The Principles of Clinical Cytogenetics

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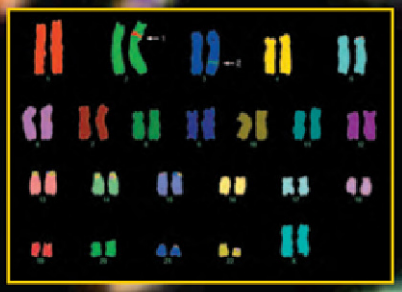

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Second Edition

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Middle inset: M-FISH of a metaphase from a patient with CML in transformation to blast crisis. Image submitted by Anette Grand, Inge-Lise Frost Andersen, and Mette Klarskov Andersen.
Bottom inset: M-FISH karyotype demonstrating a complex cryptic rearrangement in a child with multiple anomalies and developmental delay. Image submitted by Charles Lee.
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Preface

In the summer of 1989, one of us (SLG), along with his mentor, Dorothy Warburton, attended the Tenth International Workshop on Human Gene Mapping. The meeting was held at Yale University in celebration of the first such event, which also took place there.

This meeting was not open to the general public; one had to have contributed to mapping a gene to be permitted to attend. The posters, of course, were therefore all related to gene mapping, and many were covered with pretty, colorful pictures of a novel, fluorescent application of an old technology, *in situ* hybridization. Walking through the room, Dorothy remarked that, because of this new FISH technique, chromosomes, which had become yesterday's news, were once again "back in style."

Approximately three years later, a commercial genetics company launched a FISH assay for prenatal ploidy detection. A substantial number of cytogeneticists across the country reacted with a combination of outrage and panic. Many were concerned that physicians would be quick to adopt this newfangled upstart test and put us all on the unemployment line. They did not at the time realize what Dorothy instinctively already knew—that FISH would not spell the doom of the cytogenetics laboratory, but it would, rather, take it to new heights. In the early 1990s we didn't know where FISH would end up being performed, but because of the number of FISH applications that require metaphase chromosomes, it has landed, either literally or functionally, squarely in the cytogenetics laboratory, securing its place in an increasingly "molecularized" laboratory environment. Add to this the explosion of cytogenetic and FISH data to become available in oncology in recent years, and it becomes apparent that chromosomes are here to stay.

This brings us to the revision of *The Principles of Cytogenetics*. After the first edition was printed, it seemed possible that we had achieved our goal of assembling the basic concepts of clinical cytogenetics for the "end user" physician or student who needed to understand what we do, and that perhaps no update would be necessary. However, FISH and cancer cytogenetics continued to march on, and new data have become available even for such basic concepts as chromosome rearrangements, sex chromosome abnormalities, and autosomal aneuploidy. Combine these with all that has been learned about uniparental disomy and imprinting in the last five years, plus the regulatory changes we are all subject to, and it becomes obvious that what was needed was not a second printing, but a second edition.

Our one concern is that, as *The Principles of Cytogenetics* goes to press, the nomenclature committee has met but has not set any date for a revision of the ISCN, the nomenclature that forms the core of reporting in clinical cytogenetics. The best guess is that this will be available sometime in 2005, ten years after the last revision and a year after this

book will have been published. Though we are not comfortable with the notion that part of this book could be out of date shortly after its printing, most if not all updates will involve details of FISH nomenclature that could not have been envisioned when ISCN 1995 was released, and we therefore decided not to delay the publication of this update merely to wait for that one.

This edition of *The Principles of Clinical Cytogenetics* is organized much like the first, though there are several important changes. First, because of its increasing importance, the FISH chapter is now its own section in the book. Next, the increasing importance of cancer cytogenetics has prompted us to separate this subject into two distinct chapters, covering hematological disorders and solid tumors, also as a separate section. Because the ever-increasing popularity of computerized imaging and karyotyping systems has resulted in a waning popularity and likely eventual elimination of standard photography in the cytogenetics lab, this topic has been eliminated. Finally, two new chapters have been added, covering chromosome instability and the cytogenetics of infertility.

We would like to take this opportunity to thank the authors who contributed to this book, and to the readers who made the first edition a success. We hope this edition will prove to be equally valuable.

Steven L. Gersen, ***PhD***
Martha Keagle, ***MEd***

Preface to First Edition

The study of human chromosomes plays a role in the diagnosis, prognosis, and monitoring of treatment involving conditions seen not only by medical geneticists and genetic counselors, but also by pediatricians, obstetrician/gynecologists, perinatologists, hematologists, oncologists, endocrinologists, pathologists, urologists, internists, and family practice physicians. In addition, cytogenetic testing is often an issue for hospital laboratory personnel and managed care organizations.

Few esoteric clinical laboratory disciplines have the potential to affect such a broad range of medical specialists, yet cytogenetics is often less well understood than most "specialized" testing.

One can attribute this to several causes:

- The cytogenetics laboratory is essentially the only setting in which living cells are required for traditional testing (fluorescence *in situ* hybridization [FISH] provides an exception to this rule). This unusual sample requirement is a potential source of confusion.
- Cytogenetics is still perceived, and rightly so, to be as much "art" as it is science in an era when most clinical testing is becoming more and more automated or "high tech."
- Genetics in general still does not receive sufficient emphasis in the training of medical personnel.

This issue has been complicated in recent years because, in an era of molecular medicine, chromosome analysis has become somewhat less of a stand alone discipline; as genes are mapped to chromosomes, traditional cytogenetics is often augmented with DNA analysis and/or FISH. The latter, often referred to as "molecular cytogenetics," represents the single most significant advance in this field in decades, and has become such an integral part of the typical cytogenetics laboratory, with such a wide variety of applications, that it warrants its own chapter in *The Principles of Clinical Cytogenetics.*

It is impossible to completely separate the relationships that exist today between the cytogenetics and the molecular genetics laboratories, from cases involving fragile-X-syndrome to those dealing with cancer patients, and for this reason, relevant molecular concepts are discussed in several chapters.

Entire volumes have been devoted to some of the topics covered in *The Principles of Clinical Cytogenetics*; these often serve as references or how-to manuals for those involved in providing genetics services, and in most cases provide a greater level of detail than is needed here. The purpose of the present book is to provide a comprehensive description of the basic concepts involved in chromosome analysis in a single volume, while at the same time producing a summary of sufficient depth to be of value to the practicing genetics

professional. We hope that it will serve as a valuable reference to any health care provider, from the individual who utilizes cytogenetics routinely to someone who has need of it on rare occasions.

The Principles of Clinical Cytogenetics is divided into four sections. The first section provides an historical perspective and explanation of the concepts involved, including a detailed description of cytogenetic nomenclature and examples of its use. The second section is an overview of the processes involved. The purpose of this section is to provide a fundamental understanding of the labor-intensive nature of chromosome analysis. It is not, however, a "laboratory manual"; detailed protocols for laboratory use are available elsewhere and are not appropriate in this setting. The third section comprises the main focus of this book, namely, the various applications of chromosome analysis in clinical settings and the significance of abnormal results. The final section connects cytogenetics to the broader field of clinical genetics, with discussions of synergistic technologies and genetic counseling.

We gratefully acknowledge the hard work and attention to detail provided by the individuals who authored each chapter of *The Principles of Clinical Cytogenetics*, and thank our publisher for supporting this effort.

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I Basic Concepts and Background

1

History of Clinical Cytogenetics

Steven L. Gersen, PhD

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" *(2)*, and several prominent scientists of the day began to formulate the idea that determinants of heredity were carried on chromosomes. 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*.

Owing in part to improvements in optical lenses, stains, and tissue manipulation techniques during the late 19th and early 20th 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 have 48 *(5)*. Then, in 1923, 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, Hsu reported that, rather than depending on 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 ideogram *(9)* of all 48 human chromosomes!

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.

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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 1 year, Hsu and Pomerat, 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 the metaphase *(11)*. Joe Hin Tjio, an American-born Indonesian, learned about these procedures and worked with Hamerton and Ford to further improve upon them.

In November 1955, Tjio was invited to Lund, Sweden to work on human embryonic lung fibroblast cultures in the laboratory of his colleague, 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 1956 (after carefully reviewing images from decades of previously reported work), they 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 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.

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 3 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 *(14)*, but the science of the time could neither prove nor disprove his idea; this would take almost three decades. In 1958, Lejeune studied the chromosomes of fibroblast cultures from patients with Down syndrome, and in 1959, Lejeune and colleagues 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 1962, he received one of the first Joseph Kennedy Jr. Foundation International Awards for his work (see **Fig. 1**).

Three more chromosomal syndromes, all believed to involve the sex chromosomes, were also described in 1959. Ford et al. reported that females with Turner syndrome have 45 chromosomes, apparently with a single X chromosome and no Y *(16)*, 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 *(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 Turner syndrome,

Fig. 1. Jérôme Lejeune receives a Joseph P. Kennedy, Jr. Foundation International Award for demonstrating that Down syndrome results from an extra chromosome. (Photo provided by the John F. Kennedy Library, Boston, MA.)

Klinefelter syndrome, and putative XXX patients had 0, 1, and 2 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 chromosome mechanism of X-dosage compensation in mammals was developed by Lyon *(20)* and has been known since then as the Lyon hypothesis.

It was not long after Lejeune et al.'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's syndrome or "D trisomy" and the latter as Edward's syndrome or "E trisomy." Patau et al.'s article 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 the "Philadelphia chromosome" in chronic myelogenous leukemia, demonstrating, for the first time, an association between chromosomes and cancer *(23)*.

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 *(24,25)*. Within two years, Jacobs et al. described "aggressive behavior, mental subnormality and the XYY male" *(26)*, and the chromosomal instabilities associated with Bloom syndrome and Fanconi anemia were reported *(27,28)*.

Additional technical advancements had facilitated the routine study of patient karyotypes. In 1960, 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 *(23,29)*, permitting a peripheral blood sample to be used for chromosome analysis. 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 *para*centric 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.

For three 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 *(30)*.

Caspersson and colleagues then turned their attention from plants to the study of human chromosomes. They 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 they initially referred to them, whereas 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 and co-workers had successfully produced and reported a unique "banding" pattern for each human chromosome pair *(31,32)* (see **Fig. 2**).

For the first time, each human chromosome could be positively identified. The method, however, was cumbersome. It required a relatively expensive fluorescence microscope 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 *(33)*. 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. Although 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 and mosaicisms, plus an ever-increasing collection of rearrangements and other cytogenetic anomalies associated with neoplasia, and a seemingly infinite number of patient- and family-specific rearrangements.

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

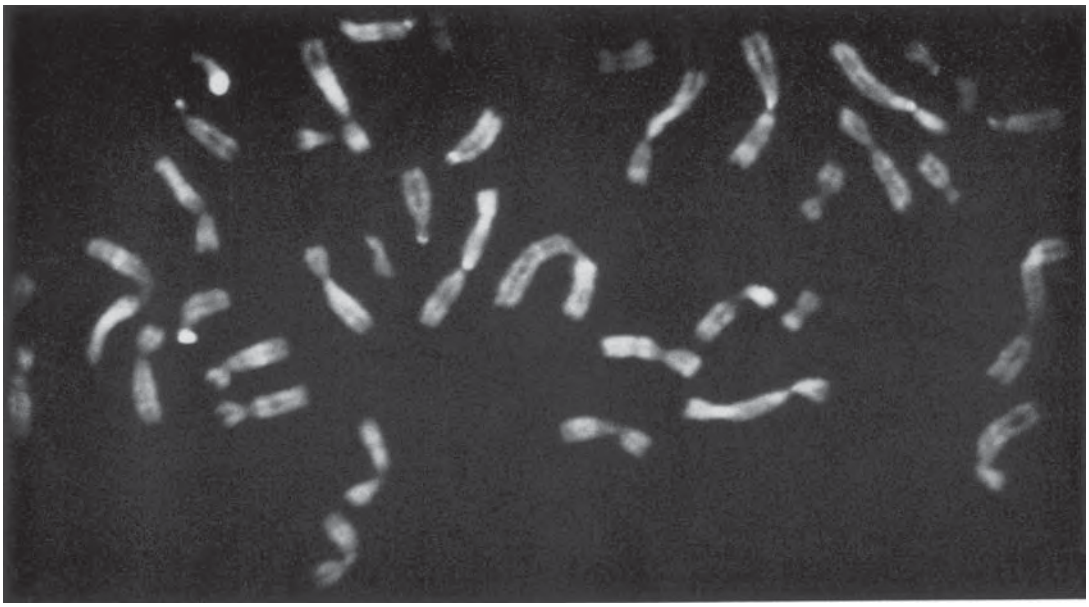

Fig. 2. The first photograph of a Q-banded cell published by Caspersson and co-workers in 1970. The figure was originally labeled "Quinacrine mustard treated human metaphase chromosomes (male) from leukocyte culture. Fluorescence microscope. ×2000." (Reprinted from ref. *31* with permission from Elsevier.)

exciting development and the many innovative procedures derived from it have created even more interest in the human karyotype.

This brings us to the present. More than 1 million cytogenetic and molecular cytogenetic analyses are now performed annually in over 400 laboratories worldwide *(34,35)*, and this testing is now often the standard of care. 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 work-up, and FISH has permitted us to visualize changes that are too subtle to be detected with standard chromosome analysis. Cytogenetics and FISH also provide information vital to the diagnosis, prognosis, therapy, and monitoring of treatment for a variety of cancers.

It was really not so long ago that we had 48 chromosomes. One has to wonder whether Flemming, Waldeyer, Tjio, Levan, Hsu, or Lejeune could have predicted the modern widespread clinical use of chromosome analysis. However, perhaps it is even more exciting to wonder what lies ahead for medical cytogenetics and molecular cytogenetics now that we have entered the 21st century.

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2

DNA, Chromosomes, and Cell Division

Martha B. Keagle, MEd

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

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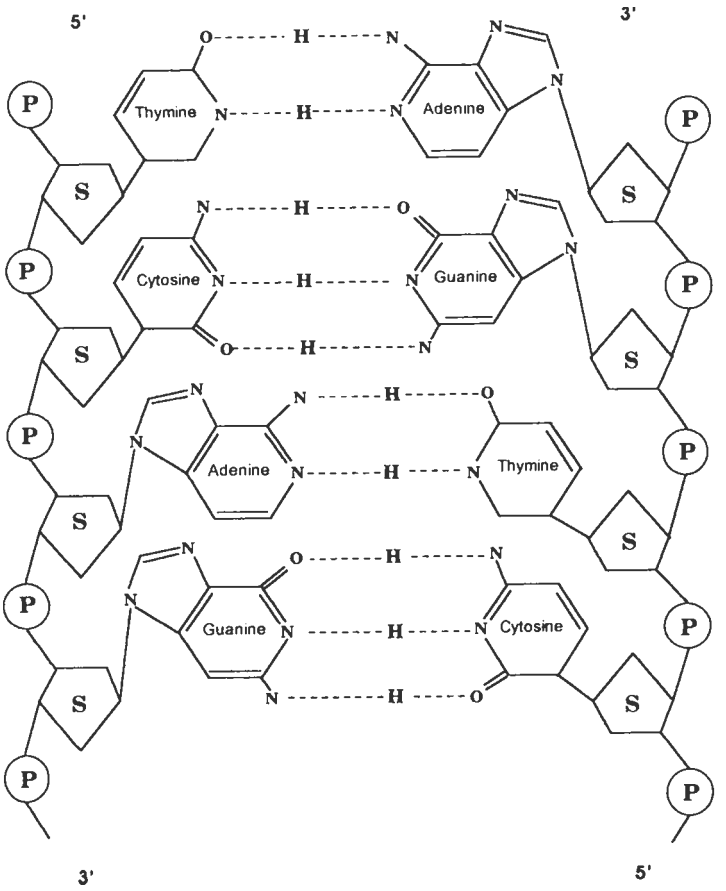

Fig. 1. DNA structure. Schematic representation of a DNA double helix, unwound to show the complementarity of bases and the antiparallel structure of the phosphate (P) and sugar (S) backbone strands.

nitrogenous basepairs 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 (see **Fig. 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 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 basepairing.

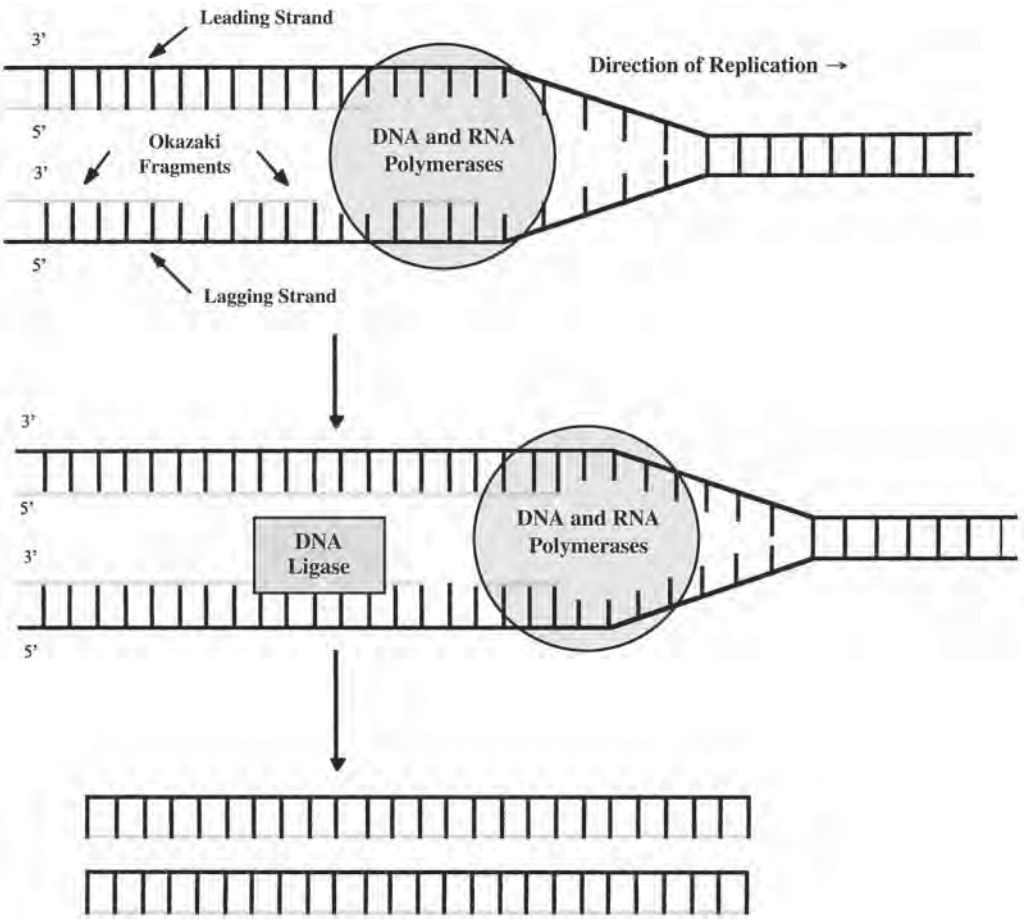

Fig. 2. Semiconservative replication. Complementary nucleotides are added directly to the 3' end of the leading strand, whereas the lagging strand is copied by the formation of Okazaki fragments.

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 (see **Fig. 2**).

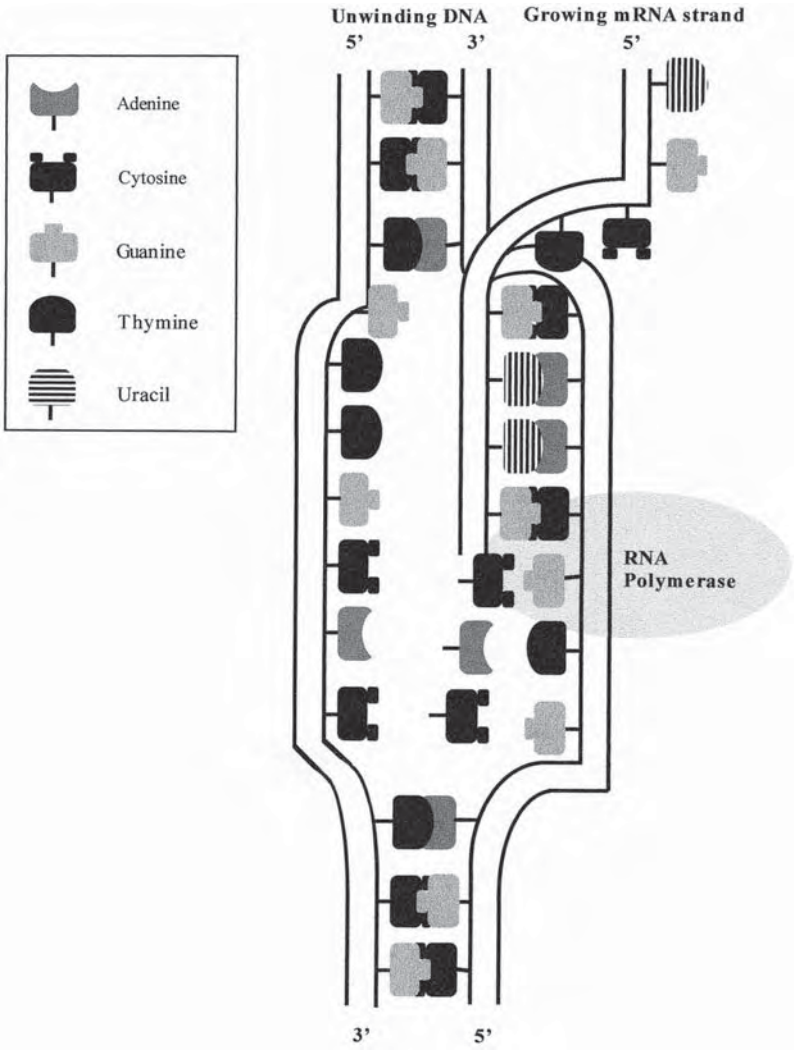

Fig. 3. Transcription. A DNA molecule is copied into mRNA with the help of RNA polymerase.

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 a mRNA molecule (see **Fig. 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

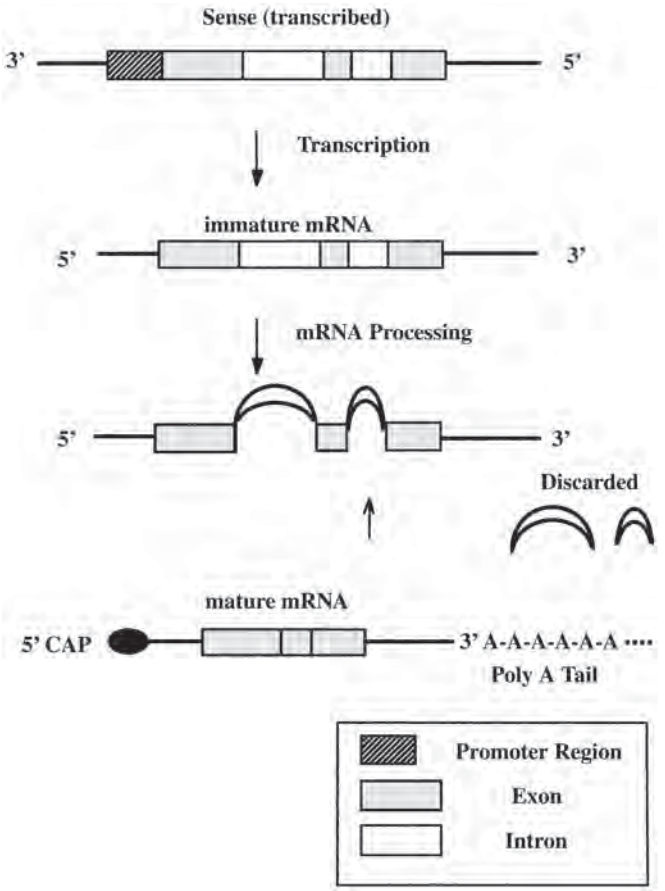

Fig. 4. Messenger RNA processing. The transcribed strand of DNA is modified to produce a mature mRNA transcript.

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 (see **Fig. 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 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 *(5)* and Khorana *(6)* determined that three nitrogen bases on an mRNA molecule constitute a *codon*. With 4 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 is referred to as degeneracy of the DNA code.

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.

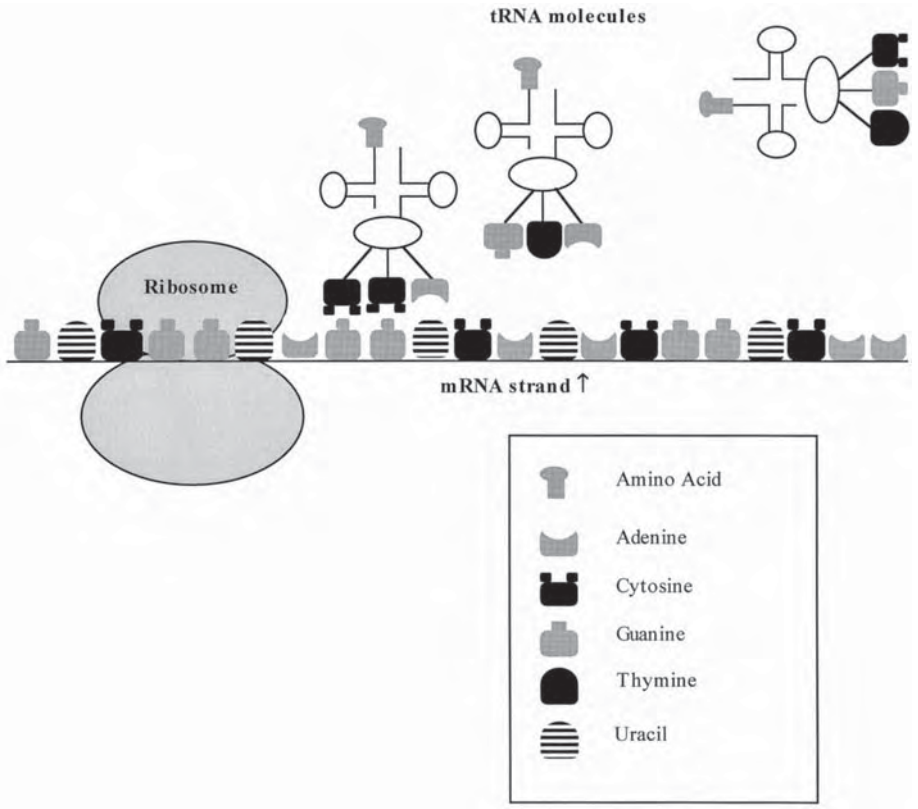

Fig. 5. Translation. Transfer RNA molecules bring in specific amino acids according to the triplet codon instructions of mRNA that are read at the ribosomes.

Ribosomes read mRNA one codon at a time. Transfer RNA molecules transfer the specific amino acids to the synthesizing protein chain (see **Fig. 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 (see **Fig. 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 *(7)* and therefore must be condensed considerably to fit within the cell nucleus. 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 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

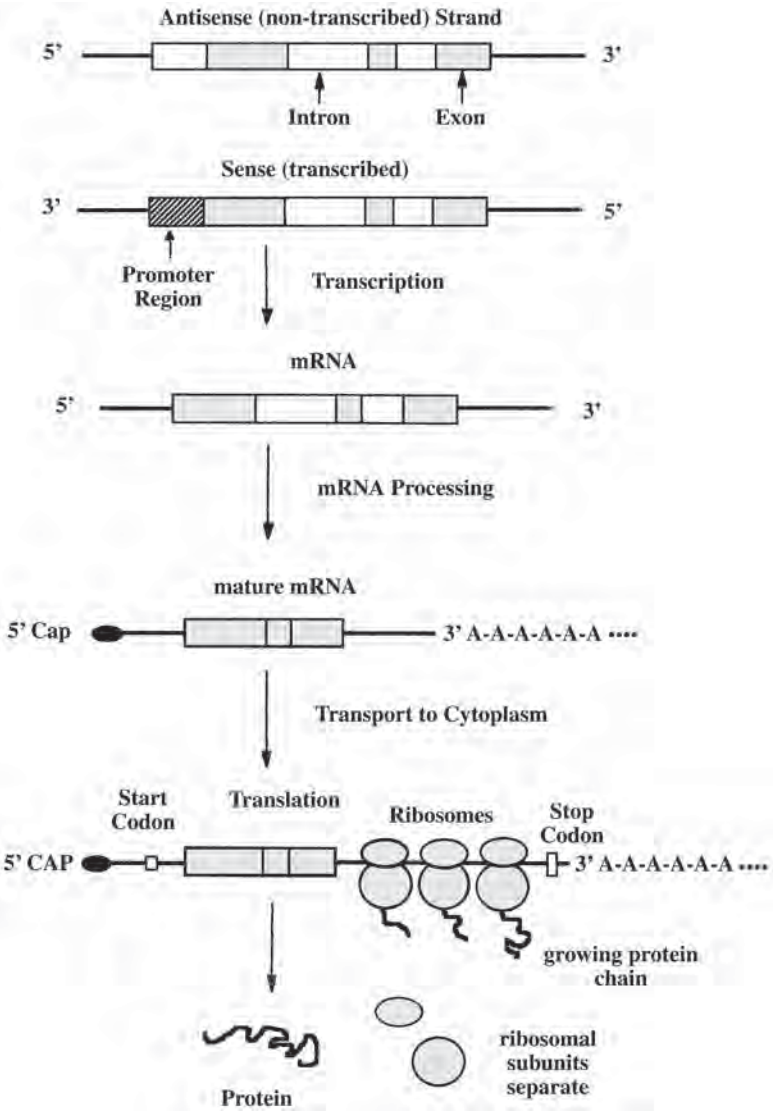

Fig. 6. Overview of protein synthesis. DNA is transcribed to mRNA, which is modified to a mature transcript and then transferred to the cytoplasm of the cell. The codons are read at the ribosomes and translated with the help of tRNA. The chain of amino acids produced during translation is joined by peptide bonds to form a protein molecule.

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 the mitotic metaphase (see **Fig. 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 organizing regions are functionally differentiated areas of the chromosomes (see **Fig. 8**).

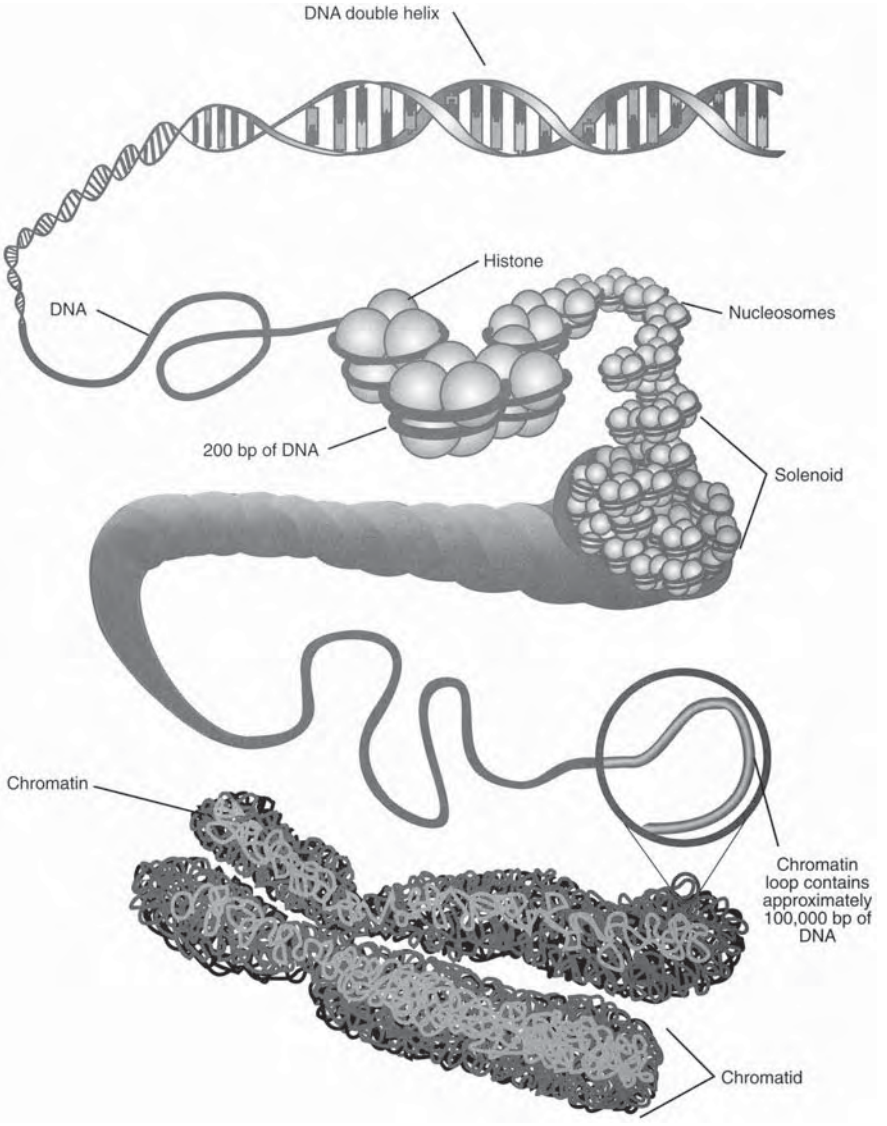

Fig. 7. The levels of DNA organization. (Reprinted from ref. *21* with permission from Elsevier).

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 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 grouped 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 it is 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

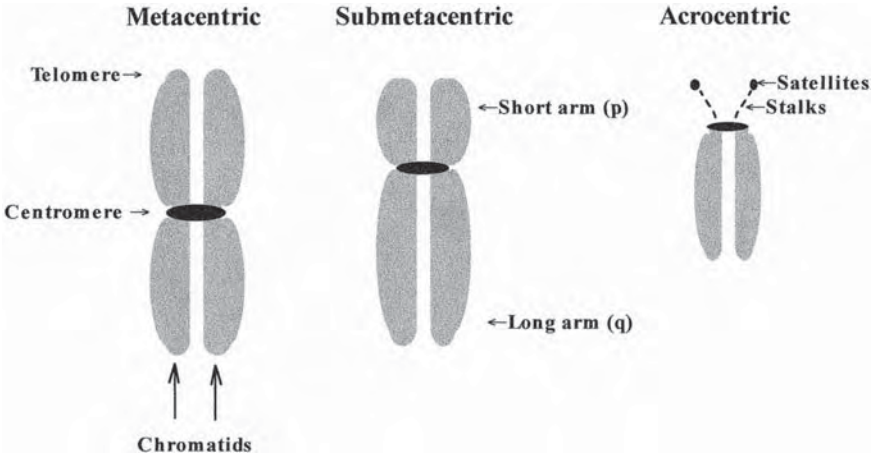

Fig. 8. The functional and structural components of metaphase chromosomes.

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 10 nucleolar organizer regions, although all may not be active during any given cell cycle.

The Telomeres

The telomeres are the physical end 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. The 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 (>10^5 copies), and middle repetitive sequence DNA (10^2–10^4 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 *(13)* and is classified according to the number of repeats and whether the repeats are tandem or interspersed among unique sequence DNA.

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-satellite, minisatellite, and microsatellite DNA.

Alpha-satellite DNA is a repeat of a 171-basepair sequence organized in a tandem array of up to a million basepairs 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 below). 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-basepair portion of some α-satellite DNA, but the functional significance of this has not been determined *(16)*.

Minisatellites have repeats that are 20–70 basepairs in length, with a total length of a few thousand basepairs. Microsatellites have repeat units of 2, 3, or 4 basepairs and the total length is usually less than a few hundred basepairs. 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 basepairs. 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 7000 bases. The predominant member of the LINEs 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 Chapters 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.

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 that 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)*.

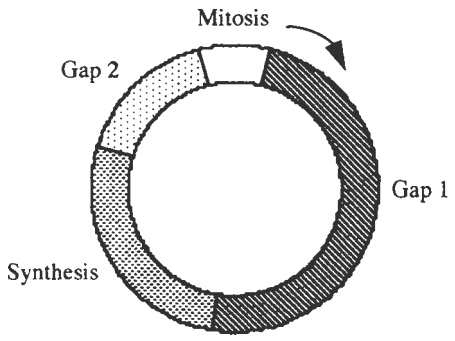

Fig. 9. The cell cycle: gap 1, Synthesis, gap 2, and mitosis.

Facultative Heterochromatin

One X chromosome of every female cell is randomly inactivated. The inactivated X is condensed during the 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)*.

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 hours and is the transition of a cell from one interphase through cell division and back to the 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 the interphase. The fourth and final stage of the cell cycle is mitosis (M) (see **Fig. 9**).

The first stage, G1, is the longest and typically lasts about 9 hours *(21)*. Chromosomes exist as single chromatids during this 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 its 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 hours 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 the 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 hours *(21)*. During this phase, the cell prepares to undergo cell division. The completion of G2 represents the end of the interphase.

The final step in the cell cycle is mitosis. This stage lasts only 1–2 hours 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 (see **Fig. 10**).

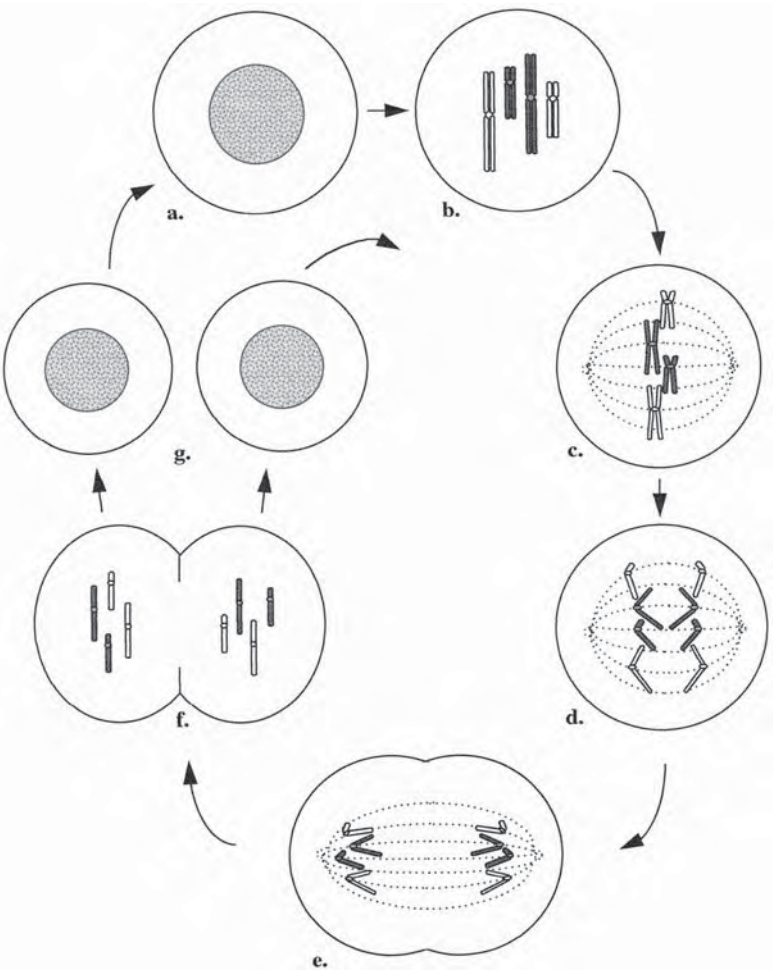

Fig. 10. Mitosis. Schematic representation of two pairs of chromosomes undergoing cell division: **(a)** interphase, **(b)** prophase, **(c)** metaphase, **(d)** anaphase, **(e)** telophase, **(f)** cytokinesis, and **(g)** interphase of the next cell cycle.

MITOSIS

Prophase

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

Prometaphase

Prometaphase is a short period between the prophase and the 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 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.

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 the 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 (see **Fig. 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 threadlike 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

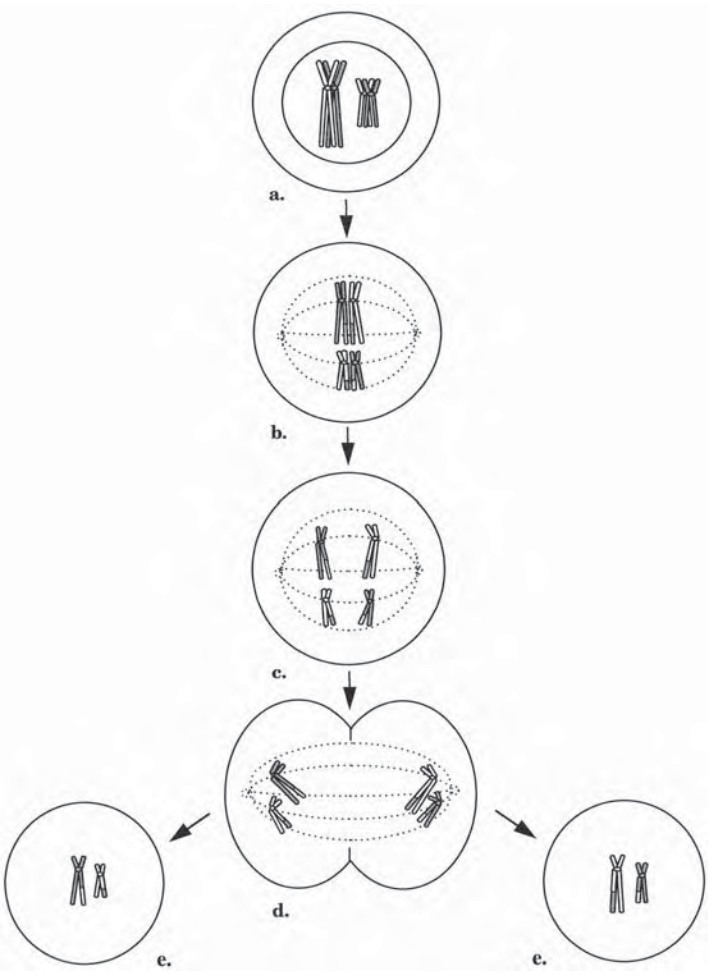

Fig. 11. Schematic representation of two chromosome pairs undergoing meiosis I: **(a)** prophase I, **(b)** metaphase I, **(c)** anaphase I, **(d)** telophase I, and **(e)** products of meiosis I.

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. Repelling 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.

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.

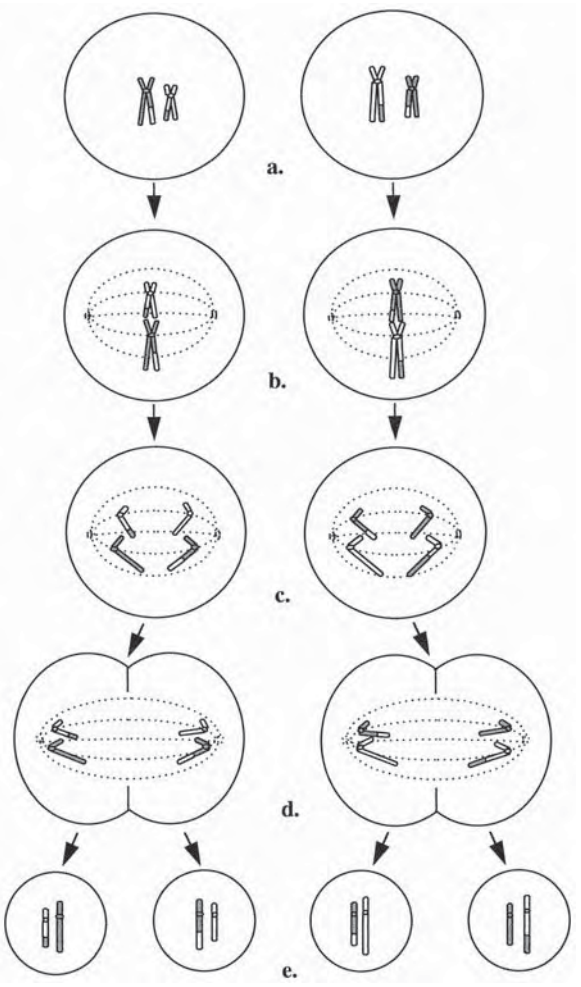

Fig. 12. Schematic representation of two chromosome pairs undergoing meiosis II: **(a)** prophase II, **(d)** metaphase II, **(c)** anaphase II, **(d)** telophase II, and **(e)** products of meiosis.

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 composed 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 (see **Fig. 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

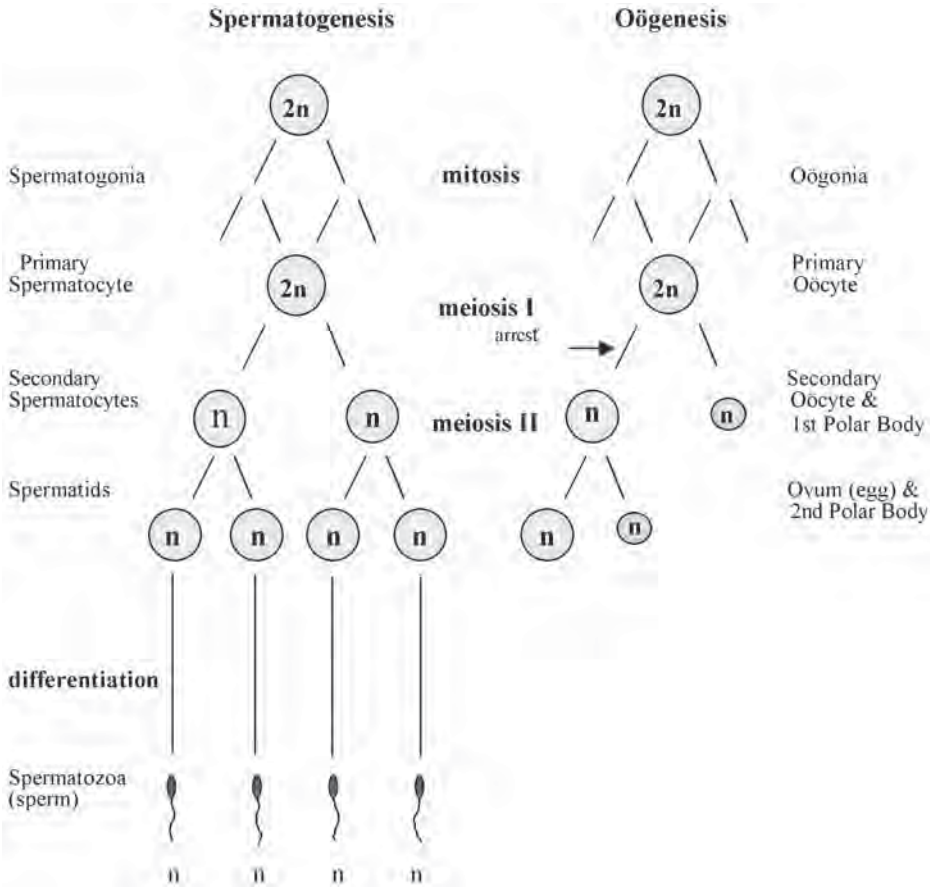

Fig. 13. Spermatogenesis and oögenesis. The events of spermatogenesis and oögenesis are the same, but the timing and net results are different. Oögenesis begins prenatally and is arrested in meiosis I until the postpubertal life of a woman.

effects crossing-over and random assortment of homologs, each of the new cells differ 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 (see **Fig. 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, 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. The pronuclei fuse to form the diploid nucleus of the zygote and the first mitotic division begins.

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3

Human Chromosome Nomenclature

An Overview and Definition of Terms

Avirachan Tharapel, PhD

INTRODUCTION

Advancements in methodology and discovery of the diploid human chromosome number invigorated further research in human cytogenetics *(1,2)*. The eventful years that followed witnessed the birth of a new specialty—human cytogenetics—which provided answers to many intriguing phenomena in medicine. Little was known at the time that human cytogenetics would form the backbone of present-day "human genetics," providing answers to questions regarding human reproduction, behavior, aging, and disease while generating knowledge that could be applied to the treatment and prevention of many disorders.

The discovery of the chromosomal etiology of Down syndrome, Turner syndrome, Klinefelter syndrome, Edwards syndrome, and Patau syndrome further added to the knowledge that variations from the normal diploid chromosome number and structure can cause severe phenotypic malformations and mental impairment. The investigators responsible for these early discoveries came from both sides of the Atlantic. Working independently, they devised their own terminology and nomenclature to describe chromosome abnormalities. Confusion in the scientific literature was the result. The need for guidelines and standardization of terminology thus became imperative. At a conference held in Denver, CO, 14 attendees from different countries argued for 3 days. In the end, they agreed upon guidelines for describing human chromosomes and chromosome abnormalities. This historic document is called the *Denver Conference (1960): A Proposed Standard System of Nomenclature of Human Mitotic Chromosomes (3)*.

Although the basic principles adopted in Denver have prevailed to date, new technologies and ever-increasing knowledge in human cytogenetics necessitated periodic revision and update of the nomenclature document (see **Table 1**). The Chicago Conference nomenclature *(4)* was widely used from 1966 to 1971 during the prebanding era (see **Fig. 1**). At the Paris Conference *(5, 6)* the document was expanded so that banded chromosomes (see **Fig. 2**) could be described. With the Stockholm Conference in 1977, the proceedings came to be known as the International System for Human Cytogenetic Nomenclature or ISCN *(7)*. Each ISCN is identified by the year of its publication *(8–10)*. The document currently in use is *An International System for Human Cytogenetic Nomenclature (1995),* abbreviated as ISCN 1995 as agreed upon by the conferees in Memphis, TN, in October, 1994 *(11)*. ISCN 1995 established a uniform code for designating both constitutional (congenital) and acquired chromosome abnormalities as well as one for describing and reporting results obtained from *in situ* hybridization methodologies.

ISCN 1995 has a certain uniqueness. It has provided a new 850-band-level ideogram based on actual measurements of bands. For comparative purposes, it includes G- and R-banded composite photographs of chromosomes at band resolutions ranging from about 400 to 850 bands. It has introduced

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Table 1
International Conferences on Human Chromosome Nomenclature

Conference/Document		*Year of Publication*
Denver Conference		1960
London Conference		1963
Chicago Conference		1966
Paris Conference		1971
Paris Conference (Supplement)		1975
Stockholm—1977	ISCN	1978
Paris—1980	ISCN	1981
ISCN		1985
Cancer Supplement	ISCN	1991
Memphis—1994	ISCN	1995

specific ways to accurately describe Robertsonian translocations, whole-arm translocations, and uniparental disomy. Keeping up with technical developments, this document for the first time has established nomenclature guidelines for the description of fluorescence *in situ* hybridization (FISH). In the following pages, I have attempted to simplify the use and point out the highlights of ISCN 1995. The examples that appear in this chapter are based on the dictates of this nomenclature document. However, for a detailed understanding of ISCN 1995, the reader is requested to refer to the original document *(11)*.

HUMAN CHROMOSOMES

Of the 46 chromosomes in a normal human somatic cell, 44 are autosomes and 2 are sex chromosomes. The autosomes are designated as pairs 1–22. The numbers are assigned in descending order of the length, size, and centromere position of each chromosome pair. In a normal female the sex chromosomes are XX, and in a normal male, they are XY.

Until the advent of certain specialized staining techniques, arbitrary identification of individual chromosome pairs was based on the size and position of the centromere *(4)*. Variability in the centromere position of different chromosomes allowed them to be classified into three basic categories. A chromosome with its centromere in the middle is *metacentric,* one with the centromere closer to one end is *sub-metacentric,* and one with the centromere almost at one end is *acrocentric* (see **Fig. 3**). Based on decreasing relative size and centromere position, a karyotype comprised of seven groups labeled A through G was devised. The X chromosome belonged to the third or "C" group, whereas the Y was often placed separately. Although still used occasionally, these letter group names are now considered obsolete.

Chromosome Banding and Identification

Unequivocal identification of individual chromosomes and chromosome regions became possible with the technical developments of the late 1960s (refer to Chapters 1 and 4). When chromosome preparations are treated with dilute solutions of proteolytic enzymes (trypsin, pepsin, etc.) or salt solutions (2X SSC) and treated with a chromatin stain such as Giemsa, alternating dark and light stained demarcations called *bands* appear along the length of each chromosome. The banding patterns produced are specific for each chromosome pair, thus enabling the identification not only of individual chromosomes but also of regions within each chromosome. Methods commonly used to produce these discriminative banding patterns include Giemsa or G-banding, quinacrine mustard or Q-banding, reverse or R-banding and constitutive heterochromatin or C-banding, each with its own uniqueness. In the United States and Canada, the most frequently used methods for routine cytoge-

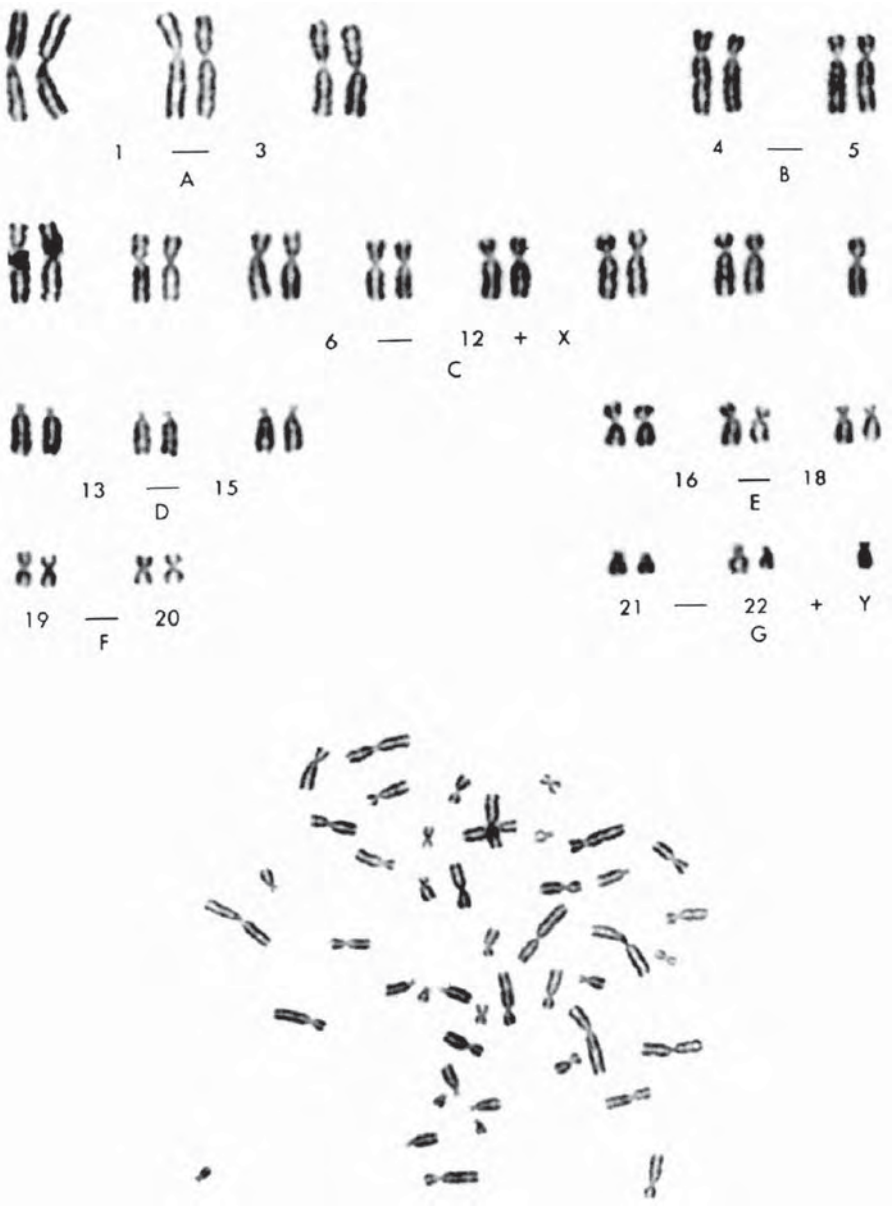


Fig. 1. Unbanded metaphase spread **(bottom)** and corresponding karyotype **(top)** per the Chicago conference.

netic analysis are G- and Q-bands (see **Fig. 2**), whereas in other countries (France, for example), R-banding is more common. Additional banding methods are occasionally employed to exemplify specific abnormalities or chromosome regions. Abbreviations commonly used to denote the various banding techniques appear in **Table 2**.

Chromosome Regions and Band Designations

The chromosomal details revealed by the new banding techniques necessitated the introduction of additional terminology and modifications of certain existing ones. This task was accomplished by a

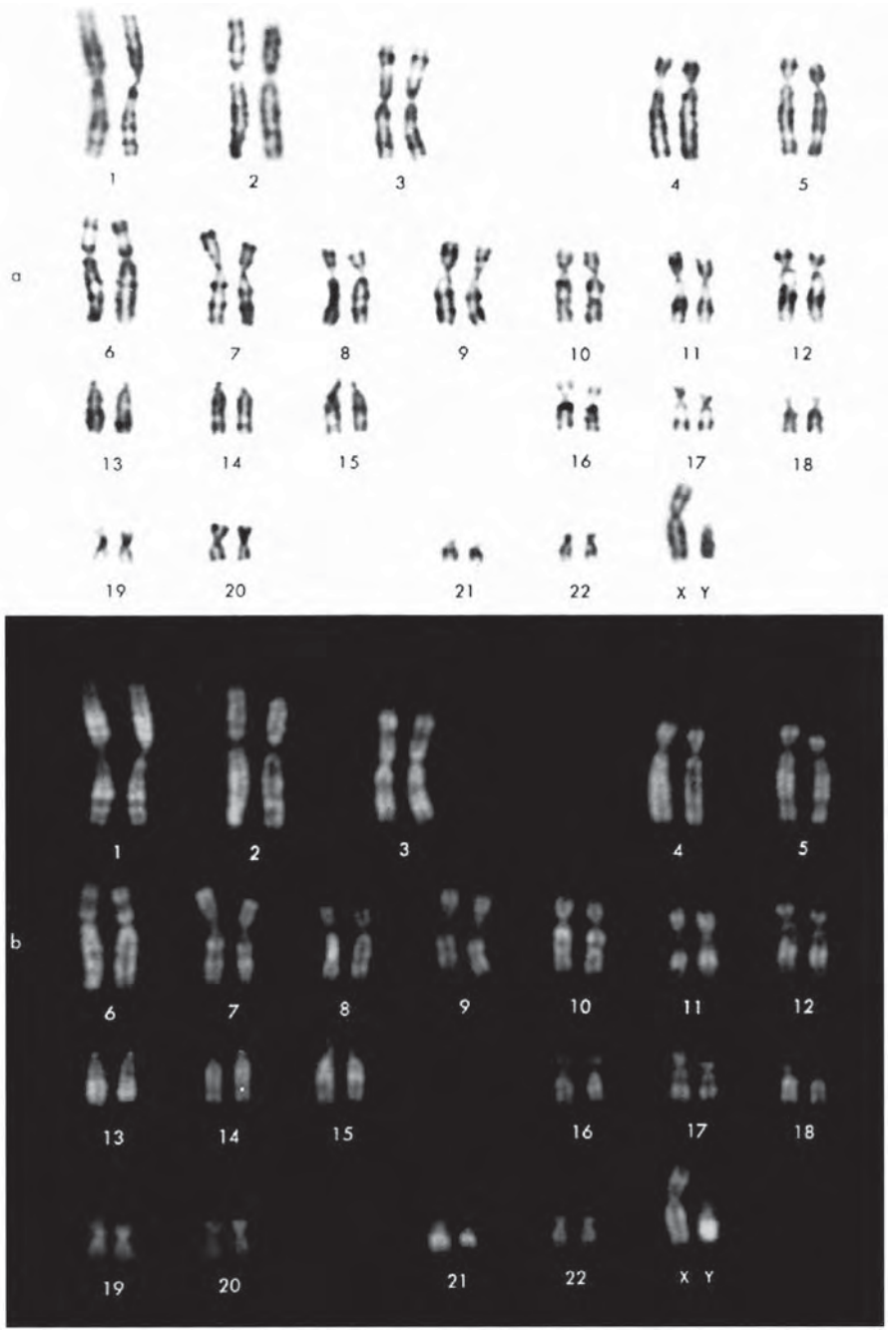


Fig. 2. Normal 46,XY male karyotype. Characteristic G-band pattern **(a)** and fluorescent Q-banding **(b)**. The same cell was used for both methodologies to demonstrate the complementary banding patterns.

standing committee appointed at the Fourth International Congress of Human Genetics in Paris. The recommendations of the committee were published as *Paris Conference (1971): Standardization in Human Cytogenetics*. Through a diagrammatic representation of banding pattern, the document elucidated the typical band morphology for each chromosome *(5)* (see **Fig. 4**). The Paris Conference

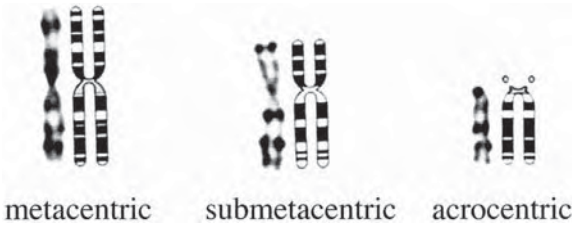


Fig. 3. Examples of metacentric, submetacentric, and acrocentric chromosomes.

