens [313]. A cause-and-effect relationship has not yet been proven. The type of Y isochromosome does not predict the risk of development of gonadoblastoma in a female.

A review of prenatally diagnosed cases of isodicentric chromosome Yp that compared the percentage of mosaicism in peripheral blood postnatally suggested that idic(Yp) may be preferentially present in blood and that the proportion in amniocytes is not a reliable indicator of sexual phenotype [314]. Fetal ultrasound is more important in predicting phenotypic sex [314].

Isochromosome Yp

Without a demonstrable 45,X cell line, most cases with monocentric i(Yp) will have a male phenotype but have infertility [281]. Cases with normal fertility typically have mosaicism for a 46,XY cell line. In one study, all seven cases with an i(Yp) (one centromere) were phenotypic males. This may be the result of greater mitotic stability with a single centromere [305].

Isochromosome Yq

Hsu reviewed seven reported cases with nonmosaic, monocentric isochromosome Yq. All were phenotypic females (expected due to the absence of *SRY*), with sexual infantilism and streak gonads. Approximately half had Turner syndrome features and short stature. The lack of Yp in a case with monocentric i(Yq) without a demonstrable 45,X cell line leads to a female phenotype with typical or atypical Turner syndrome [281].

Ring Y Chromosome

The brightly fluorescent heterochromatic region of Yq is usually deleted during formation of a ring Y [r(Y); see Chap. 3] making Q-banding (see Chap. 4) an unreliable tool for identification (Fig. 10.8). The most accurate way to determine origin of a ring sex chromosome in a patient with a 46,X,-X or Y,+r karyotype is with FISH, using probes for X and Y (see Chap. 17), or with chromosome microarray (see Chap. 18). Because of the instability of ring chromosomes, multiple different cell lines might be seen [315, 316].

In a review of 34 cases with r(Y), 25 had a 45,X cell line. Nine cases were nonmosaic: eight were phenotypic males,

one of whom had proven azoospermia. Other variable features described included small testes, small penis, hypospadias, and short stature. One patient was a phenotypic female with streak gonads and sexual infantilism. Of cases with mosaicism, phenotype varied from normal male to ambiguous genitalia to normal female. Phenotypes were similar to the nonmosaic cases. Because of the different degrees of deletion of Yp and Yq in ring formation, phenotype-karyotype correlation is difficult [281].

In a series of five males with 45,X/46,X,r(Y) mosaicism and bilateral scrotal testes, the three adults had azoospermia, and one of the three had short stature. All had normal puberty and testosterone levels. Two prepubertal males had short stature, one of whom had testes removed due to concern for gonadoblastoma. Pathology exam of the gonadal tissue showed calcinosis that was thought to be an early sign of gonadoblastoma [317]. There has been a report of a male with normal phenotype, oligozoospermia, and a 45,X/46,X,r(Y) karyotype who fathered a son with 46,XX/47,XX,r(Y). The additional X was paternal in origin. Sperm analysis in the father showed a high percentage of XY aneuploid sperm compared to controls [318]. There has been another report of a man with ring Y having a son with ring Y through intracytoplasmic sperm injection [319] (see also Chap. 11). Although men with a ring Y chromosome have a high incidence of infertility, those who father children appear to have a high risk of passing the ring Y to their offspring and may have an increased risk of chromosome aneuploidy in their offspring.

Men with a 45,X/46,X,r(Y) mosaicism with scrotal testes have a relatively low risk of gonadoblastoma or other testicular tumors. Their testes are often functional in terms of testosterone production despite poor sperm production. Monitoring for tumors with physical examination, Doppler ultrasound, and serum markers should be considered, as opposed to prophylactic gonadal removal.

Y-Chromosome Polymorphisms

Heterochromatic Length

The Y chromosome varies in size in the normal male population owing to variability in size of the heterochromatic portion of Yq (Yqh or Yq12). This is not associated with phenotypic abnormalities or infertility (see the section “**Yq Deletions**” earlier).

Satellited Y Chromosome

The presence of satellites on the end of the long arm of the Y chromosome (Yqs) is considered to be a normal variant not associated with phenotypic abnormalities. Transmission through several generations has been reported. These chromosomes arise from translocations involving the short arm of an acrocentric autosome, most

commonly chromosome 15 [320, 321]. All have an active nucleolar organizer region. Loss of the pseudoautosomal region (PAR2) on distal Yq has been shown in several cases of Yqs [321].

At least two cases of satellited short arm of the Y chromosome have been reported both in phenotypically normal males, including one adult with normal fertility [322, 323]. In one case, the satellite Y was inherited from the father who had a satellited X chromosome, presumably from a meiotic crossover event [323].

Inverted Y Chromosome

Pericentric inversion of the Y chromosome—inv(Y) (p11.2q11.2)—is estimated to occur as a normal variant in 0.6 in 1,000 males [324] (see Fig. 10.8). A very high frequency of 30.5% was found in the Gujarati Muslim Indian population of South Africa [325]. In most cases, inverted Y chromosomes are inherited. These are not usually associated with any phenotypic or reproductive abnormalities, although pericentric inversions of the Y have been reported in males with infertility as a result of a small deletion in Yq11.1-2 or interruption of the *DAZ* gene area. Rivera et al. have characterized pericentric inversions of the Y chromosome into two types: “true” inversions with a single active centromere juxtaposed to Yqh and accounting for the majority and “false” inversions with a neocentromere at the most proximal heterochromatin in addition to a classic centromere [325–329].

Knebel et al. demonstrated heterogeneity of the breakpoints in nine cases of pericentric inversion Y using FISH probes. The inversion breakpoints in the short arm were all in a gene-poor region of X-transposed sequences proximal to PAR1 and *SRY*. There were three different types of breakpoints in the long arm. One type was identified in both familial cases and associated with infertility [330].

There have been rare reports of paracentric inversions of the long arm of the Y [253, 331]. One of the cases had ambiguous genitalia with Müllerian structures present [331]. The inverted Y was present in the father and grandfather who were normal fertile males. Abnormalities in *SRY*, *ZFY*, *TSPY1*, or *NROB1* were not found. The cause of the sexual ambiguity could not be identified. A possible position effect related to the inversion or a coincidental cause was hypothesized. A case identified prenatally was inherited from a normal father, and the infant was reported as normal [332].

Disorders of Sex Development with “Normal” Sex Chromosomes

Although visible structural abnormalities of the sex chromosomes are often associated with phenotypic abnormalities of the internal or external genitalia, there are other disorders of

sexual development in which the sex chromosomes may appear structurally normal. Despite discovery of several sex-determining genes, the majority of patients with disorders of gonadal development, often referred to as intersex conditions, still remain genetically unexplained. (For excellent reviews of this complex process, see references [333] and [334]).

Along with discoveries of genes involved in sexual differentiation has come the realization that female development is not merely the default state. Genes that are critical for male sex determination include *SOX9* (SRY-box 9) located on chromosome 17 and thought to be the primary testis-inducing gene and *SRY* the sex-determining genetic factor in males on the Y chromosome. Disruption of the process that *SRY* initiates can lead to an XY female. Candidate genes for the sex-determining factor in females include *RSPO1* (human R-spondin-1 gene), *NROB1* (nuclear receptor subfamily 0 which encodes DAX1), and *WNT4* (wingless-type MMTV integration site family, member 1). Additional as-yet-unknown genes are also thought to be important in female sex differentiation [335].

One in 20,000 newborns is 46,XX with sex reversal, whereas 46,XY with sex reversal is more common, occurring in 1 in 3,000 newborns [336]. In approximately 75% of patients with sex reversal, the cause remains unknown [337].

For parents, genital ambiguity is one of the most stressful problems encountered at birth. Adding to this stress can be the well-meaning physician or nurse who makes premature pronouncements about the infant’s sex [338]. Evaluation by a team of specialists including pediatric endocrinologists, geneticists, and urologists with appropriate endocrine, cytogenetic, pathology, and imaging studies is critical in differentiating the various types of these conditions. It is best to delay sex assignment until there is sufficient information from these studies and after extensive discussions are held with the family. In the past, sex assignment in cases of genital ambiguity was often based on what would give the most potential for sexual function and fertility. Early studies suggested that sex of rearing different from genetic sex did not make a difference in terms of gender identification and adjustment [339]. Many experts stressed the importance of assigning gender as soon as possible in the newborn period. Recently, however, there have been reports of major psychological difficulties with gender identity for some adult patients with disorders of genital ambiguity. The effect of androgen exposure on the developing brain may be the strongest predictor of sexual identity [340]. This has led physicians to reexamine their treatment of such patients and some experts to argue that the central nervous system dictates the sexual identity and that surgical procedures should be postponed until the patient can participate in the decision-making process [340]. These varying opinions emphasize the need for more long-term studies [340, 341]. Recent reviews of patients with ambiguous genitalia have reported

that either male or female sex of rearing can lead to successful long-term outcome [342, 343].

Confusing and, to some, pejorative terminology such as “intersex,” “hermaphroditism,” and “pseudohermaphroditism” has been replaced by the recommended overall term of “disorder of sex development” (DSD), defined as a congenital condition in which development of chromosomal, gonadal, or anatomical sex is atypical. These have been grouped into categories that include sex chromosome DSD, 46,XY DSD, and 46,XX DSD [344]. The category known as sex chromosome disorder of sex development includes 47,XXX; 45,X; 45,X/46,XY (mixed gonadal dysgenesis); and 46,XX/46,XY chimerism. This nomenclature recognizes the importance of the karyotype in differentiating this often very complex and heterogeneous group of conditions and should help in proper classification and diagnosis. 46,XX DSD and 46,XY DSD will be summarized here [344–346].

Accurate diagnosis of the underlying etiology in these conditions is critical for proper medical management and genetic counseling. Many have an increased risk of germ cell malignancy, other tumor types, and serious medical problems [344]. The genetic etiology is extremely variable and often difficult to determine.

46,XX Disorders of Sex Development

Using the new classification system, this group consists of (1) disorders of ovarian development; (2) disorders of androgen excess, with the most common cause of 46,XX DSD being congenital adrenal hyperplasia; and (3) other disorders that include cloacal exstrophy, vaginal atresia, MURCS (Müllerian, renal, cervicothoracic somite abnormalities), and other rare syndromes. It is estimated that 1 in 20,000 newborns has 46,XX sex reversal [336].

Disorders of Gonadal (Ovarian) Development

This category includes gonadal dysgenesis in a 46,XX individual, ovotesticular DSD (previously referred to as true hermaphroditism), and testicular DSD in a 46,XX individual due to presence of *SRY* and duplication of *SOX9* or *RSP01*.

46,XX Testicular Disorders of Sex Development

Previously termed XX male or XX sex reversal, this is a genetically heterogeneous group of conditions involving individuals who have bilateral testes while lacking a Y chromosome. Most have normal male external genitalia, although 10–15% have some degree of genital ambiguity, cryptorchidism, and/or hypospadias and are more likely to be diagnosed in childhood [347]. Others present in adulthood with infertility or gynecomastia. Most have small testes and some signs of androgen deficiency, similar to Klinefelter syndrome patients [348]. The seminiferous tubules are decreased in number and

size, and there are interstitial fibrosis and hyperplastic Leydig cells and usually no spermatogonia [348]. There are also 46,XX sex-reversed patients with both testicular and ovarian tissue in gonads, either separately or, more commonly, as an ovotestis. They usually have ambiguous external and internal genitalia depending on the amount of functional testicular tissue present (see section “[Ovotesticular Disorders of Sex Development](#)” later).

There are at least four different mechanisms to explain the male phenotype in XX DSD: (1) translocation of Y sequences, including the *SRY* gene, to an X chromosome or autosome; (2) gain of *SOX9* through a duplication; (3) a mutation in an as-yet-unknown X-linked or autosomal gene in the testis-determining pathway; or (4) cryptic Y-chromosome mosaicism [349, 350]. The majority of patients (90%) fall into category 1, most often with Y sequences including *SRY* translocated to the X chromosome. The pseudoautosomal regions of Xp and Yp pair during male meiosis, and there sometimes may be unequal interchange of material extending beyond the pseudoautosomal boundaries. This theory has been used to explain the origin of XX males with *SRY* and other portions of Yp translocated to Xp [351]. Ten percent of XX males have no detectable *SRY* or other Y sequences [350].

Most *SRY* positive XX males have normal male external genitalia, while those lacking Y-derived sequences are more likely to have ambiguous genitalia [352, 353]. However, some 46,XX testicular DSD patients with *SRY* present may have ambiguous genitalia and evidence of ovotesticular DSD. This variability may be due to differential inactivation of the X chromosome carrying *SRY* or the size of Yp present on Xp [354, 355, 359].

There have been familial cases of 46,XX testicular DSD, suggesting autosomal recessive inheritance [360]. There have also been families reported with both XX testicular DSD and XX ovotesticular DSD, so that there may be a common origin for both [357]. Others have also found evidence that full virilization requires the expression of a second Y-linked gene, near *SRY*, which may be expressed outside the testis [361, 362].

Ovotesticular Disorders of Sex Development

This term replaces that previously used of “true hermaphroditism.” This is a rare condition where both testicular and ovarian tissues are present either as separate structures or as an ovotestis. Most patients have ambiguous external genitalia with a phallus of variable length and urogenital sinus and are reared as males. Secondary sex characteristics in each patient will be the result of the predominant steroid hormone produced. Ovulation and pregnancy have been reported in a few cases [350]. A few patients who were chimeras with 46,XX and 46,XY cell lines arising from the fusion of two zygotes have been described, although not all 46,XX/46,XY individuals have ovotesticular DSD [348, 351]. The *SRY* gene is present

in 33% of cases [352]. At least 50% of cases of ovotesticular DSD are 46,XX with no Y DNA [348]. The cause of testicular tissue in these patients is unclear. Possible etiologies are gonadal mosaicism for a Y-bearing cell line and alterations in unknown X-linked or autosomal sex-determining genes [350, 353].

Gonadal neoplasia and breast cancer have been reported in these patients [73, 344, 351].

Disorders of Androgen Excess

This group of conditions includes those previously termed female pseudohermaphroditism. Patients with disorders of androgen excess with a 46,XX chromosome complement have gonadal tissue of one sex but ambiguous external genitalia. This is most commonly the result of congenital adrenal hyperplasia. It is critical to identify these patients early due to the risk of hypovolemic shock in untreated 21-hydroxylase deficiency, the most common type of congenital adrenal hyperplasia that leads to salt wasting. This is an autosomal recessive condition, and prenatal diagnosis and treatment are possible.

There are other causes of androgen excess such as 11 β -hydroxylase (11beta-hydroxylase) deficiency and 3 β -hydroxysteroid (3beta-hydroxysteroid) dehydrogenase deficiency. Exogenous hormones from the mother may also cause masculinization of genitalia in a 46,XX fetus [353].

46,XY Disorders of Sex Development

This category includes (1) disorders of gonadal (testicular) development such as 46,XY complete gonadal dysgenesis or Swyer syndrome (previously known as XY sex reversal or XY females), partial gonadal dysgenesis (previously termed male pseudohermaphroditism), and ovotesticular DSD (previously termed true hermaphroditism); (2) disorders in androgen synthesis or action such as complete androgen insensitivity syndrome (the current term for what was previously known as “testicular feminization”), which is an X-linked condition due to mutations in the androgen receptor gene (*AR*), Smith-Lemli-Opitz syndrome, an autosomal recessive condition due to mutations in the gene *DHCR7*, and 5-alpha-reductase deficiency, an autosomal recessive condition that causes ambiguous genitalia at birth but normal virilization at puberty; and (3) others that include syndromic associations of male genital development such as cloacal anomalies and Robinow and Aarskog syndromes [74]. This category also includes undervirilization of an XY male and undermasculinization of an XY male [344]. There are many causes of this including partial androgen insensitivity [355, 369].

It is more difficult to make a specific diagnosis in 46,XY DSD as compared to the category of XX DSD despite the fact that it is more common [336, 354]. There are more numerous etiologies as compared to XX DSD.

Disorders of Gonadal (Testicular) Development

This includes complete or partial gonadal dysgenesis associated with mutations in sex-determining genes including *SRY*, *WT1* on 11p, *DMRT1* (doublesex- and MAB3-related transcription factor 1) on 9p, and *SF1* (splicing factor 1) on 11q in 46,XY individuals [334, 350]. Only 10–15% of cases of complete gonadal dysgenesis are caused by mutations in *SRY*. Some cases are due to loss of *SRY* [364]. Heterozygous mutations of *SF1* (steroidogenic factor 1) appear to be a relatively frequent cause of XY DSD without adrenal insufficiency [344]. There is also a dosage-sensitive region on Xp that, when duplicated, leads to female external genitalia in a 46,XY individual. *DAX1* (dosage-sensitive sex reversal/adrenal hypoplasia congenita/critical region on the X chromosome, gene 1) appears to be the gene responsible for this [231, 365–367]. Mutations in this gene are associated with congenital adrenal hypoplasia and hypogonadotropic hypogonadism [367, 368]. Familial cases of disorders of testicular development suggesting X-linked sex-limited, autosomal recessive, or autosomal dominant inheritance have been reported [363]. Malformation syndromes such as Smith-Lemli-Opitz and campomelic dysplasia also produce female or ambiguous genitalia with a 46,XY karyotype. These are due to mutations or deletions in the autosomal genes *DHCR7* (7-dehydrocholesterol reductase) and *SOX9*, respectively. Other syndromes associated with gonadal dysgenesis are alpha-thalassemia/mental retardation (*ATRX*) and Denys-Drash syndrome, due to mutations in *WT1* (Wilms' tumor 1 gene) on 11p. Denys-Drash syndrome is a condition with Wilms' tumor, diffuse mesangial sclerosis of the kidneys leading to nephrotic syndrome, and gonadal dysgenesis with ambiguous genitalia or hypospadias (*WT1*) on 11p13 [354]. *WT1* is also associated with WAGR syndrome (Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation) and Frasier syndrome (focal segmental glomerular sclerosis, male-to-female sex reversal, and low risk of Wilms' tumor). Many other malformation syndromes associated with testicular DSD have been described including X-linked lissencephaly due to mutations in *ARX* [370–372].

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Introduction

The World Health Organization has described infertility as a health problem of global concern. One in seven couples experiences infertility or subfertility [1]. Infertility is commonly defined as absence of a pregnancy after a year of unprotected intercourse. For information about cytogenetic aspects of spontaneous abortions, please refer to Chap. 13. Male factor and female factor infertility each accounts for about 40% of cases of infertility, and the remaining 20% is a combination [2]. In this chapter, an overview of known causes of infertility will be presented so that the cytogenetic component's relative contribution can be placed into context.

Causes of Female Infertility

Female infertility falls roughly into four categories:

- Fallopian tube obstruction and/or adhesion
- Anatomic abnormalities of the genital tract
- Endometriosis
- Ovulation disorders, which include hypothalamic, pituitary, and ovarian causes. Most cytogenetic abnormalities fall into this category

In this chapter, only the latter two categories will be discussed. An algorithm for the evaluation of delayed puberty/amenorrhea and secondary amenorrhea is shown in Fig. 11.1. Primary amenorrhea is the condition of never having had menses; secondary amenorrhea is described as discontinuation of menses. The majority of cytogenetic abnormalities in women

with infertility are in those with primary amenorrhea. Nonetheless, most women with infertility have experienced normal menarche. In the evaluation of female infertility, if the serum estrogen level is low and the gonadotropins are high, ovarian failure is likely, and chromosome analysis is indicated. The gonadotropins include follicle-stimulating hormone (FSH) and luteinizing hormone (LH). They are secreted by the pituitary gland in response to gonadotropin-releasing hormone (GnRH), which in turn is secreted by the hypothalamus gland. If the estrogen level is low, and gonadotropins FSH and LH are also low, the likelihood is that hypothalamic or hypopituitary issues are the cause. In this case, there are several gene abnormalities known to cause these problems, but the yield of cytogenetic investigation is very low.

Cytogenetic Findings in Female Infertility Due to Ovarian Dysfunction

45,X and 45,X Mosaicism

As discussed in Chap. 10, one in about 2,500 baby girls is born with 45,X or a mosaic variant thereof. Ninety percent of women with 45,X or with 45,X mosaicism with 46,XY, 46,XX, 47,XXX, or 46,X,i(Xq) cell lines present with primary amenorrhea and lack of pubertal development. Up to 25% have some breast development [3]. Two to 3% of 45,X women and 10–15% of women with mosaic 45,X experience normal pubertal development and menarche but are highly likely to undergo secondary amenorrhea [3, 4].

Of those who undergo menarche, their only manifestation of a sex chromosome abnormality may be short stature. Thus, blood chromosome analysis is recommended when a woman has short stature and reproductive failure.

More than 100 pregnancies have been reported in women with a 45,X cell line who did not use assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF). Kaneko et al. reviewed the literature on 138 pregnancies in 62 women with a 45,X cell line, many of whom had a second cell line [5]. Thirteen 45,X patients had 21 pregnancies, 22

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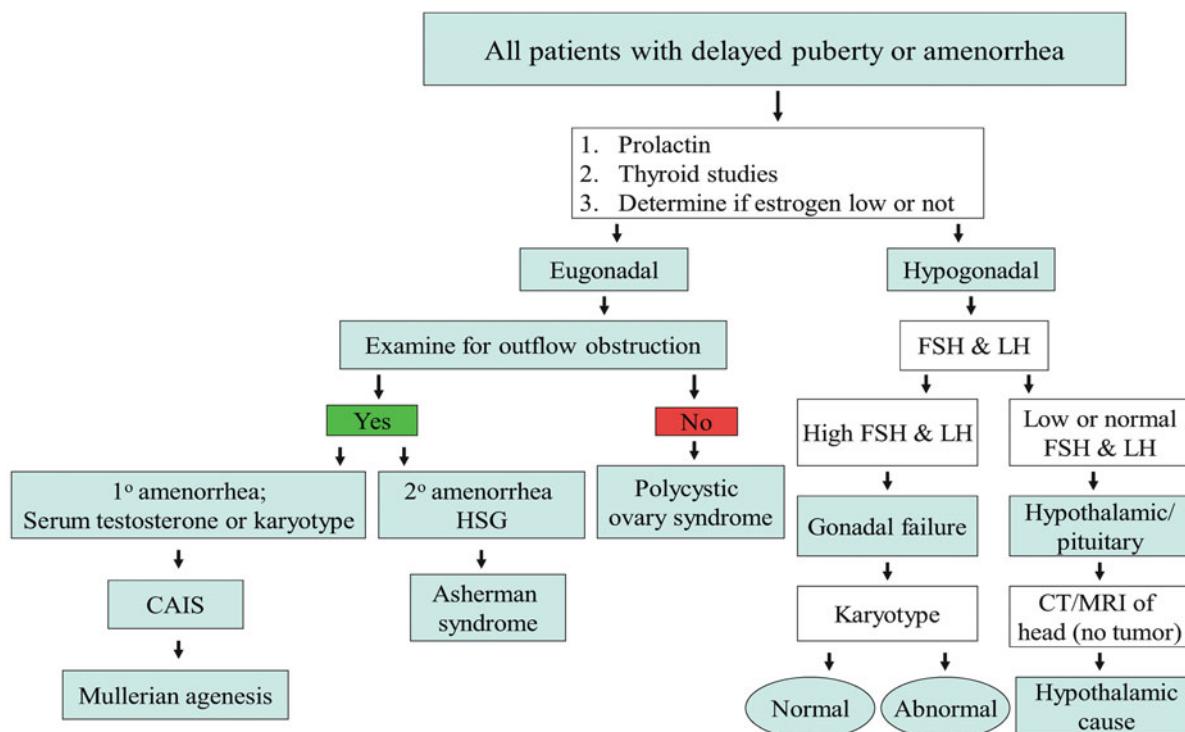


Fig. 11.1 Flow diagram for the workup of a woman with delayed puberty (Courtesy of Dr. Lawrence Layman.) PCOS polycystic ovary syndrome, CAIS complete androgen insensitivity syndrome, FSH

follicle-stimulating hormone, LH luteinizing hormone, *Asherman syndrome* adhesions from prior uterine curettage

45,X/46,XX women had 50 pregnancies, 12 patients with 45,X/47,XXX had 20 pregnancies, 44 pregnancies were from 14 patients with 45,X/46,XX/47,XXX, and one patient with 45,X/46,XX/47,XXX/48,XXXX had three pregnancies. None was reported in women with a 46,XY cell line without IVF.

Among 13 women with 45,X without mosaicism, six of 21 pregnancies ended in spontaneous abortions, and two of 21 ended in stillbirth, including one with hydrocephalus. Another three culminated in a pregnancy and/or offspring with a chromosome abnormality or significant birth defect (one with Down syndrome, one with 45,X, one with a partial cleft of the soft palate).

Among the liveborn for 45,X and mosaic 45,X patients, 23/102 (23%) had chromosome abnormalities or birth defects. For 16 others in the mosaic group, no outcome information was available. Ten percent of liveborn of pregnant women with a 45,X mosaic cell line had a female child with ovarian failure with reported karyotypes in their offspring including 45,X (3%), 45,X/46,XX (4%), 45,X/46,XY (1%), and 45,X/46,XX/47,XXX (2%). From these data, it appears that nonmosaic 45,X live-born offspring are less likely to have abnormalities than live-born offspring of women with

mosaic 45,X. In this group, 12 nonmosaic 45,X women's live born infants had no abnormalities.

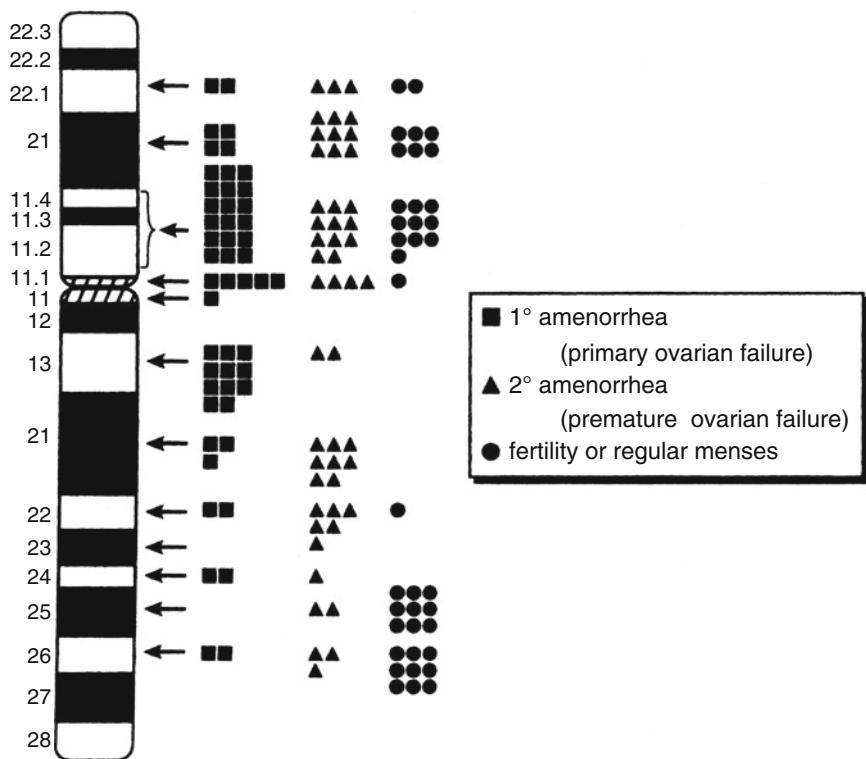
Magee et al. described a patient with nonmosaic 45,X, based on several tissue studies, who appeared to have had seven pregnancies. Three of them were confirmed. These three culminated in a missed abortion, a fetus with 45,X that was terminated, and a healthy baby boy [6].

The approximately 30% incidence of fetal loss and other abnormal outcomes among offspring of 45,X mosaic and nonmosaic women should be stressed when providing genetic counseling to these patients, and prenatal diagnosis by chorionic villus sampling or amniocentesis is indicated.

Women with 45,X and 45,X Mosaicism and *In Vitro* Fertilization

Pregnancy rates using ovum donors in centers specializing in *in vitro* fertilization report pregnancy rates of 50–60%, with the endometrial response to estrogen treatment not significantly different from that of women with secondary ovarian failure [3]. Cardiovascular and kidney functions are to be assessed prior to instituting a pregnancy in these patients, given the high baseline risks of heart and kidney abnormalities in women with 45,X and mosaic 45,X.

Fig. 11.2 An idiogram of the X chromosome with locations of various deletions and the corresponding clinical characteristics (Reprinted with permission from Simpson and Rajkovic [9])



Detection of Y Chromosome Sequences in 45,X and Mosaic 45,X Patients

Among the hypotheses as to why all but one percent of 45,X fetuses die *in utero* and why some women with apparent nonmosaic 45,X have some fertility is that 45,X individuals may actually be cryptic mosaics for another cell line that supports survival. It is important to consider too that the detection of mosaicism is limited by the numbers of tissues and cells examined. Sometimes, mosaicism is inferred by cytogenetic findings in the offspring. In one such case, described by Magee et al., a woman with 45,X had two pregnancies—one ending in spontaneous abortion at 8 weeks of gestation and the other resulting in a female with 46,X,del(X)(p21) [6]. In another case, a woman with apparent nonmosaic 45,X had a baby girl with 46,X,der(X). Using fluorescence *in situ* hybridization (FISH), one cell of 450 examined in maternal lymphocytes showed a der(X). Kocova et al. note in their paper describing Y chromosome sequences in Turner syndrome that when others evaluated both peripheral lymphocytes and fibroblasts, only about 21% of karyotypes of 87 live-born Turner syndrome patients were found to be 45,X. Kocova's group evaluated 18 females with nonmosaic Turner syndrome by performing chromosome analysis on blood and/or skin fibroblasts. In six of these patients, presence of the SRY (testis-determining factor, or gene) was detected [7].

X Chromosome Deletions

X chromosome deletions are usually sporadic, although familial cases have been reported. Deletions affecting the short arm of the X chromosome at band p11 result in ovarian

failure in about half of women, and the other half experience menstrual irregularities. Fertility is rare even if menstruation occurs. If the deletion occurs more distally, such as at band p21, patients usually have a milder phenotype with normal menarche, even though secondary amenorrhea or infertility is common. Most women with Xp deletions are short, even if ovarian function is normal.

Deletions of the long arm of the X chromosome generally are associated with ovarian failure if they involve the so-called critical region—the region between Xq13 and Xq26. As with deletions of the short arm, more distal Xq deletions are associated with a milder phenotype. These women may have menarche with or without ovarian failure. Women with deletions in Xq13 have primary amenorrhea, no breast development, and ovarian failure with high levels of FSH and LH. Davison et al. performed cytogenetic analyses on 79 women with primary or secondary amenorrhea, and two of the 79 had an abnormal karyotype. One of them was a woman with primary amenorrhea and a 46,XY karyotype. The other was a woman with secondary amenorrhea and a deletion at Xq26.1. This woman had a family history of premature ovarian failure, and her mother, who had undergone premature ovarian failure at 28 years, also had this deletion [8]. See Fig. 11.2, which shows locations of different deletions of the X chromosome and the associated phenotype. See also Chap. 10.

X Chromosome; Autosome Translocations

In a balanced X-autosome translocation, the normal X is generally inactivated. If the abnormal X were inactivated, autosomal material would be inactivated along with it.

Inactivation of autosome genes would probably be a lethal event.

In an unbalanced X-autosome translocation, the normal X chromosome remains active, while the abnormal X is inactivated in an attempt to compensate for the imbalance.

Translocations involving the X chromosome and an autosome are rare, occurring in one in about 30,000 live births [4]. This relates in part to the fact that all males and half of females with this finding are infertile. For women, the phenotypic effects depend on the breakpoint and the status of inactivation of the X chromosomes. If the derivative X is active in all cells and the breakpoint does not interrupt a functional gene, about half have a normal phenotype and half have ovarian failure. In general, those with ovarian failure have breakpoints within the Xq13–26 region.

For women with an active derivative X, when the breakpoints interrupt important genes on either the X or the autosome, a single-gene disorder, such as Duchenne muscular dystrophy, may result. When the derivative X is active in only a portion of cells, multiple anomalies and mental retardation usually result.

The breakpoints on the X chromosome vary widely in X-autosome translocations. The most common autosomes involved include chromosomes 15, 21, and 22. The pericentromeric regions of these chromosomes are predisposed to pairing with the X chromosome.

For non-cytogenetic-inherited causes of ovarian dysfunction, please see Table 11.1.

Endometriosis

Endometriosis is a common disorder that accounts for infertility in 6–10% of women of reproductive age. It is characterized by the formation of collections of endometrial

tissue outside of the uterus in so-called chocolate cysts. These cysts occur in the ovary and elsewhere in the pelvis and body. The name of the cysts is due to the appearance of chocolate syrup within the cysts. Endometriosis causes painful menses as well as infertility and has been reported in sisters fairly often [10, 11].

Endometriosis is characterized by monoclonal growth and can exhibit features of malignant behavior, including local invasion and metastasis. Comparative genomic hybridization (CGH), a molecular cytogenetic method that facilitates screening of the entire genome for chromosome gains and/or losses (see Chap. 17), showed recurrent copy number losses on several chromosomes in 15 of 18 cases of endometrial tissue. Losses of 1p and 22q were each detected in half of the cases. Chromosome 7p was lost in one-fifth of the cases. These results were validated by selective dual-color FISH and were interpreted as indicating that genes localized to certain chromosome regions play a role in the development and progression of endometriosis [12]. A subsequent case-control study showed that gene variants on chromosome 7 and chromosome 1 were found to be associated with endometriosis [11].

Hypothalamic and Pituitary Causes of Female Infertility

Several genes have been identified as the cause of infertility involving malfunction of the hypothalamus or pituitary gland. Because no cytogenetic testing is helpful in these cases, a summary of gene-level conditions associated with hypothalamic malfunction is provided in Table 11.2. Table 11.3 indicates gene-level conditions associated with pituitary malfunction, and Table 11.4 lists gene-level conditions associated with uterine development abnormalities.

Table 11.1 Gene mutations that affect ovarian function [4]

Gene	Locus	Phenotype	Inheritance
<i>FMRI</i>	Xq27.3	Fragile X syndrome; ovarian failure	X-linked dominant
<i>SRY</i>	Yp11.3	Swyer syndrome; sexual infantilism, normal vagina and uterus, streak gonads with risk for gonadoblastoma +/– germ cell tumor	Sporadic; Y-linked
<i>FSHR</i>	2p21-p16	Primary amenorrhea, half with breast development; men have oligospermia	Autosomal recessive
<i>LHCGR</i>	2p21	Anovulation in women; undermasculinization in men	Autosomal recessive
<i>CYP17A1</i>	10q24.3	17-hydroxylase deficiency; delayed puberty in women, absent breast development, primary amenorrhea, and elevated gonadotropins	Autosomal recessive
<i>CYP19A1</i>	15q21.1	Aromatase deficiency; cannot convert androgens to estrogens; females with sexual ambiguity, clitoromegaly; no breast development or menses	Autosomal recessive
<i>AIRE</i>	21q22.3	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED); multisystem autoimmune disease; adrenal, ovarian, and testicular failure may occur	Autosomal recessive
<i>NR5A1</i>	9q33	Steroidogenic factor 1; adrenal failure, sex reversal in men; presents as female phenotype; rare	Autosomal recessive
<i>GALT</i>	9p13	Galactose-1-phosphate uridylyltransferase; causes galactosemia; normal puberty; half with primary and half with secondary amenorrhea; 67% of women have ovarian failure and testicular function apparently not affected. Heterozygotes (carriers) not affected	Autosomal recessive

Table 11.2 Gene mutations affecting hypothalamic functions in females [4]

Gene	Locus	Phenotype	Inheritance
<i>KAL</i>	Xp22.32	Kallmann syndrome; hypogonadotropic hypogonadism, anosmia; affects only males	X-linked recessive
<i>NROB1</i>	Xp21.3	Adrenal hypoplasia; congenital and hypogonadotropic hypogonadism	X-linked recessive
<i>LEP</i>	7q31.3	Obesity, hypogonadotropic hypogonadism; delayed puberty	Autosomal recessive
<i>LEPR</i>	1p31	Obesity, hypogonadotropic hypogonadism, and elevated serum leptin	Autosomal recessive

Table 11.3 Gene mutations affecting pituitary function in females [4]

Gene	Locus	Phenotype	Inheritance
<i>GNRHR</i>	4q21.2	Hypothalamic hypogonadism	Autosomal recessive
<i>HESX1</i>	3p14.3	Septo-optic dysplasia	Autosomal recessive
<i>LHB</i>	19q13.3	Isolated luteinizing hormone deficiency	Autosomal recessive
<i>FSHB</i>	11p13	Isolated follicle-stimulating hormone deficiency	Autosomal recessive
<i>PROPI</i>	5q35.3	Short stature, hypothyroidism, and hypogonadotropic hypogonadism	Autosomal recessive

Table 11.4 Gene mutations affecting uterine development [4]

Gene	Locus	Phenotype	Inheritance
<i>AR</i>	Xq12	Androgen insensitivity syndrome (male, 46,XY, phenotypic female)	X-linked recessive
<i>HOXA13</i>	7p15.2	Hand-foot-uterus syndrome	Autosomal dominant

Causes of Male Infertility

A standardized approach to the evaluation of an infertile man was published by the World Health Organization (WHO) in 2004 [13]. This manual delineates several diagnostic categories for male infertility, including acquired and idiopathic causes. Among genetic causes are chromosome disorders, genetic disorders that affect reproduction only, and genetic disorders with other effects which also are associated with infertility. Although many advances have been made in the field of male factor infertility, it is estimated that the cause of about 30% of male infertility is still not known [14].

This chapter will provide an overview of causes but provide detail only on cytogenetic and molecular cytogenetic causes.

The *SRY* Gene and Genetic Sex

The presence of the *SRY* (sex-determining region Y) gene on the short arm of the Y chromosome induces differentiation of precursor cells into Sertoli cells, which express anti-Müllerian hormone. Anti-Müllerian hormone, which is also known as Müllerian-inhibiting substance, causes regression of Müllerian structures—the fallopian tubes, uterus, and upper vagina—and the production of testosterone in the Leydig cells. The Leydig cells are thought to differentiate because of messages from the Sertoli cells. Testosterone leads to the formation of internal male genitalia, such as epididymis, vas deferens, seminal vesicles, and ejaculatory duct. The production of dihydrotestosterone results in the formation of the penis, testes, prostate gland, and urethra. Secretion of insulin-like hormone 3 by the Leydig cells causes the descent of the testes [15].

About 10% of infertile men have severe defects in sperm production, and it is in this group of men that many of the cytogenetic and genetic disorders are concentrated [16]. Hackstein et al. note that in the fruit fly *Drosophila*, there is evidence that up to 1,500 genes contribute to male fertility [17]. Much more work remains to be done in humans, in whom several genes have been found to be involved in early sexual development, but many remain to be discovered. In this chapter, cytogenetic and molecular cytogenetic causes of male infertility will be discussed. See Table 11.5 for information on other genetic causes of male infertility.

In general, men with infertility and a normal semen analysis are less likely to have a cytogenetic or molecular cytogenetic basis for their infertility. However, men with normal spermatozoa concentrations but whose spermatozoa do not fertilize also have an increased risk of a constitutional chromosome abnormality. In a study of 400 men who were to undergo intracytoplasmic sperm injection (ICSI), 6.1% of the azoospermic men and 2.7% of the oligospermic men were found to have constitutional chromosome abnormalities, and 7.4% of the men with normospermic analysis also had constitutional cytogenetic abnormalities [20].

Semen Analysis

Semen analysis is usually performed on a sample that has been ejaculated into a specimen cup. The volume and pH of the semen are measured, and the concentration, morphology, and motility of the spermatozoa are analyzed under a microscope. Cellular debris is examined to determine whether an infection is present, and fructose is measured as

Table 11.5 Percentage of certain cytogenetic or microdeletion findings in various studies of men with infertility

Cytogenetic finding	% of men with chromosome abnormality	Sperm count	Motility	Morphology
Robertsonian translocations	6 ^a ; 0.7 ^b ; 1.6 ^c	$\leq 5.6 \times 10^6/\text{mL}^a$; 0.8–12 $\times 10^6/\text{mL}^b$; 1 of 15 with 0 and 14 of 15 with <10 ⁶ per mL ^c	Normal to very low ^a ; NS ^b ; 13 of 15 with OA or OAT ^c	Poor ^a ; NS ^b ; 11 of 15 with OAT ^c
Reciprocal translocation	0.97 ^a ; 0.3 ^b ; 0.94 ^c	0 ^a ; 0–1 $\times 10^6/\text{mL}^b$; 4 of 9 with 0 sperm ^c	5 of 9 with OA or OAT ^c	2 of 9 with OAT ^c
46,X,del(Y)(q11q22); del(Yq) ^b ; 46,X,del(Y)(q12) ^c	1.9 ^a ; 2.1 ^b ; 0.21 ^c	0 ^a ; 0 in 7 of 17 ^b ; “oligo” in 10 of 17 ^b ; <10 ⁶ per mL ^c	2 of 2 with OAT ^c	2 of 2 with OAT ^c
47,XXY	2.5 ^b ; 5.4 ^c	0 ^a ; 0 in 49 of 52 ^c ; <10 ⁶ per mL in 3 of 52 ^c	3 of 52 with oligo only ^c	3 of 52 with oligo only ^c
47,XYY;46,XY/47,XXY; 47,XXY/48,XXXY;46,XX; 45,X/46,XY	0.8 ^b ; 1.0 ^c	0	—	—
46,XY,inv(1)—pericentric	0.97 ^a ; 0.1 ^c	0 ^a	OA ^c	—
46,XY,inv(9)(p23q33)	0.1 ^b	6 $\times 10^6$ per mL ^b		
Other pericentric inversions:	Together, 0.4 ^c	<10 ⁶ per mL for all but inv(Y), which had 0 sperm count ^c	inv(1) and inv(3), OA ^c	inv(2) and inv(5), OAT ^c
Two with inv(2)(p11q13), one inv(3)(p13q25), one inv(5)(p13q13), and one 46,X,inv(Y)(p11q11)				
46,XY,inv(9)(p11q12)	2.7 ^b	0 in 9 of 22 ^b ; “oligo” in 13 of 22 ^b	—	—
Variants: Yqh+, 21 ps+, 22 ps+, 1qh+, 13 ps+, 14 ps+, 15 ps+, 16qh+	7.2 ^b	0 in 35 of 78 ^b ; “oligo” in 43 of 78 ^b	—	—
46,XY	—	0 ^a	0 ^a	—

OA oligoasthenospermia, OAT oligoasthenoteratospermia

^aRetrospective study over 5-year period of normally androgenized men with azoospermia, oligospermia, and normospermia with normal or subnormal testicular volume [18]

^bCytogenetic study of 820 men with 2-year infertility and who had azoospermia or oligospermia. The common inversion of chromosome 9 and various chromosome variants were included in their study [19]

^cBor et al [20]

an indicator of obstruction. Spermatozoa counts are designated as the number present per mL. A normal number as defined by WHO is 20 $\times 10^6$ sperm per mL of semen [13]. However, in a study of 430 couples in Denmark having unprotected sex, the probability of conception increased with increasing spermatozoa concentration to 40 $\times 10^6$ per mL. Above that level, there was no additional likelihood of pregnancy. The authors suggested that the WHO guidelines should be used with caution, as some men above the normal range may be subfertile [21].

Oligospermia, Non-obstructive Azoospermia, and Teratozoospermia

Oligospermia, also called oligozoospermia, is defined as having a low spermatozoa count in an ejaculate. Azoospermia is the absence of spermatozoa, and teratozoospermia indicates abnormally formed spermatozoa. The concentration, morphology, and motility of spermatozoa are important factors in achieving conception.

Gunduz et al. performed chromosome analysis on 41 men with azoospermia and 61 men with oligospermia. Fourteen

of the azoospermic men (34.1%), and two of the oligospermic men (3.3%), had a constitutional chromosome abnormality. The most common abnormality was 47,XXY [22].

In a review of the chromosomal contribution to male infertility, Van Assche et al. reported on the chromosome constitution of about 8,000 infertile men and compared the findings to the chromosome constitution of a group of newborn children. In the infertile group, the incidence of sex chromosome abnormalities was 27 times higher (3.8 vs. 0.13%), and the incidence of autosome abnormalities was five times higher (1.3 vs. 0.25%) [23]. When considering men with oligospermia only, pooled data show a frequency of chromosome abnormalities of 4.6%. For men with azoospermia, the pooled data show a frequency of 13.7% [23].

In a separate cytogenetic study of 1,007 infertile men, major chromosome abnormalities were seen in 62 (6.2%). Of those, 38 (3.8%) had sex chromosome abnormalities, and 24 (2.4%) had autosomal chromosome abnormalities. Of those with sex chromosome abnormalities, 28 were 47,XXY, three were 47,XYY, and seven had a Y chromosome with a structural abnormality. Of the autosomal abnormalities,

10 were reciprocal translocations, eight had Robertsonian translocations, five had an inversion, and one had a ring chromosome. The likelihood of a chromosome abnormality was higher in men with a sperm density of $<5 \times 10^6/\text{mL}$, an FSH $\geq 30.1 \text{ mLU/mL}$, an LH $\geq 8.9 \text{ mLU/mL}$, and a testosterone value $\leq 2.69 \text{ ng/mL}$ [24].

In a review by Martin, the author noted that men with any type of infertility, whether low count, abnormal motility, or abnormal form, had an increased frequency of sperm chromosomal abnormalities, from 2 to 10 times higher than that of controls. She concluded that any abnormality of spermatogenesis confers an increased risk of aneuploid sperm. The highest risk was seen in macrocephalic, multinucleated, multiflagellate sperm, in which cases the risk of aneuploidy and polyploidy is 50–100% [25].

Sex Chromosome Abnormalities

(See also Chap. 10.)

Among men with infertility, the most frequent cytogenetic findings are 47,XXY and 47,XXY/46,XY. Men with this chromosome constitution commonly have the clinical features of Klinefelter syndrome, which include essentially normal appearance at birth but for a slightly small head; delayed puberty; higher incidence of gynecomastia than other males have; and small, firm testes with hyalinization of seminiferous tubules. Intelligence is usually normal, with performance IQ normal and verbal IQ below normal on average. Reading skills may be a problem [24]. These men have hypergonadotropic hypogonadism and azoospermia or very severe oligospermia. Although many of these men are diagnosed as boys, others are not diagnosed until such time as they are seeking the cause for their infertility. Given the incidence at newborn screening, it appears most males with 47,XXY or 47,XYY do not come to diagnosis. Studies of sperm chromosomes have shown that the frequency of aneuploidy for sex chromosomes varies from 1.5 to 7% in sperm from 47,XXY mosaics and from 2 to 45% in the sperm of non-mosaic 47,XXY men [25]. The majority of the babies born are chromosomally normal, but there is an increased risk for aneuploidy in the offspring of these men.

Men with 47,XYY or 47,XXY/46,XY karyotypes are usually fertile and typically have normal semen analyses [24]. They are slightly taller than their chromosomally normal brothers on average and on average have a normal IQ. About half have learning disabilities requiring special education. [24]. The incidence of men with 47,XYY is about the same as that of 47,XXY in the general population; each is present in about one in 1,000 newborns [24]. However, in infertility surveys—for example, the study by Gunduz et al., noted previously—the finding of 47,XXY is about nine times as frequent as that of 47,XYY [22]. Men with a 47,XYY karyo-

type are represented more frequently among infertile men (0.26%) than in newborn males (0.07%). Their semen analyses are usually normal, as previously noted, but in a minority of cases, they have severe abnormalities of spermatozoa number, motility, and/or morphology [26]. This was observed in a study of 47,XYY males by Martin, in which no sperm was disomic for sex chromosomes by chromosome analysis; 10,000 sperm studied by FISH showed an increase for XY disomy to 0.6%. Martin noted other labs had reported increased frequencies of sperm aneuploidy for sex chromosomes ranging from 0.3 to 15% [25].

Autosomal Abnormalities

The most common autosomal abnormalities seen in infertile men are Robertsonian translocations (see Chap. 9). In the aforementioned review by Van Assche, the incidence of infertile men with this finding was 0.7% [23]. This was 8.5 times the incidence in the newborn survey used for comparison. It appears that the increased frequency of the X-Y bivalent and the trivalents formed by the chromosomes involved in Robertsonian translocations is correlated with the extent of germ cell impairment [27] (see also Chaps. 2, 9, and 10). Martin noted in seven men with Robertsonian translocations that the actual frequency of unbalanced sperm by chromosome analysis is lower than theoretically expected, with 3–27% of sperm being unbalanced because of the translocation. FISH studies showed 7–40% of Robertsonian translocation heterozygotes were unbalanced, with a mean of 15% [25]. When Huang et al. looked at characteristics of embryo development in Robertsonian translocation carriers, they noted that by day 3, whether or not the embryos have a chromosome abnormality could not be determined based upon morphology, but by days 5–6, this was evident. On day 3 after fertilization, there were 161 high-grade embryos, including 59 normal/balanced embryos and 102 abnormal ones, whereas by days 5–6, the blastocyst percentage in the normal/balanced embryo group was significantly higher than that in the abnormal embryo group (44 vs. 20%, $p=0.0000$) [28]. Huang also noted that several studies had shown a strong prevalence of alternate segregation in the gametes of Robertsonian carriers.

The review by Van Assche, which pooled data from several studies, also indicates that 0.5% of men with infertility had reciprocal translocations, as compared to 0.1% in the newborn population. The association between reciprocal translocations involving chromosomes 3–7, 9, 11, 13–15, 16, 17, and 19–22 and the impairment of sperm production has been documented in several studies [14]. Chromosomes from men with reciprocal translocations involving these chromosomes have been observed, at the pachytene stage of meiosis, to have a high frequency of centromeric contacts and chain configurations between the translocation quadrivalent and

the X-Y bivalent (see Chap. 10). These were not seen to any significant degree in the chromosome preparations of the men with reciprocal translocations involving other chromosomes. Martin confirmed this finding in her review, noting that this significant number of quadrivalents was attached to the sex body in the azoospermic carrier and not in the normozoospermic carrier. Her interpretation is that unpaired regions within the quadrivalent are likely to be detected by the pachytene checkpoint, so unsynapsed regions seek each other out and try to pair in order to escape the checkpoint and apoptosis of the cell. She also noted that sperm karyotyping studies of 37 reciprocal translocation heterozygotes have shown that 19–77% of sperm are unbalanced. FISH analyses in 99 reciprocal translocation heterozygotes have also shown a large range in the frequency of unbalanced sperm, from 37 to 91% [25].

Inversions

It has been suggested that paracentric inversions are harmless, but recombinant chromosomes have been observed in newborns, and the risk of viable recombinants has been estimated at 3.8% [25]. Two men with paracentric inversions were studied by sperm karyotyping. Neither showed recombinant chromosomes in sperm; one case was studied by FISH, and 1% of sperm were recombinant, with both dicentric and acentric chromosomes seen [29]. So the risk for paracentric inversions appears to be low.

Sperm karyotyping has been performed in seven men with pericentric inversions. Four had no recombinant chromosomes, and three had frequencies of imbalance from 11 to 31%. FISH studies have been performed in 24 pericentric inversion carriers with the frequency of recombinant chromosomes varying from zero to 54%. The inversions that produce recombinant chromosomes are large ones encompassing more than half of the length of the chromosome in most cases. An overall risk at prenatal diagnosis has been estimated at 10–15%, but the risks are clearly dependent on the individual inversion.

In Zuffardi and Tiepolo's review of 7,277 men, the range of autosomal abnormalities was 0.6–1.6%, with an average of 1.1% [30]. Overall, the incidence of balanced translocations was 8.9 per thousand, which is six times greater than 1.4 per thousand newborns they used as a control population. For Robertsonian translocations, the incidence in infertile men was 10 times higher than in newborns—5.9 per thousand vs. 0.6 per thousand [30]. For a comparison of chromosome abnormalities seen in studies of infertile men, see Table 11.6.

Microdeletions of the Y Chromosome

The fact that genes necessary for spermatozoa production are on the long arm of the Y chromosome was first described in a study published in 1976 by Tiepolo and Zuffardi [32]. They studied six azoospermic males and found deletions at Yq11

that were not present in the fertile fathers and brothers of the men. These were the first deletions found on the Y chromosome; some are detectable by conventional cytogenetic testing, whereas others are not (see Chap. 9). Thus, molecular or molecular cytogenetic testing is required to detect these deletions. This deleted region was called AZF for azoospermia factor. The AZF region has 31 Y-specific genes, 14 of which are protein coding and the rest non-protein coding genes, which have been subdivided as described later [33]. It is now estimated that microdeletions of the Y chromosome are present in 8–15% of men with non-obstructive azoospermia or severe oligozoospermia—that is, men with a spermatozoa count of $<5 \times 10^6$ per mL [34, 35].

The Y chromosome has been continually subdivided over the years into more refined regions. These subdivisions have been developed on the basis of nonoverlapping deletions in patients with non-obstructive azoospermia or oligospermia. The original AZF region now consists of AZFa, AZFb, and AZFc [35]. These microdeletions are associated with various histopathologies and abnormal semen parameters, as shown in Table 11.7. These are not strict categories, but some generalizations can be made.

Many studies have been published about the incidence of microdeletion of the Y chromosome in men with infertility. The results have varied significantly, probably because of selection criteria and because of differing numbers of sequence-tagged sites (STS), known stretches of DNA that can be amplified by polymerase chain reaction (PCR). However, some findings appear consistent. These deletions are found primarily in men with azoospermia or severe oligospermia. The most common deletions are AZFc or AZFc plus AZFb, which together comprise more than three-fourths of deletions. AZFa deletions occur in fewer than 5% of men with AZF deletions [15].

These deletions are thought to arise *de novo* from fertile fathers with an intact Y chromosome; as such, they represent one of the most frequent structural chromosome abnormalities, affecting one in about 5,000 males [36]. Previous reports have shown that boys born from oligospermic men treated using ICSI have an increased risk of carrying a Y chromosome microdeletion [34]. This suggests that these deletions can exist in a mosaic state in the testes of some men. This was seen to a limited degree in a study by Le Bourhis et al. in a study of 181 infertile men with azoospermia or severe oligospermia (sperm count $<3 \times 10^6$ spermatozoa/mL) [36]. Of these, 18 had an abnormal karyotype, and of the remaining 163, six (5.5%) were shown to have a microdeletion of the Y chromosome. Two of the men, both with oligospermia, had germ cell mosaicism of 1.97 and 4.13%, respectively, of spermatozoa with a deleted Y chromosome.

A telling study was performed by Krausz et al., who studied 131 infertile males for the presence of a Y chromosome

Table 11.6 Constitutional cytogenetic studies of infertile men (includes men with azoospermia and oligospermia) [30, 31]

N	47,XXY (%)	47,XXX/46,XY (%)	Other sex chrom abn(%)	Y chrom struct abn(%)	46,XX (%)	Rob T (%)	Rec T (%)	Inv (%)	+ Mar (%)	Other (%)
2,247	139 ^a (6.2)	—	6 (0.26)	15 (0.67)	3 (0.13)	25 (1.1)	5 (0.22)	1 (0.04)	2 (0.09)	—
153	9 (5.8)	3 (2.0)	2 (1.3)	3 (2.0)	1 (0.65)	2 (1.3)	2 (1.3)	—	—	—
342	2 (0.58)	2 (0.58)	2 (0.58)	—	—	2 (0.58)	1 (0.29)	1 (0.29)	—	—
2,372	24 (1.0)	—	7 (0.30)	2 (0.08)	—	4 (0.17)	10 (0.42)	—	4 (0.16)	—
281	57 (20.3)	1 (0.36)	6 (2.1)	1 (0.36)	2 (0.71)	3 (1.1)	5 (1.8)	—	—	—
57	9 (15.8)	—	1 (1.8)	1 (1.8)	—	—	3 (5.2)	1 (1.8)	2 (3.5)	—
1,000	21 (2.1)	—	? ^b	? ^b	—	2 (0.2)	—	—	—	—
2,542	147 (5.8)	NA	7 (0.26)	18 (0.7)	3 (0.10)	26 (1.0)	6 (0.24)	1 (0.04)	3 ^c (0.12)	4 ^d (1.5)
1,363	57 (4.2)	NA	—	—	1 (0.07)	11 (0.81)	3 (0.22)	—	4 ^e (0.29)	20 ^f (1.5)
Total 10,357	465 (4.5)	6 (0.06)	31 (0.30)	40 (0.39)	10 (0.10)	75 (0.72)	35 (0.34)	4 (0.04)	15 (0.15)	24 (0.23)
Newborns	39/36,855 (0.11)	—	—	—	—	51/59,514 (0.09)	55/59,514 (0.09)	7/59,514 (0.01)	13/59,514 (0.02)	—

Abbreviations: N number of men studied in that series, Other sex chrom abn other sex chromosome abnormalities, Y chrom struc abn Y chromosome structural abnormalities, Rob T Robertsonian translocation, Rec T reciprocal translocation, inv inversion, mar marker chromosome NA not applicable; mosaics included in 47,XXX column in these surveys

^aIncludes 47,XXY and 47,XXX/46,XY

^bIncludes six cases described as XYY, deletions, and rings

^cDescribed as an "extra G"

^ddel(14p); del(15p); fragile site at 16q22; "ring E"

^eIncludes several heteromorphisms and other variants not generally reported in other series

^fFrequency of XYY in newborn series was 33 in 36,855, or 0.09%

Table 11.7 AZF gene regions and their usual phenotypes [35]

Gene region	Phenotype
AZFa	Absence of germ cells, aka Sertoli cell-only syndrome (SCOS)
AZFb	Maturation arrest at spermatocyte stage
AZFc	Variable from SCOS to severe oligospermia

microdeletion. Of this group, 46 were idiopathic and 85 were not. Nineteen percent of idiopathic males with normal 46,XY chromosomes had microdeletions of the AZFa, b, or c region. Of the group with known causes of infertility, 7% were found to have microdeletions and a 46,XY chromosome complement, including deletions of the AZFb and c regions. They recommend that all males with reduced or absent sperm counts seeking assisted reproductive technologies be screened for microdeletions of the Y chromosome [37].

The presence of the deletions has been suggested to create instability of the Y chromosome, as noted by Martin [25]. She described 12 mosaic 45,X/46,XY patients with Turner syndrome traits or sexual ambiguity, and one-third had Y chromosome microdeletions. In another study described in the review, FISH studies were performed on 11 men with AZFc deletions, and only 33% of Y-bearing sperm were found compared with 49% in controls. It was suggested that men with a Y chromosome microdeletion might have an increased risk of 45,X and 47,XXY offspring as well as mosaic offspring because of loss of the Y chromosome.

Several genes and gene families have been isolated from the AZFb and AZFc regions [33, 38, 39]. One family is called RBMY, or RNA-binding motif. It consists of genes and pseudogenes that encode proteins involved in pre-mRNA processing and transport [33, 40]. Unlike other such genes, however, it is expressed only in the testes. The functional copies of RBMY are in the AZFb region [41]. A homolog for RBMY exists on the X chromosome [42, 43]. Delbridge et al. suggested that RBMY and its homolog on the X chromosome, RBMX, evolved from a gene on the mammalian proto-X and proto-Y pair at least 130 million years ago before the divergence of eutherian and metatherian mammals [42]. Other important genes are the *KDM5D* gene on AZFb and in AZFb/c the *CDY* gene [33].

Another family of genes is from the AZFc region. It is called the *DAZ* (deleted in azoospermia)/*SPGY* (spermatogenesis gene on the Y) family. This group of genes, thought to have four copies on the Y chromosome, also codes for RNA-binding proteins. A homologous gene to *DAZ* called *DAZL1*, for DAZ-like 1, is on chromosome 3 at band p24. This gene is expressed in ovarian cells as well as in the testes. Most of the genes on the Y chromosome are expressed more widely and have homologs on the X chromosome [33, 43, 44].

In the AZFa region, two main genes have been localized. They are known as *USP9Y* (formerly known as *DFFRY*,

DBY, and *UTY*) and *DDX3Y*, both thought responsible for the infertility phenotype observed. Men with deletions of the AZFa region have lost the *USP9Y* gene, resulting in no germ cells being present. These genes are expressed widely and have homologs on the X chromosome [15].

In summary, the genes *KDM5D* in AZFb and *DAZ* and *CDY* in AZFb/c are thought to represent key determinants for spermatogenesis, in addition to *DDX3Y* in AZFa, although much is yet to be learned [33].

Intracytoplasmic Sperm Injection

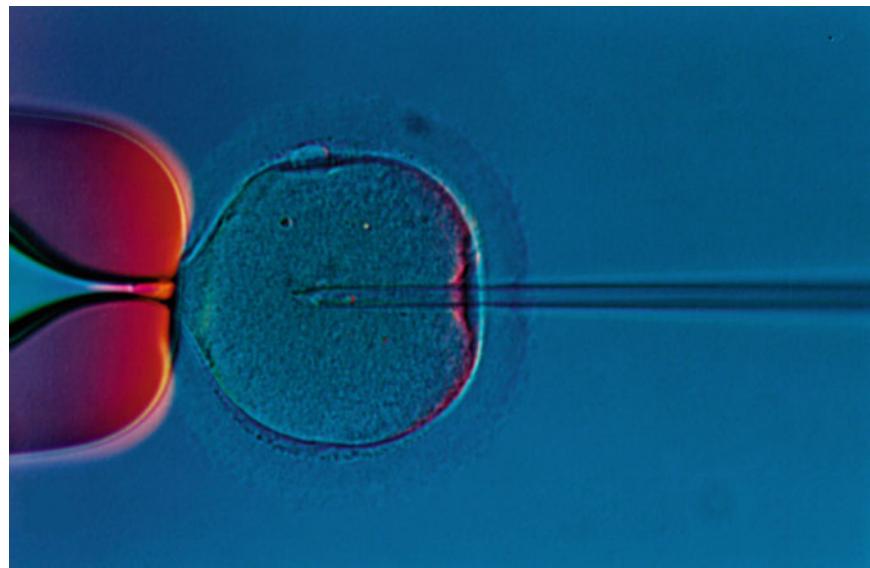
Intracytoplasmic sperm injection (ICSI) is the physical insertion of a spermatozoan into the ovum in a laboratory setting. Figure 11.3 is a photograph demonstrating ICSI. The spermatozoan may be extracted prior to the site of obstruction or from the testis, or it may be taken from several collections of semen that have been ultracentrifuged to collect any spermatozoa that might be present. Using micromanipulation techniques, an embryologist grasps the ovum with one instrument and the spermatozoan with another and injects the spermatozoan into the cytoplasm of the ovum. This has been used for several years for men with 47,XXY and for men with severe male factor infertility for other reasons.

Although this technique has allowed people to have biological children who would not otherwise have been able to, serious consideration of transmissible genetic conditions to offspring must be given, and genetic counseling should be offered to couples prior to undergoing the procedure.

Some of the reasons for this have been discussed previously. A man with a microdeletion of the Y chromosome would be expected to pass on the Y chromosome with the deletion to his sons—in other words, to make his infertility an inherited trait. One group studied the Y chromosome of 86 men who fathered 99 sons by ICSI to determine the incidence of vertical transmission and *de novo* deletions in the sons [45]. Two of the men, or 6.9%, were found to have an AZFd/c deletion, and identical deletions were seen in their sons. No *de novo* deletions were detected in any of the remaining 97 sons of the other men, who did not have deletions themselves.

Levron et al. evaluated the risk for cytogenetic abnormalities in offspring of men with nonmosaic 47,XXY or Klinefelter syndrome [46]. They obtained testicular biopsies from 20 patients and found testicular sperm in eight of them. Four couples became pregnant following ICSI. Sperm chromosomes were analyzed in five patients. Of 112 sperm, seven (6.3%) had chromosome abnormalities, of which five were sex chromosome abnormalities and two had monosomy 18. Six children were born, all with normal karyotypes. The authors discussed the probability of normal germ cell lines as the origin of sperm with normal chromosomes in these men.

Fig. 11.3 Intracytoplasmic sperm injection (ICSI). In this photograph, a technologist is viewing an ovum and spermatozoan under a microscope and injecting the spermatozoan into the cytoplasm of the egg (Courtesy of ViaGeneFertility.com.)



As for the studies that show a high risk of sex chromosome abnormalities in men with gonadal failure and low FSH undergoing ICSI, they postulate that their spermatozoa undergo meiotic errors as part of their underlying disorder and therefore have a higher risk of having offspring with sex chromosome abnormalities.

This suggestion was supported by the findings of Giltay et al., who examined semen specimens from seven severely oligospermic ICSI candidates and compared results to three normal specimens and to ten ICSI candidates with normal constitutional karyotypes but with oligoasthenoteratozoospermia (OAT) [47]. Six of the ICSI candidates had a numerical sex chromosome abnormality, including 45,X/46,XY mosaicism, 47,XXY/46,XY mosaicism, and 47,XXY. One man had an inversion of the Y chromosome. Chromosome aneuploidy rates for chromosomes 18, X, and Y by FISH were high in the ICSI candidates with and without constitutional chromosome abnormalities, both for the sex chromosomes and chromosome 18, compared to the normal controls. They conclude that males with sex chromosome abnormalities have no higher risk of producing offspring with a sex chromosome abnormality by ICSI than do OAT males with normal karyotypes.

Viville et al. examined the role of morphology of spermatozoa and chromosome abnormalities of the spermatozoa [48]. They examined specimens from a patient with shortened flagella syndrome, a patient with globozoospermia, a patient with spermatozoa with irregular acrosomes, and a patient with macrocephalic spermatozoa with associated multiple flagella. From 1,656 to 5,000 spermatozoa were analyzed from patients and 5,064 to 7,423 spermatozoa from controls. They employed three-color FISH and found that patients one through three had signals that compared with normal controls. Patient 4—the one with macrocephalic

spermatozoa—showed an elevated Y to X ratio and elevated aneuploidy to diploidy rate. The authors concluded that patients with the first three forms of teratozoospermia are good candidates for ICSI, and patients with macrocephalic spermatozoa are not.

However, in his review of genetic risks of ICSI, Johnson cited a publication that suggested spermatozoa with amorphous, round, and elongated heads are associated with an increased frequency (26%) of structural chromosome abnormalities when compared with that of morphologically normal spermatozoa [27].

Bonduelle et al. performed a study to determine whether prenatal cytogenetic abnormalities after ICSI could be related to sperm parameters [49]. Of 1,586 fetuses, chorionic villus sampling (CVS) was performed on 698, and amniocentesis was performed on 888. Of these, 47 (3%) had abnormal karyotypes; 25 of these (2%) were *de novo*. They found a 2.1% *de novo* prenatal chromosome abnormality rate for sperm concentrations, of $<20 \times 10^6$ per mL and 0.24% abnormality rate for sperm concentrations of 20×10^6 per mL or greater. The likelihood of a chromosome abnormality was associated with spermatozoa motility and concentrations, but not morphology in this study. The *de novo* chromosome abnormality rate of 1.6% vs. 0.5% risk for women aged 33.5 years ($p < 0.007$) was seen in ICSI offspring; most of the increase was in sex chromosome abnormalities, while some were due to autosomal chromosome abnormalities.

Effect of Chromosome Abnormalities on Preimplantation Embryo Development

A study to evaluate the influence of aneuploidy upon preimplantation embryo development was conducted by Rubio

et al. [50]. They evaluated 6,936 embryos from 1,245 women undergoing PGD for various indications. Embryo biopsy was performed on day 3. Normal euploid embryos showed significantly higher blastocyst rates of 68.2% compared to chromosomally abnormal (42.8%) and mosaic (53.7%) embryos (all $p < 0.0001$). Among autosomes, higher blastocyst rates were seen in trisomies than monosomies, although statistically significant only in women over 36 years of age. For sex chromosome aneuploidy, similar blastocyst rates were seen between trisomies and monosomy X.

Chromosomal Mosaicism in Pregnancies from Couples with Infertility

Huang et al. evaluated 5,337 consecutive chorionic villus sampling specimens and found 1.29% were mosaic. Those from spontaneous pregnancies versus from infertility treatment were 1.22 vs. 1.32%, respectively. A subgroup of infertile couples comparing *in vitro* with *in vivo* fertilization showed rates of 1.84 and 0.41%, respectively, which is not statistically significant [51].

Pregnancy Rates, Obstetric Outcomes, Chromosome Abnormalities, and Birth Defects After ICSI

In 2006, 138,198 ART procedures were reported to the Centers for Disease Control, resulting in 41,343 deliveries of liveborn and 54,656 babies. They accounted for 1% of live births nationwide and 18% of multiple births. All but 12% of the centers providing ART provided outcomes data to the CDC. The question of ICSI was evaluated separately. No clear advantage was found of using ICSI when treating couples with no indication of male factor infertility. Cycles using ICSI with no indication of male factor infertility were less likely to fail before transfer but more likely to result in implantation failure, pregnancy loss, and a lower overall chance of live birth [52].

Palermo et al. performed a study in New York City of 751 couples in whom 987 ICSI cycles were undertaken [53]. The male partner was thought to be the cause of repeated *in vitro* fertilization (IVF) failures. The pregnancy rate was 44.3%, defined as the detection of a fetal heartbeat, with a delivery rate per ICSI cycle of 38.7%. In eight of the 11 spontaneous abortions for which cytogenetic information was available, an autosomal trisomy was found, and seven additional pregnancies were terminated because of a chromosome abnormality after prenatal diagnosis. An equal number were delivered vaginally versus by Caesarean section; about half of those delivered by Caesarean section were multiple gestations.

Fifteen of 578 newborns in this study had birth defects—nine major and six minor—and this frequency of 2.6% compared to the frequency seen in the IVF frequency seen in that center. The major birth defects were Goldenhar syndrome, ventricular septal defect (VSD), hypoplastic right heart and pulmonary stenosis, pyloric stenosis, cleft palate, aqueductal stenosis, spina bifida, and hydronephrosis [2]. All of those were seen in multiple gestations except the VSD, which was present in a singleton. The minor birth defects were hypospadias, urethral obstruction, and double ureter [4]. Hypospadias was seen in two singletons and a set of twins, and the double ureter was present in a twin. The urethral obstruction was seen in a singleton. The conclusion of this study was that standard IVF and ICSI are similar in pregnancy evolution and in incidence of birth defects.

More recently, Simpson et al. examined ICSI data from the United States in 1997 [54]. Of 6,077 ICSI cycles begun, there were 17.5% pregnancy losses, and that same cohort showed a malformation rate of 1.7% in the live-born babies. In their paper, they note that the Swedish IVF Registry of 1,139 ICSI babies listed a relative risk of 2.9 for hypospadias. Birth weight and prematurity rates in ICSI were found to be similar to those of conventional IVF. In a cohort of 1987 pregnancies in Brussels, *de novo* autosomal rearrangements of 0.36% and *de novo* sex chromosome abnormalities of 0.83% were seen, both higher rates than are expected in the general population. This raises the question of whether these abnormalities are *de novo* in fact or would have been considered to be inherited if more intensive genetic study of their parents had been carried out before ICSI had been initiated.

After Bonduelle et al. published a prospective follow-up study of 423 children born after ICSI in 1996, Kurinczuk and Bower published a different interpretation of their data [55, 56]. They applied the Western Australian classification system of birth defects to their own population registry of children and to the Belgian data reported by Bonduelle et al. They determined that the Belgian children were twice as likely as Western Australian babies to have a major birth defect (7.4 vs. 2.3%, odds ratio 2.3) and almost 50% more likely to have a minor birth defect (odds ratio 1.49). These reports highlight the importance of using a standard system of classification when reporting outcomes measures. As an example, the Belgian data considered coronal hypospadias and renal duplication to be minor defects, while in the Western Australian system these were considered to be major defects. Results suggested an excess occurrence of cardiovascular, gastrointestinal, and genitourinary defects generally. The authors report their findings with caution, as the numbers are small.

The Centers for Disease Control reported that septal heart defects, cleft lip with and without cleft palate, esophageal atresia, and imperforate anus were more common in babies conceived by ART, with and without ICSI. The relative risks

were 2, 2.4, 4.5, and 3.7, respectively. The data were derived from mothers of about 13,500 babies born with birth defects and mothers of more than 5,000 babies without birth defects born from October 1997 through December 2003 in 10 states. The findings applied to singletons only. About 1% of the babies without birth defects were conceived through ART, compared to 2.4% of those with birth defects [57].

The contribution of male age to outcomes in ART was studied by Whitcomb et al., who reviewed 1,392 cycles from 1,083 women and their male partners. They found no significant association with the likelihood of live birth after adjustment for female recipient age [58].

Imprinting, IVF, and ICSI

A review of the troublesome phenomenon of imprinting errors in the offspring conceived by *in vitro* fertilization, with or without ICSI, was published by Gicquel et al. [59]. In this report, they note that in their series of 149 patients of Beckwith-Wiedemann syndrome (BWS), six were born following IVF, two of whom involved ICSI. All six showed demethylation of *KvDMR1*, a finding seen in 90 of the 149 patients. Demethylation of *KvDMR1* is an epigenetic, or imprinting, abnormality. In the same review, they note other reports of BWS after IVF, and in all patients studied, the same demethylation finding was present. They estimate an odds ratio of 3.2 for the risk of BWS after IVF compared to that of the general population. In addition, they comment on three patients born with Angelman syndrome (AS) and imprinting defects, which in Angelman syndrome is a rare finding. Menezo et al. studied some of the biochemical and metabolic pathways in oocytes and embryos that might have relevance to methylation and imprinting (see Chap. 20) during IVF. They noted that ovarian stimulation leads to elevated follicular homocysteine, which might affect methylation. This should be balanced by taking folic acid and other B vitamins. In addition, they comment on a trend to culture early human embryos in culture medium lacking essential amino acids. As a result, methionine is not present in the first 3 days of culture, when methylation is important. They recommend the use of culture medium with essential amino acids in IVF, and they also urge caution in extrapolating mouse data to humans [60].

In two reviews of imprinting disorders and ART, Owen et al. and Manipalviratn et al. summarize the literature, including mention of animal studies that have shown that ART procedures can alter normal imprinting, specifically DNA methylation patterns [61, 62]. Collectively, the studies suggest a possible association between ART and loss of maternal methylation. Owen's group noted the estimated prevalence of ART in Beckwith-Wiederman syndrome (BWS) populations ranging from 2.9 to 5.6% from case series reports and two

survey studies, whereas the only case-control study had shown the estimated prevalence of ART in BWS populations to be as high as 10.8% [61]. Their assessment based upon studies to date was that there is likely an association between BWS and ART. The association between AS and ART was described as more tenuous, and for Russell-Silver syndrome, it is inconclusive. For maternal hypomethylation, although evidence is limited, there is no suggestion of an association between ART and Russell-Silver syndrome. For retinoblastoma, only one study has shown a significant association, although the sample sizes have been small.

Manipalviratn et al. agreed with the conclusions regarding BWS, AS, and retinoblastoma and did not comment on the other disorders [62]. Both expressed the need for prospective, larger studies to address these questions, and neither recommended testing of pregnancies for these disorders, although counseling was recommended.

This discussion makes it clear that more careful, prospective follow-up of children born after IVF and other assisted reproductive techniques must be carried out and that information should be made readily available to people who are contemplating such procedures. In the meantime, prospective parents should be told that the birth defect rate after ART may be twice that of the general population and that much remains to be learned.

And What About the Mitochondria of the Spermatozoa?

Does the offspring inherit paternal mitochondria after ICSI? After all, the entire spermatozoa is injected into the ovum. Two groups have examined this question, and to a sensitivity level of 0.001 and 0.5%, no paternal mitochondrial DNA has been detected in the offspring, placenta, or umbilical cords after ICSI [63, 64].

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Linda Marie Randolph

Introduction and History

Amniocentesis

Amniocentesis, the transabdominal or transcervical puncture of the uterus for the purpose of removing amniotic fluid, has been practiced since the 1930s [1]. It was used in the early 1950s in the prenatal evaluation of Rh sensitization [2].

A key event that laid the foundation for prenatal cytogenetic analysis was the discovery of the ability to determine gender on the basis of the incidence of the sex chromatin body observed in the nuclei of oral mucosa smears [3, 4]. In 1956, James described the use of amniotic fluid sediment to determine fetal sex by Papanicolaou and Giemsa stains, and Fuchs and Riis showed in amniotic fluid of term pregnancies that they could accurately determine the fetal sex in 20 of 21 cases [5, 6]. It is of interest that they concluded,

Although transabdominal puncture of the uterus has been carried out often for therapeutic and experimental reasons without accidents, mere curiosity does not justify the procedure, and its practical value is probably limited in the human. If the results are confirmed in animals, however, it might become of great significance in veterinary practice [6].

Other investigators confirmed the accurate determination of fetal gender by similar procedures, staining amniotic fluid obtained at term by various techniques [7, 8].

In 1966, Steele and Breg demonstrated, in a study of amniotic fluid obtained from women because their fetuses were at risk for erythroblastosis fetalis, that human amniotic cells could be cultured and the chromosomes analyzed [9]. They foresaw that this "...would allow more practical genetic

counseling of mothers with high risks of having children with chromosome abnormalities or inborn errors of metabolism" [9].

Further refinement of the technique and timing of amniocentesis were demonstrated in a 1967 paper by Jacobson and Barter, and they proposed that the optimal timing of amniocentesis is 16 weeks after performing the procedure from 5 weeks to term in 85 women [10]. Of these 85, 57 were successfully cultured. In a thoughtful discussion after the paper, Edward C. Hughes noted that, "Speculation might go so far as to suggest that, although chromosome constitution cannot be changed, a specific DNA that would carry the coding information lacking in certain diseases might replace the missing element," and in the same discussion, S.R.M. Reynolds pointed out that, "...in the future there will be even more refined methods of evaluating gene abnormalities in which the karyotype appears normal."

In 1968, Nadler and Gerbie described the use of amniocentesis for the detection of cytogenetic and biochemical abnormalities in 155 women at increased risk for these disorders. They reported a highly successful culture rate of 97% and uniformity of timing of the procedure, from 13 to 18 weeks [11].

By 1986, more than a quarter of a million amniocenteses had been performed for cytogenetic analysis, and the number to date is undoubtedly in the millions [12]. Although other means of prenatal cytogenetic testing have been developed, amniocentesis is by far the most common technique performed today for prenatal genetic diagnosis. In the United States in 2003, 66,901 procedures were reported from 31 states and the District of Columbia [13].

Chorionic Villus Sampling

Although techniques for transcervical and transabdominal placental biopsy, or late chorionic villus sampling (CVS), were described in the 1950s and 1960s for the diagnosis of hydatidiform mole, the first paper describing a technique for

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fetal genetic diagnosis was published in 1968 [14–16]. Mohr developed techniques for sampling fetal cells no later than the third month of pregnancy by a transabdominal approach. Due primarily to the absence of real-time ultrasound, low culture success rate, and the risks of endoscopic approaches, as described by Kullander and Sandahl in 1973 and by Hahnemann in 1974, the technique was not widely used in the United States [17, 18]. In Kullander and Sandahl's experience, 19 of 39 specimens (48.7%) were successfully cultured, which they described as a "high percentage" [17]. In Hahnemann's experience, there was a 38% success rate, with causes of failure being puncture or biopsy of the amniotic membrane and bleeding [18]. The optimal time of performing the procedure was the 10th week of gestation, and although the procedure had a low success rate in terms of obtaining tissue, the culture success rate was 91%. All but one of the pregnancies was terminated by previous intention, and in the one continuing pregnancy, the newborn was normal.

In China, transcervical CVS was widely used in the 1970s as a method of fetal sex prediction and selection. A report of the Chinese experience was published in 1975 [19]. The accuracy of their fetal sex prediction, based on X chromatin, was 94%. Efforts to replicate this success were unsuccessful for several years [20, 21].

In their 1981 paper, Niazi and Loeffler reported an improved technique using trypsin for culturing trophoblastic cells obtained by transcervical CVS, minimizing the risk of maternal cell admixture in fetal cells [22].

The first use of real-time ultrasound scanning in CVS was reported in 1982 by Kazy et al [23]. Of their 165 patients, 139 had biopsies performed prior to induced abortion, and in 26 patients, biopsy was performed for genetic reasons. Of the eventual 13 continuing pregnancies, none was spontaneously aborted, and all 11 babies who had been born to date were normal. Fetal sex prediction by X chromatin was accurate in all cases. This was the first study that brought CVS out of the experimental category and into the world of a promising prenatal diagnostic test. As of 1996, more than 200,000 CVS cases from more than 100 centers worldwide had been entered into an informal World Health Organization-sponsored CVS registry in Philadelphia, which has not been continued [24].

Percutaneous Umbilical Cord Sampling (PUBS)

In the early 1970s, in an effort to develop a method for prenatal diagnosis of hemoglobinopathies, investigators sought to establish safe techniques for fetal and/or placental blood sampling. In his preliminary report of 1973, Valenti demonstrated in 11 women scheduled for abortion that, in the second trimester, a surgical "endoamnioscope" with a

flexible needle introduced through it could be operated under direct vision [25]. This required regional or general anesthesia and an abdominal wall incision. Three of the women had umbilical cord puncture, and the blood obtained was shown to be of fetal origin. Hobbins and Mahoney performed fetoscopy in 34 women scheduled for abortion [26]. Local anesthesia was provided, and the cannula was smaller than the endoscope used by Valenti. In eight of these, successful blood sampling of a placental vessel was attempted and achieved. However, in only one of these cases was the composition of the blood 100% fetal. Placentocentesis was essentially replaced by cordocentesis thereafter.

Daffos et al. demonstrated in 50 women referred for abortion that by using local anesthetic, real-time ultrasound, and puncture of the umbilical vein, pure fetal blood was obtained in 46 cases [27]. Sixteen of the 50 women underwent abortion 2–10 days later, by which time none of these fetuses had died. Twelve other women delivered healthy babies, and 22 pregnancies were ongoing.

The technique was later applied, by the same group, in 606 samplings of 562 women with a variety of indications for prenatal diagnosis [28]. Complications were seen in 15%, including a 2% rate of fetal death or spontaneous abortion. By obtaining larger volumes of fetal blood, these investigators were able to perform physiologic and hematologic assays that helped provide the basis for normal values in fetal blood, and they showed that percutaneous umbilical cord sampling (PUBS) deserved a place in the prenatal diagnostic-testing world.

The Incidence of Chromosome Abnormalities

Combining surveys from 1969 to 1982 of 68,159 liveborn babies, one in 156 live births were found to have a major chromosome abnormality (see Table 12.1) [27]. The most common is trisomy 21, or Down syndrome, with an incidence from these surveys of 1 in 833 live births. The next most common are sex chromosome aneuploidies, with one XYY or XXY per 1,000 male liveborns and one XXX per 1,000 female liveborns [29]. Because non-banded chromosome preparations were used in the early survey years (from 1969 to 1975), it was thought, when Giemsa banding (G banding) was introduced, that the incidence of chromosome abnormalities would be found to be higher. However, in a 1980 study by Buckton et al. of 3,993 newborns, no significant difference in the frequency of rearrangements or of other chromosome aneuploidies was found [30].

It is clear that the incidence of most fetal chromosome abnormalities increases with maternal age. Data for women ages 35–49 were compiled by Hook based on North American collaborative studies and the New York State registry [31]. His analysis of the data indicated a 30% differential between

Type of abnormality	Total abnormalities (%)
<i>Sex chromosomes, males</i>	
47,XYY	45 (0.103)
47,XXY	45 (0.103)
Other	32 (0.073)
<i>Sex chromosomes, females</i>	
45,X	6 (0.024)
47,XXX	27 (0.109)
Other	9 (0.036)
<i>Autosomal trisomies</i>	
47,+21	82 (0.120)
47,+18	9 (0.013)
47,+13	3 (0.004)
Other	2 (0.002)
<i>Structural balanced arrangements</i>	
Robertsonian translocation	
der(D;D)(q10;q10) ^a	48 (0.070)
der(D;G)(q10;q10) ^b	14 (0.020)
Reciprocal and insertional translocation	64 (0.093)
Inversion ^c	13 (0.019)
<i>Structural unbalanced arrangements</i>	
Robertsonian	5 (0.007)
Reciprocal and insertional	9 (0.013)
Inversion	1 (0.001)
Deletion	5 (0.007)
Supernumerary	14 (0.020)
Other	9 (0.013)
Total abnormalities	442 (0.648)
<i>Total babies surveyed</i>	
Males	43,612
Females	24,547

Data from reference [27]

^ader(D;D) refers to Robertsonian translocations involving chromosomes 13, 14, and/or 15

^bder(D;G) refers to Robertsonian translocations involving chromosomes 13, 14, or 15 and 21 or 22

^cExcludes common pericentric inversion of chromosome 9

the rates observed at amniocentesis and those seen at birth, a figure that is still valid almost 20 years later.

In 1982, Schreinemachers et al. analyzed data on the results of 19,675 prenatal cytogenetic diagnoses on women aged 35 and older for whom there was no known cytogenetic risk for a chromosome abnormality except parental age [32]. The expected rates at amniocentesis of clinically significant cytogenetic abnormalities by maternal age were obtained and compared with previously estimated rates by maternal age in live births. A differential between amniocentesis and live birth incidences was shown for trisomies 21, 18, and 13 but not for 47,XYY and 47,XYY (see Table 12.2) [29–34]. In the following year, Hook confirmed and refined the differences in the incidences for trisomies 21, 13, and 18 and also found a difference between fetal and newborn rates of

47,XXY, 47,XYY, 45,X, and 45,X/46,XX, but not for 47,XXX (see Table 12.3) [33]. Contrary to what was found in other studies, there was no significant maternal age effect in the incidence of fetal death of chromosomally abnormally fetuses.

The incidence of *de novo* balanced structural rearrangements in 337,357 amniocenteses was reported by Warburton [35]. Another survey of *de novo* balanced chromosome rearrangements in prenatal diagnosis was published by Giardino et al. [36]. The results are shown in Tables 12.4 and 12.5.

Spontaneous Abortions

It is a well-established fact that the incidence of major chromosome abnormalities is much higher in first-trimester spontaneously aborted fetuses than later in pregnancy and at birth. The incidences in various studies range from 20 to 60%, with the average in pooled data of unselected spontaneous abortions being 41% (see Table 12.6) [29, 37]. A cautionary note in consideration of this high incidence range is that the tissue cultured and analyzed may not represent the fetus. It has been shown that 45,X cells and some lethal trisomies seen in chorionic villus samples may not be seen in the fetus, so this may lead to spurious elevation of estimates of chromosome abnormalities in spontaneous abortion tissue [38]. Notwithstanding this caveat, the following frequencies of chromosome abnormalities are reported in spontaneous abortions: autosomal trisomies comprise the largest group of 52% of chromosome abnormalities, followed by 45,X at 19%, triploidy at 16%, and tetraploidy at 6% [37].

The association between advanced maternal age and the incidence of trisomies has been demonstrated in spontaneous abortions. Of interest is that in a study of 494 girls with Turner syndrome born in Sweden from a population of 1.6 million girls, among women older than 40 years, 3.2% gave birth to an affected daughter, compared to 1.8% of the population controls. This amounted to an odds ratio of 1.83 [39]. In previous publications, 45,X appeared to be associated with younger maternal age, with, for example, about one-third of 45,X spontaneous abortions coming from women 20–24 years of age [40]. The distribution of trisomies is quite different from that seen at birth or even at amniocentesis, with 30% being trisomy 16, compared to almost negligible rates of trisomy 16 at amniocentesis (see Table 12.7) [37].

This topic is also covered in detail in Chap. 13.

Stillbirths and Neonatal Deaths

Fetal loss from 28 weeks on in pregnancy is defined as stillbirth, and neonatal death refers to death occurring within the first 4 weeks after birth. Chromosome studies in such cases

Table 12.2 Maternal age-specific rates (%) for chromosome abnormalities

Maternal age (years)	From liveborn studies ^a			From amniocenteses			From CVS	
	47,+21 ^b	47,+21 ^c	All chromosome abnormalities ^b	47,+21 ^b	47,+21 ^c	All chromosome abnormalities ^b	47,+21 ^c	All chromosome abnormalities ^d
33	0.16	—	0.29	0.24	—	0.48	—	—
34	0.20	—	0.36	0.30	—	0.66	—	—
35	0.26	—	0.49	0.40	—	0.76	—	0.78
36	0.33	0.35	0.60	0.52	0.31	0.95	0.42	0.80
37	0.44	0.43	0.77	0.67	0.80	1.20	0.68	2.58
38	0.57	0.42	0.97	0.87	0.73	1.54	0.45	3.82
39	0.73	0.79	1.23	1.12	0.84	1.89	2.05	2.67
40	0.94	1.21	1.59	1.45	1.03	2.50	1.20	3.40
41	1.23	2.67	2.00	1.89	1.50	3.23	3.12	6.11
42	1.56	4.28	2.56	2.44	2.92	4.00	2.88	8.05
43	2.00	1.82	3.33	3.23	3.05	5.26	1.20	5.15
44	2.63	—	4.17	4.00	1.52	6.67	2.63	10.00
45	3.33	—	5.26	5.26	2.50	8.33	8.33	7.14

^aEstimated liveborn statistics [32]^bData compiled from 19,675 genetic amniocenteses [32]^cData compiled from 3,041 CVS; 7,504 amniocenteses; and 13,139 with no test [34]. These are observed prevalences^dData compiled by L. Hsu [29]**Table 12.3** Fetal deaths subsequent to amniocentesis

Fetal deaths			
Abnormalities	Number	Proportion (%)	95% confidence interval (%)
47,+21	73	30.1	19.0–42.0
47,+18	25	68.0	46.5–85.1
47,+13	7	42.9	9.9–81.6
47,XXX	39	0.0	0.0–9.0
47,XXY	37	8.1	0.8–11.0
47,XYY	33	3.0	0.08–15.8
45,X	12	75.0	42.8–94.5
45,X/46,XX	19	10.5	1.3–33.1
Balanced translocations and inversions	71	2.8	0.3–9.8
Markers, variants, fragments	27	0.0	0.0–12.8

Data from reference [33]

Note: Proportion refers to the number of fetal losses compared to the total number of fetuses diagnosed with the given abnormality

Table 12.4 The incidence of *de novo* balanced structural rearrangements in 337,357 genetic amniocenteses

De novo rearrangement	Number of cases	Percentage
Reciprocal translocation	176	0.047
Robertsonian translocation	42	0.011
Inversion	33	0.009
Supernumerary small marker chromosome	162	0.040
Satellited marker	77	0.020
Nonsatellited marker	85	0.023
Total	413	0.109

Data from reference [35]

have shown that the incidence of chromosome abnormality is about ten times that in the rest of the population. Combining three studies of stillbirths and neonatal deaths, of those in which chromosome analysis was performed, 52 of 823 (6.3%) studied had a chromosome abnormality. Of these 823, 59 macerated stillbirths were studied, of which seven (11.9%) had a chromosome abnormality. Of 215 nonmacerated stillbirths, nine (4.2%) were chromosomally abnormal, and of 549 neonatal deaths, 33 (6.0%) had a chromosome abnormality [29]. Given the value it provides families in terms of understanding more about their losses and in providing recurrence risks, it is recommended that consideration of chromosome analysis be given in all such cases (see Table 12.8).

Prenatal Cytogenetic Diagnosis

Genetic Amniocentesis

With increased public awareness, number of practitioners, laboratory capacity, proportion of women older than 35 having babies, and use of maternal serum screening, the utilization rate of amniocentesis has grown. It was estimated that in 1974, 3,000 women underwent genetic amniocentesis, and the number now is in the millions. The increased utilization has extended to women of lower socioeconomic status who previously did not have access to or finances for the procedure [41, 42]. With improvements in laboratory procedures, including sterile technique, plasticware, enriched cell culture media, and automated harvesting and imaging systems, the turnaround time for reporting results of an amniocentesis has dropped

Table 12.5 The incidence of *de novo* balanced structural rearrangements in 269,371 prenatal diagnoses

Specimen	Reciprocal translocation (%)	Robertsonian translocation (%)	Inversion (%)	Complex chromosome rearrangement (%)	Total (%)
AF	160 (73)	38 (17)	15 (7)	7 (3)	220 (0.9)
CVS	15 (63)	7 (29)	2 (8)	—	24 (0.8)
FBS	2 (100)	—	—	—	2 (0.5)
Total	177 (72)	45 (18)	17 (7)	7 (3)	246 (0.9)
% Total prenatal diagnoses	0.7	0.2	0.1	0.03	

Data from reference [36]

Table 12.6 Frequencies of chromosome abnormalities in unselected spontaneous abortions

Number of abortuses studied	Number of abortuses (%) with chromosome aberrations	Different types of chromosome abnormalities (% of all chromosome abnormalities)						Reference
		Autosomal trisomy	45,X	Triploid	Tetraploid	Other		
8,841	3,613 (40.87%)	1,890 (52.29%)	689 (19.06%)	586 (16.21%)	119 (5.51%)	249 (6.89%)	[29] ^a	
3,300	1,312 (39.8%)	645 (49.2%)	201 (15.3%)	198 (15.1%)	78 (5.9%)	190 (14.5%)	[37]	

^aData compiled from more than ten studies

dramatically, from several weeks in the 1970s and 1980s to less than a week in some laboratories today. The cost of the laboratory test has dropped as well due to increased efficiency and competition. Thus, prenatal diagnosis by amniocentesis has become, and probably will remain, by far the most common mode of prenatal diagnosis until such time as a reliable, cost-effective noninvasive procedure is developed.

The accuracy of amniocentesis for the detection of recognized chromosome abnormalities is greater than 99%. Diagnostic accuracy has been enhanced by the recent use of fluorescence *in situ* hybridization (FISH) and chromosome-specific probes. These are of particular value in marker chromosome, translocation, and deletion cases, when microscopic findings require further study for clarification [43–50] (see Chap. 17).

Conventional Amniocentesis:

15–24 Weeks of Gestation

Midtrimester, defined here as the 15th through the 24th week of gestation, is by far the most common time period for performing the amniocentesis procedure. Culture of amniotic fluid cells is optimal in this time period, both from the perspective of rapidity of cell growth (and therefore sample turnaround time) and because the culture failure rate is less than 0.5% in experienced laboratories [51, 52].

The risks associated with midtrimester amniocentesis include leakage of fluid, cramping, bleeding, infection, and miscarriage. The risk of miscarriage following midtrimester amniocentesis is related to practitioner experience, number of needle insertions, size of the needle, and other factors [53]. The appropriate risk figure to provide patients is still debated. In spite of the millions of amniocentesis procedures performed and the importance of an accurate risk figure to

provide patients, there has been only one large prospective controlled study performed regarding the risks of amniocentesis. In this paper, known as “the Danish study,” 4,606 women comprised the final study population [54]. Of these, half were randomized to have amniocentesis, and the other half were randomly assigned to the control, non-amniocentesis group. At the conclusion of the study, it was found that the total rate of spontaneous abortion was 1.7% in the study group and 0.7% in the control group ($p < 0.05$). When the women with a high maternal serum alpha-fetoprotein (AFP) were considered, it was found that they had a relative risk of spontaneous abortion after amniocentesis of 8.3 compared to women with a normal maternal serum alpha-fetoprotein level. This equated to an overall relative risk of 2.3. Other factors found to increase the risk of spontaneous abortion were transplacental passage of the needle (relative risk of 2.6) and discolored amniotic fluid (relative risk of 9.9).

Another study from Denmark in 2009 reported on results of a national registry-based cohort study, including all singleton pregnant women having an amniocentesis or CVS between 1996 and 2006 [55]. The fetal loss rate was defined as that occurring before 24 weeks’ gestation. The miscarriage rate after amniocentesis was 1.4% after amniocentesis and was not correlated with maternal age. The number of procedures performed at each center had a significant effect on the risk of fetal loss. In those performing fewer than 500 amniocenteses, the odds ratio for fetal loss was 2.2 (95% CI, 1.6–3.1) when compared to those performing more than 1,500 per year. There was no control group in this study.

In reviews of procedure-related risks from many publications, in which five included a control group, the authors concluded that the procedure-related miscarriage rate from amniocentesis is 0.5–1.0% [56, 57].

Table 12.7 Frequency of autosomal trisomy for each human chromosome among aborted specimens

Trisomy chromosome	Number of trisomies (%)
1	0
2	34 (5.2)
3	6 (0.93)
4	15 (2.3)
5	5 (0.78)
6	5 (0.78)
7	27 (4.2)
8	23 (3.6)
9	18 (2.8)
10	11 (1.7)
11	0
12	2 (0.31)
13	53 (8.2)
14	32 (5.0)
15	52 (8.1)
16	202 (31.3)
17	4 (0.62)
18	23 (3.6)
19	0
20	18 (2.8)
21	54 (8.4)
22	55 (8.5)
Total	645 (100)

Data from reference [37]

Table 12.8 Frequencies of chromosome abnormalities in stillbirths and neonatal deaths: combined data from three studies

Macerated stillbirths	Nonmacerated stillbirths		Neonatal deaths		Total	
	Number karyotyped	Abnormal	Number karyotyped	Abnormal	Number karyotyped	Abnormal
59	7 (11.86%)	215	9 (4.18%)	549	33 (6.0%)	823

Data from reference [29]

Odibo et al. reported on the fetal loss rate after second-trimester amniocentesis in a single center in a retrospective cohort study comparing the fetal loss rate in women having amniocentesis with those not having any procedure [58]. Of the 88% for whom complete outcome data were available, fetal loss in the amniocentesis group was 0.4% compared with 0.26% in those without amniocentesis (relative risk of 1.6, 95% CI, 1.1–2.2). Fetal loss less than 24 weeks occurred in 0.97% of the amniocentesis group and 0.84% of the no-procedure group, so the fetal loss rate less than 24 weeks attributable to amniocentesis was 0.13% (95% CI, 0.07–0.20%), or 1 in 769. The only subgroup with a significantly higher amniocentesis-attributable fetal loss rate was women with a normal serum screen (0.17%, $p=0.03$).

An important and often overlooked component of providing risk assessments to patients is the underlying incidence and timing of pregnancy losses. A prospective study of 220 ultrasonographically normal pregnancies in women recruited prior to conception (in order to avoid bias of selection) found

a pregnancy loss rate after 8 weeks of 3.2% [59]. Other studies have shown a maternal age factor in the loss rate [38]. The prevalence of trisomies is about 50% higher at 16 weeks compared to term pregnancies (*ibid.*), so selection against chromosomally abnormal abortuses is still occurring at 16 weeks. The incidence of spontaneous pregnancy loss after 16 weeks is 1%.

Some genetic counselors and amniocentesis practitioners counsel patients regarding the risk of the amniocentesis relative to the risk of a fetal chromosome abnormality and in effect use this as a decision point. In this way, a woman with a risk of fetal chromosome abnormality of 1 in 200 might be inclined to decline amniocentesis if the risk of miscarriage as a result of the procedure was quoted as 1 in 100 and the risks compared during the counseling session. A maternal age of 35 has traditionally been used as a cutoff for the definition of advanced maternal age because the risk of a fetal chromosome abnormality at this age is roughly equivalent to the originally reported risk of miscarriage as a result of the amniocentesis. This is not sound reasoning because the burdens of

the risks are quite different—one burden being the potential lifetime task of caring for an individual with mental retardation and physical/health problems and the other being miscarriage of a potentially healthy fetus [60].

Early Amniocentesis

Interest in early amniocentesis (EA) rose in the 1980s, due in large part to the continued desire to provide and receive prenatal diagnosis at an earlier gestation without some of the risks and limitations associated with chorionic villus sampling, which are outlined in the following paragraphs. An increase in sophistication in ultrasound technology has also made earlier imaging of fetuses more feasible and has added to the confidence level of the physicians performing the procedure. Adding to this is the opportunity to measure amniotic fluid alpha-fetoprotein and acetylcholinesterase, which is not possible with CVS. One center reported a rise in EA procedures from 3.2% of their 495 procedures in early 1985 to 6.5% of 980 procedures in late 1987 [61].

Early amniocentesis is usually described as one that occurs before 15 weeks' gestation. It has been shown that the earlier a prenatal diagnosis procedure is performed, the higher the fetal loss rate is [62]. One should therefore further divide the periods at which amniocentesis is performed to provide better comparative data for a variety of procedures since, "...true risks ...appear to be a function of gestational age and less related to the procedure performed" [62].

Although the procedure by which EA is performed is similar to that of midtrimester amniocentesis, practitioners report several challenges unique to EA. The placenta is more widely spread, the amniotic fluid volume is lower, and the amniotic membrane is not yet totally adherent to the uterine wall, leading to the "tenting" reported by some physicians [63].

Background

In one study conducted from 1979 through 1986, 4,750 amniocenteses were performed, 541 of which were performed before the 15th week since the last menstrual period [64]. Outcome data were available for 298 women, of whom 108 were under 35 years of age. Fetal loss within 2 weeks of the procedure was seen in five pregnancies, all in the 14th week, when 228 of the 308 women had the procedure. When all spontaneous fetal losses were accounted for, there were eleven spontaneous abortions (3.6%), two stillbirths (0.7%), and one neonatal death (0.3%), resulting in a total post-procedure loss rate of 14/298 (4.7%). No culture failures were seen. The needle gauge was 20, and no difference in outcome was seen in transplacental versus placental passage.

In 1988, the combined experience of six groups, including the study previously mentioned, was reviewed [65]. The total loss rate in 1,240 pregnancies of known outcome ranged from 1 to 4.7%. Cell culture and amniotic fluid alpha-fetoprotein measurements were satisfactory. The conclusion was that EA

is feasible but that other safety issues had not been adequately addressed, such as congenital orthopedic anomalies and neonatal pulmonary compromise, which had been seen in some babies born after midtrimester amniocentesis [66].

Several other studies were published in the early 1990s [67–73]. In one paper, 505 amniocentesis procedures were performed between 11 and 15 weeks' gestation. In all but three pregnancies, follow-up information was available, including 16 fetal losses (3.1%)—ten in the 2 weeks after the procedure and six within the 28th week of gestation. The authors reported a significantly higher risk for fetal loss when the amniocentesis was performed at the 11th–12th week of gestation compared with the 13–15-week group. The fetal loss rate between the 12–13-week and the 14–15-week groups showed no statistically significant difference. They concluded that early amniocentesis is, "a valid alternative to traditional amniocentesis" [67].

In their 1990 paper, Elejalde et al. performed a prospective controlled study involving 615 amniocenteses performed between weeks 9 and 16 of gestation, and they reviewed previous EA studies [68]. Their results showed that amniocentesis after the 9th week of pregnancy does not appear to differ significantly in its complications and outcome from the results of the same procedure at 15–16 weeks or later. The issue of pseudomosaicism was also addressed and will be covered more fully later in this chapter.

Penso et al. in 1990 performed amniocentesis in 407 women between gestational ages of 11–14 weeks and compared the safety and accuracy with data obtained from collaborative studies of amniocentesis performed later in the second trimester [69]. Theirs was the first report to provide information regarding neonatal outcome associated with EA. The spontaneous abortion rate within 4 weeks of the procedure was 2.3%, and the fetal loss rate was 6.4%. Orthopedic postural deformities, including club feet, scoliosis, and congenital dislocation of the knees and hips, were seen in eight newborns, three of whose mothers had post-amniocentesis leakage of amniotic fluid. A total of ten women in the study (2.6%) had post-procedure fluid leakage. It appeared that the orthopedic deformities might be related to a post-procedure history of amniotic fluid loss. They concluded that the accuracy, risks, and complications were similar to those of traditional amniocentesis.

In 1990, Hanson et al. reported their increased practitioner experience and use of continuous ultrasonographic guidance in EA of gestations from 10 to 14 weeks [70]. The needle gauge was changed from the 20 gauge used in their 1987 study to 22, and the volume of fluid removed was generally less. Pregnancy outcome was reported for 523 patients, of whom 12 (2.3%) had a post-procedural loss. This compared favorably with their previously reported loss rate of 4.7%. Of eight women with post-procedure amniotic fluid leakage, one had a baby at term with a dislocated knee.

Another experienced fetal death 3 weeks after the amniocentesis, and the rest had normal term deliveries.

In a smaller series, 105 EA procedures were performed [71]. There were two pregnancy losses in the 64 patients for whom outcome information was available at the time of publication, and four congenital anomalies were seen in the 66 delivered babies: one imperforate anus, one hemangioma of the tongue, and two cases of positional talipes that required no treatment. These were apparently unrelated to amniotic fluid leakage.

Crandall et al. retrospectively studied 693 consecutive EA (prior to 15 weeks) cases, which had a spontaneous abortion rate (to 28 weeks' gestation) of 1.5%, compared with a non-randomized, later control group of 1,386 women having traditional amniocentesis, whose spontaneous abortion rate was 0.6%, a statistically significant difference [72]. In their review of background risk of pregnancy loss in the second trimester, they concluded that "at least some of the pregnancy loss subsequent to early amniocentesis is independent of the

procedure but the risk may be minimally higher than that for standard amniocentesis." There were no significant differences in congenital anomalies in the EA group (1.8%) versus the traditional amniocentesis group (2.2%). Interestingly, in the EA group, 4 of the 12 abnormalities involved congenital hip dislocation/subluxation or club feet, and three of the 30 congenital anomalies seen in the traditional amniocentesis group were congenital hip dislocation or club feet. They concluded that EA is a, "relatively safe prenatal diagnostic test and an alternative to CVS and later amniocentesis." See Table 12.9 for a comparison of fetal loss rates.

In all these studies, the investigators concluded that, apart from a higher rate of pseudomosaicism seen in some EA cases, the laboratory analysis of EA specimens compares favorably in validity and reliability compared to traditional amniocentesis specimens. This was confirmed in two laboratory studies of a combined 1,805 EA specimens of 10–14 weeks' gestation [73, 74]. The culture success rate was 99.8% for EA versus 100% for traditional amniocentesis

Table 12.9 Outcome in early (11–14 weeks) amniocentesis studies

Group	Study period	# of patients with outcome data in EA group	Fetal loss rate within 2 weeks of procedure (%)	Fetal loss rate (%) ^a , week(s) gestation at time of amniocentesis	Total fetal loss rate, %	Needle gauge	Comments
Hanson (1987) [64]	1979–1986	298	1.7	5/80 (6.3), 11–13 weeks; 5/228 (2.2), 14 weeks	4.7	20	Loss rate was 3.3% if patients with pre-amniocentesis history of bleeding were eliminated
Johnson and Godmilow (1988) [65]	Review of six studies, including Hanson [64]; 1979–1987	1,240	N/A ^b	N/A ^b	1–4.7	22 in 5 centers, 20 in 1 center [64]	
Strippardo (1990) [67]	1987–1988	397	1.98 ^c	9/208 (4.3), 11–13 weeks; 0/176 (0), 14 weeks ^d	3.9	22	
Penso (1990) [69]	1986–1989	389	0.8 ^e	6/365 (1.6), 11–13 weeks; 3/42 (7.1), 14 weeks	3.96	22	3 of 8 newborns with postural deformities born after post-amniocentesis fluid leak
Hanson (1990) [70]	1986–1987	517	0.8	6/272 (2.2), 11–13 weeks; 5/255 (1.96), 14 weeks	2.5	20	
Crandall (1994) [72]	1988–1993	681	0.9	13/681 (1.9), 11–14 weeks 13/1,342 (0.97), 15–22 weeks	1.9% for EA, 0.97% for conventional amniocentesis	22, sometimes 25	EA was compared to conventional amnio; spontaneous abortion rate was significantly higher in EA group. 0.6% of EA group had hip dislocation or clubfeet compared to .22% in conventional amnio group

^aThis figure includes spontaneous abortions, stillbirths, and neonatal deaths

^bN/A not available

^cOne hundred and eight 15-week-gestation amnios were included in this figure

^dData based on status at 28 weeks' gestation

^eFetal loss within 4 weeks

in one study and 98.6% for EA versus 99.9% for traditional amniocentesis in the other study. The turnaround times for reporting results were 1–2 days longer in the EA group. In one study, the EA group showed a significant increase in the number of structural and numerical single-cell abnormalities and an increase in numerical multiple-cell abnormalities compared to amniocenteses performed at 16–18 weeks. These were dealt with by examining parallel cultures.

More recent studies are mixed in their conclusions. Diaz Vega's group performed 181 amniocenteses at 10–12 weeks' gestation and reported a fetal loss rate within 2 weeks of the procedure of 0.5%, with a total fetal loss rate during pregnancy of 1.6% [75]. However, the culture success rate was only 94.5% overall, with one culture failure out of three 10-week amniotic fluid specimens.

Brumfield's group performed a retrospective matched-cohort study using a study group of 314 patients who had amniocentesis at 11–14 weeks versus a control group of 628 women who had amniocentesis at 16–19 weeks [76]. With the same practitioners, ultrasound equipment, and technique, they found a significant difference in the fetal loss rate within 30 days of amniocentesis (2.2% vs. 0.2%) in the EA group compared to the later-amniocentesis group. This was attributed at least in part to higher post-procedure amniotic fluid leakage (2.9% vs. 0.2%) and vaginal bleeding (1.9% vs. 0.2%) rates. The culture success rates were not reported.

Bravo et al. examined whether transplacental needle passage is a factor in fetal loss after EA [77]. They reviewed 380 consecutive EA procedures performed for advanced maternal age and found that transplacental needle passage had occurred in 147 cases (38.7%). Although the frequency of "bloody taps" was significantly increased in this group, there was no difference in fetal loss rates (3.4% in both groups, including stillbirths).

Wilson's review states that there have been no studies that have adequately addressed the critical question of the safety of EA relative to traditional amniocentesis, pointing out that to date only two randomized trials had been performed, and they differed in their methodologies and their conclusions [78]. He also stated that procedures at less than 13 weeks' gestation should be considered experimental. Certainly, the cumulative experience with 13–14-week EA procedures is much greater than that with under-13-week EA procedures. In addition, the two randomized EA studies he cited evaluated 11–12-week gestations and thus are not comparable to the 13–14-week-gestation studies.

Comparison of Early Amniocentesis with Chorionic Villus Sampling

In order to compare first-trimester prenatal diagnostic modalities, a number of investigators have published studies comparing CVS with early amniocentesis. Shulman et al. reported on 500 women, half of whom had transabdominal CVS

(TA CVS) from 1986 to 1988, and half of whom had EA from 1987 to 1991 [79]. Of the EA specimens, all but 11 were obtained from weeks 12 to 14, and the rest were from weeks 9 to 11. Of the continuing pregnancies, loss rates of 3.8 and 2.1% for EA and TA CVS, respectively, were seen. This was not statistically significant. The culture failure rate for both procedures was 0.8%. This study has limited applicability inasmuch as the numbers were small and the patients not randomized, and the time intervals were different. Although all procedures were listed as initial cases, the relative degree of prior individual practitioner experience in the two procedures was not addressed.

In 1994, Nicolaides et al. reported on a prospective, partially randomized study comparing EA and TA CVS in 1,870 women [80]. The spontaneous loss rate was significantly higher after EA at 5.3% than with the CVS group (1.2%). The rate of successful sampling was the same at 97.5%. Culture failure occurred in 2.3% of the EA group, compared to 0.5% in the CVS group. Confined or true mosaicism was seen in 1.2% of the CVS group, compared to 0.1% of the EA group. The authors concluded that although EA and CVS are equally likely to produce valid cytogenetic results, CVS would probably become the "established technique" due to the 2–3% excess risk of fetal loss in the EA group.

In response to this study, Saura et al. stated that EA could be a "true alternative" to CVS after the 13th week, when the disadvantages of culture failure and fetal losses decrease [81]. Bombard et al. reported one loss in 121 procedures (0.83%) performed by one practitioner at 10–13 weeks using a 22-gauge needle [82]. They suggested that Nicolaides' higher EA fetal loss rate could be related to the needle gauge and the multiple practitioners in his study, compared to one practitioner in Bombard's center.

Similar results were reported by Vandenbussche et al., who, in a partially randomized study, reported eight fetal losses among 120 EA procedures, compared to none among the 64 CVS patients with a follow-up of 6 or more weeks [83].

Another response to these reports proposed the idea that the main drawback to the studies was the very small numbers of EA procedures performed and the evident greater practitioner experience with CVS than with EA. The authors reported a spontaneous abortion rate after EA of 1% up to week 24 on the basis of 1,800 pregnancies. The culture failure rate was 0.3% for gestations ranging to 10 weeks 4 days [84].

An important consideration raised by some investigators is that the banding quality of amniocentesis specimens of any gestation is generally superior to that of CVS specimens, which increases the informativeness of the cytogenetic analysis [78, 84]. The fact that amniotic fluid AFP levels and multiples of the median have been established in many laboratories down to 12 or 13 completed weeks of gestation adds another advantage to the diagnostic power of EA compared to CVS [85].

A 14-center study of 3,775 women randomized to having either CVS or EA was conducted to try to provide more answers to the questions as to the safety and accuracy of EA and transabdominal CVS at 11–14 weeks' gestation [86]. Both types of procedures were performed by the physicians in each center. Early in the trial, reports of clubfoot at 11–12 weeks in EA patients caused procedures at these weeks to be discontinued.

Criteria for inclusion included advanced maternal age, serum marker screen positive, and prior trisomy [86]. The primary outcome was deemed to be preterm delivery or pregnancy loss of a cytogenetically normal fetus at less than 28 weeks' gestation. Secondary outcomes included total fetal loss, including neonatal death; amniotic fluid loss; pregnancy outcome; limb and other congenital defects; and cytogenetic diagnostic success and accuracy. Multiple procedures were required for EA at 11–12 weeks (2.4% vs. 1.2% for CVS). Maternal cell contamination was seen in EA specimens at 11–12 and 13 weeks' gestation (0.6% in both cases vs. 0% in CVS). Pseudomosaicism was seen in 1.2% of EA 11–12-week specimens versus 0.6% of CVS specimens. CVS specimens were harvested at 5.9–6.5 days across the sampling period, compared to 12.3–9.8 days for 11–12- or 14-week EA specimens, respectively. As for complications, the only difference that reached significance at the $p < 0.001$ level was EA with a 9.6% amniotic fluid leakage rate. Gestational hypertension/preeclampsia was seen in 5.4% of the CVS patients compared to 3.5% of the EA patients, for a p value of 0.005. Of 1,914 CVS procedures, 34 had cytogenetic abnormalities, two were lost to follow-up, and 1,878 were cytogenetically normal. Thirty-nine, or 2.1%, were lost or delivered before 28 weeks. This compares to 1,861 EA procedures, of which 38 had cytogenetic abnormalities, three were lost to follow-up, and 1,820 were cytogenetically normal. Forty-two, or 2.3%, were lost or delivered before 28 weeks. Clubfoot was seen in 0.2% of CVS patients; in EA patients, it was seen in 1.2% of 11–12-week procedure offspring, 0.8% of 13-week offspring, and 0.2% of 14-week offspring for a relative risk of EA versus CVS of 4.1 (1.17–14.6). The authors concluded that, in general, CVS is the preferred prenatal diagnostic procedure between 12 and 14 weeks [86]. This conclusion was shared by Evans and Wapner and by Tabor and Alfirevic in their reviews of prenatal diagnostic procedures [56, 57]. Tabor and Alfirevic stated, "Amniocentesis should...not be performed before 15+0 weeks' gestation" due to increased fetal loss rates compared to conventional amniocentesis and the risk of talipes [57].

Specimen Requirements

The volume of amniotic fluid obtained for prenatal diagnosis varies with the stage of gestation, with 15–20 mL conventionally removed by midtrimester amniocentesis practitioners. In one report, data from several small studies was

Table 12.10 Volume of amniotic fluid (mL) calculated using all the values for a given week from published data

Week	n	Mean	SD	Range
10	7	29.7	11.2	18–33
11	9	53.5	16.4	64–76
12	13	58.0	23.4	35–86
13	13	71.4	21.3	38–98
14	14	124.1	42.1	95–218
15	15	136.8	43.7	64–245
16	16	191.2	59.7	27–285
17	20	252.6	98.5	140–573
18	4	289	150	70–410
19	14	324.5	65.2	241–470
20	3	380	39	355–425

Data from reference [68]

pooled, and the volume of amniotic fluid for weeks 10–20 was calculated [68] (see Table 12.10). At gestations under 15 weeks, many practitioners have adopted the practice of removing 1 mL per week of gestation, and others have found excellent culture success rate and turnaround time with less fluid removed. For example, one group withdrew 4–12 mL in gestations of 9–14 weeks and obtained a 100% culture success rate in 222 specimens, while others withdrew 5–8 mL in pregnancies of 10 weeks and 4 days to 13 weeks and 6 days for an overall culture success rate of 99.7% [84, 87]. It has been observed that the total cell numbers rise exponentially from 8 to 18 weeks' gestation, but the number of viable cells increases only slightly during that time [78]. This probably explains the comparable culture success rate of EA compared to midtrimester amniocentesis.

Chorionic Villus Sampling

Associated Risks, Limitations, Benefits, Turnaround Time

Risks associated with CVS have been extensively studied. Perhaps the issue receiving the most attention in the past few years was raised by Boyd et al. involving one case and then more extensively by Firth et al., who reported five babies with severe limb abnormalities out of 289 pregnancies in which TA CVS had been performed at 56–66 days' gestation [88, 89]. Four of these had oromandibular-limb hypogenesis syndrome. They hypothesized that CVS undertaken up to 66 days' gestation may be associated with an increase in the risk of oromandibular-limb hypogenesis syndrome and other limb-reduction defects. This report generated many others, with mixed conclusions.

A flurry of letters to the editor of *Lancet* in 1991 followed Firth's report. Reporting evidence to support the association between CVS and limb-reduction defects were Mastroiacovo et al. and Hsieh et al. [90, 91]. Monni et al. suggested that the

incidence and severity of limb defects was related to the gauge of the needle since they used a 20-gauge needle while Firth used an 18-gauge needle [92]. In a series of 525 CVS procedures done before 66 days' gestation, no severe limb defects were seen, and only two mild defects were seen in 2,227 procedures that were done later [92]. Mahoney then reported on two multicenter studies that compared transcervical CVS with amniocentesis, and another comparing transabdominal CVS with transcervical CVS [93]. Of 9,588 pregnancies studied, 88% of the CVS procedures were performed after 66 days' gestation. Significant limb-reduction defects were present in seven babies. Two of these defects were longitudinal, and five were transverse. Another baby had minor reduction defects of the toes. They compared these abnormalities to those reported to the British Columbia registry and found no significant increase in these birth defects. The timing of the CVS procedures that resulted in babies with abnormalities ranged from 62 to 77 days' gestation.

Similar conclusions were reached in a study in which 12,863 consecutive CVS procedures were performed [94]. Five limb-reduction defects were seen, which were found not to be significantly different from the incidence observed in the British Columbia registry of birth defects. Of the 12,863 procedures, 2,367 were done at 56–66 days, and one of the limb defects was seen in this group. The authors observed no gestational time-sensitive interaction related to CVS and postulated that this was due to their larger experience base.

In 1993, Jahoda et al. reported on 4,300 consecutive transabdominal and transcervical CVS cases for which newborn follow-up information was obtained [95]. Of the 3,973 infants born in this group, three (0.075%) had a terminal transverse limb defect. Two of these occurred in the transcervical CVS group sampled before 11 weeks' gestation (1,389 patients), and the other one was in the transabdominal CVS group, sampled after 11 weeks (2,584 patients). The authors found the latter figure to be comparable to the prevalence figure given in population studies. They concluded that postponement of CVS to the late first or early second trimester of pregnancy would contribute to the safety of the procedure.

In the same year, a report of the National Institute of Child Health and Human Development Workshop on Chorionic Villus Sampling and Limb and Other Defects was issued [96]. The conclusions, based on a review of the literature, were mixed; some concluded that exposure to CVS appeared to cause limb defects, while others did not. All agreed that the frequency of oromandibular-limb hypogenesis appeared to be more common among CVS-exposed infants. This seemed to correlate with CVS performed earlier than 7 weeks post-fertilization (9 weeks post last menstrual period). Whether or not a distinctive type of limb defect was associated with CVS could not be determined, and it also was unclear whether the CVS-exposed infant had an increased frequency of other malformations, including cavernous hemangiomas.

A five-center retrospective cohort study was performed by the Gruppo Italiano Diagnosi Embrio-Fetali to examine this issue, with results published in 1993 [97]. Of 3,430 pregnancies in which CVS had been performed, outcome information was available for 2,759. Of these, three had transverse limb-reduction defects, two among 804 CVS procedures performed at 9 weeks and one among 1,204 CVS procedures performed at 10 weeks. There were no limb-reduction defects noted in 2,192 amniocenteses with completed follow-up performed during the same study period. The authors concluded that performing CVS at less than 10 weeks' gestation, "should be discouraged until further evidence against this association can be obtained," while noting that their follow-up rate was only 80%.

Hsieh et al. surveyed 165 obstetric units in Taiwan regarding the incidence of limb defects with and without CVS [98]. Of these, 67 hospitals responded, representing 78,742 deliveries. The incidence of limb defects was found to be 0.032% in the general population and 0.294% in the CVS population. The abnormalities seen in the CVS group included amelia, transverse reductions, adactylia, and digit hypoplasia, much like the abnormalities reported by Firth et al. [89]. The 25 limb abnormalities in the non-CVS group involved syndactyly or polydactyly. In addition, oromandibular-limb hypogenesis was seen in four of 29 CVS cases with limb abnormalities but in none of the non-CVS cases with limb abnormalities. The severity of the post-CVS limb abnormalities appeared to correlate with timing of the procedure, and the authors recommended performing CVS only after 10 full gestational weeks to minimize the risks.

In 1995, Olney et al. reported on a United States multi-state case-controlled study comprising the years 1988–1992 [99]. The case population was 131 babies with nonsyndromic limb deficiency born to women 35 and older, and control subjects were 131 babies with other birth defects. These were drawn from a total of 421,489 births to women greater than 34 years of age. The odds ratio for all types of limb deficiency after CVS was 1.7, and for transverse digital deficiency, an odds ratio of 6.4 after CVS was observed. They estimated that the absolute risk for transverse digital deficiency in babies after CVS was one per 2,900 births (0.03%).

Froster and Jackson reported on outcome data in a World Health Organization (WHO) study on limb defects and CVS in 1996 [100]. From 1992 to 1994, 77 babies or fetuses with limb defects from 138,996 pregnancies exposed to CVS were reported to the WHO CVS registry. This group represented the entire experience of 63 European and American centers reporting to the registry. They found that the overall incidence of limb defects in the CVS cohort did not differ from that in the general population, and they did not see a different pattern of distribution of limb defects between the groups. No correlation between limb-reduction defects and gestational age was identified. They indicated that other studies

finding an association between limb defects and CVS are confusing because of different methodologies and interpretations and that the numbers reported are too small to draw firm conclusions.

Larger numbers were collected by Kuliev et al., who summarized the accumulated experience of 138,996 cases of CVS from the same 63 centers that report cases to the World Health Organization CVS registry [24]. They reported an overall incidence of limb-reduction defects after CVS of 5.2–5.7 per 10,000, compared with 4.8–5.97 per 10,000 in the general population. They also found no difference in the pattern distribution of limb defects after CVS and similarly concluded that their data provide no evidence for any risk for congenital malformation caused by CVS.

Heterochromatin Decondensation in Chorionic Villus Sampling

The spontaneous decondensation of the constitutive heterochromatic regions of chromosomes 1, 9, 16, and Y has been observed in 46.6% of chorionic villus samples, per a study by Perez et al. [101]. This type of decondensation is occasionally observed in amniotic fluid cells (9%) and has never been found in fetal lymphocytes. This decondensation can lead to breaks, fragile sites, and loss of the chromosome, including, for example, the loss of 1q in culture.

Maternal Age: A Confounder?

Because CVS is usually performed on women 35 and older, the issue of whether the limb deficiencies seen after CVS were related to maternal age was raised by Halliday et al. in a study from Victoria, Australia [102]. A congenital malformations registry maintained there was reviewed by a medical geneticist, who classified all cases using the International Classification of Diseases, 9th revision [103]. All babies born with limb defects in 1990–1991 were identified, and the number of those whose mothers had amniocentesis, CVS, or no invasive study was known. Excluding babies with chromosome abnormalities, recognized inherited syndromes, or amniotic bands, the authors found a twofold relative risk of having a baby with a limb deficiency of any type among women at age 35 or older, compared to women under 35. They also discuss the difficulty in interpreting studies of limb defects and CVS, as others had, pointing out the importance of 100% follow-up, inclusion of all recognized cases of limb deficiencies (induced abortions as well as all other births), recognition of the heterogeneity of the condition, and the different risk estimates at different gestational ages [100].

A subsequent study found no maternal age confounding effect in interpretation of CVS/transverse limb deficiency data [104]. The authors analyzed the maternal age-specific rates of transverse limb deficiencies in the Italian Multicentric Birth Registry and used a case-control model for maternal age. No difference in the relative risk was seen between the

35-and-older group, whether or not CVS had been performed, and the under-35 group. The risk estimate for transverse limb defects associated with CVS was 12.63 and did not change after stratification for maternal age or for gestational age.

After 1991, the utilization of CVS dropped significantly, due in large part to the concern regarding limb deficiencies [105, 106]. Although national utilization numbers are not available, a large national prenatal genetic counseling company affiliated with a cytogenetics laboratory reported a decrease of CVS of 3% per year from 36 to 14% from the years 2007 to 2001. In their patient population of 55,019 women, 34% were offered CVS. The decline was statistically significant. The acceptance rate increased again in 2008 to 24%, thought due to American College of Obstetrics and Gynecology Practice Bulletin #77, 2007a, supporting first-trimester screening for fetal chromosome abnormalities, as well as likely being due to such factors as increased access to first-trimester screening [107].

Fetal Loss in CVS

In the first large controlled study of the safety of CVS, Rhoads et al. reported on seven centers' experience with transcervical CVS in 2,235 women compared to that of 651 women who had amniocentesis at 16 weeks' gestation [108]. They found an overall excess loss rate of 0.8% in the CVS group after statistical adjustments for gestational age and maternal age. CVS procedures in which more than one attempt was made were associated with a substantially higher loss rate, supporting the observation by Silver et al. and others that increased operator experience is a key factor in assessing the risks of CVS [109]. Silver's group found that the number of placental passes and increased sample weight/aspiration attempt ratio may be more sensitive indicators of competence than the fetal loss rate.

Results of a randomized international multicenter comparison of transabdominal and transcervical CVS with second-trimester amniocentesis were reported in 1991 [110]. Outcome information was available for 1,609 singleton pregnancies in the CVS group and 1,592 in the amniocentesis group. Thirty-one centers participated, and the numbers of cases submitted ranged from 4 to 1,709. Significantly fewer surviving newborns were seen in the CVS group than in the amniocentesis group (4.6% difference, $p < 0.01$). Most of the difference was in the significantly greater number of spontaneous fetal deaths before 28 weeks: 86/1,528 in the successfully sampled CVS group and 25/1,467 of the successfully sampled amniocentesis group (rate difference of 2.9%, $p < 0.02$).

In a report from the Centers for Disease Control, an overall risk of spontaneous abortion attributed to CVS is reported from a literature survey as 0.5–1.0%, compared to 0.25–0.50% for amniocentesis procedures [111].

In the WHO study, registry participants reported a spontaneous pregnancy loss rate after transabdominal or transcervical CVS of 2.5–3.0%, with several large-volume operators having loss figures of less than 2% [24]. This risk was deemed comparable to that of amniocentesis. Tabor et al. documented fetal loss rates in an 11-year national registry study in Denmark of the outcomes of 31,355 CVS procedures at 24 weeks' gestation in a 2009 report [55]. The overall fetal loss rate was 1.9% (95% CI, 1.7–2.0) and was not correlated with maternal age. The number of procedures done in each center had a significant effect on the loss rate; in departments performing 1,500 or fewer during the 11 years, the risk was 40% greater than in those performing more than 1,500 procedures per year. There was no control group.

In reviews of procedure-related risks from many publications, in which five included a control group, the authors concluded that the procedure-related miscarriage rate from CVS is 0.5–1.0% [56, 57].

Transabdominal Versus Transcervical CVS

Efficacy and risks associated with transcervical CVS (TC CVS) and transabdominal CVS (TA CVS) have been studied at several centers [110, 112–114] (see Fig. 12.1). The majority of CVS had been performed transcervically until the late 1980s, when more centers began using TA CVS to avoid cervical microorganisms and to reach placentas more easily. In their pilot study in 1988, Smidt-Jensen and Hahnemann reported on 100 TA CVS cases at 8–12 weeks' gestation followed to term, compared to 200 amniocentesis cases [114]. In all CVS cases, a sample was successfully obtained and cultured, and the fetomaternal complication rates were found not to be significantly different from those of previous TC CVS reports.

Transabdominal CVS has been increasingly used in recent years compared to TC CVS. Brambati et al. reported on efficiency and risk factors in 2,411 patients; 1,501 of whom had TC CVS and 910 of whom had TA CVS [112]. The two approaches had comparable success rates and complication rates, but TA CVS was considered easier to learn and less likely to be contraindicated by clinical and anatomical conditions. Subsequently, this group published results of a randomized clinical trial of TA and TC CVS [113]. All CVS procedures were performed by the same practitioner, who had prior similar experience in both techniques. The procedures were found to be equally effective, although TA CVS required significantly fewer insertions. The authors concluded, "...transabdominal and transcervical CVS appear equally effective, and by and large the choice may be based on the operator's preferences."

Confined Placental Mosaicism

Chromosomal mosaicism is characterized by the presence of two or more karyotypically different cell lines within

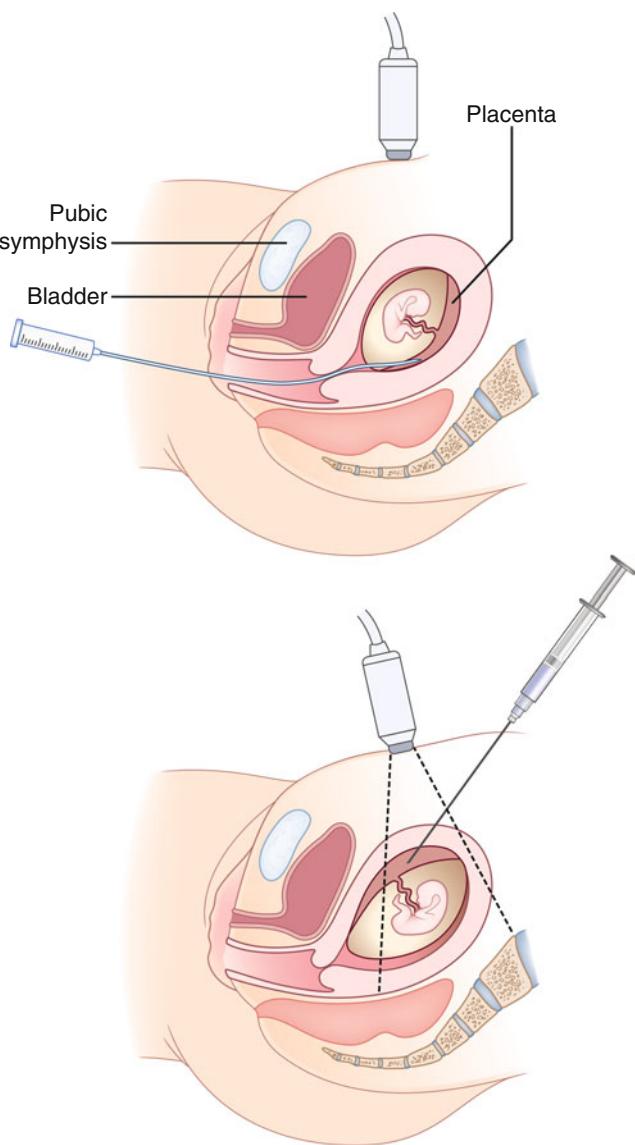


Fig. 12.1 Illustration of transcervical and transabdominal CVS. *Upper:* transcervical CVS. A flexible catheter is introduced into the chorionic villi, or future placenta. *Lower:* transabdominal CVS. A spinal needle is inserted through the abdominal wall for sampling

one individual. Confined placental mosaicism (CPM) is defined as a discrepancy between the chromosomal constitutions of placental and embryonic/fetal tissues. CPM results from viable mitotic mutations occurring in the progenitor cells of trophoblast or extraembryonic mesoderm during early embryonic development. In 1983, Kalousek and Dill reported on numerical discrepancies between the karyotypes of fetal and placental cells, either full trisomies or mosaic aneuploidies, and similar reports followed [115, 116]. Based on six cases in which placental/CVS cells had a different chromosome constitution from that of amniotic fluid cells, the authors concluded that the results of cytogenetic analysis from placental tissue may not be representative of

the fetus. Their figures, though small, were similar to the 2% incidence of this phenomenon as previously reported [117]. Since then, others have found CPM to occur in 0.8–2% of viable pregnancies studied by CVS at 9–11 weeks' gestation and in 0.1% or less in amniocentesis specimens [80, 118–125].

The outcomes of pregnancies in which CPM is diagnosed vary from apparently normal outcomes to severe intrauterine growth restriction (IUGR), although few follow-up reports are yet available in the literature. Kalousek et al. found six cases of IUGR among 17 gestations with CVS-detected CPM, 5 in liveborns, and one associated with intrauterine death [126]. They noted that others had found a 22% fetal loss rate among pregnancies with CPM. Wolstenhome et al. found 73 cases of CPM in 8,004 CVS specimens from women referred for advanced maternal age, previous child with aneuploidy, or family history thereof [125]. Comparison at delivery with the control population did not show a marked increase in adverse pregnancy outcome. In 108 other cases referred for ultrasound detection of isolated IUGR, seven were shown to have CPM involving the following chromosomes: 2 and 15 (1 case), 9 (1 case), 16 (3 cases), del(13) (1 case), and 22 (1 case).

Hahnemann and Vejerslev evaluated cytogenetic outcomes of 92,246 successfully karyotyped CVS specimens from 79 laboratories from 1986 to 1994 [127]. CVS mosaicism or nonmosaic fetoplacental discrepancy was found in

1,415 (1.5%) of the specimens. Table 12.11 shows the mosaic and nonmosaic chromosome findings seen. Updated CVS mosaicism reports are shown from other studies for specific chromosomes. Hahnemann and Vejerslev's work on several cell lineages indicated that mosaic or nonmosaic trisomies found in cytotrophoblasts, with a normal karyotype in the villus mesenchyme, were not seen in fetal cells. However, if such trisomies were seen on cultured preparations, a risk of fetal mosaic or nonmosaic trisomy existed. They recommended amniocentesis in all pregnancies involving mosaic autosomal trisomy in villus mesenchyme.

A thoughtful study on this topic was published by Daniel et al., in which rare trisomies detected at the time of CVS and amniocentesis were analyzed [128]. The authors comment on the likelihood of cryptic fetal mosaicism as the cause of abnormal phenotypic findings as opposed to CPM, given the lack of phenotypic effect of maternal uniparental disomy (UPD) for chromosomes 1, 7, 9, 10, 13, 21, and 22 or for paternal UPD for chromosomes 1, 5, 6, 7, 8, 13, 17, and 21. The effect of maternal and paternal UPD 20 is still unclear, and for chromosomes 1 and 16, effects are less clear than previously believed. In the series, they reported there was some evidence for cryptic fetal mosaicism and none for UPD. They describe other similar findings in the literature and conclude that the finding of even very low-grade mosaicism in amniocytes should be regarded as significant.

Uniparental Disomy in Confined Placental Mosaicism

When a conceptus is trisomic, this aneuploidy is said to be “corrected” if by chance there is early loss of one of the trisomic chromosomes. Depending upon the parental origin of the trisomy and of the chromosome that is lost, this can lead to an apparently normal diploid cell line with uniparental disomy (both chromosomes in a pair from one parent) for that chromosome. Because most trisomies are maternally derived, the disomy seen is often maternal, as was the case in two previously reported cases of trisomy 15 mosaicism seen at CVS in which the neonates subsequently manifested Prader-Willi syndrome due to maternal disomy 15 [125]. The authors also note the reports of several cases of chromosome 16 CPM-associated IUGR in which maternal disomy 16 was seen in most of the cases. The presence of mosaic trisomy 16 itself may be of most significance in such cases, however. In this regard, Daniel et al. presented data questioning the clinical significance of UPD 16 and CPM versus that of true cryptic fetal mosaic trisomy 16 [128].

The evaluation of parental disomy in all CPM cases involving chromosome 15 should be offered, and this recommendation has extended to other chromosomes as more information has become available.

For a thorough discussion of UPD, refer to Chap. 20.

Table 12.11 Distribution of specific single autosomal trisomies in each of the groups of mosaicism/discrepancy in chorionic villus tissue

Trisomy	CPM (# of cases) ^a	True fetal mosaicism (# of cases)
2	11	
3	10	
5	3	
7	32	
8	11	1
9	9	1
10	6	
11	1	
12	2	1
13	15	2
14	3	
15	11	1
16	11	
17	1	
18	29	4
20	12	1
21	22	9
22	3	
All	192	20

^aIncludes all types of confined placental mosaicism, including direct only, long-term culture only, and both. Data from reference [127].

Interphase FISH in Confined Placental Mosaicism

Interphase fluorescence *in situ* hybridization (FISH, see Chap. 17) can be useful for the diagnosis of CPM, given that interphase FISH is rapid and has the great advantage of not requiring growing, dividing cells to obtain results. Harrison et al. examined the placentas of 12 pregnancies in which nonmosaic trisomy 18 had been diagnosed and found significant levels of mosaicism, confined to the cytotrophoblast, in 7 of the 12 [118]. Based on their observation that most of the mosaic results were seen in stillborn or newborn trisomy 18 babies, and on the fact that the great majority of trisomy 18 conceptuses spontaneously abort, they suggested that a normal diploid trophoblast component in placental tissue may be necessary to facilitate the prolonged survival of trisomy 18 conceptuses.

Schuring-Blom et al. used FISH to document CPM in three pregnancies in which mosaic trisomy 8, mosaic trisomy 10, and nonmosaic monosomy X were observed following CVS but which were found to be chromosomally normal at amniocentesis [129]. In all three cases, FISH showed the presence of the mosaic cell line confined to one part of the placenta.

Henderson et al. performed a cytogenetic analysis using a “mapping” technique of nine term placentas after CPM had been diagnosed and found tissue-specific and site-specific patterns of mosaicism [130]. In addition to metaphase chromosome analysis, they employed interphase FISH to examine several areas of the placentas. Noting that the outcomes of pregnancies are highly variable after CPM is diagnosed, they proposed a wider study involving extensive analysis of term placentas when this occurs in order to obtain more information regarding the outcome of such pregnancies.

Direct and Cultured Preparations

Direct CVS preparations involve the rapid metaphase analysis of villous cytotrophoblastic tissue. Cultured preparations involve the mesenchymal cells in the villi. Some laboratories use only cultured cell preparations, and others utilize both methods. Investigations into the outcomes of pregnancy after CVS support the use of both techniques to maximize the accuracy of the test [121, 123, 124]. These studies documented false-negative and false-positive results using direct and cultured preparations, and the first two groups concluded that results from both direct and cultured techniques were necessary in a substantial number of cases to accurately predict the fetal karyotype. In one study, long-term culture was advocated as having higher diagnostic accuracy, and the direct method was said to be a useful adjunct to the culture method [121]. In a study by Los et al. of 1,829 consecutive CVS procedures with direct and long-term cultures, one conclusion was that using both modalities decreased the necessity for follow-up amniocentesis by 35% compared to that of long-term culture alone [131]. In part at least, the finding that both techniques add to the diagnostic accuracy appears to be related to the nonrandom findings of some trisomies in direct

versus long-term cultured tissues. Trisomy 2 is seen more in cultured cells, and trisomy 3 is more often seen in direct preparations [124, 125]. False-positive trisomy 7 or 18 can occur with either technique. To add to the complexity, it should be kept in mind that true trisomy 2 and trisomy 7 mosaicism have been documented in liveborn children after having been diagnosed prenatally by amniocentesis [132, 133].

Maternal cell contamination (MCC) in CVS is generally due to the lack of complete separation of chorionic villi from maternal decidua, and it is reported in an estimated 1.0–1.8% of cases [121, 123, 124]. The MCC reported in these studies is about half of the figures above, reflecting the XX/XY admixtures, and is doubled to account for the likely equal incidence of MCC in female fetuses. MCC occurs more often in cultured cells than in direct preparations, thus underscoring the importance of using both methods in a full CVS cytogenetic analysis. In one report, the rate of MCC was significantly higher in specimens obtained by the transcervical method (2.16%) than in samples obtained by the transabdominal method (0.79%) [121].

A note of caution is prudent here. Generally, when there is a discrepancy between the direct and the cultured preparations, a subsequent amniocentesis is considered to provide the “true” result. However, a case of mosaic trisomy 8 reported by Klein et al. illustrates the fact that a true low-level tissue-specific mosaicism can exist [134]. In this case, the CVS showed a normal direct preparation and mosaic trisomy 8 in culture. Subsequent amniocentesis showed normal chromosomes, but peripheral blood cultures of the newborn showed trisomy 8 mosaicism. Therefore, when considering amniocentesis or PUBS as follow-up studies because of possible CPM observed in CVS, factors such as the specific aneuploidy involved, the likelihood of detecting it using a given sampling technique, and the risks of the additional invasive procedure need to be weighed.

Specimen Requirements

The minimum amount of chorionic villus material necessary to obtain diagnostic results and the transport medium should be established in advance with the laboratory. In general, a minimum of 10 mg of tissue is needed to obtain both a direct and a cultured cell result, and 20 mg is ideal. If possible, the specimen should be viewed through a dissecting microscope to ensure that villi are present. The specimen should be transported at ambient temperature to the cytogenetics laboratory as soon as possible.

Percutaneous Umbilical Blood Sampling (PUBS)

Risks, Limitations, and Benefits

Percutaneous umbilical blood sampling (PUBS) is also known as periumbilical blood sampling, fetal blood sampling, or cordocentesis. The largest series in the literature

regarding risks of PUBS included outcomes of 1,260 diagnostic cordocenteses among three fetal diagnosis centers and 25 practitioners [135]. A fixed needle guide was used in this study, and prospective data was compared to the published experience of large centers that use a freehand technique, where a 1–7% fetal loss rate has been reported. The procedure-related loss rate at a mean gestation of 29.1 \pm 5 weeks at the time of sampling was 0.9%, leading to the conclusion that technique is a variable in the loss rate for cordocentesis.

PUBS experience at an earlier gestation was described by Orlandi et al. in 1990, who pointed out that, while cordocentesis was a technique largely confined to the middle of the second trimester to term, in their experience it could be performed as early as the 12th week with acceptable results [136]. They evaluated the outcomes of 500 procedures performed between 12 and 21 weeks for thalassemia study (386), chromosome analysis (97), fetomaternal alloimmunization (10), and infectious disease diagnosis (7). One practitioner performed the procedures, and the volume of blood obtained ranged from 0.2 to 2.0 mL, depending on the gestational age. Of the 370 pregnancies not electively terminated and for which outcome information was available, the fetal loss rate was 5.2% for fetuses of 12–18 weeks' gestation and 2.5% between 19 and 21 weeks. Indicators of adverse outcome included cord bleeding, fetal bradycardia, prolonged procedure time, and anterior insertion of the placenta. Fetal bradycardia is a commonly reported complication after PUBS and is associated with a higher likelihood of fetal loss. In a review of 1,400 pregnancy outcomes after PUBS, the overall incidence of recognizable fetal bradycardia was estimated at 5% [137]. It was significantly more likely to occur when the umbilical artery was punctured. Boulot et al. performed 322 PUBS and noted fetal bradycardia, usually transitory, in 7.52% of their cases [138]. Fetal bradycardia occurred in 2.5% of cases with normal outcome and in 12.5% of cases of fetal loss in one study, while in another, 11 of 12 fetal losses were associated with prolonged fetal bradycardia [136, 137].

The underlying fetal pathology is a significant factor in fetal loss rate. Of these 12 losses, 10 were fetuses with a chromosome abnormality or severe fetal growth restriction. In gestations from 17 to 38 weeks, Maxwell et al. compared the loss rates within 2 weeks of the procedure with the indications [139]. Of 94 patients having prenatal diagnosis with normal ultrasound findings, one pregnancy of the 76 that were not electively terminated was lost. Of the group with structural fetal abnormalities, 5 in 76 were lost; and in the group of 35 with nonimmune hydrops, 9 were lost. It is important to take this factor into account when counseling patients before the procedure.

It has been said that no other fetal tissue "...can yield such a broad spectrum of diagnostic information (cytogenetic, biochemical, hematological) as fetal blood" [136]. As a means of fetal karyotyping, it has the advantage of generating results in 2–4 days, compared to 6–14 or more for

amniotic fluid and CVS cells. When pseudomosaicism or mosaicism is seen in amniotic cell cultures, PUBS can provide valuable additional information regarding the likelihood of true mosaicism and thereby assist the couple in their decision making [140–143].

Although pseudomosaicism in amniotic fluid cell cultures is usually associated with normal chromosome analysis after PUBS, the absence of trisomic cells in fetal blood does not guarantee that mosaicism has been definitely excluded [144]. For example, fetal blood karyotyping is not useful for the evaluation of mosaic or pseudomosaic trisomy 20. For further discussion of mosaicism, see "Special Issues" later, and see also Chap. 8.

Because PUBS is associated with a significantly higher fetal loss rate than other prenatal diagnostic procedures, use of this technique should be recommended and provided with great care and only in certain high-risk situations such as those mentioned previously.

Specimen Requirements

Ideally, 1–2 mL of blood should be obtained and put into a small sterile tube containing sodium heparin. Results can usually be obtained from 0.5 mL, and in some cases 0.2 mL, so even small amounts obtained should not be discarded. A Kleihauer-Betke test may be useful in evaluating the possibility of maternal cell admixture, particularly when a 46,XX karyotype results.

Indications for Prenatal Cytogenetic Diagnosis

Advanced Maternal Age

Advanced maternal age, generally defined in the United States as 35 or older at delivery, is probably the most common indication for prenatal cytogenetic diagnosis. For women in this age group, this indication alone provides the advantage of greater than 99% accuracy for detection of chromosome abnormalities. The chief disadvantage lies in the fact that, overall, it results in the detection of only 20% of chromosomally abnormal fetuses, given that 80% of chromosomally abnormal babies are born to women under age 35. Advanced maternal age is the most significant determinant of the risk of a chromosome abnormality for all trisomies, structural rearrangements, marker chromosomes, and 47,XXY (Klinefelter syndrome, see Chap. 10). Maternal age is not a factor in 45,X (Turner syndrome), triploid (69 chromosomes instead of 46), tetraploid (92 chromosomes instead of 46), or 47,XYY karyotypes.

Very young women are also at increased risk of fetal chromosome abnormality. A 15-year-old has a 1 in 454 risk of having a term infant with a chromosome abnormality, compared to a 1 in 525 risk for a 20-year-old and a 1 in 475 risk for a 25-year-old woman [145] (see Fig. 12.2).

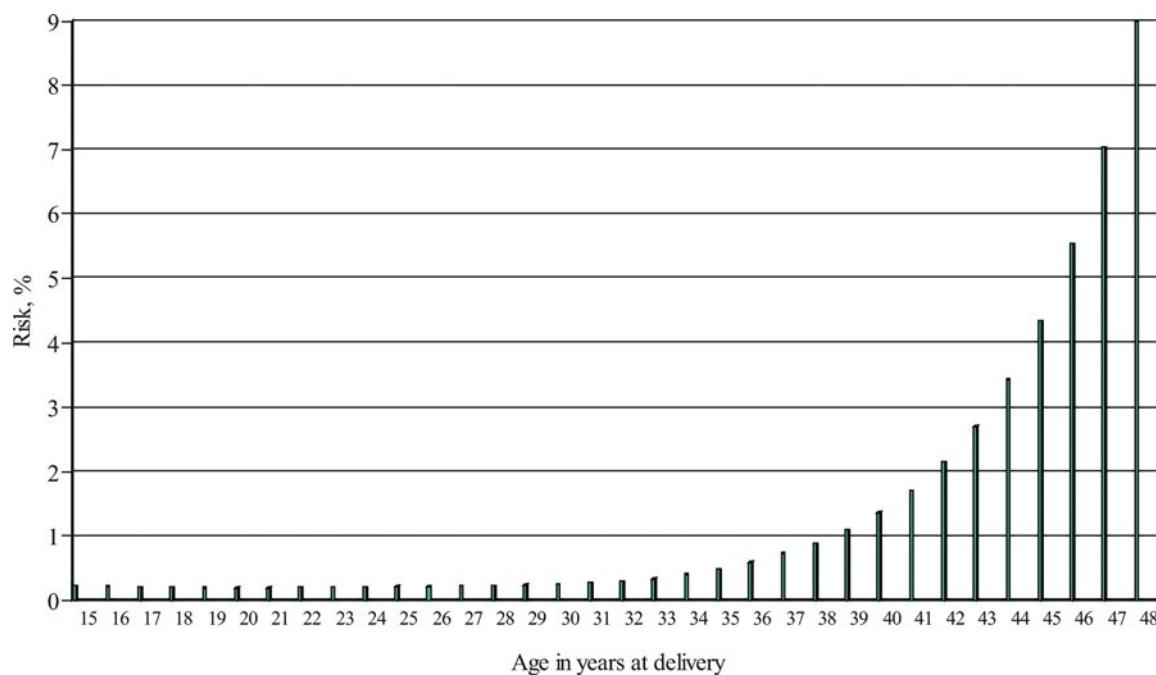


Fig. 12.2 Risk of chromosomally normal women to deliver chromosomally abnormal offspring [145]

Women 31 and Older with Twin Pregnancies

A 31-year-old with a twin gestation of unknown zygosity has a risk comparable to that of a 35-year-old woman. This is calculated as follows: given that two-thirds of such twins are dizygotic, the risk that one or the other has a chromosome abnormality is about 5/3 times that of a singleton pregnancy for that age. Thus, given that a 31-year-old woman's risk is 1 in 384 at term for any chromosome abnormality, if she is carrying twins of unknown zygosity, the risk that one or the other has a chromosome abnormality is $5/3 \times 1/384$, or 1 in 231. This is between the risk of a 34-year-old (1 in 243) and that of a 35-year-old.

The risk of a chromosome abnormality is not significantly greater for monozygotic pregnancies compared to singletons. For pregnancies known to be dizygotic, the risk that one or the other twin has a chromosome abnormality is about twice that of a singleton. See below for information on Down syndrome risk calculations in twin pregnancies taking into account the nuchal translucency in the co-twin.

Abnormal Fetal Ultrasound Findings

Many fetal ultrasound findings are associated with an increased risk for chromosome abnormalities. This list will continue to grow as the skill of practitioners and the resolution of ultrasound machines improve and also as the search for indicators of increased risk other than advanced maternal age continues.

Nuchal Thickening

Six causes have been proposed for nuchal thickening/folds:

- Cardiac defects with heart failure related to abnormal ductus venosus flow velocity.
- Abnormalities in the extracellular matrix of the nuchal skin of fetuses, which may be the leading cause of this finding in fetuses with connective tissue disorders.
- Abnormal lymphatic development and obstruction, which appears to be the case in some fetuses with Turner syndrome.
- Venous congestion in the head and neck due to constriction of the fetal body in amnion rupture sequence or superior mediastinal compression or the narrow chest in some skeletal dysplasias.
- Failure of lymphatic drainage due to impaired fetal movement in fetuses with neurologic disorders such as fetal akinesia.
- Congenital infection, acting through anemia or cardiac dysfunction [146].

The fluid collects in the posterior neck fold, causing the appearance of a nuchal membrane separation on ultrasound examination (Fig. 12.3). With resolution of the fluid collection, a nuchal fold or thickening develops.

Nuchal membranes have been recorded as early as 9 weeks' gestation. Measurement of the nuchal thickness, with or without first-trimester serum screening, has become the most sensitive first-trimester ultrasound finding used for Down syndrome detection [146–148]. Nicolaides, a pioneer of first-trimester nuchal thickness ultrasound scans, cites a detection rate of 90% for chromosome abnormalities when

Fig. 12.3 Ultrasound image of increased nuchal fold (*NF*) measuring 6.1 mm in a second-trimester fetus (Courtesy of Gregory DeVore, MD)



performed in conjunction with pregnancy-associated plasma protein A (PAPP-A) and free β -hCG at 11–14 weeks of pregnancy, with an invasive pregnancy testing rate of 5% [146].

Nuchal folds and cystic hygromas have been known to be associated with chromosome abnormalities since 1966, with an incidence of chromosome abnormalities ranging from 22% to more than 70% in various series [149]. Based on 22 other studies, plus their own data, Landwehr et al. found that 32% of 1,649 karyotyped fetuses with nuchal folds or membranes and/or cystic hygromas had a chromosome abnormality. These included 207 cases of trisomy 21; 108 cases of trisomy 18; 30 cases of trisomy 13; 131 cases of 45,X; and 48 other chromosome abnormalities. This study included first- and second-trimester ultrasound scans, which employ different criteria for nuchal thickness.

In a 12-center study designed to determine the sensitivity and specificity of second-trimester soft-tissue nuchal fold measurement for the detection of trisomy 21, 3,308 fetuses of 14–24 weeks' gestation were evaluated [150]. Using 6 mm as a cutoff, a nuchal skin fold was seen in 8.5% of chromosomally normal fetuses and in 38% of those with trisomy 21. A false-positive rate below 5% was obtained by 81% of the investigators. The authors concluded that this sign is useful in skilled hands in the second trimester, but it does not appear suitable for population screening because of the high variability in the results among the investigators.

A nuchal thickness cutoff of 4 mm was chosen by Nadel et al. in a study of 71 fetuses of 10–15 weeks' gestation, of which 63 were karyotyped [151]. Abnormal karyotypes were found in 31 of 37 hydropic fetuses and in 12 of 26 nonhydropic fetuses. The nonhydropic fetuses also had no septations in the hygromas. Twenty-two of the fetuses with septated hygromas had chromosome analysis, and 19 had abnormal chromosomes. Of fetuses with hydrops and no septations, 11 of the 14 had abnormal chromosomes.

There have been several first-trimester ultrasound studies of nuchal thickening. Van Vugt et al. karyotyped 102 first-trimester fetuses with a nuchal translucency of 3 mm or more and found that 46% had an abnormal karyotype: 19 had trisomy 21; 9 had trisomy 18; 13 had 45,X; 1 had 47,XXX; and 5 had other chromosome abnormalities [152]. Multiple logistic regression analysis was used to take into account data modifiers such as gestational age and maternal age. The authors examined the septated versus the nonseptated nuchal translucencies. Septa were seen in 45 (44%) of the fetuses, of whom 36 (80%) had chromosome abnormalities. Of 57 fetuses with no septation, 11 (19%) had abnormal chromosomes. This compared to a 56% incidence of chromosome abnormalities in first-trimester fetuses with septation and 23% incidence of chromosome abnormalities in first-trimester fetuses without septation in Landwehr's study [149].

In 1,015 fetuses of 10–14 weeks' gestation with nuchal fold thicknesses of 3, 4, 5, and >5 mm, Pandya et al. found incidences of trisomies 21, 18, and 13 to be approximately 3 times, 18 times, 28 times, and 36 times higher than the respective numbers expected on the basis of maternal age alone [153]. This corresponded to risks of one of these chromosome abnormalities to be 5, 24, 51, and about 60%, respectively.

Using a 4-mm cutoff in fetuses of 9–13 weeks, Comas et al. detected 57.1% of aneuploidies with a false-positive rate of 0.7% and a positive predictive value of 72.7% [154].

Szabó et al. evaluated 2,100 women under 35 years of age by ultrasound at 9–12 weeks' gestation [155]. Women were offered CVS if the nuchal fold was 3 mm or greater. The authors found an incidence of first-trimester nuchal fold to be 1.28% in women under 35, with a corresponding percentage of chromosome abnormalities being 0.43%. This indicated a 1 in 3 risk for chromosome aneuploidy in this age group when a thickened nuchal fold was seen.

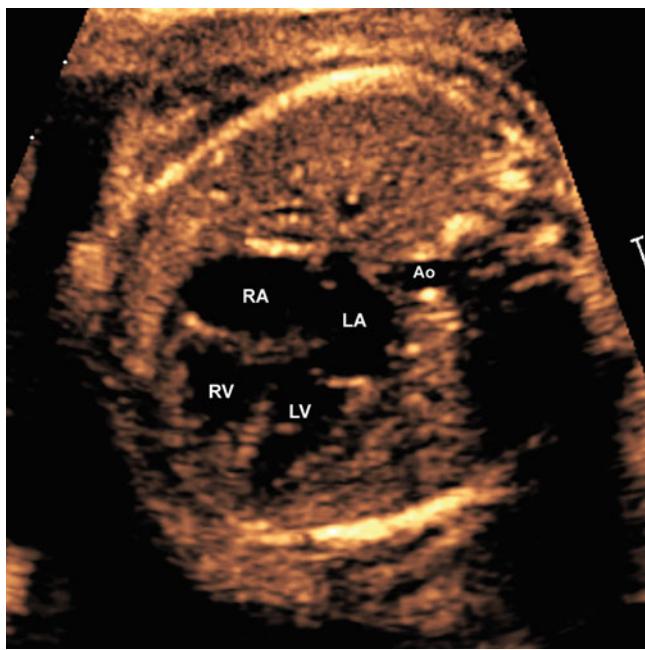


Fig. 12.4 Fetal Echocardiogram: Normal 4 Chamber View of the Fetal Heart (obtained using an iE33 system and 5-MHz curvilinear probe; Philips Healthcare, Bothell, WA) in a breech fetus at 26 weeks gestational age. Ant, anterior chest wall; Ao, Aorta; Post, Posterior Spine; RA Right Atrium; RV Right Ventricle; LA Left Atrium; LV Left Ventricle. Image courtesy of Jay Pruetz, M.D., Assistant Professor of Pediatrics, Division of Cardiology, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California

Given that nuchal thickening is clearly associated with chromosome abnormalities, most commonly trisomy 21, and that it is the most common abnormal ultrasound finding in the first trimester, ultrasound evaluation of nuchal thickness in the first trimester in combination with maternal serum markers has proven to be one of the most important early screening tools to evaluate an increased risk of aneuploidy [156]. In a review of ultrasound diagnosis of fetal abnormalities in the first trimester, Dugoff cites the work of Hyett et al., who reported on an association between increased nuchal translucency and heart abnormalities. In that study, the prevalence of major cardiac defects increased with nuchal thickness from 5.4 per 1,000 for translucency of 2.5–3.4 mm to 233 per 1,000 for translucency ≥ 5.5 mm. The authors recommended that when fetuses have a thickened nuchal fold and normal chromosomes, fetal echocardiography at 18–22 weeks' gestation is merited, besides close scrutiny of cardiac anatomy in the first trimester [157] (Fig. 12.4). This topic was reviewed by Clur et al. in 2009 [158]. They concluded that an increased NT is associated with an increased risk for congenital heart disease with no bias for one form or another. The risk increases with increasing NT measurement. In combination with tricuspid regurgitation and an abnormal ductus venosus Doppler flow profile, however, it is a strong marker for congenital heart disease. The authors recommended a fetal echo at 18–22 weeks' gestation in fetuses

with a nuchal translucency of ≥ 95 th percentile but less than the 99th percentile. In fetuses with a nuchal translucency measurement ≥ 99 th percentile or in which tricuspid regurgitation and/or an abnormal ductus venosus flow pattern had been found, an earlier fetal echo was recommended.

Sau et al. evaluated the significance of a positive second-trimester serum screen in women who were screen negative after a first-trimester nuchal translucency scan. Of 2,683 women screened, eight cases of trisomy 21 were detected, all of which had a positive nuchal screen result. Serum screening of 1,057 women who screened negative by nuchal translucency showed 46 high-risk results, all of which proved to be false-positive. The authors concluded that second-trimester biochemistry screening following a negative nuchal translucency screen did not increase the detection of trisomy 21 [159].

Cuckle and Maymon reported on a method whereby fetus-specific Down syndrome risks in twins could be assessed taking the other fetal nuchal fold into account [160]. This was based upon the previous report by Wøjdemann et al. that there is a correlation coefficient of 0.34 between the pairs of NTs, expressed in log multiples of the median for crown-rump length [161]. This was seen in both monochorionic and dichorionic twins. Cuckle and Maymon found a correlation coefficient in unaffected pregnancies of 0.45 ($P < 0.0001$) and estimated to be 0.12 and 0.04 in discordant and concordant twins, respectively [160].

Cystic Hygroma and Cytogenetic Evaluation of Cystic Hygroma Fluid

Women whose second- or third-trimester fetuses have large cystic hygromas may not have an easily accessible fluid pocket in which to perform an amniocentesis. In such cases, paracentesis of the hygroma may yield a cytogenetic result, and at fetal demise or delivery, chorionic villus or placental cell cultures may prove beneficial in obtaining chromosomal diagnosis. The yield from amniocentesis is still the greatest, so if it can be accomplished, this is still the procedure of choice for cytogenetic diagnosis in such cases [162].

Heart Abnormalities

Structural Heart Abnormalities

Structural heart abnormalities are a well-established risk factor for chromosome abnormalities. Postnatal data indicate a frequency of chromosome abnormalities in infants with congenital heart diseases to be 5–10%, and 2–8 per 1,000 live births have a structural cardiac abnormality [163]. Prenatal data indicate that up to 32–48% of fetuses with cardiac abnormalities are chromosomally abnormal [163–165]. The difference between prenatal and postnatal data probably reflects the high incidence of *in utero* demise in fetuses with chromosome abnormalities.

The most frequent prenatally and postnatally diagnosed heart abnormality is the ventricular septal defect, followed by tetralogy of Fallot (TOF), right or left hypoplastic heart, and

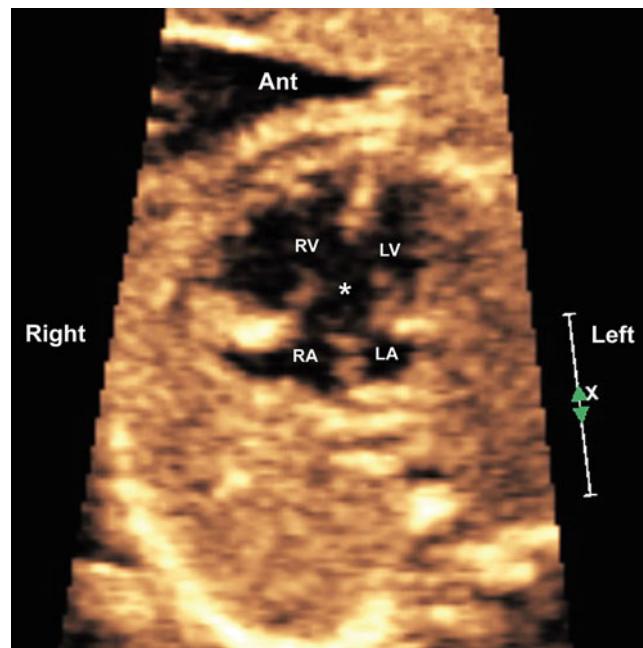


Fig. 12.5 Fetal Echocardiogram: Abnormal 4 Chamber View of the Fetal Heart (obtained using an iE33 system and 5-MHz curvilinear probe; Philips Healthcare, Bothell, WA) in a fetus with confirmed trisomy 21 showing a complete atrioventricular (AV) canal defect with large inlet ventricular septal defect and primum atrial septal defect (*). Note the AV valves are located at same level and the crux of the heart

is not formed. There was also mild mitral regurgitation on color Doppler assessment (not shown). RA Right Atrium; RV Right Ventricle; LA Left Atrium; LV Left Ventricle. Image courtesy of Jay Pruetz, M.D., Assistant Professor of Pediatrics, Division of Cardiology, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California

transposition of the great arteries. Many investigators use the four-chamber view (Fig. 12.4) to evaluate the fetal heart, with an 80–92% sensitivity claimed by this method [166]. However, the four-chamber view alone will not detect TOF or transposition of the great arteries, and only detects approximately 59% of heart abnormalities. A complete atrioventricular canal defect is seen in a fetus with trisomy 21 in Fig. 12.5.

Extracardiac abnormalities are seen, depending on the gestational ages at which the ultrasound evaluations are performed and what is considered an abnormality, in 36% to 71% of fetuses with heart abnormalities [165–167]. The presence of extracardiac abnormalities increases the risk of a chromosome abnormality from 32–48% to 50–71%.

Conotruncal heart abnormalities are those related to faulty conotruncal septation, or division, of the single primitive heart tube into two outflow tracts that in turn result from the fusion of two swellings that arise in the truncal region at 30 days' gestation. With increasing awareness of the strong association between conotruncal heart abnormalities and chromosome 22q11 deletions or microdeletions, it is now recommended that FISH analysis of this region be performed when a conotruncal heart abnormality is seen on fetal ultrasound and fetal chromosomes are normal. In five patients whose fetuses had fetal cardiac abnormalities and a prenatal diagnosis of 22q11 deletion [del(22)(q11.2)], the heart abnormalities included TOF with absent pulmonary valve, pulmonary atresia with VSD, truncus arteriosus, and left

atrial isomerism with double outlet right ventricle. One of the fetuses had an absent kidney, and the others had isolated cardiac abnormalities [166].

A population-based study of the 22q11.2 deletion was undertaken by a group from Atlanta, Georgia. They evaluated data on babies born from 1994 to 1999 in the Atlanta area and matched those records with the Metropolitan Atlanta Congenital Defects Program, a local heart center, and the genetics division at Emory University in Atlanta. Among 255,849 births, 43 children were found to have 22q11.2 deletions for an overall prevalence of 1 in 5,950 births [167]. Thirty-five of the children had heart abnormalities as shown in Table 12.12. What the investigators found was that about one of every two cases of interrupted aortic arch, one of every five cases of truncus arteriosus, and one of every eight cases of tetralogy of Fallot in the population were due to the deletion. See Tables 12.12 and 12.13 for a listing of the data from this study.

Intracardiac Echogenic Foci

Echogenic lesions within the fetal cardiac ventricles have been recognized since 1987, when they were described in the left ventricles of 3.5% of fetuses examined by ultrasound [170]. The foci were attributed to thickening of the chordae tendinae. Others have reported a 20% incidence of left ventricular echogenic foci and right ventricular foci in 1.7% [171]. See Fig. 12.6.

Table 12.12 Cardiovascular abnormalities in children with 22q11.2 deletion in Atlanta study, 1994–1999

Finding	Total no.	Percent ^a (%)	% of total ^b
<i>Cardiac abnormalities^c</i>	35	100	81
Interrupted aortic arch type B	8	23	19
Truncus arteriosus	4	11	9
Tetralogy of Fallot and variants	15	43	35
Pulmonary atresia with VSD	6	17	14
Tetralogy of Fallot, absent pulmonary valve	3	9	7
Tetralogy of Fallot, simple	6	17	14
D-transposition of great arteries	1	3	2
Valve pulmonic stenosis, apical VSDs, ASD	1	3	2
Ventricular septal defect	7	20	16
<i>Vascular abnormalities</i>	22	63	51
Right aortic arch	15	43	35
Mirror image of brachiocephalic vessels	5	14	12
Vascular ring	2	6	5
Aberrant origin subclavian artery	7	20	16
Left superior vena cava	4	11	9

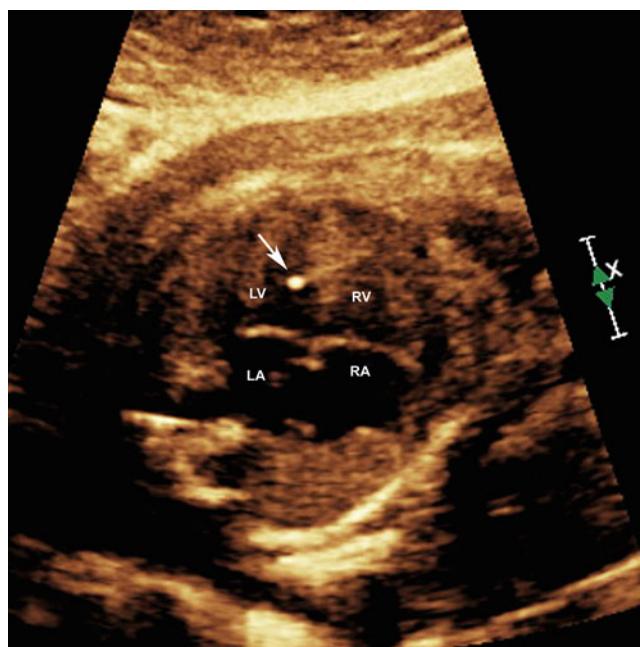
Data from reference [167]

VSD ventricular septal defect, ASD atrial septal defect

^aPercentage among children with 22q11.2 deletion and cardiovascular findings (*n*=35)^bPercentage among all children with 22q11.2 deletion (*n*=43)^cOne child had interrupted aortic arch and truncus arteriosus**Table 12.13** Clinical findings amenable to ultrasound detection that are consistent with 22q11.2 deletion

Finding	Number	Percent	one in
Any major diagnostic finding	43	100	
Cardiovascular			
Heart and great arteries	35	81	1.2
Vascular (branch arteries and great veins)	22	51	2.0
Spina bifida	2	4.7	22
Brain stem anomaly	1	2.3	43
Communicating hydrocephalus	1	2.3	43
Eventration of diaphragm	1	2.3	43
Thoracic hemivertebrae	2	4.7	22
Rib abnormalities	1	2.3	43
Polydactyly of hands	1	2.3	43
Hydronephrosis	3	7.0	14
Renal atrophy	1	2.3	43
Renal cyst	1	2.3	43

Data from reference [167]

**Fig. 12.6** Fetal Echocardiogram: Normal 4 Chamber View of the Fetal Heart (obtained using an iE33 system and 5-MHz curvilinear probe; Philips Healthcare, Bothell, WA) showing a single left ventricular echogenic focus or LVEF (arrow). Note the LVEF is brighter than the surrounding myocardium and is located in the mitral valve apparatus inside

the LV chamber and not within the myocardium itself. *RA* Right Atrium; *RV* Right Ventricle; *LA* Left Atrium; *LV* Left Ventricle. Image courtesy of Jay Pruetz, M.D., Assistant Professor of Pediatrics, Division of Cardiology, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California

The association between left ventricular echogenic foci and chromosome abnormalities was noted in a study of 2,080 fetuses at 18–20 weeks' gestation; 33, or 1.6%, had an echogenic focus. Four of these had chromosome abnormalities (two trisomy 18, one 45,X, and one trisomy 13). All had other abnormalities, including heart defects [170].

The natural history of echogenic intracardiac foci was studied in a cohort of 1,139 patients [171]. Echogenic foci were seen in 41 of 1,139 fetuses, or 3.6%. In 38, the foci were in the left ventricle; in two, they were in the right ventricle; and in one, they were in both. None of these fetuses had other abnormalities. The echogenic foci were again seen in the 27 newborns having echocardiograms up to 3 months of age. The authors pointed out that the key clinical significance of echogenic lesions is that they should be differentiated from intracardiac tumors and ventricular thrombi.

The outcomes of 25,725 ultrasound examinations were reported in a retrospective study from 12 to 24 weeks' gestation [172]. Echogenic intracardiac foci were seen in 44 cases (0.17%). Of the 35 fetuses with left-sided isolated foci, all had uneventful neonatal courses. In nine others, multiple foci were seen, involving the right ventricle in five cases. Of these, two had uneventful courses, but the other seven had additional findings, including five with structural or functional cardiac disease (including one with trisomy 13), one with GM₁ gangliosidosis, and one with echogenic bowel and missed abortion. The paper includes a useful discussion of the various possible causes of the echogenic foci, and the authors conclude by agreeing with the consensus that isolated left ventricular echogenic foci are a benign finding, but other echogenic intracardiac findings may not be.

Two subsequent publications, in contrast, found a significantly increased risk of trisomy 21 in fetuses with an echogenic intracardiac focus. In a study by Bromley et al. of 1,334 high-risk second-trimester patients, 66 (4.9%) had an echogenic intracardiac focus [173]. Four of twenty-two (18%) trisomy 21 fetuses had an echogenic focus, compared with 62 (4.7%) of 1,312 fetuses without trisomy 21. The presence of this finding increased the risk of trisomy 21 four-fold. In two of the trisomy 21 fetuses, no other ultrasound abnormalities were seen.

In a retrospective blinded study of pregnancies at 15–21 weeks' gestation, Norton et al. found an echogenic focus of unspecified location in the heart in five of 21 (24%) trisomy 21 fetuses compared to four of 75 (5%) controls, yielding an odds ratio for trisomy 21 of 5.5 (1.12 < OR < 28.4) when an echogenic focus is seen [174].

The variations in reported incidences of echogenic intracardiac foci (EIF) probably reflect the differences in definition of echogenic foci and in ultrasound machines. Ranzini et al. note that visualization depends on the orientation of the four-chamber view. In 89 fetuses with intracardiac echogenic foci, the foci were seen in only 29 with a lateral four-

chamber view, and they were seen in all 89 with an apical four-chamber view [175]. Wax et al., in a study of second-trimester high-risk pregnancies, classified the foci by their echo amplitude and found that fetuses whose echogenic foci images were lost at the same gain setting as that of the thoracic spine had a 40% risk of aneuploidy (two of five fetuses, $p=0.005$) [176]. That some centers report an association between the foci and an increased incidence of trisomy 21 and other chromosome abnormalities, and others do not, may reflect differences in the populations studied—whether small or large, whether high risk or not. Another difference that has been documented is that an isolated echogenic intracardiac focus is more prevalent among Asian fetuses compared to non-Asians, making it much less helpful in the risk assessment in this population [177].

In a review of second-trimester ultrasound evaluations over the past 30 years, Benacerraf reviewed the literature regarding EIF and concluded that the presence of an EIF alone in a patient previously found by screening to be at low risk does not constitute an indication for prenatal diagnosis [178]. She pointed out in her review that one-third of US practitioners in a survey indicated they do not tell their patients of the finding when it is the only ultrasound finding.

Nuchal Translucency and Echogenic Intracardiac Foci

To test the hypothesis that increased first-trimester nuchal translucency is associated with isolated intracardiac foci in the second trimester, Prefumo et al. evaluated 7,686 normal singleton fetuses who had a nuchal translucency scan and either a subsequent normal follow-up scan at 18–23 weeks ($n=7,447$) or isolated intracardiac foci ($n=239$) [179]. They found that the prevalence of echogenic intracardiac foci in fetuses with normal nuchal translucency was 2.9% versus 8.1% in the fetuses with abnormal nuchal translucency. The adjusted odds ratio was 2.92. The authors concluded that an association exists between first-trimester nuchal translucency and second-trimester echogenic intracardiac foci, so they should not be used independently in risk calculations.

Nasal Bone

Hypoplasia or “absence” of the nasal bone is a more recently described ultrasound finding that appears to improve the detection of fetuses with trisomy 21 [182–186]. In 2001 Cicero et al. reported that in about 70% of fetuses with trisomy 21 from 11 to 14 weeks' gestation, the nasal bone is not visible. In a follow-up study to determine whether fetal nuchal thickness and the level of maternal serum biochemical markers is independent of the presence or absence of the nasal bone, Cicero's group performed a retrospective case-control study of 100 trisomy 21 fetuses and 400 chromosomally normal fetuses. The nasal bone was absent in 69 and present in 31 of the trisomy 21 fetuses. There were no significant differences in any of the other study variables.

It was concluded that for a false-positive rate of 5 percent, screening with nuchal thickness, nasal bone, maternal free β -hCG and PAPP-A would be associated with a detection rate of 97%. For a false-positive rate of 0.5%, the detection rate was 90.5% [187].

Another study by Cicero et al. attempted to answer the question concerning the association between “absence” of the nasal bone at 11–14 weeks and chromosome abnormalities [183]. In this study, 3,829 fetuses were studied. Maternal ethnic origin was recorded. The fetal profile was successfully recorded in 98.9% of cases. In 3,358 of 3,788 cases, the fetal chromosomes were normal, and in 430, they were abnormal. In the chromosomally normal group, the incidence of absent nasal bone was related primarily to the ethnic origin of the mother. It was absent in 2.8% of Caucasians, 10.4% of Afro-Caribbeans, and 6.8% of Asians.

The nasal bone was absent in 66.9% of fetuses with trisomy 21. In trisomy 18 fetuses, it was absent in 57.1%, and with trisomy 13, it was absent in 31.8%. In Turner syndrome and in other chromosome abnormalities, the rate was 8.3–8.8%.

A study in Denmark showed the combination of nuchal translucency and visualization of the nasal bone between 11 and 14 weeks to be as good a predictive marker as nuchal translucency and biochemical markers [186]. Zoppi et al. evaluated other fetal chromosome abnormalities with regard to nonvisible nasal bone and found the bone not to be visible in four out of five trisomy 18 fetuses, in two out of three Turner syndrome fetuses, and in 0.2% of fetuses with normal karyotypes [182].

The literature to date suggests that when adequate visualization is possible, which occurs in 91.9–98.9% of series, absent or hypoplastic nasal bone is seen in 60–80% of fetuses with trisomy 21. Bunduki et al. performed ultrasound examinations on 1,923 consecutive singleton pregnancies at 16–24 weeks and noted that nasal bone length increased as a function of gestational age, showing a linear relationship [187]. Screening for trisomy 21 using the 5th percentile as a cutoff value resulted in a sensitivity of 59.1% for a 5.1% screen-positive rate. The likelihood ratio was 11.6.

A review of “promises and pitfalls” of first-trimester sonographic markers in the detection of fetal aneuploidy by Borrell in 2009 described the challenges inherent in imaging the fetal nasal bone, including incorrect insonation angle and nonsagittal section [188]. He cited a detection rate of trisomy 21 with a 2.5% false-positive rate based upon Cicero’s studies.

In Benacerraf’s review of current practice of second-trimester sonographic markers for the detection of trisomy 21, she also notes the importance of proper insonation angle, as otherwise the nasal bone might appear short, thus increasing the false-positive rate for this marker [178]. Using the criterion of absent nasal bone ossification, the detection rate in the second trimester is lower (30–40%), but the false-positive rate is very low. If a hypoplastic nasal bone is used,

in which different criteria exist for this measurement, the detection rate might be as high as 70%, but the false-positive rate rises to 5%. She cited a prevalence of 8.8% for small or absent nasal bone in the Afro-Caribbean population as opposed to 0.5% of Caucasian fetuses, based upon Cicero’s studies.

Renal Pyelectasis

Renal pyelectasis is mild dilation of the renal pelvis. A possible link between fetal renal pyelectasis and trisomy 21 was described in 1990 [189]. This led to other studies with conflicting results. In 1996, Wickstrom et al. published a prospective study of 7,481 patients referred for prenatal ultrasound evaluation [190]. Of these, 121 (1.6%) had isolated fetal pyelectasis (defined as ≥ 4 mm before 33 weeks’ gestation and ≥ 7 mm at 33 weeks’ gestation). This compares with prevalences of 1.1–18% in other studies. Of the 121, 99 karyotypes were available. One of these was trisomy 21, and the other was mosaic 47,XYY/46,XY. Based on maternal age and the baseline risk for trisomy 21 in the population, the authors calculated a relative risk of 3.9 for trisomy 21 when isolated renal pyelectasis is seen and a 3.3-fold increase in risk for all chromosomal abnormalities in the presence of isolated fetal pyelectasis.

Corteville et al. studied 5,944 fetuses for the presence of pyelectasis, defined as an anteroposterior renal pelvic diameter of 4 mm or greater before 33 weeks or 7 mm or greater after 33 weeks, the same definition as was used by Wickstrom et al. [190, 191]. Pyelectasis was seen in 4 of 23 (17.4%) trisomy 21 fetuses and in 120 of 5,876 (2%) normal controls. This was statistically significant at $p < 0.001$. When fetuses with other ultrasound abnormalities were excluded, the predictive value of pyelectasis fell from 1 in 90 to 1 in 340. They recommended that amniocentesis should be reserved for those cases presenting other risk factors such as advanced maternal age, abnormal maternal serum screening results, or other ultrasound abnormalities. They did not adjust the risk for trisomy using maternal age.

In a literature review study, Vintzileos and Egan found that isolated pyelectasis was not associated with an increased risk for trisomy 21 unless other markers were present, such as those noted previously [191, 192]. In Benacerraf’s review, she agreed, noting that the sensitivity for this marker is low, at 17–25%, with a false-positive rate of 2–3%, “making this a minor marker, used almost exclusively in conjunction with others or in patients already at high risk” [178].

Degani et al. evaluated the recurrence rate of fetal pyelectasis in subsequent pregnancies [193]. They studied 420 women with two consecutive normal uncomplicated pregnancies screened at 15–24 weeks by ultrasound. Pyelectasis was defined as a fetal pelvis of 4 mm or more in its anteroposterior dimension. Of 64 women with fetuses with pyelectasis, 43 (67%) had a recurrence in the next pregnancy.

Compared with normal fetuses, those with pyelectasis had a relative risk of 6.1 to have a recurrence (95% confidence interval=4.3–7.5, $p<0.001$). This study has implications for determining the clinical significance of pyelectasis. In this regard, Johnson et al. studied 56 pregnant women with fetal pyelectasis or cystic lesions identified from 7,500 ultrasound examinations [194]. They found that none of 50 kidneys 15 mm or smaller in anteroposterior diameter had obstruction, and 11 of 14 (79%) kidneys larger than 15 mm were obstructed or showed vesicoureteral reflux on postnatal examination. Noting that other studies have found the need for intervention in the child after a prenatal ultrasound finding of 10-mm dilation, they recommended complete radiological evaluation after birth for infants with pelvic diameters exceeding 10 mm. For children with mild to moderate unilateral hydronephrosis, evaluation may be delayed for 1–2 weeks because oliguria in the first 2 days of life leads to an underestimation of the degree of hydronephrosis.

Choroid Plexus Cysts

The existence of choroid plexus cysts (CPC) has become recognized, along with several other fetal ultrasound findings, due to improvements in ultrasound imaging. CPC were first described in 1984 [195]. The choroid plexuses are round or oval anechoic structures within the choroid plexus of the lateral ventricle derived from neuroepithelial folds. CPC are seen in 0.18–2.3% of pregnancies [196]. These cysts usually disappear in the second trimester in normal pregnancies but may also disappear in chromosomally abnormal pregnancies [197].

The first association between CPC and fetal trisomy 18 was published in 1986 by Nicolaides et al. [198]. In the intervening years, many publications on the association between CPC and chromosome abnormalities have come out. Consensus has been reached as to the positive association between CPC and chromosome abnormalities. However, investigators have differed in their conclusions as to whether an isolated CPC confers a risk of chromosome abnormality high enough to warrant amniocentesis or whether the risk is not high enough to routinely recommend amniocentesis unless other risk factors are present [196, 199–206]. Gross et al. prospectively studied patients at their institution and reviewed literature to include a meta-analysis of other studies prospectively done with more than ten cases of CPC. From these data, they estimated the risk of trisomy 18 in fetuses with isolated CPC to be one in 374. From the incidence of trisomy 18 and of isolated CPC, plus these data, they estimated the positive predictive value of CPC with trisomy 18 in the general prenatal population to be one in 390. [205]

Nyberg et al. reviewed 47 consecutive cases of trisomy 18 and found that 12 of 47 fetuses (25%) had CPC, two of whom had no other ultrasound abnormality [207]. Although trisomy 18 is the chromosome abnormality most often associated with CPC, seen in about three-fourths of aneu-

ploid fetuses with CPC, trisomy 21, mosaic trisomy 9, triploidy, 47,XXY and 45,X/46,XX, trisomy 13, unbalanced (3;13) translocation, and cri du chat syndrome [del(5p)] have also been seen in fetuses with CPC [196, 199, 202, 204, 205, 208].

Shields et al. include mention of two issues in CPC, namely size and uni- versus bilaterality [199]. They conclude, based on a review of the literature, that neither size nor laterality plays a part in the risk assessment. Size varies with gestational age, and laterality can be difficult to determine due to near-field artifact on ultrasound examination. These conclusions were also reached by Meyer et al. in a retrospective review of 119 pregnancies with CPC [209].

Demasio et al. performed a meta-analysis of eight prospective trials of 106,732 women under 35 years of age with pregnancies affected by isolated choroid plexus cysts [210]. If serum screening was positive, the woman was excluded from analysis, although those data were not available for all in the study. A total of 1,235 fetuses had choroid plexus cysts for an incidence of 1.2%. None had chromosome abnormalities. The authors contend that amniocentesis is not warranted in women with otherwise normal ultrasound examinations who are less than 35 years old or the equivalent by serum screening.

Another meta-analysis was performed by Yoder et al. to assess the risk of trisomies 18 and 21 with isolated choroid plexus cysts [211]. Women of all ages were included in the 13 prospective studies, comprising 246,545 second-trimester scans. The likelihood ratio for trisomy 18 was 13.8 and for trisomy 21 was 1.87. The authors concluded that their data support offering women amniocentesis to evaluate trisomy 18 when maternal age is 36 or older or when the risk for trisomy 18 detected by serum marker screening is greater than or equal to 1 in 3,000. In another study by Ghidini et al., a likelihood ratio for trisomy 18 for isolated choroid plexus cysts in the second trimester was 7.09. They advocate multiplying the patient's prior risk by this figure to decide on whether amniocentesis is indicated [212].

Benacerraf's review of this topic concluded that the incidence of choroid plexus cysts in fetuses with and without trisomy 21 is the same [178].

On balance, counseling regarding isolated CPC clearly cannot be undertaken in a vacuum. A young woman with a negative triple marker screen for trisomy 18 and no other ultrasound abnormalities is much less likely to be carrying a fetus with trisomy 18 than is a 39-year-old woman with a triple marker screen result positive for trisomy 18 and no other fetal ultrasound abnormalities.

Even without other ultrasound abnormalities and with normal chromosomes, CPC can be frightening to prospective parents, who often are concerned about a "hole in my baby's head." It is important to explain their significance in a balanced way, to indicate that in the majority of fetuses, they are an incidental finding and that they are likely to

disappear before birth. Results of a follow-up study (mean 35.5 ± 16.2 months) on 76 children who as fetuses were found to have CPC are also reassuring; no effect on development was found as measured by the Denver II Developmental Screening Test [213].

Short Humerus or Femur

Measurement of the long bones of the fetus does not require the same level of expertise as evaluating more subtle structural malformations. Thus, because shortness of the long bones is associated with an increased risk of chromosome abnormalities and because the length is relatively easy to measure, several investigators have focused on this finding as a way of increasing or decreasing a woman's *a priori* risk of having a fetus with a chromosome abnormality.

Shortness of the humerus and the tibia might have greater sensitivity in detecting trisomy 21 than shortness of the femur and fibula, as was found in a prospective study of 515 patients between 14 and 23 weeks' gestation who were at increased risk for a chromosome abnormality because of age or triple marker screening results or both [214]. Tables of risk for trisomy 21 for maternal age and maternal serum screening positive status were developed that take into account all four long bones' lengths being normal versus one, two, three, or four bone lengths being normal. Use of this approach led to the conclusion that if all long-bone lengths are normal, amniocentesis may not be recommended to women under age 40. Others have not found femur length to be reliable in ultrasound screening of trisomy 21, although humerus length does appear to be associated [215, 216]. The positive predictive value for trisomy 21 in women with risks of 1 in 500 and 1 in 1,000 was found to be 2.3 and 1.2%, respectively.

A significant confounder, however, is that long-bone length varies with race, and this factor has not been taken into account in most studies. In a fetal biometry study of Asians, the long-bone lengths were measured in more than 6,000 fetuses, and the conclusion was that the reference charts derived should be used in all Asian fetuses [217]. In a study of 110 Korean fetuses with trisomy 21 and 602 euploid controls, the femur length was found to be a poor predictor of trisomy 21 in the second trimester, with a sensitivity of 32.8% and false-positive rate of 5% [218]. Thus the use of fetal biometric measures should be cautiously interpreted with racial factors in mind.

As Benacerraf has pointed out in her review of second-trimester ultrasound evaluation of trisomy 21, this marker is best used in combination with other markers to predict risk [178].

Hyperechoic Bowel

Hyperechoic bowel (HEB), also known as echogenic bowel and hyperechogenic fetal bowel, is a qualitative ultrasound finding of unclear significance. It has been described as a normal variant with an incidence of 0.2–0.56%, as reviewed

by several authors [219–222]. It is also associated with several adverse outcomes, including fetal chromosome abnormalities, fetal cytomegalovirus infection, other infections, cystic fibrosis (CF), intrauterine growth restriction, fetal demise, and intestinal obstruction possibly related to CF [219, 220, 222–230]. The presence of coexisting elevated maternal serum AFP increases the risk of adverse outcome, particularly fetal IUGR and demise [220, 230]. See Table 12.14. The studies referenced previously describe the finding of HEB on second-trimester ultrasound examination. Third-trimester HEB associated with trisomy 21 has also been reported in a fetus in which the second-trimester scan did not show HEB [231].

The incidence of HEB in second-trimester fetuses with trisomy 21 is 7% [232]. The relative risk of adverse outcome in isolated HEB is 6.5 [230]. Benacerraf notes the sensitivity of hyperechoic bowel in trisomy 21 as ranging from 3.3 to 27%, likely due to the subjectivity in assessment of this marker, but as it has a very low false-positive rate of less than 1%, it remains an important marker for the detection of trisomy 21 [178].

Part of the reported variation in outcome of HEB is due to different degrees of brightness of the finding and also to inter-machine and inter-observer variability (Fig. 12.7). Grades of echogenicity, from 0 (isoechoic) to 3 (bone-like density) have been used [222, 231], but even those compare the finding to different fetal parts—liver versus iliac crest, for example. The more hyperechoic, the higher the risks. Another reason for variability in reported outcomes relates to the *a priori* risks. For example, Caucasian non-Hispanic patients have a much higher *a priori* risk of CF than individuals of other races.

What causes the finding of HEB? One group commented on the decreased microvillar enzymes in amniotic fluid in pregnancies affected by trisomy 21, trisomy 18, and CF [230]. It was thought that the low levels in CF may be due to delayed passage of meconium, and in trisomy 18 and 21, the delayed passage may be due to decreased bowel motility or abnormal meconium. Fetuses with intra-amniotic bleeding have a four- to sevenfold increase in HEB [222, 232]. These investigators hypothesized that swallowing of amniotic fluid containing heme pigments after intra-amniotic bleeding seemed to be the cause of the echogenicity.

Other Ultrasound Markers of Aneuploidy

A summary of several series of ultrasound studies indicating risks of chromosome abnormalities in association with specific ultrasound findings is shown in Table 12.15. Clearly, some ultrasound markers in isolation indicate a significant risk of chromosome abnormality, and others may not achieve significance unless other ultrasound abnormalities or other maternal risk factors are present. Less commonly used markers and abnormalities for trisomy 21 risk assessment include wide iliac angle, flat facies, sandal gap foot, short

Table 12.14 Clinical outcome of second-trimester finding of isolated bright hyperechoic bowel^a

	Scioscia [219] ^b	Nyberg [220] ^c	Bromley [222] ^b	Slotnick [229] ^c	Muller [224] ^b	MacGregor [225] ^c
1. No. of cases with isolated bright HEB	18	64	42	102	182	45
2. No. of cases with normal outcome (%)	13 (72)	41 (75)	26 (62)	—	111 (67)	34 (76)
3. No. of cases with chromosome abn. (%)	2 (11) ^d	7 (11) ^f	0	5 (4.9) ^g	8 (4.5)	0/16 (0)
4. No. of cases with cystic fibrosis mutations (%)	0/17 (0)	NT ^e	NT ^h	7/65 (11)	10/116 ⁱ (8.6)	2/15 (13)
5. No. of cases with infections (%)	NT ^h	1 (1.6)	—	—	7/? (?)	2/45 (4)
6. No. of cases with IUGR (%)	1 (5.6)	6 (9.3)	8 (19)	—	10/121 (8)	NR ^j
7. No. of cases with nonelective demise (%)	2 (11)	3 (4.7)	15 (36)	—	24/104 (23)	3/45 (6.7)

^aThis table excludes fetuses with ultrasound abnormalities other than isolated HEB

^bRetrospective study

^cProspective study

^dBoth trisomy 21

^eSeven CF mutations tested

^fFive trisomy 21, one 47,XXX, one trisomy 13

^gAll trisomy 21

^hNot tested

ⁱOne ΔF508 homozygote, 9 heterozygotes; 7 of the 9 were unaffected, and the other 2 had no follow-up information

^jOne to eight mutations tested

^kNot reported



Fig. 12.7 Ultrasound image of moderately hyperechoic bowel (indicated by plus signs (+) and circle of dots) in a 17-week gestation fetus. ST = stippling, referring to pattern of hyperechogenicity. (Courtesy of Gregory DeVore, MD.)

frontal lobe, clinodactyly/hypoplastic midphalanx of the fifth digit, brachycephaly, small ears, and small cerebellar diameter [178].

In the past 15 years, medicine in the United States has evolved from recommending amniocentesis to women 35 and older to refining risks based upon a variety of ultrasound and maternal serum screening markers. This has led

to increased detection of chromosome abnormalities while not significantly increasing the use of amniocentesis since some women 35 and older now have their *a priori* risks altered downward and choose not to have amniocentesis as a result. This was alluded to previously as well [106]. Several scoring indices have been developed to provide individualized risk assessments [216, 233–239]. The fact is that anyone with an ultrasound machine in the office may do an ultrasound examination, and the range of expertise and resolution vary significantly among practitioners and machines. Optimally, each practitioner should develop his or her own index based on the prospective evaluation of a large series of patients. These indices will be much more valid in that practice than those derived from the literature.

Limitations Imposed by Maternal Habitus

It is universally recognized that women with high body mass indices (BMI) present a challenge to visualization of fetal findings. This was shown in the FASTER trial, in which a BMI of 30 or higher was the cutoff for this assessment [240]. To quantify the degree of difficulty, Tsai et al. undertook a study to determine the degree of completeness of ultrasound surveys for aneuploid markers in women classified as normal, overweight, and obese [241]. Within the obese group, women were further divided into three classes based upon their BMI. The rates of completion of

Table 12.15 Ultrasound markers of fetal aneuploidy

Finding	Risk(s) of aneuploidy if isolated finding	Risk(s) of aneuploidy if other ultrasound abnormalities are present	Comment
Structural heart defect	32–48% [163–165]	50–71% [163–165]	
Echogenic intracardiac foci	Not increased [170]; four- to fivefold baseline risk [173, 174]	1.6-fold baseline risk [170]	Isolated left ventricular foci appear more likely to be benign than multiple or right-sided foci [172]
Renal pyelectasis (≥ 4 mm before 33 weeks and ≥ 7 mm at 33 weeks)	Not increased [192]; 3.3-fold increase over baseline [190]; 1 in 340 [191]	1 in 90 [191]	In Ref. [191], no adjustment was made for maternal age
Choroid plexus cyst(s)	Not increased [196, 204–206]; 1 in 374 for trisomy 18 [204]; 1–2% [199]; 0.6% [200]; 2.4% [209]; 3.1% [201]; 4% [202]; 1 in 82 [203]; 1 in 150 [204]	82% [199]; 3.5% [200]; 5% [112]; 9.5% [201]; one in three [106]	No adjustment for maternal age in Ref. [203]
Septated nuchal membrane, 9–20 weeks; >3 mm, <15 weeks and >5 mm, 15–20 weeks	56–60% [149]	Not studied	Ref. [149] is a retrospective database analysis. All pregnancies included in study had isolated nuchal finding and karyotype
Simple nuchal membrane, 9–20 weeks; >3 mm, <15 weeks and >5 mm, 15–20 weeks	10–25% [149]	Not studied	
Nuchal fold, >5 mm, 15–20 weeks	19–33% [149]	NS*	
Nuchal thickening, 10–15 weeks, ≥ 4 mm	46% [151]	84% if hydrops and/or septations are present [151]	Best outcome was in nonseptated, nonhydropic; worst was in septated/hydropic fetuses
Nuchal thickening, 9–15.5 weeks, ≥ 3 mm	80% [152]		
Septated Simple	19% [152]; 27-fold risk for 34-year-old women and ninefold risk for women 35 and older	Other abnormalities only reported for chromosomally normal fetuses	If all long bones are of normal length and other ultrasound findings normal, some feel amniocentesis is not indicated in women under 40. [214]
Short humerus, femur	Positive predictive value for trisomy 21 in women with risks of 1 in 500 and 1 in 1,000 = 2.3 and 1.2%, respectively, for short humerus [214]	Increased to variable degrees	Racial factors should be considered in any long-bone measurement [217]
Absent nasal bone	For short femur, some studies found very little increased risk [215, 216]	Appears to be independent finding from nuchal translucency, so can be used as independent markers in multiple marker algorithm [185]	Also seen more frequently in other chromosome abnormalities [183]

evaluation among the groups varied from 64% for normal to 47% for class II, $p < 0.001$, and the screen-positive rates, meaning more than one marker was found, differed significantly as well. In this category 16% of normal women were positive compared to 10% of class III, $p < 0.02$. They also noted an increased rate of return visits due to lack of visualization of fetal structures. They recommended that ultrasound results in obese patients should be interpreted with caution and patients counseled accordingly. Aagaard-Tillery et al. evaluated a subset of participants in the FASTER trial in a separate study of the effect of BMI on detection of fetal ultrasound abnormalities [242]. In their evaluation of 8,555 women, the detection rate for heart defects, echogenic foci, and choroid plexus cysts was significantly decreased. In their discussion they point out that patients are often counseled that the risk of aneuploidy is higher if two or more “soft signs” are present. If the likelihood of seeing such soft signs is decreased due to maternal habitus, the rate of detection of aneuploidy among these patients is also decreased. As with the study cited previously, they suggest this factor be taken into account in the counseling of patients.

Positive Maternal Serum Marker Screen

High Maternal Serum Alpha-Fetoprotein

The association between an elevated level (2.0 or 2.5 multiples of the median) of maternal serum alpha-fetoprotein (AFP) and fetal neural tube defects has been known for many years. More recently, the presence of an unexplained elevated level of maternal serum AFP has been found to be associated with an increased risk for fetal chromosome abnormalities, with an incidence of 10.92 per 1,000 amniocenteses [243]. Of these, fetal sex chromosome abnormalities were seen in 47%. Thus, although some practitioners discourage patients from having an amniocentesis with an elevated AFP and a normal ultrasound study, the facts that sex chromosome abnormalities other than 45,X and its related karyotypes have no significant associated ultrasound abnormalities and that they are quite common (with incidences of 47,XXX; 47,XXY; and 47,XYY each ≥ 1 in 1,000 liveborns) support consideration of amniocentesis in this group.

Low Maternal Serum AFP and Multiple Marker Screening

The association between low maternal serum AFP and fetal Down syndrome was established in 1984; and in 1987, the association between high maternal serum human chorionic gonadotropin (hCG) and low unconjugated estriol and fetal Down syndrome was established [244–246]. These three

substances, or markers, were combined now in what was commonly known as triple marker screening (TMS). Hundreds of thousands of women in the United States had TMS in the second trimester of pregnancy, with a resultant increase in detection of trisomy 21 before age 35 and what appeared to be a decrease in the incidence of Down syndrome births due to abortion of affected fetuses. The overall detection of trisomy 21 with TMS is about 65% with a midtrimester risk cutoff of 1 in 190, with a much lower detection in young women (about 44% in 18-year-olds) and a much higher detection in older women (about 78% in 36-year-olds) [247].

TMS detects 60% of trisomy 18 fetuses as well, when a midtrimester risk cutoff of 1 in 100 is used [248].

Less recognized is the fact that TMS detects many chromosome abnormalities nonspecifically, for unknown reasons. Thus for every trisomy 21 fetus found by TMS, a fetus with a different chromosome abnormality is also detected [249]. This is important to keep in mind when counseling patients. Triple marker screening has been supplanted by quadruple and integrated screening, with increased sensitivity and specificity of detection of trisomy 21 [250–255].

Quadruple Screening

Dimeric inhibin A, referred to as simply inhibin A or inhibin in some studies, was added to the triple marker screen panel in recent years and has been shown in several studies to increase the detection of trisomy 21 in the second trimester. In one study of 72 second-trimester fetuses with trisomy 21 and 7,063 unaffected fetuses, the detection of trisomy 21 at a risk cutoff of 1 in 270 was 81.5% with a screen-positive rate of 6.9% and a positive predictive value of 1 in 42 [250]. In other words, 1 in 42 amniocentesis procedures yielded a result of trisomy 21. In a second, larger study of 23,704 women with unaffected pregnancies and 45 women with trisomy 21-affected pregnancies, the sensitivity of the quadruple screen was 85.8%, with an initial screen-positive rate of 9.0%, corrected to 8.2% after gestational age error corrections [253]. The positive predictive rate was 1 in 51. Women who were true-positives had very high risks (median risk of 1 in 22) compared to risks in women with false-positive results (median risk of 1 in 111) [253]. Hackshaw and Wald evaluated the increase in detection of trisomy 21 by performing the triple marker screen followed by the quadruple screen in a series of patients. They found an increase in detection of 3–5% at a 5% screen-positive rate. Their interpretation was that the “modest increase...is probably not worthwhile in the light of the extra cost and delay” [252]. It should be pointed out, however, that similar arguments were made when unconjugated estriol was added to what at the time was a double screen. Based upon a study by Spencer et al. in 45 cases of trisomy 18 and 493 control pregnancies at

10–14 weeks' gestation, inhibin A was found not to add to the detection of trisomy 18 over triple marker screening alone [253]. Nevertheless, second-trimester quadruple screening has largely replaced triple marker screening.

First-Trimester Screening

In 1995 and 1996, first-trimester detection of trisomy 21 using free β -hCG and pregnancy-associated plasma protein A (PAPP-A) was reported [254, 255]. Several more papers have been published since then that have shown first-trimester screening using those biochemical markers plus maternal age alone or in combination with nuchal translucency measurements to be the most sensitive screening method for the detection of trisomy 21 and trisomy 18. Chasen et al. studied a US population of 2,131 pregnancies in 2003 in New York [148]. By using nuchal translucency measurement from 11 to 14 weeks' gestation plus maternal age, the detection of trisomy 21 was 83.3% and the detection of trisomy 18 was 90%. A large multicenter study called the BUN study—short for Biochemistry, Ultrasound, Nuchal translucency was undertaken to screen pregnancies between 74 and 97 days of gestation for trisomies 21 and 18 using maternal age, maternal levels of PAPP-A and free β -hCG, and fetal nuchal translucency measurements in 8,514 patients with singleton pregnancies [257]. In this study, the detection rate for trisomy 21 was 85.2% with a screen-positive rate of 9.4%. If the screen-positive rate was set at 5%, the detection rate of trisomy 21 was 78.7%. Of the trisomy 18 cases, screening identified 90.9% with a screen-positive rate of 2%. For women 35 years or older, 89.8% of fetuses with trisomy 21 were detected with a screen-positive rate of 15.2% and 100% of fetuses with trisomy 18 were detected [257].

Integrated and Combined Screening

The concept of integrated and combined screening examines first- and second-trimester screening in combination or conjunction to improve the detection of aneuploidy while lowering the screen-positive rate. This can be done in varying ways, including using PAPP-A in the first trimester and the aforementioned second-trimester biochemical markers. This methodology was used in a 2003 study to detect 90% of trisomy 18 cases with a screen-positive rate of 0.1% [258].

A multicenter study called the First- and Second-Trimester Evaluation of Risk (FASTER) published its findings in 2005 regarding its comparison of first- and second-trimester screening modalities [240]. The rates of detection of trisomy 21 in 38,167 patients, at a 5% false-positive rate, were as follows: with first-trimester combined screening, 87, 85, and 82% for measurements performed at 11, 12, and 13 weeks, respectively. With second-trimester quadruple screening, rate of detection was 81%; with step-

wise sequential screening, 95%; with serum integrated screening, 88%; and with fully integrated screening with first-trimester measurements performed at 11 weeks, 95%. The authors concluded that first-trimester combined screening at 11 weeks' gestation is better than second-trimester quadruple screening, but at 13 weeks has results similar to second-trimester quadruple screening. Both stepwise sequential screening and fully integrated screening have high rates of detection of trisomy 21, with low false-positive rates.

A study to determine patient choices and screening performance when three trisomy 21 screening protocols were introduced was described by MacRae et al. [259]. In this study, called the SAFER study (second- and first-trimester estimation of risk), integrated screening protocols were chosen 4.6 times more often than four-marker screening (82% vs. 18% uptake). Overall detection was higher and false-positives lower. Overall, of 8,571 women screened and 23 cases ultimately diagnosed with trisomy 21, 21 were detected in the study (91%, 95% CI, 73–98%) at a 4.2% false-positive rate (95% CI, 33.3–5.1%).

Previous Pregnancy or Child with a Chromosome Abnormality

Having a previous pregnancy or child with certain chromosome abnormalities is associated with an increased risk of a future fetal chromosome abnormality [259–262]. The risk is not only for a recurrence of the same trisomy, or homotrisomy, but also for heterotrisomy [261]. The study that examined this question effectively showed that not only gonadal mosaicism is responsible for trisomy recurrence, but some women have a risk for nondisjunction higher than do others of the same age. The reasons for this are not known. Studies in this regard were summarized by Robinson et al. and included polymorphisms in genes involved in folic acid metabolism and folic acid intake, mutations in *MSH2*, a mismatch repair gene, and environmental factors such as caffeine intake and cigarette smoking [262].

Chromosome abnormalities known to increase the future risk of aneuploidy include all nonmosaic trisomies, structural rearrangements, and marker chromosomes. In Warburton's study of recurrence of homotrisomy and heterotrisomy, there was no increased risk of X-chromosome aneuploidy [261]. Genetic counseling is suggested for couples who have had a pregnancy or child with any higher-risk karyotype, and ultrasound plus amniocentesis or CVS are recommended for consideration in future pregnancies [259].

Also not known to be associated with an increased recurrence risk are triploidy, tetraploidy, and 45,X. However, couples who have undergone the experience of having a

pregnancy with one of these findings may wish to have genetic counseling, ultrasound, and prenatal chromosome analysis due to anxiety.

The recurrence risk in *de novo* structural chromosome rearrangements is less than 0.5–2% and takes into account recurrence by chance, gonadal mosaicism, and somatic-gonadal mosaicism, but the numbers have not been derived by extensive study [259–262]. To evaluate this, Röthlisberger and Kotzot performed a literature search and found 29 case reports of recurrence of *de novo* structural chromosome rearrangements. Thirteen of them were due to a *i(21q)* replacing one normal chromosome 21, and in eight of them low-level mosaicism in one of the parents was found. The authors stated, therefore, that the recurrence risk should be reduced to less than 1% for *de novo i(21q)* and to less than 0.3% for all other *de novo* structural chromosome rearrangements. They recommended that prenatal diagnosis be performed only if requested by parents after genetic counseling [263].

Mosaicism presents complicated counseling issues. It is prudent to apprise the couple of this and offer them the opportunity for prenatal diagnosis since the risk of recurrence may be increased. Mosaicism is discussed in more detail later in this chapter.

Other Indications for Prenatal Cytogenetic Diagnosis

Previous Pregnancy or Child with Open Neural Tube Defect

Rates of open neural tube defects (NTD) vary geographically. In California, NTDs occur in 1.05 per 1,000 Hispanic women and 0.66 per 1,000 Asian women, with non-Hispanic Caucasians falling between [96]. The risk of recurrence of an isolated NTD is 3–5%. Folic acid supplementation of 0.4 mg/day periconceptionally decreases the risk by 50–70%, so the increased fortification of grains with 1.4 mg folate per pound of enriched cereal-grain products by the US Food and Drug Administration was announced in 1997 [264]. Having a previous affected pregnancy or child merits offering genetic counseling, ultrasound, and amniocentesis. Such women are advised to take 4 mg folic acid periconceptionally. All women of childbearing age, particularly those at increased risk for NTDs, should receive information about folic acid supplementation.

Chromosome abnormalities are associated with spina bifida and encephalocele, but do not appear to any significant degree to be associated with isolated anencephaly [265, 266]. In Harmon's study of 55,366 pregnancies in which isolated spina bifida was seen by ultrasound, outcome information was available for 43 of 77 cases [267]. Of the 43, seven chromosome abnormalities were detected. One was a balanced Robertsonian translocation and therefore of uncertain

significance. The others were two trisomy 18, two 69,XXX; two 69,XXY; and one X-chromosome inversion. The control population's theoretical risk of chromosome abnormality was 0.3%. The combined findings were statistically significant ($p=0.12$). Other reports have noted mosaic trisomy 14 and full trisomy 9 in association with nasal encephalocele and spina bifida, respectively [268, 269]. Trisomy 13 is also associated with increased risk for spina bifida [270]. NTDs have also been associated with 22q11.2 microdeletion syndrome [271, 272]. Several other microdeletions and microduplications detected by array comparative genomic hybridization (see Chap. 18) have also been reported in association with various types of neural tube defects [273–276].

Chromosome Rearrangement in Either Member of a Couple

Some balanced structural rearrangements (see Chap. 9) predispose a couple to an increased risk of fetal chromosome abnormality. The risk depends on the rearrangement and how it was ascertained.

For balanced reciprocal translocations, if the rearrangement was ascertained through multiple spontaneous abortions, the risk of having a child with abnormal chromosomes is 1.4–4.8%, with the lower risk associated with a paternal carrier. If it was ascertained by a previous child or stillborn with unbalanced chromosomes, the risk increases to 19.8–22.2% [277].

For balanced Robertsonian translocations, the risk of unbalanced chromosomes in the fetus is much less and appears to be negligible when chromosome 21 is involved and if the translocation is paternal [277]. See Table 12.16.

Most pericentric inversions (see Chap. 9), except the population variant *inv(9)*, are associated with an increased risk of unbalanced offspring due to deletions/duplications, and individuals with such inversions should be offered amniocentesis. The risk of unbalanced offspring depends on the length of the inversion segments [278]. See Table 12.17. Whether this recommendation applies to individuals with the common pericentric *inv(2)* is debatable. This inversion is so common that some cytogenetics laboratories do not report it.

Paracentric inversions in a carrier parent may give rise to acentric fragments or dicentric chromosomes, either of which would be expected to be lethal *in utero*. However, amniocentesis is generally to be recommended, given the possibility of viability of a fetus with structurally unbalanced chromosomes and the occasional difficulty in distinguishing between a paracentric inversion and an insertion [279].

Because of the observation that marker chromosomes can interfere with meiosis, leading to aneuploidy, prenatal diagnosis is also recommended to individuals with marker chromosomes, even when these apparently confer no adverse phenotypic effect.

Table 12.16 Prenatal results for Robertsonian translocations involving chromosomes 13, 14, or 15 and 21

Robertsonian translocation type	Maternal carrier				Paternal carrier				Total	Grand total
	Balanced	Normal	Unbalanced	Total	Balanced	Normal	Unbalanced	Total		
der(13;21)(q10;q10)	6	4	2 (16.7%)	12	5	4	0	9	21	
der(14;21)(q10;q10)	36	25	10 (14.1%)	71	9	13	1 (4.3%)	23	94	
der(15;21)(q10;q10)	5	4	0	9	4	2	0	6	15	
Total	47	33	12 (13.0%)	92	18	19	1 (2.6%)	38	130	

Data from reference [277]

Table 12.17 Prenatal results for pericentric inversions ($n=173$)

Method of ascertainment	Maternal carrier				Paternal carrier				Total	Grand total
	Balanced	Normal	Unbalanced	Total	Balanced	Normal	Unbalanced	Total		
Through term unbalanced progeny	6	1	1 (12.5%)	8	2	3	0	5	13	
Through recurrent miscarriages	10	4	0	14	4	2	0	6	20	
Other	63	4	2 (2.9%)	69	68	3	0	71	140	
Total	79	9	3 (3.3%)	91	74	8	0	82	173	

Data from reference [278]

Men with 47,XYY karyotypes usually have normal fertility and may be at increased risk for chromosomally unbalanced offspring. Some of the reported chromosome abnormalities occurring in pregnancies of 47,XYY males include markers, trisomy 21, 47,XYY, and others [280].

Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis, or PGD, involves the use of FISH or molecular testing on embryos for the purpose of implanting healthy ones into the uterus. Given the high risk of spontaneous abortion and of chromosomally abnormal offspring for couples where one member carries a balanced chromosome translocation, PGD increases the likelihood of bearing an unaffected baby [281, 282]. Otani et al. studied couples with balanced translocations in whom no births had occurred after 117 pregnancies and an average of 3.5 spontaneous abortions had occurred. After PGD, 18% of embryos were normal or balanced, and 5% of pregnancies were lost compared to 100% before PGC ($p < 0.001$) [281]. Munné et al. evaluated 35 couples in whom one partner carried a chromosome translocation [282]. They noted a statistically significant reduction in spontaneous abortions from 95 to 13% after PGD. They also noted that the chances of achieving pregnancy were correlated with 50% or more of the embryos being chromosomally normal. Robertsonian translocations caused fewer abnormal embryos than reciprocal translocations, resulting in higher rates of implantation [282].

Also refer to Chaps. 11 and 18 for other discussions regarding the use of PGD.

Advanced Paternal Age

A body of old literature in genetics suggests an increased risk of fetal chromosome abnormality with advanced paternal age, but the most carefully constructed analyses do not support this association [283–287]. Advanced paternal age is not definitively associated with fetal chromosome abnormalities. It is, however, associated with a linearly increased risk of some autosomal dominant new mutations in the offspring due to mutations in the form of single base-pair mutations, particularly in the *FGFR3* and *RET* genes [288]. Less common are mutations due to point mutations and base-pair deletions. Some autosomal dominant conditions show no paternal age association, according to a policy statement on the subject issued by the American College of Medical Genetics [289]. In the statement, the authors point out the four- to fivefold risk of some conditions in offspring of men in their 40s versus those of men in their 20s. The relative increased risk for these defects is related to advanced age of the father for autosomal dominant conditions and the maternal grandfather for X-linked conditions. Family histories will not provide clues, as these types of mutations are sporadic. Examples of autosomal dominant conditions associated with advanced paternal age include achondroplasia, neurofibromatosis, Marfan syndrome, Treacher Collins syndrome, Waardenburg syndrome, thanatophoric dysplasia, osteogenesis imperfecta, and Apert syndrome. Examples of X-linked conditions associated with increased maternal grandfather's age include fragile X syndrome (see Chap. 19), hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), Duchenne muscular dystrophy,

incontinentia pigmenti, Hunter syndrome, Bruton agamma-globulinemia, and retinitis pigmentosa [289]. Genetic counseling is indicated so the expectant couple may understand the issues, and it is prudent to offer detailed fetal ultrasound examination in pregnancies involving men 40 years and older [289]. The American College of Medical Genetics acknowledges the risk but says that ultrasound examination is usually of little benefit.

Special Issues

True Mosaicism and Pseudomosaicism

Mosaicism, or the presence of two or more cell lines originating from a stem cell line, is one of the most complex and challenging issues in prenatal diagnosis. There are three levels of mosaicism in amniotic fluid and CVS culture: levels I, II, and III. Level I is defined as a single-cell abnormality. Level II is defined as a multiple-cell abnormality or (with an *in situ* culture method) a whole colony abnormality in one culture not seen in any other cell cultures. Level III mosaicism is “true” mosaicism—the presence of a second cell line in two or more independent cultures. The incidences of these in amniotic cell cultures range from 2.5 to 7.1% for level I, 0.6–1.1% for level II, and 0.1–0.3% for level III mosaicism [290–292].

The origin of the mosaic cell line cannot be determined without molecular studies. In general, however, it appears that the majority of 45,X/45,XX cases occur after a normal disomic fertilization, most mosaic trisomies are due to postzygotic loss of the trisomic chromosome, and, for trisomy 8, most cases are due to somatic gain of the third chromosome 8 postzygotically [293].

In addition to the level of mosaicism, the chromosome involved is an important consideration. True mosaicism has been reported in liveborns with almost all trisomies [37]. However, true mosaicism for trisomies 8, 9, 21, 18, 13, 16, X, and Y and for monosomies X and Y has potentially great significance. For chromosomes 8 and 9, mosaicism is the most common form in which trisomies occur in liveborns, perhaps because the full trisomy is not compatible with fetal survival in the majority of cases [294, 295].

Three prenatal cases with mosaic trisomy 9 were reported by Takahashi et al., in which two showed severe IUGR and had fetal demise in the third trimester and the third was born with diaphragmatic hernia and mild IUGR [296]. The authors note that in nine previously reported continuing pregnancies with trisomy 9 mosaicism, eight were live births and one died in utero. Even one cell with trisomy 8 may be significant. For trisomies of chromosomes 13, 18, 21, X, and Y and monosomy X and Y, mosaicism has been fairly commonly reported, and the clinical manifestations may vary from no apparent abnormality, at least in the newborn period, to more characteristic features of the full trisomy. The degree of mosaicism is not related to the outcome [29]. See Table 12.18 for incidences of mosaicism for specific chromosomes.

Schuring-Blom et al. evaluated first-trimester cytrophoblast cell preparations—direct preparations—showing full or mosaic trisomy 13 or 18, with the purpose of determining how often the result was a true-positive in the fetus or newborn [297]. Cultured mesenchymal tissue was available only for about half of the cases. Of the 51 cases, five false-positives were seen in those with full trisomy 18 and three with mosaic trisomy 18. One false-positive was seen in full trisomy 13, and two false-positives were seen in mosaic trisomy 13. Their conclusions were that:

Table 12.18 Outcome of cases with rare autosomal trisomy mosaicism diagnosed in amniocytes

Type	Abnormal outcomes/total no. of cases	Abnormal phenotype (no. with IUGR) ^a	Fetal demise or stillborn
46/47,+2	10/11 (90.9%)	7 (2)	3
46/47,+3	1/2 –	1	0
46/47,+4	1/2 –	1	0
46/47,+5	2/5 (40.0%)	2 (2)	0
46/47,+6	0/3 –	0	0
46/47,+7	1/8 (12.5%)	1	0
46/47,+8	1/14 (7.1%)	1	0
46/47,+9	14/25 (56.0%)	14 (2)	0
46/47,+11	0/2 –	0	0
46/47,+12	6/23 (26.1%)	4	2
46/47,+14	2/5 (40.0%)	2	0
46/47,+15	6/11 (54.5%)	6 (3)	0
46/47,+16	15/21 (71.4%)	15 (8)	0
46/47,+17	0/7 –	0	0
46/47,+19	0/1 –	0	0
46/47,+22	7/11 (63.6%)	6 (2)	1

Data from reference [292]

^aIUGR intrauterine growth restriction

- Full trisomy 13 or 18 in a short-term culture preparation is a reliable result only in combination with abnormal ultrasound findings or trisomic cells in mesenchyme or amniotic fluid.
- Mosaic trisomy 13 or 18 in a short-term culture preparation merits further prenatal testing by amniocentesis.

In a multicenter study evaluating karyotype-phenotype correlations when mosaic trisomy 13, 18, 20, or 21 was seen at amniocentesis, Wallerstein et al. found an abnormal outcome in 40% of mosaic trisomy 13, 54% of mosaic trisomy 18, 6.5% of mosaic trisomy 20, and 50% of mosaic trisomy 21 [298]. The risk of abnormal outcome in pregnancies with less than 50% trisomic cells and greater than 50% trisomic cells differed also, with better outcomes for lower levels of mosaicism, although the numbers were too small for statistical significance. Repeat amniocentesis was not useful in predicting clinical outcome, although it might be useful when there is insufficient number of cells or cultures to establish a diagnosis. The authors suggested PUBS as an adjunct study, as the risk for abnormal outcome increased with positive confirmation. One of five normal cases was confirmed versus five of eight abnormal cases. The authors also recommended high-resolution ultrasound.

Mosaicism for trisomies 12 and 20 poses unique problems. For both of these trisomies, mosaicism has been reported that appeared to have no discernible effect on the fetus or liveborn, and yet in other cases the mosaicism was associated with an abnormal outcome. A case report and survey of a decade of literature showed a total of 13 reported cases in which trisomy 12 mosaicism was observed in amniocytes [299]. In nine cases, the pregnancy was terminated, and in seven of the nine, no phenotypic abnormalities were reported. One fetus was not described, and one had only two lobes in each lung and appeared otherwise normal. In seven cases, confirmatory cytogenetic studies on skin, blood, rib, placenta, kidney, liver, lung, and/or villi were performed, and in the six cases in which fetal tissue was known to be cultured, five showed confirmation of mosaic trisomy 12.

In five cases in which the pregnancy was continued after diagnosis of trisomy 12 mosaicism in amniocytes, the diagnosis was confirmed in urinary cells or skin in two children. One of them had mild dysmorphic features with near-normal development at 3 years, and the other was dysmorphic and died in the first weeks of life with cardiac abnormalities. In the other three, the diagnosis was not confirmed in fetal skin and/or blood; one had normal development at 5 months, and the other two died in the newborn period with heart, kidney, vertebral, tracheoesophageal, and other abnormalities.

It is interesting to note that the terminated fetuses were described as normal, and the liveborns were almost all abnormal. This was not related to degree of mosaicism. It may be due to unrecognized abnormalities in second-trimester fetuses, or there may have been a bias toward reporting live births with congenital abnormalities.

Outcomes of 144 cases of trisomy 20 mosaicism indicate that 112 of 123 cases (91%) were associated with a normal phenotype; 18 of these were abortuses [29]. In most cases, the cells with trisomy 20 are extraembryonic or largely confined to the placenta. Of the eleven abnormal outcomes, three were in liveborns and eight in abortuses. Three abortuses with urinary tract abnormalities and two with heart abnormalities represent the only consistent, serious abnormalities associated with such mosaicism. Of 21 children followed for one to two years, all were normal except for two with borderline psychomotor delay. It was also apparent that attempted cytogenetic confirmation of the finding should not be limited to analysis of fetal blood, because trisomy 20 has not been observed in blood cells. Confirmation studies in newborns should be done on placental tissues, skin, cord blood, and urine sediment and in abortuses, on kidney, skin, and placental tissues. Finally, true mosaic trisomy 20 may be associated with a mild phenotype. A case was reported in which nonmosaic trisomy 20 was diagnosed by CVS, and the term placental karyotype showed the same finding. Mosaic trisomy 20 was seen in foreskin cultures and in a second skin culture, while lymphocyte culture chromosomes were 46,XY. Aside from diffuse hypopigmentary swirls along the lines of Blaschko on his extremities and trunk, he was considered clinically normal at 8 years of age [300].

Trisomy 16 mosaicism has attracted a great deal of interest in the past several years, inasmuch as it was previously thought that the finding of mosaic or nonmosaic trisomy 16 was thought to result always in pregnancy loss; now it is known that this is not always the case. Of recognized conceptions that spontaneously abort in the first trimester, 6% have trisomy 16 [37]. Most conceptuses abort between 8 and 15 weeks' gestation, and the extra chromosome is usually of maternal meiosis I origin. The mosaicism is thought to arise from either failure of bivalent formation or the precocious separation of bivalent homologs with or without crossing over, during meiosis I. These unpaired univalents then enter a second premature division, separating into constituent chromatids. During the second meiotic division, these chromatids cannot take part in a normal anaphase and would therefore be partitioned at random [301]. This would be misinterpreted as a maternal meiosis I error by DNA marker analysis. Virtually all mosaic trisomy 16 is thought to arise from trisomic zygote rescue of error of maternal origin [302]. Nonmosaic trisomy 16 has not been observed in a liveborn child, although it was documented in a third-trimester fetus at 32 weeks' gestation. That fetus was stillborn with a birth weight of 783 g, indicating severe IUGR, and the diagnosis was confirmed in skin chromosomes [303].

Mosaic trisomy 16 is commonly reported in CVS cultures and has been reported to result in the birth of liveborn infants with maternal uniparental disomy or with normal biparental inheritance of the normal cell line [304, 305]. When CVS

detection of mosaic trisomy 16 occurred, in one series of continued pregnancies, 13 of 63 resulted in fetal death, with three of those occurring after 37 weeks' gestation. One baby was stillborn. Preterm delivery occurred in 11 cases, often associated with fetal or maternal complications. Among the 50 liveborns, IUGR was seen in 27, or more than half. Birth defects or fetal abnormalities were seen in 13, or 18%, of cases; multiple abnormalities were seen in six; and in seven, the abnormalities were isolated to a single organ. Of the continuing pregnancies, only 17 of 60, or 28%, appeared to be full-term, normal pregnancy outcomes [305].

Finding mosaic trisomy 16 at amniocentesis appears to be associated with an elevated maternal serum AFP (MSAFP, see above). Hsu et al. reported on a series of 11 cases diagnosed via amniocentesis ascertained after an elevated MSAFP [306]. In their series, 9 of the 11 pregnancies affected with mosaic trisomy 16 were referred for this reason or because of elevated maternal serum human chorionic gonadotropin.

In another series of 29 amniocentesis-diagnosed cases of trisomy 16 mosaicism not referred due to abnormal CVS results, the indication was elevated MSAFP in twelve; in only three was the indication of abnormal ultrasound findings. Preterm delivery was seen in 12 of the 19 pregnancies, and IUGR was seen in 13 of the 19 continuing pregnancies. Multiple abnormalities were seen in 18 of the 29 cases, or 62%, and isolated abnormalities were seen in two other babies. Only four appeared to have a totally normal outcome [305]. It is important to study skin fibroblasts, as often the trisomic cell line does not appear in lymphocytes. Placental tissue should also undergo chromosome or FISH analysis [307].

Yong et al. evaluated 162 cases of prenatally diagnosed mosaic trisomy 16 [308]. Among live births, 45% had at least one malformation, most commonly VSD, ASD, and hypospadias. The level of trisomy on direct CVS, or cytrophoblast, was associated with more severe IUGR and higher risk of malformation, while the level of trisomy on cultured CVS, or chorionic villous stroma, was associated only with more severe IUGR. The degree of trisomy in placental tissues was independent of the degree of trisomy in amniotic fluid and amniotic mesenchyme [308].

A follow-up review of 17 patients diagnosed by amniocentesis and 19 by chorionic villus sampling with mosaic trisomy 16 was published by Langlois et al. [309]. Of the 17 amniocentesis cases, 11 had congenital anomalies, and almost all had growth delay. In 13 for which birth weight was available, it was low in 11, and catch-up growth occurred in about 90%. Four of the 17 had global development delay. Birth defects correlated with delay. Uniparental disomy did not correlate with developmental delay [309].

There is a phenotype associated with trisomy 16 mosaicism. Some abnormalities have occurred more than once

in affected fetuses and newborns—namely VSD, complex heart disease, hypospadias, imperforate anus, inguinal hernia, clubfoot, and IUGR. The combination of an elevated maternal serum hCG or AFP plus IUGR and one or more of the structural abnormalities previously listed merits the clinical suspicion of mosaic trisomy 16. [302, 304–306, 309]

Other Mosaic Trisomies and Monosomies

Trisomy 22 mosaicism was reported in a collection of 11 cases [29]. Of these, four continued and five terminated. Four of eight reported cases showed a normal outcome, and in the others, one fetal demise, one neonatal death with IUGR, one liveborn with IUGR, and one abortus with multiple congenital abnormalities were seen. Another report of five cases was published by Leclercq et al. [310]. They note that 19 prenatal and 21 postnatal cases of mosaic trisomy 22 have been reported. The phenotype of postnatal cases often includes growth restriction; dysmorphic features; mental retardation; hemiatrophy; plus heart, eye, ear, and limb defects. The five cases they added included four prenatal cases diagnosed by amniocentesis and one diagnosed at 4 years of age. One prenatal case showed normal results at 4 years of age. Two prenatal cases suffered *in utero* demise. The others showed multiple congenital abnormalities, as did the deceased fetuses. No predictive factors were helpful to determine the prognosis in such cases and those they reviewed, including confirmation of true fetal mosaicism. Of note is that the normal outcome reported was after a normal ultrasound reported during pregnancy. The others had IUGR and other findings.

In a study of chromosome mosaicism of chromosomes other than 13, 18, 20, and 21, one to 25 cases each of mosaic trisomies 2–9, 11, 12, 14, 15, 16, 17, 19, and 22 were reported in one series [306]. The outcomes were stratified by very high, high, moderately high, moderate, low, and undetermined. Most abnormalities were detectable by ultrasound. The authors also stressed the importance of obtaining fibroblasts and placental tissues. See Table 12.19 for more information on these mosaic trisomies. Hsu reported on 13 cases of autosomal monosomy mosaicism that had been prenatally diagnosed [29]. These included five cases of monosomy 21, three of monosomy 22, two of monosomy 17, and one case each of monosomies 9, 19, and 20. Of seven cases with phenotypic information and four cases with confirmatory cytogenetic studies, only one case with monosomy 22 was reported to have multiple congenital abnormalities, including congenital heart disease. Another case of monosomy 21 was confirmed but was reported to be phenotypically normal. If autosomal mosaic monosomy is detected, particularly of chromosomes 21 or 22, further workup, such as PUBS and/or ultrasound examination, is indicated.

Table 12.19 Rare trisomy mosaicism cases diagnosed in amniocytes involving autosomes other than chromosomes 13, 18, 20, and 21, along with risk of abnormal outcome as determined by ultrasound and/or physical examination at termination or birth

Chromosome number	Number of cases	Degree of risk for abnormal outcome
2	11	Very high
3	2	Undetermined
4	2	Undetermined
5	5	High
6	3	Undetermined
7	8	Moderate
8	14	Moderate
9	25	High
11	2	Undetermined
12	23	Moderately high
14	5	High
15	11	High
16	21	Very high
17	7	Low
19	1	Undetermined
22	11	Very high

Very high=>60% risk; high=40–59% risk; moderately high=20–39% risk; moderate=up to 19% risk; low risk, no abnormalities seen; and undetermined, no cases for evaluation. From reference [29]

As Phillips et al. described, the common autosomal trisomies of 21, 18, and 13 make up a smaller number of cases of mosaicism detected on CVS but are confirmed in fetal tissue in 19% of cases [307]. The uncommon autosomal trisomies are more common but are less often confirmed in 3.2% of fetal tissues. They also confirmed that the type of chromosome abnormality is a predictive factor as to whether it will be confirmed in fetal tissues. In 28 cases of mosaic polyplodiy detected on CVS, fetal mosaicism was confirmed in one case, compared to marker chromosomes found on CVS, in which mosaicism was confirmed in one-fourth of the fetuses [307].

Mosaicism of an Autosomal Structural Abnormality

In 78 reported cases of mosaicism for a balanced autosomal structural abnormality, phenotypic information was available in 16 cases, and all were associated with a normal phenotype [29]. However, for unbalanced autosomal structural abnormality mosaicism, 25/52 (48%) were reported to be phenotypically abnormal, and 28/48 (58.3%) were cytogenetically confirmed. Such a finding thus merits consideration of PUBS and ultrasound examination.

Culture Failure

Rates of culture failure vary from lab to lab, and guidelines for acceptable rates exist (see Chap. 6). Cell culture failure is more likely to occur in advanced-gestation amniocentesis

specimens, since the number of nonviable cells in the fluid is very high, and they appear to slow the growth of the viable cells. The usual counseling provided in such cases is that the fetal outcome is not related to the lack of cell growth. However, there is a report describing 32 of 7,852 (0.4%) amniocentesis specimens that were classified as unexplained growth failures, and, in this group, ten women did not repeat the procedure while twenty-two did [311]. Of the ten who did not, a fetal bladder-outlet obstruction, two stillbirths, and one acardiac twin resulted. Of the twenty-two who repeated, eighteen had normal fetal karyotypes, but four were aneuploid. Of these, two had trisomy 21, one had trisomy 13, and one had Pallister-Killian syndrome, or tetrasomy 12p.

Maternal Cell Contamination

After cell culture and cytogenetic analysis of amniotic fluid specimens, maternal cell contamination (MCC) is rarely found. Maternal cells were detected in 0.17% of 44,170 cultured amniotic fluid samples in one study [312]. Since detection of MCC would only be expected in male pregnancies (as a mixture of XX and XY cells), the frequency of maternal contamination was estimated at twice this rate, or 0.34%. If *in situ* hybridization techniques are used on uncultured cells, thus identifying both maternal and fetal nuclei, the proportion of MCC increases dramatically, being present at a level of 20% in half of amniotic fluid specimens. This was found to be strongly associated with the sampling technique in a survey of 36 amniotic fluid specimens [313]. Maternal cell contamination of less than 20% was seen in 19 specimens in which the placenta was posterior. In two other bloody specimens from pregnancies with posterior placentas, more than 20% MCC was seen. In cases in which the placenta was anterior, less than 20% MCC was seen in two cases and more than 20% in 13 cases. It was thought that the maternal cells were introduced into the amniotic fluid specimen as a result of placental bleeding during amniocentesis. The authors stated that molecular cytogenetic analysis, or FISH, should not be performed on uncultured amniotic fluid cells without preselecting fetal cells. The preselection could consist of simultaneous analysis of the morphology of the nuclei and of the *in situ* hybridization findings.

Microarray Analysis

Microarrays are standard sets of short DNA sequences, or targets, which are compared to an unknown DNA specimen using computer software that reads and interprets the array differences between the two. Arrays are made of short DNA sequences from 45 to 65 kilobases in length—in which case they are called oligonucleotide arrays, or oligoarrays—or

shorter stretches of nucleotides of 1.8 million or more such targets, called single nucleotide polymorphism (SNP) arrays. These can be read and interpreted by allowing controlled hybridization of the fragmented DNA specimen being studied. The resulting report describes whether there is a deletion or duplication of any genetic material; its location; if any disease-associated genes are involved; and, in the case of SNP arrays, whether loss of heterozygosity has occurred. The advantage of arrays is clear—they have much greater resolution than conventional cytogenetic analysis, at 50 kb or so, which is at least 100 times smaller than changes that can be seen by cytogenetic methods [314].

Although microarrays have been available for several years, their use in prenatal medicine has been more slowly adopted due, in part, to concerns regarding the clinical significance of copy number variations in the human genome [315]. In 2009, the American College of Obstetricians and Gynecologists released a position paper indicating that the "...use of array CGH technology in prenatal diagnosis is currently limited by several factors, including the inability to detect balanced chromosomal rearrangements, the detection of copy number variations of uncertain clinical significance, and significantly higher costs than conventional karyotype analysis. Although array CGH has distinct advantages over classic cytogenetics in certain applications, the technology is not currently a replacement for classic cytogenetics in prenatal diagnosis" [316].

Friedman echoes concerns about adoption of array technology, given the little that is known about the natural history and range of clinical variability associated with most pathogenic submicroscopic copy number variants (CNVs). His recommendation is that it be offered only if the pregnancy is at very high risk of having a pathogenic CNV or it is being done as part of a clinical trial [314].

Increased use of prenatal microarray has nevertheless been increasingly occurring. A survey of genetic counselor current practice was reported by Smith et al. in 2009 [317]. In the survey responses, 84% indicated their employer had no guidelines for the use of arrays in prenatal diagnosis, and over 75% were not familiar with available literature. More than 50% were not familiar with ACMG guidelines. About 57% had offered array within the past 2 years, and pretest counseling was performed by a genetic counselor in about 75% of cases. The type of array was approximately equal among prenatal BAC, targeted oligoarray, and whole genome oligoarray. Of those who offered it, 77% only did so following normal chromosome analysis, and all offered it for abnormal ultrasound findings. Fewer than 30% offered it for other indications for prenatal diagnosis, and fewer than 1% offer it to everyone, regardless of indication [317].

A comparison of the use of prenatal versus whole genome array was described in a study by Rickman et al. [318]. In their study, abnormalities were detected in 22 of 30 of specimens with genome-wide array and 29 of 30 with a prenatal array.

Several commercial laboratories market oligoarrays designed for prenatal use. An evaluation of 300 amniocentesis or CVS specimens using bacterial artificial chromosome (BAC) or oligoarrays from women with advanced maternal age or abnormal ultrasound findings showed 58 CNVs, or 19.3% [319]. Of these, 40 of 58 (13.3%) were interpreted as likely benign, 15 (5%) were clearly abnormal, and three (1%) were of uncertain clinical significance. For seven (2.3%), microarray contributed important new information, and for two of these (1%), the abnormality would not have been detected without microarray analysis. In one case, after the microarray result showed an abnormality, a targeted ultrasound showed an anatomic abnormality consistent with the genetic defect found by array; for this and other reasons, the authors stated that the use of prenatal array only for an indication of abnormal ultrasound might not be the optimal diagnostic strategy.

Another group evaluated fetuses with abnormal ultrasound findings and compared normal cytogenetic results with array results. They found clinically significant CNVs in one of 50 cases of those pregnancies [320]. In three other cases, CNVs were seen that were inherited from a parent. As with the previous study, they note that many genetic syndromes present postnatally with growth disorders and facial abnormalities, and hypothesize that evaluating a subset of fetuses with growth abnormalities and another anomaly might yield more findings. They also raise an important question: if a parent has an identical CNV, does that mean it is truly benign? Careful evaluation of the parent carrying the same CNV is vital in distinguishing true benign variants from those that cause disease [320].

Hillman et al. published a meta-analysis of 751 prenatal array CGH cases, of which 409 had an ultrasound abnormality [321]. Array CGH detected an additional 2.9% of chromosome abnormalities in 2.9% of cases, regardless of the indication, and when the indication was abnormal ultrasound, the additional predicted causative chromosome abnormalities found on array CGH was 5.2%. Variants of unknown significance were detected in 1.1% of specimens [321].

An evaluation of low-level chromosome mosaicism by array CGH was reported by two groups [322, 323]. In the Ballif et al. study, mosaicism was detected in 14 cases of 3,600 analyzed and was not detected by cytogenetic testing. Because 20%, and possibly 10%, mosaicism may be detectable by microarray, and the same degree of detection by cytogenetics would require analysis of 29 or 63 metaphases, respectively, microarray might circumvent some of challenges associated with detecting low-level mosaicism by conventional cytogenetics. They also comment on the different percentages of mosaic cells found in stimulated cell cultures compared to unstimulated blood smears, indicating that the culturing process might introduce selection bias that distorts the percentage of abnormal cells.

Microarray technology is covered in detail in Chap. 18.

Conclusion

Noninvasive Fetal Diagnosis

The Holy Grail for prenatal diagnosis is the ability to detect fetal abnormalities through maternal blood testing. One of the limitations in the field has been isolating the small amount of fetal DNA in maternal circulation. In a new model reported by Chiu et al., 753 pregnant women at high risk for fetal trisomy 21 had cell-free DNA from the fetus isolated and subjected to massively parallel genomic sequencing for sequences specific to chromosome 21 [324]. The z-scores were analyzed in which the percentage of chromosome 21 in the test case minus the mean percentage chromosome 21 in reference controls was divided by the standard deviation of percentage chromosome 21 in reference controls. They found 86 cases of trisomy 21. One of the protocols resulted in 100% sensitivity and 97.9% sensitivity. They also detected trisomy 13 and 18 pregnancies but did not have the assay ready for those analyses. The results were validated against karyotyping. In future, this could be an approach that supplants much of invasive prenatal diagnosis [324].

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The Cytogenetics of Spontaneous Abortion

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Introduction

Pregnancy loss is quite common, with 15–20% of recognized pregnancies resulting in failure. The majority of these occur early in gestation, though losses in the second and third trimester are not rare. Approximately 2–5% of women will experience two or more losses. The majority of pregnancy failures are associated with cytogenetic abnormalities, with over 50% of early miscarriages and as many as 5% of stillbirths exhibiting abnormal karyotypes.

Loss of a wanted pregnancy is always stressful for both the patient and her partner. A number of questions and concerns may be raised regarding the loss, including: What happened and why? How likely is it to happen again? What can be done to improve the chances of a successful future pregnancy? Is this even possible? Answering such concerns is important in helping the patient through the grieving process and in facilitating resolution. The answers that are provided may ultimately impact family planning and management of any future pregnancies the couple may undertake.

Unfortunately, early pregnancy losses are often given less attention than they merit, both by medical care providers and by society. The patient who loses an older child or who experiences a stillbirth at term can expect an attempt at explanation for the loss from her health care provider. She will also be offered sympathy and support from family and friends. Rituals associated with mourning and with disposition of the remains help bring closure. However, the patient who experiences an early loss often feels isolated and alone. Her friends

may be uncomfortable with discussing the loss, if they are even aware of it, and so may avoid the issue altogether. She may have been told by her caregiver that such early losses are common and that there is no reason she cannot have a successful pregnancy, but this does not explain why the loss happened to her and usually does little to alleviate her sense of guilt and failure. These feelings of inadequacy are often amplified in the patient with recurrent losses [1–5]. Answering the patient's questions, whether verbalized or not, will help bring about closure to the loss and may open dialog with the patient and her partner about their specific concerns for the future. This in turn may have significant impact on management of future pregnancies. Thus, anyone caring for women of childbearing potential should be familiar with the causes and recurrence risks for pregnancy loss.

The Scope of the Problem

When examining the chances for success of a given conceptus, the results of human reproduction are quite poor. Approximately 78% of all conceptions fail to go to term [6]. Combined data from three studies of women attempting pregnancy revealed a postimplantation loss rate of 42% in documented conceptions confirmed by positive human chorionic gonadotropin (hCG) levels [7–9]. A 4-year follow-up of 3,084 pregnancies demonstrated a 23.7% loss rate following the first missed period [10]. The net overall fecundity for patients 20–30 years of age has been estimated at 21–28%, a level that is quite low compared with most other mammalian species [11]. Leridon provides a useful summary table of pregnancy survival from fertilization to term, with only 31 survivors among 100 ova exposed to fertilization (Table 13.1) [12]. Although most of the losses occur very early in gestation, losses continue to occur throughout the second and third trimesters of pregnancy with a slight increase in mortality at term.

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Table 13.1 Intrauterine mortality per 100 ova exposed to fertilization [12]

Week after ovulation	Embryonic demise	Survivors
-	16 (not fertilized)	100
0	15	84
1	27	69
2	5.0	42
6	2.9	37
10	1.7	34.1
14	0.5	32.4
18	0.3	31.9
22	0.1	31.6
26	0.1	31.5
30	0.1	31.4
34	0.1	31.3
38	0.2	31.32

Live births: 31

Natural wastage: 69

Relationship Between Cytogenetic Abnormalities and Gestational Age

Multiple studies have suggested that approximately 50% of early pregnancy losses are associated with cytogenetic abnormalities. Evaluation of 1,205 pregnancy losses of varying gestational ages submitted to the author's laboratory between 1992 and 1996 revealed 539 (45%) cases with identified cytogenetic abnormalities (Yusuf and Naeem, 1997, personal communication). The likelihood of a cytogenetic abnormality varies with the gestational age and morphology of the abortus. In evaluating products of conception, the developmental age at which growth arrest occurred is a more useful parameter than gestational age at the time of miscarriage, since products of conception are often retained *in utero* for several weeks following embryonic demise.

Overall, the earlier the developmental age, the greater the likelihood of an abnormal karyotype in a spontaneous pregnancy loss. Boué and colleagues found that approximately two-thirds of losses under 8 weeks and nearly one-fourth of those between 8 and 12 weeks had abnormal karyotypes (Table 13.2) [13].

It is also of interest to note that the earlier the pregnancy undergoes growth arrest, the more likely it is for there to be anomalous development and that there will be an abnormal karyotype (Table 13.3).

Gestational Age

Examination of induced abortuses confirms the greater incidence of karyotypic abnormalities earlier in pregnancy [14] (Table 13.4). A total of 1,197 pregnancies were examined. The rate of chromosomal abnormality varied with gestational

Table 13.2 Chromosomal abnormalities and gestational age

Gestational age (weeks)	Number of cases	Abnormal cases	Percent abnormal (%)
2	23	18	78.0
3	374	258	69.0
4	203	125	61.6
5	139	85	62.2
6	302	211	69.9
7	56	27	48.2
<i>Total weeks 2–7</i>	<i>1,097</i>	<i>724</i>	<i>66.0</i>
8	36	8	22.2
9	42	6	14.3
10	14	7	50.0
11	8	1	12.5
12	8	3	37.5
<i>Total weeks 8–12</i>	<i>108</i>	<i>25</i>	<i>23</i>
<i>Total</i>	<i>1,205</i>	<i>749</i>	<i>62.2</i>

Adapted from [13]

age; 9.3% of cases were abnormal at 3–4 weeks, falling to 5.4% at 9–10 weeks.

The likelihood of detecting congenital anomalies in therapeutic terminations is variable and may be a reflection of the thoroughness of the examination and the skill of the examiner. However, identification of anomalous development may have considerable impact upon future reproduction, and it is the opinion of the author that careful anatomic evaluation of aborted products of conception should be considered regardless of whether the pregnancy is aborted spontaneously or induced.

In the second trimester, ascending infection becomes more frequent as a cause of spontaneous pregnancy loss. Abnormal karyotypes become less prevalent as pregnancy progresses since many of the less viable abnormal gestations have already undergone growth arrest and miscarriage. Gaillard et al. studied 422 consecutive second trimester losses [15]. Of these, 78.6% were recent demises without extensive maceration. Ascending infection could explain 85% of these. Structural anomalies were seen in 7.6% of fetuses. Cytogenetic abnormalities were confirmed or suspected in half of these. The majority of abnormal fetuses showed maceration consistent with long-standing intrauterine fetal demise. This again confirms the observation that cytogenetic abnormalities are associated with early demise but that there is also frequent retention of the products of conception for some time prior to spontaneous abortion. The macerated fetus is at significant risk for chromosomal abnormality whereas the fresh fetal demise without gross congenital anomalies is more often due to other etiologies including but not limited to infection, endocrine disorders, abnormal uterine anatomy, and immunological factors.

Although cytogenetic abnormalities are frequent in early pregnancy, they are much less common at term. Approximately

Table 13.3 Abnormal development and gestational age

Study	4 weeks or less		5–8 weeks		9–12 weeks	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Milamo	0	48	21	40	71	10
Miller and Poland	10	73	51	71	121	56
Total	10 (8%)	121 (92%)	72 (39%)	111 (61%)	192 (74%)	66 (26%)

Adapted from [13]

Table 13.4 Chromosomal abnormalities in induced abortuses [14]

Developmental age	Number of cases	Abnormal cases (percent)
3–4 weeks	108	10 (9.3%)
5–6 weeks	570	37 (6.5%)
7–8 weeks	389	25 (6.4%)
9–10 weeks	130	7 (5.4%)

Table 13.5 Prenatal loss of chromosomally abnormal fetuses

Autosomal monosomy	100.0%
Tetraploid	100.0%
Triploid	99.9%
Monosomy X	99.8%
Autosomal trisomy	96.5%
Mosaics	68.8%
Structural rearrangements	53.4%
Sex chromosomal trisomy	11.0%

Adapted from [17]

Table 13.6 Percent of chromosomal anomalies among spontaneous abortions and live births

Anomaly	Spontaneous abortions	Live births
<i>Autosomal trisomies</i>		
13	1.10%	0.01%
16	5.58%	0.00%
18	0.84%	0.02%
21	2.00%	0.11%
Other	11.81%	0.00%
<i>Total trisomies</i>	21.33%	1.34%
<i>Monosomy X</i>	8.35%	0.01%
<i>Sex chromosome trisomies</i>	0.33%	0.15%
<i>Triploids</i>	5.79%	0.00%
<i>Tetraploids</i>	2.39%	0.00%
<i>Total abnormal</i>	41.52%	0.60%
<i>Number karyotyped</i>	3,353	31,521

Adapted from [18]

one in 200 live newborns exhibits readily identified aneuploid karyotypes, and one study estimates that with moderate levels of banding, 0.061% of infants will show unbalanced structural abnormalities and 0.522% will harbor balanced

rearrangements [16]. The rate of unbalanced karyotypes showing numerical or structural abnormality is much higher in stillbirths, approximating 5–7% overall. Here, too, the risk is greatest for macerated stillbirths, especially in the presence of congenital anomalies. Cytogenetic abnormalities and associated congenital anomalies are also a significant factor in neonatal deaths.

The likelihood of survival for a pregnancy with an aberrant karyotype is a reflection of the particular cytogenetic abnormality and the extent of its deleterious effects on embryonic growth and development. Davison and Burn examined the likelihood of loss for various chromosomal abnormalities, confirming virtual 100% loss for autosomal monosomies and tetraploids [17]. Autosomal trisomies resulted in a 96.5% loss rate. Although greater than 99% of monosomy X pregnancies failed, only 11% of sex chromosome trisomies were lost spontaneously. Mosaic and structurally rearranged karyotypes show intermediate loss rates of 68.8 and 53.4%, respectively (Table 13.5).

Although there have been several reports of tetraploid conceptuses and near complete autosomal monosomies surviving into the third trimester, these are exceptionally rare.

Summarizing data from several series, Kline and Stein compared the frequency of chromosomal anomalies of spontaneous abortions and live births (Table 13.6) [18].

Summary

These data indicate that the majority of chromosomally abnormal pregnancies fail, that the losses are selective rather than random, and that the differing survival potential is dependent upon the particular cytogenetic abnormality involved.

Cytogenetic abnormalities are a significant factor in human pregnancy wastage at all stages of gestation, as well as into the neonatal period. However, the incidence of karyotypic abnormalities is greatest during early pregnancy, with the majority of aberrant gestations resulting in early spontaneous loss. Very early pregnancy loss is most likely to be the result of chromosomal abnormalities, especially when there is evidence of marked embryonic growth arrest at the time of delivery. The clinical significance of the loss and the potential

impact on future reproductive risks for the couple is dependent upon the type of chromosomal error.

Types of Errors Leading to Chromosomally Abnormal Conceptuses

Although most chromosomal abnormalities are associated with poor outcome early in pregnancy, the underlying mechanisms leading to an aberrant karyotype and the risk for recurrence vary considerably depending on the particular abnormal chromosomal complement. Generally speaking, most karyotypic abnormalities fall into one of four classes: errors in meiosis (gametogenesis), errors in mitosis leading to mosaicism, errors in fertilization, and structural abnormalities and rearrangements.

A classic study of 1498 abortuses by Boué and colleagues revealed 921 abnormal karyotypes (61.5%) [19, 20]. Among the chromosomally abnormal losses were 636 nondisjunctional events: 141 monosomies (15.3%), 479 trisomies (52.0%), and 16 double trisomies (1.7%). There were 183 triploids (19.9%), 57 tetraploids (6.2%), and ten cases of mosaicism (1.1%). Structural abnormalities were identified in 35 abortuses (3.8%). With improved cytogenetic and molecular methods being used today, the incidence of detectable abnormalities may have been even higher. However, the study clearly shows that cytogenetic abnormalities are present in the majority of early spontaneous losses, and the data provide a useful breakdown of the types of abnormalities that are observed. Normal karyotypes were seen in 577 abortuses (38%), although there may have been a few undetected underlying abnormalities such as subtle rearrangements, uniparental disomy, or tissue-specific mosaicism that could have gone undetected in this sample.

Analysis of 1,205 products of conception of varying gestational ages received in the author's laboratory between 1992 and 1996 revealed 539 (47.2%) abnormal karyotypes. Of these, 50.6% were trisomies, 11.3% were monosomies, 4.2% were tetraploid, and 14.8% were triploid (Yusuf and Naeem, 1997, personal communication). Although the total percentage of abnormal karyotypes is lower in our series than that of Boué and colleagues, this can be explained by a higher proportion of cases from later in gestation in our population, giving a greater number of losses due to nonchromosomal etiologies. The distribution of types of abnormalities among the aberrant karyotypes is similar, however.

Errors in Meiosis

During meiosis, the usual parental diploid chromosome complement of 46 is reduced to the haploid number of 23. Nondisjunctional events during meiosis I or II of either oögenesis or spermatogenesis can result in monosomic or

trisomic conceptuses due to formation of a gamete with fewer or greater than the usual number of chromosomes (see Chaps. 2 and 8). With the exception of monosomy X, complete, apparently non-mosaic monosomies are almost invariably lethal early in gestation and are not usually identified in recognized pregnancies. Gene dosage effects or imprinting failure may be factors contributing to the high embryonic lethality of the autosomal monosomies.

Trisomies, on the other hand, are relatively common and represent the most frequently encountered group of abnormalities leading to spontaneous pregnancy loss. Approximately 25% of karyotyped spontaneous abortions will be trisomic [20–22]. All autosomal trisomies have been reported in multiple studies with the unique exception of chromosome 1. Trisomy 1 appears to be lethal prior to implantation and thus would be unlikely to survive long enough to be seen in routine series of spontaneous abortions. The majority of trisomic conceptuses, even those with karyotypes that may be viable in the neonate, result in miscarriage.

Trisomies

The frequency of particular autosomal trisomies varies with gestational age. At term, trisomy 21 (Down syndrome) is the most common and is seen in approximately 1 in 700 live births. Trisomy 18 (Edwards syndrome) and trisomy 13 (Patau syndrome) are seen in approximately 1 in 6,000–8,000 and 1 in 12,000 births, respectively. Trisomy 8 is much less frequent and most cases are mosaic. Although individual case reports indicate that other unusual autosomal trisomies and rare autosomal monosomies do occasionally occur in the neonate, these aneuploidies are typically seen only in a mosaic state and generally appear to be lethal when a normal cell line is absent. See also Chap. 8.

The distribution of trisomies in spontaneous abortions is quite different from that seen at term. The most common trisomy observed in spontaneous abortuses is trisomy 16, accounting for 31.0% of trisomic conceptuses and 7.27% of all spontaneous abortions. This is followed by trisomy 22, seen in 11.4% of trisomies and 2.26% of all spontaneous abortions. Trisomy 21 (Fig. 13.1) is third most frequent, accounting for 10.5% of trisomies and 2.11% of spontaneous abortions (Table 13.7) [17, 22].

Double trisomies also occur and show a strong association with advancing maternal age, even more so than the age effect seen with the viable trisomies such as trisomy 21 [13].

Identification of trisomic conceptuses is of clinical importance because of the question of possible increased risk for aneuploidy in subsequent pregnancies. The recurrence risk for a couple with a previous trisomic infant is often cited as approaching 1% [23, 24]. Verp and Simpson combined data from several smaller studies to suggest that the risk for an aneuploid liveborn following a trisomic abortus may also



Fig. 13.1 Trisomy 21 in a spontaneous abortion. Note the hydropic appearance of the fetus, a frequent finding in trisomy 21. This may lead to confusion with the Turner syndrome phenotype in some cases. The

facial features and short hands with a single transverse palmar crease are typical of Down syndrome

Table 13.7 Distribution of individual trisomies among trisomic spontaneous abortions [17, 22]

Chromosome number	% of trisomies	% of abortuses
1	Single case report	0
2	4.0	1.11
3	0.9	0.25
4	2.4	0.64
5	0.3	0.04
6	0.9	0.14
7	4.0	0.89
8	4.6	0.79
9	2.3	0.72
10	2.0	0.36
11	0.3	0.04
12	1.2	0.18
13	4.1	1.07
14	4.8	0.82
15	7.4	1.68
16	31.0	7.27
17	0.3	0.18
18	4.6	1.15
19	0.2	0.01
20	2.2	0.61
21	10.5	2.11
22	11.4	2.26

approximate 1% [25]. Connor and Ferguson-Smith offer an empirical risk of 1.5% for a trisomy (not necessarily a viable trisomy) in any subsequent pregnancy following a trisomic abortus [26]. This raises the issue as to whether prenatal diagnosis should be offered to couples who have experienced a previous trisomic abortus.

There may be increased susceptibility to trisomic concep-tuses in some patients with a history of trisomic pregnancies. The risk for these patients would be for nondisjunction in general, not for a specific trisomy. The author's laboratory has seen several patients with three or more consecutive trisomies, each involving different chromosomes, suggesting a population of couples who are at significant risk for recurrence. At this time, however, it is difficult to determine which women with a trisomic abortus are more likely to experience recurrent nondisjunctional events. Thus, couples may benefit from genetic counseling following a trisomic or any other chromosomally abnormal pregnancy (see Chap. 21).

The majority of autosomal trisomies are maternal in origin, with errors in meiosis I being more frequent than meiosis II, although there appears to be some variability depending upon the chromosome involved. Of 436 informative cases reviewed by Hassold and colleagues, 407 trisomies were maternal in origin [27]. All cases of trisomy 16 and trisomy 22 were also maternal in origin, 19% of trisomies involving

chromosomes 2 through 12 were paternal in origin, and 27% of trisomies of chromosomes 13 through 15 were paternally derived. Paternal nondisjunction was also associated with sex chromosome aneuploidies, being responsible for 44% of XXY and 6% of 47,XXX conceptions.

Examination of oocytes reveals a significant percentage of cytogenetic abnormalities. Kamiguchi and colleagues found abnormal chromosomal complements in 24.3% of unfertilized oocytes [28]. Aneuploidy was most commonly observed, followed by diploidy and structural abnormalities. A review of 1,559 published cases revealed chromosomal abnormalities in 24% of mature oocyte karyotypes [29]. The majority were aneuploid (22.8%); fewer had structural aberrations (1.2%). It is of interest to note that only one oocyte with an extra chromosome 16 was identified, although this is the most common trisomy in spontaneous abortions. The difference in distribution of trisomies suggests that postmeiotic viability may be as significant as meiotic error in determining the incidence of particular trisomies in the human species.

Cytogenetic studies of human spermatocytes also reveal abnormalities in paternal gametogenesis. The reported studies have used several different methods for karyotype preparation. In 1987, Martin et al. reported that 3–4% of sperm exhibited aneuploidy due to nondisjunctional events, and 10% had structural abnormalities [30]. More recently, fluorescence *in situ* hybridization (FISH) techniques have been used, allowing for examination of far greater numbers of sperm. FISH does have inherent limitations based on the particular chromosome-specific probes utilized; only the specific aneuploidies being probed for will be detected. Using FISH techniques, Miharu and colleagues analyzed 450,580 sperm from 9 fertile and 12 infertile men [31]. Disomy for chromosomes 1, 16, X, and Y ranged from 0.34–0.84% in infertile subjects and 0.32–0.61% in fertile subjects. Guttenbach and colleagues examined 16,127 sperm from eight healthy donors for disomy of chromosome 18 and found a range of 0.25–0.5% [32]. Examination of 76,253 sperm from seven donors revealed a range of 0.31–0.34% of disomy for chromosomes 3, 7, 10, 11, 17, and X [33]. Although FISH studies have inherent limitations, the data suggest that the rate of paternal meiotic nondisjunction appears relatively constant for the various chromosomes studied.

Overall, maternal age is the best known predictor of risk for nondisjunctional events, in particular those resulting from errors in meiosis I. The association between maternal age and risk for Down syndrome has long been established, and risk for trisomic abortuses also increases with advancing maternal age [18, 34, 35] (Table 13.8).

Not all chromosomal trisomies appear to have the same association with maternal age. Warburton et al. found that age-associated nondisjunction appeared to have a greater

Table 13.8 Effect of maternal age

Maternal age (years)	Number karyotyped	Percent abnormal	Percent trisomic	Percent non-trisomic
20	104	18.3	4.8	13.5
20–24	256	28.5	12.1	16.4
25–29	339	26.3	10.6	15.6
30–34	161	32.3	19.3	13.0
35–39	99	34.3	25.3	9.0
40+	32	65.6	50.0	15.6

Adapted from [35]

effect on the smaller chromosomes, with mean maternal age increasing with decreasing size of the trisomic chromosome [21]. Susceptibility to nondisjunction may not be the same for all chromosomes, and recurrence risks may be dependent upon the particular chromosome involved in the trisomy, the parent contributing the extra chromosome, and the background risk associated with maternal age. Regardless of the exact risk, many couples who have experienced a trisomic conceptus find the availability of prenatal diagnosis reassuring in planning subsequent gestations.

Sex Chromosome Aneuploidy

Sex chromosome aneuploidies are among the most common chromosomal abnormalities, both in spontaneous pregnancy loss and in liveborn infants. By far, the most frequent sex chromosome aneuploidy at conception is 45,X, accounting for approximately 1–2% of clinically recognized pregnancies. It is the single most frequent abnormal karyotype seen in spontaneous abortions. The vast majority of monosomy X conceptuses terminate in miscarriage, less than 1% of affected pregnancies surviving to term [17, 36]. The incidence of Turner syndrome in surviving pregnancies is approximately 1 in 1,000 female live births. No 45,Y karyotypes have been reported. This is not an unexpected finding, considering the important contributions of genes located on the X chromosome.

The three sex chromosome trisomies, 47,XXX, 47,XXY, and 47,XYY, are much less frequent than monosomy X in spontaneous pregnancy loss but are similar to monosomy X in frequency at term, each affecting approximately 1 in 1,000 infants of the appropriate sex. Affected infants with sex chromosome trisomies are not usually markedly dysmorphic and are often not identified unless cytogenetic studies are performed for other reasons. These conditions are frequently not recognized until later in life when behavioral changes or, in the case of 47,XXY, infertility may cause the patient to present for evaluation. Some affected individuals may never be identified. The mild phenotypic expression at birth appears to reflect an absence of markedly deleterious effects during embryogenesis. This would explain the relatively low

frequency of sex chromosome trisomies of 0.2% among spontaneous abortuses [22].

Monosomy X gestations vary considerably in phenotype and may exhibit marked dysmorphism. The majority undergo early embryonic growth arrest and present as an empty gestational sac or as an umbilical cord ending with a small nodule of necrotic embryonic tissue (Fig. 13.2). A lesser number survive into the second trimester, at which time, the phenotype is often that of a hydropic fetus with massive cystic hygroma (Fig. 13.3). Renal and cardiac anomalies are frequently seen as well. During the third trimester, the appearance may be similar to that seen in the second trimester, with cystic hygroma and dorsal edema over the hands and feet, the classic Turner syndrome phenotype. There are also 45,X infants who appear minimally affected and may not be recognized at birth, presenting later in childhood or adolescence with hypogonadism and short stature.

Several explanations have been proposed for the wide variability in phenotype. Although the majority of 45,X conceptuses surviving to term appear to have a maternally derived X, parental origin of the monosomy does not appear to affect phenotype or viability [37, 38]. Rather, survival of the early pregnancy may be dependent upon presence, in some tissues, of a second sex chromosome, either another X

or a Y. The non-mosaic 45,X conceptus appears unlikely to survive, whereas a mosaic gestation with a second sex chromosome, regardless of whether it is an X or a Y, has a better chance of undergoing orderly morphogenesis early in gestation and of surviving to term [38, 39]. This second cell line may be absent from many tissues and is often difficult to detect with routine cytogenetic studies but can sometimes be identified using multiple sampling sites or FISH techniques. Although extensive efforts at identification of a second cell line may not be justified in routine evaluation of a monosomy X abortus, such techniques are often helpful in evaluation of Turner syndrome patients with suspected low-level Y chromosome mosaicism. The presence of genes originating on the Y chromosome may place the patient at increased risk for gonadoblastoma.

Whereas the mean maternal age for most trisomic conceptuses is increased over the normal population, this is not the case with monosomy X. Rather, the mean maternal age for monosomy X is the same or lower than expected for the reproductive age population as a whole [13]. The evidence that many cases of monosomy X are the result of post-zygotic nondisjunction may possibly explain the difference in maternal age between aneuploid pregnancies with monosomy X and those with autosomal trisomies. Mitotic nondisjunction



Fig. 13.2 Gestational sac with very small embryo, consistent with an underlying cytogenetic abnormality, often a nonviable trisomy or, as in this case, monosomy X



Fig. 13.3 45,X spontaneous loss at mid-gestation. Note marked cystic hygroma and generalized edema

during embryogenesis appears to be a different process, which may not exhibit the same maternal age effect; hence, the maternal age for monosomy X would not be expected to be increased over the mean reproductive age of the population. Although patients who have experienced a pregnancy with monosomy X often choose to have prenatal cytogenetic evaluation in subsequent gestations, the recurrence risk for post-zygotic/mitotic nondisjunctional events has not been established.

Errors in Mitosis

Malsegregation in the first mitotic division can give rise to tetraploidy. Tetraploid conceptions are usually lost relatively early in gestation, although there are rare exceptions.

Mitotic nondisjunction often results in mosaicism—the presence of two or more cell lines with a different genetic makeup. As has been suggested for Turner syndrome, mosaic aneuploidy may be better tolerated by the developing conceptus than complete aneuploidy, and there is evidence that survival of a trisomic fetus to term may be more likely if there is a normal cell line present within the placenta.

The question of tissue-specific mosaicism has long been an issue in prenatal diagnosis, especially with the advent of chorionic villus sampling (CVS; see Chap. 12). Early non-disjunction can result in a generalized pattern of mosaicism, whereas divergence later in gestation can lead to mosaicism confined to either the fetus or the placenta. Mosaicism confined to the amnion may present a dilemma in interpretation of amniotic fluid karyotype yet may not pose a problem for the fetus [40]. Within the placental chorionic villous tissue, there may be karyotypic differences between the direct preparation and long-term culture methods. This is a reflection of the different origins of the trophoblast cells and the extraembryonic mesodermal cells.

Confined placental mosaicism is a potential concern even in the fetus with a normal karyotype. The presence of confined placental mosaicism has been associated with abnormal mid-trimester hCG levels, and with increased risk for adverse pregnancy outcome, including growth retardation and fetal demise [41, 42]. Confined placental mosaicism may also be a factor leading to spontaneous abortion. A normal fetal karyotype does not rule out a cytogenetic abnormality in the placenta as a factor leading to pregnancy failure, suggesting the need for karyotype analysis of both fetal and placental tissues in unexplained stillbirths [42]. Although the incidence of mosaicism in CVS series is often cited in the 1–2% range, Kalousek and colleagues detected confined placental mosaicism in 11 of 54 spontaneous abortions studied and have suggested that the frequency may be especially high in growth-disorganized embryos [43]. The cytogenetic contribution to human pregnancy failure may thus be even

higher than estimates based on early series, since those cases were often examined using only a single tissue source, and some morphologically aberrant conceptuses classified as euploid may actually have been the result of undiagnosed mosaicism.