Table 2
Frequently Used Banding Methods and Their Abbreviations

Banding Method	*Abbreviation*
Q-bands	Q
Q-bands by quinacrine derivatives and fluorescence microscopy	QFQ
G-bands	G
G-bands by trypsin and Giemsa	GTG
C-bands	C
C-bands by barium hydroxide and Giemsa	CBG
R-bands	R
R-bands by acridine orange and fluorescence microscopy	RFA
R-bands by BrdU and Giemsa	RBG
Telomere bands or T-bands	T

(1971) introduced a numbering system helpful in designating specific bands and regions. New terminology and abbreviations were introduced to help explain chromosome abnormalities in a more meaningful way. Other conferences then followed, with the latest held in Memphis in 1994. Descriptions of human chromosomes and their abnormalities utilize a series of symbols and abbreviations. A partial list of recommended symbols and abbreviations in ISCN 1995 appear in **Table 3**.

The centromere "cen" divides a chromosome into a short or "p" arm (from the French petit) and a long or "q" arm. For descriptive purposes, the centromere is composed of two portions. The portion of the centromere lying between its middle and the first band on the short arm is designated as "p10." Similarly, the portion of the centromere lying between its middle and the first band on the long arm is designated as "q10." The designations p10 and q10 allow us to describe accurately the nature and organization of centromeres in isochromosomes, whole-arm translocations, and Robertsonian translocations (see below). Each arm ends in a terminus ("ter," thus "pter" and "qter"), where telomeres are present to prevent the chromosomes from having "sticky ends."

Each chromosome arm is divided into *regions*. This division is based on certain *landmarks* present on each chromosome. By definition, a landmark is "a consistent and distinct morphologic area of a chromosome that aids in the identification of that chromosome." A *region* is an area that lies between two landmarks. The two regions immediately adjacent to the centromere are designated as "1" (p1 and q1), the next distal as "2," and so on. Regions are divided into *bands* and the bands into *subbands* (see **Fig. 5**). A band is that part of a chromosome that is distinctly different from the adjacent area by virtue of being lighter or darker in staining intensity. Sequential numbering of chromosome arms and

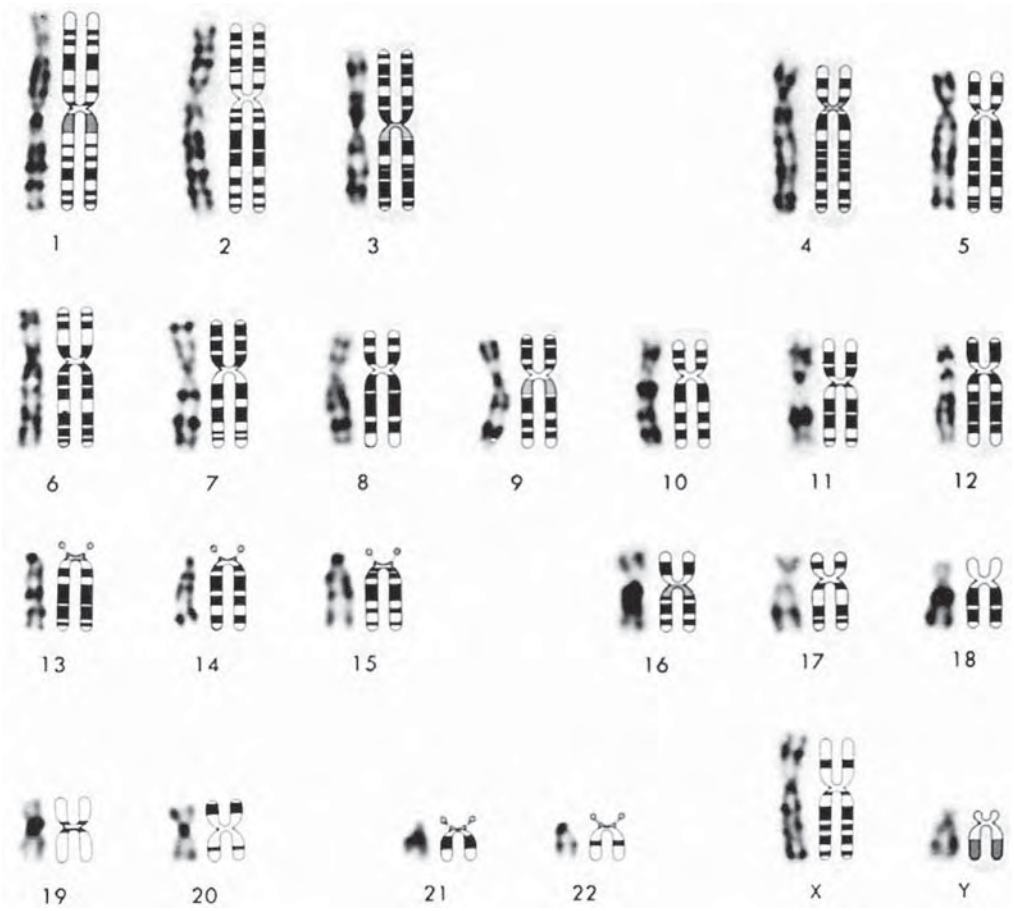


Fig. 4. A composite karyotype of G-banded chromosomes **(left)** along with the corresponding 1971 Paris Conference ideograms **(right)**.

bands helps make the designation of specific bands easy. For example, the terminal band on the long arm of chromosome 2 can be written as 2q37 to mean chromosome 2, long arm, region 3, band 7 and is referred to as "two q three-seven," not "two q thirty-seven".

Karyotype Descriptions

Karyotype descriptions follow certain basic rules. When designating a karyotype, the first item specified is the total number of chromosomes, including the sex chromosomes present in that cell, followed by a comma and the sex chromosomes in that order. Thus, a normal female karyotype is written as 46,XX and a normal male karyotype as 46,XY. The characters are contiguous, without spaces between items. Chromosome abnormalities, when present, follow the sex chromosome designation using abbreviations or symbols denoting each abnormality (see **Table 3**). These are listed in a specific order: Sex chromosome abnormalities are described first, followed by autosomal changes in numerical order. For each chromosome described, numerical changes are listed before structural abnormalities.

Most karyotypes can be described using the "short form" of the nomenclature, which is used in this chapter. However, it should be noted that for certain complex rearrangements this can produce ambiguity. ISCN therefore provides for a "long form," in which abnormal chromosomes can be

Table 3
Selected List of Symbols and Abbreviations Used in Karyotype Designations

Abbreviation or Symbol	*Description*
add	Additional material, origin unknown
arrow (_ or →)	From – to, when using long form
[ ] square brackets	Number of cells in each clone
cen	Centromere
chi	Chimera
single colon (:)	Break
double colon (::)	Break and reunion
comma (,)	Separates chromosome number, sex chromosomes, and abnormalities
del	Deletion
der	Derivative chromosome
dic	Dicentric
dmin	Double minute(s)
dup	Duplication
fis	Fission
fra	Fragile site
h	Heterochromatin
i	Isochromosome
inv	Inversion
ins	Insertion
mar	Marker chromosome
mat	Maternal origin
minus sign (–)	Loss
mos	Mosaic
multiplication sign (×)	Multiple copies; also designates copy number with ish
p	Short arm of chromosome
pat	Paternal origin
Ph or Ph1	Philadelphia chromosome
plus sign (+)	Gain
q	Long arm of chromosome
question mark (?)	Uncertainty of chromosome identification or abnormality
r	Ring chromosome
rcp	Reciprocal
rec	Recombinant chromosome
rob	Robertsonian translocation
s	Satellite
slash (/)	Separates cell lines or clones
semicolon (;)	Separates chromosomes and breakpoints in rearrangements involving more than one chromosome
stk	Satellite stalk
t	Translocation
upd	Uniparental disomy

Note: For a complete listing of symbols and abbreviations, refer to ref. *11*.

described from end to end, with all structural changes "spelled out" in detail. Some examples are provided throughout the chapter; the reader is encouraged to refer to the original document *(11)* for additional information.

The remainder of this chapter discusses the current method of using the ISCN nomenclature to describe chromosome abnormalities. A section on interpretation of karyotype descriptions follows.

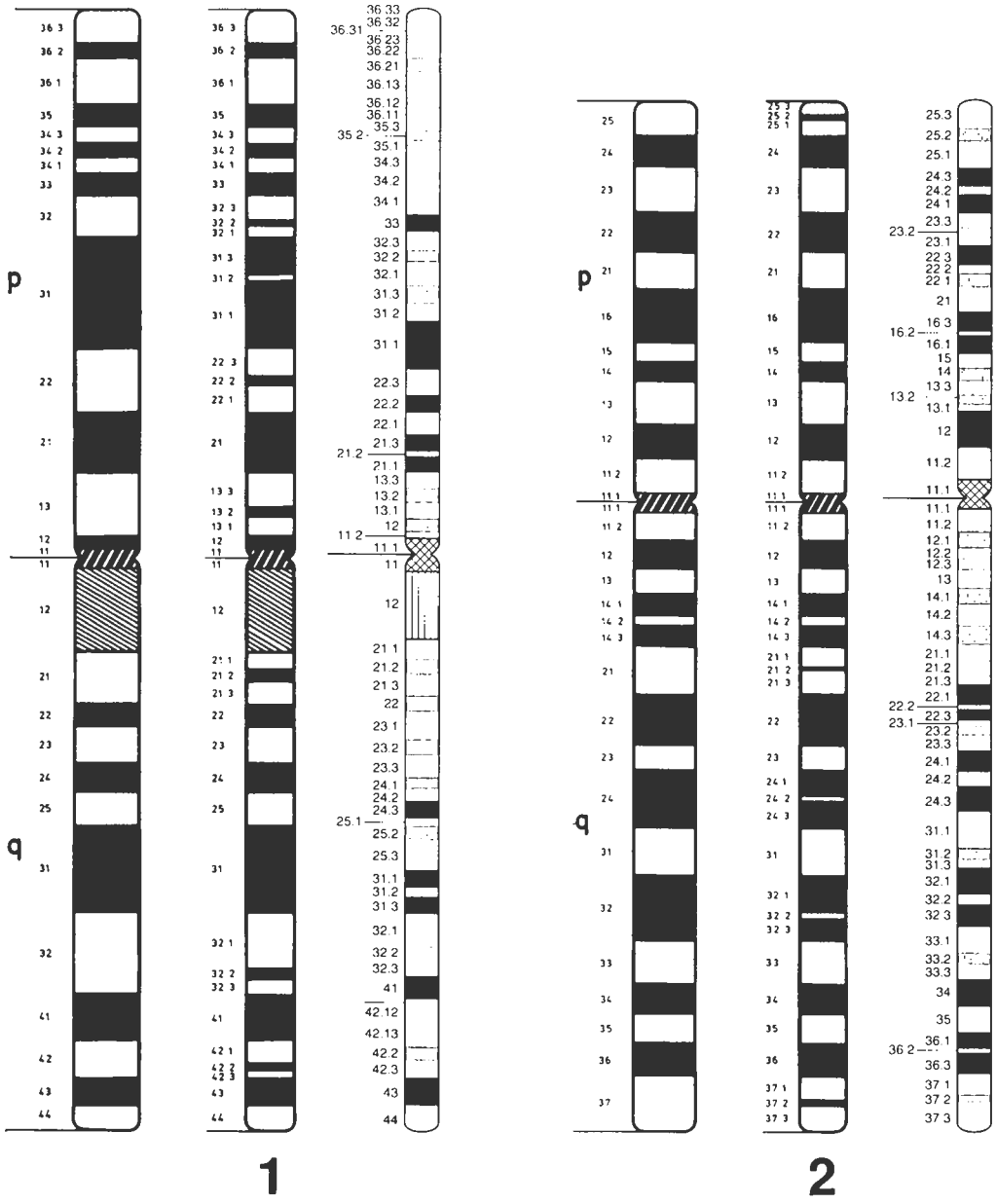


Fig. 5. Ideogram showing the G-banding pattern for normal human chromosomes at three different band resolutions. The left chromosome represents a haploid karyotype (one of each chromosome) of approximately 400 bands. The middle chromosome is at an approximately 550-band level, and the right chromosome represents about 850 bands. (Reproduced from ref. *11* with permission of S. Karger AG, Basel.)

NUMERICAL ABNORMALITIES OF CHROMOSOMES

The term "numerical abnormality" refers to gain or loss of chromosomes. As outlined above, all such abnormalities are presented in numerical order with the exception of the X and Y, which are always listed first. To designate an additional or a missing chromosome plus (+) and minus (–) signs

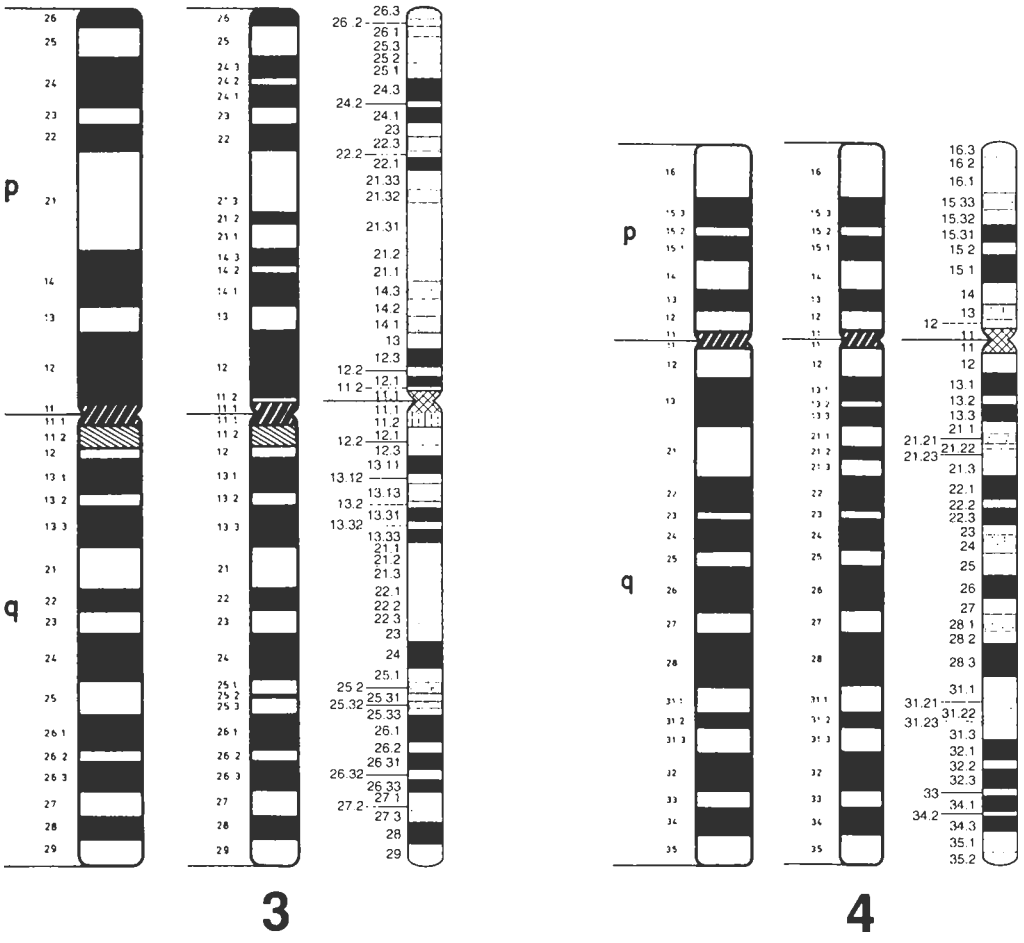


Fig. 5. (*continued*)

are placed before the specific chromosome number. Thus, –7,+18 would mean a missing chromosome 7 and an extra chromosome 18. Note that these abnormalities are presented in numerical order, regardless of whether they involve gain or loss of a chromosome. A + sign can also be used to denote additional copies of derivative chromosomes or accessory marker chromosomes, e.g., +der(6) or +mar (see below).

Numerical Abnormalities Involving the Sex Chromosomes

These can be constitutional (congenital) or acquired. ISCN 1995 provides special ways to distinguish between the two. As shown in the examples below, the + and—signs are not needed to designate constitutional sex chromosome aneuploidies.

Constitutional Sex Chromosome Aneuploidies

45,X	Classical monosomy X or Turner syndrome
47,XXY	Classical Klinefelter syndrome
47,XXX	A female with three X chromosomes
48,XXYY	Variant of Klinefelter syndrome with two X and two Y chromosomes

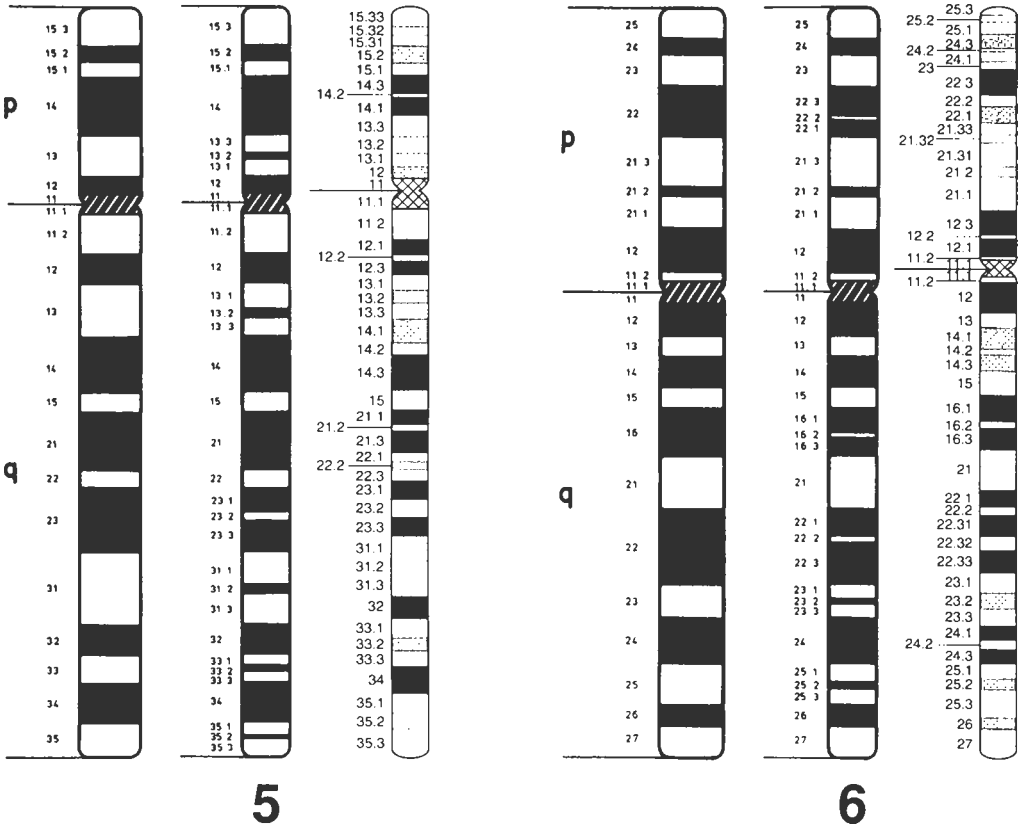


Fig. 5. (*continued*)

Acquired Sex Chromosome Aneuploidies

These involve chromosome changes seen in certain leukemias and solid tumors and are restricted to the affected tissues:

45,X,–X

This describes a normal female with two X chromosomes but with the loss of one X chromosome in her tumor cells.

47,XX,+X

This is a normal female with two X chromosomes and gain of an extra X chromosome in her tumor cells.

45,X,–Y

This is a normal male with XY chromosomes and loss of the Y chromosome in his tumor cells.

48,XY,+X,+Y

This describes a male with acquired X and Y chromosomes in his tumor cells.

48,XXYc,+X

Here, we have a patient with Klinefelter syndrome who has an acquired X chromosome in his tumor cells. The letter "c" is placed next to XXY to show that the patient's sex chromosome complement is XXY and *not* XY or XXXY.

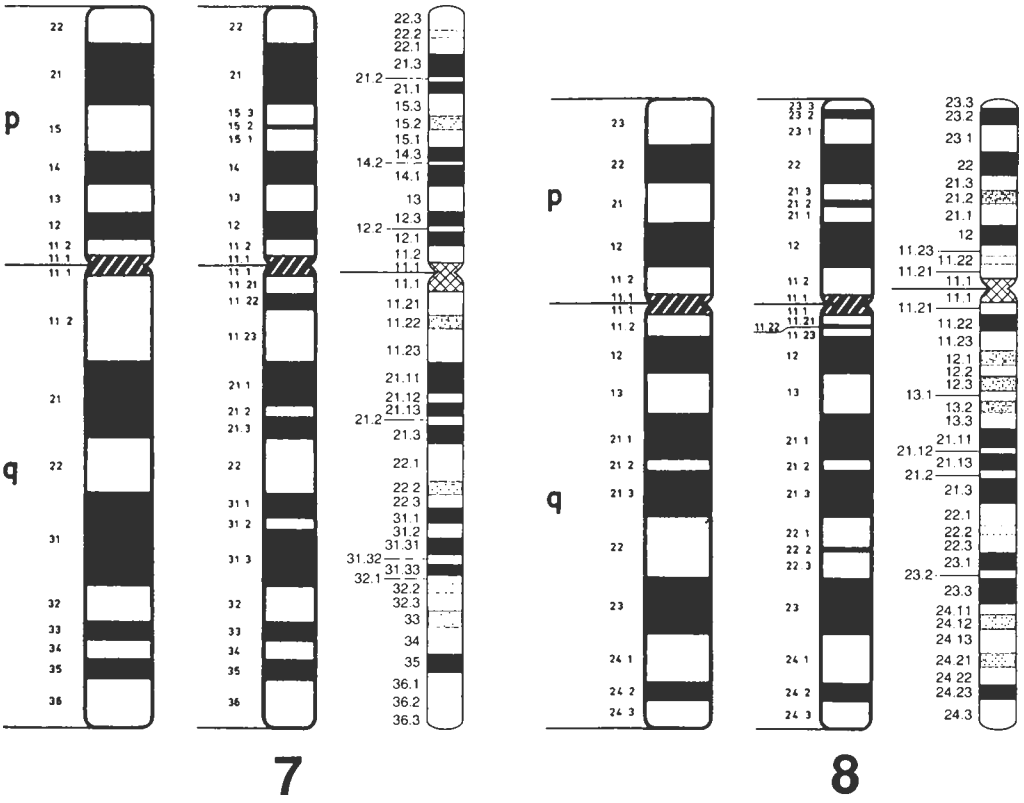


Fig. 5. (*continued*)

46,Xc,+X

This is a Turner syndrome patient (45,X) with gain of an X chromosome in her tumor cells.

Numerical Abnormalities of the Autosomes

The situation here is similar to that involving the sex chromosomes, with the exception that (+) and (–) signs are used to designate constitutional abnormalities:

47,XY,+18	Male with trisomy 18
48,XX,+18+21	Female with both trisomy 18 and trisomy 21
45,XY,–21	Male with monosomy 21
46,XY,+21c,–21	Male trisomy 21 patient with loss of one chromosome 21 in his tumor cells
48,XX,+21c,+21	Female with trisomy 21 and gain of an additional chromosome 21 in her tumor cells

Mosaics and Chimeras

An individual with two or more cell types, differing in chromosome number or structure is either a mosaic or a chimera. If the two cell types originated from a single zygote, the individual is a mosaic (mos). If the cell types originated from two or more zygotes that subsequently fused, the individual is a chimera (chi). In designating mosaic or chimeric karyotypes, a slash (/) is used to separate the cell lines. The actual number of cells detected in each clone can be given within square brackets [ ]. The largest clone is recorded first, then the next largest, and so on. Whenever a normal cell line is present, it is always recorded last, irrespective of the number of normal cells detected.

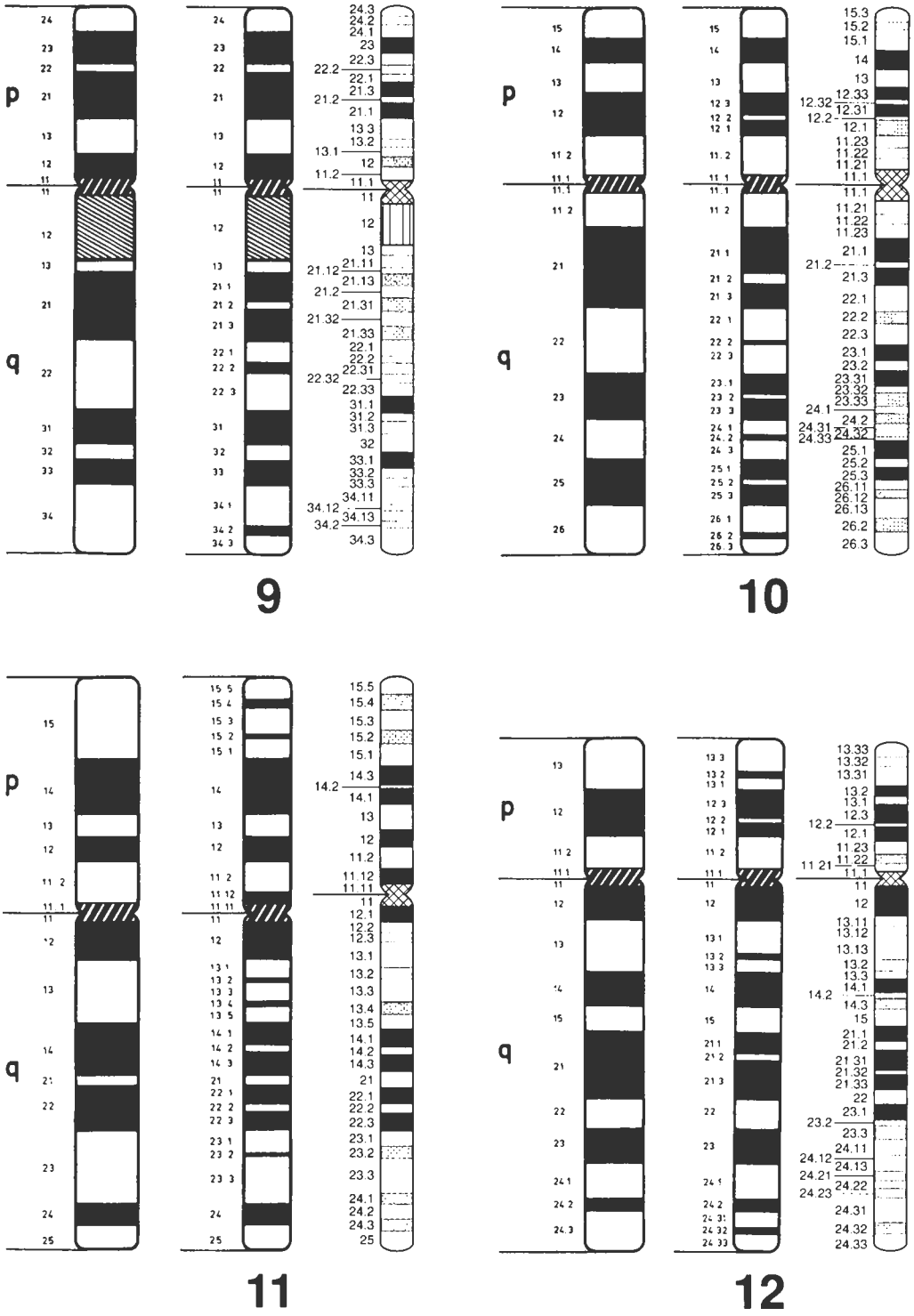


Fig. 5. (*continued*)

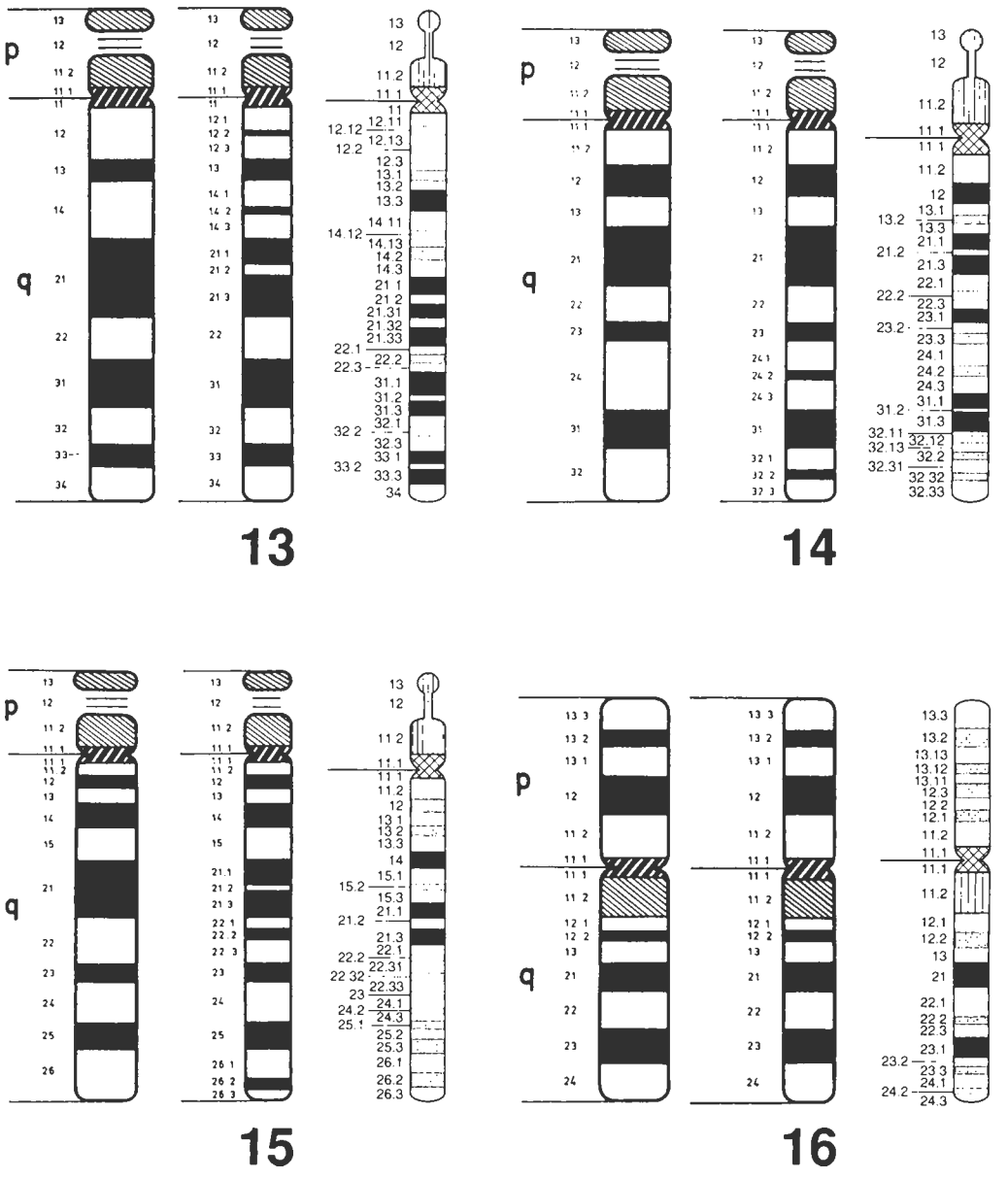


Fig. 5. (*continued*)

Examples are as follows:

mos 45,X[4]/46,XX[16]
: This is a Turner mosaic with two cell lines. Analysis of 20 cells showed that this individual has 4 cells that are 45,X and 16 cells that are 46,XX.

mos 45,X[5]/47,XYY[5]/46,XY[10]
: This represents a mosaic with three cell lines.

mos 47,XX,+13[15]/46,XX[5]
: This is a mosaic with both trisomy 13 and normal cell lines.

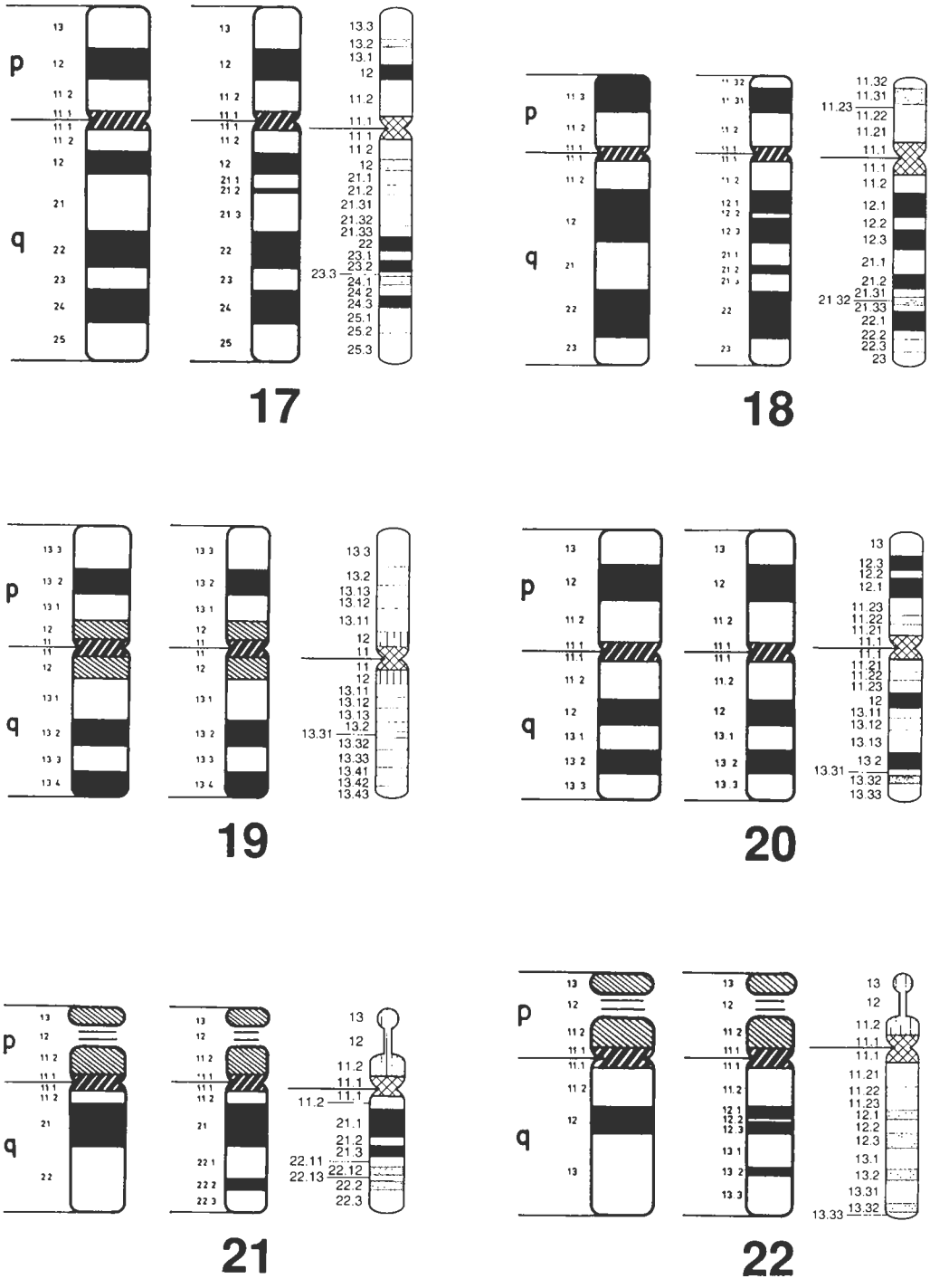


Fig. 5. (*continued*)

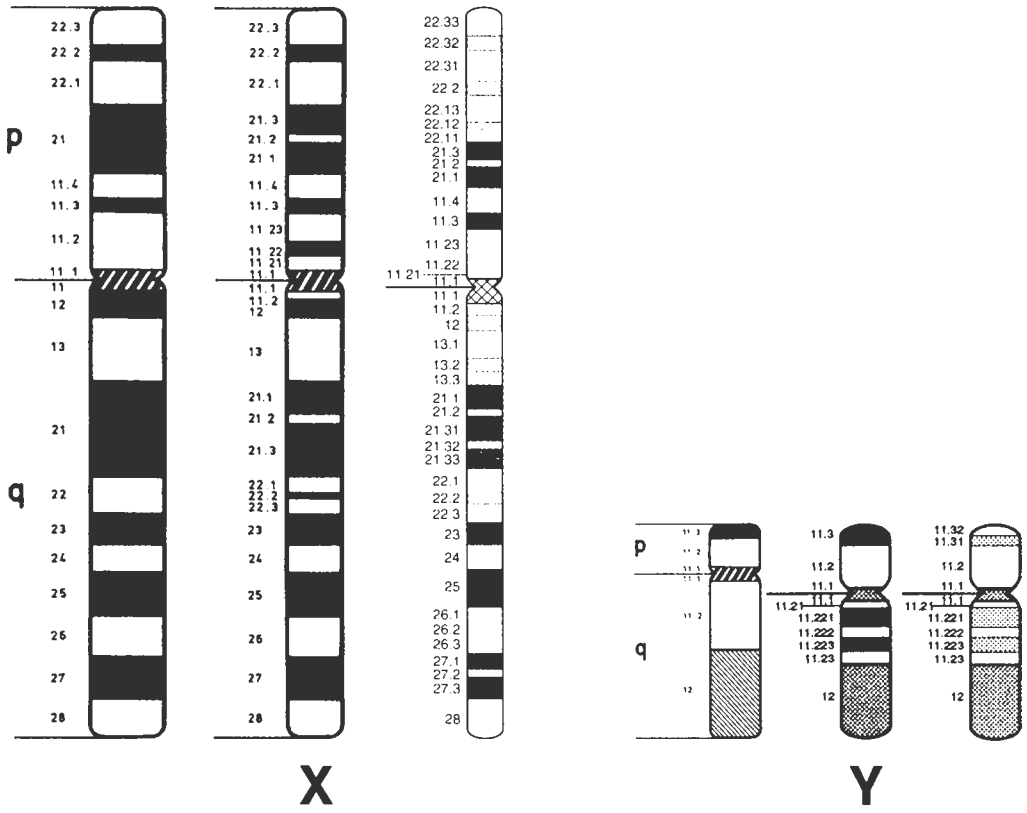


Fig. 5. (*continued*)

In a chimera where the two cell lines are normal (46,XX and 46,XY) and both are present in equal proportions, either one of them can be listed first. If one cell line is larger than the other, the larger clone is listed first.

chi 46,XX[10]/46,XY[10]
: This describes a chimera with female and male cells in equal number.

chi 47,XX+21[15]/46,XY[5]
: This is a chimera with both female and male cell lines. The female cell line shows trisomy 21, whereas the male cell line is normal.

chi 69,XXX[20]/46,XY[5]
: This represents a chimera with triploid and diploid cell lines. The triploid line is XXX, whereas the diploid line is XY.

Use of the abbreviations chi and mos is optional, as the presence of chimerism or mosaicism is usually evident from the karyotype.

STRUCTURAL CHROMOSOME ABNORMALITIES

This category of abnormalities includes several subclasses that will be discussed under separate headings. Again, as previously stated, all chromosomes involved in abnormalities are designated in numerical order, except for the X and Y, which are listed first.

When designating an abnormality that is limited to a single chromosome, the abbreviation for that abnormality is used, followed by the chromosome number in parentheses [e.g., r(X), del(2), ins(4), dup(5)]. If two or more chromosomes are involved in a rearrangement, as with translocations, a semicolon (;) is used to separate chromosome numbers within parentheses [e.g., t(3;4), t(2;5;10;) or t(15;17)]. Again, chromosomes are listed in numerical order unless a sex chromosome is involved [e.g., t(X;1) or t(Y;15)]. If, in the same cell, a specific chromosome is involved in both a numerical and a structural rearrangement, the numerical abnormality is designated first [e.g., +13,t(13;14)].

For ease of reference, the abnormalities covered will be presented in alphabetical order. For a thorough description of the mechanisms and clinical significance of structural chromosome abnormalities, see Chapter 9.

Additional Material, Origin Unknown (add)

When a chromosome has additional material attached to it, the origin of this material might not be identifiable with conventional banding methods. This is especially likely if the abnormality is subtle and originated *de novo* or is acquired. The abbreviation "add" (from the Latin *additio*) is used.

46,XX,add(17)(p13)
Additional material of unknown origin is attached to chromosome 17 at band p13.

46,XX,add(9)(q22)
Additional material of unknown origin attached to chromosome 9 at band q22. The region 9q22 → qter is missing and has been replaced by this material.

Deletions (del)

A deletion is an aberration in which a part of a chromosome is lost. Deletions can be either terminal, where all chromosomal material from the breakpoint on is lost, or interstitial, in which an internal section of one arm is missing. To introduce the reader to the long form of the nomenclature, a few of the following abnormalities will be presented using both the short and long forms.

Terminal Deletions

46,XY,del(1)(q32) (short form)
46,XY,del(1)(pter → q32:) (long form)
This karyotype describes a terminal deletion involving the long arm of chromosome 1. The colon present in the long form indicates a break at band 1q32 and deletion of the region distal to it. The rest of the chromosome, from 1pter to 1q32, is present.

Interstitial Deletions

46,XY,del(1)(p21p32) (short form)
46,XY,del(1)(pter → p21::p32 → qter) (long form)
Breakage and reunion are represented in the long form by a double colon (::). Here, this occurred involving bands 1p21 and 1p32. The segment between them has been deleted.

Derivative and Recombinant chromosomes

Derivative Chromosomes (der)

A structurally rearranged chromosome generated by events involving two or more chromosomes or the result of multiple events within a single chromosome is a derivative chromosome. Thus, each unbalanced product of a translocation event is a derivative chromosome. The identity of a derivative chromosome is determined by its centromere.

Examples are as follows:

46,XY,der(3)t(3;6)(p21;q23)
The derivative chromosome 3 in this example is the result of a translocation between the short arm of chromosome 3 at band p21 and the long arm of chromosome 6 at band q23. The der(3) replaces one

normal chromosome 3, and both chromosomes 6 are normal. This unbalanced karyotype results in monosomy (loss) of region 3p21 → pter and trisomy (gain) of 6q23 → qter. This karyotype is the product of adjacent-1 segregation (see Chapter 9).

45,XY,der(3)t(3;6)(p21;q23),–6
The der(3) is same as in the above example and again replaces one of the normal chromosomes 3. However, there is only one normal chromosome 6 in the case, resulting in monosomy for both 3p21 → pter and 6pter → q23. This is the result of 3:1 segregation (see Chapter 9).

47,XY,+der(3)t(3;6)(p21;q23)mat
The der(3) is the same as in the above examples. A 3:1 segregation in the mother resulted in a normal 3 and the derivative 3 to be retained in the ovum. The father contributed a normal 3 as well. The patient is, therefore, trisomic for both3p21 → qter and 6q23 → qter.

Recombinant Chromosomes (rec)

Recombinant chromosomes are also structurally rearranged chromosomes. They arise *de novo* from *meiotic* crossing-over between homologous chromosomes when one is structurally abnormal, often, in an inversion heterozygote.

Take, for example, an individual with the karyotype 46,XY,inv(3)(p21q27). As described below, this man has one chromosome 3 with a pericentric inversion involving the segment between bands p21 and q27. During meiosis, crossing-over within the inverted segment could result in two recombinant chromosomes, each of which has a duplication of one part of the chromosome and deletion of another part; this is described in detail in Chapter 9:

46,XY,rec(3)dup(3p)inv(3)(p21q27)
One normal chromosome 3 has been replaced by a recombinant chromosome 3. The segment 3p21 → pter is duplicated, and the segment from 3q27 → qter is deleted. The key to interpreting this karyotype is "dup(3p)"; dup indicates a duplication (see **Table 3**).

46,XY,rec(3)dup(3q)inv(3)(p21q27)
Here, the other possible recombinant chromosome is present, resulting in duplication of the segment 3q27 → qter and loss of the segment 3p21 → pter. In this case, note "dup(3q)."

Fragile Sites

As discussed in Chapters 14 and 18, fragile sites exist in many areas of the human karyotype. Although the fragile site responsible for fragile X syndrome is no longer diagnosed via cytogenetic analysis, the nomenclature occasionally can still be seen. A male would be described as 46,Y,fra(X)(q27.3), and a female would be 46,X,fra(X)(q27.3). Other fragile sites are described in the same way [e.g., 46,XY,fra(12)(q13.1)].

Insertions (ins)

An insertion is a structural rearrangement in which a part of a chromosome is typically interstitially repositioned into a different area of the karyotype. Insertions can occur within a chromosome or between two chromosomes. They can also be direct, in which the inserted segment retains its orientation relative to the centromere, or inverted, where the inserted segment has been "flipped over." Although the symbols "dir" and "inv" can be used to distinguish between the two, they are optional, as the orientation of the inserted material is typically evident from the nomenclature.

Insertion Within a Chromosome

In these cases, only one chromosome need be described. The first band listed is the break at the point of insertion, followed by the breakpoints that define the inserted segment itself. No punctuation is used:

46,XX,ins(3)(p21q27q32)
This represents a direct insertion. The long-arm segment between bands 3q27 and 3q32 has broken away and has been inserted into the short arm of the same chromosome at band p21. The

orientation of the inverted segment has not changed (i.e., band q27 is still proximal to the centromere relative to band q32).

46,XX,ins(3)(p21q32q27)
: In this case, the inserted segment is inverted; band q32 is now closer to the centromere than band q27.

Insertion Between Two Chromosomes

Here, both chromosomes are listed, with the recipient chromosome presented first, irrespective of numerical order. As with other rearrangements, a semicolon separates the chromosome numbers.

46,XX,ins(4;9)(q31;q12q13)
: The long-arm segment between bands 9q12 and 9q13 has been inserted, in its original orientation, into the long arm of chromosome 4 at band q31.

Inversions (inv)

A chromosomal aberration in which a segment of a chromosome is reversed in orientation but not relocated is called an inversion. There are two types of inversion. Paracentric inversions involve only one arm of a chromosome, whereas pericentric inversions involve both arms of a chromosome and, therefore, *include the centromere*. The type of inversion does not have to be specified, as this will be evident from the breakpoints.

Paracentric Inversions

46,XY,inv(3)(q21q27)
: Break and reunion occurred at bands q21 and q27 in the long arm of chromosome 3. The segment lying between these breakpoints has been reattached with its bands in reverse (inverted) order.

Pericentric Inversions

46,XY,inv(2)(p21q31)
: Break and reunion occurred at bands p21 (short arm) and q31 (long arm) of chromosome 2. The segment between these bands, including the centromere, was reattached with its bands in inverted order.

Isochromosomes (i)

An abnormal chromosome in which one arm is duplicated (and the other lost) is an isochromosome, abbreviated as "i" in the nomenclature. The breakpoint in an isochromosome is assigned to the centromere, at band p10 or q10, depending on which arm is duplicated:

46,XX,i(18)(p10)
: This describes an isochromosome for the short arm of chromosome 18, as evident by assigning the breakpoint to band p10.

46,XX,i(18)(q10)
: This describes an isochromosome for the long arm of a chromosome 18; the breakpoint is assigned to q10.

Isodicentric Chromosomes (idic)

Unlike isochromosomes, isodicentric chromosomes contain two copies of the same centromere. One of the two centromeres might be inactive, in which case the chromosome is pseudodicentric (psu dic). The breakpoints in isodicentric chromosomes are usually on the band adjacent to the centromere *on the opposite arm*:

46,XX,idic(18)(q11.2)
: Here, we have an isodicentric chromosome comprised of two copies of the entire short arm of chromosome 18, two copies of the centromere, and two copies of the small portion of the long arm between the centromere and band q11.2.

Marker Chromosomes (mar)

Marker chromosomes (mar) are supernumerary, structurally abnormal chromosomes of which no part can be identified. If any part of such a chromosome is identifiable, it is not a marker but a derivative chromosome. The presence of a "mar" in a karyotype is always recorded by a plus (+) sign:

47,XY,+mar
: This is a male karyotype with a marker chromosome.

48,XY,+2mar
: This is a male karyotype with two marker chromosomes.

48,XY,t(5;12)(q13;p12),+21,+mar
: This describes a male karyotype with a translocation involving chromosomes 5 and 12, an extra chromosome 21, and a marker chromosome.

Ring Chromosomes (r)

A structurally abnormal chromosome with two breaks, one on the short arm and one on the long arm, in which the broken ends are attached to form a circular configuration is a ring chromosome. The net result is deletion of at least the terminal ends of both arms, and potentially more (or most) of either or both arms:

46,X,r(X)
: This is a female karyotype with only one normal X chromosome and a ring X chromosome with no information on breakpoints.

46,X,r(X)(p22q24)
: This describes a female karyotype with one normal X chromosome and a ring X chromosome with break and reunion at bands p22 and q24. The material distal to both breakpoints is lost.

Translocations (t)

The interchange or transfer of chromosomal segments between two nonhomologous chromosomes is defined as a translocation.

Reciprocal Translocations

If the translocation involves mutual exchange of segments between two chromosomes, it is referred to as a reciprocal translocation. To describe a reciprocal translocation, the abbreviation "rcp" can be substituted for the "t," but this is generally not done, as all translocations are, in one sense, theoretically reciprocal, even if this is not readily apparent visually. As always, sex chromosomes are listed first, with autosomes presented in numerical order. If a translocation involves three or more chromosomes, the same rule applies to the first chromosome listed; however, in these rearrangements, the second chromosome specified will be the one that received the segment from the first and so on:

46,XX,t(7;10)(q22;q24)
: Break and reunion occurred at bands 7q22 and 10q24. The segments distal to these bands were interchanged. The translocation event has not altered the total DNA content of this cell. Therefore, the translocation is microscopically (cytogenetically) balanced.

46,X,t(X;1)(p21;q32)
Break and reunion occurred at bands Xp21 and 1q32. The segments distal to these bands were interchanged. The translocation is balanced. Note that the X chromosome is specified first.

46,X,t(Y;15)(q11.23;q21.2)
Break and reunion occurred at subbands Yq11.23 and 15q21.2. The segments distal to these bands were interchanged. This translocation is cytogenetically balanced. Here, again, the sex chromosome is specified first.

46,XY,t(9;22)(q34;q11.2)
Break and reunion has occurred at bands 9q34 and 22q11.2. The segments distal to these bands have been interchanged. This represents the typical "Philadelphia" rearrangement associated with CML and also seen in ALL and AML.

46,XX,t(1;7;4)(q32;p15;q21)
This is an example of a complex translocation involving three chromosomes. The segment on chromosome 1 distal to band q32 has been translocated onto chromosome 7 at band p15, the segment on chromosome 7 distal to band p15 has been translocated onto chromosome 4 at band q21, and the segment on chromosome 4 distal to band q21 has been translocated onto chromosome 1 at band q32. The translocation is cytogenetically balanced.

These same general principles also apply to describing translocations involving more than three chromosomes.

Whole-Arm Translocations

Whole-arm translocations are a type of reciprocal translocation in which the entire arms of two nonacrocentric chromosomes are interchanged. Such rearrangements are described by assigning the breakpoints to the arbitrary centromeric regions designated as p10 or q10, as the actual ultimate composition of the centromeres is not known. If both chromosomes have exchanged the same arms, so that the resultant rearranged chromosomes are still comprised of one short arm and one long arm, the breakpoint p10 is assigned to the chromosome with the lowest number (or a sex chromosome, if applicable). Consequently, the other chromosome will have the breakpoint at q10:

46,XX,t(3;8)(p10;q10)
This represents a balanced whole-arm translocation between chromosomes 3 and 8. In this example, the short arm of chromosome 3 and the long arm of chromosome 8 have been fused. Reciprocally, the long arm of chromosome 3 has fused with the short arm of chromosome 8, but only one combination need be written. The composition of the resultant centromeres is not known.

46,XX,t(3;8)(p10;p10)
This is a balanced whole-arm translocation in which the short arms of chromosomes 3 and 8 have been fused, as have both long arms of these chromosomes. Note that the breakpoints designate the short arms of both chromosomes. Here, again, the reciprocal product [t(3;8)(q10;q10)] need not be written, as its presence is obvious from the chromosome number of 46.

Whole-arm translocations are not always balanced, as in the following examples:

45,X,der(X;3)(p10;q10)
Here, we have a derivative chromosome consisting of the short arm of an X and the long arm of chromosome 3. The reciprocal product consisting of the long arm of the X and the short arm of 3 is missing. Note: The total chromosome number is 45, indicating the loss of the reciprocal product; no (–) sign is used. The net result is monosomy for both the long arm of X and short arm of 3.

47,XX,+der(X;3)(p10;q10)

This karyotype has an extra derivative chromosome consisting of the short arm of an X and the long arm of chromosome 3, the same derivative chromosome as in the previous example. However, in this case, two normal X chromosomes and two normal chromosomes 3 are also present, and so the derivative chromosome is extra (note that the total number of chromosomes is 47). The net result is trisomy for both the short arm of the X and the long arm of chromosome 3.

Robertsonian Translocations

Although long believed to originate through centric fusion of the long arms of acrocentric chromosomes (pairs 13, 14, 15, 21, and 22), recent data suggest this might not always be so (see Chapter 9). They were first described by Robertson, whose name they have been given. The short arms, which all contain redundant copies of ribosomal genes, are lost in these rearrangements; this is of no clinical significance. Because Robertsonian translocations are still treated as a type of whole-arm translocation, they can be adequately described using the same nomenclature:

45,XX,der(13;14)(q10;q10)

This describes a Robertsonian translocation between chromosomes 13 and 14. The centromere origin is unknown, and so the breakpoints are designated as 13q10 and 14q10 to indicate that both long arms are involved. This derivative chromosome has replaced one chromosome 13 and one chromosome 14; there is no need to indicate the missing chromosomes. The karyotype now contains one normal 13, one normal 14, and the der(13;14). The short arms of the 13 and 14 are lost, which is why the abbreviation "der" is used instead of "t" to describe the translocation. One can also use "rob" to describe Robertsonian translocations. The loss of these short arms is not clinically significant and, therefore, this description represents a balanced Robertsonian translocation (an individual with this karyotype is referred to as a balanced carrier) even though only 45 chromosomes are present.

46,XX,+13,der(13;14)(q10;q10)

The derivative chromosome consists of the long arms of chromosomes 13 and 14, as in the above example. However, in this karyotype, there are two normal 13s and one normal 14, plus the der(13;14). The net result is trisomy for the long arm of chromosome 13, clinically identical to trisomy 13. The additional chromosome 13 is shown by the designation +13. Here, we have an example of both numerical and structural abnormalities that involve the same chromosome number, and so the numerical abnormality is designated first.

Uniparental Disomy (upd)

Representation of both maternally and paternally inherited genes is required in many areas of the genome in order for normal development to occur. This phenomenon is referred to as genomic imprinting and involves selective inactivation of certain genes by methylation. Uniparental disomy is a situation in which both homologs of a specific chromosome pair are inherited from the same parent and, in some cases, is associated with an abnormal phenotype. Uniparental disomy can occur, for example, in an embryo that starts out trisomic for a given chromosome and then loses one copy of this chromosome early enough in development to "rescue" what would have been a pregnancy doomed to abort spontaneously. If, by chance, the two remaining copies were inherited from one parent, the individual is said to have upd for that chromosome. For example, some patients with Prader–Willi/Angelman syndrome and no deletion of chromosome 15 have been shown to have upd for this chromosome. Inheriting two paternal chromosomes 15 results in Angelman syndrome, whereas receiving two maternal 15s results in Prader–Willi syndrome. See Chapter 19.

Nomenclature examples are follows:

46,XY,upd(15)pat

This is a male patient with uniparental disomy for paternally derived chromosomes 15.

46,XY,upd(22)pat[10]/47,XY,+22[6]
: This represents a mosaic male karyotype involving one cell line that contains two paternally derived chromosomes 22 and the other with trisomy 22. Here, both cell lines are abnormal and, therefore, the larger clone is recorded first.

46,XX,upd pat
: This describes a complete hydatidiform mole with XX sex chromosomes (very rare). All 46 chromosomes are paternally derived.

46,XY,upd pat
: This describes a complete hydatidiform mole with XY sex chromosomes. All 46 chromosomes are paternally derived.

46,XX,upd mat
: This is an ovarian teratoma. All 46 chromosomes are maternally derived.

NEOPLASIA

The basic rules for using the nomenclature apply when describing the karyotypes associated with cancer. However, special situations, requiring additional guidelines, might arise in these cases. Therefore, special ISCN definitions and rules have been devised for use with neoplasia.

Clones

A clone is defined as two cells that share the same abnormality or abnormalities, unless the change involves loss of a chromosome, in which case three such cells are required (because of the possibility of coincidental random chromosome loss). During tumor progression, related subclones can evolve; related or unrelated clones are separated by slashes "/" and the number of cells observed for each is given in square brackets "[ ]".

Mainline, Stemline, Sideline, and Clonal Evolution

These terms can be confusing and are often misunderstood. The mainline (ml) is the term used to describe the most common clone (i.e., the one represented by the most cells). This is a quantitative issue only. It does not necessarily indicate the most basic clone in tumor progression, which is referred to as the *stemline* (sl). Clones that evolve from the stemline are referred to as *sidelines* (sdl):

46,XY,t(9;22)(q34;q11.2)q34;q11.2)[5]/47,XY,+8,t(9;22)(q34;q11.2)[11]/46,XY,t(9;22)(q34;q11.2),i(17)(q10)[4]
⇔ ⇑ ⇒
stemline mainline sidelines

When more than one clone is present but no clear clonal progression is evident, the mainline is listed first, followed by each clone in order of relative size. When clonal evolution is present, the stemline is listed first, with sidelines listed in order of increasing complexity whenever possible, or by clone size when more than one sideline evolves independently from the stemline, as in the preceding example.

Composite Karyotype (cp)

When a clone contains multiple abnormalities, a frequent occurrence is that not all changes are present in every cell, yet the interpretation can be made that these cells do, in fact, represent a single abnormal clone rather than an evolving process. To report such a phenomenon, the clone is described as a *composite*, using the abbreviation "cp" before the number in brackets. It should be noted that this can occasionally produce seemingly contradictory data, as some cells will contain additional copies of a chromosome that is missing in others.

INTERPRETING A KARYOTYPE DESCRIPTION

Receiving a cytogenetic report that contains the description of a patient's karyotype can create confusion, particularly if complex rearrangements or multiple clones are present. Interpretation of the description of a karyotype can be facilitated by breaking this description into its component parts.

First, determine whether more than one cell line is present. This will happen if constitutionally the patient is a mosaic or a chimera as is often the case with acquired cytogenetic abnormalities, particularly in patients whose neoplasm is progressing. Because the first item described is always the number of chromosomes present, each clone or cell line present will start with this number, and each is separated by a slash (/). Each cell line can then be examined individually. If abnormalities present in the first clone listed are also present in others, the description can be simplified by using the abbreviation "idem" to indicate this; note that idem always refers to the first cell line described, which will be the stemline in these cases.

As discussed above, the sex chromosome complement follows the chromosome count. Sex chromosome abnormalities are listed first, followed by autosomal abnormalities in numerical order. When abnormalities involve the same chromosome, numerical changes are presented first, followed by structural abnormalities listed in alphabetical order, using the abbreviations listed in **Table 3**.

Commas separate each abnormality listed, and so by examining the karyotype from comma to comma, the abnormalities involved can be interpreted.

Consider the following example from a patient with AML:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)[4]/48,idem,+9,i(17)(q10)[12]/46,XY[4]

At first blush, receiving a report with this karyotype might be enough to scare away even the most confident clinician! However, let us break this karyotype down into its component parts, which will simplify its interpretation.

The slashes, brackets, and listings of number of chromosomes tell us that three different clones are present:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)**[4]**
/48,idem,+9,i(17)(q10)**[12]**
/46,XY**[4]**

Of the 20 cells examined, the first clone has 47 chromosomes and is represented by 4 cells. The second clone has 48 chromosomes; 12 of these cells were observed. Finally, four normal 46,XY cells are present.