Recent molecular studies have shown that mosaic autosomal trisomies can arise either from errors in meiosis, with subsequent loss of one of the chromosomes leading to production of a euploid cell line, or from the post-zygotic duplication of one of the chromosomes in an originally euploid cell line. The likelihood of one or the other mechanism may vary depending on the particular chromosome involved. Robinson and colleagues suggest that the mosaic trisomies involving chromosomes 13, 18, 21, and X most often result from somatic loss of a supernumerary chromosome that arose from meiotic nondisjunction [44]. Mosaic trisomy 8, on the other hand, may be more likely to survive when the aneuploid line is derived later, as a result of a post-zygotic error in mitosis in a conceptus that was originally chromosomally normal.

Mosaicism in the placenta may be a significant determining factor in survival of the trisomic conceptus. Those cases of trisomies 13 and 18 that survive to term appear to have a diploid cell line in the cytotrophoblasts, whereas those lost early in gestation are less likely to show a normal cell line [45, 46]. Mosaicism does not appear to be necessary for survival in trisomy 21, possibly due to a less deleterious effect of this trisomy on placental function [45].

The presence of a euploid cell line in the fetus does not necessarily imply a genetically normal fetus. If the mosaicism is the result of “rescue” of a trisomic cell line, the possibility of both remaining chromosomes of the pair originating from a single parent becomes a concern. This condition, uniparental disomy, can often have severe consequences in the affected fetus due to potential loss of heterozygosity with expression of recessive traits only carried by one parent or due to effects of inappropriate imprinting (see Chap. 20). Thus, multiple sampling sites should be evaluated in cases where a cytogenetic abnormality is strongly suspected, even if a normal karyotype is identified on initial evaluation. Molecular studies may be indicated to rule out uniparental disomy in ongoing pregnancies that have been identified as mosaic. More study regarding the effects of uniparental disomy on embryogenesis is clearly needed.

Chimerism

Another possible cause for the presence of more than one cell line in a fetus is chimerism. The chimera of classical mythology was a creature with the head of a lion, the body of a goat, and the tail of a serpent. Although the mythical chimera composed of several unrelated species is purely fanciful, individuals with cells derived from two separate fertilized eggs are known to exist in humans and other mammals.

Post-zygotic fusion of dizygotic twin zygotes results in a single chimeric individual.

Chimerism can explain the presence of two cell lines, in a single individual, where neither can be derived from the other. This is the most likely mechanism underlying 46,XX/46,XY hermaphroditism and may also explain a 45,X/69,XXY fetus described by Betts and colleagues [47]. A number of diploid/triploid mosaics have also been reported [48]. Some of these are probably chimeras, although another possible mechanism here is dispermy, in which a single maternal haploid pronucleus is fertilized by a haploid sperm in the usual manner, resulting in the diploid line. A second fertilization event then occurs in one of the daughter cells after the first cell division, leading to the triploid cell line [49].

Errors in Fertilization

Errors in fertilization can lead to pregnancies with an extra complete set of chromosomes (triploidy; see Chap. 8) and also abnormal diploid pregnancies in which both sets of chromosomes come from one parent (hydatidiform or complete molar pregnancies). Because paternal triploids may exhibit changes in the villi that resemble hydatidiform moles, these are sometimes referred to as partial moles. Both partial and complete molar pregnancies have been instrumental in advancing our understanding of imprinting (see Chap. 20) and the role imprinting plays in fetal development and carcinogenesis. Imprinting may have functions not only in gene expression early in embryogenesis but may also play a significant role in surveillance for chromosome loss later in life and thus may help reduce the risk of cancer [50].

An extra haploid set of chromosomes from either the mother (digyny) or the father (diandry) can result in a triploid conceptus. A 69,XYY karyotype is indicative of a paternal origin for the extra chromosomal set, whereas a 69,XXX or 69,XXY karyotype could represent either digyny or diandry. A variety of events can lead to the presence of an extra set of chromosomes.

The paternally derived triploid usually results either from fertilization of a normal haploid egg by two separate sperm (dispermy) or from fertilization of the egg by a diploid sperm. Fertilization by a haploid sperm with subsequent endoreduplication of the paternal chromosomal complement is also a possible mechanism. The latter process would result in isodisomy for all paternal chromosomes, as would an error in meiosis II [51]. The maternally derived triploid, on the other hand, most often originates from an error during maternal meiosis I or II, resulting in a diploid egg, although other mechanisms including fertilization of a primary oocyte have also been postulated [52]. Together, triploidy accounts for 1–3% of all recognized pregnancies and 15–20% of all chromosomally abnormal miscarriages, placing the triploidy

among the most frequent chromosomal aberrations in human conception [53, 54].

Although the net result of either diandry or digyny is a pregnancy with 69 chromosomes, the phenotype of the paternal triploid is quite different from that of the maternal. On microscopic section, paternal triploids will often show a mixture of hydropic villi together with smaller, more normal appearing villi, a phenotype sometimes referred to as a “partial mole.” Most present as a “blighted ovum” with an empty gestational sac in the first trimester. Those that survive into the second trimester exhibit an abnormal fetal to placental weight ratio with a very large placenta showing grossly hydropic villi (Fig. 13.4). Alpha-fetoprotein (AFP) and hCG levels are characteristically elevated.

The maternal triploid fetus is growth retarded with a disproportionately large cranium. The placenta is small and fibrotic in appearance, with none of the hydropic degeneration seen in the paternal triploid (Fig. 13.5). In contrast with the paternal triploid, AFP and hCG levels are low.

The risk for triploid gestations appears to decrease with advancing maternal age. A decline in survival of aberrant conceptuses, in older women, to the stage of recognized pregnancy is one possible explanation. Younger patients appear more likely to present with paternal triploids, whereas maternal triploids are more frequent in older patients.

The complete mole is a pregnancy characterized by marked placental overgrowth with large, cystic-hydropic villi (Fig. 13.6). The fetus is absent and the villi do not exhibit fetal vascularization. The trophoblastic layers on the surface of the villi show varying degrees of proliferation. Patients usually exhibit markedly elevated hCG levels, although a method-dependent artifact can result in falsely low levels [55]. Despite the markedly abnormal phenotype, molar pregnancies usually exhibit a diploid karyotype of 46,XX in approximately 90% of cases and 46,XY in 6–10% of cases [56]. Both haploid sets of chromosomes are of paternal origin, however. Mechanisms are probably similar to paternal triploids but with fertilization of an “empty” egg. Duplication of the chromosomes of a haploid sperm appears frequent and may explain the preponderance of 46,XX karyotypes, whereas fertilization by a diploid sperm could result in either a 46,XY or a 46,XX karyotype. The 46,YY karyotype appears to be nonviable [54].

Hydatidiform moles pose a risk of undergoing malignant transformation, becoming choriocarcinomas. Because of this, the diagnosis is critical for patient management. The triploid conceptus does not appear to have the same malignant potential [57, 58]. The mechanism for malignant transformation in the complete mole appears to be relaxation of imprinting with expression of genes that would normally be repressed [59, 60]. Imprinting has also been suggested as an explanation for the difference in phenotype between the maternal and paternal triploids [61].

Fig. 13.4 Paternal triploid, 69,XXY karyotype. Patient presented with markedly elevated β hCG at 16 weeks. Note very large placenta in relation to the size of the fetus

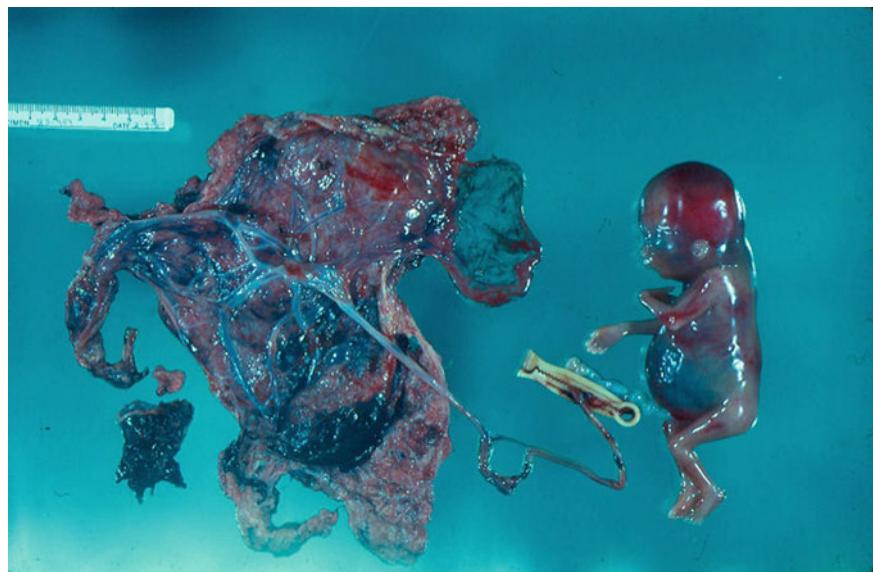


Fig. 13.5 Maternal triploid. Note very small placenta in relation to fetal size and fetus with micrognathia, syndactyly, and disproportionately large cranium in relation to body

Although the experienced perinatal pathologist should have little difficulty in recognizing the true hydatidiform mole based on the histologic appearance of the villi, cytogenetic evaluation should be considered whenever there is a question of the diagnosis, since follow-up with serial hCG levels is

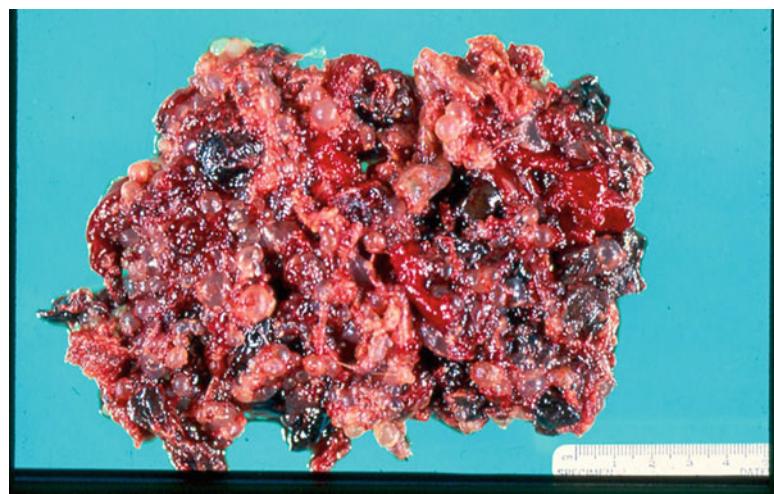
crucial in order to prevent a malignancy in cases of complete mole.

Both complete hydatidiform moles and most triploids appear to represent random errors at the time of fertilization. As such, a significant impact on the risk for other chromosomal abnormalities in subsequent pregnancies is not expected. Berkowitz et al. studied 1,205 pregnancies following a complete molar pregnancy and found no increase in risk for stillbirth, prematurity, ectopic gestations, malformations, or spontaneous abortion [62]. However, there appears to be a recurrence risk of about 1–2% for a future mole following a molar pregnancy [62–65]. Early sonographic surveillance is suggested for future gestations to rule out recurrent mole, and postnatal hCG determinations are recommended to rule out persistent trophoblastic disease [62]. Several pedigrees suggesting familial predisposition to molar pregnancies have also been reported, although the significance of family history on risk has not yet been established with certainty [66–69].

Structural Rearrangements

Structural rearrangements are less common than the other types of chromosomal abnormalities in pregnancy losses. Approximately 1–2% of spontaneous abortions show structural rearrangements. Jacobs summarized 5,726 karyotyped spontaneous abortions, revealing 0.28% balanced and 1.54% unbalanced rearrangements [70]. Balanced rearrangements include Robertsonian translocations, reciprocal translocations, and inversions (see Chap. 9). A survey of the literature by Dewald and Michels revealed translocations in 2.1% of couples with recurrent miscarriage [71]. Translocations were found in 1.7% of male patients and 2.6% of female patients.

Fig. 13.6 Complete hydatidiform mole with 46,XX (paternal disomy) karyotype. Note the hydropic villi with absence of gestational membranes and embryonic tissue



This compares with an incidence of 1 in 500 (0.2%) in newborns [72]. The frequency of balanced rearrangements in spontaneous abortions is not markedly increased over that seen in live births. This is not unexpected, since balanced rearrangements are typically not associated with significant phenotypic alterations and are usually compatible with embryonic and fetal life.

The most frequent unbalanced rearrangements result from Robertsonian translocations (see Chaps. 3 and 9). These may occur *de novo* or be familial in origin. The incidence of unbalanced Robertsonian translocations is much higher in spontaneous abortuses than live births, reflecting the uterine mortality of trisomic conceptuses.

Other unbalanced structural rearrangements seen in spontaneous abortions include abnormal chromosomes with extra or missing material, ring chromosomes, and small supernumerary chromosomes. *De novo* rearrangements are more frequently paternal in origin [73]. Analysis of human sperm revealed considerable variability among donors (0–17.8%) with a median of 9.3% abnormal sperm, consisting primarily of breaks, fragments, and small deletions. Increased susceptibility of sperm to chromosomal damage could explain the paternal origin of the majority of rearrangements.

Although many structural rearrangements arise *de novo*, the majority appear to be familial. Numerous studies of patients experiencing recurrent pregnancy loss have shown that these individuals are at increased risk of carrying a balanced chromosomal rearrangement. Cytogenetic analysis to rule out structural rearrangements and genetic counseling are indicated for couples who have experienced two or more losses. Since most balanced rearrangement carriers can produce both balanced and unbalanced gametes, a combination of normal and abnormal conceptions is frequently seen in such couples, and rearrangements may be more likely in those who have experienced both miscarriages and live births than in those with only miscarriages [74, 77].

Campana and colleagues note that the chromosomes and breakpoints involved in structural rearrangements do not appear to be random [75]. Survival of pregnancies with unbalanced chromosomal complements appears to be dependent upon the particular chromosome and segment(s) involved.

Structural rearrangements appear to occur with greater frequency in females than in males. Braekeleer and Dao found translocations or inversions in 2.6% of females with a history of reproductive failure compared with 1.4% in males, and Gadow and colleagues found that 3.5% of women and 1.7% of men with recurrent loss had balanced translocations [76, 77]. Both reports suggest increased risk for sterility in male carriers as a possible explanation. Chromosomal rearrangement appears to be associated with increased risk for infertility as well as for pregnancy loss.

The risk for poor pregnancy outcome when one member of a couple carries a structural rearrangement varies considerably depending upon the particular type of rearrangement and the chromosome(s) involved. Counseling must be individualized for each family with attention given to potential viability of any unbalanced meiotic products.

The risk figures that are used in counseling are often based on pooled data from translocations involving various chromosomes and breakpoints. Generally, it has been suggested that a male carrier is at lower risk for abnormal offspring than a female carrier. However, such generalizations may not be applicable in all cases and more specific risks figures based on the particular chromosomes involved may be beneficial in evaluating reproductive options for a family in which a balanced translocation has been identified [78].

The cause of reproductive failure in patients with balanced translocations is most likely the production of unbalanced gametes as a result of abnormal segregation during meiosis. Inversions can also lead to unbalanced gametes through crossover events involving the inverted segment.

A discussion of the implications of specific rearrangements with regard to abnormal segregation products can be found in Chap. 9; see also [24].

Chromosomally Normal Pregnancy Loss

Identification of the cytogenetically normal spontaneous abortion may be more important clinically than identification of the aberrant gestation. The risk of repeat miscarriage is higher when the prior loss is chromosomally normal [79]. Boué and colleagues found a risk of repeat loss of 23% after a chromosomally normal miscarriage compared with 16.5% following a chromosomally abnormal loss [13]. Morton and colleagues found that in women under 30, the risk for miscarriage was 22.7% following a chromosomally normal loss, 15.4% following a trisomy, and 17% following other chromosome abnormalities. In women over 30, these risks were 25.1, 24.7, and 20.3%, respectively [80]. Cytogenetic study of repeated spontaneous abortions suggests that those patients who experience a chromosomally normal pregnancy loss are more likely to show normal karyotypes in subsequent losses [81, 82].

Women with recurrent pregnancy losses and normal fetal karyotypes may be more likely to have underlying uterine abnormalities or endocrine dysfunction (see Chap. 11). Menstrual irregularities and elevated luteinizing hormone levels are more common in women with normal fetal karyotypes than in women with abnormal fetal karyotypes [83].

Immunological disorders have also been linked with recurrent normal pregnancy loss [84]. Systemic lupus erythematosus is perhaps the best known, but other autoimmune conditions have also been implicated [85]. Since patients with antiphospholipid antibodies and pregnancy failure frequently respond to treatment with prednisone and low-dose aspirin or heparin, it is important to recognize autoimmune disease as a frequent cause of recurrent chromosomally normal pregnancy losses [86–88]. Alloimmune disorders are less well understood but also appear to play a role in recurrent pregnancy failure. Several therapies including immunization with paternal white cells and administration of intravenous immunoglobulin have been suggested [89, 90].

Mutations that are lethal in the embryo are known from animal models and may also be a factor in recurrent euploid abortion in man [91]. Mutations in genes responsible for early organization of the embryo can have devastating effects on embryogenesis, with resultant pregnancy failure. Parental sharing of HLA antigens may also increase risk for spontaneous abortion, although the mechanism is not yet clearly understood [92]. More study of such genes and their effects on embryonic development is needed in order to determine

the frequency of their contribution to poor pregnancy outcome.

Specimens for Cytogenetic Studies

Although cytogenetic studies may be very helpful in managing patients with recurrent pregnancy loss, fetal karyotypes are infrequently performed. Cowchock and colleagues reported a success rate of 84% in a series of 100 samples, showing that chromosome analysis is indeed feasible in most specimens [79]. Chorionic villi are often the tissue of choice, as skin biopsies from deceased fetal tissue can be associated with a higher failure rate. As previously mentioned, with spontaneous pregnancy loss, it is frequently the case that the tissue is retained *in utero* for several days or even weeks following embryonic or fetal demise. Because of this, fetal tissue is often autolyzed and is unlikely to respond to standard culture methods, although chondrocytes appear to survive longer than skin and other soft tissues following fetal demise and may offer a greater chance of success [93]. Placental tissue, on the other hand, often remains viable for a much longer period of time, since necessary substrates for survival are provided by contact with the maternal blood supply. Ideally, both fetal and placental sources should be utilized. The advantage of the fetal tissue is that there is little risk for maternal cell contamination. If the fetus appears macerated, however, a high success rate is not to be expected. Placental tissue usually grows well but adds the risk of maternal cell contamination. This risk is reduced if the technologist processing the sample is experienced in the identification of membrane and chorionic villi.

Direct preparations using the *in situ* method of tissue culture work well with cells derived from spontaneously aborted tissues and have the advantage of rapid results with a high success rate and minimal risk for maternal cell contamination [94, 95]. However, if maternal cells are present in the original sample, trypsinization of slow-growing cultures to increase cell yield appears to increase the risk for maternal cell overgrowth. Careful tissue selection and washing to decrease the number of maternal cells may be helpful in decreasing the likelihood of maternal cell contamination [96].

Fluorescence *in situ* hybridization (FISH, see Chap. 17) using either tissue sections or disaggregated cells may be used in cases in which the tissue was accidentally fixed in formalin prior to receipt in the cytogenetics laboratory, since it does not require dividing cells [97]. It must be remembered, however, that this method will detect only those chromosome abnormalities for which specific probes are available. FISH can be useful in diagnosing suspected aneuploidies, similar to its use in prenatal screening of uncultured amniocytes, but the resulting information is limited to those

specific chromosomal regions for which probes are applied. Chromosomal rearrangements not involving numeric changes are not generally amenable to this type of FISH analysis in interphase cells.

Flow cytometry can also provide useful information in cases that are not amenable to cell culture, as it allows quantification of DNA [98]. This may be especially useful for products of conception with hydropic changes seen with a histological examination, as it can differentiate between complete hydatidiform moles (paternal diploids) and partial moles (usually triploid), an important distinction with regard to patient management because of the risk for persistent trophoblastic disease with complete moles. DNA image cytometry has also been shown to be useful in the diagnosis of molar pregnancies [99].

Newer methodology that has been proven useful for diagnosis of unbalanced karyotypes in cases for which dividing cells are not available uses microarray-based technology, either comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) analysis [100–102]. These methods are dependent upon DNA extraction but do not require viable or intact cells and thus may be used for formalin-fixed frozen or paraffin-embedded tissues as well as fresh samples. Although both methods are useful for detection of aneuploidy, they do differ and may not always provide the same results. See also Chap. 18.

In CGH, test specimen and reference DNA samples are hybridized together using different fluorochromes. The intensities between the test and reference samples are compared, enabling identification of gains or losses of individual chromosomes or chromosomal regions. This technique can detect unbalanced karyotypes such as trisomies, the largest group of chromosomally abnormal pregnancies. However, CGH is not useful for the detection of balanced structural rearrangements or polyploidy. Thus, a triploid or tetraploid conceptus would not be recognized by CGH unless the sex chromosomes differed because the ratio of intensity between the sample being tested and the control would be constant across the genome. However, additional studies such as flow cytometry could be used if abnormal ploidy were of concern clinically [103]. SNP array may be an alternative to consider.

Unlike CGH, a SNP array compares the patient sample with a control database rather than a single control sample. Like CGH, gains and losses are readily detected using SNP arrays. However, SNP technology offers the advantage of being able to detect loss of heterozygosity that may reflect uniparental disomy and will also permit the detection of triploid gestations, conditions that may be missed using CGH methods [104]. However, as with CGH, a balanced translocation or inversion would not be recognized using this technique.

Because of this, peripheral blood cytogenetic studies should still be considered for any couple experiencing recurrent pregnancy loss. In examining parental chromosomes, structural rearrangements including translocations and inversions are the obvious focus. Such rearrangements may have significant impact on the couple's risk for miscarriage or infants with anomalies.

Cytogenetic abnormalities that are less clear in terms of their implications for future reproduction may also be seen. It is not uncommon to find mosaic aneuploidy in couples with recurrent pregnancy loss. Low-level sex chromosome aneuploidy is sometimes seen in lymphocytes but is not usually found in cultured fibroblasts. The risk appears to increase with age but does not appear to be correlated with reproductive history [105]. Discussion with a cytogeneticist can be invaluable in interpreting whether unexpected findings are of potential clinical significance or artifact unrelated to the reproductive history.

Evaluation of Pregnancy Losses

Although a complete evaluation of a pregnancy loss requires extensive specialized testing, including cytogenetic studies, such tests are costly and labor intensive. With increasing emphasis on delivery of cost-effective health care, cytogenetic studies simply cannot be justified for every unsuccessful pregnancy. However, a careful examination by a pathologist can often go a long way toward answering the patient's questions about the loss, and a more thorough evaluation by a pathologist with training and interest in developmental anatomy can often provide considerable information without significant increase in cost.

Such an examination can establish how far the pregnancy proceeded prior to developmental arrest and whether the pregnancy appears to have been developing normally. The developmental age is especially helpful since the earlier the growth arrest, the more likely it is that the conceptus will exhibit an abnormal karyotype.

Any embryo or fetus should be examined closely for evidence of congenital anomalies. Embryos with malformations and growth-retarded embryos are more likely to exhibit abnormal karyotypes. Some isolated anomalies, such as cleft palate or neural tube defects, may be associated with significant recurrence risks yet may have normal karyotypes. Specific anomalies may also be indicative of an underlying syndromal process, with or without an abnormal karyotype. Single gene defects with significant recurrence risk can sometimes be identified from a careful fetal examination. Evidence of infectious processes or teratogen exposure may also be present, with their own implications for future pregnancy management. Anatomic evaluation can therefore play a useful role when traditional cytogenetic studies either are

Table 13.9 Phenotype of abortus and incidence of abnormal karyotypes [35]

Appearance of abortus	% Chromosomally abnormal
Incomplete specimen, no embryo	47.3
Incomplete embryo/fetus	40.0
Intact empty sac	64.3
Severely disorganized embryo	68.6
Normal embryo	54.1
Embryo with focal abnormalities	55.0
Normal fetus	3.3
Fetus with malformations	18.2

not indicated, as in a first loss with no other risk factors, or are not possible, for example, a formalin-fixed or otherwise nonviable specimen.

Although most chromosomal abnormalities are not associated with distinct phenotypes, especially in very early losses, there does appear to be some correlation between specimen morphology and the likelihood of an abnormal karyotype. Creasy studied the prevalence of chromosomal abnormalities and phenotype [35]. The results are summarized in Table 13.9.

Even though the degree of correlation between specimen types and risk for chromosomal abnormalities is far from ideal, some information regarding the likelihood of a karyotype abnormality can be gained from the embryonic pathology. Absence of abnormalities in a pregnancy that has progressed to the fetal stage is a good predictor for a normal chromosomal complement.

Although morphology can help predict the likelihood of a chromosomal etiology for the loss, it cannot be expected to identify the particular karyotype abnormality involved. However, even distinguishing probable chromosomal from nonchromosomal losses is of considerable benefit to the patient, as it can help in determining need for further studies and in predicting risk for recurrence.

There is a strong correlation between the chromosomal constitutions of first and subsequent abortions. The patient with a chromosomally abnormal abortus is more likely to experience abnormal karyotypes in subsequent losses, whereas a patient with a normal karyotype in one loss is more likely to show normal karyotypes in any future pregnancy losses [81, 82, 106].

Chromosome studies are especially useful for stillbirths suspected of having cytogenetic abnormalities, such as infants with congenital anomalies or intrauterine growth restriction. There may also be increased risk in the presence of fetal hydrops, maceration, or a history of prior losses [107, 108]. Cytogenetic studies should probably be performed in any case in which a pathophysiologic explanation for the demise is not identified [109]. A careful anatomic evaluation of both fetus and placenta is indicated in all stillbirths, as are photographs and radiographic studies to document

morphology when there is question of a skeletal dysplasia or other anomalies. These can be reviewed later by a specialist in fetal dysmorphology if there is any question of anomalous development. Additional special studies for congenital infection, hematologic disorders, or metabolic disease may also be indicated in some cases. Overall, a cause of death can be assigned in approximately 80% of cases [107, 109].

A wide range of problems can result in decreased fertility or pregnancy failure, and the work-up for an infertile couple can be extensive and costly [110]. Identifying those couples whose losses are explained as being due to karyotypic abnormalities may be a cost-effective alternative. Cowchock and colleagues argue that if cytogenetic studies cost \$500, with an 84% chance of culture success and a 40% chance of detecting a chromosomal abnormality that would explain the loss, one of every three women with multiple miscarriages would be spared further costly and invasive evaluations for recurrent pregnancy loss [79]. This would save approximately \$2,000 in expenses for testing that would otherwise be done as part of a multiple miscarriage protocol. Given the availability of therapy for many patients with nonchromosomal causes of pregnancy loss, the cost-benefit ratio may actually be even better.

Although recurrent spontaneous abortion is often defined as three consecutive losses, today, many couples find that three miscarriages are more than they are willing to accept before looking for answers. There may indeed be justification for initiating further evaluation after the second failed pregnancy. Coulam compared 214 couples with a history of two or more consecutive abortions with 179 couples with a history of three or more abortions [111]. Both groups showed 6% of losses that were chromosomal, 1% that were anatomic, and 5% that were hormonal. Sixty-five percent of the group with two losses and 66% of the group with three losses had immunologic causes. Twenty-three percent of the group with two losses and 22% of the group with three losses were unexplained. The absence of any significant difference in prevalence between the two groups suggests that there is little to be gained by delaying evaluation until after the third pregnancy loss.

Tharapel and colleagues reviewed published surveys of couples with two or more pregnancy losses (8,208 women and 7,834 men) and found an overall prevalence of major chromosome abnormalities of 2.9% [112]. They go on to suggest that even with normal parental chromosomes, prenatal diagnosis should be offered because of the high incidence of chromosomal abnormalities in spontaneous pregnancy loss. Drugan and colleagues identified five anomalous fetuses, including one trisomy 18, two trisomy 21, one trisomy 13, and one monosomy X fetus among 305 couples with recurrent pregnancy loss [113]. This 1.6% risk is greater than the risk usually cited for amniocentesis. A control group of 979 patients revealed only three abnormalities

(0.3%), all sex chromosome aneuploidies. This would suggest an increased risk for nondisjunction among couples experiencing repeated pregnancy failure. Their conclusion is that prenatal diagnosis is sufficiently safe and the risk for an abnormal result is sufficiently high to justify offering prenatal diagnosis to couples with a history of two losses. Although this conclusion is based on a relatively small sample size and not all obstetrical caregivers would agree, a discussion of risks and benefits of prenatal diagnosis would appear to be justified in this patient population.

Although considerable advances have been made in understanding the causes underlying pregnancy failure and there is considerable hope for more specific therapies for couples experiencing nonchromosomal losses, there is unfortunately little to offer the couple who may be at increased risk for cytogenetically abnormal pregnancies. When a rearrangement is incompatible with normal pregnancy outcome (such as an isochromosome 21), use of donor ova or sperm may be an option. The issues are not so clear for the couple with recurrent aneuploidy or polyploidy.

Preimplantation assessment of the fetal karyotype using FISH may be a consideration for some patients undergoing *in vitro* fertilization for other reasons. Simultaneous use of probes for chromosomes 13, 18, 21, X, and Y can enhance the likelihood of transfer of normal embryos; however, some mosaic aneuploid conceptions and aneuploidy for other chromosomes would still be missed [114]. It is important to remember that the majority of embryos with cytogenetic abnormalities will be lost spontaneously, thus the unknowing transfer of cytogenetically abnormal embryos potentially contributes to the less than optimal success rate for IVF procedures. Better methods for identifying chromosomally normal embryos for transfer are needed [115].

Summary

Humans experience a wide range of chromosomal abnormalities at conception. The incidence is surprisingly high when compared with other mammals, such as the mouse. When considering pregnancy loss in this context, spontaneous abortion can be seen as a means of “quality control” in an otherwise inefficient reproduction system [13]. Current knowledge of the mechanisms involved in meiosis, fertilization, and mitosis is still quite limited, and the factors affecting survival of the embryo are not yet fully understood. Maternal age appears to increase the incidence of abnormal conceptions but may also decrease the efficiency of this control process.

Although current understanding of pregnancy loss is limited and risks cannot be fully predicted, it is often possible to offer patients some explanation as to why a given pregnancy has failed and whether there is any treatment that might

improve chances for future success. Prenatal diagnosis can also be made available in those cases in which there is increased risk for cytogenetic abnormalities or when additional reassurance of a normal fetal karyotype is needed. It is important to keep in mind that even with a history of a chromosomally abnormal pregnancy, most couples have a good chance for a subsequent successful outcome.

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Xiao-Xiang Zhang

Introduction

Two levels of genetic instability have recently been characterized in human cancers: subtle sequence changes observed at the nucleotide level and instability that is visible at the chromosomal level [1]. The high incidence of chromosome instability reported in neoplastic processes has made this an area of active investigation.

Chromosome instability describes a variety of chromosome alterations, including numerical and structural chromosomal rearrangements observed at an increased rate when compared with normal controls. Numerical changes can be the consequence of abnormal segregation at the metaphase/anaphase transition. Dysregulation of genes involved in chromosome condensation, sister chromatid cohesion, kinetochore structure and function, and centrosome/microtubule formation and dynamics have been implicated in the formation of aneuploidy, hypodiploidy, and polyploidy, as have cell cycle checkpoint genes. Chromosome breaks and telomere dysfunction can result in various structural rearrangements (deletions, duplications, inversions, insertions, and translocations). Impairment of DNA repair, DNA replication, or DNA recombination is responsible for causing sister chromatid exchanges, fragile sites, chromatid/chromosome breaks, and mutagen sensitivity.

The most common forms of chromosome instability are seen in cancers. Virtually all malignant human tumors contain chromosome rearrangements, and in many instances, these chromosomal changes were considered to have occurred in the late stages of tumorigenesis. However, recent evidence has suggested that chromosome instability was present in premalignant head and neck lesions and that high levels of such instability were associated with subsequent tumor progression [2].

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The acquisition of chromosome abnormalities by target cells is a central event that contributes to malignant transformation and tumor development (see Chap. 16). This chapter will focus on other forms of chromosome instability: fragile sites and chromosome breakage associated with chromosome instability syndromes.

Fragile Sites in Humans

Definition and Classification

Chromosomal fragile sites are specific chromosome loci that usually appear as nonstaining gaps and breaks on metaphase preparations, either spontaneously or in response to special agents or tissue culture conditions (Fig. 14.1). All fragile sites are part of the chromosome structure and are inherited as Mendelian codominant traits. Fragile site loci can be normal variants or can be associated with specific genetic conditions/phenotypes. They are named by the letters “fra” followed by designation for the specific chromosomes and bands where the fragile sites are located. For example, fra(10)(q25.2) describes a fragile site on chromosome 10 at band q25.2 [3]. The HUGO Gene Nomenclature Committee refers to this fragile site as FRA10B.

Fragile sites are grouped into two classes, rare and common, based on their frequency of occurrence and means of induction. Common fragile sites are expressed in all individuals at various rates. The most frequently observed common fragile sites occur, in decreasing order, at 3p14.2 (FRA3B), 16q23 (FRA16D), 6q26 (FRA6E), 7q32 (FRA7H), and Xp22 (FRAXB) [4]. The rare fragile sites are found only in some families, with a population frequency of less than 5%. The fragile site at 16q22 (FRA16B) is seen most often, with an occurrence of 1 in 20 in the German population, while 1p21.3 (FRA1M) is the rarest, having been reported only once.

Most fragile sites are not expressed spontaneously but require induction using special chemical agents. Both the rare and the common fragile sites are further subdivided



Fig. 14.1 An example of fragile sites on human chromosomes (arrows) in response to folate/thymidylate depletion using FUdR. See text for details

according to the culture conditions required for expression. While the majority of common fragile sites are induced by aphidicolin, some are induced by 5-azacytidine or bromodeoxyuridine (BrdU). Most of the rare fragile sites are observed when cells are grown in folic acid-deficient medium, but some are expressed when cells are grown in the presence of BrdU or distamycin. To date, 89 common fragile sites and 30 rare fragile sites are described in the Human Genome Database (GDB) (<http://www.gdb.org/>). More than 150 fragile sites were recorded using the criteria formulated at a session known as the Chromosome Coordinating Meeting [5, 6].

Fragile sites have been found on every chromosome with the exception of chromosome 21. Table 14.1 shows the classification and a list of the common and the rare fragile sites [7, 8]. It is interesting to note that the locations of many common fragile sites are highly conserved in man, gorilla, chimpanzee, and orangutan, while none of the rare folate-sensitive fragile sites have been identified in species other than humans [9, 10].

The physical basis of the cytogenetic expression of fragile sites is not yet completely understood. However, advances in the characterization of DNA sequences of fragile sites and cell biology have shed some light on the mechanisms responsible for fragile site expression. All members of the three

classes of rare fragile sites that have been cloned thus far contain tandem repeat sequences (e.g., a CCG repeat in FRAXA, a 33 bp AT rich in FRA16B, and an approximately 42 bp variable AT-rich repeat in FRA10B). In contrast, no dinucleotide or trinucleotide repeat expansion has been found at any of the common fragile sites that have been studied, even though all fragile sites cloned are relatively AT rich. Sequence analysis of FRA3B, FRA7G, and FRA7H shows no striking molecular structure that explains the fragility in these regions. It has been proposed that delayed DNA replication underlies expression of fragile sites and that cytogenetic manifestation of these fragile sites is due to incomplete DNA replication, which leads to a failure of chromatin compaction [11]. This becomes more obvious when DNA replication is perturbed by aphidicolin or folate induction.

Clinical Significance

The discovery of the fragile X syndrome (see Chap. 19) has dramatically stimulated the search for other fragile sites that might be associated with abnormal phenotypes. It has frequently been suggested that breakage and recombination at these sites may be mechanistically involved in constitutional

Table 14.1 Common and rare fragile sites

Mode of induction	Common fragile sites				Rare fragile sites	
					Mode of induction	
Aphidicolin inducible	1p36.1	3p14.2	7q22	12q24	Folate-sensitive	2q11.2
	1p32	3q27	7q31.2	13q13.2		2q13
	1p31.2	4p16.1	7q32.3	13q21.2		2q22.3
	1p31	4p15.2	7q36	14q23		5q35
	1p22	4q31.1	8q22.1	14q24.1		6p23
	1p21.2	5p14	8q24.1	15q22		7p11.2
	1q21	5q15	8q24.3	16q22.1		8q22.3
	1q25.1	5q21	9q22.1	16q23.2		9p21
	1q31	5q31.1	9q32	17q23.1		9q32
	1q44	6p25.1	10q22.1	18q12.2		10q23.3
	2p24.2	6p22.2	10q25.2	18q21.3		11q13.3
	2p16.2	6q15	10q26.1	20p12.2		11q23.3
	2p13	6q21	11p15.1	22q12.2		12q13.1
	2q21.3	6q26	11p14.2	Xp22.31		12q24.13
	2q31	7p22	11p13	Xq22.1		16p12.3
	2q32.1	7p14.2	11q13	Xq27.2		16p13.11
	2q33	7p13	11q14.2			19p13
	2q37.3	7q11.2	11q23.3			20p11.23
	3p24.2	7q21.2	12q21.3			22q13
						Xq27.3
5-Azacytidine inducible	1q12				Distamycin A inducible	8q24.1
	1q44					11p15.1
	9q12					16q22.1
	19q13					17p12
						10q25.2
5-Bromodeoxyuridine (BrdU) inducible	4q12	6q13	13q21		BrdU requiring	12q24.2
	5p13	9p21				
	5q15	10q21				

Source: Data from references [6, 7]

rearrangements or the deletions observed in many tumors. The finding of a fragile site during the course of chromosome analysis often raises questions regarding the potential clinical significance and can create uncertainty regarding patient care. Currently, with the exceptions of FRAXA and FRAXE (two well-known causes of familial mental retardation), and possibly FRA11B in relation to the breakpoint associated with Jacobsen syndrome, no other rare fragile site has to date been shown to predispose to any heritable chromosome abnormality or malignancy [12]. In a study of 10,492 cases available from the literature, no statistical association between fragile sites and constitutional breakpoints was noted [13]. The occurrence of folate-sensitive autosomal rare fragile sites (ARFS) was compared in populations of mentally retarded, mentally subnormal, and mentally normal children, and the frequencies did not differ significantly [14].

On the other hand, compelling evidence has suggested that common fragile sites are highly unstable regions in the human genome, associated with cancer predisposition and

progression. The theory that the common fragile sites might play a role in tumor development was initially proposed by Yunis et al. in 1984, soon after fragile sites were discovered [15]. It has well been recognized that 50–70% of common fragile sites co-localize with oncogenes, tumor suppressor genes, and breakpoints in cancer rearrangements [16]. Subsequent experimental evidence has revealed that fragile sites appear to be preferential targets for viral integration [17]. The observation of intrachromosomal amplification of the *MET* oncogene in a human gastric carcinoma via a breakage-fusion-bridge within the FRA7G region further supports the hypothesis that fragile sites play a key role in the amplification of some oncogenes during tumor progression [18, 19]. More direct evidence was reported by Egeli et al., who noted a significantly higher expression of fra(3)(p14) in squamous cell lung cancer patients and their relatives than in healthy control subjects, and they suggested that the high expression of fra(3)(p14) in these patients and their relatives may be a valid marker for genetic predisposition to lung cancer [20].

However, arguments downplaying the role of common fragile sites in the tumorigenic process are based on the fact that these sites are present in virtually everyone's genome, and therefore it would be unreasonable to suggest that any one individual is at a particularly higher risk of developing a malignancy. Since there is no convincing evidence implicating common fragile sites in the cancer process, the following guidelines provided by Sutherland et al. can be used when dealing with patients who express fragile sites:

With the definite exceptions of FRAXA and FRAXE and possibly FRA11B, patients with any other fragile site, either rare or common, can be strongly reassured the fragile site will not affect their personal health or increase their risk of having chromosomally abnormal children. [21]

Chromosome Instability Syndromes

The chromosome instability syndromes, formerly known as chromosome breakage syndromes, comprise a number of rare but distinct clinical entities. The classic chromosome instability syndromes are Fanconi anemia, ataxia telangiectasia, Nijmegen syndrome, ICF syndrome, Robert syndrome, Werner syndrome, and Bloom syndrome. They are all autosomal recessive, show increased frequency of chromosome changes (spontaneous or induced) and, with the exception of Robert syndrome, are all associated with an increased risk of development of malignancies. This higher incidence of neoplasia may also apply to family members of affected individuals.

These disorders were initially described as clinical syndromes, independent of their mechanisms of action. However, recent progress in molecular genetics and biochemistry indicates that, despite their clinical characteristics, they essentially constitute disorders of DNA recombination. Although each has its own specific molecular defect related to abnormalities of DNA repair, cell cycle control, or apoptosis, the common result is chromosomal instability leading to a neoplastic phenotype.

Fanconi Anemia

Fanconi anemia (FA) is a rare disorder characterized by diverse congenital anomalies and a predisposition to bone marrow failure and malignancy. FA patients present with a wide range of clinical heterogeneity, and many major organ systems can be affected. Approximately 50% of patients have radial ray anomalies ranging from bilateral absent thumbs and radii to unilateral hypoplastic thumb or bifid thumb. Malformations of the heart and kidney and anomalies of the skeleton and limbs show considerable overlaps with some clinical syndromes, such as VATER, TAR, and Holt-

Oram syndromes. Bone marrow failure leading to progressive pancytopenia and predisposition to cancers, especially AML, is the major cause of death in FA patients. Auerbach suggested that the cellular defect in FA results in chromosome instability, hypersensitivity to DNA damage, and hypermutability for allele-loss mutations, predisposing to leukemia as a multistep process [22].

The condition has a worldwide prevalence of 1–5 per million and is found in all races and ethnic groups, with an estimated carrier frequency of 0.3–1% [23]. The carrier frequency in the Ashkenazi Jewish population is approximately one in 90 [24]. Recent studies indicate that there are at least 13 genetically distinct complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, and N). The manner in which these function has been identified [25]. The majority of FA patients (60–80%) are assigned to group A, followed in frequency by groups C (8–14%) and G (~9%).

The gene for the FA-D1 subtype, *FANCD1*, is identical to the breast cancer susceptibility gene *BRCA2*. Patients with heterozygous mutations of *FANCD1/BRCA2* have an increased risk for breast/ovarian cancers while those with homozygous mutations will suffer from the FA-D1 subtype of Fanconi anemia. All FA genes are recessive and are transmitted autosomally except *FANCB*, which is X-linked.

Increasing evidence indicates that multiple FA proteins, including a ubiquitin ligase (FANCL), a monoubiquitinated protein (FANCD2), and DNA helicases (FANCJ and FANCD1/BRCA2), cooperate in a biochemical pathway involved in cell cycle regulation and response to DNA damage (the FA pathway). The interaction of *BRCA1* with the FA protein pathway likely plays a critical role as a caretaker of genomic integrity. Genetic defects of FA proteins result in a failure of recognition of interstrand DNA cross-links and leave damaged DNA unrepaired. In a *FANCA* gene mutation analysis conducted on a panel of 90 patients, Wijker et al. found no hot spots, and the mutations were scattered throughout the gene. Most mutations were predicted to result in the absence of the *FANCA* protein [26].

The determination of the complementation group for each patient has become increasingly important as more data on genotype–phenotype relationship has been collected. Patients in the FA-C group showed a significantly poorer survival than those in groups A (FA-A) and group G (FA-G), and patients in the FA-D1 group have an increased risk of developing medulloblastoma, Wilms tumor, and an early onset of acute leukemia. Certain genotype–phenotype correlations have been noted; for example, *FANCA*-null patients tend to have more severe hematological manifestation and develop AML more often than non-null patients [27].

The International Fanconi Anemia Registry (IFAR) was established at the Rockefeller University in 1982 to study

this rare genetic disorder (database available online) [28]. In a survey of the clinical features gathered from the IFAR, the extreme variation of the phenotypes of FA makes the clinical diagnosis difficult and unreliable.

Fanconi anemia was the first disease in which spontaneous chromosome breakage was detected, both *in vitro* and *in vivo*. Chromatid breaks and gaps are the most common spontaneous aberrations. Acentric and dicentric fragments, rings, and endoreduplicated chromosomes are also seen in the cells from FA patients, as are multiradial formations (Fig. 14.2). A quantitative FISH analysis showed an accelerated telomere shortening in both arms of FA chromosomes; this may explain a tenfold increase in chromosome end fusions observed in FA cells [29]. Other cellular features of FA include retardation of *in vitro* growth of FA cells, delay during the G2 phase of the cell cycle, and hypersensitivity to cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB). Because the heterogeneity of the mutation spectrum and the frequency of intragenic deletions present a considerable challenge for the molecular diagnosis of FA, *in vitro* enhancement of chromosome breakage by DEB and MMC has been the gold standard for diagnosing FA. Currently, the best treatment is bone marrow transplantation.

Ataxia Telangiectasia

Ataxia telangiectasia (A-T) is an autosomal recessive disorder associated with cerebellar degeneration, oculocutaneous telangiectasias, immunodeficiency, chromosome instability, radiosensitivity, and cancer predisposition. A-T patients present in early childhood with progressive cerebellar ataxia that can be misdiagnosed as ataxic cerebral palsy before the appearance of oculocutaneous telangiectases. Serum IgG2 or IgA levels are diminished or absent in 80 and 60% of patients, respectively [30]. An elevated alpha-fetoprotein (APF) level is observed in a majority of A-T patients, who have a strong predisposition to develop lymphocytic leukemias and lymphomas. In general, lymphomas tend to be of B-cell origin, whereas leukemias tend to be T-cell type. Other solid tumors, including medulloblastomas and gliomas, are also seen in A-T patients.

The incidence of A-T has been estimated at 1 in 89,000 in the US Caucasian population and the A-T heterozygote frequency is approximately 2.8% [31, 32].

The responsible gene, *ATM* (ataxia telangiectasia mutated), encodes a large protein kinase with a phosphatidylinositol 3-kinase-like domain and was cloned in 1995. *ATM* is thought to play a central role in a signal transduction



Fig. 14.2 Metaphase from a Fanconi anemia patient, observed in a clastogen-exposed lymphocyte culture. Note the chromosome breakage and radial formations (Photo courtesy of Dr. Susan Olson)

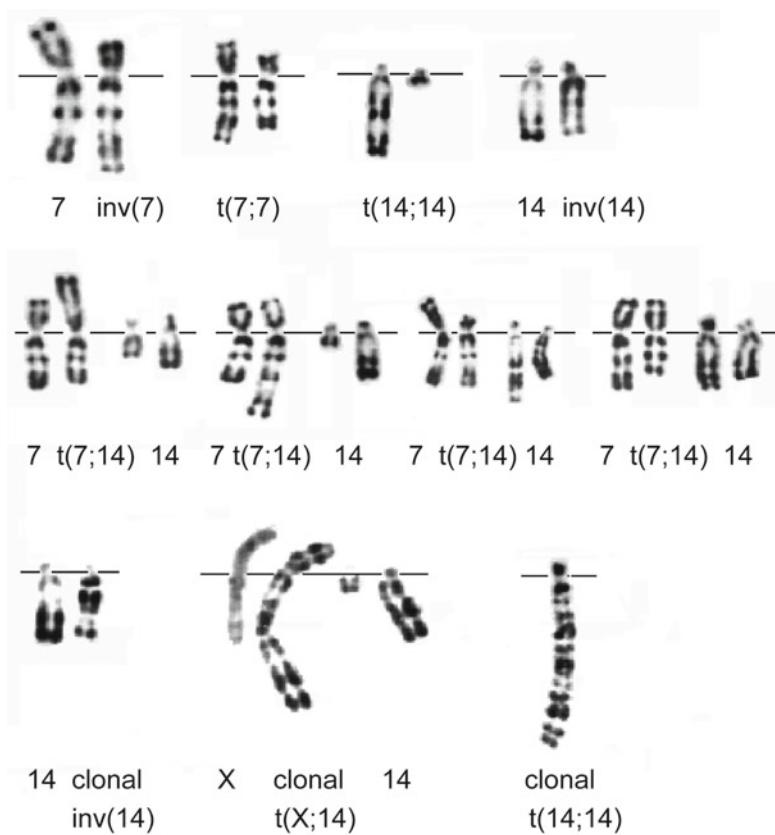
network that regulates cell cycle checkpoints, genetic recombination, apoptosis, and other cellular responses to DNA damage. Heterozygous carriers of an *ATM* mutation have a 6.1-fold relative risk of developing cancer, most commonly breast cancer. Such carriers may account for 5% of all cancer patients in the United States [33]. Although *in vitro* cell fusion studies had suggested that A-T was genetically heterogeneous, all A-T patients studied to date have been found to harbor one of 200 different mutations in *ATM* [34]. However, a single mutation was observed in A-T patients of Jewish Moroccan or Tunisian origin [35]. Greater than 70% of mutations are predicted to lead to protein truncation. Approximately 90% of affected individuals showed no detectable ATM protein while about 10% of A-T patients demonstrated trace amounts of ATM protein.

Elevated spontaneous chromosome breakage has been observed in fibroblasts and peripheral lymphocytes from A-T patients, and tissue-specific chromosome aberrations are noted in A-T patient cells. For example, a high frequency of balanced rearrangements involving chromosomes 7 and 14 at loci for immunoglobulin and T-cell receptor genes (Fig. 14.3) is often seen in lymphocytes from A-T affected individuals. A greatly increased sensitivity of A-T cells to

X-ray and radiomimetic substances, such as bleomycin, is another characteristic cytogenetic hallmark. In a study that utilized two recombination vectors, spontaneous intrachromosomal recombination rates were 30–200 times higher in an A-T fibroblast cell line than in normal cells, but extra-chromosomal recombination frequencies were near normal [36]. Therefore, the defects in ataxia telangiectasia seem to be related primarily to the processes of DNA recombination, and increased recombination may contribute to the high cancer risk seen in A-T patients. Repair deficiencies after ionizing irradiation are secondary by-products of such recombination defects. Nevertheless, treatment of malignancies with conventional dosages of radiation can be fatal to A-T patients.

The presence of early-onset ataxia along with oculocutaneous telangiectasias facilitates a clinical diagnosis of A-T, which can be problematic before the appearance of such telangiectasias. The large size of the *ATM* gene, together with the diversity and broad distribution of *ATM* gene mutations in A-T patients, greatly limits the utility of direct mutation analysis as a diagnostic tool, except where founder effect mutations are involved. Immunoblotting for intracellular ATM protein depletion is to date the most sensitive and rather

Fig. 14.3 Sporadic (rows 1 and 2) and clonal (row 3) rearrangements in ataxia telangiectasia (R-banding). Row 1, from left to right: inv(7)(p14q35), t(7;7) (p14;q35), t(14;14)(q11;q32), inv(14)(q11q32). Row 2, from left to right: t(7;14) (p14;q11), t(7;14)(q35;q11), t(7;14)(p14;q32), t(7;14) (q35;q32). Row 3, from left to right: inv(14)(q11q32), t(X;14)(q28;q11) (note the late replicating X on the left), t(14;14)(q11;q32) (Courtesy Alain Aurias and the Atlas of Genetics and Cytogenetics in Oncology and Hematology. Modified from [36])



inexpensive test for establishing a diagnosis of A-T syndrome. A newly developed flow cytometric assay that measures the intranuclear phosphorylation of SMC (structural maintenance of chromosomes) proteins claims to provide a clear distinction between *ATM* mutation heterozygotes and homozygotes [37].

Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is a rare disorder characterized by progressive microcephaly, a “birdlike” face, growth retardation, lack of secondary sex development in females, recurrent sinopulmonary infections due to immunodeficiency, and cancer predisposition, with the highest risk being for B-cell lymphoma. Because cells from NBS patients share cytogenetic features with those from A-T patients, such as clonal rearrangements preferentially involving chromosomes 7 and 14 in PHA-stimulated lymphocytes and hypersensitivity to ionizing radiation, NBS was originally thought to be a variant of A-T [38]. Clinical features differ, however. NBS patients have microcephaly, but neither cerebellar ataxia nor telangiectasias, and have normal serum levels of AFP. Complementation studies and, moreover, the recent identification of the genes responsible for A-T (*ATM*) and for NBS, *NMN* (*NBS1*, Nibrin, or p95 protein of the *NBS1/Mre11/Rad50* complex) have proven that A-T and NBS are related but separate entities [39, 40].

Several lines of evidence suggest that *ATM* and *NMN* functionally interact in response to DNA damage induced by ionizing radiation (IR). Zhao et al. showed that *NMN* is phosphorylated by *ATM* in response to DNA damage. This involves S-phase checkpoint activation, formation of *NBS1/Mre11/Rad50* nuclear foci, and reversal of IR damage [41]. This observation links *ATM* and *NMN* in a common signaling pathway and provides an explanation for the phenotypic similarities between these two disorders.

Diagnosis is based on molecular testing of *NMN*, the only gene known to be associated with Nijmegen breakage syndrome. Disease-causing mutations are detected in almost 100% of NBS patients, and a 5-base pair truncating deletion (657Del5) has been identified in 90% of patients. A German group recently found a high carrier frequency (1/177) of 657Del5 mutations in three Slavic populations [42, 43].

ICF Syndrome

ICF syndrome (immunodeficiency, centromere instability, and facial anomalies) is a recessive disorder characterized by facial dysmorphism, immunoglobulin deficiency, and centromeric region instability involving chromosomes 1, 9, and 16. The most common clinical features of ICF syndrome are



Fig. 14.4 Chromosome 1 multiradial configuration from a patient with ICF syndrome. Some stretching of the pericentromeric heterochromatin can also be seen (Photo courtesy of Dr. Jeffrey Sawyer)

hypertelorism, low-set ears, epicanthal folds, macroglossia, recurrent respiratory infections, and variable immune deficiency with a decreased level of IgA.

ICF syndrome is the only genetic disorder known to involve constitutive abnormalities of genomic methylation patterns. Mutations in the DNA methyltransferase gene *DNMT3B* at 20q11.2 were identified in 75% of affected individuals [44]. *DNMT3B* mainly affects *de novo* methylation of the GC-rich classical satellite DNAs 2 and 3, which are components of constitutive heterochromatin. In ICF patients, there is constitutive hypomethylation of satellite 2 DNA, mostly located at the juxtacentromeric heterochromatin of chromosomes 1 (1qh) and 16 (16qh), and of satellite 3 DNA, located on chromosome 9 (9qh).

Cytogenetic analysis of peripheral blood lymphocytes reveals multiradial configurations and a stretching of the pericentromeric heterochromatin of chromosome 1, 9, and 16 (Fig. 14.4). An increase in formation of micronuclei is also noted in ICF patients. Using FISH probes specific for alphoid satellite (centromere) DNA and classical satellite II DNA (paracentric heterochromatin), Sumner et al. showed that it is always the paracentromeric heterochromatin of chromosomes 1, 9, and 16 that becomes decondensed and fused to form multiradial configurations [45]. The centromeric regions remain outside the regions of interchange. These same juxtacentromeric heterochromatin regions are subjected to persistent interphase self-associations and are extruded into nuclear blebs or micronuclei. By an unknown mechanism, the *DNMT3B* deficiency that causes ICF interferes with lymphogenesis (at a step after class switching) or lymphocyte activation. The stretched heterochromatic blocks appear to be restricted to PHA-stimulated T-cells only.

When 35 individuals with ICF syndrome were screened for *DNMT3B* gene mutations, only 20 affected patients showed positive results, indicating genetic heterogeneity [46]. The majority of mutations are missense mutations; others include nonsense mutations and splice-site mutations. No genotype–phenotype correlation was found.

Robert Syndrome (RS)

Robert syndrome (RS) is characterized by craniofacial anomalies, limb defects, and pre- and postnatal growth retardation. RS patients present with various degrees of limb malformations, involving symmetric phocomelia or hypomelia. Hypertelorism and cleft lip and palate are often seen in affected individuals. Many of the malformation features are similar to those observed in affected children whose mothers took thalidomide during pregnancy; thus, RS is sometimes called pseudothalidomide syndrome. RS is an extremely rare disorder with only about 150 reported cases. It is an autosomal recessive condition, and parental consanguinity is common. The carrier frequency for RS is unknown.

Despite the heterogeneous clinical presentation, complementation studies of cells derived from RS patients defined a single complementation group [47]. Using a candidate gene approach, Vega et al. found a variety of mutations in the *ESCO2* gene at 8p21.1, including missense, nonsense, and frameshift mutations in 18 RS patients from 15 families of different ethnic backgrounds [48]. N-acetyltransferase *ESCO2*, also known as establishment of cohesion 1 homolog 2 or ECO1 homolog 2, is an enzyme required for the establishment of sister chromatid cohesion during the S phase of mitosis. Mutations of *ESCO2* leading to loss of *ESCO2* acetyltransferase activity is the only known cause for RS thus far identified. No clear genotype–phenotype correlation has been found.

Cytogenetic analysis using solid staining or C-banding (see Chap. 4) to detect chromosome abnormalities involving the heterochromatic regions has been used as a diagnostic test for RS. Premature centromere separation (PCS; a phenomenon of chromosome centromeres separating during metaphase instead of anaphase) and heterochromatin repulsion (HR; centromere splitting and puffing of heterochromatic regions near centromeres), particularly of chromosomes 1, 9, and 16, are commonly seen in metaphases of 80% of RS patients (Fig. 14.5). Other cytogenetic abnormalities such as aneuploidy with random chromosome loss, micronuclei, and abnormal nuclear morphology are also observed. Barbosa et al. demonstrated asynchronous replication of homologous alpha-satellite DNA that was more evident in chromosomes 1, 9, and 16, in cells from RS patients [49]. This asynchrony, in turn, prevents the establishment of proper cohesion between sister chromatid heterochromatin,

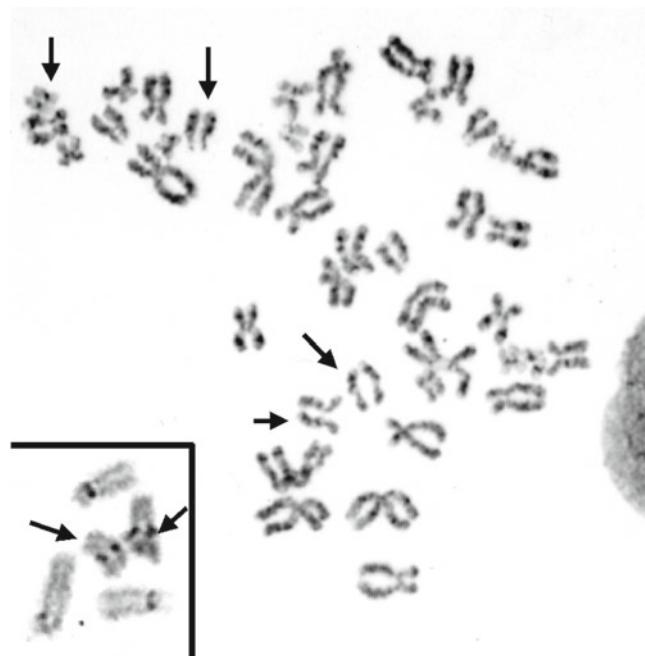


Fig. 14.5 G-banded and C-banded (insert) image of cells from a patient with Robert syndrome, demonstrating premature centromere separation (arrows) (Photo courtesy of Dr. Mazin Qumsiyeh)

leading to chromosome lag and aneuploidy. RS has been designated as a human mitotic mutation syndrome that leads to secondary developmental defects.

Clinical diagnostic criteria for RS were published by Vega et al. [50]. Cytogenetic analysis or a molecular assay to identify *ESCO2* mutations are required for confirmation of the diagnosis [51]. Prenatal diagnosis requires an ultrasound examination in combination with cytogenetic testing or prior identification of an *ESCO2* mutation in the family. Cytogenetic testing is uninformative for carrier status.

Werner Syndrome

Werner syndrome (WS) is a human premature aging syndrome manifested by scleroderma-like skin changes, especially in the extremities, and cancer predisposition. Individuals with WS generally show normal development until the end of the first decade. Lack of growth, graying and/or thinning of scalp hair, and scleroderma-like skin changes begin to manifest in the teens and 20s. Wizened and prematurely aged faces, described as “birdlike,” are often observed in individuals with WS (Fig. 14.6).

The most consistent feature of WS is bilateral cataracts. Variable features include diabetes mellitus, hypogonadism, osteoporosis and atherosclerosis, and an increased incidence of neoplasia. Malignant sarcomas, meningiomas, and carcinomas are seen in approximately 10% of WS patients; cancer

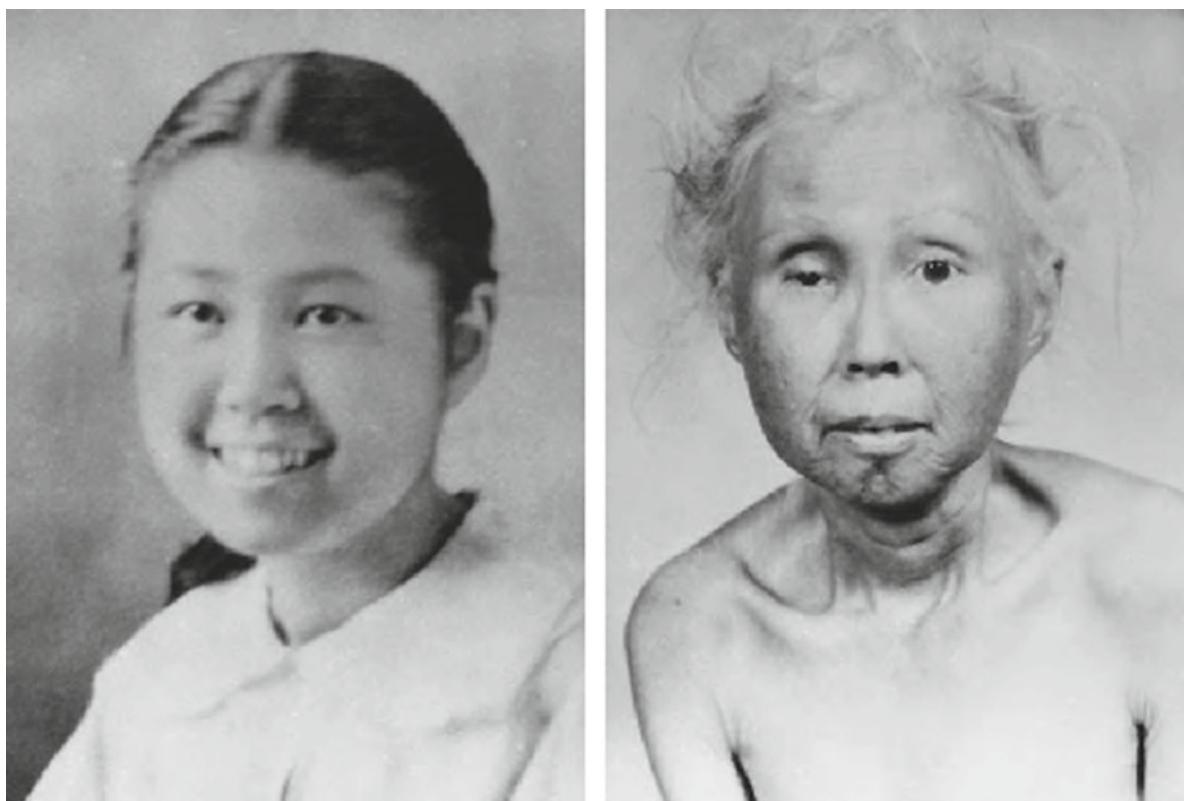


Fig. 14.6 A Werner syndrome patient at ages 15 and 48 years of age (Reprinted with permission from Epstein et al. et al. [52]. Photo courtesy of Nancy Hanson)

is the leading cause of death in these patients. The prevalence of WS varies with the level of consanguinity, ranging from one in 20,000–40,000 in the Japanese population to an estimated one in 200,000 in the US population. The carriers frequency is reported to be as high as one in 150–200 [53].

The frequency of spontaneous chromosome damage in WS is not as striking as in other chromosome instability syndromes. A variety of somatic chromosome rearrangements, including translocations, inversions, and deletions, were noted in lymphocytes and cultured skin fibroblasts from WS patients. Variegated translocation mosaicism (VTM), used to designate apparently balanced translocations, not numerical rearrangements, has been observed in WS cells. In addition, skin fibroblast lines established from WS patients have a diminished *in vitro* life span. WS cells usually achieve only about 20 population doublings, in contrast with the approximately 60 doublings seen in normal control cells. Studies of cultured cells from an obligate heterozygote revealed that these cells exited the cell cycle at a faster rate than did normal cells. Wyllie et al. demonstrated that forced expression of telomerase in WS fibroblasts confers extended cellular life span. Telomerase activity and telomere extension are sufficient to prevent accelerated cell aging in WS fibroblast cultures [54].

The gene responsible for WS, *WRN* at 8p12, which contains a total of 35 exons, was identified by positional cloning

[55]. *WRN* is a DNA helicase belonging to the RecQ family and is an exonuclease that participates in the pathways of DNA repair, recombination, transcription, and replication. Loss of *WRN* function may promote genetic instability and disease via recombination-initiated mitotic arrest, cell death, or gene rearrangement. Crabbe et al. proposed that lack of *WRN* helicase activity can result in dramatic telomere loss, which leads to chromosome fusion and breakage. They demonstrated that telomere elongation by telomerase significantly reduced the appearance of new chromosomal aberrations in cells lacking the *WRN* helicase, similar to complementation of Werner syndrome cells with the *WRN* helicase [56].

Mutations of the *WRN* gene have been identified in approximately 90% of affected WS individuals, and these mutations are located at different sites across the coding region. All *WRN* mutations found to date either create stop codons or cause frameshifts that lead to premature termination and complete loss of function of the *WRN* gene product. No missense mutations have been identified. A splice-junction mutation is found in 50–60% of Japanese WS patients; no genotype–phenotype correlation has been observed [57]. Following the identification of mutations by sequencing analysis, a western blot has been developed to demonstrate the absence of WS protein in the majority of affected patients.

Bloom Syndrome (BS)

Bloom syndrome (BS) is a rare genetic disorder characterized clinically by severe pre- and postnatal growth restriction, proportionately short stature, sun sensitivity, erythematous facial skin lesions, immunodeficiency, and increased predisposition to cancer. Although BS occurs in many ethnic groups and a single complementation group exists among patients of diverse ethnic origin, Ashkenazi Jews have a significantly higher incidence, with a gene frequency estimated to be one in 110.

Genomic instability is manifested by formation of quadri-radial configurations of symmetric shape with centromeres in opposite arms, seen in approximately 1–2% in cultured lymphocytes from BS patients (versus none in controls). These rearrangements occur before mitosis and are a consequence of an equal exchange of chromatid segments near the centromeres of two homologous chromosomes.

The most characteristic and consistent cytogenetic feature of BS is the greatly elevated (~10-fold) level of sister chromatid exchange (SCE; a reciprocal exchange of homologous segments between the two sister chromatids of a chromosome) in various cell types, including lymphocytes, fibroblasts, and bone marrow cells in affected individuals [58] (Fig. 14.7). Increased SCE still represents the most distinctive cytogenetic diagnostic marker for BS.

BS arises from mutations in *BLM* at 15q26.1, a gene encoding a protein with RecQ helicase function, and *BLM* is the only gene yet identified as causing BS. Hyper-recombination in BS is explained by a model in which *BLM* disrupts potentially recombinogenic molecules that arise at sites of stalled replication forks, promoting branch migration

at the Holliday junction [60]. Gruber et al. demonstrated that carriers of a *BLM* mutation are at increased risk for colorectal cancer [61]. Mutation analysis indicates that the cause of most BS is the loss of enzymatic activity of the *BLM* gene product.

Multiple *BLM* mutations have been identified. A specific 6-bp deletion/7-bp insertion at position 2,281 in exon 10 of *BLM*, 2281del6ins7, often designated *BLM*^{Ash}, was identified in 98% of affected individuals and in ~1% of unaffected individuals of Ashkenazi Jewish origin [62]. A PCR-based mutation analysis is available for the Ashkenazi Jewish population. Sequence analysis of the entire coding region of *BLM* is required to detect other mutations.

The clinical diagnosis of BS is confirmed either by demonstrating a quadriradial formation in cultured lymphocytes or a highly increased rate of sister chromatid exchange in cultured cells of any type. The diagnosis can also be confirmed by *BLM* mutation analysis.

Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) is an autosomal recessive disorder. Sensitivity to sunlight and the tendency to develop skin cancer at an early age are the key features of XP. The initial symptoms in most affected individuals are an abnormal reaction to sun exposure, including severe sunburn with blistering and persistent erythema after minimal sun exposure. Freckling in exposed areas occurs by two years of age in most patients.

Neoplasms are predominantly basal cell or squamous cell carcinomas and malignant melanomas. Approximately 90% of squamous and basal cell carcinomas appear in the regions

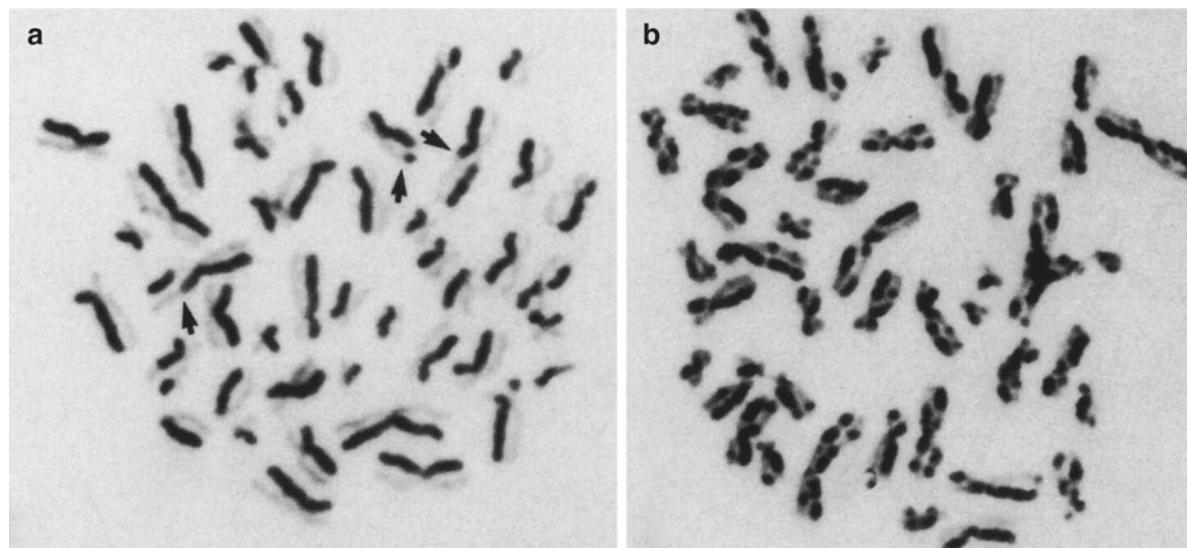


Fig. 14.7 Sister chromatid exchange (SCE). (a) Two SCEs in a normal cell (arrows). (b) Multiple SCEs in a cell from a patient with Bloom syndrome (Reprinted from Gardner and Sutherland [59]. By permission of Oxford University Press, Inc)



Fig. 14.8 *Above:* Lesions of the face in an XP patient. Note multiple scars of carcinomas and an aged aspect of the skin. *Below:* multiple basocellular carcinomas on the face of an XP patient. Thick arrow points to a recent lesion and thin arrow to a scar of an old lesion (Reprinted from Viguié [63]. Image courtesy of Daniel Wallach and used with permission of the Atlas of Genetics and Cytogenetics in Oncology and Haematology [63])

of greatest sunlight exposure, such as the face, head, and neck. The median age of onset of the first skin cancer is 8 years, nearly 50 years younger than that in the general population in the United States. The rate of skin cancer in XP patients is nearly 2,000 times higher than in the general population under 20 years of age (Fig. 14.8).

Ocular involvement is also common, and as a result, XP patients tend to develop photophobia. Abnormalities are restricted to sun-exposed anterior portions of the eye. Approximately 30% of XP patients also show progressive neurologic degeneration including microcephaly, diminished deep tendon stretch reflexes, sensorineural deafness, and cognitive impairment.

Although the disorder is transmitted in an autosomal recessive manner, heterozygous carriers may be predisposed to skin cancers. Swift et al. reported that in 31 families of XP patients, blood relatives had a significantly higher frequency of nonmelanoma skin cancer than their spouses [64].

XP has been found in all races, with a frequency of approximately 1 in one million in the United States and considerably higher in Japan and North Africa. Consanguinity has been reported in nearly 30% of cases. Seven complementation groups (XPA to XPG) have been identified in one class of XP patients with defective excision of pyrimidine dimers (excision-deficient XP). A xeroderma pigmentosum variant (XPV) with impaired replication of damaged DNA has also been identified. Groups A and C are the most common forms worldwide, XPD and XPF have intermediate frequency, group F has exclusively been described in Japan. XPA, XPB, XPD, and XPG are associated with neurological disorders, such as progressive mental deterioration, abnormal motor activity, hearing loss, deafness, and primary neuronal degeneration. The lowest level of DNA repair is found in patients from group A. This may explain the clinical severity involving both the skin and central nervous system seen in these patients.

Elevated spontaneous chromosome breakage, a cytogenetic hallmark for some chromosome instability syndromes, is not seen in XP. However, an increased rate of sister chromatid exchange and chromosome aberrations after exposure to ultraviolet light and chemical carcinogens has been reported [65].

UV sensitivity in the form of deficient DNA repair is the primary cellular feature of XP. Cells from XP patients lack the ability to repair DNA damage by inserting new bases into damaged DNA after UV irradiation. Colony-forming ability after UV irradiation, as visualized under the microscope, can be used as an *in vitro* sensitivity test for XP. Nucleotide excision and repair (NER)-deficient XP fibroblasts are more sensitive than normal cells, and those from patients who have neurological defects generally exhibit the highest sensitivity. Fibroblasts from patients with defects in XPV do not show a significant increase of UV sensitivity under standard test conditions, but a dramatically increased sensitivity is seen when XPV fibroblasts are incubated with caffeine after UV exposure. Measurement of UV-induced unscheduled DNA synthesis (UDS) is required for a definitive diagnosis of NER-deficient XP. Carrier detection and prenatal diagnosis are possible if an unequivocal NER defect or the responsible mutation in the family has been characterized.

All XP genes have been cloned, and all reside on different chromosomes. Some have the same names as the complementation groups they are associated with (e.g., *XPA* and *XPC*), while others have excision repair cross-complementing (*ERCC2*, 3, 4, and 5) or damage-specific DNA binding protein names (e.g., *DDB1*). With the exception of XPV, the products of the XP genes are all involved in different steps of the NER system, a major cellular defense against the carcinogenic effects of UV exposure [66]. Cockayne syndrome and the photosensitive form of trichothiodystrophy, two other NER-deficiency syndromes, should be considered in any differential diagnosis due to the common feature of extreme sensitivity to sunlight shared by these disorders.

The diagnosis of XP is made clinically based on skin, eye, and neurologic manifestations. Sequencing analysis for *XPA* and *XPC* mutations is clinically available, and molecular analysis for the remainders is still considered as research testing. Successful treatment for XP using a topical DNA repair enzyme has been reported [67].

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Part IV

Cancer Cytogenetics

Aurelia Meloni-Ehrig

Introduction

The knowledge that cancer is a malignant form of uncontrolled growth has existed for over a century. Several biological, chemical, and physical agents have been implicated in cancer causation. However, the mechanisms responsible for this uninhibited proliferation, following the initial insult(s), are still object of intense investigation.

The first documented studies of cancer were performed over a century ago on domestic animals. At that time, the lack of both theoretical and technological knowledge impaired the formulations of conclusions about cancer, other than the visible presence of new growth, thus the term *neoplasm* (from the Greek *neo*=new and *plasma*=growth). In the early 1900s, the fundamental role of chromosomes in heredity and reproduction was already valued by a number of biologists. During that period, the most comprehensive view of the role played by chromosomes in heredity was held by Boveri and Sutton, who independently theorized that it was necessary to have all chromosomes present in the cells for proper embryonic development to take place [1, 2]. This innovative concept was later applied to the origin of tumor cells by Boveri himself. Although he never experimented with tumors, Boveri obviously sensed that tumors began from a single cell in which defects in the chromosome makeup led cells to divide uncontrollably. He formulated his theories in the book *Zur Frage der Entstehung maligner Tumoren* (*On the Problem of Origin of Malignant Tumors*), published in 1914 [3]. This book is probably the most important early contribution on the genetics of cancer, as it offered some of the concepts still applicable today, specifically that chromosome imbalances, mitotic disturbance, and monoclonality are all attributes found in cancer cells. The thought

that errors during cell division were the basis for neoplastic growth was most likely the determining factor that inspired early researchers to take a better look at the genetics of the cell itself. Thus, the need to have cell preparations good enough to be able to understand the mechanism of cell division became of critical importance.

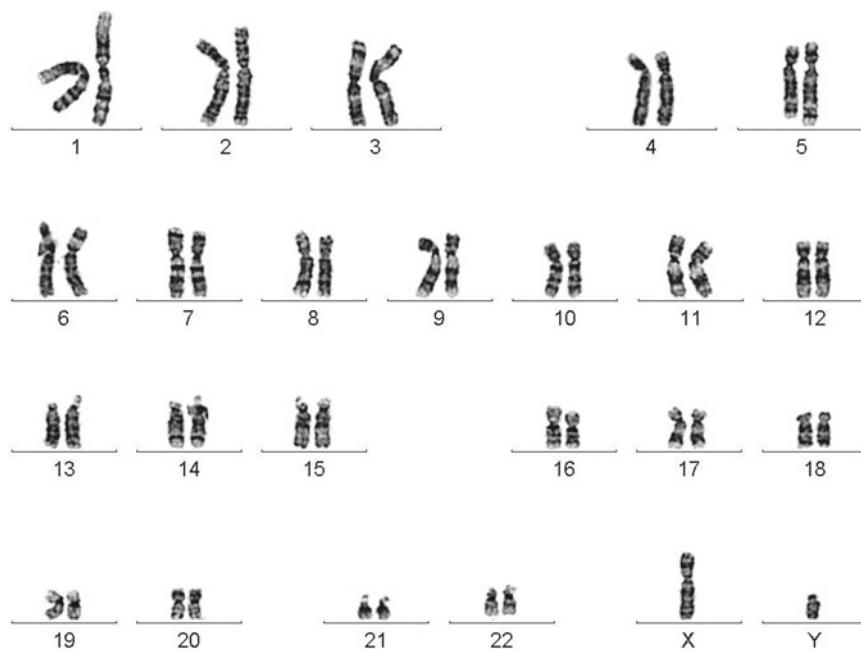
About 50 years after Boveri's chromosome theory, the first manuscripts on the chromosome makeup in normal human cells and in genetic disorders started to appear, followed by those describing chromosome changes in neoplastic cells. A milestone of this investigation occurred in 1960 with the publication of the first article by Nowell and Hungerford on the association of chronic myelogenous leukemia with a small size chromosome, known today as the Philadelphia (Ph) chromosome, to honor the city where it was discovered (see also Chap. 1) [4]. This finding stimulated subsequent research on chromosome aberrations in human neoplasms that still continues to augment our understanding about cancer. This chapter will focus on the visibly recognizable chromosome abnormalities in human hematologic neoplasms and their implication in diagnosis, prognosis, and therapeutic strategies.

Cytogenetic Methods for Diagnosis of Hematologic Neoplasms

Cytogenetics requires the presence of live cells or at least intact nuclei (for FISH studies; see Chap. 17). Although it is understood that human cancer cells divide spontaneously and that culturing might not be a necessary step, it is also true that neoplastic cells are regulated by different growth cycles, and therefore longer times in culture, as well as mitogen stimulation (in the case of mature lymphoid neoplasms), may be beneficial [5–8]. Cytogenetics starts with proper sample collection, which in the case of hematologic neoplasms includes bone marrow aspirate, peripheral blood, as well as various body fluids and solid tissues in which infiltration by the neoplastic hematologic cells has occurred [5, 9].

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Fig. 15.1 G-banded karyogram of a normal bone marrow cell



Collection of samples should be performed aseptically, and in the case of solid tissues, samples should be placed in a room temperature medium, preferably enriched with growth factors and antibiotics. Longer transit times (>48 h) might affect the viability of the neoplastic cells and should be avoided when possible [5]. Analyzable chromosome preparations are obtained by first exposing the cells to mitotic inhibitors and subsequently treating them with hypotonic solution and fixation [10, 11]. Chromosome preparations are then subjected to banding techniques, the most widespread of which is the trypsin-Giemsa banding method [12, 13]. See Fig. 15.1; see also Chap. 4. The terms used in cancer cytogenetics are listed in Table 15.1, and the karyotypes are described according to *An International System for Human Cytogenetic Nomenclature* (the most recent version appeared in 2009; see also Chap. 3) [14].

Importance of Conventional Cytogenetics in the Diagnosis and Prognosis of Hematologic Neoplasms

There is no question that the development of sophisticated techniques such as fluorescence *in situ* hybridization (FISH), multicolor karyotyping (M-FISH, SKY), and, to some extent, array comparative genomic hybridization (array CGH) has enhanced the knowledge of chromosome abnormalities in hematologic neoplasms [15–23] (see also Chaps. 17 and 18). These techniques have immensely contributed to the discovery of significant cryptic rearrangements as well as to the detection of such rearrangements in nondividing cells of

various tissue preparations. Their invention was seen as a potential competitor to conventional cytogenetics, due to their higher resolution. Nevertheless, several years after the introduction of these sophisticated technologies, conventional cytogenetic analysis is still the best method for the diagnosis of most hematologic neoplasms since it has the advantage of an overall examination of all chromosomes, compared to the more focused detection of abnormalities with the other molecular genetic methods. Undisputed, in fact, is the ability of conventional cytogenetics to identify related and distinct clonal populations, which is challenging for FISH and practically impossible for array CGH [24, 25]. Furthermore, the presence of abnormalities acquired during clonal evolution, an important indicator of disease progression, might be missed during a targeted FISH analysis [26–29].

Chromosome Abnormalities in Hematologic Neoplasms

Cytogenetics began in 1956, when Tijo and Levan, and soon after them Ford and Hamerton declared that normal human cells contained 46 chromosomes and not 48, as previously believed (see Chap. 1) [30, 31]. From that point on, experimental work on cell cultures and banding was geared to the improvement of chromosome spreading and morphology and was presented in subsequent publications [4, 32]. It was the detection of the Philadelphia chromosome by Nowell and Hungerford, however, that definitively established that chromosome abnormalities in leukemia are acquired and as such they are present exclusively in the neoplastic cells [4]. But it

Table 15.1 Glossary of cytogenetics terminology used in this chapter

Acentric fragment	A chromosome fragment lacking a centromere and therefore incapable of attaching to the spindle. Acentric chromosomes are distributed randomly among daughter cells
Aneuploidy	Deviation of the chromosome number that is characteristic for a particular species caused by either gain or loss of one or more chromosomes
Autosome	Any chromosome other than the sex chromosomes
Banding	Alternating intrachromosomal light and dark segments along the length of chromosomes
Breakpoint	Specific band on a chromosome containing a break in the DNA as the result of a chromosome rearrangement
Centromere	An area of chromosomal constriction that holds the two chromatids together and is needed for spindle site attachment. Based on the position of the centromere, chromosomes are classified as metacentric (middle position), submetacentric (above the middle), and acrocentric (extremely small short arm consisting of satellites and stalks)
Chromosome	Arrangement of nuclear genetic material into formations containing a centromere and two chromosome arms. The normal chromosome number in human somatic cells is 46, whereas in germ cells it is 23
Chromosome rearrangement	Structural aberration in which chromosomes are broken and rejoined. These rearrangements can occur on a single chromosome or involve multiple chromosomes
Clonal evolution	A stepwise evolution characterized by the acquisition of new cytogenetic abnormalities
Cytogenetics	The examination of chromosomes
Deletion	Loss of a chromosome segment. Deletions can either be terminal or interstitial
Dicentric	A chromosome containing two centromeres
Diploid	Normal chromosome complement (two copies of each autosome and two sex chromosomes) in somatic cells
Double minute	Cytogenetic visualization of gene amplification. So called because of their appearance as two adjacent dots. Each double minute is thought to contain hundreds of copies of a particular oncogene
Duplication	Two copies of the same segment present on a single chromosome
Haploid	Half (i.e., 23 chromosomes) of the normal human chromosome complement in somatic cells. This is the number of chromosomes present in normal germ cells
Homogeneously staining region	Cytogenetic visualization of gene amplification. Multiple copies of a particular oncogene are inserted into one of more chromosome region giving the appearance of a uniform staining
Hybrid gene	Fusion of two different genes as a result of a structural chromosomal rearrangement. A hybrid gene leads to a hybrid protein with abnormal function
Hyperdiploid	Gain of one or more chromosomes
Hypodiploid	Loss of one or more chromosomes
Idiogram	Diagrammatic representation of a partial or complete karyogram
Insertion	Balanced or unbalanced relocation of chromosomal material into a different or the same chromosome
Inversion	Structural rearrangement affecting a single chromosome. This is generated by a 180° rotation of a segment included between 2 breaks along a single chromosome. Inversions can be paracentric (breaks involving a single arm) or pericentric (breaks involving both arms)
ISCN	Suggested guidelines of An International System of Human Cytogenetic Nomenclature used for the description of karyotypes
Isochromosome	Structural rearrangement affecting a single chromosome generated by the misdivision of the centromere in transverse plane resulting in loss of one arm and duplication of the other
Karyogram	Arrangement of metaphase chromosomes according to size, position of centromere, and banding patterns
Karyotype	Description of the chromosome complement according to ISCN guidelines
Locus	Location of a particular gene on a chromosome
Marker chromosome	Chromosome whose origin cannot be identified using standard banding methods
Metaphase	Arrangement of chromosomes in one plane at the equator of the cell. This phase of mitosis is characterized by the disappearance of the nuclear membrane and appearance of the spindle with subsequent attachment of the centromeres to the spindle
Monosomy	The absence of one member of a homologous pair of chromosomes
Oncogene	Gene that promotes cell growth and development. One abnormal allele is sufficient to cause uncontrolled growth and lead to tumor formation
Polypliod	A cell containing a multiple of the haploid chromosome complement
Pseudodiploid	Approximate diploid number of chromosomes, often accompanied by structural rearrangements
Recurrent abnormality	Structural rearrangement or numerical abnormality detected in multiple patients with the same or similar disease
Ring chromosome	A circular formation of a chromosome originating from two breaks on opposite arms and reunion of the broken ends

(continued)

Table 15.1 (continued)

Sex chromosomes	The X and the Y chromosomes. With some exceptions, XX is observed in females and XY in males
Translocation	Chromosome abnormality resulting from a break in two or more chromosomes and exchange of the material distal to the breaks. In a balanced translocation, there is exchange but no loss of DNA, whereas in an unbalanced translocation there is gain or loss of DNA. With unbalanced translocations, abnormal chromosomes are referred to as derivatives if the exchanged material is known. The term add is used if the origin of the exchanged material cannot be identified
Trisomy	Three copies of a chromosome
Tumor suppressor gene	Locus that inhibits tumor growth when at least one allele is functional. Loss of both alleles is associated with tumor growth

was not until the middle 1970s that reports of cytogenetic abnormalities in cancer started to increasingly populate the scientific literature [33–35]. Today, a complete list of these abnormalities can be found in *Mitelman Database of Chromosome Aberrations in Cancer* [36]. It is immediately evident from consulting Mitelman's database that the most common rearrangements in hematologic neoplasms are balanced translocations [37–39]. In the majority of cases, translocations represent the sole abnormality, whereas in other cases, they are identified during disease progression [40–44]. The significance of a primary translocation versus a later-appearing abnormality differs, the latter usually suggestive of a more aggressive clinical course. Similarly, the significance of the same translocation in *de novo* and treatment-related hematologic neoplasms differs, with the latter, again, carrying a worse prognostic outcome and, in some cases, a greater resistance to therapy [45]. Balanced translocations are often the sole abnormality in the majority of acute and chronic myeloid leukemias and in a large number of acute and mature lymphoid neoplasms [46, 47]. It is interesting to note that the product of a translocation in leukemia is almost always a hybrid protein with abnormal function, whereas in lymphoma no hybrid protein is produced [48–50]. In lymphoma, the relocation of an oncogene to a site under the control of an immunoglobulin promoter often leads to overproduction of a protein with oncogenic activity [51–53]. Translocations appear to be less frequent in myelodysplastic syndromes and classical myeloproliferative neoplasms where partial or full unbalances, leading to loss of tumor suppressor genes and/or gain of oncogenes, dominate [54–60]. Apart from balanced translocations, practically every abnormality known today has been observed in hematologic neoplasms, including ring chromosomes, double minutes (dmin), and homogeneously staining regions (hsr), which for some time were considered to be present exclusively in solid tumors [61–66]. The specificity and recurrence of chromosome abnormalities in hematologic neoplasms have gained significance to the point that the latest version of the World Health Organization (WHO) guidelines focuses intensively on the genetic and cytogenetic features of hematologic neoplasms as predictors of diagnostic and prognostic outcome [67].

Myeloid Neoplasms

The classification of myeloid neoplasms has recently been modified [67]. This reclassification more than ever before takes into account the genetic and cytogenetic changes associated with these neoplasms. Consequently, neoplasms with similar morphologic and genetic features have been grouped together. The myeloid neoplasms include the myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs), MDS/MPN, and acute myeloid leukemias. These are described in more detail in the following sections.

Myelodysplastic Syndromes

The term myelodysplastic syndrome (MDS) refers to a fairly heterogeneous group of hematopoietic stem cell neoplasms characterized by a series of similar features such as dysplastic cellular morphology, defect in cellular maturation, and increased risk of transformation into acute myeloid leukemia (AML) via a multistep process [68, 69]. MDS is rare in children as it makes up approximately 5% of the pediatric hematologic neoplasms. MDS occurs mainly in adults with a median age of 70 years, and although there is a risk for developing AML, about 50% of deaths occur as a result of unrelated causes, such as bleeding or infection [70].

There are two main types of MDS: primary or *de novo* MDS, and secondary or therapy-related MDS. Although secondary MDS occurs as a result of treatment with radiation and/or alkylating agents or treatment with DNA topoisomerase inhibitors for an unrelated malignancy, the initial insults leading to the development of primary MDS are still being debated. Some of the possible triggers include exposure to radiation, tobacco, and benzene.

Classification of MDS

Cytogenetic studies, which are routinely performed in patients with these neoplasms, are useful since chromosome abnormalities provide both diagnostic and prognostic information [57, 58, 70]. Table 15.2 describes the subdivision of

Table 15.2 Subdivision of MDS neoplasms according to the 2008 WHO classification and percent of chromosome abnormalities in each category

Neoplasm	Marrow blasts (%)	Cytogenetics (%)
RCUD	<1	25–50
RARS	<5	5–20
RCMD	<5	50
RAEB-1	5–9	50–70
RAEB-2	10–19	50–70
MDS-U	<5	50
MDS with isolated del(5q)	<5	100
Childhood MDS	5–10	5–10
t-MDS	<5	90

Abbreviations: *RCUD* refractory cytopenia with unilineage dysplasia, *RARS* refractory anemia with ring sideroblasts, *RCMD* refractory cytopenia with multilineage dysplasia, *RAEB-1* refractory anemia with excess blasts-1, *RAEB-2* refractory anemia with excess blasts-2, *MDS-U* myelodysplastic syndrome—unclassifiable, *t-MDS* therapy-related MDS

MDS neoplasms according to the 2008 World Health Organization classification [67]. Chromosome abnormalities have been observed in approximately 50% of patients with *de novo* MDS and in as many as 90% of patients with therapy-related MDS. There appears to be a correlation between the frequency of chromosomal abnormalities and the severity of disease, and this is evident in this Table [57, 69]. About 25% of patients with low-grade MDS, such as refractory anemia and refractory anemia with ring sideroblasts, have an abnormal karyotype, compared with 50–70% of patients with refractory anemia with excess blasts (RAEB-1 and RAEB-2). The karyotypes observed in MDS are variable as they present with single or complex chromosome rearrangements [56–58].

Chromosome Abnormalities in MDS

The most frequent chromosome abnormalities are complete or partial loss of chromosomes 5 and/or 7, deletions on the long arm of chromosome 20, and gain of chromosome 8 [36, 37, 71–75] (see Table 15.3 and Fig. 15.2). In general, aggressive neoplasms are characterized by more complex karyotypes than those seen in low-grade MDS. Furthermore, as a general rule, dosage aberrations appear to be more represented in primary MDS, whereas balanced translocations are encountered more frequently in secondary MDS [56] (see Table 15.3). Among therapy-related MDSs, complex karyotypes with loss/deletion of chromosomes 5 and/or 7 together with deletions of 6p, 12p, and/or 16q are typical of alkylating agent-induced MDS, whereas balanced translocations involving 11q23 (*MLL*) and 21q22.3 (*RUNX1*) are associated with preceding therapy with DNA topoisomerase II inhibitors [76–79].

Table 15.3 Recurrent chromosome abnormalities in primary MDS and t-MDS

Abnormality	Primary MDS	t-MDS	Fig. 15.2
+1/+1q	+		a
der(1;7)(q10;p10)	+	++	b
del(3p)	+		c
3q21.3 rearrangements	+		d
3q26.2 rearrangements	+		d
-5/del(5q)	+	++	e
+6	+		—
del(6p)	+	++	f
-7/del(7q)	+	++	g
t(7;12)(q36.3;p13.2)	+		h
+8	++	+	—
del(9q)	+		i
+10	+		—
+11	+	++	—
del(11q)	+		j
11p15.4 rearrangements		+	k
11q23 rearrangements		+	l
12p13 rearrangements	+	++	m
+13	+		n
del(13q)	+		—
+14	+		—
+15	+		—
del(15q)	+		o
del(16q)	+		p
t(16;21)(q24.3;q22.3)	+		q
del(17p)	+	++	r
dic(17;20)(p11.2;q11.2)	+	++	s
i(17)(q10)	+		t
+19	+		—
del(20q)	+		u
ider(20)(q10)del(20q)	+		v
-21	+		—
i(21)(q10)	+		w
idic(X)(q13)	+		x

+ indicates the presence of an abnormality and ++ indicates increased frequency

MDS with Deletion of 5q

The significance of del(5q) in MDS has to take into account not only the presence of this abnormality but also the associated morphologic picture [72, 75, 80]. The size of the deleted portion of the long arm of chromosome 5 is highly variable. The critical deleted region is approximately 1.5 Mb in size and is located at 5q31.2, where the *EGFR* gene is located [75].

del(5q) can be associated with the so-called 5q– syndrome. In this hematologic syndrome, patients present with refractory macrocytic anemia and demonstrate hypolobulated micromegakaryocytic hyperplasia in the marrow [73, 80]. A female predominance has been noted (sex ratio: 1M/3F). The clinical course is said to be relatively



Fig. 15.2 Partial karyograms of recurrent abnormalities in MDS (Refer to Table 15.3 for additional information on the various rearrangements illustrated in this figure)

indolent, with a very low-risk of developing acute leukemia. In the International Prognostic Scoring System (IPSS), del(5q) MDS patients are placed in the most favorable prognostic category [81, 82]. About 15% of patients do not fit into this category but still have a del(5q) as the sole abnormality [83, 84]. These cases do not appear to have the same favorable prognostic outcome, demonstrating the importance of the specific deletion for the prognosis and response to therapy [58, 85]. Similarly, del(5q) together with other abnormalities is no longer associated

with the most favorable prognostic outcome that is typically seen in patients with 5q- syndrome. Deletion 5q and/or complete loss of chromosome 5 in the context of a complex karyotype is frequently seen in high-grade as well as therapy-related MDS [63, 65]. Here, deletions of 5q might be derived from unbalanced translocations with a variety of chromosome regions. The most common of these is a dic(5;17)(q11.2;p11.2), a result of which is loss of TP53 at 17p13.1, a marker of poor prognostic outcome in numerous neoplasms [86].

MDS with Deletion of 7q or Monosomy 7

Deletion of 7q/monosomy 7 has been viewed as a marker of poor prognostic outcome [56, 57, 81]. However, the 2007 prognostic score criteria places patients with this rearrangement in an intermediate risk [82]. As a sole abnormality, del(7q) occurs in approximately 1% of cases [87]. Three regions are most frequently deleted: 7q22, 7q31.1, and 7q31.3 [88, 89]. Some studies indicate that retention of band 7q31 may be found in patients with longer survival, suggesting that 7q31 might be the location of a tumor suppression gene [89, 90]. More often, del(7q) or -7 occurs as part of a complex karyotype (approximately 5–10% of cases), characterized by recurrent abnormalities that include one or more of the following: rearrangements of chromosome 3, -5/del(5q), del(6p), +8, +9, del(9q), del(11q), del(12p), del(17p), +19, del(20q), +21 [56]. Monosomy 7 as a sole abnormality is seen in pediatric patients with all MDS subtypes as well as in juvenile myelomonocytic leukemia (JMML) [91]. Loss of chromosome 7 is also seen in siblings with the so-called -7 syndrome and a predisposition to develop juvenile MDS [92, 93]. Literature shows that the lost chromosome 7 can come from either parent, suggesting that some other genetic defect that predisposes these children to lose one chromosome 7 is at the origin of this phenomenon [94, 95].

MDS with Trisomy 8

Gain of one copy of chromosome 8 is recurrent in all myeloid neoplasms. In MDS, it is found in over 10% of patients [96–98]. According to both the old and new prognostic scoring systems for MDS, trisomy 8 is associated an intermediate risk when detected as the sole abnormality [97]. The presence of additional abnormalities generally worsens the prognostic outcome [96]. Trisomy 8 is often present as an additional abnormality, particularly in addition to del(5q). In about 2% of cases, four copies of chromosome 8 (tetrasomy 8) might be seen. These patients are given a high prognostic risk [99].

MDS with Other Chromosome Abnormalities

Rearrangements of chromosome 3, specifically bands 3q21.3 and/or 3q26.2, occur in about 5% of cases [100]. They have been observed in *de novo* as well as therapy-related MDS, in AML, and in accelerated phase or blast crisis CML [101, 102]. The most common rearrangements include inv(3)(q21.3q26.2), t(3;3)(q21.3;q26.2), and del(3)(q21.3q26.2) [103]. Generally, these patients present with trilinear dysplasia in their bone marrow with dysmegakaryopoiesis. These rearrangements are associated with an adverse prognostic risk in both MDS and AML. This adverse prognosis probably correlates to the highly increased *MECOM* (*EVII*, at 3q26.2) expression, detectable in the vast majority of these patients [104, 105]. Some patients with the 3q21.3q26.2 rearrangement do not have detectable *MECOM* expression, suggesting that the poor prognosis in these patients may be independent of such expression [106].

Another recurrent abnormality in MDS is del(17p). This deletion, which often is observed in the context of a complex

karyotype, can be the result of various rearrangements, including simple deletions, unbalanced translocations, formation of an isochromosome, and monosomy 17 [86, 107]. Deletion of 17p is recurrent in myeloid disorders, mainly refractory anemia with excess of blasts (RAEB-1 and RAEB-2) and AML. About 30% of AML and MDS cases with 17p deletion are therapy related [108]. Deletion of 17p has been found to correlate with a particular form of morphological dysgranulopoiesis, sometimes associated with *TP53* mutation [109].

The clinical significance of sex chromosome loss in the bone marrow of patients with hematologic neoplasms is still questionable [110, 111]. Loss of the Y chromosome is observed in approximately 10% of MDS cases, but since it is seen also in males of increasing age without evidence of a hematologic neoplasm, it is generally interpreted to represent an age-related phenomenon of no clinical significance [112]. However, it is interesting to note that some elderly males with MDS and loss of the Y chromosome show the Y chromosome in their marrow cells when they achieve complete hematologic remission. Loss of an X chromosome in the bone marrow of female patients is less frequent than loss of the Y chromosome in males, and tends to be viewed as being associated with a hematologic neoplasm rather than as an age-related phenomenon [113].

An interesting association is loss of the Y chromosome together with gain of chromosome 15, which is also characteristically seen in males with increasing age [114] (Fig. 15.3). The significance of trisomy 15 with or without the loss of the Y chromosome is not fully understood. In some cases, particularly when only a few abnormal metaphase cells are present, this finding is thought to be a transient phenomenon by some but not all authors [115–117].

Apparently balanced translocations have been reported in MDS, but they appear to be less common than the unbalanced rearrangements. Chromosomes 1, 2, 3, 5, 6, 7, 13, 15, 17, 18, 19, and 20 appear to be more frequently involved [118]. Table 15.3 shows some of the most well-characterized translocations. From this Table, it is apparent that balanced translocations have been found in both *de novo* and therapy-related MDS.

Due to the variety of chromosome abnormalities reported in MDS, it is understandable that, at present, the best genetic test at diagnosis is conventional cytogenetics [119]. FISH is unquestionably useful when a limited number or no metaphase cells are available or as a follow-up tool for a patient with a known cytogenetic abnormality, but adds little to a normal conventional chromosome study based on the analysis of 20 metaphase cells [120].

Prognostic Significance of Chromosome Abnormalities in MDS

The 1997 International Prognostic Scoring System (IPSS; Table 15.4), which was constructed with data gathered from

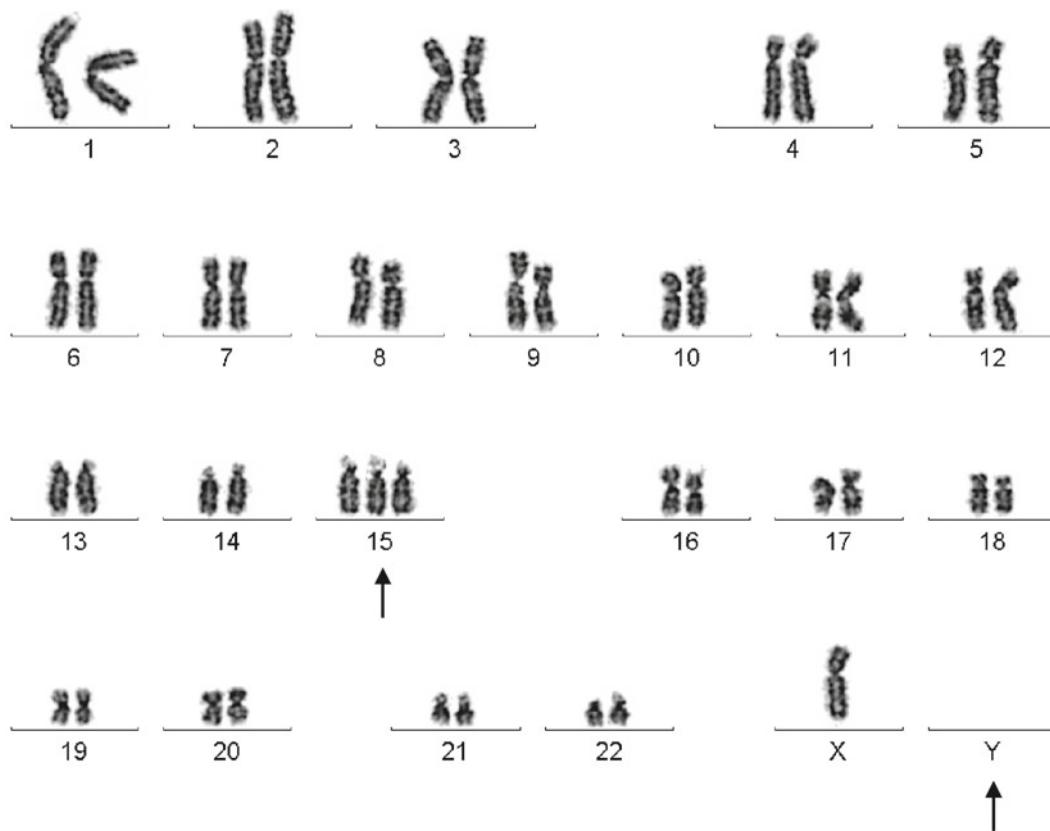


Fig. 15.3 Karyogram showing the simultaneous gain of chromosome 15 and loss of the Y chromosome (arrows). The significance of trisomy 15, particularly when present in few cells, is not clear

Table 15.4 International Prognostic Scoring System of common abnormalities in MDS [81]

Abnormalities	Risk	Median survival (mo.)
Normal, isolated – Y, isolated del(5q), isolated del(20q)	Favorable	42
Complex with ≥ 3 abnormalities, –7/del(7q)	Unfavorable	8
Other abnormalities	Intermediate	28

patients with *de novo* MDS, was revised in 2007 to also include patients who received established treatments for MDS [81, 82]. This new prognostic scoring system named IPSS-IMRAW (International MDS Risk Analysis Workshop) is a combined effort by European and American institutions and lists 22 groups of chromosome abnormalities compared with only 7 listed in the 1997 IPSS (Table 15.5). These guidelines are still a work in progress, and experts from around the world are intensively working on a more updated and satisfactory version.

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are stem cell disorders characterized by proliferation of one or more myeloid cellular

elements in the marrow and mostly affect adult individuals [121]. These neoplasms are known by different names, depending on the lineage affected. The classic MPNs include chronic myelogenous leukemia (CML), polycythemia vera (PV), primary myelofibrosis (PMF), and essential thrombocythemia (ET) [122]. Other hematologic disorders included in the MPN category are chronic eosinophilic leukemia (CEL), systemic mastocytosis, chronic neutrophilic leukemia (CNL), and the unclassifiable MPNs [67, 123, 124]. Except for CML, which is characterized by the presence of the t(9;22)(q34;q11.2)—the Philadelphia (Ph) chromosome translocation—the classic MPN exhibits similar cytogenetic abnormalities, such as gain of 1q, +8, +9, del(13q), and/or del(20q) [59, 71, 125, 126]. Two or more of these abnormalities might be present in the same karyotype (Table 15.6).

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a stem cell neoplasm that can occur at any age but is most frequent in the 5th and 6th decades of life [127, 128]. It is characterized by high white blood cell count with increased levels of granulocytes and megakaryocytes, often in the presence of eosinophilia and basophilia. CML is characterized by the t(9;22)(q34;q11.2), which leads to the formation of a chimeric

Table 15.5 Revised prognostic scoring system of common chromosome abnormalities in MDS based on the combined German-Austrian, Spanish MDS Registry, and IMRAW cohorts (IPSS-IMRAW) [82]

Abnormalities	Risk	Median survival (mo.)
Normal, -Y, isolated del(5q), del(11q), del(12p), del(20q), t(11;V)(q23;V), +21, any 2 abnormalities including del(5q)	Favorable	51
+1q, t(3q21.3;V), t(3q26.2;V), +8, t(7q;V), +19, -21, any other single abnormality, any 2 abnormalities not including 5q or 7q	Intermediate-1	29
-X, -7 or del(7q), any 2 abnormalities with -7 or del(7q), complex with 3 abnormalities	Intermediate-2	15.6
Complex with >3 abnormalities	Unfavorable	5.9
V variable translocation partners		

Table 15.6 Classification of myeloproliferative neoplasms according to the WHO, including the most common chromosome abnormalities associated with them

Neoplasm	Frequent abnormalities	Abnormalities during progression
CML	t(9;22)(q34;q11.2)	+8, i(17q), +der(22)t(9;22)
PV	+8, +9, del(20q)	-5/del(5q), -7/del(7q), del(17p)
PMF	+8, 13q-, del(20q)	+1q, -5/del(5q), -7/del(7q), del(17p)
ET	+1q, +8, +9, del(20q)	+1q, -5/del(5q), -7/del(7q)
SM	4q12 rearrangements (<i>KIT</i> mutations)	-7/del(7q), +8, +9, del(11q), del(20q)
CNL	+8, +9, del(11q), del(20q), +21	del(12p)
CEL, NOS	No specific abnormalities	Unknown
MPN, U	No recurrent abnormalities	Unknown

Abbreviations: *CML* chronic myelogenous leukemia, *PV* polycythemia vera, *PMF* primary myelofibrosis, *ET* essential thrombocythemia, *SM* systemic mastocytosis, *CNL* chronic neutrophilic leukemia, *CEL, NOS* chronic eosinophilic leukemia, not otherwise specified, *MPN, U* myeloproliferative neoplasm, unclassified

transcript between the *ABL1* and *BCR* genes at 9q34 and 22q11.2, respectively [129, 130] (Fig. 15.4). The derivative chromosome 22 is also known as the Philadelphia (Ph) chromosome and is the first abnormality to have been associated with a specific malignant neoplasm (see also Chap. 1). The Ph chromosome was described in 1960 by Nowell and Hungerford and is named after the city in which it was discovered [4].

The *BCR-ABL1* rearrangement is also the first reported example of a “hybrid” gene leading to the production of an abnormal tyrosine kinase [131, 132]. Three fusion proteins

derived from different breakpoints in the *BCR* gene are known: P210^{BCR-ABL1}, P190^{BCR-ABL1}, and P230^{BCR-ABL1}. The P210^{BCR-ABL1} is found in the majority of patients with CML and in 30% of patients with Ph-positive acute lymphoblastic leukemia (ALL); the P190^{BCR-ABL1} is found in about 20% of adults and 80% of children with ALL, in Ph-positive AML, and rarely in CML; and the rare P230^{BCR-ABL1} is found only in cases of neutrophilic-chronic myeloid leukemia (CML-N), which has been described as a CML variant associated with a more benign clinical course than classic CML [133, 134].

There are three main clinical phases of CML: chronic, accelerated, and blast crisis [135]. The chronic phase of CML is characterized by mild or no symptoms and less than 5% blasts. At this stage, the only abnormality is the t(9;22). About 6% of cases have a variant translocation due to the involvement of one or more additional chromosomes, whereas in approximately 3% of cases the translocation cannot be identified by routine cytogenetics mostly due to cryptic insertions of *ABL1* sequences from chromosome 9 into the *BCR* region on chromosome 22 or vice versa [136, 137]. These variants and cryptic rearrangements generally have the same prognostic outcome of the standard t(9;22), but some are associated with a more aggressive course. This may be due to the fact the variant translocations might be the result of one, two, or more events or they might lead to a deletion of either *BCR* or *ABL1* sequences adjacent to the translocation breakpoints. Fluorescence *in situ* hybridization (FISH) has revealed small deletions adjacent to the *ABL1* and *BCR* breakpoints in approximately 16 and 8% of cases, respectively [138] (see also Chap. 17).

Conventional cytogenetic analysis can sometimes reveal abnormalities in addition to the t(9;22). It is important to note, however, that an additional balanced rearrangement in all metaphase cells in chronic phase CML (or any neoplasm, for that matter) might be constitutional in origin. This should be investigated and removed from the equation when determined to be the case. When the abnormality in addition to the t(9;22) is obviously (or proven to be) acquired, it is indicative of clonal evolution. At the clinical level, such clonal progression is associated with the accelerated phase or blast crisis, both characterized by an increase in the number of blasts and worsening of clinical symptoms [139]. The most recurrent chromosome abnormalities (about 90% of cases) in these phases are an additional Ph chromosome, +8, i(17q10), and/or +19 [140] (Fig. 15.5). Other abnormalities, such as -Y, -7, del(7q), t(8;21)(q22;q22.3), t(15;17)(q24.1;q21.2), inv(16)(p13.1q22.1), as well as 3q21.3, 3q26.2, and 11q23 rearrangements, have been reported but only in a small number of cases.

Polycythemia Vera

Polycythemia vera (PV) is a myeloproliferative neoplasm of adults (50–60 years of age) characterized by a proliferation

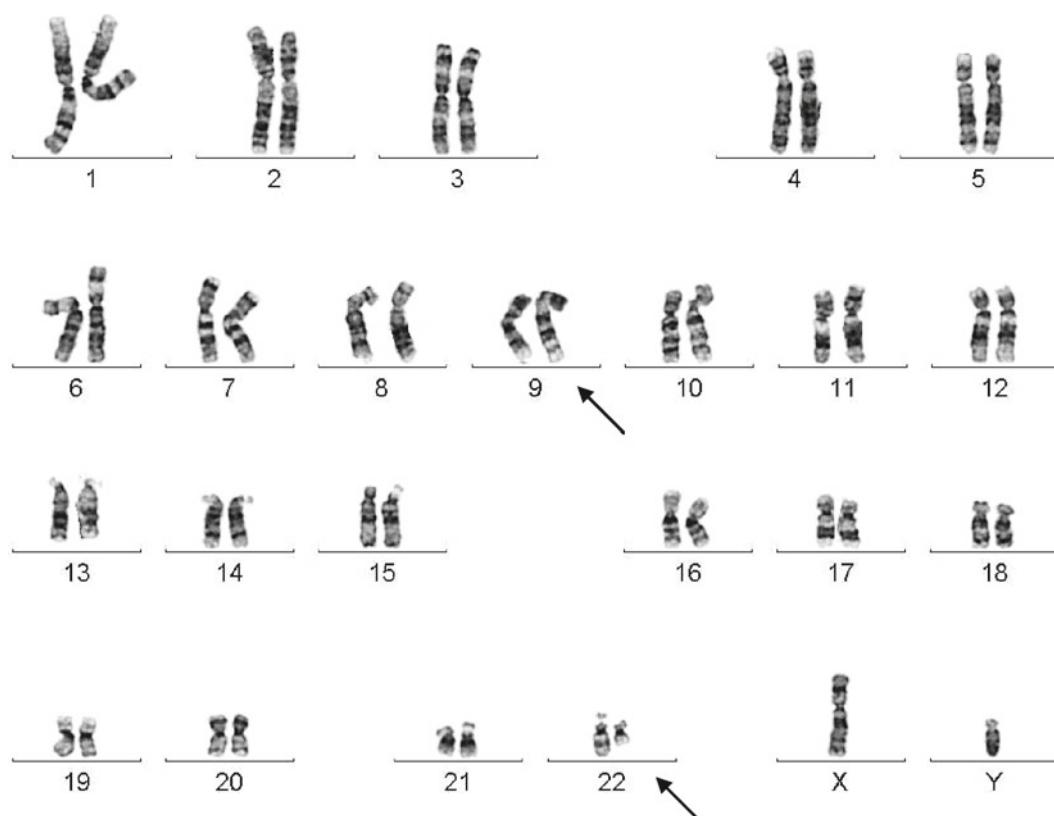


Fig. 15.4 Karyogram of a patient with CML in chronic phase. The abnormal chromosomes involved in the $t(9;22)(q34;q11.2)$ are indicated with arrows. The derivative 22 is the Philadelphia (Ph) chromosome

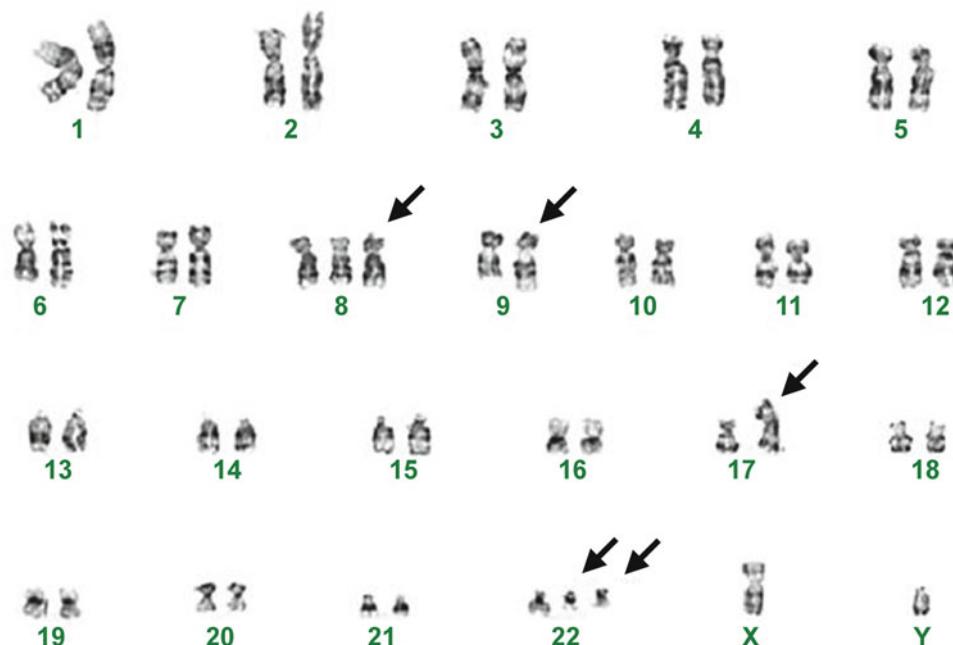
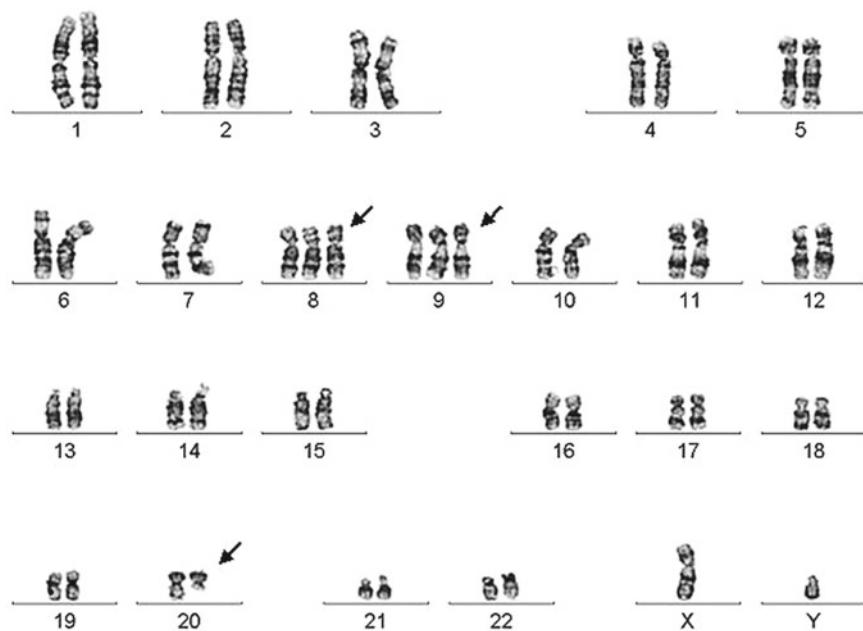


Fig. 15.5 Karyogram of a patient with CML in blast crisis. This karyogram contains the three most common additional abnormalities observed in the progressive phases of CML, specifically +8, $i(17q)$, and +Ph

Fig. 15.6 Karyogram of a patient with polycythemia vera. The three most common abnormalities are present in this karyogram, specifically +8, +9, and del(20q)



of red blood cells, which in some patients leads to bleeding and thrombosis [141]. At the chromosome level, patients are *BCR-ABL1* fusion-negative, and most, if not all, cases have a mutation at codon 617 in the Janus kinase 2 gene (*JAK2*, located at 9p24.1) that results in a substitution of phenylalanine for valine (V617F) [60, 142]. Mutations in exons 12 and 13 have also been described in patients negative for *JAK2* V617F mutations [143, 144]. Other mutations involving the *MPL*, *TET2*, and *CBL* genes have been found in some of these patients [143, 144]. These mutations are receiving increasing attention, particularly in the area of possible targeted therapy using tyrosine kinase inhibitors. About 20% of cases have an abnormal karyotype at diagnosis, mostly characterized by +8, +9/+9p, and/or del(20q) [98, 145] (Fig. 15.6). Furthermore, gain of 9p is usually the result of a derivative chromosome, the most common of which is a der(9;18) (p10;q10) [146–148]. Gain of chromosome 9 or 9p is assumed to represent a gain-of-function mechanism with respect to *JAK2* [149]. Less frequently gain of 1q, or partial trisomy 1q, might be seen. This gain is often the result of unbalanced translocations involving chromosome 1 and various chromosome regions [150]. The detection of chromosome abnormalities in PV increases as the disease progresses to MDS or AML [151]. The most common abnormalities during disease progression are del(5q), del(7q), and/or del(17p) [152, 153].

Primary Myelofibrosis

Primary myelofibrosis (PMF), also known as idiopathic myelofibrosis and agnogenic myeloid metaplasia, is characterized by marrow fibrosis with an increased number of

megakaryocytes and immature granulocytes and associated anemia. Affected patients are generally in their 5th and 6th decade of life [154, 155]. Approximately 50% of patients with PMF have the *JAK2* V617F mutation, but unlike PV, no mutations of *JAK2* other than V617F have been found. A small number of patients have mutations of other genes, particularly *MPL* [156]. At diagnosis, about 40–50% of cases show chromosome abnormalities, the most common of which are del(13q), del(20q), and gain of chromosome 8 [157, 158]. Additional abnormalities are detected during disease progression, including del(5q), del(7q), gain of 1q, and del(17p) [159].

Essential Thrombocythemia

Essential thrombocythemia (ET) is associated with an increased number of platelets and megakaryocytes, plus fibrosis in the marrow. Patients are generally asymptomatic, with about 50% presenting with circulation problems such as bleeding and thrombosis [160]. Similar to the other classic MPN, *JAK2* mutations are also detected in these patients. Approximately 50% have the characteristic *JAK2* V617F mutation found in PV and PMF, whereas another 4–5% of patients have mutations of *MPL* [161]. Only 10% of cases have chromosome abnormalities, which are similar to those seen in PV and PMF. Specifically, +8, +9, del(13q), and del(20q) are the most common, followed by gain of 1q, del(5q), and del(7q) [162]. As in other MPNs, karyotypic abnormalities are more frequent during disease progression to MDS or AML. Because ET is often a diagnosis of exclusion, some clinicians prefer to definitively rule out CML by testing for t(9;22) or a *BCR-ABL1* rearrangement in these patients when the karyotype is normal.

Chronic Eosinophilic Leukemia, Not Otherwise Specified (NOS)

Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS), is characterized by hypereosinophilia and represents a rare MPN [163]. The diagnosis is usually achieved by the exclusion of conditions that might be causing the abnormal increase of eosinophils in the marrow and blood. Two entities exist: CEL, not otherwise specified, and CEL with rearrangements involving the platelet-derived growth factor receptors (*PDGFRA* and *PDGFRB*) [123, 155]. Pertinent literature indicates that CEL should be distinguished from idiopathic hypereosinophilia by the presence of leukemic blasts. No specific abnormalities have been reported in CEL, NOS. Among the CELs with *PDGFR* rearrangements, the most common abnormality is deletion of *CHIC2* located at 4q12, which leads to a *FIP1L1-PDGFR*A fusion [164]. See later section: “Myeloid and Lymphoid Neoplasms Associated with *PDGFRA*, *PDGFRB*, and *FGFR1*.”

Systemic Mastocytosis

Patients with systemic mastocytosis (SM) present with proliferation of mast cells in the bone marrow and/or other organs [165]. Most patients are characterized by symptoms such as hepatomegaly, osteoporosis, and ascites, among others. This is a very complex disease, as it comprises several distinct entities and is also found in association with neoplasms such as MPN and leukemia [165]. The disease course can vary from indolent to aggressive. A large number of cases have rearrangements involving chromosome 4, most likely due to the fact that this disease is often associated with mutations in *KIT* located at 4q12 [166]. The most common *KIT* mutation, which results in substitution of valine for asparagine, occurs at amino acid position 816 and is thus known as D816V. This mutation leads to relative resistance to the tyrosine kinase inhibitor imatinib mesylate (Gleevec®) and therefore provides relevant information for treatment selection [167].

Some cases, particularly those associated with hypereosinophilia, present with the same *FIP1L1-PDGFR*A fusion and other rearrangements involving *PDGFR*A observed in CEL [168]. Other detectable chromosome abnormalities are similar to those reported for other MPNs and leukemias, specifically +8, +9, del(7q), del(11q), del(20q), t(8;21), and inv(16)/t(16;16). The association of mastocytosis with core binding factors AML, specifically those leukemias with t(8;21) and inv(16)/t(16;16), makes it necessary to investigate these patients for *KIT* mutations [169].

Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia (CNL), as the name implies, is characterized by an increase in mature neutrophils [170]. Patients often present with splenomegaly, but no fibrosis is present in the marrow. Approximately 20% of cases have an abnormal karyotype. The abnormalities observed so far include +8, +9, del(11q), del(20q), +21, and less frequently del(12p) [171, 172].

Some CNL patients present with a t(9;22)(q34;q11.2) as seen in typical CML but with a p230 *BCR-ABL1* transcript [173]. According to the WHO 2008 classification, these cases should be considered CML with a variant *BCR-ABL1* transcript and not CNL.

Myeloid and Lymphoid Neoplasms Associated with *PDGFRA*, *PDGFRB*, and *FGFR1*

This is a rare group of stem cell myeloid and lymphoid neoplasms that have in common the presence of eosinophilia and the involvement of genes that code for a tyrosine kinase [174]. In the WHO 2008 classification, these neoplasms are grouped together under the name, “myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*” [67]. Various translocations involving the *PDGFRA* (4q12), *PDGFRB* (5q33.1), and *FGFR1* (8p12) genes have been reported (Fig. 15.7). It is essential to

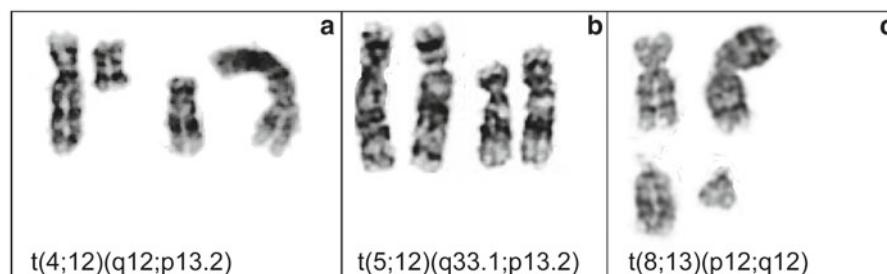


Fig. 15.7 Partial karyograms showing some of the most common translocations involving *PDGFRA*, *PDGFRB*, and *FGFR1*. In this particular figure, t(4;12)(q12;p13.2) fuses *PDGFRA* with *ETV6* (a), and

t(5;12)(q33;p13.2) fuses *PDGFRB* with *ETV6* (b), whereas t(8;13)(p12;q12) leads to fusion of *FGFR1* and *FLT3* (c)

clarify that although some earlier publications position the *FGFR1* gene locus at 8p11, the present chromosome location following more precise mapping is at 8p12 [175]. The most common translocation observed in these neoplasms is t(5;12)(q33.1;p13.2) leading to a *PDGFRB-ETV6* fusion [176, 177]. Some of the rearrangements are cryptic at the chromosome level. Since the presence of translocations involving *PDGFRA* and *PDGFRB* is associated with responsiveness to tyrosine kinase inhibitors, it is important, when one of these particular MPNs is suspected, to perform appropriate molecular studies to investigate whether any are present. Some translocations involving 4q12, 5q33.1, or 8p12, but not resulting in a rearrangement of the *PDGFRA*, *PDGFRB*, and *FGFR1* genes, respectively, have been also reported. In these cases, as well, the final interpretation should be dependent on the presence or absence of the molecular rearrangement. The rearrangement involving *PDGFRA* and *FIP1L1* at 4q12 is cryptic with conventional cytogenetics and can be detected only by FISH or by RT-PCR. However, FISH appears to be superior as it can provide information about other rearrangements involving the 4q12 region [178]. Rearrangements involving *PDGFRB*, located at 5q33.1, include various translocations, the most common of which is t(5;12)(q33.1;p13.2), which fuses the *PDGFRB* and the *ETV6* genes [179]. FISH is useful and should be performed on these patients since the presence of these rearrangements requires a specific alternative treatment. See Chap. 17, Fig. 17.12a, b and discussion on tyrosine kinases that follows.

Myeloproliferative Neoplasms, Unclassifiable

This category includes stem cell neoplasms that do not have the morphologic characteristics typically seen in any particular MPN [67]. They might have overlapping features seen in various MPNs but nothing specific enough to be classifiable as a specific MPN. Genetically, no rearrangements of *PDGFRA*, *PDGFRB*, or *FGFR1* are present, and no recurrent chromosome abnormalities have been associated with these neoplasms.

Myeloid Neoplasms with Translocations Involving Genes Coding For Tyrosine Kinases

A number of myeloid neoplasms exhibit translocations involving genes that code for tyrosine kinases other than *PDGFRA*, *PDGFRB*, or *FGFR1*. These neoplasms are not at this time included in a specific group but deserve some consideration, particularly in view of the increasing interest in these genes for therapeutic advancements. See Table 15.7 for a list of these translocations and associated neoplasms. The majority of neoplasms where these translocations have been observed fall into the category of atypical CML (aCML), and the rest have been observed in other myeloid or lymphoid neoplasms [16, 180–182].

Table 15.7 Rearrangements involving genes that code for tyrosine kinases and neoplasms associated with them

Abnormality	Gene fusions ^a	Neoplasms
t(1;12)(q25;p13.2)	<i>ABL2-ETV6</i>	AML
t(2;13)(p16;q12.2)	<i>SPTBN1-FLT3</i>	aCML
t(5;9)(q33.3;q22)	<i>ITK-SYK</i>	T-Cell lymphoma
t(8;9)(p22;p24.1)	<i>PCM1-JAK2</i>	aCML, AML, CEL, ALL
t(9;12)(p24.1;p13.2)	<i>JAK2-ETV6</i>	aCML, ALL
t(9;12)(q34;p13.2) or ins(12;9)(p13.2;q34q34)	<i>ABLI-ETV6</i>	aCML, AML, ALL
t(9;12)(q22;p13.2)	<i>SYK-ETV6</i>	MDS
t(9;22)(p24.1;q11.2)	<i>JAK2-BCR</i>	aCML
t(12;13)(p13.2;q12.2)	<i>ETV6-FLT3</i>	MPN, AML, ALL
t(12;15)(p13.2;q25.3)	<i>ETV6-NTRK3</i>	AML

Abbreviations: *AML* acute myeloid leukemia, *aCML* atypical chronic myeloid leukemia, *CEL* chronic eosinophilic leukemia, *ALL* acute lymphoblastic leukemia, *MDS* myelodysplastic syndrome, *MPN* myeloproliferative neoplasm

^aGenes that code for tyrosine kinases are in **bold**

Myelodysplastic/Myeloproliferative Neoplasms

This group includes neoplasms with morphologic features that can be seen in both MDS and MPN [183]. Generally, the bone marrow is hypercellular, but there is also some degree of dysplasia. The number of blasts is always below 20%. The neoplasms included here are chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML), juvenile myelomonocytic leukemia (JMML), and myelodysplastic syndrome/myeloproliferative neoplasm, unclassifiable (MDS/MPN, U). Table 15.8 presents some clinical and cytogenetic data for each of these neoplasms. The workup of the diagnosis includes the absence of *BCR-ABL1* fusion and of rearrangements of *PDGFRA*, *PDGFRB*, and *FGFR1*. On the other hand, mutations involving transcription factors such as *CEBPA*, *NPM1*, or *WT1* are frequent in these neoplasms, and one or more of these mutations might be present at the same time. Other significant gene mutations involve *TET2*, *RUNX1*, *ASXL1*, and *CBL* [184]. The prognosis associated with MDS/MPN is considered, in most cases, unfavorable since these patients rapidly progress to acute leukemia and are generally resistant to chemotherapy with associated short survivals after transformation [183].

Chronic Myelomonocytic Leukemia

Chronic myelomonocytic leukemia (CMML) is an MPN characterized by persistent monocytosis and variable degree of dysplasia [185]. The cases that were described previously as having a t(5;12)(q33.1;p13.2) leading to a *PDGFRB-ETV6* fusion are now included in the group of neoplasms with rearrangements of *PDGFRA*, *PDGFRB*, and *FGFR1* [67, 177, 186]. Although no specific abnormality has been associated with CMML, recurrent chromosome abnormalities, such as

Table 15.8 MDS/MPN according to WHO 2008 and most common chromosome abnormalities in order of frequency

Neoplasm	Percent of blasts (%)	Recurrent chromosome abnormalities
CMM _L	<20	-7/del(7q), +8, 12p rearrangements, i(17q), del(5q)
aCML	<5	+8, del(20q), involvement of chromosomes 12, 13, 14, and 17
JMML	<5	-7/del(7q), del(5q)
MDS/MPN,U	<20	del(5q) (infrequent)

Abbreviations: *CMM_L* chronic myelomonocytic leukemia, *aCML* atypical chronic myeloid leukemia, *JMML* juvenile myelomonocytic leukemia, *MDS/MPN*, *U*: myelodysplastic syndrome/myeloproliferative neoplasm, unclassified

-7/del(7q), gain of chromosome 8, and less commonly del(5q), 12p rearrangements, and i(17)(q10), have been observed [187–189]. See Table 15.8.

Atypical Chronic Myeloid Leukemia

Atypical chronic myeloid leukemia (aCML) is an interesting neoplasm that presents with features seen in classic CML as well as with myelodysplastic characteristics [190]. Although this neoplasm has many similarities with classic CML, it lacks the typical t(9;22)(q34;q11.2). Chromosome abnormalities are detected in the majority of cases and are similar to the ones described for CMM_L, except for losses involving chromosomes 6 and 7 and i(17)(q10), which seem to be confined to CMM_L. Thus, gain of chromosome 8 and rearrangements resulting in deletions of 12p are the most frequent aberrations [191, 192]. Furthermore, the t(8;9)(p22;p24) (leading to a *PCM1-JAK2* fusion) that was previously associated with aCML is no longer associated with this neoplasm but most likely belongs with chronic neutrophilic leukemia (CNL). In fact, neoplasms with *JAK2* mutations should not be considered as aCML [193].

Juvenile Myelomonocytic Leukemia

As the name implies, juvenile myelomonocytic leukemia (JMML) is an MPN of childhood, characterized by an abnormal proliferation of myelocytes and monocytes in the bone marrow [190]. As with the other MPNs in this category, the final diagnosis is based on the exclusion of the *BCR-ABL1* fusion [67]. The most common abnormality is -7/del(7q) and less frequently del(5q) [55, 194, 195].

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is defined by the presence of myeloblasts in the bone marrow, peripheral blood, and other tissues [196, 197]. At least 20% blasts should be present in the marrow. However, <20% blasts and presence of a

specific/recurrent abnormality associated with a particular subtype of AML is sufficient to make the diagnosis. The classification of these hematologic neoplasms has been revised by the WHO to account for the various genetic and cytogenetic changes that characterize this neoplasm [67]. See Table 15.9 and Fig. 15.8. Although AML more frequently affects adults in their 6th decade of life, it has been described in children and young adults as well [198]. Among the myeloid neoplasms, this is the group that accounts for the majority of specific abnormalities and for a large number of balanced rearrangements, most of which are translocations [39, 199–201].

Acute Myeloid Leukemia with Recurrent Genetic Abnormalities

The AMLs included in this group are characterized by the presence of well-established genetic abnormalities, the most common of which are t(8;21)(q22;q22.3), inv(16)(p13.1q22.1) or (16;16)(p13.1;q22.1), t(15;17)(q24.1;q21.2), t(9;11)(p22;q23), t(6;9)(p23;q34.1), inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2), and t(1;22)(p13.3;q13.1). These translocations/inversions belong to the so-called class 2 mutations, which have the ability to arrest differentiation of the lineage affected by the rearrangement [202]. This results in proliferation of only a particular subset of myeloid cells. As such, these chromosome abnormalities have been associated with particular subtypes of AML.

AML with t(8;21)(q22;q22.3)

This represents one of the core binding factor (CBF) myeloid leukemias and affects approximately 8–10% of AML patients, mainly adults [203]. The t(8;21) leads to a *RUNX1-RUNXT1* (formerly *AML1-ETO*) fusion and is generally associated with a favorable prognostic outcome [46, 47]. This particular AML is also known as AML with maturation and as subtype M2 according to the French-American-British (FAB) classification [204, 205]. Less commonly, t(8;21) can be also seen in AMML (FAB M4) and in therapy-related MDS/AML [206]. Variant translocations, usually affecting a third chromosome, have been reported in 3% of cases [26, 207–212]. The presence of additional abnormalities is common (about 70% of cases). The most frequent additional abnormality is loss of a sex chromosome (the Y in males), followed by del(9q), del(7q), +8, and/or +21. Although most of these additional abnormalities do not appear to affect the favorable prognostic outcome associated with t(8;21), gain of chromosome 6 is also seen, and some reports indicate a less favorable disease course when trisomy 6 is part of the karyotype [26, 213].

Regardless of the presence or lack of additional abnormalities, patients exhibit a good response to chemotherapy together with a high rate of complete remission and disease-free

Table 15.9 Acute myeloid leukemia (AML) classification and associated chromosome abnormalities according to the World Health Organization [67] (See Fig. 15.8)

Neoplasm	Frequency (%)	Chromosome abnormality (typical and variants)	Common additional abnormalities (in order of frequency)
<i>AML with recurrent genetic abnormalities</i>			
AML with t(8;21)	5–10	t(8;21)(q22;q22.3)	–X or –Y, del(9q), del(7q), +8
AML with inv(16) or t(16;16)	5–8	inv(16)(p13.1q22.1) or t(16;16)(p13.1;q22.1)	+22, +8, del(7q)
AML with t(15;17)	5–8	t(15;17)(q24.1;q21.2)	+8, del(7q), del(9q)
AML with t(9;11) 2 (adult)	9–12 (pediatric) 2 (adult)	t(9;11)(p22;q23)	–X or –Y, +8
AML with t(6;9)	1–2	t(6;9)(p23;q34.1)	+8, +13, +21
AML with inv(3) or t(3;3)	1–2	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)	–7, del(5)
AML (megakaryoblastic) with t(1;22)	<1	t(1;22)(p13;q13)	del(5q), del(7q), +21
AML with <i>NPM1</i> mutation	25–30	Normal or no specific abnormality	No specific abnormality
AML with <i>FLT3</i> mutation	20–40	Normal or no specific abnormality	No specific abnormality
AML with <i>CEBPA</i> mutation	5–15	Normal or no specific abnormality	No specific abnormality
AML with <i>KIT</i> mutation		t(8;21) or inv(16)/t(16;16)	Same are for t(8;21) and inv(16)/t(16;16)
AML with <i>WT1</i> mutation	20–25	Normal or no specific abnormality	No specific abnormality
<i>AML with myelodysplastic-related changes</i>			
Therapy-related myeloid neoplasms	10–20	Complex with del(5q), del(7q), +8, 3q21.3 or 3q26.2 rearrangements, 11q23 rearrangements	del(6p), del(12p), del(17p), del(9q), del(20q), +21
<i>AML, not otherwise specified</i>			
AML with minimal differentiation	<5	Complex with del(5q), del(7q), +8, <i>MLL</i> rearrangements, <i>RUNX1</i> rearrangements	del(17p), del(12p)
AML without maturation	5–10	+8, del(9q)	No recurrent abnormality
AML with maturation	8–10	+8	No recurrent abnormality
AML (myelomonocytic)	5–10	+8	No recurrent abnormality
AML (monoblastic/monocytic)	<5	t(8;16)(p12;p13.3)	No recurrent abnormality
AML (erythroid)			
Pure erythroid leukemia	<5	Complex with –5/del(5q), –7/del(7q), +8, del(20q) ^a	del(6p), del(12p), del(17p)
Erythroleukemia (erythroid/myeloid)	<5	Complex with t(3;3)/inv(3), –5/del(5q), –7/del(7q), +8, del(20q) ^a	del(6p), del(12p), del(17p)
AML (megakaryoblastic)	<5	Children: t(1;22)(p13;q13), +21 Adults: Complex with –5/del(5q), –7/del(7q), t(3;3)/inv(3), +8, del(20q) ^a	del(6p), del(12p), del(17p)
AML (basophilic)	<1	No recurrent abnormality	No recurrent abnormality
AML (panmyelosis with myelofibrosis)	Rare	Complex with –5/del(5q), –7/del(7q), +8, del(20q) ^a	del(6p), del(12p), del(17p)
Myeloid sarcoma		Abnormalities similar to AML with recurrent genetic abnormalities	Similar to AML with recurrent genetic abnormalities
<i>Myeloid proliferation related to Down syndrome</i>			
Transient abnormal myelopoiesis	10 newborns	Additional copies of chromosome 21 (in addition to constitutional trisomy 21)	+8
Myeloid leukemia associated with Down syndrome	1–2 children (<5 years of age)	Additional copies of chromosome 21 (in addition to constitutional trisomy 21)	–7, +8
Blastic plasmacytoid dendritic cell neoplasms	Rare	del(4q), del(5q), del(12p), del(13q), del(6q), del(15q), del(9p), del(9q)	No recurrent abnormalities

survival. However, the favorable prognostic outcome is without exception altered by the presence of *KIT* mutations [214, 215].

AML with inv(16)(p13.1q22.1) or t(16;16)(p13.1;q22.1)
The characteristic of this AML is the presence of myelomonocytic blasts and atypical eosinophils. Also known as

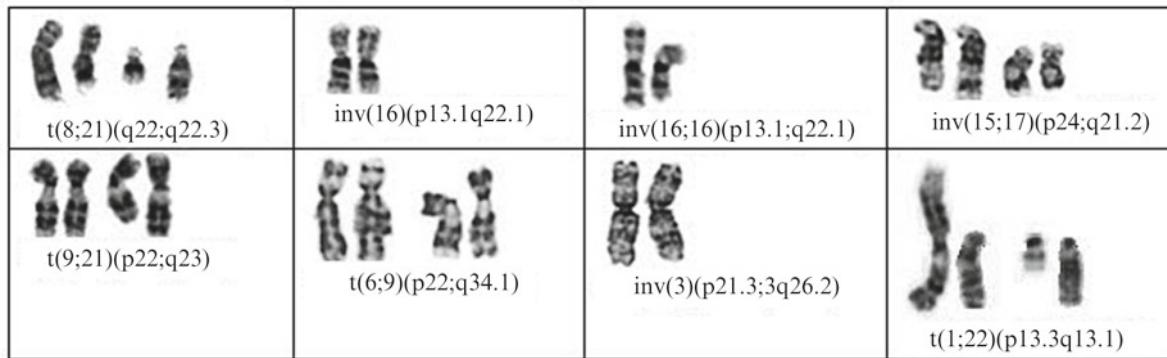


Fig. 15.8 Partial karyograms showing recurrent (or specific) rearrangements in AML. These translocations/inversions define particular AML subtypes in the WHO classification

AML M4_{EO} according to the FAB classification, this leukemia makes up 7–10% of AML cases and is generally associated with a favorable prognostic outcome [203]. However, patients have a higher risk of central nervous system (CNS) involvement at diagnosis or at relapse than patients with other types of AML. Adults are more frequently affected than children. The hallmark of this AML is the inv(16)(p13.1q22.1) or, less commonly, the t(16;16)(p13.1;q22.1). Either abnormality leads to the fusion of *MYH11* at 16p13.1 with *CBFB* at 16q22.1 [216]. The identification of these rearrangements by conventional cytogenetics might be challenging, particularly when the chromosome morphology is not optimal. In those cases, FISH or RT-PCR can be helpful [217]. These rearrangements have been reported occasionally in tMDS and tAML [218]. Chromosome abnormalities in addition to inv(16) or t(16;16) are detected in approximately 30% of cases [219]. The most common is +22, which is considered a clue by many cytogeneticists, particularly when the presence of inv(16) or t(16;16) is not obvious. Other additional chromosome abnormalities include +8, del(7q), and/or +21. Although this leukemia has been associated with complete remission and improved long-term survival, molecular testing for *KIT* mutations is necessary, as these are associated with adverse prognosis and necessitate more aggressive therapy [214].

Acute Promyelocytic Leukemia with t(15;17)(q24.1;q21.2)

The vast body of research of the past 30 years has contributed to the successful management of acute promyelocytic leukemia (APL) [42, 220]. Originally considered one of the most aggressive leukemias, it is now a model for targeted therapy [221]. Due to the high risk of early death and the potential for high cure rate, it is essential to immediately identify this leukemia. The t(15;17) is the specific abnormality that characterizes this subtype of AML [42]. The formation

of this translocation leads to a fusion between *PML* at 15q24.1 and *RARA* at 17q21.2 [48]. The *PML-RARA* fusion is associated with a favorable prognosis and response to treatment with all-trans retinoic acid (ATRA) [220]. Translocations with additional rearrangements involving either chromosome 15 or 17 or complex translocations involving a third chromosome occur in approximately 5% of cases [222, 223]. In these cases, it is important to determine that the *PML-RARA* fusion is intact. RT-PCR can easily be used to verify this as well as determine the size of transcript, which could negatively influence the prognostic outcome [224]. Other variants involving 17q21.2 and not 15q24.1 exist but are rare. The most known of these variants are t(5;17)(q35.1;q21.2) leading to a fusion of *NPM1* and *RARA* and t(11;17)(q23.2;q21.2) leading to a fusion of *ZBTB16* (*PLZF*) and *RARA* [225]. The t(5;17) seems to respond to ATRA, whereas t(11;17) does not. The presence of t(15;17) and variants in therapy-related neoplasms is infrequent, but it has been reported [226]. These cases show dysplastic features and often are associated with additional chromosomal and molecular changes.

Additional abnormalities have also been observed in *de novo* APL, of which +8, del(9q), and del(7q) are the most frequent. The presence of chromosome abnormalities in addition to t(15;17) does not appear to affect the prognosis associated with this neoplasm [227].

AML with t(9;11)(p22;q23) and Other Translocations Involving *MLL*

This translocation leads to fusion of *MLLT3* at 9p22 with *MLL* at 11q23 and is found in AML with a monocytic or myelomonocytic phenotype, lack of CD34 expression, and frequent *RAS* mutations [228]. This is the most common translocation involving *MLL* [67]. Among the approximately 85 known *MLL* translocations, t(9;11) is thought to be associated with a better prognostic outcome [229]. However, large-scale retrospective studies could not confirm this earlier result [230].

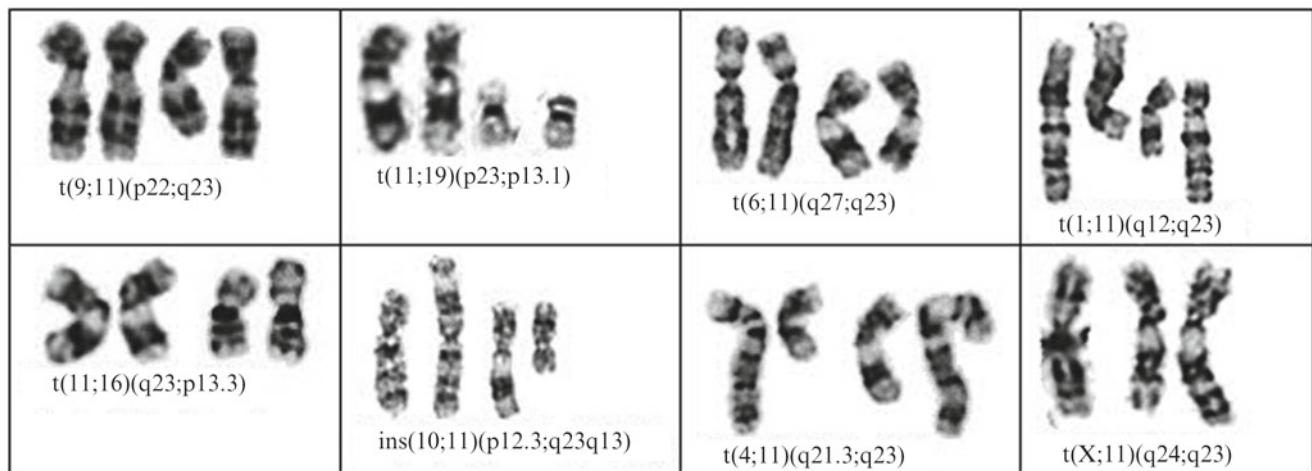


Fig. 15.9 Partial karyograms illustrating common *MLL* (11q23) translocations observed in AML. Some of these translocations have been observed also in therapy-related MDS/AML

With conventional cytogenetics, *MLL* translocations are usually present in all or almost all metaphase cells analyzed. Additional abnormalities can be seen, and the most common are loss of a sex chromosome (-Y in males) and +8 [231]. Another frequent *MLL* translocation is t(11;19) with variant breakpoints on chromosome 19. Specifically, the breakpoint at 19p13.1 (*ELL*) is seen mainly in adults, whereas the breakpoint at 19p13.3 (*MLLT1*) is typical of childhood AML [232]. t(6;11)(q27;q23) which involves the *MLLT4* and *MLL* genes, respectively, is at times difficult to identify and often has been erroneously identified as del(11q) [233].

Two recurring translocations involving chromosomes 10 and 11 have been observed. The most common involves the *MLLT10* gene at 10p12.3. The fusion of this gene with *MLL* is often the result of an inverted insertion of a variant segment of chromosome 11 containing the 3' portion of *MLL* into the short arm of chromosome 10 rather than a reciprocal translocation [234]. The other is t(10;11)(q21.3;q23), which fuses *TET1* with *MLL*.

Due to the cryptic nature of some of these translocations, it is always good practice to perform FISH to look for an *MLL* rearrangement when the karyotype appears to be normal. See also Chap. 17, Fig. 17.8.

In addition to *de novo* AML, *MLL* translocations have also been reported in therapy-related MDS/AML [79] (Fig. 15.9).

AML with t(6;9)(p23;q34.1)

This somewhat rare translocation results in the fusion of *DEK* at 6p23 with *NUP214* at 9q34.1. t(6;9) is probably the abnormality most frequently associated with basophilia and had been seen in both pediatric and adult patients [235]. In most of the cases, this abnormality is present as the only change, but it can also be seen in a complex karyotype, par-

ticularly together with gains of chromosomes 8, 13, and/or 21 [236].

AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)

These two abnormalities have in common the involvement of two genes associated with an unfavorable prognostic outcome, *RPN1* at 3q21.3 and *MECOM* (*EVI7*) at 3q26.2 [103]. There are several other balanced and unbalanced rearrangements involving these two regions, including 1p36, 2p15, 3p12, 3p24, 3q23, 5q31.2, 5q34, 7q21, 8q24, 11p15, 12p13, 12q21, 17q22, 18q11, and 21q22.3 [104–106]. Some of these rearrangements are also common in therapy-related MDS/AML. Rearrangements of 3q21.3 and 3q26.2, particularly t(3;21)(q26.2;q22.3), are also seen as additional abnormalities during progression of CML to accelerated phase and blast crisis. The most common additional abnormalities that accompany cases with rearrangements of 3q21.3 and 3q26.2 are -7 and, less frequently, del(5q) [237].

Megakaryoblastic AML with t(1;22)(p13.3;q13.1)

Acute megakaryoblastic leukemia (AMKL; FAB M7) is a clonal stem cell neoplasm that makes up about 3–15% of all AML cases [238]. This leukemia is seen mostly in children, with the median age at presentation between 1 and 8 years [238, 239]. The incidence of developing this subtype of AML is much higher in children with Down syndrome (DS) than in children without DS [240]. Interestingly, DS children generally have a more favorable prognosis compared to patients without constitutional +21 [240].

Three entities of AMKL have been described. The first subtype is observed in Down syndrome (DS) children and is characterized by mutation of *GATA1* and also by t(1;22)(p13.3;q13.1), leading to a fusion of *RBM15* at 1p13.3 with *MKL1* at 22q13.1 [241]. *GATA1* mutations are rare in non-DS

AMKL [242]. The few non-DS cases with *GATA1* mutation are characterized by the presence of an acquired trisomy 21 in the karyotype [242]. This is intriguing and raises the possibility that *GATA1* mutation might be dependent on the presence of an additional copy of chromosomes 21. The second subtype is observed in about 20% of infants with Down syndrome and transient myeloproliferative disease (TMD) who subsequently develop AMKL [240]. *GATA1* is likely to play a critical role in the etiology of TMD and mutation of this gene represents a very early event in the development of AMKL. The karyotype of these DS patients typically contains additional copies of chromosome 21 (four or more copies of chromosome 21 can be seen), as well as gain of chromosome 8. The third subtype of AMKL is found in infants that show the t(1;22) but do not have Down syndrome [241, 242]. Detection of the t(1;22) is diagnostic in this group. The prognosis associated with the t(1;22) used to be considered unfavorable but is now considered intermediate since these patients are responsive to AML therapy and exhibit long clinical remission times.

In adults, this leukemia is often secondary in nature, either posttreatment or during leukemic transformation [239]. Approximately 50% of adult patients have chromosome abnormalities at diagnosis. The most common rearrangements involve the regions 3q21.3 and 3q26.2. In addition, frequently, the abnormal karyotype includes -5/del(5q), -7/del(7q), and +8 [238]. t(1;22) has not been observed in adults.

Acute Myeloid Leukemia with Gene Mutations

This new category of AML is characterized by a normal karyotype and recurrent gene mutations involving genes such as nucleophosmin (*NPM1*) located at 5q35.1, fms-like tyrosine kinase 3 (*FLT3*) located at 13q12.2, CCAAT/enhancer-binding protein- α (alpha) (*CEBPA*) located at 19q13.1, and mixed lineage leukemia (*MLL*) located at 11q23 [243, 244]. Specifically, 45–60% patients have mutations involving *NPM1*, 20–35% patients show mutations of *FLT3*, 10–20% have *CEBPA* mutations, and 5–25% have *MLL* tandem duplications. Some of these mutations are not mutually exclusive; most notably, *NPM1* and *FLT3* might be present at the same time. The prognosis is variable and depends on which gene is mutated. Patients with *NPM1* or biallelic *CEBPA* mutation alone have a favorable prognosis, whereas the presence of *FLT3* or *MLL* mutations is associated with an unfavorable prognostic outcome [245]. Therefore, patients with both *FLT3* and *NPM1* mutations have an adverse prognosis. Other less frequent mutations observed in AML with normal or abnormal karyotype involve *KIT*, *WT1*, *KRAS*, and *NRAS*. Of note, *KIT* is also associated with abnormalities involving the core binding factor genes, such as t(8;21)(q22;q22.3) and inv(16)(p13.1q22.1)/t(16;16)(p13.1;q22.1) [214]. Since *KIT* mutations affect the clinical course, a suggestion to investigate for the presence of *KIT*

mutations should be provided for patients characterized by one of these chromosome abnormalities.

Acute Myeloid Leukemia with Myelodysplasia-Related Changes

As the name implies, these are myeloid leukemias characterized by abnormalities typically seen in MDS, specifically -5/del(5q), -7/del(7q), and +8, as well as translocations involving 3q21.3, 3q26.2, and 11q23. The majority of these AML have complex karyotypes similar to what has been reported in high-grade MDS and tMDS/tAML. However, according to the WHO, this group should not include patients that have a prior history of cytotoxic or radiation therapy [246].

Myeloid Sarcoma

Myeloid sarcoma or granulocytic sarcoma is the name given to a myeloid leukemic process that forms a mass at an anatomical site outside of the bone marrow; the term extramedullary myeloid tumor is therefore also used to describe this leukemia [247]. Other terms used include granulocytic sarcoma and chloroma. This leukemia occurs at any age and affects males more than females. It can arise *de novo*, represent a relapse of a known leukemia, or occur as a transformation of a chronic myeloproliferative neoplasm or myelodysplastic syndrome.

Several cytogenetic abnormalities have been observed in myeloid sarcoma. The most common are -7, +8, del(5q), del(20q), +4, +11, del(16q), inv(16)/t(16;16), *MLL* rearrangements, and t(8;21)(q22;q22.3) [248–250]. The prognosis is variable as it is influenced by several factors including but not limited to age, morphology, and cytogenetic abnormality.

Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastic plasmacytoid dendritic cell neoplasm is a very aggressive leukemia derived from the plasmacytoid monocytes and usually involves the skin, bone marrow, and peripheral blood. This neoplasm is best known as blastic natural killer lymphoma [251]. The median survival is around 12 months. Patients are typically in their sixth decade of life at presentation. Around 20% of cases transform into acute myeloid leukemia, preferentially acute myelomonocytic leukemia. The majority of cases have an abnormal karyotype that is usually complex. The most common abnormalities include del(5q), del(12p), del(13q), del(6q), del(15q), del(4q), del(9p), and del(9q) [252].

Acute Leukemia of Ambiguous Lineage

This group of neoplasms includes acute undifferentiated leukemia (AUL) and mixed phenotype acute leukemia (MPAL) that have not differentiated into a particular lineage or expressed cell surface markers of more than one lineage, respectively [253]. In other words, AUL blasts express nei-

ther lymphoid nor myeloid markers, whereas MPAL blasts express markers of different lineages. Although no specific or recurrent abnormalities are observed in AUL, MPAL is characterized by two recurrent abnormalities, t(9;22) (q34;q11.2) and 11q32 (*MLL*) rearrangements [254]. The prognosis associated with these leukemias is poor. However, patients characterized by the *BCR-ABL1* fusion are expected to have a better course due to response to imatinib.

Lymphoid Neoplasms

This group of hematologic neoplasms includes immature and mature neoplasms of B-cell, T-cell, and natural killer (NK) cell subtypes. Neoplasms of B-cell origin are more frequent than those of T-cell origin [255]. Immature B-cell neoplasms include precursor B-cell lymphoblastic leukemia/lymphoma (pre-B-ALL/LBL) and precursor T-cell lymphoblastic leukemia/lymphoma (pre-T-ALL/LBL) [256]. The yearly incidence of these immature neoplasms is estimated to be 1–4.75/100,000 individuals worldwide. They are by far more common in children than adults. Approximately 85% are of B-cell origin and present as ALL, whereas precursor T-cell lymphoblastic neoplasms present mostly as lymphoma and affect mainly adolescent males.

Disorders of mature cells make up 90% of all lymphoid neoplasms [255]. These lymphomas are more frequent in developed countries with 33 cases/100,000 individuals diagnosed each year.

The majority of lymphoid neoplasms (both precursor and mature types) are characterized by recurrent chromosome abnormalities. Some of the most common subtypes are discussed as follows.

Acute Lymphoid Neoplasms

Acute B-cell Lymphoblastic Leukemia/Lymphoma

This neoplasm is defined as leukemia when it involves the bone marrow and peripheral blood and as lymphoma when it presents as a lesion without evidence of bone marrow and peripheral blood involvement [256]. There is often extramedullary involvement, particularly of the central nervous system, lymph nodes, spleen, liver, and testis in cases of B-ALL and of skin, soft tissue, bone, and lymph nodes in cases of LBL. A large percentage of ALL cases, especially those involving children, are classified as precursor B-cell ALL (pre-B-ALL).

Several factors impact the prognosis. Approximately 85% of B-ALL patients are children [257]. In general, older age (≥ 10 years) and high WBC are factors associated with high-risk B-ALL, compared with younger age and low WBC, which are associated with low-risk disease [256]. Chromosome abnormalities have been reported in the majority of cases and are useful for prognostic stratification [258, 259]; conventional metaphase cytogenetics is still considered the basic method for the detection of these abnormalities. Pediatric cases with t(9;22)(q34;q11.2), 11q23 (*MLL*) rearrangements, t(1;19)(q23.3;p13.3), and hypodiploidy (≤ 45 chromosomes) are known to have an unfavorable prognosis, whereas t(12;21)(p13.2;q22.3) and hyperdiploidy (> 50 chromosomes) are associated with a favorable prognostic outcome particularly if trisomies 4 and 10 are present in the latter [258] (see Table 15.10). Cytogenetic and FISH analyses are indicated for proper risk stratification.

Children and young adults (generally up to 21 years of age) enrolled in the Children's Oncology Group (COG) program are required to have their bone marrow or informative peripheral blood sample analyzed by conventional cytogenetics and FISH [260]. The latter is mainly geared toward

Table 15.10 Recurrent chromosome abnormalities and involved genes in B-ALL

Cytogenetic abnormality	Gene(s) involved	Common additional abnormalities	Prognosis	% of patients
t(9;22)(q34;q11.2)	<i>ABL1-BCR</i>	+der(22)t(9;22), -7	High risk	2.5% children 25% adults
t(12;21)(p13.2;q22.3)	<i>ETV6-RUNX1</i>	del(6q), del(11q), 12p rearrangements, del(16q), +21	Low risk	30% children Absent in adults
Hyperdiploidy (≥ 50 chromosomes)	Dosage	Rare structural rearrangements	Low risk	25% children 5% adults
Hypodiploidy (≤ 45 chromosomes)	Dosage	Few structural rearrangements	High risk	2% children and teenagers
t(1;19)(q23.3;p13.3)	<i>PBX1-TCF3</i>	dup(1q), del(6q), +8, i(9q), i(17q), +21	High risk	Children: 25% pre-B-ALL and 5% B-ALL Adults: 3% pre-B-ALL
del(9)(p21.3)	<i>CDKN2A</i>	del(6q), del(12p)	Undetermined	10% children and adults
<i>RUNX1</i> amplification	<i>RUNX1</i>	Generally none	High risk	5% children 2% adults
11q23 rearrangements, including partial deletions and duplications	<i>MLL</i>	Generally none	High risk	80% infants 10% children and adults

the detection of prognostic markers such as *BCR-ABL1* and *ETV6-RUNX1* fusions, *MLL* rearrangements (including partial deletions/duplications), as well as trisomies 4 and 10. Some of these recurrent abnormalities are discussed at length as follows. Other FISH probes are available to detect and/or clarify less common, atypical, or prognostically less informative chromosome abnormalities. See also Chap. 17.

The Philadelphia Chromosome

The Philadelphia (Ph) chromosome derived from the t(9;22) (q34;q11.2) occurs in approximately 2.5% of children and approximately 25% of adults with B-ALL [261]. At the molecular level, the breakpoints in B-ALL and CML differ, and this variation leads to the production of p190 and p210 fusion proteins, respectively. Approximately 20% of Ph-positive B-ALL patients, however, have been found to generate both the p190 and p210 fusion transcripts, possibly as a result of alternative splicing or missplicing events in the *BCR* gene [262, 263]. Alternatives to the typical translocation include insertions of *ABL1* into the *BCR* locus and vice versa to form the *BCR-ABL1* fusion [264, 265]. Some of these variants and most of the insertions will not result in a classic Ph chromosome with conventional chromosome analysis. However, the presence of the gene fusion will be revealed by FISH and/or PCR testing.

Chromosome abnormalities in addition to the Ph chromosome are seen in greater than 60% of patients and are similar to those observed in CML during progression to accelerated phase or blast crisis, specifically +8 and one extra copy of the Ph chromosome [266]. However, i(17)(q10) is seen primarily in CML, while -7, +X, and del(9p) are seen primarily in B-ALL [267] (Fig. 15.10). While additional abnormalities are associated with disease progression in CML, they do not appear to modify the disease's course in B-ALL. However, patients with loss of chromosome 7 seem to have a much worse prognosis than patients without this abnormality, probably due to its association with resistance to therapy [268, 269].

MLL Rearrangements

Rearrangements involving *MLL* at 11q23 have been reported in infants, children, and adults with B-ALL [228, 268, 269]. They have been observed in approximately 80% of infants (<1 year old) and 5–10% of children and adults with B-ALL. In children, the cells have a pre-B immunophenotype that express myeloid antigens and are CD19+/CD10– by flow cytometry. The CD10– immunophenotype, high WBC, and young age are helpful clues suggestive of the presence of an *MLL* rearrangement.

The most frequent *MLL* translocations include t(4;11) (q21.3;q23) leading to *MLL-AFF1* fusion and t(11;19)

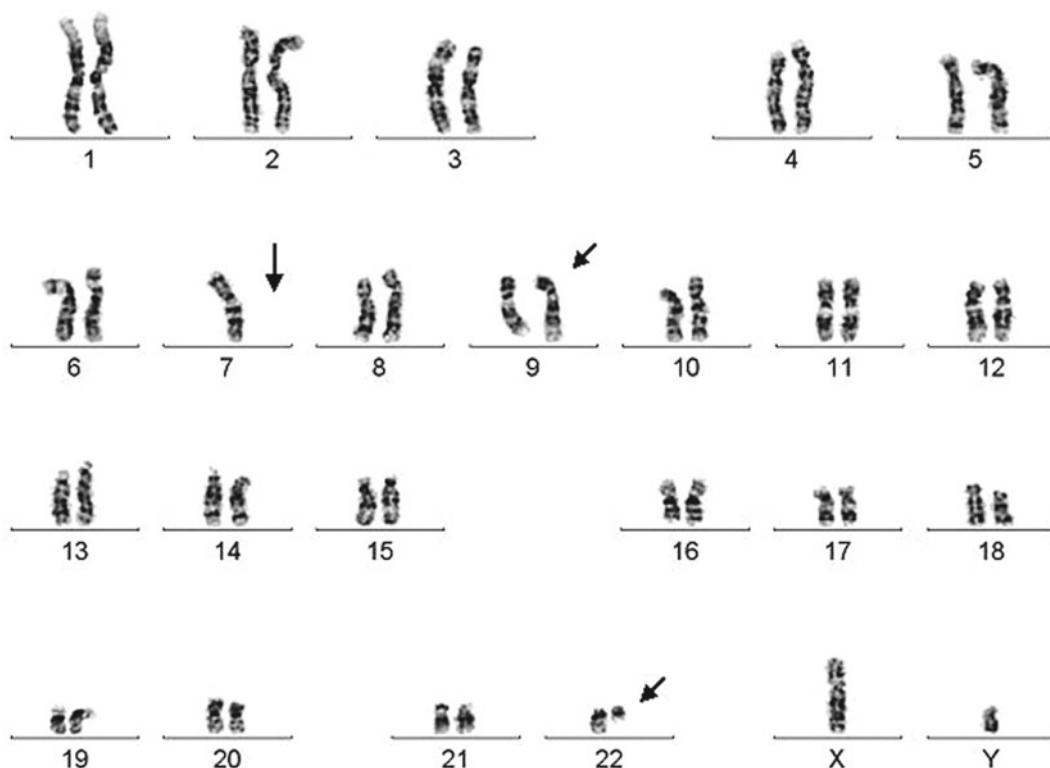


Fig. 15.10 Karyogram of a patient with B-ALL. The t(9;22)(q34;q11.2) is rarely the sole abnormality in B-ALL. In this case, there is also loss of one copy of chromosome 7

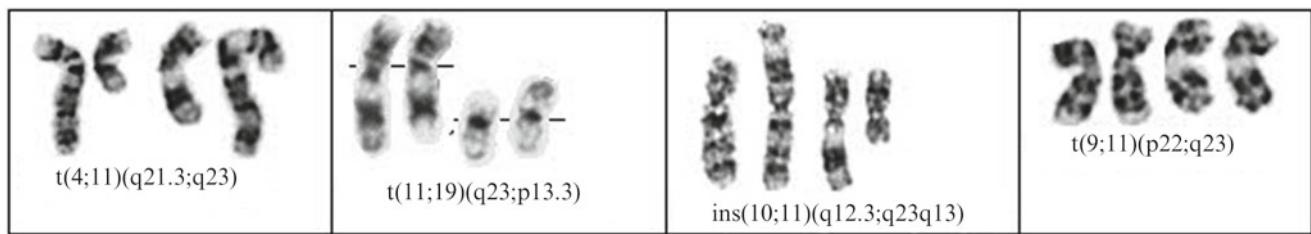


Fig. 15.11 Partial karyograms illustrating some of the most recurrent *MLL* (11q23) translocations in B-ALL. These are presented in order of frequency. The majority of patients with *MLL* rearrangements are infants

(q23;p13.3) leading to *MLL-MLLT1* fusion [15, 270, 271]. Occasionally, t(9;11)(p22;q23) involving *MLLT3* and *MLL* or other less frequent translocations might be seen (Fig. 15.11). *MLL* rearrangements are associated with an unfavorable prognostic outcome in both children (particularly infants) and adults, and bone marrow transplant is still the treatment of choice.

MLL rearrangements have also been reported in T-ALL [272]. Even though these rearrangements are uncommon, recent studies indicate that *MLL* rearrangements are one of the early leukemogenic hits in T-ALL [273].

t(1;19)(q23.3;p13.3)

Approximately 5% of children with pre-B-ALL and 5% of children with B-ALL have a t(1;19)(q23.3;p13.3), which leads to a fusion of *TCF3* located at 19p13.3 with *PBX1* at 1q23.3 [274]. This translocation is rare (3% of cases) in adults, who also present with a pre-B immunophenotype. The majority of patients (75% of cases) have an unbalanced form of the translocation, der(19)t(1;19)(q23.3;p13.3), whereas 25% have the balanced t(1;19). Whether the unbalanced form in pediatric B-ALL patients is associated with a better prognostic outcome than the balanced t(1;19) is still controversial [275]. A variant form of t(1;19) is t(17;19)(q22;p13.3), which leads to a fusion between the *HLF* and *TCF3* genes located at 17q22 and 19p13.3, respectively, and has been observed in approximately 1% of pediatric B-ALL patients. This variant translocation is associated with poor prognostic outcome [276]. Both t(1;19) and t(17;19) are easily identifiable with conventional cytogenetics. However, since cells with these abnormalities are characterized by low mitotic activity in culture, their detection might require the analysis of more than the 20 metaphase cells typically examined. Probes that target both *TCF3* (break-apart probe) and the actual *TCT3-PBX1* fusion are available for initial detection and monitoring [277]. These FISH studies have also proven useful to detect cryptic rearrangements such as inv(19)(p13.3q13.4), which leads to a fusion of the *TCF3* and *TFPT* genes. This inver-

sion has been reported in approximately 5% of pediatric B-ALL cases [274].

Hypodiploidy

Hypodiploidy is associated with an unfavorable prognosis. Fortunately, only 2% of pediatric patients with B-ALL and rare cases of adult patients are found to have a hypodiploid chromosome complement [278–280].

Three separate groups have been observed. The most common is the near-haploid karyotype, with a chromosome count ranging from 26 to 29 [281]. The loss and retention of chromosomes in this group is not random. In fact, invariably, the karyotypes with 26 chromosomes retain two copies of chromosomes 14, 21, and the sex chromosomes, with a single copy of all other chromosomes. The chromosomes that are preferentially lost include chromosomes 3, 7, 15, and 17 [281]. Therefore, the investigation of hypodiploidy by FISH should target regions on the preferentially lost chromosomes. The second and third groups include karyotypes with a chromosome count ranging from 30 to 39 and 40 to 44, respectively. Generally, a lower number of chromosomes correspond to a worse prognosis. A peculiarity of hypodiploid karyotypes is their tendency to double the chromosome complement via endoreduplication. This is not a culture-induced (*in vitro*) doubling, but rather it occurs *in vivo*, as demonstrated by DNA index studies of uncultured specimens [282] (Fig. 15.12a, b). These studies have shown the presence of three distinct populations: a hypodiploid complement with DNA index below 1, a diploid complement with DNA index equal to 1, and a hyperdiploid complement with DNA index above 1 [283].

From the DNA index, it is possible to estimate the number of chromosomes present in the karyotype by using the simple mathematical formula $46 \times \text{DNA index} = \text{number of chromosomes in the karyotype}$. The reason(s) that cells with a hypodiploid complement endoreduplicate is not clear. Some authors have suggested that hypodiploid cells are unstable and doubling their complement gives them the

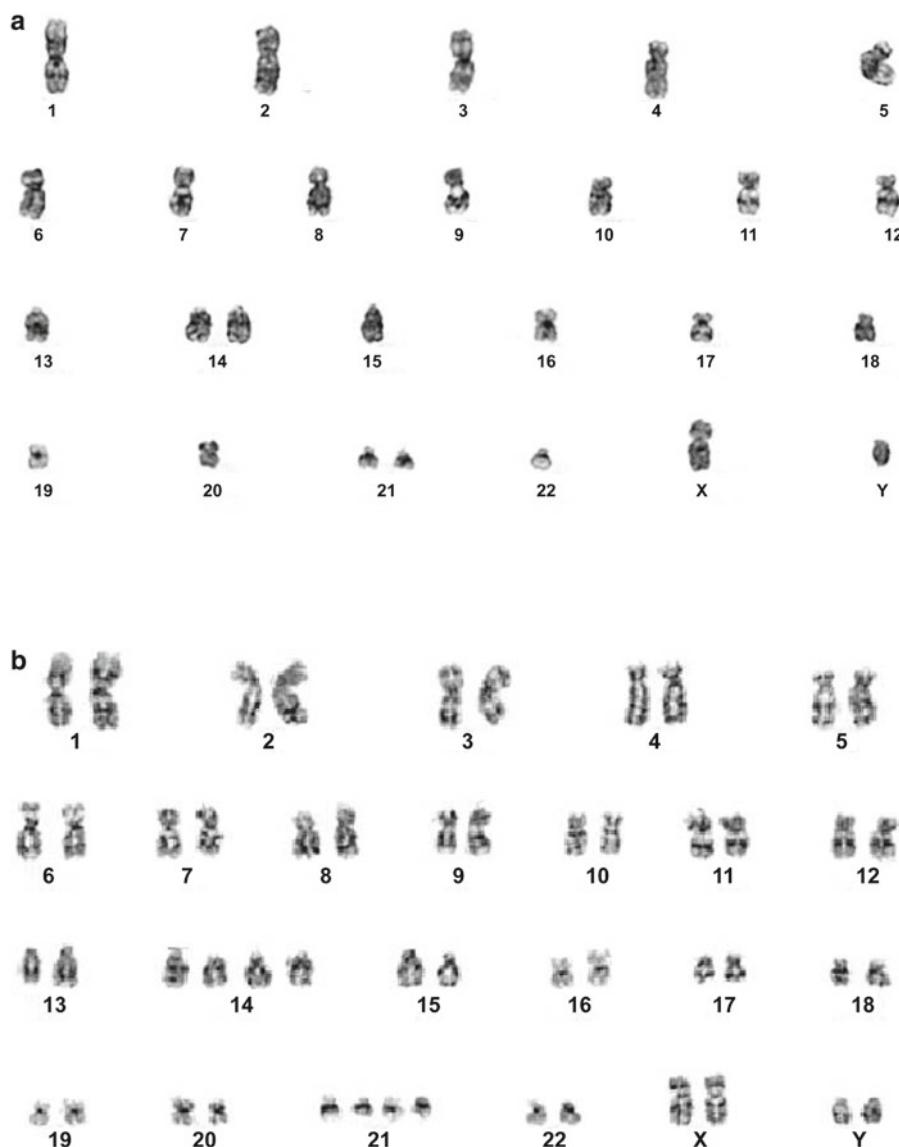


Fig. 15.12 Karyograms of a patient with B-ALL showing (a) a near-haploid karyotype and (b) its doubling version. Note the retention of chromosomes 14, 21, and the sex chromosomes. This is the one

of the most basic doubling, from which it is easy to suspect the presence of the hypodiploid counterpart

ability to survive longer. This might be the reason why, with conventional cytogenetics, more cells with the doubled complement than with the hypodiploid complement are observed. It is imperative to distinguish a doubling from a true hyperdiploid clone since they are associated with different prognostic outcomes. Molecular testing, such as microsatellite markers and SNP array, could be useful to distinguish true hyperdiploid from an endoreduplicated hypodiploid cell population.

Hyperdiploidy

True hyperdiploidy (51–68 chromosomes) occurs in approximately 25% of children and 5% of adults with B-ALL [282].

A relatively young age (2–10 years) is associated with a favorable prognosis; children also tend to present with favorable features such as low WBC and a pre-B immunophenotype. Modal chromosome numbers between 51 and 55 are thought to be associated with a relatively less favorable prognosis than those from 56 to 68 chromosomes [259]. The better prognosis of the latter seems to correlate with the presence of trisomies 4 and 10. The most common gains involve chromosomes 4, 6, 8, 10, 14, 17, 18, 19, and 21 (Fig. 15.13). Gain of chromosome 21 (often tetrasomy 21) is the most common numerical abnormality in B-ALL, present in more than 95% of cases with hyperdiploidy. This is followed by gains of chromosomes 6 (85% of cases, especially in adults), X and 14 (80% of cases),

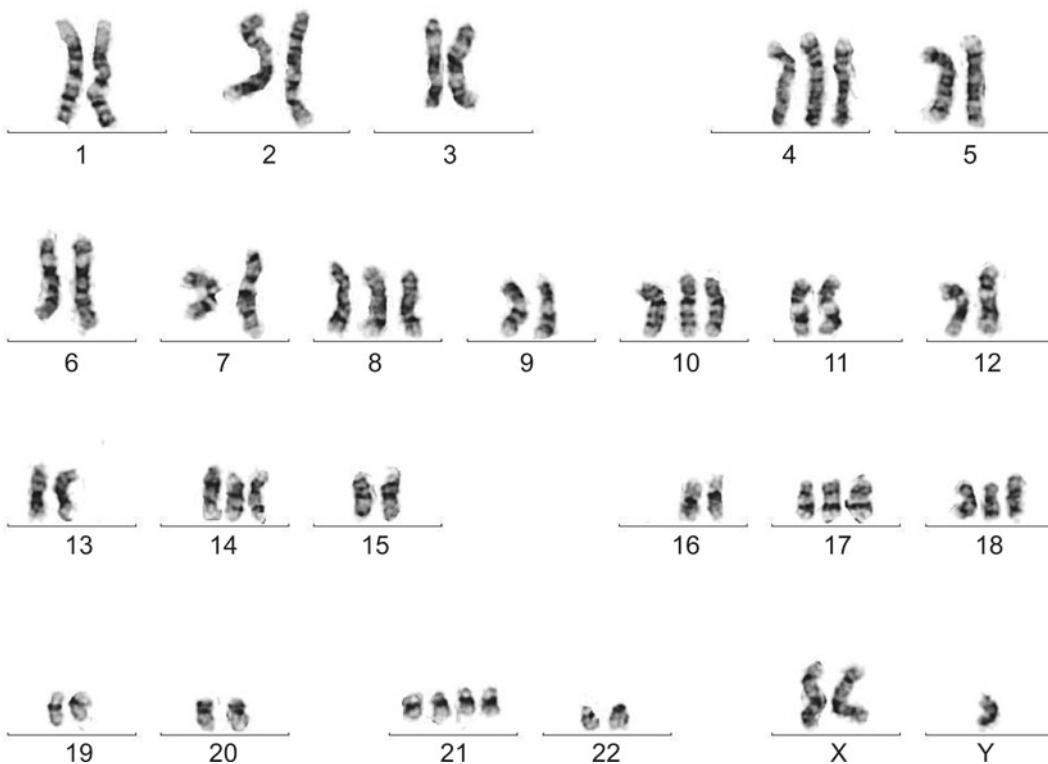


Fig. 15.13 Hyperdiploid karyogram of a patient with B-ALL. Chromosomes X, 4, 10, 14, 17, 18, and 21 are usually overrepresented. The best prognosis is associated with the presence of trisomies 4 and 10, as seen here

17 and 18 (70% of cases), and 10 (55% of cases). For reasons that are not completely clear, the prognostic outcome of adult B-ALL patients with hyperdiploidy is not as favorable as in children.

High hyperdiploidy is sometimes associated with the presence of poor prognostic markers such as t(9;22) and t(1;19) [284]. Another hyperdiploid group with 47–50 chromosomes has been described in approximately 10–15% of children and 2–5% of adults with B-ALL [259]. Trisomy 21 is again the most common numerical abnormality. Furthermore, patients in this group often also exhibit structural chromosome rearrangements [285].

t(12;21)(p13.2;q22.3)

Prior to the discovery of the cryptic t(12;21), which fuses *ETV6* at 12p13.2 with *RUNX1* at 21q22.3, at least 30% of B-ALL cases were thought to have a normal or prognostically informative karyotype [286]. This translocation is most often seen in children between 2 and 12 years old, with a peak at 3–5 years of age. These children have disease that is characterized by a long duration of first remission and excellent cure rates. t(12;21) is rare in adults with an incidence of 2–5% of cases.

After the first detection of the *ETV6-RUNX1* fusion by FISH, a large number of studies demonstrated that the t(12;21) is rarely the only abnormality present. Additional abnormalities include del(6q), del(11q), rearrangements of 12p, and del(16q), and often these abnormalities provide a clue that a t(12;21) might be present [287, 288] (Fig. 15.14).

Variant *ETV6-RUNX1* fusion patterns can be seen, as demonstrated by FISH studies. The most common of these variants is loss of the native *ETV6* allele, generally subsequent to a translocation or other rearrangement involving 12p [289]. Molecular studies have demonstrated that fusion with *ETV6* converts *RUNX1* from an activator to a repressor of transcription [290]. Molecular studies have also demonstrated that the presence of t(12;21) occurs early and is most likely present *in utero*. Using PCR, researchers confirmed the presence of t(12;21) in cord blood and perinatally obtained blood samples (Guthrie cards) of patients who later developed t(12;21)-associated ALL. Furthermore, these studies demonstrated the presence of both the typical and several variant *ETV6-RUNX1* fusion patterns at levels higher than what is seen in overt B-ALL [291, 292]. This implies that the t(12;21)-carrying cells present in those early samples

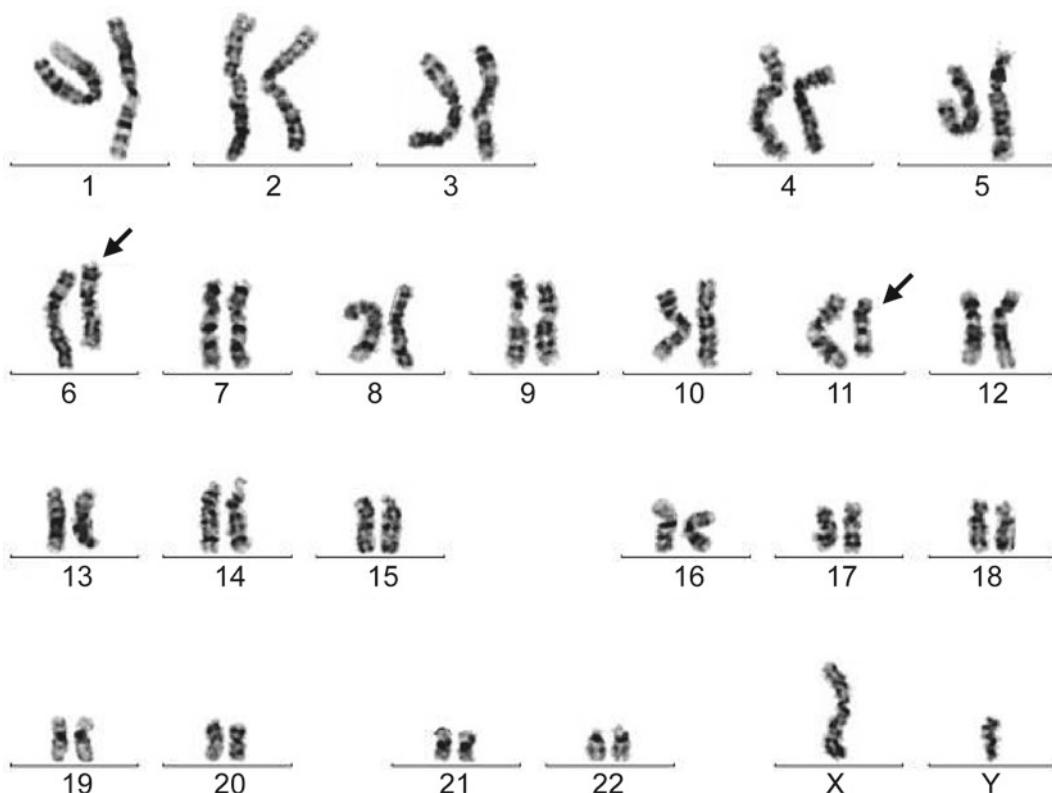


Fig. 15.14 Karyogram of a patient with B-ALL and cryptic t(12;21). Even if the translocation is cryptic at the conventional cytogenetic level, additional abnormalities, in this case deletions of 6q and 11q (arrows), often serve as a clue

most likely represent clones predisposed to leukemia development and that the acquisition of more genetic aberrations is needed for one of these clones to become fully malignant.

The usefulness of *ETV6-RUNX1* FISH studies goes beyond the ability to merely detect the fusion. Two additional abnormalities in particular can be detected with the *ETV6/RUNX1* FISH probes—deletions of *ETV6* and amplification of *RUNX1*. Specifically, a small group of B-ALL patients with a median age of 9 years have been found to have amplification of *RUNX1*, defined as multiple copies of this gene clustered in a marker chromosome. In the majority of the cases, this amplification is actually concentrated on an abnormal chromosome 21 (iAMP21) [293]. This abnormality is associated with an unfavorable prognosis characterized by high risk of relapse and a decreased event-free and overall survival at 5 years.

Rearrangements of 9p

Unbalanced rearrangements of the short arm of chromosome 9, usually leading to loss of 9p, have been observed in various neoplasms and are particularly frequent in ALL of both B- and T-lineages [294, 295]. In B-ALL, these deletions have been observed in approximately 10% of patients and often are not easily detected with conventional

cytogenetics. Therefore, FISH testing targeting the *CDKN2A* gene located at 9p21.3 is very helpful to demonstrate the deletion [296]. Homozygous deletions of this gene, frequently unsuspected, are also confirmed by FISH. Although the *CDKN2A* gene is thought to play a key role, other genes located at 9p, such as *MLLT3*, *PAX5*, *MTAP*, *IFN*, *JAK2*, and *PTPLAD2*, may play important roles as well [296]. In adults with B-ALL, the presence of del(9p) appears to be associated with improved outcome, whereas in children the same deletion is associated with poor outcome [297]. Besides pure deletions, other unbalanced rearrangements include dic(9;20)(p13.2;q11.2), dic(9;12)(p13.2;p12.2), and i(9)(q10), all of which are associated with an excellent prognostic outcome [298].

This being a textbook dedicated to cytogenetics, it is important to mention some of the “tricks of the trade.” One of these involves the apparent loss of chromosome 20 in some of the karyotypes of patients with B-ALL. FISH has shown that the apparent loss of chromosome 20 is actually the result of a dic(9;20)(p13.2;q11.2). This abnormality, which results in loss of most of 9p and 20q, might be difficult to detect in a sample with poor chromosome morphology, and the apparent loss of chromosome 20 can provide a helpful hint [299] (Fig. 15.15).

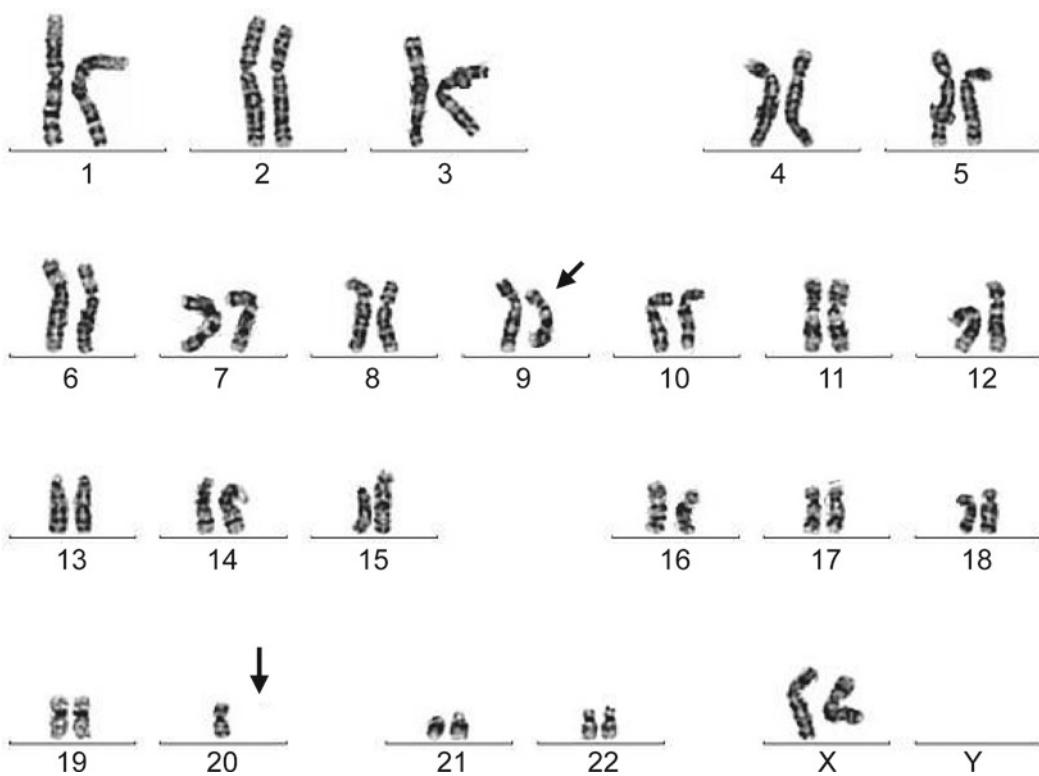


Fig. 15.15 Karyogram of a patient with B-ALL showing apparent loss of chromosome 20. Upon careful review, it is possible to recognize that the right chromosome 9 is in reality a dic(9;20)(p13.2;q11.2)

Rearrangements of 14q32.3 (*IGH@*)

Most of the *IGH@* rearrangements observed in B-ALL are balanced translocations. The most common are t(8;14) (q11.2;q32.3), inv(14)(q11.2q32.3), t(14;14)(q11.2;q32.3), t(14;19)(q32.3;q13.1), and t(14;20)(q32.3;q13.1) [300–302]. These translocations appear to be more frequent in adults (approximately 10% of cases) than in children (approximately 2% of cases) with B-ALL.

These recurrent *IGH@* translocations have in common the deregulated expression of unmutated CEBP genes (CCAAT enhancer-binding protein transcription factors) [303]. A rare t(5;14)(q31.1;q32.3), which leads to an *IL3-IGH@* fusion, has also been observed in B-ALL and is often associated with eosinophilia [304, 305]. Other reported *IGH@* translocations include t(6;14)(p22.3;q32.3) and t(9;14)(p13.2;q32.3), leading to a fusion of *IGH@* with *ID4* and *PAX5*, respectively [306, 307].

Rarely, deletions involving *IGH@* have been reported. Based on the small number of cases reported so far with this abnormality, the prognosis is unknown [308].

Two cryptic translocations, t(X;14)(p22.3;q32.3) and t(Y;14)(p11.3;q32.3), have recently been described in B-ALL, especially in patients with Down syndrome. These translocations lead to overexpression of the cytokine receptor gene, *CRLF2*, located in the X/Y pseudoautosomal

region, via juxtaposition of this gene to the *IGH@* enhancer. This deregulation can also arise via a cryptic interstitial deletion within the pseudoautosomal region, that is, del(X) (p22.3p22.33)/del(Y)(p11.3p11.32) via juxtaposition of *CRLF2* to the *P2RY8* promoter also located in the X/Y pseudoautosomal region. The deregulation of *CRLF2* is associated with activating mutations in *JAK2* [309]. Therefore, performing FISH with an *IGH@* probe is a valuable option, particularly in Down syndrome patients with a karyotype lacking acquired chromosome abnormalities.

Acute T-Cell Lymphoblastic Leukemia

Approximately 15% of children and 25% of adults diagnosed yearly with ALL have T-cell ALL/LBL [67]. There appears to be a prevalence of adolescent males (age range 12–19 years), but this is by no means exclusive [310]. Patients with this leukemia often present with mediastinal mass, CNS involvement, and leukocytosis [256].

The cytogenetic abnormalities most often found in T-ALL involve the T-cell receptors, specifically *TRA@* and *TRD@* at 14q11.2, *TRB@* at 7q35, and *TRG@* at 7p14 [311]. *TRD@* is contained within the *TRA@* locus, and thus *TRA@* is commonly used as reference.

Several translocations involving these genes have been reported; some are cryptic with conventional cytogenetics [312].

Table 15.11 Recurrent chromosome abnormalities and involved genes in T-ALL

Abnormality	Genes	% Children	% Adults
t(1;7)(p32;q34)	<i>TAL1-TRB@</i>	5	
t(1;14)(p32;q11.2)	<i>TAL1-TRA@</i>	10	
t(4;11)(q22.3;p15.4)	<i>RAP1GDS1- NUP98</i>		2–5
t(5;14)(q35.1;q32.2) (cryptic)	<i>TLX3-BCL11B</i>	20	10–20
del(6q)	Unknown	10–20	5–10
inv(7)(p15.2q34) or t(7;7)	<i>HOXA10-TRB@</i>	1–2	2
t(7;9)(q34;q31.2)	<i>TRB@-TAL2</i>	Rare	
t(7;9)(q34;34.3)	<i>TRB@-NOTCH1</i>	Rare	
t(7;10)(q34;q24.3)	<i>TRB@-TLX1</i>	7	30
t(7;11)(q34;p13)	<i>TRB@-LMO2</i>	5–10	
t(7;12)(q34;p13.3)	<i>TRB@-CCND2</i>	3–5	
t(7;14)(q34;q32.1)	<i>TRB@-TCL1A</i>	Rare	
t(7;19)(q34;p13.2)	<i>TRB@-LYL1</i>	Rare	
t(8;14)(q24.2;q11.2)	<i>MYC-TRA@</i>	2	
del(9)(p21.3) (homozygous/hemizygous)	<i>CDKN2A</i>	30	3
		80 by FISH	8–10
t(9;12)(p24.1;p13.1)	<i>JAK2-ETV6</i>	Rare	
t(10;14)(q24.3;q11.2)	<i>TLX1-TRD@</i>	5–10	
t(11;14)(p13;q11.2)	<i>LMO2-TRA@</i>	5–10	
t(11;14)(p15.4;q11.2)	<i>LMO1-TRA@</i>	Rare	
t(12;14)(p13.3;q11.2)	<i>CCND2-TRA@</i>	2–5	
12p rearrangements	<i>ETV6</i> , others	10–15	5
inv(14)(q11.2q32.1) or t(14;14)(q11.2;q32.1)	<i>TRA@-TCL1A</i>	Rare	Rare

Except for the Y chromosome, virtually every chromosome has been involved with one or more of these T-cell receptors. The most common translocations involve chromosomes 1, 7, 9, 10, 11, 12, and 14 [313] (see Table 15.11).

A rare but recurrent abnormality seen in T-ALL is inv(14)(q11.2q32.1) or t(14;14)(q11.2;q32.1). Either of these rearrangements generally leads to overexpression of *TCL1A/TCL1B* at 14q32.1 via relocation to the *TRA@/TRD@* locus at 14q11.2. *TCL1A* and *TCL1B* have approximately 65% homology, and therefore overexpression of one affects the other [314]. Other genes in the vicinity of *TCL1A/TCL1B*, such as *BCL11B* at 4q32.3, can also be involved, and thus overexpressed, as a result of this inversion [315]. The prognosis associated with this abnormality is unknown.

Another frequent abnormality is deletion of 9p21.3 leading to loss of *CDKN2A*. This gene encodes the p14ARF protein, which binds to and inactivates HDM-2. HDM-2 in turn targets the TP53 tumor suppressor protein for degradation [316]. Therefore, deletions of 9p21.3 result in reduction in the amount of p14ARF, loss of inhibition of HDM-2, and subsequent inhibition of TP53 protein production. This deletion can be seen in about 30% of cases by conventional cytogenetics and about 80% of cases with FISH [317]. This indicates that the majority of deletions involving 9p are cryptic. Approximately 50% of these deletions affect both

chromosome 9 homologs. This deletion is less frequent (8–10%) in adults with T-ALL.

A relatively new abnormality, discovered by chance while investigating the frequency of *BCR-ABL1* fusion in T-ALL, is the amplification of *ABL1* [318, 319]. The rearrangement is a cryptic episomal *NUP214-ABL1* translocation and occurs in approximately 6% of patients, most of whom are children. FISH with the *BCR-ABL1* probes and RT-PCR can both detect T-ALL patients with *ABL1* amplification. The quick identification of this rearrangement is fundamental in the clinic because this T-ALL subset is Gleevec® sensitive but may become resistant due to the development of additional mutations (9p21.3 deletions often accompany this amplification) [320]. *ABL1* quantitative RT-PCR may be easily applied to monitor minimal residual disease.

Mature B-Cell Neoplasms

Culturing of Mature B-Cell Neoplasms for Cytogenetic Analysis

The detection of chromosome abnormalities in mature B-cell lymphomas by conventional cytogenetics is dependent upon the culturing method used. Historically, the utilization of B-cell mitogens has proven effective in promoting the growth of the abnormal clonal population in culture [321, 322].

Table 15.12 Neoplasms stimulated with DSP-30/IL-2 and associated cytogenetic abnormality rates. A. Meloni-Ehrig, personal data

Diagnosis ^a	Number of cases	Abnormal cases	Normal cases	% abnormal
v-CLL	14	14	0	100
HCL	13	13	0	100
b-MCL	8	8	0	100
B-PLL	2	2	0	100
sMZBCL	29	28	1	97
DLBCL	36	32	4	89
CLL	430	367	63	85
LPL	10	8	2	80
MCL	19	15	4	78
BL	6	4	2	66
MALT	14	9	5	64
HL	3	1	2	33
FL	35	9	26	26
NHL ^b	34	1	33	3
LPD ^b	12	0	12	0
Totals	665	511	154	77

Abbreviations: *BL* Burkitt lymphoma, *CLL* chronic lymphocytic leukemia, *v-CLL* variant type, *DLBCL* diffuse large B-cell lymphoma, *FL* follicular lymphoma, *HCL* hairy cell leukemia, *HL* Hodgkin lymphoma, *LPD* lymphoproliferative disorder, *MCL* mantle cell lymphoma, *b-MCL* mantle cell lymphoma, blastoid type, *MALT* mucosa-associated lymphoid tissue, *sMZBCL* marginal zone B-cell lymphoma, splenic type, *NHL* non-Hodgkin lymphoma, *B-PLL* B-cell prolymphocytic leukemia, *LPL* lymphoplasmacytic lymphoma

^aDefined according to immunophenotypic and/or morphologic analyses

^bNo specific subtype

The recent introduction of CpG-oligodeoxynucleotides (CpG-ODNs) has increased the detection of clonal abnormalities. CpG-ODNs are made of short single-stranded DNA, are known to activate cells of the immune system in a sequence-dependent manner, and are also known to promote proliferation of chronic lymphocytic leukemia (CLL) cells [323]. One of these CpG-ODNs is DSP-30. In combination with interleukin-2 (IL-2), DSP-30 has proven to be effective in increasing the detection of chromosome abnormalities with conventional cytogenetics when compared to other traditional well-established B-cell mitogens, not only in CLL but also in other mature B-cell lymphoid neoplasms [8, 324, 325]. Table 15.12 shows the author's experience using the DSP-30/IL-2 cocktail. Table 15.13 lists recurrent chromosome abnormalities in B-cell neoplasms.

Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma (NHL) comprises a heterogeneous group of disorders characterized by localized proliferation of lymphocytes. The WHO recognizes that genetic anomalies represent one of the most reliable criteria for classification of malignant lymphomas [67]. Some tend to be confined to a particular lymphoma—for example, t(14;18)(q32.3;q21.3) in follicular lymphoma—whereas others are nonspecific and can be seen in a variety of lymphomas, such as del(6q) and del(14q).

Most NHL cases are of B-cell origin and are characterized by rearrangements involving the immunoglobulin genes: *IGH@* at 14q32.3, *IGK@* at 2p12, and *IGL@* at 22q11.2 [321]. Translocations can be simple or complex and at times have partial deletions or partial duplications of the genes involved in the translocation. By far, the majority of translocations involve *IGH@* [36, 37].

The most common associations between chromosome anomalies and specific lymphomas include t(14;18) (q32.3;q21.3) and follicular lymphoma (FL), t(8;14) (q24.2;q32.3) and Burkitt lymphoma (BL), t(11;14) (q13;q32.3) and mantle cell lymphoma (MCL), and t(11;18)(q21;q21.3) and mucosa-associated lymphoid tissue (MALT) lymphoma [37].

Follicular Lymphoma

Follicular lymphoma (FL) affects 1 in 24,000 individuals per year in the USA [322]. The majority of patients have an indolent disease, and few develop a more aggressive form [326]. Approximately 85–90% of patients with FL and 25–30% of patients with diffuse large B-cell lymphoma (DLBCL) exhibit the t(14;18)(q32.3;q21.3), which results in fusion of *BCL2* at 18q21.3 and *IGH@* at 14q32.3 [327] (Fig. 15.16a). This translocation, which is one of the most common abnormalities in NHL, repositions the *BCL2* oncogene so that it is under the control of *IGH@* promoter, leading to overexpression of *BCL2* and therefore overproduction of *BCL2*, one of the proteins involved in regulation of apoptosis [52]. Variant translocations, such as t(2;18)(p12;q21.3) and t(18;22)(q21.3;q11.2) involving *IGK@* at 2p12 or *IGL@* at 22q11.2, respectively, have been described in both FL and DLBCL [328]. These translocations also lead to the overexpression of *BCL2*.

Numerous additional chromosome abnormalities are identified by conventional cytogenetics. In addition to t(14;18), certain numerical abnormalities, specifically trisomies 2, 7, and/or 8, are associated with a more favorable course of disease when compared with patients with structural abnormalities, specifically del(1p), del(1q), del(6q), +der(18), or del(22q), or gain of an X chromosome or chromosome 12, which are associated with an unfavorable outcome [29, 329]. Progression of FL to DLBCL occurs in 60–80% of cases and is accompanied by accumulation of secondary abnormalities, including +7, del(10q), del(6q), and/or +der(18) [330] (Fig. 15.16b).

The lifespan of the FL cells is very short; therefore, longer transit times and longer culture times should be avoided whenever possible. B-cell mitogens do not appear to promote the mitotic activity of the clonal population and more success is obtained with overnight cultures. In contrast to what is seen in adults, neither the t(14;18) or variant translocations nor overexpression of *BCL2* is observed in children with FL, who are found to show

Table 15.13 Mature B-Cell lymphoid neoplasms and associated recurrent chromosome abnormalities, according to the World Health Organization

Mature B-cell neoplasm	Primary chromosome abnormalities	Chromosome abnormalities during progression
CLL/SLL	del(6q), del(11q), del(13q), +12, del(17p), t(2;14), t(14;19), t(14;18),	del(14q), +18, t(8;14) or variants, 13q rearrangements
PLL	del(6q), del(11q), +12, del(13q), del(17p)	<i>MYC</i> translocations
MCL	t(11;14) and other <i>CCND1</i> variants; t(6;14) and <i>CCND3</i> variants; t(12;14) and <i>CCND2</i> variants	Gains of chromosomes 3, 8, and 15q; losses of 1p, 8p, 9p, 11q, and 13q.
Splenic MZBCL	del(7q); +3, +12, +18	del(17p)
Nodal MZBCL	+12, +18, 3q27 rearrangements	del(17p)
MZBCL (MALT type)	t(11;18) or t(14;18); +3 with or without +18; t(1;14) and variants; (3;14)	del(17p)
HCL	+5, del(6q), del(7q), +12, del(17p)	Variable
LPL	del(6q), +4, +3, +7	del(17p)
FL	t(14;18) and variants	Gains of chromosomes X, 2, 7, 8, and 12; del(1p), del(1q), del(6q), del(10q), +der(18), del(22q)
DLBCL	t(14;18), 3q27 rearrangements	1q and 14q rearrangements, del(6q), del(10q), del(11q), del(13q), del(17p), +X, +7, +12, +18
BL	t(8;14) and variants	Gain of 1q
PCM	<i>High risk</i> : hypodiploidy, 1p/1q rearrangements, del(13q), t(4;14), t(14;16), t(14;20), del(17p) <i>Standard risk</i> : hyperdiploidy with gain of odd number chromosomes (+5, +9, +11, +15), without high-risk markers; t(11;14)	del(4q), del(6q), del(16q), del(20q), <i>MYC</i> translocations
PBL	Similar to high-risk PCM: hyperdiploidy with gain of odd number chromosomes (+5, +9, +11, +15) and high-risk markers [rearrangements of chromosome 1, del(13q), del(17p)]	<i>MYC</i> translocations
Unclassifiable—DLBCL/ Burkitt	t(8;14) and variants, t(14;18) and/or 3q27 rearrangements, and/or t(11;14)	Same as DLBCL
Unclassifiable—DLBCL/ Hodgkin	3q27 rearrangements, del(17p)	Variable
HL	Hyperdiploidy, del(1p), del(6q), del(7q), del(13q), del(16q), del(17p), gain of 2p, 9p, +12, rearrangements of 3q27	Variable

Abbreviations: *CLL* chronic lymphocytic leukemia, *SLL* small lymphocytic lymphoma, *PLL* prolymphocytic leukemia, *MCL* mantle cell lymphoma, *MZBCL* marginal zone B-cell lymphoma, *MALT* mucosa-associated lymphoid tissue, *HCL* hairy cell leukemia, *LPL* lymphoplasmacytic lymphoma, *FL* follicular lymphoma, *DLBCL* diffuse large B-cell lymphoma, *BL* Burkitt lymphoma, *PCM* plasma cell myeloma, *PBL* plasmablastic lymphoma, *HL* Hodgkin lymphoma

variable rearrangements of 14q32.3 (*IGH@*) and 3q27 (*BCL6*) [331].

Burkitt Lymphoma

This lymphoma is named for Dr. Denis Burkitt, who first described it in 1958 [332]. Burkitt lymphoma (BL) has an incidence of approximately 1,200 cases per year in the USA and affects children as well as adults [333].

Three immunoglobulin gene translocations, all affecting *MYC* at 8q24.2, are seen in BL [334] (Fig. 15.17). The most common of these, t(8;14)(q24.2;q32.3), is detected in about 75–80% of patients. Two variant translocations, t(8;22)(q24.2;q11.2) and t(2;8)(p12;q24.2), are seen in ten and 5% of patients, respectively. All three translocations involve *MYC* and one of the three immunoglobulin genes (*IGH@* at 14q32.3, *IGK@* at 2p12, or *IGL@* at 22q11.2) and lead to

overexpression of *MYC*. In the majority of BL cases, the reciprocal translocation involving *MYC* and one of the immunoglobulin genes is the sole cytogenetic abnormality.

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of neoplasms characterized by a diffuse infiltrate of large lymphoid cells. Some of these lymphomas are clearly defined, while the classification of others remains challenging [67, 335]. In general, these lymphomas affect individuals of increasing age, and their presence in children and young adults is uncommon.

In addition to being heterogeneous at the cellular level, these neoplasms are also cytogenetically diverse. Among the recurrent abnormalities is t(14;18)(q32.3;q21.3), which involves *IGH@* and *BCL2*. This translocation is observed in

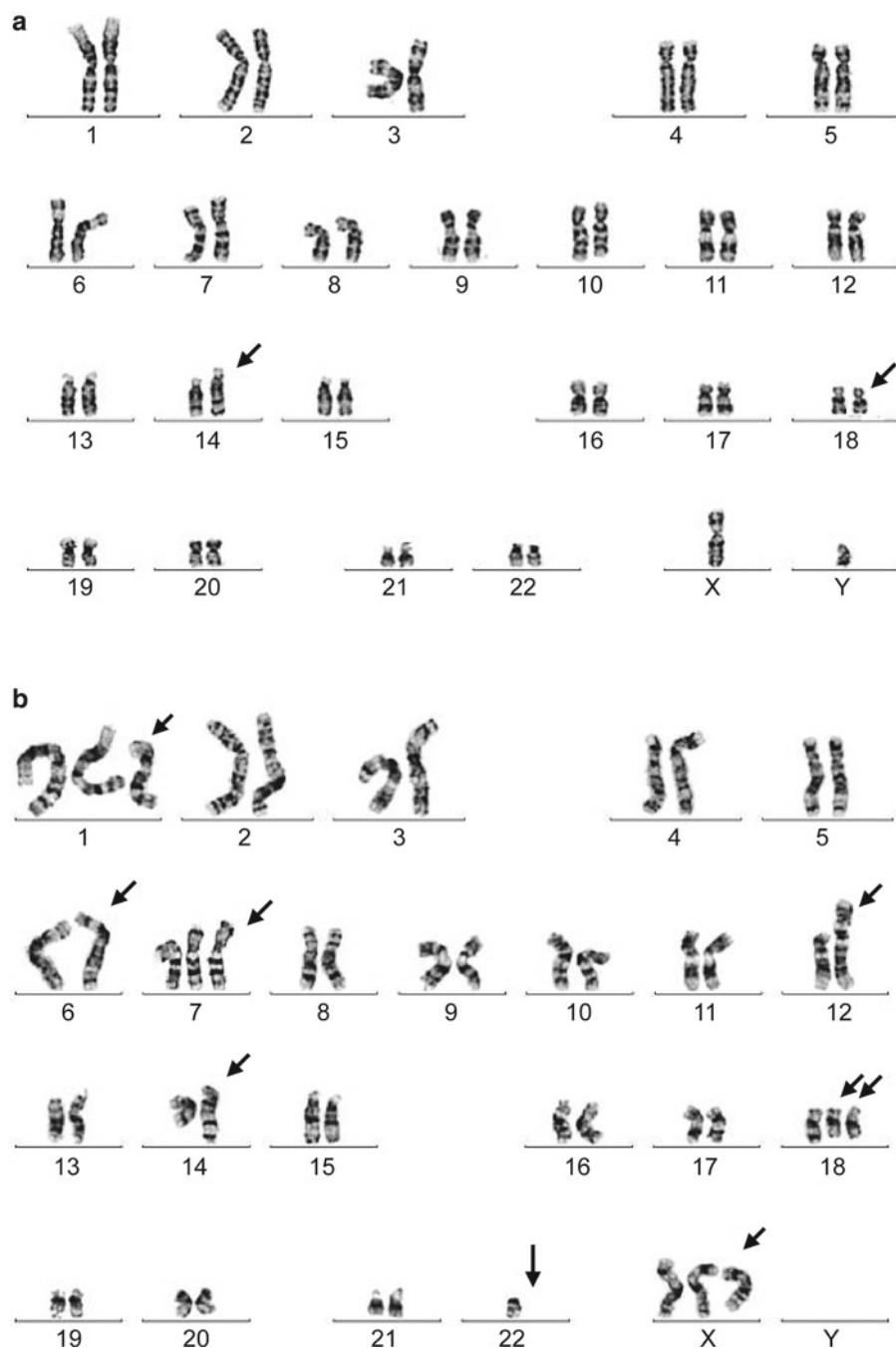


Fig. 15.16 (a) Karyogram of a patient with follicular lymphoma showing t(14;18)(q32.3;q21.3) as the sole abnormality and (b) together with other rearrangements in a patient in progression to diffuse large B-cell lymphoma. Typical abnormalities during progression are the gain of chromosomes X, 7, and the der(18), as seen in this case

25–30% of DLBCL cases [336]. t(14;18) in DLBCL is usually present in the context of a complex karyotype. Additional abnormalities include rearrangements of 1q and 3q, del(6q), +7, +8, del(10q), del(11q), +12, del(13q), rearrangements of 14q and 17p, +der(18)t(14;18), and +X (Fig. 15.16b). It is believed that the more complex the karyotype the worse the

prognostic outcome [337]. Translocations involving 3q27 (*BCL6*) have been detected in approximately 35% of patients with DLBCL. More than 30 different partner genes have been translocated with this locus, the most recurrent of which include 2p12, 3q29, 4p13, 6p21.2, 6p22, 7p12, 8q24.2, 11q23, 13q14, 14q32.3, 15q22, 16p13, 17q11.2, 18p11.2,

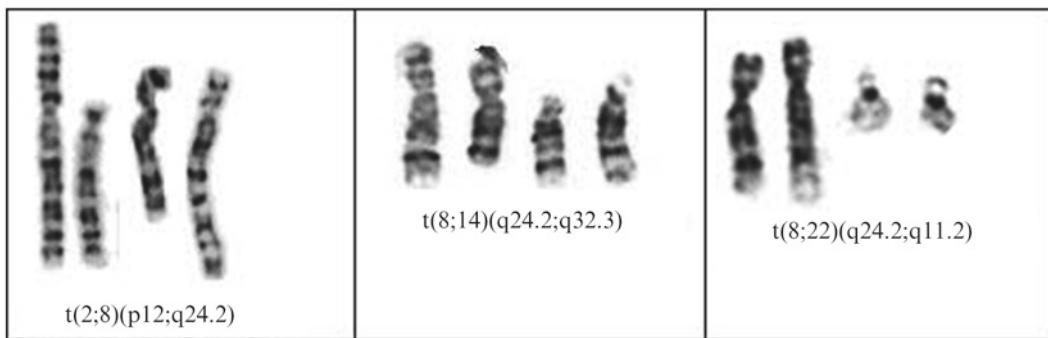


Fig. 15.17 Partial karyograms illustrating the three translocations involving *MYC* (at 8q24.2) in Burkitt lymphoma. In these translocations, the relocation of *MYC* to the immunoglobulins *IGK@*, *IGH@*,

and *IGL@* loci, located at 2p12, 14q32.3, and 22q11.2, respectively, leads to its overexpression

and 22q11.2 [338]. These translocations juxtapose different promoters derived from partner chromosomes to the *BCL6* coding domain causing overexpression of this oncogene. Since *BCL6* functions as a transcriptional repressor of genes containing its binding sites, its overexpression leads to transcription deregulation [339]. Many 3q27 rearrangements are not detectable with conventional cytogenetics. Therefore, FISH or PCR are useful to provide evidence of them. The prognostic significance of *BCL6* rearrangements is not clear, and different outcomes have been reported [340].

Other recurrent abnormalities observed in DLBCL include partial or complete gain of chromosome 3, specifically 3q; loss of chromosome 6; and gain of chromosome 18 and t(14;15)(q32.2;q11.2), which leads to fusion of the *BCL8* at 15q11.2 with *IGH@* at 14q32.3. It is thought that among these abnormalities, only gain of chromosome 3 is associated with an adverse prognosis [341].

B-Cell Lymphoma, Unclassifiable, with Features Intermediate Between Diffuse Large B-Cell Lymphoma and Burkitt Lymphoma

This is a new lymphoma category described by the WHO in 2008 [67]. As its name implies, this lymphoma does not meet the criteria for either classical BL or DLBCL [342]. This neoplasm occurs in fewer than 2% of lymphoma patients and is associated with aggressive histology and poor prognosis [343].

These cases have translocations involving *MYC* with either immunoglobulin or non-immunoglobulin genes; therefore, t(8;14), t(2;8), and t(8;22) have been observed. Among the translocations involving *MYC* and non-immunoglobulin genes, the most common is t(8;9)(q24.2;p13) [344].

The differentiator between typical BL and these unclassifiable lymphomas is the simultaneous presence of rearrangements involving *MYC*, *BCL2*, and/or *BCL6* [345]. As such, this entity is also known as double-hit or triple-hit lymphoma (Fig. 15.18). FISH to investigate the

presence of rearrangements involving *MYC*, *BCL2*, and/or *BCL6* is strongly suggested. FISH is very helpful in these cases, as rearrangements are often complex and difficult to sort out.

B-Cell Lymphoma, Unclassifiable, with Features Intermediate Between Diffuse Large B-Cell Lymphoma and Hodgkin Lymphoma

This second category of unclassifiable lymphomas includes DLBCL with features of classic Hodgkin lymphoma (HL) [342]. The diagnosis of these cases is difficult. However, a large B-cell lymphoma with a strong expression of CD15, or a classic HL with a strong and diffuse expression of CD20 and CD79, would support inclusion in this category. Similarly to what has been observed with DLBCL/BL, the prognosis of this neoplasm is worse than that of DLBCL or HL. The genetics of this entity is not well established. However, earlier reports have implicated rearrangements of 3q27 (*BCL6*) and 17p13.1 (*TP53*) [346].

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is an aggressive lymphoma that affects primarily adult male patients (median age 65 years) and represents 5–10% of all lymphoma cases in the USA [347]. Typical immunophenotypic findings are positivity for CCND1, CD20, CD19, and CD5 and negativity for CD10, CD23, and BCL16.

With conventional cytogenetics, about 70% of MCL patients exhibit a t(11;14)(q13;q32.3) (Fig. 15.19). This translocation relocates the *CCND1* gene at 11q13, a gene involved in cell cycle control, to the *IGH@* locus on chromosome 14. Similar to what occurs with other immunoglobulin gene translocations, t(11;14) leads to overexpression of *CCND1* [348].

The use of B-cell mitogens such as DSP-30/IL-2 has proven useful to promote the growth of the abnormal lymphocytes in culture [8]. The best method, however, to detect

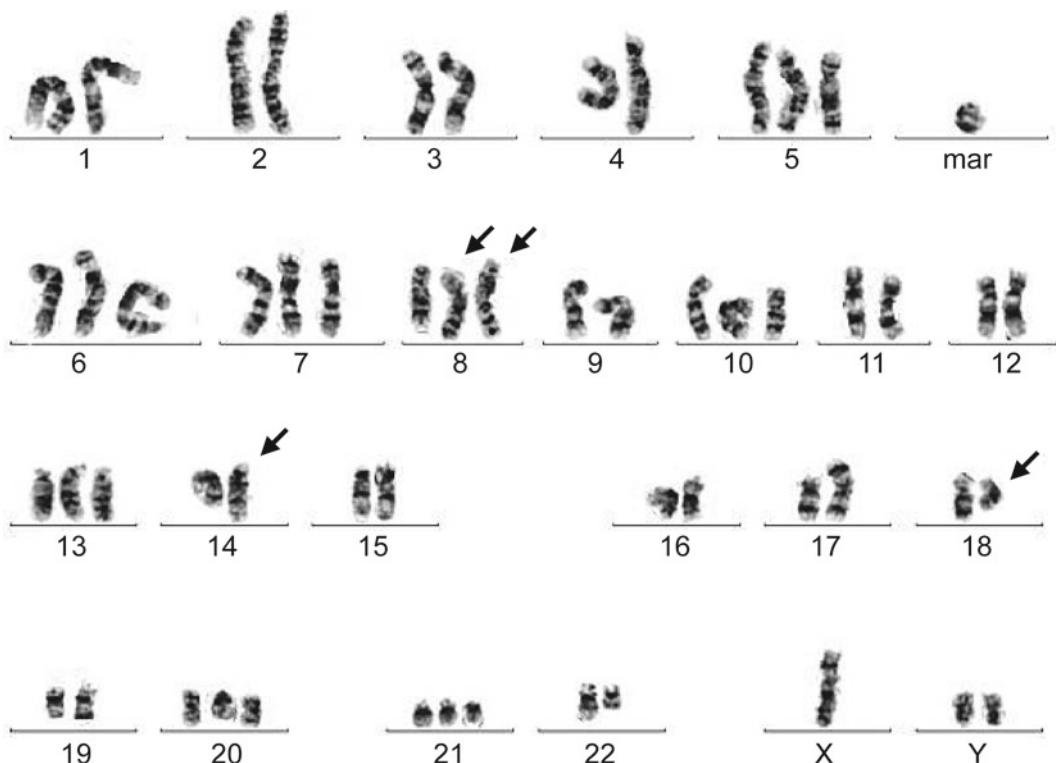


Fig. 15.18 Karyogram of a patient with the aggressive “double-hit” lymphoma. In this particular case, the complex karyogram shows the simultaneous involvement of *MYC* and *BCL2* rearrangements in the form of t(8;14;18)(q24.2;q32.3;q21.3). Arrows point to the abnormal chromosomes

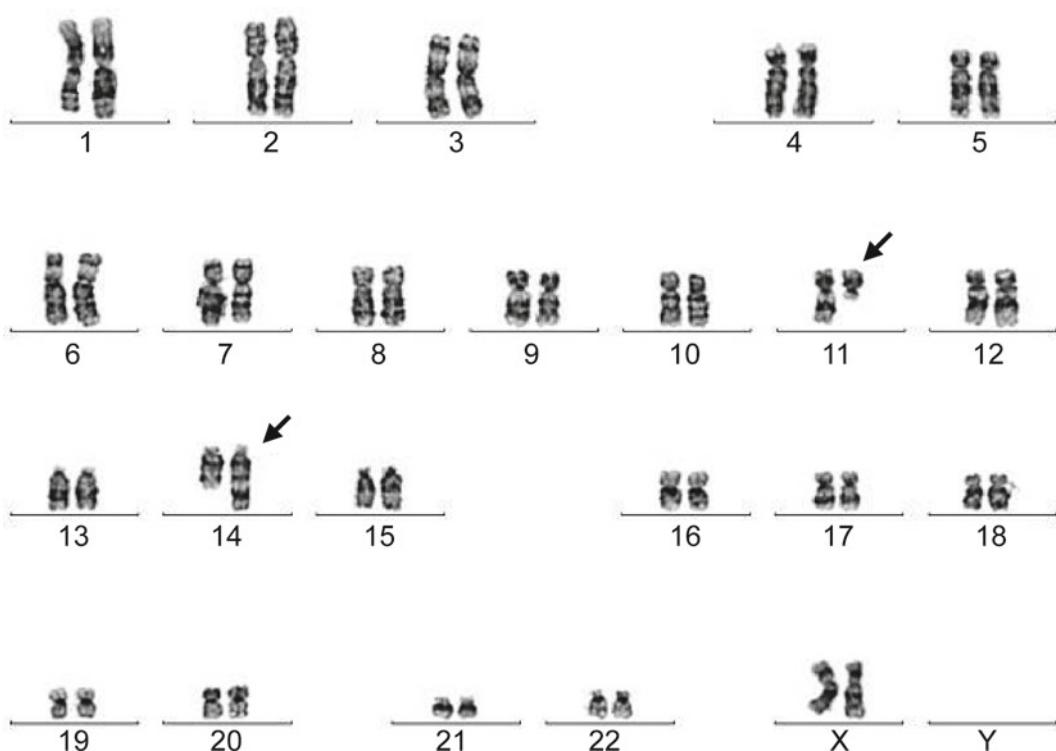


Fig. 15.19 Karyogram of a patient with mantle cell lymphoma at diagnosis. In these cases, t(11;14)(q13;q32.3) is usually the sole abnormality

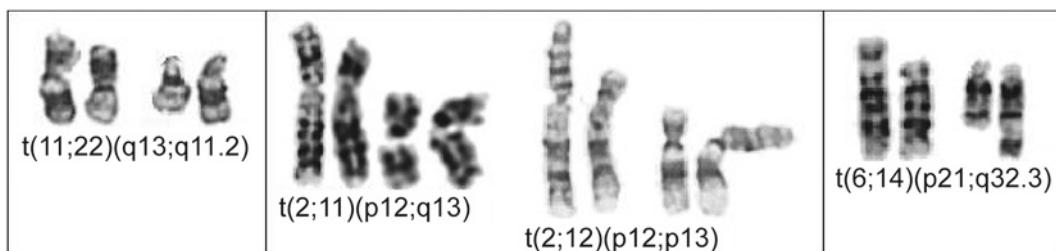


Fig. 15.20 Partial karyograms illustrating translocations observed less frequently in mantle cell lymphoma. The translocations (11;22) (q13;q11.2) and (2;11)(p12;q13) fuse *CCND1* with *IGL@* and *IGK@*, respectively. The t(2;12) (p12;p13) fuses *CCND2* with *IGK@*, and

t(6;14)(p21;q32.3) leads to fusion of *CCND3* with *IGH@*. Other combinations of these key genes are possible but much less frequent than those illustrated here

the presence of t(11;14) is FISH, with a detection rate of up to 95% [349]. Various archival samples, including touch preparations and non-decalcified paraffin-embedded tissue, can be used for FISH testing.

Variant translocations involving *CCND1* and *IGK@* [t(2;11)(p12;q13)], or *CCND1* and *IGL@* [t(11;22) (q13;q11.2)], have been observed in a limited number of cases, but their detection is equally important for the diagnosis of MCL [350, 351] (Fig. 15.20). An *IGH@*-*CCND1* FISH study showing three signals for *CCND1* should be evaluated further if MCL is a concern since the third *CCND1* signal could be the result of a splitting of this region due to a translocation involving an immunoglobulin gene other than *IGH@*.

About 5% of patients have been found to be *CCND1*-negative because they lack *CCND1* expression by IHC and do not exhibit t(11;14)/*CCND1-IGH@* by either conventional cytogenetics or FISH [351, 352]. Some of these cases involve other cyclin genes such as *CCND2* at 12p13 and *CCND3* at 6p21, specifically the cryptic t(12;14)(p13;q32.3) and the t(6;14)(p21;q32.3), respectively (Fig. 15.20). MCL rarely displays variant translocations involving *CCND2* or *CCND3* with either *IGK@* or *IGL@* [353].

Furthermore, a novel cryptic t(2;14)(p24;q32.3) involving *MYCN* and *IGH@* has been observed in two patients with blastoid MCL [354]. The blastoid variant of MCL, as the term implies, is associated with a very aggressive clinical course and shorter survival than typical MCL [355].

Both *CCND1*-positive and *CCND1*-negative patients might have the same secondary chromosome abnormalities, which include partial or complete gain of chromosomes 3 and 8, gain of 15q, and losses of 1p, 8p, 9p, 11q, and 13q. Highly complex chromosome complements, particularly those with loss of 9p, 17p, and gain of 3q and 8q, have been described in blastoid variant of MCL [355] (Fig. 15.21).

The t(11;14) has been previously associated with other B-cell lymphomas, including atypical CLL, sMZBCL, and B-PLL, and with PCM [356]. However, presently, most MCL and some PCM cases are thought to be characterized by the t(11;14). The others are now considered variants or leukemic variants of MCL [67].

t(11;14) is not limited to MCL as it is also found in other B-cell neoplasms, including CLL, sMZBCL, B-PLL, and PCM [356]. See following sections.

Marginal Zone B-Cell Lymphoma

Marginal zone B-cell lymphoma (MZBCL) comprises a group of indolent NHL arising from the marginal zone of lymphoid tissues and accounts for approximately 10% of NHL [357]. According to the 2008 WHO classification, this lymphoma is subdivided into three subtypes: splenic (sMZBCL), nodal, and MALT or extranodal [67]. The splenic and nodal subtypes are rare (1% of NHL), whereas MALT is relatively frequent (8% of all NHL).

Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

This particular subtype of MZBCL has received much attention particularly because of its association between low-grade gastric MALT lymphoma and *Helicobacter pylori* infection [358]. There is also evidence that suggests that chronic antigenic stimulation in autoimmune diseases, such as Hashimoto thyroiditis, contributes to an increased risk of developing MALT lymphoma [357].

One of the specific aberrations occurring in 50% of MALT lymphoma cases is t(11;18)(q21.3;q21.3), which leads to a fusion between *BIRC3* (*API2*) at 11q21.3 and *MALT1* at 18q21.3 [359]. When present, this translocation is usually the only chromosome abnormality. Interestingly, it is also the only recurrent translocation in NHL that does not involve an immunoglobulin gene. The *BIRC3-MALT1* fusion is easily identified using a dual-color *BIRC3-MALT1* FISH probe or the “break-apart” strategy of the dual-color *MALT1* probe

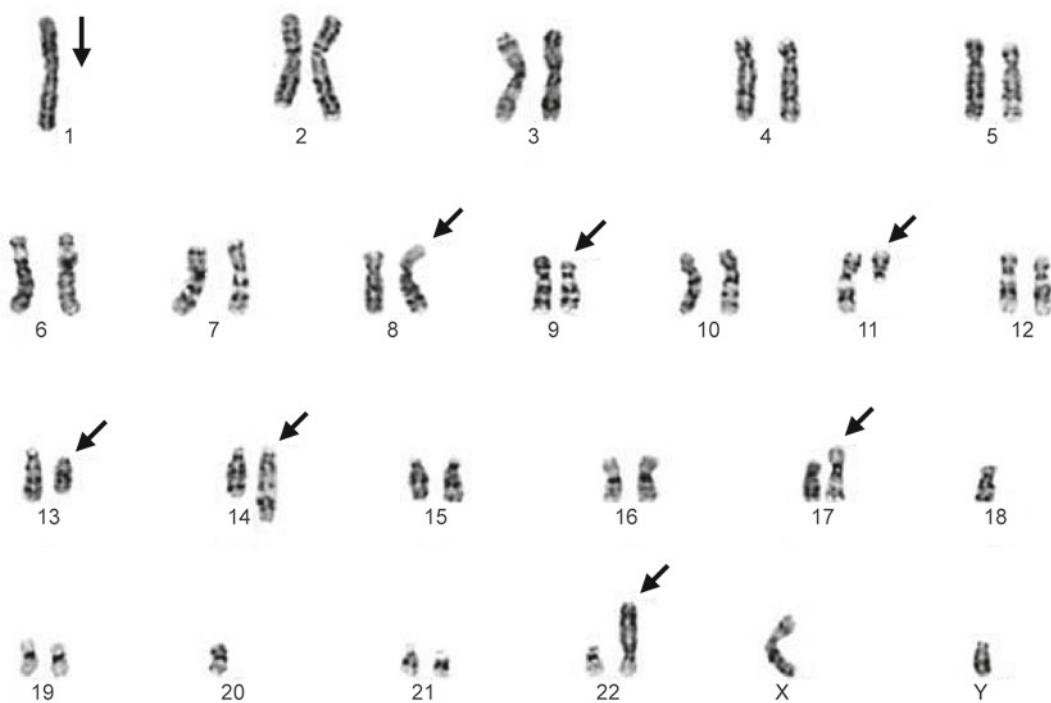


Fig. 15.21 Complex karyogram showing various abnormalities in addition to t(11;14)(q13;q32.3). Recurrent abnormalities in these karyograms include losses of 1p, 8p, 9p, 13q, and 17p, and gain of 3q. Similar karyotypes have been described in blastoid variant of mantle cell lymphoma

(see Chap. 17). The other specific *MALT1* translocation is t(14;18)(q32.3;q21.3), which relocates the *MALT1* gene from 18q21.3 to the *IGH@* locus at 14q32.3. This translocation has been observed in approximately 2% of MALT lymphoma cases.

In contrast to *BIRC3-MALT1*-positive cases, patients with *IGH@-MALT1* fusion have disease outside the gastrointestinal tract, usually presenting with ocular, skin, liver, or salivary gland tumors [360]. With conventional cytogenetics, this translocation is indistinguishable from the t(14;18) with involvement of *BCL2* seen in follicular lymphoma. Therefore, FISH studies are useful to distinguish the two translocations. The combination of the *BIRC3-MALT1* dual fusion and *IGH@* break-apart probes is most practical to detect the presence of either of these rearrangements.

Two other translocations seen in approximately 5% of the cases include t(1;14)(p22.3;q32.3) and its variant t(1;2)(p22.3;p12), which relocate *BCL10* from 1p22.3 to the *IGH@* locus at 14q32.3 or the *IGK@* locus at 2p12, respectively [361].

All four translocations described previously are known to activate the nuclear factor (NF)-κB activation pathway [362]. Another translocation seen in rare cases of MALT lymphoma is t(3;14)(p13;q32.2), which leads to fusion of the *FOXP1* gene on chromosome 3p14.1 with *IGH@* resulting in overexpression of FOXP1F [361].

Splenic Marginal Zone B-Cell Lymphoma

Splenic marginal zone B-cell lymphoma (sMZBCL) is a rare neoplasm that typically affects individuals aged greater than 60 years. It represents 1–2% of non-Hodgkin lymphomas. As the name implies, the primary site of involvement is the spleen, but bone marrow and peripheral blood might be involved as well [363].

Immunophenotypically, this lymphoma is positive for CD19, CD20, CD22, and CD79b. The majority of cases also lack expression of CD5, CD10, and CD23 [363]. Cytogenetically, they are characterized by recurrent numerical and structural abnormalities [364]. One of the most common structural abnormalities is deletion of 7q (30–40% of cases). The deletion appears to be concentrated to bands 7q22~q32 and can be detected with conventional as well as molecular cytogenetics [365]. Other recurrent chromosome abnormalities include partial or complete trisomy 3, particularly involving the long arm (30–50% of cases), and partial or complete trisomy 12 (20–30% of cases) [366, 367]. In addition to these abnormalities, the karyotype of some aggressive cases often includes a deletion of 17p which leads to loss of *TP53* [368].

Nodal Marginal Zone B-Cell Lymphoma

Nodal marginal zone B-cell lymphoma (MZBCL) is a rare low-grade neoplasm comprising 1–2% of all NHL in adults. This lymphoma contains a heterogeneous group of cells that

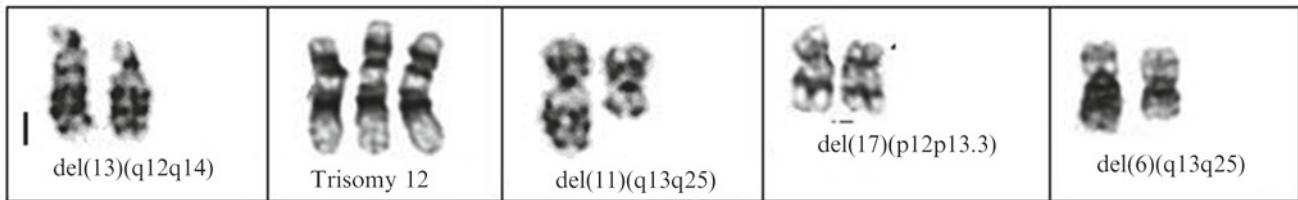


Fig. 15.22 Partial karyograms illustrating recurrent abnormalities observed in CLL, in order of frequency

express CD19, CD20, and CD79a, with variable expression of CD138 depending on the degree of plasma cell differentiation. Like other MZBCLs, it is negative for CD5, CD10, and CD23 [369]. Cytogenetically, it is not well defined, but partial or complete trisomy 12 appears to be more common than in sMZBCL with a frequency of 40–50% [369].

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) is an indolent neoplasm characterized by the expansion of small B-lymphocytes with variable plasmacytoid differentiation that are negative for CD5, CD10, and CD23 [370]. LPL represents 5% of all NHL affecting adult individuals and often is associated with Waldenström macroglobulinemia (WM). There is also an association between this lymphoma and hepatitis C virus infection [371].

Although most cases of LPL appear to be sporadic, familial cases have been reported. A familial history of at least one first-degree relative with either LPL or another B-cell disorder has been observed in approximately 20% of patients [372].

Chromosome abnormalities have been observed in this neoplasm. However, it is important to specify that these rearrangements tend to occur in bone marrow-based LPL and are not typically seen in nodal LPL [67]. The most common rearrangement is deletion of 6q, which is found approximately 50% of the cases, followed by gain of chromosome 4 (20%), and other less frequent abnormalities such as del(17p) and gains of chromosomes 3 and 7 [373–375]. The clinical or pathogenic significance of chromosome abnormalities in LPL is unclear.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) is a B-cell neoplasm that leads to proliferation of mature, normal-appearing lymphocytes in the peripheral blood, bone marrow, spleen, and lymph nodes. These lymphocytes are typically positive for CD5, CD19, CD20, and CD23 and negative for CD10 [376]. The term SLL is used to indicate the aleukemic counterpart that is morphologically and genetically comparable to CLL but without significant lymphocytosis and mostly nodal involvement [376]. Although CLL accounts for 30% of all adult leukemias, the apparent incidence of this neoplasm has fluctuated

noticeably over the years [377]. According to the WHO, the current incidence rate is estimated to be 2–6 cases per 100,000 individuals per year, increasing to up to 12.8 cases per 100,000 for individuals at age 65 and older [67].

The most important risk factor for the development of CLL is family history. In fact, among patients with newly diagnosed CLL, 8–10% have one or more blood relatives with this neoplasm [378]. The prognosis of familial and nonfamilial cases is highly variable, with survival varying from months to several years, and is highly dependent on the presence of recurrent chromosome abnormalities (specifically del(6)(q23.3)/*MYB*, del(13q)(q14.3), +12, del(11)(q22.1)/*ATM*, and del(17)(p13.1)/*TP53*) [379–381] (Fig. 15.22; see also Chap. 17, Fig. 17.10), and also gene mutations. Other less frequent abnormalities have been reported in CLL and are discussed at the end of this section. Morphologically, the worst prognosis is associated with the presence of prolymphocytes and the development of Richter syndrome (RS). RS corresponds to progression of CLL to a high-grade non-Hodgkin lymphoma, prolymphocytic leukemia, Hodgkin disease, or acute leukemia [382].

Deletion of 13q

From the cytogenetic point of view, the best prognosis is related to the presence of del(13q) as a sole abnormality, with a survival of more than 130 months [383]. The deleted portion of chromosome 13 can vary in size, but it always involves band 13q14.3 (Fig. 15.22). In a significant proportion of cases, del(13q) involves both the *RBL* gene and the D13S25/D13S319 loci, while in others only the latter are deleted. Because of the minimal size of the deletion, this abnormality is often cryptic at the chromosome level and can only be detected by FISH [383]. In fact, the frequency of del(13q) with conventional cytogenetics is 10–15%, but with FISH it can be detected in over 70% of cases [384]. The occurrence of this abnormality surpasses that of trisomy 12, previously believed to be the most common chromosome abnormality in CLL.

FISH has also demonstrated the existence of biallelic 13q deletions [385]. Although patients with deletions of one or both copies of chromosome 13 seem to have similar prognosis, it is believed that the presence of a high number of cells with del(13q) is characterized by a more aggressive clinical course, equivalent to that seen in the “intermediate”-risk

FISH category [385]. Furthermore, del(13)(q14.3) leads to deletion or reduced expression of two microRNAs, *miR15a* and *miR16*, which have been identified as regulators of *BCL2* mRNA [384]. Therefore, lack or reduced expression of these two miRs is considered to be the basis of the consistent overexpression of *BCL2* in CLL exhibiting del(13)(q14.3).

Trisomy 12

After the discovery of the high incidence of del(13q) in CLL, gain of chromosome 12 has settled in as the second most common abnormality [386] (Fig. 15.22). Patients with this abnormality often present with atypical lymphocyte morphology and intermediate risk, with a survival of about 115 months [387]. Factors that worsen the prognostic outcome include advanced age, male gender, elevated CD38 expression, and unmutated *IGHV@*. At least 50% of patients with trisomy 12 have been found to carry unmutated *IGVH* and to progress to Richter syndrome [388]. By conventional cytogenetics, this abnormality is detected in 10–15% of all cases. With the use of FISH, its detection has increased to 30% [389]. In approximately 30% of cases, gain of chromosome 12 is associated with additional abnormalities, the most common of which is del(13q) (12% of cases). Less frequent is the association of trisomy 12 with del(11q) and del(17p), believed to occur mostly as clonal evolution [389]. The presence of trisomy 12 together with del(14q) or t(14;18) has also been reported [390, 391] (Fig. 15.23). These patients appear to have somewhat a more aggressive disease due to the frequent association of this cytogenetic entity with unmutated *IGHV@* (see later section on “*IGH@ Rearrangements*”).

Deletion of 11q

CLL patients with del(11q) have a progressive disease course [386]. Survival in this group is about 80 months [387]. The detection frequency of this deletion is 5–10% by conventional cytogenetics (Fig. 15.22) and 20–25% by FISH [392]. The deleted region at 11q22.3 includes the ataxia-telangiectasia-mutated (*ATM*) gene and is also the site of *MIR34B*. Approximately 10% of all CLL patients and about 30% of those with del(11q) have an *ATM* mutation on the homologous chromosome 11. Furthermore, in up to 12% of patients with *ATM* deletions, these are large enough to also result in loss of *MLL* at 11q23 [392].

ATM is an important checkpoint gene involved in cell damage control. The *ATM/TP53* interaction has been shown to have an important impact on cell proliferation. Therefore, deletion of *ATM* removes this checkpoint and blocks the activation of *TP53*. As a result, even if *TP53* is present, there is no attempt at repairing damaged cells [393]. Additional abnormalities are present in approximately 60–70% of patients with del(11q) and often include those that are recurrent in CLL, such as del(13q), +12, del(6q), and del(17p) [392].

Deletion of 17p

Patients with del(17p) and associated loss of *TP53* at 17p13.1 are characterized by a poor response to chemotherapy (e.g., alkylating agents) and short survival (24–32 months after diagnosis) [394]. This deletion is seen in approximately 5% of cases with conventional cytogenetics (Fig. 15.22) and in about 7–20% by FISH (see also Chap. 17, Fig. 17.10) [386].

The majority of abnormalities leading to del(17p) are unbalanced translocations. Generally, the loss of 17p is present in the context of a complex karyotype. However, a few cases with i(17)(q10) as the only change have been described [395].

Mutations of *TP53* are found in 8–10% of patients with untreated CLL. Some reports indicate that the clinical behavior of cases with *TP53* mutation only is very similar to cases with deletion of one allele and mutation of the remaining allele. However, the overall survival after treatment was significantly improved in patients with del(17p) but without *TP53* mutation [396].

Deletion of 6q

del(6q) occurs in approximately 6–7% of patients with CLL [397]. The deletion is associated with atypical lymphocyte morphology, higher white blood cell count, splenomegaly, and short survival compared to patients with normal karyotype or 13q deletion [397]. Thus, this abnormality is considered an intermediate marker in CLL.

Deletion of 6q is rarely the sole abnormality. More often, it is present in the context of a complex karyotype. At least two regions of minimal deletion have been observed, one involving bands 6q21–q23.3, which includes *MYB*, and the other involving bands 6q25–q27 [398, 399]. The majority of deletions were found to occur in the 6q21–q23.3 region (Fig. 15.22). However, not all of these deletions involve *MYB*. Therefore, it is important to point out that since CLL is characterized by variable 6q deletions, probes targeting solely the *MYB* locus will not detect all 6q deletions.

IGH@ Abnormalities

It is important to distinguish the *IGH@* rearrangements that occur in CLL from those that are seen in other neoplasms. t(11;14)(q13.q32.3) had been reported in CLL, but these cases are now believed to belong to a different entity, and therefore the terminology atypical MCL is used to refer to these neoplasms [68, 400, 401]. Other *IGH@* translocations that are observed in CLL include t(2;14)(p16.1;q32.3), t(14;19)(q32.3;q13), and t(14;18)(q32.3;q21.3), and their variants (Figs. 15.23 and 15.24). These translocations, respectively, relocate *BCL11A*, *BCL3*, and *BCL2* to the *IGH@* locus (or less commonly to one of the immunoglobulin light chain loci) leading to their upregulation [391, 402, 403].

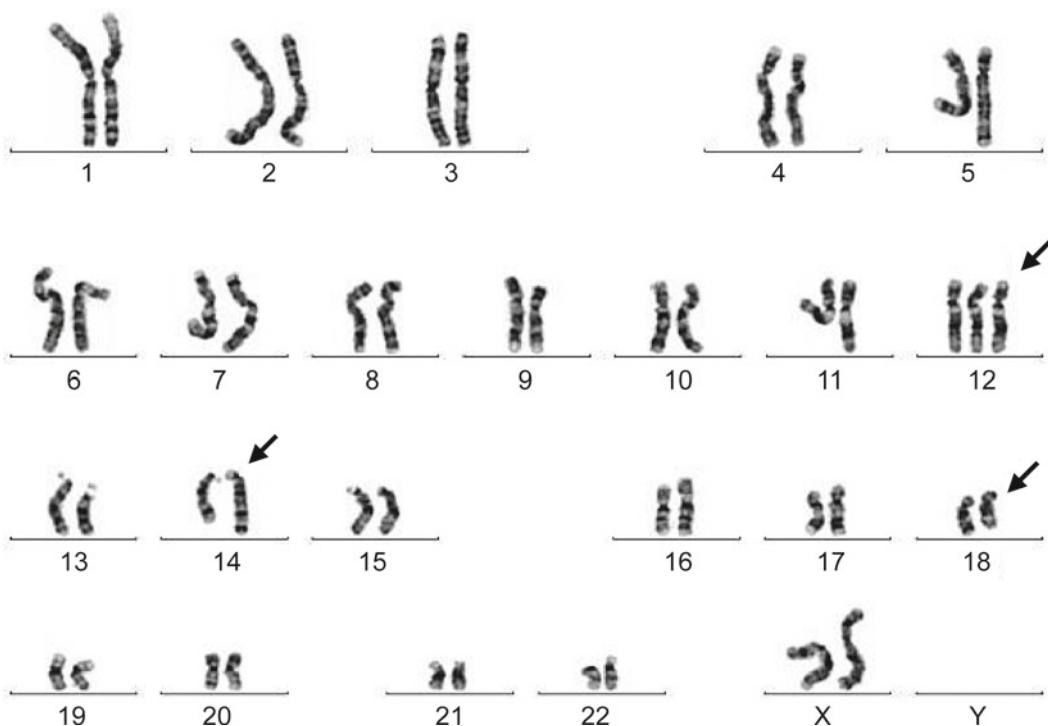


Fig. 15.23 Karyogram showing the simultaneous presence of trisomy 12 and t(14;18)(q32.3;q21.3) in a patient with atypical CLL

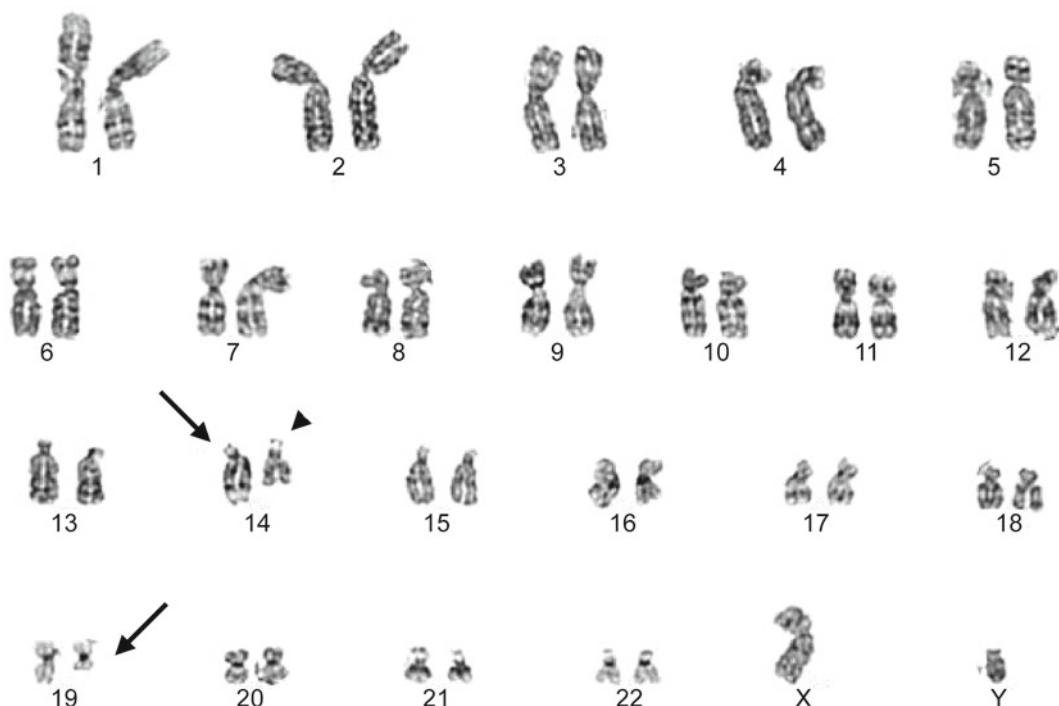


Fig. 15.24 Karyogram showing del(14)(q24) and t(14;19)(q32.3;q13) in a patient with CLL. Arrows indicate the chromosomes involved in the t(14;19). The arrowhead indicates the deletion of 14q

Deletions of *IGH@* are apparently more frequent but are often cryptic; some reports have indicated that *IGH@* deletions, whether cryptic or not, are present in over 30% of CLL cases [404]. FISH studies have demonstrated that up to 80% of *IGH@* deletions involve the 5' portion of the gene. Loss of one complete copy of *IGH@* is indicative of a larger deletion, generally involving band 14q24.1 (Fig. 15.24). Detecting these deletions, either by conventional cytogenetics or with FISH, is important since in over 60% of cases del(14)(q24.1) is found in association with unmutated *IGHV@* [405]. Furthermore, in approximately 50% of cases it is found associated with gain of chromosome 12 [390, 406]. Given the unfavorable prognosis associated to deletion of *IGH@*, it is good practice to perform FISH to investigate the status of this gene in patients with CLL.

Non-*IGH@* Reciprocal Translocations

Balanced reciprocal translocations, particularly those that do not involve *IGH@*, are rare in CLL [407]. Occasionally, t(8;14)(q24.2;q11.2) involving *MYC* and *IGH@* (or less frequently another immunoglobulin gene) is observed as an additional abnormality in some CLL cases [408]. The recent introduction of new mitogenic stimulants such as CpG-oligodeoxynucleotides (CpG-ODNs) has increased the detection of these rearrangements with conventional cytogenetics [325]. Interestingly, several of these apparently balanced translocations are found to involve chromosome 13. FISH has demonstrated, however, that the majority of these translocations are not balanced but often have a cryptic deletion of 13q14.3, suggesting that they are most likely secondary to the deletion [409]. Very few of these—for example, t(6;13)(p21;q14.1) or t(10;13)(q24;q14)—are recurrent [409, 410]. The reason that these translocations are being detected only now with the use of CpG-ODNs is not clear. What is clear is that the broader use of ODNs is likely to increase the number of cases with these apparently balanced translocations, and a review of these cases might ultimately help elucidate their incidence and significance in the prognosis of patients with CLL.

Prognostic Markers

A set of genetic markers has significant prognostic value in CLL. The most well-recognized are CD38, *IGHV@* (*IgVH*), and ZAP70 [411].

CD38 is a 45-kDa transmembrane glycoprotein that acts as both as an enzyme and a receptor [412]. CLL patients with expression of CD38 in over 30% of cells tend to have unmutated *IGHV@*, resistance to chemotherapy, and shorter overall survival compared with patients with low expression of CD38 [324, 411, 412].

IGHV@ mutation status is a good parameter for assessing the prognosis of CLL patients at presentation [324].

While patients with unmutated *IGHV@* follow an unfavorable course with rapid progression and short survival, those with mutated *IGHV@* often show slow progression and long survival [411].

Zeta-chain (TCR)-associated protein kinase 70 kDa (ZAP70) is a molecule involved in T-cell receptor signaling. It is coded for by *ZAP70* on the proximal long arm of chromosome 2, and is abnormally expressed in some CLL patients. The presence of ZAP70 in at least 20% of leukemic cells is associated with unmutated *IGHV@* [411]. The clinical reliability of ZAP70 analysis has been questioned by some experts [413]. However, prognostic determinations can be made based on the combinatorial status of these markers, where ZAP70 can provide useful information. In general, CLL patients with unmutated *IGHV@* and/or CD38 expression, but without expression of ZAP70, will not require therapy by current criteria for many years after diagnosis [414].

Due to genetic variability of CLL, the combination of conventional cytogenetics, FISH, mutation analysis of *IGHV@*, and expression studies of ZAP70 and CD38 provides the best prognostic tool for the diagnosis and management of patients with CLL.

B-Cell Prolymphocytic Leukemia

B-cell prolymphocytic leukemia (B-PLL) is a rare chronic lymphoproliferative neoplasm accounting for only 1% of all chronic leukemias of lymphoid origin. This neoplasm is characterized by the presence of >55% prolymphocytes in the peripheral blood. The bone marrow and/or spleen are often involved [415].

Patients present with severe leukocytosis and prominent splenomegaly. Lymphocytes show the immunophenotypic expression of B-cell markers such as CD19, CD20, CD22, CD79a, and FMC7. There is weak expression of CD5 and absent expression of CD10 and CD23. Approximately 50% of the cases express CD38 and/or ZAP70 [415].

B-PLL has overlapping features with other mature neoplasms such as CLL and MCL. In fact, patients are diagnosed as having either *de novo* PLL or PLL that progressed from CLL or MCL [415, 416]. This overlapping is also evident in the cytogenetic findings. Approximately 25% of patients were reported to have the t(11;14) that is the hallmark of MCL; PLL cases with t(11;14) are now considered a leukemic variant of MCL (Fig. 15.25). Other abnormalities include gain of chromosome 12 and deletions of 6q, 11q, 13q, and 17p, abnormalities that are often seen in CLL, and thus these cases are thought to have progressed from CLL.

An additional abnormality seen in some cases of PLL is the t(8;14) characteristic of Burkitt lymphoma. Less frequently, a variant of the t(8;14), either t(2;8) or t(8;22), might be seen [417]. Rearrangements of chromosome 17 leading to loss of 17p13.1 (*TP53*) have been reported in approximately 50% of PLL cases [418].

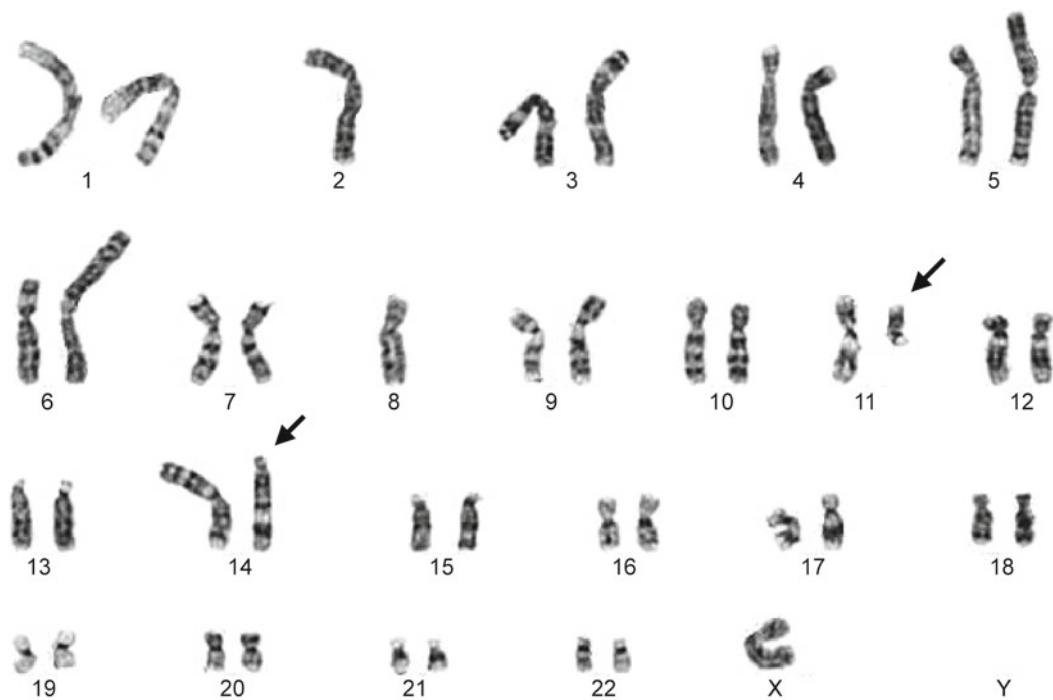


Fig. 15.25 Karyogram with t(11;14) in the context of a complex karyotype in a patient diagnosed with B-cell PLL. These cases are now considered to be a leukemic variant of MCL

Hairy Cell Leukemia (Typical and Variant)

Hairy cell leukemia (HCL) is an indolent mature B-cell lymphoproliferative neoplasm that affects adult individuals aged 50 years and over and accounts for 2% of all B-cell lymphomas [419]. The sites involved include primarily the bone marrow and spleen [419, 420].

The circulating peripheral blood B-lymphocytes are small to medium in size and have a characteristic morphology with prominent cytoplasmic projections termed hairs, thus the name “hairy cell” used to refer to this entity [420]. Flow cytometric analysis shows expression of CD20 (bright), CD22, CD25, CD103, and CD11c. CD5 and CD10 are often negative [419, 420].

The variant counterpart of HCL (HCLv) shares many similarities with typical HCL, except that patients present with leukocytosis and the hairs are less evident in the affected blood lymphocytes [421].

There are no specific chromosome abnormalities in either typical or variant HCL. However, conventional cytogenetics has demonstrated the recurrent gain of chromosome 5, specifically the region 5q13-q31, and deletion of chromosome 7, specifically the region 7q22-q36. Of importance is the discovery of the *BRAF* V600E mutation in HCL. Interestingly, the *BRAF* gene is located on 7q34, a chromosome region often implicated in HCL. This mutation results in the production of an aberrant protein that is most likely suitable for targeted therapy [422]. Other less frequent abnor-

malities involve chromosomes 1, 6, 14, and 19 [419, 421, 423]. HCLv often shows a more complex karyotype than that seen in typical HCL and tends more frequently to exhibit gain of chromosome 12 and rearrangements of chromosome 17 leading to loss of *TP53* [423, 424] (Fig. 15.26).

Plasma Cell Myeloma

Plasma cell myeloma (PCM), also known as multiple myeloma, is a neoplasm that affects the terminally differentiated plasma cells in the bone marrow and accounts for approximately 12% of hematologic neoplasms. Generally, patients are in their seventh decade of life and present with an excess of plasma cells in the bone marrow, monoclonal proteins in the blood and urine, osteolytic bone lesions, and multiorgan dysfunction [425].

Conventional cytogenetics and FISH have been crucial in the characterization of prognostically significant markers in PCM. However, due to the short life and low proliferative rate of plasma cells, it is challenging to consistently obtain abnormal metaphases for analysis; the detection of chromosome abnormalities by conventional cytogenetics is believed to be between 25 and 40% [426]. This detection rate has been somewhat increased by the use of specific mitogens, the most widely used of which is interleukin-4 (IL-4) [427].

FISH with a subset of probes targeting common abnormalities in PCM has certainly improved the detection of

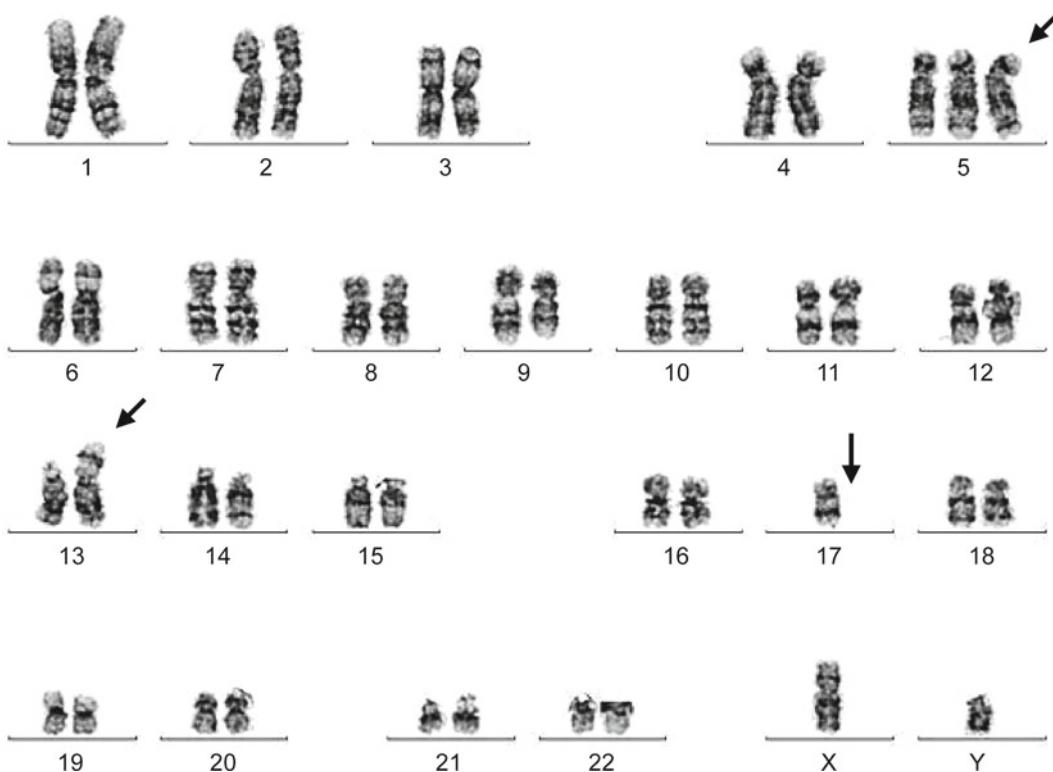


Fig. 15.26 Karyogram of a patient with hairy cell leukemia. Some of the recurrent abnormalities include gain of chromosome 5 and rearrangements leading to deletion of 17p, as seen in this case. Here, the derivative (13;17) leads to the net loss of 17p

chromosome abnormalities of prognostic significance in this neoplasm [428]. More recently, the use of plasma-cell-enriched fractions obtained with the use of CD138-coated immunomagnetic beads has provided a concentrated number of plasma cells for easy identification of chromosome abnormalities by FISH analysis [429]. See also Chap. 17.

PCM is characterized by distinct karyotypic entities, each with an associated prognostic outcome [430].

Hypodiploidy

Hypodiploidy (<46 chromosomes) generally includes the loss of chromosome 13, specifically 13q14.3, and/or chromosome 17, specifically 17p13.1 (*TP53*), both of which are associated with unfavorable prognosis [430, 431]. As such, patients with these karyotypes are placed in a high-risk category. In the majority of cases, the hypodiploid chromosome complement includes structural abnormalities, involving, in particular, chromosomes 1, 4, 6, 14, 16, and 20. Specifically, loss of 1p and/or gain of 1q, losses of 4q and 6q, loss and/or rearrangements of 14q and 16q, and partial or complete loss of chromosome 20 are most commonly seen [431].

Translocations involving chromosome 14, specifically 14q32.3 (*IGH@*), are seen in approximately 85% of the cases. The most common *IGH@* translocations in this group

are t(4;14)(p16.3;q32.3), t(14;16)(q32.3;q23.1), and t(14;20)(q32.3;q12) which are associated with an unfavorable prognosis [430].

An interesting characteristic of PCM with hypodiploidy is the tendency to double the abnormal chromosome complement, similar to what is seen in hypodiploid acute lymphoblastic leukemia cases. Karyotypes with 70–90 chromosomes and a double content of structural rearrangements, including the relative losses of chromosomes 13 and 17, most likely represent the doubling of a hypodiploid clone [431, 432] (Fig. 15.27a, b). The original hypodiploid clone has a very low mitotic proliferation in culture compared to the clone with the near-tetraploid (doubled) karyotype. Both carry the same adverse prognosis.

Hyperdiploidy

Another group of PCM patients is characterized by hyperdiploidy and few or no structural abnormalities. Gains are nonrandom and often involve chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 [430] (Fig. 15.28). Patients with the presence of these additional chromosomes are placed in a standard-risk category, as long as there is no deletion of 13q or 17p [432].

The most common translocation in this group involves *IGH@*; it is t(11;14)(q13;q32.3) and is present in approximately 25% of cases.

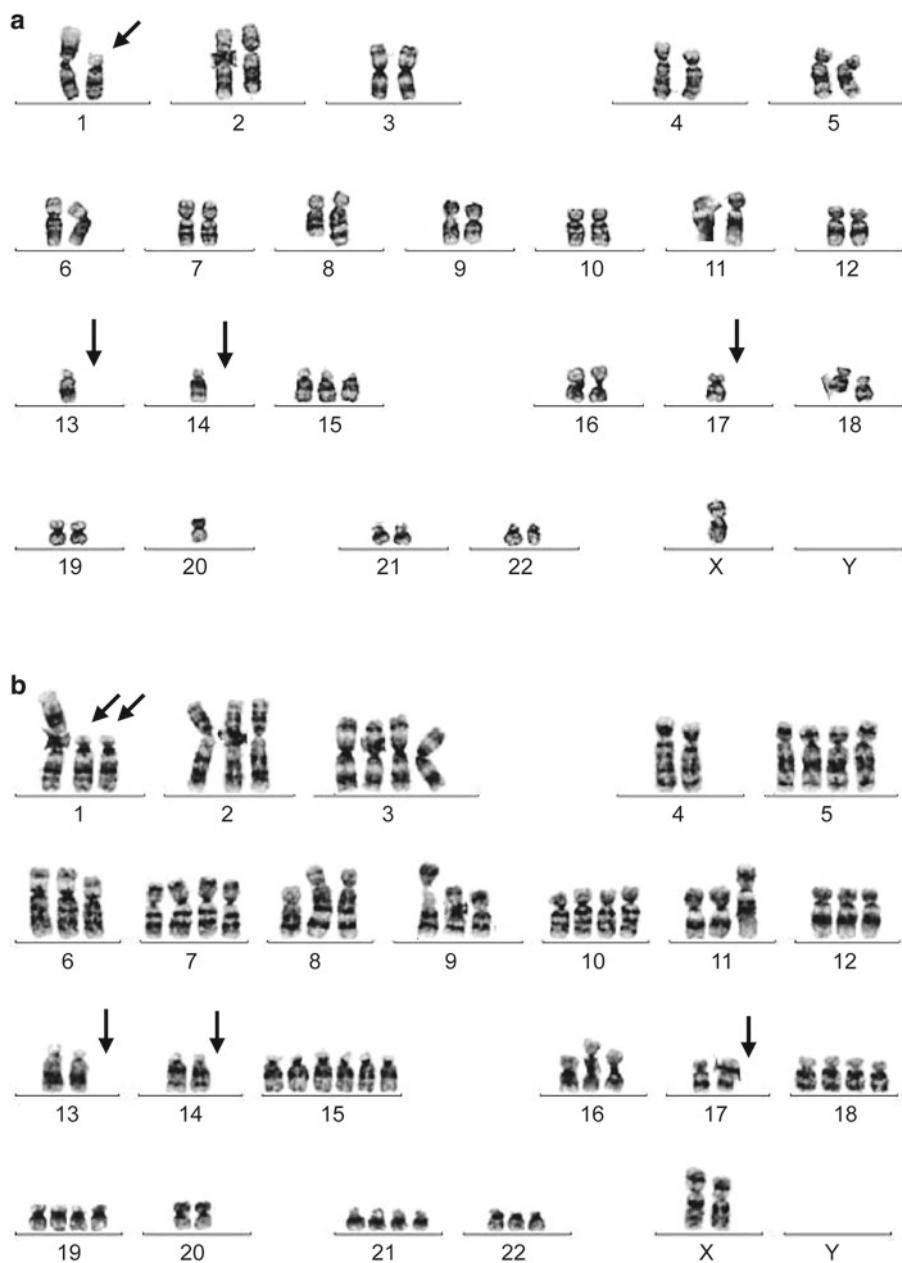


Fig. 15.27 (a) Hypodiploid karyogram in a patient with high-risk plasma cell myeloma showing, among other abnormalities, the simultaneous loss of chromosomes 13, 14, and 17. (b) The hypodiploid cells

often undergo reduplication and give origin to a doubling version. The prognosis associated with these karyograms is unfavorable

Patients with this translocation are placed in a separate prognostic category because of their specific treatment and their improved prognostic outcome compared to patients with other *IGH@* translocations [430].