Now, let us look again at the first cell line, the *stemline* in this case. It has an XY sex chromosome complement. It also has three cytogenetic abnormalities: It has one chromosome 5 with an interstitial deletion of the material between bands q13 and q33 (on the long arm):

47,XY,**del(5)(q13q33)**,+8,t(9;22)(q34;q11.2)[4]/48,idem,+9,i(17)(q10)[12]/46,XY[4]

It has an extra copy of chromosome 8,

47,XY,del(5)(q13q33),**+8**,t(9;22)(q34;q11.2)[4]/48,idem,+9,i(17)(q10)[12]/46,XY[4]

and it has a translocation involving the long arms of chromosomes 9 and 22, at band q34 of chromosome 9 and band q11.2 of chromosome 22:

47,XY,del(5)(q13q33),+8,**t(9;22)(q34;q11.2)**[4]/48,idem,+9,i(17)(q10)[12]/46,XY[4]

Yes, this is the "Philadelphia" rearrangement, which is sometimes also seen in patients with AML.

The second cell line contains the sex chromosomes and all of the abnormalities present in the first:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)[4]/48,**idem**,+9,i(17)(q10)[12]/46,XY[4]

plus an additional copy of chromosome 9:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)[4]/48,idem,**+9**,i(17)(q10)[12]/46,XY[4]

and an isochromosome for the long arm of chromosome 17:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)[4]/48,idem,+9,**i(17)(q10)**[12]/46,XY[4].

Because this is the largest clone present (with 12 cells), it represents the *mainline*.

Finally, as mentioned above, the third cell line represents cells with a normal male karyotype:

47,XY,del(5)(q13q33),+8,t(9;22)(q34;q11.2)[4]/48,idem,+9,i(17)(q10)[12]/**46,XY**[4]

Thus, we see that by examining the components of a reported karyotype using the above-outlined rules, together with the abbreviations listed in **Table 3** or in the nomenclature document itself *(11)*, what initially might appear as an indecipherable compilation of numbers and symbols becomes a concise, universal method of describing the results of a patient's chromosome analysis.

FLUORESCENCE AND OTHER *IN SITU* HYBRIDIZATION

Recent advances in human cytogenetics include the development and application of *in situ* hybridization (ish) protocols to incorporate and bind labeled, cloned DNA or RNA sequences to cytological preparations. These techniques facilitate the localization of specific genes and DNA segments onto specific chromosomes, ordering the position and orientation of adjacent genes along a specific chromosome, identification of microduplications or microdeletions of loci that lie beyond the resolution of conventional cytogenetics but manifest themselves as abnormal clinical phenotypes, and the detection of aneuploidies involving whole chromosomes or chromosomal regions (see Chapter 17). For these reasons, nomenclature to designate various ish applications was introduced in ISCN 1995. The symbols and abbreviations used in ish nomenclature are listed in **Table 4**.

Prophase or Metaphase Chromosome In Situ *Hybridization (ish)*

Even though fluorescence microscopy is most commonly used to view *in situ* hybridization signals, the abbreviation ish, not FISH, is used in the karyotype description. If chromosome analysis was done prior to ish, the karyotype is first designated using conventional rules. A period (.) is then placed to record the end of the cytogenetic findings. This is then followed by the ish results. If a standard cytogenetic analysis was not done and only ish studies were done, the ish results are presented directly.

When presenting an abnormal ish result, the abbreviation for that specific abnormality is recorded (e.g., ish del) followed by the chromosome number, the breakpoints, and a designation for the probe used, all listed in separate parentheses [e.g., ish del(4)(p16.p16)(D4S96–]. Whenever possible, Genome Data Base (GDB) designations for loci are used. These consist of the letter "D" (for DNA), the chromosome of origin, the letter "S" (segment), and the GDB number of the probe. The above example uses the 96th DNA segment assigned to chromosome 4, D4S96. The locus designation must be given using capital letters only. When a GDB designation is not available, a probe name can be

Table 4
Selected List of Symbols and Abbreviations
Used for *In Situ* Hybridization (ish) Nomenclature

Abbreviation or Symbol	*Description*
–	Absent on a specific chromosome
+	Present on a specific chromosome
++	Duplication on a specific chromosome
x	Precedes the number of signals seen
.	Period, separates cytogenetic results from ish results
con	Connected or adjacent signals
ish	Refers to *in situ* hybridization; when used by itself, ish refers to hybridization to chromosomes
nuc ish	Nuclear or interphase *in situ* hybridization
pcp	Partial chromosome paint
sep	Separated signals (which are usually adjacent)
wcp	Whole chromosome paint

Note: For a complete listing of symbols and abbreviations, refer to ref. *11*.

used. If more than one probe from the same chromosome is used, these are listed in order from pter to qter. If probes from two different chromosomes are used, they are separated by a semicolon.

Given below are a series of examples that illustrate the ish karyotype designations one might see, using as examples patients suspected of having various disorders.

Patients with Possible DiGeorge/VCF Syndrome

46,XX.ish 22q11.2(D22S75x2)

This example illustrates the basic rules in describing an ish karyotype when both chromosome and ish results are normal. The test was performed on prophase/metaphase chromosomes. The probe used, D22S75, detects about 80% of deletions leading to DiGeorge/velocardiofacial (VCF) syndrome. First, the cytogenetics result 46,XX is recorded, followed by a period and then the abbreviation ish. A single space is left, after which the chromosome and region numbers are given together without parentheses, 22q11.2, followed by the GDB locus designation of the probe used, within parenthesis (D22S75), and the number of times the probe signal is observed (x2, because in a normal cell, neither chromosome 22 would have a deletion).

46,XX.ish del(22)(q11.2q11.2)(D22S75–)

This patient has a normal karyotype resulting from standard chromosome analysis, but a deletion in the DiGeorge region of chromosome 22, at band q11.2, was detected by ish using a probe for that locus, D22S75. Note: The chromosome 22 and the region tested are now placed within parentheses because an abnormality (no signal, indicated by a minus sign) is being described.

46,XX,del(22)(q11.2q11.2).ish del(22)(q11.2q11.2) (D22S75–)

Here, we have a karyotype in which a deletion was identified with standard chromosome analysis and confirmed with ish using a probe for locus D22S75.

Patients with Possible Prader–Willi/Angelman Syndrome

46,XX.ish 15q11.2q13(D15S11x2,GABRB3x2)

This patient has normal chromosomes, but was suspected of having a microdeletion in the Prader–Willi/Angelman syndrome region. She was studied by *in situ* hybridization using probes D15S11 and GABRB3, both of which map to the region 15q11.2 → q13. Hybridization showed two copies each of the two probes, suggesting no deletion for either locus.

46,XX.ish 15q11.2q13(D15S11x2,GABRB3x2D15S10x2,SNRPNx2,)
Because of the negative ish results but continued clinical suspicion, the above patient was retested using the additional probes D15S10 and SNRPN. There is still no deletion detected. Because of the high degree of suspicion, this patient is a candidate for uniparental disomy analysis (see Chapter 19).

46,XX,del(15)(q11.2q13).ish del(15)(q11.2q13)(D15S10–,SNRPN–)
Here, a deletion of 15q11.2 → q13 was detected with cytogenetic analysis and was confirmed with ish. The probes used were D15S10 and SNRPN. Both were absent from one chromosome 15. The ish "deletion" is denoted by a minus sign.

Patients with Possible Williams Syndrome

46,XX.ish 7q11.23(ELNx2)
This patient had a diagnosis of Williams syndrome and normal chromosomes. *In situ* hybridization with a probe for the Elastin–Williams syndrome (ELN) locus produced hybridization at band 7q11.23 on both chromosomes 7. There is no deletion.

46,XX.ish del(7)(q11.23q11.23)(ELN–)
As above, this is a patient with Williams syndrome and normal cytogenetic results. *In situ* hybridization with a probe for the ELN locus showed a deletion on one chromosome 7.

46,XX.ish del(7)(q11.23q11.23)(ELN–x2)
Again, this is a patient with Williams syndrome and normal cytogenetic results. However, in this case, ish with a probe for the ELN locus showed deletions on both chromosomes 7.

Patients with Possible Charcot–Marie–Tooth Syndrome

46,XX.ish 17p11.2(CMT1Ax2)
This is a patient with Charcot–Marie–Tooth syndrome and normal chromosomes. *In situ* hybridization with a probe for the CMT1A locus showed normal hybridization on both chromosomes 17 and, thus, no deletion or duplication of the locus.

46,XX,ish dup(17)(p11.2p11.2)(CMT1A++)
This Charcot–Marie–Tooth syndrome patient also has normal chromosomes. *In situ* hybridization with a probe for the CMT1A locus showed duplication of the locus on one chromosome 17(++).

Chromosome Abnormalities Identified with Whole Chromosome Paints

In situ hybridization can be performed using a cocktail of chromosome-specific probes that will hybridize along the entire length of that chromosome pair, effectively "painting" them. Because the procedures used ensure that no other chromosomes are "painted," these probes provide a way to identify or confirm the identity of chromosomal material:

46,XX,add(20)(p13).ish dup(5)(p13p15.3)(wcp5+)
In this patient, one chromosome 20 has extra material attached to it at band p13. By using a whole chromosome paint for chromosome 5 (wcp5), the extra segment was identified as a partial duplication of chromosome 5. Subsequent analysis of the band morphology using Giemsa banded preparations allowed the duplicated material to be identified as the segment 5p13 → p15.3. The diagnosis is essentially made by going from G-banding to ish and then back to G-banding.

Both whole chromosome paints and locus-specific ish probes can be used in combination in order to determine the composition of an abnormal chromosome:

46,X,r(X).ish r(X)(p22.3q13.2)(wcpX+,DXS1140+,DXZ1+,XIST+,DXZ4–)
This is an example of a ring X that was identified with G-banding and then further defined by ish. First, a whole chromosome paint for the X confirmed the origin of the ring. Next, probes

localized to Xp22.3 (DXS1140), the X centromere (DXZ1), Xq13.2 (XIST), and Xq24 (DXZ4) were used. The last probe, DXZ4, produced no hybridization signal (–), narrowing down the portion of the X that was lost during formation of the ring.

46,X,r(?).ish r(X)(DYZ3–, wcpX+)
In this case, a small ring chromosome of indeterminate origin was detected with G-banding. Hybridization with the Y probe DYZ3 showed that the ring was not derived from the Y. Follow-up hybridization with a whole chromosome paint for the X showed that it originated from an X.

47,XX,+mar.ish der(12)(wcp12+,D12Z1+)[10]/46,XX[10]
This patient is a mosaic, with both normal cells and cells with an extra marker chromosome. The marker was identified with ish as being derived from chromosome 12, using a whole chromosome paint for chromosome 12 and chromosome 12 centromere probe D12Z1.

Painting probes that hybridize to specific parts of chromosomes have also been developed and are referred to as partial chromosome paints (pcp). Consider the following example:

46,XX.ish inv(16)(p13.1q22)(pcp16q sp)
In this case, what appeared to be a normal female karyotype with routine G-banding was found to have a pericentric inversion of chromosome 16 using ish. When a partial chromosome paint for band 16q22 was used, this band was shown to be split (sp) between the long and short arms.

Identification of Cryptic Translocations Using ish

Some translocations are beyond the limits of microscopic resolution. Take, for example, an individual who has a child with Miller-Dieker syndrome. Although routine chromosome analysis produced a normal karyotype, the child was shown to have a microdeletion for this locus on chromosome 17 using ish:

46,XX.ish del(17)(p13.3p13.3)(D17S379–)
The patient and her husband wish to know if this condition could have been inherited as the result of a microscopically undetectable (cryptic) translocation carried by one of them. Both of their karyotypes are normal with standard chromosome analysis, but ish analysis demonstrates that the mother carries such a cryptic translocation.

46,XX.ish t(16;17)(q24;p13.3)(D17S379+;wcp16+)
After hybridizing the Miller–Dieker locus probe D17S379 to previously banded cells, one signal was observed in its proper location on the short arm of chromosome 17, but the other appeared on the long arm of chromosome 16. Subsequent hybridization with a whole chromosome paint for chromosome 16 showed that part of this chromosome is now on chromosome 17, confirming the presence of a reciprocal cryptic rearrangement.

Sometimes, a translocation appears to be present with standard cytogenetics, but it is so subtle and must be confirmed with ish:

46,XX,?t(4;7)(p16;q36).ish (wcp7+,D7S427+,D4S96–;wcp4+,D4S96+,D7S427–)
Here, a cryptic translocation between the short arm of chromosome 4 and the long arm of chromosome 7 was suspected with G-banding, but was not a certainty via cytogenetics alone, hence the "?." The presence of this rearrangement was confirmed with ish using probe D7S247 localized to 7qter, D4S96 localized 4pter, and whole chromosome paints for both chromosomes 4 and 7. The distal short arm of the der(4) was wcp7+, D7S247+, and D4S96–. The distal long arm of the der(7), on the other hand, was wcp4+, D4S96+, and D7S247–.

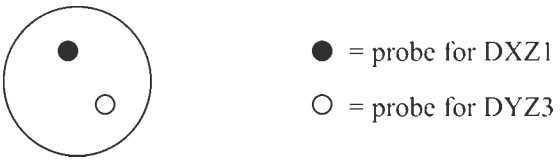
Interphase or Nuclear In situ *Hybridization (nuc ish)*

In situ hybridization can be performed on interphase nuclei to provide information concerning the number and/or relative positions of the probes (and therefore the loci) involved. Thus, it can be used as a screening method for the rapid detection of aneuploidies and gene rearrangements. Typically performed prior to or in the absence of standard chromosome analysis, interphase ish results are abbreviated nuc ish.

Designation of the Number of Signals

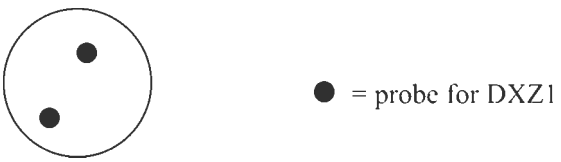
When designating interphase ish results, the abbreviation nuc ish is followed by a space, the chromosome band to which the probe is mapped, and then, in parentheses, by the GDB locus designation, a multiplication sign, and the number of signals detected:

nuc ish Xcen(DXZ1x1,DYZ3x1)



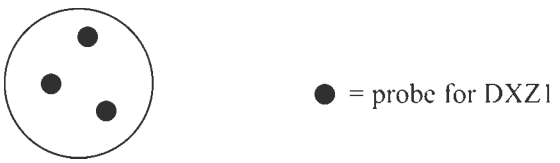
One copy of the X centromere probe DXZ1 is detected, as is one copy of the Y chromosome probe DYZ3. This implies the presence of one X and one Y chromosome, suggesting an XY sex chromosome complement. No other information is presented, and so this report cannot specify whether these sex chromosomes are normal. These types of data are therefore generally used when the sex chromosome information itself is of value (e.g., when monitoring the progress of a mixed-gender bone marrow transplant).

nuc ish Xcen(DXZ1x2)



Here, two copies of the DXZ1 locus were detected. This implies the presence of two X chromosomes.

nuc ish Xcen(DXZ1x3)



Three copies of the DXZ1 locus are detected, implying the presence of three X chromosomes.

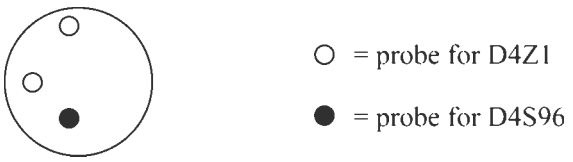
nuc ish 13q14(Rb1x1)

Only one copy of the retinoblastoma locus probe Rb1 is detected. This implies a deletion of the Rb1 gene from one chromosome 13.

nuc ish Yp11.2(DYZ3x2)

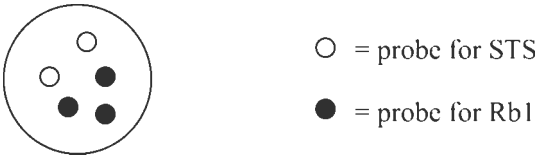
In this case, two copies of DYZ3 are detected, implying that an extra copy of this locus is present. It is not clear whether the extra copy is the result of the presence of two Y chromosomes or an isochromosome involving the short arm of the Y.

nuc ish 4cen(D4Z1x2),4p16.3(D4S96x1)



Here, we have an example of a structural abnormality identified with ish. Two copies of chromosome 4 are implied by two D4Z1 signals. However, only one copy of D4S96 is detected, implying a deletion of this locus from one chromosome 4. In the nomenclature, two or more probes for the same chromosome are separated by commas.

nuc ish Xp22.3(STSx2),13q14(Rb1x3)

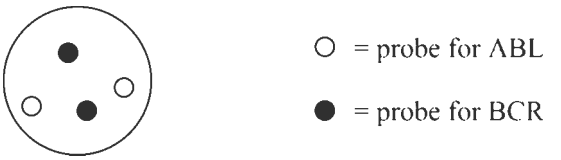


This is an example of ploidy detection. Two copies of the steroid sulfatase locus on the X chromosome and three copies of the Rb1 locus are detected. This implies the presence of two X chromosomes and trisomy 13. Probes from different chromosomes are separated by commas.

Designation of Relative Positions of the Signals

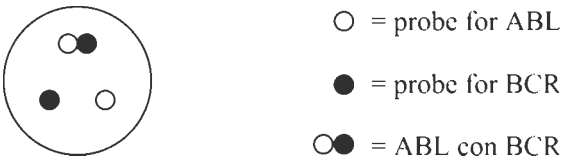
Under normal conditions, if probes from two chromosomes are tested simultaneously, the signals are expected to appear separated. However, chromosome rearrangements, such as the BCR/ABL gene fusion, can bring signals together:

nuc ish 9q34(ABLx2),22q11.2(BCRx2)



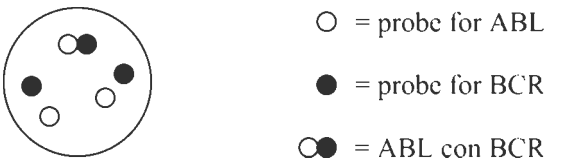
Two ABL and two BCR loci seen and they are well separated. No gene rearrangement is evident.

nuc ish 9q34(ABLx2),22q11.2(BCRx2)(ABL con BCRx1)



Here, signals from two ABL and two BCR loci are seen. However, one ABL signal and one BCR signal are juxtaposed (or connected, "con"), suggesting that they now reside on the same chromosome. This is the pattern observed when a t(9;22) or "Philadelphia rearrangement" is present.

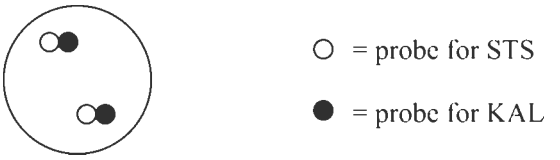
nuc ish 9q34(ABLx3),22q11.2(BCRx3)(ABL con BCRx2)



Here, three ABL and three BCR signals are present. However, two pairs of BCR/ABL signals are juxtaposed. This is the pattern observed when both a t(9;22) and an additional der(22) ["Philadelphia chromosome"] are present.

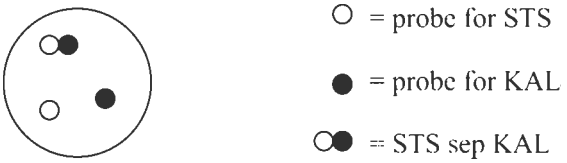
Sometimes, a rearrangement can be detected when signals that are normally juxtaposed become separated:

nuc ish Xp22.3(STSx2,KALx2)



STS and KAL are two loci on Xp22.3 that are adjacent to each other. Because they map to the same band, they are reported within parentheses. Under normal circumstances, ish signals will appear side by side, as they do here. However, the signals might appear to be independent of each other.

nuc ish Xp22.3(STSx2,KALx2)(STS sep KALx1)



In this example, one STS and one KAL locus are separated, most likely as the result of a rearrangement involving this area of the X chromosome. The nomenclature term "sep" is used to designate this change.

ISCN 1995 has, indeed, made a good beginning concerning nomenclature for various *in situ* hybridization scenarios. However, it has become obvious in clinical practice that this document requires substantive revision in order to accommodate the technical explosion we are witnessing in this arena (see Chapter 17). Primed *in situ* labeling (PRINS), comparative genomic hybridization (CGH), use of five or more probes simultaneously for rapid aneuploidy screening, multiplex FISH, neoplasia panels, and arm-specific subtelomeric detection screening, to name a few, will all need to be included in future versions. At the time of publication, the next revision of ISCN is not expected until 2005.

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II Examining and Analyzing Chromosomes

Editors' Foreword to Section II

Despite a prolific increase in machinery available to the clinical laboratorian, involving both the actual number of devices themselves as well as an ever-increasing variety of tasks they can perform, cytogenetics is still a relatively labor-intensive discipline. The basic tool of the cytogenetics technologist remains the microscope, and the number of steps involved in generating chromosomes for analysis has essentially not changed for decades.

Although it is sometimes difficult to make a distinction between "how it's done" and "how to do it," the former is our goal in presenting the following four chapters. It is not our intention to prepare the reader for a career in the cytogenetics laboratory or to provide the cytogeneticist with yet another lab manual. Our aim, rather, is to impress upon the reader the amount of effort and attention to detail required in order to accurately diagnose a myriad of cytogenetic conditions from the variety of tissue types routinely submitted to the laboratory.

However, given the diverse background of the individuals likely to be referring to this book, a certain amount of technical data has been retained for those who might find it interesting and/or informative.

It is our hope that this section will leave the reader with a newfound respect for the effort required to perform this often critical aspect of patient care.

4

Basic Laboratory Procedures

Martha B. Keagle, MEd and Steven L. Gersen, PhD

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, and chorionic villi. If there are not enough naturally dividing cells for a chromosome analysis, these specimen types can also be cultured in the laboratory. Peripheral blood lymphocytes, tissue biopsies, and amniotic fluid samples are routinely cultured to obtain dividing cells; 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	• Arrest division
• Sterility	• Optimal temperature	• Swell cells
• Proper growth medium	• Optimal pH	• Fix cells
• ± Mitotic stimulant	• Optimal humidity	• Prepare slide
• Microbial inhibitors	• Optimal time interval	• Stain/band

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. The laboratory may reject specimens that are improperly labeled or unlabeled (see Chapter 6).

SPECIMEN COLLECTION AND HANDLING

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 hours 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

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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 hours old, etc.). 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, although the number and quality of cells may be compromised.

Requirements: 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.

Requirements: Amniotic Fluid Specimens

Amniocentesis can be performed from as early as 10 weeks gestation until term (see Chapter 12). From 15 to 30 mL of amniotic fluid should be obtained under sterile conditions and collected in a sterile container approved for cell culture. For amniocentesis 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 tap.

Requirements: Solid Tissue Specimens

Solid tissue sources include skin biopsies, chorionic villi, products of conception, and stillbirth biopsies. Products of conception and stillbirths are one-of-a-kind specimens that cannot be recollected, and repeat collection of chorionic villi increases the risk of abortion, 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, most solid tissue specimens are not sterile prior to collection. In addition, viable cells might 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 can be delivered with no liquid added at all. Larger samples can be sent to the laboratory *in toto* for dissection. Solid tissue samples should 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® or Chang® for

amniocytes, Giant Cell Tumor Conditioned Medium for malignancies, PANDIS for breast tumors, etc.), whereas 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, whereas 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 types 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 are generally 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; when observed under an inverted microscope, it appears 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 hours after the addition of PHA, with additional waves of division at 24-hour intervals. The culture period for blood specimens is based on this knowledge. For routine peripheral blood cultures, 72 hours is usually optimal. Blood specimens from newborns might require a shorter culture period.

Some leukemia and lymphoma studies require stimulation of B-lymphocytes. There are a number of B-cell mitogens available, including Epstein–Barr virus, LPS (lipopolysaccharide from *Escherichia coli*), protein A, TPA (12–O-tetradecanoyl-phorbol-13-acetate) and pokeweed. A cocktail including PHA and interleukin-2 (IL-2) has proven successful as a lymphoid mitogen for bone marrow samples.

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

The 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) can 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 can 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, chorionic villus (CVS) and other tissue samples can be grown directly on cover slips 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 subcultured) when adequate numbers of dividing cells are present, and cells do not have to be enzymatically removed prior to harvest. Therefore, the cells can be analyzed as they grow *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 might result in an equivocal diagnosis. Low-level mosaicism cannot be completely ruled out in such cases. Clinical correlation can help clarify the picture. A repeat amniocentesis might confirm the presence of true mosaicism, but cannot, of course, eliminate the results of the first study. See also Chapter 12.

No inference can be made about the origin of cells when using the flask method, because 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 an 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) because only primary cultures are harvested. Flask cultures are often subcultured, adding days to the culture time.

Preparation of Specimens for Culture

Amniotic fluid specimens, whole blood samples, 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 (800–1000 rpm) to retrieve the small number of viable cells. The cell pellet is then used to establish the cultures. The supernatant can 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 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 in either "open" or "closed" systems, depending on 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 CO_2 incubator is required for open systems to maintain the 5% CO_2 level necessary to sustain the ideal pH of 7.2–7.4. A humidity level of 97% should be maintained to prevent cell death as a result of 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, because of the moist warm surfaces in the incubator. An open system is required for samples grown on cover slips by 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 CO_2 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 on 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 hours.

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-hour culture time can be used to increase the mitotic index. Longer culture periods are generally not advised because the abnormal cancerous cells might be lost over time or be diluted out by normal precursor cells that might 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. Certain B-cell mitogens require increased 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, which 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 might 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 on 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 can 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. 1**.

Mitotic Inhibitor

A mitotic inhibitor must be used to obtain adequate numbers of cells in the 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 the 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 chromosomes is affected by the hypotonic solution used. The choice of hypotonic solution is based on specimen type and laboratory protocol.

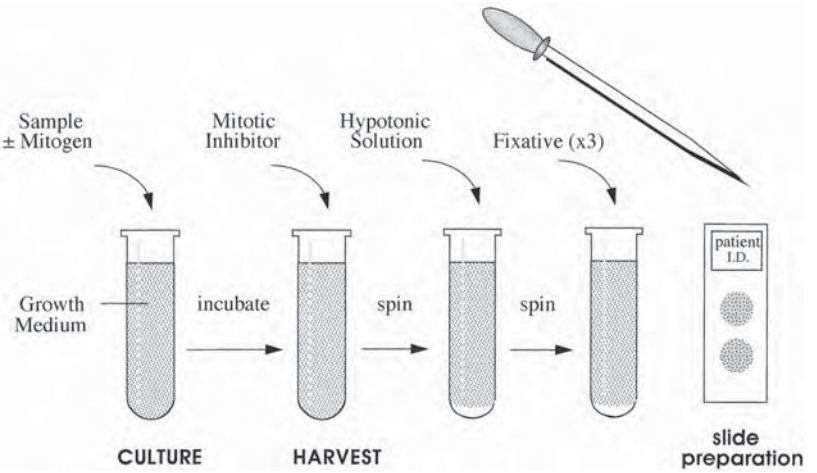

Fig. 1. Overview of culture and harvest for chromosome analysis. This procedure, with minor variations, is utilized for all specimen types.

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 because 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. Fixed cells from suspension cultures are dropped onto glass slides to allow for subsequent staining and analysis. The concentration of the cell suspension can be adjusted to achieve optimal results. Fixed cells from *in situ* cultures are not dropped because they are already attached to a cover slip or other solid surface. The prepared slides or cover slips are dried under conditions that favor optimal chromosome spreading and are checked with a phase contrast microscope for metaphase quality and number. A good slide preparation 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.

A number of variables affect the rate of evaporation of fixative from the slide, the spreading of chromosomes, and the overall quality of the slide preparation. Ambient temperature and humidity and length of time in hypotonic treatment all affect spreading of chromosomes. Increased temperature and humidity enhance chromosome spreading, whereas cooler temperature and lower humidity decrease it. Longer exposure to hypotonic solution makes cells more fragile and increases spreading, but an inadequate exposure can result in cells that are difficult to burst. Every technologist must have an arsenal of techniques to effectively deal with these variables.

Other variables in slide making include the height from which the cells are dropped, the use of wet or dry slides, the use of cold, room temperature, or warm slides, the use of steam, air, or flaming the slides, and the angle at which the slide and/or pipet is held.

After slides are prepared they are aged overnight at 60°C or 1 hour 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 the 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 Chapter 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 progresses, the ability to visualize subtle abnormalities is reduced. Chromosomes with a greater number of visible bands and subbands (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 Chapter 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 400 bands. Well-banded, moderately high-resolution metaphases are usually in the 500- to 550-band range, whereas prometaphase cells can achieve resolutions of 850 or more bands.

G-Banding (Giemsa Banding)

G-banding is the most widely used routine banding method in the United States. GTG banding (G bands produced with trypsin and Giemsa) is one of several G-band techniques. With this method, prepared and "aged" slides are treated with the enzyme trypsin and then stained with Giemsa. This produces a series of light and dark bands that allows for the positive identification of each chromosome (*see* **Fig. 2**). The dark bands are A-T-rich, late replicating, heterochromatic regions of the chromosomes, whereas 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, whereas the G-dark bands contain relatively few active genes. There are also G-banding techniques that actually utilize stains other than Giemsa, 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. Certain fluorochromes, such as quinacrine dihydrochloride, will bind to DNA and produce

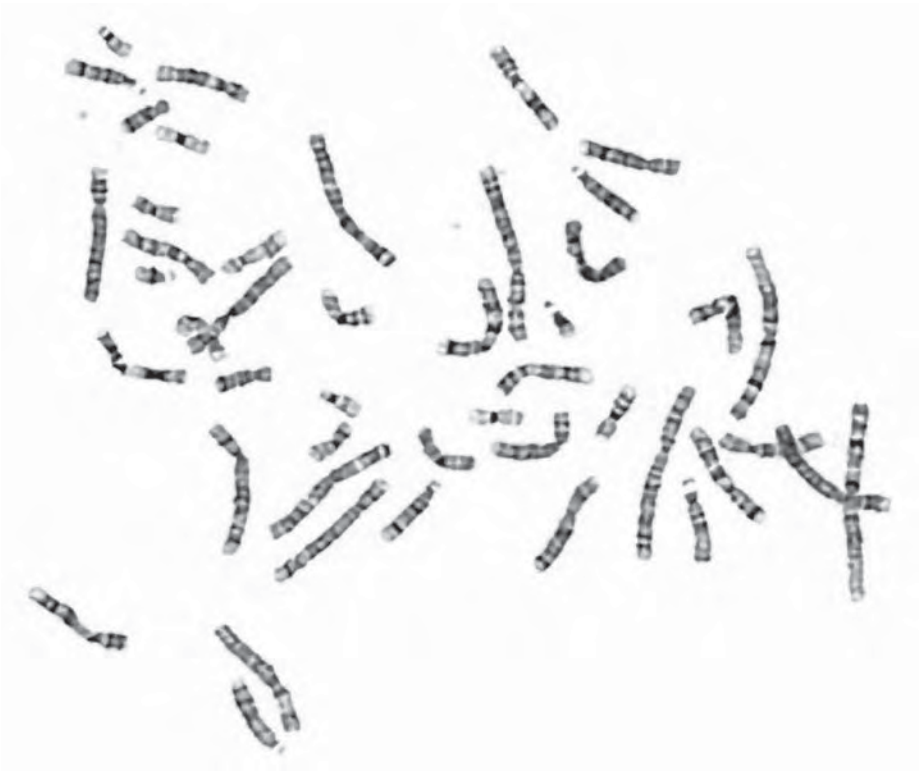

Fig. 2. G-Banding (Giemsa banding). Note the light and dark bands along the length of each chromosome. (Image provided by Alma Ganezer.)

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. Q-banding is, therefore, useful to confirm the presence of Y material or when studying the cited polymorphic regions. (See **Fig. 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.

R-Banding (Reverse Banding)

R-banding techniques produce a banding pattern that is the opposite or reverse of the G-banding pattern. There are fluorescent and nonfluorescent methods. The C-G-rich, euchromatic regions stain darkly or fluoresce brightly, whereas 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 because the pale telomeres might 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 (see **Fig. 4**).

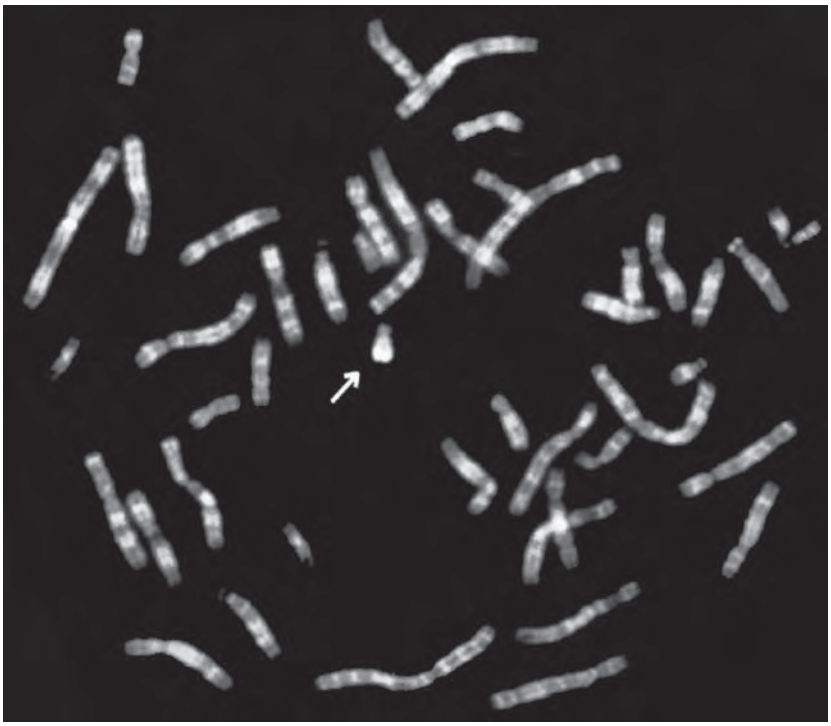

Fig. 3. Q-Banding. The fluorescence banding pattern is essentially the same as with G-banding. Note, however, the bright fluorescence on the long arm of the Y chromosome (arrow).

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, and 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 ghostlike 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 (see **Fig. 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 harsher treatment of the chromosomes diminishes staining except at the heat-resistant telomeres. There are fluorescent and nonfluorescent T-banding techniques.

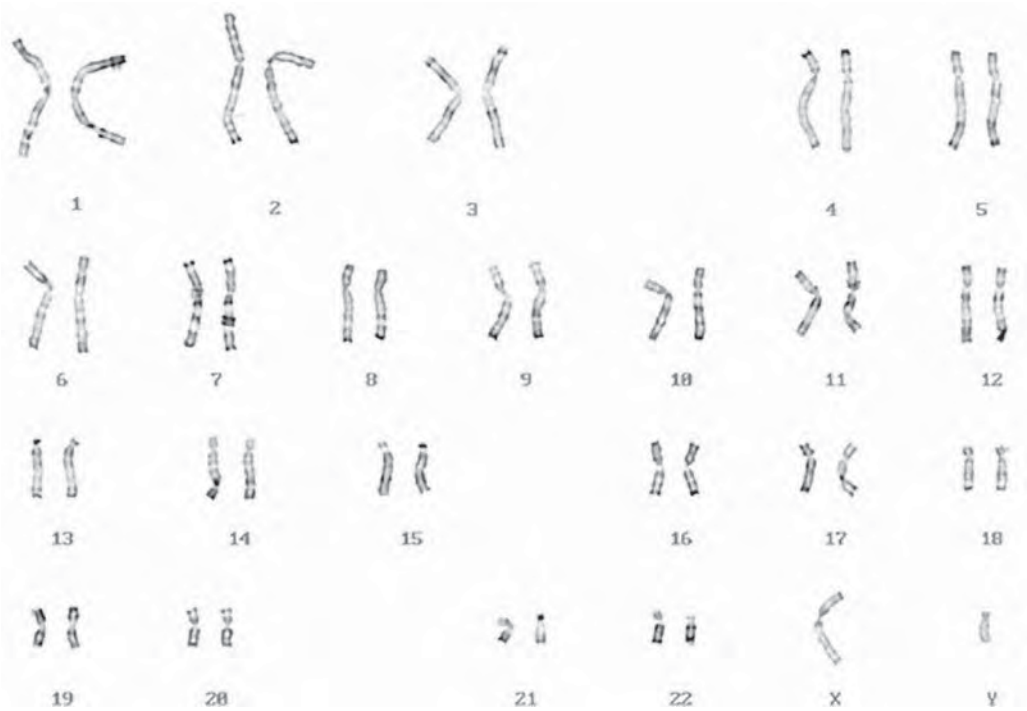

Fig. 4. R-Banding (reverse banding). The light and dark bands are the opposite of those obtained with G-banding. R-Banding can also be performed with fluorescent staining. (Image courtesy of Dr. Sylvie Szpiro-Tapia, Laboratoire cerba, Val d'Oise, France.)

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, which 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, and 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 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 above, staining them magenta. G-11 banding is used to delineate these heterochromatin polymorphisms.

G-11 banding also has research applications. It is used to differentiate between human and rodent chromosomes in hybrid cells. The human chromosomes stain pale blue, whereas the rodent chromosomes stain magenta.

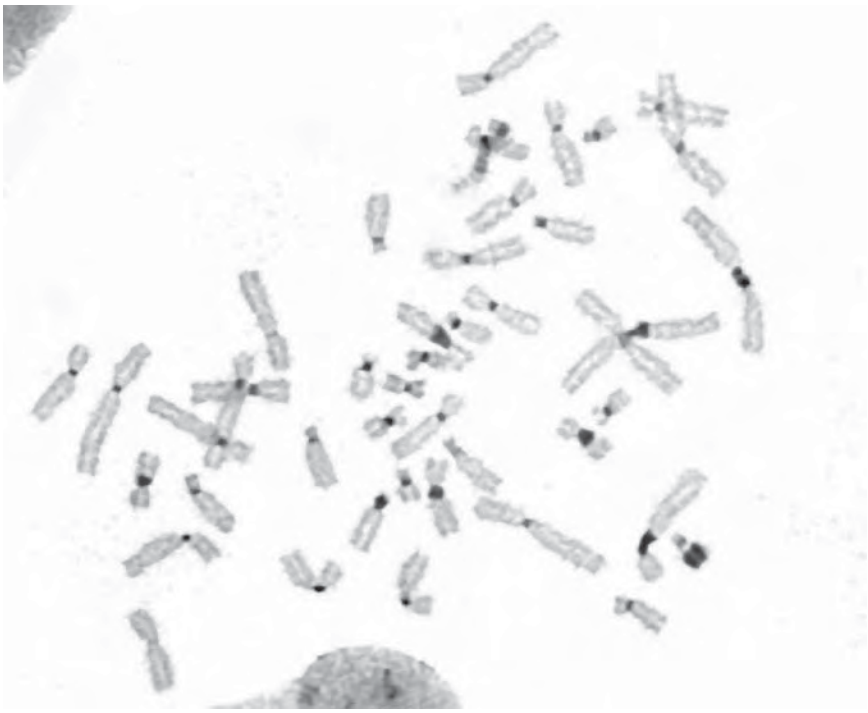

Fig. 5. C-Banding. This technique stains the constitutive heterochromatin found in each chromosome (hence the term C-banding) and is useful for clarification of polymorphisms. Note the large heterochromatic regions in some of the chromosomes. (Image provided by Alma Ganezer.)

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 10 NORs per cell, 1 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. 6**.)

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

This stain combines DAPI, a fluorescent dye, with distamycin A, a nonfluorescent 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 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. Chapter 17 deals with this topic in depth.

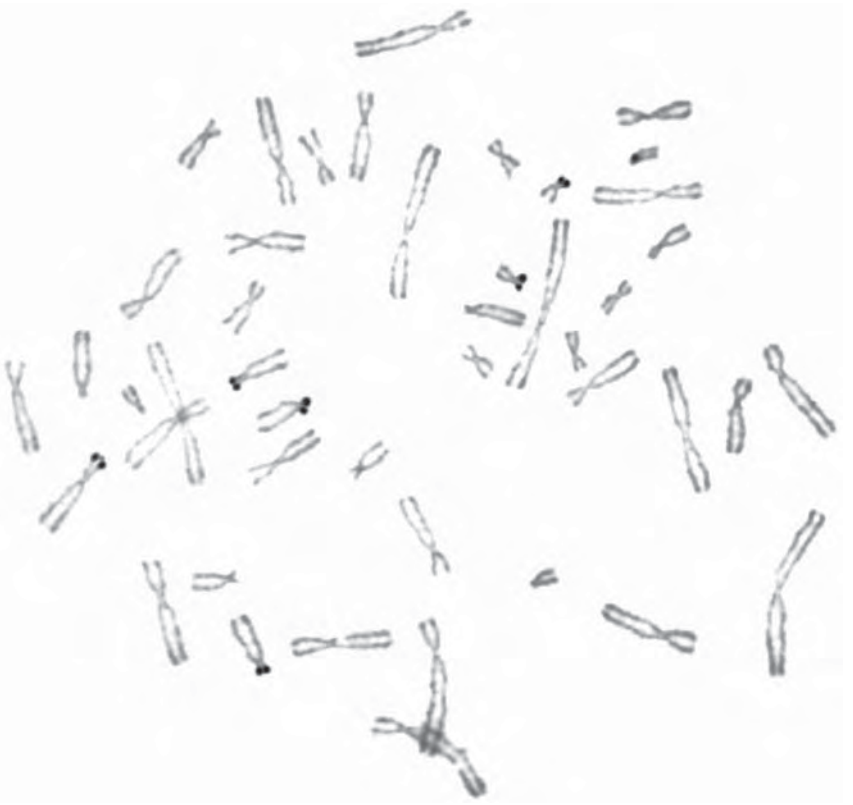

Fig. 6. NOR staining (silver staining). This procedure identifies active NORs found on the stalks of acrocentric chromosomes. Silver nitrate produces dark staining in these areas.

HIGH-RESOLUTION STUDIES

Chromosomes are routinely examined during metaphase, when they are at their most contracted state. Although this is often sufficient for chromosomal analysis, small structural abnormalities might not be detected in chromosomes of metaphase length. In such cases, longer, less contracted prophase or prometaphase chromosomes are needed. 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 hours, the accumulated cells are released from the block by the addition of a high level of thymidine. The peak prometaphase index occurs 5–6 hours later and this is when the harvest is performed.

(+) Amethopterin or methotrexate (MTX) can also be used to achieve cell synchrony, 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.

In previous decades, before the introduction of molecular analysis for fragile X syndrome (see Chapter 18), the diagnosis of this disorder was made in the cytogenetics lab, using special culture conditions. Among these was the inclusion of FUdR, described above. 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 hours 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 Chapter 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 on-going quality assurance program (see Chapter 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 the result of 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 nonsterile 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. Therefore, it is 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, CO_2 level, or pH of the culture can have deleterious results. The temperatures and CO_2 levels of all incubators must be 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 older) could 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 could suggest fetal death or threatened miscarriage. In such samples, there might 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, immunosupression, 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 might 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 the result of the samples themselves. In the case of products of conception or stillbirths, the sample might not contain viable cells or the wrong tissue type might have been collected. Additionally, microbial contamination is a frequent contributing factor because 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 might 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 can be placed in a –70°C freezer for 1–4 hours. After this initial freezing has been accomplished, the cells are stored in liquid nitrogen 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 α-fetoprotein (AFP) 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 chose among traditional amniocentesis, early amniocentesis, chorionic villus sampling, or, sometimes, percutaneous umbilical blood sampling (see Chaper 12). A decision concerning whether ploidy analysis via FISH is warranted must be made, and acetylcholinesterase (AChE) 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 prenatally diagnose late-onset disorders such as Huntington's disease. Screening for increased potential or predisposition to develop certain cancers or other diseases will create new moral and ethical pitfalls. Each of these might 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 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.

Instead of a child or an adult suspected of having a constitutional chromosome abnormality, a blood sample, therefore, could also be from a patient with leukemia or a fetus. 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 degree of 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, should there be a need to relocate a cell, that the correct metaphase has been found. 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 Chapter 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 karyotypes are prepared. Regulations notwithstanding, it is clearly 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 on the results obtained and/or initial diagnosis, additional cells might be examined in order to correctly identify all cell lines present.

Once the appropriate number of mitotic cells has been examined and analyzed, a representative sample must be selected for imaging and ultimate preparation of karyotypes. This will involve either traditional photography and manual arrangement of chromosomes (becoming increasingly rare) or computerized image capture and automated karyotyping (see Chapter 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 and imaged, etc.) is recorded in the patient's file and is used, either manually or via computer, in the clinical report.

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 karyotypes 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 analyzed in detail, the number of karyotypes prepared, the patient's karyotype, and a 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. Although 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 on individuals with the appropriate expertise and dedication to patient care.

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5

The Fundamentals of Microscopy

Christopher McAleer

INTRODUCTION

Microscopy is an essential technique for clinical cytogenetic analysis and is an integral part of the cytogenetics laboratory. Computer imaging (see Chapter 7) is a newer technology that has also become an essential component of most cytogenetics laboratories over the last decade.

There are many variables involved in obtaining good microscope images. By themselves, the individual processes might have little impact on a specimen image, but together they can have a significant influence on the final product. It is key to recognize that each step in microscopy tends to build upon those before it. Achievement of the best image quality thus requires a full understanding and application of many principles. This chapter will explore a process that many take for granted.

BRIGHTFIELD MICROSCOPY

The brightfield microscope is arguably the most important piece of equipment in the cytogenetics laboratory. Knowledge of its component parts and their proper use is fundamental. The basic components of a brightfield microscope are illustrated in **Fig. 1**.

The Transmitted Light Source and Power Supply

A transmitted light source is found in the base of a microscope, often in the rear, but occasionally in the front of the scope. The bulb housings of many microscopes will automatically center the light bulb, but some microscopes might require the bulb to be manually centered and focused.

The power supply for transmitted light sources is typically located within the base of a microscope, but might be an external unit. In either instance, the intensity of the microscope light is usually controlled by regulating the bulb voltage through a rheostat.

Even illumination and precise control of the light supply are important for good microscope images and are critical for the proper function of computer imaging systems. Proper alignment of a bulb and use of dispersion filters are requirements for illumination that is free of shadows. The quality and age of a light bulb, the setting of the microscope rheostat, and the quality of the power supplied to the microscope can quickly degrade images if they do not permit a stable supply of light.

Microscope Filters for Brightfield Microscopy

In general, the resolution of a microscope image improves with the quantity of focused light passing from the smallest details of a specimen into the objective lens. In addition, the color of light plays an important role in the resolving capacity of a microscope. Resolving power is the ability to visually distinguish two separate objects that lie in close proximity. As the distance between two such objects approaches the wavelength of light, the ability to see separate points becomes highly dependent on

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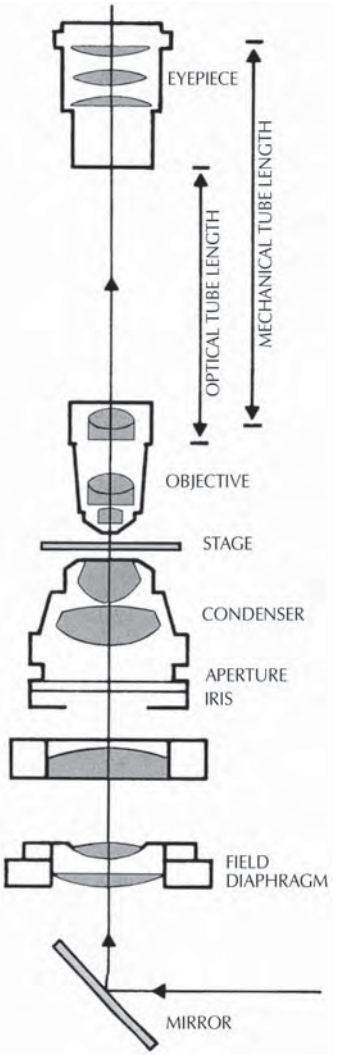

Fig. 1. Schematic diagram of a brightfield microscope illustrating the basic components. (Reprinted with permission of Olympus America, Inc.)

the wavelength used for viewing. For this reason, shorter wavelengths of light (green–blue) have greater resolving capabilities than longer ones (yellow–red).

Resolving power is also influenced by the *color and brightness difference* (contrast) between two objects, as well as the contrast of the background on which the objects appear. When an image has a low range of contrast, it is more difficult for the eye to detect structures resolved by the microscope components. Stains used for G-banding act to increase the contrast range of a chromosome by absorbing transmitted light in areas where the stain has bound. This produces bands of varying intensity, as regions with more stain allow less light to be detected. Green light enhances this absorption effect, as G-banding stains strongly absorb green wavelengths. This increases the overall contrast range of the image, allowing the eye to detect more of the subtle details of chromosome morphology and banding. For these reasons, a green filter is recommended for routine chromosome analysis.

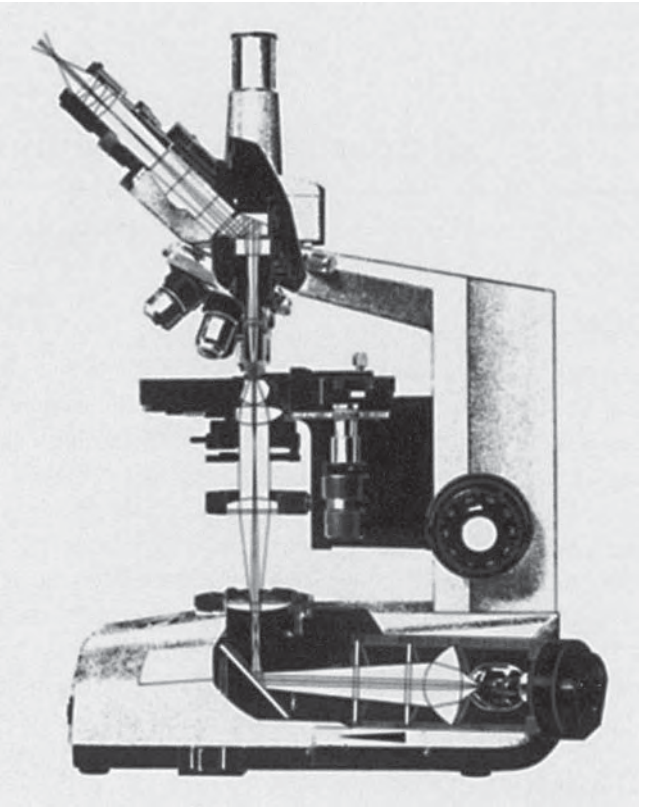

Fig. 2. Cut-away view depicting the light path of a brightfield microscope. (Reprinted with permission of Olympus America, Inc.)

A variety of filters are available for improving the image contrast of cytogenetic specimens. "Green glass" filters are the least expensive option and will improve the contrast range of G-banded chromosomes. Green interference filters absorb all but a single wavelength to a narrow band of green wavelengths, but are more expensive. Because the optics of some microscopes rely upon the use of monochromatic green light to produce quality images, investment in a green interference filter may be required. Interference filters are easily identified by their partial reflective quality and unusual tint (often orange) when viewed at an angle.

Field Diaphragm, Condenser, and Condenser (Aperture) Diaphragm

The field diaphragm, condenser, and condenser (aperture) diaphragm gather and focus the microscope light, passing it through the specimen and into the objective lens. These components play an important role in image contrast and resolution.

As light passes through a specimen, the light rays bend or diffract from their original path. It is important to understand that the smaller structures of a specimen diffract light to a greater degree relative to the diffraction of larger structures. To obtain well-resolved images, a microscope must gather as many of these highly diffracted light rays as possible for viewing *(1)*.

The process of seeing an image begins with the field diaphragm, which is used to control the area of specimen illumination. From here, light passes into the condenser, through the specimen, and into the small opening (aperture) of the objective lens. The path of light through a brightfield microscope is depicted in **Fig. 2**. The numerical apertures (NAs) of the condenser and objective are

measurements of their ability to gather highly diffracted light, thus a measure of a microscope's resolving potential.

How the condenser is used to illuminate the specimen will influence the number of light rays passing into an objective lens. A properly adjusted condenser will illuminate the small structures of a specimen from many different angles. This increases the likelihood of producing light rays with an angle of diffraction that will pass through the small opening of an objective and, thus, be present for viewing.

Microscope condensers come in a variety of NAs, ranging from 1.4 to less than 1.0. Because the operating NA of an objective lens cannot be greater than that of the condenser, these components should have similar NA values.

Condensers require correction for two basic groups of optical imperfections (aberrations) that are created as light passes through any lens. Monochromatic aberrations are those that can occur with any wavelength of light, whereas chromatic aberrations are problems unique to a specific range of wavelengths. A microscope is equipped with one of three of condensers: abbe, aplanatic, or aplanatic/achromatic. These differ in their ability to correct optical aberrations. Condensers are generally labeled with the type of optical correction they make and their NA.

Abbe condensers are the most basic type of condenser and are not corrected for either type of optical aberration. They are not recommended for use in the cytogenetics laboratory.

Aplanatic condensers are corrected solely for monochromatic aberrations and rely upon green light for the greatest degree of correction *(2)*. The performance of aplanatic condensers is highest when a monochromatic green interference filter is used.

Aplanatic/achromatic condensers are corrected for both types of optical aberration and do not require the use of green light for the correction *(2)*.

The aperture diaphragm of a condenser is adjusted to achieve a balance between the resolving power of the microscope and image contrast. When the aperture diaphragm is completely open, the small structures of a specimen are illuminated by light from the greatest number of angles and resolving capacity is at its highest. Unfortunately, the details of these structures are so well illuminated that they lose the "shadowing" or contrast variation that give such structures perspective.

As the aperture diaphragm is closed, the structures of the specimen are illuminated from fewer angles, resulting in a loss of resolving power, but an improvement in the "shadowing" or contrast of the image. Considering this, the aperture diaphragm should be set to produce a suitable balance between image contrast and resolution. Many microscope manufacturers recommend setting the condenser aperture between 66% and 75% open to achieve the best balance.

Köhler Illumination

Centering and focusing the condenser (Köhler illumination) are crucial for optimum image quality. The process begins by closing the field diaphragm so that light traveling through the condenser can be visually centered and focused. Once this is achieved, the field diaphragm is opened so that the light illuminates the specimen just beyond the field of view. Finally, the aperture diaphragm is adjusted to generate the desired image contrast.

The Phase-Contrast Condenser

Phase-contrast microscopes use a special condenser and objective lens to increase the contrast range of a specimen by darkening areas of greater density and lightening areas of lesser density. Phase contrast is often used for visualizing living or other unstained cells, but can be used successfully to increase the contrast range of G-banded specimens. A microscope equipped for phase contrast makes use of a special condenser and a phase objective lenses. Proper use of a phase-contrast microscope requires achievement of Köhler illumination, followed by an adjustment to align the phase components of the microscope. Of key importance is selection of a phase condenser setting that matches the "Ph" number of the phase objective.

Immersion Oil

As light rays enter and exit a lens, they can reflect (refract) off of its outer and inner surfaces. This occurs because the lens and air each have a different refractive property (refractive index).

Most microscopes are engineered to reduce the incidence refraction to that occurring between the condenser and microscope slide and between the specimen and objective lens. Immersion oil can be used to greatly reduce the remaining refraction by removing the air from these spaces, filling them with a substance that has a nearly identical refractive index. As a result, more light rays will exit one surface and pass into the next than would occur if air occupied these spaces, thus increasing the resolving capacity of the microscope.

A question that is often raised is whether oiling a condenser will actually improve the image quality to the extent that it is worth having to clean an oiled condenser. The highest operating NA of an unoiled condenser and objective lens is slightly less than 1.0, as the refractive index of the air (1.0) prevents a higher operating NA *(3)*. If the NA of a condenser or objective lens is less than 1.0, no benefit results from applying immersion oil.

Even with higher NAs, the loss might not be of practical significance. Remembering that resolving capacity must be balanced with image contrast, the act of applying immersion oil increases the operating numerical aperture, but also lowers image contrast. In turn, closing the aperture diaphragm improves image contrast but also lowers the resolving capacity of the microscope. Thus, the application of immersion oil to a condenser might only be beneficial when a specimen can be viewed at a reasonable contrast while the aperture diaphragm of the condenser is nearly wide open.

Immersion oil comes in several types that vary in viscosity and fluorescent properties. Each is formulated to have a refractive index of 1.5150 ± 0.0002 at 23°C (essentially the refractive index of glass).

Non-cover-slipped microscope slides should be cleaned of immersion oil at the completion of microscopic analysis. Immersion oil can cause fading of unprotected G-banded chromosomes if left on the slides for prolonged periods of time.

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 along *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-and-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, Cover Slips, and Mounting Media

The microscope slides, cover slips, and mounting media play a significant role in the contrast and resolution of an image. Microscope slides and cover slips 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. Cover-slip thickness can be 0.17–0.18 mm, depending on the recommendation of the microscope manufacturer. It is important to note that high-NA 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) *(3)*. 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.

Magnification and Objective Lenses

The objective lens (often referred to as the objective) plays the largest role in the magnification and resolution of the microscope image. Choice of objective often begins by selecting a lens that allows a specimen's detail to be comfortably analyzed at the microscope. For most cytogenetic analyses, this is either a ×63 or ×100 lens.

Magnification of the microscope image as it is projected onto a recording medium is also an important criterion for lens selection. Images that are too small as they emit onto an electronic chip of a camera will have their resolution limited by the resolving capacity of the camera. This will result in a significant reduction in image quality when later viewed on an electronic monitor or hard-copy print. Because of this, ×100 objectives have the capacity to allow the greatest degree of enlargement by a computer imaging system (see Chapter 7). Although ×63 objectives can also be used with computer imaging systems, they will often require a secondary means of magnification in order to produce printed enlargements of equal feature size and quality.

There are a variety of objective lens types: plan, achromat, apochromat, and fluorite. Markings indicating the type of objective lens are engraved on the objective barrel.

Plan Objective Lenses

Plan objective lenses are corrected for flatness of field, a term that describes an image that is in focus at its center and its periphery. A flat field is critical for imaging.

Achromat Objective Lenses

As light passes through any lens, each wavelength comes into focus at a different distance from the lens (red at the farthest point, green in the middle, and blue at the nearest point). This phenomenon is referred to as chromatic aberration. Achromatic objectives are engineered to bring the red and blue wavelengths into focus at nearly the same point, greatly reducing the depth of the overall focal plane and producing sharper detail for all colors.

Achromat objectives are often engineered for correction of monochromatic aberrations and require green light for the greatest degree of correction *(4)*. Thus, use of a monochromatic green interference filter greatly reduces the incidence of both chromatic and monochromatic aberrations experienced with achromat objectives.

Apochromat Objective Lenses

Apochromat objectives are regarded as the highest-quality lenses. Apochromatic objectives bring three or four colors into focus at nearly the same focal plane. Apochromatic objectives also correct for monochromatic aberrations and do not rely upon green light for the correction *(4)*.

Fluorite Objective Lenses

Fluorite objectives (also referred to as FL, Fluars, Neofluars, or semiapochromat objectives) are lenses of intermediate quality between that of achromatic and apochromatic objectives and are more commonly used for fluorescent microscopy where maximum light gathering power is required.

Tube Length

The tube length is the distance from the top of the objective to the back lens of the eyepiece and is the distance at which an objective lens will bring an image into focus within the microscope. Tube

lengths have been standardized by some microscope manufacturers at 160 mm and by others at 170 mm *(3)*. When appropriate, the tube length of an objective is engraved on its outer surface. Objectives with identical tube lengths can sometimes be exchanged between different microscopes, but should only be done after first consulting with the microscope manufacturer.

The objectives of many modern microscopes have been engineered with infinity correction. The tube length of an infinity-corrected system is established internally with a tube lens, allowing the objective to move independent of the eyepiece. A key feature of infinity-corrected systems is that a change in the tube length will not significantly impact the size of the image as it projects onto the back lens of the eyepiece or onto a camera. Remembering the importance of image size, infinity-corrected systems allow the addition of optional microscope components without significantly impacting image quality.

Cover-Slip Correction

A predictable working distance between the condenser, specimen, and objective lens is required for microscopy. Microscope slides are made with a specific thickness to place the specimen at the proper working distance between these components. When a slide is coated with mounting medium and a cover slip is attached, the objective lens must be corrected for this additional thickness.

The correction factor is engraved on the objective immediately following the tube length (often 0.17 or 0.18 mm). A cover slip and mounting medium of that thickness must be used in order for the objective to maintain its optimal resolving power. If the value is listed as "–," the objective may be used with or without a cover slip.

Non-Cover-Slip-Corrected Lenses

Non-cover-glass (NCG)-corrected lenses are occasionally found in cytogenetics laboratories and are used exclusively when cover slips are not used.

Numerical Aperture

The NA of an objective is a statement of its resolving capacity. Objectives of differing magnification but identical NA values have similar resolving potential.

Many factors can reduce the operating NA of an objective lens, resulting in a decrease in the microscope's overall resolving capacity. These include the alignment of the light bulb, the wavelength of light, the quality of the condenser, the setting of the aperture diaphragm, the presence of immersion oil between the condenser and slide, and the characteristics of the microscope slide and mounting medium.

Oil Immersion Lenses

The designation "Oil" or "Oel" engraved on an objective denotes an oil immersion lens. These lenses use oil as a means of increasing the refractive index of the space between the specimen and objective lens, thus increasing the operating NA and resolving capacity of the microscope.

High-Dry Objective Lenses

High-dry objective lenses are not used with immersion oil. High-dry lenses substantially sacrifice the resolving potential of a microscope and are not recommended for diagnostic use in a cytogenetics laboratory. They are, however, commonly used to check the morphology of nonstained chromosomes. They can also be used to check the banding characteristics of stained chromosomes before the application of immersion oil.

The spaces between the condenser, specimen, and objective lens are filled with air when a high-dry lens is used. Therefore, the operating numerical aperture of high-dry lenses is limited to that of air, 1.0. In practice, the operating NA of a high-dry objective is typically 0.95 or less, compared with the NA of 1.3–1.4 found in oil immersion lenses.

High-dry objectives typically have a very low tolerance to slide thickness *(4)*. In addition, when a high-dry lens is used on a cover-slipped specimen, a correction collar should also be present to allow compensation for the additional thickness generated by the mounting medium and cover slip.

Infinity Correction

The symbol "∞" engraved on an objective identifies it as an infinity-corrected lens. Infinity-corrected objectives project parallel light rays into the microscope. These rays do not come into focus until they pass through a special tube lens, where they are brought into focus at the back lens of the microscope eyepiece. As the tube length for the objective is not fixed, a variety of features can be added to a microscope without significantly impacting image magnification. Infinity-corrected lenses are typically engineered for a specific microscope and are not usually interchangeable between microscopes.

Objective-Correction Collar

Objectives with a correction collar are often designed for use with materials that have a range of refractive properties, as well as a variation in overall specimen thickness. For brightfield microscopy, it is very important to ensure that a correction collar is adjusted appropriately for the specimen preparation. Typically, correction collars are labeled with a scale that should be adjusted to the appropriate cover-slip or plating material thickness. Often, the correction collar is left in a specific position for cytogenetic analysis, as even a slightly misadjusted collar can result in a poorly resolved image.

The quality of fluorescent images can also be impacted by use of a correction collar and one is sometimes used for controlling image contrast. Again, it is important to realize that the collar has been designed to allow optimization of the resolving capacity of a lens for the plating materials and overall thickness of the specimen. Although closing a collar might improve contrast, it might also lower the resolving capacity of the lens (see the Fluorescence Microscopy section for additional information).

Optivar Lenses and Magnification Changers

A microscope could have additional lenses that increase the total magnification of the image. A magnification changer or Optivar lens can appear as a rotating control or a sliding bar located between the objective lenses and the eyepieces. Optivar lenses allow microscopes using a mid-range objective (×63) with a high NA to increase the size of an image without the loss of resolution.

Use of Optivar lenses to increase the magnification of an image beyond that provided by the objective should be done so with care, as image magnification that exceeds the resolving capacity of a microscope will result in "empty magnification."

Eyepieces

Microscope eyepieces increase the magnification of the microscope image and position the image so that it can be seen by each eye. Eyepieces may also be engineered with a variety of features, including those that correct chromatic aberrations (C, K), those that provide a wide field of view (WF), and those that allow viewing from a greater than standard distance (H) (thus allowing the microscopist to wear corrective eyeglasses) *(5)*. Most eyepieces are adjustable so that the focus characteristics of each eye can be optimized for the individual viewing an image. This allows the images at each eyepiece to be brought into simultaneous focus, permitting individuals with vision deficits to be able to use the microscope without the need for corrective lenses. Finally, cross-hairs may also be present in an eyepiece to provide an indication of the image focus at the microscope compared to the image focus at a photographic camera.

Beam Splitter

A beam splitter is present on microscopes capable of photomicrography or electronic image capture. A beam splitter allows the light to be diverted between the eyes and the photographic port at various relative intensities.

FLUORESCENCE MICROSCOPY

Microscopes used for brightfield microscopy can also be equipped for fluorescence microscopy. The additional components for fluorescence microscopy include an epifluorescence lamp housing, a horizontal attachment for the fluorescent light path, fluorescence filters, and fluorescence objective lenses.

Essentials of the Fluorescence Microscope

Epifluorescence Lamp Housing and Microscope Attachment

The housing for the epifluorescent light source is mounted on the rear of many microscopes and is located just above the housing for the brightfield light source. The fluorescence housing is mounted to an epifluorescent microscope attachment, which is used to direct fluorescent light into the microscope and to house the fluorescence filters. Most fluorescent lamp housings include bulb alignment controls and an adjustable collector lens to control the dispersion of the light across the microscope field of view. The epifluorescent microscope attachment also includes a light shutter to block the passage of light into the microscope, a field diaphragm to control the area of illumination, and a means of inserting infrared or neutral density filters.

Filters

Infrared (IR) filters can be used with electronic cameras to block IR light emitted by the lamp and keep it from from reaching the camera and producing a high background. IR filters can also reduce the overall intensity of the fluorescent light and should therefore be used on an "as-needed" basis. In addition, IR filters should not be used with fluorescent dyes that rely on infared or near-infared wavelengths for excitation.

Neutral density (ND) filters allow the intensity of the fluorescent light of all wavelengths to be reduced (attenuated) by a fixed amount. Neutral density filters come in several attenuations and are labeled to indicate the degree of reduction. Neutral density filters are often used with fluorescent preparations that have unusually bright fluorescence intensity or that produce a great deal of fluorescent flare. Neutral density filters can dramatically reduce the intensity of a limited fluorescent light supply and should be used only when necessary.

Fluorescence Filters and the Fluorescence Filter Housing

The basic principal of fluorescence microscopy involves the excitation of a fluorochrome (fluorescent stain) with one wavelength of light, causing the emission of a second wavelength of light. The wavelength used for excitation will vary for each fluorochrome, but will be of a higher energy (shorter wavelength) than the emission wavelength it produces (e.g., green excitation wavelengths can produce red emission wavelengths).

Fluorescent filters include three basic components: the excitation filter, the dichroic mirror, and the emission filter (see **Fig. 3**). The excitation filter and dichroic mirror work together to produce precise excitation wavelengths and to direct the light down into the objective lens and onto the specimen. The resulting emission wavelengths then travel up through the objective and pass through the mirror and emission filter so that precise wavelengths reach the eyes of the microscopist or camera.

Protocols for the various staining techniques will specify the fluorochromes and filters required for their specific use.

Viewing Fluorochromes at the Microscope

The best fluorescent stain and filter combination for specimens stained with more than one fluorochrome is one in which the color of each fluorochrome contrasts strongly against all other fluorochromes present.

Single-emission fluorescence filters limit the wavelengths of light so that only one fluorochrome is visible at a time. Dual- and triple-emission filters allow multiple fluorochromes to be seen at the same time.

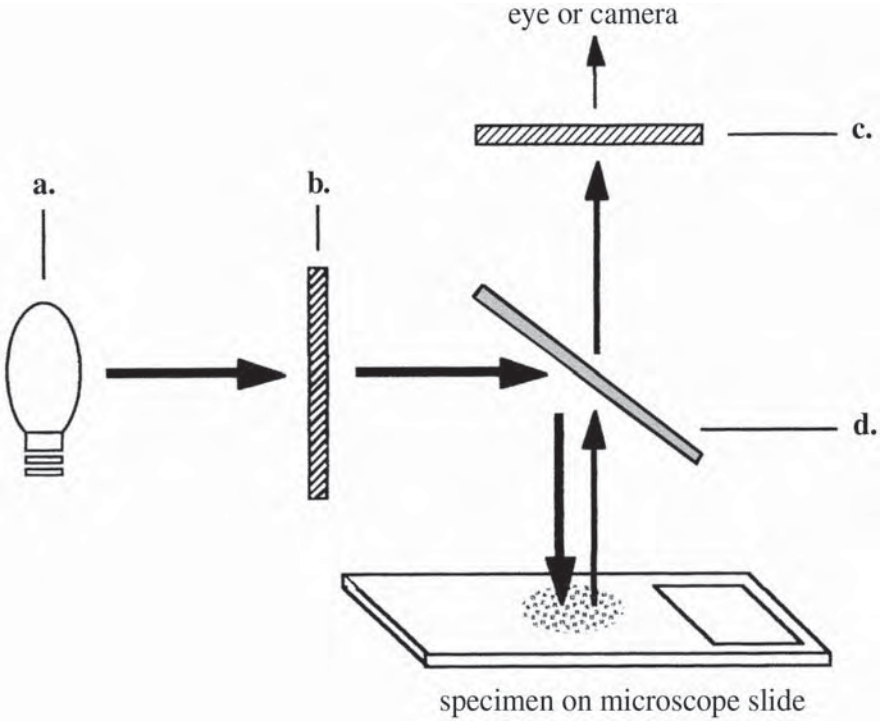

Fig. 3. Schematic of the basic components of a fluorescence microscope: **(a)** light source, **(b)** excitation filter, **(c)** dichroic mirror, and **(d)** emission or barrier filter.

Single-emission filters offer several advantages over dual- or triple-emission filters, including the use of peak excitation wavelengths for particular fluorochromes, resulting in stronger emission intensities. In addition, single-emission filters usually allow a wider band of wavelengths to emit to the eyes or camera, thus increasing the overall intensity of the fluorescent image. Finally, viewing only one fluorochrome at a time allows for the visualization of very-low-intensity fluorescence, without the signal becoming lost in the fluorescence of other fluorochromes. The disadvantage of single-emission filters is that the fluorescence filters must be frequently changed to allow all fluorochromes to be seen when multiple fluorochromes are used simultaneously. Scanning and analysis can be less tedious when dual- or triple-emission filters are used in such situations.

Fluorescence Objective Lenses

Fluorescence objective lenses are made of fluorite or quartz, not glass. This extends the useful range of the objective into the UV spectrum (a requirement for DAPI microscopy), as UV wavelengths are absorbed by glass.

As previously mentioned, many fluorescence lenses are equipped with a correction collar designed to allow use of the objective with materials with a range of refractive properties and that must be adjusted to the thickness of the plating material. Other lenses are equipped with an aperture diaphragm. Although this diaphragm can be adjusted to reduce the intensity of fluorescence and image flare, closing it will result in a significant loss of resolution and is therefore not recommended. A slight adjustment of the collector lens, use of an ND filter, or other controls present on the epifluorescent microscope attachment are better solutions to control image intensity.

Immersion Oil

Immersion oil is very important for fluorescent imaging, as the fluorescent light source is very limited. Low-fluorescing oil is recommended to allow the contrast of the fluorescing objects to stand out against a dark background.

Beam Splitters

Because fluorescent specimens produce low-intensity images, it is very important to equip the microscope with a beam splitter that allows all light to be directed either to the eyes for microscopic analysis or to photographic ports for photography or electronic image capture.

The Brightfield Condenser

The brightfield condenser is not used for epifluorescent microscopy and can allow ambient light to interfere with the fluorescent image quality. For this reason, it is recommended that the condenser be blocked off or defocused so that ambient light will not be passed into the objective lens.

ACKNOWLEDGMENTS

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6

Quality Control and Quality Assurance

Michael S. Watson, PhD and Steven L. Gersen, PhD

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. Practice standards (American College of Medical Genetics [ACMG], 2003) based on the consensus of professionals and common sense are the basis for oversight by regulatory agencies, to prevent clinical and clerical errors. These comprise the area of laboratory medicine known as quality assurance and quality control (QA/QC). These are supplemented by both total quality management (TQM) and complete quality improvement (CQI) programs that seek to minimize errors where the laboratory interfaces to 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, and others are specific to cytogenetic laboratory tests.

A proper QA/QC program requires that policies for validation of protocols and reagents, training and credentials of individuals performing chromosome 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, microscopic analysis, photography or electronic imaging, karyotype 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 in order to reach a proper diagnosis.

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: physical space and mechanical requirements, sample amount and collection, transport requirements, personnel experience and credential requirements, and safety and protection requirements for those personnel. It includes sections on training and compliance with the various regulatory agencies that monitor and inspect laboratories, and, finally, it might contain a section pertaining to quality assurance and quality control. The majority of these issues pertain to the analytic component of testing. The format of the SOP manual is specified by the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS), which requires

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that, in addition to containing a detailed protocol for every procedure the laboratory performs, the manual also includes the clinical and technical rationale behind each one.

With the rapid growth of knowledge and expansion of genetic testing, the laboratory has become increasingly involved in ensuring that the preanalytic and postanalytic aspects of testing also are 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 laboratory's clients to interface with it. These aspects are commonly encompassed in a complete quality improvement program.

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

PREANALYTICAL TESTING COMPONENTS

Before a test specimen arrives in the laboratory, there are a number of steps that must be done correctly to ensure that an accurate and useful test result is provided. Laboratories often develop and provide to their clients materials 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 can be done by the laboratory developing the test or 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. Furthermore, all new tests must be revalidated 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 (ACMG, 2003). These are most often applied to fluorescence *in situ* hybridization (FISH) tests (see Chapter 17), particularly when interphase-based, but also are important when mosaicism is considered. Requirements for validation can vary with the regulatory status of a product. When a test is Food and Drug Administration (FDA) approved, 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, perform particular tests, or to detect a particular abnormality by testing samples from individuals with those abnormalities.

Submitting a Specimen

Specimens are almost always collected by individuals who rely upon the laboratory to provide a requisition form and instructions for specimen collection and transport. Considering this, QA/QC begin through an interaction with the health care providers who 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 the following:

- 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, as well as 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 submit samples infrequently to the laboratory. Regular interaction will promote a complete understanding of collection requirements, as well as general expectations for a sample submitted for cytogenetic analysis. It will also provide an opportunity to discuss questions, concerns, or suggested improvements of collection or submission procedures.

Specimen Labeling and Requisition Forms

Accurate specimen identification is perhaps 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, date of collection, and so forth 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, 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 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 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 or FISH 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 might 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, 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 is often considered preanalytical, we include it here because labeling

and tracking of specimens through a test are among the most common causes of error in a clinical laboratory testing. This phase usually ends when a laboratory test result is apparent.

Accessioning a Specimen

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 log book and/or database, and the culture and analysis requirements for the studies requested are identified.

Assessing the Condition of the Specimen and Requisition

Once a specimen is received, the accessioning individual 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 a specimen as it enters a laboratory, distinguishing it from other lab specimens, as well as distinguishing it 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 or future studies. In addition, other data can be entered into a database record as a study progresses, allowing the laboratory to track the following:

- Culture conditions
- Results
- Turnaround times
- Dates and individuals issuing reports
- Cytogenetic results versus the findings of patients with similar histories or abnormalities
- Culture failures, labeling errors, transcription errors, and misdiagnoses
- Incidence of submission problems

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

Once a specimen has been logged into the cytogenetics laboratory, the individual who has done so should follow the procedure for preparing it for cell culture. This might include storing a specimen under appropriate conditions, creation of culture records and container labels, notification of the appropriate individuals, if others, that the samples have been received, and creation of a patient folder for paper records.

It is also important to have a system for identifying specimens that require special handling such as an accelerated study, a preliminary report, or completion by a certain date to meet anticipated turnaround 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. Up to this point, labels have been assigned to the specimen container and requisition form. After the accessioning process, specimen labels can take on many forms, involving a culture worksheet, culture flasks or Petri dishes, microscope slides, a microscope analysis worksheet, photographic film, photographic or electronically produced prints, karyotypes, and reports. With these things in mind, it is important for a labora-

tory to establish a labeling policy so that patient identification can 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

When preparing a specimen for culture, it is important, when possible, to generate duplicate or independently established cultures for all samples. In addition, it is also important to place duplicate cultures in separate incubators, each equipped with its own power, CO_2 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 CO_2 (automatic gas tank supply change) failure.

As a specimen is added to culture medium, a culture is transferred between containers, or reagents are added to a specimen culture, it is important to take precautions to prevent contamination.

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, pipets, or other measuring devices to a flame will also reduce the likelihood of contamination.

Working with *one specimen at a time* and disposing of all used pipets 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 done 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 similar changes in established protocols should be done prior to their use with patient specimens. For critical reagents that may be of variable quality from manufacturer to manufacturer or 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 might 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 is an important consideration and 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 follow regular cleaning 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. Laminar flow hoods also should be checked and certified annually for airflow and bacterial contamination. 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 (CO_2) concentration should be monitored continuously and documented daily. Incubators should be on a regular cleaning schedule and, as discussed above, 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.

Automation of the harvesting procedure is used by many cytogenetics laboratories as a way of increasing laboratory productivity and improving consistency (see Chapter 7). However, automation does not imply "care-free"! Laboratories that utilize such technology must closely follow the manufacturers' recommended operational guidelines and closely monitor the equipment for acceptable performance. A procedure for the use of automated equipment must be prepared that details the procedural steps for operation, appropriate reagents, calibration and cleaning requirements, and preventive maintenance. 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 (mitotic arrest, osmotic swelling of cell membranes, and fixation), spreading of chromosomes on microscope slides, and staining chromosomes with one of various methods that produce an appropriate banding pattern (see Chapter 4). Each of these steps must be optimized to facilitate correct diagnoses.

Protocols

Harvesting, slide making, and banding/staining determine the quality of metaphase chromosomes made available by successful cell culture. Following validated protocols is very important for these procedures, but frequent modifications could 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 or microscope slides, and so forth must be validated under controlled conditions. The method of validation should be one that is appropriate for the reagent or technique being tested and might 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 will be uniform and plane of focus will not be affected, and they must be aged (literally or artificially) in order for most banding and staining procedures to work properly.

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. Consider the following variables:

- Harvesting method (centrifuge tubes versus *in situ* processing; see Chapter 4)
- The humidity and temperature of the laboratory or drying chamber utilized (See Chapter 7)
- The number of fixations and the method of fixing the specimen

- The slide temperature
- Wet or dry slide? 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 significantly contributes to the success of slide preparation. As theses can be variable from day to day or between individuals, close observation and documentation of technique can allow the highest proficiency of these skills.

Banding and Staining

Slide preparation and aging are deciding factors in the lab's ability to successfully stain a specimen, by affecting adjustments to solution concentration, the time a slide is left in a staining solution, and so forth. Careful reagent preparation and documentation of adjustments made to staining procedures helps laboratories to refine 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, the date received, and an 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 important for microscope analysis, it is also necessary to consider the microscope on which a specimen will be analyzed and the staining requirements of the recording media (photography or electronic image capture). When a laboratory has a variety of microscopes, each might have a light source, contrast or interference filters, objectives, or other lenses that produce images with a unique set of visual characteristics. Additional variables are introduced with the use fluorescence microscopy, such as excitation and barrier filters, and features such as the numerical aperture of lenses or bulb intensity could be critical. Individual taste will also play an important factor in identifying a staining intensity that is well suited for microscope analysis.

For either photography or electronic capture of traditional G-banded images, it is important to identify staining intensities that produce the following:

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

It is not unusual to find that a staining protocol might not be well suited for both microscope analysis and photography/electronic capture.

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

Specimen Analysis

Any chromosome analysis must begin by identifying specific requirements for the specimen type being examined. Following this, the basic steps involved are microscope analysis itself (location of metaphase spreads suitable for analysis, counting chromosomes and determining sex chromosome complement, and band-pattern analysis), photographic or computerized imaging, preparation of karyotypes, 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.

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, sex chromosomes examined, and bands analyzed in detail must be clearly stated, including whether each type of examination is to occur at the microscope, on an image, or via a karyotype. 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 items, 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 be karyotyped, 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, both during analysis and once analysis has been completed.

Personnel Requirements

In addition to understanding specimen analysis requirements, it is important to identify personal criteria to be met for and by individuals performing the analysis. The experience level, credentials, and workload of each technologist are 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 turnaround time and the number of cases to be completed in a week, are important aspects 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 within the training an individual receives on the components of a microscope and their proper use. One of first subjects detailed in any protocol for microscopy should, therefore, be training, including quality checks to identify equipment in need of service or adjustment or individuals in need of additional training.

The selection of a microscope 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 individuals need to understand the limiting factors of any given scope. “Newer” does not necessarily imply “better” in this instance, as many “veteran” microscopes can produce excellent images, and it is often the resolution of the objective (lens), not extraneous accessories, that is the key to image clarity. Also, keep in mind that 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 Chapter 5.

General Analysis Requirements

Analysis requirements have evolved as a mix of “conventional wisdom” and statistically validated needs for specific types of study. Professional organizations have developed consensus-based standards for different types of analysis (ACMG, 2003) and regulatory bodies have typically used these

as a guide when they specify minimum requirements for each sample type processed for chromosome analysis. Laboratories themselves and individual states of the United States frequently augment these. It should be noted that minimum requirements are just those; 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 might be necessary for correct diagnosis. It is also important to realize that a patient's clinical indications could dictate that analytical resolution should be higher than the stated minimums

Some general guidelines follow:

Sample Type	*Basic Analysis Requirements*
PHA-stimulated Blood (non-neoplastic disorders)	Chromosome count and sex chromosome complement determination of at least 20 cells, or at least 30 cells when a mosaic condition is suspected or detected. At least five metaphase cells should be completely analyzed and at least two karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each. A minimum resolution of 400–450 bands (500–550 is preferable) should be attained; this number should greater for high-resolution or focused studies of a specific chromosome pair.
Amniotic fluid, *in situ* method	Chromosome count and sex chromosome complement determination of 1 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 confirm pseudomosaicism. Cells must originate from at least two independent cultures (initiated 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 karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each. A minimum resolution of 400–450 bands is recommended.
Amniotic fluid, flask method	Chromosome count and sex chromosome complement determination from at least 20 cells from at least 2 independent cultures as above. Other requirements are the same as for the *in situ* method.
Chorionic villus samples	Chromosome count and sex chromosome complement determination of least 20 cells from at least 2 independent cultures. Altough opinions differ, most laboratories examine both "direct" preparations and cultured cells when possible. Additional cells should be examined when mosaicism is detected, particularly when there are discrepancies between the direct and cultured preparations, often an indication of confined placental mosaicism (see Chapter 12). At least five metaphases should be completely analyzed, and at least two karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each. A minimum resolution of 400 bands should be achieved.
Solid tissues (non-neoplastic studies)	Chromosome count and sex chromosome complement determination of least 20 cells from at least 2 independent cultures. At least five metaphases should be completely analyzed, and at least two karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each. A minimum resolution of 400 bands should be achieved.

Neoplastic studies (bone marrow, tumor biopsy or aspirate, unstimulated peripheral blood)	Thorough examination of 20 cells when possible. All metaphases should be analyzed, and at least two karyotypes should be prepared. If more than one cell line is present, at least one karyotype must be prepared from each. The resolution should be at least 400 bands. When fewer than 20 cells can be analyzed and an abnormality has been detected, the number of abnormal and normal (if any) cells is reported. When fewer than 20 cells can be examined and an abnormality is not detected, the number of cells studied is reported and additional procedures (FISH, molecular analysis) or a repeat study, when clinically appropriate, might be recommended. For studies of minimal residual disease or engraftment studies, additional metaphases can be examined.
Fragile X syndrome	Although guidelines were created for the diagnosis of this disorder via cytogenetic analysis, current standard of care now involves analysis via molecular methods. See Chapter 18.

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 performing the analysis and the date should both 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 sex chromosome complement are typically noted, along with other relevant data such as quality of banding, abnormalities, polymorphisms, or chromosome breakage observed, whether the cell was analyzed and/or imaged, which cells should be considered for karyotype, and so forth. Finally, a summary of the results, including the patient's karyotype, can be included, along with indications of clerical review.

Photography, Imaging Systems, and Karyotype Production

During or upon completion of the microscopic analysis, a specimen is ready to be photographed or imaged electronically, printed, and karyotyped. Photography or electronic capture are the tools used to record the microscope image, allowing the chromosomes to be documented and reanalyzed as necessary. Understanding how to operate, optimize, and maintain the materials and equipment used in these processes is necessary in order to achieve optimum results from any sample.

Photography

Like microscopy, good photography is not a difficult technique, but is one that relies heavily upon proper training. Technologists must understand the factors that go into successful transfer of a microscopic image to film and must be thoroughly familiar with their photomicrographic equipment, as well as with the chemicals, print papers, equipment, and procedures for developing film and preparing photographic prints. Knowledge of the resolution and contrast characteristics of print papers and related developer characteristics are important to those considering individualizing their darkroom capabilities. Photography could soon become a "lost art," as more and more cytogenetics laboratories implement computerized imaging and karyotyping systems (see below).

One of the most important quality controls in photographic processes is accurate labeling of film and developed prints. A protocol for the laboratory and its darkroom should include a system for labeling these items, as well as information that will allow a label to be cross-checked against the microscope analysis worksheet.

Computerized Imaging Systems

Computer-driven imaging systems are essentially the digital equivalent of photography; otherwise, the steps involved are similar. Instead of photographing a cell, it is electronically captured in digital form. 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 laser or other type of printer provides a hard copy. Finally, images are stored not as photographic negatives but as digital files on tape drives, optical disks, DVD-R, or other digital storage media.

Finally, as with photography, an understanding of theory and hardware, generated by the appropriate amount of training, are required so that laboratory staff can utilize an imaging system properly and efficiently. (See Chapter 7.)

Karyotype Production

Although not used in all countries, the final laboratory manipulation required for chromosome analysis is typically the generation of the ordered arrangement of chromosomes known as a *karyotype*.

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 karyotype 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), one 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, lab aides, interns, or other students are often employed to generate karyotypes. A good rule to follow when this occurs is that no such individual is permitted to karyotype an entire case without supervision or review by a trained technologist.

Karyotype production is one method laboratories use to divide analyses between two or more technologists. This can be accomplished with a guideline that specifies that the technologist(s) who performed the microscopic analysis cannot prepare or review the karyotypes for that patient. When one adds to this a review by the laboratory supervisor or another senior individual, followed by final review by the laboratory director, it can be seen that 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 karyotypes. In the past this essentially involved "cutting and pasting" the chromosomes with a trackball or mouse; however, pattern-recognition software has improved to the point that many sophisticated systems can now arrange the chromosomes with little or no human input (see Chapter 7). This, of course, creates a quality concern. Laboratories deal with this by putting in place protocols that require appropriate review of all computer-generated karyotypes. When properly monitored, such systems can increase laboratory efficiency by markedly reducing the time required for karyotype production.

POSTANALYTICAL TESTING COMPONENTS

Preliminary and Final Reports

Reporting the results of chromosome analysis can have a direct impact on the diagnosis and treatment of a patient. Considering this, it is important to establish a reporting procedure that accomplish the following:

- Summarize the findings of the laboratory
- Crosscheck the findings against the various specimen labels for labeling errors
- Interpret the test results, where appropriate
- Establish a reporting process to outside individuals so that the data, individual issuing the report, 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 have been collected to formulate a likely indication of the final result or once the data already available are clinically critical and must be communicated to a physician. 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, the 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 might change once the study is completed.

Final Reports

A final report will summarize and interpret the results of the study. Some states and regulatory agencies also require a statement describing the limitations of chromosome analysis, and many laboratories choose to include such a statement whether required or not. A procedure for the creation of final reports should include a checklist to ensure that all appropriate procedures have been completed and that all data are clerically correct. Once completed, final reports can be generated electronically or on paper. If a preliminary report was provided, any variations from that 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 samples themselves; some of these are inevitable and therefore are not preventable (insufficient volume, wrong sample type, no living cells present, etc.). Others may be the result of collection or transport of the specimen, incorrect labeling, or other human error at sample collection, during transport, or in the laboratory. There are also a number of difficulties that can arise in the laboratory after an appropriate specimen has been received without incident.

Any of these can result either in an incorrect diagnosis or in failure to reach one at all. Therefore, it is very important for a laboratory to document all problems that arise and, by determining ways to prevent similar occurrences, improve overall quality.

It is also important to monitor specific types of laboratory test outcome in order to judge one's laboratory's performance. This is most commonly done when a laboratory can expect a particular distribution of outcomes. In studies of products of conception, review of distribution of results can inform the laboratory 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 rate and abnormality detection rates. For instance, among patients entered in national cancer cooperative group studies, there is usually a groupwide expectation based on prior performance of laboratories in the group.

Specimen Failures

The inability of a laboratory to provide a diagnostic result is typically the result of one of two basic reasons: cells from the sample do not grow in culture and, therefore, no mitotic cells are produced, or a problem occurs in one of the many postculture steps, rendering the processed material useless. The purpose of this subsection is not to convince the reader that problems are inevitable, but rather to impress upon him or her the amount of care and attention to detail required and the critical role quality assurance plays in the cytogenetics laboratory.

Culture Failure

As described in Chapter 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 from fetal demise or in necrotic or aplastic bone marrow samples. Other times, one can deduce the cause (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 still other instances, 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 could involve peripheral blood with no circulating blasts being collected instead of bone marrow. (Without blasts in the periphery, there are no spontaneously dividing cells present and the unstimulated cultures used for hematopoietic disorders will not produce metaphases.) It might be the result of the wrong collection tube being used, or of 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*. For example, "2 mL of extremely bloody amniotic fluid" or "0.5 mL of watery bone marrow" is the type of description that frequently accompanies a culture failure record. It should be pointed out, however, that *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, placed in separate incubators, should be initiated whenever possible. There should also be appropriate backup power, redundant CO_2 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), this risk can be minimized.
- *Human error*. Although 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.

Postculturing Errors

There are few things as frustrating to the cytogenetics laboratory as having seemingly good cell cultures or routine blood cultures produce no usable metaphases. Although these are admittedly rare events, they do occur and, as with culture failures, must be fully investigated and documented. Some examples are as follows:

- *Harvesting errors.* As outlined in Chapter 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 amount 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 via gravity will be removed via aspiration or pouroff. Other errors, such as adding the wrong hypotonic, 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 a robotic harvester.* As described in Chapter 7, cultures that are processed with the *in situ* method are amenable to harvesting on a robotic fluid handling device. Although the concept when using such a machine is freeing up the technologist's time for other vital functions, it is not 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 periodic checkups until the cultures are in fixative is a good policy for the laboratory to adopt.
- *Slide making/culture 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 Chapter 4 and is also discussed in Chapter 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.* This is another example 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 Chapter 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 postculture steps has been covered, there are still strange 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, 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

Perfection is always a goal in medicine, but it is never achieved. Every laboratory discipline strives to eliminate all mistakes, but given the fact that human beings are involved, each also has an "acceptable" error limit. A small cytogenetics laboratory processing 2000 samples per year that achieves a 99.97% accuracy rate (far in excess of the performance of the typical excellent pathology lab) will make 6 misdiagnoses in a 10-year period.

Misdiagnosis in the cytogenetics laboratory can occur in three ways: as the result of incorrect specimen labeling (described above), by incorrect interpretation of a chromosome abnormality, or by missing an abnormality that is present. Despite the many "pairs of eyes" that typically see each specimen in many labs, as described above, some things can 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 chromosome morphology often produced by bone marrow aspirates or solid tumors or the complex abnormalities frequently present in such samples, it is often difficult, if not impossible, to correctly identify every change. It is not uncommon for a laboratory to receive serial bone marrow aspirates from a patient, only to discover that, because of improved resolution in the current sample, an abnormality can be more accurately characterized and that a previous interpretation was not quite correct. This is typically of little clinical consequence and can easily be addressed in the current clinical report. On the other hand, misidentification of 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 used to be, because most such changes can be confirmed or further characterized via FISH (see Chapter 17). Many rearrangements are family-specific, and predicting the phenotype likely to result from an unbalanced translocation 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" is subjective in the field of cytogenetics.

Failure to identify a chromosome abnormality that is, in fact, present can be a serious issue should it ultimately be discovered. Such is not always the case. As discussed above, an abnormality might 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 laboratory receives both specimens, this can be detected and interpreted correctly, and it is possible that the referring physician(s) might 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, and in such a scenario, the initial diagnostic failure might never be revealed.

Perhaps the most serious example of a missed diagnosis is the 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 Chapters 9 and 20), but rarely has an impact on the current pregnancy. However, failure to identify an unbalanced abnormality will almost always result in the birth of an abnormal child and, should the parents believe that they would have interrupted the pregnancy had the abnormality been caught, can result in a lawsuit. 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 the abnormality "should have been detected." The latter often involves presenting uninvolved professionals

with the karyotypes, to determine whether or not they can identify the abnormality (a biased process, because 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

In order to realize the benefits of having tracked the function of the laboratory and monitored its performance and problems, an organized process of review, communication, and staff education is required. This could involve subsets of the laboratory personnel but at certain times, it is 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 ensure and improve quality. These vary from country to country and even from state to state in the United States. Oversight of clinical laboratories is in two main forms. 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 of CLIA '88 regulation with specialty specific requirements (CFR§493.1276).

United States

Accreditation, Inspections and External Proficiency Testing

Under the Clinical Laboratory Improvement Amendment of 1988 (CLIA '88), every laboratory performing moderate- to high-complexity testing (i.e., every cytogenetics laboratory) must enroll in Health and Human Services approved external inspection and testing programs. In fact, virtually all clinical laboratories in the United States do so under the auspices of the CLIA-deemed program of the College of American Pathologists (CAP). This accrediting organization inspects laboratories and provides the American College of Medical Genetics (ACMG)/CAP proficiency testing survey program, according to CLIA requirements, several times a year. A lab's ability to perform and be reimbursed for testing depends on successful participation in each aspect of this process, as repeated failure can lead to loss of accreditation. As of this time, no areas of genetic testing have mandated performance requirements for their proficiency testing programs.

The College of American Pathologists 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 unknowns in the form of banded metaphase preparations plus sufficient clinical information for the lab to make a diagnosis. A fifth unknown, in the form of a peripheral blood sample, is also frequently submitted, but the reader will appreciate the logistical and medical challenges of this procedure; there are enough cytogenetics laboratories in the United States that care must be taken not to exsanguinate the individual (typically a carrier of some rearrangement) who has volunteered to be the test subject!

State requirements can be quite variable. Several require participation in the CAP programs. However, this is often difficult for private-sector laboratories. One of the more rigorous programs is admin-

istered by the New York State Department of Health, which conducts its own inspections and proficiency tests of all labs in the United States that process specimens from New York State residents. This body also has its own certification process (see below).

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 as among the boards required of laboratory directors. Such certification is awarded to a doctor (MD or PhD) who passes a comprehensive examination in General Genetics as well as a specialty exam (in this case, Clinical Cytogenetics). Both exams must be passed for an individual to be Board-Certified. Diplomates certified prior to 1993 when maintenance of certification became a requirement must recertify after 10 years. Other need not recertify but are encouraged to do so.

Many technologists, supervisors, and even directors in clinical cytogenetics labs across the United States learned how to perform chromosome analysis on the job, and such experience was all that was needed in order to find employment. Today, degreed programs in cytogenetics exist in several colleges and universities, and a technologist can now be certified as Clinical Laboratory Specialist in Cytogenetics [CLSp(CG)] by the National Credentialing Agency for Laboratory Personnel (NCA). Initial certification results from passing an examination and lasts for 5 years. Recertification can be accomplished either via continuing education (every 2 years) or re-examination (every 4 years).

The State of New York requires documentation of sufficient postdoctoral experience in order to receive a Certificate of Qualification (CQ) in various laboratory disciplines, including cytogenetics. Individuals, even ABMG Board-Certified ones, who do not have such a COQ in cytogenetics, are not permitted to sign chromosome analysis reports for samples from New York State residents.

International QA/QC

There are cytogenetics laboratories located in North America, South America, Central America, eastern and western Europe, Africa, Australia, and Asia. Although a comprehensive listing would not be feasible here, the following are some examples of the way quality issues are handled around the world.

Canada

Cytogenetics laboratories in Canada fall under provincial jurisdiction, and regulations vary from province to province. In some, quality control is legislated; in others, it is optional. However, many laboratories voluntarily follow in CAP guidelines and/or participate in CAP proficiency testing. There is also no uniformity in the credentials needed to be a cytogenetics laboratory director, although many are certified by the Canadian College of Medical Genetics (CCMG).

Europe

For many years, only the United Kingdom had a comprehensive, formal quality control program in place. Criteria are similar to those found in the United States. However, because the British do not prepare hard-copy karyotypes, greater emphasis is given to slide quality.

In November 1996, 24 geneticists representing 15 European countries met in Leuven, Belgium to discuss quality control and quality assurance guidelines for prenatal genetic diagnosis in Europe. This committee endorsed the formation of "pan-European external quality assessment (EQA) networks" and produced "Quality Guidelines and Standards for Genetic Laboratories/Clinics in Prenatal Diagnosis on Foetal Samples Obtained by Invasive Procedures—an attempt to establish a common European framework for quality assessment."

Since this meeting, the French have already modified their accreditation procedures under the auspices of the Ministry of Health, and formal national guidelines are under development. At this

point in time, the major difference between France and the United States is that only an MD can interpret and report cytogenetic results in France, whereas in the United States, many laboratory directors are PhDs

Requirements and guidelines for other European countries vary widely at the present time. Some have detailed policies, some use those of other countries, and some have no formal guidelines in place, although individual laboratories may.

Japan

In 2001, the Japan Clinical Reference Laboratory Association polled 442 reference laboratories and found that 33 were conducting genetic tests. However, no distinction was made between cytogenetics and molecular analysis, and so the number of laboratories actually performing chromosome analysis remains unclear. It should also be noted that since the poll was administered independently of the Japanese Society of Human Genetics (JSHG), a portion of the facilities conducting such tests may not have been included.

As of April 1, 2000, the JSHG had certified 43 facilities as cytogenetics laboratories. These include both commercial laboratories and hospital laboratories. However, the Ministry of Health and Labor, which oversees clinical testing in Japan, does not recognize clinical cytogenetics laboratories as distinct entities despite the fact that the tests themselves are so recognized. Beginning in 2004, national laboratory inspection guidelines are to include a category for cytogenetics, although the cytogenetics laboratory itself continues to go unrecognized officially as a distinct entity, in contrast, for example, to the pathology laboratory.

The JSHG has several policies that are designed to ensure that clinical cytogenetics laboratories achieve and maintain the highest possible standards. It has developed a Board that is comprised of Medical Geneticists (MDs) and "Instructors of Clinical Cytogeneticists" (PhDs). This Board has formulated requirements for training and qualifications for certified clinical cytogeneticists (technologists) and instructors, and has created an examination for certifying those individuals who have completed the prescribed requirements.

Candidates must go through a training period during which they must process at least 100 samples representing various tissue types. They must demonstrate proficiency in all phases of specimen preparation, culture and harvest, and analysis. A candidate must also be a member of the JSHG, and must be listed as an author on at least three papers.

The JSHG holds annual seminars designed to keep these certified individuals up to date, and regional seminars are also held.

Despite these recent developments, only physicians are still allowed to make clinical interpretations of test results. Many reference laboratories get around this problem by retaining the services of qualified MDs as technical advisors. The relationship between doctor and laboratory in Japan is certainly still quite different from that in the United States.

Australia

In 1979, Australia established its National Pathology Accreditation Advisory Council (NPAAC). This body makes recommendations concerning the accreditation of pathology laboratories, introduces and maintains uniform standards of practice, and formulates guidelines and educational programs relating to the performance of laboratory testing throughout the commonwealth. Based on guidelines provided by the Human Genetics Society of Australia (HGSA), the NPAAC produced guidelines for cytogenetics labs in 2001 and recommended that these be reviewed in 3 years time.

These guidelines are quite extensive, dealing with every aspect of laboratory function from staffing and staffing levels to equipment, confidentiality, laboratory, chromosome analysis and karyotyping protocols, clinical reports, and quality. There is also a section on FISH, and one on acceptable turnaround times.

In Australia, the creation of karyotypes is recommended, but not considered essential. Qualifications of individuals directing and supervising a cytogenetics laboratory were created by the HGSA in

1984, which created certification examinations in this field. Australia has its own confidentiality policy that is summarized in an NPAAC document entitled *Guidelines for Data Communication*. For more information, see www.health.gov.au/npaac/pdf/cytogen.pdf.

Related Topics

We have covered most issues involved in the generation of clinical results in the cytogenetics laboratory, which was the goal of this chapter. 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 decades past, laboratory design and protocols put the specimen first and the technologist second. Mouth pipetting was common, even with potentially toxic reagents (Giemsa stain, for example, 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 Chapter 4). 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 AIDS.

  Today's hoods feature split vertical airflow and protective glass windows. Pipetting devices are typically required, and, in the United States, MSDS (material safety data sheets) for every reagent used in the lab 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.

- *FISH*. Many of the multitudinous and ever-expanding uses of this versatile technology are employed in the clinical cytogenetics laboratory (and many, we should remember, are used in other settings). Guidelines typically involve probe validation, use of controls, and training.

- *Reference laboratories*. Not every cytogenetics lab performs every type of test on every type of sample. Some specimens require additional noncytogenetic 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.

- *Ethics policies*. Although most laboratories that perform prenatal testing consider themselves to be "pro choice" concerning a patient's right to make informed decisions, many feel compelled to contribute only clinically relevant data to such a process. Prenatal analysis for "gender selection" does not fall into this category, and such studies are, therefore, often refused by laboratories with this type of policy. 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.

- *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 of 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 are fully in effect as of 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, the requirements apply to all information,

including that in a nonelectronic form. There are three main areas of requirement. These include 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 polices 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 acknowledgement. In order to extend a clinician's patient privacy protections to third parties, they might seek to enter into a "Business Associate Agreement." However, where a geneticist is only analyzing specimens to provide treatment services, they should not need to sign such an agreement. There is a wide range of information that could be considered "individually identifiable," including names, social security numbers, geographic subdivisions smaller than a state, and so forth. Care also must be taken in the use of photographs. The HIPAA privacy rules are likely to evolve as their intent is interpreted over time. They set the floor for the protection of individual's information. About half of the states have enacted more specific genetic information privacy statutes. Consultation with local or institutional compliance officers for one's specific needs is recommended.

- *Compliance training*. Many labs, particularly those in commercial settings, find themselves subjected to an increasing number of restrictions designed to prevent kickbacks" or other potentially fraudulent finance-related practices. Although the average technologist is unlikely to be faced with decisions that may involve such regulations, training in this area is becoming common as a precaution.

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7

Instrumentation in the Cytogenetics Laboratory

Steven L. Gersen, PhD and Lotte Downey

INTRODUCTION

Ask anyone to envision a typical clinical laboratory, and a host of blinking, whirring, computer-controlled machines that analyze samples and spit out results usually comes to mind. Even traditionally labor-intensive settings, such as the cytology laboratory are frequently populated by automatic stainers, and machines that prepare and automatically analyze pap smears are becoming ever more popular.

This image does not hold up when one takes a closer look at the modern cytogenetics laboratory. Although certain procedures have been automated in recent years, most processes are still performed manually. One message that the reader should take away from this chapter, therefore, is that although technology can be utilized in any setting, the world of cytogenetics is still essentially one of manual manipulation and diagnosis.

Nevertheless, no description of the steps involved in producing a cytogenetic diagnosis would be complete without mention of the instrumentation that has been developed to assist the chromosome laboratory. Such instrumentation can help with both sample preparation 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 fluorescence *in situ* hybridization (FISH) analysis. It should be pointed out that some cytogenetics laboratories use all of these devices, most use one or two, and some do not use any.

ROBOTIC HARVESTERS

As described in Chapter 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, a process that, by its very nature, is not amenable to any form of automation. 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. Therefore, reagents are removed and added without the need to collect the cells in a tube that can be centrifuged. If the culture vessel is a Petri dish with a removable cover or a similar type of "chamber slide," the harvest process does lend itself 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 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. What is required, then, is a device that can both aspirate and dispense liquids, monitor the

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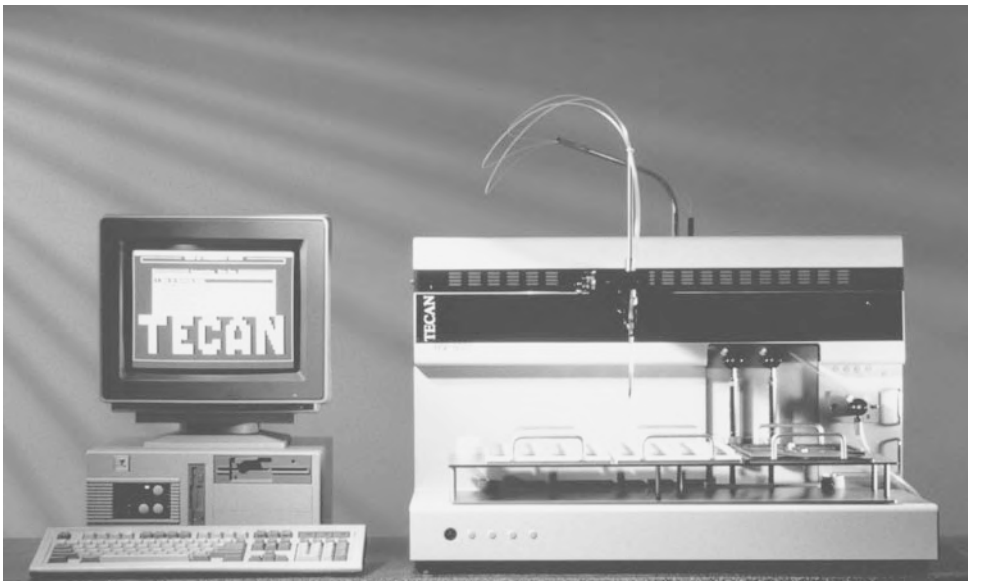

Fig. 1. Robotic harvester for processing *in situ* cultures. (Courtesy of Tecan U.S., Inc.)

timing of each step, and control these steps correctly regardless of the number of cultures being processed at any one time (i.e., some form of computer control that can be "told" how many dishes there are and where on the device they are).

Such automatic liquid-handling devices have been available for many years, and only a minor modification was required for Petri dishes to be accommodated. An example is shown in **Fig. 1**. Two of the racks that sit on the base of the robot are designed to accept 35mm Petri dishes. The arm moves along both the *x*- and *y*-axes and contains, in this case, three plastic tubes: one for aspiration, one for dispensing the hypotonic solution, and one for dispensing the fixative. The incubation times for each step are programmed into a computer that controls the robot, as is the number of dishes and their locations on the racks. After Colcemid treatment, the dishes are placed on the harvester. The robot will then aspirate the culture medium, add hypotonic solution, and proceed to the next dish. The process continues until all dishes are filled with hypotonic solution. After the first dish has incubated for the proper amount of time, the hypotonic solution is aspirated and fixative is added; some protocols call for addition of a small amount of a "prefix" first. It should be pointed out that the computer program will not accept more dishes than it can process without perturbing the timing of these steps. The end result is culture dishes that contain fixative, which must then be removed in order to properly spread the chromosomes.

A different approach would be to design an instrument specifically for this purpose, rather than modifying one that had already existed. Such a device is now available. In addition to being built specifically for cytogenetics laboratories, this 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. (See **Fig. 2**.)

A robotic processor has also been modified to facilitate automation of the blood/bone marrow harvest procedure. At the present time, this device is only available in Europe.

DRYING CHAMBERS

Again, as described in Chapter 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

Fig. 2. 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 on-board programming, which eliminates the need for an external computer, reducing bench space requirements. (Courtesy of Genial Genetic Solutions.)

one or more drops of this suspension on a number of microscope slides, and it 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 the case with *in situ* harvesting. Most cytogenetics laboratories initiate four to six cultures from each sample, 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; in many cases, three 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 usable metaphases. The concept of discarding one and trying again, as is so often done 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*.

Fig. 3. Benchtop drying chamber. (Courtesy of Percival Scientific, Inc.)

As the 3:1 methanol:acetic acid fixative used in cytogenetics laboratories dries, it "pulls" the cell membrane across the slide or cover slip 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 airflow over the cells (and possibly, as suggested by some studies, the barometric pressure) all affect the rate of drying; therefore, when utilizing *in situ* processing, controlling these parameters is the only way to control chromosome spreading *(1)*.

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.

There are probably as many solutions to this situation as there are cytogenetics laboratories. Some have constructed enclosed chambers in which airflow, humidity, and temperature can be varied, although these are typically prone to failure whenever the air conditioning breaks, because it is easy to warm the air inside the chamber but extremely difficult to cool it. Some labs have designed climate-controlled rooms; these frequently function well, but the drawbacks here are 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 conditions. Such rooms are also often costly to build.

Recently, several companies have developed self-contained chambers specifically for the purpose of drying *in situ* cultures; an example is shown in **Fig. 3**. 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

Fig. 4. Floor model drying chamber. (Courtesy of Thermotron Industries.)

as heating), this chamber has been modified to control humidity as well, and fans have been installed to allow for control of airflow over the cover slips. 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. 4**. Here, the entire drying process, including aspiration of fixative, can take place inside the chamber. The technologist sits at the unit and manipulates the processing with a glove-box approach. 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.

These condition-controlled chambers are gaining in popularity in cytogenetics laboratories, and some use them not only for *in situ* processing but also for routine slide making because of the consistency they provide.

INSTRUMENTATION FOR FISH

Although FISH (see Chapter 17) represents one of the most exciting and clinically significant developments of the last decade, 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 enormous increase in FISH sample volume most cytogenetics laboratories are experiencing, any device that can reduce the labor component of the process becomes indispensable.

Fig. 5. VP 2000 processor. The device automates various laboratory protocols, such as a pretreatment or deparaffinization step prior to performing a FISH assay. (Courtesy of Vysis, Inc.)

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. However, newer applications of the technology (e.g., *HER2* analysis for breast cancer or ploidy analysis for bladder cancer recurrence; see Chapter 17) utilize specimen types not routinely handled in the cytogenetics laboratory, 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. Although not difficult or complicated, these procedures are repetitive and time-consuming. Fortunately for the laboratory, devices that automate such steps have been developed (see **Fig. 5**). These devices also offer the laboratory the flexibility of performing other FISH pretreatment 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

Fig. 6. HYBrite denaturation/hybridization 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. (Courtesy of Vysis, Inc.)

a modest number of slides at one time to facilitate volume testing, and they can store several user-defined programs for analytical flexibility. (See **Fig. 6**.)

The drawback to these devices is the large volume or frequent use of probes that require different programming, necessitating the purchase of more than one unit. They have, however, come down in price in recent years.

AUTOMATED IMAGING SYSTEMS

Introduction

The traditional method of imaging chromosomes has always been photomicrography. A photograph of metaphase chromosomes is taken, the film is developed and photographs are printed in a darkroom, and the chromosomes are cut out and arranged to form a karyotype. Although a standard technique for a long time, this process increases the already time-consuming nature of clinical cytogenetics. Because of increasing workload in cytogenetics laboratories around the world, automated imaging is increasing in popularity.

Automated imaging systems dramatically reduce the time it takes to produce a karyotype, 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 FISH, interphase FISH, and comparative genomic hybridization (CGH) can also be attributed to automated imaging. (See Chapter 17.)

Currently, the primary application of an imaging system in a cytogenetics laboratory is still the production of karyotypes, either from brightfield (G-band) or fluorescence (Q-band or R-band) images, although the use of automated imaging systems in FISH (painting probes, single-locus probes, multicolor FISH, CGH, etc.) is rapidly gaining popularity. Rare event detection (e.g., automated

metaphase finding or fluorescent spot counting) also represents a growing application for imaging systems in cytogenetics.

Benefits

Reduction in the time it takes to complete an analysis is unquestionably the major benefit of an automated imaging system. Laboratories can save operator time by automating metaphase scanning, karyotyping, and FISH applications, 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. Some states require storage of patient cases for up to 100 years! With current compression technologies and digital storage devices, this is easier and less space-consuming than with photographs. In addition, photographs can deteriorate over time, making them harder to re-examine if necessary.

Automated imaging systems also provide consistency, especially when performing interphase FISH assays. Whereas manual spot counting can be highly subjective and error-prone, an automated system will use predefined parameters for spot counting and, using those parameters, will produce consistent results.

Sharing of data is important in a clinical lab setting and is clearly facilitated by the use of digital images versus traditional photography. With the growing use of the Internet and electronic mail, digital images are more easily shared for consultation and discussion (*2*). However, with data sharing via the Internet comes the need for compression, and a more pressing need for patient record security. Partly to address this need for patient record security, the US Congress recently passed the Health Insurance Portability and Accountability Act (HIPAA) (*3*). See also Chapter 6.

Although traditional photographic techniques offer some degree of contrast and other image adjustment, automated imaging systems further offer easy image enhancements, visualization techniques, and quantification, providing additional information. As stated earlier, recent advances in cytogenetic applications, especially in FISH applications such as M-FISH, CGH, and interphase FISH can be attributed to imaging systems.

Limitations

Of course, there are limitations to automation in the cytogenetics laboratory. Probably the greatest limitation is the interpretation of the karyotype or FISH results and the diagnosis based on the analysis of the image. This will still be a task for the Director. Another limitation is that despite image enhancement features, the quality of the final image is still dependent on the quality of the original microscope image (*2*). An image might be improved through background elimination, contrast, and color enhancement or even longer exposure times, but all of these will not make up for a poor image resulting from poor microscope configuration or slide preparation.

Imaging System Components

In general, an imaging system for cytogenetics contains the following components: a microscope with camera adapter, a camera, computer and software, a printer, and an archival device. (See **Fig. 7**.)

Microscope with Camera Adapter

A detailed discussion of microscopes and microscopy can be found in Chapter 5. As the name already implies, the camera adapter 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.

Camera

Although a wide range of 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 (*4*).

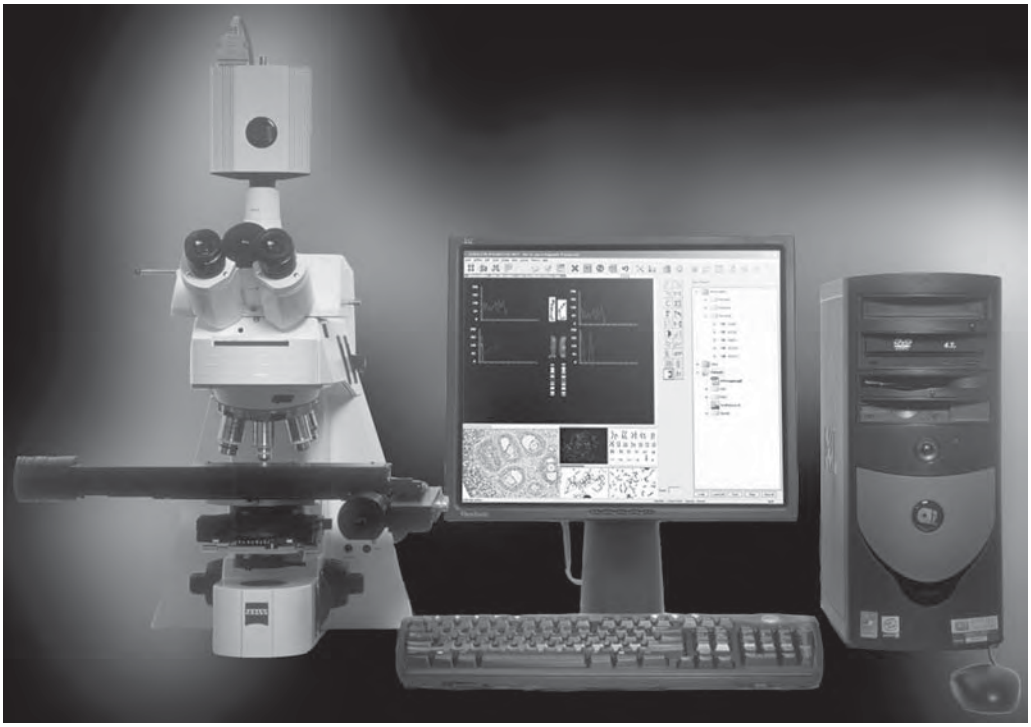

Fig. 7. CytoVision automated chromosome imaging system. (Courtesy of Applied Imaging.)

Computer

Although 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.

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, and shading correction. After image capture and enhancement, the user can analyze the image using the analysis applications of the software.

Although 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 karyotypes and the automated scoring of interphase FISH slides.

Printer

A high-quality print of the image is still important in the cytogenetics laboratory. Although the trend might be moving to a so-called paperless laboratory, a hard-copy print is often needed to for diagnostic and/or archival purposes. In addition to the high-resolution black and white images of karyotypes prepared by all cytogenetics laboratories, a printer used for FISH applications must be capable of reproducing the range of colors generated by modern FISH software.

Archival Device

As mentioned earlier, there is a need (often legally imposed) for long-term archiving of patient data. With the use of automated imaging systems, the data are in digital form and are easier to store. There are three basic categories of archival devices for digital data: tape, optical disk, and magnetic disk. Which type of archival device is best depends on several factors, including the expected volume of data to store, the duration of storage, and how often the data need to be accessed in the future. Currently, DVDs are used more and more as the storage device of choice.

Cytogenetics Applications of Automated Imaging

Karyotyping

The predominant application of automated imaging systems is karyotyping. Karyotyping involves separating and classifying the chromosomes based on the length of the chromosome, location of the centromere, and the banding pattern (see Chapter 3). Automated systems for karyotyping need to provide at least the following benefits to the user: ease of use, speed, image quality, and accuracy (*4*).

Less automated systems require the user to "cut out" the chromosomes using the mouse and then to place them into a karyotype. In semiautomated systems, the system will "cut out" the chromosomes, and the user classifies them into a karyotype. On the other hand, a fully automated imaging system will capture the metaphase chromosomes (either brightfield for G-banding or fluorescent for Q-banding), separate or "cut out" the chromosomes, classify them, and arrange them in a karyotype (see **Fig. 8**). However, some metaphases contain very complex clusters of overlapping chromosomes, and the user might still need to intervene and manually separate the chromosomes using the mouse.

A fully automated karyotyping system can also be used in conjunction with a so-called metaphase finding capability. This means that the system will automatically scan the slide in search of good metaphase spread that can be used for karyotyping. This application will be discussed later in this chapter.

From a software perspective, automated karyotyping systems need to include the following capabilities:

- *Separation of chromosomes*. Chromosomes in a metaphase might be touching or overlapping, and the software will not be able to classify the individual chromosomes until they are separated. Cytogenetics software will include features such as "split," "overlap" and "draw axis" to allow for the segmentation of such chromosomes.
- *Automatic classification of the chromosomes into a karyotype*. Using pattern recognition, the system will assign the classification of each chromosome based on length of the chromosome, location of the centromere, and the banding pattern. However, in cases where there are chromosomal abnormalities, the system might not recognize a chromosome, so then the user can assign or change the classification.
- *Image enhancement to facilitate the interpretation of the banding pattern*. Image enhancement features include the ability to change the contrast and brightness to bring out the banding pattern.