The prognostic relevance of hyperdiploid karyotypes might be difficult to ascertain when structural abnormalities are present. For the sake of proper prognostic stratification of PCM patients, every effort should be made to differentiate true hyperdiploidy from the doubling of a hypodiploid clone.

FISH can be useful in the differentiation of these clonally different populations.

Deletion of 13q/Loss of Chromosome 13

An interstitial deletion of 13q, involving either 13q14.2 (*RBL*) or 13q14.3 (D13S319), is one of the most common abnormalities in PCM and has been detected by FISH in over 50% of cases [426, 427, 433] (see also Chap. 17, Fig. 17.11c). The region of deletion in these cases can be very

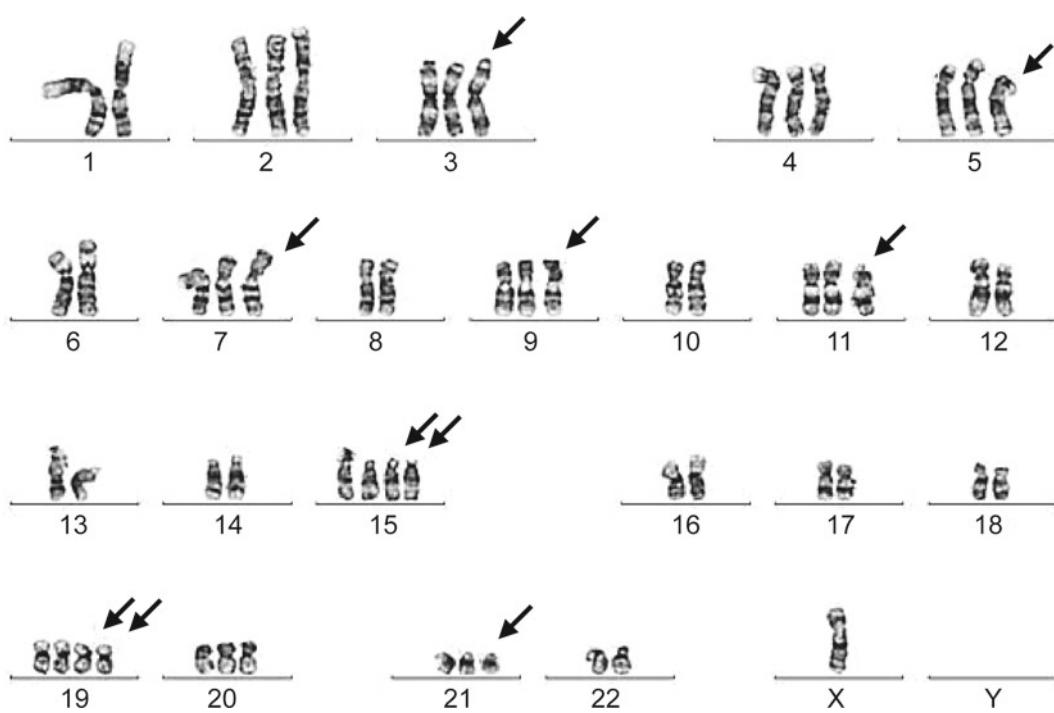


Fig. 15.28 True hyperdiploidy in plasma cell myeloma. These karyograms are characterized by gain of odd-numbered chromosomes (+3, +5, +7, +9, +11, +15, +19, and +21)

small and in some cases has been found to involve either D13S319 or *RBL1*, but not both. In these cases, therefore, the deletion is cryptic and so is not detected with conventional cytogenetics; only 10–20% of PCM cases exhibit del(13q) with chromosome analysis.

In other instances, when a sufficient number of FISH probes are used, it can be shown that approximately 80% of cases with abnormal signal patterns for chromosome 13 actually show complete loss of one copy of the chromosome, generally in the context of a hypodiploid karyotype [434].

The significance of del(13q) as the sole abnormality, when detected by FISH only, is unclear. It appears that the poor prognosis originally attributed to del(13q) might be the result of other abnormalities present in the karyotype [435–437] (Fig. 15.29). In fact, studies have shown that the prognostic outcome of a hyperdiploid karyotype typically associated with standard-risk myeloma is not altered by the presence of del(13q). On the other hand, in a hypodiploid karyotype, del(13q) or loss of chromosome 13 indicates a poor prognosis.

Deletion of 17p

Deletion of 17p in PCM generally leads to deletion of *TP53* (17p13.1), and it has been observed in approximately 10% of patients [438]. This deletion, when it involves

TP53, is considered a marker of adverse prognostic outcome associated with aggressive disease, short survival, and resistance to treatment. It is believed that in PCM, deletions of 17p13.1 occur as secondary events during disease progression. In fact, this deletion has been observed in both hypodiploid and hyperdiploid karyotypes (Fig. 15.29; see also Chap. 17, Fig. 17.11d). Occasionally, it has also been observed together with t(11;14), which is known to be a standard-risk marker [439]. Contrary to what is seen with deletion of 13q, deletion of *TP53* has a negative impact, irrespective of the presence of favorable prognostic markers.

Chromosome 1 Abnormalities

Abnormalities involving chromosome 1 in PCM include deletions of 1p, gains of 1q, and/or translocations involving either arm (Fig. 15.29, see also Chap. 17, Fig. 17.11b). Deletions of 1p most frequently involve the segment between bands 1p12 and 1p31, whereas gain of 1q involves the segment q21→qter or the entire long arm [440]. Gain of 1q represents the second most frequent chromosomal abnormality in PCM after del(13q). In fact, it has been observed in approximately 40% of newly diagnosed PCM patients and in approximately 70% of relapsed PCM cases [440, 441].

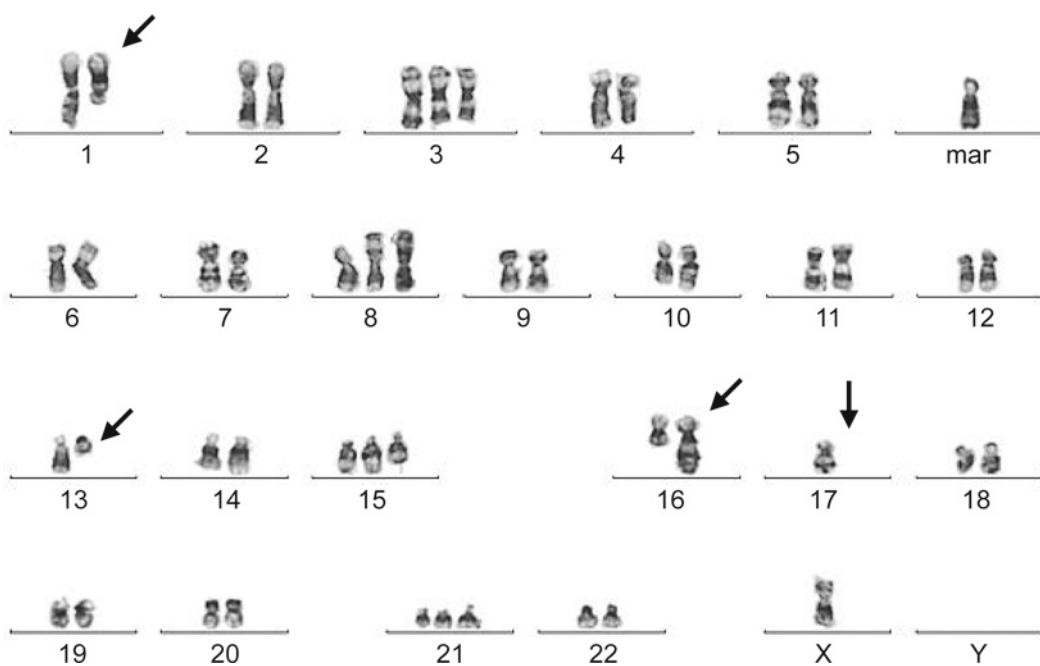


Fig. 15.29 Complex karyogram in plasma cell myeloma. Although deletion 13q is thought to be one of the high-risk markers, other abnormalities often present in such karyotypes, specifically rearrangements

of chromosomes 1 and 16, and loss of chromosome 17, are most likely to blame for the poor prognosis

Among the translocations involving chromosome 1, the majority are derivatives of rearrangements involving various chromosomes, resulting in gain of 1q. The most recurrent unbalanced translocations leading to gain of 1q are der(1;15)(q10;q10), der(1;16)(q10;p10), and der(1;19)(q10;p10) [440]. However, other derivative chromosomes have been described. Gain of 1q can also be the result of an isochromosome of the long arm of chromosome 1 [441]. More recently, FISH probes and CGH targeting the region q21→q23 have detected multiple copies (amplification) of genes located in this region, such as *MUC1*, *BCL9*, and *ARNT* [442]. The segmental amplification of 1q is thought to be associated with an unfavorable prognostic outcome.

IGH@ Rearrangements

Some PCM patients exhibit a rearrangement of an immunoglobulin (Ig) gene, most often of the heavy chain gene *IGH@* at 14q32.3. The primary translocations are the result of somatic hypermutation or recombination errors in the VDJ portion of the switch region [440].

At least 20 nonrandom chromosomal partners have been found in translocations with *IGH@* at 14q32.3. The most frequent of these are t(11;14)(q13;q32.3), t(4;14)(p16.3;q32.3), and t(14;16)(q32.3;q23.1) [443–446]. The latter two are cytogenetically cryptic and are detected only by FISH or PCR. Two other translocations, t(6;14)(p21.1;q32.3) and t(14;20)(q32.3;q12), have also been described [447, 448]. All of these translocations lead to overexpression of the gene translocated

to *IGH@* or other Ig loci, for example, *CCND1* at 11q13, *FGFR3/MMSET* at 4p16.3, *MAF* at 16q23.1, *CCND3* at 6p21.1, and *MAFB* at 20q12.

t(11;14), which is detected in approximately 20–25% of PCM patients, is not exactly the same as the one seen in mantle cell lymphoma (MCL) [449]. In MCL, the breakpoints on 11q13 are clustered to a specific minimal region, whereas in PCM they are scattered over a large portion of the q13 region [450]. Therefore, to increase the targeted area and improve the ability to detect the *CCND1-IGH@* fusion, two commercial *CCND1-IGH@* fusion probes are available. The one suggested for PCM includes another gene, *MYEOV*, which is distinct from the *CCND1-IGH@* probe used for detection of t(11;14) in MCL [451]. An association has been found in PCM between the presence of t(11;14), CD20 expression, and lymphoplasmacytic morphology [452]. Therefore, a clinical laboratory should consider performing FISH to investigate the presence of a *CCND1-IGH@* fusion in patients with such morphologic characteristics.

t(4;14)(p16.3;q32.3) is the second most frequent translocation involving *IGH@*. It is detected in approximately 15% of PCM patients and is cryptic by conventional cytogenetics [444, 445]. This translocation is thought to involve two genes located at 4p16.3, fibroblast growth factor receptor 3 (*FGFR3*) and the Wolf-Hirschhorn syndrome candidate 1 (*WHSC1*) gene, which in myeloma is known as multiple myeloma SET domain (*MMSET*) [453]. The detection of this translocation is

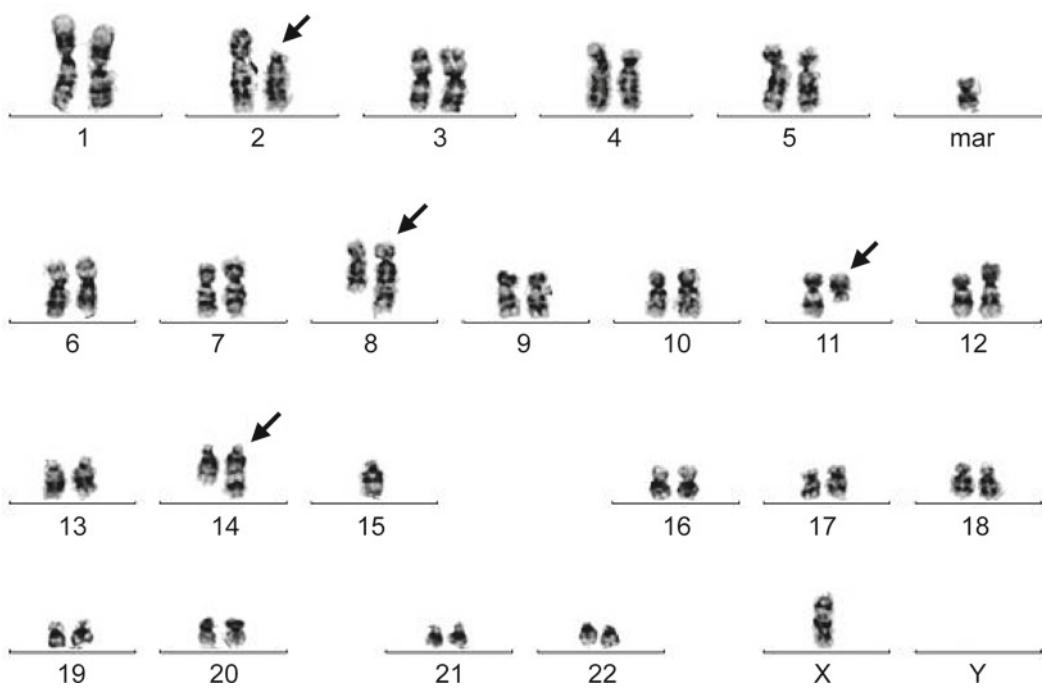


Fig. 15.30 Karyogram of a patient with plasma cell myeloma (PCM) showing a *MYC* rearrangement, specifically t(2;8)(p12;q24.1), together with t(11;14)(q13;q32.3). PCM with t(11;14) are thought to be associated

with favorable or neutral prognostic outcome. However, *MYC* rearrangements might be seen in these cases, and when present, they appear to be associated with a more aggressive course

possible with FISH using the commercially available *FGFR3-IGH@* probes, or with PCR. This translocation tends to be very frequent in hypodiploid karyotypes, particularly together with del(13q) and/or del(17p). As such, patients with this translocation are placed in a high-risk prognostic category [444, 445].

t(14;16)(q32.3;q23.1) is observed in approximately 5–7% of PCM patients [446]. This translocation is also cryptic by conventional cytogenetics, and FISH or PCR are used to determine its presence. In this case, the gene involved is *MAF* at 16q23.1 [446]. Similarly to t(4;14), this translocation tends to occur in hypodiploid karyotypes, together with deletions of 13q and/or 17p. As a result, it has been suggested that patients with this translocation should be placed in a high-risk prognostic category. However, a recent large study of myeloma patients indicates that the overall survival of patients with t(14;16) did not significantly differ from that of patients lacking this abnormality [454].

MYC Rearrangements

Approximately 15% of PCM patients, particularly those with high-risk PCM, have a translocation involving *MYC* at 8q24.2 and an immunoglobulin gene, leading to overexpression of the *MYC* oncogene [455, 456]. Some translocations do not involve an immunoglobulin gene, but they still lead to overexpression of *MYC*. In the majority of cases, however, there is gain or amplification of *MYC* without an apparent translocation involving this region [457].

Because abnormalities involving *MYC* are detected in hypodiploid and hyperdiploid clones and in clones with t(11;14) (Fig. 15.30), it is believed that they are not primary abnormalities but rather occur during disease progression.

MYC translocations in the presence of a highly complex karyotype have also been observed in plasmablastic lymphoma, an aggressive lymphoma with plasmacytic differentiation (see next section). Other clinical, morphologic, and immunophenotypic data should therefore be used to differentiate aggressive forms of PCM from plasmablastic lymphoma.

Plasmablastic Lymphoma

Plasmablastic lymphoma (PBL) is an aggressive B-cell lymphoma that accounts for approximately 3% of human immunodeficiency virus (HIV)-related lymphomas and is characterized by plasma cell differentiation and an immunoblastic cellular morphology [458]. The first described cases involved the oral cavity of patients infected with HIV [459]. However, this lymphoma also affects other sites, such as the gastrointestinal mucosa, skin, and soft tissues of HIV and non-HIV patients [460].

With conventional cytogenetics, plasmablastic lymphomas are quite interesting. They have chromosome abnormalities similar to those observed in PCM, particularly rearrangements of chromosome 1, deletions of 13q and 17p, and simultaneous gains of odd-numbered chromosomes, specifically +3, +5, +7, +9, +11, and/or +15 [461]. Invariably, they are also characterized by

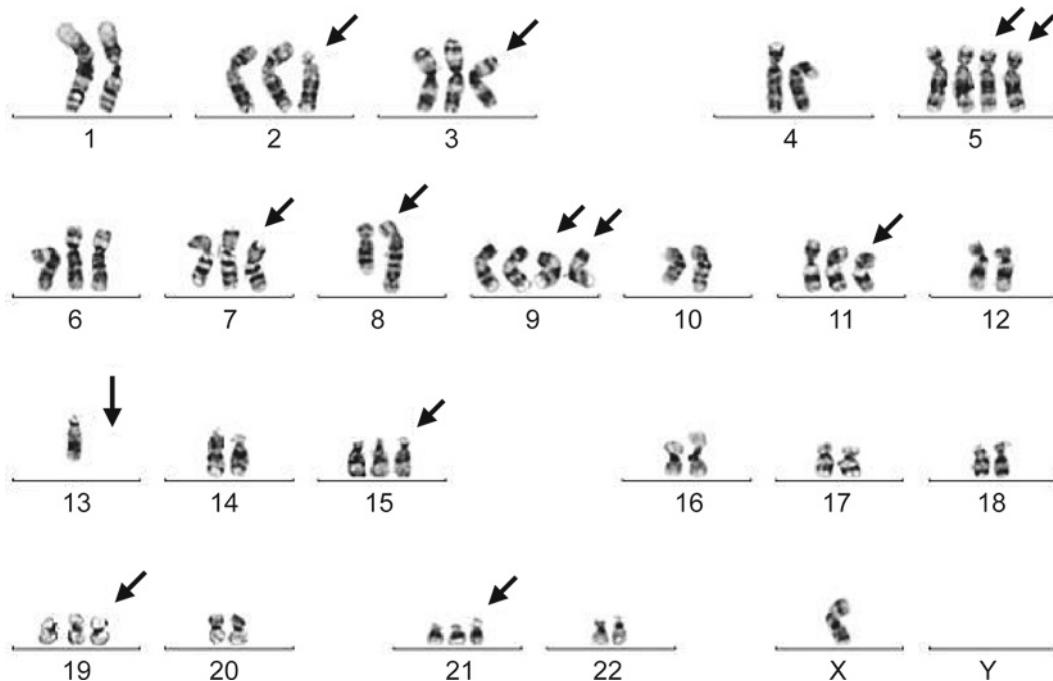


Fig. 15.31 Typical karyogram of a patient with plasmablastic lymphoma. Of interest is the presence of chromosome gains similar to what is seen in plasma cell myeloma, in this case gain of odd-numbered

chromosomes, loss of chromosome 13, and the simultaneous presence of a *MYC* translocation, in this case $t(2;8)(p12;q24.1)$

one of the *MYC* translocations, possibly occurring during progression [461] (Fig. 15.31). Since plasmablastic morphology, plasma cell immunophenotype, and *MYC* translocation can also be observed in some aggressive (anaplastic) PCM, it is important to use all available clinical information to differentiate these two entities. Some of the main differentiating factors in PBL are the high association with Epstein-Barr virus (EBV) and the lack of monoclonal paraproteinemia and lytic bone lesions typically seen in PCM [458].

Hodgkin Lymphoma

Hodgkin lymphoma (HL) is an indolent neoplasm of the lymphatic system that makes up approximately 30% of all lymphoma cases [462]. This lymphoma owes its name to Dr. Thomas Hodgkin who first described it in 1832 [463]. HL affects individuals of all ages with two preferential peaks, one occurring between the ages of 15 and 30 years and the other at ≥ 60 years [462, 464].

One of the morphologic characteristics of HL is the presence of giant cells called Reed-Sternberg cells. These cells comprise only approximately 1% of the affected tissue; the remaining tissue is composed of inflammatory cells [462].

HL is subdivided into two morphologically and clinically distinct subgroups, nodular and classic [464]. Classic HL (CHL) is itself subdivided into four histologic entities known as lymphocyte rich, nodular sclerosis, mixed cellularity, and lymphocyte depleted [464]. CHL accounts for about 95% of all Hodgkin lymphomas and is therefore the most common type

analyzed with conventional cytogenetics. Although the majority of HL patients have a normal karyotype, a minority are found to have an abnormal chromosome complement. The first karyotype was described in 1967 [465]. Subsequently, other cytogenetic cases have been described, and still no specific abnormalities have been detected. The common feature is that the karyotypes tend to be hyperdiploid, with 60–70 chromosomes. Some recurrent abnormalities include losses of 1p, 6q, 7q, 13q, 16q, and 17p; gains of 2p, 9p, and chromosome 12, as well as rearrangements of 3q27 (*BCL6*) [466, 467].

Mature T-Cell Lymphomas

A complete description of all mature T-cell and natural killer cell (NK) lymphomas, some of which are quite rare, is provided by the WHO [67]. In this chapter, only the most common T-cell lymphomas, particularly those that have been reported to have recurrent chromosome abnormalities, are described. These T-cell neoplasms have many characteristics in common, such as location (skin is the most common site of involvement) and genes involved (T-cell receptors are most frequently rearranged) [468, 469]. Bone marrow and peripheral blood samples usually require mitogen stimulation, the most frequently used of which is phytohemagglutinin (PHA). Other tissues such as lymph nodes, spleen, or liver produce analyzable metaphase cells in overnight cultures without stimulation. See Table 15.14.

Table 15.14 Biologic and genetic characteristics of mature T-cell lymphomas

Neoplasm	Sites involved	Frequency	Chromosome abnormalities	
			Primary	Additional
T-PLL	PB, BM, LN, SP, LV	2% of lymphomas; 20% of PLL	inv(14), t(7;14), t(14;14), t(X;14)	i(8)(q10), t(8;8), del(6q), del(11q), del(12p), del(17p)
NKCL	PB, BM, SP, LV	Rare	del(6q), del(11q)	del(17p)
ATLL	LN, PB, BM, SK	2–5% of lymphomas	Complex with +X, +3, and +7; rearrangements of 1q, 3q, 6q, 9p, 14q	del(17p)
HSTL	SP, LV, BM	<1% of lymphomas	i(7)(q10), gain of 7q	+8
MF	SK, LN	50% of all cutaneous lymphomas	Complex with rearrangements of 1p, del(6q), del(9p)	Rearrangements of 3q, 10q, 11q, 12p, and 14q
SS	SK, PB, BM	5% of cutaneous lymphomas	Complex with del(1p), del(6q), del(10q), +17, +18	del(9p), del(17p), -19
PTCL-NOS	LN, BM, SP	30% of PTCL	TCR gene rearrangements, very complex karyotypes with +3q, +7q, +8q, del(4q), del(5q), del(6q), del(9p), del(10q), del(13q)	Variable
AITL	LN	1–2% of T-cell lymphomas	TCR gene rearrangements; +X, +3, +5, +11q, del(10q), del(12q), del(13q)	Variable
ALCL-ALK+	LN, SK, LV	3–5% adults, 15–20% children among all lymphomas	t(2;5)(p23.1;q35.1), or variant translocations involving 2p23 (ALK): t(1;2), inv(2), t(X;2), t(1;2), t(2;3), t(2;17), t(2;19), t(2;22)	+X, +7, +9; variable structural rearrangements

Abbreviations: *T-PLL* T-cell prolymphocytic leukemia, *NKCL* NK-cell leukemia (aggressive type), *ATLL* adult T-cell leukemia/lymphoma, *HSTL* hepatosplenic T-cell lymphoma, *MF* mycosis fungoides, *SS* Sézary syndrome, *PTCL-NOS* peripheral T-cell lymphoma, not otherwise specified, *AITL* angioimmunoblastic T-cell lymphoma, *ALCL-ALK+* anaplastic large cell lymphoma, ALK-positive, *PB* peripheral blood, *BM* bone marrow, *LN* lymph node, *SP* spleen, *LV* liver, *SK* skin

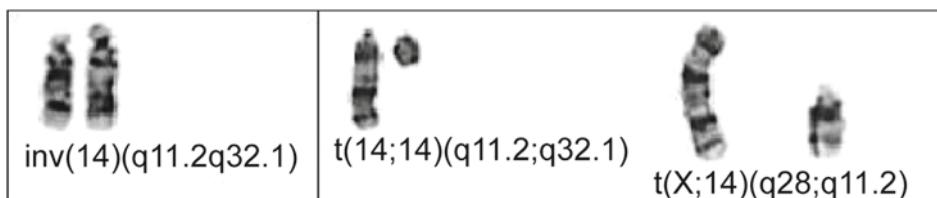


Fig. 15.32 Three common rearrangements in T-PLL. inv(14) and t(14;14) relocate the *TCL1* gene from its normal location at 14q32.1 to 14q11.2 under the control of the *TRA/D@* promoter. Less frequently, the *MTCPI* at Xq28, instead of *TCL1*, relocates to the *TRA/D@* locus

T-Cell Prolymphocytic Leukemia

T-cell prolymphocytic leukemia (T-PLL) is an aggressive neoplasm that affects approximately 2% of adults aged 30 years and over [469–471]. The most common sites of involvement include peripheral blood, bone marrow, lymph node, and other hematopoietic organs such as spleen and liver [469]. The most common chromosome abnormalities are inv(14)(q11.2q32.1), t(14;14)(q11.2;q32.1), and t(7;14)(q34;q32.1) [472] (Fig. 15.32). All of these translocations involve the T-cell receptor (TCR) genes (*TRA@/TRD@* at 14q11.2, and *TRB@* at 7q34) and the T-cell leukemia 1 (*TCL1*) gene located at 14q32.1 [472, 473]. In a minority of cases, translocations involve a TCR gene and *MTCPI*, located at Xq28, instead of *TCL1*. The most common translocation in this group is t(X;14)(q28;q11.2) [474].

Additional abnormalities are present in the majority of cases; these include i(8)(q10) or other rearrangements leading to gain of 8q, deletion or rearrangements of 11q, and deletions of 6q, 12p, and 17p [475] (Fig. 15.33).

Natural Killer Cell Leukemia

Natural killer cell leukemia (NKCL) is a rare form of T-cell leukemia that has a strong association with the Epstein-Barr virus (EBV) and tends to affect younger individuals, with a median age at diagnosis of 40 years [476]. Although a large number of these patients have an abnormal chromosome complement, no specific cytogenetic abnormalities have been identified. Recurrent chromosome abnormalities include deletions of 6q and 11q. Gain of 1q and loss of 17p are also seen [252].

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATLL) is a lymphoid neoplasm that it is known to be associated with early exposure to the human T-cell lymphotropic virus type 1 (HTLV-1) and affects approximately 3% of individuals who carry the virus (median age: 60 years) [469]. Although HTLV-1 is considered to be a primary insult, neoplastic transformation requires additional genetic changes [477]. In fact, the karyotypes of patients with ATLL are very complex.

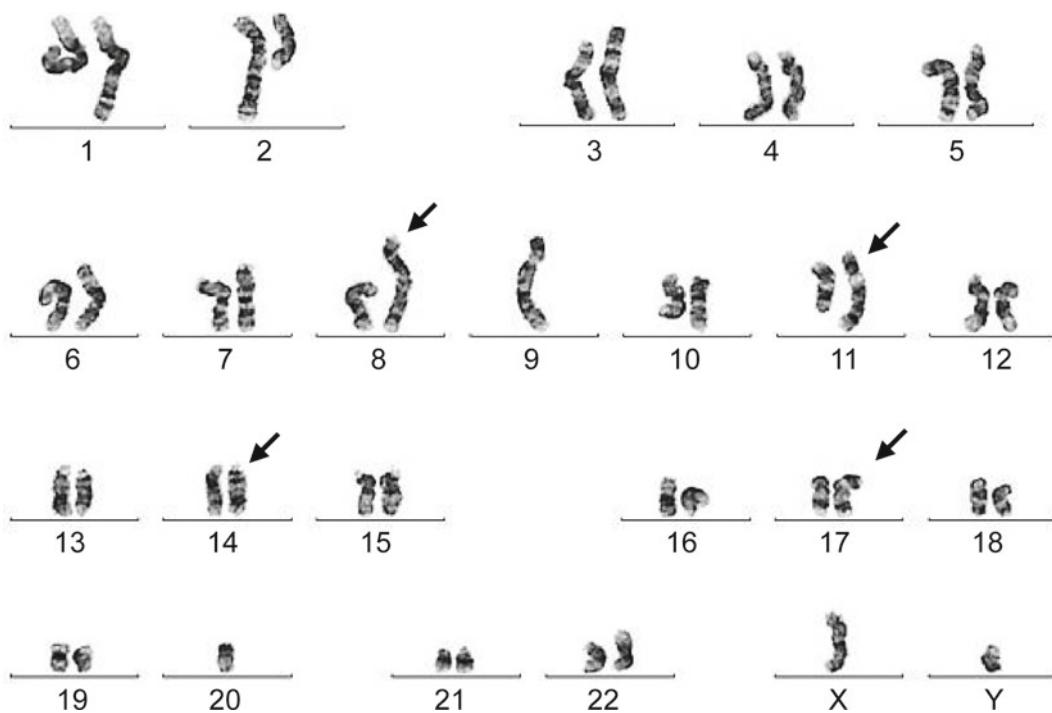


Fig. 15.33 Karyogram of a patient with T-cell prolymphocytic leukemia. In addition to the inv(14)(q11.2q32,1), the recurrent abnormalities observed in the majority of cases include gain of 8q, mainly in the form of isochromosome 8q as seen here, deletion of 11q, and deletion of 17p

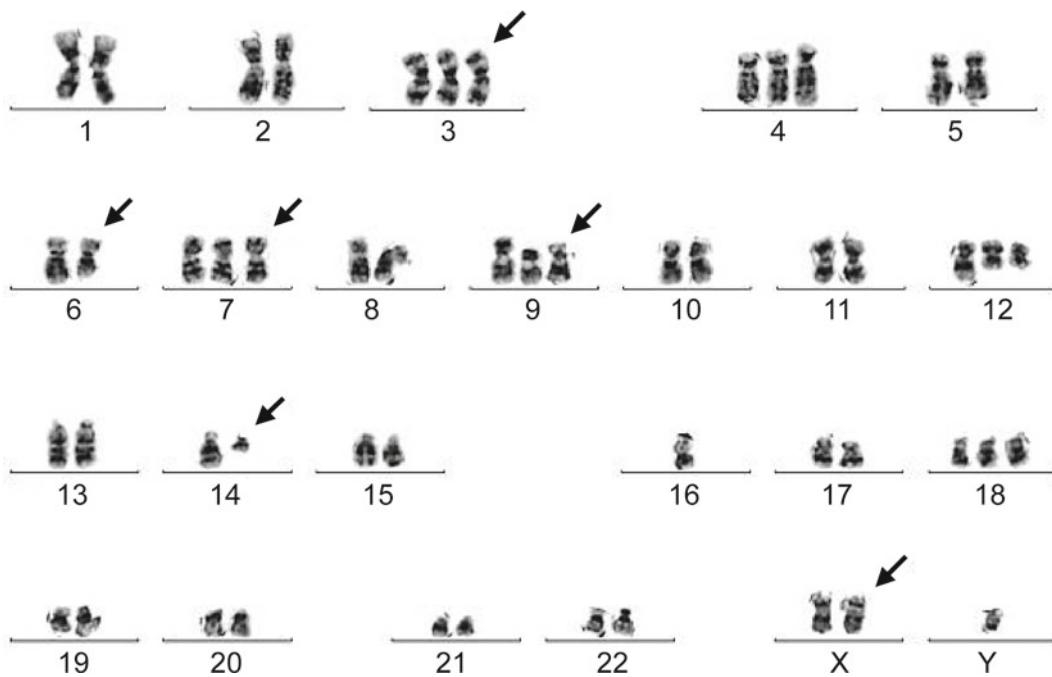


Fig. 15.34 Complex karyogram with several chromosome rearrangements typically seen in adult T-cell leukemia. Gain of chromosomes X, 3, and 7 and deletions 6q and 9p are present in most cases

The most frequent genetic abnormalities include rearrangements of the T-cell receptor genes *TRG@* at 7p14.1, *TRB@* at 7q34, and *TRA/D@* at 14q11.2; gains of the X chromosomes and chromosomes 3 and 7; rearrangements of 1p, 1q, 2q, 3q, and 17q; and deletions of 6q, 9p,

13q, and 17p [478] (Fig. 15.34). The prognosis associated with these karyotypes is considered unfavorable. In particular, abnormalities of 1p, 1q, 3q, and 14q and deletions of 2q, 9p, 14q, and 17p are thought to be associated with shorter survival [479].

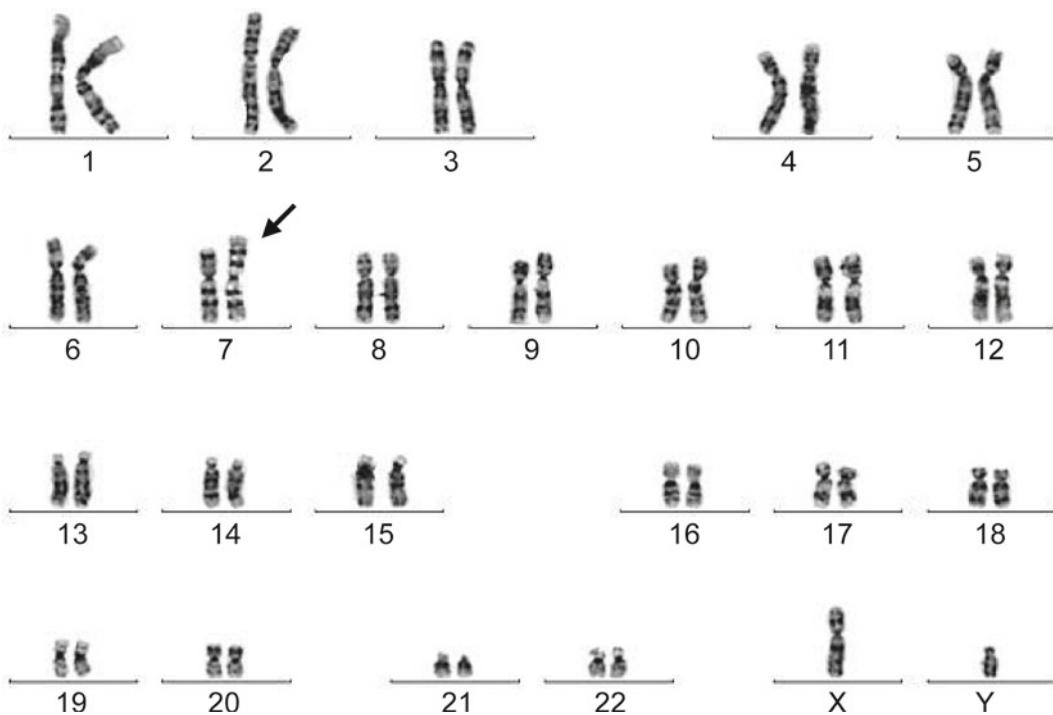


Fig. 15.35 Isochromosome 7q is often the sole abnormality in the karyogram of patients with hepatosplenic lymphoma

Hepatosplenic T-Cell Lymphoma

Hepatosplenic T-cell lymphoma (HSTL) is a rare and aggressive T-cell lymphoma that usually affects adolescents and young adults [469]. A male predominance has been noted. As the name implies, there is obvious involvement of the spleen and liver with evident hepatosplenomegaly, neutropenia, and thrombocytopenia [469, 480]. A characteristic recurrent abnormality of this lymphoma is the presence of an isochromosome for the long arm of chromosome 7 [i(7)(q10)] or less frequently alternative rearrangements leading to gain of 7q [481, 482] (Fig. 15.35). Additional abnormalities, most commonly +8 and loss of a sex chromosome, have also been reported [482].

Mycosis Fungoides

Mycosis fungoides (MF), the most common form of cutaneous T-cell lymphoma, is characterized by an increased number of CD4+ T-cells in the skin [470, 483]. The annual incidence is around 0.3 cases per 100,000 in Western countries, and the median age at diagnosis is between 55 and 60 years, with a 2:1 male to female ratio [470]. MF is an indolent disease, and given the high average age at diagnosis, it therefore has a high incidence among the elderly. However, more advanced cases have an unfavorable prognosis that resembles the clinical behavior seen in patients with Sézary syndrome, a closely related lymphoma (see next section) [483].

The karyotypes of patients with MF tend to be complex and often include rearrangements of the short arm of chromosome 1, particularly of the critical regions 1p32-p36, as

well as other numerical and/or structural abnormalities involving chromosomes 3, 6, 10, 11, 12, and 14 [484, 485].

Sézary Syndrome

Sézary syndrome (SS) is a cutaneous T-cell lymphoma similar in many ways to mycosis fungoides except for the presence of erythroderma and lymphadenopathy with circulating malignant cells in the peripheral blood and in the bone marrow [470, 483]. This neoplasm occurs in adults over the age of 60 and is associated with an unfavorable prognosis [470]. Karyotypes tend to be complex, with rearrangements involving chromosomes 1, 6, 10, 14, 17, 18, and 19 [484–486].

Peripheral T-Cell Lymphoma, Not Otherwise Specified

Peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS), represents any nodal or extranodal mature T-cell lymphoma that does not fit into the current system of classification. These neoplasms account for approximately 30–70% of all T-cell lymphomas worldwide [470, 487, 488].

The majority of these lymphomas have a nodal histology and occur primarily in adults, with a peak at around 60 years if age [470, 487, 488].

These lymphomas have similar genetic abnormalities. Karyotypes are usually highly complex, with rearrangements that often lead to losses of 6q, 9p, 10q, and 13q and to gains of 3q, 7q, and 8q [489]. The prognosis is poor for most patients. However, the identification of a new recurrent translocation, t(5;9)(q33.3;q22.2), has given hope for the possible

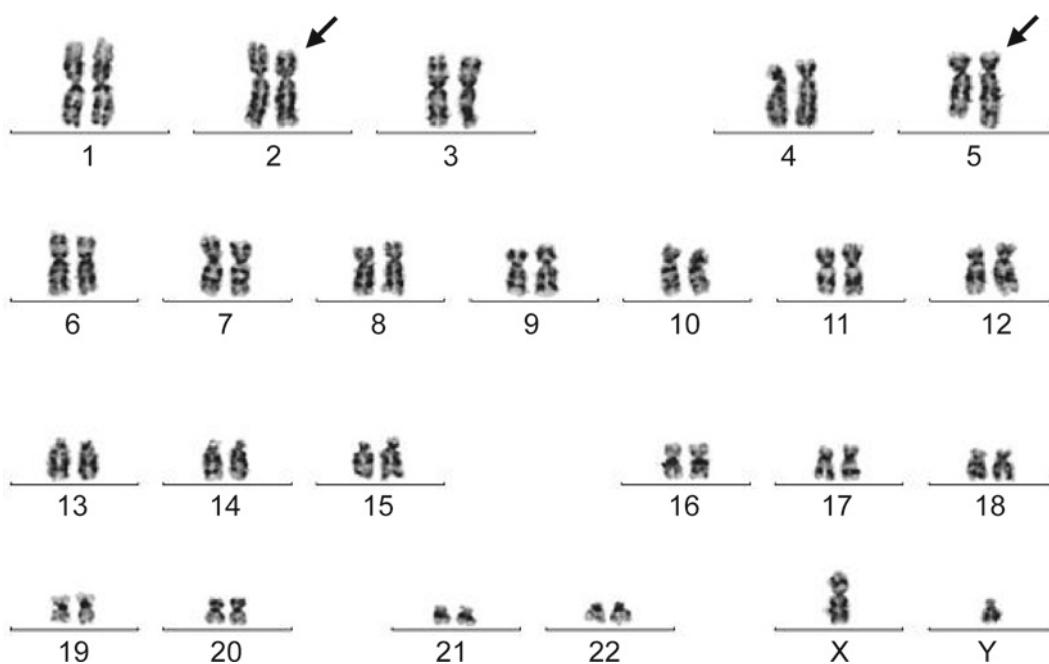


Fig. 15.36 t(2;5)(p23.1;q35.1) in a patient with anaplastic large T-cell lymphoma. This translocation leads to fusion of *ALK* at 2p23.1 and *NPM1* at 5q35.1. Other translocations involving *ALK* and other partner chromosomes have been reported in this lymphoma (see Table 15.13)

development of a new treatment for some patients with these lymphomas [490]. This translocation leads to fusion of the inducible T-cell kinase (*ITK*) gene located at 5q33.3 and the spleen tyrosine kinase (*SYK*) located at 9q22.2 and subsequent tyrosine kinase activation [490]. Patients with the *ITK-SYK* fusion appear responsive to treatment with *SYK* inhibitors.

Angioimmunoblastic T-cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AITL) is an aggressive nodal T-cell lymphoma that accounts for approximately 2% of all non-Hodgkin lymphomas but represents the most common subtype (15–20%) of peripheral T-cell lymphomas [470]. Affected patients average 60 years of age and present with an array of symptoms including skin rash, pleural effusion, and eosinophilia [470, 491]. One of the characteristics of this lymphoma is the presence of the EBV genome in the lymph nodes [492]. Karyotypes are complex and often show gain of 11q13 and gains of chromosomes 3, 5, and an X chromosome, as well as losses of 5q, 10q, and 12q [493, 494]. Gain of 11q13 may represent a primary event in angioimmunoblastic T-cell lymphoma.

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) accounts for approximately 3% of all lymphomas [470]. This lymphoma includes two main subtypes: ALK+ and ALK− [495]. Both entities characteristically express CD30 [496].

Approximately 60% of cases are positive for *ALK* (ALK+ ALCL). This subtype tends to have an aggressive course with extranodal involvement. Fifteen to twenty percent of patients are children, and 3–5% are young adults (around 30 years of age) [497–500]. The cytogenetic hallmark of ALK+ ALCL is the presence of specific translocations involving the anaplastic lymphoma kinase gene (*ALK*) and various partner chromosomes [499] (see Table 15.14). The most common *ALK* translocation is t(2;5)(p23.1;q35.1) (approximately 80% of cases), which fuses part of the nucleophosmin gene (*NPM1*) located at 5q35.1 with *ALK* located at 2p23.1, leading to activation of *ALK* [500] (Fig. 15.36). This has promoted the development of specific therapies with the function of inhibiting tyrosine kinase activity [498].

ALK−ALCL on the other hand, affects older individuals (around 60 years of age) and has a relatively favorable prognosis. This subtype does not involve the *ALK* gene and no recurrent chromosome abnormalities have been associated with it [497, 498].

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Linda D. Cooley and Kathleen S. Wilson

Introduction

All neoplasms have genetic abnormalities; most have visible nonrandom chromosome abnormalities. Clonal chromosome aberrations in both benign and malignant neoplasms define particular tumors. These can make or refine the histopathologic diagnosis, provide prognostic information, and inform therapeutic decisions. Examples include distinguishing an atypical teratoid/rhabdoid tumor from a medulloblastoma and documenting *MYCN* gene amplification in a neuroblastoma.

Today, more and more therapeutic drugs are designed to target a specific genetic anomaly. Examples include imatinib mesylate for patients with t(9;22) or *BCR-ABL1* fusion, ATRA (all-trans retinoic acid) for patients with t(15;17) or *PML-RARA* fusion, trastuzumab for *ERBB2* amplified intraductal breast carcinoma, tyrosine kinase inhibitors for gastrointestinal stromal tumors, and others. For tumors without a known targetable genetic anomaly, chromosome aberrations provide information to determine optimal therapies. Examples include oligodendroglial brain tumors with der(1;19) or 1p/19q deletion and Wilms tumors with 1p/16q deletion. Collaborative oncology groups have, over the past several decades, dedicated their efforts toward the discovery of the most effective, least

toxic therapeutic regimens to increase patient survival and quality of life. The genetic anomalies of tumors and their correlation with response to therapy have altered therapies such that the survival rates, length of survival, and even cure rates have significantly increased. The pace of discovery has been faster for hematologic tumors due to the ease of culture and yield of genetic information through chromosome analysis (see Chap. 15).

While slower to accumulate, the knowledge of the genetic aberrations in solid tumors has proven to be as valuable as for those in hematologic disorders. It is vitally important to continue this acquisition of genetic information to persevere toward finding effective therapies. Conventional chromosome analysis of solid tumors is a valuable tool that continues to discover genetic anomalies that influence current patient therapy. Chromosome data and that from newer technologies—e.g., various types of microarrays (see Chap. 18) and molecular methods—contribute to the growing genetic databases that are used to further efforts to design new therapeutic trials and discover new more effective drugs through understanding the genes involved.

Solid Tumor Culture and Analysis

Cytogenetic analysis of solid tumors (STs) is challenging. While most STs can be grown in tissue culture with good results, STs require more time and effort than the typical tissues like products of conception and skin that are cultured and analyzed in cytogenetics laboratories. Solid tumors are many and diverse, with more different names than there are tumor types. Understanding the “diagnosis” is fundamental to a successful outcome. The diagnosis directs the processing of the sample, preparation, culture type (monolayer vs. suspension), growth medium, and times and methods of harvest. ST cultures require close observation for growth and growth patterns to determine when to flood the culture, change the medium, and harvest. Harvest should begin as soon as the cultures are mitotic in order to capture

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the malignant cell population and avoid cell confluence and growth of normal cells. Harvest time and method differ for various cell types.

Chromosome analysis of STs can also be challenging. Some STs have a single diagnostic abnormality, while others may be complex with many structural and numerical aberrations. Perseverance and experience are factors in analysis of any complex hematologic or solid tumor in order to discern which anomalies have significance for diagnosis and/or therapeutic management.

Factors Affecting Growth of Solid Tumors

Tissue culture simulates the *in vivo* environment of the tumor so as to support viability and growth. Knowledge of the tumor type influences how it is processed; the referring physician or pathologist can provide the tumor type or working diagnosis.

The condition of the sample is also critical to culture success. A surgeon or pathologist obtains the specimen; thus, it is imperative that he or she knows how to handle a sample for cytogenetic culture. A working relationship with the pathologist(s) can be very beneficial and is encouraged.

Critical aspects of the tumor sample collection that the referring physician or pathologist controls and should understand are:

- Sterility must be maintained. Non-sterile tumors must be handled in a way that minimizes further contamination. If the sample is contaminated, this should be communicated to the cytogenetics laboratory.
- The sample needs to be tumor without surrounding normal tissue.
- The sample must be viable, i.e., not necrotic, frozen, or fixed.
- The sample must be delivered to the laboratory ASAP. The sample should be stored in supplemented culture medium at room temperature or 37°C until delivered.
- Adequate sample size; a 1 cm³ piece of tumor is optimal. However, even very small pieces can be successfully cultured.
- Touch preparations of tumor often provide a good sample for fluorescence *in situ* hybridization (FISH) analysis and, therefore, rapid results.

Tissue culture factors that impact tumor cell growth include:

- Type of culture: suspension or monolayer. Tumors that need attachment and/or cell-to-cell contact for growth require a monolayer culture, while small round cell tumors may grow better in suspension.
- Type of medium: monolayer cultures do fine with basic supplemented medium (e.g., minimum essential medium, MEM), while suspension cultures work well using

supplemented medium prepared for bone marrow cultures (e.g., Roswell Park Memorial Institute medium, RPMI).

- Monolayer culture on coverslips has advantages that include: facilitates growth of tiny samples, avoids trypsinization for splitting and harvesting, avoids making slides for banding, requires less medium, allows growth and mitotic activity to be easily monitored with an inverted microscope, etc.
- Tumor disaggregation (physically with scalpel blades and/or enzymatically with collagenase) usually promotes faster growth. Some tumors grow better with physical disaggregation alone. Cultures of tumor with and without enzymatic disaggregation can be initiated with sufficient sample.
- Sample initiation into culture should occur ASAP to preserve viability.
- Initiation of multiple cultures provides opportunities to feed and harvest at different times to promote growth and capture metaphase cells.
- Initiation with a small amount of medium allows cells to attach faster. Be patient and wait for evidence of cell attachment before flooding. Waiting several days can yield success.
- Daily observation is needed to detect optimal times for harvest and prevent confluent growth.
- Harvest with different lengths and strengths of Colcemid® exposure may capture more metaphase cells.

Laboratories that grow and analyze solid tumors must recognize and provide the extra time for the work it takes to culture and analyze these samples. As noted, the genetic information gleaned from this process is critical to optimal patient management. Both pathologists and oncologists use this information to refine diagnoses and therapeutic decisions.

This chapter will cover certain solid tumors with known chromosomal abnormalities that have documented pathological and clinical significance for diagnosis, diagnostic subtype, prognosis, and therapeutic intervention. The chapter does not comprehensively cover all tumors and does not present all chromosomal or genetic anomalies of those discussed.

Central Nervous System Tumors (Table 16.1)

Gliomas

Gliomas, the most common primary central nervous system (CNS) tumors, include astrocytomas, oligodendrogiomas, and ependymomas. Histopathologic features used for pathological classification and grading of the tumor correlate with prognosis and guide therapy. The annual incidence of primary brain tumors is ~8–12 in 100,000 and for intraspinal tumors it is 1–2 in 100,000. CNS tumors are the most common (~20%) cancers of childhood [1].

Table 16.1 Chromosome abnormalities with diagnostic or clinical significance in tumors of the central nervous system

Tumor	Chromosomal aberration(s)	Gene(s) involved	Clinical significance	References
<i>Glia</i>				
Pilocytic astrocytoma	Gain of 5, 7/7q common, followed by 6, 8, 11, 12, 17, 19, 22	KIAA1549-BRAF from BRAF tandem duplication	Constitutive kinase activity	[3–5]
Diffuse grade II/anaplastic astrocytoma grade III	Loss of 10q, 13q, 17p, 19q	IDH1, IDH2, RB1 and TP53 mutation	TP53 mutation correlates with IDH mutations	[6]
Glioblastoma	Loss of 9p, 10q; EGFR amplification, MDM2 amplification	CDKN2A/B, PTEN, EGFR, MDM2	Short survival, aggressive course	[8–10]
<i>Oligodendroglial</i>				
Oligodendrogloma (OD)	der(1;19)(q10;p10), loss of 1p, 19q	1p36, 19q13.3, IDH mutation	Longer survival, sensitive to therapy	[11–15]
Anaplastic OD	der(1;19)(q10;p10), loss of 1p, 19q, 9p	1p36, 19q13.3, CDKN2A/B, IDH	Longer survival, sensitive to therapy	[11–15]
<i>Oligoastrocytic</i>				
Oligoastrocytoma (OA)	der(1;19)(q10;p10), TP53 mutation	1p36, 19q13.3, TP53, IDH mutation	Outcome is better with der(1;19)	[1, 16]
Anaplastic OA	der(1;19)(q10;p10), loss of 9p, 10q	1p36, 19q13.3, TP53, CDKN2A/B	Overall survival is better with der(1;19)	[20, 21]
<i>Ependymoma</i>				
Spinal	Loss of 22q		More common in adults	[22]
Posterior fossa	Gain of 1q, balanced karyotypes		Young age, unfavorable prognosis	[23, 24]
Posterior fossa	Loss of 6q, 22q, gain of 9q, 15q, 18q		Favorable prognosis	[23, 24]
Intracranial	Gain of 1q, 7, loss of 6q, 9p	EGFR, CDKN2A	Gain of 1q, CDKN2A homozygous deletion is unfavorable	[25]
<i>Choroid plexus</i>				
Papilloma (CPP)	Normal karyotype		Distinguish from atypical CPP	[26–28]
Atypical papilloma	Hyperdiploid with gain of 7, 12, 20	Chromosome enumeration	Distinguish from CPC	[26–28]
Carcinoma (CPC)	Hyperhaploid with gain of 1, 12, 20, 4, 7	Chromosome enumeration	Distinguish from papilloma	[26–28]
<i>Embryonal</i>				
Medulloblastoma, pediatric	Gain of 6q, MYC or MYCN amplification	MYC, MYCN, ERBB2	Very high risk, correlates with large cell/ anaplastic histology	[29–33]
	i(17q), gain of 17q		Intermediate risk	[29–33]
	Loss of 6 or 6q		Excellent prognosis, desmoplastic histology	[29–33]
Medulloblastoma, adult	Loss of 10q with gain of 17q, CDK6 amplification	CDK6 (7q21.2)	High risk, poor OS	[30]
	Either loss of 10q or gain of 17q		Intermediate risk	[30]
	Neither loss of 10q nor gain of 17q		Good prognosis	[30]
PNET, supratentorial	Gain of 1q, loss of 14, 16, 19, rare EGFR amplification	EGFR	Lacks i(17q), poor prognosis	[31, 35]
Atypical teratoid/rhabdoid tumor	Loss of 22, del(22q)	SMARCB1-INI1	Distinguishes AT/RT from MB, PNET, CPC	[36–39]
<i>Meningioma</i>				
Atypical/anaplastic	Loss of 22, del(22q)	22q, NF2	Primary abnormality	[43, 44]
	loss of 1p, 14/14q, 18, 10, 6q		Increased risk of recurrence	[43, 44]
<i>CPC</i> choroid plexus carcinoma, <i>CPP</i> choroid plexus papilloma				

Astrocytomas

Astrocytomas have a spectrum of histologic features that correlate with the clinical course [2]. Grade I, pilocytic astrocytoma (PA), is a slow-growing, noninfiltrating tumor with a relatively benign course. Most PAs have a normal diploid karyotype; ~30% show gain of one or more chromosomes. Approximately half of children younger than 15 years old show a single extra chromosome, while those greater than 15 years of age show gain of multiple chromosomes. Gains of chromosomes 5 and 7 are the most frequent, followed by chromosomes 6, 8, 11, 12, 17, 19, and 22. See Fig. 16.1. Tandem duplication of *BRAF* at 7q34 is found in >50% of pediatric PA and in up to 66% of all PA [3, 4]. Tandem duplication produces a novel fusion gene, *KIAA1549-BRAF*, which has constitutive kinase activity [4, 5].

Grade II diffuse astrocytoma and grade III anaplastic astrocytoma show increasing cellularity and nuclear pleomorphism. Gliomas of World Health Organization (WHO) grade II or III are invasive, progress to higher-grade lesions, and have a poor outcome. As the tumor progresses, acquired genetic abnormalities include loss of 10q, 13q, 17p, and 19q, and *TP53* and *RBI* mutations. The majority of WHO grade II and III gliomas, including astrocytomas and oligodendroglomas, have *IDH1* (2q34) or *IDH2* (15q26.1) mutations. Most also have *TP53* mutation. Patients with mutations of *IDH* genes have longer median overall survival, 65 versus 20 months [6].

Grade IV, glioblastoma (GB), is the most malignant astrocytoma. In addition to increases in cellularity, nuclear atypia, and pleomorphism, mitotic activity, vascular proliferation, and necrosis are characteristic. Genetic changes in primary glioblastomas, which account for >90% of GBs, include loss of 9p (*CDKN2A* deletion), loss of 10q (*PTEN* deletion), *PTEN* mutation, frequent *EGFR* amplification, and infrequent *MDM2* amplification. The secondary glioblastomas (5–10%) that result from progression of grade II–III tumors have mutations of *IDH1* or *IDH2* and *TP53*. Tumors with wild-type *IDH1* and *IDH2* have fewer *TP53* mutations and frequent alterations of *PTEN*, *EGFR*, *CDKN2A*, or *CDKN2B* [1, 6–8]. Median overall survival in patients with *IDH* mutations is twice that of patients with wild-type *IDH*, 31 versus 15 months [6]. Double minutes (dmin) with *EGFR* amplification have been demonstrated in up to 50% of GBs [9]. Current clinical trials are evaluating several targeted molecular therapies [10].

Oligodendroglial Tumors

Oligodendrogloma (OD), a diffusely infiltrating well-differentiated (grade II) to anaplastic (grade III) glioma, is a relatively rare primary brain tumor, comprising 2–5% of all primary brain tumors. ODs characteristically show an unbalanced der(1;19)(q10;p10) with loss of 1p and 19q (Fig. 16.2). These aberrations may be detected by conventional chromosome analysis, fluorescence *in situ* hybridization (FISH), loss

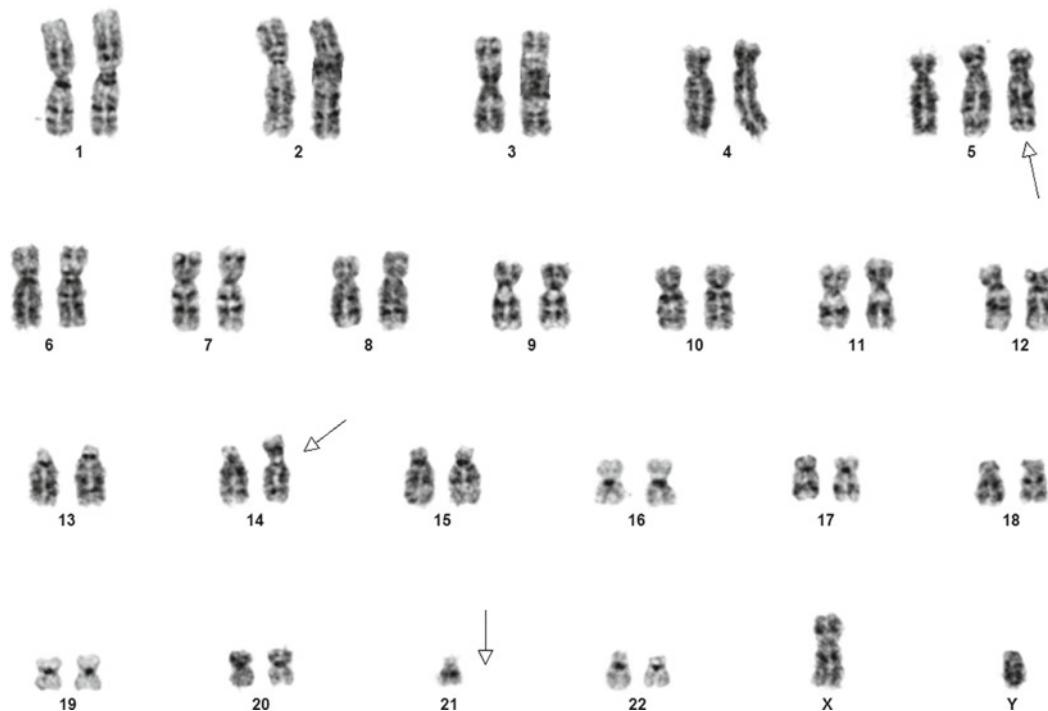


Fig. 16.1 Pilocytic astrocytoma, grade I, from the posterior fossa of a 12-year-old boy: 47,XY,+5,der(14;21)(q10;q10)

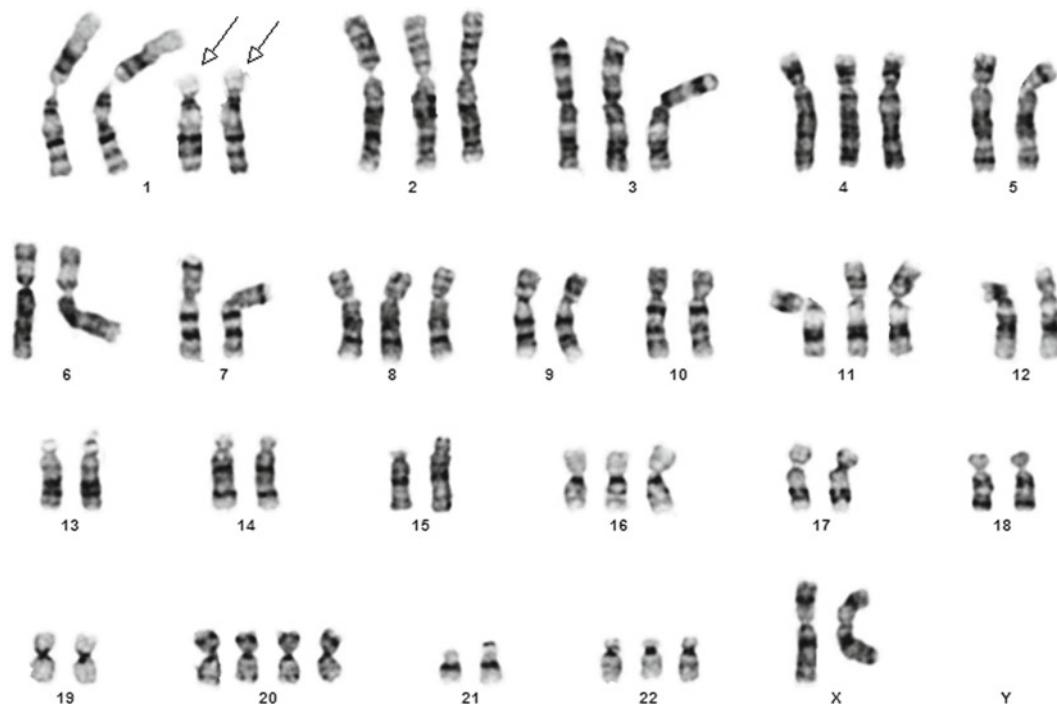


Fig. 16.2 Oligodendrogloma, grades II–III, from the brainstem of an 8-year-old girl: 57,XX,+der(1;19)(q10;p10)x2,+2,+3,+4,+8,+11,+16,+20,+20,+22. Arrows indicate the der(1;19); (see text for details)

of heterozygosity (LOH) studies, or microarray comparative genomic hybridization (aCGH) [11, 12]. 1p/19q codeletion is associated with a longer median survival (~10 years vs. 2 years) in patients with OD and anaplastic OD [13]. Anaplastic oligodendroglomas show loss of 9p (*CDKN2A/B*) in addition to 1p/19q loss [14]. Recent studies have shown that *IDH1* mutations are strongly associated with 1p/19q codeletion (~85%) and that *IDH1* mutation and 1p/19q codeletions are independent prognostic factors [6, 15].

Oligoastrocytic Tumors

Oligoastrocytomas (OA) are composed of a mixture of distinct cell types found in oligodendrogloma and astrocytoma. Genetic analysis reveals 1p/19q codeletion in ~30–50%, *TP53* mutations in ~30%, and *IDH* mutations in 100% of OA. Progression-free survival (PFS) was 60 versus 30 months in patients with and without 1p/19q codeletion, respectively [1, 16]. Few studies have attempted to evaluate the genetics of the distinct cellular components. Rare tumors have been shown to have different genetic aberrations in the two histologic elements [17–19]. Anaplastic OA (grade III) acquire additional genetic alterations—e.g., 9p (*CDKN2A/B*) loss, which is also implicated in the progression of astrocytoma and OD. Recent studies of anaplastic OD and OA confirm that presence of 1p/19q codeletion correlates with significantly longer survival than presence of genetic abnormalities other than 1p/19q codeletion [20, 21].

Ependymomas

Ependymomas are slow-growing neuroepithelial neoplasms that arise from the wall of the intracranial ventricles and the central canal of the spinal cord. Little is known about the genetics of myxopapillary ependymoma, a grade I tumor that arises predominately in the terminal spinal cord of young adults. The grade II ependymoma may arise at any site in the ventricular system and spinal canal with the posterior fossa the most common site in children. The most frequent cytogenetic aberrations include losses of 22q and 6q and gains of 1q and 9q (Fig. 16.3).

Alterations of 22q are more frequently identified in spinal tumors. *NF2* may be a candidate tumor suppressor gene in spinal ependymoma. However, few mutations in candidate genes, including *NF2*, *TP53*, *PTEN*, *MEN2*, and *CDKN2A*, have been identified in pediatric intracranial tumors [1, 3, 22].

Recent studies have identified two clinically and genetically different posterior fossa subgroups [23, 24]. Group A patients are younger (median age 4 years), more often male, more often have higher grade tumors (grade III), have more metastases at recurrence, and have a diminished prognosis compared with group B patients. Group A tumors have predominately balanced karyotypes; some show gain of 1q. Group B tumors frequently show loss of 6q and 22q and gain of 9q, 15q, and 18q. Intracranial tumors are more common in children than adults, with spinal tumors more common in adults. Tumors with gain of 1q correlate with higher grade, tumor recurrence, and worse prognosis [25].

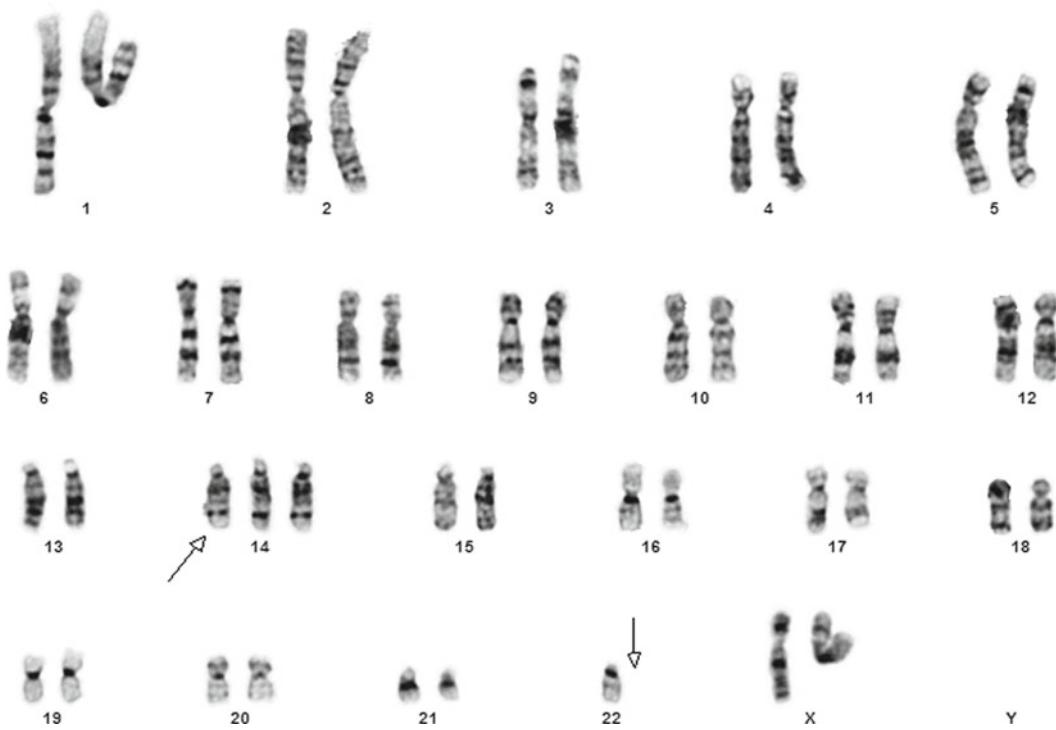


Fig. 16.3 Ependymoma, grade II, from the posterior fossa of a 2-year-old girl: 46,XX,+14,-22

Choroid Plexus Tumors

Choroid plexus (CP) tumors arise from the choroid plexus in the ventricles of the brain. Choroid plexus papilloma (CPP) is a benign (grade I) neoplasm with very low mitotic activity that closely resembles normal choroid plexus. Atypical CPP (grade II) is a CPP with increased mitotic activity that may have increased cellularity and nuclear pleomorphism. Grade III choroid plexus carcinoma (CPC) has frequent mitoses, increased nuclear pleomorphism, cellular density, necrotic areas, and sheets of tumor cells. The cytogenetics of these lesions helps to distinguish them from each other and from entities with which CP tumors may be confused. The CPP is karyotypically normal, the atypical CPP is hyperdiploid (Fig. 16.4), while CPC is hyperhaploid (Fig. 16.5). The most common recurrent chromosomal gains in atypical CPP are of chromosomes 7, 12, and 20, followed by gains of whole chromosomes 8, 9, 11, 15, 17, 18, and 19. Hyperhaploid CPCs characteristically have between 32 and 35 chromosomes; the most common gains (relative to a haploid background) in decreasing frequency order are of chromosomes 1, 12, 20, 4, and 7 [26–28].

Embryonal CNS Tumors

Medulloblastoma and Supratentorial Primitive Neuroectodermal Tumor

Medulloblastoma (MB) is a primitive small round cell tumor, which may show glial or neuronal differentiation. MB, also referred to as an infratentorial primitive neuroectodermal tumor (iPNET), is located in the cerebellum. MB is the most common malignant brain tumor in children, accounting for ~20% of all pediatric brain tumors. MB is rare in adults, comprising ~1% of primary adult intracranial malignant tumors. Supratentorial primitive neuroectodermal tumors (sPNETs) are histologically very similar to MBs, but are located in the cerebrum, and are clinically more aggressive.

Cytogenetically, i(17q) is the most common chromosome abnormality in pediatric MBs, present in ~35% of cases, but it is not found in pediatric sPNET. Recent studies identified characteristic and prognostic cytogenetic subgroups [29–31]. Highest risk pediatric MBs are characterized by gain of 6q, *MYC* amplification, *MYCN* amplification, and *ERBB2* amplification; intermediate risk MB by 17q gain; and low risk MB by loss of 6q [29, 32]. High-risk genetic abnormalities

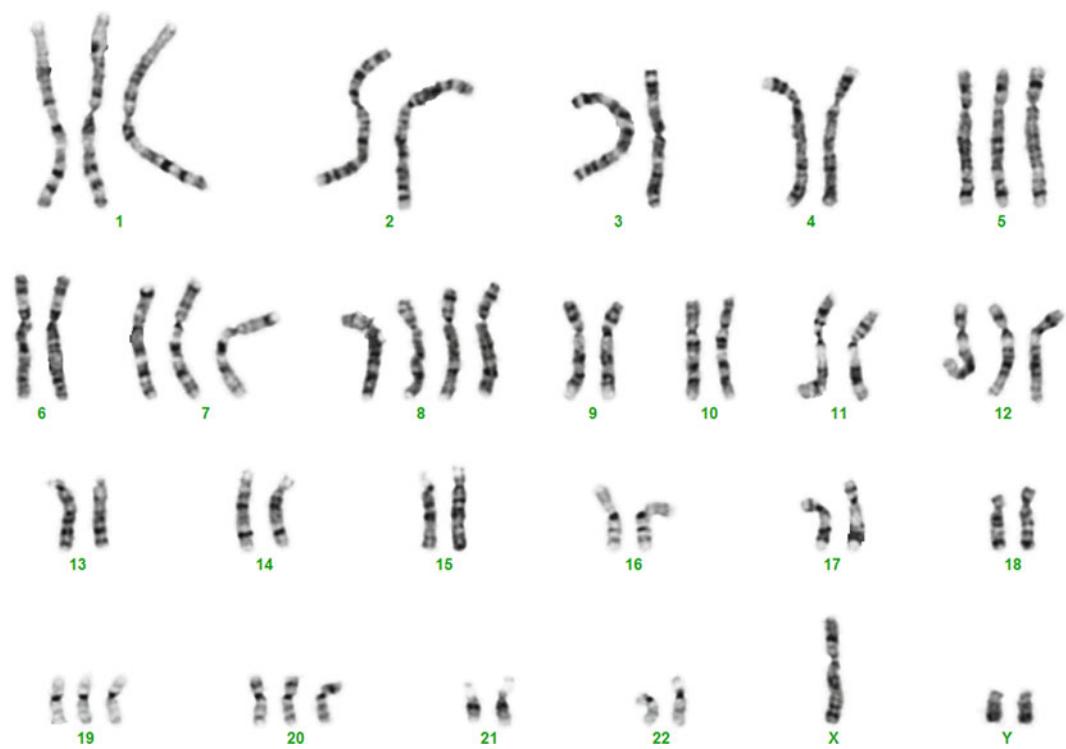


Fig. 16.4 Choroid plexus papilloma, grade II, from the lateral ventricle of an 8-month-old boy: 55,XYY,+1,+5,+7,+8,+8,+12,+19,+20. The constitutional karyotype is 47,XYY

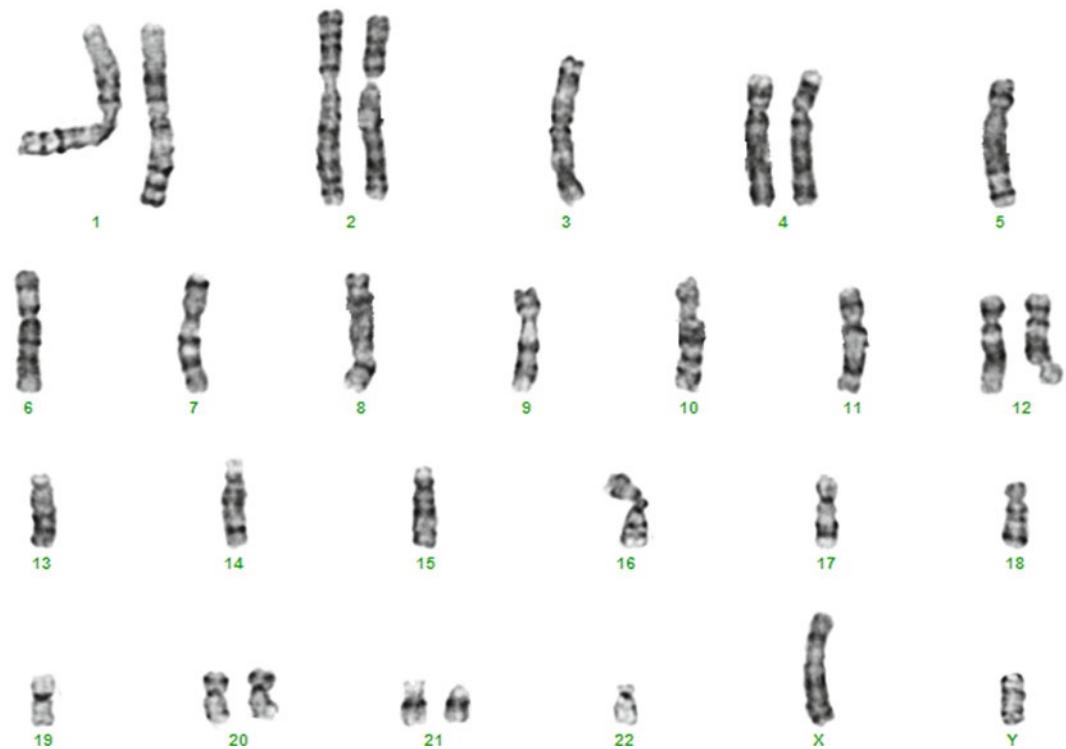


Fig. 16.5 Choroid plexus carcinoma, grade III, from the lateral ventricle of a 4-month-old boy: 30<1n>,XY,+1,+2,+4,+12,+20,+21

strongly correlate with the large cell/anaplastic histology found in ~7% of MBs [29, 33]. *MYC/MYCN* amplification is rare (~4–10% of MBs). Patients with low-risk genetic abnormalities, loss of chromosome 6 or 6q, and nodular desmoplastic histology have an excellent 5-year overall survival (OS) of ~90%. Most (~90%) MBs have classic histology with a 60% OS. Gain of chromosome 7, common in MB, is not associated with OS [34].

Cytogenetic abnormalities associated with poor OS in adult MBs include high-level amplification of the oncogene *CDK6* at 7q21.3 (more frequent than *MYC*), chromosome 17 aberrations including i(17q), and loss of 10q [30]. Oncogene amplification frequently associates with aberration of chromosome 17. Independent significant predictors for poor prognosis are loss of 10q and gain of 17q. Combined 10q loss and 17q gain show the poorest OS of ~16%; either 10q loss or 17q gain is associated with a 44% OS, and absence of both 10q loss and 17q gain is associated with the best OS (92%).

Supratentorial PNETs are less well characterized due to a smaller number of sPNETs as compared with MBs. The most frequent cytogenetic aberrations so far associated with sPNET are gain of 1q and losses of 14q, 16p, and 19p/q. Gain of 17q is not found in sPNET. Rare amplifications of 1q, 4q12-q13, 8q22-q24, 19q12-q13, and *EGFR* are reported [31, 35].

Atypical Teratoid/Rhabdoid Tumor

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly malignant tumor in young (<5 years of age) children that may arise infratentorially (posterior fossa) or supratentorially. AT/RTs account for ~10% of CNS tumors in infants. The tumor is defined by the rhabdoid cell, which resembles a rhabdomyosarcoma cell. Pathologically, AT/RTs may be difficult to recognize by histology alone due to variable components of primitive neuroectodermal, mesenchymal, and epithelial features. AT/RT may be distinguished from other poorly differentiated and anaplastic CNS tumors (e.g., sPNET, MB, or ependymoma) by a characteristic loss of chromosome 22 or 22q deletion [36]. The region of loss includes the *SMARCB1/INI1* gene at 22q11.23. The overall prognosis for patients with AT/RT is poor, particularly in patients diagnosed at <3 years of age. AT/RT may be sporadic or part of the rhabdoid tumor predisposition syndrome [36, 37]. Germline deletion or mutation of *SMARCB1* has been identified in up to 35% of patients with rhabdoid tumors [38]. Investigation of familial cases has revealed other affected relatives, unaffected carrier parents, and gonadal mosaicism. Individuals with germline mutations of *SMARCB1* present at a younger age (5 vs. 18 months), may have multiple primary tumors, or affected siblings as expected with a germline tumor suppressor gene mutation [36, 38, 39].

Meningeal Tumors

Meningioma, a tumor that arises from membranes surrounding the brain, accounts for ~20–30% of primary intracranial tumors. The World Health Organization classification of brain tumors recognizes three grades of meningioma: WHO grade I, the most common (70–80%); grade II, atypical (5–25%); and grade III (1–3%), the anaplastic type [1]. Grade I tumors are slow growing and benign with a low risk of recurrence, while grade II and III tumors have a greater likelihood of recurrence and/or aggressive behavior. Meningiomas occur most commonly in middle-age to older patients with a peak in the sixth and seventh decades. Female-to-male ratio is ~2:1, with males overrepresented in grade II and III tumors [40, 41]. Pediatric (<20 years old) meningiomas are rare, comprising ~2% of all meningiomas [42].

Meningioma was the first solid tumor to be associated with a nonrandom cytogenetic abnormality (monosomy 22). Loss of or interstitial deletion of chromosome 22q is the most common and often the sole anomaly in benign meningioma. Atypical and anaplastic meningiomas often show additional chromosome losses, i.e., 1p, 14q, 18, 10, and 6q. Spinal meningiomas are usually low grade with monosomy 22 as the sole anomaly [43, 44]. Pediatric meningiomas show similar chromosome anomalies as those in adults. A complex karyotype is more common in pediatric meningiomas, but there is insufficient data to correlate karyotype with biologic behavior in pediatric tumors [26, 42]. Mutations and/or deletions encompassing the *NF2* gene at 22q12.2 are present in *NF2*-associated meningiomas and in ~60% of sporadic meningiomas [1].

Genitourinary Tumors (Table 16.2)

Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common malignant tumor arising from the kidney. Prognosis is related to histologic subtype and tumor stage at diagnosis. Histologic subtypes include clear cell RCC (70%), papillary RCC (10–15%), chromophobe RCC (4–6%), Xp11.2 translocation RCC, and others. Different subtypes are characterized by different genetic abnormalities. Hereditary syndromes with RCC as a feature include von Hippel-Lindau, Birt-Hogg-Dube, tuberous sclerosis, hereditary papillary RCC, familial clear cell RCC, hereditary leiomyomatosis and RCC, and familial oncocytoma. Hereditary RCCs account for 4% of RCCs [7].

Clear Cell RCC

Clear cell RCC (ccRCC) histology shows cells with clear or granular cytoplasm without a papillary growth pattern. The majority of ccRCCs have deletion or rearrangement of the

Table 16.2 Chromosome abnormalities with diagnostic or clinical significance in genitourinary tumors

Tumor	Chromosomal aberration(s)	Gene(s) involved	Clinical significance	References
<i>Renal</i>				
Clear cell RCC	Loss of 3 or 3p Loss of 3p with gain of 5q Loss of 9p, 14q	<i>VHL, PBRM1, PTH1R</i>	Non-papillary RCC Favorable prognosis Unfavorable, shorter survival	[45–48]
Papillary RCC	Gain of 7, 17, loss of Y	<i>MET</i>	Adult papillary RCC	[45, 49]
t(Xp11.2) RCC	t(X; 1)(p11.23;q23.1)	<i>TFE3-PRCC</i>	Xp11.2 RCCs infrequent in adult RCC, common in pediatric RCC	[50–54]
	t(X;17)(p11.23;q25.3)	<i>TFE3-ASPSCR1</i>	Balanced t(X;17) in RCC; Unbalanced t(X;17) in ASPS	[50–54, 184]
	t(X;17)(p11.23;q23.1)	<i>TFE3-CLTC</i>		[50–54]
	t(X;1)(p11.23;p34.3)	<i>TFE3-SFPQ</i>		[50–54]
t(6;11) RCC	t(6;11)(p21.1;q13.1)	<i>TFEB-ALPHA</i>	Subset of RCC, children, young adults	[54, 55]
Chromophobe	Loss of 1, 2, 6, 10, 13, 17, 21		Distinguish from oncocytoma	[56, 57]
Oncocytoma	Loss of 1 or 1p, Y		Distinguish from chromophobe	[57]
Wilms tumor	Loss of 16q, 1p, 4q, 14q, 17p, 22; gain of 1q; der(1;16)(q10;p10)	<i>TP53</i>	Unfavorable histology; augmented chemotherapy if loss of 1p, 16q	[58–64]
Clear cell sarcoma (CCSK)	t(10;17)(q22.3;p13.3), del(14)(q24.1q31.1)	<i>FAM22-YWHAE</i>	t(10;17) in 12% of CCSK; t(10;17) also seen in endometrial stromal sarcoma	[65–68]
CMN	t(12;15)(p13.2;q25.3), gain of 11, 20, 17, 8	<i>ETV6-NTRK3</i>	Diagnostic; t(12;15) also seen in CFS/IFS and secretory breast cancer	[69, 70]
Rhabdoid tumor (RTK)	Loss of 22, del(22q)	<i>SMARCB1-INH1</i>	Diagnostic	[71–73]
Prostate	Gain of 7q, 8q, loss of 8p, 10q, 13q, 17p, 7p, 16q, 6q	<i>MYC, PTEN, TP53, TMPRSS2-ERG</i>	Poor OS with <i>ERG</i> amplification, <i>TMPRSS2-ERG</i> fusion+; poorest OS with <i>PTEN</i> , <i>TP53</i> .	[75–80]
Bladder	Gain of 3, 5p, 7, 17, 20, loss of 9, 9p	<i>CDKN2A</i>	Homozygous deletion <i>CDKN2A</i> higher grade and stage; recurrence, progression	[81–87]
<i>Reproductive</i>				
Endometrial stromal tumor (EST)	t(7;17)(p15.2;q11.2), t(6;7)(p21.3;p15.2), t(6;10) (p21.3;p11.22), t(10;17)(q22.3;p13.3)	<i>JAZF1-SUZ12, PHF1-JAZF1, PHF1-EPC1, FAM22-YWHAE</i>	Distinguish from non-EST uterine tumors	[88–91]
<i>Germ cell</i>				
Postpubertal GCTs	12p overrepresentation, i(12p) or 12p amplification	12p	i(12p), amplification of 12p distinguishes GCTs	[95, 100–104]
Ovarian dysgerminoma; testicular, seminoma, nonseminoma; extragonadal seminoma, nonseminoma, infantile fibrosarcoma, rhabdoid tumor of kidney	12p overrepresentation, i(12p) or 12p amplification, gain of X, 7, 8, 12, 21, loss of 1p, 11, 13, 18	Chromosome enumeration	Mediastinal GCT associated with Klinefelter syndrome	[2, 7, 95, 98, 100, 102]
Prepubertal GCTs	Loss of 1p36, 4q, 6q; gain of 1q, 3p, 16p, 20q; rare gain of 12p	Chromosome enumeration	12p gain rare in prepubertal GCT	[96, 101, 102]

ASPS alveolar soft part sarcoma, CFS/IFS congenital fibrosarcoma/infantile fibrosarcoma, CMN congenital mesoblastic nephroma, CCSK clear cell carcinoma of kidney, GCT germ cell tumor, OS overall survival, RCC renal cell carcinoma, RTK rhabdoid tumor of kidney

short arm of chromosome 3 that results in loss of part or all of 3p, often including the von Hippel-Lindau (*VHL*) gene at 3p25.3. Mutations of *VHL* are present in ~90% of sporadic tumors. No association between *VHL* status, tumor grade, and stage has been found. Other genes at 3p21.1 (*PBRM1*) and 3p21.31 (*PTH1R*) are reported to be mutated or lost in 40 and 76% of ccRCCs, respectively [45–47]. In addition to 3p loss, ccRCCs may show gain of chromosome 5 or gain of 5q, gain of chromosome 7, and loss of 9p and/or 14q [48].

Recent work by Dondeti et al. further elucidated two subtypes of *VHL* deficient ccRCC—H1H2 and H2—based on *HIF1A* expression, with each group having its own specific pattern of copy number alterations. The H2 subtype showed gain of 5q and loss of 14q more frequently than H1H2, while the H1H2 subtype more frequently showed gain of 16p and 19p and loss of 6q. Gain of 5q, present in ~30% of ccRCCs, is reported as a favorable prognostic factor, while losses of 9p and 14q are associated with a poor outcome [45]. Two genes identified on 5q—*STC2* at 5q35.2 and *VCAN* at 5q14.3—are thought to be important in the tumorigenesis of ccRCCs [47].

Papillary RCC

Papillary RCC (pRCC) is characterized by a papillary growth pattern and occurs in familial and sporadic forms. Cytogenetically, pRCC shows gains of chromosomes 7 and 17 and loss of the Y chromosome (Fig. 16.6). Gains of

chromosomes 12, 16, and 20 are also frequent. The *MET* proto-oncogene located at 7q31.2 is mutated in a subset of sporadic pRCC and is responsible for hereditary pRCC [45, 49].

TFE3 and *TFEB* Translocation RCC

RCC with Xp11.2 translocations/*TFE3* gene fusions is seen in children and adults, but is more predominant in the pediatric age group. These tumors have a papillary architecture and resemble pRCC. Xp11.2 RCCs have been misclassified as chromophobe and ccRCCs.

Several partner genes fuse with *TFE3* at Xp11.2. The two most common translocations are t(X;1)(p11.23;q23.1)/*TFE3-PRCC* and t(X;17)(p11.23;q25.3)/*TFE3-ASPCR1*. Variants include t(X;1)(p11.23;p34.3)/*TFE3-SFPQ* and inv(X) (p11.23q13.1)/*TFE3-NONO* and others. The Xp11.2 translocation tumors are reported to be aggressive in both pediatric and adult patients, which may in part be due to higher stage disease at diagnosis [50–54].

TFE3 is a member of the MiT transcription factor family. Another member, *TFEB*, is involved in a subset of RCCs. These RCCs show a t(6;11)(p21.1;q13.1). *TFEB* at 6p21.1 fuses with *alpha* at 11q13.1. Tumors with the t(6;11) are a distinctive subset of RCCs in children and young adults. The tumor has nests of epithelioid cells with clear cytoplasm along with a second population of smaller cells usually clustered around hyaline nodules [54, 55].

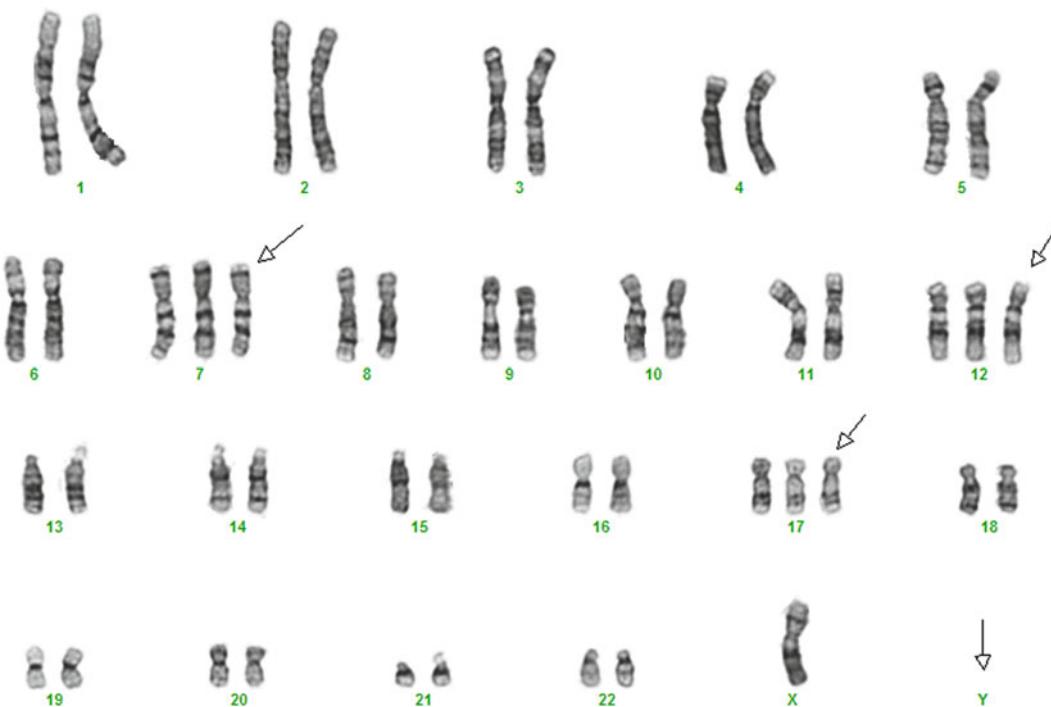


Fig. 16.6 Papillary renal cell carcinoma from the kidney of a 67-year-old male: 48,X,-Y,+7,+12,+17

Chromophobe RCC and Oncocytoma

Chromophobe RCCs are composed of cells with prominent cell membranes and eosinophilic cytoplasm. They may be difficult to distinguish from the benign renal oncocytoma or the granular variant of ccRCC. Chromophobe RCC is characteristically hypodiploid with loss of multiple chromosomes including chromosomes 1, 2, 6, 10, 13, 17, and 21. Renal oncocytomas, composed of large eosinophilic cells, show a normal karyotype in 60% of tumors and partial or complete loss of chromosome 1 in 40%. Loss of the Y chromosome and chromosome 14 may be seen together with chromosome 1 loss [56, 57].

Wilms Tumor

Wilms tumor (WT) or nephroblastoma is the most common primary malignant renal tumor of childhood and the fourth most common pediatric malignancy overall. The classic WT is triphasic with blastemal, epithelial, and stromal components [2]. Most tumors are sporadic and unilateral.

Approximately 5–10% of patients with Wilms tumor have a germline predisposing gene mutation. WT is associated with congenital syndromes including Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome, Denys-Drash syndrome, Perlman's syndrome, and Beckwith-Wiedemann syndrome. Patients with a germline mutation come to medical attention ~10 months earlier than patients with sporadic tumors and may have bilateral tumors. With the treatment protocols of two large cooperative groups that prospectively study children with Wilms tumor (the National Wilms Tumor Study Group and the International Society of Paediatric Oncology), survival of patients with WT is now >85%. Prognosis correlates with histopathologic features; survival rates are lower with unfavorable histology (UFH) versus favorable histology (FH). Cytogenetic analysis and aCGH and LOH studies of Wilms tumor tissue have found that loss of 16q consistently correlates with UFH and higher mortality [58, 59]. Loss of 16q often results from an unbalanced der(1;16)(q10;p10). Deletions of 1p, 4q, 14q, 17p, and 22q and gain of 1q are associated with adverse outcome [60–63]. Mutations of TP53 at 17p13.1 are associated with anaplasia, a feature of UFH tumors [63, 64].

Clear Cell Sarcoma of the Kidney

Clear cell sarcoma of the kidney (CCSK), the second most common pediatric renal tumor, is an unfavorable histology tumor with a propensity for recurrence and metastasis to bone, brain, and soft tissue. Little is known about the genetics of CCSK. Several individual cases have been reported with t(10;17)(q22.3;p13.3) and/or del(14)(q24.1q31.1) [65, 66]. O'Meara et al. recently identified the genes involved in the t(10;17) as *FAM22* at 10q22.3 and *YWHAE* at 17p13. The translocation produces an in-frame fusion gene that is comprised of exons 1–5 of *YWHAE* and exons 2–7 of *FAM22*.

Of 50 CCSKs studied by RT-PCR, only 12% were *FAM22-YWHAE* fusion positive [67]. This same translocation and gene fusion was also recently characterized and reported in high-grade endometrial stromal sarcomas [68].

Congenital Mesoblastic Nephroma

Congenital mesoblastic nephroma (CMN), the third most common pediatric renal tumor, is an uncommon spindle cell tumor diagnosed in infancy (60%) to <2 years of age. Infantile or congenital fibrosarcoma (CFS) is a soft tissue spindle cell tumor usually located in an extremity in children under 2 years old. Both CFS and CMN share a common translocation, t(12;15)(p13.2;q25.3), and gains, in decreasing frequency order, of chromosomes 11, 20, 17, and 8 (Fig. 16.7). t(12;15) fuses *ETV6* with *NTRK3*. These chromosomal abnormalities distinguish CFS and CMN from other childhood spindle cell tumors, such as benign infantile fibromatosis or malignant adult-type fibrosarcoma [69, 70].

Rhabdoid Tumor of the Kidney

Rhabdoid tumor of the kidney (RTK), a neoplasm different from Wilms tumor, was given the name “rhabdoid” because microscopically it resembled a rhabdomyosarcoma (see later). RTK, a highly malignant neoplasm that occurs perinatally, during the first year of life, and occasionally in older individuals, is characterized by early metastases and a high mortality rate. Malignant rhabdoid tumors (MRT) occur in soft tissues, skin, CNS, and other extrarenal sites. Concomitant brain tumors are present in about one third of fetuses and neonates with RTK [71]. Loss of chromosome 22, 22q deletions, *SMARCB1/INI1* mutations, and lack of *INI1* immunostaining in histopathologic sections facilitate the diagnosis of MRTs [72, 73].

Prostate Cancer

Prostate adenocarcinoma is the most common cancer in males, representing 29% of cancers and causing 9% of cancer deaths in men [74]. Prognosis for patients with prostate cancer correlates with stage and grade of disease at diagnosis. Frequently reported chromosome alterations include loss of 8p, 10q (*PTEN*), 13q (*RBL1*), 17p (*TP53*), 7p, 16q, 6q, and gain of 8q24.1 (*MYC*) and 7q31. Hemizygous or homozygous deletion of *PTEN* at 10q23.3 correlates with disease stage, disease progression, and survival [75–77]. *PTEN* loss is among the most frequent recurring abnormalities in prostate cancer and is seen in preinvasive prostatic intraepithelial neoplasia as well as in invasive prostate cancer.

In 2005, *TMPRSS2-ETS* gene fusions were discovered in prostate cancer. *TMPRSS2*, transmembrane protease serine 2 gene, is androgen-regulated. The fusion of *TMPRSS2* at 21q22.3 with an *ETS* gene results in overexpression of the 5' truncated *ETS* oncogene. The most common

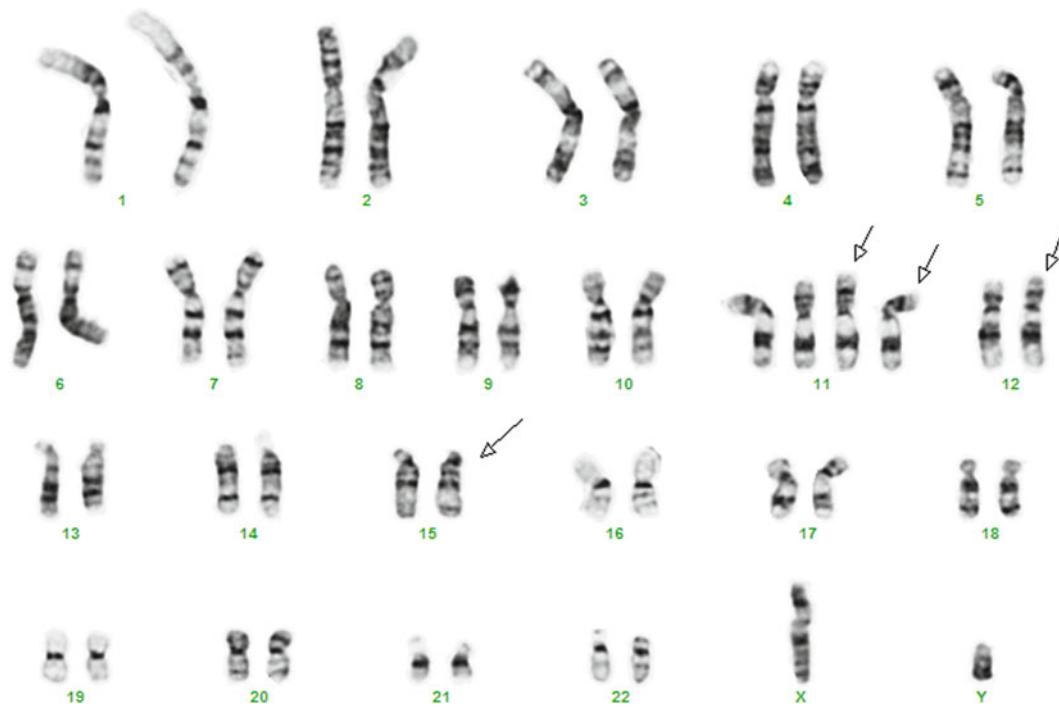


Fig. 16.7 Cellular congenital mesoblastic nephroma, grade III, from the kidney of a 6-week-old boy: 48,XY,+11,+11,t(12;15)(p13.2;q25.3). This same t(12;15), which results in *ETV6-NTRK3* fusion, can also be

seen in congenital/infantile fibrosarcoma and in secretory breast carcinoma (see text for details)

fusion (*TMPRSS2-ERG*, present in >50% of prostate specific antigen-screened localized cancers) results from an interstitial ~2.8 Mb deletion within 21q22; *ERG* is located at 21q22.2 [78, 79]. *TMPRSS2-ERG* fusion is associated with *PTEN* deletion and with earlier disease recurrence of localized prostate cancer after surgical resection. A study by Markert found that the poorest overall survival (57 months mean OS) correlated with *TP53* and *PTEN* inactivation. *TMPRSS2-ERG* fusion positive tumors had a mean OS of 93 months compared with a mean OS of >103 months in the most favorable group. Data from this study and others suggests that a subset of *TMPRSS2-ERG* fusion-positive tumors with amplification of *ERG* have a poorer outcome [80].

Bladder Cancer

Bladder cancer can generally be divided into superficial, invasive, and *in situ* categories, which correspond to the TNM (“tumor, nodes, metastasized”) staging scheme. Tumor stage is the most important independent predictor of patient prognosis. Most tumors are superficial at diagnosis. However, during the course of the disease, multiple recurrences are common, with a minority of superficial tumors progressing to muscle invasion or metastatic disease. Identification of genetic changes in exfoliated cells from the bladder has shown utility in detecting disease

recurrence. Primary noninvasive (Ta) or superficially invasive (T1) transitional cell carcinoma of the bladder can be monitored using FISH with centromeric and locus-specific DNA probes. UroVysion®, a US Food and Drug Administration (FDA)-approved FISH probe set, detects aneusomy of chromosomes 3, 7, and 17, and 9p21 (*CDKN2A*) loss in patients with hematuria or a history of bladder cancer [81, 82]. Recent studies support FISH analysis for use in monitoring and predicting recurrence risk in patients with non-muscle-invasive bladder cancer and predicting residual tumor load after transurethral resection [83–86]. FISH analysis in combination with cytology and the telomeric repeat amplification protocol (TRAP) assay to detect telomerase activity increases the sensitivity of detection in low-grade and early-stage cancers [87]. See also Chap. 17, Fig. 17.16.

Tumors of the Reproductive System (Table 16.2)

Endometrial Stromal Tumor

Endometrial stromal tumors (EST) are rare uterine mesenchymal tumors that occur in women of reproductive and postmenopausal age. In 2003, the World Health Organization (WHO) divided ESTs into three categories: endometrial stromal nodules (ESN), endometrial stromal sarcomas (ESS),

and undifferentiated endometrial sarcomas (UES). ESNs are benign, circumscribed tumors. ESSs are low-grade, malignant tumors that invade the myometrium. Both can have variant histologic features, but most have a classic morphology. UESs are highly aggressive, malignant tumors with cytologic atypia and high mitotic activity. ESSs may be primary uterine, metastatic uterine, or primary extrauterine. The tumors are characterized by reciprocal translocations and gene fusions with t(7;17)(p15.2;q11.2)/*JAZF1-SUZ12* in ESN, ESS, and rarely UES, and t(6;7)(p12.3;p15.2)/*JAZF1-PHF1* or t(6;10)(p12.3;p11.22)/*EPC1-PHF1* and variants in ESS [88–91]. Lee et al. recently identified a t(10;17)(q22.3;p13.3)/*FAM22-YWHAE* in high-grade ESS [68]. This same t(10;17) is found in clear cell sarcoma of the kidney (see earlier) [67].

Germ Cell Tumors (Table 16.2)

Germ cell tumors (GCTs) are a heterogeneous group of rare benign and malignant tumors. GCTs may arise in the gonads and at extragonadal sites found primarily in the body midline (intracranial, mediastinal, retroperitoneal, sacrococcygeal, and others). Primordial germ cells are thought to give rise to these tumors with aberrant germ cell migration responsible for the extragonadal tumors. While the tumors may arise from the same cell type, the clinical course and outcome of the various GCTs differ depending on tumor site and histology [92–94]. Tumors are found prenatally into old age with diverse groups: neonates, infants and children ≤5 years of age, postpubertal to the fifth decade, and older age.

Gonadal Germ Cell Tumors

Testicular GCTs (TGCT), divided into seminomas and non-seminomas, are the most common tumor of men in the second to fourth decades and are responsible for ~10% of cancer deaths of men in this age bracket. The US incidence is ~6/100,000 with a 5:1 white: black ratio. Cryptorchidism is associated with ~10% of TGCTs. Seminomatous GCTs, ~40–50% of GCTs, are composed of cells that resemble primordial germ cells. Nonseminomatous GCTs (NSGCT) may be composed of undifferentiated cells that resemble embryonic stem cells. Malignant cells can differentiate to generate yolk sac (endodermal sinus) tumor, embryonal carcinoma, choriocarcinoma, teratomas, and mixed malignant GCTs. Approximately 60% of TGCTs are composed of more than one of these cell types [95]. TGCT of infants are rare neoplasms occurring in boys 0–4 years of age, the majority of which are pure yolk sac tumors [96].

GCTs account for 15–20% of all ovarian tumors. Most are benign cystic teratomas. The remaining, primarily found in children and young adults, may be malignant with histologic types similar to those in the testis. The counterpart to the testicular seminoma is the ovarian dysgerminoma, which is always malignant. Dysgerminomas account for ~2% of all ovarian tumors, but ~50% of GCTs. They may occur in patients with gonadal dysgenesis.

Extragonadal Germ Cell Tumors

Rare primary brain GCTs occur in the midline, pineal (male predominance), or suprasellar regions. They account for 0.2–1% of brain tumors in those of European descent, but up to 10% in those of Japanese origin.

Primary mediastinal germ cell tumors account for 10–15% of all mediastinal tumors [97]. Pediatric mediastinal GCTs represent ~5% of all GCTs [98]. These tumors have the same histologies as gonadal GCTs, but have a worse prognosis. The mediastinum is the most common site of extragonadal tumors in young males. Mediastinal NSGCTs are associated with Klinefelter syndrome in ~20% of cases [7]. Patients with Klinefelter syndrome have a 50-fold higher risk for a mediastinal GCT, but do not develop testicular GCT [2].

Sacrococcygeal GCTs, the most common extragonadal GCT in children, present prenatally to ~4 years of age. Most (~90%) external lesions are benign, while intrapelvic or intra-abdominal tumors are more likely to be malignant (60–90%) [99].

Chromosome Abnormalities in Germ Cell Tumors

Cytogenetically, additional copies of all or part of 12p are the characteristic chromosome abnormality associated with GCTs in adults and postpubertal children [95, 100]. Additional copies of 12p are present as i(12p) in 80–90% of cases, while the remaining tumors show 12p amplification. Additional cytogenetic anomalies in this group include gains of an X chromosome and chromosomes 7, 8, 12, and 21 and loss of 1p, 11, 13, and 18 (Fig. 16.8).

GCTs in infants and prepubertal children characteristically show loss of 1p36, 4q, and 6q, and gain of 1q, 3p, 16p, and 20q. Gain of 12p is rarely reported in prepubertal children and infants [101, 102]. Array CGH has shown proximal 12p11.2-p12.1 gain associated with adult TGCTs and distal 12p12-pter gain in yolk sac tumors of very young children [102–104]. GCTs are rare in children between 5 and 9 years of age; thus there is a paucity of tumor genetic information in this age range.

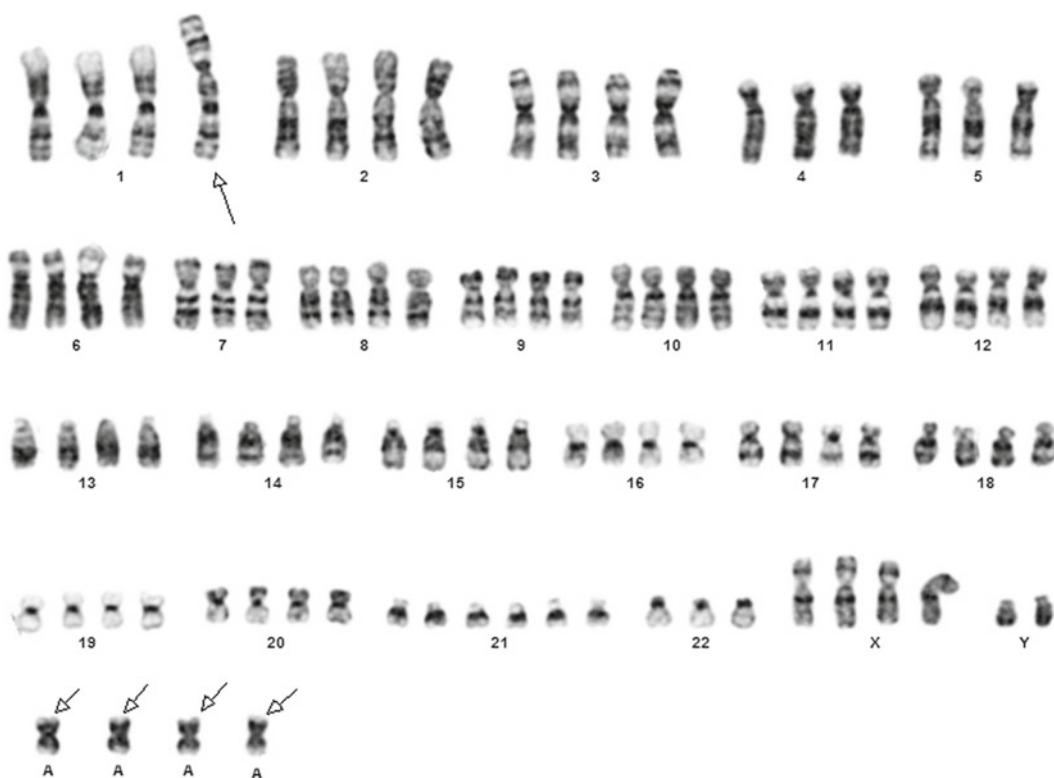


Fig. 16.8 Malignant mixed germ cell tumor from the mediastinum of an 8-year-old boy: 96<4n>,XXYY,+X,+X,+1,idic(1)(p22),-4,-5,-7,+i(12)(p10)x4,+21,+21,-22. In addition to other abnormalities, this karyotype

results in gain of 12p in the form of isochromosomes for the short arm (lower left) (see text for details)

Gastrointestinal Tumors (Table 16.3)

Gastrointestinal Stromal Tumors

Gastrointestinal stromal tumors (GIST), the most common mesenchymal tumor of the gastrointestinal (GI) tract, arise from the connective tissue of the GI wall. Approximately 90% of GISTs have activating mutations of the *KIT* or *PDGFRA* receptor tyrosine kinase genes [105]. In addition to *KIT* or *PDGFRA* mutations, other genetic events involved in tumorigenesis include primarily chromosomal losses (14q, 22q, 1p, 15q, 13q), nuclear/mitochondrial microsatellite instability, LOH at 9p21 (*CDKN2A*), methylation of *CDKN2B* (*p15*), homozygous loss of *TLX2* (*HOX11L1*), and rare gene amplification (*MYC*, *MDM2*, *EGFR*, *CCND1*, *KIT*) [106–109]. The *KIT* and *PDGFRA* mutations are diagnostic, and tyrosine kinase inhibitors (TKIs) are used as targeted therapy. The molecular aberrations of *KIT-PDGFRA* are correlated with cell histomorphology, metastasis, prognosis, and efficacy of targeted therapy. Genotyping these tumors helps to guide therapy, as the effects of TKIs vary with the presence or absence and site of *KIT-PDGFRA* mutation. Metastatic GISTs often have secondary *KIT* kinase mutations,

and some have *KIT-PDGFRA* genomic amplifications, which are responsible for therapeutic resistance [105]. Disease-free survival correlates with mutation (*KIT*=poor), site (stomach=best), cytogenetic complexity (≥ 3 abnormalities=poor), and losses of 1p and/or 22q and gains of 1q and 12q (shorter survival) [110, 111]. GISTs in patients with neuro-fibromatosis type I (*NFI*) lack *KIT* and *PDGFRA* mutations. Rare families have been reported with germline *KIT* or *PDGFRA* mutations [105, 110].

Liver Tumors

Hepatoblastoma

Hepatoblastoma (HB) is the most common primary malignant tumor of the liver in children. This rare tumor accounts for $\sim 1\%$ of all pediatric malignancies, with ~ 100 –150 new tumors per year in the United States.

Cytogenetic analysis of hepatoblastomas has found that the most common anomalies are numerical, with gain of whole chromosomes, specifically of chromosomes 20, 2, and 8, in decreasing order of frequency (Fig. 16.9). The most common structural abnormalities result in gain of chromosome 1 long-arm material. An unbalanced der(4)t(1;4) that

Table 16.3 Chromosome abnormalities with diagnostic or clinical significance in gastrointestinal and other tumors

Tumor	Chromosomal aberration(s)	Gene(s) involved	Clinical significance	References
<i>GIST</i>	Loss of 14q, 22q, 1p, 15q, 13q; gain of 1q, 12q	<i>KIT, PDGFRA</i>	<i>KIT, PDGFRA</i> mutation diagnostic, response to TKIs	[105–111]
<i>Liver</i>				
Hepatoblastoma	Gain of 1q, 2, 8, 20, der(4)t(1;4), loss of 4q		Distinguish from HCC, HMH	[112, 113]
Hepatic mesenchymal hamartoma (HMH)	t(11;19)(q13;q13.4), t(19q13.4)	Unknown genes	Distinguish from hemangioma or malignant tumor	[120]
<i>Salivary gland</i>				
Pleomorphic adenoma	t(3;8)(p22.1;q12.1), t(12q14.3), +8	<i>CTNNB1-PLAG1, HMGA2</i>	Diagnostic; benign tumor	[127–129]
Ca-ex-PA	<i>HMGA2, MDM2</i> amplification	<i>HMGA2, MDM2</i>	Amplification contributes to malignant transformation of PA	[128]
Mucoepidermoid cancer	t(11;19)(q21;p13.11) in 40–80%; gain of 7, 8, X, loss of 6q	<i>MAML2-CRTC1</i>	Malignant; t(11;19) assoc with better outcome	[130, 131]
Warthin's tumor	t(11;19)(q21;p13.11) in low percentage	<i>MAML2-CRTC1</i>	Benign; t(11;19) w/metaplasia	[132]
<i>Dermal</i>				
DFSP and variants (GCF, Bednar, other)	t(17;22)(q22;q13.1), der(22)t(17;22) or r(22)t(17;22)	<i>COL1A1-PDGFB</i>	Diagnostic for DFSP; response to TKIs	[135–140]
Hidradenoma	t(11;19)(q21;p13.11), gain of 7, 8, X, loss of 6q	<i>MAML2-CRTC1</i>	Clear cell variant	[133, 134]
Cutaneous melanoma	Gain of 6p, 1q, 7, 8q, 17q, 11q, 20q; loss of 9, 9p, 10q, 6q	<i>CDKN2A, BRAF, PTEN</i>	<i>CDKN2A</i>	[143, 144]
Uveal melanoma	Loss of 3, gain of 8q	<i>GNA11, GNAQ</i>	Monosomy 3 correlates with metastatic disease	[145, 146]
<i>Breast</i>				
Invasive intraductal	dmin, hsr (<i>ERBB2</i> amplification)	<i>ERBB2</i>	Improved outcome with targeted therapy	[149–151]
Secretory breast	t(12;15)(p13.2;q25.3)	<i>ETV6-NTRK3</i>	Favorable; distinguish from other breast lesions	[152]
<i>Lung</i>				
NSCLC	<i>EGFR</i> high copy number or amplification, loss of 3p, gain of 7 inv(2)(p21p23.2)	<i>EGFR</i>	Response to TKIs	[155]
		<i>EML4/ALK</i>	Response to TKIs	[156, 157]

Ca-ex-PA Carcinoma ex Pleomorphic Adenoma, *DFSP* dermatofibrosarcoma protuberans, dmin double minutes, *GCF* giant cell fibroblastoma, *GIST* gastrointestinal tumor, *HCC* hepatocellular carcinoma, hsr homogeneously stained regions, *NSCLC* non-small cell lung carcinoma, *TKI* tyrosine kinase inhibitor

results in gain of 1q and loss of 4q is the most common recurring structural abnormality [112]. Rare genomic and expression profiling studies have confirmed these abnormalities and further refined the regions of gain and loss. Single nucleotide polymorphism (SNP) array analysis revealed paternal 11p uniparental disomy (UPD, see Chap. 20) that included the *IGF2-H19* region at 11p15.5 [113]. Molecular studies have discovered mutations in key genes that are important in the genetic pathways of the developing liver [114]. These studies may help elucidate the pathogenetic mechanisms responsible for the development of hepatoblastoma. *SMARCB1 (INI1)* testing helps differentiate hepatoblastoma from a more aggressive variant that mimics rhabdoid tumor.

Most cases of HB are sporadic. However, HB is associated with several cancer predisposition syndromes including

Beckwith-Wiedemann syndrome (BWS), familial adenomatous polyposis, and Li-Fraumeni syndrome. HB can also be seen in glycogen storage disease type I. Premature infants, particularly those with low or very low birth weight, are at increased risk of developing hepatoblastoma [115].

Familial adenomatous polyposis (FAP), a syndrome of early-onset colonic polyps and adenocarcinoma, results from germline mutations in the *APC* tumor suppressor gene at 5q22.2. Children with a family history of FAP have a significantly increased risk for hepatoblastoma [116]. One study estimated that 1 in 20 hepatoblastomas is probably associated with FAP. *APC* mutations, common in patients with hepatoblastoma and FAP, are rare in patients with sporadic hepatoblastomas [117, 118]. Children who survive HB should be considered for evaluation of FAP, and those patients found

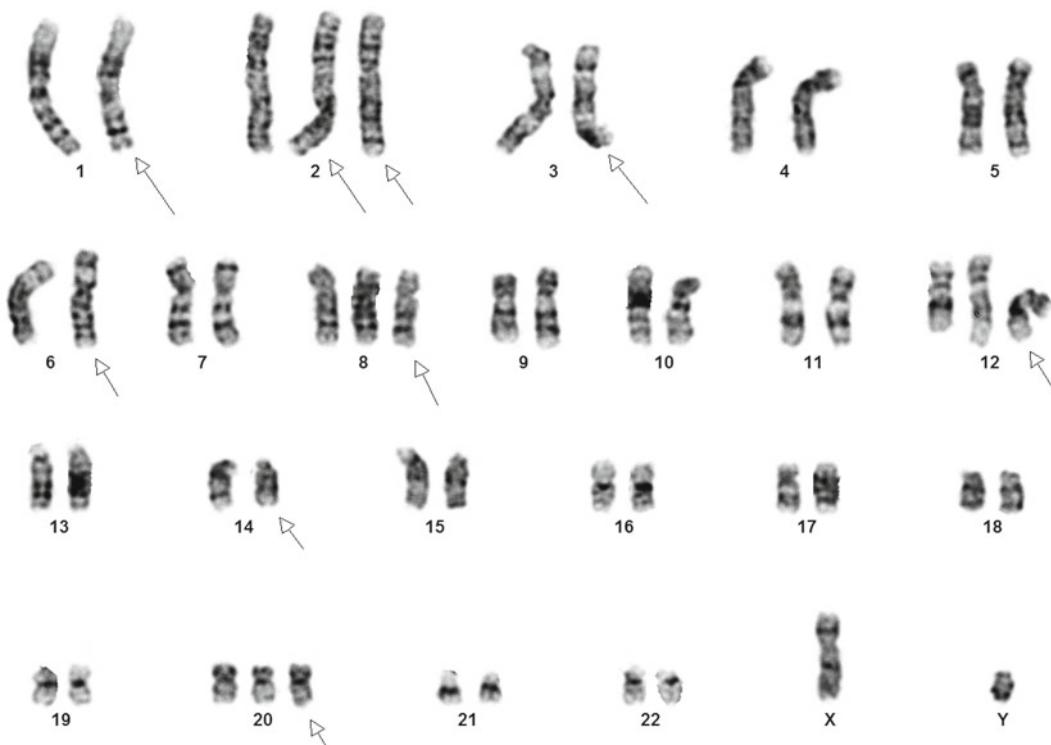


Fig. 16.9 Hepatoblastoma, mixed embryonal and fetal, from the liver of a 9-year-old extremely premature boy: 50,XY,del(1)(q32q42),add(2)(q23),+add(2)(q31),add(3)(p21),add(6)(q23),+8,+12,add(14)(q13),+20

to carry an *APC* mutation need close surveillance because of their increased risk for colonic polyps and adenocarcinoma.

Patients with hemihypertrophy or Beckwith-Wiedemann syndrome should be screened using α -fetoprotein (AFP) as a marker to detect hepatoblastoma. AFP monitoring should be performed every 3 months until the child is at least 4 years. Loss of heterozygosity of 11p markers occurs commonly in hepatoblastoma associated with BWS and hemihypertrophy.

Children with HB often have very elevated AFP levels and may have anemia and thrombocythemia. Complete surgical removal effects a cure. However, ~70% of tumors are metastatic or unresectable at diagnosis. Even with aggressive chemotherapy, 25–30% remain resistant. AFP levels return to normal with tumor removal and rise if the tumor returns, thus providing a monitor for disease [115, 119].

Hepatic Mesenchymal Hamartoma

Mesenchymal hamartoma of the liver (HMH) is a rare benign lesion that occurs mainly in infants. Histologically, the lesion has cystic and solid areas with islands of hepatocytes and poorly defined or dilated biliary ducts in a myxoid stroma. Complete surgical removal is curative. In the few reported cases, a common denominator has been involvement of chromosome band

19q13.4 with t(11;19)(q13;q13.4) so far the most common reciprocal translocation [120].

HMH has been associated with placental mesenchymal dysplasia (PMD), an uncommon disorder of the placenta. Placental changes include cystic villi with dilated/thick-walled vessels, which can mimic a partial hydatidiform mole. In contrast to a partial mole, PMD can coexist with a normal viable fetus [121]. Both HMH and PMD have been considered developmental aberrations rather than true neoplasms [120].

Further analysis suggests PMD may be a disease of dysregulated imprinting with mosaic placental and fetal paternal UPD [122–124]. HMH and PMD have been associated with Beckwith-Wiedemann syndrome (BWS), an imprinting disorder [125]. One cause of BWS is paternal UPD at 11p15.5. A paternally imprinted gene, *PEG3*, is within the locus at 19q13.43 that is commonly disrupted in HMH [123].

While HMH is considered to be benign, there is a low risk of malignant transformation. Several malignant tumors of undifferentiated embryonal sarcoma (UES) of the liver have been reported to arise from HMH. Cases of UES have been reported to have involvement of the same 19q13.4 locus as that of HMH [120, 126].

Salivary Gland Tumors

Pleomorphic Adenoma

Pleomorphic adenoma (PA), a benign mixed salivary gland tumor, has been associated with abnormal karyotypes in the majority of cases, with nonrandom involvement of 8q12.1 (locus of the pleomorphic adenoma (*PLAG1*) gene), 3p22.1 (*CTNNB1*), 12q14.3 (*HMG A2*), and gain of chromosome 8 [127, 128]. t(3;8)(p22.1;q12.1) is the most common translocation (Fig. 16.10). Reported partner genes for *PLAG1*, *CTNNB1*, and *HMG A2* vary.

Few PAs (~6%) undergo malignant transformation to carcinoma ex pleomorphic adenoma (Ca-ex-PA). Recent work has shown that *HMG A2* translocations are often associated with gene amplification. There is an increased risk of malignant transformation with *HMG A2* amplification. *HMG A2* is usually co-amplified with other genes, most often *MDM2*.

Other genetic abnormalities thought to contribute to malignant transformation of PA to Ca-ex-PA include deletions of 5q23.2-q31.2 and *TP53*, gains of 8q12.1 (*PLAG1*) and 8q22.1-q24.1 (*MYC*), and *ERBB2* amplification [129].

Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) accounts for ~15% of salivary gland tumors and is the most common primary malignant tumor of the salivary gland. MEC arises predominantly,

but not exclusively in the parotid gland. At(11;19)(q21;p13.11) that results in *CRTC1-MAML2* fusion is found in 40–80% of tumors (Fig. 16.11). Evidence from several studies found that fusion positive tumors are less likely to recur or metastasize and are associated with an overall better survival [130, 131]. t(11;19) negative cases show gain of chromosomes 7, 8, and X and 6q deletion [131].

Warthin's Tumor

Warthin's tumor, the second most common salivary gland tumor, is a benign neoplasm that arises almost exclusively in the parotid gland. Warthin's tumor, also referred to as papillary cystadenoma lymphomatosum, is composed of polyclonal lymphoid cells and neoplastic epithelium. Recurrence and malignant transformation occur very rarely [2]. t(11;19)(q21;p13.11), which results in *CRTC1-MAML2* fusion, is found in a low percentage of Warthin's tumors [132]. Tumors that exhibit the translocation or are fusion-positive characteristically have metaplasia of the oncocytic epithelium. There is ongoing discussion regarding the association of the t(11;19) *CRTC1-MAML2* fusion with both Warthin's tumor and MEC; there is morphologic overlap between metaplastic Warthin's tumor and MEC [130]. Further, clear cell hidradenoma, a benign sweat gland tumor, also demonstrates the t(11;19) and *CRTC1-MAML2* fusion [133, 134].

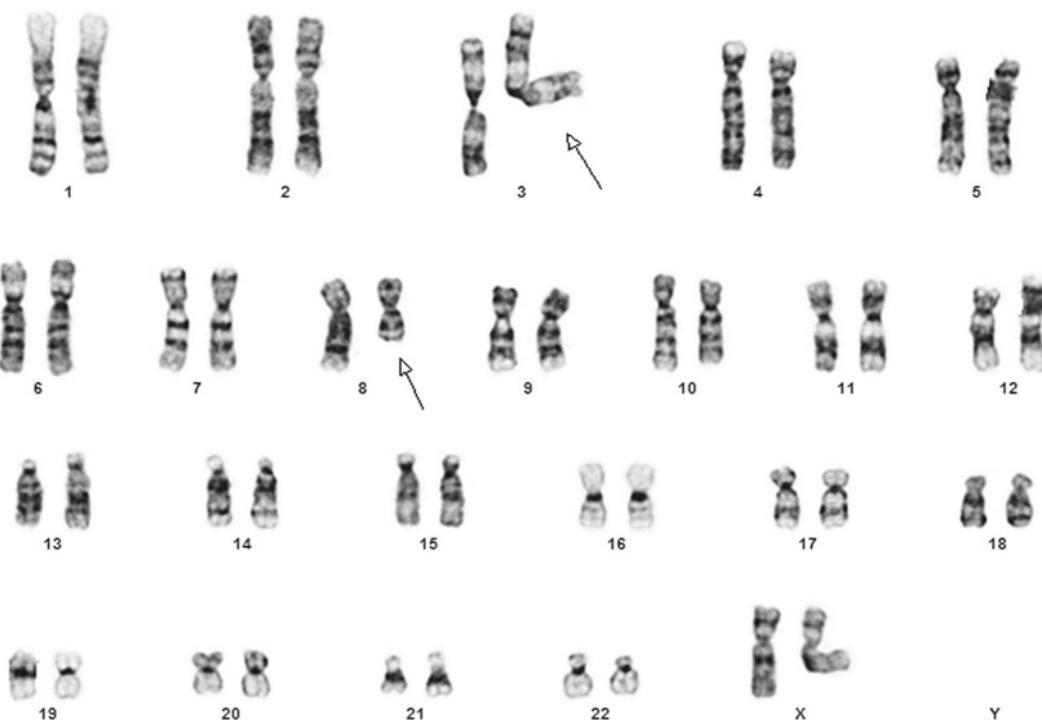


Fig. 16.10 Pleomorphic adenoma, submandibular, from a 10-year-old girl: 46,XX,t(3;8)(p22.1;q12.1). The translocation results in *CTNNB1-PLAG1* fusion

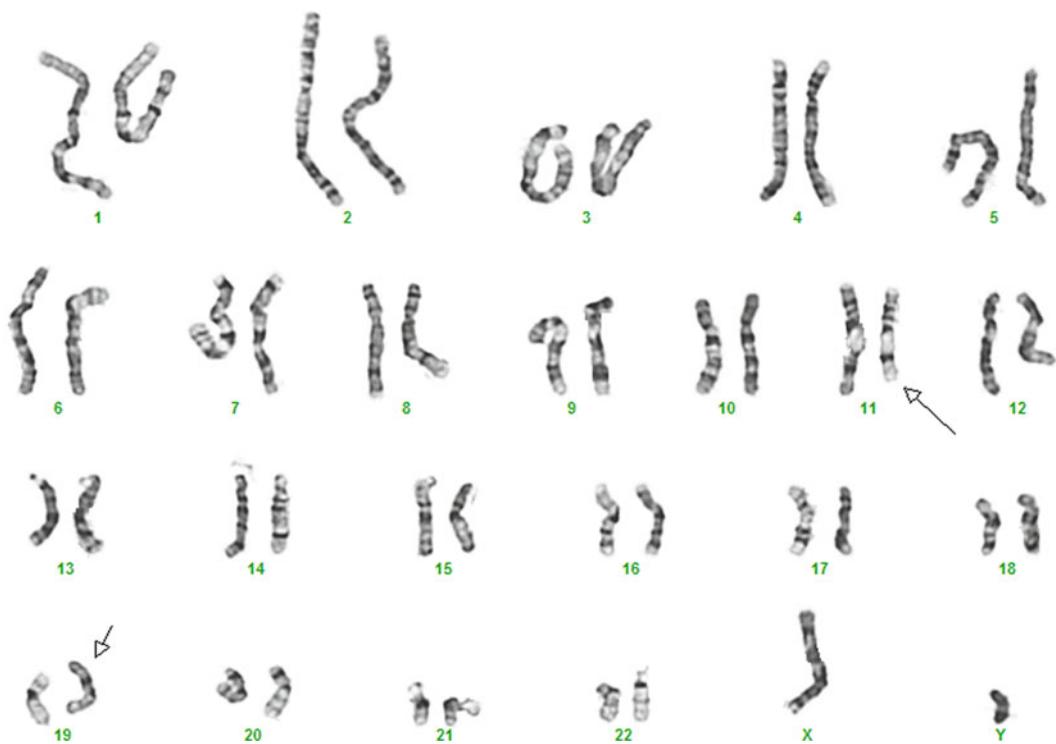


Fig. 16.11 Mucoepidermoid carcinoma from the parotid gland of a 9-year-old boy: 46,XY,t(11;19)(q21;p13.11). This same t(11;19), which results in *MAML2-CRTC1* fusion, can be seen in hidradenoma, a benign sweat gland tumor

Dermal Tumors (Table 16.3)

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is an intermediate-grade soft tissue malignancy that usually arises in the dermis and subcutaneous tissue of adults and rarely in children [135]. DFSP is a slow-growing infiltrative dermal neoplasm with a propensity to recur locally after surgical resection, but is rarely metastatic (1–4%). Tumor-related deaths are very rare. There are several histologic variants of DFSP, such as giant cell fibroblastoma, Bednar tumor, and other fibrohistiocytic tumors, which should be considered in the differential diagnosis.

DFSP is characterized by a balanced or unbalanced form of a translocation t(17;22)(q21.3;q13.1) or by supernumerary ring chromosomes derived from this chromosome 22 [der(22)r(17;22)] that contain low-level amplified sequences from 17q21.31-qter and from 22q11.1-q13.1 [136]. The unbalanced form is usually a der(22)t(17;22) (Fig. 16.12). Both the ring and linear forms of the derivative chromosome 22 result in fusion of the α (alpha)-1 chain of type 1 collagen gene (*COL1A1*) at chromosome 17q21.3 with the second exon of the platelet-derived growth factor- β (*PDGFB*) gene

at chromosome 22q13.1. Variability of the *COL1A1* break point has no correlation with any clinical or histological parameter [135, 137–139]. However, cytogenetically, ring chromosomes are common in adult DFSP, while the translocation derivatives are seen in all childhood cases [140]. The *COL1A1-PDGFB* chimeric gene protein causes unregulated expression of platelet-derived growth factor leading to abnormal activation of the platelet-derived growth factor receptor beta (*PDGFRB*) tyrosine kinase through an autocrine loop. Tyrosine kinase inhibitors are the current therapy for recurrent, metastatic, or inoperable tumors [141, 142]. Demonstration of the *COL1A1-PDGFB* fusion is necessary for the diagnosis of DFSP or DFSP variants [137].

Variants of DFSP

Several histologic variants of DFSP are described including giant cell fibroblastoma (GCF), pigmented Bednar tumor (BT), DFSP with fibrosarcoma (FS)-like changes (DFSP-FS), and others.

Giant cell fibroblastoma (GCF), also called juvenile DFSP, more commonly affects infants and children. Bednar tumor, a pigmented form of DFSP with additional melanin-containing dendritic cells, occurs in early to middle adulthood. DFSP-FS is a more cellular form with higher mitotic rate. All variants are characterized by *COL1A1-PDGFB* fusion [137, 140].

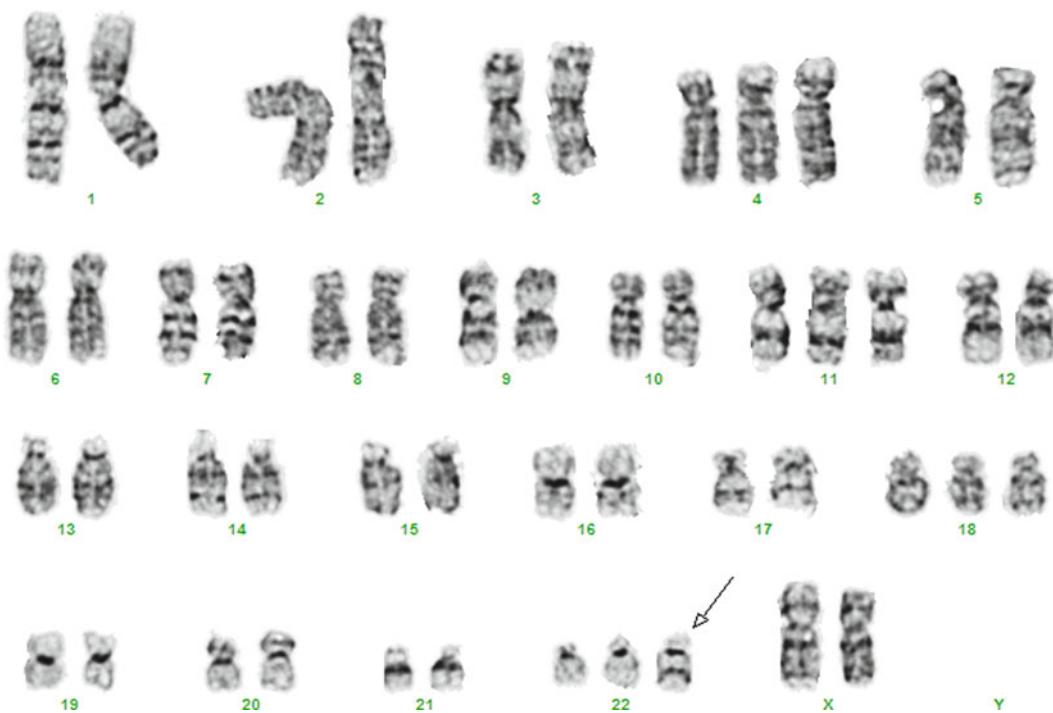


Fig. 16.12 Dermatofibrosarcoma protuberans from the breast of a 2-year-old girl: 50,XX,+4,+11,+18,+der(22)t(17;22)(q21.3;q13.1). The arrow indicates the chromosome 22 derived from the translocation, which results in *COL1A1-PDGFB* fusion (see text for details)

Hidradenoma

Hidradenoma, a benign sweat gland tumor, often presents as a solitary, slow-growing, solid, or cystic intradermal nodule. Malignant transformation is uncommon. t(11;19)(q21;p13.11) is characteristic of a subset of these tumors (Fig. 16.11). The *CRTC1-MAML2* fusion was demonstrated in 50%, specifically in tumors with clear cells, representing the clear cell variant of hidradenoma [133]. As noted earlier, salivary gland Warthin's tumor and mucoepidermoid carcinoma also demonstrate the t(11;19)/*CRTC1-MAML2* fusion. The common glandular denominator for these different tumors suggests they may originate from a common progenitor cell in salivary, bronchial, and sweat glands [130, 133].

Malignant Melanoma

Melanomas are malignant lesions, primarily cutaneous, but may occur on mucosal surfaces and in the eye. Cutaneous malignant melanoma, a pigmented skin lesion, may be lethal if not recognized and completely excised prior to metastasizing. Malignant melanomas spread superficially before progressing to invade the deeper dermal tissues. Malignant melanoma may be recognized by visible changes of a pigmented lesion (mole) characterized by changes in size and color and irregular borders, and may evolve from dysplastic

nevi. Individuals with dysplastic nevus syndrome have a 50% risk for developing melanoma by 60 years of age. Frequent aberrations found in melanomas include gains of 6p, 1q, 7p, 7q (*BRAF*), 8q, 17q, 11q, and 20q and losses of 9p (*CDKN2A*), 9q, 10q (*PTEN*), 10p, and 6q [143, 144].

Uveal melanoma, the most common form of primary eye cancer, is characterized by loss of chromosome 3 in ~50% of tumors; metastasis is correlated with such loss. Chromosome 3 loss is often accompanied by i(8q); tumors without loss of chromosome 3 have 6p abnormalities.

Two regions of chromosome 3, 3p25 and 3q24-26, appear to harbor tumor suppressor genes. More than 80% of uveal melanomas have been found to have a constitutively active somatic mutation of one of two genes, *GNA11* at 19p13.3 and/or *GNAQ* at 9q21.2. These genes appear to contribute to the development of these tumors [145, 146].

Epithelial Cancer (Table 16.3)

Breast Cancer

Breast carcinoma is the most common cancer in women and the second leading cause of cancer deaths in women. The lifetime risk for breast cancer for women in the general population is 1 in 8. A positive family history of breast cancer increases this risk. A germline mutation of one of the known

breast cancer predisposing genes greatly increases risk [2]. Currently known breast cancer genes explain only ~30% of the heritability. Mutations of the breast cancer predisposing genes *BRCA1* and *BRCA2* account for ~16–20% of the familial risk of breast cancer in the general population [147, 148].

Invasive or Metastatic Breast Cancer

Prognosis for patients with breast cancer correlates with stage, histologic type and grade, hormonal (estrogen and progesterone) receptor, and *ERBB2* (*HER2*) status. Amplification and/or protein overexpression of *ERBB2*, found in ~20% of new diagnosis breast cancer, is associated with more aggressive disease and decreased survival time. Accurate assessment of *ERBB2* oncogene status is critical to care of the patient with invasive or metastatic breast cancer as it is used in selection of therapy. The risk of recurrence and mortality are reduced by ~50% and ~33%, respectively, in patients with early-stage *ERBB2*-positive tumors treated with trastuzumab (Herceptin®). Data indicate that patients with tumors that show *ERBB2* overexpression (3+ by IHC) or gene amplification (by FISH) be considered a candidate for anti-*ERBB2* therapy [7] (see also Chap. 17, Fig. 17.15). Because correlation between the IHC and FISH is <100%, guideline recommendations for *ERBB2* testing were established by an expert panel of members from the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) [149–151].

Secretory Breast Carcinoma

Secretory breast carcinoma (SBC), a rare subtype of breast cancer, is characterized by abundant eosinophilic secretions in intracellular vacuoles and intercellular spaces. SBC occurs in both sexes and in children and adults, but is most often seen in young adult females. Most tumors are hormone receptor and *ERBB2* negative. SBC is associated with a favorable prognosis, even in cases with local recurrence or ≤3 positive lymph nodes. SBC is characterized by t(12;15) (p13;q26)/*ETV6-NTRK3* fusion, which results in a chimeric tyrosine kinase fusion product. This same t(12;15) *ETV6-NTRK3* fusion is also seen in congenital (infantile) fibrosarcoma and congenital cellular mesoblastic nephroma [152] (see Fig. 16.7).

Lung Cancer

Lung cancer, the most common cancer worldwide, is largely due to tobacco products. Incidence and mortality rates of lung cancer have been declining since ~1990 secondary to decreased smoking rates over the past 30 years. The most common types are non-small cell carcinomas (which include adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma) and small-cell carcinomas [7].

Non-small Cell Lung Carcinoma

Adenocarcinoma is the most common type of lung cancer in women and nonsmokers. *KRAS* mutations occur primarily in adenocarcinoma, while *TP53*, *RBI*, and *CDKN2A* mutations occur in squamous cell and adenocarcinoma. *EGFR* mutations and amplification occur more frequently in patients with adenocarcinoma histology, no history of smoking, East Asian ethnicity, and female gender [2].

Up to 20% of non-small cell lung carcinomas (NSCLC) have *EGFR* mutations and/or amplification, and ~80–85% of patients with *EGFR* mutations respond to therapy with tyrosine kinase inhibitors. FISH analysis detects *EGFR* amplification (defined as a gene:chromosome ratio ≥2, or ≥15 copies per cell in ≥10% cells) and polysomy (defined as ≥4 copies in ≥40% of cells) [153, 154]. Fukuoka et al. reported that *EGFR* mutation was the strongest predictive biomarker for benefit of gefitinib over carboplatin/paclitaxel on progression-free survival (PFS) and overall response rate (ORR). PFS was significantly longer with gefitinib for patients whose tumors had both high *EGFR* gene copy number and *EGFR* mutation [155].

A subset of NSCLCs exhibit *ALK* gene rearrangement, e.g., *EML4-ALK*, which results from an inv(2)(p21p23.2). The *EML4-ALK* fusion is found predominantly in younger (average 52 years) nonsmokers with adenocarcinoma histology. The fusion protein causes constitutive activation of the *ALK* tyrosine kinase. Most reports show no overlap with *EGFR* or *RAS* gene mutations. *ALK* positive patients have shown significantly better overall survival at 1 and 2 years when treated with crizotinib, a drug targeted against the constitutively active tyrosine kinase. The FDA has approved treatment with crizotinib with FISH testing as a companion diagnostic test for *ALK* detection [156, 157]. See also Chap. 17.

Bone and Soft Tissue Tumors (Table 16.4)

Congenital Fibrosarcoma/Infantile Fibrosarcoma

Congenital fibrosarcoma (CFS), also referred to as infantile fibrosarcoma (IFS), is a rare soft tissue tumor composed of malignant fibroblasts in a collagen background. It is highly cellular and composed of spindle cells arranged in fascicles. Tumor cells are diffusely positive for vimentin and may show focal positivity for actin but are negative for S-100, desmin, and myoglobin [158]. The cellular variant of congenital mesoblastic nephroma (CMN) is histologically similar to CFS, and both tumors share a similar cytogenetic aberration, t(12;15) (p13.2;q25.3), that rearranges the *ETV6* and *NTRK3* genes (Fig. 16.7). Because of the histologic and cytogenetic similarities, as well as the fact that both tumors are low-grade and highly responsive to chemotherapy, cellular CMN is considered a visceral form of CFS. Fusion of the promoter region of the transcription factor *ETV6* with the tyrosine kinase receptor

Table 16.4 Chromosome abnormalities with diagnostic or clinical significance in bone and soft tissue tumors

Tumor	Chromosomal aberration(s)	Gene(s) involved	Clinical significance	References
CFS/IFS	t(12;15)(p13.2;q25.3), gain of 11, 20, 17, 8	<i>ETV6-NTRK3</i>	Distinguish from malignant adult type fibrosarcoma	[69, 158–161]
Synovial sarcoma	t(X;18)(p11.2;3;q11.2) Gain of 12q, 21q22	<i>SSX1-SS18, SSX2-SS18</i> <i>TSPAN31 amp</i>	Biphasic, unfavorable monophasic Unfavorable prognosis	[162–165] [166]
Lipoma			Distinguish from LPS	[167, 168]
Conventional	12q14.3 65% of lipomas; t(3;12)(q28;q14.3) in 25%; many variant translocations	<i>HMGAA2</i>		
Spindle/pleomorphic	Loss of 13q, 6p21 or 6p rearrangement		15–20% lipomas w/o 12q abnormalities	[167, 168]
	Loss of 13q, 16q, karyotype more complex		Distinguish from LPS	[167]
Atypical lipoma	Rings, giant markers		Deep seated, larger tumors, older age	[167, 168]
Lipoblastoma	8q12.1 rearrangements, gain of 8	<i>PLAG1</i>	Distinguish from LPS	[169]
Liposarcoma (LPS)				
Myxoid or myxoid round cell	t(12;16)(q13.3;p11.2), t(12;22)(q13.3;q12.2)	<i>DDIT3-FUS, DDIT3-EWSR1</i>	Diagnostic of M/R/C LPS	[171, 172]
Well-differentiated (WDLPS)	Rings, giant markers, dmin	<i>MDM2, CDK4, HMGAA2</i>	Distinguish from lipoma	[171, 173–175]
Dedifferentiated (DDLPS)	Rings, giant markers, dmin	<i>MDM2, CDK4, HMGAA2</i>	Aggressive, poor prognosis	[171, 173–175]
Pleomorphic LPS	Loss of 13q, 17p, 17q	<i>RB1, TP53, NF1, MAD2L1</i> amp	Aggressive, myxofibrosarcoma-like histology	[171, 173]
Leiomyoma	t(12;14)(q14.3;q24), loss of 7q22-q31.1, +12	<i>HMGAA2</i>	Distinguish from leiomyosarcoma, 7q, 12 associated w/tumor size	[176–178]
Leiomyosarcoma	Loss of 17p	<i>TP53</i>	Higher tumor grade	[179]
Alveolar soft part sarcoma (ASPS)	der(17)t(X;17)(p11.23;q25.3)	<i>ASPS-CRI-TFE3</i>	Diagnostic of ASPS	[182–184]
Osteosarcoma	Loss 6q, 10, 13, 18; gain/amp 17p11.2, 6p12, 1p32;p36, 8q24	<i>RB1, TP53, MYC</i> amp	<i>TP53</i> mutation correlates with genomic instability	[185, 186]
Aneurysmal bone cyst	t(16;17)(q21;p13.2), t(1;17)(p34.3;p13.2), t(3;17)(q21.3;p13.2), t(9;17)(q22.3;p13.2), inv(17)(p13.2;q21.33)	<i>CHD11-COL1A1, THRAP3-USP6, CNBP-USP6, OMD-USP6, USP6-COL1A1</i>	Benign, locally aggressive, recurrences common	[188–192]
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12.2), t(9;17)(q22;q12), t(9;15)(q22;q12.3)	<i>NR4A3-EWSR1, NR4A3-TAF15, NR4A3-TCF12</i>	Diagnostic	[195, 196]
Angiomyoid fibrous histiocytoma	t(2;22)(q33.3;q12.2), t(12;16)(q13.12;p11.2), t(12;22)(q13.12;q12.2)	<i>CREBB1-EWSR1, ATF1-FUS, ATF1-EWSR1</i>	t(2;22) in majority; t(12;16) is a variant; t(12;22) atypical presentation in organs	[197–199]
Inflammatory myofibroblastic (IMT)	t(1;2)(q21.3;p23.2), t(2;19)(p23.2;p13.12), t(2;17)(p23.2;q23.1), t(2;2)(p23.2;q12.3)	<i>TPM3-ALK, ALK-TPM4, ALK-CLTC, ALK-RANBP2</i>	May recurrence, rarely metastatic	[200, 201]
Epithelioid variant	t(2;2)(p23.2;q12.3)	<i>ALK-RANBP2</i>	Rapid recurrence, poor prognosis	[202]
Malignant rhabdoid tumor (MRT)	Loss of 22q, rare t(1;22)(p36;q11.2)	<i>SMARCB1-INII</i>	Rare tumor prenatal/perinatal; poor prognosis	[38, 71, 203, 204]

aCGH array comparative genomic hybridization, CFS/IFS congenital fibrosarcoma/infantile fibrosarcoma, dmin double minute, M/R/C LPS myxoid/round cell liposarcoma, w/ with, w/o without

NTRK3 results in dysregulated *NTRK3* tyrosine kinase activity and is postulated to be the initial transforming event [69, 159]. Both CFS and CMN have a favorable prognosis and are documented to have the potential for spontaneous regression [160]. Congenital fibrosarcomas usually present in the perinatal period in the subcutaneous tissues of the extremities. Wide local excision is the treatment of choice unless size, anatomic location, or metastases dictate otherwise [69, 161].

Cytogenetic aberrations in addition to t(12;15)(p13.2;q25.3) include gains of chromosomes 8, 11, 17, and 20. Tumors with these additional chromosome aberrations often have a more cellular histology, and acquisition of these polysomies has been associated with progression from a classic to cellular histology in mixed histology CMN. Therefore, these chromosomal polysomies are considered secondary oncogenic events [69].

Synovial Sarcoma

Synovial sarcomas (SS) account for approximately 10% of all soft tissue sarcomas and are the fourth most common sarcoma. The lower extremity is involved in 60–70% of cases. There are two histologic subtypes of synovial sarcoma: monophasic and biphasic. Biphasic tumors have two types of differentiation: epithelial and mesenchymal. The epithelial cells are cuboidal to columnar and grow in solid cords or

may form glands. The spindle cells are arranged in fascicles and surround the epithelial cells. Monophasic synovial sarcoma is composed of only spindle cells, or rarely only epithelial cells. Tumors composed of only spindle cells can be histologically misdiagnosed as fibrosarcomas or malignant peripheral nerve sheath tumors. Immunohistochemically, synovial sarcoma shows positivity for keratin and epithelial membrane antigen, distinguishing this entity from other sarcomas. These tumors require aggressive treatment. Common sites of metastases include lung, bone, and lymph nodes [2].

A characteristic cytogenetic aberration, t(X;18)(p11.23;q11.2), is present in greater than 95% of tumors (Fig. 16.13). This translocation fuses *SS18* at 18q11.2 with *SSX1*, *SSX2*, or *SSX4*, all in Xp11.2, producing chimeric transcription factors. Both RT-PCR and FISH utilizing a break-apart probe for *SS18* may be helpful adjuncts in diagnosing this entity [162, 163] (see also Chap. 17, Fig. 17.14). The two most common fusion transcripts appear to be associated with specific histologic subtypes, the *SS18-SSX2* rearrangement presents in the monophasic tumors, and the more aggressive biphasic tumors are associated with the *SS18-SSX1* transcript [164, 165]. Whether the fusion transcript type has prognostic significance as an independent variable remains unclear. Studies by aCGH have associated tumors with gains of 12q and 21q22 and gain and/or amplification of *TSPAN31* at 12q14.1 with unfavorable clinical outcomes [166].



Fig. 16.13 Synovial sarcoma from the thigh of a 46-year-old man: 45,Y,t(X;18)(p11.23;q11.2),tas(13;19)(p13;p13.3). The translocation (arrows) results in *SSX1-SS18* fusion (see text for details)

Lipoma

Lipomas are benign neoplasms of fat. They are the most common soft tissue tumors of adults and are categorized based on morphologic features. Subtypes include conventional lipoma, fibrolipoma, angiolioma, spindle cell lipoma, myelolipoma, and pleomorphic lipoma. The most common subtype is the conventional lipoma. Histologically, it is a well-encapsulated mass of mature adipocytes with variability in size. It most frequently presents in mid-adulthood and arises in the subcutis of the proximal extremities and trunk. Lipomas are soft, mobile tumors that are usually cured by complete excision. Some of the morphologic subtypes are associated with particular cytogenetic aberrations, distinguishing them from liposarcoma [2]. Conventional lipomas are associated with rearrangement of 12q14.3, most commonly as a result of a t(3;12)(q28;q14.3) resulting in rearrangement of *HMGA2*. Rearrangements of 13q and 6p21 are also common aberrations seen in these tumors. Supernumerary rings or giant marker chromosomes, which characterize atypical lipomatous tumors, are associated with an older age and larger tumor size at presentation as well as with a propensity to recur locally. Atypical lipomatous tumor is also called well-differentiated liposarcoma. Spindle cell and pleomorphic lipomas are characterized by losses in 13q and 16q. The presence of these genetic aberrations aids in distinguishing this benign neoplasm from the malignant liposarcoma [167, 168].

Lipoblastoma

Lipoblastoma, a rare benign soft tissue mesenchymal tumor, arises from fetal white fat and occurs almost exclusively in children under 3 years of age. Lipoblastomas are superficial, lobulated, small, localized masses. When diffuse, rather than localized, the tumor is referred to as lipoblastomatosis. The tumors are most often found in the extremities, but may involve other sites and organs. They have no malignant potential, but may recur with incomplete surgical excision. Histologically, lipoblastoma may be difficult to distinguish from an atypical lipomatous tumor/well-differentiated liposarcoma or myxoid liposarcoma.

For cases with ambiguous clinical or histologic features, chromosomal analysis provides the only unequivocal diagnostic confirmation. Cytogenetically, ~70% of lipoblastomas have 8q12 rearrangements with involvement of *PLAG1* that results in transcriptional upregulation of the *PLAG1* oncogene. Another ~20% have trisomy or polysomy of chromosome 8 with or without 8q12 rearrangement [169].

Liposarcoma

Liposarcoma (LPS) is the most common soft tissue sarcoma, accounting for approximately 20% of mesenchymal malignancies [170]. The tumor is most common in adults, presenting between 40 and 60 years of age. Tumors arise in the deep soft tissues of the proximal extremities and retroperitoneum and may become large masses [2]. There are three histologic subtypes: myxoid and round cell, well-differentiated, and pleomorphic [171].

Myxoid LPS accounts for one third of all LPS. It is a malignant tumor composed of round- to oval-shaped primitive nonlipogenic mesenchymal cells with small signet-ring lipoblasts in myxoid stroma with a characteristic branching vascular pattern [170]. Some myxoid liposarcomas undergo histologic change to a hypercellular, round cell morphology, which is associated with a poorer prognosis than those with pure myxoid histology. Tumors with myxoid histology only or those with myxoid and round cell histology are characterized by a t(12;16)(q13.3;p11.2) that fuses *DDIT3* at 12q13.3 and *FUS* at 16p11.2. Rare cases of myxoid LPS demonstrate a variant translocation, t(12;22)(q13.3;q12.2), which fuses *DDIT3* with *EWSR1* [171]. FISH analysis using a breakapart probe set for *DDIT3* has been shown to have high sensitivity and specificity in the diagnosis of this neoplasm [172].

Well-differentiated liposarcoma (WDLPS), also referred to as atypical lipomatous tumor, comprises 40–45% of all LPS. WDLPS is a low-grade tumor that may recur after removal, but rarely metastasizes. It occurs in the retroperitoneum and deep soft tissues of the extremities—commonly the thigh. It has a peak incidence in the sixth decade. WDLPS is characterized cytogenetically by supernumerary ring chromosomes, giant marker chromosomes, and double minutes that are associated with amplification of *MDM2* and *CDK4* (Fig. 16.14). The modal chromosome number of abnormal clones in WDLPS is usually near-diploid but may be near-tetraploid [171]. Dedifferentiated liposarcoma (DDLPS) represents progression from low- to high-grade nonlipogenic morphology within a WDLPS. DDLPS is more aggressive, with a metastatic rate of 10–20% and overall mortality of 50–75%. Cytogenetically, DDLPS shows the same supernumerary ring and giant marker chromosomes as WDLPS.

Several studies have now compared the genetic aberrations of WDLPS and DDLPS, seeking differences to elucidate the genes that influence progression. The majority of WDLPS and DDLPS show amplification of *MDM2*, *CDK4*, *HMGA2*, and *TSPAN31* within the 12q13-q15 region. Chromosome regions with identified differences so far include 1p32.2 amplification (*JUN*), 6q23 amplification (*MAP3K5* [*ASK1*]), loss of 11q23-24, loss of 19q13, and amplification of *GLII* at 12q13.3 detected in DDLPS but not in WDLPS by aCGH studies [173–175]. Tap et al. found amplification of *GLII*, *JUN*, and *MAP3K5* to be mutually



Fig. 16.14 Dedifferentiated liposarcoma from the retroperitoneum of a 76-year-old male: 47,XY,t(3;20)(q27;q12),+r(12),add(16)(p13.3). The arrow indicates the ring chromosome 12. Interphase

FISH analysis was positive for amplification of *DDIT3*, *CDK4*, and *MDM2* (not shown). This same karyotype and amplification may also be seen in well-differentiated liposarcoma (see text for details)

exclusive [174]. Loss of 11q23-24 was associated with histologic features similar to undifferentiated pleomorphic sarcoma and myxofibrosarcoma, while loss of 19q13 was associated with an unfavorable outcome [175].

Pleomorphic LPS, comprising 5% of all LPS, is an aggressive, high-grade sarcoma with histologic features similar to malignant fibrous histiocytoma (MFH) with the addition of a variable number of pleomorphic lipoblasts. Pleomorphic LPS usually arises in patients older than 50 years of age with an equal gender distribution. Tumors commonly present in the extremities. Cytogenetically, this tumor type is characterized by complex structural rearrangements, often with high chromosome counts. Deletions of 13q encompassing *RB1* are found in ~60% of pleomorphic LPS. Additional described abnormalities include amplification of *MAD2L1* at 4q27 and deletions of 17p (*TP53*) and 17q (*NFI*). Aberrations of the 12q14-q15 region with associated *MDM2* amplification are not characteristic of pleomorphic LPS [171, 173].

Leiomyoma

Uterine leiomyomas are one of the most common tumors in women. They are benign smooth muscle tumors that usually occur in multiple uterine sites. The tumors are sharply circumscribed, round, firm neoplasms that may vary in size

from small, barely visible, to large tumors that fill the pelvis. Histologically, they are characterized by a whorled pattern of smooth muscle bundles. Two rare benign variants include metastasizing leiomyoma, a uterine tumor that metastasizes hematologically, and disseminated peritoneal leiomyomatosis. Both are considered benign despite their biologic behavior. Neither has been associated with specific genetic aberrations [2]. Approximately 40% of leiomyomas harbor cytogenetic aberrations that include t(12;14)(q14.3;q24), deletion in the 7q22 to q31.1 band regions, gain of chromosome 12, rearrangements of 6p21 and 10q, and deletion in 3q [176, 177]. t(12;14)(q14.3;q24) characterizes the leiomyoma and is useful in distinguishing it from leiomyosarcoma. Cytogenetic aberrations involving 7q and gain of chromosome 12 have been associated with tumor size, those tumors with deletions in 7q being of smaller size than those with an additional copy of chromosome 12 [178].

Leiomyosarcoma

Leiomyosarcomas account for approximately 10% of mesenchymal neoplasms, have a female predilection, and can occur in a number of body sites. Tumors are characterized by smooth muscle differentiation. Immunohistochemical and ultrastructural evaluation play an important role in the

diagnosis. The tumors can be categorized into three groups: those that arise in deep soft tissues such as the retroperitoneum and female genital tract, subcutaneous and cutaneous tumors, and those tumors with a vascular presentation. Both conventional cytogenetic and aCGH analyses have identified recurrent aberrations in these tumors including losses at 1p36, 2p, 11q23-q24, 13q, and 17p. Aberrations at 17p that cause *TP53* mutations or loss have been associated with high-grade leiomyosarcomas and a poor prognosis [179]. Upregulation of a receptor tyrosine kinase, *ROR2*, in leiomyosarcomas has been associated with a poor clinical outcome. Because of the tyrosine kinase receptor activity, it is a potential therapeutic target [180].

Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma (ASPS) is a rare tumor of young adults. It occurs in the soft tissues of the extremities and has a unique histology with nest-like or organoid patterns of cells in fibrovascular septae. The tumors also commonly have cytoplasmic crystals identified with periodic acid-Schiff staining with diastase digestion or by electron microscopy [181]. The diagnosis of ASPS may be difficult, however, in tumors that arise in an atypical location or lack the typical crystal morphology. Distinguishing ASPS from tumors that can mimic ASPS histology, such as granular cell tumor, paraganglioma, clear cell sarcoma, and metastatic clear cell renal cell carcinoma, is important because the treatment is different for each tumor type.

The der(17)t(X;17)(p11.23;q25.3) that fuses *TFE3* and *ASPSCR1* is considered diagnostic of the entity and has significant diagnostic utility in cases that lack typical presentation and histology [182, 183]. RT-PCR for the tumor-specific fusion transcript *ASPSCR1-TFE3* has proven to be an important diagnostic adjunct for this entity. Immunohistochemical evaluation for nuclear immunoreactivity of *TFE3* can also be a useful diagnostic adjunct, although it is considered to be less sensitive [184].

Osteosarcoma

Osteosarcoma (OS), the most common primary malignant bone tumor, is a high-grade malignant mesenchymal tumor that produces bone matrix. OS occurs at all ages, but 75% occur prior to age 20 years, with males more commonly affected than females. OS arises in the metaphyseal regions of the extremity long bones with ~50% involving the knee [2].

The karyotype of most osteosarcomas is very complex. Recurrent chromosome abnormalities are difficult to detect; however, 13q (*RBI*) and 17p (*TP53*) deletions are most frequent. Comparative genomic hybridization analysis of primary OS

found gain and losses in all chromosomes, with the exception that no losses were seen on chromosome 21. Recurrent losses most commonly involved chromosomes 18, 6q, 10q, and 13; recurrent gains or amplifications most commonly involved 17p11.2, 6p12, 1p32p36, and 8q24 [185, 186]. Osteosarcoma in young children may warrant consideration of a predisposing germline gene mutation. Children in families with a germline *RBI* mutation have a 2,000-fold risk of developing OS. Three percent of OSs are found in families with Li-Fraumeni syndrome and a germline *TP53* mutation [187].

Aneurysmal Bone Cyst

Aneurysmal bone cyst (ABC) is a benign, locally aggressive bone lesion found in all age groups, with most occurring in the first two decades of life. The lesion is characterized by multiloculated blood-filled cystic spaces that rapidly expand the bone and look aggressive radiologically. Surgical removal is curative, though recurrences are frequent. ABC was considered nonneoplastic until a clonal t(16;17)(q22;p13) was discovered in separate tumors [188]. The t(16;17) was shown to result in *CDH11-USP6* fusion. Other chromosomal translocations, e.g., t(1;17)(p34;p13.2), t(3;17)(q21;p13.2), t(9;17)(q22;p13.2), and t(17;17)(q22;p13.2), involve *THRAP3* (*TRAP150*), *CNBP* (*ZNF9*), *OMD*, and *COLIA1*, respectively, with *USP6* at 17p13.2. [189–191]. Variants of the t(17;17) include inv(17)(p13.2q11) and inv(17)(p13.2q21.33) [192]¹ (see Fig. 16.15). The common theme in each of the 17p13.2 translocations is juxtaposition of the *USP6* coding sequences to the noncoding promoter regions of highly expressed genes. These fusion oncogenes result in *USP6* transcriptional upregulation [190].

Extraskeletal Myxoid Chondrosarcoma

Extraskeletal myxoid chondrosarcoma (EMC) is a rare malignancy that comprises 2.5% of soft tissue sarcomas. It has a male predilection with 50% of cases occurring between the fifth to sixth decades of life [193]. The tumor is more indolent than skeletal myxoid chondrosarcoma, is late to metastasize, and has favorable survival rates [194]. Current treatment includes wide local excision as the principal modality.

The majority of EMC have the translocation t(9;22)(q22;q12.2) or variants t(9;17)(q22;q11.2) and t(9;15)(q22;q21.3). Each of these translocations involves *NR4A3* resulting in the chimeric fusion genes of *NR4A3-EWSR1*, *NR4A3-TAF15*, and *NR4A3-TCF12*, respectively. These translocations are considered to be diagnostic of extraskeletal myxoid chondrosarcomas [195, 196].

¹and personal observation of LDC.

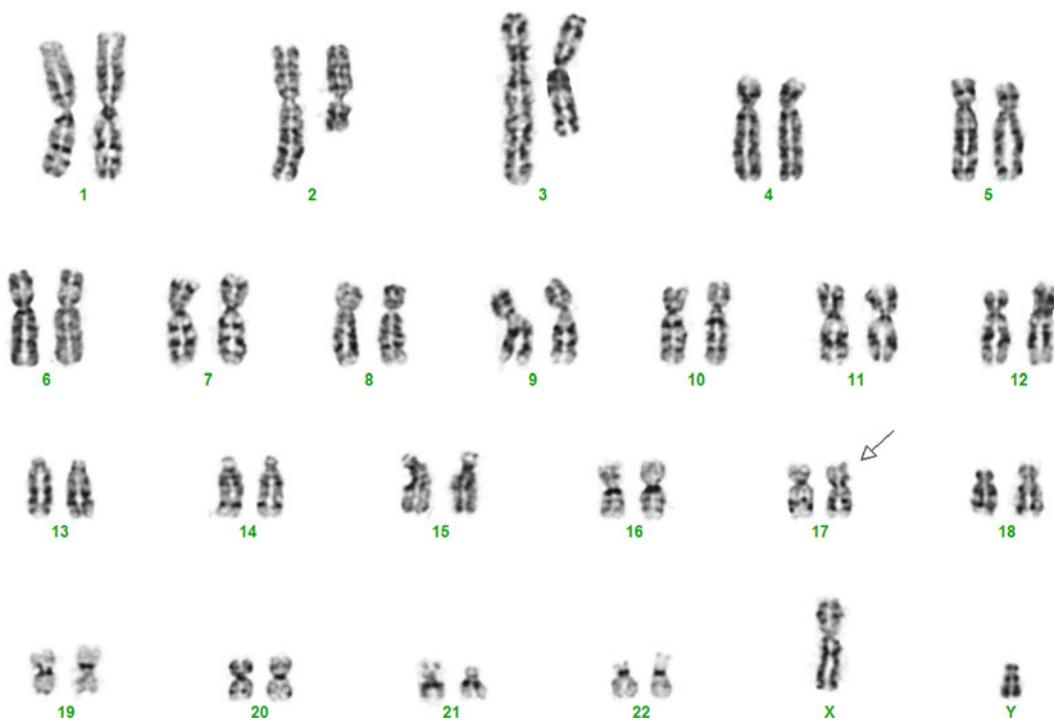


Fig. 16.15 Aneurysmal bone cyst from the olecranon of a 5-year-old boy: 46,XY,t(2;3)(q11.2;q25),inv(17)(p13.2q21.33). The inversion (arrow) results in *USP6-COL1A1* fusion (see text for details)

Angiomatoid Fibrous Histiocytoma

Angiomatoid fibrous histiocytoma (AFH) is a rare, slowly growing multinodular tumor of the deep dermis or subcutis in children and young adults. The extremities are the most common location, followed by the trunk, head, and neck regions. Three characteristic cytogenetic aberrations have been reported in AFH: t(2;22)(q33.3;q12.2) that rearranges *CREB1* with *EWSR1* (Fig. 16.16), t(12;22)(q13.12;q12.2) that rearranges *ATF1* with *EWSR1*, and t(12;16)(q13.12;p11.2) that rearranges *ATF1* with *FUS* [197, 198]. Tumors bearing the *ATF1-EWSR1* fusion and/or the concomitant t(12;22)(q13.12;q12.2) are associated with rare presentation outside of soft tissues in organs such as lung, mediastinum, and retroperitoneum. Such AFH tumors with mediastinal/retroperitoneal presentation demonstrate a higher mean age, larger size, higher frequency of systemic symptoms, higher recurrence rate, and a higher incidence of myxoid change [199].

Inflammatory Myofibroblastic Tumor

Inflammatory myofibroblastic tumor (IMT) is a rare mesenchymal neoplasm that is more common in the pediatric and young adult population with an equal gender ratio. It is characterized by a proliferation of spindle-shaped fibroblasts and myofibroblasts admixed with lymphocytes,

plasma cells, and peripheral fibrosis [2]. IMTs may recur but rarely metastasize. Approximately 50% of IMTs harbor a cytogenetic aberration that rearranges the *ALK* gene at 2p23.2. The clonal chromosome abnormalities indicate this is a neoplastic rather than a reactive inflammatory process. The *ALK* gene has a diversity of partner genes, e.g., t(1;2)(q21.3;p23.2)/*TPM3-ALK*, t(2;19)(p23.2;p13.12)/*ALK-TPM4*, t(2;17)(p23.2;q23.1)/*ALK-CLTC*, and t(2;2)(p23.2;q12.3)/*ALK-RANBP2* [200]. *ALK* rearrangement results in upregulation of *ALK* expression and constitutive gene activation [200].

Absence of *ALK* expression in IMTs has been associated with higher age and a higher mortality rate in younger patients from disease or distant metastases [201].

Recently, a subgroup of IMTs with aggressive behavior and poor outcome has been described [202]. These IMTs had epithelioid morphology, were intra-abdominal, arose in the mesentery or omentum, and were often multifocal at diagnosis. Neutrophils and lymphocytes were prominent, but plasma cells were absent. All were *ALK* positive by immunohistochemistry and/or FISH analysis. All patients had rapid local recurrences and most died of disease. RT-PCR identified *RANBP2-ALK* chimeric fusion with exon 18 of *RANBP2* fused to exon 20 of *ALK* in three tumors with available DNA. One patient treated with an *ALK* inhibitor had no evidence of disease. The authors propose this group of tumors be designated as “epithelioid

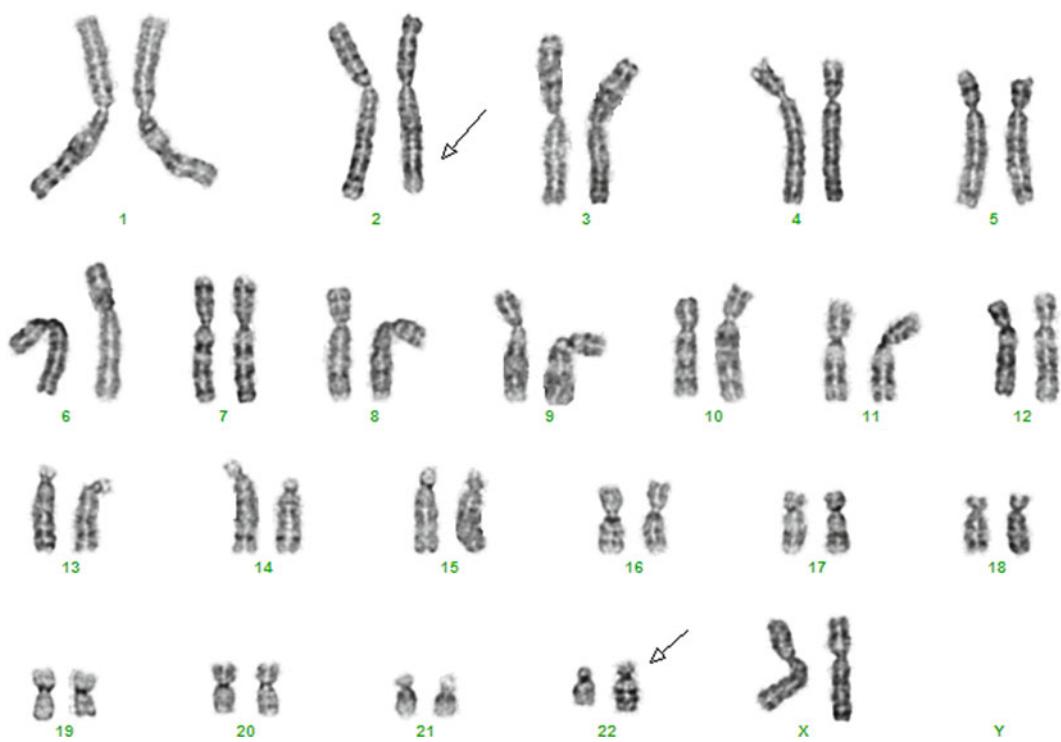


Fig. 16.16 Angiomatoid fibrous histiocytoma from the inguinal region of a 12-year-old girl: 46,XX,t(2;22)(q33;q12.2). The translocation results in *CREB1-EWSR1* fusion (see text for details)

inflammatory myofibroblastic sarcoma” to convey the malignant behavior of this tumor type and its close relationship with IMT [202].

Malignant Rhabdoid Tumor

Malignant rhabdoid tumors (MRT) are uncommon highly aggressive tumors that occur in the neonate, infant, or young child and occasionally at older ages. MRTs occur in many body sites, including the kidney, central nervous system, soft tissues, skin, liver, lungs, and others [71, 203]. A 40-year review of reported cases of fetal and neonatal MRTs found that of 72 cases, 12 presented prenatally and 60 in the neonatal period. There were 12 CNS, 27 renal, and 33 non-CNS, non-renal tumors. Metastatic disease was present at diagnosis in 33% of CNS, 52% of renal, and 70% of non-CNS, non-renal tumors. There was a concomitant renal tumor in 25% of patients with CNS tumors, a concomitant CNS tumor in 30% of patients with renal tumors, and a concomitant CNS tumor in 6% of patients with non-renal, non-CNS tumors. MRTs, non-renal non-CNS, occur more often in the perinatal period than in older children. Prognosis was poor, with ~10% overall survival [71]. Cytogenetic and molecular analysis of MRTs shows 22q11.23 deletion, loss of chromosome 22, or *SMARCB1/INI1* gene deletion or mutation [38, 204].

Small Round Cell Tumors (Table 16.5)

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) usually presents before 20 years of age and is the most common sarcoma of children and adolescents. It most commonly occurs in the head, neck, or genitourinary tract, with a paucity of normal skeletal muscle in the tumor mass. Morphologically, rhabdomyosarcomas are characterized by the rhabdomyoblast. Ultrastructural evaluation shows the presence of sarcomeres, and the tumors stain with antibodies to the myogenic markers desmin, *MYOD1*, and myogenin [2]. There are three histologic subtypes of RMS: alveolar (ARMS), embryonal (ERMS), and pleomorphic, which are associated with differences in outcome and prognosis. The ARMS and ERMS subtypes are characterized by certain molecular cytogenetic aberrations. Because the subtypes may have overlapping histologic features, particularly between ERMS and solid pattern ARMS, molecular cytogenetic analysis has diagnostic utility [205, 206].

Alveolar Rhabdomyosarcoma

Alveolar rhabdomyosarcoma accounts for 20% of all RMS, presents in adolescence, and most commonly arises in the extremities. Histologically, the tumor has a crude resemblance to pulmonary alveolae, with a network of fibrous

Table 16.5 Chromosome abnormalities with diagnostic or clinical significance in small round cell tumors

Tumor	Chromosomal aberration(s)	Gene(s) involved	Clinical significance	References
Rhabdomyosarcoma				
Alveolar RMS	t(2;13)(q36.1;q14.11), t(X;2)(q13.1;q36.1), t(2;2)(p23.3;q36.1) t(1;13)(p36.13;q14.11)	<i>PAX3-FOXO1</i> , <i>FOXO4-PAX3</i> , <i>NCOA1-PAX3</i> <i>PAX7-FOXO1</i>	Older youth, unfavorable, poor outcome with metastatic disease Younger, extremity location, 75% survival with metastatic disease	[207–210]
		<i>PAX</i> fusion negative	Favorable, comparable to ERMS	[210]
Embryonal RMS	Gain of 2, 7, 8, 11, 12, 13, 20 15q26.3 11p15.5	Chromosome enumeration <i>IGF1R</i> amplification Loss of heterozygosity	Distinguish from alveolar RMS Gene amplification with anaplasia Implicates imprinting	[212] [212] [213]
Neuroblastoma	del(1p) with or without <i>MYCN</i> amplification 2p24.3 11q Gain of 17q with or without <i>MYCN</i> amplification	1p <i>MYCN</i> amplification 11q23 band region 17q	Unfavorable Unfavorable Unfavorable; inversely associated with <i>MYCN</i> amplification Unfavorable	[215–217] [214, 217] [215, 217] [214, 219]
EWS/pPNET	Triplex without above abnormalities t(11;22)(q24.3;q12.2), t(21;22)(q22.3;q12.2), t(7;22)(p21.2;q12.2), t(17;22)(q21.31;q12.2), t(2;22)(q35;q12.2) del(9p), 17p-, der(1;16)(q10;p10)	Chromosome enumeration <i>FLI1-EWSR1</i> , <i>ERG-EWSR1</i> , <i>ETV1-EWSR1</i> , <i>ETV4-EWSR1</i> , <i>FEV-EWSR1</i> <i>CDKN2A</i> , <i>TP53</i>	Favorable Diagnostic, distinguish from other SRCTs Unfavorable prognosis	[217] [220, 221]
DSRCT	t(11;22)(p13;q12.2)	<i>WT1-EWSR1</i>	Distinguish from other SRCTs; peritoneal location	[225, 226]
Clear cell sarcoma	t(12;22)(q13.12;q12.2)	<i>ATF1-EWSR1</i>	Absent in cutaneous MM	[228, 229]
Retinoblastoma	del(13q14.2) Gain of 1q21-22; gain of 1q32.1q32.2 Gain of 6p22 16q24 loss	<i>RB1</i> <i>SHC1</i> ; <i>MDM4</i> , <i>GACI</i> <i>DEK</i> , <i>E2F3</i> Multiple genes	Retinoblastoma hallmark Implicated in cellular proliferation Potential therapeutic targets Associated with vitreous seeding	[233] [234] [235, 236] [237]
Adrenal cortical carcinoma	Complex karyotype, loss of 11q22.3	<i>ATM</i>	Common to ACC, not adenoma; associated with hereditary cancer syndromes	[239, 240]

ACC adrenal cortical carcinoma, DSRCT desmoplastic small round cell tumor, ERMS embryonal rhabdomyosarcoma, EWS/pPNET Ewing sarcoma/peripheral primitive neuroectodermal tumor, MM malignant melanoma, RMS rhabdomyosarcoma, SRCT small round cell tumor

septae traversing the tumor mass [2]. The tumor is characterized by three genetic subgroups: *PAX3-FOXO1* fusion and variants (60% of cases), *PAX7-FOXO1* fusion (20% of cases), and *PAX3-FOXO1* negative (20%) [207]. t(2;13)(q36.1;q14.11) fuses *PAX3* to *FOXO1* (Fig. 16.17). Variant translocations that also rearrange *PAX3* include t(X;2)(q13.1;q36.1) with concomitant *FOXO4-PAX3* fusion and t(2;2)(p23.3;q36.1) with *PAX3-NCOA1* fusion [208]. *PAX7* is fused to *FOXO1* by the variant t(1;13)(p36.13;q14.11) (Fig. 16.18). In patients with metastatic ARMS, a translocation involving *PAX3* has been associated with a significantly shorter overall survival than those with *PAX7* translocations [207, 209]. Patient outcomes for *PAX* fusion negative ARMS are comparable to those of ERMS patients [210].

About 20% of *PAX3-FOXO1* fusion positive tumors show amplification of the fusion gene [211]. Data indicate a significantly larger percentage of *PAX7-FOXO1* cases show

amplification of the fusion gene than *PAX3-FOXO1* cases (Personal communication). FISH analysis of a *FOXO1* breakapart probe set will show amplification of the 3' *FOXO1* probe in cases with fusion gene amplification. Concurrent chromosome analysis is needed to confirm which fusion gene is amplified (Fig. 16.18). The prognostic significance of fusion gene amplification still requires elucidation.

Embryonal Rhabdomyosarcoma

Embryonal rhabdomyosarcoma accounts for 60% of all RMS. The tumor occurs in young children and commonly presents in the nasal cavity, orbit, middle ear, prostate, and paratesticular region. The tumors are soft, gray infiltrative masses that histologically resemble skeletal muscle with the presence of round and spindled cells in a myxoid stroma [2]. ERMS is characterized by segmental or whole chromosome gains, in decreasing order of frequency, of chromosomes 2, 7, 8, 11,



Fig. 16.17 Alveolar rhabdomyosarcoma from the hand of a 14-year-old boy: 47,XY,t(2;13)(q36.1;q14.1),+der(13)t(2;13). The translocation (arrows) results in *PAX3-FOXO1* fusion (see text for details)



Fig. 16.18 Alveolar rhabdomyosarcoma from the thigh of a 4-year-old girl: 91,<4n>,XXXX,t(1;13)(p36.1;q14.1),der(1)t(1;13),-13. The translocation (arrows) results in *PAX7-FOXO1* fusion (see text for details). FISH analysis was positive for amplification of the fusion product (not shown)

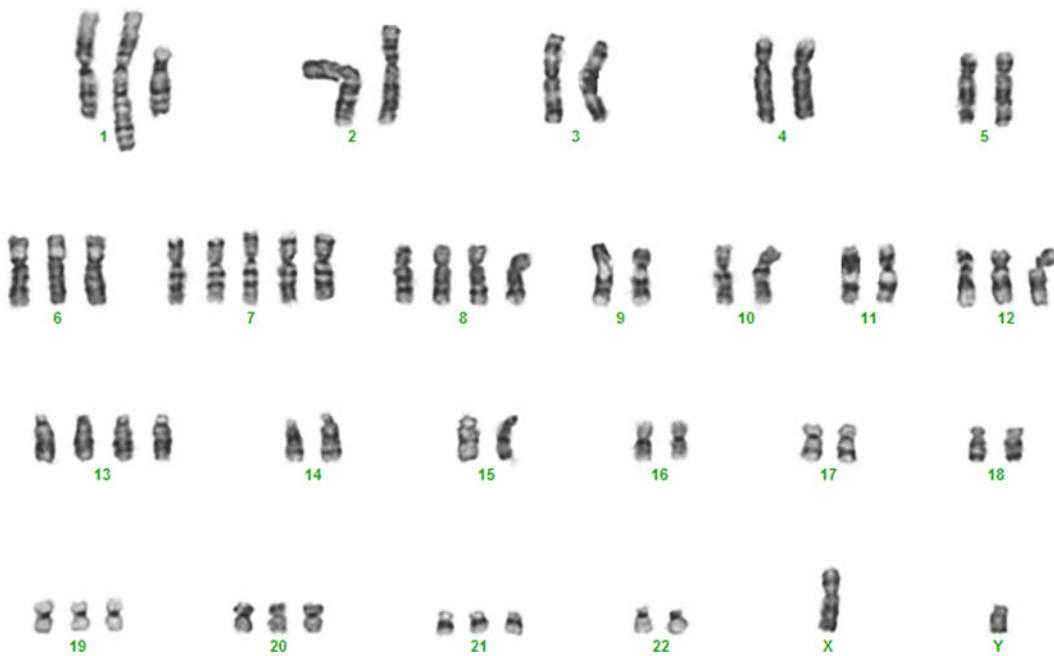


Fig. 16.19 Embryonal rhabdomyosarcoma from the nasal cavity of an 8-year-old boy: 59,XY,dup(1)(q23q43),+der(1;19)(q10;p10),+6,+7,+7,+7,+8,+8,+12,+13,+13,+19,+20,+21

12, 13, and 20 [212] (Fig. 16.19). Amplification of the insulin-like growth factor 1 receptor (*IGF1R*) at 15q26.3 has been associated with progression of ERMS with development of anaplastic histology [212]. Loss of heterozygosity in 11p15.5 is also a frequent finding, implicating genomic imprinting as a potential mechanism of tumorigenesis [213].

Neuroblastoma

Neuroblastoma (NB) is the most common extracranial pediatric solid tumor. Approximately 700 cases are diagnosed annually in the United States. It originates from neural crest cells within the sympathetic nervous system, and approximately 40% of NBs arise in the adrenal medulla. Histologically, the classic neuroblastoma exhibits solid sheets of small cells with dark nuclei, scant cytoplasm, and poorly defined cell borders. Cytoplasmic catecholamine-containing granules are often present by ultrastructural analysis, consistent with the fact that 90% of NBs produce catecholamines [2]. A morphologic classification has been developed, which associates specific morphology with prognostic features. The presence of Schwannian stroma and gangliocytic differentiation and low number of mitotic or karyorrhectic cells is favorable. Metastases can develop early with pronounced hematologic spread. A standardized staging system has been developed which is widely used internationally. Age and stage at diagnosis are considered the two most important prognostic features. Children younger than

18 months of age have an excellent prognosis as do those patients with ipsilateral disease (no extension across the midline). Patients are grouped into low-, intermediate-, or high-risk groups based on age, stage, and genetic characteristics. Overall survival for younger children and those with ipsilateral disease is 80–90%, while OS for those with higher stage and metastatic disease is 60% and <15%, respectively [7].

A number of specific genetic aberrations have prognostic significance in NB. Amplification of *MYCN* at 2p24.3 is associated with a poor prognosis and puts the patient in the high-risk category regardless of age, stage, or histology [214]. Specific segmental chromosomal aberrations (SCAs) that are evaluated by conventional cytogenetic analysis can also be characterized by aCGH analysis. Deletion of the distal short arm of chromosome 1 (1p36.3) and deletion of the long arm of chromosome 11 in a minimal deletion interval including band 11q23 are associated with a poor prognosis as independent prognostic variables, i.e., in the absence of *MYCN* amplification [215, 216]. In fact, deletion in 11q is inversely associated with *MYCN* amplification and has emerged as an important prognostic marker [217]. Gain of the long arm of chromosome 17 is often present together with loss of 1p and is also associated with a poor prognosis [214, 218] (Fig. 16.20a). Deletion of 1p is strongly associated with *MYCN* amplification [219] (Fig. 16.20b). The presence of an abnormal clone with a triploid or near-triploid chromosome number is associated with a more favorable prognosis, while near-diploid clones are generally associated with more aggressive disease and a poorer prognosis [217]. The presence of

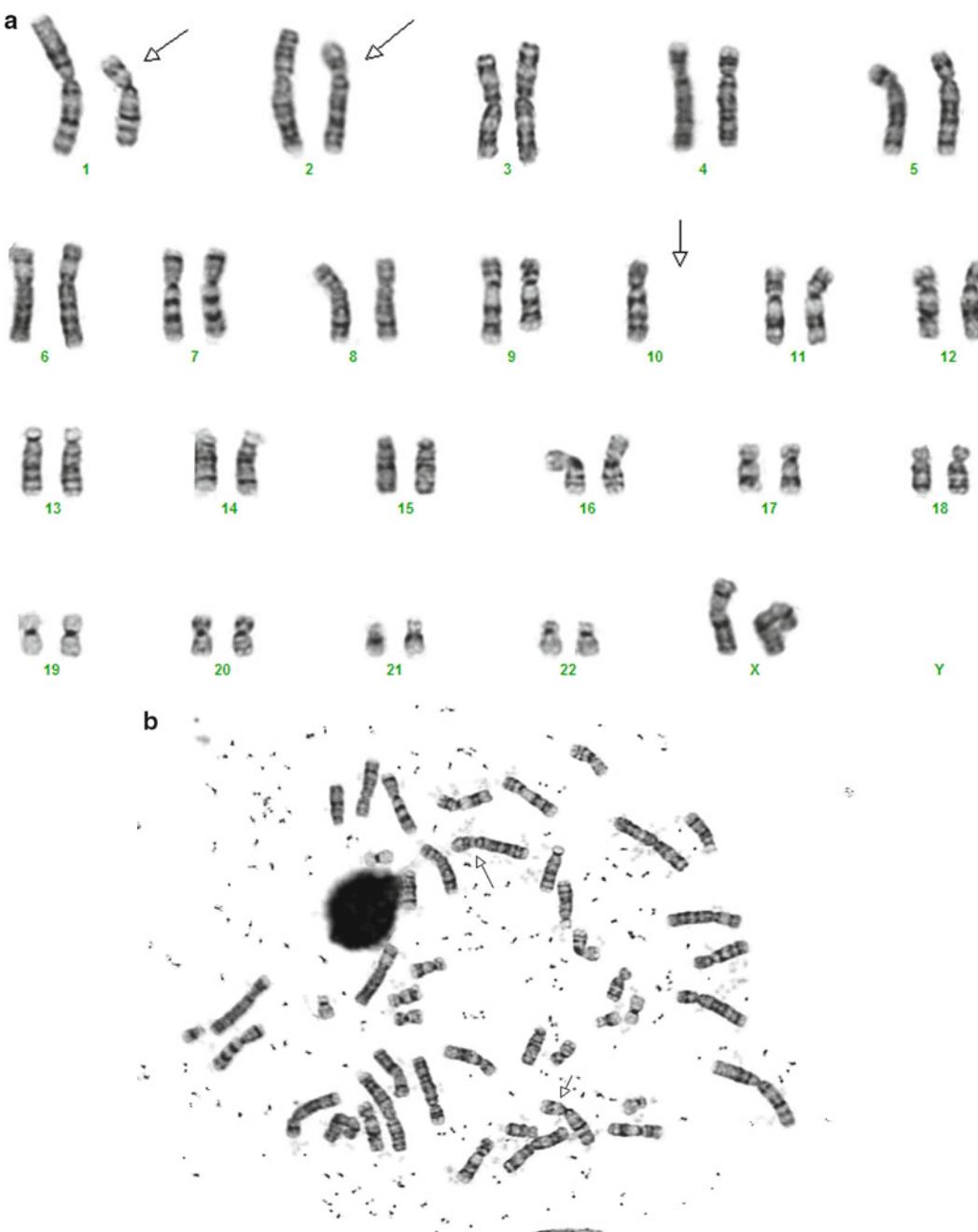


Fig. 16.20 (a) Neuroblastoma from the adrenal gland of a 2-year-old girl: 45,XX,der(1;17)(q10;q10),der(2)(qter→q24.2::p16→q11.2::?)-,−10,+17,>100dmin. (b) The double minutes are shown in the metaphase image. FISH analysis was positive for *MYCN* amplification (not shown)

genome-wide SCAs, with or without *MYCN* amplification, has been shown to be a significant predictor of relapse [219].

Ewing Sarcoma/Peripheral Primitive Neuroectodermal Tumor

The Ewing sarcoma family of tumors (EWSFT) includes Ewing sarcoma (EWS) and peripheral primitive neuroectodermal tumor (pPNET), small round cell tumors of bone and

soft tissue. EWS and pPNET share a similar neural phenotype and genetic features and thus are viewed as variants of the same tumor, differing in their degree of neural differentiation. pPNET has a greater degree of neural differentiation, while tumors with less differentiated histology are designated as Ewing sarcoma. The EWSFTs comprise the third most common sarcoma of bone, accounting for approximately 10% of primary bone tumors. There is a slight male preponderance, with the majority of all patients presenting between the ages of 5 and 30 years [7].



Fig. 16.21 Ewing sarcoma from the thoracic spine of a 14-month-old girl: 46,XX,t(11;22)(q24.3;q12.2). The translocation (arrows) results in *FLII-EWSR1* fusion (see text for details)

EWS arises from the medullary canal of bone and usually presents in the diaphysis of long tubular bones, particularly the femur or the flat bones of the pelvis. Classic Ewing sarcoma is composed of sheets of monotonous small round cells with faintly eosinophilic cytoplasm, nuclei with granular chromatin and inconspicuous nucleoli, and a low mitotic rate. The tumors are vimentin positive, contain cytoplasmic glycogen, and strongly express the cell-surface glycoprotein CD99 in a “chain-mail” pattern. pPNETs often exhibit positivity for neuron-specific enolase (NSE) and exhibit rosette formation, characteristics of neural differentiation [2, 220].

The tumor is characterized by cytogenetic aberrations involving the *EWSR1* gene at 22q12.2 that fuses *EWSR1* to various genes on different partner chromosomes. The most common is the *FLII-EWSR1* rearrangement present in 85% of tumors due to a t(11;22)(q24.3;q12.2) (Fig. 16.21). *EWSR1* fusion to *ERG* is present in 10% of tumors and is due to a t(21;22)(q22.3;q12.2). Additional rearrangements include fusion of *EWSR1* to *ETV1* at 7p21.2, *ETV4* at 17q21.31, or *FEV* at 2q35 (<1% each). The resulting fusion genes differ by partner gene and also by intragenic variability in break point location. While the prognostic impact of these various fusion transcripts has been evaluated, no clear impact of the partner gene or intragenic fusion architecture on disease progression and relapse has been elucidated [221].

Desmoplastic Small Round Cell Tumor

Desmoplastic small round cell tumor (DSRCT) is a rare aggressive tumor present in the pediatric and young adult population. Patients are most commonly 5–30 years of age at presentation and 90% are male [222]. DSRCT was first described in 1991, and fewer than 200 cases have subsequently been reported [222, 223]. Overall survival at 5 years is approximately 15% and no consensus has evolved regarding optimal treatment because of the rarity of the tumor and the aggressive clinical course [224].

Clinical presentation usually includes significant peritoneal or retroperitoneal involvement with hundreds of tumors studying the peritoneal cavity. Treatment requires a multidisciplinary approach and aggressive surgical extirpation in conjunction with adjuvant chemotherapy. Morphologically, the tumor consists of small round blue cell nests that are separated by desmoplastic stroma. Immunohistochemical findings demonstrate the trilinear coexpression of the epithelial marker keratin, the mesenchymal markers desmin and vimentin, and occasionally the neuronal marker neuron-specific enolase [225].

The diagnostic cytogenetic finding of t(11;22)(p13;q12.2) results in fusion of exon 7 of the Ewing sarcoma gene (*EWSR1*) and exon 8 of the Wilms tumor gene (*WT1*). The *EWSR1-WT1* chimeric gene encodes a transcriptional activator protein that fails to suppress tumor growth. RT-PCR shows high sensitivity for detection of the fusion transcript [225, 226].

Clear Cell Sarcoma

Clear cell sarcoma (CCS) of tendons and aponeuroses or malignant melanoma of soft parts is a rare soft tissue tumor of mesenchymal derivation. It most commonly occurs in the tendons and aponeuroses of young adults, has a propensity to metastasize to bone, and has a poor overall prognosis. Histologically, the tumor cells may demonstrate morphologic characteristics similar to malignant melanoma: the presence of melanin as well as positivity for the melanocyte immunohistochemical markers HMB-45 and S-100. It is therefore important to distinguish between malignant melanoma and CCS since the treatment and prognosis of the two is different [227].

t(12;22)(q13.12;q12.2), which characterizes CCS, fuses the *ATF1* and *EWSR1* genes to create a chimeric EWSR1-ATF1 protein that constitutively activates transcription. Molecular evaluation for *EWSR1* rearrangement by either a breakapart FISH probe set specific for *EWSR1* or RT-PCR for the fusion transcript is recommended to definitively distinguish clear cell sarcoma from malignant melanoma in melanotic tumors of soft tissue [228, 229].

Retinoblastoma

Retinoblastoma is the most common pediatric intraocular neoplasm. It is reported to have an incidence of 6 in 100,000 live births and comprises approximately 10% of cancers during the first year of life. Approximately 300 new cases are diagnosed in the United States each year [230, 231]. One third of retinoblastoma tumors are bilateral [232].

Retinoblastoma arises as a result of mutational inactivation of both retinoblastoma (*RBI*) alleles. The *RBI* gene maps to 13q14.2, and gene inactivation by cytogenetic rearrangement is a major mechanism for retinoblastoma development. *RBI* encodes a tumor suppressor gene that regulates the cell cycle at the G1/S checkpoint, blocking entry into S phase and therefore cell growth. Inactivation of *RBI* function as a tumor suppressor gene results in uncontrolled cell growth and the malignant phenotype. The Knudson “two-hit” model posits that retinoblastoma develops when both *RBI* alleles are inactivated, most commonly by a chromosomal mechanism. Patients with hereditary retinoblastoma inherit their first “hit” as a germline mutation in every cell. The second hit is somatic, with loss of the second *RBI* allele by mutation or deletion. Patients with a germline *RBI* mutation have a 10,000-fold increased risk for retinoblastoma over the general population and a significantly increased risk for other neoplasms including sarcomas, lymphomas, and brain tumors [7, 233]. Patients with sporadic retinoblastoma have two somatic *RBI* mutations in the tumor. These patients

do not carry the same increased risk for other malignancies, as they lack the inherited *RBI* mutation [7].

Additional molecular cytogenetic aberrations reported in retinoblastoma patients include a gain in the 1q21-q22 band region, encompassing the *SHC1* gene, and a gain in the 1q32.1-q32.2 band region, encompassing *MDM4* and *LRRN2*. These genes are implicated in cellular proliferation pathways [234]. A 0.6 Mb copy number gain in 6p22 encompassing the *DEK* and *E2F3* genes has also been identified as a recurrent aberration. *E2F3* is a cell cycle promoting gene and *DEK* encodes for a nuclear protein. These genes are thought to be potential therapeutic targets [235, 236]. A 6.6 Mb region of loss in 16q24 involving multiple genes including cadherin 13 (*CDH13*) has been associated with diffuse vitreous seeding [237].

Heredity and nonhereditary retinoblastomas are morphologically indistinguishable. The tumors may contain both undifferentiated and differentiated elements. Undifferentiated areas include small, round cells with hyperchromatic nuclei. Well-differentiated tumors may have rosettes reflecting photoreceptor differentiation [2]. Leukocoria (cat’s eye reflex or white pupil) and strabismus are the two most common presenting signs of retinoblastoma, present in 55 and 20% of patients, respectively [238]. Retinoblastoma is now considered curable if diagnosed early [7].

Adrenal Cortical Carcinoma

Adrenal cortical carcinomas (ACC) are rare and occur in all age groups. They are commonly functional and are associated with characteristics of hyperadrenalinism including virilism. The tumors are usually large and invasive. Metastases by hematogenous spread to lungs and other viscera are common. The tumors can show histologic variability ranging from well-differentiated tumors with cells that resemble cortical adenomas to undifferentiated tumors with significant anaplasia and large giant cells [2].

Carcinomas that metastasize to the adrenal gland are more common than primary adrenal cortical carcinomas. They may be difficult to distinguish histologically from primary ACC, and a distinguishing genetic aberration would have diagnostic utility. Deletion of the *ATM* gene at 11q22.3 as well as lower gene expression is more commonly present in ACC than in the benign adrenal cortical adenoma, implicating *ATM* in the oncogenesis of ACC [239].

Cytogenetically, ACCs generally have complex karyotypes without a diagnostic nonrandom recurrent abnormality. ACC is a feature of patients with hereditary tumor syndromes with germline mutations: Li-Fraumeni syndrome and *TP53*, as well as Beckwith-Wiedemann syndrome and 11p15.5 rearrangement. Both of these genetic aberrations, at *TP53* and 11p15.5, may also occur in sporadic forms of ACC [240].

Summary

Solid tumor cytogenetic analysis, while labor intensive for the cytogenetics laboratory personnel, has in the past and continues today to provide pathologists and oncologists with valuable genetic information that is used to refine patient diagnoses and therapeutic interventions. The chromosome rearrangements and imbalances that occur in tumor chromosomes point to genes that are responsible for oncogenic events that initiate and propel the cellular proliferation, resulting in tumor growth and patient morbidity. Conventional cytogenetic analysis, FISH analysis, and now array CGH analysis of tumor tissues provide clinically usable genetic information that is employed daily to improve the care and the therapeutics available for and offered to patients with many different benign and malignant neoplasms. This chapter discusses only the tip of the genetic iceberg of information that we still need to understand to manage and cure these diverse disorders that we call cancer.

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Part V

Adjunct Technologies

Daynna J. Wolff

Introduction

Dr. Seuss's eloquent *One Fish, Two Fish, Red Fish, Blue Fish* may have been describing one of the most significant advancements in clinical cytogenetics, fluorescence *in situ* hybridization or FISH [1]. While the basic *in situ* technology was developed more than 30 years ago, the application involving fluorescent detection of probe DNA hybridized to chromosomal target sequences was introduced to the clinical cytogenetics laboratories in the late 1980s [2, 3]. The overall hybridization process was essentially the same as that used for radioactive probes, but the major advantage was the incorporation of fluorescent detection of the probe sequences that allowed for high sensitivity in a simple and quick assay. In the ensuing years, "molecular cytogenetics," as it has come to be called, has become an integral part of the clinical cytogenetics laboratory and has been accepted as standard-of-care for the study of a host of chromosomal aberrations. FISH allows for the study of genetic aberrations that are too small to visualize by routine cytogenetic studies and too large to detect using standard DNA sequencing. In addition, as the complexity of copy number variation in the human genome has been appreciated, FISH has become an important tool for visualizing the copy number changes, determining the etiology of the change, and correlating the clinical significance. Standardized nomenclature rules for FISH were published in An International System for Cytogenetic Nomenclature [4] (see Chap. 3). In addition, the American College of Medical Genetics and Genomics (ACMG) and the Clinical Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), have developed standards and guidelines for the use of FISH in clinical laboratory testing [5, 6].

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Methodology

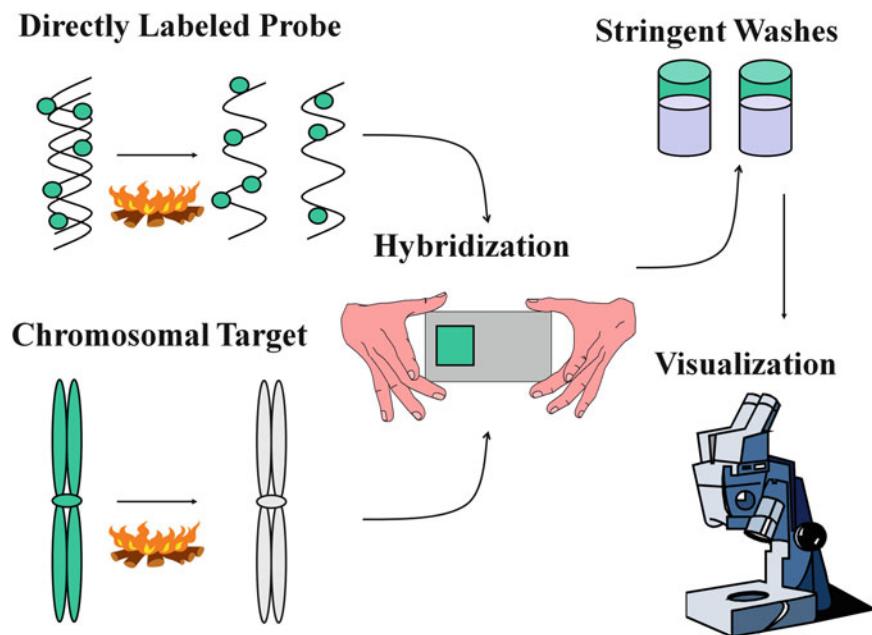
Basic Procedure

The FISH method that is widely employed in clinical laboratories involves the hybridization of a labeled DNA probe to an *in situ* chromosomal target. Probe and target DNAs are denatured using high temperature incubation in a formamide/salt solution. The probe is applied in great excess, ensuring that the probe anneals to the specific target DNA. Probe detection is accomplished by ultraviolet (UV)-light excitation of a fluorochrome, such as fluorescein-5-thiocyanate (FITC) or rhodamine, that is directly attached to the probe DNA or by incubation of a hapten (biotin or digoxigenin)-labeled probe with a fluorescent conjugate (Fig. 17.1). FISH signal patterns may be scored manually by qualified technologists, or computerized automated "spot" counting may be incorporated into the analysis (see also Chap. 7).

Probe Types

Given the abundance of sequence data available from the Human Genome Project, probes amenable for FISH procedures may be produced for the study of almost any human chromosomal site. However, the majority of probes used for clinical purposes are commercially manufactured and sold as analyte-specific reagents (ASRs) that must be validated by each laboratory. Most FISH probes fall into one of three categories: repetitive sequence, whole chromosome, or unique sequence. The most widely used repetitive sequence probes are for the alpha satellite sequences located at the centromeres of human chromosomes. Alpha satellite DNA is composed of tandemly repeated monomers, thus the sequences targeted by the probes are present in several hundreds or thousands of copies, producing strong signals. Each chromosome's alpha satellite sequence (with the exception of chromosomes 13 and 21 and chromosomes 14 and 22) is sufficiently divergent to allow for the development of centromere-specific probes.

Fig. 17.1 Schematic representation of the basic steps of the FISH procedure. Both the probe and chromosomal target are heat-denatured. Probe sequences hybridize to the complementary target sequences, and nonspecific binding is eliminated via stringent washing. The probe hybridization is detected with fluorescence microscopy



These probes are particularly useful for detection of aneuploidy in both metaphase and interphase cells. In addition, alpha satellite probes are useful for the detection of acquired monosomy or trisomy in malignancies, such as trisomy 12 in chronic lymphocytic leukemia, or monosomy 7 or trisomy 8 in myeloid disorders (see Chap. 15). Other types of repetitive sequences for which probes have been developed include the beta satellite sequences (located in the short arms of the acrocentric chromosomes), “classical” satellite sequences (found at various locations including the heterochromatic region of the Y chromosome), and telomeric repeat sequences (TTAGGG) that mark the physical ends of each human chromosome. These latter probes are not as routinely used in the clinical setting but are valuable for the study of structural aberrations.

Whole chromosome probes (WCP), also known as chromosome libraries or chromosome “painting” probes, are composed of unique and moderately repetitive sequences from an entire chromosome or chromosomal region. The generation of this type of probe requires that DNA from a particular chromosome be isolated from the rest of the genome. This may be accomplished using flow sorting, somatic cell hybrids containing a single human chromosome or area of a chromosome, or microdissected chromosomes and subsequent amplification of the dissected DNA sequences via the polymerase chain reaction (PCR) [7]. WCPs are commercially available for each human chromosome and are most frequently used for the study of structural aberrations. For example, WCPs may be used to identify the chromosomal origin of additional unknown material of derivative chromosomes and also to confirm the cytogenetic interpretation of translocations (Fig. 17.2).

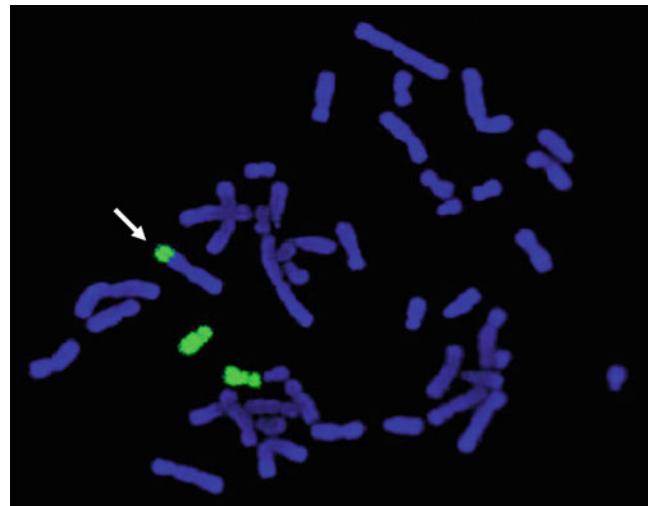


Fig. 17.2 Characterization of a structurally abnormal chromosome 7 in a patient with an unbalanced translocation. A chromosome 17 library (“painting” probe) was applied to peripheral blood metaphase cells. Both normal chromosomes 17 hybridized entirely, and the unidentifiable material attached to the short arm chromosome 7 (arrow) is also painted. The patient therefore has three copies of sequences from chromosome 17

The third and most widely used type of probe is for unique sequence DNA. These probes are generated from regions of the genome that are either cloned into various vectors (e.g., cosmids, yeast artificial chromosomes [YACs], bacterial artificial chromosomes [BACs]) or are made by PCR using sequence-specific primers. Some probes include extraneous repetitive sequences, and Cot-1 DNA must be added to the hybridization mixture to block nonspecific binding so that

only the unique sequences are visualized. Other probes, termed single-copy probes, are designed and developed based on genomic sequences that are devoid of repetitive sequences [8, 9]. Unique sequence probes, which range in size from approximately 1 kilobase (Kb) to >1 megabase (Mb), may be used to examine a particular area for copy number or location. For example, probes developed to span a translocation breakpoint, such as a probe for the 5' and 3' regions of the *MLL* gene, allow for detection of cryptic translocations involving this important cancer locus. These probes are also useful for delineating chromosomal breakpoints and for allowing the visualization of copy number changes detected by genomic microarray hybridization.

Labeling and Detection

The majority of probes that are used in the clinical cytogenetics laboratory are directly labeled and commercially available. However, probes can be indirectly labeled via incorporation of a hapten (such as biotin or digoxigenin) into the DNA via nick translation or PCR. The haptens are attached to the probe nucleotides and are detected by a secondary reaction using a fluorescently labeled antibody. The most common indirect systems involve biotin-streptavidin or digoxigenin-antidigoxigenin. Fluorochromes, such as rhodamine, Texas Red, or fluorescein, may be conjugated to the streptavidin or antidigoxigenin and detected upon excitation with a fluorescence microscope. Alternatively, directly labeled probes, with the fluorochrome attached to the probe nucleotides, require no secondary detection and may be directly visualized after fluorescent excitation.

Specimen Types

FISH can be applied to a variety of specimen types depending upon the study of interest. Metaphase preparations from cultured cells (amniocytes, chorionic villous cells, lymphocytes, cells from bone marrow aspirates or solid tumors, fibroblasts) that are routinely utilized for cytogenetic analysis are optimal preparations for FISH studies as well. FISH on metaphase cells is considered the “gold standard” because the chromosomes and the exact position of the signals can be visualized directly. However, one major advantage of FISH is that it can also be performed on interphase cells. Interphase nuclei assessment from uncultured preparations allows for rapid screening for prenatal diagnosis (amniocytes for ploidy analysis), for newborn studies (peripheral blood smears for ploidy analysis), or for cancer studies (bone marrow aspirate smear or direct harvest for translocation or copy number analysis). In addition to uncultured cells, interphase analysis may also be performed on slides prepared for routine

chromosome analysis, paraffin-embedded tissue block sections, disaggregated cells from paraffin blocks, and touch preparations of cells from lymph nodes or solid tumors. For cases in which metaphase chromosomes are limited, of poor quality, or unavailable, FISH provides a means for assessment when the routine chromosome analysis would otherwise be considered a failure. Analysis of interphase cells also allows for an increased number of cells to be assessed. Given that interphase studies cannot be verified by visualization on *in situ* chromosomes, interpretation may be compromised by hybridization inefficiency, and quality assurance is of the utmost importance.

Clinical Applications

Constitutional FISH Studies

One major advantage of FISH is its ability to detect and characterize chromosomal abnormalities that are not routinely delineated with standard banding studies. This technology allows for the detection of subtle deletions or duplications, identification of marker chromosomes, and characterization of other chromosomal rearrangements. In addition, FISH is used to visualize aberrations that are detected by copy number microarray analyses and to assess parental samples for the copy number change and/or definition of a balanced rearrangement.

Microdeletions and Microduplications

Microaberrations or contiguous gene syndromes are caused by the deletion or duplication of genetic material, usually involving multiple contiguous genes on a chromosome (Table 17.1). Breaks often occur at consistent locations and are mediated by low copy repeats (LCRs) that permit nonallelic homologous recombination. These contiguous gene syndromes, which often involve deletions or duplications that are 2 Mb or less in size, cannot be identified with routine chromosome studies. Therefore, FISH analysis provides a definitive diagnostic test for these disorders.

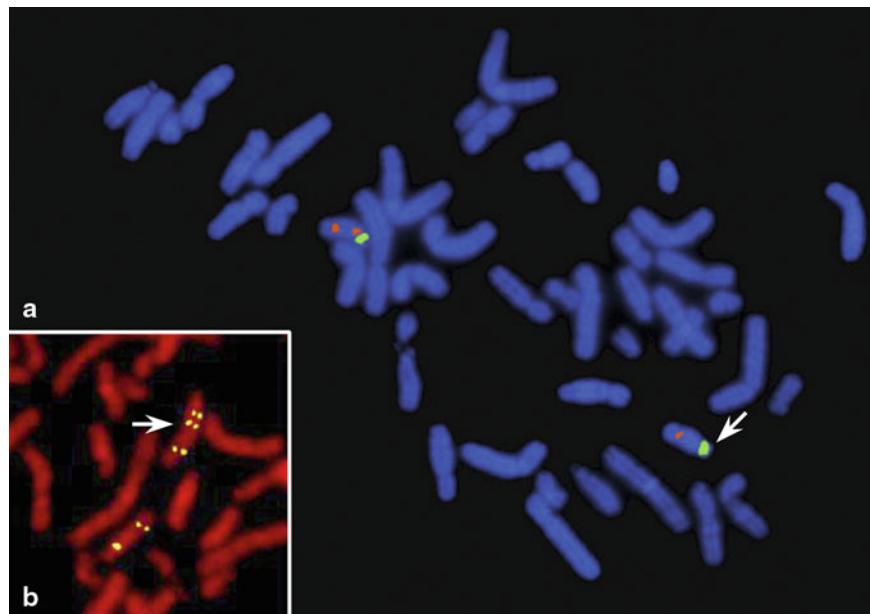
Angelman and Prader-Willi syndromes, each of which occurs in approximately 1/10,000 individuals, involve the loss of expression of the maternal or paternal genes, respectively, in 15q11.2–15q13. Approximately, 70% of cases are due to a deletion (Fig. 17.3a). Other causes include uniparental disomy (UPD), imprinting mutations (see Chap. 20), and, for Angelman syndrome, mutations of the *UBE3A* gene. The deletions involve approximately 2–5 Mb of DNA and may be detected by FISH with a probe for the *SNRPN* gene or other genes in the region. Approximately 90% of the deletions occur at the same distal breakpoint and involve one of two proximal breakpoints [10, 11]. The reciprocal product of the unequal crossing-over event, resulting in duplications of 15q11-q13, has been associated with autism (Fig. 17.3b).

Table 17.1 Microdeletion syndromes

Syndrome	Deletion	Probe ^a	Phenotype
Angelman	15q11.2–15q13	<i>SNRPN</i> <i>D15S10</i>	Severe mental retardation; hypotonia; ataxia; lack of speech; hypopigmentation; seizures; inappropriate laughter; and dysmorphic features
DiGeorge	22q11.2	<i>D22S75</i> <i>HIRA</i>	Dysmorphic features; congenital heart disease; absence of thymus; growth failure; cognitive deficits
Miller-Dieker	17p13.3	<i>PAFAH1B1</i>	Severe mental retardation; lissencephaly; dysmorphic facial features
Prader-Willi	15q11.2–15q13	<i>SNRPN</i>	Mental retardation; hypotonia; feeding difficulty; genital hyperplasia; obesity; hyperphagia; and dysmorphic features
Smith Magenis	17p11.2	<i>SHMT1</i> <i>TOP3A</i> <i>FLII</i> <i>LLGL1</i>	Mental retardation; speech delay; bizarre behavior; peripheral neuropathy; and dysmorphic facial features
Velo-Cardio Facial	22q11.2	<i>HIRA</i>	Delayed development; pharyngeal deficiency; abnormal facies; palatal defects; and congenital heart defects
Williams	7q11.2	<i>ELN</i>	Mental retardation; hypercalcemia; elfin facies; gregarious personality; and congenital heart disease

^aThe FISH probes used to diagnose the syndrome are listed in this column and are all commercially available

Fig. 17.3 Example of FISH to a single-copy target using a cosmid (*SNRPN*) to the Prader-Willi “critical region” localized to 15q11-13. (a) A metaphase in which one normal chromosome 15 has three hybridization signals from a centromeric control probe (green), a distal control probe (red), and a probe to the critical region (red). The other chromosome 15 (arrow) revealed hybridization signals only for the two control probes. Thus, this chromosome was deleted for the critical region, and this patient was diagnosed with Prader-Willi syndrome. Chromosomes were counterstained blue with DAPI. (b) In this partial metaphase, a *SNRPN* probe and a control probe (both yellow) were utilized (current standards and guidelines require the use of different fluorochromes; see Chap. 6). Chromosomes were counterstained orange with propidium iodide. The arrow indicates the chromosome 15 with a duplicated *SNRPN* signal. This patient was referred for a diagnosis of autism. See text



Several disorders involving unequal crossing-over mediated by LCRs in the short arm of chromosome 17 are routinely studied by FISH analysis [12]. Miller-Dieker syndrome involves the loss of ~2 Mb of DNA in 17p13.3 including the *PAFAH1B1* (formerly *LIS1* or lissencephaly 1) gene and other gene(s) responsible for the dysmorphic features [13]. FISH with a probe for the *PAFAH1B1* gene allows for the detection of the Miller-Dieker syndrome deletion and may also be useful for a proportion of cases with isolated lissencephaly. Another LCR-mediated mechanism results in a deletion of chromosome 17 at band p11.2 causing Smith-Magenis syndrome or a duplication of this region resulting in dup(17)(p11.2p11.2) syndrome, both of which can be diag-

nosed using FISH with probes for the critical region [14]. Similarly, interphase FISH with a probe for a 1.4 Mb area of 17p12 may be used to detect the duplication associated with Charcot-Marie-Tooth disease 1A. This same region is deleted in patients with hereditary neuropathy with liability to pressure palsies (HNPP).

Microdeletions of 22q11.2, resulting in velocardiofacial (VCF) or DiGeorge syndrome, are seen in about 1/2,000–1/3,000 individuals. Because of the relatively high frequency of this syndrome and its association with congenital heart disease, fetuses and newborns with a heart defect routinely undergo FISH testing for a 22q deletion. This syndrome, in contrast to other microdeletion syndromes, is inherited in

about 10% of the cases. Therefore, FISH studies are recommended for parents of an affected individual. Most patients with VCF or DiGeorge syndrome have an LCR-mediated deletion of ~3 Mb; some patients have a smaller ~1.5 Mb deletion that is caused by an internal LCR [15]. Mutations in the *TBX1* gene have been correlated with the abnormal phenotype, and this gene is a candidate for the psychiatric disease associated with VCF and DiGeorge syndrome [16, 17]. The reciprocal syndrome caused by duplication of 22q11.2 is associated with dysmorphic features, growth failure, cognitive deficits, hearing loss, and velopharyngeal insufficiency [18]. Both the microdeletion and the microduplication of 22q11.2 are easily detected by FISH with a probe for the *HIRA* gene or a probe for the DNA segment D22S75.

Williams syndrome involves the loss of genes in the long arm of chromosome 7 at band q11.23. The deletion has two major breakpoints that are mediated by LCRs. The deletion usually cannot be detected by G-banding, but can routinely be detected by FISH with a probe for the elastin (*ELN*) gene. Phenotypic features seen in this syndrome elegantly demonstrate the definition of a contiguous gene syndrome, as Williams syndrome involves both the central nervous system and connective tissue abnormalities. Abnormalities include mental retardation, infantile hypercalcemia, elfin facies, dysmorphic facial features, a gregarious personality, premature aging of the skin, and a congenital heart disease (supravalvular aortic stenosis) [19, 20].

Cryptic Subtelomeric Rearrangements

It is generally accepted that even with “high-resolution” chromosome analysis, alterations of chromosomal material of less than 2–4 Mb cannot be detected. In particular, due to the small size of aberration and exchange of similarly banded (G-negative) material, visualization of abnormalities in the telomeric regions is difficult. Given that these regions are gene-rich, they have particular relevance for clinical studies.

Aberrations of the subtelomeric regions have been documented in a significant percentage of patients with idiopathic mental retardation with an overall frequency of approximately 5% (range of 0–13.3%) [21, 22]. The majority of subtelomeric studies have been performed using FISH, and, in general, these studies have confirmed the efficacy of using subtelomeric probes for the assessment of individuals with mental retardation, with some cautionary notes. Not all studies used the same set of probes, and depending on the location of some probes, there was a high likelihood of detection of polymorphisms with no clinical significance, skewing the detection rates reported. Polymorphisms resulting in deletions, duplications, and other rearrangements of subtelomeric regions have been confirmed with family studies. Of note, small terminal deletions detected cytogenetically are also commonly detected by subtelomeric FISH probes. These areas of involvement include 1p, 1q, 2q, 8p, 10q, and 22q [23–26].

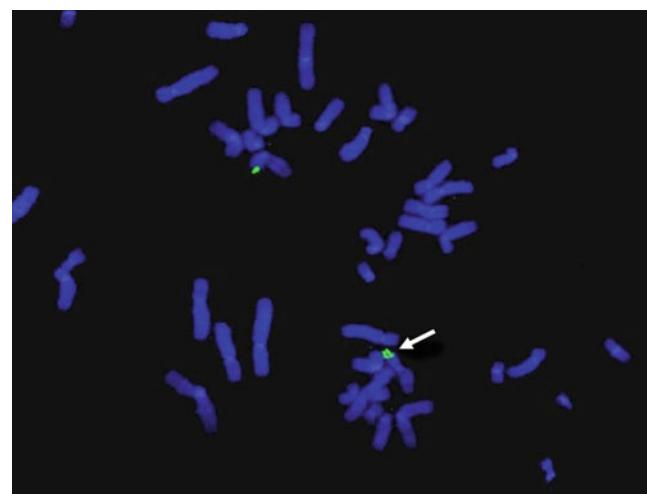


Fig. 17.4 Partial metaphase spread from a patient with a duplication involving chromosome 11. A BAC localized to chromosome 11p15.5 produced one signal on the normal chromosome 11 and a double signal on the duplicated chromosome 11 (arrow). The duplication in the short arm of chromosome 11 was detected in a newborn that was large for gestational age. The infant also had an omphalocele and was diagnosed with Beckwith-Wiedemann syndrome

While FISH has historically been the method of choice to study the subtelomeric areas, chromosomal microarray analysis that targets the entire genome, including the subtelomeric areas, has largely replaced FISH testing for patients with mental retardation/development delay and autism (see Chap. 18). Subtelomeric FISH probes are still valuable, however, as an aid.

Duplications and Marker Chromosomes

Characterization of *de novo* duplications and marker chromosomes has valuable implications with respect to phenotype/karyotype correlation. Approximately 70% of chromosomal duplications are intrachromosomal (Fig. 17.4), while 30% involve a nonhomologous chromosome [27]. Although chromosomal microarray studies are the optimal method to determine the genomic content of duplicated segments, FISH with chromosomal paints probes, locus-specific probes, and/or M-FISH (see section “**Specialized and Evolving FISH Technologies**” later in this chapter) may also be used to identify the chromosomal origin of extra material.

Chromosomes that are unidentifiable by routine banding are termed “markers” (see Chaps. 3 and 8). Marker chromosomes represent a heterogeneous group and are typically extra structurally abnormal chromosomes (ESACs). The most common types of markers, for which clinical phenotypes have been defined, may be fully characterized using FISH (Table 17.2). Other types of markers may be partially defined by FISH, and the impact of these chromosomes on the clinical phenotype often cannot be reliably predicted. Many marker chromosomes are present in mosaic form and cannot be characterized by use of chromosomal microarray analysis.

Table 17.2 Marker chromosome assessment

Type of marker	FISH probe result	Associated syndrome/phenotype (estimated risk for abnormality) ^a
ESAC	Pan-centromeric, no alpha satellite	High risk for abnormality; phenotype dependent upon euchromatin present
Bisatellited/monocentric idic(15)	Alpha satellite +: 13/21, 14/22, 15 <i>SNRPN</i> – positive <i>SNRPN</i> – negative	General risk for bisatellited = 11% 95% – MR ~0% risk 5% – MR (Usually due to UPD)
idic(22)	<i>ATP6VIE1</i> – present	Cat eye syndrome
Monosatellited	Alpha satellite +: 13/21, 14/22	No general risk, dependent on whether euchromatic material present
Nonsatellited metacentric	Alpha satellite present Alpha satellite present for 8, 9, 12, or 18 centromere	General risk for nonsatellited = 11% If metacentric, risk for MR = ~100%
Sex chromosome	DXZ1 (X centromere) + <i>XIST</i> – positive <i>XIST</i> – negative DYZ3 (Y centromere) <i>SRY</i> – positive <i>SRY</i> – negative	Turner syndrome only >95% Majority – MR Male phenotype Female phenotype

^aFrom [101, 102]

Identification of chromosomal origin can be accomplished by using M-FISH (see later in this chapter), or utilizing individual chromosomal libraries or alpha satellite DNA probes. Characteristics, such as shape and size of the marker chromosome, determine what probes are best for FISH studies. If the marker is metacentric, it is likely to be an isochromosome (see Chap. 3) and should be studied with alpha satellite probes from chromosomes 8, 9, 12, and 18, as these are the most likely isochromosomes to be present. These are all associated with an abnormal phenotype. If the marker is satellited (or bisatellited), DNA probes from the centromeres of chromosomes 13/21, 14/22, and 15 should be used. Once the origin is determined, that information, along with the structure, dictates the additional studies to be done. For example, regardless of its origin, a monocentric, bisatellited chromosome is often not associated with an abnormal phenotype, whereas a monocentric, monosatellited chromosome may be. If a satellited marker is derived from a chromosome 15, *SNRPN* status should be determined (Fig. 17.5). If *SNRPN* is present, the karyotype would be associated with an abnormal phenotype [28].

Sex chromosome markers are usually found in individuals who have 46 chromosomes, with only one normal X and a marker chromosome in place of a second sex chromosome. These abnormal chromosomes should be initially studied with X and Y alpha satellite probes. If the marker originates from an X chromosome, it should be studied with a probe for the *XIST* gene (the gene responsible for initiation of X inactivation; see Chap. 10). If *XIST* is absent, the phenotype will

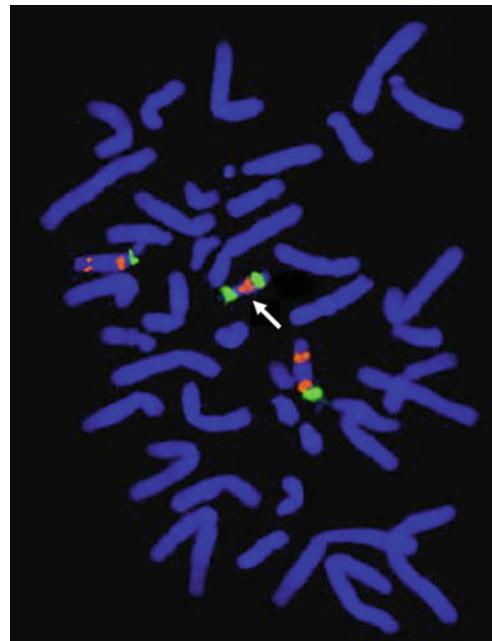


Fig. 17.5 A dicentric chromosome hybridized with dual-color chromosome 15 probes, including both an alpha satellite DNA probe (green) and a single-copy *SNRPN* probe (red). Signals from both probes are present on the normal chromosomes 15. The marker chromosome (arrow) has two alpha satellite DNA signals, confirming that it is dicentric. In addition, the marker contains two copies of the *SNRPN* probe. A control probe for the distal long arm was also included; signals are only present on the normal chromosomes 15 and not on the marker chromosome. This abnormality was ascertained in a 6-year-old female with hypotonia, behavior and learning problems, and autism

likely be associated with mental retardation/developmental delay [29]. If the marker originates from a Y chromosome, FISH with a probe for *SRY* should be performed to better understand the marker's effect on phenotype. Patients with marker chromosomes that are Y-derived are at risk for gonadoblastoma; thus, it is of significant importance to document the origin of sex chromosome markers.

The last category of markers involves ring or marker chromosomes that cannot be placed into any of the other groups. M-FISH or FISH along with each alpha satellite probe is useful for determining the chromosomal origin of such markers. However, this information does not usually allow for specific clinical risk estimations for genetic counseling (see Chap. 21).

Follow-Up Studies for Copy Number Aberrations Detected by Microarray Analysis

Microarray analysis, using comparative genomic hybridization or single nucleotide polymorphism platforms, has proven to be the most sensitive and highest resolution assessment of copy number changes in the genome, detecting aberrations in approximately 20% of individuals referred for developmental delay [30] (see Chap. 18). This genomic analysis can detect gains and losses of chromosomal material but cannot identify the mechanism underlying the change. Thus, while microarrays offer high-resolution analysis, further studies are often necessary to assist with the interpretation of the result. FISH with a probe or probes contained within the region designated on the microarray can be used to evaluate members of the proband's family for the presence of balanced rearrangements or to detect familial copy number changes that are likely of no clinical significance.

Prenatal Studies

FISH has been widely used for the detection and analysis of prenatal chromosomal abnormalities (see Chap. 12). One major advantage of FISH technology is the ability to study uncultured material to produce a rapid result. In addition, FISH is useful to characterize or detect subtle abnormalities not delineated by routine banding (e.g., deletions, markers, or duplications).

Ploidy Analysis

The vast majority of abnormalities detected prenatally are aneuploidies, involving chromosomes 13, 18, 21, or the sex chromosomes. FISH provides rapid ploidy assessment of these chromosomes by utilizing probes on uncultured interphase cells from amniotic fluid or chorionic villi [31–39] (Table 17.3). In most cases, five probes are used and applied to two different slides (or two different sections of a single slide). α -satellite DNA for the X chromosome and chromosome 18 is used together with a classical satellite probe for the Y chromosome, using three different probe colors. The other mix consists of single-copy probes for both chromosomes 13 and 21, using two different colors. These studies

Table 17.3 Prenatal ploidy analysis

Study	No.	False (+)	False (-)	Uninformative
Ward et al. (1993) [31]	4,500	.1%	.2%	6.1%
Mercier et al. (1995) [32]	630	0	(1).2%	
Bryndorf et al. (1997) [33]	2,000	0	0	7%
Jalal et al. (1998) [34]	508	0	0	
Eiben et al. (1999) [35]	>3,000	0	0	
Weremowicz et al. (2001) [36]	911	(1).1%	(5).5%	3.0%
Tepperberg et al. (2001) [37]	5,197	(1).003%	(7).024%	
Sawa et al. (2001) [38]	2,639	0	0	6.0%
Witters et al. (2002) [39]	5,049	0	0	0.26%

will ascertain numerical abnormalities for these chromosomes (Fig. 17.6) and will also detect triploidy.

While FISH assessment on uncultured cells can provide answers within 24 h of obtaining a sample, these studies are limited in that only aneuploidies for a select number of chromosomes (13, 18, 21, X, and Y) can be detected. In a 5-year collaborative study, a total of 146,128 amniocenteses were performed revealing a total of 4,163 abnormalities; however, only 69.4% of these would have been detected using interphase analysis of uncultured amniotic fluid cells [40]. A detection rate (65–70%) has been proposed in a position statement by the American College of Medical Genetics (ACMG)/American Society of Human Genetics (ASHG). The statement indicates that the sensitivity would increase to 80% with increasing age because of the association of increased age and nondisjunction.

Overall, prenatal FISH technology has been found to be effective, sensitive, and specific. Tepperberg et al. reported on a 2-year multicenter retrospective analysis and review of literature of the AneuVysion assay (Abbott Molecular) [37]. Of the 29,039 studies able to be documented, there was only one false-positive (0.003%) and 7 false-negative (0.024%) results. The results suggested that this was an effective test for aneuploidy of the testable chromosomes in cases of advanced maternal age or pregnancies indicated to be at increased risk due to maternal screening results or ultrasound findings. As this test is an adjunct test to standard cytogenetic analysis, the position statement by the ACMG/ASHG states that decisions to act on laboratory test information should be supported by two of three possible pieces of information, i.e., (1) FISH results, (2) routine chromosome analysis, and (3) clinical information (e.g., ultrasound examination).

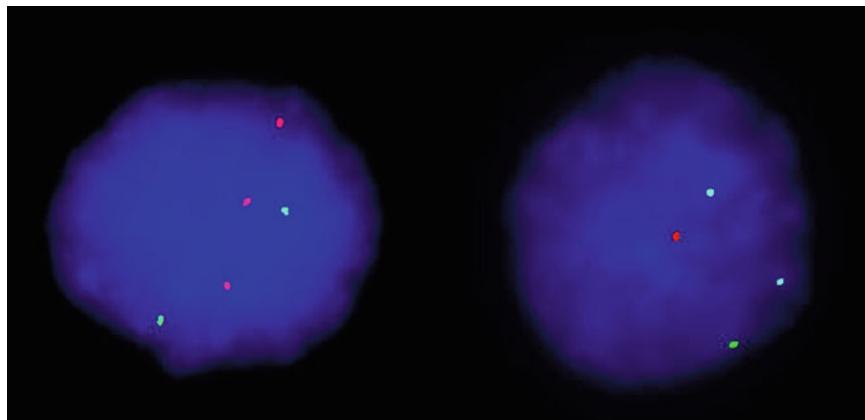


Fig. 17.6 Prenatal ploidy assessment utilizing Abbott Molecular AneuVysion™ analysis of uncultured amniotic fluid cells using unique copy probes for the long arms of chromosomes 13, 18, 21, X, and Y. The results in these interphase cells are consistent with a XY fetus with trisomy 21. *Left:*

probes for chromosomes 13 (2 green signals) and 21 (3 orange signals). *Right:* probes for chromosomes 18 (2 aqua signals), X (green signal), and Y (orange signal). Nuclei are counterstained blue with DAPI

Although much less common, these probes are also used with chorionic villus samples (CVS), *in vitro* fertilization (IVF) specimens, and fetal cells found in maternal blood. They can also be utilized to detect aneuploidy in paraffin-embedded specimens from pregnancy losses.

Preimplantation/Embryo Studies

Preimplantation genetic diagnosis (PGD) is the early diagnosis of genetic disorders, prior to the onset of pregnancy. Embryos or oocytes are biopsied during culture *in vitro* and genetic analysis is performed on the blastomeres or polar bodies. Embryos shown to be free of the genetic disease under investigation are transferred to the uterus. Multicolor FISH may be used to diagnose numerical and certain structural abnormalities of chromosomes in the embryo, and this methodology has been adopted by most PGD centers worldwide as the method of choice for sex determination and for diagnosis of aneuploidy [41–43]. Some test centers use only five probes (for chromosomes 13, 18, 21, X, and Y) for ploidy assessment, but most centers increase accuracy by using 12–24 probes. For translocation carriers, FISH with subtelomeric probes is useful for detecting unbalanced zygotes.

Although FISH is the most widely used method for PGD for some genetic diagnoses, there are several limitations with this technology [41]. FISH is generally limited to diagnosis at the chromosome level rather than the single-gene level. Therefore, other methods are needed for single-gene defects such as cystic fibrosis. Also, misdiagnosis (both false-positive and false-negative) is relatively common and has been reported in as many as 21% of single cell assessments [44]. In addition, analysis is often limited due to the restricted number of fluorochromes and the need to eliminate technical artifact (overlapping signals) in a single cell. Even with these limitations, for couples with a high risk of having a child with a genetic disease, PGD using FISH is very valuable for

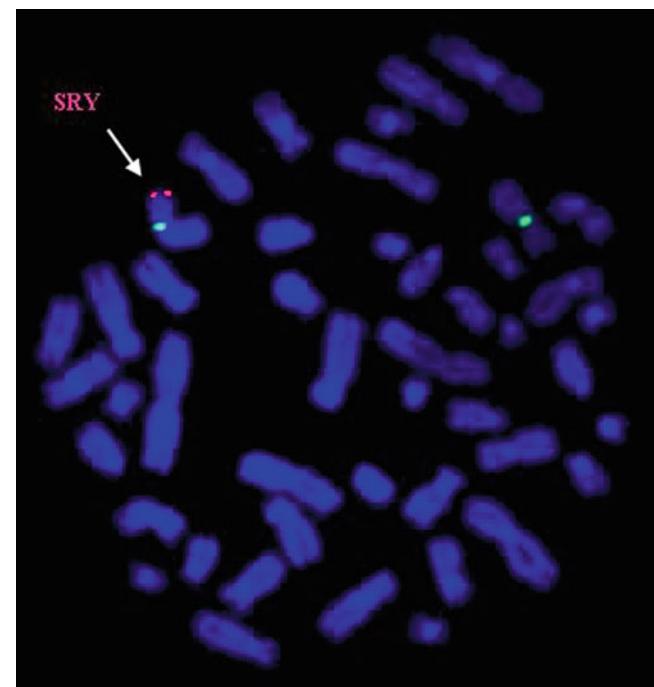


Fig. 17.7 Metaphases from an XX sex-reversed male were hybridized with probes for the X centromere (green) and a probe for the SRY gene (red). Results demonstrated a cryptic translocation in which SRY was present on the short arm of one X chromosome. Chromosomes were counterstained blue with DAPI

assessing embryo sex and chromosome number so that selective abortion and/or the birth of an affected child can be avoided.

Sex Chromosome Abnormalities

Certain sex chromosome abnormalities, such as the XX male (see Chap. 10), cannot be satisfactorily diagnosed with cytogenetics alone. Because most such patients are SRY positive, FISH analysis with probes for the X chromosome and SRY is typically necessary to confirm the diagnosis (Fig. 17.7).

For patients with a 45,X karyotype, FISH studies are recommended to determine if there is hidden mosaicism for Y-chromosomal material that could predispose the patient to gonadoblastoma [45].

FISH Applications for Studies of Acquired Chromosomal Aberrations

One major area that has been advanced greatly by FISH is the study of chromosomal abnormalities associated with cancer (see Chaps. 15 and 16). Probes have been developed for the majority of recurrent translocations found in hematologic malignancies, and there are many probes for the genetic study of solid tumors. Cancer-specific FISH probes and their characteristics are presented in Table 17.4. Several of these diseases and appropriate probes are discussed in detail as follows.

Acute Myeloid Leukemia

Approximately 40–60% of AML patients exhibit genetic aberrations that are readily detected by FISH, and in 2001, the World Health Organization (WHO) established an AML classification system that was based on recurrent genetic

abnormalities; this was updated in 2008 [46] (see also Chap. 15 and Fig. 15.8). For each category, classical cytogenetics identifies the majority of aberrations; however, FISH may be used to detect cryptic abnormalities and variant rearrangements and to monitor disease states during and following treatment.

The t(8;21) juxtaposes the *RUNX1* (*AML1*) gene on chromosome 21 and the *RUNXIT1* (*ETO*) gene on chromosome 8. A dual color, dual fusion (DCDF) probe set has been developed to detect the fusion products on the derivative 8 and the derivative 21 chromosomes (Fig. 17.8). Similarly, a DCDF probe may be used for AML with t(15;17) in which there is a juxtaposition of the retinoic acid receptor alpha (*RARA*) gene at 17q21.1 and the *PML* (promyelocytic leukemia) gene at 15q24.1. FISH with the dual fusion probes provides a definitive diagnostic test and a sensitive assay for minimal residual disease assessment. Rapid FISH diagnosis (8–48 h) of the *PML-RARA* fusion is of utmost importance, so that patients may begin receiving appropriate therapy with all-trans retinoic acid (ATRA). In addition, FISH studies allow for the differentiation of promyelocytic leukemia with t(15;17), as opposed to a variant such as t(11;17). This is clinically significant, since patients with variant translocations may not respond to ATRA treatment. The t(11;17) and

Table 17.4 FISH for hematologic malignancies

Chromosomal aberration ^a	Chromosome – gene(s) involved	Disease association ^b	Probe type(s) ^c
t(9;22)(q34;q11.2)	9 – <i>ABL1</i> 22 – <i>BCR</i>	CML, ALL, AML	DCDF, DCSF, DCES, FCDF
t(15;17)(q22;q21.1)	15 – <i>PML</i> 17 – <i>RARA</i>	AML	DCDF, DCSF, BAP
t(*;11)(*.*; q23)	11 – <i>MLL</i>	ALL, AML	BAP
t(8;21)(q22;q22)	8 – <i>RUNXIT1</i> 21 – <i>RUNXI</i>	AML	DCDF
inv(16)(p13q22) or t(16;16)(p13;q22)	16q22 – <i>CBFB</i>	AML	BAP
t(12;21)(p13;q22)	12 – <i>ETV6</i> 21 – <i>RUNXI</i>	ALL	DCES
Trisomy 8	8 – 8cen	AML, CML	SC
t(8;14)(q24;q32)	8 – <i>MYC</i> 14 – <i>IGH@</i>	ALL, NHL	DCDF
t(11;14)(q13;q34)	11 – <i>CCND1</i> 14 – <i>IGH@</i>	NHL, PCM	DCDF
t(14;18)(q32;q21)	14 – <i>IGH@</i> 18 – <i>BCL2</i>	NHL	DCDF
t(*;14)(*.*; q32)	14 – <i>IGH@</i>	NHL, PCM	BAP
del(13)(q14) or -13	<i>MIR16-1</i> , <i>MIR15A</i> (CLL); unknown for PCM	CLL, PCM	SC, PP
Trisomy 12	12 – 12cen unknown gene(s)	CLL	SC, PP
del(11)(q23)	<i>ATM</i>	CLL	SC, PP
del(17)(p13.1)	<i>TP53</i>	CLL, PCM, NHL	SC, PP

^aAn asterisk (*) is used to delineate multiple loci or breakpoints

^bAbbreviations include *ALL* acute lymphoid leukemia, *AML* acute myeloid leukemia, *CLL* chronic lymphocytic leukemia, *CML* chronic myelogenous leukemia, *NHL* non-Hodgkin lymphoma, *PCM* plasma cell myeloma

^cAbbreviations include *BAP* break-apart probe, *DCDF* dual color, dual fusion, *DCES* dual color, extra signal, *DCSF* dual color, single fusion, *FCDF* four color, dual fusion, *PP* probe panel, *SC* single color (Fig. 17.8)

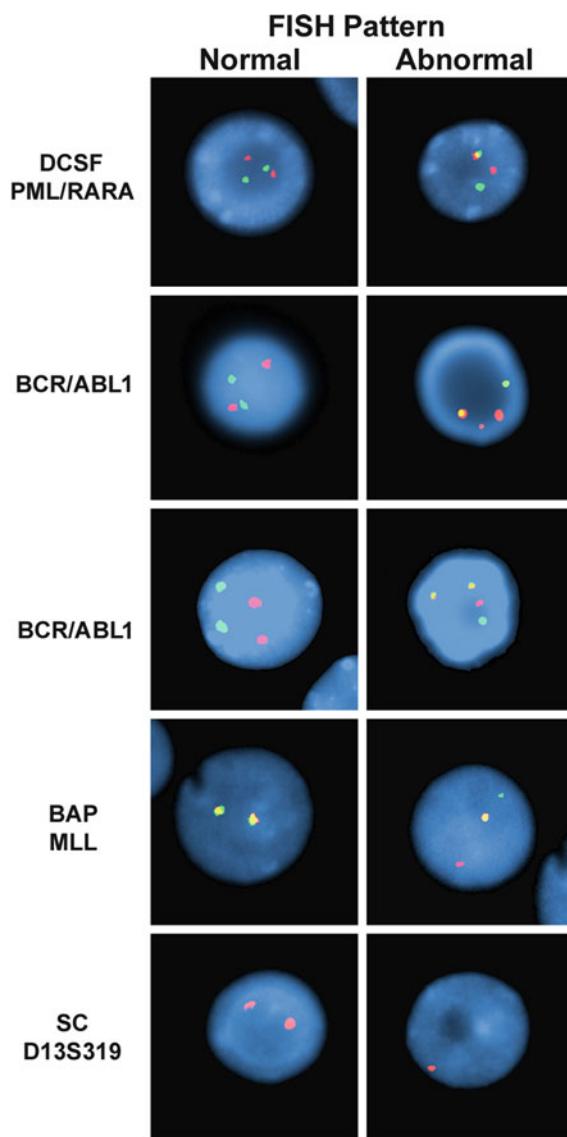


Fig. 17.8 Examples of normal (column A) and abnormal (column B) results for hematologic malignancies with various FISH probe types. The probe type and a chromosomal abnormality exemplifying typical results are given

other *RARA* variants may be identified with a *RARA* break-apart (BAP) probe.

Acute myeloid leukemia with inv(16)(p13q22) or t(16;16)(p13;q22) results from the fusion of the core-binding factor β (beta) (*CBFB*) gene at 16q22 to the muscle myosin heavy chain (*MYH11*) at p13. The fusion product interferes with the core-binding transcription pathway that is needed for normal hematopoiesis. Break-apart FISH probes have been developed that bind to the 3' and 5' regions of the *CBFB* gene, producing a yellow fusion signal in the normal situation and a single red and a single green signal when the gene is disrupted by inversion or translocation. Given that the inversion produces a subtle change in the banding pattern of chromosome 16, the aberration is often difficult to distinguish

using routine cytogenetics, particularly for cases with suboptimal chromosome preparations. Thus, FISH or other molecular techniques are recommended for definitive diagnostic and residual disease assessments.

Abnormalities of the *MLL* gene are seen in a small percentage of AML and are common in acute lymphoid leukemias (ALL). The majority of rearrangements of 11q23 involve the translocation of the 5' region of *MLL* to the 3' region of a partner gene. More than 60 different partner genes have been identified, and FISH provides an efficient screen for detection of all aberrations involving *MLL*. Dual-color break-apart probes that span the 5' and 3' regions of the gene produce a yellow fusion signal for the normal situation with no disruption of the *MLL* gene or a single red and a single green signal when any translocation involving *MLL* has occurred (Fig. 17.8). In addition, the break-apart probe allows for the assessment of copy number of *MLL* to determine if deletions or duplications/amplifications of the gene have occurred.

Chronic Myelogenous Leukemia

The t(9;22)(q34;q11.2) (see Chap. 15, Fig. 15.4) fuses the 5' region of the *BCR* (breakpoint cluster region) gene at 22q11.2 to the 3' region of the Abelson (*ABL1*) proto-oncogene at 9q34, producing a novel protein with tyrosine kinase activity. Multiple commercial FISH probe combinations are available to detect the *BCR-ABL1* fusion *in situ* including a dual color, single fusion (DCSF) probe set that detects *BCR-ABL1* on the “Philadelphia chromosome” [der(22)], a dual color, single fusion with an extra signal (DCES) probe set that detects the der(22) *BCR-ABL1* fusion and a residual signal on the der(9), and a DCDF probe set that detects the fusion products on both derivative chromosomes (Fig. 17.8). Each probe set is useful for identifying the *BCR-ABL1* fusion event in diagnostic samples. However, the ES and the DCDF probe sets offer increased sensitivity for posttreatment residual disease detection since the abnormal signal patterns produced by the latter probes rarely occur by random chance. The dual fusion probe format is particularly useful for detection of the 10–20% of patients with a t(9;22)(q34;q11.2) with atypical FISH patterns, including those with a deletion on the derivative chromosome 9 [47]. Among these patients, there is loss of a portion of *BCR* or *ABL1* or both of these hybridization sites normally associated with the break and fusion point on the abnormal chromosome 9. The loss of DNA associated with the break and fusion point on chromosome 9 in cells with a t(9;22)(q34;q11.2) has been associated with an adverse prognosis and reduced response to treatment, although more recent studies do not support this relationship [48–51]. Nevertheless, FISH for CML often includes a probe for argininosuccinate synthetase (*ASS1*) at chromosome 9q34 to detect such deletions and/or help clarify the signal patterns if possible. See Fig. 17.9.

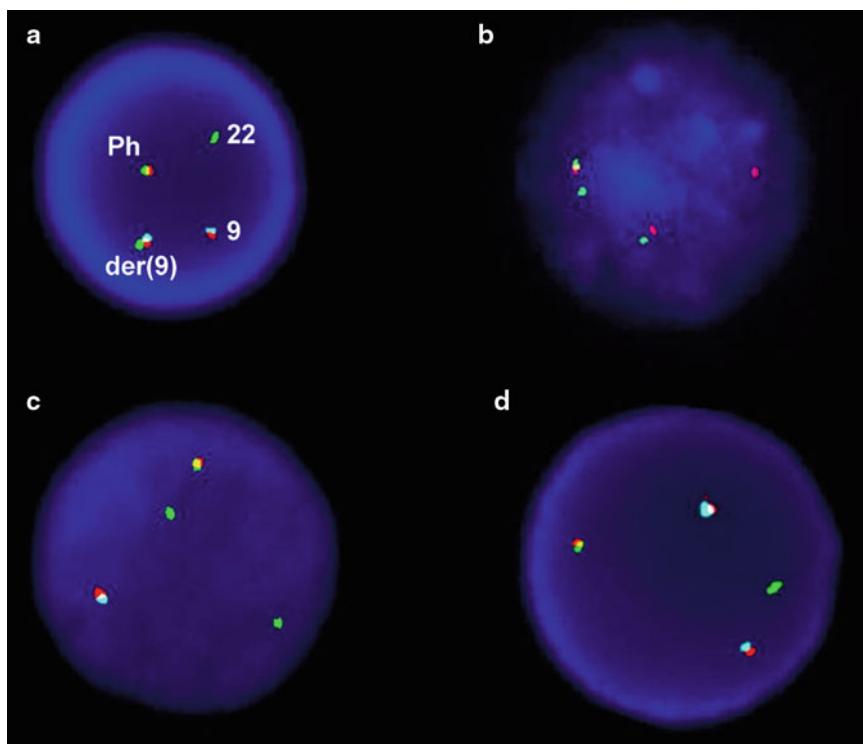


Fig. 17.9 Detection of deletions of the derivative chromosome 9, which have been associated with decreased long-term survival in CML, using dual color dual fusion BCR/ABL1 probes along with a probe for the argininosuccinate synthase gene (*ASS1*) on 9q34 (Abbott Molecular). The probe for *ABL1* is labeled *orange*, *BCR* is *green*, and the *ASS1* probe, labeled with an *aqua* fluorophore, hybridizes adjacent to *ABL1* at 9q34. *a*: A positive cell with no deletion of the der(9). The normal chromosome 9 shows *orange* and *aqua* signals, and the single *green* signal represents *BCR* on the normal chromosome 22. The “Philadelphia”

chromosome (Ph) results in a BCR/ABL1 (*green/orange*) fusion, and the derivative chromosome 9 [der(9)] produces all three signals. *b*: A positive cell with a deletion of the *aqua* signal from the der(9). *c*: A positive cell with deletions of both the *orange* and *aqua* signals from the der(9). *d*: A positive cell with loss of the *green* signal from the der(9). It should be noted that with all three deletions the derivative chromosome 9 cannot be distinguished from either the Philadelphia chromosome (*b*), the normal chromosome 22 (*c*), or the normal chromosome 9 (*d*) (Images provided by Melissa Anderson)

Acute Lymphoblastic Leukemia (B Lymphoblastic Leukemia/Lymphoma)

Routine cytogenetic studies for acute lymphoblastic leukemia (ALL) often produce suboptimal preparations and chromosomal aberrations may be missed; therefore, FISH is a useful and necessary adjunct to routine testing. Most cases of ALL are pediatric, and the identification of recurrent chromosomal aberrations is important for risk stratifying these patients.

One of the most important probe sets is for the (12;21) translocation that juxtaposes the *ETV6* (*TEL*) gene at 12p13 and the *RUNX1* (*AML1*) gene at 21q22. This translocation is present in approximately 30% of childhood precursor B-ALL, and it cannot be detected by standard cytogenetics unless a more complex rearrangement is present. Therefore, FISH with probes for *ETV6* and *RUNX1* provides a definitive diagnostic assay, as well as a means for treatment monitoring, for this subgroup of patients.

Clinical trials, including those established by the Children’s Oncology Group (COG), typically require all

newly diagnosed ALL patients to undergo both conventional cytogenetic testing as well as molecular cytogenetic characterization utilizing a FISH panel to identify *ETV6-RUNX1* and/or *BCR-ABL1* fusions, *MLL* gene rearrangements, and chromosomes 4 and 10 double trisomy. Other probes that some clinical laboratories offer as part of an ALL FISH screening panel include probes for *MYC* and a common ALL-associated deletion in 9p21-22 [52, 53].

For adult patients with ALL, *BCR-ABL1* fusion and *MLL* rearrangement are most commonly assessed by FISH. ALL FISH panels will detect the majority of genetic aberrations associated with ALL, particularly since the screen is also useful for unmasking hidden numeric abnormalities (i.e., hyperdiploidy).

t(1;19) juxtaposes *TCF3* at 19p13 with *PBX1* at 1q23. This translocation can be present in balanced form or as an unbalanced rearrangement in which only the derivative chromosome 19 is present. A *TCF3* break-apart probe and probes for the *TCF3-PBX1* fusion are both available.

See also Chap. 15, Figs. 15.10, 15.11, 15.12, 15.13, and 15.14.

Table 17.5 Cytogenetic aberrations in CLL

Cytogenetic aberration	Gene(s) involved	Percent (%) cases detected by FISH [56, 103]	Prognosis (median survival) [56]
del(13)(q14)	<i>MIR16-1, MIR15A</i>	55–64	Good (133 months)
Trisomy 12	Unknown	16–25	Intermediate (114 months)
del(6)(q21–q23)	Unknown	0–6	Intermediate
del(11)(q22.3–q23.1)	<i>ATM</i>	15–18	Poor (79 months)
del(17)(p13)	<i>TP53</i>	7–8	Poor (32 months)

B Cell Neoplasms

B cell disorders have traditionally presented challenges to the cytogenetics laboratory. The mitotic index of the cells in question is usually quite low, and while they have improved in recent years, the mitogens available to augment this can be expensive, toxic, and frequently marginally effective. The use of appropriately constructed panels of FISH probes that target the common changes seen in these diseases without adding the unnecessary cost of routinely attempting to diagnose rare events can detect chromosome abnormalities in the majority of patients.

Chronic Lymphocytic Leukemia/Lymphoma

CLL is a chronic lymphoproliferative disorder, primarily of B cell origin. As a result of the low mitotic rate of affected cells in CLL, metaphase cytogenetics traditionally only detects aberrations in approximately 40–50% of cases, although use of the synthetic oligonucleotide DSP-30 has improved this [54]. Interphase FISH is a more sensitive assay, and FISH has largely replaced conventional cytogenetics for the detection of prognostically significant genetic aberrations in CLL. FISH studies reveal that the most common abnormalities include deletions of 13q14, gain of chromosome 12, deletions of 11q22.3–q23.1, deletions of 17p13, and deletions of 6q21–q23 (Table 17.5). These genomic aberrations confer important independent predictors of disease progression and survival, thus, FISH analysis with a panel of probes for relevant aberrations is recommended for CLL patients [55]. A commercially available panel includes probes used to detect deletions of 13q14, 11q22–23 (*ATM*), and 17p13 (*TP53*) and gain of chromosome 12. A probe for *MYB* on chromosome 6 can also be used to detect deletions of this chromosome. See Fig. 17.10a, b. Abnormalities of 13q14 are present in approximately 50–60% of CLL patients, and this deletion is associated with a favorable prognosis [55, 56]. This region contains microRNAs that are thought to upregulate important cell proliferation genes resulting in CLL [57]. Deletions of the *ATM* gene at 11q22–23 have been identified in 13–18% of CLL cases assessed by FISH. Loss of *ATM* is associated with an advanced disease state and relatively rapid rate of disease progression [55, 58]. Gain of chromosome 12, originally thought to be the most common genetic aberration by routine cytogenetic analysis, is seen in

approximately 20% of B-CLL cases studied using FISH. This aberration has been associated with an intermediate prognosis in some patients presenting with what appears to be advanced stage disease [55]. del(6q) is seen in 5–10% of B-CLL patients, and in a similar percentage, the *TP53* gene at 17p13 is deleted. The latter confers the worst prognosis for CLL patients and is associated with decreased survival and increased drug resistance [59]. Since it is hypothesized that CLL clones accumulate genetic aberrations as the disease advances, FISH is appropriate for initial and follow-up studies [56]. See also Chap. 15, Fig. 15.22.

Plasma Cell Myeloma (Multiple Myeloma)/Plasmacytoma

Chromosomal abnormalities have been reported in approximately 30–50% of plasma cell disorders using routine banding studies, while interphase FISH detects deletions and translocations in at least 90% of cases studied [60]. Since plasma cell myeloma can be stratified based upon the cytogenetic findings, FISH is an important test to determine prognosis and therapy [61]. Plasma cells are typically either hyperdiploid with extra copies of the odd-numbered chromosomes or pseudodiploid with rearrangements involving the immunoglobulin heavy chain gene [62]. As with B-CLL, a panel of probes may be useful for defining the subgroup of myeloma and for staging of disease in patients with plasma cell diseases as the frequency and extent of genetic aberrations appears to correlate with clinical disease state [62]. See Fig. 17.11. Translocations involving the immunoglobulin heavy chain locus (*IGH@*) include t(4;14)(p16.3;q32) (~20% of cases), t(6;14)(p21;q32) (~3%), t(11;14)(q13;q32) (~15%), t(14;16)(q13;q21) (~8%), and t(14;20) (rare) [63]. Patients with t(4;14), t(14;16), and t(14;20) fall within a poor prognosis subgroup, while those with t(11;14) have a standard risk [62, 63]. A FISH analysis with a commercially available break-apart probe specific for the 3' and 5' ends of the *IGH@* gene provides an efficient screen for these rearrangements, while DCDF probes have been developed that detect rearrangements involving 4, 11, and 16. Deletions of 13q14 are found in approximately 40% of cases had been associated with a poor prognosis; however, more recent data has shown that this deletion is only associated with an adverse prognosis when it occurs in conjunction with t(4;14) or a

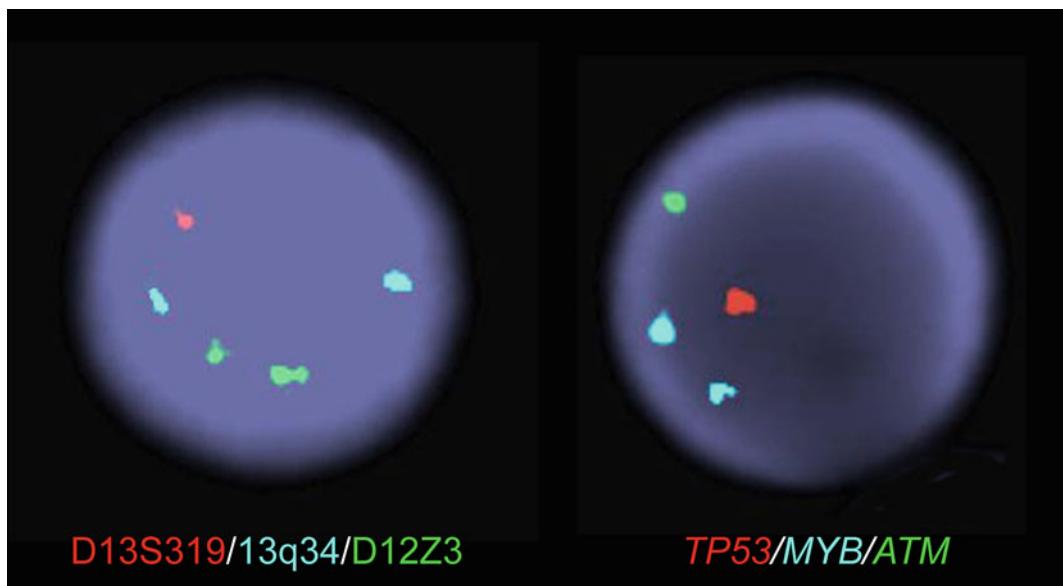


Fig. 17.10 FISH panels for B cell disorders. Results from a peripheral blood sample from a patient with CLL, hybridized with the Abbott Molecular CLL probe panel with addition of an *MYB* probe. *Left panel* shows a deletion of 13q14 (single red signal) and two *aqua* signals for

the locus on 13q34) and a normal signal pattern for the chromosome 12 centromere probe. *Right panel* shows deletion of *TP53* (single red signal), deletion of *ATM* (single green signal), and a normal signal pattern for the *MYB* probe at 6q23

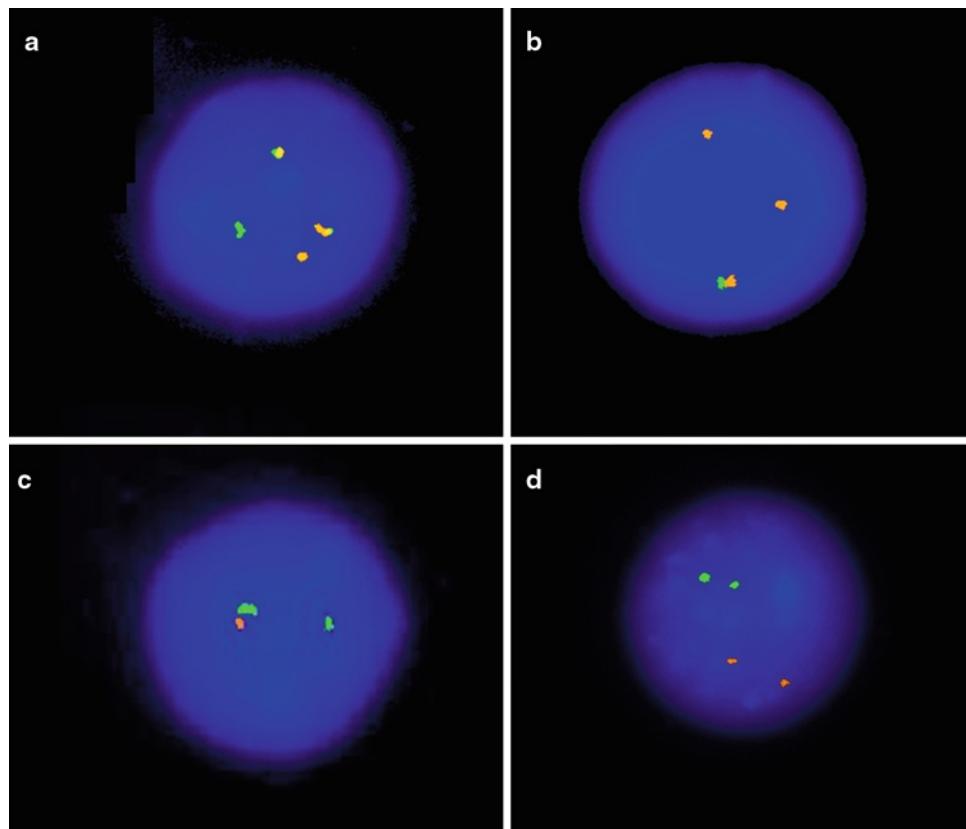


Fig. 17.11 Example of results from a panel of probes on a patient diagnosed with plasma cell myeloma with (a) a fusion (yellow) of the *FGFR3* (red) and *IGH@* (green) genes [$t(4;14)$], (b) an extra copy of the *CKS1B*

(red) gene on 1q and normal *CDK2NC* (green) gene on 1p, (c) loss of one copy of the D13S319 (red) probe (13q14), and (d) a normal signal pattern for *TP53* (red) gene—control *BCR* gene (green)

deletion of 17p [61, 64, 65]. Two important regions that confer a poor prognosis are deletion 17p (*TP53*) and duplication of 1q (possibly *CKS1B*) [61, 64]. FISH with a probe for *TP53* and for loci on 1q21 are needed to identify patients for whom more aggressive treatment may be needed [61, 64]. Given that hyperdiploidy with extra copies of the odd-numbered chromosomes is associated with a favorable prognosis, FISH with probes for chromosomes 1q, 11 (*CCND1*), 13q14, and 17 (*TP53*) is useful for the detection of probable hyperdiploidy [64, 67]. Probes for chromosomes 5, 9, and 15 are also sometimes added to confirm hyperdiploidy in these cases. It is important to note that the number of plasma cells in a sample can be low, so enrichment techniques may need to be employed prior to performing FISH. Two methods have been successfully used to enrich for plasma cells: the cIg FISH method that utilizes a fluorescently labeled antibody to cytoplasmic immunoglobulins and magnetic-assisted cell selection that allows cells to be separated based upon binding of cells to magnetic CD 138 positive particles [66, 67].

Non-Hodgkin Lymphoma

The genetic hallmarks of many non-Hodgkin lymphomas (NHLs) are translocations involving the immunoglobulin (IG) and T-cell receptor (TCR) genes resulting in inappropriate expression of genes at the reciprocal breakpoints, and FISH presents an effective test for rearrangement assessment.

A break-apart probe can be used to screen for recurrent chromosomal aberrations associated with the tumorigenesis of subtypes of B-cell lymphomas involving the immunoglobulin heavy chain gene (*IGH@*) at 14q32. Several translocations represent the primary event producing the initial disease state. t(14;18)(q32;q21) (see Chap. 15, Fig. 15.16a), which juxtaposes the *IGH@* locus with the *BCL2* gene, is virtually pathognomonic for follicular lymphoma and may also be seen in a percentage of diffuse large cell lymphomas. For mantle cell lymphoma, *IGH@* is positioned next to the *CCND1* (*BCL1*) gene by a t(11;14)(q13;q32) (see Chap. 15, Fig. 15.19). FISH with DCDF probes provides the most sensitive diagnostic assay for these rearrangements, detecting the specific gene fusions in an estimated 95–100% of cases [60]. Burkitt lymphoma, an aggressive disease of B-cell origin, harbors a t(8;14)(q24;q32) or variant translocation [t(8;22)(q24;q11), t(2;8)(p11;q24)] in all cases (see Chap. 15, Fig. 15.17). Juxtaposition of an immunoglobulin gene and *MYC* at 8q24 results in overexpression of the *MYC* transcription factor. In high-grade lymphomas, the utility of FISH with DCDF probes for *MYC-IGH@* or with a BAP probe for *MYC* is in the rapid diagnosis of rearrangements involving *MYC*, since treatment strategies differ between Burkitt lymphoma and other high-grade lymphomas. A dual color break-apart probe for the *ALK* gene at 2p23 may be used to detect the t(2;5) or variant translocations involving *ALK* that are characteristic of anaplastic large cell lymphoma.

FISH is useful for establishing the diagnosis for NHLs on primary lymph node tissue, both in cultured cells and with touch preparations, paraffin-embedded tissues, and in bone marrow to assess for involvement of this tissue.

Chronic Disorders with Eosinophilia

Chronic eosinophilic leukemia and hypereosinophilic syndrome are primary eosinophil disorders characterized by marked and persistent blood eosinophilia and organ damage. Mast cell disease is a clinically heterogeneous disorder with an accumulation of mast cells that also often has marked eosinophilia. A subset of patients with these difficult to diagnose disorders have a deletion in the *CHIC2* region and fusion of the *FIP1L1* and *PDGFRA* genes on chromosome 4q12 [68, 69]. Identification of the *FIP1L1-PDGFR*A rearrangement is a useful diagnostic tool, but more importantly, the rearrangement has been shown to be a therapeutic target of the tyrosine kinase inhibitor imatinib mesylate (Gleevec®). Thus, FISH detection of the *CHIC2* region deletion leads to appropriate therapy for this subset of patients [68]. As discussed in Chap. 15, rearrangements involving *PDGFRB* on chromosome 5 and *FGFR1* on chromosome 8 are now included in this category. See Fig. 17.12 and Chap. 15, Fig. 15.17.