Scanning and Metaphase Finding

Finding metaphases acceptable for analysis is an integral part of cytogenetics. In normal samples, good quality metaphases are abundant. However, in some samples, 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. Although 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

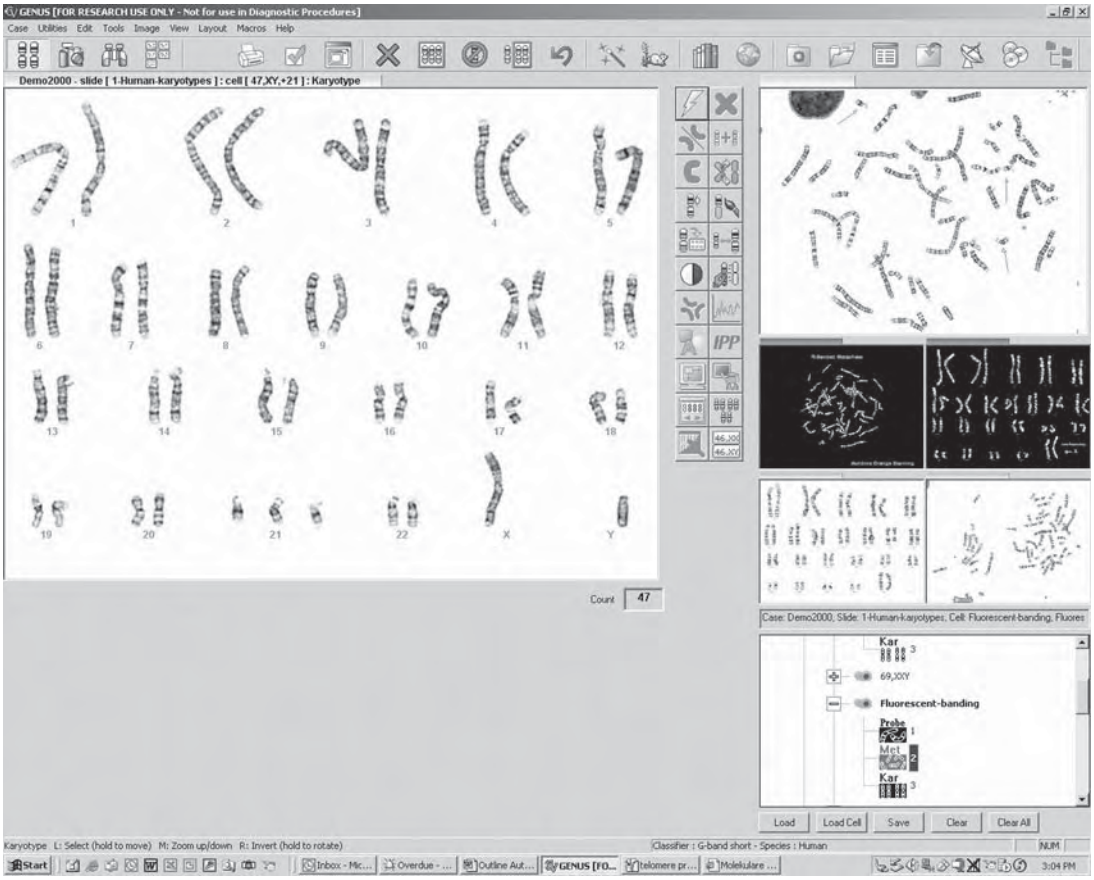

Fig. 8. An example of karyotyping software. The original metaphase is in the upper right. (Courtesy of Applied Imaging.)

(see **Fig. 9**). Based on several parameters, the system images metaphase spreads (fluorescent or brightfield) and presents them to the user for review and analysis (see **Fig. 10**).

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).

Software features important for metaphase finding include the following:

- Definition of rare event classification parameters to ensure optimum scan results. The user can define the parameters that are utilized by the system to identify the rare event.
- Ability to quickly relocate to a metaphase or rare cell for review.
- Sort function to organize metaphases or cells after scanning based on specific parameters.

Because of the general nature of the scanning system, it can also be used in other applications that require scanning for particular cells (rare events), such as FISH spot counting (see below) for detection of tumor cells in body fluids.

Laboratories are using scanning systems more and more for streamlining their workflows. The systems are set up to continuously scan slides for metaphases or rare events while technologists are analyzing the detected metaphases or cells on remote review stations. This increases throughput while using the technologists' time where it is most valuable: analyzing cases.

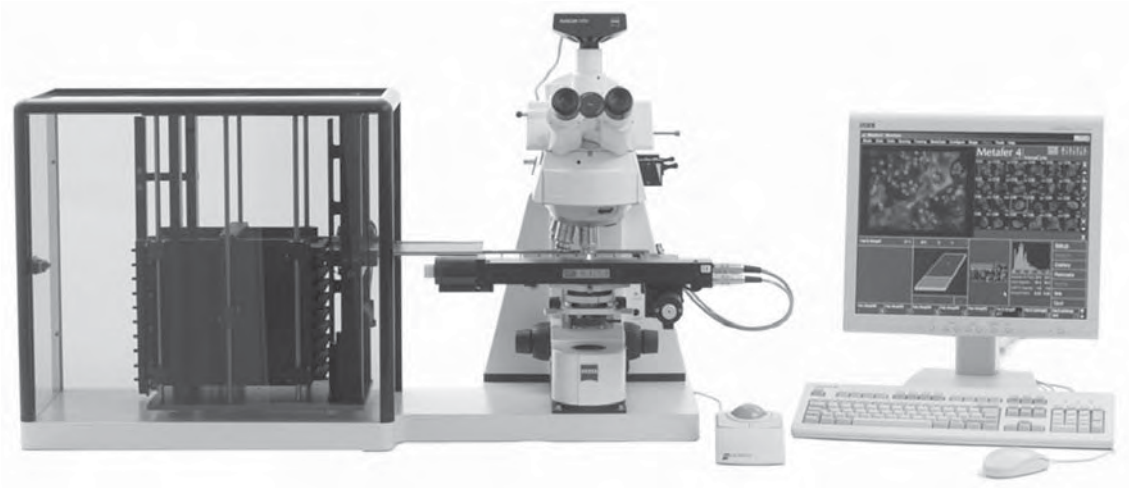

Fig. 9. Automated slide scanning and metaphase finding system. (Courtesy of MetsSystems Group, Inc.)

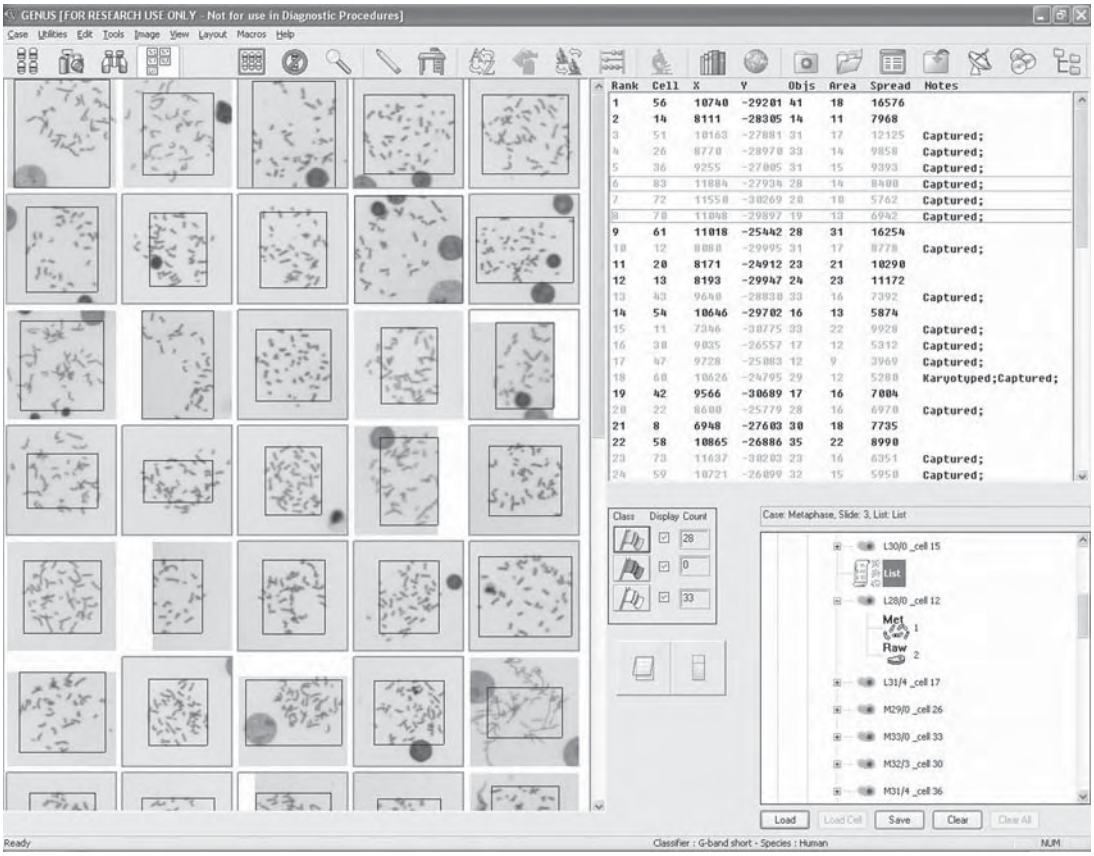

Fig. 10. Software interface of a metaphase finding system, showing thumbnails of the metaphase spreads located by the system. (Courtesy of Applied Imaging.)

FISH and Fluorescent Spot Counting

Fluorescence *in situ* hybridization is based on fluorescently labeled probes that hybridize to unique DNA sequences along the chromosomes. There are many different applications of FISH; see Chapter 17 for more detail on this technology.

Fluorescence *in situ* hybridization can be performed on either metaphase preparations or interphase cells. One of the applications is fluorescent spot counting used for translocation and copy number analysis performed on interphase cells (see **Fig. 11**). An example of an interphase FISH kit is the Vysis UroVysion® kit for the detection of chromosomal abnormalities associated with the recurrence and progression of bladder cancer (see Chapter 17, Fig. 14).

Generally, an imaging system for FISH needs to be able to capture low-light-level images, quantify the number of each fluorescent signal, and estimate the intensity ratio of the different signals.

Because interphase cells are three-dimensional (3-D) structures, the fluorescent signals in interphase FISH and spot counting 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, 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 Chapter 5, Fig. 3). 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 (*5,6*). (See **Fig. 12**.)

The microscope focus, camera, and filter wheel are 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.

To ensure consistent scoring and analysis of interphase FISH, the software should include the following:

- 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 dimensional) and the number of cells to score.
- The ability to reprocess the images under different scoring rules without having to rescan the slide.
- A reporting function that presents the results for review by the clinician. 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.

M-FISH

M-FISH, also referred to as multicolor FISH or multiplex FISH, can be viewed as fluorescent multicolor karyotyping and is mainly used for the detection and classification of interchromosomal aberrations (see Chapter 17). In this form of FISH, probes labeled with a combination of different fluors are hybridized with the chromosomes in a metaphase spread. Currently, 5 different fluorochromes are being used (*7*). The five different fluors give 31 (2^n –1) color combinations, enough to uniquely identify the 24 different chromosomes in the human genome. However, lately it has been shown that the resolution of using a five-fluorochrome set is not high enough, and certain small aberrations might be missed (*8*). To improve resolution, a set of eight fluorochromes, to facilitate labeling each probe with a unique combination of two fluors, has been suggested. However, this would require nine filters (eight for the fluors and one for the DAPI counterstain) and would involve

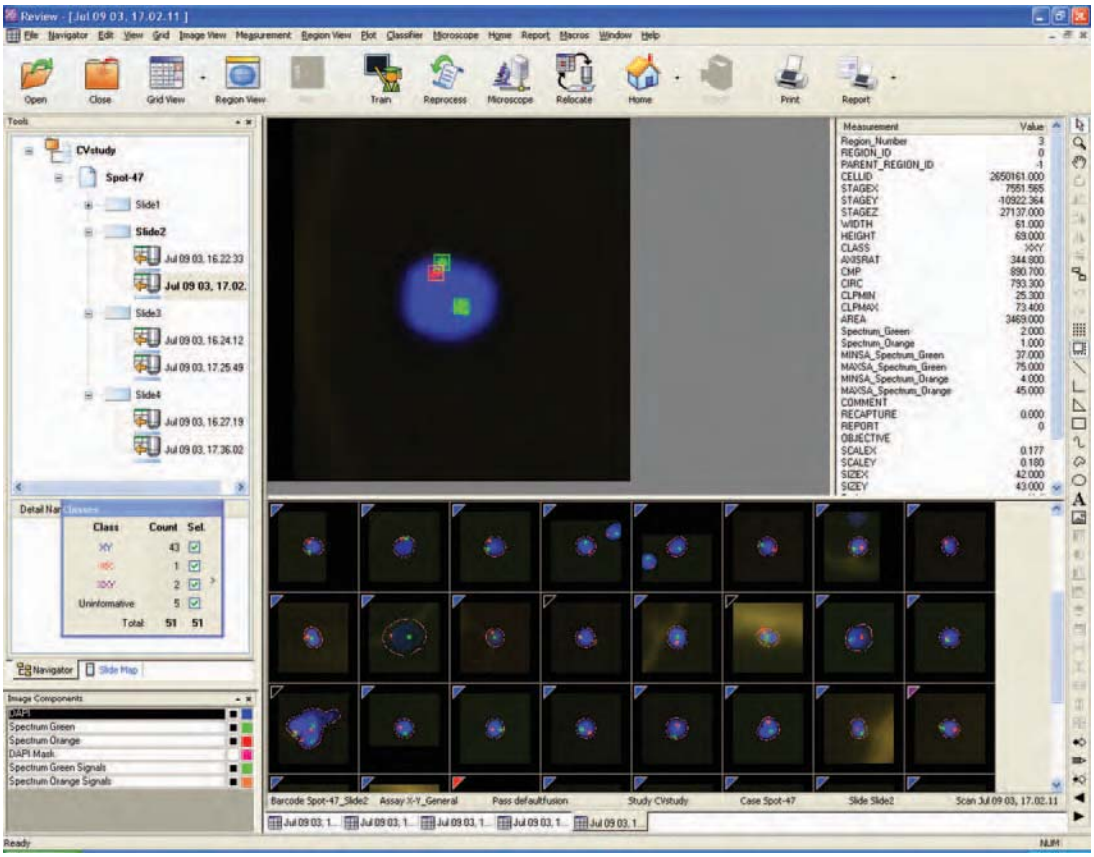

Fig 11. Software interface of a spot counting or interphase FISH system, showing thumbnails of cells and spots located by the system. (Courtesy of Applied Imaging.)

some manual filter changing, as microscopes currently only accommodate eight-position filter wheels. Therefore, experiments with a seven-fluorochrome set have been performed, with promising results (*8*).

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 (see **Fig. 13**) incorporates the following:

- Sophisticated algorithms that analyze the images to determine the fluor combination a chromosome is labeled with and then assign a pseudocolor to each fluor combination. These pseudocolors should be user-changeable to improve visualization of rearranged chromosomes.
- Karyotyping capabilities so that the colored chromosomes can be arranged in a karyotype (see Chapter 17, Fig. 17).
- Individual pseudocolor display of a single chromosome to facilitate visualization of chromosomal aberrations.

High-Resolution Comparative Genomic Hybridization and Microarray CGH

The last forms of FISH discussed here are HR-CGH (high-resolution comparative genomic hybridization), and microarray CGH. Whereas M-FISH is a useful technique for determining inter-

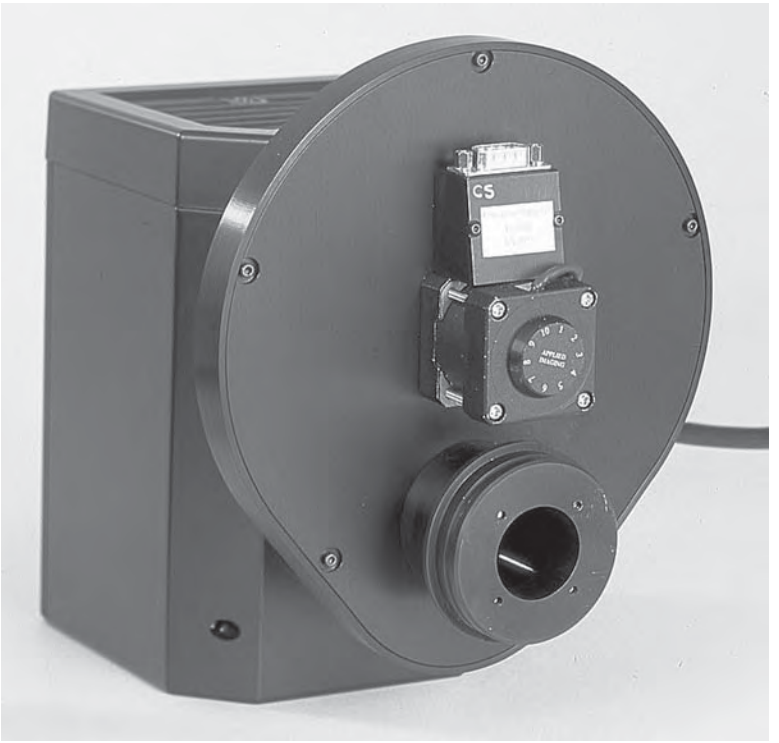

Fig 12. Computer-controlled automated filter wheel. (Courtesy of Applied Imaging.)

chromosomal rearrangements, CGH will give insight in losses or gains of DNA within a chromosome (see Chapter 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 with normal metaphases. As the name indicates, the two probe sets will compete for hybridization to the corresponding loci. The ratio of the of patient DNA to 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.

Until recent, this technique was able to pick up additions and deletions in the order of 10 megabase pairs (Mbp). However, current work has increased the resolution to the order of 3 Mbp (*9*).

Comparative genomic hybridization requires the use of a high-quality and quantitative FISH imaging system with a dedicated CGH suite. This software suite will perform the following:

- 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 Chapter 17, Fig. 15).

With microarray CGH, specific DNA targets are "printed" onto a microscope slide and CGH is performed *in situ* on the slide (see Chapter 17). A scanner reads the slide and sends the data to a computer for analysis (see **Fig. 14**).

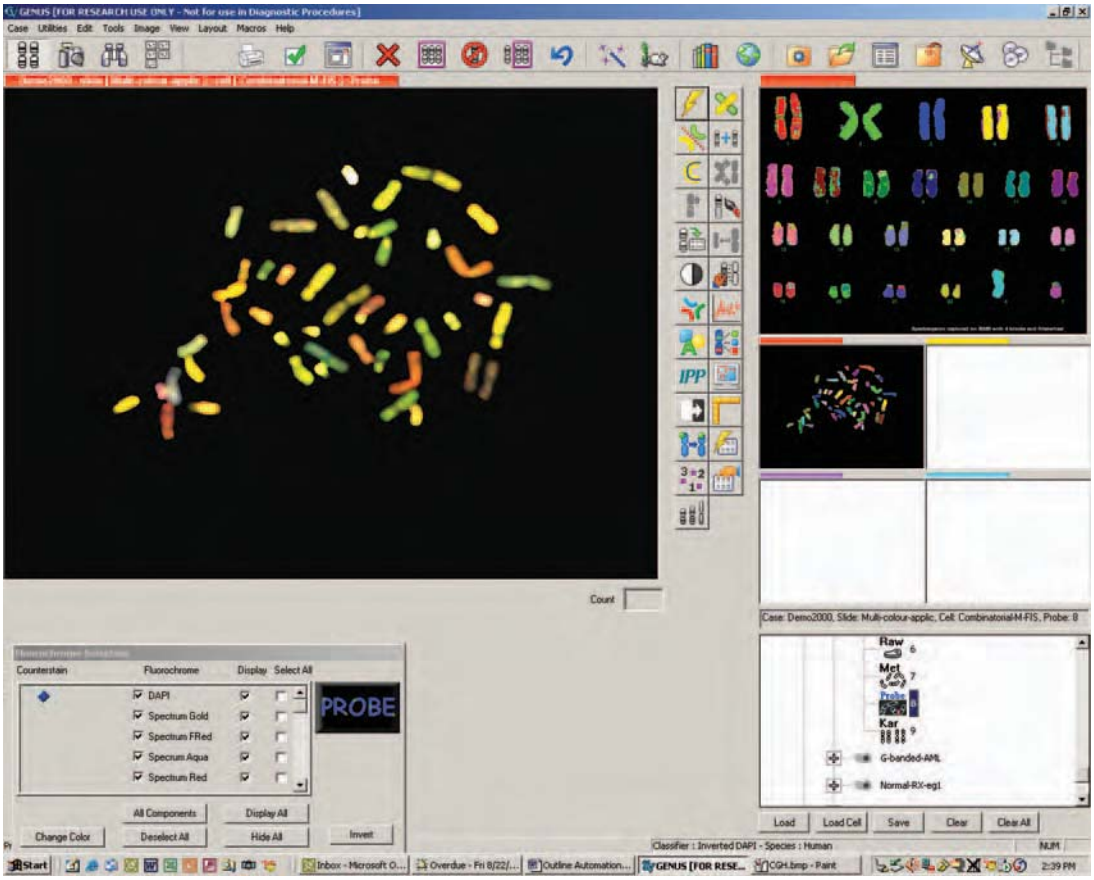

Fig. 13. M-FISH capture and analysis. The software analyzes the signals produced by the various combinations of fluors, produces a false color for each chromosome, and arranges the chromosomes into a karyotype. See text for details. (Courtesy of Applied Imaging.)

Discussion

As discussed, automated imaging and metaphase finding systems can be used in many applications in a cytogenetics laboratory. More significantly, some cytogenetic applications, such as HR-CGH, array CGH, and M-FISH, would hardly be possible if there were no automated imaging systems available.

Whereas such automation currently affects most of the possible applications in a cytogenetics laboratory, the next area automated systems are bound to impact is laboratory workflow, especially in large, high-volume laboratories. Scanning systems are used to locate and capture cells, whereas technologists analyze and review them on remote stations. This more efficient workflow will increase throughput and improve turnaround time while reducing cost.

SUMMARY

All laboratory procedures were essentially manual at one time. Primitive centrifuges were hand operated, and even the earliest microscopes (examples of "new technology" in their own right) utilized 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

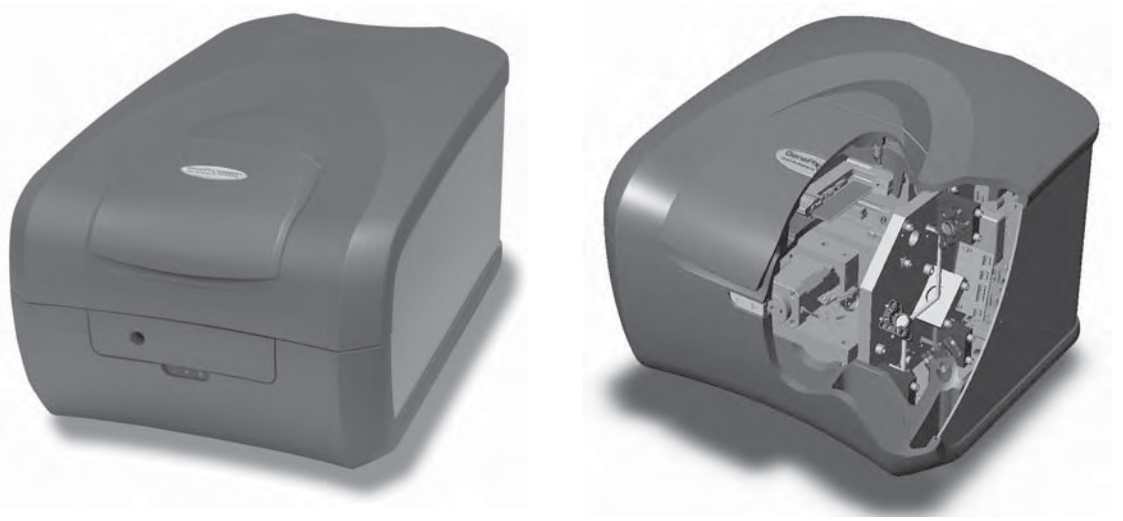

Fig. 14. GenePix microarray scanner. This benchtop unit features an eight-position emission filter wheel to allow the user flexibility in choosing fluorescent dyes. The image on the right shows a schematic of the light path through the scanner. (Courtesy of Axon Instruments, Inc.)

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 (and sometimes accuracy) at that tasks can be performed
- Performance of tasks which cannot be accomplished manually

Nevertheless, the world of cytogenetics is still essentially one of manual manipulation and diagnosis. We have, however, seen that there are notable exceptions, assisting with both sample preparation and chromosome analysis. These fall into several basic categories: robotic harvesters, environmentally controlled drying chambers, automation of certain aspects of the FISH procedure, and computerized imaging systems. We have further seen how the latter represents the single most significant example of automation in the cytogenetics laboratory. The major benefits of a computerized imaging system are a reduction in the amount of time required to complete each standard analysis and the ability to perform some FISH analyses (e.g., M-FISH) that require a computer. Whether automatically locating suitable metaphases, automating the karyotyping process, enabling the use of low-light fluorescence techniques, or eliminating the need for photomicrography and darkroom processing, computerized imaging systems can save valuable operator time.

As technology continues to advance, there seems little doubt that most of the manual tasks required for chromosome analysis today will eventually be automated.

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III Clinical Cytogenetics

8
Autosomal Aneuploidy

Jin-Chen C. Wang, MD

INTRODUCTION

The term *aneuploidy* refers to cytogenetic abnormalities in which all or part of one or more chromosomes is added 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 can 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 below and covered in detail in Chapter 12.

Cytogenetic studies of human oöcytes and sperm reveal that the overall frequency of abnormalities is approximately 20% and 10%, respectively (reviewed in ref. *5)*. More than 90% of the abnormalities observed in oöcytes and less than 50% of those seen in sperm are numerical. Studies using fluorescence *in situ* hybridization (FISH) *(6)* or primed *in situ* labeling (PRINS) *(7,8)*, 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, and 21), with a range of 0.26–0.34%. On the other hand, one study using FISH *(9)* 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, and 20). It is therefore possible that meiotic nondisjunction is random for all autosomes, with the possible exception of chromosome 21. However, this is not the case for aneuploidy actually observed in spontaneous abortuses or liveborns.

Trisomies for all autosomes have been reported in spontaneous abortuses *(3,10,11)*, including trisomy 1, which has been reported in a clinically recognized pregnancy at 8–9 weeks of gestation *(11)* and in a clinically recognized in vitro fertilization (IVF) pregnancy at 6 weeks of gestation *(12)*. However, no fetal pole ever developed in either case. The observed frequency of each trisomy, however, varies greatly. 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 pre-embryonic stage and thus result in unrecognized and, therefore, unstudied spontaneous abortions. The lethality of a particular autosomal aneuploidy correlates with the gene content of the chromosome involved *(10)*. Aneuploidies for

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"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.

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 *(2,13–26)*. 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 and 18, nondisjunction in maternal meiosis stage I accounts for the majority of cases (see **Table 1**).

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 *(27)*. Why aneuploidy is maternal-age-dependent and what constitutes the mechanism and etiology of chromosomal nondisjunction have been topics of much research, as summarized below.

Nondisjunction can occur during either meiosis I (MI) or meiosis II (MII). In MI, homologous chromosomes pair and form bivalents (see Chapter 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 (see **Fig. 1d,e**). This mechanism, as shown by Angell, can be a very rare occurrence *(28)*. 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 *(29)* (see **Fig. 2d,e**). In MII, sister chromatids separate. Malsegregation occurs when both chromatids go to the same pole (see **Fig. 3g,h**). Cytogenetic studies of oöcytes, performed mostly on unfertilized or uncleaved specimens obtained from IVF programs, have provided conflicting results regarding whether the frequency of aneuploidy actually increases with maternal age *(30–33)*. However, a FISH study of human oöcytes using corresponding polar bodies as internal controls demonstrated that nondisjunction of bivalent chromosomes (MI error) does increase with maternal age *(34)*, and a recent study using multiplex FISH on fresh, noninseminated oöcytes also indicated an increase in premature separation of the sister chromatids in MI with increasing maternal age *(35)*. More data are needed before a firm conclusion can be drawn. If confirmed, these latter studies will provide direct evidence that a maternal-age-dependent increase in the frequency of MI errors is the basis for the observation that the risk of having a trisomic offspring is greater in older women. In contrast, the "relaxed selection" hypothesis assumes that older women are less likely to spontaneously abort trisomic conceptions *(36,37)*. However, data obtained from fetal death *(38)* and from comparison of frequencies of trisomy 21 between the time of chorionic villus sampling and the time of amniocentesis *(39)* suggest that selective miscarriage is actually enhanced with increasing maternal age.

If maternal MI nondisjunction does increase in older women, what then causes this? Different mechanisms have been proposed. One example is the "production line" hypothesis *(40)*. This theory proposes that oöcytes mature in adult life in the same order as the corresponding oögonia entered meiosis in fetal life. Oögonia that enter meiosis later in development might be more defective in the formation of chiasmata and, thus, more likely to undergo nondisjunction. The first direct cytological support for this hypothesis was provided by a study that examined the frequency of unpaired homologs in MI pachytene and diplotene in oöcytes obtained from abortuses at 13–24 weeks and 32–41 weeks of gestation *(41)*. 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

Table 1
Parental and Meiotic/Mitotic Origin of Autosomal Trisomies Determined by Molecular Studies (Number of Cases)

	Maternal					Paternal					
Trisomy	MI	MII	MI or MII	Mitotic	Total[a]	MI	MII	MI or MII	Mitotic	Total[a]	Reference
2	4	1	6	1	13	5				5	*26*
7	2	3	1	6	12				2	2	
13	4		17		21	1	1	1		3	*20*
14	3	4	2		9		2			2	*20*
15	21	3		3	27				5	5	*16* (UPD study)
	10				10				2	2	*18* (UPD study)
	17	2	10		29		4	1		5	*20,26*
16	56		6		62					0	*21*
18	11	17			56		1			6	*24*
					17					5	*17*
	16	>35		3	61				2	2	*22*
21	9	1			22					3	*13*
					91					6	*14*
	128	38			188	2	7			9	*15*
						7	15		8	36	*19* (paternal study only)
	174	58	79		311	9	15	8		32	*20*
	62				81		10			13	*23*
	67	22			97	4	4			10	*25*
22	20	1	15		37			1		1	*20,26*

[a]Total numbers might not add up because not all origins of error can be determined.

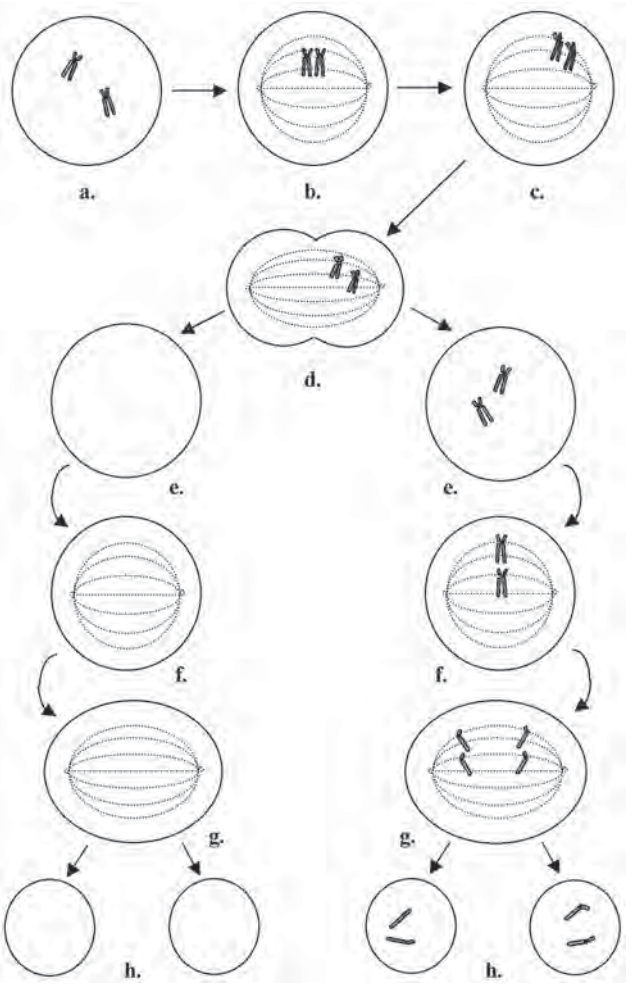

Fig. 1. Schematic representation of meiosis I nondisjunction: **(a)** prophase I, **(b)** metaphase I, **(c)** anaphase I, **(d)** telophase I, with both homologs of one chromosome pair segregating together, **(e)** products of meiosis I, **(f)** metaphase II, **(g)** anaphase II, **(h)** meiotic products—two gametes lack one chromosome and two gametes contain two copies of one chromosome.

(1.3–5.5%). Further studies are needed to substantiate this observation. Another example is the "limited oöcyte pool" model *(38)*. 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 oöcyte that is not at the optimal stage will be selected for ovulation. If such "less optimal" oöcytes are more likely to undergo MI nondisjunction, then the ovulated oöcytes of older women will have higher rates of aneuploidy. Recent data, however, do not appear to support this hypothesis *(42)*.

One probable factor that predisposes gametes to nondisjunction is aberrant recombination (see Chapter 2, reviewed in ref. *43)*. Data on recombination patterns are available for trisomies 15, 16, 18, and 21. Studies of chromosome 15 nondisjunction in uniparental disomy (see Chapter 19) revealed that there was a mild reduction in recombination in association with maternal nondisjunction, with an

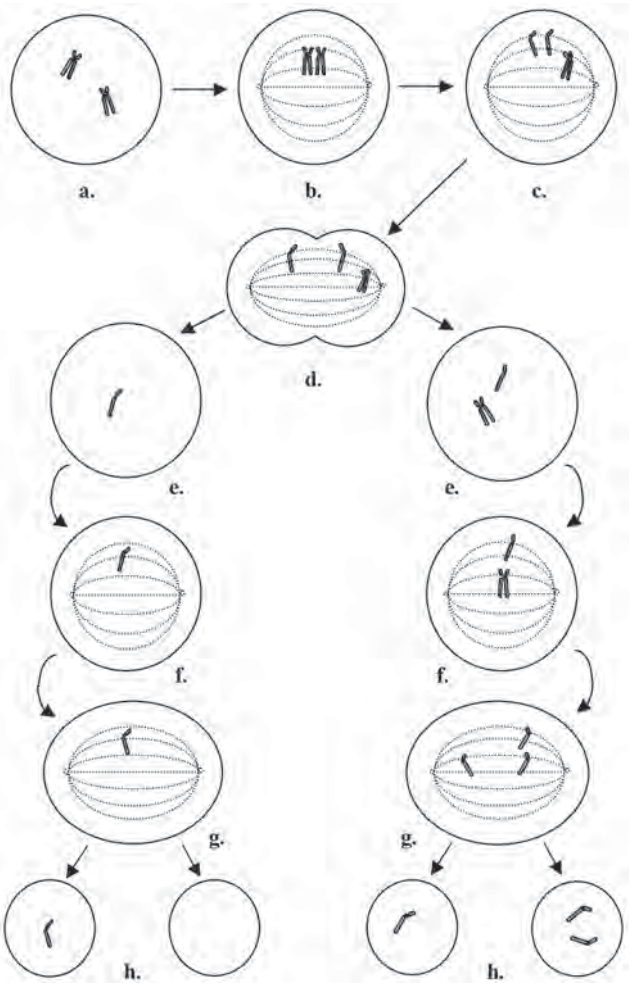

Fig. 2. Schematic representation of meiosis I error resulting from premature sister chromatid separation: **(a)** prophase I, **(b)** metaphase I, **(c)** anaphase I, with premature separation of centromere of one chromosome, **(d)** telophase I, with one prematurely separated chromatid segregating with 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.

excess of cases that have zero or one crossover and a deficiency of cases that have multiple crossovers *(16)*. 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 *(22)*. 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 *(21)*. In trisomy 21, there was an overall reduction in recombination with an increase in both zero and one crossover in maternal MI nondisjunction *(44)*. Lamb et al. *(45)* 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. When at least one crossover was present, it occurred largely at distal 21q. This

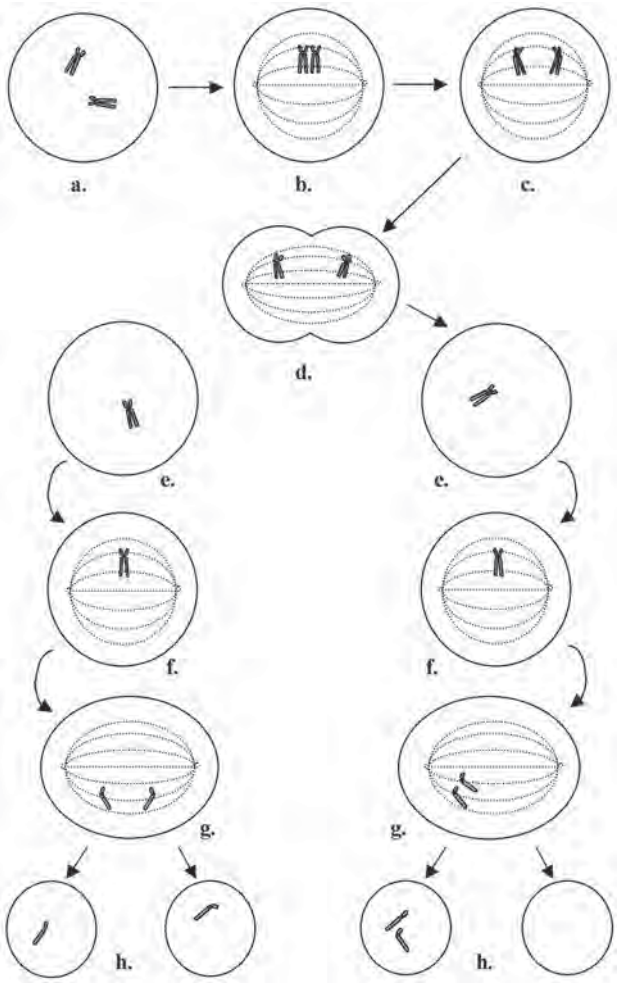

Fig. 3. Schematic representation of meiosis II nondisjunction: **(a)** prophase I, **(b)** metaphase I, **(c)** anaphase I, **(d)** telophase I, **(e)** products of meiosis I, **(f)** metaphase II, **(g)** anaphase II, with both sister chromatids segregating together, **(h)** meiotic products—two gametes with a normal chromosome complement, one gamete lacking one chromosome, and one gamete containing two copies of one chromosome.

study, together with one on trisomy 16 *(21)*, suggests that, at least for trisomies 16 and 21, distal chiasmata are less efficient in preventing nondisjunction in MI. In contrast, in maternal MII nondisjunction, the number of recombination events appeared to be increased, especially in proximal 21q. These proximal recombinations could 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 could be initiated during MI. Lamb et al. showed that the alteration in recombination pattern was not maternal age dependent. They proposed a "two-hit" model *(45,46)* and hypothesized that certain recombination configurations are less likely to be processed properly in older women. 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 *(47)*. The same argument was used by Hassold et al. to explain their findings with trisomy 16 *(21)*.

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 *(48)*. 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 might be genetically determined.

Our understanding of the mechanism and etiology of nondisjunction is not complete. It is possible that more than one mechanism contributes to the observed maternal age effect. Thus, the "two-hit," "limited oöcyte pool," and "production line" models, along with other hypothetical explanations, could explain a portion of the cases involving some chromosomes. Further studies are needed.

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] (see **Fig. 4**) was the first chromosomal abnormality described in humans *(49)*. The phenotype was delineated by John Langdon Down (1828–1896) in 1866 and is referred to today as Down syndrome *(50)*. 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 *(51)*. This finding was consistent with earlier reports showing an excess of males among trisomy 21 conceptuses that resulted from paternal meiotic errors *(19)*. 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 Chapter 9) comprise the remaining 5%. As described previously, the incidence of trisomy 21 in newborns is closely associated with maternal age (see **Table 2**).

Phenotype

The clinical phenotype of Down syndrome has been well described *(54,55)*. 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, overfolded ears. Hands and feet are small and might demonstrate clinodactyly, hypoplasia of the midphalanx of the fifth finger, single palmar crease (see **Fig. 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 (VSDs), patent ductus arteriosus (PDA), and auricular septal defects (ASDs). 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 has been observed in

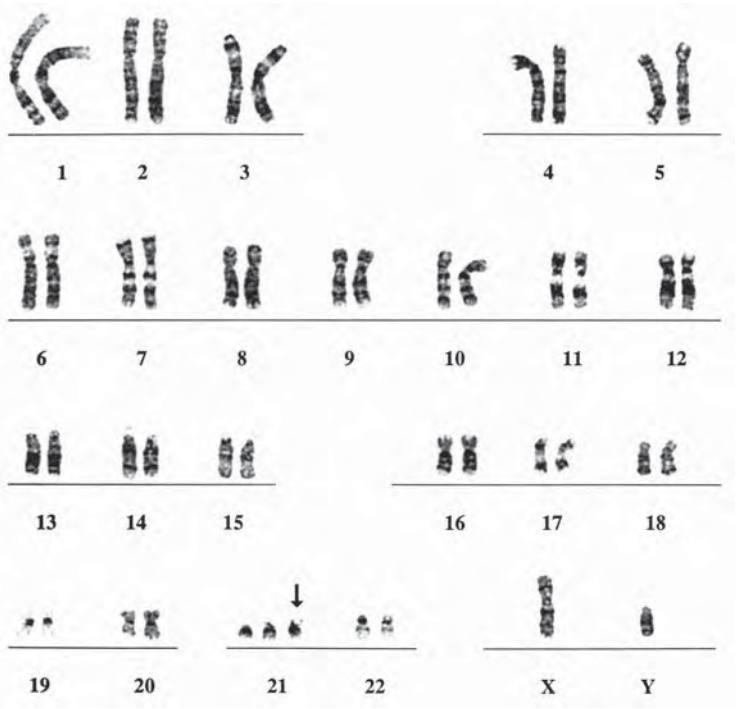

Fig. 4. Trisomy 21 Down syndrome male karyotype (47,XY,+21).

Table 2
Maternal Age-Specific Risks for Trisomy 21 at Birth

Maternal age (Years)	Incidence at bbirth (1 in)	Maternal age (Years)	Incidence at birth (1 in)
15	1580	33	570
16	1570	34	470
17	1565	35	385
18	1555	36	307
19	1540	37	242
20	1530	38	189
21	1510	39	146
22	1480	40	112
23	1450	41	85
24	1400	42	65
25	1350	43	49
26	1290	44	37
27	1210	45	28
28	1120	46	21
29	1020	47	15
30	910	48	11
31	800	49	8
32	680	50	6

Note: Data were based on eight pooled studies. Restriction of analysis to two studies with the most complete ascertainment yielded higher rates *(53)*.

Source: Modified from ref. *52*.

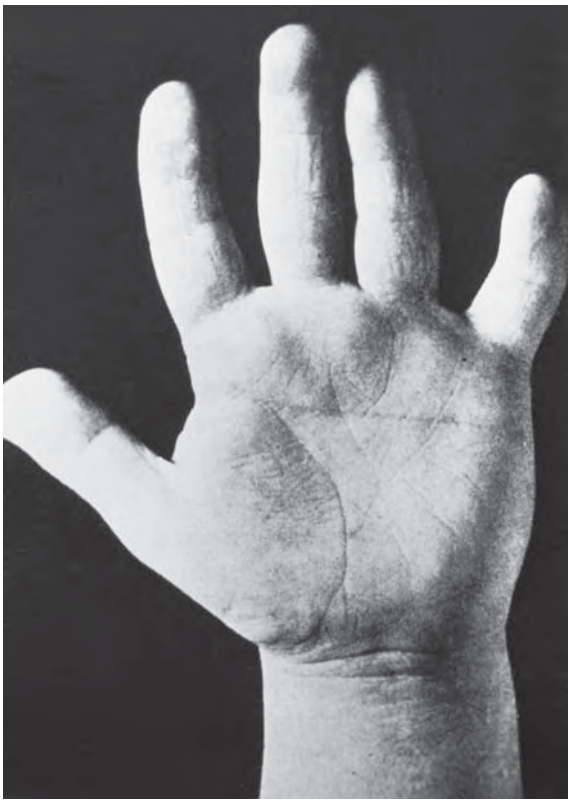

Fig. 5. The hand of a Down syndrome child showing small hand, clinodactyly, only one crease in the fifth finger, and single palmar crease.

Down syndrome patients of all ages, with a bimodal age of onset in the newborn period and again at 3–6 years *(56)*. Moreover, a transient myeloproliferative disorder (TMPD) in the newborn period, characterized by a high spontaneous remission rate with occasional relapse, occurs more frequently in children with Down syndrome. Of interest is the observation of the presence of a trisomy 21 clone in association with TMPD in 15 phenotypically normal children, at least four of whom were determined to be constitutional mosaics for Down syndrome *(57)*.

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 who present clinically with various features of the syndrome *(58–63)*. These studies suggest that the genes for CuZn–superoxide dismutase (*SOD1)* and amyloid precursor protein (*APP*), located proximal to band 21q22.1, can be excluded from a significant contribution to the Down syndrome phenotype, whereas parts of bands 21q22.2 and 21q22.3, including locus D21S55, can be the minimal region necessary for the generation of many Down syndrome features (see Chapter 3 for a discussion of band nomenclature). Studies by Korenberg et al. suggest that, instead of a single critical region, many chromosome 21 regions are responsible for various Down syndrome features *(64)*. They used a panel of cell lines derived from 16 partial trisomy 21 individuals to construct a "phenotypic map" correlating 25 Down syndrome features with regions of chromosome 21.

Recurrence

The 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 is not significantly different from the age-specific risk *(65)*.

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) *(66)*. 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 *(67)*. 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 *(68)*. Thus, 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. When present, the recurrence risk will be high and will depend on the proportion of trisomy 21 cells in the gonad.

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 *(69,70)*. 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. *(71)*. The incidence is 1 in 6000–8000 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 dysmorphism (prominent occiput, narrow bifrontal diameter, short palpebral fissures, small mouth, narrow palate, low-set malformed ears, micrognathia) (see **Fig. 6**), clenched hands with a tendency for the second finger to overlap the third and the fifth finger to overlap the fourth, short dorsiflexed hallux, hypoplastic nails, rocker bottom feet, short sternum, hernias, single umbilical artery, small pelvis, cryptorchidism, hirsutism, and cardiac anomalies (mainly VSD, ASD, and PDA). Recent studies show that median survival averages approximately 5 days, with 1-week survival at 35–45% *(72–75)*. 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 *(76)*, have been described *(77,78)*, however, the presence of a normal cell line in these patients was not always investigated.

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 *(79–84)*.

Two recent molecular studies, performed on a total of ten patients with partial trisomy 18, suggest that the region proximal to band 18q12 does not contribute to the syndrome, whereas two critical regions, one proximal (18q12.1 → q21.2) and one distal (18q22.3 → qter), could work in cooperation to produce the typical trisomy 18 phenotype *(85,86)*. In addition, severe mental retardation in these patients could be associated with trisomy of the region 18q12.3 → q21.1.

Recurrence

There are not enough data regarding the recurrence risk for trisomy 18. Single-case reports of trisomy 18 in sibs (e.g., ref. *87)* and of trisomy 18 and a different trisomy in sibs or in prior or subsequent abortuses (e.g., refs. *88* and *89)* are recorded. For genetic counseling purposes, a risk figure of less than 1% for another pregnancy with any trisomy is generally used and might be appropriate.

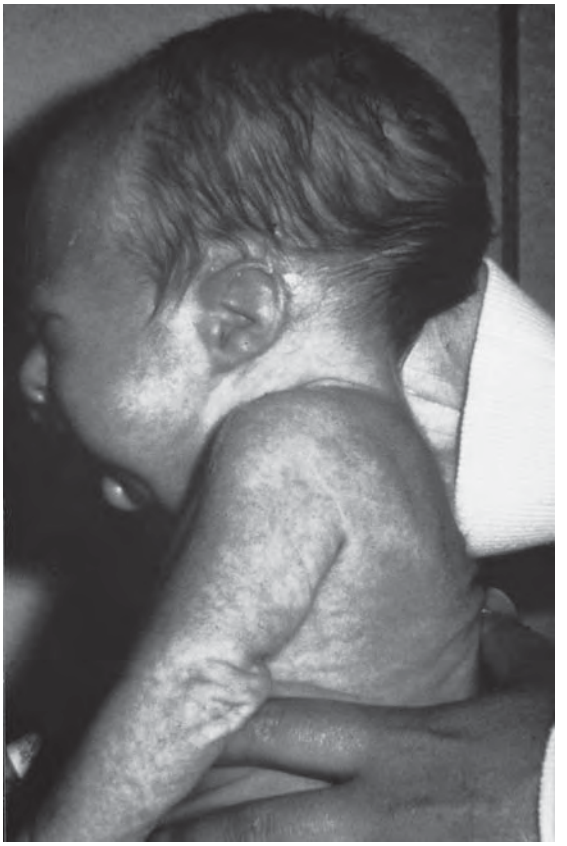

Fig. 6. Profile of a trisomy 18 child showing prominent occiput, low-set malformed ear, and micrognathia.

Trisomy 13

Incidence

Trisomy 13 [47,XX or XY,+13] was first described by Patau et al. in 1960 *(90)*. 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 (see **Fig. 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 days and a 6-month survival of 5% *(74)*. 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 *(91,92)*. These studies were based on cytogenetic

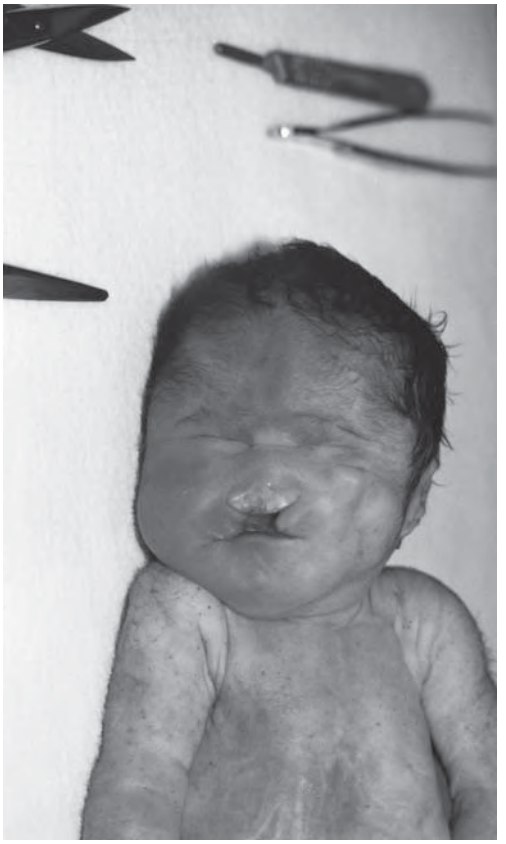

Fig 7. Trisomy 13 stillborn with midline cleft lip and holoprosencephaly.

methods and suggested that the proximal segment (13pter → q14) contributes little to the trisomy 13 phenotype, whereas the distal segment (all or part of 13q14 → qter) is responsible for the complete trisomy 13 features. Molecular studies for a more accurate delineation of the breakpoints have not been done.

Recurrence

Again, no empirical recurrence risk data are available. The risk is likely to be very low. A less than 1% risk is generally quoted for genetic counseling purposes, as with trisomy 18.

Trisomy 8

Trisomy 8 [47,XX or XY,+8] was first reported by Grouchy et al. in 1971 *(93)*. It is rare, with an unknown incidence. More than 100 cases have been reported in the literature *(94–97)*, most of them mosaics [47,+8/46]. The male-to-female ratio is 2–3 : 1.

Growth and the degree of mental deficiency are variable. Mild to severe retardation is seen, and a proportion of patients have normal IQs. Craniofacial dysmorphism (see **Fig. 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 antihelices. 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 few cases of hematological malignancy

Fig. 8. An infant with mosaic trisomy 8. Note prominent forehead, strabismus, broad nasal bridge, upturned nares, long upper lip, and everted lower lip.

have been reported in mosaic trisomy 8 patients *(98,99)*. This is of particular interest because trisomy 8 is a frequently acquired cytogenetic abnormality in myeloid disorders (see Chapter 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 could 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 *(100,101)*. More than 40 cases of liveborns or term stillborns with trisomy 9 have been reported. Most were mosaics *(102–106)*. 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. 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.

A recent study showed that the mean maternal age of women bearing trisomy 9 offspring is significantly increased over that of the general population *(103)*. This suggests that the occurrence of trisomy 9 might 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 Chapter 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 *(107)*.

Mosaic trisomy 16 fetuses, however, can occasionally survive to term. At least ten such cases have been reported *(108–114)*. Intrauterine growth retardation is invariable. An elevated maternal serum human chorionic gonadotropin (hCG) or α-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. 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 to date 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 *(115)*. 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. Very few liveborns with documented mosaic trisomy 20 have been reported and all were phenotypically normal at birth *(116–121)*. In cases with long-term follow-up, hypopigmentation was reported in three, but no major malformation or intellectual impairment was observed. 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) *(122)*.

Trisomy 20 cells have been found in various fetal tissues, including kidney, lung, esophagus, small bowel, rectum, thigh, rib, fascia, and skin *(115,122,123)*. Postnatally, they have been detected in cultured foreskin fibroblasts and urine sediments *(116–121)*. 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 *(118)*. 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 *(124)*. Since then, more than 20 liveborns have been reported in the literature (reviewed in ref. *125,126–129)*. 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 *(130)*.

The most consistent phenotypic abnormalities include intrauterine growth retardation, 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 are also common. Skin hypopigmentation (hypomelanosis of Ito) is usually present in mosaic cases. Most nonmosaic patients die in the first months of life. The longest survival reported is 3 years *(131)*. That patient had severe growth and developmental delay and died a few days before his third birthday. Prolonged survival to over 20 years has been observed in mosaic patients.

Trisomy 22 cells can be detected in both blood lymphocytes and skin fibroblasts. The risk for recurrence is unknown.

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.

A single case of liveborn mosaic trisomy 2 has been reported *(132)*. 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. 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 *(133)*. Three cases of mosaic trisomy 3 have been reported *(10,134,135)*; one of these, a severely mentally retarded woman, was alive at age 32. Clinical features in the three cases vary, except all had prominent forehead, ear and eye anomalies. One case each of postnatally confirmed mosaic trisomy 4 *(136)* and mosaic trisomy 5 *(137)* has been reported. In both cases, the trisomic cells were detected in prenatal amniocytes and confirmed postnatally in skin fibroblasts, but not in blood lymphocytes. Both patients had multiple congenital anomalies. One case of mosaic trisomy 6 has recently been reported *(138)*. This child 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 2. Trisomy 6 cells were detected in skin fibroblasts but not in blood. At lease six cases of cytogenetically documented mosaic trisomy 7 in skin fibroblasts have been recorded (reviewed in ref. *139, 140,141)*. All patients were phenotypically abnormal. Common features included growth and developmental delay, skin pigmentary dysplasia with hypopigmentation 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 (reviewed in ref. *142,143)*. 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. 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 six cases of trisomy 12 have been reported in liveborns; all were mosaics *(144–148)*. The earliest reported case was that of an infertile man, whereas the most recent case involved an infant

with multiple malformations and pigmentary dysplasia who died at 2 monts of age. Phenotypic presentation was variable 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, and thymus.

At least 15 cases of mosaic trisomy 14 have been reported in liveborns (reviewed in ref. *149*,*150)*. 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 *(150)*. 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 (reviewed in refs. *151* and *154*;*153,154)*, two of them were reportedly nonmosaics *(155,156)*. 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 Chapter 19) in the normal cell line was reported in two of the cases *(152,154)*. 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.

Two cases of confirmed mosaic trisomy 17 have been reported *(157,158)*. The trisomic cells were not seen in lymphocytes, but were found in high percentage in skin fibroblasts. One patient, age 8 years at the time of reporting, had mental and growth retardation, microcephaly, minor dysmorphism, seizures, hearing loss, attention deficit hyperactivity disorder, and autistic behavior. The other patient had mild dysmorphic features and moderate neurological involvement that the authors suggested could be related to prematurity. Two cases of mosaic trisomy 19 are 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 *(159,160)*.

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, mosaic monosomy 22, and a single case reported in an abstract of a possible mosaic monosomy 20 in a 3½ year old boy with atypical speech/language delay, behavior problems, microcephaly, and patchy hypopigmentation of the skin *(161)*.

Monosomy 21

Mosaic monosomy 21 was reported in four liveborns in the early literature *(162–165)*. The most prominent features included intrauterine growth retardation, 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 *(166)*. The above-described facial abnormalities were present in this abortus. In addition, a complex cardiac malformation, malrotation of the bowel, uterus didelphys, small dysmature ovaries, and focal cystic dysplasia of the lung were noted.

Approximately ten cases of apparently nonmosaic monosomy 21 have been reported in liveborns (reviewed in ref. *167,168,169)*. Some of these cases have subsequently been shown to represent partial monosomy 21 resulting from an undetected subtle translocation *(170)*, explaining the observation that mosaic monosomy 21 is less commonly observed than apparently nonmosaic monosomy

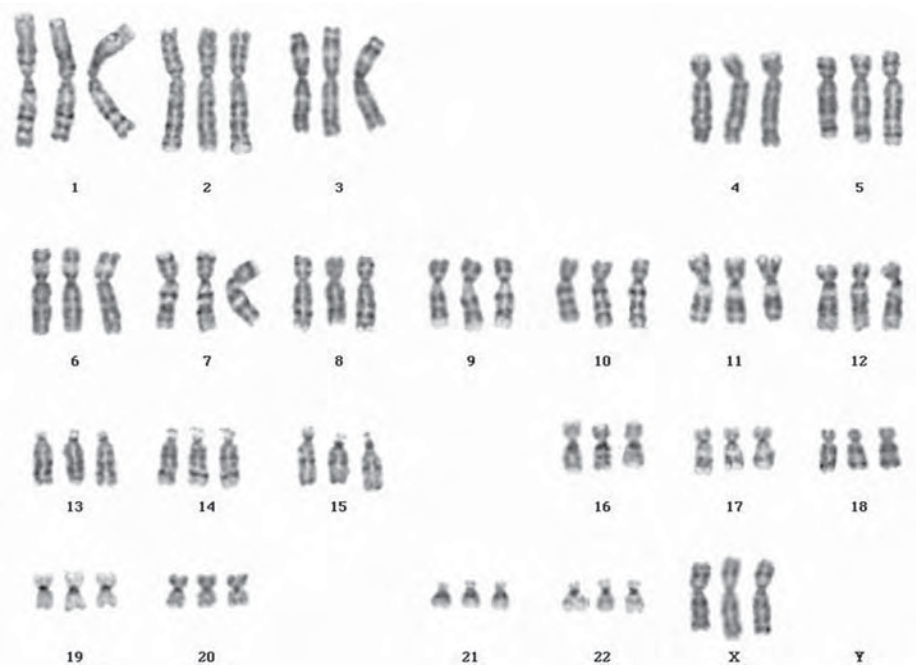

Fig. 9. Karyotype of a triploid fetus (69,XXX).

21 and indicating that complete monosomy 21 is almost always incompatible with life. Most patients died before 2 years of age, although one male child survived to 11 years. The phenotypic features were similar to those observed in the mosaics and included intrauterine growth retardation, postnatal growth and mental deficiencies, microcephaly, hypertelorism with downward slanting palpebral fissures, large low-set ears, prominent nose, cleft lip/palate, and micrognathia. Abnormal muscle tone, mostly hypertonia, was common. Cardiac anomalies were present in a few cases.

Monosomy 22

Four cases of mosaic monosomy 22 in liveborns have been reported *(171–174)*. 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 an autopsy was not performed *(173)*. 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.

POLYPLOIDY

Polyploidies 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 (see **Fig. 9**). It is estimated to occur in approximately 1% of all human conceptions and is found in 17–18% of all chromosomally abnormal abortuses *(175,176)*. Only very rarely do triploid conceptuses survive to term. Two distinct phenotypes have been recognized *(177)*. One type presents as a relatively well-grown fetus with or without

microcephaly and an abnormally large and cystic placenta usually classified as partial hydatidiform moles. The parental origin of the extra haploid set of chromosomes in such cases is determined to be paternal (diandry) by analysis of cytogenetic heteromorphisms *(177,178)* or DNA polymorphisms *(179)*. 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 retardation with relative macrocephaly and a small and noncystic placenta. The extra haploid set of chromosomes in such cases is maternal (digyny) *(177–180)*. Digyny can result from a failure of the first maternal meiotic division, generating a diploid egg, or from retention of the second polar body. Although the occurrence of triploidy does not appear to be associated with maternal age, digyny can play a major role in the generation of triploidy in the advanced maternal age group *(176)*. Early cytogenetic studies indicated that the majority of triploid conceptuses were diandric partial moles *(178,181)*. Later studies based on DNA polymorphisms have suggested that a maternal contribution to triploidy could occur more frequently than was previously realized *(179,182)*. Yet, in a most recent study of 87 informative cases of triploid spontaneous abortuses at 5–18 weeks of gestation, Zaragoza et al. *(183)* showed that approximately two-thirds are androgenetic in origin and that many, but not all, androgenetic triploids developed a partial molar phenotype. The sex chromosome complement in triploidy is either XXX or XXY, with XYY occurring only rarely. For example, the reported numbers of XXX : XXY : XYY cases in two studies performed on spontaneous abortuses were 82 : 92 : 2 *(3)* and 26 : 36 : 1 *(176)*, and in one study performed on amniotic fluid cells, this ratio was 6 : 8 : 0 *(184)*. 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 *(185,186)*. 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 *(187–189)* (see Chapter 19).