Sex-Mismatched Bone Marrow or Stem Cell Transplant

For many hematologic malignancies, bone marrow or stem cell transplantation may be a reasonable treatment

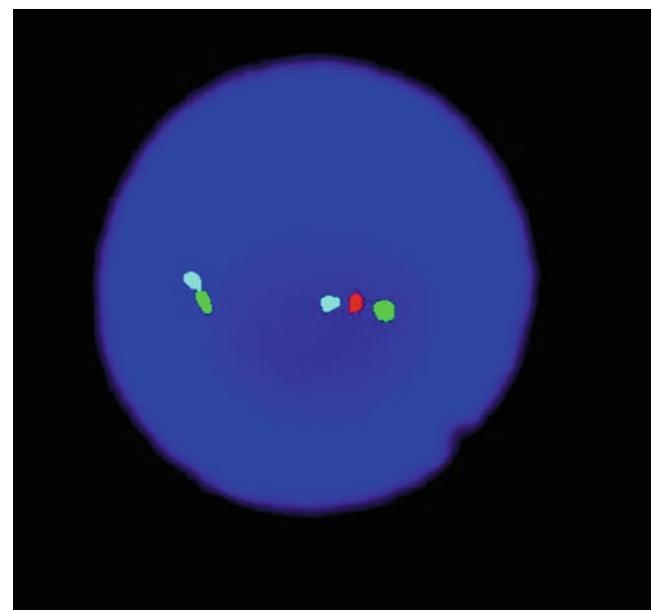
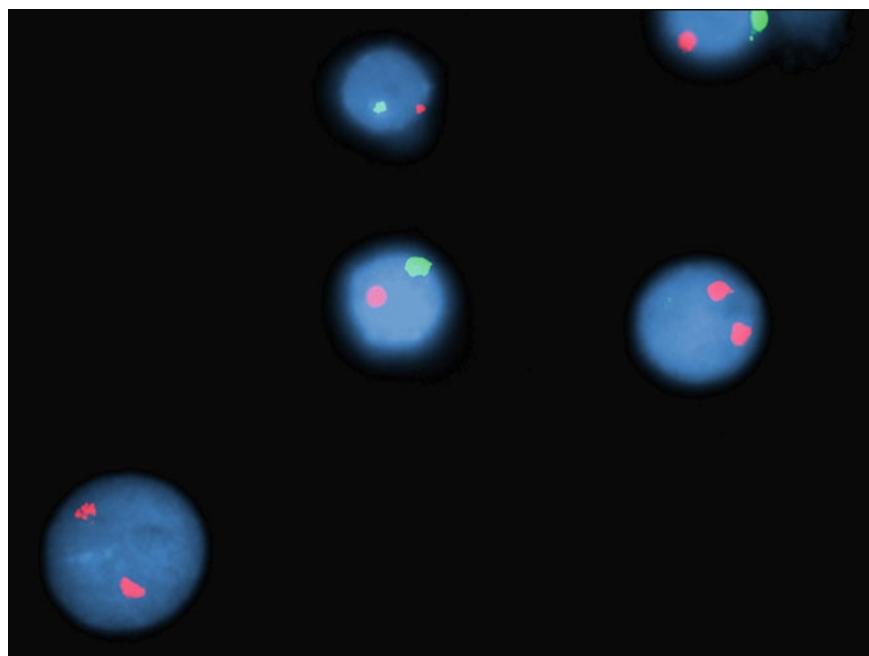


Fig. 17.12 Tricolor FISH probes for detection of the *PDGFRA-FIP1L1* fusion associated with hypereosinophilia. The green probe is centromeric to *FIP1L1*, the orange probe is telomeric to *FIP1L1* and centromeric to *PDGFRA* and contains *LNX*, and the aqua probe begins in *PDGFRA*, extends toward the telomere, and contains *KIT*. Deletion of the orange probe is a surrogate for the *PDGFRA-FIP1L1* fusion

Fig. 17.13 FISH is the most sensitive assessment for opposite sex bone marrow transplantation engraftment status studies. For this female patient who was transplanted with marrow from a male, both cell types (two red signals consistent with two X centromeres; one red signal and one green signal, consistent with one X centromere and one Y heterochromatic region) were seen, consistent with partial engraftment



and/or the only hope to cure the patient of disease. FISH is particularly useful for patients who receive bone marrow cells with an opposite sex chromosome complement. Most often, dual color probes for the X centromere (DXZ1) and the Y heterochromatic region (DYZ1) (Fig. 17.13) are employed in the analysis of interphase cells to assess for bone marrow engraftment, or engraftment status. This methodology provides a very sensitive and specific assay.

Solid Tumors

Conventional cytogenetic studies of solid tumors are limited by the ability to culture appropriate cells and to obtain metaphases for chromosome analysis. Analyses from tumors often reveal complex karyotypes with multiple numerical and structural aberrations that may not be well defined by banding. FISH is a useful tool for detecting abnormalities that allow for proper diagnosis of tumors and/or for providing prognostic information. One major advantage of FISH is the ability to study interphase nuclei of touch preparations and paraffin-embedded tissue, allowing for assessment of fresh and archival samples. M-FISH and/or comparative genomic hybridization (CGH) (see section “Specialized and Evolving FISH Technologies” later in this chapter) has proven particularly useful for characterizing the complex karyotypes associated with some solid tumors.

Soft Tissue Tumors

Many soft tissue tumors have characteristic chromosomal rearrangements or gene amplifications that can be detected using FISH, which is important given that these are often difficult to diagnose by morphology alone [70]. In particular, FISH with break-apart probes has been used to detect rearrangement of genes involved in the t(11;22)(q24;q12) that fuses the *FLII* and *EWSR1* genes associated with Ewing sarcoma [64] (see also Chap. 16, Fig. 16.21), the t(X;18)(p11.2;q11.2) that juxtaposes the *SS18* (*SYT*) and *SSX1* or *SSX2* genes in synovial sarcoma [65] (Fig. 17.14; see also Chap. 16, Fig. 16.13), and the t(2;13) (q35;q14) or t(1;13)(p36;q14) that fuses *PAX3* or *PAX7* with *FOXO1* in rhabdomyosarcomas [71] (see also Chap. 16, Figs. 16.17 and 16.18). In addition, FISH has been used to identify amplifications of the *MYCN* oncogene on chromosome 2p that are associated with a poor prognosis in children with neuroblastoma [72, 73] (see also Chap. 16, Fig. 16.20a, b). Molecular cytogenetic techniques can detect chromosomal translocations and/or other rearrangements that are used to stratify/diagnose various types of adipocytic tumors. Atypical lipomatous tumor/well-differentiated liposarcoma and dedifferentiated liposarcoma contain amplification of 12q13-15 involving *MDM2*, while myxoid/round cell liposarcoma is characterized by a translocation t(12;16)(q13;p11) that fuses the *DDIT3* (*CHOP*) and *FUS* genes. As more genes that play a role in the pathophysiology of solid tumors are identified, it is likely that clinical FISH applications for these neoplasms will be developed and marketed.

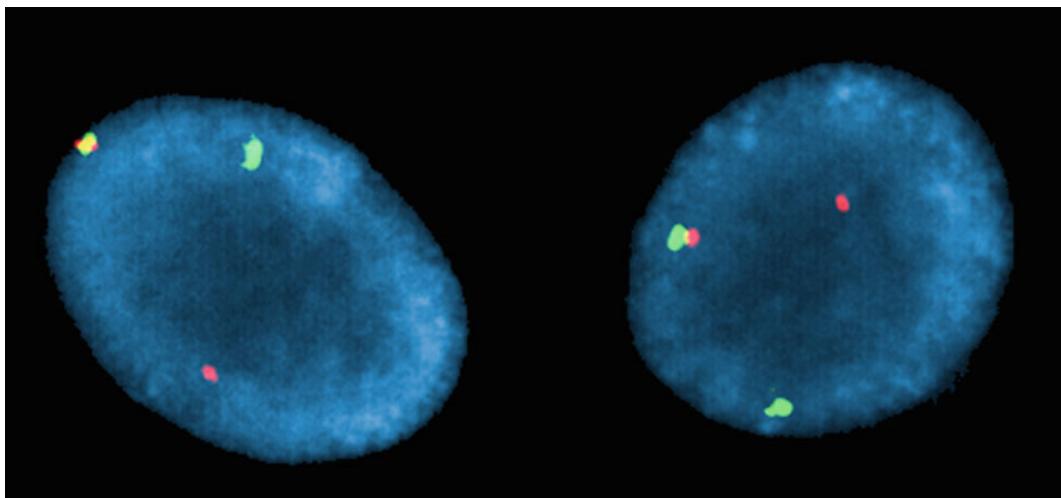


Fig. 17.14 FISH for rearrangement of the *SS18* (*SYT*) locus in a synovial sarcoma. Probes on the telomeric and centromeric sides of *SS18* are detected with FITC (green) and Texas Red, respectively. One pair of

green-red probe signals is split apart in each cell, due to rearrangement of the *SS18* gene

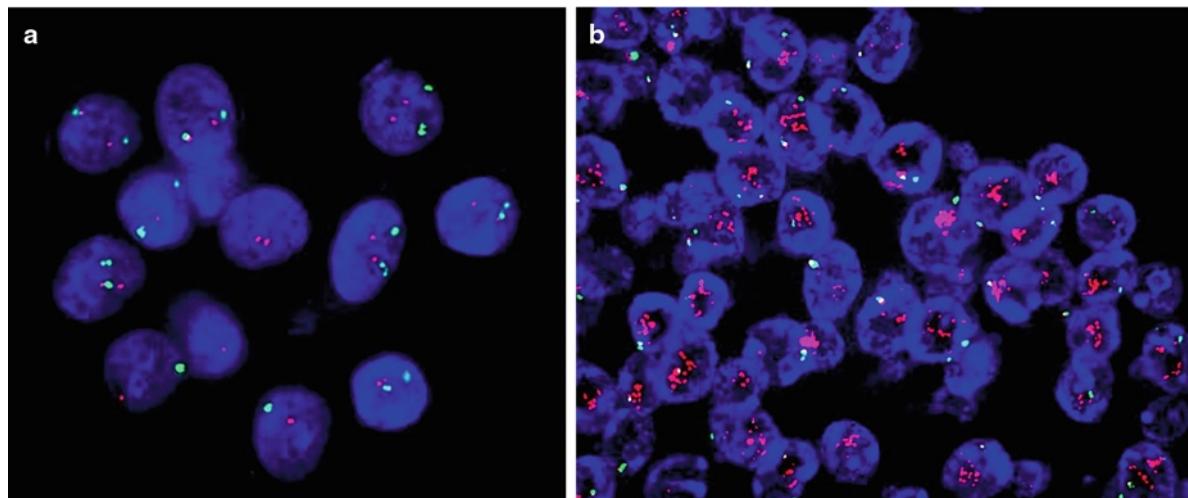


Fig. 17.15 *ERBB2* (*HER2*) analysis for carcinoma of the breast. Green signals represent the chromosome 17 centromere probe, while the *ERBB2* probe signals are red. An *ERBB2*:17 centromere ratio of ≥ 2.0

represents amplification of the *ERBB2* gene. See text for details. (a) Normal cells, with two red and two green signals. (b) *ERBB2* amplification

***ERBB2* and Breast Cancer**

Amplification of *ERBB2* (*HER2*) and/or overexpression of the protein product, which has been demonstrated in approximately 25% of breast cancers, has been associated with poor prognosis, increased risk for recurrence, and shortened survival in breast cancer patients [74, 75]. *HER2* assessment is useful for prognosis, chemotherapy responsiveness, and selection for targeted monoclonal antibody therapy—trastuzumab (Herceptin®) [75]. FISH is the most sensitive and specific US Food and Drug Administration (FDA)-approved methodology for

HER2 detection [76]. FISH with a probe for *ERBB2* (17q11.2) and, usually, an alpha satellite probe for the centromere of chromosome 17 (in a second color) are hybridized to 4- μ m sections of paraffin-embedded tumor samples. The invasive component of the cancer, as identified by a pathologist, is scored for the number of signals, and an *ERBB2*:17 centromere ratio is calculated. A ratio of ≥ 2.0 indicates *ERBB2* gene amplification (Fig. 17.15a, b). These results are used in conjunction with clinical findings to guide treatment options for the patients [77, 78].

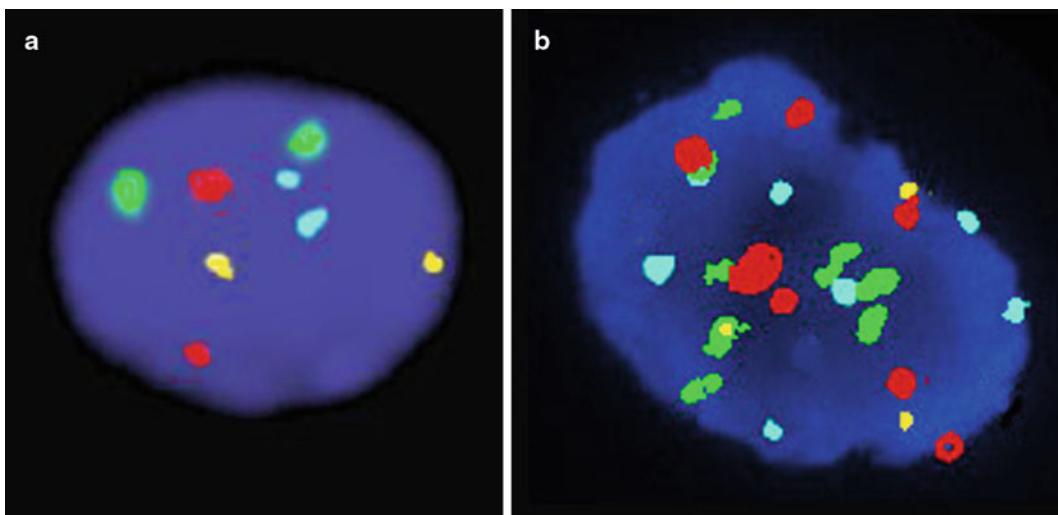


Fig. 17.16 Examples of normal (a) and abnormal (b) results for the Abbott Molecular UroVysion assay used to monitor for bladder cancer recurrence in urine or bladder wash samples. The normal signal pattern reveals two red signals for the chromosome 3 centromere, two green signals for the chromosome 7 centromere, two gold signals for 9p21, and

two aqua signals for the chromosome 17 centromere. These cells were from a male with microhematuria. The abnormal cell exhibits aneuploidy for chromosomes 3 (red), 7 (green) and 17 (aqua), consistent with urothelial carcinoma. These results confirmed a recurrence in a 70-year-old male with a history of bladder cancer

Urothelial Cancer Screening

Bladder cancer is a relatively common cancer that has a >70% chance of tumor recurrence [79]. A multitarget FISH assay consisting of alpha satellite probes for chromosomes 3, 7, and 17 and a locus-specific probe for 9p21 (Fig. 17.16) can be used in conjunction with cystoscopy to assess for bladder cancer (UroVysion®, Abbott Molecular, Des Plaines, IL) [80]. The probes are hybridized to cells from voided urine or bladder-washing samples and are used to detect aneuploidy for chromosomes 3, 7, and 17 and homozygous loss of the 9p21 locus. The overall specificity is estimated to be greater than 94% in patients without bladder cancer, and the sensitivity is approximately 71%, which is considerably better than the standard cytology testing that has an estimated 40% overall sensitivity. The FISH methodology has been shown to be particularly useful for detection of transitional cell carcinoma in cytologically equivocal and negative urine samples, often providing the earliest measure of bladder cancer recurrence (anticipatory positives) [81].

1p/19q Loss in Brain Tumors

Loss of sequences on 1p and 19q, typically mediated by a whole arm translocation between chromosomes 1 and 19 with subsequent loss of the (1;19)(p10;q10) derivative chromosome, is a hallmark feature of most oligodendroglomas, where it is associated with response to chemotherapy and radiation and improved survival [82–85]. FISH with probes for 1p36 and 19p13 is a sensitive

method to detect this aneusomy and to distinguish 1p/19q loss in a polysomic background and in many cases can be superior to molecular detection [84]. It is important to determine the 1p/19q status in brain tumors to provide the correct diagnostic and prognostic information and to allow for the appropriate therapy. See also Chap. 16, Fig. 16.2.

Non-small Cell Lung Cancer

As the molecular basis of non-small cell lung cancer (NSCLC) has become better understood, several genetic mutations have been elucidated that stratify patients for therapy. Approximately 2–7% of patients with NSCLC, and as high as 20–30% of never smokers with NSCLC, have a rearrangement of the *ALK* gene resulting in the production of an aberrant tyrosine kinase fusion protein [86, 87]. Eligibility for crizotinib, a targeted inhibitor of the abnormal tyrosine kinase, is dependent on demonstration of rearrangement of the *ALK* gene [88, 89]. Currently, the only FDA-approved method for detection of *ALK* rearrangement in NSCLC is FISH with an *ALK* break-apart probe that detects the most common *EML4-ALK* fusion, as well as several other less common rearrangements [90, 91]. The clinical trial results showed a remarkable response rate in *ALK*-rearranged patients who were treated with crizotinib, and FISH testing is now considered part of the routine screening performed on patients with NSCLC [89–92].

Special Quality Considerations for FISH

Although a few commercially manufactured probe kits have been approved by the FDA for *in vitro* diagnostic FISH testing, the majority of materials used for clinical FISH studies are considered analyte-specific reagents (ASRs) that are exempt from the FDA and must be independently validated in each laboratory. In accordance with the standards and guidelines for Clinical Genetics Laboratories from the American College of Medical Genetics and Genomics (ACMG) and the CLSI document, prior to utilizing a probe for clinical purposes, probe validation must be performed [5, 6]. The validation should consist of localizing the probe to the correct chromosomal band on normal metaphase spreads and determining sensitivity and specificity. For probes that will be used for interphase analysis, normal ranges must also be calculated from a database of cytogenetically characterized cases to establish the percent of cells with an apparent “abnormal” pattern that might occur randomly. Thus, depending on the normal cut-off point, probes may or may not be useful for detecting aberrations for interphase cell analysis. Biannual or continuous evaluation of performance characteristics of each probe is required.

It is recommended that FISH tests be analyzed by two or more non-color-blind technologists who have been trained in the scoring of the resulting signal patterns. For metaphase studies, at least 10 intact cells should be scored with one image saved for documentation. A large number of nuclei (~200) are generally scored for an interphase study, with at least one image documenting results. Many commercially available probe mixes contain internal control probes that identify the chromosome of interest. In addition, the normal homolog signal may often be used as a control as well. For tests without internal controls, for example, a Y chromosome probe on a newborn with ambiguous genitalia, a control sample (for the example given, a sample known to have a Y chromosome present) needs to be studied along with the test case. Reports should include the names of probes used and proper ISCN nomenclature (see Chap. 3).

When ASRs are employed for FISH studies, the disclaimer “This test was developed and its performance characteristics determined by <laboratory name>. It has not been cleared or approved by the U.S. Food and Drug Administration” must be included on the final report.

Specialized and Evolving Technologies

There are a number of technologies that involve variations of the standard FISH applications already discussed in this chapter. These include comparative genomic hybridization

(CGH), multiplex FISH (M-FISH), fiber FISH, m-banding, primed labeling (PRINS), and reverse hybridization [93]. While these techniques can be used for clinical analysis, typically for identification of abnormalities that cannot be elucidated with chromosome analysis, most are more regularly used on a research basis.

In addition, while this chapter deals exclusively with fluorescence *in situ* hybridization, there are several other technologies that use a different method to detect the hybridization of probes, including chromogenic *in situ* hybridization (CISH) and silver precipitation (SISH) [94, 95]. These technologies have the advantages of allowing for permanent storage of material and the use of bright-field microscopy but lack the sensitivity and/or specificity of most fluorescent probes. These ISH techniques have not been widely applied to clinical laboratory testing and are not discussed in detail here.

Comparative Genomic Hybridization (CGH) on Metaphase Cells

Comparative genomic hybridization is a technique that uses DNA from the cells of interest, rather than using a standard karyotype, for chromosomal analysis. This can be very useful, especially in some cancers when only DNA is available rather than any growing cells. DNA from the sample of interest (e.g., tumor DNA) that is labeled in one color and DNA from a normal control labeled in a different color are hybridized, in equal proportions, to metaphase chromosomes from a normal control. The ratio of fluorophores generated by photometric analysis of the two samples of DNA as determined by a computer algorithm indicates gains and/or losses of material from the DNA being examined. Where there is a normal amount of genetic material, the equal hybridization of patient and control DNA will give a 1:1 ratio; the ratio will be skewed to the patient color if there is a gain of material in the sample of interest or be skewed to the control color if there is loss of genetic material in the patient sample. This technology has been used successfully for clinical analysis, particularly with cases that have a low (or no) mitotic index; however, it is limited in that its resolution is about 10–20 Mb. It is not useful for detecting balanced rearrangements [96]. See Fig. 17.17.

M-FISH

Multiplex FISH (M-FISH) (Fig. 17.18a, b; see also Chap. 7, Fig. 7.17) is a technique that allows the investigator to view a karyotype so that each chromosome is “painted” with a different color. Combinatorially or ratio-labeled probes are used

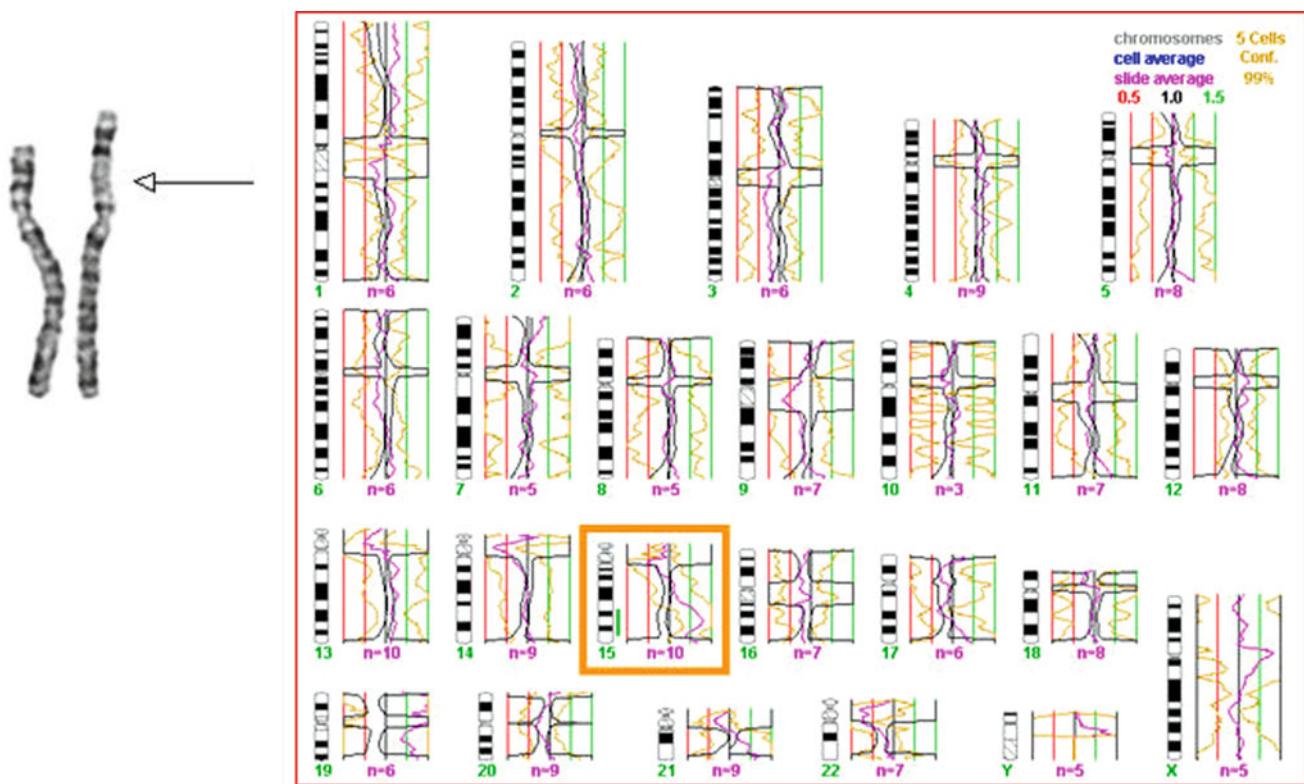


Fig. 17.17 The utility of metaphase CGH is illustrated by the CGH profiles of a case with an insertion of unknown material into the short arm of chromosome 4. The chromosomal profiles reveal

a gain of 15q (highlighted in *orange*) (This figure was kindly provided by Dr. Brynn Levy, Columbia University)

to create a distinct computer-generated false color for each chromosome [97].

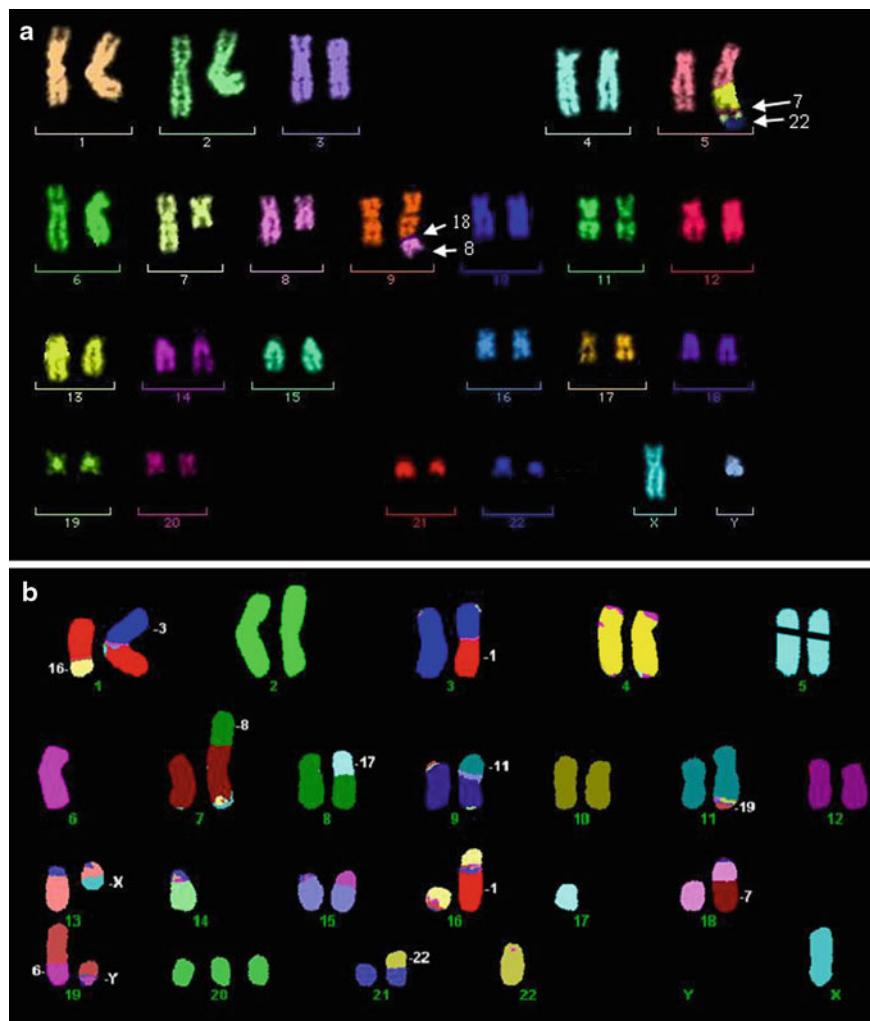
As described earlier, the use of different fluorophore colors (and the appropriate bandpass filters, see Chaps. 5 and 7) allows for the examination of more than one probe simultaneously. For example, using two colors (red and green) permits the examination of three probes at the same time (red, green, and red + green = yellow). The addition of a third color (e.g., blue) increases the number of colors to 7(red, green, blue, red + green = yellow, red + blue = magenta, green + blue = cyan, and red + green + blue = white). The formula for the number (n) of possible combinations is $n = 2^{c-1}$, where c is the number of colors used. In order, therefore, to “paint” each of the 24 human chromosomes a different color, five different fluorophores are needed. Specific computer software analyzes the data acquired from the probes and pseudocolors the chromosomes for analysis (the multiple colors can only be detected and analyzed by utilizing such software). This type of analysis is

especially useful for complex rearrangements, such as those seen in neoplastic disorders and solid tumors. As described earlier, this technology is also very useful for determining the origin of duplications and marker chromosomes.

mBAND Analysis

Multicolor banding uses chromosome-specific mixtures of partial chromosome paints that are labeled with various fluorochromes [98]. A computer program analyzes metaphase chromosome data and produces a pseudocolored, banded karyotype with an estimated resolution of 550 bands, regardless of chromosome length. This methodology is advantageous for the determination of breakpoints and the analysis of intrachromosomal rearrangements (Fig. 17.19a, b) and can be particularly useful in preparations with shorter chromosomes.

Fig. 17.18 Multiplex or multicolor FISH (M-FISH) analysis for cancer. **(a)** Metaphase from a leukemia patient with complex rearrangements. **(b)** Analysis of a hypodiploid colon cancer line with multiple numerical and structural chromosome abnormalities. The multicolor approach is useful and successful for identifying both rearrangements and aneuploidies. The origin of the different chromosomes in the rearrangements is noted on the karyotypes



Fiber Fish

Fiber FISH is a technique that is almost entirely used for research. This technology allows the chromosomes to be stretched out and elongated [99]. The probes are applied and can be physically ordered on the fibers. This provides a much higher spatial resolution and allows for correct orientation and placement of probes and for precise mapping of probes.

Primed *In Situ* Labeling (PRINS)

Primed *in situ* labeling (PRINS) is essentially PCR on a slide. Primers of interest are hybridized on a slide and then subjected to cycles of denaturation, reannealing, and elongation that are used to incorporate labeled nucleotides. The labels are then detected fluorescently, or labeled nucle-

otides are incorporated during the reaction. This technology has been used successfully with both repetitive and single-copy probes. One of the more useful applications of this technology is that it can differentiate hybridization with the alpha satellite sequences for chromosomes 13 and 21, something that cannot be done with traditional FISH. See Fig. 17.20.

Reverse FISH

Reverse FISH is used to identify material of unknown origin [100]. This unidentified material, such as a marker chromosome or duplication, is flow sorted or microdissected off of a slide after G-banding. The DNA from this material is extracted, PCR-amplified and labeled with a fluorochrome. This is then used as a probe and hybridized

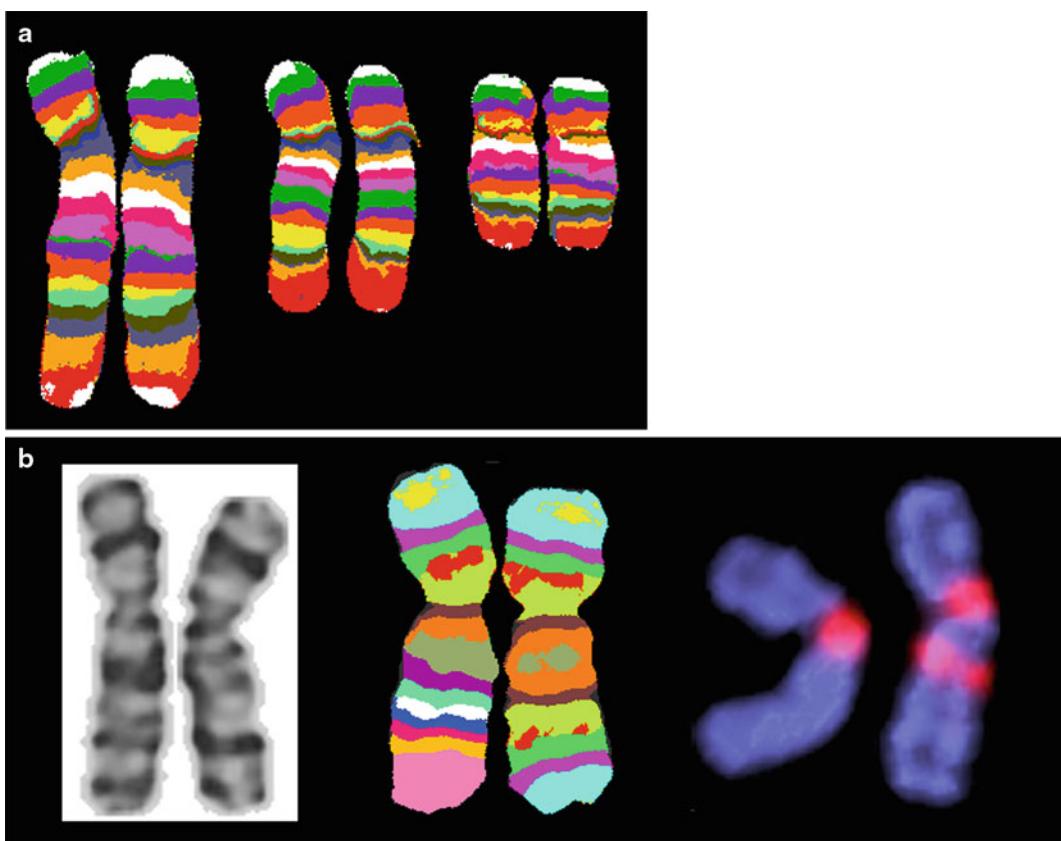


Fig. 17.19 Multicolor banding. (a) Region-specific probes labeled with different partial chromosome paints (PCP) and computer false color (MetaSystems' mBAND) produces a definable number of colored bands per chromosome, regardless of chromosome length. (b) This example shows an abnormal X chromosome (*right* homolog of each pair). Using GTG-banding, the chromosome was initially diagnosed as a paracentric inversion of the long arm (*left*). mBAND analysis, however, suggests an isodicentric chromosome X (*center*). Note the identical band colors in both chromosome arms. An X centromere probe supports this interpretation (*right*) (Images provided by MetaSystems Group, Inc)

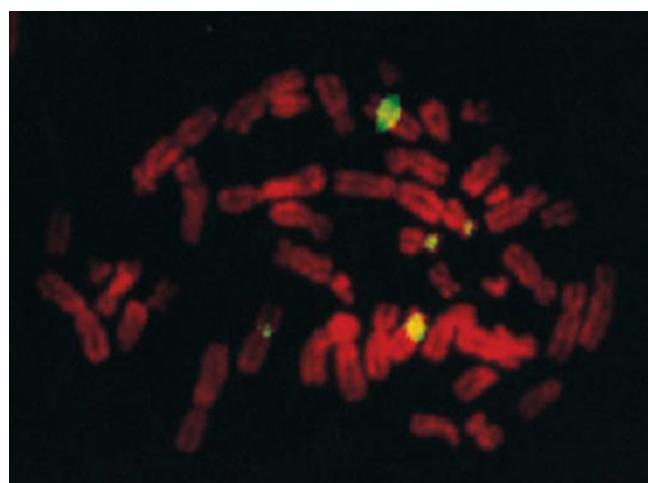


Fig. 17.20 Primed random *in situ* hybridization (PRINS). Metaphase chromosomes are subjected to PRINS with alpha satellite oligonucleotides specific for chromosomes X, 11, and 17. Bright yellow fluorescein staining is seen at the centromeres of these chromosomes. See text for details. Photo courtesy of Drs. Steen Kolvraa and Lars Bolund, Aarhus University, Sweden

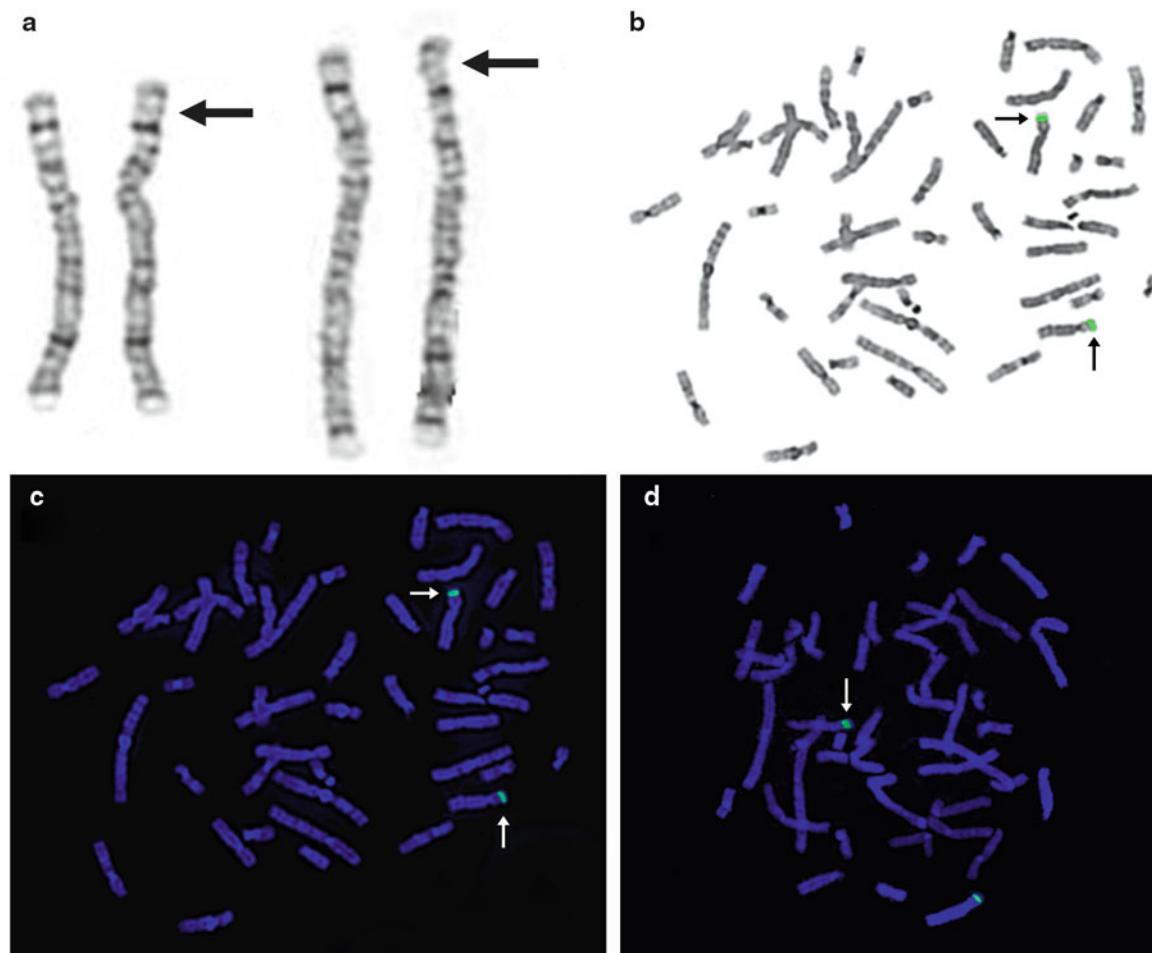


Fig. 17.21 Reverse FISH of a patient with an abnormal chromosome 8 (a) G-banding suggested a duplication of bands 8p23.1 → p23.3. Two pairs of chromosomes 8 are shown; arrows indicate the additional band. This band was microdissected, and the DNA was amplified, labeled, and used as a FISH probe (b, c) Hybridization to normal chromosomes. (b) The same metaphase is imaged with reverse DAPI to approximate G-banding patterns and identify the two chromosomes 8, and with (c)

typical DAPI staining. Arrows indicate both chromosomes 8 (d) Hybridization back to a metaphase from the patient, demonstrating that one chromosome 8 contains a duplication (arrow). The reverse FISH results confirm the initial interpretation (G-banded images courtesy of Lisa Plumley and Alma Ganezer. Reverse FISH was performed by Dr. Jingwei Yu)

to normal or patient metaphase chromosomes to identify the origin of the unknown material (Fig. 17.21a–d). This procedure has been successfully used to identify a variety of different chromosome abnormalities and is appropriate when M-FISH would be excessive. As for many of the specialized FISH techniques, copy number microarray technology offers a better resolution and characterization of chromosome abnormalities.

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Lisa G. Shaffer

Introduction

The field of cytogenetics has experienced many revolutions including hypotonic solution, banding, high-resolution preparation and analysis, and fluorescence *in situ* hybridization (FISH). However, none of these advances resulted in the rapid identification of novel cytogenetic aberrations that microarray analysis has achieved. This chapter will review the various types of genomic microarrays available to identify copy number gains and losses of the genome that result in chromosomal abnormalities. As with any new technology, there are advantages and challenges that accompany innovation. However, even with these challenges, the enormous potential of microarray testing for uncovering the etiologies of intellectual, developmental, and physical disabilities is staggering. While cytogeneticists are accustomed to unusual findings in the laboratory, the amount of data and the interpretive challenge of microarray data were not anticipated. These challenges, as well as the advantages of microarrays, will be explored.

Types of Microarrays for Cytogenetic Analysis

Microarrays are constructed from various-sized targets ranging from bacterial artificial chromosomes (BACs), which are 80–200 kilobases in size, to synthetic oligonucleotides, which are 25–85 base pairs in length. The targets, representing various segments of the genome, can number in the thousands and up to more than a million targets on some commercially available arrays. Depending on the genomic coverage (backbone) and regions targeted on the array (overall termed the microarray content), microarrays can quickly identify genomic gains

and losses that are indicative of chromosome abnormalities. Depending on the software used, the diagnostician can quickly discern the aberration's position in the genome, allowing for the identification of terminal and interstitial gains and losses of the genome (Fig. 18.1). Because microarrays can only identify copy number alterations (CNAs) of the genome and cannot distinguish the molecular etiology, a chromosome rearrangement should be visualized by chromosome banding or FISH to characterize it. This is especially important for identified gains of the genome, which may represent a duplication, extra supernumerary marker chromosome, unbalanced translocation, or insertion [1, 2].

Of the arrays that are available, two general types exist: microarray-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism-based arrays (SNP arrays). Both types of arrays will detect copy number gains and losses. In addition, both aCGH and SNP arrays will detect mosaicism with greater than about 20–30% abnormal cells [3, 4]. However, there are some unique differences between these two types of arrays, discussed in the next section.

Microarray-Based Comparative Genomic Hybridization

In aCGH, the patient sample and a control sample are each labeled with a distinct fluorescent dye and hybridized in the same concentration to the same microarray, and the fluorescent intensity of each dye is then captured by computer imaging. The amount of fluorescence, or dosage of the dyes for a particular locus, is compared between the patient and a control, and the ratio of the two dyes is plotted on a graph [5–10]. When the patient has a genomic gain or loss, as compared to the control, the difference in the fluorescent intensity of the dyes at this genomic location can be visualized (Fig. 18.1). aCGH can be applied to both BAC-based arrays and oligonucleotide-based arrays (Figs. 18.2a, b and 18.3a, b). Depending on the genomic coverage contained

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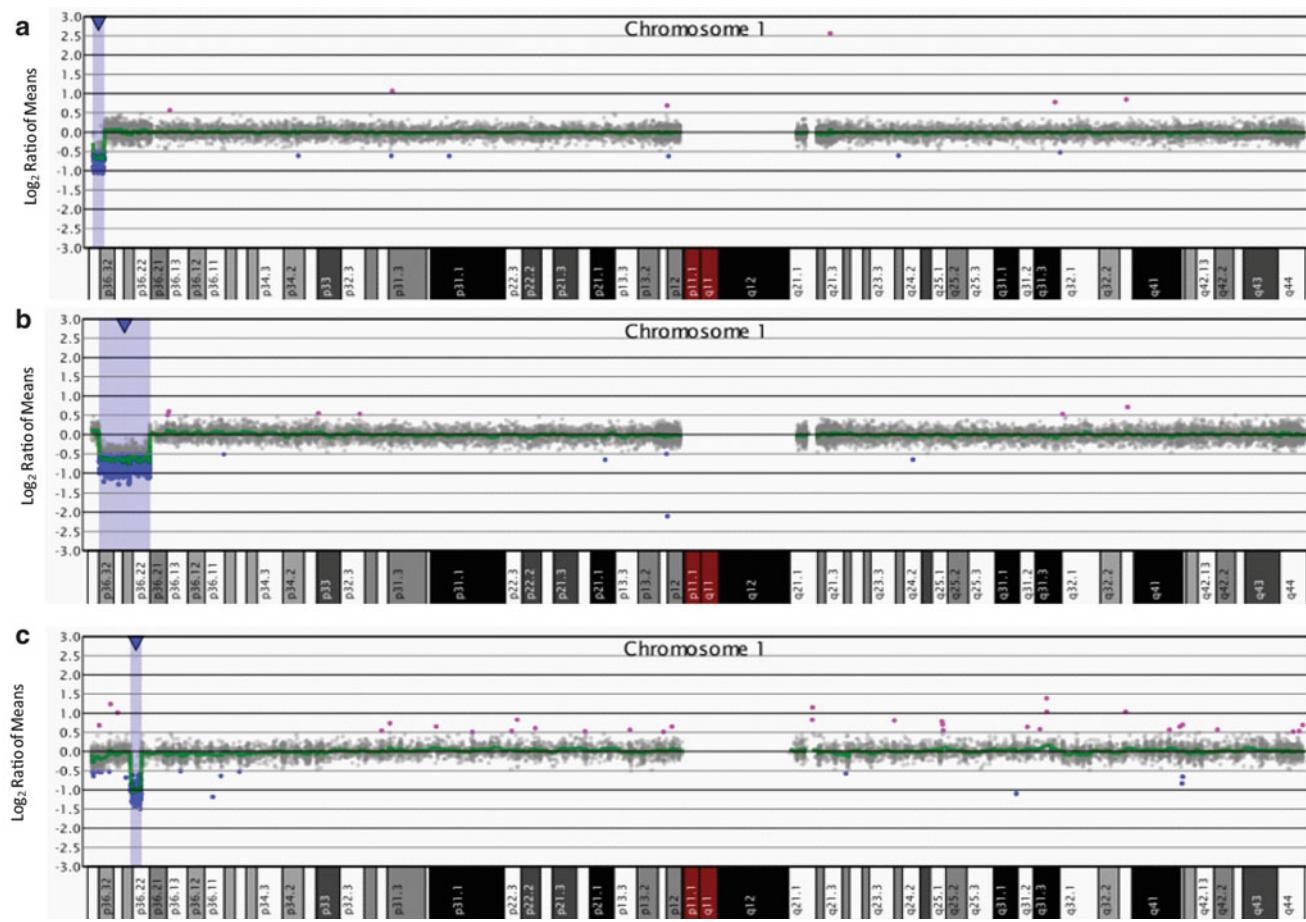


Fig. 18.1 Examples of various deletions of 1p36.3. In each, probes are ordered on the *x*-axis according to physical mapping positions, with the short arm oriented to the *left* and the long arm to the *right*. The *y*-axis indicates the log₂ ratio of the patient:control fluorescence intensity. A log₂ ratio of zero indicates a normal (1:1 ratio) result. The *blue* spots

indicate deviation from a log₂ ratio of zero. (a) ~2.5-Mb terminal deletion. (b) ~10.3-Mb interstitial deletion. (c) ~2.1-Mb interstitial deletion. Microarray analysis was performed at Signature Genomic Laboratories (Spokane, WA). Results were visualized using Genoglyphix® (Signature Genomic Laboratories, Spokane, WA, USA)

within the targets on the array, in general, oligonucleotide arrays have the potential for higher-resolution analysis, with the ability to detect smaller alterations than can be identified by BAC arrays.

Single Nucleotide Polymorphism-Based Microarrays

In contrast to aCGH, SNP-based arrays do not directly compare a patient and a control specimen. SNP arrays compare the dosage of the patient at any given locus to a database of control individuals. As with aCGH, gains and losses of the genome are readily detectable using this method (Figs. 18.2c, d and 18.3c, d). FISH should also be used after SNP array analysis to visualize a chromosome rearrangement. SNP arrays have the added advantage of being able to detect DNA base alterations, or genotyping, for any given SNP. The combination of multiple SNPs can identify regions with absence

of heterozygosity that can result from uniparental isodisomy (Fig. 18.4; see also Chap. 20). SNP arrays cannot distinguish heterodisomy from a normal result without comparison to parental SNP results. Thus, in order to suggest uniparental heterodisomy, significant stretches of homozygosity must be present. In addition, absence of heterozygosity may indicate homozygosity in offspring from closely related parents (from consanguineous unions). Although aCGH can detect some cases of triploidy based on the dosage over the sex chromosomes, SNP arrays readily detect triploidy because of the separation of the various allele combinations [11].

For cancer specimens, SNP arrays allow for the detection of loss of heterozygosity (LOH) or copy number neutral loss (CNNL) that cannot be detected using CGH-based arrays. These arrays also provide more information regarding ploidy changes, often associated with certain types of cancers. This unique ability to detect single base changes will be important as the understanding of the contribution of LOH/CNNL to disease and disease progression increases. In addition,

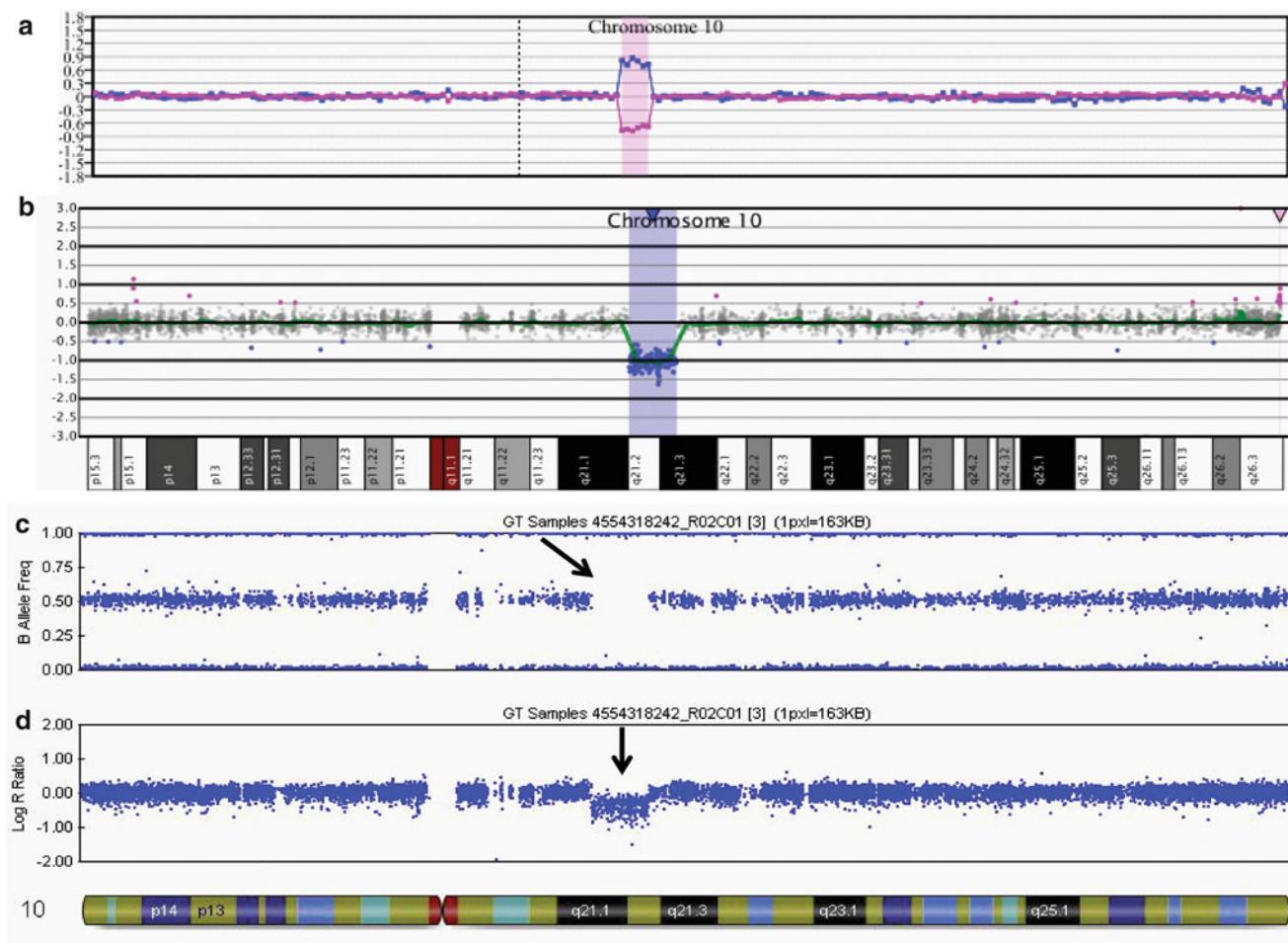


Fig. 18.2 Examples of microarray results for three different deletions of 10q21. In each, probes are ordered on the *x*-axis according to physical mapping positions, with the short arm oriented to the *left* and the long arm to the *right*. (a) Bacterial artificial chromosome (BAC) array. Two experiments were performed, one shown as a *pink line* (patient:control) and one shown as a *blue line* (control:patient). Regions of chromosome 10 in a normal dose of two copies plot at a log₂ ratio of zero and the lines come together. Region of deletion shows a deviation of the two lines, as the *pink* shading indicates. The deleted region in 10q21.2q21.3 is ~3 Mb in size (b) Oligonucleotide aCGH. All of the data points at zero indicate

normal dosage of two copies. The *blue* data points and shading indicate a loss of chromosome region 10q21.2q21.3, ~5.4 Mb in size. (c) SNP array plot shows the B-allele frequencies. Deletions show either A or B alleles, but no AB alleles, as indicated by the gap in the middle of the plot (*arrow*). (d) SNP array plot shows the dosage of the SNPs on chromosome 10. The depression in the probes over 10q21.1q21.2 indicates deletion and is ~6.4 Mb in size. Microarray analysis was performed at Signature Genomic Laboratories (Spokane, WA). BAC and oligo results were visualized using Genoglyphix (Signature Genomic Laboratories, Spokane, WA, USA)

because ploidy changes and clonal evolution can make the interpretation of arrays challenging, the ability to sort out the various alleles (sometimes referred to as the B-allele frequency) can aid in the identification of complex karyotypes that involve hyper- or hypodiploidy.

Clinical Applications of Microarray Analysis

The Use of Microarrays to Detect Cytogenetic Abnormalities in Children

The genomic content of the microarray and the density of the location of the probes (resolution) will determine the array's

ability to detect copy number changes. The first genomic microarrays were constructed from BACs and were targeted to known chromosomal syndromes, the subtelomeric regions, which are known to be involved in deletion and unbalanced translocations, and the pericentromeric regions, which are usually retained in marker chromosomes [12]. In those early years, the samples received by the laboratory were usually from children whose phenotypes were suggestive of a chromosome abnormality (typically complex presentations, with multiple systems involvement), yet no chromosomal diagnosis had been established through karyotyping. Thus, even with these fairly limited, targeted arrays, the detection rate of chromosomal abnormalities was quite high, ~6% above that detected by chromosome analysis [12, 13]. Whole genome

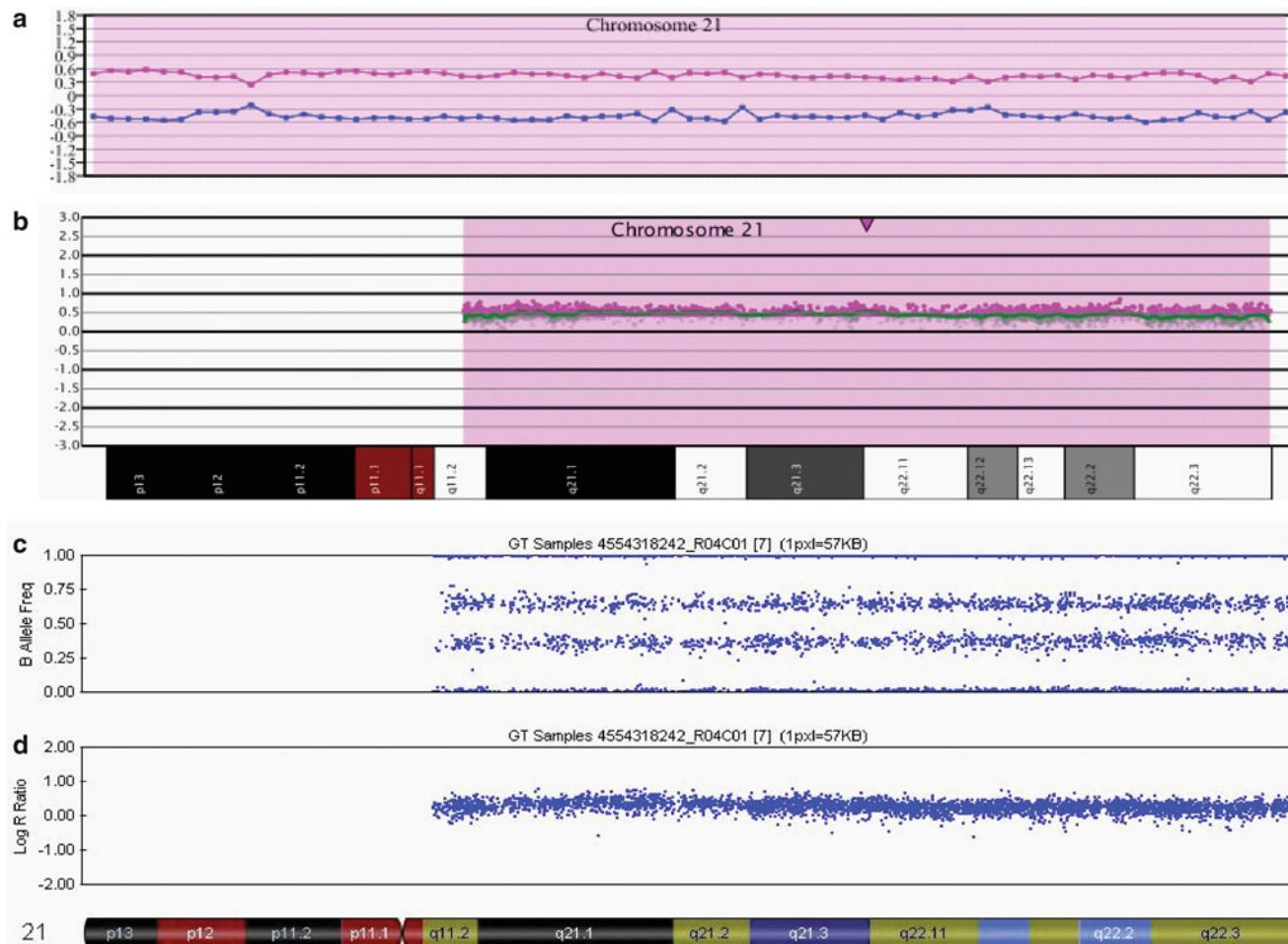


Fig. 18.3 Three examples of trisomy 21. In each, probes are ordered on the x-axis according to physical-mapping positions, with the proximal long arm oriented to the left and the distal long arm to the right. The \log_2 ratio of the patient-labeled DNA fluorescence to the control-labeled DNA fluorescence is shown on the y-axis. A \log_2 ratio greater than zero indicates a gain. (a) Bacterial artificial chromosome (BAC) array. Two experiments were performed, one shown as a *pink line* (patient:control) and one shown as a *blue line* (control:patient). The entire chromosome 21 shows a gain which is evident by the separation of the two experiments

and the *pink* shading. (b) Oligonucleotide array. The *pink* data points and shading indicate a gain of chromosome 21. (c) SNP array. The plot shows the B-allele frequencies for AAA, AAB, ABB, and BBB alleles, indicating a trisomy. (d) SNP array. The plot shows the dosage of SNPs on chromosome 21 with a \log_2 ratio of greater than zero indicating a gain across all probes. Microarray analysis was performed at Signature Genomic Laboratories (Spokane, WA). BAC and oligo results were visualized using Genoglyphix (Signature Genomic Laboratories, Spokane, WA, USA)

BAC and oligonucleotide arrays, with more comprehensive coverage of the genome, proved to have much higher detection rates (~15–20%) than the targeted arrays (reviewed in [14]), although the detection of results of unclear clinical significance also increased dramatically [15] (see section “[Interpretation of Microarray Data](#)”). Recently, physicians and diagnosticians recommended that microarray analysis be the first-tier test for children suspected of having a chromosome disorder [14, 16]. Although microarrays cannot detect balanced rearrangements because there is no net gain or loss of DNA, the ability of arrays to detect other abnormalities that can be seen through the light microscope and those alterations that are submicroscopic, and the comprehensive nature of a whole genome assay, makes microar-

rays an attractive alternative to karyotyping. Thus, microarrays can detect the copy number gains and losses associated with deletions, duplications, and amplifications; unbalanced translocations and insertions; and marker and ring chromosomes. In addition to the clinical utility, there are technical advantages to microarray testing. For example, microarrays do not require tissue culture; rather, they can use DNA extracted directly from tissues, making this assay substantially faster than karyotyping.

The resolution of the array is determined by the number of targets and the genomic coverage or density of probes within and between the targets. The clinical utility of the array is determined by the specific genomic coverage and the potential pathogenicity of the particular loci targeted. Gains

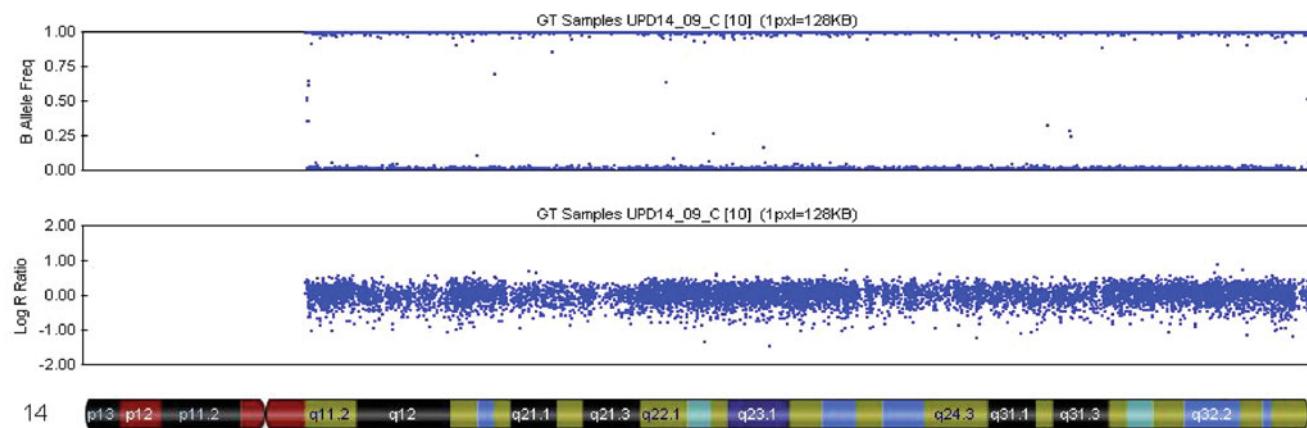


Fig. 18.4 An example of uniparental isodisomy for chromosome 14 using a SNP array. The upper panel shows the B-allele frequencies. Complete isodisomy (homozygosity) shows either A or B alleles, but no AB alleles,

as indicated by the large gap in the middle of the plot. The lower panel shows normal dosage of SNPs on chromosome 14. Microarray analysis was performed at Signature Genomic Laboratories (Spokane, WA)

and losses will be detected wherever there is a sequence represented on the microarray. Thus, while microarrays detect copy gains and losses, they cannot discriminate between potentially pathogenic loci and segmental duplications, which can be found throughout the genome of normal individuals. Consequently, if a repetitive region, such as a segmental duplication, is represented on the array, gains and losses of this region can be detected and potentially misinterpreted. Therefore, arrays for clinical use should be designed specifically for particular applications by individuals knowledgeable in the intended use and interpreted by experts in cytogenetics.

The Use of Microarrays to Detect Fetal Chromosome Anomalies

Microarray analysis has been used to detect chromosome anomalies in products of conception, terminated pregnancies, and ongoing pregnancies. Some of the first studies utilized products of conception to demonstrate the power of microarray testing, whereas other studies focused on retrospective analysis of terminated pregnancies with abnormal ultrasound findings [17–22]. These studies showed abnormalities detected by microarray analysis in about 8–16% of cases.

More recently, microarray analysis has been incorporated into the diagnostic evaluation of fetuses in the prenatal setting. Prenatal microarray testing can be performed on cultured and direct specimens from both amniotic fluid and chorionic villus sampling (CVS). Several prospective studies have demonstrated the diagnostic yield of chromosome abnormalities to range from about one to approximately 9% [23–29]. The reported detection rates varied considerably depending on the genomic coverage of the array being used, whether the authors

included known chromosome abnormalities in their prospective study, and the selection of fetal samples, with the highest yield in those fetuses with abnormal ultrasound findings in these limited studies (reviewed in [26]). However, to date, less than 1,000 prospective prenatal specimens tested by microarray have been reported in the literature. The adoption of array testing in the prenatal setting has been slower than that seen for postnatal testing. Recently, the American College of Obstetrics and Gynecology released a committee opinion for aCGH, endorsing the use of arrays in pregnancies with abnormal ultrasound findings [30]. This endorsement may raise awareness among obstetricians and result in an increased usage of microarrays for prenatal testing (see also Chap. 12).

The Use of Microarrays in Oncology

In cancer and related disorders, identifying chromosome and chromosomal region gains and losses is important for disease detection and classification, providing prognostic information and assessing disease progression, and can guide therapeutic decisions [31–33].

Microarrays are well suited to provide this information, as they readily detect copy number alterations. Copy number assessment has been achieved for many hematologic malignancies, including chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), myelodysplastic syndromes (MDS), plasma cell myeloma, follicular lymphoma, polycythemia vera, acute lymphoblastic leukemia (ALL) in childhood, and adult ALL [31, 32, 34–55]. In all studies, the number of chromosomal aberrations identified was significantly increased beyond that found with routine cytogenetics. This is especially evident in cases with normal karyotypes and submicroscopic alterations found by microarray analysis [41, 52].

Microarrays have also been used to identify copy gains and losses in solid tumors, including medulloblastoma, meningioma, breast cancer, gastric cancers, and renal cell carcinoma [56–62]. Given that many solid tumors show chromosome aberrations, arrays will continue to have an impact on the diagnosis. However, because solid tumors are quite often fixed by embedding in paraffin, the extraction of sufficient quality and quantity of DNA for microarray analysis is challenging [63].

As discussed throughout this chapter, microarrays are designed to detect genomic gains and losses; balanced translocations cannot be routinely detected using either aCGH or SNP-based arrays. A modification of aCGH analysis, called translocation-CGH, provides a new approach to the detection of prognostically important translocations in neoplastic disorders [64, 65]. In this analysis, a linear amplification is performed over the translocation breakpoint using primers in the vicinity of the junction. After labeling the reaction for an aCGH experiment and hybridizing to a microarray with the proper content over the translocation breakpoints, a peak can be visualized at the site of the translocation breakpoint (Fig. 18.5). In theory, by using one of the known or potential partner chromosomes, the recipient chromosome involved in the translocation can be identified. Thus, a large pool of primers might be used in a multiplex assay to identify balanced translocations and stratify patients into specific diagnoses based on the rearrangements identified.

Interpretation of Microarray Data

The ability of microarrays to interrogate thousands of loci simultaneously has changed the practice of medical genetics, and although some copy number alterations can be clearly classified as pathogenic while others can be classified as benign, some loci are now known to confer susceptibility to some abnormal phenotypes, while others remain of unclear clinical relevance. Copy number variations (CNVs) across the genome have been demonstrated in normal individuals and in some cases likely represent normal (benign) population variation [66–68]. Cytogeneticists have been aware of genomic variation for decades, often encountering heteromorphic acrocentric short arms and staining variability of certain pericentromeric regions. However, the identification of multiple small genomic gains and losses in the normal population has drawn much attention. These benign variants are identified in both patient and control populations in roughly equal frequencies.

There are other regions of the genome that appear to confer susceptibility to certain phenotypes [68, 69]. These susceptibility loci can be carried by an apparently unaffected parent, are enriched in patient populations, and have a relatively low frequency in control populations. However, in some individuals who carry these copy number changes, abnormal phenotypes result. Recent examples include

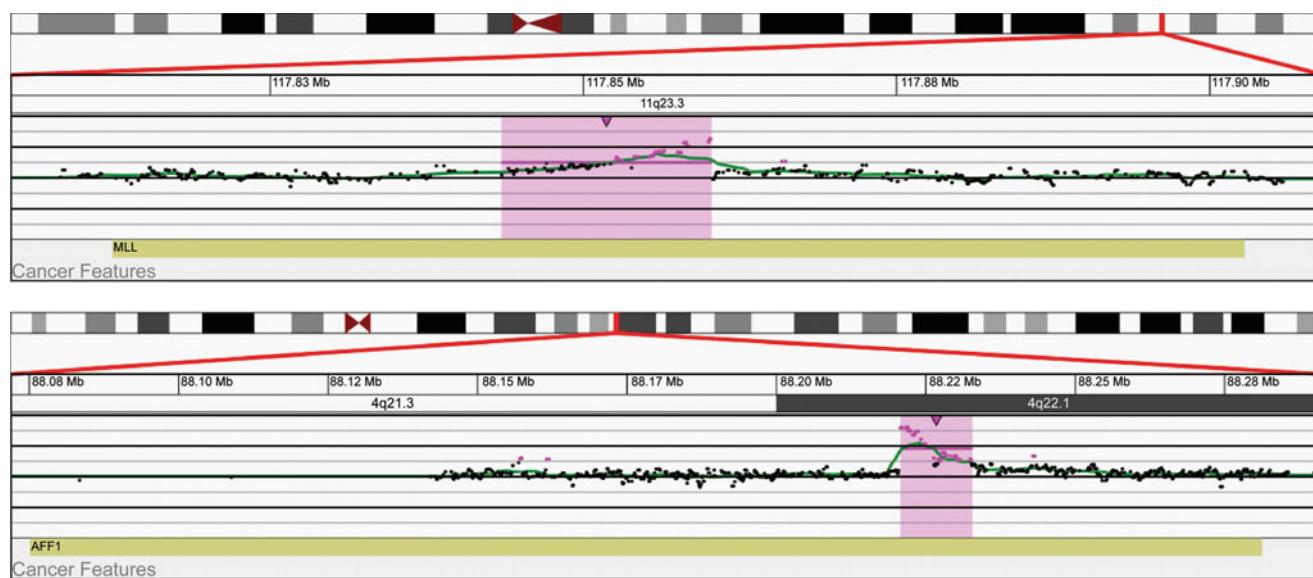


Fig. 18.5 Microarray results showing a translocation between chromosomes 4 and 11. Probes are ordered on the x-axis according to physical mapping positions. **(a)** Zoomed-in view of chromosome 11 plot at 11q23.3 showing the proximal 11q23.3 probes on the left and the more distal 11q23.3 probes on the right. The software displays a number of features including the Cancer Features track (gold) showing the genes in the region, including *MLL*. The pink shading indicates the

translocation breakpoint. **(b)** Zoomed-in view of the breakpoint area of 4q22.1 and the *AFF1* gene. The Cancer Features track (gold) in the region shows the inclusion of the *AFF1* gene. The pink shading indicates the breakpoint in *AFF1*. Microarray analysis was performed at Signature Genomic Laboratories (Spokane, WA), and results were visualized using Oncoglyphix™ (Signature Genomic Laboratories, Spokane, WA, USA).

deletions and duplications of 1q21, deletions and duplications of 16p11.2, and deletions of 16p13.1 [68–76].

In addition, CNVs of unclear clinical significance are often found in patient populations that undergo microarray analysis [28, 77]. These alterations are considered unclear because of their very low frequency in patient populations, inheritance from a clinically normal parent, and absence in control populations. The interpretation of these CNVs as causative to the patient's phenotype is challenging, and the study of parents is often not helpful because a rare, novel change, inherited from a normal parent, may represent a susceptibility locus, and this possibility cannot be excluded without further population studies. Even the finding of a small, *de novo* change in a patient's DNA may not indicate a causal relationship between the alteration and the phenotype; however, it is generally accepted that *de novo* changes are more likely to be causative than inherited CNVs. Even with these challenges in interpretation, most CNAs have clear clinical implications, and those that are determined to be pathogenic do provide answers to families seeking the reason for their child's medical problems.

It is well worth the arduous endeavor of trying to interpret these rare copy number changes because identifying the genetic etiology of disease allows for accurate genetic counseling, reproductive management, and anticipation of potentially serious medical problems in the child [78]. Databases are being established that may aid in the interpretation of these alterations. Some examples include: DECIPHER,¹ the Database of Genomic Variants,² the database at the Children's Hospital of Philadelphia, the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA),³ and the Signature Genomics Genoglyphix⁴ Chromosome Aberration Database [79, 80].⁴

Challenges with, and Advantages of, Microarray Testing

Laboratory Challenges with Microarray Testing

The choice of array used may impact the information gained. Therefore, the first challenge for the diagnostician is choosing the right array. The microarray should be designed with a specific use in mind—including targets to particular regions of the genome. For example, in cancer, BAC arrays may not detect small, single gene deletions; this usually requires higher density and smaller targets and can be achieved by using oligonucleotide-based CGH or SNP arrays [52]. Because balanced translocations cannot be routinely

identified with microarrays, the addition of FISH may increase the detection of additional cytogenetic changes, especially in leukemia and lymphoma in which certain translocations are the hallmark of the disease.

Hyperdiploidy, hypodiploidy, clonal evolution, and other mechanisms leading to complex karyotypes may be difficult to identify or interpret with microarray testing. The reason for this is that a mixture of complex genomic changes, multiple clones, will all be extracted together into a single sample and may produce a complex pattern on the array that may not be interpretable. Therefore, the use of chromosome analysis and FISH may be more appropriate for highly complex cancer genomes.

In addition to complex karyotypes, microarrays may not be sensitive enough to detect low-level clonal changes. Thus, changes in a very minor clone may not be apparent after microarray analysis. In this respect, minimal residual disease should not be assessed using microarrays but rather should be evaluated using FISH or PCR (if possible) or cytogenetics to identify the presence of a low-level clone. At initial disease presentation or relapse, microarray analysis is useful for identifying genomic changes characteristic for a particular patient. These changes can be monitored using FISH or PCR during treatment or remission.

Quite often in cancer diagnosis, chromosomal changes are observed, but the banding pattern is not sufficient to identify the chromosomal origin of the structural change. Microarray analysis is very useful for the identification of the chromosomal origin of derivative chromosomes, additional chromosomal material, and marker chromosomes. The challenge with marker chromosomes is that the presence of multiple marker chromosomes in a cell does not necessarily mean that they were all derived from a common chromosome source. Thus, the microarray pattern may be complex, which may indicate multiple origins or evolution of the markers. In addition, the presence of a marker chromosome in a minority of cells may not be identified by microarray analysis due to loss of sensitivity at levels below a certain threshold, typically less than ~20% of cells with that particular clone.

Advantages of Microarray Testing

The clear advantage of microarray analysis over conventional cytogenetics is the high resolution and genomic coverage that will uncover chromosome aberrations at detection rates of 15–20% in children with intellectual and developmental disabilities. In comparison to karyotyping, which will detect a chromosome abnormality in about 3–4% of children with global developmental delay, an additional 6.4% of children with global delays had a pathogenic copy number alteration by microarray analysis [81, 82]. There are other advantages in addition to the higher detection rate for chromosome abnormalities.

¹<http://decipher.sanger.ac.uk/>

²<http://projects.tcag.ca/variation/>

³<http://agsserver01.azn.nl:8080/ecaruca/ecaruca.jsp>

⁴<http://www.genoglyphix.com>

The turnaround times can be exceptionally fast. The rate-limiting step is the hybridization, which is usually 24–48 h with most arrays. Thus, if the DNA from the sample is extracted on day one and the DNA is labeled and hybridized to the array on the same day, the array can be washed, analyzed, interpreted, and reported on the morning of day three, slightly more than 48 h from receipt of the specimen.

Culture failures are unfortunately quite common in the laboratory with tissues from products of conception and peripheral blood samples or bone marrow aspirates for cancer diagnosis. Because DNA can be extracted directly from the tissue, arrays can be useful for identifying genomic changes in cases of culture failure. Although copy gains and losses can be readily detected, the lack of cultured cells will hinder the ability to confirm array findings and visualize the rearrangements by performing FISH to metaphase chromosomes.

Summary

Cytogenetics laboratories have a number of tools that can be used to detect chromosome abnormalities. No longer is the cytogeneticist restricted to peering through the microscope. Today, the well-tooled laboratory has the ability to use microarrays to identify genomic alterations. Copy number detection can be achieved with either an SNP-based or CGH-based microarray assay. In either case, gains and losses of individual chromosomes or genomic regions are readily identified. Both approaches can provide accurate and rapid diagnoses, and the DNA can be extracted directly from samples without necessitating cell culture prior to extraction. Appropriate samples for study are the same as those used for chromosome analysis, including peripheral blood, amniotic fluid, chorionic villus samples, products of conception, bone marrow aspirates, lymph nodes or solid tumors, or skin or other tissue biopsies, depending on the suspected disorder or tissue involved. In addition, paraffin-embedded or formalin-fixed tissues are rich sources of archived specimens for research or a stabilized resource for clinical assessment.

As the resolution of arrays increases, computational tools must be developed to handle the additional information, and databases must be developed and maintained for reference. The excitement of making a diagnosis in nearly a quarter of cases submitted to the cytogenetics laboratory is somewhat quelled by the overwhelming task of sifting through, perhaps, hundreds of benign and unclear CNVs, depending on the array used. Computer software must be developed and utilized to store the data and allow it to be easily retrieved and re-reviewed as knowledge of the genome matures. Nonetheless, microarrays have had a significant positive impact on the practice of medical genetics and have enabled the identification of chromosome abnormalities in individuals who would have otherwise gone undiagnosed.

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Part VI

Beyond Chromosomes

Fragile X—A Family of Disorders: Changing Phenotype and Molecular Genetics

Elaine B. Spector

Genetics of Fragile X Syndrome, Prior to the Availability of Molecular Analysis

X-Linked Mental Retardation

In 1938, Penrose noted a higher incidence of mental retardation (MR) in males and reports of families with only affected males [1]. These observations were compatible with X-linked inheritance, and numerous reports appeared in the literature [2]. Based on this early work, a clinically nonspecific X-linked MR disorder was delineated and called Renpenning's syndrome, Martin-Bell syndrome, or nonspecific X-linked MR. In 1959, Lubs described the first family with cytogenetic expression of the "marker X," which became the fragile X (fraX), and the heterogeneity of this nonspecific X-linked MR disorder became apparent [3]. Numerous disorders have been delineated from this original subgroup of MR males. The fraX subgroup was unique because there was a diagnostic laboratory test; the name Martin-Bell syndrome was attached when this family, first described in 1943, was shown to be positive for the fraX [4, 5]. However, the popular name for this disorder became fragile X syndrome (FXS).

Inheritance of fraX

Soon after the cytogenetic test became available, it became apparent that the inheritance pattern and penetrance of FXS were unlike those of any previously described X-linked disorder, although it came closest to an X-linked dominant with reduced penetrance. It was determined that some males who inherited the fraX were clinically normal, but passed the disorder to their normal daughters and frequently had affected

grandchildren, usually grandsons. Sherman, et al. observed that the mothers of such males are much less likely to have affected offspring than are their unaffected daughters [6, 7]. This became known as the Sherman paradox. The term "transmitting male" (TM) was coined to describe such unaffected carrier males. These TMs were thought to be the "missing" 20% of affected males described by Sherman et al. from 206 fraX families. Other unusual features of FXS are that TMs have fewer mentally retarded daughters than do unaffected carrier females, affected females occur more frequently (about one of three) than in other X-linked disorders, and affected females have more affected offspring than do unaffected carrier females.

FXS is now known to be the most prevalent cause of inheritable mental retardation, often presenting as an autism spectrum disorder, with a frequency of approximately one in 4,000 males and one in 6,000 females (see also section, "[Epidemiology](#)").

Cytogenetic Expression of fraX

The fraX site (FRAXA) is located in band Xq27.3, one of six fragile sites located on the X chromosome (Table 19.1). It can be visualized in both solid stained and banded preparations (Fig.. 19.1). However, banded preparations are required because other fragile sites and lesions can mimic fraX [8–10]. Three other fragile sites have been found in bands Xq27–28: FRAXD, FRAXE, and FRAXF [11–13]. The latter two sites cannot be cytogenetically distinguished from fraX.

FraX is not a chromosome abnormality. It is a chromosomal "marker" that facilitated the diagnosis of FXS until better techniques were developed.

Cytogenetic Expression in Affected Males and Carrier Females

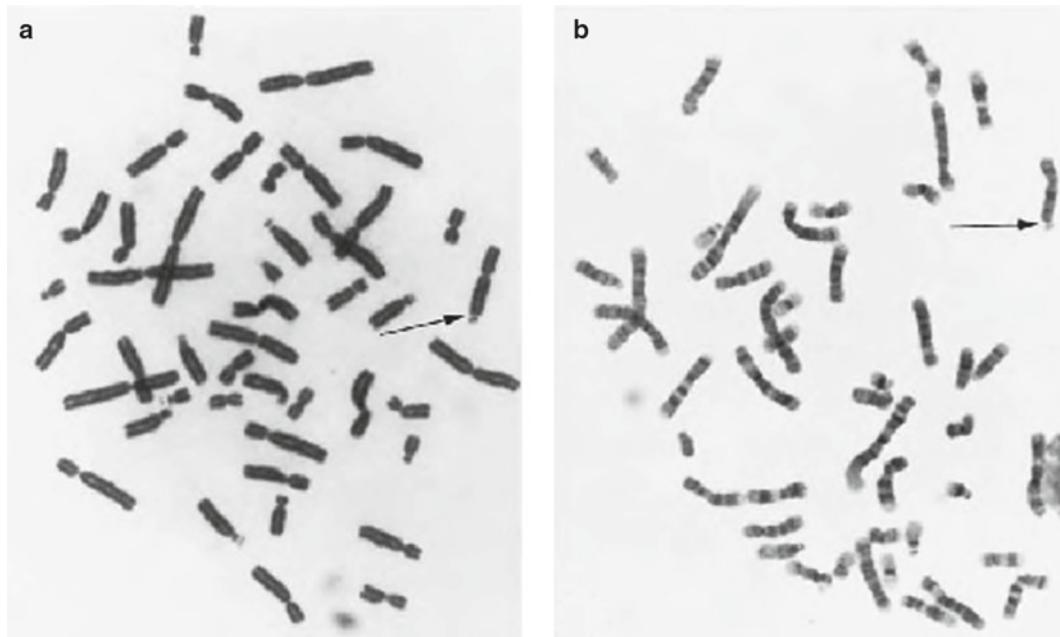
In affected males, fraX expression varied from less than 49% to 50%, with the low-expressing males comprising a minority of the diagnosed cases. However, this group represents the

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Table 19.1 Fragile sites on X chromosome

Name	Location	Type
FRAXA	Xq27.3	Rare
FRAXB	Xp22.31	Common
FRAXC	Xq22.1	Common
FRAXD	Xq27.3	Common
FRAXE	Xq28	Rare
FRAXF	Xq28	Rare

**Fig. 19.1** Appearance of FRAXA. (a) Conventional stain (Giemsa) and (b) GTG-banded. The arrow indicates the location of the fraX site

false-negative males diagnosable with molecular techniques. Why fraX does not express in >50% of metaphases is still not known. Cytogenetic testing of carrier (heterozygous) females was even more problematic. Among obligate carriers, only about 50% tested positive, and about one-third of these carriers were clearly affected to some degree. In general, fraX expression was easier to demonstrate (although lower than in males) in affected females than in those with normal intelligence. Guidelines were established for interpretation of these data [14, 15].

Prenatal Diagnosis

Prenatal testing was available on an experimental basis beginning in 1981 using cytogenetic techniques. Testing was done on fetal blood, amniocytes, or chorionic villus cells with varying degrees of success. False-negative males were reported with all three tissue types. In the United States, amniocentesis was the major procedure, while chorionic villus sampling (CVS) was the standard in Europe and

Australasia at this time. England had the major experience with fetal blood sampling. Worldwide experience with prenatal diagnosis by cytogenetic analysis or cytogenetic analysis plus DNA polymorphism analysis (see discussion later) exceeded 400 cases. The “state of the art” was summarized at the Fourth International Workshop on Fragile X and X-Linked Mental Retardation. Fortunately, with the identification of the molecular defect in FXS, prenatal diagnosis of the syndrome became much more accurate.

Molecular Aspects of Fragile X Syndrome

Analysis Using Linked Polymorphisms

From the mid-1980s through 1991, molecular (DNA) analysis using linked polymorphisms was used in confirmed fraX families to help with prenatal diagnosis and carrier status. Although the gene for FXS had not been identified,

its relative location on a linear map of the distal X long arm was known. Using genes and polymorphisms on both sides of fraX allowed molecular geneticists to track fraX chromosomes through families. The risks of inheriting the fraX chromosome were expressed as probabilities. Success with the method depended on the distance between the tested polymorphism/gene and the FXS gene, the size of the family, and which polymorphism/genes were informative. Regardless of these limitations, the combination of cytogenetic and linkage analysis allowed many families to receive more reliable results than with chromosome analysis alone.

Trinucleotide Repeats: Classification

The early 1990s marked the discovery of a new type of genetic mutation in humans: the trinucleotide or dynamic repeat. The mechanism causing the FXS mutation was first identified in 1991 and revealed that the mutation results from the expansion of a trinucleotide repeat located in or near an expressed sequence [16–19]. For the fragile X syndrome, the trinucleotide repeat is cytosine-guanine-guanine or CGG. This revelation was soon followed by the discovery that a similar mechanism causes myotonic dystrophy (DM) and spinocerebellar ataxia type 1 (SCA1). To date, more than 30 human diseases known to be associated with the expansion of a trinucleotide repeat [20–22].

Trinucleotide repeat disorders can be categorized in one of two ways: (1) according to the specific trinucleotide sequence or (2) according to the location of the expansion in relation to the coding sequence. Here, we have chosen to describe trinucleotide repeat disorders based on the position of the expansion in relation to the coding sequence. The repeats may be located in the 5' untranslated region, in an intron, in an exon, or in the 3' untranslated region. The list of disorders continues to grow. One characteristic of these disorders, that each generation shows an earlier age of onset and increasing severity, is known as *anticipation*. All the disorders are either X-linked or autosomal dominant except Friedreich ataxia, which is autosomal recessive [22].

The CGG trinucleotide repeats are located at folate-sensitive fragile sites, and their characteristics are summarized in Table 19.2 [23, 24]. Based on the trinucleotide repeat size in FRAXA, an individual's status can be classified as *normal* (5–44 CGG repeats), *indeterminate* or *gray zone* (45–54 CGG repeats), *premutation* (55–200 CGG repeats), or *full mutation* (>200 CGG repeats) [25]. An individual with a *normal* repeat size is characterized by stability of the repeat length and normal intelligence, while an individual with a *premutation* repeat size shows instability of the repeat length from generation to generation but normal intelligence. In contrast, *full mutation*

Table 19.2 Classification of trinucleotide repeat diseases

Class	n	Repeat	Position of Repeat	Examples
1	3	CGG	5' Untranslated region	FXS
				FRAXE syndrome
1	CAG			Spinocerebellar ataxia type 12 (SCA12)
2	2	CTG	3' Untranslated region	Myotonic dystrophy (DM)
3	8	CAG	Inside coding region	Huntington's disease (HD) Spinocerebellar ataxia type 1 (SCA1) Kennedy's disease
4	1	GAA	In first intron	Friedreich Ataxia

individuals have massive repeat sizes differing in lengths (“mosaic”) in a pattern that is often conserved across tissues, resulting in FXS.

Instability of the CGG Repeat

Through observational studies of families with FXS, several factors involved in CGG repeat instability have been proposed, including the sex of the transmitting parent, the size and structure of the CGG repeat, and other yet-to-be-identified factors. With the resolution of the Sherman paradox, it is now known that a premutation-sized repeat has the propensity to expand when passed through a female germ line, and the size of the resulting expansion is positively correlated with the maternal repeat size [26–30]. In contrast, when passed through a male germ line, the premutation does not dramatically change in repeat size and often remains the same or even contracts [29–31].

In addition to the sex of the transmitting parent, the size and structure of the CGG repeat play a role in instability. Sequencing of the CGG repeat revealed that the repeat is not pure and is interspersed with one to three AGGs (adenine-guanine-guanine sequences) every 9–10 CGGs in the general population. Among families with FXS, premutation-sized repeats usually have one AGG at the most proximal end of the repeat or none at all [32–34]. Transmission studies of families with premutation- or intermediate-sized repeats demonstrate that these are unstable if >34 repeats at the 3' end of the repeat structure are uninterrupted by an AGG [29, 32, 34]. To date, all known expansions have occurred at the 3' end of the repeat. This polarity of expansion further demonstrates the importance of the 3' end of the repeat in the expansion process. While the role of the AGG interruption has only been minimally defined by experimental studies, these observational and population studies suggest that the AGG sequence acts as an anchor during DNA replication to prevent expansions or deletions that are the result of slips or misalignments of the repeat sequence during replication

[35–38]. Despite the identification of these factors, the exact mechanism of the formation of the repeat expansion has not been completely elucidated.

Many models have been proposed to explain the expansion of trinucleotide repeats. One of the first proposed mechanisms involved in repeat instability at the molecular level was slippage of the replication fork during DNA synthesis. Unpaired bases form loops, which result in expansions or contractions in the next round of replication, depending on whether the looped repeats are located in the newly synthesized or template strand [39]. However, slippage alone cannot explain all aspects of repeat expansions, especially large expansions and contractions. It is clear that other yet-to-be-identified factors are involved in the expansion process. Experimental support has come from studying a yeast model deficient in *rad27* [40]. The Rad27 protein is involved in removing DNA loops, such as those arising during displacement synthesis of the Okazaki fragments. Propagation of a CGG repeat in *rad27* null yeast results in a highly significant increase of repeat expansions [40]. The human homolog of this gene is *FEN1*.

Recognition of the unusual structural properties of trinucleotide repeats yielded new insights. Disease-causing repeats are almost exclusively formed by (CNG)n triplets. Single-stranded (CNG)n can form hairpin-like structures that can include both Watson-Crick and mismatched base pairs. Due to their different sequences, the leading and lagging strands have different tendencies to form hairpins. The secondary structures are likely to affect recognition and subsequent repair or recombination of the structure [41, 42]. Unusual DNA structures may stall DNA polymerase, leading to instability. A complex model based on replication fork stalling and restarting has been described in detail [43].

Unlike other trinucleotide repeat disorders, there is absence of repeat instability in somatic cells in FXS. Methylation may stabilize the CGG repeats in full mutations (see section, “Epigenetic Changes in the *FMR1* Gene”). Repeat expansion from pre- to full mutation occurs exclusively in females due to sex-specific factors. It has long been known that sperm from full-mutation male patients possess only premutation alleles [44]. This must result from reduction of repeat instability occurring during a limited time in early development. Both prezygotic and postzygotic models, directly after separation of the germ cells, have been proposed [45]. Material is not available from premutation females to support either hypothesis.

The Fragile X Gene and Its Product: *FMR1* and *FMRP*

In 1991, the responsible gene was identified by positional cloning and named the fragile X mental retardation-1 (*FMR1*) gene [17–19]. *FMR1* encompasses 38 kb of Xq27.3 and consists of

17 exons [46]. The polymorphic CGG repeat exists in the 5' untranslated region (UTR) of *FMR1*. Among the general population, the CGG repeat ranges from 6 to 55 repeats and usually the size does not change in size when passed from parent to offspring [26]. The most common forms of the repeat sizes found in human populations studied are 21 and 28–30 CGG repeats [47–50]. Although the CGG repeat has no known function, it is found in all species of mammals investigated [51, 52].

The common CGG-repeat sizes have not proven to be associated with a disease phenotype; however, the consequence of an expanded CGG repeat (>200 repeats) in *FMR1* is the fragile X syndrome. The hyperexpanded CGG repeat signals the hypermethylation and deacetylation of the *FMR1* promoter, the CGG repeat, and a nearby CpG island, which transcriptionally silences the gene [17, 53–56]. Recent *in vitro* experiments demonstrated that it is methylation and chromatic modification triggered by the expansion that are responsible for the transcriptional silencing of *FMR1* rather than the CGG repeat expansion itself [57, 58] (see also section, “Epigenetic Changes in the *FMR1* Gene”).

Because FXS is essentially caused by the loss of the *FMR1* gene product, there is much interest in gathering information on the normal expression patterns of the gene and its product’s function for the development of interventions or therapies. The *FMR1* transcript is approximately 4.4 kb in size and is alternatively spliced at the 3' end, giving rise to various isoforms [46, 59]. Expression studies in human and mouse tissues demonstrated that *FMR1* is widely expressed, with the highest levels localized to the brain, testes, ovaries, esophageal epithelium, thymus, spleen, and eye [60–62]. High expression of *FMR1* in regions of the brain such as the neurons of the hippocampus and the granular layer of the cerebellum is consistent with the mental retardation phenotype typical of FXS [63, 64] (see also section, “Clinical Aspects of Fragile X Syndrome”). Identification of other mutations (e.g., deletions and point mutations in patients with FXS) has confirmed that *FMR1* is the only gene involved in the pathogenesis of the disorder and that the loss of the *FMR1* product causes the syndrome.

A search for genes similar to *FMR1* within the human genome found two identified autosomal homologs, fragile X-related (FXR) genes 1 and 2, located at 3q28 and 17p13.1, respectively [65, 66]. Analysis of mouse and human genomic sequences demonstrates similarities in gene structure among *FMR1*, *FXR1*, and *FXR2*, suggesting that the three genes have an ancestral gene in common [67]. The functions of *FXR1* and *FXR2* are presently unclear; neither gene has been shown to be associated with human disease. Many investigators have postulated that, because of their similarity to *FMR1*, the FXR genes are somewhat redundant, but although there are similarities, significant differences have been noted [68]. Furthermore, *FXR1* and *FXR2* are not overexpressed in cells from persons with

FXS, suggesting that neither gene product compensates for the loss of the *FMR1* gene product [69, 70].

The full-length protein product of *FMR1* is 69 kilodaltons in size and is known as the fragile X mental retardation protein, or FMRP [71]. At the protein level, FMRP is highly conserved across humans, mice, the African clawed frog (*Xenopus laevis*), and chickens [18, 59, 72, 73]. Although not as highly conserved as among vertebrates, a homolog for the *FMR1* coding sequence has also been identified in *Drosophila melanogaster* [74].

Much has been accomplished in elucidating the function of FMRP and how its absence leads to the development of the FXS phenotype. Several properties of FMRP were the first clues to its function. First, FMRP contains two ribonucleoprotein K homology domains (KH domains) and clusters of arginine and glycine residues (RGG boxes), features typical of RNA-binding proteins [71, 75]. Second, FMRP contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) [76]. Two coiled coils and a G-quartet-binding structure have been identified (Fig. 19.2) [77]. FMRP is primarily a cytosolic protein, but its presence in the nucleus has been reported by nuclear staining experiments [63, 78]. Furthermore, FMRP has been detected in the nuclear pore [79]. Taken together, current evidence suggests that FMRP shuttles between the nucleus and the cytoplasm affecting protein synthesis in dendrites (dendritic spines) and synapses [76, 80–82].

Dendritic spines are small membranous extensions on neuronal dendrites [83]. They serve as synaptic storage sites,

support the electric signal transmission, and increase the number of possible contacts between neurons [84].

On their surface, the dendritic spines express glutamate receptors (GluR) of two types: the ionotropic receptors alpha-amino-3-hydroxy-*t*-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D aspartic acid (NMDA), and the metabotropic receptors (mGluRs). A broad variety of proteins mediate the signaling from the GluRs [85].

Cognitive function, motivation, learning, and memory are based on spine plasticity. After the formation of numerous dendritic spines during fetal cortical neurogenesis, the spines need to mature or be pruned; immature spines show a significant impairment in signal transduction. Abnormalities in spine formation have been observed in FXS, which can be directly correlated to the cognitive impairment. It has been postulated that the loss of FMRP in FXS leads to an excessive expression of mRNA near synapses, making it impossible to regulate protein synthesis adequately, thus increasing long-term depression due to receptor loss. FMRP is considered to be a repressor of specific mRNA translation and numerous proteins are upregulated, particularly in the hippocampus, when FMRP is absent [86].

FMRP colocalizes primarily with polyribosomes and ribosomes at/in the endoplasmic reticulum membrane and appears to play an important role in the regulation of translation of specific target mRNAs. A subset of mRNAs containing a G-quartet (a nucleic acid structure in which four guanine residues are arranged in a planar configuration) has been identified that are potential targets for FMRP, including

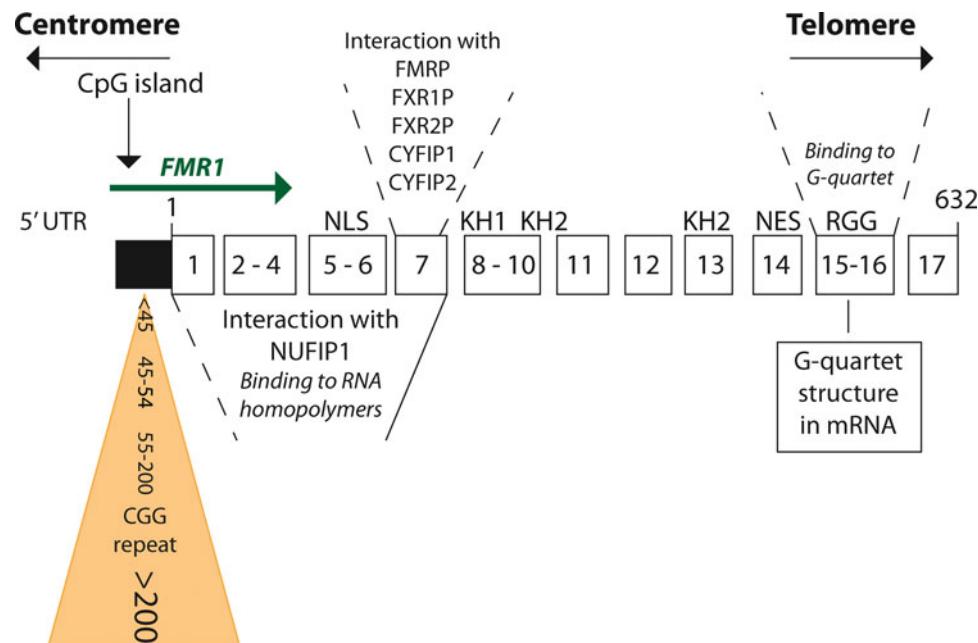


Fig. 19.2 The *FMR1* gene-coding exons (numbered boxes) and protein domains. *NLS* nuclear localization signal, *KH1/KH2* RNA-binding domains, *NES* nuclear export signal, *RGG* RGG box RNA binding. The triangle indicates the untranslated CGG repeat alleles, <45=normal range, 45–54=gray zone, 55–200=fragile X premutation, >200=fragile X full mutation (Modified from Schneider et al. [77]; reprinted with permission)

cates the untranslated CGG repeat alleles, <45=normal range, 45–54=gray zone, 55–200=fragile X premutation, >200=fragile X full mutation (Modified from Schneider et al. [77]; reprinted with permission)

important neuronal proteins like microtubule-associated protein 1B (MAP1B) and semaphorin 3F [87–89]. FMRP also can bind to mRNAs that do not contain a G-quartet.

In addition, experimental evidence suggests that FMRP is involved in suppression of translational activities. FMRP forms complexes with messenger ribonuclear particles (mRNP) and is associated with translating ribosomes [76, 90, 91]. Because RNPs are formed in the nucleus, this observation further supports the hypothesis that FMRP shuttles between the nucleus and the cytoplasm. Recent experiments suggest that FMRP may play a role in regulation of translation for certain messages. Laggerbauer et al. demonstrated that FMRP suppresses translation by preventing the assembly of the 80S subunit of the ribosome on the target RNAs [92]. New evidence suggests that translational control may be mediated through the RNA interference (RNAi) and/or microRNA (miRNA) pathways [93, 94].

The two major activities identified for FMRP, cytoplasm-nucleus shuttling and translational regulation, imply that FMRP is a facilitator for the expression and localization of several messages and proteins. The search for FMRP's partners has identified at least seven such proteins, one of which includes FMRP itself [68]. In contrast, very few specific mRNAs that bind FMRP have been identified.

FMRP was shown to bind its own mRNA and also approximately 4% of fetal brain mRNAs [71]. Nearly a decade would pass before the identity of the specific mRNAs (other than the *FMR1* transcript) binding to FMRP would be identified [91–93]. These mRNAs contain a G-quartet structure that facilitates binding to FMRP. FMRP can be phosphorylated, a mechanism that possibly affects the binding of specific mRNAs [94].

The ability of FMRP to bind RNA and suppress translation has definite clinical relevance. As an RNA-binding protein, FMRP is found to form a messenger ribonucleoprotein (mRNP) complex that associates with translating polyribosomes [95]. FMRP is also known to be involved in translational control and could suppress translation both *in vitro* and *in vivo* [86]. In 1987, Davis et al. demonstrated that the mRNAs were transported into dendrites of cultured hippocampal neurons [96]. Since then, a large number of dendritic localized mRNAs have been identified, and it is suggested that the translation of those mRNAs can be regulated in a spatially restricted manner in response to stimulation [97]. At the cellular level, abnormal dendritic spines are found in the brains of both human patients with FXS and *Fmr1* knockout (KO) mice, implying that synaptic plasticity is affected in the absence of FMRP. Based on these observations, it has been proposed that FMRP is involved in synaptic plasticity via regulation of mRNA transport and local protein synthesis of specific mRNAs at synapses. Transport and regulated translation of mRNAs in dendrites are important for neuronal function, including modulation of synaptic plasticity. This is essential in

memory consolidation and learning [84, 98–103]. Altered spine morphology (long and thin dendritic spines) has been observed in postmortem brains of fragile X patients and in *Fmr1* KO mice [84, 98–102]. The presence of the protein machinery near synaptic connections allows neurons to rapidly respond to signals at particular synapses through local translation of specific mRNAs in the vicinity of the synapse, and FMRP plays a crucial role. The response is mediated through the action of mGluR activation [77, 104, 105].

It has been proposed that FMRP located at the synapse represses translation of mRNAs encoding proteins that regulate endocytic events involving the AMPA receptor. Upon synaptic stimulation, FMRP may dissociate from these mRNA targets to allow translation and facilitation of AMPA receptor internalization. The model predicts that in the absence of FMRP, the upregulated translation of a subset of mRNAs would result in the perturbation of AMPA receptor internalization dynamics (Fig. 19.3) (see Oostra and Willemens [44] for further discussion).

Protein kinases are crucial for the regulation of neuronal development and synaptic transmission upon response to extracellular or intracellular signals. The mGluR theory is in line with the translation control pathways within the dendritic spines; a simplified version is depicted in Fig. 19.4 [44]. Strong evidence supports the postsynaptic FMRP signaling model. Data suggest that dephosphorylation of FMRP may regulate FMRP and that the release of FMRP-induced translational suppression may involve a dephosphorylation signal. Rapid dephosphorylation of FMRP allows target mRNAs to be translated, whereas rephosphorylation represses translation. Several proteins involved in this process have been identified [106, 107].

Further research is needed to characterize the cascade of signaling upon mGluR activation and the mechanism whereby FMRP phosphorylation regulates translation of target mRNAs.

Epigenetic Changes in the *FMR1* Gene

Methylation of the CGG repeat, which occurs in the promoter region of the *FMR1* gene, takes place early in embryonic development and is a dynamic process. In early germ cells from female full-mutation fetuses, the *FMR1* repeat is fully expanded and unmethylated [108]. In chorionic villus samples from full-mutation fetuses, the expanded repeat is methylated to an increasing degree as development progresses [109]. Almost all FXS patients carry a fully methylated full expansion.

Infrequently, individuals are identified who carry full mutations that are not methylated. These patients often do not show the full spectrum of the fragile X syndrome, demonstrating that methylation and not repeat elongation alone causes the phenotype [110, 111].

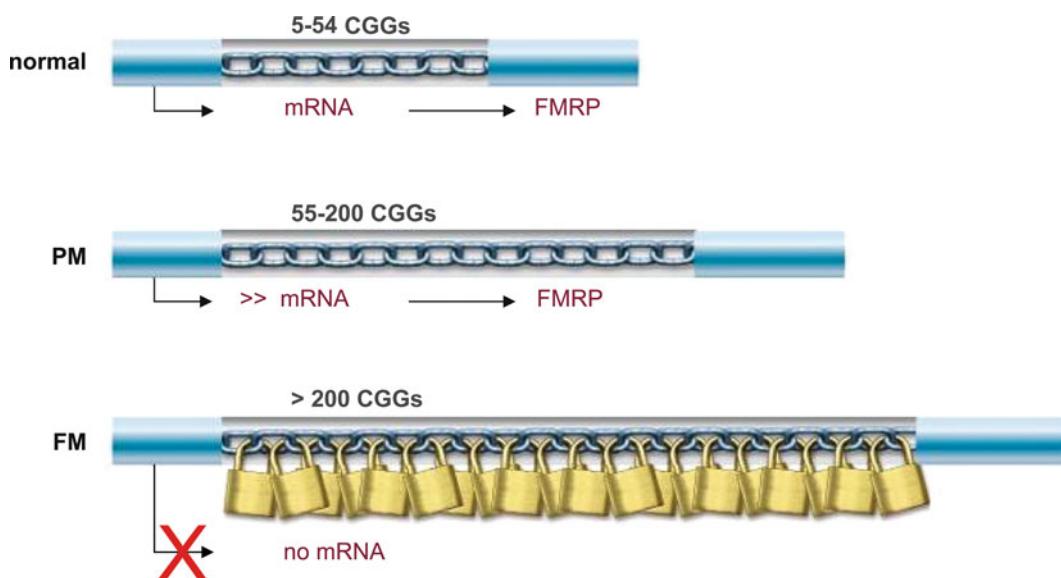


Fig. 19.3 The CGG repeat in the *FMR1* gene. Schematic representation of normal, PM (premutation), and FM (full mutation) alleles of the *FMR1* gene and the effect of the expansion on transcription and translation.

Methylation due to extensive elongation of the CGG repeat in the 5'-UTR of the *FMR1* gene is depicted as a lock (Modified from Oostra and Willemse [45]; reprinted with permission)

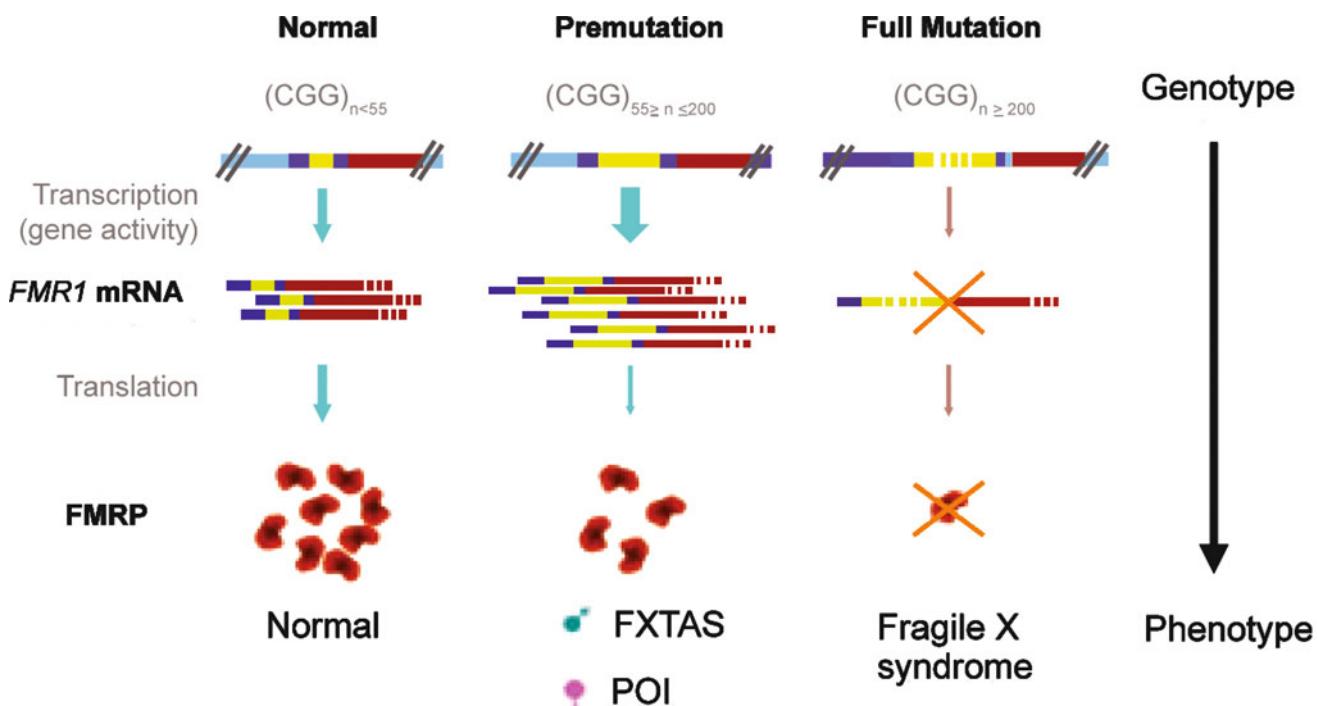


Fig. 19.4 The mGluR theory: in the absence of FMRP, as in fragile X syndrome, the balance between FMRP and Gp1 mGluRs is lost, and unchecked protein synthesis at the synapse leads to the characteristic

features of the disease (Reprinted with permission from Willemse R, Levenga J, Oostra BA. CGG repeat in the *FMR1* gene: size matters. Clin Genet. 2011;80(3):214–5).

FMR1-Associated Disorders

Allelic Forms of the *FMR1* Gene

There are four allelic forms of the gene: normal, intermediate, premutation, and affected. The associated numbers of CGGs for each are defined here; however, the cutoffs for

each allelic type have evolved over time and may change with increased empirical data and research.

- **Normal Alleles:** Normal alleles have a range of 5–44 repeats. The most common repeat lengths are 29 and 30 CGG repeats.
- **Intermediate (Gray Zone, Inconclusive, Borderline):** The range from 45 to 54 repeats is intermediate. Alleles in this range can be considered normal in the sense that such

alleles have not been observed to expand to a full mutation in a single generation. Moreover, there is no observed increased risk for the specific premutation-associated disorders, although data are limited.

- **Premutation:** Premutation alleles range from 55 to ~200 repeats. These alleles are long repeat tracks that are unstably transmitted from female parent to child. Premutations are not associated with somatic variation, are not hypermethylated, and are not associated with classic features of fragile X syndrome. Women with alleles in this range are at risk to have affected children, although all known mothers of affected children have alleles of 59 repeats or higher [112]. Female members of families with CGG repeats in this range benefit from genetic counseling and prenatal diagnosis.
- **Full Mutations:** Full mutations associated with the fragile X phenotype exhibit more than 200 CGG repeats and typically several hundred to several thousand repeats. There is usually broad somatic variation within each patient. Hypermethylation is typically present on most or all copies.

Clinical Aspects of Fragile X Syndrome

Several disorders are now associated with mutations in the *FMR1* gene and are reviewed as follows.

Full-Mutation Phenotypes

Physical Phenotype

In males, the classic features of FXS are X-linked mental retardation, macroorchidism, and minor dysmorphic facial features including a long, oblong face with a large mandible and large and/or prominent ears. Pectus excavatum, mitral valve prolapse, and strabismus have also been described in males with FXS. At least 80% of affected males have one or more of these features, but expression varies with age. Other frequent features are a high-arched palate, hyperextensible finger joints, velvet-like skin, and flat feet. Females with a full mutation may express these same features of FXS, depending upon X-inactivation status [22].

Behavioral Phenotype

The behavior of males with the FXS can be quite variable. They show distinct behavioral features in the areas of attention, hyperarousal, social function, anxiety, and aggression [113, 114]. Additionally, they are often diagnosed with autism because they exhibit poor eye contact, hand flapping, and social deficits that are the most prominent features of autism. They also exhibit various degrees of speech delay. Other complicating features can include irritability, hypo-

tonia, and perseveration in speech and behavior. Social anxiety and avoidance are prominent features of FXS in both sexes.

Hagerman reviewed in detail the physical and behavioral phenotype of FXS [113]. The variability of expression makes clinical diagnosis difficult. Therefore, FXS should be considered in the differential diagnosis of all mentally retarded individuals.

Cognitive Phenotype

In males, preliminary evidence suggests that there are specific deficits in arithmetic, visual motor, and spatial skills; short-term auditory, visual, and working memory; executive function; visuospatial-processing abilities; processing of sequential information; and sustained attention. Approximately 85% of males and 25–30% of females with the full mutation have an intelligence quotient (IQ) less than 70. The severity of intellectual impairment is related to FMRP deficiency [77, 114, 115]. Some individuals with only a mild decrease of FMRP may present with a normal or borderline IQ with or without learning disabilities (LD). LD with a normal or borderline IQ is a typical presentation in females with FXS. IQ decreases with age, although the reason for this longitudinal decline is unclear [116]. Adult males with FXS function within the moderate to severely retarded range. IQ is not correlated with the size of the CGG repeat. However, it does appear to be correlated with the mosaic status of the male. Affected males with both somatic full mutation and premutation size repeats or those who are methylation mosaics have higher IQs than the affected males who are nonmosaic or fully methylated. On occasion, such males will test in the normal/low normal range [117].

In females, with FXS cognitive studies indicate specific weaknesses in arithmetic as well as short-term auditory memory and visual-spatial tasks. They also have significant deficits in executive function. Full-mutation females have mean IQs in the low-average range (74–91), and, as in males, the IQ is not correlated with CGG-repeat size. Most studies have found a relationship between IQ and X-inactivation ratios.

Aging in FXS

There is an ever-increasing group of patients with fragile X syndrome. Until recently, there were no studies on the behavior and cognitive problems in aging for those with FXS. Utari et al. studied a group of individuals (44 males and 18 females) with the syndrome who were over 40 years of age [118]. The most frequent difficulties faced by these patients were neurological problems (38.7%), gastrointestinal problems (30.6%), obesity (29.8%), and heart problems (24.2%), which include mitral valve prolapse (MVP), cardiac conduction abnormalities, heart attack, and heart rhythm disorder. Males had a significantly higher percentage of neurologic problems compared with females. However, only movement disorders,

including Parkinson's disease, were significantly different in prevalence between males and females; some of these may be the result of long-term treatment with antipsychotics. An increase in seizures was observed in the older patients as well. It is not known if this represents a second peak in seizure onset or if seizures are a complication of aging.

Other Clinical Aspects

Several recent reviews explore the neurologic and pathologic findings in FXS and related disorders [77, 113, 119]. Medical follow-up, pharmacotherapy, treatment of emotional and behavioral problems, and intervention approaches for FXS have also been reviewed and are ever expanding [120].

Premutation Carrier Phenotypes

Unlike the full mutation, the existence of a phenotypic consequence of the premutation in males was controversial for some time. However, a specific phenotype has been identified and is referred to as fragile X tremor ataxia syndrome (FXTAS) [121]. Approximately 40% of older males with the premutation will eventually develop FXTAS. The features of this disorder include an intention tremor, ataxia, Parkinsonism, neuropathy, cognitive deficits (particularly executive function deficits with eventual cognitive decline to dementia in some), and autonomic dysfunction including hypertension, impotence, and eventual bladder and bowel incontinence [118, 122–126]. Although some patients with FXTAS have a rapid decline over 5–6 years, others are stable for a decade or two. More rapid decline typically occurs when the features of FXTAS are combined with another disorder. FXTAS occurs in approximately 8% of female carriers [127].

The neuroanatomical hallmark of FXTAS is intranuclear eosinophilic inclusions in neurons and astrocytes throughout the brain, with highest numbers in the hippocampus and limbic system [128]. These inclusions have also been found in Leydig and myotubular cells of the testicles and in peripheral nerve ganglia throughout the body [126]. They contain the excess mRNA and also a number of proteins including lamin A/C and MbP that are dysregulated by the elevated mRNA [129]. The inclusions are probably not pathognomonic; they are a marker for RNA toxicity.

While cognitive or behavioral deficits have not been definitively attributed to the premutation in males, a molecular phenotype related to this repeat size range has emerged. Early on, investigators examined levels of *FMR1* mRNA and FMRP from the lymphocytes of carriers of premutation alleles and found that the levels were not significantly different compared with controls [55, 63]. Recent changes in technology, however, have made measurements of *FMR1* mRNA more sensitive and accurate [130, 131]. Using this technology, Tassone et al. reexamined the levels of *FMR1* mRNA

and FMRP in premutation male carriers and found that carriers with 100–200 CGG repeats had a fivefold increase in *FMR1* mRNA levels while carriers with 55–100 repeats had a twofold increase compared with controls [132, 133]. Moreover, these high-end premutation carriers (100–200 repeats) had reduced levels of FMRP compared with controls [132]. Additional experiments suggest that the elevated level of *FMR1* mRNA is correlated with CGG-repeat size and is not simply a response to decreased levels of FMRP [133, 134].

An RNA gain of function mechanism has been suggested for FXTAS based on the aforementioned observation of elevated levels of CGG containing *FMR1* mRNA, along with either no detectable change in FMRP or slightly reduced FMRP levels, observed in peripheral blood leukocytes and brain regions [132, 134–138] of premutation carriers.

Many conflicting reports exist in the literature concerning cognitive, behavioral, and physical phenotypes among female premutation carriers; these reports have been reviewed [139, 140]. For reports on cognitive ability, studies of varying designs have shown that the prevalence of mental retardation, the range of cognitive ability, and the range of IQ scores among adult female premutation carriers did not differ compared with control groups [141, 142]. However, at least two studies have suggested differences among female carriers compared with controls in specific subsets of IQ scores. In terms of a behavioral phenotype related to the premutation, several studies suggest a difference based on specific behavioral or psychological measures among women with premutations compared with controls [123, 143]. However, many of these suggested differences were not replicated in other studies. Lastly, for physical or anthropometric measures, two studies suggest that female premutation carriers do not have the same facial dysmorphic features typically observed in patients with the full mutation, while two studies suggest otherwise [138, 139].

While the existence of a cognitive, behavioral, or physical phenotype among premutation females remains controversial, one consequence is consistently associated with the premutation: Fragile X primary ovarian insufficiency (FXPOI), referred to as premature ovarian insufficiency (POI) in older literature. FXPOI is defined as the cessation of menses before the age of 40 years. In contrast, the mean age of menopause in the general population is 51 years. The first reports of female carriers of the fragile X mutation having FXPOI were anecdotally noted at the first International Fragile X Conference (1987) in Denver, Colorado [144–149]. Schwartz et al. were the first to report an association between the fragile X premutation and POI in a multicenter study [145]. The relationship between the fragile X premutation and POI was eventually confirmed by a large, multicenter study, which demonstrated that 16% of premutation carriers experienced POI, while only 0.4% of noncarriers and none of the

full-mutation carriers experienced POI [146]. Results from this collaborative effort conclusively demonstrated that the premutation form of the CGG repeat, not the full mutation, is associated with POI. Also, these data, combined with additional reports from other sites, suggest that the rate of POI among premutation carriers is 21% (95% confidence interval: 15–27%) [147]. Overall, approximately 14% of idiopathic familial POI and 2% of sporadic POI in the general population can be attributed to the fragile X premutation allele [147].

The cause of POI among premutation carriers is related to excess mRNA produced by the cells. Many models have been proposed to explain the role of the premutation allele (as opposed to the full-mutation allele) in the development of POI among many (but not all) premutation carriers, but recent studies have yielded few clues to lend support to any one model. Regardless of the cause, the occurrence of POI is one of the factors that can limit the usefulness of preimplantation genetic testing (PGT) as a reproductive option for carrier females [150–152]. In fact, recent hormonal studies suggest that female premutation carriers may unknowingly be experiencing ovarian dysfunction at an early age and may be facing a poorer prognosis for future pregnancy much earlier than expected [153]. The objective of PGD for FXS is to utilize only those embryos that receive the normal X chromosome from the mother. Donor egg, where available, is another reproductive option that allows carrier females, even those with POI, to have unaffected children [154].

It is important to recognize that there are other medical and psychiatric problems that can occur in some carriers, and these are not necessarily part of FXTAS or FXPOI but may nevertheless be related to mRNA toxicity. Neuropathy is relatively common in older carriers and can occur without other symptoms of FXTAS. Hypertension is seen in the majority of older carriers and may be secondary to the autonomic dysfunction related to RNA toxicity [127]. Autoimmune problems may be more frequent in female premutation carriers [127].

Psychopathology that is more common in those with the premutation includes anxiety, depression, and obsessive compulsive behavior; these problems are clinically significant for 25–40% of carriers [155, 156]. In addition, there is newer evidence that the premutation has a neurodevelopmental component in some children, especially boys, causing a higher incidence of ADHD, shyness, and social deficits including autism spectrum disorder [123, 157, 158]. Further studies of premutation carriers identified during newborn screening may further delineate the percentage of carriers with these problems.

Intermediate Carriers

Intermediate alleles, also known as “gray-zone” alleles, range from 45 to 54 CGG repeats and are classified differently than premutation or common alleles in that they may or may not be

transmitted unstably from parent to offspring [29]. Intermediate alleles, like premutation alleles, do not cause hypermethylation of the CpG island near *FMR1* and are not thought to affect cognitive or behavioral development. However, a recent study from Wessex, United Kingdom, found that boys placed in special education had a higher frequency of alleles in the intermediate and premutation range compared with controls [159]. The results from these data suggested, for the first time, that large CGG repeats smaller than premutations were somehow responsible for the child’s placement in special education [49, 159]. Although an excess of intermediate and premutation alleles has not been observed in other special education populations, new cognitive and molecular data warrant further research to identify and define a phenotypic consequence of intermediate-repeat alleles of *FMR1*, if one exists [132, 160, 161].

Timing of the Premutation Expansion

One of the yet unsolved questions is when in development the expansion from premutation to full mutation occurs. Expansion could occur during oogenesis (meiotic) or after fertilization (mitotic). Reyniers et al. showed that full-mutation or mosaic full/premutation males produce only premutation sperm and therefore premutation daughters, since repeat expansion occurs only in females [26, 162]. Testicular selection against full-mutation sperm is unlikely, since male *Fmr1* knockout mice show fertility [163]. These data support a model of expansion only in somatic cells and protection of the premutation in the germ line cells. However, Malter et al. showed that, in full mutation fetuses, only full-mutation alleles (in the unmethylated state) were found in oocytes from intact ovaries or in immature testes from 13-week fetuses, but that both full and premutation alleles were found in the germ cells of a 17-week male fetus [164]. They hypothesize that the full mutation contracts in the fetal testes, with subsequent selection for the premutation sperm. In females, the expansion could occur during maternal oogenesis or very early in embryogenesis prior to general methylation. The answer requires analysis of oocytes from premutation females.

Current Genetic Aspects of Fragile X Syndrome

Epidemiology

Crawford et al. provided an extensive review of the literature and indicated a prevalence of FXS ranging from 1 in 3,717 to 1 in 8,198 in Caucasian males in the general population [165]. The female prevalence rate is presumed to be approximately one-half of the male rate. In another study carried out over 4 years in metropolitan Atlanta, Crawford et al. determined

the prevalence of the FXTAS to be 1 in 2,545 African-American males and 1 in 3,717 Caucasian males [166]. However, the prevalence estimate for Caucasian males, determined from this and from other studies, fell within the 95% confidence interval for African-American males. The prevalence of the fragile X mutation in an Afro-Caribbean population in the French West Indies was similar (1 in 2,539) to that in the African-American population in Atlanta [167]. Falik et al. have suggested that the Tunisian Jewish population is the only other ethnic group to have a higher prevalence of FXTAS than the Caucasian population [168]. However, these studies were not supported by the data of Tolodano-Alheder et al. [169]. Further studies are required to determine if the frequency of FXTAS differs in ethnic populations. A recent systematic review of population screening for fragile X syndrome summarized the data and suggested that if population screening is to be instituted more psychosocial support will be required [170].

The premutation is common in the general population with a prevalence of 1 in 130–260 females and 1 in 250–810 males [171]. There is variability in the prevalence figures depending on where the study was done and the ethnic or racial background of the patients. The study recently reported by Cronister et al. in 2008 has shown that the premutation is less common in those of Chinese background and more common in the Middle East, particularly in Israel, as reported by others [169, 172].

The premutation form of the CGG repeat is the precursor to the full mutation in that the repeat is very unstable when transmitted from parent to offspring, eventually expanding to the full-mutation form when passed through a female germ line [27]. Using this definition, premutations can range from 50 to <200 repeats. The absolute lower boundary of the premutation repeat size that is at risk for expanding to the full mutation in a single generation is still under debate [25, 173]. Studies of premutations among families with a member reported affected by FXTAS suggest that the smallest premutation to expand to the full mutation in a single generation is 59 repeats [173]. However, small premutation alleles (~50–65 repeats) ascertained from the general population have proven to be more stable than those ascertained from families with FXTAS [173]. Given the uncertainty in the lower boundary of the premutation, the prevalence of the premutation varies from study to study, depending on the ranges of CGG repeats that are considered premutations.

Molecular Rules of Inheritance

DNA analysis of the *FMR1* allele can detect all stages of the trinucleotide repeat expansion. Reduced penetrance, the Sherman paradox, and other unusual characteristics of FXTAS were explained by the silent premutation state.

The rules of inheritance, as currently understood, include the following [174]:

1. Every affected individual has a carrier mother with an observable expansion. No new mutation has gone directly from normal to full. Full-mutation males do not pass a full mutation to their daughters.
2. Affected females have a full mutation, and unaffected females may have premutations or nonpenetrant full mutations. As a result, a female with a full mutation has an obligate carrier mother, but a female with a premutation could have received that X chromosome from either parent.
3. The risk that a female carrier will have a child with a full mutation is directly related to the size of her expansion. A repeat size of 99 appears to be the point of significance, as nearly all premutations with ≥99 repeats become full mutations in subsequent offspring [173].
4. Premutations appear to be inherited silently for many generations. No family has been documented in which a normal allele expanded to a premutation allele. Thus, many present families may have the same ancestral premutation, but this cannot be traced reliably. Using polymorphism analysis, Smits et al. showed one family with five living males with FXTAS who share an X chromosome to be related through their last common ancestor six or more generations in the past [175].

Diagnostic Laboratory Testing for Fragile X Syndrome

Cytogenetic Testing

From 1977 to 1992, the standard laboratory test for diagnosis of FXTAS was cytogenetic scoring for expression of the fraX in metaphase cells (Fig. 19.1).

Compared to routine chromosome analysis, fraX testing was fraught with technical difficulties as well as biological limitations. Culture conditions had to be altered to facilitate expression of the fragile site via folate stress (see Chap. 14). fraX expression was variable (between 1 and 50%), with females usually having fewer positive cells than males, and obligate carriers often tested negative. Expression of the marker tended to be easier to appreciate in unbanded (or under-banded) cells, which in turn created the potential for uncertainty of X chromosome identification. As a result, many cells had to be scored. Also, the presence of the other three fragile sites on Xq reduced the reliability of the assay. Lastly, lower expression in cell types other than lymphocytes made prenatal diagnosis difficult if not impossible.

One significant advantage, however, was that the test was combined with routine chromosome analysis, and as a result, chromosome abnormalities could be diagnosed as well.

Molecular Testing

By the time DNA-based diagnosis of FXS became available, the problems with cytogenetic testing had become apparent [176]. DNA-based testing has solved these problems, and therefore cytogenetic fraX testing has been retired. In fact, the reimbursement (CPT) code for such testing has been deleted.

The objective of all DNA-based methods for FXS is to identify a piece of DNA containing the CGG repeat and determine its length and methylation status in order to classify it as normal, premutation, or full mutation.

DNA-Based Methods

The two DNA-based methods available for *FMR1* testing are Southern blot, with or without methylation, and PCR (polymerase chain reaction). PCR is more sensitive for premutations or carrier testing, and the results are usually expressed as total repeat number. PCR is followed by capillary electrophoresis (CE) or polyacrylamide gel electrophoresis (PAGE) for size resolution for the detection of up to 100–150 CGG repeats. For the last 20 years, Southern blots have been the preferred method for detecting full mutations and, if double digestion is utilized, the methylation status can be determined. The results are expressed as Δ kb (delta kb, defined as the difference between the patient and a normal reference). Both DNA-based methods are considered diagnostic and are 99% sensitive and 100% specific. Detailed descriptions and illustrations of these techniques are provided in the American College of Medical Genetics Standards and Guidelines for fragile X testing [174]. The most current version can be obtained from www.acmg.net [174].

Latest PCR-Based Methods

Standard PCR of GC-rich regions is difficult and special amplification conditions are required. The difficulty increases with increasing numbers of CGG repeats; therefore, in the past, many PCR strategies did not attempt to detect large alleles. It is not possible to use standard PCR to distinguish between a female who is homozygous for a normal allele and a female who has one normal allele and a second, large nonamplifiable allele. Similarly, patients who are mosaic for premutations and full mutations will appear to have only premutations. Even though a standard strategy can detect alleles in the premutation range, amplification usually favors the smaller allele, and mosaicism may be missed. Thus, because of disproportionate amplification, standard PCR is not reliable for determining the ratio of different allele species in a mosaic individual.

PCR amplification of the relevant portion of the *FMR1* gene is not affected by methylation. Although PCR-based tests that are specifically modified to detect methylation status have been described, the common PCR strategies that have been in use for many years are completely independent

of methylation [175, 176]. A genotype classification method using a methylation-specific triple PCR method that distinguishes all normal and premutation males and females and all full-mutation males and females has been described [177, 178]. This method may provide a suitable alternative for Southern blot analysis and yield estimates of allele sizes similar to other PCR-based methods.

Recently, a PCR-based screening method has been reported for detection of carrier females and affected newborns. These assays use CGG-repeat primed PCR and automated capillary electrophoresis and detect the presence or absence of an expanded *FMR1* allele with high sensitivity and specificity, minimizing the need for Southern blot analysis [177–179]. Importantly, the use of triplet primers allows females who are homozygous for a single allele to be distinguished from females with a normal allele and an expanded allele. This screening method can also detect the expanded allele in affected males. The technique uses two gene-specific primers (forward and reverse) and a CGG-repeat primer in a single tube. After amplification, the products, which include the full-length amplicon that completely encompasses the triplet repeat region and a multiplicity of CGG-repeat primed products, are resolved by capillary electrophoresis. The resulting electropherogram supports quantification of the number of CGG repeats, the determination of the allele zygosity, and the sequence context of any AGG spacer elements [178].

Commercially available reference materials have been characterized [180, 181]. These reference materials are available as DNA isolated from cell lines or the cell lines themselves for normal, premutation, and full mutations from the Coriell Repository [182]. The genotypes of these cell lines are listed at the Centers for Disease Control (CDC) Get-RM Web site [183].

An important caveat for DNA-based methods is the fact that a small percentage (<1%) of patients with FXS have a normal CGG-repeat size. To date, numerous deletions have been reported in the literature [180]. Also, two other types of mutations have been reported: a two-base-pair substitution that alters splicing and leads to altered levels of FMRP, and a missense mutation that leads to dysfunctional FMRP [181, 184]. Prior et al. reported a case of germ line mosaicism, an important issue when counseling deletion families [185].

Protein/mRNA-Based Diagnosis

Monoclonal antibodies against FMRP have been used with success to diagnose affected males and some affected females [186]. This earlier method is more rapid than DNA-based testing but cannot be used for premutation testing. It has been successfully utilized for prenatal diagnosis and may also be used for a patient with the physical and mental features of the FXS without evidence for an expanded CGG repeat [80]. The protein test can be performed on a variety of samples, including blood and hair root [187]. Iwahashi et al. introduced a

quantitative sandwich ELISA assay for the FMRP1 protein. This assay can be used in peripheral blood lymphocytes, is quantitative and scalable, is specific for the FMRP, and could potentially be used for diagnosis of various *FMRI*-associated clinical phenotypes as well as newborn screening [188].

FRAXE Syndrome

Cytogenetically, FRAXE was described in 1992 [12]. The gene (*AFF2*, formerly *FMR2*) is located 600 kb distal to *FMRI*, and the repeat sizes in normal, premutation, and full-mutation individuals are similar (Table 19.3). FRAXE expansion can decrease or increase in both males and females, and two deletions have been identified [189]. No point mutations within *AFF2* have been reported. The phenotype of FRAXE syndrome appears to be mild MR (IQ=60–80); however, the collection of cognitive and behavioral data from FRAXE families may further differentiate this phenotype from that of FRAXA [190, 191]. A knockout mouse model for *AFF2* (*fmr2*) exhibiting impaired learning and memory may also help to further define the mild phenotype of FRAXE observed in humans [192]. Preliminary work suggests that *AFF2*'s gene product acts as a transcription activator, but its function in relation to the phenotype remains largely unknown [192]. FRAXE expansions are not common in human populations (approximately 1 in 23,500 individuals), and, although available, DNA analysis for the FRAXE expansion is not widely utilized [193].

Indications for Prenatal Diagnosis and Carrier Testing

Carrier Testing

Women who have affected children are obligate carriers. Determining DNA status for these women is indicated if future pregnancies are planned. Other family members who could share an X chromosome with an obligate carrier are at risk and should be referred for counseling and possible testing.

Carrier testing could be elected by any individual whether he or she has a positive family history or not, especially since the frequency of premutation carriers in human populations appears to be high. Family members whose carrier status was determined by DNA linkage should be tested to confirm the result. Likewise, DNA testing is recommended for low-expressing family members who were diagnosed cytogenetically.

Prenatal Diagnosis (See Also Chapter 12)

Prenatal DNA testing is indicated in families where the mother is a known carrier of a premutation/full-mutation CGG repeat. This is the only situation in which the offspring is at risk to inherit a full mutation. Specimens from either amniocentesis or CVS (direct or tissue culture) can be used to determine the allele size of the fetus. Timing and availability are issues that help determine the procedure selected.

CVS is done early in pregnancy and, if sufficient tissue is obtained, testing can be performed on uncultured cells. In CVS tissue, full mutations are not always methylated, so interpretation must be based on the size of the allele, not its methylation pattern. Maternal cell contamination, if present, can be detected via fetal to maternal comparison.

Interpretation of results of testing is usually unremarkable, except in the case of full-mutation females. The severity of the disorder cannot be predicted in an individual female, but is based on the risk probabilities developed in family studies of such females.

Genetic Counseling

Genetic counseling is a vital part of a multidisciplinary approach to helping families adjust to and cope with the stresses of FXS and its impact on the family (see the excellent review by Gane and Cronister [194]). Genetic counseling covers a multitude of areas such as diagnosis, prognosis, recurrence risks, family planning options, management, and psychosocial issues, to name a few. It provides the family

Table 19.3 Characteristics of the cloned folate-sensitive fragile sites

Symbol	Location	Disease	Copy Number		
			Normal	Premutation	Full mutation
FRAXA	Xq27.3	FXS	6–54	61–200	230 to >1,000
FRAXE	Xq28	Fragile XE syndrome	6–25	?50–200	200 to >800
FRAXF	Xq28	None	6–29	?	300–1,000
FRA16A	16p13.1	None	16–50	?50–200	?1,000–2,000
FRA11B	11q23.3	Offspring predisposed to Jacobsen syndrome	11	85–100	100–1,000

Adapted from Howard-Peebles [32]

with educational and emotional support so they can adjust to and cope with present as well as future circumstances.

General genetic counseling is covered in detail in Chap. 21.

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Jin-Chen C. Wang

Introduction

Genomic imprinting refers to the process of differential modification and expression of parental alleles; the parental origin of the allele dictates whether it is transcribed. It is an epigenetic form of gene regulation that allows expression of only one parental allele. As a result, the same gene functions differently depending on whether it is maternally or paternally derived. This concept is contrary to that of the traditional Mendelian inheritance in which genetic information contributed by either parent is assumed to be equivalent.

The term “imprinting” was coined by Crouse to describe the modification and the selective elimination of paternal X chromosomes from somatic and germline cells of the fly *Sciara*, in which the “imprint” a chromosome bears is determined only by the sex of the parent through which the chromosome has been inherited. It has since been used in many other species, including man [1, 2].

Evidence for the existence of genomic imprinting is manifold. Initial experimental approaches included studies in mouse embryos using nuclear-transplantation techniques [3–7]. These experiments involved the removal and reintroduction of pronuclei into zygotes, thus creating embryos that had either only the maternal or paternal genome. In parthenogenetic eggs—i.e., eggs that contain two maternal pronuclei and no paternal pronucleus—fetal development was relatively good, but extraembryonic tissue development was poor. In contrast, in androgenetic eggs—i.e., eggs containing two paternal pronuclei and no maternal pronucleus—the development of extraembryonic tissue was good, but fetal development was poor. In either case, the embryos failed to reach term. Thus, both maternal and paternal genomes are required for normal development, and it appears that, at least

in mice, the maternal genome is essential for embryogenesis, while the paternal genome is essential for placental development.

The human equivalents to these observations in mice are the ovarian teratoma and the complete hydatidiform mole and the two types of triploidy, namely, digynic triploidy and diandric partial hydatidiform mole (see Chap. 8). Ovarian teratoma is an embryonal tumor that contains tissues predominantly derived from ectodermal but also mesodermal and endodermal germ layers. The ovarian teratoma has been shown to be parthenogenetic and contains two sets of the maternal genome and no paternal genome [8]. The complete mole, on the other hand, is androgenetic and contains two sets of the paternal genome and no maternal genome [9, 10]. Studies of the parental origin of the extra haploid set of chromosomes in triploids reveal that this is maternal (digynic triploidy) when severe intrauterine growth restriction and abnormally small placentas are seen, while it is paternal (diandric triploidy) in partial hydatidiform moles, in which the placenta is abnormally large [11–13]. Intercross experiments in mice between either Robertsonian or reciprocal translocation carriers further demonstrate that maternal duplication/paternal deficiency or maternal deficiency/paternal duplication of certain mouse chromosomes or regions of chromosomes results in different phenotypic abnormalities [14].

Observations of X-chromosome inactivation in different species and different tissues provide further evidence of imprinting. Although inactivation of the X chromosome in females of placental mammals is in general random in somatic cells, studies in interspecies crosses between marsupials reveal that the paternally derived X chromosome is preferentially inactivated in female kangaroo somatic tissues [15, 16]. In extraembryonic tissues, the paternally derived X chromosome is preferentially inactivated in mice [17–19]. Further, the paternal X in mice is imprinted to become inactive early during embryonic development, perhaps as early as the two-cell stage. Although apparently incomplete, this early form of inactivation insures dosage compensation throughout

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development. Silencing of the paternal X chromosome persists in cells of extraembryonic tissues, but it is erased and followed by random X inactivation in cells of the embryo proper [20]. Earlier studies on the pattern of X inactivation in trophoblastic cells of human placenta yielded conflicting results; both preferential inactivation of the paternal X and random X inactivation have been reported [21–25]. Those studies analyzed only one or two X-linked loci to infer the activity of the entire X chromosome. By analyzing allele-specific expression of 22 X-linked genes, a recent study concluded that X inactivation is random in the human placenta and that the placenta is arranged in relatively large patches of cells with either maternal or paternal inactive X chromosome [26].

Direct evidence that genomic imprinting exists in man is provided by the observation of a variety of human conditions or diseases such as Prader-Willi syndrome (PWS) and Angelman syndrome (AS), certain types of cancer, and uniparental disomy. These are discussed in detail later.

Not all chromosomes or all regions of one chromosome are involved in genomic imprinting [27]. It is estimated that <1% of the mammalian genome is imprinted [28, 29]. In the mouse, approximately 100 genes undergo genomic imprinting and hundreds more are predicted by examining specific epigenetic features rather than local sequence features [30]. Approximately 70 imprinted genes have been identified in humans (on chromosomes 1, 2, 4, 6, 7, 8, 10, 11, 14, 15, 16, 18, 19, 20), and many more possibly imprinted genes are waiting to be identified and confirmed [31, 32].

Mechanism

Imprinting is a phenomenon that is reversible from generation to generation. The process must therefore involve the establishment of the imprint during gametogenesis, the maintenance of the imprint through embryogenesis and in adult somatic tissues, and then the erasure of the imprint in the germline [33–35] (see Fig. 20.1). Thus, stable and differential modification of chromatin is required. Differential methylation, controlled by DNA methyltransferase enzymes, of the cytosine residues of DNA on maternal and paternal chromosomes appears, at least in part, to fulfill this role.

DNA methylation is involved in human X-chromosome inactivation. Using 5-azacytidine, which causes hypomethylation of DNA, Mohandas et al. were able to achieve reactivation of an inactive human X chromosome [36]. Yen et al. showed that the human HPRT (hypoxanthine phosphoribosyltransferase) gene is hypomethylated on the active X chromosome relative to the inactive X [37]. Furthermore, DNA methylation has been shown, in experiments involving gene insertion into mouse L cells, to render these sequences insensitive to both DNase I and restriction endonucleases, by directing DNA into an inactive supranucleosome structure

[38]. These observations suggest that DNA methylation may exert its effect on gene transcription by altering interactions between DNA and nuclear proteins.

The involvement of methylation in the initiation and/or maintenance of genomic imprinting has been examined extensively. Experiments with transgenic mice, in which a foreign gene was inserted into the mouse genome by microinjection, have demonstrated that some transgenes show different states of methylation specific to the parent of origin and that the methylation pattern of those transgenes changes from generation to generation depending upon the sex of the transmitting parent [39–41]. In most cases, a paternally inherited transgene is less methylated than one that is maternally inherited. In a study of transgene-bearing elements of the Rous sarcoma virus (RSV) and a fused *c-myc* gene, the paternally inherited transgene was undermethylated in all tissues and was expressed only in the heart [41]. This observation suggests that methylation status alone does not determine the expression of a transgene and that undermethylation may be necessary, but not sufficient, for gene expression. In this same study, the somatic organs of a male animal with a maternally inherited transgene exhibited a methylated transgene pattern, but in the testes the transgene was undermethylated, suggesting that the maternally derived methylation pattern is eliminated in the testes of male offspring during gametogenesis.

The role of DNA methylation in genomic imprinting is further demonstrated by observations made in three imprinted endogenous genes in mice: insulin-like growth factor 2 (*Igf2*), *H19* (these two genes are closely linked on mouse chromosome 7) and the *Igf2* receptor gene (*Igf2r*, on mouse chromosome 17).

Studies of mouse *H19* showed that it is subject to transcriptional regulation by genomic imprinting, with the maternal allele expressed and the paternal allele silent [42]. By comparing CpG methylation and nuclease sensitivity of chromatin in mouse embryos, Ferguson-Smith et al. showed that hypermethylation and chromatin compaction in the region of the *H19* promoter are associated with repression of the paternally inherited copy of the gene [43]. This normally silent paternal *H19* allele is activated in DNA methyltransferase-deficient embryos, providing *in vivo* evidence that a direct correlation is present between DNA methylation and gene activity [44].

Studies of the mouse *Igf2* gene showed that, contrary to *H19*, the paternal allele is expressed in embryos, while the maternal allele is silent, but both parental alleles are transcriptionally active in the choroid plexus and leptomeninges [45]. Therefore, imprinting of *Igf2* may also be tissue specific. In addition, studies using mouse embryos with maternal duplication and paternal deficiency of the region of chromosome 7, which encompasses *Igf2*, showed that the chromatin of the 5' region of the repressed maternal *Igf2* allele is potentially active for transcription; i.e., it is hypomethylated and

contains DNase I hypersensitive sites [46]. Recently, a region of paternal-specific methylation between *H19* and *Igf2* has been postulated to function as the imprint control region. This imprint control region, when unmethylated, acts as a chromatin boundary or insulator that blocks the interaction of *Igf2* with its enhancer, thus resulting in silencing of the *Igf2* gene, as is observed on the maternal chromosome. On the paternal chromosome, this region is methylated, resulting in the loss of enhancer-blocking activity and allowing the expression of *Igf2* [47, 48]. A deletion within this imprint control region results in loss of imprinting of both *H19* and *Igf2*.

Studies of the mouse *Igf2r* gene indicated that the maternal allele is expressed and the paternal allele is silent [49]. The parental-origin-specific difference in methylation for this gene has been demonstrated in two distinct CpG islands [50]. Here, while the promoter is methylated on the inactive paternal allele, an intronic CpG island is methylated only on the expressed maternal allele, suggesting that methylation of the latter site is necessary for expression of the *Igf2r* gene.

In humans, the methylation patterns of the parental alleles have been determined for several imprinted loci on chromosome 15 at bands 15q11.2-q13. These include the *MKRN3* gene (D15S9) studied in PWS and AS patients and in complete hydatidiform moles, the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene, and the DNA sequence PW71 (D15S63) [51–55]. Distinct differences in methylation of the parental alleles are observed in all instances. This is also true for some of the other known imprinted genes in humans: *H19* (maternal allele active) and *IGF2* (paternal allele active), both located on the short arm of chromosome

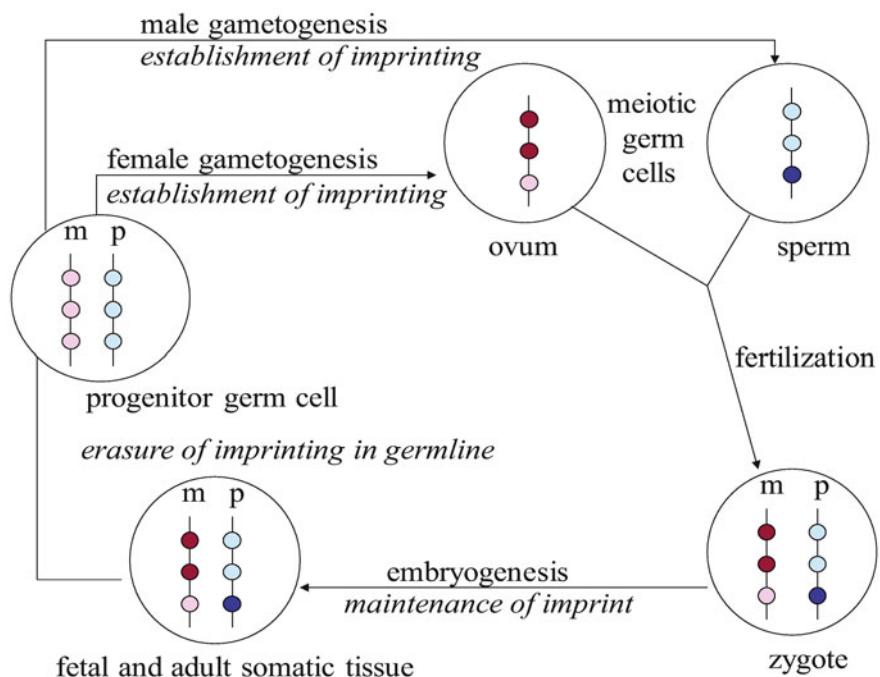
11 at band 11p15.5 [56–58]. In the case of *IGF2*, although it is the paternal allele that is active, the maternal allele is hypomethylated while the paternal allele is methylated at the 5' portion of exon 9, similar to the findings in mouse studies. Unlike this gene in mice, the human *IGF2R* gene is not imprinted [59].

The differentially methylated domains (DMDs) of imprinted genes contain CpG-rich imperfect tandem repeats with similar predicted secondary structures. It is this repeat-related DNA structure, not the sequence, that is implicated in the imprinting mechanism—the establishment and maintenance of parent of origin-specific methylation patterns. It is suggested that a structural feature or features of these tandem repeats are the conserved DMD imprinting signal (reviewed in [35]).

In summary, epigenetic modifications by methylation of alleles of each imprinted gene are established during oogenesis and spermatogenesis. This imprint pattern is maintained throughout embryogenesis and in adult somatic tissues. In fetal gonads, global demethylation of the progenitor germ cells occurs and the inherited imprinting pattern is erased. This is followed by reestablishment of methylation of imprinted genes during gametogenesis, depending on the sex of the fetus. This reprogramming in germ cells ensures that sex-specific genomic imprinting is initiated and that an accurate imprinting cycle is achieved through each generation (reviewed in [60]) (refer to Fig. 20.1).

A difference in DNA replication timing of maternal and paternal alleles of imprinted genes has also been observed [61–65]. Cell-cycle replication timing has been shown to

Fig. 20.1 Diagrammatic representation of the imprinting process. Light-colored circles represent undermethylated genes, dark-colored circles represent hypermethylated genes. *m* maternally derived chromosome, pink. *p* paternally derived chromosome, blue. See text for details



correlate with gene activity: genes that are expressed generally replicate earlier [66, 67]. Furthermore, most genes on homologous chromosomes replicate synchronously [68]. This is not the case for imprinted genes. Using fluorescence *in situ* hybridization (FISH; see Chap. 17) on interphase nuclei and scoring for the stage of the two alleles in S phase, Kitsberg et al. showed that the imprinted genes *H19*, *Igf2*, *Igf2r*, and *Snrrpn* in mice and their corresponding positions in the human genome all replicate asynchronously, with the paternal allele replicating early [61]. Studies of genes in the 15q11.2-q13 region in humans demonstrated that most show a paternal-early/maternal-late pattern, with some exhibiting the opposite pattern [62, 63]. Therefore, it appears that imprinted genes are embedded in DNA domains with differential replication patterns, which may provide a structural imprint for parental identity [62]. This asynchronous replication timing is established in the gametes during late gametogenesis and maintained throughout development [65].

Thus, the process of genomic imprinting is very complex, and while DNA methylation plays a critical role in genomic imprinting, the process is much more complex than simply inactivating a gene by methylation. It may involve an interaction between DNA methylation, histone modification including acetylation and methylation, chromatin compaction, DNA replication timing, and potentially other mechanisms [69, 70].

Genomic Imprinting and Human Diseases

Genomic imprinting provides an explanation for the observation that the transmission of certain genetic diseases cannot be explained by traditional Mendelian inheritance, but that rather the phenotype depends upon whether the gene involved is maternally or paternally inherited. Conversely, the existence of such diseases provides evidence that genomic imprinting occurs in man. Human conditions that fall into this category include certain deletion/duplication syndromes, a number of cancers, and many disorders arising from uniparental disomy. In addition, imprinted genes may also contribute to modification of disease phenotype, such as is observed in Albright hereditary osteodystrophy, language development, and some psychiatric disorders and complex behavioral phenotypes, including bipolar affective disorder and catatonic schizophrenia [71–74].

Albright hereditary osteodystrophy (AHO) is characterized by short stature, obesity, brachydactyly, mild-to-moderate mental handicap, and subcutaneous ossifications. A proportion of patients with AHO have associated end-organ resistance to parathyroid hormone (PTH), known as “pseudo-hypoparathyroidism type I” (PHP Ia, also known as AHO with multiple hormone resistance, OMIM #103580). Individuals with AHO and normal endocrine responsiveness have “pseudo-pseudohypoparathyroidism” (PPHP, also

known as AHO without multiple hormone resistance, OMIM #612463). AHO is caused by heterozygous deactivating mutation in the *GNAS* gene located at 20q13. *GNAS* is imprinted (paternal allele inactive); the imprinting appears to be tissue specific, with maternal expression only in certain cells, such as cells of the proximal renal tubule [75]. In families with AHO, the strongest predictor of the endocrine phenotype is the parent of origin. PHP1A occurs only after maternal inheritance of the molecular defect, either *GNAS* mutation or *GNAS* imprinting defects, whereas PPHP occurs only after paternal inheritance of the molecular defect [76]. These observations indicate involvement of imprinting in disease phenotype.

Chromosome Deletion/Duplication Syndromes

Prader-Willi Syndrome/Angelman Syndrome

The best-studied examples of genomic imprinting in human disease are the Prader-Willi and Angelman syndromes. These are clinically distinct disorders; both map to the chromosome 15q11.2-q13 region, but they involve different genes [77–81]. The etiologies of these disorders include the absence of a parent-specific contribution of this region due to either deletion or uniparental disomy (UPD), disruptions in the imprinting process, and mutations within the gene [82–96].

The clinical phenotype of PWS has been well characterized [97, 98]. Briefly, it includes hypotonia during infancy, obesity, hyperphagia, hypogonadism, characteristic facies, small hands and feet, hypopigmentation, and mental deficiency. Approximately 70–75% of cases have an interstitial deletion of a 4 Mb sequence at 15q11.2-q13 on the paternally derived chromosome 15 [69]. Approximately 20–25% of cases are due to maternal uniparental disomy for chromosome 15 and 1% or so as a result of an abnormality of the imprinting process, causing a maternal methylation imprint on the paternal chromosome 15 [86, 89, 93, 94] (Table 20.1). Many paternally expressed transcripts have been identified in a cluster in the proximal part of the 15q11.2-q13 region. These include *MKRN3*, *MAGEL2*, *NDN*, *PWRN1*, *NPAP1*, *SNURF-SNRPN*, a number of C/D box small nucleolar RNA (snoRNA) genes, and other additional transcripts (reviewed in references [99–103]). This clustering of paternally expressed transcripts suggests strong regional control of the imprinting process [103]. It has been recently demonstrated that deficiency of *SNORD116* (previously *HBII-85*) snoRNAs causes the key characteristics of the PWS phenotype [104, 105]. Other imprinted genes in the 15q11.2-q13 region, such as *MAGEL2* and *NDN*, probably also contribute to the PWS phenotype.

The clinical phenotype of AS patients is distinct from that of PWS [106, 107]. Briefly, it includes microcephaly, ataxia, characteristic gait, spontaneous laughter, seizures, severe

Table 20.1 Etiology and recurrence risk of Prader-Willi syndrome and Angelman syndrome

Etiology	PWS	AS	Recurrence risk
Deletion	~70–75% Paternal chr 15	~70% Maternal chr 15	<1%
UPD	~20–25% Maternal UPD	~6% Paternal UPD	<1%
IC abnormality	~1%	~3–6%	50%
			(PWS: when present in father) (AS: when present in mother)
Gene mutation	–	~10% <i>UBE3A</i>	50%
			(AS: when present in mother)
Unknown	–	~10%	–

mental retardation, and hypopigmentation. Approximately 70% of AS patients have a deletion of the same 4 Mb sequence at 15q11.2-q13 on the maternally derived chromosome 15 [83–85]. Approximately 6% are due to paternal uniparental disomy for chromosome 15; 3–6% as a result of an abnormality of the imprinting process, causing a paternal methylation imprint on the maternal chromosome 15; and approximately 10% as a result of a mutation within the AS gene (reviewed in Refs. [81, 87–93, 95, 96, 108, 109]) (Table 20.1). In contrast to PWS, mutation of a single gene, the gene for E6-associated protein (E6-AP) ubiquitin-protein ligase (*UBE3A*) (maternal allele active) has been identified in some AS families and is considered the candidate gene for AS [81, 96]. The imprinting of *UBE3A* is tissue specific, being restricted to the brain [110–112]. More recently, another imprinted gene *ATP10A*, mapped within 250 kb telomeric to *UBE3A*, has also been shown to be expressed only on the maternal allele [113]. It is speculated that *ATP10A* may be involved in phospholipid transport and may also contribute to the AS phenotype. Both *UBE3A* and *ATP10A* are located at the distal part of the 15q11.2-q13 region.

In both PWS and AS patients with abnormalities of the imprinting process, Buiting et al. identified inherited microdeletions in the 15q11.2-q13 region [114]. They proposed that these deletions probably affect a single genetic element that they called an “imprinting center (IC).” This AS/PWS-IC has been shown to have a bipartite structure and overlaps the *SNRPN* promoter with the AS-IC being only 35–40 kb upstream of the PWS-IC [115–117]. Mutations or disruptions of the imprinting center impair the imprinting process. These mutations can be transmitted silently through the germline of one parent, the one in whom the gene is normally silent, but appear to block the resetting of the imprint in the germline of the opposite sex. Thus, a female with a PWS-IC mutation will not have affected children. Her sons, however, if they inherit the mutation and are therefore unable to reactivate the cluster of PWS genes in their germ cells, will be at risk of having PWS children, both male and female. The opposite is true for AS; i.e., a male with an AS-IC mutation will not have affected children, but his daughters, if they inherit the mutation, will be at risk of having AS children.

These observations in PWS and AS indicate that the PWS genes are active only on the paternal chromosome 15 and the AS gene is active only on the maternal chromosome 15. These two syndromes serve as classical examples of genomic imprinting in humans.

Deletion, UPD, or IC disruption can all result in an abnormal methylation pattern of the PWS/AS parental alleles. Therefore, the most cost-effective approach to laboratory diagnosis of PWS/AS is to perform DNA methylation studies first. This will detect virtually all cases of PWS and approximately 80% of the cases of AS. If the result is abnormal, fluorescence *in situ* hybridization (FISH) to detect 15q11.2-q13 microdeletion, followed by UPD studies, should be performed to determine the exact etiology. In the case of AS, *UBE3A* mutation analysis can be considered when the methylation study is normal (Fig. 20.2).

Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS) is an overgrowth disorder associated with neonatal hypoglycemia, abdominal wall defects, macroglossia, visceromegaly, gigantism, mid-face hypoplasia, and a predisposition to embryonal tumors (seen in 7.5–10% of patients) including Wilms tumor (most common, see next section), rhabdomyosarcoma, and hepatoblastoma [118, 119] (see next section). Most cases (85%) are sporadic. BWS is a multigenic disorder resulting from dysregulation of a number of imprinted genes at the chromosome 11p15.5 region and is caused by several molecular mechanisms. These include:

- Paternal UPD for the p15 region of chromosome 11 in approximately 20% of sporadic cases [120, 121].
- Cytogenetic abnormalities involving 11p15, present in a small number (~1%) of all BWS patients. These include duplication of the paternal 11p15 region as a result of either a *de novo* rearrangement or a familial translocation/inversion and maternally inherited balanced rearrangements involving 11p15 [122–124].
- IC mutation in the gene cluster *IGF2/H19* or *KCNQ1/KCNQ1OT1* [125] (see later). In familial cases, the segregation appears to be autosomal dominant with incomplete penetrance [119]. Furthermore, penetrance appears to be

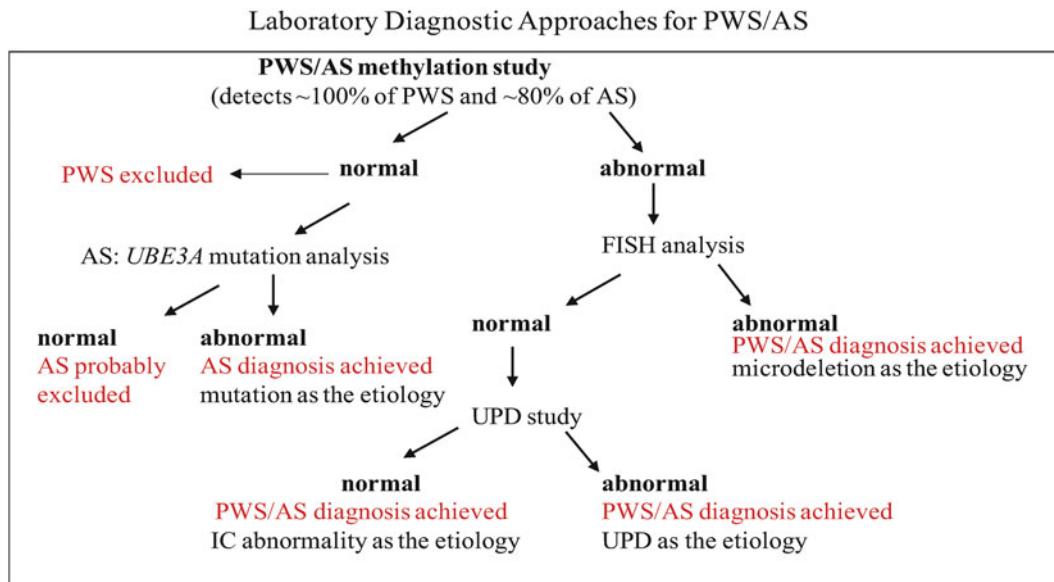


Fig. 20.2 Laboratory diagnostic approaches for Prader-Willi syndrome/Angelman syndrome

more complete with maternal inheritance; i.e., there is an excess of transmitting females [126, 127].

- Mutation in the maternally active *CDKN1C* (p57^{KIP2}) gene [128]. *CDKN1C*, a cyclin-dependent kinase inhibitor, is a negative regulator of cell proliferation; its overexpression arrests cells in G1. Germline *CDKN1C* mutations have been found in 40% of familial and 5% of sporadic BWS cases [129].

Linkage studies confirm that BWS maps to 11p15.5 [130, 131]. Imprinted genes in this region have been shown to consist of two domains separated by non-imprinted genes [132, 133]. The proximal centromeric domain contains *CDKN1C* (p57^{KIP2}) (maternal allele active), *KCNQ1* (maternal allele active), and *KCNQ1OT1* (*LIT1* or *KvDMR1*) (paternal allele active) [125, 134–136]. The distal telomeric domain contains *H19* (maternal allele active) and *IGF2* (paternal allele active, located approximately 130 kb centromeric to *H19*). The paternally expressed genes are growth promoter genes, while the maternally expressed genes are growth suppressor genes. Functional imbalance between the growth promoter and growth suppressor genes causes the phenotype seen in BWS. In some BWS patients who inherited an 11p15.5 allele from both parents, an altered pattern of allelic methylation of *H19* and *IGF2* has been reported [121, 137]. In these patients, a paternal imprint pattern is seen on the maternal allele, which results in the non-expression of *H19*, while *IGF2* is expressed from both parental alleles. This switching from normally monoallelic expression to biallelic expression is known as loss of imprinting (LOI) and is caused by imprinting center abnormalities. As in PWS/AS, an IC abnormality prevents the resetting of imprinting in the maternal germline and explains the observation that the affected individuals are

usually born to carrier mothers in familial cases. The same explanation can be applied to the observation that in BWS patients with balanced rearrangements involving 11p15, the rearrangements are usually maternally inherited. A disruption/mutation of the IC has occurred in the rearrangement process, preventing the resetting of imprinting in the maternal germline, and *H19/CDKN1C/KCNQ1* remains inactive on the maternal allele.

In addition to these abnormalities involving 11 p15, other not yet well-defined mechanisms or genetic loci may also cause the BWS phenotype.

Laboratory diagnostic approaches for BWS include cytogenetic analysis to rule out an 11p15 abnormality, UPD study for the 11p15 region, mutation analysis of the *CDKN1C* gene, and methylation studies of *H19/IGF2* and *KCNQ1OT1*. One study reported that by analyzing the methylation status of the *H19* and *KCNQ1OT* genes in leukocytes, more than 70% of the 97 patients could be diagnosed [138]. Of all cases with abnormal methylation, 80% involved the promoter region of the *KCNQ1OT* gene and 20% the *H19* gene.

Imprinting Disorders and Assisted Reproductive Technology

Assisted reproductive technology (ART), including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), accounts for 1–3% of all births in developed countries (see Chap. 11). A possible link between ICSI and Angelman syndrome was first reported in 2002 [139]. Two unrelated children conceived by ICSI developed AS. In normal individuals, the maternal *SNRPN* allele is methylated,

and the paternal allele is unmethylated. In these two patients, hypomethylation of the *SNRPN* promoter region was detected; only an unmethylated band was present by methylation-specific PCR studies, and the normally methylated maternal band was absent. Microdeletion, uniparental disomy, and AS-IC mutation were all excluded as an etiology. The authors suggested that hypomethylation of the *SNRPN* locus in these two patients resulted from a sporadic imprinting defect on the maternal chromosome. Subsequently, three additional ART-conceived cases of AS were reported: two by ICSI and one using ovarian hyperstimulation alone [140, 141]. These findings are suggestive but not conclusive for an association between ART and AS due to an imprinting defect.

An association between ART and another imprinting disorder, Beckwith-Wiedemann syndrome (see previous section), was reported in 2003. Three case series from BWS registries reported an estimated sixfold increase of BWS cases among children born after ART (reviewed in [142, 143]). More than 60 such BWS patients have been reported. In the vast majority of these BWS patients, loss of methylation at the *KCNQ1OT1* gene at 11p15 is observed. Since *KCNQ1OT1* is normally maternally methylated/paternally unmethylated, this finding indicates that hypomethylation of the allele on the maternal chromosome is the cause of BWS in these patients. BWS remains the imprinting disorder with the strongest evidence for an association with ART.

A novel imprinting syndrome resulting from maternal hypomethylation at multiple loci was proposed in 2006 [144]. In a cohort of 12 patients with transient neonatal diabetes resulting from loss of maternal methylation at the transient neonatal diabetes locus, the authors found that six patients had hypomethylation at other loci. None of the patients were conceived following ART, although one was born following a period of subfertility. Similarly, studies of BWS patients with loss of maternal methylation at *KCNQ1OT1* showed that approximately 25% had hypomethylation at multiple, additional maternally imprinted loci [145, 146]. Again, only a proportion of patients were conceived following ART. These observations suggest that ART is not specifically associated with maternal hypomethylation syndromes.

An association between ART and other imprinting disorders such as Silver-Russell syndrome and retinoblastoma has also been suggested but not established.

The association of imprinting disorders and ART appears to be related to subfertility, ovulation induction, and/or embryo culture. The exact mechanism for the association is not clear. *In vitro* embryo culture might predispose to loss of methylation. Alternatively, imprinting defects and subfertility might have a common cause, and treatment for infertility with ovarian hyperstimulation may further increase the risk of imprinting defects [141].

To date, the imprinting disorder that has the strongest evidence for an association with ART is BWS. Nevertheless, the

absolute risk of BWS after ART is estimated to be <1%; thus, routine screening of BWS in children born after ART does not appear to be warranted [142].

Cancer

Normal epigenetic modifications of DNA involve three types of changes: chromatin modifications, DNA methylation, and genomic imprinting. These are altered in cancer cells. The epigenetic dysregulation in cancer cells includes global genome hypomethylation, regional hypo- and hypermethylation, histone modification, and disturbed genomic imprinting [147, 148]. Thus, an altered genomic imprinting process is a common mechanism for cancer development.

Paraganglioma

A type of non-childhood tumor, paraganglioma (PGL) of the head and neck (glomus tumor), has been mapped to chromosome 11 at two distinct loci, 11q23 and 11q13.1, by linkage analysis [149, 150]. Approximately 30% of cases are familial. Mutation in *SDHD* (succinate dehydrogenase subunit D), a gene mapped to 11q23 that encodes a mitochondrial respiratory chain protein, has recently been reported in families with PGL [151–153]. Inheritance of PGL is autosomal dominant with both males and females affected. However, transmission is almost exclusively through the father, and only male gene carriers will have affected offspring. The disease is only very rarely observed in the offspring of affected females [149, 154–157]. These observations suggest genomic imprinting. However, expression of *SDHD* is biallelic (i.e., it is expressed from both maternal and paternal alleles) in all tissues studied to date (lymphoblastoid cell lines, adult brain, fetal brain, and kidney) [151]. Therefore, the mechanism for the observed genomic imprinting inheritance pattern of this tumor is as yet uncertain. It remains possible that imprinting of *SDHD* is tissue specific and may be restricted to the carotid body, the most common tumor site of PGL, and other paraganglionic cells.

Wilms Tumor/Rhabdomyosarcoma

In a number of embryonal tumors, loss of heterozygosity (LOH) of a specific parental allele has been observed. In all cases studied, the maternal allele is preferentially lost. This suggests that duplication of some paternal alleles results in enhanced cell proliferation, while duplication of certain maternal alleles may inhibit cell proliferation.

In Wilms tumor and rhabdomyosarcoma, LOH involves chromosome 11 [158–160]. LOH does not involve markers for 11p13, the proposed Wilms tumor locus, but only markers on 11p15.5 [159]. Known imprinted genes in the 11p15.5 region include *H19*, *IGF2*, and *CDKN1C* (*p57^{KIP2}*) (see previous). The expression of *CDKN1C* is reduced in Wilms

tumor [135]. In addition, by using several overlapping subchromosomal transferable fragments from 11p15 distinct from *H19* and *IGF2*, Koi et al. were able to obtain *in vitro* growth arrest of rhabdomyosarcoma cells [161]. These observations suggest that *CDKN1C*, which is normally active on the maternal allele only, may be a candidate for a tumor suppressor gene. Loss of the active *CDKN1C* allele on the maternal chromosome results in tumor development. Besides LOH, another possible mechanism, loss of imprinting (LOI; see BWS), has been proposed. Ogawa et al. reported biallelic *IGF2* RNA synthesis in 4 of 30 Wilms tumors they studied [162]. Thus, “relaxation” of *IGF2* gene imprinting on the maternal allele has occurred, resulting in its expression. This would be equivalent to having two copies of an active *IGF2* gene, as would occur with a paternal duplication or with paternal UPD. A similar biallelic expression of *IGF2* was reported in 30% of breast cancer patients studied [163]. Disruption of the imprinting mechanism (i.e., LOI) may therefore also play a role in tumorigenesis. A third possible mechanism has also been proposed in a proportion of Wilms tumor patients. In some patients, LOI was observed in both the Wilms tumor tissue and the normal adjacent kidney tissue, but *IGF2* expression was significantly higher in tumor tissue. The overexpression in tumor tissue was accompanied by activation of all four *IGF2* promoters [164]. These studies indicate that while genomic imprinting plays an important role in tumorigenesis, a single mechanism does not account for all cases.

Retinoblastoma/Osteosarcoma

In retinoblastoma and osteosarcoma, loss of both functional copies of the retinoblastoma gene (*RBI*) on chromosome 13 at band q14, usually by mutation or deletion, has been observed [165]. In familial cases, a mutation in one of the alleles is present in the germline. *De novo* mutations in the germline occur preferentially in the paternal chromosome, consistent with the general observation that new germline mutations arise predominantly during spermatogenesis [166, 167]. In sporadic, nonfamilial tumors, loss of function of both alleles occurs somatically. In sporadic osteosarcomas, the initial mutation occurs preferentially on the paternal chromosome 13, suggesting that genomic imprinting may be involved [168]. In sporadic retinoblastoma, epigenetic change with hypermethylation of the *RBI* gene was reported in 9% of cases [169]. *RBI* is a tumor suppressor gene, and it has been recently shown to be imprinted [170]. These observations suggest a role of genomic imprinting in retinoblastoma.

Neuroblastoma

Neuroblastoma is the most common extracranial tumor of childhood. Deletions of chromosome 1p and amplification of the *MYCN* gene on chromosome 2p are frequently seen in

neuroblastoma [171]. Preferential amplification of the paternal *MYCN* allele in neuroblastoma tumor tissues has been reported [172]. In tumors with *MYCN* amplification, loss of parental 1p alleles was found to be random [172, 173]. In tumors without *MYCN* amplification, loss of 1p was previously reported to be preferentially maternal (16 of 17 cases) but random in a later study that suggested no imprinted gene in this region [173, 174]. On the other hand, the *TP73* tumor suppressor gene located at 1p36.32 has been shown to be imprinted (maternal active) [32].

An imprinted gene cluster at 14q32.2 that includes two closely linked but reciprocally imprinted genes, *DLK1* (paternal active) and *MEG3* (also known as *GTL2*) (maternal active), has been identified [175]. These two genes have similarities to *IGF2* and *H19*, genes involved in BWS (see previous section), respectively. Both *MEG3* and *H19* (gross suppressors) are maternally expressed, and both *DLK1* and *IGF2* (growth promoters) are paternally expressed. Hypermethylation of the *MEG3* promoter differentially methylated region was associated with *MEG3* transcriptional repression and was detected in 5 of 20 (25%) neuroblastomas tumors [176]. Therefore, loss of *MEG3* expression may also contribute to tumorigenesis in a subset of human cancers.

Uniparental Disomy

The term uniparental disomy (UPD) was introduced by Engel in 1980 [177]. It describes a phenomenon in which both homologs or homologous segments of a chromosome pair are derived from a single parent. An example of the latter is the paternal UPD for 11p15 in BWS described previously. Discussion here will be restricted to uniparental disomies for entire chromosomes, of which there are two types. Uniparental isodisomy describes a state in which both copies of a chromosome are not only derived from one parent but also represent the same homolog (i.e., two copies of the same exact chromosome). Uniparental heterodisomy refers to both of one parent’s homologs being represented (i.e., both chromosomes of the pair from the same parent). The type of UPD present is not always readily apparent, and it should be noted that, because of the recombination that takes place during meiosis, UPD along the length of an involved chromosome pair can be iso- for certain loci and hetero- for others.

UPD for an entire chromosome can occur as a result of gamete complementation, as suggested by Engel [177]. Since aneuploidy is relatively frequent in gametes, the chance union of two gametes, one hypo-, the other hyper-haploid for the same chromosome, will result in a diploid zygote with UPD for that chromosome. Structural rearrangements, such as Robertsonian or reciprocal translocations (see Chap. 9),

increase the chance of meiotic malsegregation and thus may predispose to UPD. This is best illustrated by the case reported by Wang et al., in which UPD for chromosome 14 was observed in a child with a paternal (13;14) Robertsonian translocation and a maternal (1;14) reciprocal translocation [177] (see Fig. 20.3 and Chaps. 3 and 9). Studies in animals also support this concept. Maternal or paternal disomies are readily produced in mice with intercrosses between either Robertsonian or reciprocal translocation carriers [14].

Another mechanism for the occurrence of UPD is by “trisomy rescue” [178]. The vast majority of trisomic conceptuses are nonviable; they may survive to term only if one of the trisomic chromosomes is postzygotically lost. In one-third of these cases, such loss will result in UPD in the now disomic cells (Fig. 20.4). Since the loss occurs postzygotically, mosaicism in such conceptuses is often observed, with the trisomic cell line sometimes confined to the placenta (see Chap. 12). Another way of “rescuing” a

trisomic conceptus is by forming a smaller marker chromosome from one of the trisomic chromosomes after losing most of its active genetic material. If the one chromosome that rearranged and became the marker chromosome is the single chromosome contributed by one parent, the remaining two of the trisomic chromosomes will be from the same parent and thus represent UPD for this chromosome pair.

A third possible mechanism for the occurrence of UPD is “monosomy rescue,” the duplication of the single chromosome in monosomic conceptuses [179]. In this case, uniparental isodisomy for the entire chromosome would be observed.

Two mechanisms contribute to the phenotypic effects of UPD. Unmasking of a recessive gene can occur as a result of uniparental isodisomy, in which the disomic chromosomes are homozygous. This was illustrated initially in an individual with cystic fibrosis who had maternal uniparental isodisomy for chromosome 7 and later in many other patients with

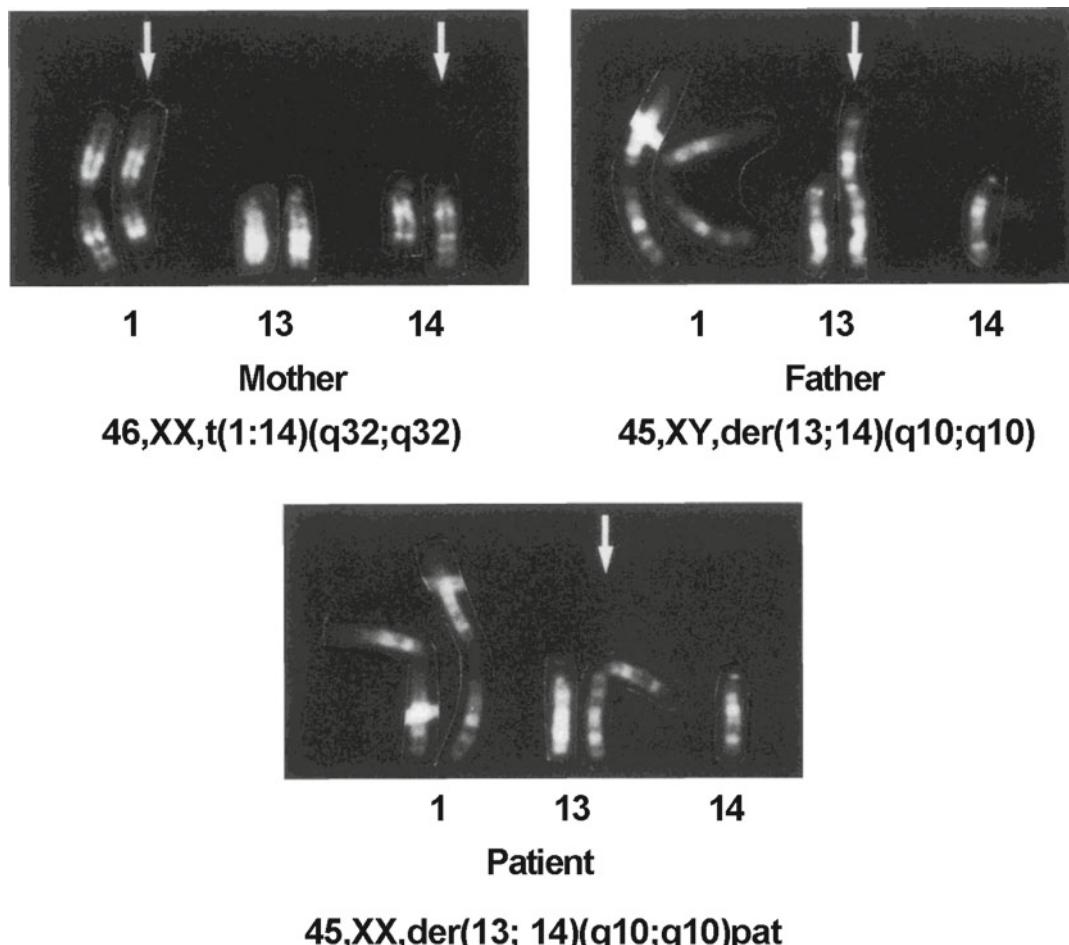
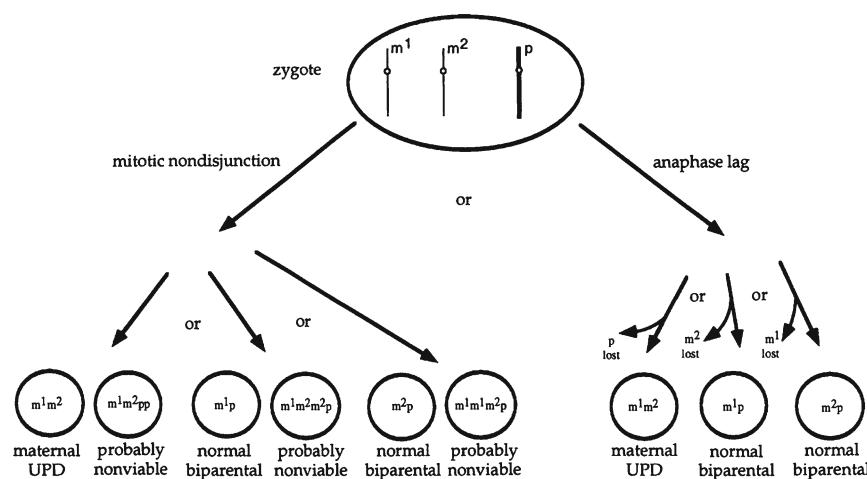


Fig. 20.3 An example of paternal UPD formation by gamete complementation. Malsegregation involving chromosome 14 occurred in both parents as the result of structural rearrangements. *Mother*: reciprocal translocation t(1;14)(q32;q32). *Father*: Robertsonian translocation

der(13;14)(q10;q10). The patient inherited both chromosomes 14 from the father and neither from the mother. Segregation is normal for chromosome 13 in the mother and for chromosome 1 in the father. Chromosomes are Q-banded

Fig. 20.4 A diagrammatic representation of maternal UPD formation by “trisomy rescue.” A trisomic zygote resulting from maternal meiosis I nondisjunction is depicted here. Loss of one of the trisomic chromosomes through either mitotic nondisjunction or anaphase lag results in euploidy. Uniparental disomy occurs in one-third of these cases. m^1 and m^2 maternally derived chromosomes, p paternally derived chromosome



recessive disorders and UPD (see later in chapter) [179]. The second mechanism is the effect caused by imprinted genes on the involved chromosome. This is best illustrated by PWS/AS patients who have no deletion of 15q11.2, but rather have UPD, as discussed previously. In addition to these two mechanisms, in cases where UPD arises as a result of “trisomy rescue,” the presence of a mosaic trisomic cell line in the placenta and/or fetus may modify the phenotype.

Of the 47 possible types of UPD of whole chromosomes, 36 have been reported to date. Some provide clear evidence for imprinting and some seem to suggest no such effect, while others will require accumulation of additional data before their status in this regard can be determined.

upd(1)mat

At least seven cases of maternal UPD for chromosome 1 have been reported. One patient had lethal autosomal recessive Herlitz-type junctional epidermolysis bullosa as a result of homozygosity for a nonsense mutation in the *LAMB3* gene on chromosome 1 [180]. The mother was a heterozygous carrier for the mutation and the father had two normal *LAMB3* alleles. The patient died at 2 months of age. Autopsy was not performed but weight and length were reportedly normal, and no overt dysmorphisms or malformations were noted. Another child with Chediak-Higashi syndrome (CHS) was found to be homozygous for a nonsense mutation in the *LYST* gene for CHS on chromosome 1 [181]. The mother was a carrier of the mutation, while the father had two normal *LYST* alleles. Two additional unrelated patients had lethal trifunctional protein deficiency due to homozygous alpha-subunit mutations. In both patients, the mothers were heterozygous for the mutation and the fathers did not have the mutation [182]. One patient had Zellweger syndrome due to homozygosity for a maternally inherited mutation in *PEX10*, one of

the peroxisome biogenesis genes [183]. A boy had autism whose mother and brother also had autistic features; the brother did not have UPD [184]. Another case involved a patient with insulin-dependent diabetes mellitus. Maternal UPD for chromosome 1 was accidentally discovered during a family linkage study [185]. This patient was developmentally and mentally normal at age 23. Therefore, maternal UPD for chromosome 1 does not appear to have an imprinting effect.

upd(1)pat

At least seven cases of paternal UPD for chromosome 1 have been reported. A 7-year-old boy presented with pycnodysostosis as a result of a homozygous mutation of the cathepsin K gene, for which the father was a heterozygote and the mother was normal [186]. The child was otherwise developmentally normal. Five additional patients, one with congenital insensitivity to pain with anhidrosis, two with Herlitz junctional epidermolysis bullosa, one with Leber congenital amaurosis, and one with hemolytic-uremic syndrome, were reported [187–191]. All five had paternal isodisomy for chromosome 1. None had any overt dysmorphisms or malformations. Their phenotype resulted from having two copies of the mutated recessive genes, both inherited from their fathers. Another patient was a 43-year-old female with short stature, ptosis, micro-/retrognathia, scoliosis, hearing loss, myopathy, and infertility. She has isochromosomes for the short arm and long arm of chromosome 1 [i(1)(p10), i(1)(q10); see Chaps. 3 and 9] [192]. It was not clear whether the abnormal phenotype in this woman resulted from an imprinting effect or from homozygosity for some undetected recessive alleles. These observations provide no clear evidence for an imprinting effect of paternal UPD 1.

upd(2)mat

Maternal UPD for chromosome 2 has been reported in at least ten cases. Four cases were associated with confined placental mosaicism (CPM) for trisomy 2. Two cases, one with and one without phenotypic abnormalities, resulted from *de novo* isochromosome formation of the short arm and long arm of chromosome 2 [i(2)(p10),i(2)(q10); see Chaps. 3 and 9]. One case with no phenotypic abnormalities was discovered at age 3 during paternity testing. Three cases had an autosomal recessive disorder due to a maternally inherited homozygous mutation; two of these were unrelated patients with lethal mitochondrial trifunctional protein deficiency as a result of mutations in the *HADHA* gene, and one patient had infantile-onset ascending spastic paralysis caused by mutations in the gene *ALS2* [182, 193–200]. No phenotypic abnormalities were reported other than those associated with the specific autosomal recessive disorders. A common phenotype was observed in the four cases associated with CPM and one of the two cases with isochromosomes. This includes intrauterine growth restriction (IUGR), oligohydramnios, pulmonary hypoplasia, hypospadias (in two patients), and normal development in the four surviving patients at ages 6, 20, 31 months, and 8 years, respectively. IUGR, oligohydramnios, and pulmonary hypoplasia can be explained by placental dysfunction as a result of trisomy 2 mosaicism. However, these same features were also present in one of the two cases with isochromosomes, suggesting a possible imprinting effect of maternal UPD 2 [198]. In another case reported recently, UPD for maternal 2q and paternal 2p was detected in a 36-year-old woman with normal physical and mental development [201]. Therefore, it is not clear whether maternal UPD 2 confers an imprinting effect.

upd(2)pat

At least three cases of paternal isodisomy for the entire chromosome 2 have been reported. A 34-year-old woman diagnosed with retinitis pigmentosa was found to have a homozygous *MERTK* mutation [190]. She was otherwise phenotypically normal. The patient's father was heterozygous for the mutation and the mother did not carry the mutation. Two other cases, one with Crigler-Najjar syndrome type I due to a paternally inherited homozygous *UGT1A1* mutation at 2q37 and one with Donnai-Barrow syndrome (faciooculoacousticorectal syndrome) due to homozygous mutation in the *LRP2* gene at chromosome 2q31.1, had no phenotypic abnormalities other than the disease-related findings [202, 203]. These cases further illustrate unmasking of autosomal recessive disorders as a result of uniparental disomy. An additional case with isodisomy for paternal 2p as described under upd(2)mat was

phenotypically normal. Paternal UPD 2 therefore does not appear to have an imprinting effect.

upd(3)mat

At least two cases of maternal isodisomy for chromosome 3 have been reported. Both cases presented with autosomal recessive disorder as a result of homozygous mutations: one dystrophic epidermolysis bullosa with no additional phenotypic abnormalities and one Fanconi-Bickel syndrome, a rare disorder with growth failure, hepatomegaly, renal Fanconi syndrome, and abnormal glucose homeostasis caused by mutations in *SLC2A2* (previously *GLUT2*) at 3q26.1-3q26.3 [204, 205]. Another case with confined placental mosaicism (CPM, see Chap. 12) for trisomy 3 detected by chorionic villous biopsy and a marker of chromosome 3 origin detected by a subsequent amniocentesis was found to have maternal uniparental disomy for the two chromosomes 3 [206]. The fetus had IUGR and microcephaly that could be attributed to CPM and the marker chromosome. There is no clear evidence that maternal UPD 3 has an imprinting effect.

upd(3)pat

A case of paternal UPD for chromosome 3 was detected serendipitously during a whole genome linkage study [207]. No apparent phenotypic abnormality was observed.

upd(4)mat

A case of maternal UPD for chromosome 4 as a result of isochromosome formation of the short arm and long arm of chromosome 4 [i(4)(p10),i(4)(q10); see Chaps. 3 and 9] was reported in an abstract [208]. Cytogenetic studies were performed because of multiple early miscarriages. The patient was otherwise phenotypically normal. Two additional cases of maternal isodisomy have been reported: one patient had afibrinogenemia as a result of a maternally inherited homozygous mutation of the fibrinogen alpha-chain gene at 4q28; the other adult female patient had a history of major depressive disorder and multiple suicide attempts but normal fertility and no major medical complaints [209, 210]. Another case with confined placental mosaicism for trisomy 4 in a fetus with intrauterine growth restriction and oligohydramnios followed by intrauterine fetal death at 30 weeks of gestation was determined to have maternal UPD 4 [211]. No external malformations were detected in this stillborn. There is no clear evidence to date that maternal UPD for chromosome 4 confers an imprinting effect.

upd(5)pat

Paternal UPD for chromosome 5 was reported in a child with autosomal recessive spinal muscular atrophy [212]. The child had no other developmental abnormalities. Spinal muscular atrophy in this case can be explained by the paternal transmission of two copies of the defective gene. There is no evidence for an imprinting effect.

upd(6)mat

Maternal uniparental isodisomy for chromosome 6 was first identified in a renal transplant patient in the process of HLA typing [213]. Another patient with congenital adrenal hyperplasia resulting from unmasking of the maternally inherited mutation in the 21-hydroxylase gene had intrauterine growth restriction but good catch-up growth [214]. There is no clear evidence for an imprinting effect.

upd(6)pat

More than 15 cases of paternal uniparental disomy for chromosome 6 have been reported (reviewed in references [215–219]). All except one were isodisomy. Many patients had transient neonatal diabetes mellitus (TNDM) associated with very low birth weight. Two genes, an imprinted cell cycle control gene *PLAGL1* (also known as *ZAC*) and hydatidiform mole associated and imprinted gene *HYMA1* (untranslated with unknown function) at 6q24 with differential methylation of parental alleles, have been identified [220–222]. These genes are expressed only from the paternal allele and are potential candidate genes for TNDM. Increased expression of this gene by paternal UPD appears to result in the diabetic phenotype. It was estimated that paternal UPD 6 accounts for approximately 15–20% of cases of TNDM [221, 223]. Paternal UPD 6 clearly has an imprinting effect.

upd(7)mat

More than 60 patients with maternal UPD for chromosome 7 have been reported in the literature [27, 224–227]. This was the first documented UPD in humans, identified initially in two individuals with cystic fibrosis and short stature [179, 228]. Approximately 10% of patients with Silver-Russell syndrome (SRS) are noted to have maternal UPD 7 [225, 226, 229, 230]. SRS is a heterogeneous disorder. The clinical phenotype includes intrauterine growth restriction and persistent postnatal growth delay, body asymmetry, triangular face, prominent forehead, decreased subcutaneous tissue, delayed bone age, and usually normal intelligence.

Three regions on chromosome 7 have been shown to contain imprinted genes [231, 232]. One region at 7p12 contains an imprinted gene *GRB10* (growth factor receptor-binding protein 10), a known growth suppressor that is expressed on the maternal allele and is therefore one of the candidate genes for SRS [233–235]. A second region at 7q32.2 contains a number of other imprinted genes including *CPA4*, *MEST* (also known as *PEG1*), and *COPG2IT1*. *CPA4* was proposed to be a candidate for SRS [236]. The role of the other genes is not yet clear [236–239]. A third region at 7q21.3 contains three imprinted genes *SGCE* (epsilon-sarcoglycan, maternally imprinted), *PEG10*, and *PPP1R9A*. Mutation of the *SGCE* gene causes an autosomal dominant movement disorder myoclonus-dystonia. A 36-year-old man with both SRS and myoclonus-dystonia has been reported [232].

SRS is also the first human disorder with imprinting disturbances that affect two different chromosomes: chromosomes 7 and 11 [240]. Approximately 40% of SRS show hypomethylation in the IC region upstream of *H19* at 11p15.5, which results in overexpression of the growth suppressor *H19*. Hypermethylation of the same IC region, on the other hand, is associated with the overgrowth disorder, BWS (see section “[Chromosome Deletion/Duplication Syndromes](#)”). These two developmental syndromes, SRS and BWS, are therefore clinically and epigenetically opposite diseases (reviewed in [241]).

Maternal UPD 7 clearly has an imprinting effect.

upd(7)pat

At least four cases of paternal isodisomy for the entire chromosome 7 have been reported. One patient had recessive congenital chloride wasting diarrhea with normal growth and development [242]. One patient had cystic fibrosis as a result of inheriting two copies of the ΔF508 mutation from his father. This patient also had complete *situs inversus* and immotile cilia with growth retardation and significant respiratory disease [243]. The other two patients also had cystic fibrosis. One of them had normal growth and the other had overgrowth and developmental delay [244, 245]. In addition, two patients had paternal isodisomy 7p and maternal isodisomy 7q [246, 247]. These two patients had similar phenotypes that resembled the phenotype seen in maternal UPD 7, and their growth retardation was considered to be a result of maternal isodisomy for 7q. It is not clear whether paternal UPD 7 confers an imprinting effect.

upd(8)mat

One case of maternal isodisomy for the entire chromosome 8 has been reported [248]. The patient was a 39-year-old male with normal appearance, stature, and intelligence. He had

early-onset ileal carcinoid, slight thoracic scoliosis, and numerous pigmented nevi. More cases are needed before a conclusion can be drawn as to whether maternal UPD 8 has an imprinting effect.

upd(8)pat

A single case of paternal uniparental isodisomy for chromosome 8 has been reported [249]. This five-and-a-half-year-old girl had normal development and lipoprotein lipase (LPL) deficiency due to a mutation of the *LPL* gene. The patient was ascertained due to a diagnosis of chylomicronemia. The father was a heterozygous carrier for the same mutation. It appears that normal development can occur in paternal UPD 8 and that an imprinting effect of this UPD may not exist.

upd(9)mat

At least seven cases of maternal UPD for chromosome 9 have been reported. Two patients had recessive cartilage-hair hypoplasia, a disorder that maps to the short arm of chromosome 9 [250]. Two homozygotic female twins had Leigh syndrome as a result of inheriting two copies of the mutated *SURF1* gene from their mother [251]. Both twins died of respiratory failure at age 3. No gross dysmorphic features or malformations were noted apart from Leigh syndrome. One case involved a fetus associated with confined placental mosaicism (see Chap. 12) for trisomy 9 [252]. Pathological examination of the abortus was not possible. One case of syndromic congenital hypothyroidism, characterized by thyroid dysgenesis, cleft palate, spiky hair, and choanal atresia and bifid epiglottis who was homozygous for a maternally inherited *FOXE1* (9q22) mutation, has been reported recently [253]. Another 34-year-old healthy woman with recurrent spontaneous abortions had isochromosomes of the short and long arms of chromosome 9 [i(9)(p10),i(9)(q10); see Chaps. 3 and 9]. Molecular analysis demonstrated maternal isodisomy [254]. The available data indicate that maternal UPD 9 may not have an imprinting effect.

upd(10)mat

A case of prenatally diagnosed maternal UPD for chromosome 10 associated with confined placental mosaicism (see Chap. 12) has been reported [255]. The infant was phenotypically and developmentally normal at 8 months of age. Another case of familial hemophagocytic lymphohistiocytosis characterized by lethal primary immunodeficiency was reported in a patient who was homozygous for the perforin gene (*PRF1*) at 10q22 as a result of maternal uniparental disomy for chromosome

10 [256]. Two other cases of maternal UPD 10 reported were associated with either a marker chromosome 10 or a trisomy 10 cell line, and the abnormal phenotypes were attributed to the karyotypic abnormalities. There is no evidence to date that this UPD confers an imprinting effect.

upd(11)pat

Paternal UPD for the entire chromosome 11 has been reported in at least three cases. One patient had hemihypertrophy, congenital adrenal carcinoma, and Wilms tumor [257]. The second had associated confined placental mosaicism (see Chap. 12) for trisomy 11, and intrauterine death occurred between 19 and 20 weeks gestation. This fetus had growth restriction, aberrant intestinal rotation, and hypospadias [258]. The third patient had possible mosaic paternal isodisomy along the entire chromosome 11. The clinical findings in this patient did not differ from that of other BWS patients [259]. In addition, many cases of paternal segmental UPD for distal 11p associated with BWS have been observed (see previous section). The existence of an imprinting effect due to paternal UPD 11 is clear.

upd(12)mat

A case of maternal UPD for chromosome 12 was reported [260]. The infant had normal somatic and psychomotor development with no congenital anomalies or dysmorphic features at 6 weeks of age. Chromosome analysis demonstrated mosaicism with the presence in some cells of a small marker chromosome consisted of chromosome 12 centromeric heterochromatin with no euchromatic material. This suggests that the mechanism for the occurrence of UPD in this case is by trisomy rescue. It appears that maternal UPD 12 may not have an imprinting effect.

upd(13)mat

At least two cases of maternal UPD for chromosome 13 have been reported [261, 262]. In both cases, a normal phenotype was associated with the presence of an isochromosome for the long arm of chromosome 13. These indicate that an imprinting effect due to maternal UPD 13 is very unlikely.

upd(13)pat

At least six cases of paternal UPD for chromosome 13 have been reported. One was the mother of one of the maternal UPD 13 patients described previously [261]. This phenotypically normal individual presumably received the isochromosome

13q from her father, who was not available for study, but DNA polymorphism studies of her mother revealed the absence of maternal chromosome 13 alleles in this patient [263]. Four cases had *de novo* der(13;13)(q10;q10) translocations (see Chaps. 3 and 9) [264–266]. Three of them were complete isodisomies, and one exhibited evidence of recombination with proximal isodisomy and distal heterodisomy. All four patients were phenotypically normal. Another case of maternal UPD 13 was reported in a patient with hearing loss as a result of unmasking of the recessive gap junction protein *GJB2* gene at 13q11-q12 [266]. Therefore, paternal UPD 13 does not appear to have an imprinting effect.

upd(14)mat

Maternal UPD for chromosome 14 has been reported in many cases (reviewed in [267–275]). Although many are associated with Robertsonian translocations involving chromosome 14, cases with a normal karyotype have also been observed. A distinct clinical phenotype is present and consists of intrauterine growth restriction, mild-to-moderate motor and/or mental developmental delay, hypotonia, short stature, and precocious puberty. Less frequent findings include hydrocephalus, dysmorphic features (prominent forehead, supraorbital ridge, short philtrum, downturned corner of mouth), small hands, hyperextensible joints, scoliosis, and recurrent otitis media.

Chromosome 14 contains a cluster of imprinted genes at 14q32.2 including *RTL1* and *DLK1* (both paternal active) and *MEG3* (also known as *GTL2*, maternal active) (see also previous “**Neuroblastoma**” section). They are regulated by a differentially methylated region (DMR) between *RTL1/DLK1* and *MEG3* genes (intergenic DMR). A number of patients with biparental inheritance of chromosome 14 but with a clinical phenotype similar to that of maternal uniparental disomy have been found to have various deletions in the imprinted region or hypomethylation of the intergenic and the *MEG3* DMRs [273–278]. These findings indicate that abnormal methylation patterns of the imprinted genes are associated with the maternal UPD 14 phenotype. Therefore, methylation analysis of the imprinted gene *MEG3* can be performed to detect upd(14)mat and to determine the molecular basis in patients with a phenotype similar to upd(14)mat but who do not have UPD [273, 274].

Evidence for an imprinting effect due to maternal UPD 14 is clear.

upd(14)pat

Many cases of paternal UPD for chromosome 14 have been reported [279–284]. Most are associated with Robertsonian

translocations involving chromosome 14; cases with a normal karyotype have also been observed [285]. A similar phenotype is present in these patients and includes polyhydramnios, low birth weight, hirsute forehead, blepharophimosis/short palpebral fissures, protruding philtrum, small ears, small thorax, abnormal ribs, simian creases, and joint contractures. Severe mental retardation was seen in a patient who was beyond 20 months of age at the time of reporting [279]. These observations indicate that an imprinting effect due to paternal UPD 14 exists.

Studies comparing maternal and paternal UPD cases with cases of partial trisomy and partial monosomy of various segments of 14q have suggested that 14q23-q32 may be the region where the imprinted genes on chromosome 14 reside [286, 287]. Further studies of segmental and full paternal isodisomy for chromosome 14 indicated imprinted genes at 14q32 as the critical components of the phenotype observed in upd(14)pat [288]. This is consistent with the observation that overexpression of the paternally active gene *RTL1* plays a major role in the upd(14)pat phenotype [277] (see also previous section discussing maternal UPD 14).

Human chromosome 14 has significant homology to mouse chromosomes 12 and 14 [289]. Mouse chromosome 12 is imprinted, and both maternal and paternal disomies cause early embryonic death [290]. Thus, the observation of imprinting effects for both maternal and paternal UPD 14 in humans is not unexpected.

upd(15)mat

More than 100 cases of maternal UPD for chromosome 15 have been reported in the literature in association with Prader-Willi syndrome [86, 89, 291, 292] (see section “**Prader-Willi Syndrome/Angelman Syndrome in Genomic Imprinting and Human Diseases**”). As previously discussed, UPD(15) mat accounts for approximately 20–25% of patients with PWS. Many patients had associated trisomy 15 mosaicism, which was confined to the placenta in most cases. Comparison of the phenotypes of PWS patients with different etiologies has shown that advanced maternal age was present in mothers of patients with maternal UPD, while a higher frequency of hypopigmentation is seen in patients due to deletion of paternal 15q11.2-q13 [291–293]. Advanced maternal age can be expected in UPDs that result from “trisomy rescue,” as advanced maternal age is associated with meiotic nondisjunction. Hypopigmentation results from mutation/deletion of the *OCA2* gene (mouse homolog pink-eyed dilution *p* gene) located at 15q11.2-q12 [294–296]. The human *OCA2* gene is not imprinted, and both copies are functional in UPD patients. Hypopigmentation is therefore more prominent in PWS patients due to deletion. Differences in other clinical features between these two groups are less clear-cut. While

there may not be a significant difference in the overall severity, female UPD patients were found to be less severely affected than female deletion patients [291]. UPD patients were found to be less likely to have “typical” facial appearance, somewhat higher IQ, and milder behavior problems and more likely to have psychosis and autism spectrum disorders [292, 297, 298]. Deletion patients have a higher frequency of sleep disturbance, hypopigmentation, and speech articulation defects [299]. These differences can again be attributed, at least partially, to the presence of two copies of non-imprinted genes in UPD cases, whereas there is haploinsufficiency of these genes in the deletion cases.

upd(15)pat

Many cases of paternal UPD for chromosome 15 associated with Angelman syndrome have been reported in the literature [88, 90, 300–302]. Paternal UPD 15 accounts for approximately 6% of AS patients. AS patients with paternal UPD may have a milder phenotype than those with a maternal deletion of 15q11.2-q13; UPD patients have better physical growth, fewer movement abnormalities, less ataxia, and a lower prevalence of seizures [90, 300–303]. One possible mechanism for the milder phenotype in UPD patients may be the presence of many non-imprinted genes in the 15q11.2-q13 region in these patients, whereas these are absent in deletion patients. Alternatively, as proposed by Bottani et al., it may be due to the “leaky” expression of the imprinted paternal genes, where two copies of the allele will result in an expression higher than in deletion cases, in which only one imprinted paternal allele is present [90].

Both maternal and paternal UPD 15 clearly confer imprinting effects.

upd(16)mat

More than 20 cases of maternal UPD for chromosome 16 have been described, and potentially many more cases are not reported [304–312]. Again, associated trisomy 16 mosaicism, usually confined to the placenta, is present in most cases. A clinical phenotype of maternal UPD 16 has not been clearly defined; the possibility of the presence of an undetected trisomy 16 cell line complicates the comparison among reported cases. IUGR is a frequent finding. IUGR may result from the presence of trisomy 16 cells in the placenta; however, no catch-up growth was observed in these patients [304]. Development has been normal in all cases, the oldest reported at 4 years of age. Imperforate anus has been reported in three cases, hypospadias in two, and congenital cardiac anomalies were observed in five cases, with an A-V canal defect in one and ASD and VSD in four. Subtle but

apparently characteristic facial dysmorphisms (slightly upslanted palpebral fissures, almond-shaped eyes, broad nasal root, upturned nares, long philtrum, thin upper lip, prominent ears, and triangular face) may exist [307, 309, 311]. In addition, in a later study, statistical analysis performed on a large series of mosaic trisomy 16 cases with molecular determination of UPD status indicated that upd(16) mat was associated with fetal growth restriction and with increased risk of major malformation [313]. One protein coding gene, *ZNF597* at 16p13.3, has been found to be imprinted (maternal allele active) [32]. Although not yet certain, the existence of an imprinting effect due to maternal UPD 16 is a distinct possibility.

upd(16)pat

A single case of paternal UPD for chromosome 16 has been reported [314]. This case was associated with confined placental mosaicism (see Chap. 12). Paternal isodisomy for chromosome 16 was prenatally diagnosed and confirmed after birth. Intrauterine growth restriction was present with catch-up growth observed at 13 months of age. Minor physical abnormalities included bilateral pes calcaneus and additional rudimentary mandibular dental arch. Psychomotor development was normal. It is not clear whether paternal UPD 16 has an imprinting effect.

upd(17)mat

A case of maternal UPD involving the entire chromosome 17 was reported in a 2-year-old boy with trisomy 17 confined placental mosaicism (see Chap. 12) [315]. His growth and psychomotor development was normal. Another case with infantile nephropathic cystinosis as a result of a homozygous 57-kb deletion encompassing the *CTNS* gene at 17p13 was recently reported [316]. The mother was heterozygous for the deletion and the father did not carry the deletion. The child had maternal UPD for chromosome 17, and the abnormal phenotype was resulted from unmasking of the recessive gene. There is no evidence that maternal UPD 17 confers an imprinting effect.

upd(20)mat

At least three cases of maternal UPD 20 have been reported [317–319]. One of them was associated with a mosaic cell line containing a small marker chromosome consisting of the pericentromeric region of chromosome 20, and another was associated with confined placental mosaicism (see Chap. 12) for trisomy 20. The common features in these three patients at

ages 4 years, 35, and 17 months, respectively, are pre- and postnatal growth retardation. Isolated findings included mild facial dysmorphism, strabismus, microcephaly, macrocephaly, developmental delay, and hyperactivity. A further case involved a live-born girl with maternal uniparental isodisomy of chromosome 20 in the diploid cell line who had moderate psychomotor retardation, central hypotonia with peripheral hypertension, multiple minor dysmorphism, and marked kyphosis [320]. However, the clinical phenotype in this patient was complicated by the presence of a trisomy 20 cell line in both blood and urine specimens. Imprinted genes have been found on chromosome 20; available clinical information in the literature unfortunately does not unequivocally support the possibility that maternal UPD 20 has an imprinting effect [32].

upd(20)pat

No pure paternal UPD involving the entire chromosome 20 has been reported. One case had a structurally abnormal chromosome 20 derived from a terminal rearrangement that joined two chromosomes 20 at band p13 [45,XY,psu dic(20;20)(p13;p13)] [321]. DNA polymorphism studies indicated that the two chromosomes 20 in this terminal rearrangement were derived from one paternal chromosome, thereby representing paternal isodisomy. The patient had multiple anomalies including microtia/anotia, micrencephaly, congenital heart disease, neuronal subependymal heterotopias, and colonic aganglionosis. However, this case was complicated by the presence of trisomy 20 cells in skin. Therefore, although an imprinting effect is possible for paternal UPD 20, a definitive conclusion cannot be drawn without further case reports.

upd(21)mat

Maternal UPD for chromosome 21 has been reported in at least two patients [322, 323]. Both had a balanced *de novo* (21;21) Robertsonian translocation (see Chaps. 3 and 9) and were phenotypically normal. Although maternal UPD 21 has been reported in early abortus specimens, it has not been possible to clearly attribute embryonal death to UPD [324]. Therefore, maternal UPD 21 may be considered at this time to have no imprinting effect.

upd(21)pat

Two cases of paternal UPD for chromosome 21 have been reported [325, 326]. In both cases, UPD resulted from *de novo* formation of a Robertsonian translocation (see Chaps. 3 and 9). Both individuals were phenotypically normal. Paternal UPD 21 does not appear to have an imprinting effect.

upd(22)mat

Maternal UPD for chromosome 22 not associated with mosaic trisomy 22 has been reported in three cases [327–329]. All three phenotypically normal individuals were ascertained via history of multiple spontaneous abortions and were found to have balanced (22;22) Robertsonian translocations (see Chaps. 3 and 9). Two other cases of maternal uniparental heterodisomy for chromosome 22 associated with prenatally diagnosed mosaic marker chromosome 22 were reported: one male infant had no dysmorphic features at birth and reportedly normal development at age 6 months and the other, a girl, had normal development at 18 months of age [330, 331]. Additionally, a prenatally diagnosed case with nonmosaic trisomy 22 in placental tissue and apparently nonmosaic normal 46,XY cells in newborn blood had severe intrauterine growth restriction, first-degree hypospadias, and other features attributed to prematurity [332]. There is no evidence that maternal UPD 22 has an imprinting effect.

upd(22)pat

A single case of paternal UPD for chromosome 22 was reported in an abstract [333]. It was observed in a phenotypically normal individual with a balanced (22;22) Robertsonian translocation (see Chaps. 3 and 9). Paternal UPD 22 is not likely to have imprinting effect.

upd(X)mat

Maternal UPD for the two X chromosomes in females has been reported in three cases [334, 335]. The first two cases were detected by screening a normal population of 117 individuals. The third patient had Duchenne muscular dystrophy due to homozygosity of a maternally inherited deletion of exon 50 of the dystrophin gene. These observations indicate that maternal UPD for the X chromosome may not have an imprinting effect. To date, no imprinted genes on the X chromosome have been identified in humans.

upd(X)pat

A single case of paternal UPD for the two X chromosomes in the 46,XX cell line of a 14-year-old girl with 45,X/46,XX mosaicism (see Chap. 10) has been reported [336]. This patient had impaired gonadal function and short stature. The presence of a 45,X cell line makes it difficult to determine if the observed clinical features in this patient can be attributed to paternal UPD for the X chromosome. Therefore, it is unknown at this time if paternal UPD X has an imprinting

effect. However, no imprinted genes on the X chromosome have been identified in humans.

upd(XY)pat

A single case of paternal contribution of both the X and Y chromosomes in a male patient was reported in an abstract [337]. This patient was ascertained because he had hemophilia A, which was transmitted from his father. No abnormalities other than hemophilia were described. Paternal UPD for XY may therefore not have an imprinting effect.

Summary

In summary, of 47 possible maternal and paternal UPDs for whole chromosomes in humans, 36 have been reported. Among them, seven clearly have imprinting effects (6pat, 7mat, 11pat, 14mat, 14pat, 15mat, and 15pat), one potentially has an imprinting effect (16mat), 20 are unlikely to have imprinting effects [1mat, 1pat, 2pat, 3pat, 4mat, 5pat, 6mat, 8pat, 9mat, 10mat, 12mat, 13mat, 13pat, 17mat, 21mat, 21pat, 22mat, 22pat, Xmat, and XYpat], and the status is not known for 2mat, 3mat, 7pat, 8mat, 16pat, 20mat, 20pat, and Xpat at this time. A better understanding of the effects of UPD will be possible as more data are accumulated.

Prenatal UPD analysis should be considered when the risk for UPD involving chromosomes with known imprinting effects is increased. These include:

- Confined placental mosaicism (CPM; see Chap. 12) with a trisomic cell line for chromosomes 6, 7, 11, 14, or 15 (and possibly also 16) found in CVS but only normal cells in amniotic fluid.
- The presence of a supernumerary marker chromosome originating from one of these chromosomes.
- *De novo* or familial Robertsonian translocations (see Chaps. 3 and 9) involving chromosomes 14 or 15, especially when homologous.
- Abnormal prenatal ultrasound findings of features seen in known UPD syndromes.

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Sarah Hutchings Clark

Introduction

Genetic counseling, in the traditional sense, has been defined as a communication process, whereby individuals and families are educated about the genetic conditions in their families and about those for which they could be at risk. Genetic counseling, as its name implies, also involves addressing the psychosocial issues that accompany the diagnosis, or possible diagnosis, of such a condition. The counselor supports the family in learning about the diagnosis and in decision making about issues surrounding the diagnosis or potential diagnosis. However, genetic counseling is still a rapidly evolving field, with many counselors becoming increasingly involved in “nontraditional” roles.

In 1983, the National Society of Genetic Counselors (NSGC), the field’s professional membership organization, defined the role of genetic counselors:

Genetic counselors are health professionals with specialized graduate degrees and experience in the areas of medical genetics and counseling. Most enter the field from a variety of disciplines, including biology, genetics, nursing, psychology, public health and social work.

Genetic counselors work as members of a health care team, providing information and support to families who have members with birth defects or genetic disorders and to families who may be at risk for a variety of inherited conditions. They identify families at risk, investigate the problem present in the family, interpret information about the disorder, analyze inheritance patterns and risks of recurrence and review available options with the family. Genetic counselors also provide supportive counseling to families, serve as patient advocates and refer individuals and families to community or state support services. They serve as educators and resource people for other health care professionals and for the general public. Some counselors also work in administrative capacities. Many engage in research activities related to the field of medical genetics and genetic counseling [1].

Genetic counselors work in a variety of settings. There are genetic counselors who work primarily in the areas of prenatal, pediatric, adult, and cancer genetics, as well as public health, administration, research, and molecular and cytogenetic testing. Genetic counselors can be found in a variety of public and private medical settings, in state and federal offices, in research and diagnostic laboratories, and in health insurance companies. Some genetic counselors are certified by the American Board of Genetic Counseling (ABGC), the organization that is also responsible for the accreditation of genetic counseling graduate programs.

The term genetic counseling was first coined by Sheldon Reed in 1947. He was also vital in establishing the respect for counselees that is a cornerstone of the field of genetic counseling. Dr. Reed had a deep concern for the feelings of his patients, and he cared about how genetic conditions influenced their lives [2]. However, the roots of the field can be traced back to the early 1900s. At that time, people were not only concerned about elucidating the genetic mechanisms behind hereditary conditions but were also interested in eugenics. This interest facilitated tragic consequences, including the killing of thousands of people with genetic conditions, along with individuals of Jewish descent, in the Holocaust. Additionally, individuals with hereditary conditions or mental retardation were encouraged or forced not to reproduce. The field of genetics later rejected eugenics and moved away from this unfortunate past [3].

Formally speaking, genetic counseling is a relatively new field. The first class with a master’s degree in genetic counseling graduated from Sarah Lawrence College in 1971. In 1975, a formal definition of genetic counseling was proposed and adopted by the American Society of Human Genetics. In 1979, genetic counselors formed a professional society, the National Society of Genetic Counselors, which has played a critical role in establishing and furthering the profession [4].

Genetic counseling is based on the principles of nondirectiveness and a client-centered approach. The principle of nondirectiveness states that genetic counselors are to provide information in a way that does not encourage, or discourage,

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a certain course of action. In other words, genetic counselors do not tell their clients what decisions to make. That is not to say that genetic counseling should be devoid of guidance, particularly in complex situations, but that the counselor should provide guidance within the framework of the patient's beliefs and values. The counselor assists and supports the individual and family as they process the information provided during the counseling session and as they attempt to reach a decision regarding the course of action that is the most appropriate for them. To effectively and responsibly accomplish this, the counselor must have some understanding of the patient's "social, cultural, educational, economic, emotional, and experiential circumstances" [3]. This is by no means a simple task, particularly in light of the complex and powerful emotions that genetic conditions often evoke. By maintaining a client-centered approach, genetic counselors seek to empower their patients and to support and encourage them in their ability to make the best decisions for themselves in their own unique circumstances [3].

Components of a Genetic Counseling Session and the Role of the Genetic Counselor

The components of a genetic counseling session can vary widely depending upon the reason for the referral and the specific needs of the patient and/or family. However, several components are frequently part of the counseling session, particularly if it is the first time that the counselor and patient are meeting.

The first step is to elicit the patient's understanding of why he or she has been referred and to clarify the reason for referral, if necessary. The counselor also seeks to establish a mutually acceptable set of goals for the session and to understand the concerns of the patient and/or family. This is referred to as contracting.

During the majority of sessions, the genetic counselor obtains a detailed family, medical, and pregnancy history in the form of a pedigree (Fig. 21.1). In medical genetics, a pedigree is the accepted, standardized method of documenting the family history in the form of a diagram, which indicates the family members, their relationships to one another, their status with regard to the genetic condition or trait in question, and any other relevant medical issues. In addition to providing valuable information about the medical aspects of the family history, obtaining the information for the pedigree allows the genetic counselor to gain useful information about the dynamics of the family in general and in relation to the condition in question [5, 6]. The pedigree also often allows the counselor to begin to establish a relationship with the patient. Pedigrees, in varying forms, have been a part of genetics since the early days of the field. Interestingly, the history of the pedigree provides valuable insights into the evolution of the field of genetics [7].

As is likely apparent at this point, one major goal of a genetic counseling session is to provide information. Genetic counselors seek to convey relevant information in a manner that is clear and understandable to each individual patient or family member. Information is provided about the clinical features, natural history, and potential variability of the particular condition. Additionally, the genetic basis of the condition and mechanism by which it occurs, recurrence risks, available options for research and clinical testing, test results, evaluation, and treatment are discussed [3].

The presence of a genetic condition or birth defect in a family can have a significant impact on family relationships and on the way that the patient and family interact with society as a whole. Individuals and families facing a genetic condition are often in an emotionally vulnerable state. The emotions experienced by the individual and family can vary widely and can be extremely powerful. Feelings of guilt, stigmatization, and altered self-esteem are relatively common, whether the diagnosis of a genetic condition is made prenatally, during childhood, adolescence, or adulthood. Therefore, the counselor seeks to support the patient and family emotionally in an empathic manner and to advocate for them. In keeping with this goal, the potential impacts of the condition, including positive and negative economic, psychological, and social effects, and available resources to assist in dealing with the condition are presented to the individual and/or family [3]. It is important to realize that different individuals may have unique perceptions of and reactions to the information discussed during a genetic counseling session. Genetic counselors are trained to be sensitive to this fact and to remain nonjudgmental in the face of it.

General Indications for Referral to a Genetic Counselor

There are many indications for an appropriate referral to a genetic counselor. Several of the more common reasons for referral are addressed here. The indications that are specifically related to cytogenetic issues are introduced and are then discussed in additional detail in the following section.

Family History or Clinical Suspicion of a Genetic Syndrome or Chromosome Abnormality

The presence of certain birth defects (also known as congenital anomalies), mental retardation, and/or other characteristic features can raise the level of suspicion that an individual is affected with a genetic syndrome or chromosome abnormality. When possible, the identification of a cause for the congenital anomalies and/or mental retardation in an individual not only allows for genetic counseling

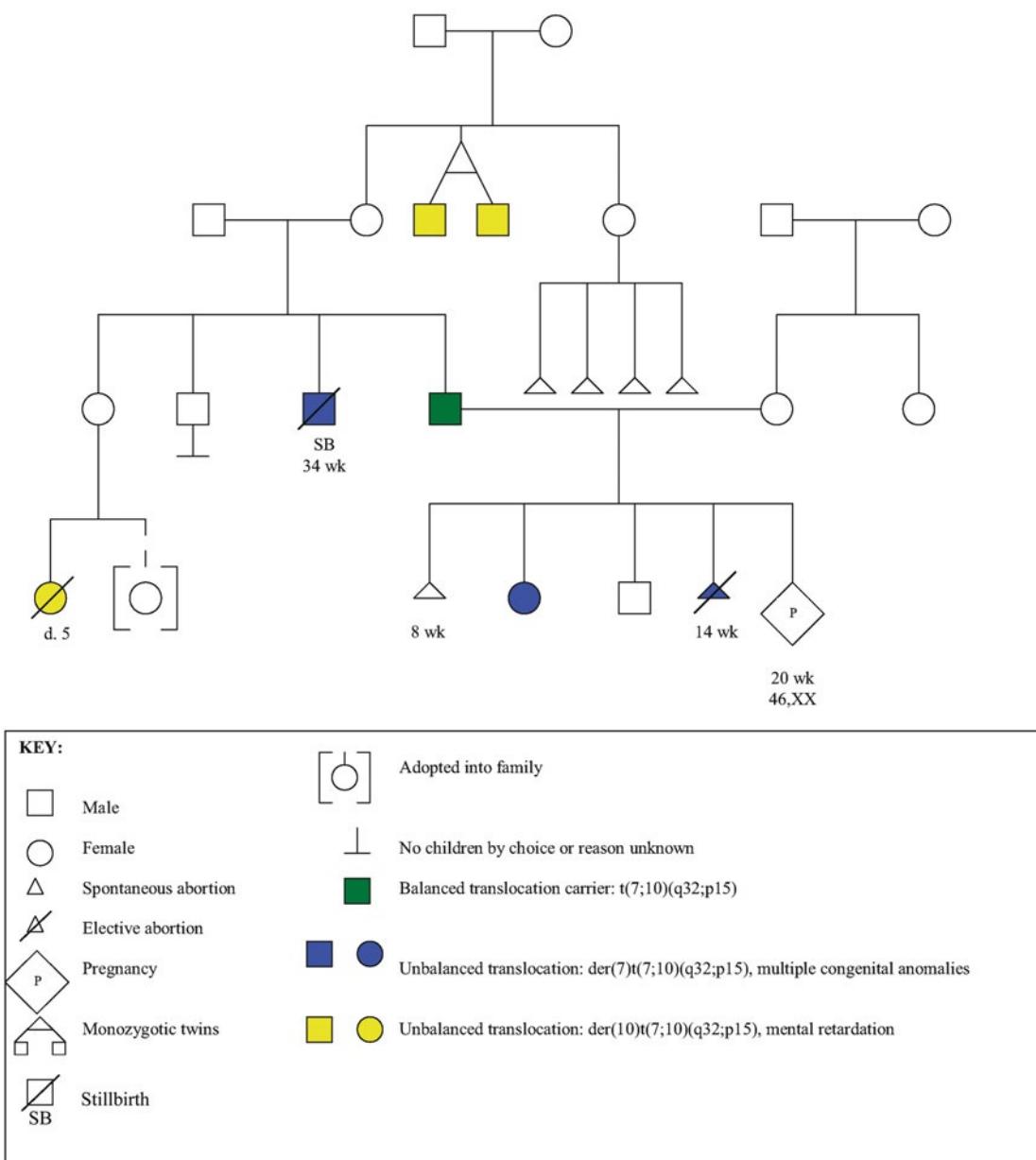


Fig. 21.1 Pedigree of a family carrying a balanced translocation involving the long arm of chromosome 7 and the short arm of chromosome 10. See key for interpretation of symbols

regarding recurrence risk but can also be important, psychologically and practically, for the individual and family. The evaluation of an individual to rule out the presence of a genetic condition often involves the evaluation of that individual by a medical geneticist. Certain biochemical, molecular, cytogenetic, and physiologic tests may also be helpful. The genetic counselor can be an important part of the health-care team that evaluates and cares for the patient. The counselor can aid the geneticist in his or her clinical evaluation of the patient, help to coordinate further testing, and help to keep the patient and/or family apprised of the need for such testing. The counselor can also help to keep the family informed of the possible conditions in the differential

diagnosis, assist in discussing test results, and support the individual and/or family emotionally.

Although beyond the scope of this book, it is important to recognize that genetic counselors routinely interact with individuals who have a personal or family history of a genetic syndrome. It is, therefore, also important to be acquainted with the more common patterns of inheritance:

In genetic syndromes that follow an autosomal recessive pattern of inheritance, a carrier has one copy of a genetic alteration, or mutation, and, as a general rule, does not exhibit symptoms of that syndrome. If both members of a couple are carriers of an autosomal recessive disorder, there is a 25% chance for them to have an affected child in each pregnancy.

Examples of autosomal recessive conditions include cystic fibrosis, which results in thickened mucus primarily affecting the lungs, digestive tract, and male reproductive tract; and Tay-Sachs disease, a fatal neurodegenerative disorder that is more common in the Ashkenazi Jewish, Cajun, and French-Canadian populations.

In autosomal dominant inheritance, there is a 50% chance for an affected individual to transmit the disease-causing mutation to each of his or her offspring. Depending upon the particular condition, inheriting the mutation might or might not mean that an individual will show features of that condition, a phenomenon known as incomplete or reduced penetrance. Additionally, there can be a wide range of clinical severity, even within a family; this is known as variable expressivity. Examples of autosomal dominant conditions include Huntington's disease, an adult-onset neurodegenerative condition, and Marfan syndrome, a condition that affects connective tissue.

In X-linked recessive inheritance, there is a 50% chance for each son of a female carrier to be affected and a 50% chance for each daughter of a female carrier to be a carrier herself. Under certain uncommon circumstances, females can be affected with X-linked recessive conditions. As in autosomal recessive inheritance, carriers have one mutation, except in this case on one X chromosome instead of on an autosome, and generally do not exhibit features of the condition. Examples of conditions that follow an X-linked recessive pattern of inheritance include fragile X syndrome, which is the most common inherited form of mental retardation (see Chap. 19), and hemophilia, a bleeding disorder.

In X-linked dominant inheritance, there is a 50% chance for each child of an affected woman to inherit the disease-causing mutation. Affected females tend to be more common and are often less severely affected than are affected males; X-linked dominant conditions, particularly those that are rare, can be prenatally lethal in affected males. Incontinentia pigmenti type 2, which affects the skin, skin derivatives, and central nervous system, is an X-linked dominant condition that is frequently lethal in affected males [8].

In multifactorial inheritance, a genetic predisposition increases the chance that an individual will develop a particular condition. Certain environmental factors, such as diet and exercise, also have a role in determining if the individual will be affected. Examples of multifactorial conditions are diabetes, heart disease, and neural tube defects. Generally speaking, the more distant the degree of relationship between the individual in question and the affected relative, the lower the recurrence risk, until such risk approximates that of the general population.

Personal or Family History of Cancer

In the majority of cases, cancer is sporadic in an individual. However, in some families, a genetic predisposition to

cancer significantly increases the chance to develop the condition. Hallmarks of hereditary cancer families include relatively early-onset cancer as compared to the general population, bilateral or multiorgan cancer, multiple affected family members (usually following an autosomal dominant pattern of inheritance), and unusual cancer or the presence of certain characteristic clinical features. When an individual is referred for cancer genetic counseling, the genetic counselor educates the counselee about the genetics of cancer predisposition. Based on personal and family history information, the counselor also provides a risk assessment for cancer or for a hereditary cancer predisposition. The risks, benefits, and limitations of appropriate, available molecular testing options and research opportunities are discussed, as are the potential results and their possible psychosocial and practical implications. Options for cancer risk reduction, such as prophylactic surgery, chemoprevention, and cancer screening, are also likely to be reviewed.

As discussed in Chap. 15, certain translocations are characteristic of certain cancers. For example, the (9;22) translocation, which results in the "Philadelphia chromosome" and the fusion of two genes, *BCR* and *ABL1*, is associated with chronic myelogenous leukemia (CML). Similarly, Burkitt lymphoma is associated with an (8;14) translocation. The identification of cytogenetic abnormalities in a cancer patient can have important diagnostic and prognostic implications and can also play a role in designing a treatment strategy [8, 9]. Occasionally, when chromosome analysis is performed for the indication of a hematological abnormality, a chromosome abnormality that may be constitutional is identified. In such a situation, this should be verified, and, if true, the patient should be counseled about the finding and the associated implications, not only for him- or herself, but for other family members as well [9].

Consanguinity

When both members of a couple share at least one common ancestor, they may be referred to a genetic counselor to discuss the possibility for an increased risk of birth defects and/or genetic conditions in their offspring. Using information about the degree of relationship between the members of the couple, their ethnicities, and family history, the counselor discusses the potential for increased risk, if any, and offers any appropriate options for carrier and/or prenatal testing [10]. Although in some cultures consanguinity is accepted and even common, in other cultures, it carries a social stigma. Not only might a consanguineous couple be dealing with an increased risk of abnormalities in their offspring, but they might also be facing criticism from their family and society. In these situations, the genetic counselor can provide emotional support and referral to an appropriate support organization.

Advanced Maternal Age

The chance of having a pregnancy or child affected with a chromosome abnormality increases with advancing maternal age (Table 21.1) [11, 12, 14]. While previous standard of care required that prenatal diagnosis (see Chap. 12) via chorionic villus sampling (CVS) or amniocentesis be offered to all pregnant women who will be 35 or older at their estimated date of delivery (EDD), it is now recommended that such diagnostic testing be offered to all women, regardless of age [14–16].

Table 21.1 Risks for chromosome abnormalities at term by maternal age [14]

Maternal age at term	Risk for trisomy 21 ^b [12]	Risk for any chromosome abnormality ^{b,c} [11]
15 ^a	1:1,578	1:454
16 ^a	1:1,572	1:475
17 ^a	1:1,565	1:499
18 ^a	1:1,556	1:525
19 ^a	1:1,544	1:555
20	1:1,480	1:525
21	1:1,460	1:525
22	1:1,440	1:499
23	1:1,420	1:499
24	1:1,380	1:475
25	1:1,340	1:475
26	1:1,290	1:475
27	1:1,220	1:454
28	1:1,140	1:434
29	1:1,050	1:416
30	1:940	1:384
31	1:820	1:384
32	1:700	1:322
33	1:570	1:285
34	1:456	1:243
35	1:353	1:178
36	1:267	1:148
37	1:199	1:122
38	1:148	1:104
39	1:111	1:80
40	1:85	1:62
41	1:67	1:48
42	1:54	1:38
43	1:45	1:30
44	1:39	1:23
45	1:35	1:18
46	1:31	1:14
47	1:29	1:10
48	1:27	1:8
49	1:26	1:6
50	1:25	Data not available

^aReference [13]

^bRisks based on maternal age at term. Term risks do not include chromosomally abnormal fetuses spontaneously lost before term

^cIncludes risk for trisomy 21. Does not include 47,XXX

Advanced Paternal Age

Advanced paternal age, frequently defined as 40 or older at the time of conception, is an acceptable, although infrequent, reason for a referral to a genetic counselor. Studies have shown an increased risk for genetic defects associated with advanced paternal age. These genetic defects include sporadic, dominant single gene mutations, most commonly Pfeiffer syndrome, Crouzon syndrome, Apert syndrome, achondroplasia, thanatophoric dysplasia, and MEN2A and MEN2B. The risk for a sporadic, autosomal dominant genetic syndrome in the offspring of men over the age of 40 is presently felt to be less than 0.3–0.5%. Studies also indicate that advanced paternal age may be associated with an increased risk of complex conditions, including some birth defects, schizophrenia, autism spectrum disorders, and some cancers. There does not, however, appear to be an increased risk of chromosome abnormalities associated with advanced paternal age with the possible exception of trisomy 21 and Klinefelter syndrome. Most of the paternal age-related birth defects cannot be reliably detected by prenatal diagnosis [17].

Abnormal Prenatal Screen

Screening can be used, along with maternal age, to estimate the possibility that a fetus is affected with Down syndrome or trisomy 18. Such aneuploidy screening can be performed through the utilization of ultrasound, maternal serum, or, frequently, a combination of the two.

Teratogen Exposure

The term “teratogen” applies to any medication, chemical, or environmental agent that has the potential to cause adverse effects, such as birth defects, on a developing fetus. When the mother or father of a current or future pregnancy has been exposed to an agent that could have a detrimental effect on that pregnancy, a referral to a genetic counselor is appropriate. Of note, certain maternal conditions, such as phenylketonuria (PKU), which is an inherited metabolic disorder, diabetes, and seizure disorders increase the risk for birth defects in a pregnancy. The counselor will consult current resources and discuss with the exposed individual or couple the potential adverse effects associated with the exposure in question. Any available options for minimizing these potential adverse effects or for identifying them prenatally are also discussed.

Infertility

Certain chromosome abnormalities and genetic conditions result in varying degrees of infertility (see Chap. 11). Therefore, when an individual or couple experiences infertility,

it is appropriate to rule out the possible genetic and cytogenetic causes. If such a cause is identified, a genetic counselor can be important in educating the individual about the condition. The genetic counselor can also assist the physician in discussing the available options that could allow for reproduction. In addition, if the individual is able to reproduce using his or her own gametes, the possible recurrence risks for future offspring should be addressed.

Recurrent Spontaneous Abortion

Miscarriage is more common than many people recognize. In fact, it is estimated that 10–15% of all recognized pregnancies end in miscarriage [18]. There are many possible causes of miscarriage, including a chromosomally abnormal conceptus. Approximately 50% of recognized first trimester miscarriages are chromosomally abnormal [8, 18, 19]. In some individuals, pregnancy loss is recurrent. In addition to having the potential to cause significant psychological distress, recurrent miscarriage warrants a complete evaluation, which could include genetic, cytogenetic, and endocrinology studies, in an attempt to identify the cause. As discussed later, some causes of recurrent miscarriage confer increased reproductive risks for the patient, as well as his or her family members.

Cytogenetic Indications for Genetic Counseling

Family History or Clinical Suspicion of a Chromosome Abnormality

As previously mentioned, congenital anomalies, mental retardation, developmental delay, or certain characteristic features are all examples of indications for chromosome analysis. Several chromosome abnormalities are detectable through conventional chromosome analysis, while others, such as microdeletion syndromes, require specialized analysis, such as fluorescence *in situ* hybridization (FISH) (see Chap. 17) or microarray (see Chap. 18). The following is a brief introduction to several of the more common chromosome abnormalities encountered in genetic counseling. The style of genetic counseling associated with the identification of a chromosome abnormality often varies depending upon the age of the affected individual. Although the clinical information is unlikely to be significantly different, the tone of the discussion often varies depending on whether the diagnosis is made prenatally, when termination of the pregnancy might be an option, or during childhood, adolescence, or adulthood. As previously mentioned, regardless of whether a chromosome abnormality is diagnosed prenatally or postnatally, the genetic counselor often plays a role in

educating the patient or family about the clinical features of the condition, recurrence risks, and available supportive treatments. Although the identification of a cause for the phenotypic abnormalities in an individual can be an empowering event for the patient and family, it can also induce significant stress. The genetic counselor, acting as a member of the team caring for the individual, often plays an important role in helping the family to cope with the diagnosis both practically and emotionally.

Autosomal Trisomies

Down Syndrome

Down syndrome, which is caused by non-mosaic trisomy 21 in approximately 94% of cases, is the most common human chromosome abnormality, affecting approximately 1 in 800 individuals [8, 20, 21]. Individuals with Down syndrome frequently have a characteristic facial appearance and frequently resemble one another more than they resemble their family members. Certain health conditions and birth defects are more common in individuals with Down syndrome, including congenital heart defects, gastrointestinal problems, leukemia, Alzheimer disease, immune dysfunction, thyroid dysfunction, and problems with hearing and vision. Poor muscle tone and delayed growth are also frequent findings. In 1997, the median age at death was noted to be 49 years with congenital heart defects presenting a major cause of early mortality. Everyone with Down syndrome has some degree of mental retardation. While the general IQ range is usually said to be 25–50, a range of mental capability exists. Children with Down syndrome often benefit from early programs aimed at stimulation, developmental enrichment, and education [20–22].

Trisomy 13

Trisomy 13 results in severe mental retardation and multiple birth defects. The abnormalities most commonly noted in this condition involve the heart (congenital defect in 80%), brain, eyes, ears, lip, and palate (cleft lip and/or cleft palate), hands and feet (such as polydactyly or extra digits), and genitalia. This condition is frequently fatal early in infancy with only 5–9% of affected individuals surviving the first year of life with a median survival of 7–10 days [8, 21, 22].

Trisomy 18

Like trisomy 13, trisomy 18 results in severe mental retardation and birth defects. Congenital heart defects and abnormalities of the hands and feet (clenched hands with overlapping fingers) defects are common, as is growth deficiency. Several other congenital anomalies, including those involving the kidneys, central nervous system, skeletal system, gastrointestinal system, and genitalia, are also associated with this condition. Approximately 5–10% of babies affected with trisomy 18 survive the first year of life with a median survival of 10–14 days [8, 21, 22].

Some cases of Down syndrome, trisomy 13, or trisomy 18 are the result of unbalanced translocations. If such a translocation is carried, in a balanced form, by one of the parents, recurrence risks are generally greater than they would be if simple trisomy 13, 18, or 21 was present in the affected individual. It should also be noted that mosaic chromosome abnormalities, with a chromosomally normal cell line, can be associated with a less severe mental and physical phenotype, although the severity of the condition cannot be predicted from the karyotype.

For more comprehensive coverage of trisomy, refer to Chap. 8.

Unbalanced Chromosome Rearrangements

A family history of birth defects and/or mental retardation, sometimes accompanied by a history of recurrent pregnancy loss, can result from the segregation of a familial chromosome rearrangement, such as a translocation or inversion (Fig. 21.1; see also Chap. 9).

Microdeletion Syndromes

Microdeletion syndromes, as their name implies, are the result of relatively small chromosomal deletions that may be undetectable via conventional cytogenetic analysis. When a clinician suspects that an individual is affected with one of these conditions, FISH or microarray techniques are generally employed to confirm, or rule out, the diagnosis. Occasionally, certain ultrasound findings raise the possibility of a particular microdeletion syndrome in the fetus, as can be the case with 22q11.2 deletion syndrome when a heart defect is noted on prenatal ultrasound. In these cases, analysis can be performed on the material obtained from a chorionic villus sampling (CVS) or amniocentesis. Several of these microdeletion syndromes occasionally result from the unbalanced segregation of a familial chromosome rearrangement. See Chaps. 12 and 17.

22q11.2 Deletion Syndrome (Including DiGeorge and Velocardiofacial Syndromes)

This syndrome results from an interstitial deletion of the long arm of chromosome 22. One interesting feature of this condition is the potential for wide clinical variability within and between families. At times, subsequent to the diagnosis of a child, one of the parents is found to be affected, although usually more mildly. The microdeletion is frequently sporadic (approximately 93% of cases) but can also be inherited in an autosomal dominant manner. A variety of features in multiple organ systems have been reported in individuals with DiGeorge syndrome. Some of the more common features include learning disabilities, heart defects, cleft palate, short stature, immune deficiency, low muscle tone in infancy, hypernasal speech, low calcium levels, renal abnormalities, psychiatric illness, and characteristic facial features [21, 23].

Prader-Willi Syndrome

Approximately 70–75% of cases of Prader-Willi syndrome result from deletion on the paternally derived copy of chromosome 15 [del(15)(q11.2q13)]. Other potential causes are maternal uniparental disomy for chromosome 15 and an imprinting mutation. Imprinting refers to certain genes being active on only the maternally or paternally derived copy of a particular chromosome (see Chap. 20). Affected individuals usually have low muscle tone and feeding difficulties during infancy. Later in childhood, however, obsessive eating and obesity develop. Other features commonly seen in individuals with this condition include short stature, mental retardation, small hands and feet, small underdeveloped genitals, characteristic facial features, and decreased sensitivity to pain. Behavior problems, such as skin picking, stubbornness, temper tantrums, obsessive-compulsiveness, and, in some, psychosis, can also be present [21, 24]. See also Chap. 9.

Angelman Syndrome

Approximately 70–75% of cases of Angelman syndrome are caused by the same microdeletion found in the majority of cases of Prader-Willi syndrome, except that the deletion occurs on the maternally derived copy of chromosome 15, and there are in fact differences at the molecular (DNA) level. The clinical features most commonly found in affected individuals include severe mental retardation, spontaneous, excessive fits of laughter, “jerky” limb movements, characteristic facial features, sleep abnormalities, and seizures [21, 25]. Imprinting also plays an important causative role in this disorder (see Chaps. 9 and 20).

Williams Syndrome

Williams syndrome is the result of a microdeletion on chromosome 7 at the q11.23 locus and involves, among others, the elastin (*ELN*) gene. The condition is usually sporadic, but as with the 22q microdeletion syndrome, can also follow an autosomal dominant pattern of inheritance. As infants, affected individuals tend to experience failure to thrive, gastrointestinal complications, delayed milestones, and delayed speech. The rate of growth is slow, and mental retardation, characteristic facial features, cardiovascular defects, urinary tract abnormalities, and joint problems are often present. One of the most interesting features of Williams syndrome is the unique, characteristic personality. Affected individuals tend to be extremely friendly and talkative. Certain behavior problems, such as a generalized anxiety and sleep difficulties, can be encountered [21, 26]. See Chap. 9.

Smith-Magenis Syndrome

Smith-Magenis syndrome, which is the result of a deletion involving the short arm of chromosome 17 [del(17)(p11.2p11.2)], is almost always sporadic. In infancy, individuals

with Smith-Magenis syndrome tend to have feeding problems and low muscle tone. Language and motor skills are delayed, and mental retardation is a feature of the condition. Other features include short stature, severe sleep disturbances, characteristic facial features that become more evident with age, and behavioral problems. The behavioral problems often include self-injury, attention deficit, and temper tantrums [18, 21, 27].

Miller-Dieker Syndrome

Miller-Dieker syndrome is also the result of an interstitial deletion involving the short arm of chromosome 17 [del(17) (p13.3p13.3)], more distal than that seen in Smith-Magenis syndrome. The abnormalities associated with this condition involve the central nervous system, with lissencephaly, or a smooth brain, being a characteristic feature. This results in severe mental retardation, seizures, low muscle tone, and a small head size. Certain characteristic facial features are also associated with Miller-Dieker syndrome. The majority of affected individuals die within the first two years of life. Approximately 80% of affected individuals have a sporadic deletion. However, the remaining 20% inherit the deletion from a parent with a balanced chromosome rearrangement [21, 28].

Subtelomere Rearrangements

Cryptic microdeletions, or subtle rearrangements near the tips of chromosomes, are estimated to be a common cause of mental retardation, with or without dysmorphic features. Unbalanced subtelomere rearrangements are reported to occur in 7.4% of individuals with moderate to severe mental retardation and can be detected with FISH probes for the unique subtelomeric regions of most chromosomes (see Chap. 17) [29]. The identification of such an unbalanced rearrangement in a phenotypically abnormal individual allows subtelomeric FISH studies to be offered to the parents, and other at-risk family members, to determine if one of them carries a balanced subtelomeric rearrangement. Based upon the results of the parental analyses, recurrence risks can be more accurately quoted. Certain other clinical indications for subtelomere analysis, such as characterization of known chromosomal abnormalities, have been noted in the literature [30, 31]. Subtelomeric abnormalities are now more often diagnosed with microarray analysis (see Chap. 18).

Chromosome Instability Syndromes

As discussed in Chap. 14, there are a number of genetic syndromes of which a notable feature is an increased incidence of chromosome breaks and instability. The majority of these syndromes, including Fanconi anemia, Bloom syndrome, ataxia telangiectasia, and Roberts syndrome, follow an autosomal recessive pattern of inheritance. Therefore, the presence of one of these conditions in a family can have significant implications for recurrence [18, 21].

Infertility

At times, when one of the members of a couple is a carrier of a structural chromosome rearrangement (see Chap. 9), the unbalanced segregation of that rearrangement can result in miscarriage before the couple is aware of the pregnancy. This can cause the couple and their physicians to suspect infertility. True infertility is also a frequent feature of certain sex chromosome abnormalities, and, therefore, the clinician and genetic counselor must also consider the possibility of a sex chromosome disorder when faced with an infertile couple. See also Chaps. 10 and 11.

Sex Chromosome Abnormalities

It has been estimated that, overall, approximately one in 400 infants have some form of sex chromosome aneuploidy [32]. A thorough discussion of sex chromosomes and sex chromosome abnormalities can be found in Chap. 10. A potentially challenging situation that genetic counselors face regarding the diagnosis of a sex chromosome abnormality is that the patient is often an adolescent. It is imperative for the counselor to discuss this finding and its implications on the patient's level of understanding. Additionally, he or she must appreciate that the diagnosis may create for a young adult a potentially unique and more delicate set of psychosocial issues, as this diagnosis may come at a time when the individual is already struggling with a developing sense of self and sexuality.

Klinefelter Syndrome

Klinefelter syndrome, 47,XXY, affects approximately one in 500 males and is a common cause of male infertility. Men who are affected with this condition tend to be tall and thin. The genitals, particularly the testes, are usually small and there can be gynecomastia (male breast enlargement). The development of secondary sex characteristics is incomplete. As testosterone production is often insufficient, testosterone replacement therapy is often utilized to minimize the features of this condition related to testosterone insufficiency. Learning difficulties are common. The IQ is usually average but may be lower than that of siblings. A wide range of IQs has been noted, including some well above and well below average. Behavioral differences, such as shyness and insecurity, can be present [8, 21, 32].

There are many chromosomal variants of this condition. Some of these variants are associated with a less severe phenotype, such as some cases of 47,XXY/46,XY mosaicism. Other variants are associated with a more severe phenotype, such as 48,XXYY, which is associated with a greater likelihood of mental retardation [21, 32].

Turner Syndrome

Turner syndrome, 45,X, is estimated to affect approximately one in 2,500 live-born females. The infertility associated with

Turner syndrome results from ovarian degeneration. Affected individuals experience delayed and/or incomplete puberty, and the majority do not menstruate. For this reason, estrogen replacement therapy is often utilized to stimulate menstruation and pubertal development. Stature is often short, with an average height of 55 inches. Growth hormone therapy may be used in an effort to increase stature. Kidney and heart defects, along with other anomalies, can be present. Congenital lymphedema can result in puffiness of the fingers and toes and a neck that appears webbed. This lymphedema, in the form of a cystic hygroma and/or hydrops, is sometimes identifiable on prenatal ultrasound. Certain health conditions, such as diabetes, high blood pressure, and thyroid disease, are more common. In addition to appearing webbed, the neck tends to be short, the chest is often broad, and the nipples widely spaced. Learning difficulties can be present, although most affected individuals have a normal IQ [8, 21, 32].

As with Klinefelter syndrome, there are several chromosomal variants of Turner syndrome. Individuals with mosaic Turner syndrome and those who are missing only part of one X chromosome can be less severely affected [8, 21, 32]. Only 50% of Turner patients present with the classic 45,X karyotype. The remainder have some form of mosaicism and/or structurally abnormal X chromosome [8].

47,XYY

Although certain phenotypic features have been associated with this condition, affected individuals frequently go undetected, as the features are generally nonspecific. Males with 47,XYY usually have an IQ that, although in the normal range, is below that of their unaffected siblings. These individuals tend to be relatively tall, frequently have severe acne, and could experience certain behavior problems in childhood, such as hyperactivity and attention deficit disorder. However, it should be noted that violence and psychopathology are not more common in these males. This is particularly important in light of the fact that some early, erroneous studies reported that 47,XYY males were over represented in prisons and mental hospitals. Fertility is usually normal [8, 21, 32].

47,XXX

Females with 47,XXX could be of above average height and experience learning disabilities, behavior problems, and delayed motor milestones with subsequent poor coordination and “awkwardness.” Otherwise, there are no remarkable phenotypic features that are associated with this condition [8, 21, 32].

Recurrent Spontaneous Abortion

One cause of recurrent spontaneous abortion is a structural chromosome rearrangement, usually found in a balanced

state, in one member of the couple. Carriers of structural chromosome rearrangements are often at increased risk to produce unbalanced gametes. When such an unbalanced gamete is fertilized, this imbalance can result in miscarriage.

It is estimated that in approximately 4% of couples with two or more miscarriages, one of the partners carries a balanced translocation [19]. Blood chromosome analysis should be offered to any individual with a personal or family history of repeated pregnancy loss.

As previously noted, an unbalanced chromosome rearrangement not only has the potential to cause miscarriage but can also result in live-born offspring with birth defects and/or mental retardation (Fig. 21.1). The risk for an abnormal live-born child associated with a given balanced chromosome rearrangement can be difficult to predict precisely. This risk depends on a number of factors, including the family history, mode of ascertainment, predicted type of segregation leading to viable gametes, sex of the carrier parent, and degree of imbalance of the viable gametes [18]. Genetic counseling can be vital in helping the individual or couple to understand the reproductive risks associated with a balanced chromosome rearrangement. Often, the finding of a chromosome rearrangement comes as a shock to the couple following the frequently frustrating and emotionally distressing loss of wanted pregnancies.

For a detailed discussion of chromosome rearrangements, refer to Chap. 9.

Advanced Maternal Age

During a genetic counseling session for advanced maternal age, the maternal age-related risks for a chromosome abnormality are discussed. The risks, benefits, and limitations of invasive diagnostic testing for chromosome abnormalities (i.e., CVS and amniocentesis) are also discussed, as are the benefits and limitations of prenatally available aneuploidy screening tests. Prenatal chromosome analysis is routinely performed via CVS or amniocentesis (see Chap. 12). It is stressed to the patient or couple that although greater than 99% of chromosome abnormalities are detectable by CVS or amniocentesis, other genetic, nonchromosomal conditions are not routinely detectable via this testing. If there is an indication for additional genetic testing, such as a positive family history, such testing can, at times, be performed on the sample obtained during one of these procedures.

The decision to pursue or decline invasive prenatal testing is a highly personal and, at times, complicated decision. It involves weighing the risks and benefits, the individual or couple's psychosocial circumstances, religious beliefs, personal experiences with disability, pregnancy history, and a multitude of other issues. Genetic counseling can be helpful as the individual or couple considers these issues, as a major goal of genetic counseling is to enable the individual or couple to make a thoughtful, well-informed decision. Two common

reasons that prenatal diagnosis is pursued are if the couple/patient would consider pregnancy termination for the condition in question and if they would want knowledge of the diagnosis to prepare for the birth of a child who could have special needs.

Chorionic villus sampling is generally performed between 9 and 12 weeks of pregnancy. During this procedure, a small sample of the placenta is removed either transabdominally or transcervically under ultrasound guidance. The chorionic villi present in this sample are then placed in culture, and the chromosomes are analyzed. The risk of a miscarriage associated with a CVS was previously quoted as approximately one in 100 or 1%, although more recent studies indicate a lower procedure related risk [14, 16, 33]. One advantage of CVS, as compared to amniocentesis, is that it is performed during the first trimester of pregnancy, allowing for an earlier termination of pregnancy if an abnormality is identified. One potential disadvantage of CVS is that approximately 1–2% of samples result in a mosaic karyotype. The cause of the mosaicism can be that the placenta has a different chromosome constitution than the fetus. This is known as confined placental mosaicism. Even when the chromosomally abnormal cells are confined to the placenta, there can still be adverse effects on the fetus, as a chromosomally abnormal placenta can cause fetal growth restriction and adverse pregnancy outcome and can raise the possibility of uniparental disomy in the fetus. In these situations, follow-up testing, such as amniocentesis, is often performed in an attempt to clarify the fetal karyotype [8, 14, 16, 18, 34, 35].

Amniocentesis is generally performed at about 16–18 weeks of pregnancy, although this procedure can be performed either earlier or later in gestation. During this procedure, a small amount of amniotic fluid is removed transabdominally under ultrasound guidance. The fetal cells (amniocytes) present in this sample are cultured, and the chromosomes are analyzed. The level of α -fetoprotein (AFP) in the amniotic fluid can also be analyzed to screen for open fetal defects, such as open neural tube defects and abdominal wall defects. The risk of a miscarriage associated with an amniocentesis was previously quoted as approximately one in 200 or 0.5%, although more recent studies indicate a lower procedure related risk [10, 14–16, 33].

When rapid information about the fetal chromosomes is needed, generally the result of a particularly high risk of aneuploidy or a late gestational age, FISH (see Chap. 17) for chromosomes 13, 18, 21, X, and Y can be performed on the direct amniotic fluid or chorionic villi. Chromosomes 13, 18, 21, X, and Y are the most common chromosomes involved in a prenatally diagnosed, potentially viable chromosome abnormality and are, therefore, the focus of prenatal FISH analysis [36–38]. Although FISH can yield important information in a short period of time, it is not a substitute for routine cytogenetic analysis. As such, it is recommended

that, in the prenatal setting, clinical decision making based upon FISH results should be supported by a confirmatory chromosome analysis and/or consistent clinical information [39]. FISH can also be performed on prenatal specimens for the detection of several microdeletion syndromes when the ultrasound findings or family history indicates an increased risk for such a condition. Additionally, FISH or microarray can be performed on prenatal specimens for the detection of submicroscopic abnormalities. See also Chap. 12.

Abnormal Prenatal Screen

While Down syndrome and trisomy 18 are commonly screened for prenatally, other chromosome abnormalities can, at times, be detected using certain screening methods, although that is not the goal of such screening. Given that maternal age alone is a poor screening criterion for fetal aneuploidy, prenatal aneuploidy screening should be offered to all pregnant women [16]. The patient or couple should be fully counseled about the benefits and limitations of screening. It is important for the patient to appreciate the distinction between screening, which is designed to provide a risk estimate, and diagnostic tests, which are designed to diagnose or rule out a chromosome abnormality. When screening indicates that there is an increased risk for a chromosome abnormality in a pregnancy, the pregnant woman or couple should be counseled about the implications of this result and the options for further testing, such as CVS or amniocentesis. An individual or couple may be referred for genetic counseling prior to pursuing a prenatal screen so that an informed decision can be made about whether or not to pursue the testing.

First trimester screening is, as its name implies, performed during the first trimester of pregnancy. This screening involves biochemical analysis of the levels of certain pregnancy-related proteins in the maternal circulation, namely, pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG). To increase the number of affected pregnancies detected by this screening, the biochemical analyses can be used in conjunction with a nuchal translucency ultrasound measurement, a measurement of the amount of fluid between the skin and soft tissue over the cervical spine of the developing fetus. Combined with additional information about the pregnancy and family history, this data is used to generate estimated risks for Down syndrome and trisomy 18 [16, 40–43]. In addition to being associated with an increased risk for aneuploidy, an increased nuchal translucency measurement is also associated with other fetal abnormalities, particularly cardiac malformations, and some genetic syndromes [16, 40, 44–47].

Second trimester maternal serum screening is generally performed between 15 and 20 weeks of gestation. This screening usually involves analyzing the maternal blood for

the levels of four pregnancy-related proteins, α -fetoprotein (AFP), human chorionic gonadotropin (hCG), unconjugated estriol (uE3), and dimeric inhibin A (DIA) and is often referred to as the quad screen. As with first trimester screening, the levels of these analytes, combined with certain other information, yield a risk estimate for Down syndrome and trisomy 18. Unlike first trimester screening, second trimester maternal serum screening also screens for the presence of open fetal defects, such as a neural tube or abdominal wall defects, through the analysis of the level of AFP present in the maternal serum [8, 16, 40].

Several screening modalities, including integrated and sequential approaches, have been developed to take advantage of both first and second trimester aneuploidy screening. These screens seek to increase the Down syndrome and trisomy 18 detection rates and, in some situations, decrease the overall chance of a woman receiving a “screen-positive” result (i.e., elevated screening risk Down syndrome and/or trisomy 18) when the fetus is unaffected. It is currently recommended that all women, regardless of age, who present for prenatal care prior to 20 weeks gestation be counseled regarding the option to pursue a fetal aneuploidy screening test. Counseling should include a thorough discussion of the benefits and limitations of such screening [16, 40, 48]. The American College of Obstetricians and Gynecologists (ACOG) specifically recommends that integrated or sequential screening be offered to women who seek prenatal care during the first trimester [40].

An ultrasound examination to evaluate a pregnancy for the presence of certain birth defects and sonographic findings associated with aneuploidy can also be used to screen for Down syndrome and certain other chromosome abnormalities. Such an ultrasound is generally performed during the second trimester of pregnancy, although some aneuploidy markers are identifiable during the first trimester, as is the case with increased nuchal translucency (see the section “First Trimester Screening”). The percentage of aneuploid pregnancies with a demonstrable abnormality on ultrasound depends upon the particular chromosome abnormality and the experience of the sonographer. Some of the aneuploidy markers that are potentially detectable with prenatal ultrasound include cardiac malformations, altered fetal growth, duodenal atresia, and cystic hygroma. In addition to conferring an increased risk for aneuploidy, certain congenital anomalies identifiable on ultrasound could be associated with certain genetic syndromes. At times, when prenatal chromosome analysis produces an ambiguous or unclear result, ultrasound is utilized in an attempt to evaluate the fetal anatomy and to search for any fetal abnormalities that could be associated with the karyotype. As with all other screening, the limitations of ultrasound should be made clear to the patient or couple [8, 22, 49, 50]. See Chap. 12.

Prenatal Identification of a Chromosome Abnormality

When a chromosome abnormality is identified prenatally, the genetic counselor provides information to the patient or couple regarding the phenotype associated with the abnormality in question. Options for continuation or termination of the pregnancy and adoption are also discussed, as is the fact that many chromosomally abnormal pregnancies are at an increased risk to miscarry, and not only in the first trimester of pregnancy [51]. For example, this risk is particularly high in pregnancies affected with Turner syndrome, with at least 99% of affected pregnancies aborting spontaneously early in pregnancy [8]. The prenatal identification of a chromosome abnormality (or any anomaly or genetic condition for that matter) can be traumatic and heartbreak for a couple as they face difficult decisions about an often much wanted pregnancy. It is especially important for the genetic counselor to support the individual, couple, and family during and after such a diagnosis. No matter what the final decision regarding the future of the pregnancy might be, the emotional support of the counselor, as well as referrals to appropriate resources and support groups, can be vital in helping the pregnant woman and/or couple cope with the diagnosis.

Although the majority of the common chromosome abnormalities are associated with a rather well-defined phenotype, results associated with unclear or ill-defined phenotypes can understandably be anxiety provoking. This is particularly true if the couple/patient is struggling to make a decision regarding termination versus continuation of the pregnancy.

The general phenotypes associated with the more common autosomal chromosome aneuploidies, trisomies 13, 18, and 21, are described in a previous section and in Chap. 8. Although these phenotypes are well defined, there is a range of severity particularly associated with Down syndrome or with mosaicism where a normal cell line is also present. As noted previously, the degree of severity of the condition cannot be predicted from the karyotype. Some individuals find this to be a difficult situation, as they may feel capable of caring for a child with mild disabilities but unable to care for a child with more severe disabilities.

The common sex chromosome abnormalities are generally associated with less severe phenotypes than the aforementioned autosomal trisomies. Although for some this is encouraging, for others the milder phenotypic features complicate the decision of whether to continue the pregnancy or terminate [52].

As previously noted, mosaicism can make the prognosis less clear. One example of this is 45,X/46,XY mosaicism. The majority of prenatally diagnosed affected individuals, approximately 85–95%, are phenotypically normal males externally. However, a range of phenotypes, from a female with Turner syndrome to ambiguous genitalia to externally

normal males, is possible. In phenotypic males, there can be variation with respect to the size of the phallus, descent of the testes, and scrotal fusion. Hypospadias and other congenital abnormalities have also been noted. There is a risk, estimated to be approximately 27%, for abnormal gonadal histology, which increases the risk for a gonadal tumor (gonadoblastoma). Therefore, close follow-up to monitor for tumor development is warranted. The degree of mosaicism does not appear to be a reliable predictor of the phenotype. Of note, the majority of cases of 45,X/46,XY mosaicism diagnosed postnatally were associated with an abnormal phenotype. The reason for this discrepancy is that the postnatally diagnosed cases reflect an ascertainment bias [22, 53, 54].

When an apparently balanced chromosome rearrangement is identified by CVS or amniocentesis, the first step is to perform chromosome analyses on the parents. If one of the parents carries the same rearrangement and is phenotypically normal, it is felt that the rearrangement is unlikely to confer a significantly increased risk of abnormality. It is important to note that there are some mechanisms, such as uniparental disomy (see Chap. 20), by which a balanced translocation inherited from a phenotypically normal parent can be associated with an increased risk for abnormalities. These mechanisms seem to be relatively uncommon [18]. If the rearrangement is *de novo*, the risk assessment becomes more difficult. It has been estimated that the risk for abnormality associated with a *de novo* reciprocal translocation is approximately 6.1%. The estimated risks for abnormality associated with a *de novo* Robertsonian translocation and inversion are 3.7 and 9.4%, respectively [55]. However, it can be difficult, if not impossible, to predict specific abnormalities.

When a structural chromosome rearrangement is unbalanced, whether it is *de novo* or results from the segregation of a balanced rearrangement in a carrier parent, the phenotype is likely to be abnormal. Again, however, it can be difficult to predict the specific abnormalities. Ultrasound examination and a literature review might lend some information about the clinical picture.

The issue of confined placental mosaicism was introduced in the previous section regarding CVS, as such mosaicism is more likely to be found at CVS than at amniocentesis (see also Chap. 12). Mosaicism is not, however, always confined to the placenta. Mosaicism is classified as follows:

- *Level I mosaicism* is defined as a single abnormal cell. This almost always represents a cultural artifact and, in the vast majority of cases, is of no clinical significance to the pregnancy [18].
- *Level II mosaicism* is defined as more than one cell with the same chromosome abnormality in one colony. This type of mosaicism is, in the majority of cases, pseudomosaicism, which is the result of cultural artifact [18]. It is important to note that cultural artifact does not mean

“laboratory error” but is, rather, an occasionally unavoidable result of growing cells *in vitro*.

- *Level III mosaicism* is defined as two or more cells with the same chromosome abnormality in two or more colonies. This finding is likely to represent true mosaicism and raises the level of concern that there is an abnormal cell line in the fetus [18].

When mosaicism is identified prenatally, particularly level III mosaicism, follow-up testing, such as a detailed ultrasound to evaluate the fetal anatomy and/or repeat chromosome analysis, via amniocentesis or percutaneous umbilical blood sampling (PUBS)—in which fetal blood is obtained from the umbilical cord under ultrasound guidance—can be pursued. It is important to realize, however, that such testing is unlikely to completely clarify the fetal karyotype. Again, the limitations of ultrasound in identifying certain phenotypic abnormalities, such as mental retardation, must be made clear to the patient or couple. Furthermore, a normal repeat chromosome analysis, although encouraging, does not guarantee the absence of an abnormal cell line in the fetus. Likewise, an abnormal repeat chromosome analysis does not necessarily mean that the abnormal cell line is present in the fetus. Genetic counseling to help the patient/couple interpret this information is particularly important in such complex situations. If the pregnancy is terminated or aborted spontaneously, chromosome analysis of a variety of fetal tissues should be considered. If the pregnancy is carried to term, follow-up analysis of blood and/or skin might also be indicated.

Although, as previously stated, mosaic chromosome abnormalities can be associated with milder phenotypes, the clinical features associated with true mosaicism cannot be entirely accurately predicted from the karyotype. One reason for this is that it is impossible to know the distribution of normal and abnormal cells in the various tissues of the body. In some cases there can, however, be a correlation between the percentage of abnormal cells and the degree of abnormality. A review of the pertinent literature might provide useful information regarding the general phenotype [56–59].

It has been estimated that the prevalence of supernumerary marker chromosomes at the time of CVS and amniocentesis is approximately 0.6–1.5 per thousand [60]. The discovery of such a marker can be frustrating for the parents, as there is a lack of substantial information about many of these. The limitations of prenatal ultrasound in identifying fetal abnormalities can often compound this frustration. The risk for abnormalities in the light of a marker chromosome can depend on the amount of euchromatin present, whether the origin of the marker is an acrocentric or nonacrocentric chromosome, whether the marker is familial or *de novo*, and, if familial, whether the marker is found in a mosaic state in the carrier parent [60]. One source quotes a 10.9% risk for abnormality associated with

a *de novo* satellited marker and a 14.7% risk for a *de novo* non-satellited marker [55].

Certain supernumerary chromosomes are, however, associated with well-defined clinical features. For example, an isochromosome for the short arm of chromosome 12 [i(12p)] causes Pallister-Killian syndrome, which is associated with profound mental retardation, seizures, characteristic facial features, and pigmentary abnormalities. Cat-eye syndrome, which is usually caused by a marker that results in tetrasomy 22q11.2, can be highly variable and can cause mental retardation, as well as abnormalities involving the eyes, heart, and urogenital system. Additionally, the “inverted duplicated 15” [inv dup(15)] can be associated with varying features, ranging from mental retardation and clinical features of Prader-Willi/Angelman syndrome to a normal phenotype [60]. See Chaps. 8 and 9.

Microarrays

Interest in the postnatal and prenatal utility of microarrays (array comparative genomic hybridization [array CGH] or single nucleotide polymorphism [SNP] arrays) has increased substantially of late. This technology has many advantages over conventional karyotyping, including a higher resolution, a faster turnaround time, and the fact that it does not require dividing cells [61, 62]. Particularly in the postnatal evaluation of individuals with congenital anomalies and/or unexplained mental disability, these advantages make microarrays an important adjunct to conventional karyotyping [61]. The ability to simultaneously analyze multiple loci dismisses the need for multiple FISH assays [63]. Furthermore, the lack of a need for dividing cells makes the microarray an important option in the evaluation of congenitally abnormal fetal demises for which a conventional karyotype result could not be obtained [62]. Array technology has proven a useful tool in defining or redefining the causative genetic mechanism in some genetic syndromes sometimes leading to the identification of new syndromes. Studies have shown that arrays are capable of detecting a causative genomic imbalance in up to 10% of individuals with unexplained mental retardation and a normal conventional karyotype [64]. Microarrays can also assist in the characterization of a chromosome rearrangement or marker chromosome identified via standard chromosome analysis [61, 63]. Based upon a small data set, microarrays appear to identify a chromosome abnormality in 5–10% of fetuses with multiple anatomic abnormalities and a normal standard karyotype via CVS or amniocentesis [65]. However, larger studies are needed to better define the utility of array technology in the prenatal setting.

Microarrays can yield results that are difficult to interpret. In an estimated 12–15% of prenatal samples, a copy number variant (i.e., a deletion or duplication of a DNA segment

larger than 1,000 bases up to several megabases) of uncertain clinical significance is identified [62]. This rate applies to targeted arrays, in which the study is designed to analyze chromosome abnormalities associated with known genetic syndromes. Genome-wide arrays are, understandably, expected to identify a higher rate of copy number variants of unknown significance, but can also identify novel pathogenic variants. While the analysis of parental specimens can assist in the characterization of these variants, as most inherited copy number variants are benign, such testing is not always informative [62, 63, 66]. Additional limitations of array technology include the inability to detect abnormalities such as balanced rearrangements, ploidies (i.e., some cases of triploidy), low-level mosaicism, and single gene mutations. Uniparental disomy cannot be detected with array CGH, but can be identified via SNP array. Furthermore, the cost of the array, which is not always covered by insurance, can be prohibitive for some patients [61–63].

The American College of Medical Genetics and Genomics (ACMG) recommends that microarrays be used as an adjunct to standard karyotyping and FISH in the evaluation of individuals with mental retardation and/or congenital anomalies [61]. The role of arrays in the prenatal cytogenetics setting is, as yet, not well defined. Both the American College of Obstetricians and Gynecologists (ACOG) and ACMG recommend against the employment of microarrays for routine prenatal diagnosis. However, both societies state that targeted arrays can be offered as an adjunct to conventional karyotyping in the evaluation of fetuses with structural abnormalities and a normal karyotype [61, 62]. Particularly given the limitations of the analysis and the potential for ambiguous results, genetic counseling is a critical component of array analysis. In fact, to this end, ACOG recommends both pretest and posttest genetic counseling [62].

Microarray technology is covered in detail in Chap. 18.

Summary

Genetic counseling is a complex, fascinating, and continuously evolving field. With the current focus of science and popular culture on genetics, genetic counseling is becoming increasingly important in medicine. As stated in the beginning of this chapter, genetic counselors are increasingly found in a wide variety of settings in clinical, research, and administrative roles. Furthermore, genetic counselors can contribute significantly, not just in the setting of prenatal genetics, but also in the pediatric and adult arenas.

Counselors not only play a vital role in explaining genetic concepts, recurrence risks, and genetic testing in understandable terms, but also in helping individuals anticipate and cope with the psychosocial consequences that can be associated with the diagnosis of a genetic condition. Although

seemingly straightforward, these can be challenging tasks, particularly when ambiguous test results, cultural differences, and/or mental handicaps are involved. The unique training that genetic counselors receive makes them especially well-suited to tackle such challenges.

Ethics and genetics are closely intertwined, as genetic counselors continuously encounter a variety of situations where ethical principles and guidelines must be consulted and followed. These situations range from the fairly routine to the more obscure. There are several resources at the counselor's disposal that provide assistance in working through such ethical dilemmas. The continuing development of new genetic technologies will facilitate the understanding of the genetic contribution to human life and disease. The public, government, and scientific communities will face a greater number of increasingly complex ethical dilemmas, particularly in the realm of genetic predisposition to adult-onset conditions.

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