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 (reviewed in ref. *190)*, with the longest being 10 months *(191)*. 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 *(192)*. 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 (reviewed in ref. *192)*. These findings suggest that, in general, digynic triploids survive longer than diandric triploids. The most frequent phenotypic abnormalities include intrauterine growth retardation, 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 *(193)*. Patients with such mixoploidy are less severely affected than nonmosaics and survival beyond 10 years has been observed. Usual clinical features include intrauterine growth retardation, psychomotor retardation, asymmetric growth, broad nasal bridge, syndactyly, genital anomalies, and irregular skin pigmentation *(194)*. Truncal obesity was seen in some patients *(195)*.

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 sperms; 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 *(196)*. 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 *(197)*.

Tetraploidy

The chromosome number in tetraploidy is 4*n*=92. It is rarer than triploidy in spontaneous abortuses, seen in approximately 6–7% of such specimens with chromosome abnormalities *(175,176)*. 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 cleavage during the first division. Other theoretically possible mechanisms require the occurrence of two independent, rare events and are, thus, highly unlikely.

At least eight apparently nonmosaic tetraploid liveborns have been reported (reviewed in ref. *198)*. The sex chromosome complement was either XXXX or XXYY. No 92,XYYY or XXXY conceptus has 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 three cases. Urinary tract abnormalities, such as hypoplastic kidneys, have also been recorded. Most patients died before 1 years of age. One girl had survived to 22 months at the time of report *(199)*.

Mosaic tetraploidy (diploid/tetraploid mixoploidy) has been reported in 12 liveborns (reviewed in ref. *200*,*201)*. 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 *(202)*. In two females, aged 11 and 21 years, with severe intellectual handicaps and skin pigmentary dysplasia, tetraploid cells were found only in skin fibroblasts *(201)*. In lymphocytes, the proportion of tetraploid cells decreases with age *(203)*. Overall, clinical features are similar to but less severe than those in nonmosaic tetraploidy patients. In addition to the longer survivals mentioned above *(201)*, survivals to 6 years at the time of reporting have also been recorded *(204)*.

PARTIAL AUTOSOMAL ANEUPLOIDIES

Partial duplication/deletion as a result of structural rearrangement is discussed in Chapter 9. Only those partial autosomal aneuploidies that result from the presence of a supernumerary chromosome 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 three liveborns, all of whom are mosaics with both normal and abnormal cell lines *(205)*. The abnormal cell line was 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 one patient. Survival was variable; one patient died at 6 months and one 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 cases reported are 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 *(206–208)*. At least 11 cases have been reported (reviewed in ref. *208*,*209)*. 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.

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 (reviewed in ref. *210,211–213)*. The isochromosome consists of either the entire short arm of chromosome 9 as described above, 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 *(210,211,214,215)* and in fibroblasts at a much lower percentage than in lymphocytes in two others *(216,217)*. 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 over 50% of patients. 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 *(210)*.

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)] (see **Fig. 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 *(218)*. In 1981, Killian and Teschler-Nicola reported a 3-year-old girl with similar clinical manifestations *(219)*. Subsequently, more than 60 cases have been reported (reviewed in ref. *220*, *221)*, and many more have been observed but not reported in the literature. 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 *(222)*. 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 six of seven cases studied by molecular analysis, the meiotic error was determined to be maternal *(223,224)*. Tissue specificity and both the in vivo and in vitro age dependencies of the i(12p) have been well demonstrated (reviewed in ref. *225)*. The i(12p) is found in a high percentage of skin fibroblasts and amniocytes, but it 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 reported in at least two newborn infants *(226,227)* and in only 6% of marrow cells in a 3-year-old child *(228)*. In lymphocytes, it has been found in fetal blood *(225,229)*, but has never been seen beyond childhood. 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 *(226)*. The isochromosome is more stable in skin fibroblasts and can be found in adults, usually at a 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 *(223,225–227,230)*. One study using FISH showed that in lymphocytes, the i(12p) was present in a significantly higher proportion of interphase nuclei than in

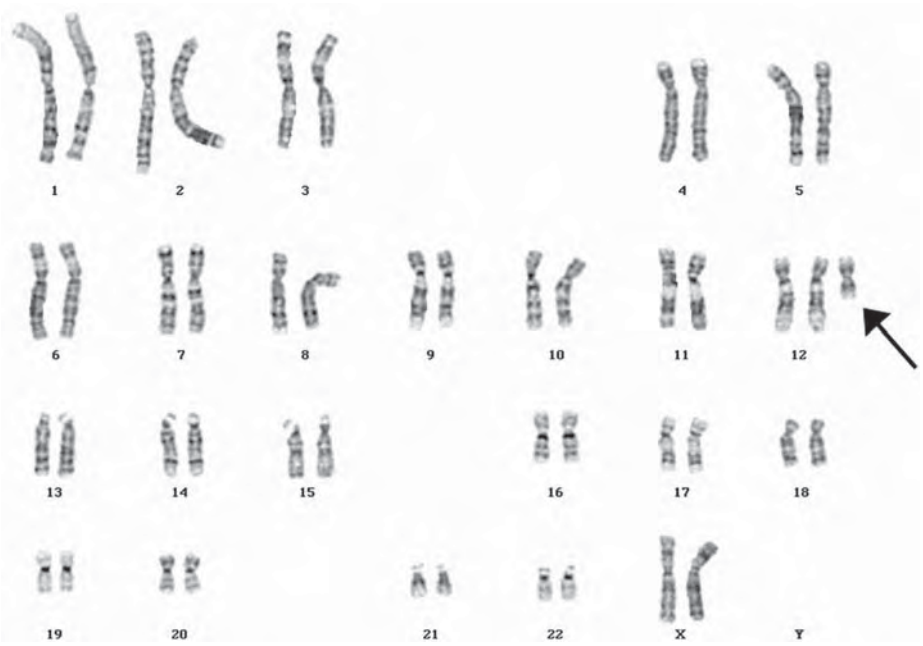

Fig. 10. Tetrasomy 12p female karyotype.

metaphase cells *(231)*. These authors proposed that lymphocytes containing i(12p) might fail to divide upon PHA stimulation. These observations suggest that tissue-limited mosaicism in Pallister–Killian syndrome could 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, whereas 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 hyperpigmentation and hypopigmentation. Other abnormalities include short neck, macroglossia, micrognathia progressing to prognathia, accessory nipples, umbilical and inguinal hernias, and urogenital abnormalities. 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 *(232)*, although identification of the marker as an i(18p) was not made until after the introduction of banding techniques in 1970. 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 α-satellite DNA in the i(18p) in one case *(233)*.

At least 50 cases have been reported *(234–238)*. Most are nonmosaics. The i(18p) is usually readily detectable in lymphocytes. Its presence in amniocytes *(239)* and cultured chorionic villus cells *(233)* has also been reported.

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 ASDs, VSDs, and PDA have been observed in some cases. Urogenital anomalies, including horseshoe kidneys, double ureter, and cryptorchidism have occasionally been seen.

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 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 *(240,241)*. 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 *(242)*. In another recent 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 *(238)*. The recurrence risk in such families will be high.

Other Partial Autosomal Aneuploidies

Supernumerary Marker Chromosomes

In addition to the tetrasomies described above, 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 1000 in newborns *(243)* and 0.8–1.5 per 1000 in prenatal specimens *(244–246)*. Because their cytogenetic origins are not initially known, these markers might represent autosomal aneuploidy. Identification of such markers is now typically achieved using FISH; this is covered in Chapter 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 (reviewed in ref. *247,248–250)*. 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 account for approximately 40% of all marker chromosomes *(249,251)*. 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 *(252–254)*. Of particular interest is the observation of a few patients with this type of marker who present clinically with Prader–Willi syndrome *(253,255–258)* or Angelman syndrome *(258,259)*. 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 *(253,258)* or a deletion of 15q11-q13 on one of the apparently cytogenetically normal 15s *(259)*.

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 *(260–262)*. 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 marker, 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 *(263)*.

Clinically definable entities have not been observed for other markers, as each is typically unique. However, this could change as data concerning the composition of marker chromosomes accumulates through the use of FISH and other molecular technologies.

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9

Structural Chromosome Rearrangements

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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 normal 23-pair arrangement with which we are familiar. Although we tend to think of the resulting structural rearrangements 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.

In this chapter, we will discuss and provide examples of the ways in which chromosome rearrangements can occur. We begin with an overview of general concepts that relate to all structural rearrangements and their association with human pathology. Each category of structural rearrangement is then 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 exchange 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. Because 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. The presence of a DNA sequence that is repeated elsewhere in the genome, a fragile site (see Chapter 14) and/or a particular secondary DNA structure all appear to influence the likelihood that a particular chromosome region is involved in a structural rearrangement *(1–6)*.

Numerous studies have shown that many rearrangements occur secondary to recombination between nonallelic regions of homology. These regions of homology sometimes represent high-copy-number repeats such as Alu or satellite DNA sequences. Recently, the role of low-copy repeats (LCRs) in the formation of structural rearrangements has begun to be elucidated. 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. The LCRs that serve as substrates for these recombination events range in size from 1 to 500 kilobasepairs (kb) and share > 97% sequence identity. Although distributed throughout the genome, LCRs might appear preferentially within pericentromeric chromosomal regions. The ultimate size and types of rearrangement that result from these nonallelic homologous recombination

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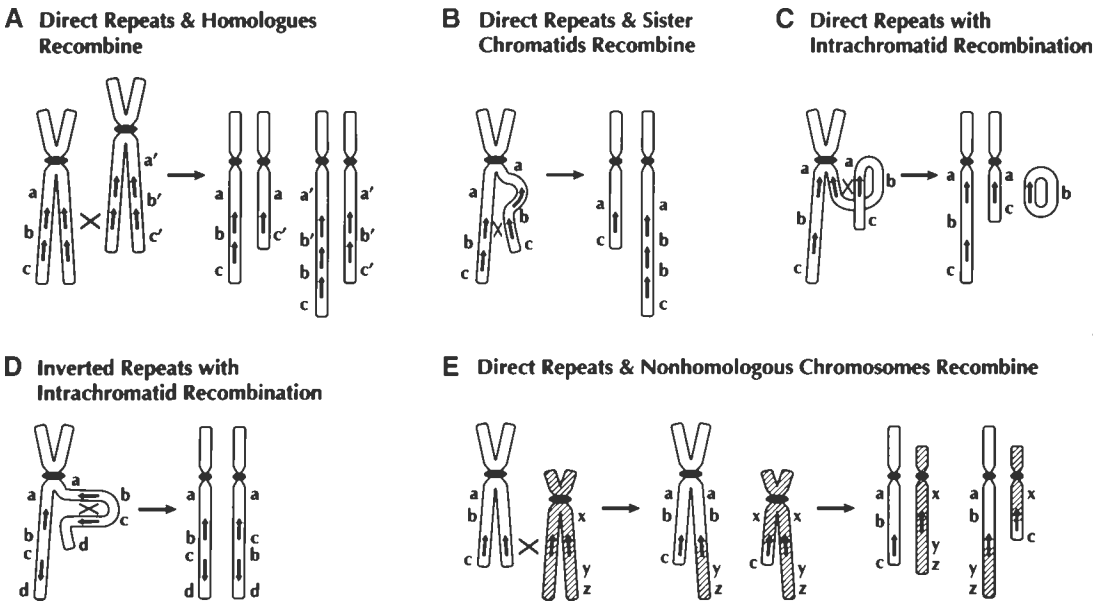

Fig. 1. Chromosome rearrangements can be produced by nonallelic homologous recombination between shared sequences or repeats of identical (direct repeats) or opposite (inverted repeats) orientation. Recombination between direct, nonallelic repeats on homologous chromosomes **(A)** or sister chromatids **(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 the lowercase letters represent unique sequences.

events appear to reflect the location, size, and orientation of the LCRs, as well as the number of crossover events that occur within the LCR.

Direct LCRs (those with the same orientation) located on the same chromosome can mediate both duplications and deletions as shown in **Fig. 1**. When a single, nonallelic, homologous recombination event involving homologous chromosomes (interchromosomal) or sister chromatids (intrachromosomal) is mediated by direct LCRs, complementary duplications and deletions occur (see **Figs. 1A,B**). Only deletions are predicted to occur, however, if nonallelic homologous recombination involving direct LCRs occurs within a single chromatid (intrachromatid; **Fig. 1C**). As shown in **Fig. 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 (see **Fig. 1E**) as well as other more complex rearrangements *(7)*.

The size of the inversions, duplications, and deletions produced by the recombination events described above are dependent on 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 Charcot–Marie–Tooth disease type 1A, hereditary neuropathy with liability to pressure palsies (HNPP), hemophilia A, and many others *(6)*. In contrast to single-gene rearrangements, recombination events that utilize nonal-

lelic homologous sequences that are separated by large regions of the genome (usually at least 3–5 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 pericentric inversion 9 with breakpoints at p11 and q13 and the (4;8) translocation with breakpoints at 4p16 and 8p23 (see inversions and reciprocal translocations below).

Although 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 *(8)*. Although 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 *(9)*. 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 Robertsonian translocations below).

In contrast to the wealth of data supporting a role for both high- and low-copy repeat sequences in the formation of many constitutional chromosomal rearrangements, the data suggesting a role for fragile sites (see Chapter 14) is modest. In fact, the only fragile site with convincing evidence for a possible role in the formation of a constitutional chromosome abnormality is that of FRA11B. FRA11B is a rare heritable folate-sensitive fragile site located within the *CBL2* oncogene in band 11q23.3. In a subset of patients with Jacobsen syndrome secondary to a terminal chromosome 11 long arm deletion, molecular studies demonstrated that the deletion breakpoints map to the chromosome 11 homolog with the FRA11B premutation or full mutation and lie in the vicinity of the fragile site *(10,11)*. The paucity of data suggesting a role for other fragile sites in the formation of constitutional chromosome rearrangements suggests that their involvement might be minimal.

That DNA architecture could create "hot spots" for chromosome rearrangements has been supported by studies addressing the recurring (11;22) translocation (see Reciprocal Translocations below). The breakpoints involved in this translocation are not associated with regions of homology, but rather with AT-rich palindromic sequences (DNA sequences that contain two inverted regions complementary to each other) that are predicted to form hairpin-shaped secondary structures. These hairpin structures are thought to be susceptible to nucleases that produce double-stranded breaks that serve as substrates for recombination and the formation of the resulting translocation. Despite the fact that the breakpoints of a number of translocations have now been examined, at this time palindromic sequences have been implicated in the formation of only one other translocation, a (17;22) translocation identified in a family with neurofibromatosis type I *(12)*.

In contrast to the maternal bias noted for numerical chromosome abnormalities, approximately 75% of structural chromosome rearrangements appear to be paternally derived *(13,14)*. 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, might promote the accumulation of mutations. Additionally, studies on mouse and *Drosophila* suggest that male gametogenesis might be more sensitive to mutagens than oögenesis *(15)*. 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 *(16,17)*. Several supernumerary isochromosomes and inverted duplicated chromosomes also appear to form primarily during maternal gametogenesis *(18–21)*. No parental bias has been noted for several other types of rearrangements, including the interstitial microdeletions associated with DiGeorge and Williams syndrome *(22,23)*. 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 Chapter 19).

In theory, chromosome breakage, rearrangement, and reunion can occur during meiosis or mitosis. Meiotic errors, because 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. Although this observation suggests that many structural rearrangements might be formed during meiosis, ascertainment bias likely plays a role as well. Because 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.

Although 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 prometaphase chromosomes is reported to be 2–5 Mb or $2–5 \times 10^6$ bp. This number will vary, however, depending on the quality of the chromosome preparations and the skill of the cytogeneticist examining the karyotype(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 comparative genomic hybridization (CGH) are currently being used to detect submicroscopic or otherwise cryptic rearrangements that cannot be detected using traditional cytogenetics (see Chapter 17).

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 children, parental karyotypes should be obtained 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. Because 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%. Therefore, it is imperative that these families be 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 could 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, from the point of view of the physician treating the patient, the presence of any acquired cytogenetic change is significant (see Chapters 15 and 16). Alternatively, demonstrating that the rearrangement is constitutional can be considered "good news," because this means that there are, in fact, no acquired chromosomal changes. Second, and equally important, however, is to consider the potential reproductive consequences for the extended family. Because it is necessary to focus on the treatment of the patient's cancer, and because many of these patients are elderly and well beyond childbearing age, reproductive issues associated with a familial chromosome rearrangement are frequently overlooked. It should be clear from this chapter, however, that these issues must be addressed. Genetic counseling is covered in detail in Chapter 20.

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, we have no idea what has occurred at the molecular level within the rearrangement and we have 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 *(24)*. Apparently balanced *de novo* rearrangements detected at amniocentesis have also been associated with a risk for congenital abnormalities that is twofold to threefold higher than 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 above, structural rearrangements that appear balanced at the microscopic level might 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

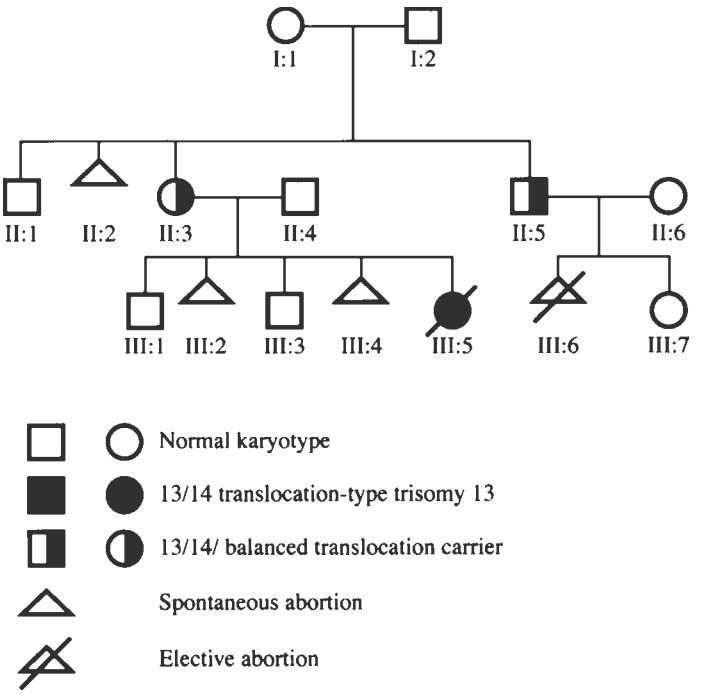

Fig 2. A pedigree of a family in which a balanced Robertsonian (13;14) translocation is segregating. Multiple spontaneous abortions (see individuals II-2, III-2, and III-4), abnormal children (III-5), and infertility are frequently observed in families segregating a balanced rearrangement.

expression is altered. This scenario has been demonstrated in several patients with Duchenne muscular dystrophy, for example *(25)*. 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 might be completely unrelated to his or her rearrangement must always be examined. Other nonchromosomal genetic disorders, prenatal exposures, birth trauma, and so forth must all be considered.

Familial Rearrangements

Balanced structural rearrangements can pass through multiple generations of a family without detection. When these families are ascertained, it is usually the result of the presence of infertility, multiple spontaneous pregnancy losses, and/or clinically abnormal family members (see **Fig. 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, on interactions between their shared 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 Chapter 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 sections Translocations, Inversions, Insertions, and Duplications). 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. Although 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 extra 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 one might expect, those families or individuals who have had a liveborn child or children with congenital abnormalities, especially those where 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 also, in some cases, 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 oögenesis, and/or the selective pressure against unbalanced gametes is greater in the male, and/or imprinting effects might cause the unbalanced embryos of male carriers to be less viable than those of their female counterparts. Male infertility could also play role *(15,26)* (see Chapter 11).

On rare occasions, an abnormal phenotype is observed in an apparently balanced carrier of a familial rearrangement. Although some of these cases could 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 *(27)* (see Chapter 19). 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 *(28)*. After 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.

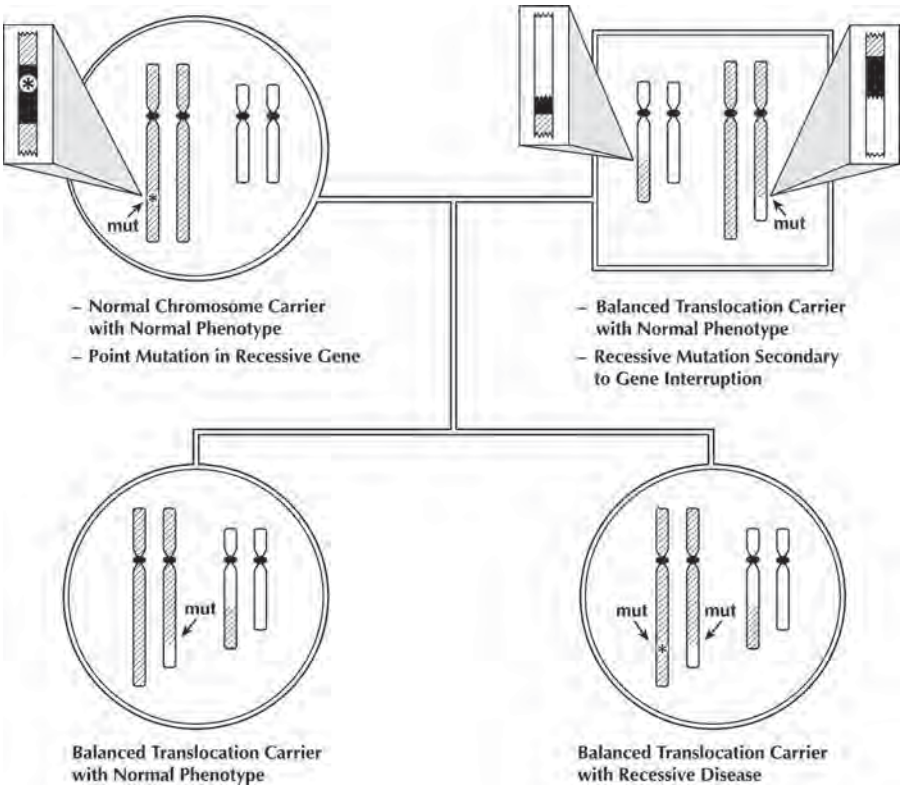

Fig. 3. In the example here, the mother **(top left)** carries a recessive point mutation (asterisk) within a gene (black box) located on one chromosome homolog (hatched). The father **(top right)** carries a mutation in the same gene secondary to interruption via a translocation event. Because the second 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, whereas the comparable paternally inherited allele has been interrupted secondary to a translocation.

Phenotypic discrepancies between child and parent could also be explained by the presence of a recessive allele that is inherited from a chromosomally normal parent. Although the parent is phenotypically normal because of 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, whereas the other is inherited from the cytogenetically normal parent (see **Fig. 3**).

DELETIONS

Autosomal deletions that can be detected by traditional, high-resolution, or molecular cytogenetic methods produce monosomies that are generally associated with significant 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

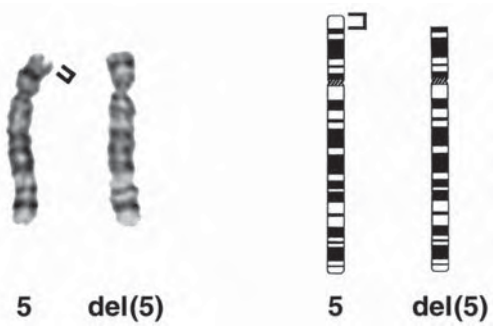

Fig. 4. A terminal deletion involving the distal short arm of chromosome 5 [del(5)(p15.3)]. Patients with similar deletions are said to have cri du chat or cat cry syndrome because of the characteristic cat-like cry present in many during infancy.

Fig. 5. An interstitial deletion involving the long arm of chromosome 13 [del(13)(q21.3q33)].

that have traditionally been considered euchromatic. Gardner and Sutherland catalog deletions of this type in bands 5p14, 11p12, 11q14, 13q21, and 16q21 *(29)*.

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 or not 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, whereas deletions of similar size involving the short arms of chromosomes 17 and 19 are rarely, if ever, seen in liveborns *(30)*.

Classic deletions have traditionally been described as either terminal (see **Fig. 4**) or interstitial (see **Fig. 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 *(31–33)*. 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 particular deletions 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 *(34,35)*. Still other deletions appear to be terminal by cytogenetics analysis, but have been shown by molecular analysis to be interstitial. It is estimated that 7–20% of apparent terminal deletions fall into this category *(36,37)*. 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 a new class of cytogenetic abnormality variously referred to as chromosomal microdeletions, contiguous gene syndromes, and, more recently, segmental aneusomy syndromes (SAS). These abnormalities are mostly very small interstitial deletions, often at or below the resolution of microscopic analysis, recur with appreciable frequency, and are associated with distinct clinical phenotypes. The term "microdeletion" is descriptive, but it fails to include the minority category of "microduplications" (e.g., CMT1A; see also the section Duplications) 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 *(38)*. Although this terminology remains appropriate for some of the disorders in this new category, recent molecular investigations have shown that others are actually single-gene disorders or the result of imprinting defects or uniparental disomy (see Chapter 19). In an effort to more accurately characterize the pathogenesis of these disorders, the term "segmental aneusomy syndrome" was proposed to imply that the phenotype is the result of "inappropriate dosage for a critical gene(s) within a genomic segment" *(39)*.

Williams syndrome is one example of an SAS that results from a small deletion. These patients typically carry an approximately 1.5-Mb deletion within the proximal long arm of chromosome 7 that encompasses a minimum of 16 different genes *(40,41)*. 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 *(40,41)*. Presumably, some or all of the remaining 14 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 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 *(42–44)*. Recent evidence suggests that these unequal exchange events could be promoted, in some cases, by the presence of heterozygosity for a submicroscopic inversion that spans the same LCR sequences that mediate the common 1.5-Mb Williams syndrome deletion *(45,46)*. Presumably, 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 the section Inversions and **Fig. 12**).

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 1**.

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 *(17,47,48)*. Although the mechanism(s) responsible for these more variable deletions is currently unknown, recent evidence suggests that LCRs might also be involved in the formation of at least some *(5,6,49)*.

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 (see **Fig. 6**), or in combination with a deletion or some other rearrangement. Examples of some types of rearrangement that involve duplications include isochromosomes, dicentrics, derivatives, recombinants, 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 *(50)*.

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. Very 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 (see **Table 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 *(30)*. Paternally derived duplications of distal 11p have also been associated, in some cases, with Beckwith–Wiedemann syndrome *(51)*. 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 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 LCR sequences. The causative unequal exchange events can occur following misalignment of either sister chromatids or homologs, as shown in **Fig. 1**. At this time, complementary microduplication/microdeletion syndromes have been documented for the Prader–Willi/Angelman, Smith–Magenis, and DiGeorge syndrome critical regions.

Several proximal chromosome 17 short arm duplications involving the same loci that are deleted in Smith–Magenis syndrome have now been reported. The same LCRs that mediate the common Smith–Magenis syndrome deletion also appear to mediate the complementary duplication. Consistent clinical features in these duplication patients include growth and developmental delay, as well as dental and behavioral abnormalities *(52)*.

Likewise, a handful of patients with a proximal chromosome 22 duplication that is complementary to the DiGeorge syndrome deletion have now been reported in the literature *(53–55)*. The phenotypes reported in these patients are variable and range from nearly normal to severe. A number of the features observed in these duplication patients, such as mental retardation and developmental delay, palate abnormalities, conotruncal heart defects, absent thymus, and T-cell deficiency, are also associated with DiGeorge syndrome. In fact, it is interesting to note that several of the reported duplication patients were identified by a fluorescence *in situ* hybridization (FISH) (see Chapter 17) study that was requested because of suspected DiGeorge syndrome. Unexpectedly, an extra fluorescence signal representing the duplication, rather than a deletion, was seen within the proximal long arm of chromosome 22.

Table 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, down-turned mouth, cleft lip and/or palate, micrognathia, cryptorchidism, hypospadias
Cri du chat	5p[a]	Mental and growth retardation, catlike cry in infancy, microcephaly, round face, hypertelorism, downslanting palpebral fissures
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 Deletion, Enlarged Foramina, Exostoses, Cranial dysostosis, reTardation)	11p11.2[a]	Mental retardation, biparental foramina, brachycephaly, turricephaly, multiple exostoses, micropenis, 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 (Tricho-rhino-phalangeal 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 deletion of 15q11-15q13[a]	Mental and growth retardation, inappropriate laughter, ataxia and jerky arm movements, seizures, maxillary hypoplasia, deep-set eyes, large mouth with protruding tongue, widely spaced teeth, prognathia

Prader–Willi	Paternal deletion of 15q11-15q13[a]	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.
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
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
Alagille	20p12[b]	Cholestasis, peripheral pulmonic stenosis, vertebral arch defects, posterior embryotoxon, abnormal facies including deep-set eyes, broad forehead, long straight nose, prominent chin, small low-set or malformed ears
DiGeorge/velocardiofacial (Shprintzen)	22q11.2[a]	Learning disabilities, short stature, overt or submucous cleft palate, velopharyngeal incompetence, prominent nose with squared nasal root and narrow alar base, conotruncal cardiac defects, psychiatric disorders in some
Monosomy 22q13.3	22q13.3[a]	Moderate to severe developmental delay, severe expressive speech delay, increased tolerance to pain, hypotonia, normal to accelerated growth, dysplastic toenails, large hands, minor dysmorphic features, including dolicocephaly, ptosis, abnormal ears, pointed chin
Kallmann[c]	Xp22.3[b]	Hypogonadotropic hypogonadism, eunuchoid habitus, anosmia or hyposmia, bimanual synkinesia
Ichthyosis (X-linked)[c]	Xp22.3 [b]	Hypertrophic ichthyosis, corneal opacities without impairment of vision

[a] Deletion is frequently visible.
[b] Typically not visible using traditional cytogenetics.
[c] This has been seen in association with several other X-linked disorders when it occurs as part of a contiguous gene syndrome.

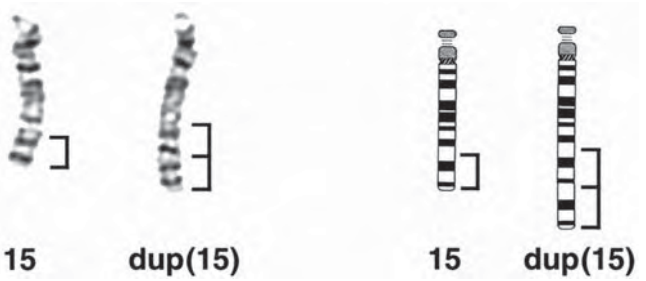

Fig. 6. A duplication involving the distal long arm of chromosome 15 [dup(15)(q24q26.3)]. This duplication was initially observed in the bone marrow of a patient with mental retardation and leukemia. By obtaining a peripheral blood karyotype, we were able to demonstrate that the duplication was constitutional and apparently unrelated to his leukemia.

Many patients have also been observed with duplications in the proximal long arm of chromosome 15 that encompass the same region that is deleted in Prader–Willi and Angelman syndrome (PWS/AS) (see **Fig. 7**). These duplications can be detected by FISH and are associated with a phenotype that includes mental retardation, decreased motor coordination, autism spectrum disorder, and mild to no dysmorphic features *(56)*. The clinical significance of these particular duplications was initially difficult to assess because many affected patients appeared to have 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 carry a maternally derived 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 not only responsible for the phenotypic differences we observe with maternal verses paternal deletions but also for the presence or absence of a clinical phenotype in patients with a duplication (see Chapter 19).

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 (see **Figs. 8**, **9**, and **11**). Paracentric inversions, on the other hand, have both breakpoints on the same side of the centromere or within a single chromosome arm (see Chapter 10, **Fig. 5**). 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 an inversion have been karyotyped, the inversion is found in a parent as often as 85–90% of the time *(57,58)*. Most inversions of both types therefore appear to be inherited.

Pericentric Inversions

Both recurring and unique pericentric inversions have been reported in humans. Some recurring inversions are considered normal variants. In these polymorphic 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 (see **Fig. 8**), and 16. A second

Table 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.
Beckwith–Wiedemann	11p15.5 (paternal)	Macrosomia, macroglossia, organomegaly, omphalocele, ear creases, hypoglycemia, tumor susceptibility; Beckwith-Wiedemann patients with cytogenetic duplications more likely to have learning difficulties.
Pallister–Killian	Mosaic tetrasomy 12p; usually secondary to an extra metacentric isochromosome	Mental retardation, streaks of hyperpigmentation and hypopigmentation, sparse anterior scalp hair, sparse eyebrows and eyelashes, prominent forehead, protruding lower lip, coarsening of face with age.
Duplication of proximal 15q	15q11.2; typically of maternal origin and complementary to the Prader–Willi and Angelman syndrome deletions	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.
Pseudodicentric 15 (inverted duplicated 15)	Tetrasomy 15pter-15q13; the result of the presence of an extra pseudodicentric chromosome	Mental and growth retardation, autism, behavioral disturbance, seizures, low posterior hairline, epicanthal folds, low-set ears, strabismus; the smaller pseudodicentric 15 chromosomes might not cause phenotypic abnormalities.
Duplication of proximal 17p	17p11.2; duplication is complementary to the Smith–Magenis syndrome deletion.	Mild to borderline mental retardation, behavioral problems, short stature, normal facies, dental abnormalities including malocclusion and crowded teeth.
Duplication of proximal 22q	22q11.2; duplication is complementary to the DiGeorge syndrome deletion	Currently no clearly established phenotype recognized; some patients noted to have features that overlapwith 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, downslanting palpebral fissures, preauricular tags and/or fistulas, anal atresia.

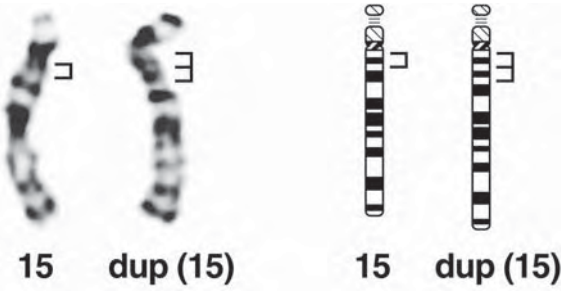

Fig. 7. A duplication involving the proximal long arm of chromosome 15 [dup(15)(q11.2q13)] that appears to be complementary to the common deletions that are observed in Prader–Willi syndrome and Angelman syndrome patients. Duplication of the Prader–Willi/Angelman syndrome region was confirmed using FISH (not shown).

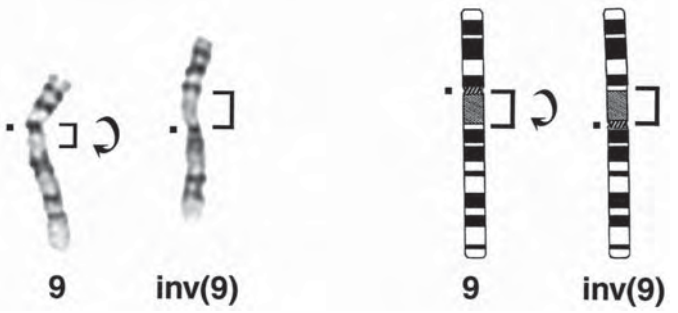

Fig. 8. This benign inversion of chromosome 9 [inv(9)(p11q13)] represents a pericentric inversion with breakpoints in both chromosome arms. The material between the two breakpoints has been inverted, the block of heterochromatin that normally sits in the long arm has been shifted to the short arm, and 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.

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); see **Fig. 9**] *(59)*. 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 *(60)*.

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 below, 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% *(29)*.

Meiotic Behavior and Risks for Carriers of Pericentric Inversions

In order to understand the reproductive risks of an inversion carrier (heterozygote), we must first consider the meiotic behavior of inverted chromosomes. During meiosis, homologous chromosomes

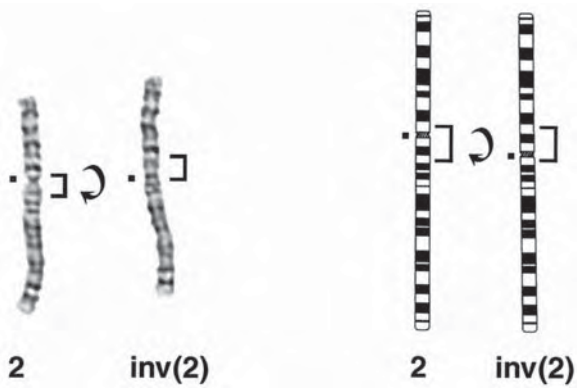

Fig. 9. Although this recurring pericentric inversion [inv(2)(p11q13)] is considered to be benign, individuals who carry this inversion might have a slightly increased risk for miscarriages.

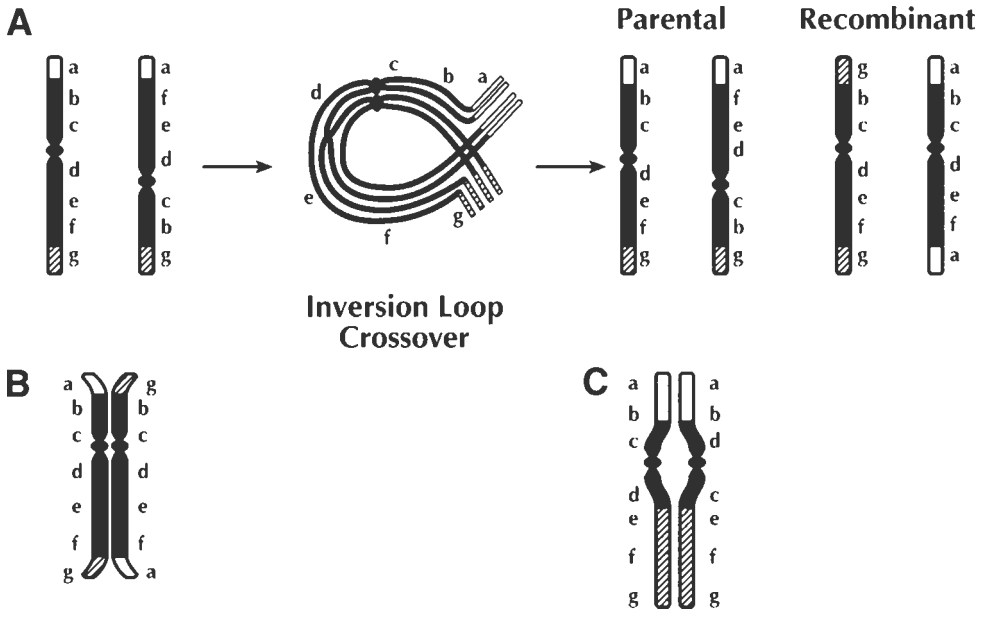

Fig. 10. Several models for meiotic pairing in a pericentric inversion heterozygote. **(A)** An inversion loop containing a single crossover and the resulting parental and recombinant chromosomes. Note that only the material that is distal to the inversion breakpoints has been duplicated/deleted in each recombinant chromosome. **(B,C)** Alternate models for pairing during which only partial pairing or synapsis occurs.

pair in close association. During this pairing phase, genetic information is exchanged between homologs through a process known as crossing-over or recombination (see Chapter 2). Crossing-over appears to be a necessary step for orderly chromosome segregation, and it 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. 10A**. In this model, the

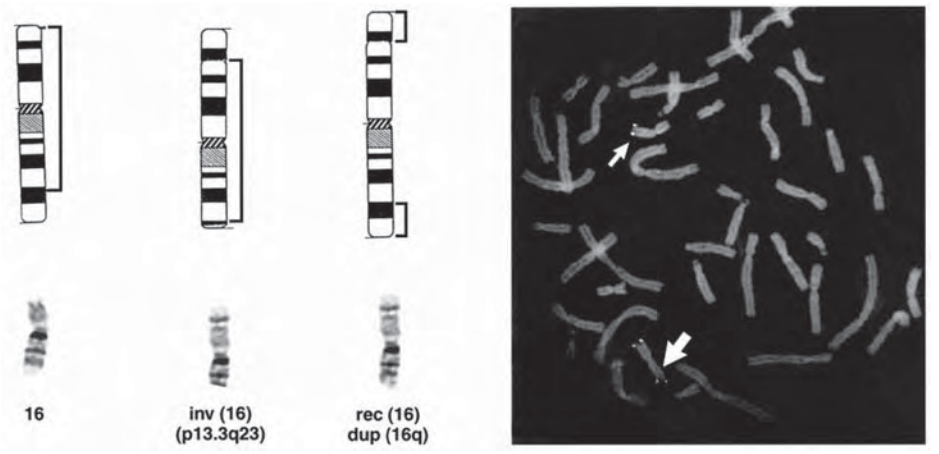

Fig. 11. Left: A normal 16, an inverted chromosome 16, and a recombinant chromosome 16 [rec(16)dup(16q) inv(16)(p13.3q23)] resulting from recombination within the inversion loop of the parental inversion carrier. The recombinant chromosome 16 is missing the material distal to the short arm breakpoint and contains a duplication of the material distal to the breakpoint within the long arm. **Right:** A metaphase that has been hybridized with a FISH probe specific for the subtelomeric region in the long arm of chromosome 16. A signal is seen on the distal long arm of the normal chromosome 16 (small arrow) and on both arms of the recombinant chromosome 16 (large arrow), confirming the duplication of long arm material.

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, whereas 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 chromosome 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. 10** and **11**). Both recombinants are known as duplication-deficiency chromosomes.

Alternate models for pairing in an inversion heterozygote are seen in **Figs. 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 could 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 could 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 because 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, whereas 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, because 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. Because monosomies are generally more lethal than trisomies, an inversion that produces a recombinant with a very small monosomy might 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, because 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 *(29)*. 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 might 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 *(61)*. Large inversions with distal segments that have been seen in liveborn children as monosomies or trisomies might be associated with high risk regardless of their mode of ascertainment in a particular family.

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 1000 *(57)*. Recurring paracentric inversions have been reported in the short arms of chromosomes 3 and 6 and in the long arms of chromosomes 7, 11, and 14. A recurring 11(q21q23) inversion has been observed in a large number of families in the Netherlands *(62)* and in Canadian Hutterites *(63)*. With the advent of FISH and other molecular techniques, a number of submicroscopic recurring inversions have also begun to be identified in the human genome.

Recent data suggest that heterozygosity for some recurring submicroscopic inversions confer susceptibility to other nearby rearrangements involving the same chromosome. For example, a submicroscopic inversion polymorphism that spans the same LCR sequences that mediate the recurring

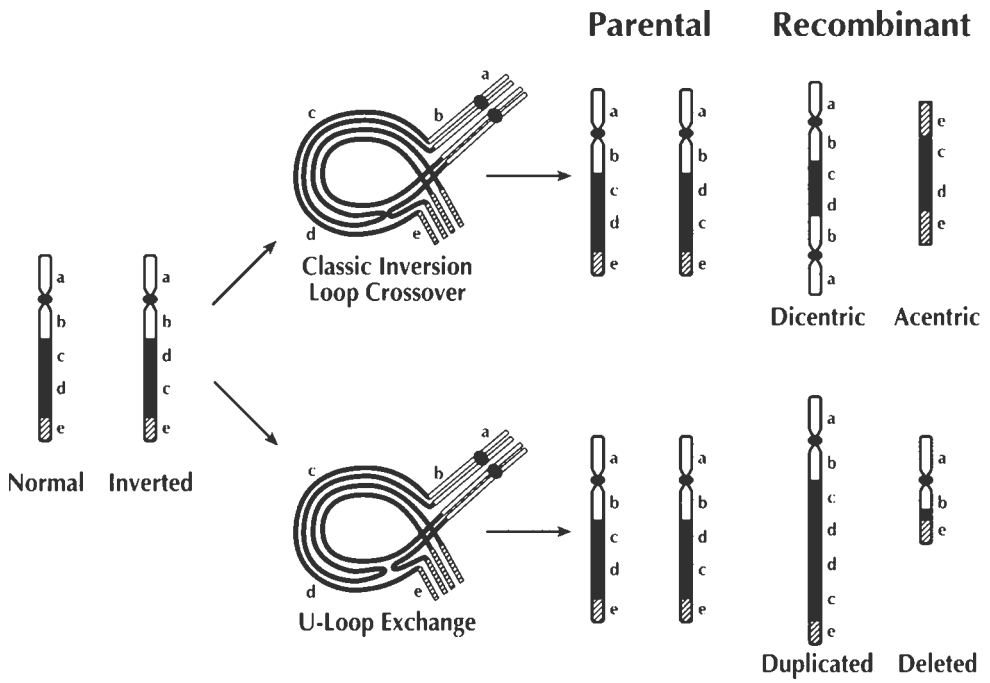

Fig. 12. The type of recombinant chromosome produced depends on which mechanism of chromosome exchange occurs within the paracentric inversion loop. A classic crossover within the inversion loop results in the formation of an acentric and a dicentric recombinant chromosome **(top)**, whereas a U-type exchange produces only monocentric chromosomes **(bottom)**.

Williams syndrome deletion has been observed in the transmitting parent of some Williams syndrome parents *(45,46)*. Similarly, heterozygosity for submicroscopic inversions that span the olfactory receptor gene clusters at both 4p16 and 8p23 and mediate the recurring 4;8 translocation has been seen in all five of the transmitting parents examined *(7)*. 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 *(64)*. Recombination between the homologous *PRKX* and *PRKY* genes located on the short arm of the X and Y chromosome respectively leads to the formation of both XX males and XY females. This particular recombination event also appears to occur preferentially in association with a Yp inversion polymorphism *(65)*.

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 (see **Fig. 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, because 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 acentric chromosomes and dicentric chromosomes below).

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 *(57)*. Whereas 2 of these recombinant chromosomes were dicentric, all of the remaining 15 were 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. 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 might be based our inability, in some cases, to distinguish between a paracentric inversion and an intrachromosomal insertion using G-banded chromosomes *(66)*. 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 might 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 *(57,67)*. 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 to 0.5%" *(29,66,68,69)*. 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 the section Robertsonian Translocations). Recombination within a paracentric inversion loop is also a well-documented method by which a dicentric chromosome can form (see the section Inversions above).

As one might suspect, 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 centromeres, the chromosome will be simultaneously pulled in two opposing directions. As a result of this bipolar pulling, the chromosome could continue to straddle both daughter cells in a state of limbo until it is ultimately excluded from both. Alternatively, the chromosome could break, allowing some portion to go 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 *(70–72)*. 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 *(70,73)*. An example of a pseudoisodicentric chromosome 9 is shown in **Fig. 13**.

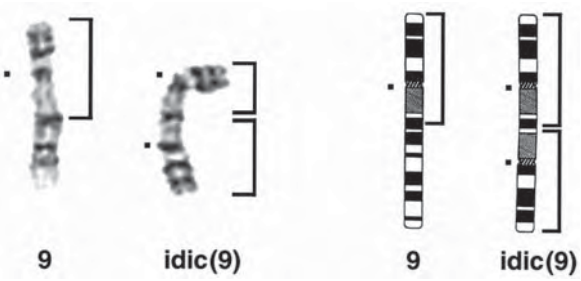

Fig. 13. A pseudoisodicentric chromosome involving the entire short arm and a portion of the long arm of chromosome 9. It appears to have one constricted active centromere *(upper dot)* and one unconstricted inactive centromere *(lower dot)*. This chromosome was found in each of the cells of a phenotypically abnormal infant with the following karyotype: 47,XY,+psu idic(9)(q21.1).

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 50 chromosomes with atypical centromeres have, however, been reported in the literature *(74)*. These atypical centromeres or neocentromeres have now been reported in association with at least 18 different chromosomes. Although clearly functional, the high incidence of mosaicism associated with neocentromere-containing chromosomes suggests that these centromeres often do not function as well as their normal counterparts.

Like traditional centromeres, neocentromeres are denoted by the presence of a primary constriction. Interestingly, however, neocentromeres are located in noncentromeric regions and they interact with only a subset of the centromeric proteins that typically bind active centromeres. Furthermore, they do not react to stains specific for centromeric heterochromatin, nor do they hybridize to centromere specific FISH 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 suggest that neocentromeres do not contain DNA sequences that we typically associate with centromeres, such as α-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 *(75)*. These data, in addition to DNA modeling studies, 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. It has been speculated that neocentromeres might represent ancient centromere sequences that have been reactivated as a consequence of chromosome rearrangement *(76,77)*.

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, 1 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 an extra (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

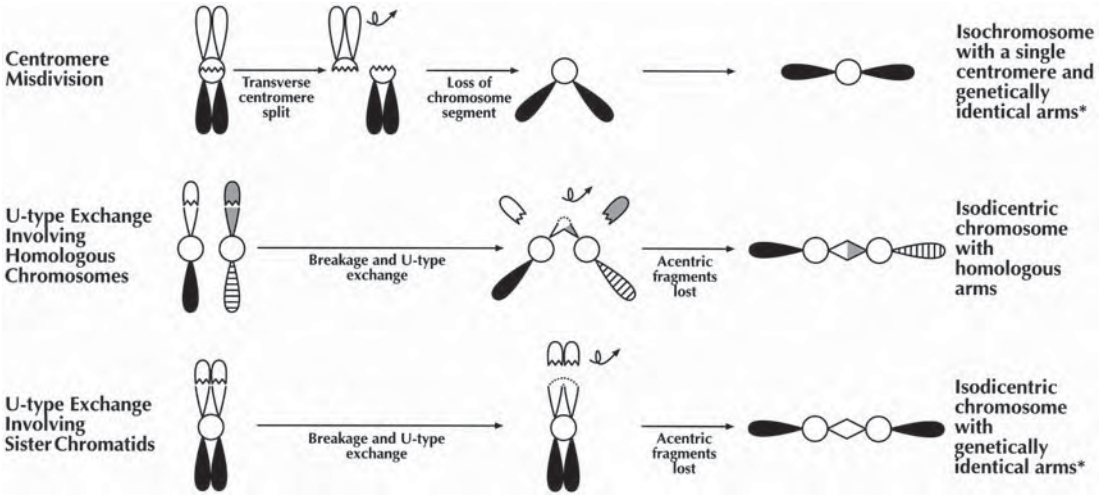

Fig. 14. Some of the mechanisms proposed for isochromosome formation. *Because recombination occurs during normal meiotic cell division, the arms of an isochromosome formed during meiosis would be identical only for markers close to the centromere.

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 Fig. 3 of Chapter 10 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 *(20,21,70,78–81)*. One of the more popular proposals has been that isochromosome formation is the result of centromere misdivision (see **Fig. 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. Recent molecular studies, however, suggest that the breakage and reunion events required to form some isochromosomes might occur predominantly within the area adjacent to the centromere, rather than within the centromere itself *(70,82,83)*. 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. Recently, 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 might rely on sister chromatid exchange *(81,82,84–87)*. Breakage and reunion involving the pericentromeric regions of sister chromatids, an event sometimes referred to as a sister chromatid U-type exchange, might therefore represent an important mechanism of isochromosome formation. Additional molecular studies suggest that most isochromosomes are maternal in origin and that nondisjunction appears to occur prior to isochromosome formation in the majority of cases involving supernumerary isochromosomes *(19–21)*. 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 could 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.

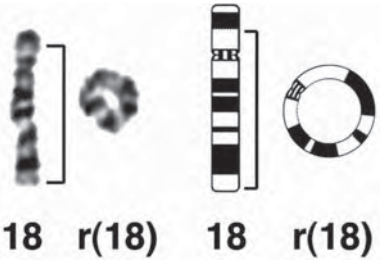

Fig. 15. A ring chromosome 18 [r(18)(p11.2q23)]. This ring chromosome is the result of fusion between two broken arms. The chromosome material distal to the breakpoints in each arm has been lost because it lacks a centromere.

RING CHROMOSOMES

Autosomal ring chromosomes are rare and usually arise *de novo* (see **Fig. 15**). Reported frequencies range from 1 in 27,225 to 1 in 62,279 in consecutive newborn and prenatal diagnosis studies *(58)*. Rings have been reported for all chromosome pairs, although those involving chromosomes 13 and 18 are among the most common *(88)*. 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.

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. Recent molecular studies, however, have suggested additional mechanisms. In a 1991 study, Callen et al. characterized 10 small supernumerary rings using FISH *(89)*. 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 *(90)*. A number of ring chromosomes that are composed of discontinuous sequences have also been reported in the literature, suggesting still other mechanisms of ring formation. Some of these discontinuous ring chromosomes are believed to represent the "breakdown" products of larger rings, whereas others appear to derived from a structurally abnormal chromosome rather than a normal one *(91–94)*.

A novel class of ring chromosomes that lack a traditional centromere has recently been identified *(74)*. In contrast to the traditional ring chromosomes described above, 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 the section Acentric Chromosomes above).

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" *(29,88)*. 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 *(29)*.

In addition to mosaicism, the genetic content and breakpoints of the rings will also have a significant impact on the patient's phenotype. A heterozygote with a 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, 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 on the phenotype of individuals with ring chromosomes is uniparental disomy (UPD) (see Chapter 19). Petersen et al. 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 (95). Uniparental isodisomy for chromosome 21 was present in the normal cell line. The authors suggested that the isodisomy developed when the normal 21 was duplicated in a cell that had lost the ring ("monosomy rescue"). Similarly, Crolla reported a patient with a supernumerary ring 6 in which the normal copies of chromosome 6 showed paternal isodisomy *(96)*. 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 *(97)*. The presence of uniparental heterodisomy (rather than isodisomy as described earlier) suggests that both of the abnormal cell lines in this patient could have arisen secondary to trisomy rescue events (see Chapter 19). Presumably, the original zygote had three copies of chromosome 1: one paternal chromosome 1 and two different maternal chromosome 1's. 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 suggest 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 *(98)*. These patients have 46 chromosomes, 1 of which is a ring chromosome with no detectable 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 *(99)*. Kosztolanyi has proposed that this phenomenon might also contribute to the severity of the phenotype in patients who have ring chromosomes with obvious deletions *(99)*.

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. Among the 32 patients with inherited rings, half had a phenotype similar to the carrier parent, whereas approximately one-third were more severely affected (100). In over 90% of inherited ring chromosome cases, the carrier parent is the mother *(29)*.

In addition to the risks associated with ring instability, carriers of ring chromosomes might 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 *(100)*.

RECIPROCAL AUTOSOMAL TRANSLOCATIONS

Reciprocal translocations represent one of the most common structural rearrangements observed in humans. Estimates of the population frequency range from 1/1000 to 1/673 *(1,101)*. A reciprocal

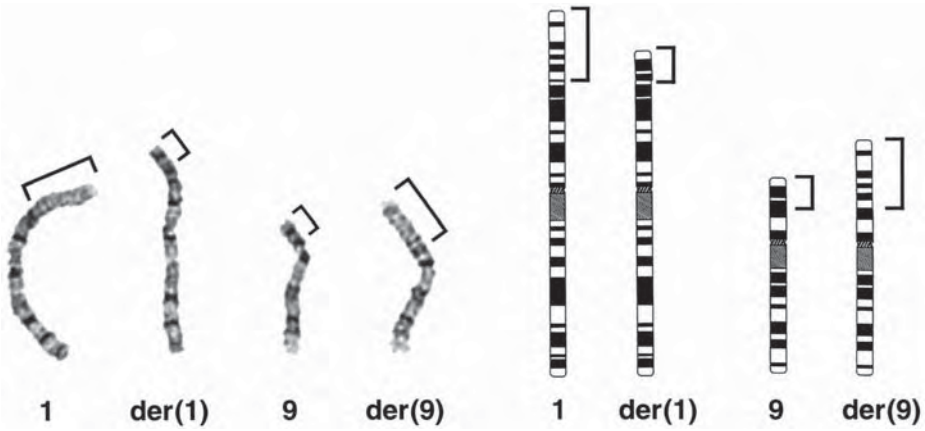

Fig. 16. A balanced reciprocal translocation involving the short arm of chromosomes 1 and 9 [t(1;9)(p32.3;p21)]. The translocated segments of each chromosome have been bracketed.

translocation forms when two different chromosomes exchange segments. In the example shown in **Fig. 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 vise versa. 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 and in Chapter 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 above is diagrammed in **Fig. 17**. Note that the four chromosomes within the quadrivalent have arranged themselves such that pairing between homologous regions is maximized.

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 and 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. The 3:1 and 4:0 segregations also occur, resulting in trisomies and monosomies or tetrasomies and nullisomies, respectively. 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 segregant. The remaining 3:1 and 4:0 segregants appear to be much rarer. Corresponding data are not available for female carriers, as large numbers of oocytes are much more difficult to obtain and study than spermatocytes. We do know, however, that 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 *(102,103)*.

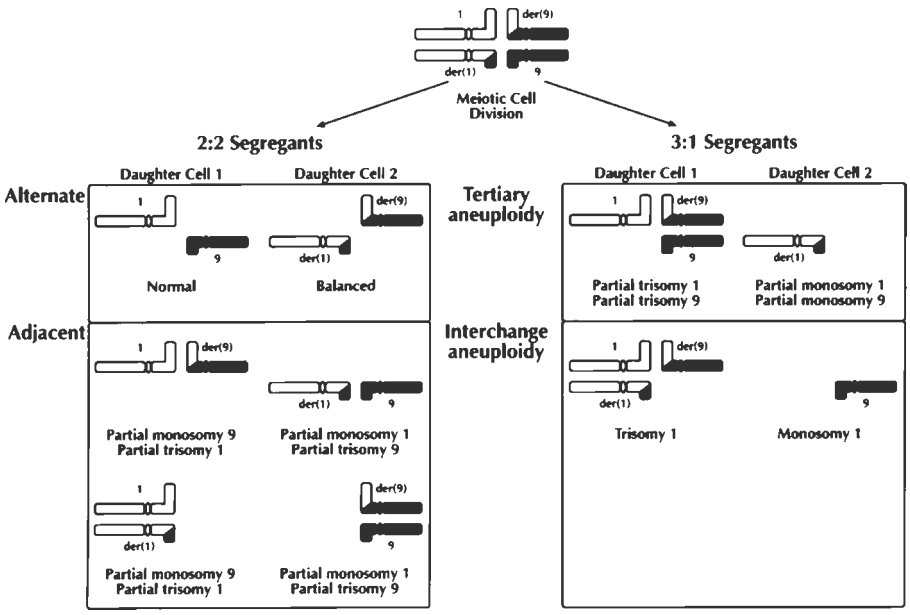

Fig. 17. The expected meiotic pairing configuration for the (1;9) translocation described in Figure 16. Each of the 2:2 and 3:1 segregants typically produced during meiotic cell division are shown.

In addition to being inherited, reciprocal translocations can also occur as new or *de novo* mutations. As discussed in the Introduction, 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 two to three 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 humans (see **Fig. 18**). Evidence for a second recurring translocation, a 4;8 translocation with breakpoints at 4p16 and 8p23.1, has only recently been reported (see below).

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 recurrence of this translocation is best explained by (1) the efficient transmission of a single ancient unique translocation through multiple generations or (2) multiple independent translocation events between two susceptible regions. However, we now know that the latter is true. Mapping studies involving many different unrelated families have demonstrated that the translocation breakpoints cluster within long AT-rich palindromic sequences. [A palindrome is a DNA sequence that contains two inverted regions that are complementary to each other *(104,105)*.] The breakpoints are located at the tip of the imperfect hairpin or cruciform structures that are predicted to form. Palindromic sequences that are predicted to form hairpinlike secondary structures have also been implicated in the formation of at least one other translocation, a nonrecurring (17;22) translocation *(12)*. Although exactly how these structures promote this translocation is unknown, it has been suggested that they might be susceptible to nicking by hairpin-specific nucleases. Once nicked, these structures would then become susceptible to other nucleases that produce double-stranded breaks and further erosion of the palindromic DNA surrounding the initial nick

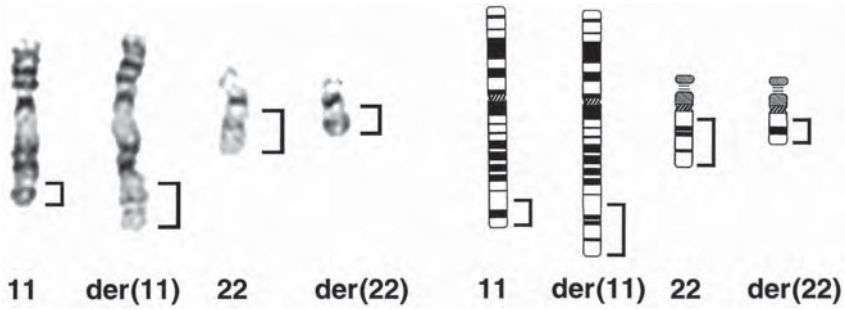

Fig. 18. A balanced reciprocal translocation involving the long arm of chromosomes 11 and 22 [t(11;22)(q23.3;q11.2)]. This is the first recurring constitutional translocation reported in multiple, apparently unrelated families.

site. Illegitimate (nonhomologous) recombination between the resulting double-stranded breaks, perhaps secondary to some existing repair mechanism, is then predicted to produce the recurring (11;22) translocation. Only future studies will determine whether this current model for the formation of the (11;22) translocation is accurate.

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. We know, for example, that a carrier's empiric risk for having a liveborn child with an unbalanced karyotype is 2–10% *(106,107)*. We also know 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. These individuals are therefore trisomic for the distal long arm of chromosome 11 and the proximal long arm of chromosome 22. Mental retardation, congenital heart disease, malformed ears with preauricular skin tags and /or pits, a high arched or cleft palate, micrognathia, anal stenosis or atresia, renal aplasia or hypoplasia, and genital abnormalities in males are common features shared by these unbalanced (11;22) segregants.

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 might have a predisposition to breast cancer *(108)*. Although, cytogenetically the breakpoints involved in this translocation appear to be identical to those identified in the acquired chromosome rearrangements seen in Ewing's sarcoma, peripheral neuroepithelioma, and Askin tumor, molecular studies have shown that they differ *(109–111)* (see also Chapter 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 *(7,112,113)*. 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-Hirshhorn patients with pure 4p deletions (see **Table 1**) *(112)*. Both groups of patients demonstrate mental retardation, poor growth, hypotonia, heart defects, and an abnormal facies, including hypertelorism, prominent forehead, broad nasal bridge, large downturned 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 *(114)*.

It has recently 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 *(7)*. Among the six individuals whose translocation breakpoints were examined, the 8p23 breakpoint was confined to a single olfactory receptor cluster, whereas 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 rearrangement that are mediated by the same 8p23 olfactory receptor gene clusters implicated in the (4;8) translocation *(64)*.

Heterozygosity for a submicroscopic inversion has also been reported in association with several other chromosome rearrangements. In approximately 30% of the Williams syndrome families studied, a submicroscopic inversion that spans the same LCR sequences that mediate the common 1.5-Mb deletion was observed in the transmitting parent (45,46). This inversion was never seen within the nontransmitting parent or within any of the 26 unrelated control individuals who were examined. Similarly, as discussed previously, Jobling et al. have reported an association between the presence of a submicroscopic Y chromosome short arm inversion and short arm translocations between the X and Y chromosomes, leading to the formation of XX males and XY females (65). How heterozygosity for small inversions promotes the formation of these chromosome rearrangements is not currently known. It has been suggested, however, that these rearrangements might be the consequence of abnormal homologous chromosome pairing caused by the presence of heterozygosity for the associated inversion (see the section Paracentric Inversions above).

ROBERTSONIAN TRANSLOCATIONS

A Robertsonian translocation occurs when the long arms of any two acrocentric chromosomes *(13,14,15,21,22)* join to produce a single metacentric or submetacentric chromosome (see **Figs. 19** and **20**). Although these translocations might 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 *(115)*. 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 could promote the formation of Robertsonian translocations.

Since Robertsonian translocations were first described by WRB Robertson in 1916, we have come to recognize that these translocations are among the most common balanced structural rearrangements in the human population *(116)*. Numerous studies examining both spontaneous abortions and liveborn individuals indicate a frequency of approximately 1/1000 *(117–119)*. Although pairwise association of the 5 human acrocentric chromosomes can form 15 different

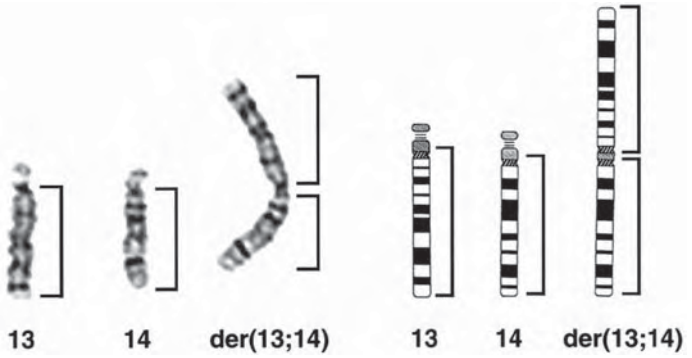

Fig. 19. This (13;14) translocation is the most common Robertsonian translocation observed in man [der(13;14)(q10;q10), sometimes described as der(13;14)(p11.2;p11.2); see Chapter 3].

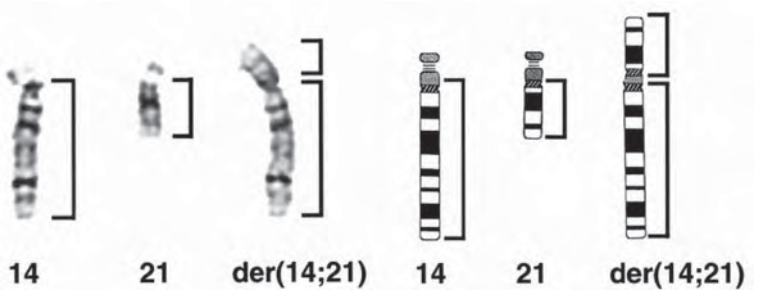

Fig. 20. Although less common than the (13;14) translocation, the Robertsonian (14;21) translocation is more clinically significant because the affected offspring of such a carrier are more likely to survive to birth. Their unbalanced offspring will inevitably have three copies of chromosome 21 long arm material or Down syndrome, a chromosome abnormality that is more compatible with survival than trisomy 13 [der(14;21)(q10;q10)].

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 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 *(29)* (see **Figs. 19** and **20**). Molecular studies performed to explore the origins of these rearrangements suggest that they occur predominantly during oögenesis *(16,120)*.

Despite the monocentric appearance of many of these chromosomes, most are, in fact, dicentric *(121–123)*. Therefore, the majority of these chromosomes 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, whereas multiple short arm breakpoint regions are utilized during formation of each of the remaining types of Robertsonian translocation *(121,124–126)*. Precisely where the breakpoint occurs within the short arm therefore seems to be dependent on the type of Robertsonian translocation being formed, and perhaps the mechanism responsible for the rearrangement. Although 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 *(73,127)*. 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 *(128)*. It should be noted that current nomenclature (see Chapter 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 *(82,123)* and several of them have been shown to form postmeiotically *(87,129,130)*. Although historically all such rearrangements were collectively called homologous Robertsonian translocations, recent molecular studies have shown that approximately 90% of the chromosomes within this category might actually be isochromosomes composed of identical rather than unique homologous arms *(82,83,86,131)*. 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 homolog 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 (UPD) or the inheritance of both copies of a chromosome pair from a single parent (see Chapter 19). The relative risk for each of these outcomes is a function of the sex of the heterozygous parent and/or the particular acrocentric chromosome 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. Because 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 could 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 Chapter 12).

Occasionally, abnormal offspring with UPD have also been observed among the children of balanced Robertsonian translocation carriers *(27)*. 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%, whereas that for a fetus with a balanced homologous Robertsonian translocation is predicted to be approximately 66% *(132)*. 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 *(133)*. 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 UPD 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 could occur as well *(132–134)*. Current data indicate that UPD is most concerning when

Robertsonian translocations containing chromosomes 14 or 15 are involved, because both chromosomes appear to have imprinted regions. Maternal and paternal UPD for chromosome 15 result in Prader–Willi syndrome and Angelman syndrome, respectively *(135,136)*. Clinically abnormal offspring have also been documented in association with paternal and maternal UPD for chromosome 14 *(137–141)*. A single reported case of maternal UPD 14 in a normal individual has created uncertainty regarding the association between maternal UPD 14 and phenotype *(138)*. Because UPD involving chromosomes 14 and 15 is associated with an adverse outcome, it has been proposed by the American College of Medical Genetics that prenatal UPD testing be offered when a fetus carrying a balanced Robertsonian translocation involving one or both of these chromosomes is ascertained.

Although 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 might influence the phenotype of all uniparental disomy offspring *(134)*. 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 Chapter 19).

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 *(29,142)*. 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. However, there is some recent evidence suggesting that female Robertsonian translocation carriers could produce greater numbers of unbalanced gametes than their male counterparts *(143)*.

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 *(144–150)*. Jewett et al. have described an individual with four different cell lines in which long arm material of chromosome 15 was translocated to five different sites *(150)*. 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 *(151,152)*. Tomkins, for example, describe a mother and daughter with different, apparently balanced translocations involving the same short arm segment of chromosome 11 *(151)*. 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 *(146,150,152,153)*. 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 might play a role. 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 orienta-

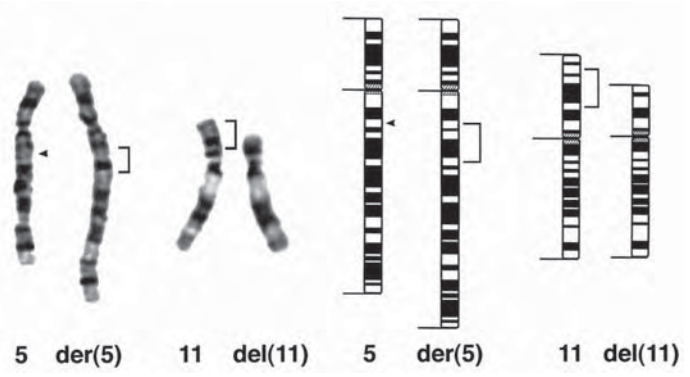

Fig. 21. Insertion. A portion of chromosome 11 short arm material has been inserted into the proximal long arm of chromosome 5 to produce an apparently balanced, inverted, interchromosomal insertion [ins(5;11)(q13.1;p15.3p13)]. The individual who carries this insertion was ascertained following the birth 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.)

tion 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, whereas 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. 21**.

Three-break rearrangements, of which insertions are an example, are extremely rare. Chudley et al. estimated that they occur 10 times less frequently than two-break rearrangements, or in approximately 1 in 5000 live births *(154)*. Madan and Menko found only 27 reported cases of intrachromosomal insertions, whereas Van Hemel and Eussen found only 87 cases of interchromosomal insertions reported in the literature *(155,156)*. Although these complex rearrangements are rare, they can be associated with a very high risk for abnormal reproductive outcome. The unbalanced offspring of insertion carriers typically have either a pure partial monosomy or a pure partial trisomy.

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 *(66,157,158)*.

During meiotic pairing, the inserted segment and its complementary region on the normal chromosome can loop out, allowing synapsis or pairing of the rest of the chromosome (see **Fig. 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

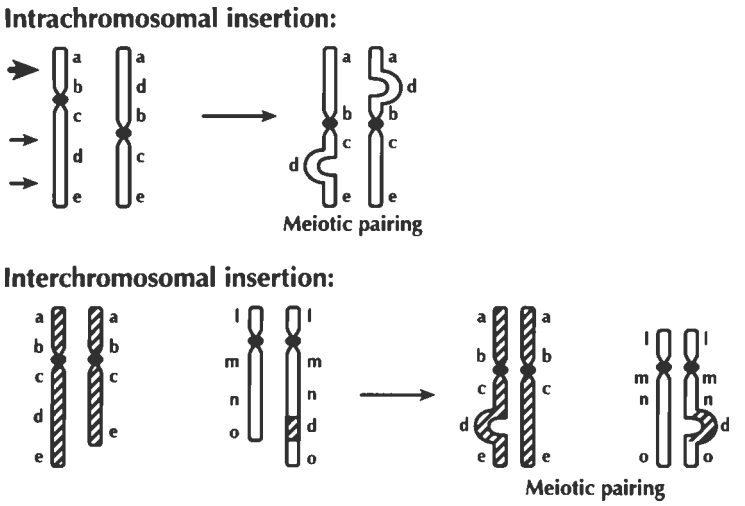

Fig. 22. Models for meiotic pairing during which partial pairing is observed between the insertion chromosome and its homolog.

insertion will have a liveborn child with an unbalanced karyotype *(155)*. This risk might 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. The incidence of interchromosomal insertions is estimated to be approximately 1/80,000. Approximately 85% are inherited, usually from a carrier mother, and no fertility differences were noted between the two sexes *(156)*.

For relatively small inserted segments, it seems most likely that the homologs involved in the rearrangement will pair independently *(159)*. The inserted segment and its homologous region on the normal chromosome can loop out, allowing full pairing of the uninvolved segments of the bivalents (see **Fig. 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 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 might be formed, allowing complete pairing of the chromosomes involved in the rearrangement *(160)*. If no crossover occurs within the insertion loop, the consequences are the same as described earlier for nonpaired bivalents. If a crossover occurs within the insertion loop, however, recombinant chromosomes that would lead to the production of gametes with duplications and deletions might 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. Van Hemel 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 *(156)*. The theoretical risk, as mentioned above, 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 *(161)*. 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. 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. 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 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 could partially explain this observation *(142,162–164)*. Selection against grossly unbalanced gametes at fertilization could also play a role. As discussed in the Introduction, the actual reproductive risks for any CCR carrier will vary depending on the precise rearrangement involved, as well as many other variables.

VARIANT CHROMOSOMES

There are a number of structural chromosome rearrangements that have no apparent clinical consequences for the patients that carry them *(165–169)*. 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 (see **Fig. 8** and the section Pericentric Inversions above). Whereas 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, whereas 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) provide another major source 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 above 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, an attempt might be made to (1) document the variant chromosome in multiple normal family members and (2) further characterize the variant chromosome using molecular techniques such as FISH (see Chapter 17). This more extensive work-up would be done to ensure that one has correctly interpreted the rearrangement and has not overlooked the presence of imprinting or a more complex rearrangement with reproductive consequences for the family.

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10

Sex Chromosomes and Sex Chromosome Abnormalities

Cynthia M. Powell, MD

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 liveborn infants, children, and adults. Physicians in many specialties, including pediatrics, obstetrics and gynecology, endocrinology, internal medicine, and surgery, commonly encounter individuals with sex chromosome abnormalities. 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 abnormalities, and disorders of sexual development with normal chromosomes.

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). 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, the gonad differentiates into an ovary. The term "phenotypic sex" refers to the appearance of the external genitalia and in some disorders might not correspond to the genetic or gonadal sex (see the section Sex Reversal).

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 *(2)*.

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 *(3)*. A

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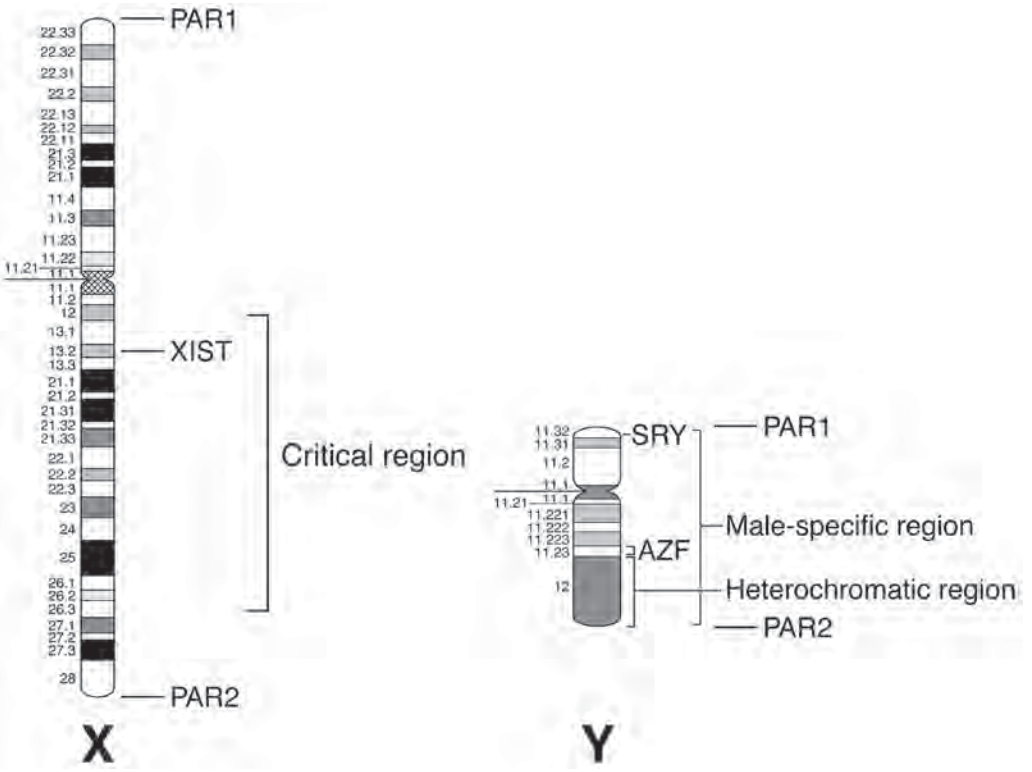

Fig. 1. Ideograms of the X and Y chromosomes showing the pseudoautosomal regions (PAR1 and PAR2), the locations of the X-inactive-specific transcript (*XIST*) gene, the critical region on Xq, the sex-determining region Y (*SRY*), the azoospermia factor region (AZF), the heterochromatic region of the Y, and the male-specific region located between PAR1 and PAR2.

gene that seems to control X inactivation is *XIST* (X-inactive-specific transcript) and is located at the X-inactivation center (XIC) at band Xq13 (see **Fig. 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 *(4)*. About 15% of genes on the X chromosome escape inactivation and remain active on both X chromosomes in females *(5)*. Many more genes on Xp escape inactivation as compared to Xq *(3)*. 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 *(3)*.

Early evidence for the existence of the inactive X was the observation of the Barr body, named for the Canadian cytologist Murray Barr *(6)*. 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 women, but not in men *(7)*.

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) *(8)*. Newer methods for detecting the inactive X involve molecular techniques often using differential methylation analysis *(9–13)*.

Pseudoautosomal Regions

The distal region of the short arms of the X and Y chromosomes contain highly similar DNA sequences. During normal meiosis in the male, an XY bivalent is formed and crossing-over occurs between these regions. Because this resembles the crossing-over that occurs between autosomes, these regions have been termed pseudoautosomal or PAR1 (see **Fig. 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 *(14)* (see also Chapter 2).

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 (see **Fig. 1**). Twelve of the MSY genes are ubiquitously expressed in many organs throughout the body, whereas 11 MSY genes are expressed predominantly in the testes *(15)*.

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 *(16)*. It is located on the short arm of the Y at band p11.3 in the MSY region (see **Fig. 1**). Deletions and mutations in this gene have been found in some 46,XY females (see below).

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) *(17)* (see **Fig. 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 *(18)* (see Chapter 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 *(19)*. Deletion mapping has localized this putative gene to a region near the centromere, but it has raised the possibility of multiple *GBY* loci dispersed on the Y chromosome *(20,21)*. One of the most likely candidate genes in this region is *TSPY*, which is expressed in gonadoblastoma tissues *(22)*.

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 abnormalities 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 abnormalities would have gone through life undetected, as they do not have physical or developmental problems that would have warranted a chromosome study *(23)*. Women undergoing amniocentesis and chorionic villus sampling (see Chapter 12) should be informed about the possibility of detecting a sex

chromosome abnormality *(23)*, 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. Cytogenetic labs reporting results to physicians and genetic counselors discussing results with patients should provide updated and accurate information about these conditions. It is important for couples faced with having a child with a sex chromosome abnormality to see the potential problems for developmental delay in the context of any chromosomally normal child having a risk of developmental delay *(23)* (by definition, a 5% chance).

There appears to have been a trend toward a higher rate of pregnancy continuation in more recent years *(24)*. This might be at least in part from results of long-term studies of individuals with sex chromosome abnormalities revealing a better prognosis than previously reported *(25)*. 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 *(26–32)*.

Turner Syndrome

45,X (and its variants) occurs in approximately 1 in 2000 to 1 in 5000 live female births, but it 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 *(33)* and a report that it was caused by a single X chromosome appeared in 1959 *(34)* (see Chapter 1). 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 (see **Fig. 2**). 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 *(35)* (see below).

Origin of the X Chromosome in Turner Syndrome

In approximately 75% of patients with 45,X, the X chromosome is maternal in origin *(36,37)*. There is no parental age effect *(37)*. Although phenotypic differences have not been found between Turner patients with a maternal or paternal X chromosome, there might be some cognitive differences, particularly in memory function *(38)*. This has been theorized to be on the basis of an imprinted X-linked locus *(39)*.

Phenotype

Clinical features of Turner syndrome in newborns could include decreased mean birth weight (average weight = 2800 g), posteriorly rotated ears, neck webbing (see **Fig. 3A**), and edema of hands and feet (see **Figs. 3C,D**), although many are phenotypically normal. Older children and adults with Turner syndrome have short stature and variable dysmorphic features that could include downslanting eyes, posteriorly rotated ears, low posterior hairline, webbed neck (see **Fig. 3A**), a broad chest, short fourth metacarpals (see **Fig. 3B**), and cubitus valgus. Congenital heart defects, especially coarctation of the aorta, and structural renal anomalies are common and should be checked for. There is primary amenorrhea and, without hormonal supplementation, lack of secondary sex characteristics. The gonads are generally streaks of fibrous tissue. Intelligence is average to above average, although there is an increased risk of cognitive and behavioral problems. Standard treatment includes use of growth hormone and estrogen. It is recommended that these patients be followed by endocrinologists familiar with Turner syndrome. The American Academy of Pediatrics has published guidelines for health supervision for children with Turner syndrome *(40)*.

The *SHOX* gene, located in the distal part of the pseudoautosomal region on Xp, escapes X inactivation. Haploin sufficiency for *SHOX* causes short stature and Turner skeletal features. *(41–43)*. A gene determining lymphedema has been proposed at Xp11.4 *(44)*

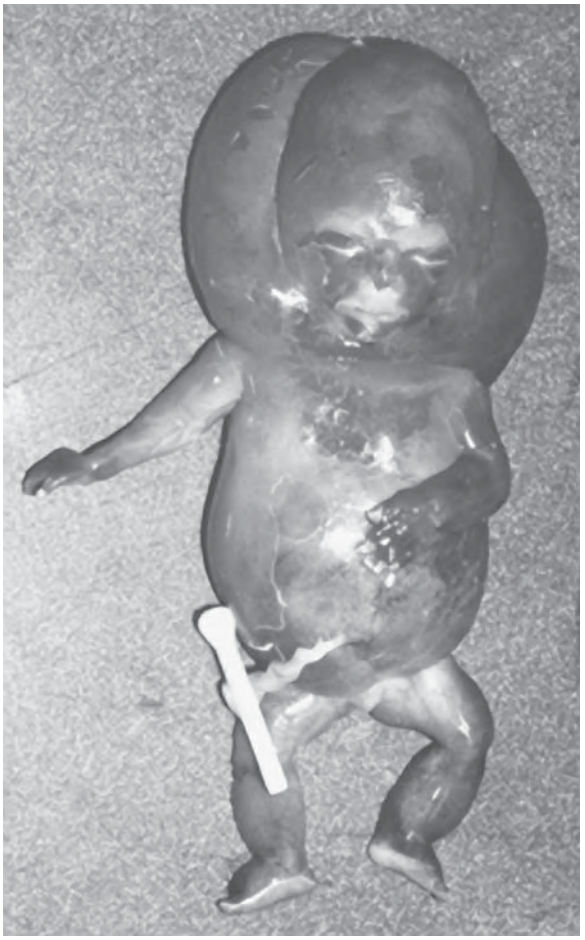

Fig. 2. A 45,X fetus with large cystic hygroma and hydrops.

Turner Syndrome Variants

Approximately half of all patients with Turner syndrome have a 45,X karyotype. The remainder exhibit mosaicism and other structural abnormalities of the X chromosome. In a study of cytogenetic and cryptic mosaicism in 211 patients with Turner syndrome, Jacobs et al. *(45)* reported pure 45,X in 46%, 47% had a structurally abnormal sex chromosome (41% with abnormal X and 6% with abnormal Y), and 7% had a 46,XX or 47,XXX cell line. 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 the lymphocytes of 15–20% of patients with Turner syndrome. A 46,XX cell line might 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% *(46)*. In a patient with several typical features of Turner syndrome but normal lymphocyte chromosome analysis, analysis of another tissue such as skin should be considered *(46)*.

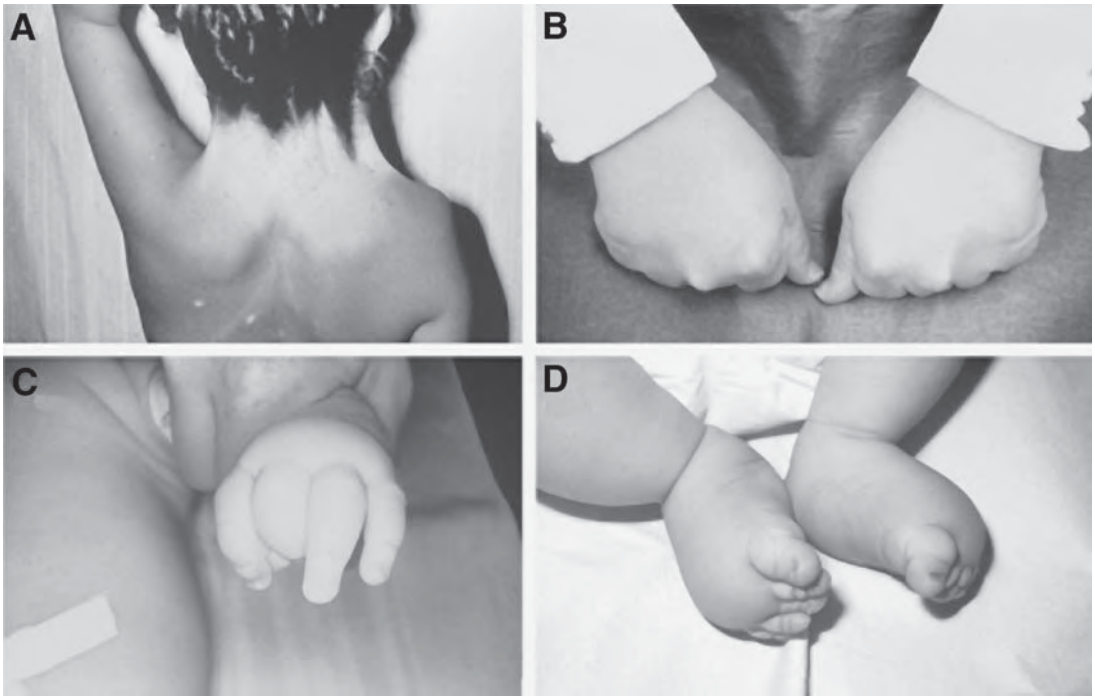

Fig. 3. Child with Turner syndrome and low posterior hairline and webbed neck **(A)** and short fourth metacarpals **(B)**. Infant with Turner syndrome and lymphedema of the hand **(C)** and feet **(D)**.

Mosaicism with a Y chromosome

Patients with 45,X/46,XY mosaicism can 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 *(47)*. 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 *(48)*. In another study, 3 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 mental retardation and autism were seen in four and two patients, respectively, in this series, although there might have been biased ascertainment *(49)*. 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 *(49)*. 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 fourteen cases of Turner syndrome with at least 1 cell line with an abnormal Y chromosome *(50)*. Ten patients had a pseudodicentric Yp chromosome, two had an isodicentric Yq, one had a pseudodicentric Yq, and one had a derived Y. 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 Y Chromosome). In a study of 211 patients with Turner syndrome, Jacobs et al. *(45)* found a clinically significant structural abnormality of the Y chromosome present in 13 patients (6%). One hundred cells were counted from each patient. No patients were found to have 45,X/46,XY 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 *(51)*. 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 *(52)*. 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. Gonadoblastoma was found in 10% of patients with Y chromosome material who had ovariectomy performed *(53)*. This patient had a 45,X/46,XY karyotype. None of the patients with only PCR-detected Y chromosome material developed gonadoblastoma. Nishi et al. studied 122 patients with Turner syndrome and compared use of nested PCR in these patients versus 100 women with no known chromosome abnormality *(54)*. 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 *(55)*. Jacobs et al. found no occult mosaicism for Y in 178 patients with Turner syndrome using Y-specific PCR primers *(45)*. Thirteen patients had Y-chromosome material detected by routine cytogenetic analysis of 100 cells. Only 2 of the 13 patients had fewer than 10% of cells with a Y chromosome: 1 had 7% and 1 had 8%. It is likely that these would have been detected with standard analysis of 30 cells *(45)*.

These studies suggest that the presence of cryptic mosaicism involving the Y chromosome is rare and might be 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 *(54)*. 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 because of 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 (see Chapter 17) to look for Y-chromosome mosaicism in 45,X patients has been recommended by the American Academy of Pediatrics *(40)*.

The risk of gonadoblastoma has been estimated at 15–20% in phenotypic females with 45,X/46,XY mosaicism *(56)*. Surgical removal of streak gonads and dysgenetic testes is recommended for these patients. Many studies looking at the incidence of gonadoblastoma have had a selection bias. In a cross-sectional population study of patients with Y-chromosome material and Turner syndrome, 10% had gonadoblastoma detected at the time of surgery *(53)*. Some but not all patients with Turner syndrome and a Y cell line will have masculinization. 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 *(57)*.

Isochromosome X

An isochromosome X [i(X)(q10)] (see **Fig. 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. The X isochromosomes can be monocentric or dicentric, although most are dicentric *(45,58)* and result from breakage of a single X chromosome in proximal Xp with

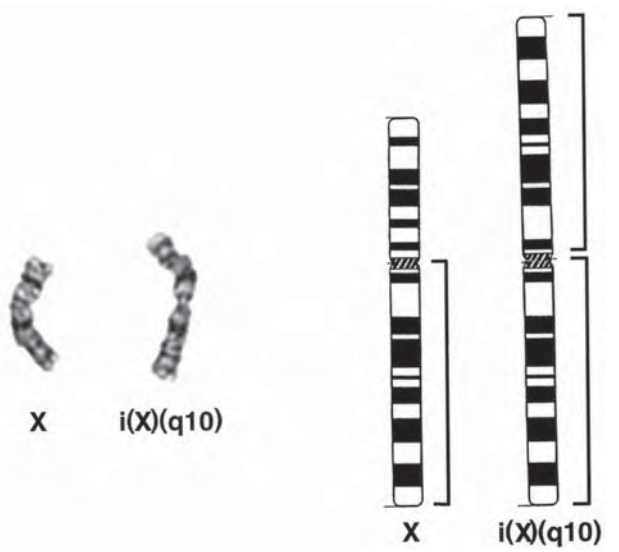

Fig. 4. Normal X chromosome and isochromosome Xq: 46,X,i(X)(q10) in a patient with Turner syndrome. Brackets indicate region of duplication on Xq.

subsequent reunion of the sister chromatids. The i(Xq)s are equally likely to be maternal or paternal *(45)*. The error occurs during male or female gametogenesis. When the error occurs postzygotically, it is likely to be at the first postzygotic division because 45,X/46,XX/46,Xi(X)(q10) mosaics are extremely rare *(45)*.

These patients are phenotypically indistinguishable from those with pure 45,X, although there have been reports of an increased risk of autoimmune problems in patients with an isochromosome X *(59)*. In a study of 145 women with Turner syndrome, 83% with an X isochromosome had positive thyroid autoantibodies compared with 33% of women with other karyotypes. The isochromosome-X women were also more likely to become hypothyroid and require thyroxine *(60)*.

Ring X

A subset of patients with Turner syndrome, estimated at approximately 15%, have one normal X chromosome and a ring X, most often as a mosaic cell line *(45)*. 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 Chapter 19). 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 *(61)*. 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 Chapter 17) with a painting probe for the X to confirm that it is derived from the X and not Y *(62)* (see the section Mosaicism with a Y Chromosome).

In general, ring X patients lack many of the classic somatic features of Turner syndrome *(63)*. Those with ring X often do not have lymphedema and, therefore, are less often ascertained at birth compared to 45,X individuals *(64)*. Although some have typical features of Turner syndrome, others have a severe phenotype with mental retardation, facial dysmorphism, and congenital malformations. Some patients with a ring X and severe phenotype have features similar to those of patients with Kabucki syndrome, a multiple malformation syndrome *(63)*. El Abd et al. reported a characteristic behavioral profile in five patients with a small active ring X that included autisticlike features, obsessive compulsive behavior, and social and communication problems *(65)*.

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 *(66–68)*. Larger rings have *XIST* present and are preferentially inactivated. However, Turner et al. *(69)* reported that in seven females with 45,X/46,r(X) and an *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 (see Chapter 19). 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 *(63,68–70)*. 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 *(70)*. Migeon et al. reported two patients with inactive ring X chromosomes, mental retardation, and a severe phenotype *(71)*. 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 *(69)*. 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 *(70)*. 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 *(71)*. 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 *(62)*.

45,X/47,XXX Mosaicism

Approximately 2% of patients with Turner syndrome have a 45,X/47,XXX mosaic karyotype *(45,72)*. 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 or 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 *(72)*.

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 45,X/46,XY Mosaicism above) 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

This is the most frequent sex chromosome abnormality present at birth in females, occurring in 1 in approximately 1000 live female births *(73)*. It was first described in 1959 by Jacobs et al. *(74)*. Unfortunately, the term originally used for this cytogenetic abnormality was “superfemale,” which gives a misconception of the syndrome and is no longer in use.

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 extra 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 *(75)*. In general, as adults, these women have moderately tall stature, with an average height of 172 cm (5 ft 7 in.). Pubertal development is normal and most have normal fertility, although a small number have ovarian dysfunction *(76)*. There is a small but slightly increased risk of chromosomally abnormal offspring of 47,XXX women *(77,78)*. 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. Most have good health, although one study found that 25% had recurrent nonorganic abdominal pain as teenagers *(79)*.

Development

47,XXX females usually have normal intelligence, but most have IQs less than their siblings. There is a great deal of variability in this syndrome, and some women have normal intelligence and are well adjusted. However, they are represented in mentally and developmentally disabled institutionalized populations at four to five times greater a rate than would be expected based on the incidence in newborns *(76,80)*. Precise predictions regarding an individual child's prognosis are not possible *(75)*, but as for all of the common sex chromosome abnormalities, there does appear to be a risk for mild to moderate developmental problems in the areas of motor, speech and language, and learning *(75,82)*.

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 *(82)*. Most showed early delays in motor, language, and cognitive development and were described as shy, withdrawn, and immature, with poor coordination *(75)*. The full-scale IQ was 26 points lower than in normal sibling controls, the average IQ was in the 85–90 range, and patients were at the 24th percentile in academic achievement scores, but mental retardation was rare. Nine of the 11 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 abnormalities, 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 *(82)*. In adulthood, most of these women were employed full time (unskilled or semiskilled jobs), had married, and had had children *(83)*. In another long-term study, 47,XXX women were found to have more work, leisure, and relationship problems compared to a control group *(84)*. They were found to have poorer psychosocial adaptation and more psychiatric impairment compared to their female siblings. However, most were self-sufficient and functioned reasonably well as adults. Severe psychopathology and antisocial behavior was rare and there was a larger variability in behavioral phenotype than originally appreciated *(84)*. 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 *(85)*.

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 *(86)*. Another longitudinal study by Linden and Bender of 17 47,XXX females, ages 7–18, initially diagnosed prenatally, found that 11 needed academic assistance and 7 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 *(87)*. 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 *(84)*.

Variants with Additional X Chromosomes

48,XXXX

As compared to 47,XXX, there is almost always mild to moderate mental retardation with an average IQ of 60, ranging from less than 30 to 75 *(88)*. One patient was reported to have a normal IQ *(89)*. Phenotypic features include mild hypertelorism, epicanthal folds, micrognathia, and midface hypoplasia *(90)*. Tall stature is common, with an average height of 169 cm (approximately 5 ft 6 in.) *(88)*. 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 *(91)*. Speech and behavioral problems are common. Fertility is reduced and primary ovarian failure has been reported, although some have had chromosomally normal offspring *(92)*.

49,XXXXX

Phenotypic features seen in penta-X females include short stature, microcephaly, up-slanting palpebral fissures, low hairline, and coarse, Down syndrome-like facial features. Congenital heart and renal anomalies have been reported *(93–95)*.

Most patients have moderate mental retardation (IQ range: 20–75; average IQ: 50) *(88,96)* and are described as shy and cooperative *(88)*. There have been no reports of pregnancy in women with this chromosomal aneuploidy *(88)*. Nondisjunction in successive meiotic divisions is the probable mechanism, and molecular studies have shown that, at least in some cases, the extra X chromosomes are all maternally derived *(97,98)*. One patient with penta-X and hyper-IgE syndrome has been reported *(99)*.

47,XXY (Klinefelter Syndrome)

Klinefelter syndrome was the first sex chromosome abnormality to be described (100) and its cytogenetic cause identified *(101)* and is the most common cause of hypogonadism and infertility in males *(96,102)*. It is found in approximately 1 in 575–1000 newborn males *(103)*.

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 *(104)*. A recent study found the extra X chromosome to be paternal in approximately 25% of cases and these were associated with increased paternal age *(105)*.

Phenotype

Males with 47,XXY 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 *(79)*. Head circumference during infancy is usually average, but beginning at age 4 years, it tends to be below the mean for age, although generally within normal limits *(106)*. 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. Testes are small in adulthood. Almost all have infertility with absent spermatogenesis, tubular hyalinization, and Leydig cell hyperplasia. Many are diagnosed in adulthood, with a chief complaint of infertility, but based on a population study, as many as 74% might never be diagnosed *(23)*. 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.

There have been 38 reports of pregnancies resulting from intracytoplasmic sperm injection (ICSI) from nonmosaic 47,XXY patients. These pregnancies produced 34 karyotypically normal neonates, two karyotypically normal pregnancy losses, one healthy unkaryotyped neonate, and one 47,XXY prenatally diagnosed fetus *(107)* (see Chapter 11).

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 differences and dyslexia *(108)*. IQs are lower than in controls and compared to siblings, with the average between 85 and 90, although there is a wide range *(79)*. 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 neuromotor deficits, shy, immature, restrained, reserved, and lacking confidence *(79)*. 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 *(82)*. 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 *(82)*. Another long-term study of this group found that as adults, 10 of 11 were employed full time and 8 had married *(83)*.

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 *(86)*. In a long-term study of 14 prenatally diagnosed boys with Klinefelter syndrome followed to 7–18 years, 10 had average school performance, 8 required educational assistance, 3 had attention deficit disorder, and 4 had speech problems. In general, they were healthy and had pleasant personalities *(87)*.

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 is strongly recommended because the prognosis can be improved significantly with appropriate therapeutic intervention *(108)*.

Variants with Additional X or Y Chromosomes

48,XXYY

This is the most common variant of Klinefelter syndrome *(88)*. There is overlap between this condition and both Klinefelter and XYY syndrome (see below). The incidence is estimated at 1 in

50,000 male births *(109)*. 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 *(88)*.

Most 48,XXYY patients have mild mental retardation, although IQs ranging from 60 to 111 have been reported. Psychosocial and behavior problems are generally more severe than in 47,XXY individuals *(110)*, although patients without significant behavior problems have been reported *(88)*. Four patients with 48,XXYY observed over a 10-year period had psychiatric disorders, including aggressiveness, self-injury, and mental retardation *(111)*.

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 *(112)*.

48,XXXY

This is a rare condition, with more abnormal features and mental retardation than 47,XXY. It was first described by Barr et al. in 1959 *(113)*. Stature is normal to tall and dysmorphic features include epicanthal folds, hypertelorism, protruding lips, prominent mandible, and 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 mental retardation, with most in the 40–60 IQ range, although an individual with an IQ of 79 has been reported *(88)*. Most have speech delay, slow motor development, and poor coordination. Behavior is immature, with personality traits described as passive, pleasant, placid, and cooperative *(88)*.

49,XXXXY

49,XXXXY has an approximate incidence of 1 in 85,000 male births and over 100 cases have been described in the literature since this karyotype was first reported by Fraccaro et al. in 1960 *(114)*.

Common features include low birthweight, short stature in some patients, craniofacial features consisting of round face in infancy, coarsening of features in older age, with hypertelorism, epicanthal folds, and prognathism *(112)*, and a short, broad neck *(88)*. Congenital heart defects are found in 15–20% *(88,115)*, with patent ductus arteriosus the most common defect described, but atrial ventricular septal defects and tetralogy of Fallot also reported *(116)*. Skeletal anomalies include radioulnar synostosis, genu valgus, pes cavus, and fifth finger clinodactyly. Muscular hypotonia and hyperextensible joints are present. Genitalia are hypoplastic, and cryptorchidism with hypergonadotropic hypogonadism is common *(88)*. Because of the distinctive phenotype, some authors have suggested classifying this condition separately from Klinefelter syndrome *(116)*.

Mild to moderate 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 *(116)*. Behavior has been described as timid, shy, pleasant, anxious, and irritable *(88)*. Testosterone replacement therapy has been found to be beneficial in some patients, with improvement in attention and behavior *(117)*.

49,XXXYY

Only five cases of liveborn males with this sex chromosome abnormality 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 autisticlike behavior *(88)*.

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,XXXXX *(97,98,118–122)*. 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 *(98,122)*.

For cases of 48,XXXY studied with Xg blood groups or other polymorphic markers, two are maternal and five are paternal in origin *(36,98,119,123–125)*. 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 *(98)*.

47,XYY

One in 800 to 1000 males has an extra Y chromosome *(103)*. This arises through nondisjunction at paternal meiosis II. 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 *(126)*. Patients often have severe facial acne. 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 abnormality. Sexual orientation is heterosexual. Individuals are described as somewhat awkward and have minor neuromotor deficits *(79)*. Most have normal fertility and are able to father children. It has been estimated that only 12% of men with XYY are ever diagnosed. One-half of those identified were karyotyped because of developmental delay and/or behavior problems *(23)*.

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 *(79,86)*. Most older boys attended college or have jobs after high school. Longer follow-up of the boys in the second group *(87)* found that most were tall and thin, and all were in good physical health. Five of 14 required extra assistance in school for academic difficulties and 2 were in special education programs for the learning disabled. 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 *(87)*.

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 are 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.

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 extra Y chromosomes. Mild mental retardation to low normal intelligence and sterility have been described *(127,128)*.

49,XYYYY

Four nonmosaic cases of 49,XYYYY have been reported. Facial features include hypertelorism, low-set ears, and micrognathia. Skeletal abnormalities include radioulnar synostosis, scoliosis, and clinodactyly. Mental retardation and speech delay, along with impulsive and aggressive behavior, were reported *(88,118,129–131)*.

SEX CHROMOSOME ANEUPLOIDY AND AGE

Increased aneuploidy with advancing age was first reported by Jacobs et al. in 1961 *(132)*. This was subsequently found to be the result of the loss of the X chromosome in females and of the Y in males *(133)*. Premature centromere division in the X chromosome and loss through anaphase lag and formation of a micronucleus is one proposed mechanism in females *(134,135)*. 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 *(136)*. It is usually the inactive X chromosome that is missing in X monosomic cells *(137)*. Another proposed mechanism is telomere shortening, as telomeres play a key role in chromosome segregation *(138)*. X chromosome aneuploidy is not observed in bone marrow preparations from older women, but it is seen in phytohemaglutinin (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 *(136,139)*.

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 *(140)*. Galloway and Buckton found a 10-fold increase in X chromosome aneuploidy in women age 25–35 compared with those between 65 and 75 *(133)*. Between 30 and 55, the rate of hypodiploid cells was 3–5% in females, increasing to 8% at age 70. 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 can be helpful in clarifying these situations.

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 *(136)*. Studies of bone marrow preparations have shown that Y chromosome loss was restricted to males over age 40–50, with a frequency of 8–10% of cells *(141,142)*.

Most studies comparing age-related sex chromosome aneuploidy were done on metaphase preparations and are, therefore, at risk for preparation aneuploidy. Guttenbach et al. *(143)* performed an *in situ* study of lymphocyte interphase nuclei to look at sex chromosome loss and aging. In males, the rate was 0.05% up to age 15, 0.24% in 16- to 20-year-olds, and then steadily increased to 1.34% at age 76–80. 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. Only women over 51 years of age showed a distinct age correlation. This study also found no difference in aneuploidy rates between cultured and noncultured cells *(143)*. 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 *(144)*.

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 Chapter 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.

The molecular X inactivation pattern seems to correlate with phenotype in women with structural abnormalities of the X. 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 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 work-up. The phenotype could be correlated with differences in X inactivation ratios *(145)*. There have been very few reports on the use of prenatal X inactivation studies in amniotic fluid or CVS (*146)* (see Chapter 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 *(147)*. 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 *(148–150)*. There are several different hypotheses concerning the cause of gonadal dysfunction in these cases, including disruption of POF-related genes *(149,151)*, a position effect resulting from local alteration of chromatin caused by the translocation *(148,150*), and incomplete pairing of X chromosomes at pachytene *(152)*. In cases in which the translocated X chromosome is the inactive X, 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;aut) 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 *(153)*. There is strong selection against such cells. In general, the normal X is preferentially inactivated in approximately 75% of such patients *(153,154)*. When the translocation disrupts a gene located on the X chromosome, a female with such a translocation could manifest a disease condition *(155,156)*. Any mutated genes on the translocated X chromosome will be fully expressed, as they would be in a male *(3)*. Several X-linked genes have been mapped in this way.

A "critical region" determining normal ovarian function has been hypothesized at Xq13-Xq26 *(150,157)*. The majority of females with balanced translocations with breakpoints in this region usually have POF (secondary amenorrhea associated with elevated gonadotropin levels before the age of

40). 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 have often shown no gene disruption *(158,159)*. 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 *(160,161)*. Schmidt and DuSart *(153)* found that most X;autosome translocation patients with phenotypic abnormalities or developmental delay had breakpoints clustered in the subtelomeric bands Xp22 and Xq28. 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 *(162)*. 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 *(162)*.

Some studies have indicated that in those patients with phenotypic and/or developmental abnormalities, the translocated X was late-replicating (inactive) *(163)*. 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 *(162)*.

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 mental retardation even though both carry the same such rearrangement. This is 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 *(155)*.

A fertile woman with a balanced X;autosome translocation is at risk for having offspring with an unbalanced rearrangement (see **Fig. 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 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 liveborn child with a structural and/or functional aneuploidy has been estimated at 20–40% *(154)*. Phenotypic abnormalities can range from mild effects to severe mental retardation and birth defects.

Males with balanced X;autosome translocations are usually phenotypically normal but almost all are infertile *(164)* (see Chapter 12). There have been reports of severe genital abnormalities in males with such translocations *(165)* and of multiple congenital anomalies in a man with an apparently balanced (X;6) translocation inherited from his mother *(166)*. As noted earlier, there is also a risk of an X-linked recessive disorder because of disruption of a gene by the translocation.

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 mental retardation and multiple congenital anomalies *(167)*. There have also been patients described who have unbalanced X;autosome translocations but no phenotypic abnormalities and only mild behavioral problems *(168)*.

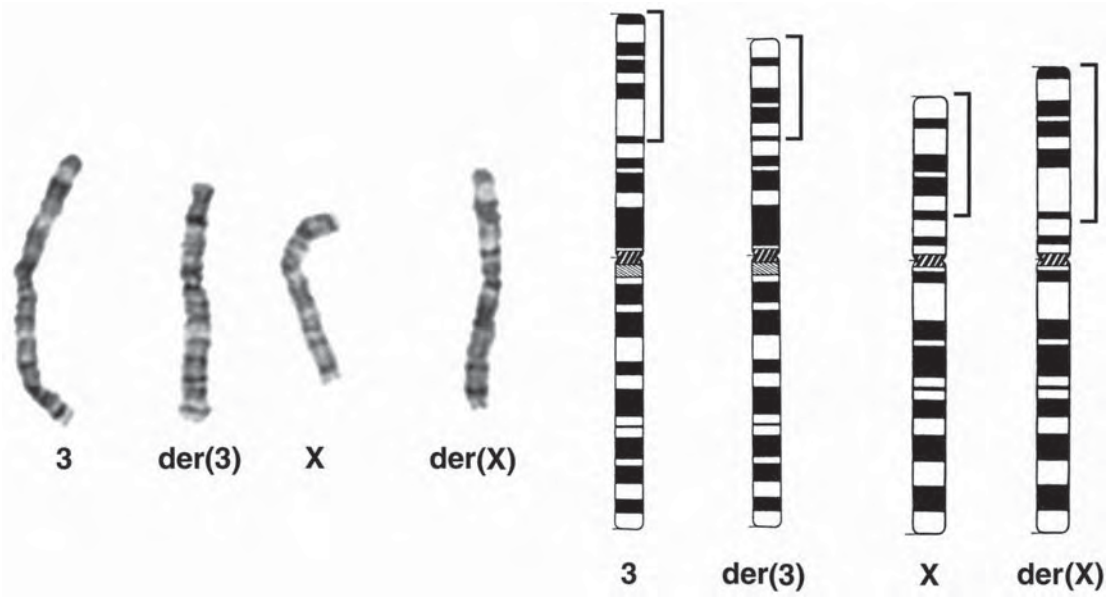

Fig. 5. Balanced reciprocal translocation between the short arms of chromosomes X and 3: 46,X,t(X;3)(p11.3;p21.2). Brackets indicate regions involved in translocation on the derivative chromosomes. The patient was a 30-year-old clinically normal, fertile female who had a daughter with an unbalanced translocation consisting of a normal X, the derivative X, and two normal chromosomes 3 (partial monosomy Xp and partial trisomy 3p).

Earlier studies relied on replication-timing studies 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 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 *(169)*. The spreading of late replication is often incomplete and might skip some autosomal bands and affect others *(170)*. This suggests that autosomal chromatin does not transmit or maintain the inactivation signal as efficiently as the X chromosome *(169)*. Sharp et al. *(169)* reported a family with both a balanced and unbalanced (X;10) translocation segregating. 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 *(171,172)*. 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 *(169)* and should not be used to make predictions of phenotype. 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 *(173)*. 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 *(173)*.

Prenatal detection of an unbalanced X;autosome translocation presents a difficult counseling problem (see Chapter 20). Although there have been reports of affected females having only secondary

amenorrhea or mild developmental delay *(169,173–175)*, many have had a more severe phenotype with mental retardation and birth defects *(173)*.

In males with unbalanced X;autosome translocations, there is *in utero* lethality or, if they survive, multiple congenital anomalies and mental retardation *(176)*.

Deletions of Xp

Males with deletions of the short arm of the X show contiguous gene syndromes characterized by different combinations of phenotypes, depending on the location and length of the deletion *(177)*. X-linked ichthyosis, Kallmann syndrome (anosmia and hypogonadism), mental retardation, and chondrodysplasia punctata (skeletal dysplasia) are seen in males with deletions involving distal Xp. Deletions in Xp21 could cause a contiguous gene syndrome of Duchenne muscular dystrophy, retinitis pigmentosa, adrenal hypoplasia, mental retardation, and glycerol kinase deficiency *(178)*. 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 *(179)*. Females with Xp deletions do not usually manifest any of the recessive disorders because of the presence of a normal X chromosome, although almost all have short stature and some have Turner syndrome phenotypic features. Short stature in these patients is likely the result of haploinsufficiency for the *SHOX* gene, within the pseudoautosomal region on Xp *(41,180)*. 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 *(43,44)*.

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 *(181)*. Females with terminal deletions originating at Xp21 are more likely to show premature rather than complete ovarian failure *(182)*, although they may have normal fertility *(181)*. The phenotypes associated with Xp deletions can vary, even within the same family *(183)*. This is most likely the result of variable X inactivation and modifying genes.

Studies have shown that most *de novo* Xp deletions originate on the paternal chromosome *(179)*. UPD (see Chapter 19) for the deleted and nondeleted X chromosomes was not found in a study of 25 females with Xp deletions *(179)*.

Deletions of Xq

Large Xq deletions in males are not compatible with survival. Smaller deletions are associated with severe phenotypes *(184)*.

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 *(185)*. 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 a variable inactivation of growth genes in Xp or proximal Xq *(186)*. 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 *(182)*. 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 *(187)*. One woman with deletion at Xq27 had fertility and menopause at 43 years. Clinical features of Turner syndrome are less common in Xq as compared to Xp deletions *(188)*, but more common in patients with deletions proximal to Xq25 *(187)*. The likelihood of an abnormal phenotype in a female with an Xq deletion is low, although primary or secondary ovarian failure is likely. 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 of the deletion.

Xp Duplications

Duplications of Xp involving bands p21.2 to 21.3 plus a Y chromosome have been reported in patients who were phenotypic females, suggesting a sex-determining gene locus on Xp *(189,190)*. 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 Females). Dosage-sensitive sex reversal is the result of duplication of the *DAX1* gene, which, when deleted or mutated, leads to congenital adrenal hypoplasia *(191)*. Males with duplications involving more distal Xp have been reported with mental retardation and autism but without sex reversal *(192)*.

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 *(189,190,193)*. The abnormal phenotype, including Turner syndrome features, short stature, seizures, amenorrhea, but normal intelligence, was seen in a female with complete inactivation of the duplicated X, suggesting that random inactivation was not the cause *(193)*. 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 *(194)*. In a review of 52 females with partial X duplications involving Xp or Xq, Matsuo et al. *(195)* found that random or skewed but not completely selective X inactivation occurred in 9 of 45 patients examined for X-inactivation pattern, independent of the size or location of the duplicated segments. For Xp duplications, 4 of 6 patients with random or skewed X inactivation had an apparently normal phenotype, and 3 of 12 patients with selective inactivation of the duplicated chromosome had clinical abnormalities *(195)*.

A dicentric inverted duplication of most of the short arm of the X [dic inv 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 *(196)*.

Females with duplications of Xp including the *SHOX* gene region have been reported with both tall *(197)* and normal stature *(198)*.

Xq Duplications

Males with duplications of the long arm of the X usually have significant mental retardation and birth defects resulting from 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 might be the size or location of the duplicated segment *(199,200)*, random (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 *(199)* (see Chapter 19).

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 *(199)*. In a review of X duplications, Zhang et al. reported that the duplicated segments in individuals with abnormal phenotypes were more frequently located in proximal Xq *(200)*. A review by Matsuo et al. *(195)* 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.

Goodman et al. reported three families with duplication of Xq27-qter on the short arm of Xp *(201)*. 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 been reported to preferentially inactivate the abnormal X chromosome *(202)*. Both normal and abnormal phenotypes can be seen even when there is preferential inactivation of the abnormal X. One

Fig. 6. Distal paracentric inversion of Xq: inv(X)(q26q28) in a woman with normal phenotype and fertility. Brackets indicate region involved in inversion.

of 3 patients with random or skewed inactivation had an abnormal phenotype and 9 of 22 cases with selective inactivation of the duplicated X had an abnormal phenotype *(195)*. The reason for the variable 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 phenotype from those with abnormal phenotype. The functional disomy of genes might affect the phenotype *(202)*.

Replication studies cannot distinguish phenotypically normal and abnormal females with Xq duplications *(193,199)*. Correlations of X inactivation pattern and phenotype in patients with small duplications should be interpreted carefully *(145)*.

Inversions of the X Chromosome

Paracentric Inversions

Paracentric inversions of the X chromosome (see **Fig. 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 *(203)*. When the inversion is outside the critical region, normal phenotype and fertility have been reported *(204)*, although there are exceptions to this *(205)*. 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 *(206)*. Males can be phenotypically normal or have mental retardation *(205,207)*. Fertility in males is also variable *(208,209)*.

Pericentric Inversions

Most females with pericentric inversions of the X have normal phenotypes and fertility *(210,213,214)*. However, pericentric inversions of the X have been reported in females with gonadal dysgenesis and with mental retardation *(211)*. Keitges et al. reported dizygotic twins with the same pericentric X inversion (p11;q22) *(211)*. One twin was phenotypically normal with normal intelligence and menses and had random inactivation of the X. 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 inversion X *(211)*. Offspring of females with pericentric inversions are at risk for inheriting a recombinant chromosome with associated phenotypic abnormalities *(210,213–215)*.

Most males with inherited pericentric inversions of the X have a normal phenotype and fertility *(210,215,216)*. 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 *(217–219)*. 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 *(216,218)*. Inactivation status of the mother might provide helpful information in cases of prenatal detection of a male fetus with a maternally inherited inversion *(220)*.

Isodicentric X Chromosomes

Isodicentric X chromosomes are formed by the fusion of two X chromosomes *(221)*. The phenotypic effects are variable and dependent on whether the chromosomes are fused at long or short arms and whether there is a deletion. No isochromosome of only the short arm is viable because of the lack of *XIST*. Patients with isodicentric X chromosomes joined at their short arms exhibit short or normal stature, gonadal dysgenesis, and, occasionally, Turner syndrome features, whereas those with long arms joined are normal or above average in stature and have gonadal dysgenesis *(222)*, normal intelligence, and no somatic abnormalities *(223)*. Explanations for the phenotype of short stature when the short arms are joined is most likely secondary to deletion of the distal short arm at the region of *SHOX*. Likewise, tall stature could be related to the presence of three copies of *SHOX* in those patients with long arms joined.

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 or homologous chromosomes *(224)*

The isodicentric X is almost always late-replicating, suggesting nonrandom inactivation of the derivative X. The second centromere is nonfunctional, making it a pseudodicentric chromosome *(225)* (see also the subsection Turner Syndrome, Isochromosome X above).

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 because of the risk of gonadoblastoma in females with Turner syndrome. FISH probes have improved the ability to recognize 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

Hsu reviewed 51 reported cases of (X;Y) translocations, 47 with a derivative X and 4 with a derivative Y *(226)*. The (X;Y) translocations with a derivative X were divided into seven types, with the most common types involving translocation of a portion of Yq11.2 → Yqter onto Xp22.3.

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 → Xp22.3::Yq11.2 → 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

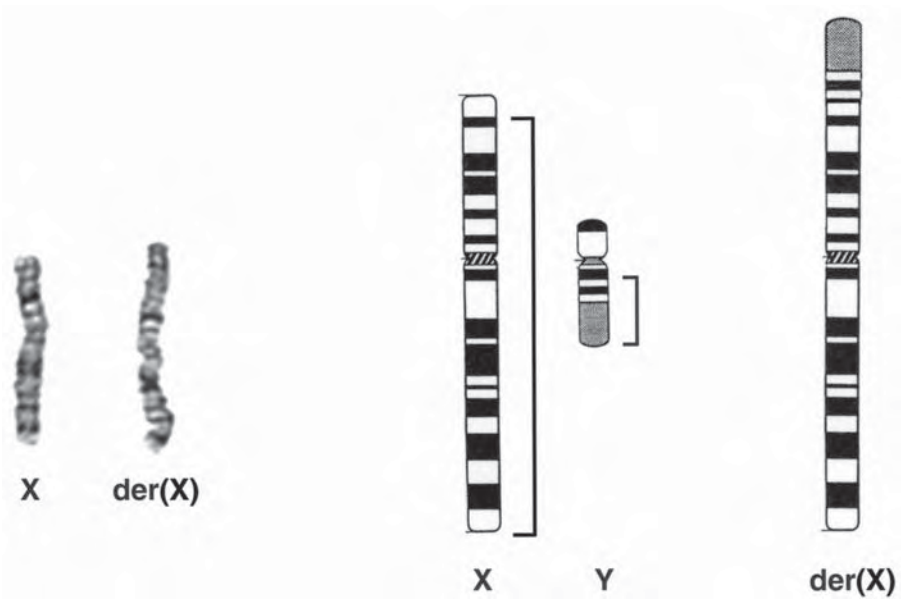

Fig. 7. Derivative X chromosome consisting of a small terminal Xp deletion and translocation of Yq: 46,X,der(X)t(X;Y)(p22.3;q11.2) mat. This was seen in a 5-year-old girl with short stature who had inherited the chromosome from her mother, who also had short stature but was otherwise normal. Brackets indicate regions on X and Y making up the derivative X.

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 (*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 dyschrondrostosis (as a result of *SHOX* deletion), chondrodysplasia punctata (*CDPX* deletion), mental retardation (presumed *MRX* locus deletion), ichthyosis (*STS* deficiency), and hypogonadotrophic hypogonadism in combination with anosmia (Kallmann syndrome) when the deletion is large and encompasses all of the genes in this region *(227)*.

With probes for the *STS* and Kallman syndrome regions on Xp, it is now possible to use FISH (see Chapter 17) 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 Yq11.2 → Yqter onto Xp22.3, with one normal X chromosome and a derivative X: 46,X,der(X)t(X;Y)(Xqter → Xp22.3::Yq11.2 → Yqter) (see **Fig. 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 *(226)*. Most have normal intelligence, but mild mental retardation has been reported *(228)*.

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 → Xp22.3::Yp11.2 → Yqter). All three patients reported were phenotypic males and had short stature and hypogonadism or azoospermia *(226)*.

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 *(226)*.

Types 5 and 6 patients had varying amounts of Yq material translocated to Xq22.3; of two patients described, both had streak gonads *(226)*.

Type 7 has a dicentric chromosome: 46,X,dic(X;Y)(Xpter → Xq22.3::Yp11.2 → Yqter), and the one case reported was a phenotypic female with streak gonads, normal stature, and secondary amenorrhea *(226)*.

Four cases reported of (X;Y) translocations with a derivative Y, 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 *(226,229)*.

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::Yp11.2 → 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 *(230)*.

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 *(226)*.

(Xp;Yp) translocations involving the testis determining factor can be found in XX males or, rarely, XY females with sex reversal (see below). These translocations are usually not seen with cytogenetic analysis and require molecular probes for diagnosis *(177)*.

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 with true hermaphroditism *(231)*.

Different phenotypes of XX males and true hermaphrodites who carry the same translocation has been explained by a different pattern of inactivation of the Y-bearing X chromosomes *(232)*. Inactivation on the X chromosome spreading into a translocated Yp fragment is the proposed mechanism for a sexually ambiguous phenotype in some 46,XX (SRY+) individuals *(233)*.

Because most males with (X;Y) translocations will inherit the translocation from their mothers, it is important to advise women with such translocations of the risk for more severe manifestations in their male offspring who inherit the derivative X.

Y;Autosome Translocations

Y;Autosome translocations are estimated to occur with a frequency of 1 in 2000 in the general population *(234)*. 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 to 15) or "G group" (21 and 22) chromosome *(226)*. 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 (226). There would be a significant risk of masculinization or sex reversal in the female (see below). Molecular studies using Yp probes are indicated in such situations.

Translocations have been reported involving all autosomes except 11 and 20. Twenty-nine of 50 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. Of the 27 without

another chromosome abnormality, 25 were male and 2 were female. 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. Two of the 27 cases had female phenotypes, with 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 *(226)*. 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 aneusomy. 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 *(226)*. 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 generally 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 *(235)*. 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 *(235)* (see also Chapter 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 *(226)*. 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.

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 *(236–238)*.

Yq Deletions

Deletions involving the heterochromatic portion of Yq are compatible with normal genital development and sexual differentiation (see the section Y Chromosome Polymorphisms below). Larger deletions involving the euchromatic portion of Yq could cause azoospermia *(239)*. 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 46,X,del(Y)(q11); 48 were phenotypic males and most were infertile with azoospermia or oligospermia *(226)*. Based on patients with Yq deletions, the azoospermia factor (*AZF*) was identified (see above). 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 *(226)*.

Interstitial microdeletions in the euchromatic portion of the Y chromosome occur in 10–15% of men with azoospermia and severe oligozoospermia *(240,241)*. AZFa in proximal Yq (Yq11.21)

contains two genes whose absence or mutation cause spermatogenic failure (*USP9Y* and *DBY*) *(242–244)*. Complete absence of AZFa is associated with complete absence of germ cells. AZFb (Yq11.23) contains seven Y genes (*CDY2, EIF1AY, PRY, RBMY1, SMCY, TTY5,* and *TTY6*). 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 associated with variable testicular pathology and occasionally are inherited (most *AZF* deletions are *de novo*). AZFc deletions are the most common chromosome abnormality in men with azoospermia or severe oligozoospermia and most are de novo *(245–247)*. Polymerase chain reaction (PCR) techniques are needed to identify various deleted regions (see also Chapter 12).

Short stature in males with Xq deletions might be the result of the loss of the *GCY* (growth control gene[s] on the Y chromosome) locus near the pericentromeric region of Yq *(248,249)*. 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 *(249)*.

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 above). Phenotypes are extremely variable because of the level of 45,X mosaicism and tissue distribution for the abnormal Y chromosome.

i(Yp)

Phenotypic features reported in patients with isochromosome for the short arm of Y include ambiguous genitalia and Turner syndrome features with normal stature *(226,250)*, although cytogenetic methods could not rule out a partial Yq deletion *(226)*. Other patients were phenotypic males. One who was an adult male had hypogonadism, short stature, mental retardation, and facial anomalies *(251)*. Without a demonstrable 45,X cell line, most cases with monocentric i(Yp) will have a male phenotype *(226)*.

i(Yq)

Hsu reviewed seven reported cases with nonmosaic, monocentric isochromosome Yq. All were phenotypic females (expected because of 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 *(226)*.

Isodicentric Y

The dicentric Y is among the most commonly detected structural abnormalities of the Y chromosome (see **Fig. 8**) *(226)*. Most (91%) are found in mosaic form, usually with the other cell line being 45,X. Therefore, it is difficult to know the phenotypic effects of a dicentric Y alone. Some dicentric Ys have the breakpoint in the long arm, with duplication of the proximal long arm and entire short arm, whereas others have the break in the short arm, with the proximal short arm and entire long arm duplicated.

Most reported patients have short stature, and external genitalia could be female, ambiguous, or male. 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 the result of the loss of the *AZF* gene *(226,252)*. Mental retardation has been reported in a few patients, and schizophrenia in two patients *(253,254)*, although there is a bias of ascertainment in postnatally diagnosed cases and there are very few reports of prenatally diagnosed cases *(252)*.

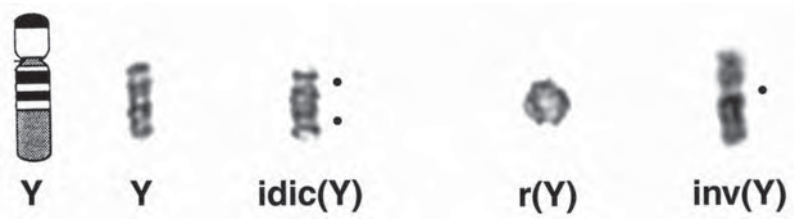

Fig. 8. (Left to right) Normal Y; isodicentric Y consisting of two copies of the short arm, centromere, and proximal long arm (q11.2); ring Y, and pericentric inversion of the Y: inv(Y)(p11q11). Dots indicate location of centromeres.

The presence of a 45,X cell line in addition to any cell line with an isochromosome Y or isodicentric Y leads to variable phenotypic manifestations, ranging from phenotypic male with azoospermia to ambiguous genitalia to phenotypic females with typical or atypical Turner syndrome features *(226)*.

Ring Y Chromosome

The brightly fluorescent heterochromatic region of Yq is usually deleted during formation of a ring Y [r(Y); see Chapter 3], making Q-banding (see Chapter 4) an unreliable tool for identification. See **Figure 8**. The most accurate way to determine the 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 Chapter 17). 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 *(226)*.

Because of the instability of ring chromosomes, multiple different cell lines might be seen *(255,256)*.

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 above).

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 *(257,258)*. All have an active nucleolar organizer region. Loss of the pseudoautosomal region (PAR2) on distal Yq has been shown in several cases of Yqs *(258)*.

At least one case of satellited short arm of the Y chromosome has been reported and was present in a phenotypically normal, fertile male *(259)*.

Inverted Y

Pericentric inversion of the Y chromosome [inv(Y)(p11q11) (see **Fig. 8**)] is estimated to occur as a normal variant in 0.6 in 1000 males (260). A very high frequency of 30.5% was found in the

Gujerati Muslim Indian population of South Africa *(261)*. In most cases, inverted Y chromosomes are inherited. It is not usually associated with any phenotypic or reproductive abnormalities *(261)*, 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 *(262–264)*. 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 *(265)*.

There have been rare reports of paracentric inversions of the long arm of the Y *(266,267)*. One of the cases had ambiguous genitalia with müllerian structures present *(267)*. The inverted Y was present in the father and grandfather, who were normal fertile males. Abnormalities in *SRY*, *ZFY*, *TSPY,* or *DAX-1* 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 were hypothesized.

DISORDERS OF SEXUAL 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 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 refs. *268* and *269*.

For parents, genital ambiguity is one of the most stressful problems encountered at the birth of a child. Adding to this stress can be the well-meaning physician or nurse who makes premature pronouncements about the infant's sex *(270)*. 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 this condition. 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 *(271)*. Many experts have 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 might be the strongest predictor of sexual identity *(272)*. This has led physicians to re-examine 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 *(272)*. These varying opinions emphasize the need for more long-term studies *(272,273)*. Recent reviews of patients with ambiguous genitalia have reported that either male or female sex of rearing can lead to successful long-term outcome *(274,275)*.

Complete Sex Reversal

46,XX males

This is a genetically heterogeneous group of conditions involving individuals who have bilateral testes while lacking a Y chromosome. Most have normal external genitalia, although 10–15% have some degree of genital ambiguity, cryptorchidism, and/or hypospadias. These are more likely to be diagnosed in childhood *(276)*. Others present in adulthood with infertility or gynecomastia. Most have small testes and some signs of androgen deficiency, similar to Klinefelter syndrome patients *(277)*. The seminiferous tubules are decreased in number and size, and there is interstitial fibrosis and

hyperplastic Leydig cells, and usually no spermatogonia *(277)*. There are also 46,XX sex-reversed patients with true hermaphroditism having 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 below).

There are at least three different mechanisms to explain the male phenotype in XX males: (1) translocation of Y sequences, including the *SRY* gene, to an X chromosome or autosome; (2) a mutation in an as yet unknown X-linked or autosomal gene in the testis-determining pathway; or (3) cryptic Y chromosome mosaicism *(278,279)*. 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, might 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 *(280)*. Ten percent of XX males have no detectable *SRY* or other Y sequences *(279)*.

Most *SRY*-positive XX males have normal male external genitalia, whereas those lacking Y-derived sequences are more likely to have ambiguous genitalia *(281,282)*. However, 46,XX sex-reversed patients with *SRY* present could have ambiguous genitalia and evidence of true hermaphroditism. This variability could be the result of differential inactivation of the X chromosome carrying *SRY (283)*. The amount of Yp present on Xp could be an additional factor *(284)*.

There have been familial cases of 46,XX males, suggesting autosomal recessive inheritance *(285)*. There have also been families reported with both XX males and XX true hermaphrodites, so that there might be a common origin for both *(281)*. Others have also found evidence that full virilization requires the expression of a second Y-linked gene, near *SRY*, which could be expressed outside the testis *(286,287)*.

46,XY Females

The etiology of 46,XY sex reversal is unclear in most cases. In approximately 15% of cases of 46,XY complete gonadal dysgenesis, there is a mutation in the *SRY* gene *(288)*. Some 46,XY females have been described with loss of *SRY (289),* whereas others have complete androgen insensitivity ("testicular feminization"), an X-linked condition. Malformation syndromes such as Smith–Lemli–Opitz and campomelic dysplasia also produce female or ambiguous genitalia with a 46,XY karyotype. These are the result of mutations or deletions in the autosomal genes *DHCR7* (7-dehydrocholesterol reductase) and *SOX-9,* respectively. There is also a dosage sensitive region on Xp (*DSS*) 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 *(191,290,291)*. Mutations in this gene are associated with congenital adrenal hypoplasia and hypogonadotropic hypogonadism *(292,293)*. *WT1* on 11p, *DMRT1* on 9p, and SF1 on 9q are other important genes in sex determination *(*reviewed in refs. *269* and *279)*. Familial cases suggesting X-linked sex-limited, autosomal recessive, or autosomal dominant inheritance have been reported (reviewed in ref. *288*).

True Hermaphroditism

This is a rare condition where both testicular and ovarian tissue is 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 *(279)*. A few patients who were chimeras with 46,XX and 46,XY cell lines arising from the fusion of two zygotes have been described *(277)*, although not all 46,XX/46,XY individuals have true hermaphroditism *(294)*. At least 50% of true hermaphrodites are 46,XX with no Y DNA *(277)*. Cryptic gonadal mosaicism for *SRY* has been found in some patients, whereas others could have alterations in unknown X-linked or autosomal sex-determining genes *(279)*.

Gonadal neoplasia and breast cancer have been reported in these patients *(56,294)*.

Pseudohermaphroditism

Patients with pseudohermaphroditism have gonadal tissue of one sex but ambiguous external genitalia. Female pseudohermaphroditism, in which there is a 46,XX chromosome complement, is usually the result of congenital adrenal hyperplasia. It is critical to identify these patients early because of 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.

Male pseudohermaphroditism (46,XY) has many causes. These include partial androgen insensitivity or Reifenstein syndrome, which is an X-linked condition *(296)*. Genetic males with 5α-reductase deficiency have ambiguous genitalia at birth but have normal virilization at puberty. This is an autosomal recessive condition *(57)*. Denys–Drash syndrome is a condition with Wilms tumor, diffuse mesangial sclerosis of the kidneys leading to nephrotic syndrome and male pseudohermaphroditism *(296)* caused by mutations in the Wilms' tumor 1 gene (*WT1*) on 11p13. *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 male pseudohermaphroditism have been described *(297–299)*.

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11
Cytogenetics of Infertility

Linda Marie Randolph, MD

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 Chapter 13. Male factor and female factor infertility each account 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
- Ovulation disorders, which include hypothalmic, pituitary, and ovarian causes; most cytogenetic abnormalities fall into this category
- Endometriosis

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. 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 Chapter 10, 1 in about 2500 to 5000 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,iXq cell lines present with primary amenorrhea and lack of pubertal development. Up

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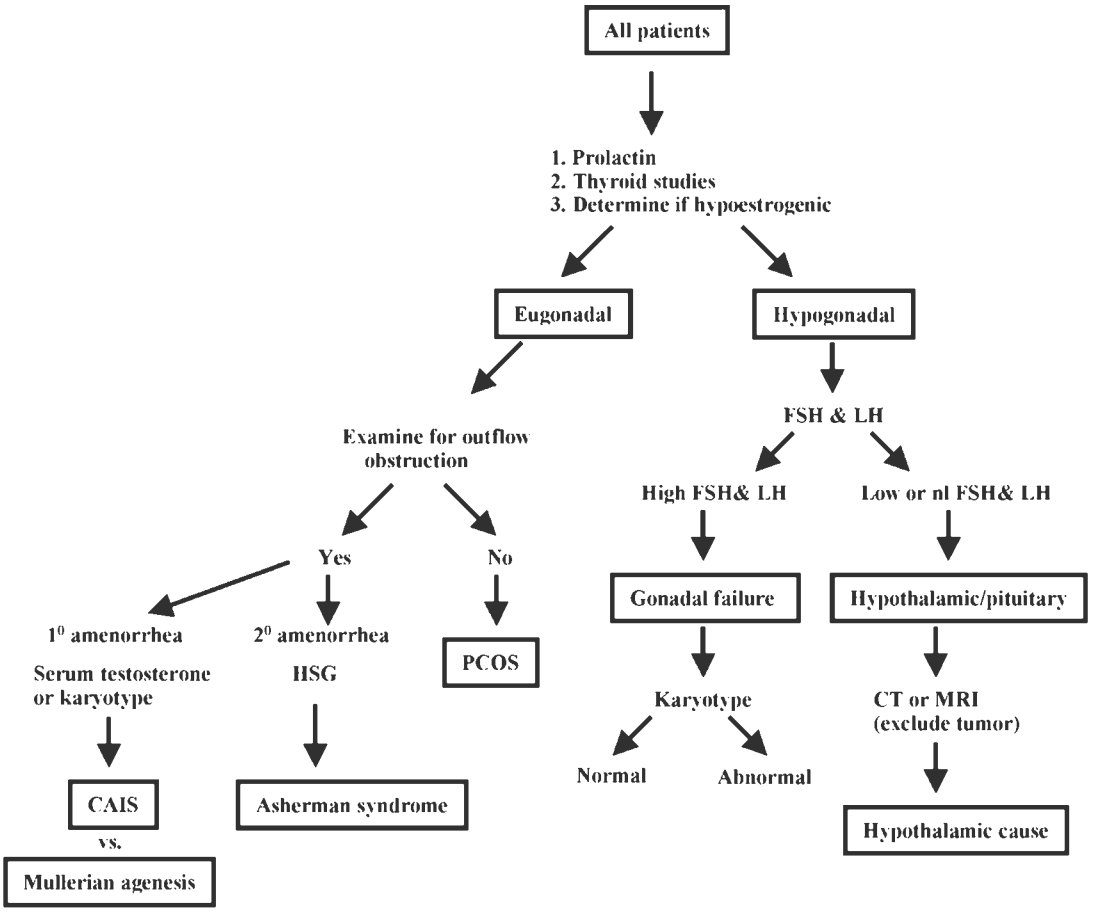

Fig. 1. Flow diagram for the workup of a woman with delayed puberty. (Courtesy of Dr. Lawrence Layman.) PCOS = polycystic ovary syndrome. CAIS = complete androgen insensitivity syndrome. FSH = follicle stimulating hormone. LH = luteinizing hormone Asherman syndrome = adhesions from prior uterine curettage.

to 25% have some breast development *(3)*. Two to three percent of 45,X women and 10–15% of women with mosaic 45,X undergo 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 might 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 that did not use assisted reproduction techniques 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 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 1 patient with 45,X/46,XX/47,XXX/48,XXXX had 3 pregnancies. None were reported in women with a 46,XY cell line without IVF.

Among 13 women with 45,X without mosaicism, 6 of 21 pregnancies ended in spontaneous abortions and 2 of 21 ended in stillbirth, including 1 with hydrocephalus. Another three culminated in a pregnancy and/or offspring with a chromosome abnormality or significant birth defect (one Down syndrome, one 45,X, one with a partial cleft of the soft palate).

Among the liveborns 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 liveborns 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 liveborn offspring are less likely to have abnormalities than liveborn offspring of women with mosaic 45,X. In this group, 12 nonmosaic 45,X women's liveborn infants had no abnormalities.

Magee et al. *(6)* 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 function 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.

Detection of Y Chromosome Sequences in 45,X and Mosaic 45,X Patients

Among the hypotheses as to why all but 1% of 45,X fetuses die *in utero* and why some women with apparent nonmosaic 45,X have some fertility is that 45,X individuals might actually be cryptic mosaics for another cell line that supports survival. It is important also to consider 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 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), 1 cell of 450 examined in maternal lymphocytes showed a der(X). Kocova et al. *(7)* 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 liveborn 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, the presence of the SRY (testis-determining factor, or gene) was detected *(7)* (see also Chapter 10).

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 p11.2 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 can 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. *(8)* performed cytogenetic analyses on 79 women with primary or secondary amenorrhea, and 2 of the 79

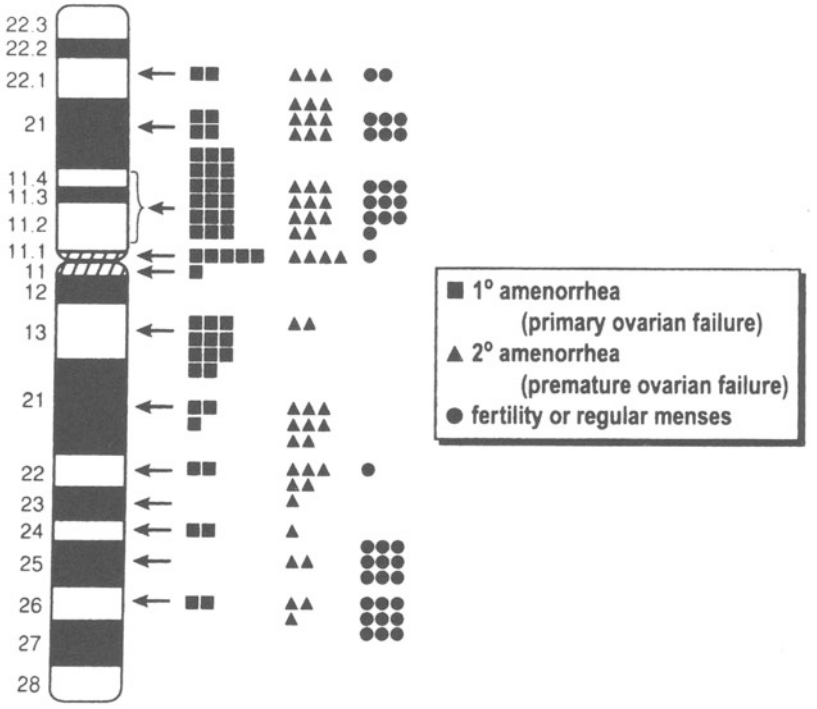

Fig. 2. An ideogram of the X chromosome with locations of various deletions and the corresponding clinical characteristics. (From ref. *9*; reprinted by permission of the author and of Wiley-Liss, Inc., a subsidiary of John Wiley Sons, Inc.)

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)*. **Figure 2** shows locations of different deletions of the X chromosome and the associated phenotype (see also Chapter 10).

X Chromosome;Autosome Translocations

In a balanced X;autosome translocation, the normal X is generally inactivated (see Chapter 10). 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, whereas 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 1 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-q26 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, could result. When the derivative X is active in only a portion of cells, multiple anomalies and mental retardation usually result.

Table 1
Gene Mutations That Affect Ovarian Function

Gene	Locus	Phenotype	Inheritance
FMR1	Xq27.3	Fragile X syndrome; ovarian failure	X-linked dominant
SRY	Yp11.3	Swyer syndrome; sexual infantilism, normal vagina and uterus, streak gonads with risk for gonadoblastoma ± germ cell tumor	Sporadic; Y-linked
FSHR	2p21-p16	Primary amenorrhea, half with breast development; men have oligospermia	Autosomal recessive
LHR	2p21	Women: anovulation; men: undermasculinization	Autosomal recessive
CYP17	10q24.2	17-Hydroxylase deficiency; delayed puberty in women, absent breast development, primary amenorrhea, and elevated gonadotropins	Autosomal recessive
CYP19	15q21.1	Aromatase deficiency; cannot convert androgens to estrogens; females with sexual ambiguity, clitoromegaly; no breast development or menses	Autosomal recessive
AIRE	21q22.3	Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED); multisystem autoimmune disease; adrenal, ovarian and testicular failure might occur	Autosomal recessive
FTZF1(SF1)	9q33	Steroidogenic factor 1; adrenal failure, sex reversal in men; presents as female phenotype; rare	Autosomal recessive
GALT	9p13	Galactose-1-phosphate uridyltransferase; causes galactosemia; normal puberty; half with primary and half with secondary amenorrhea; 67% women have ovarian failure, and testicular function apparently not affected; heterozygotes (carriers) not affected	Autosomal recessive

Source: Ref. *4*.

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 noncytogenetic inherited causes of ovarian dysfunction, see **Table 1**.

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 2**. **Table 3** indicates gene-level conditions associated with pituitary malfunction, and **Table 4** lists gene-level conditions associated with uterine development abnormalities.

Endometriosis

Endometriosis is a common disorder that accounts for infertility in 10–15% 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)*.

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

Table 2
Gene Mutations Affecting Hypothalamic Functions in Females

Gene	Locus	Phenotype	Inheritance
KAL	Xp22.3	Kallmann syndrome; hypogonadotropic hypogonadism, anosmia; *?affects only males*	X-linked recessive
AHC	Xp21	Adrenal hypoplasia congenital and hypogonadotropic hypogonadism	X-linked recessive
Leptin	7q31.3	Obesity, hypogonadotropic hypogonadism, delayed puberty	Autosomal recessive
Leptin receptor	1p31	Obesity, hypogonadotropic hypogonadism, elevated serum leptin	Autosomal recessive

Source: Ref. *4*.

Table 3
Gene Mutations Affecting Pituitary Function in Females

Gene	Locus	Phenotype	Inheritance
GNRHR	4q21.2	Hypothalamic hypogonadism	Autosomal recessive
HESX1	3p21.1–21.2	Septo-optic dysplasia	Autosomal recessive
LH–	19q13.3	Isolated luteinizing hormone deficiency	Autosomal recessive
FSH–	11p13	Isolated follicle-stimulating hormone deficiency	Autosomal recessive
PROP1	5q	Short stature, hypothyroidism, hypogonadotropic hypogonadism	Autosomal recessive
HESX1	3p21.1–21.2	Septo-optic dysplasia	Autosomal recessive

Source: Ref. *4*.

Table 4
Gene Mutations Affecting Uterine Development

Gene	Locus	Phenotype	Inheritance
AR	Xq11q12	Androgen insensitivity syndrome (male, 46,XY, phenotypic female)	X-linked recessive
HOXA13	7p15-p14.2	Hand–foot–uterus syndrome	Autosomal dominant

Source: Ref. *4*.

(see Chapter 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 fluorescence *in situ* hybridization (FISH) *(11)* and were interpreted as indicating that genes localized to certain chromosome regions play a role in the development and progression of endometriosis.

CAUSES OF MALE INFERTILITY

A standardized approach to the evaluation of an infertile couple was proposed by the World Health Organization (WHO) in 1993 *(12)*. This manual delineates 16 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 but 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 *(13)*. 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 *(14)*.

About 10% of infertile men have severe defects in sperm production *(15)*, and it is in this group of men that many of the cytogenetic and genetic disorders are concentrated. Hackstein et al. *(16)* note that in the fruit fly *Drosophila* there is evidence that up to 1500 genes contribute to male fertility. 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 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 *(17)*.

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 an indicator of obstruction. Spermatozoa counts are designated as the number present per milliliter. A normal number as defined by WHO is 20×10^6 sperm/mL of semen *(12)*. However, in a study of 430 couples in Denmark having unprotected sex, the probability of conception increased with increasing spermatozoa concentration to $40 \times 10^{6/}$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 could be subfertile *(18)*.

Oligospermia, Nonobstructive 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 number in terms of concentration, morphology, and motility of spermatozoa are important factors in achieving conception.

Gunduz et al. *(19)* performed chromosome analysis on 41 men with azoospermia and 61 men with oligospermia. Fourteen of the 41 men, or 34.1%, and 2, or 3.3%, of the oligospermic men had a constitutional chromosome abnormality. The most common abnormality was 47,XXY *(19)*.

Table 5
Genetic Conditions in Which Male Infertility Is a Feature

Condition	Mode of Inheritance	Incidence	Features
XX sex reversal	X-Y recombination in 80%; ? cause in 20%	1 : 20,000	Male appearance, gonadal dysgenesis, hypogonadotropic hypogonadism; azoospermia
Persistent Müllerian duct syndrome	Two or three types; autosomal recessive	Rare	Cryptorchidism, pseudohermaphroditism, gonadal dysgenesis, anti-Müllerian hormone deficiency or anti-Müllerian receptor deficiency; azoospermia
Testosterone synthesis defects	Three types; autosomal recessive	Rare	Pseudohermaphroditism; low testosterone
5-α-reductase deficiency	Autosomal recessive, male limited	Rare	Sex ambiguity; pseudohermaphroditism
Kennedy disease	X-linked recessive, trinucleotide repeat disorder	1 : 50,000	Spinal bulbar and muscular atrophy; gynecomastia; azoospermia and oligospermia
Luteinizing hormone deficiency	Autosomal recessive	Rare	Hypogonadism; azoospermia
Luteinizing hormone receptor defect	Two types: autosomal recessive and autosomal dominant, male limited	Rare	Recessive: female phenotype, pseudohermaphroditism Dominant: male phenotype, precocious puberty
Idiopathic hypogonadotropic hypogonadism	Two types, both autosomal recessive	? Rare	GnRH deficiency; azoospermia, pubertal delay GnRH receptor mutation; same phenotype
Cystic fibrosis Congenital bilateral (and some unilateral) absence of the vas deferens (CBAVD)	Autosomal recessive	1 : 2500 1-2% of males with infertility	Pulmonary and gastrointestinal problems; CBAVD Azoospermia, often with upper respiratory problems/mild form of cystic fibrosis

Primary ciliary dyskinesia	Autosomal recessive	?; dynein gene(s); many loci	Asthenozoospermia; bronchiectasis, other respiratory infections
Kartagener syndrome	Autosomal recessive	?; dynein gene(s); heterogeneous	Same as for ciliary dyskinesia plus situs inversus
Bardet–Biedl syndrome	Six genes, all autosomal recessive	1 : 100,000	Mental retardation, hypogonadiam, multiple congenital abnormalities
Noonan syndrome	Two or more genes, autosomal dominant	1 : 5000	Short stature, multiple congenital abnormalities, cryptorchidism, azoospermia, oligospermia
Myotonic dystrophy	Autosomal dominant, trinucleotide repeat disorder	1 : 8000	Myotonia, balding, oligoasthenospermia, cataracts, cardiac conduction problems
Kallmann syndrome	Type that affects only males is X-linked recessive; are autosomal recessive and dominant forms that affect both genders	1 : 10,000	Pubertal delay, anosmia, azoospermia
Prader–Willi syndrome	Paternal deletion (70%), maternal uniparental disomy (29%), imprinting defect (1%)	1 : 10,000	Hypotonia at birth; excessive appetite in early childhood associated with obesity, plus characteristic facies, hypogonadism, developmental delay

Source: Ref. *14*.

In a review of the chromosomal contribution to male infertility, Van Assche et al. *(20)* reported on the chromosome constitution of about 8000 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%) *(20)*. 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% *(20)*.

In a separate cytogenetic study of 1007 infertile men, major chromosome abnormalities were seen in 62, or 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, 3 were 47,XYY, and 7 had a Y chromosome with a structural abnormality. Of the autosomal abnormalities, 10 were reciprocal translocations, 8 had Robertsonian translocations, 5 had an inversion, and 1 had a ring chromosome. The likelihood of a chromosome abnormality was higher in men with a sperm density of $< 5 \times 10^6$/mL, an FSH 30.1 mLU/mL, an LH 8.9 mLU/mL, a testosterone value 2.69 ng/mL, or an average testis volume 8 mL *(21)*.

Sex Chromosome Abnormalities (See Also Chapter 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 can be a problem *(22)*. Patients 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.

Men with 47,XYY or 47,XYY/46,XY karyotypes are usually fertile and typically have normal semen analyses *(22)*. They are slightly taller than their chromosomally normal brothers on average and have, on average, a normal IQ. About half have learning disabilities requiring special education *(22)*.

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 1 in 1000 newborns *(21)*. However, in infertility surveys [e.g., the study by Gunduz et al. *(19)*], the finding of 47,XXY is about nine times as frequent as that of 47,XYY. Men with a 47,XYY karyotype are represented more frequently among infertile men (0.26%) than in newborn males (0.07%). Their semen analyses are usually normal, as noted above, but in a minority of cases, they have severe abnormalities of spermatozoa number, motility, and/or morphology *(23)*.

Autosomal Abnormalities

The most common autosomal abnormality seen in infertile men is the Robertsonian translocation (see Chapter 9). In the above-cited review by Van Assche *(20)*, the incidence of infertile men with this finding was 0.7%. 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 the Robertsonian translocation are correlated with the extent of germ cell impairment *(24)*; see also Chapters 2, 9, and 10.

The review by Van Assche, which pooled data from several studies, also indicated 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 *(13)*. 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 Chapter 10). These were not seen to any significant degree in the chromosome preparations of the men with reciprocal translocations involving other chromosomes.

In Zuffardi and Tiepolo's *(25)* review of 7277 men, the range of autosomal abnormalities was 0.6–1.6%, with an average of 1.1%. Overall, the incidence of balanced translocations was 8.9 per 1000, which is 6 times greater than the 1.4 per 1000 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 1000 vs 0.6 per 1000 *(25)*. For a comparison of chromosome abnormalities seen in studies of infertile men, see **Tables 6–8**.

Microdeletions of the Y Chromosome

The fact that genes necessary for spermatozoa production are on the long arm of the Y chromosome became evident in a study published in 1976 by Tiepolo and Zuffardi *(27)*. They studied six azoospermic males and found microdeletions at Yq11.2 that were not present in the fertile fathers and brothers of the men. These were the first microdeletions found on the Y chromosome; they are called microdeletions because they are not detectable by conventional cytogenetic testing (see Chapters 9 and 17). Thus, molecular or molecular cytogenetic testing is required to detect these deletions. This deleted region was called *AZF* for azoospermia factor. It is now known that there are several other genes on the long arm of the Y chromosome that are associated with faulty spermatogenesis, so the *AZF* region has been subdivided as described below. It is now estimated that microdeletions of the Y chromosome are present in 8–15% of men with nonobstructive azoospermia or severe oligozoospermia *(28,29)*—that is, men with a spermatozoa count of $<5 \times 10^6$/mL.

The Y chromosome has been continually subdivided over the years into more refined regions. In one system, the short arm and centromere comprise intervals 1–4, and the long arm comprises intervals 5–7. More refined subdivisions have been developed on the basis of nonoverlapping deletions in patients with nonobstructive azoospermia or oligospermia. The original *AZF* region now consists of *AZFa*, *AZFb,* and *AZFc (29)*. A fourth region, called *AZFd*, between *AZFb* and *AZFc*, is thought to exist as well. These microdeletions are associated with various histopathologies and abnormal semen parameters, as shown in **Table 9**. 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 (STSs), 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 75% of deletions. *AZFa* deletions occur in fewer than 5% of men with AZF deletions *(14)*.

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 1 in about 5000 males *(30)*. Previous reports have shown that boys born from oligospermic men treated using ICSI have an increased risk of carrying a Y chromosome microdeletion *(28)*. 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) *(30)*. Of these, 18 had an abnormal karyotype, and of the remaining 163, 6 (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. *(31)*, who studied 131 infertile males for the presence of a Y chromosome 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*, *AZFb,* or *AZFc* region. Of the group with known causes of infertility, 7% were found to have a 46,XY chromosome complement and microdeletions, including deletions of the *AZFb* and *AZFc* regions.

Table 6
Constitutional Cytogenetic Studies of Infertile Men; Includes Men with Azoospermia and Oligospermia

N	47,XXY (%)	47,XXY/ 46,XY(%)	Other sex chrom abn (%)	Y chrom struct abn (%)	46,XX (%)	Rob trans (%)	Recip trans (%)	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)		?*	?*		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[b] (0.12)	4[c] (1.5)
1,363	57 (4.2)	NA			1 (0.07)	11 (0.81)	3 (0.22)		4[b] (0.29)	20[d] (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)	—	—[e]	—		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 struct abn = Y chromosome structural abnormalities; Rob trans = Robertsonian translocation; Recip trans = reciprocal translocation; Inv = inversion; Mar = marker chromosome.

Note: NA = not applicable; mosaics included in 47,XXY column in these surveys.

[a] Includes 47,XXY and 47,XXY/46,XY.

[b] Described as an "extra G."

[c] 14p–; 15p–; fragile site at 16q22; "ring E."

[d] Includes several heteromorphisms and other variants not generally reported in other series.

[e] Frequency of XYY in newborn series was 33 in 36,855, or 0.09%.

*Includes six cases described as XYY, deletions and rings.

Source: Data from refs. *25* and *26*.

Table 7
Constitutional Chromosome Abnormalities in Men with Oligospermia Compared with Chromosome Abnormalities in Pooled Newborn Series

N	Sperm count (10^6/mL)	47,XXY (%)	47,XXY/ 46,XY (%)	Other sex chrom num abn (%)	Y chrom struct abn (%)	Rob Trans (%)	Recip Trans (%)	Inv (%)	+ Mar (%)
569	<10	3	1	5	2	14	5	5	4
390	<10	4	3	3	4	4	3	1	2
34	<20		2	1	1				
47	<20					2			
464	<20					6	1	1	
108	<20	1				1			
115	<30			1		2	1	1	1
49	<30					1	3		
53	<40	1				1	3		
233	<80	2	1	4	2	2	6	2	1
2062									
Total		11 (0.53)	7 (0.34)	14 (0.68)	9 (0.44)	32 (1.56)	19 (0.92)	10 (0.49)	8 (0.39)
Newborns		39/36,855 (0.11)	—	—[a]	—	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 num abn = other sex chromosome numerical abnormalities; Y chrom struct abn = Y chromosome structural abnormalities; Rob trans = Robertsonian translocation; Recip trans = reciprocal translocation; Inv = inversion; Mar = marker chromosome.

[a] Frequency of XYY in newborn series was 33 in 36,855, or 0.09%.

Source: Ref. *26*.

The authors recommend that all males with reduced or absent sperm counts seeking assisted reproductive technologies be screened for microdeletions of the Y chromosome *(31)*.

Several genes and gene families have been isolated from the *AZFb* and *AZFc* regions *(32,33)*. One family is called *RBMY,* or RNA-binding motif. It consists of 30–50 genes and pseudogenes that encode proteins involved in pre-mRNA processing and transport *(34)*. Unlike other such genes, however, it is expressed only in the testes. The functional copies of *RBMY* are in the *AZFb* region *(35)*. A homolog for *RBMY* exists on the X chromosome *(36,37)*. Delbridge et al. *(36)* 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.

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 7–10 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. Another 12 or more genes have been found on the Y chromosome. Some are expressed only in the testes, and others are expressed more widely and have homologs on the X chromosome *(38,39)*.

In the *AZFa* region, three genes have been localized. Formerly known as *DFFRY*, *DBY,* and *UTY*, they are known as *USP9Y*. Men with deletions of the *AZFa* region have lost the *USP9Y* and *DBY* genes, resulting in no germ cells' being present. These genes are expressed widely and have homologs on the X chromosome *(14)*.

INTRACYTOPLASMIC SPERM INJECTION

Men who were once unable to have children who were biologically their own are now able to with the advent of intracytoplasmic sperm injection (ICSI) (see **Fig. 3**). The spermatozoan could be extracted prior

Table 8
Constitutional Chromosome Abnormalities in Men with Azoospermia Compared with Chromosome Abnormalities in Pooled Newborn Series

N	47,XXY (%)	47,XXY/ 46,XY (%)	Other sex chrom num abn (%)	Y chrom struct abn (%)	46,XX (%)	Rob trans (%)	Recip trans (%)	Inv (%)	Mar (%)
383	49		1	1	3	1	4		
106	7	4	2	4	2				
68	12	1		1	2		1		1
36	1	1	1	1		1			
33	6				1				
53	13	1	2	1	1				
163	31			7	1		1	1	
8	4		31						
20	3	1	1						
356	21	4	2		1		2		
34		1						1	
53	9								
54		2	1					2	
Total 1,450	159 (11.0%)	13	12	15	11 (0.76)	2 (0.14)	11 (0.76)	1 (0.07)	1 (0.07)
Newborns	39/36,855 (0.11)	—	—[a]	—		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 num abn = other sex chromosome numerical abnormalities; Y chrom struct abn = Y chromosome structural abnormalities; Rob trans = Robertsonian translocation; Recip trans = reciprocal translocation; Inv = inversion; Mar = marker chromosome.

[a] *Source:* Ref. *26*.

Table 9
***AZF* Gene Regions and their Usual Phenotypes**

Gene region	Phenotype
*AZF*a	Absence of germ cells, aka Sertoli cell-only syndrome (SCOS)
*AZF*b	Maturation arrest at spermatocyte stage
*AZF*c	Variable from SCOS to severe oligospermia
*AZF*d	Mild oligospermia or normal cell counts with abnormal sperm morphology

Source: Ref. *29*.

to the site of obstruction or from the testis, or it could 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 a spermatozoan with another and injects the spermatozoan into the cytoplasm of the ovum. This has been used for several years for 47,XXY men and for men with severe male factor infertility for other reasons.

Although this technique has allowed for 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 (see Chapter 20).

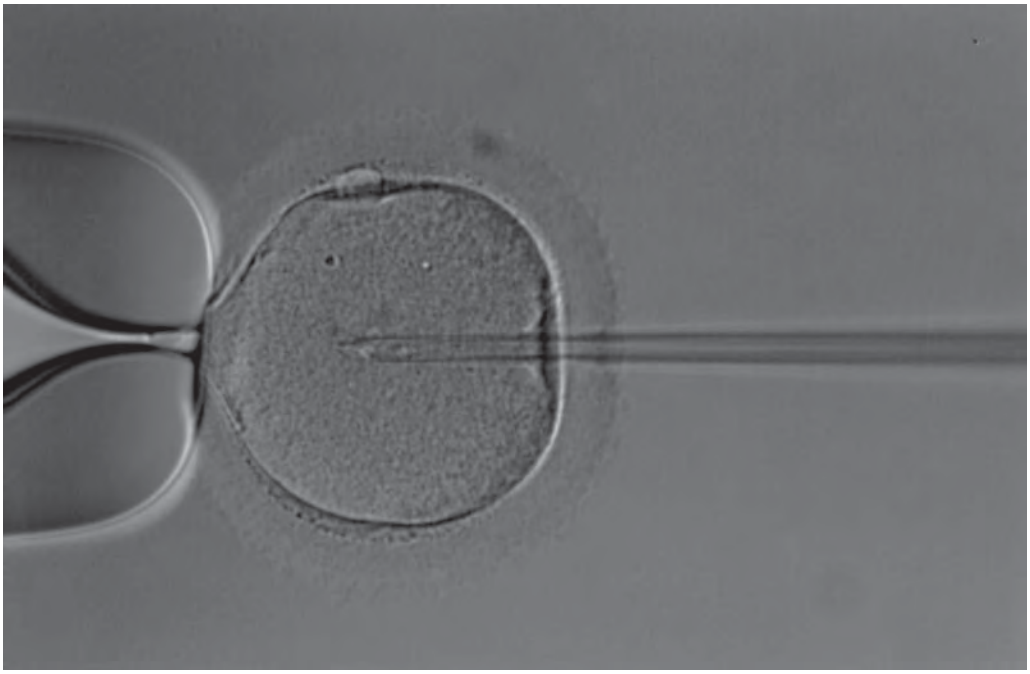

Fig. 3. Intracytoplasmic sperm injection (ICSI). In this photograph, a technologist is viewing an ovum and spermatozoan under a microscope and injecting the spermatozoan into the cytoplasm of the egg. (Courtesy of ViaGeneFertility.com.)

Some of the reasons for this have been discussed earlier. A man with a microdeletion of the Y chromosome would be expected to pass on his 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 of *de novo* deletions in the sons *(40)*. 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. *(41)* evaluated the risk for cytogenetic abnormalities in offspring of men with nonmosaic Klinefelter syndrome. They obtained testicular biopsies from 20 patients and found testicular sperm in 8 of them. Four couples became pregnant following ICSI. Sperm chromosomes were analyzed in five patients. Of 112 sperm, 7 (6.3%) had chromosome abnormalities, of which 5 were sex chromosome abnormalities and 2 had nullisomy 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.

As for the studies that show a high risk of sex chromosome abnormalities in men with gonadal failure and low FSH undergoing ICSI, it is postulated that their spermatozoa undergo meiotic errors as part of their underlying disorder, and they therefore have a higher risk of having offspring with sex chromosome abnormalities.

This suggestion was supported by the findings of Giltay et al. *(42)*, who examined semen specimens from 7 severely oligospermic ICSI candidates and compared results to 3 normal specimens and to 10 ICSI candidates with normal constitutional karyotypes but with oligoasthenoteratozoospermia (OAT). 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 determined by FISH (see Chapter 17) 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. The authors concluded 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. *(43)* examined the role of morphology of spermatozoa and chromosome abnormalities of the spermatozoan. 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 1656 to 5000 spermatozoa were analyzed from patients and from 5064 to 7423 spermatozoa from controls. They employed three-color FISH and found that patients 1–3 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 therefore 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 *(23)* 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.

Bonduelle et al. *(44)* performed a study to determine whether prenatal cytogenetic abnormalities after ICSI could be related to sperm parameters. Of 1586 fetuses, chorionic villus sampling (CVS) (see Chapter 12) was performed on 698, and amniocentesis was performed on 888. Of these, 47 (3%) had abnormal karyotypes, and 25 of the 47, or 2%, were *de novo*. They found a 2.1% *de novo* prenatal chromosome abnormality rate for sperm concentrations of $< 20 \times 10^6$/mL and a 0.24% abnormality rate for sperm concentrations of 20×10^6/mL or greater. The likelihood of a chromosome abnormality was associated with spermatozoa motility and concentrations, but not morphology in this study. A *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, and part was the result of autosomal chromosome abnormalities.

Pregnancy Rates, Obstetric Outcomes, Chromosome Abnormalities, and Birth Defects After ICSI

Palermo et al. *(45)* performed a study in New York City of 751 couples in whom 987 ICSI cycles were undertaken. The male partner was thought to be the cause of repeated failed 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 8 of the 11 spontaneous abortions for which cytogenetic information was available, an autosomal trisomy was found, and 7 additional pregnancies were terminated because of a chromosome abnormality after prenatal diagnosis. An equal number were delivered vaginally vs by caesarean section; about half of those delivered by caesarean section were multiple gestations.

Fifteen of 578 newborns in this study had birth defects (9 major and 6 minor), and this frequency of 2.6% compared to 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 (two), cleft palate, aqueductal stenosis, spina bifida, and hydronephrosis. All of those were seen in multiple gestations except the VSD, which was present in a singleton pregnancy. The minor anomalies were hypospadias (four), urethral obstruction, and double ureter. 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 both in pregnancy evolution and in incidence of birth defects.

More recently, Simpson et al. *(46)* examined ICSI data from the United States in 1997. Of 6077 ICSI cycles begun, there were 17.5% pregnancy losses, and that same cohort showed a malformation

rate of 1.7% in the liveborn babies. In their paper, they note that the Swedish IVF Registry of 1139 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, a *de novo* autosomal rearrangement rate of 0.36% and a *de novo* sex chromosome abnormality rate of 0.83% were seen, both higher rates than expected in the general population. One wonders whether these abnormalities are in fact *de novo* 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 *(47)*, Kurinczuk and Bower *(48)* published a different interpretation of their data. 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 outcome measures. As an example, the Belgian data considered coronal hypospadias, renal duplication, and coronal hypospadias minor defects, and in the Western Australian system, these were all considered major defects. Results suggested an excess occurrence of major cardiovascular, gastrointestinal, and genitourinary defects generally. The authors report their findings with caution, as the numbers are small.

Imprinting, IVF, and ICSI

A review of the troublesome phenomenon of imprinting errors (see Chapter 19) in the offspring conceived by IVF, with or without ICSI, was published by Gicquel et al. *(49)*. In this report, the authors note that in their series of 149 patients with Beckwith–Wiedemann syndrome (BWS), 6 were born following IVF, 2 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 noted 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 commented on three patients born with Angelman syndrome and imprinting defects, which is a rare finding in Angelman syndrome.

The above discussion makes it clear that more careful, prospective follow-up of children born after IVF and other assisted reproductive techniques (ART) must be carried out and that information 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 might be twice that of the general population and that much remains to be learned about such innovative procedures as ICSI.

And What About the Mitochondria of the Spermatozoa?

Does the offspring inherited paternal mitochondria after ICSI? After all, the entire spermatozoan is injected into the ovum. Two groups have examined this question, and to a sensitivity level of 0.001% *(50)* and 0.5% *(51)*, no paternal mitochondrial DNA has been detected in the offspring, placenta or umbilical cords after ICSI.

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12
Prenatal Cytogenetics

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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 *(5)* described the use of amniotic fluid sediment to determine fetal sex by Papanicolaou and Giemsa stains, and Fuchs and Riis *(6)* showed in amniotic fluid of term pregnancies that they could accurately determine the fetal sex in 20 of 21 cases. 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.

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."

Further refinement of the technique and timing of amniocentesis were demonstrated in a 1967 paper by Jacobson and Barter *(10)*, and they proposed that the optimal timing of amniocentesis is 16 weeks after performing the procedure from 5 weeks to term in 85 women. 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)*.

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By 1986, more than a quarter of a million amniocenteses had been performed for cytogenetic analysis *(12)*, and the number to date is undoubtedly in the millions. Although other means of prenatal cytogenetic testing have been developed, amniocentesis is, by far, the most common technique performed today for prenatal genetic diagnosis, with 96,698 reported in the United States in 2000 *(13)*.

Chorionic Villus Sampling

Although techniques for transcervical *(14)* and transabdominal *(15)* 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 fetal genetic diagnosis was published in 1968 *(16)*. Mohr developed techniques for sampling fetal cells no later than the third month of pregnancy by a transabdominal approach. Primarily because of the absence of real-time ultrasound, low culture success rate, and the risks of endoscopic approaches, as described by Kullander and Sandahl in 1973 *(17)* and by Hahnemann in 1974 *(18)*, the technique was not widely used in the United States. In Kullander and Sandahl's experience, 19 of 39 specimens (48.7%) were successfully cultured, which they described as a "high percentage." In Hahnemann's experience, there was a 38% success rate, with causes of failure being puncture or biopsy of the amniotic membrane and bleeding. 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 colleagues 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 in the World Health Organization–sponsored CVS registry in Philadelphia *(24)*. This number would be much larger if all of the hundreds of centers around the world that perform CVS provided their data to the registry *(25)*.

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 *(26)*. 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 *(27)*. 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. *(28)* 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. 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 *(29)*. 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 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, 1 in 156 live births were found to have a major chromosome abnormality (see **Table 1**) *(30)*. The most common remains 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 1 XYY or XXY per 1000 male liveborns and 1 XXX per 1000 female liveborns. Because nonbanded 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 3993 newborns, no significant difference in the frequency of rearrangements or of other chromosome aneuploidies was found *(31)*.

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 *(32)*. His analysis of the data indicated a 30% differential between the rates observed at amniocentesis and those seen at birth, a figure which 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 over for whom there was there no known cytogenetic risk for a chromosome abnormality except parental age *(33)*. 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,XXY and 47,XYY (see **Table 2**). 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, 47XYY, 45,X, and 45,X/46,XX but not for 47,XXX *(34)* (see **Table 3**). 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. Halliday et al. compared risks of Down syndrome only at amniocentesis, CVS, and at birth, and these also are shown in **Table 3** *(35)*.

The incidence of *de novo* balanced structural rearrangements in 337,357 amniocenteses was reported by Warburton *(36)*. The results are shown in **Table 4**.

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% *(30,37)* (see **Table 5**). A cautionary note in consideration of this high incidence range is that the tissue cultured and analyzed might not represent the fetus. It has been shown that 45,X cells and some lethal trisomies seen in chorionic villus samples might not be seen in the fetus, so this could lead to spurious elevation of estimates of chromosome abnormalities in spontaneous

Table 1
Chromosome Abnormalities in Surveys of 68,159 Liveborn Babies

Type of abnormality	Total abnormalities (%)
Sex chromosomes, males	
47,XYY	45 (0.103)
47,XXY	45 (0.103)
Other	32 (0.073)
Sex chromosomes, females	
45,X	6 (0.024)
47,XXX	27 (0.109)
Other	9 (0.036)
Autosomal trisomies	
47,+21	82 (0.120)
47,+18	9 (0.013)
47,+13	3 (0.004)
Other	2 (0.002)
Structural balanced arrangements	
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)
Structural unbalanced arrangements	
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)
Total babies surveyed	
Males	43,612
Females	24,547

[a] der(D;D) refers to Robertsonian translocations involving chromosomes 13, 14, and/or 15.
[b] der(D;G) refers to Robertsonian translocations involving chromosomes 13, 14, or 15 and 21 or 22.
[c] Excludes common pericentric inversion of chromosome 9.
Source: Data from ref. *30*.

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 45,X appears to be associated with younger maternal age, with about one-third of 45,X spontaneous abortions coming from women 20–24 year of age *(39)*. 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 *(37)* (see **Table 6**).

Table 2
Maternal Age-Specific Rates (%) for Chromosome Abnormalities

Maternal age (year)	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

[a] Estimated liveborn statistics *(33)*.
[b] Data compiled from 19,675 genetic amniocenteses *(33)*.
[c] Data compiled from 3041 CVS, 7504 amniocenteses, and 13,139 with no test *(35)*. These are observed prevalences.
[d] Data compiled by Hsu *(30)*.

Table 3
Fetal Deaths Subsequent to Amniocentesis

Abnormalities	Fetal deaths		
	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

Note: Proportion refers to the number of fetal losses compared to the total number of fetuses diagnosed with the given abnormality.
Source: ref. *34*.

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 have shown that the incidence of chromosome abnormality is about 10 times that in the rest of the population. Combining

Table 4
The Incidence Of *De Novo* Balanced Structural Rearrangements in 337,357 Genetic Amniocenteses

De novo rearrangement	No. 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

Source: Data from ref. *36*.

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 7 (11.9%) had a chromosome abnormality. Of 215 nonmacerated stillborns, 9 (4.2%) were chromosomally abnormal, and of 549 neonatal deaths, 33 (6.0%) had a chromosome abnormality *(30)*. 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 7**).

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, 3000 women underwent genetic amniocentesis *(40)*, 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)*. 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 dramatically, from several weeks in the 1970s and 1980s to less than 1 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 *(42–49)* (see Chapter 17).

Conventional Amniocentesis—15–24 Week of Gestation

Mid-trimester, 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 *(50,51)*, 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.

The risks associated with mid-trimester amniocentesis include leakage of fluid, cramping, bleeding, infection, and miscarriage. The risk of miscarriage following mid-trimester amniocentesis is

Table 5
Frequencies of Chromosome Abnormalities in Unselected Spontaneous Abortions

No. of abortuses studied	No. of abortuses (%) with chromosome aberrations	Different types of chromosome abnormality (% of all chromosome abnormalities)					
		Autosomal trisomy	45,X	Triploid	Tetraploid	Other	Ref.
8841	3613 (40.87%)	1890 (52.29%)	689 (19.06%)	586 (16.21%)	119 (5.51%)	249 (6.89%)	*30*[a]
3300	1312 (39.8%)	645 (49.2%)	201 (15.3%)	198 (15.1%)	78 (5.9%)	190 (14.5%)	*37*

[a]Data compiled from more than 10 studies.

Table 6
Frequency of Autosomal Trisomy for Each Human Chromosome Among Aborted Specimens

Trisomy chromosome	No. 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)

Source: Data from ref. *37*.

Table 7
Frequencies of Chromosome Abnormalities in Stillbirths and Neonatal Deaths; Combined Data from Three Studies

Macerated stillbirths		Nonmacerated stillbirths		Neonatal deaths		Total	
No. karyotyped	Abnormal	No. karyotyped	Abnormal	No. karyotyped	Abnormal	No. karyotyped	Abnormal
59	7 (11.86%)	215	9 (4.18%)	549	33 (6.0%)	823	52 (6.31%)

Source: Date from ref. *30*.

related to practitioner experience, number of needle insertions, size of the needle and other factors *(52)*. 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," 4606 women comprised the final study population *(53)*. Of these, half were randomized to have amniocentesis, and the other half were randomly assigned to the control, nonamniocentesis 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 α-fetoprotein 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 α-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).

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% *(54)*. 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 *(38)*, 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 *(55)*.

Early Amniocentesis

Interest in early amniocentesis (EA) has risen in recent years, as a result in large part the continued desire to provide and receive prenatal diagnosis at an earlier gestation without some of the risks and limitations associated with CVS, 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 α-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 *(56)*.

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 *(57)*. 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" *(57)*.

Although the procedure by which EA is performed is similar to that of mid-trimester 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 *(58)*.

Background

In one study conducted from 1979 through 1986, 4750 amniocenteses were performed, 541 of which were performed before the 15th week since the last menstrual period *(59)*. 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 5 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 11 spontaneous abortions (3.6%), 2 stillbirths (0.7%), and 1 neonatal death (0.3%), resulting in a total postprocedure 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 vs placental passage.

In 1988, the combined experience of six groups, including the above-mentioned study, was reviewed *(60)*. The total loss rate in 1240 pregnancies of known outcome ranged from 1% to 4.7%. Cell culture and amniotic fluid α-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 mid-trimester amniocentesis *(61)*.

Several other studies were published in the early 1990s *(62–67)*. In one paper, 505 amniocentesis procedures were performed between 11 and 15 weeks' gestation. In all but 3 pregnancies, follow-up information was available, including 16 fetal losses (3.1%)—10 in the 2 weeks after the procedure and 6 within the 28th week. 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- to 15-week group. The fetal loss rate between the 12- to 13-week and the 14- to 15-week groups showed no statistically significant difference. They concluded that early amniocentesis is "a valid alternative to traditional amniocentesis" *(62)*.

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 *(63)*. 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 *(64)* performed amniocentesis in 407 women between gestational ages of 11 and 14 weeks and compared the safety and accuracy with data obtained from collaborative studies of amniocentesis performed later in the second trimester. 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 postamniocentesis leakage of amniotic fluid. A total of 10 women in the study (2.6%) had postprocedure fluid leakage. It appeared that the orthopedic deformities might be related to a postprocedure 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 *(65)*. The needle gauge was changed from the 20-gauge needle used in their 1987 study to 22 gauge, and the volume of fluid removed was generally less. Pregnancy outcome was reported for 523 patients, of whom 12 (2.3%) had a postprocedural loss. This compared favorably with their previously reported loss rate of 4.7%. Of eight women with postprocedure 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 *(66)*. There were 2 pregnancy losses in the 64 patients for whom outcome information was available at the time of publication, and 4 congenital anomalies were seen in the 66 delivered babies: 1 imperforate anus, 1 hemangioma of the tongue, and 2 cases of positional talipes that required no treatment. These were apparently unrelated to amniotic fluid leakage.

Crandall et al. *(67)* 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 nonrandomized, later control group of 1386 women having traditional amniocentesis, whose spontaneous abortion rate was 0.6%, a statistically significant difference. 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 amnio-

centesis is independent of the procedure but the risk might be minimally higher than that for standard amniocentesis." There were no significant differences in congenital anomalies in the EA group (1.8%) vs 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 3 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 8** for a comparison of fetal loss rates.

In all of 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 2 laboratory studies of a combined 1805 EA specimens of 10–14 weeks' gestation *(68,69)*. The culture success rate was 99.8% for EA vs 100% for traditional amniocentesis 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% *(70)*. 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 *(71)*. 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 postprocedure 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 *(72)*. 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).

In Wilson's review *(73)*, he stated 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. He also stated that procedures at less than 13 weeks' gestation should be considered experimental. Certainly, the cumulative experience with 13- to 14-week EA procedures is much greater than that with under-13-week EA procedures. In addition, the two randomized EA studies he cited evaluate 11- to 12-week gestations and thus are not comparable to the 13- to 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. *(74)* 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. Of the EA specimens, all but 11 were obtained from weeks 12–14, and the rest were from weeks 9–11. 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 rates for both procedures was 0.8%. This study has limited applicability inasmuch as the numbers were small

Table 8
Outcome in Early (11–14 Wk) Amniocentesis Studies

Group	Study period	No. of patients with outcome data in EA group	Fetal loss rate within 2 weeks of procedure (%)	Fetal loss rate (%)[a] weeks' gestation at time of amniocentesis	Total fetal loss rate %	Needle gage	Comments
Hanson (1987) *(59)*	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 preamniocentesis history of bleeding were eliminated.
Johnson and Godmilow (1988) *(60)*	Review of six studies, including Hanson *(59)*; 1979–1987	1240	NA[b]	NA	1–4.7	22 in 5 centers; 20 in 1 center *(59)*	
Stripparo (1990) *(62)*	1987–1988	397	1.98[c]	9/208 (4.3), 11–13 weeks; 0/176 (0), 14 weeks[d]	3.9	22	
Penso (1990) *(64)*	1986–1989	389	0.8[e]	6/365 (1.6), 11–13 weeks; 3/42 (7.1), 14 weeks	3.96	22	Three of 8 newborns with postural deformities born after postamniocentesis fluid leak.
Hanson (1990) *(65)*	1986–1987	517	0.8	6/272 (2.2), 11–13 weeks; 5/255 (1.96), 14 weeks	2.5	20	
Crandall (1994) *(67)*	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. group. 0.6% EA group had hip dislocation or clubfeet compared to 0.22% in conventional amnio group.

[a] This figure includes spontaneous abortions, stillbirths, and neonatal deaths.
[b] NA = Not available.
[c] One hundred eight 15-weeks' gestation amnios were included in this figure.
[d] Data based on status at 28 weeks gestation.
[e] Fetal loss within 4 weeks.

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. *(75)* reported on a prospective, partially randomized study comparing EA and TA CVS in 1870 women. 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" as a result of the 2–3% excess risk of fetal loss in the EA group.

In response to this study, Saura et al. *(76)* stated that EA could be a "true alternative" to CVS after the 13th week, when the disadvantages of culture failure and fetal losses decrease. Bombard et al. *(77)* reported 1 loss in 121 procedures (0.83%) performed by 1 practitioner at 10–13 weeks using a 22-gauge needle. 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 8 fetal losses among 120 EA procedures, compared to none among the 64 CVS patients with a follow-up of 6 or more weeks *(78)*.

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 1800 pregnancies. The culture failure rate was 0.3% for gestations ranging to 10 weeks 4 days *(79)*.

An important consideration raised by some investigators *(73,79)* 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. The fact that amniotic fluid α-fetoprotein 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 *(80)*.

A 14-center study of 3775 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 to 14 weeks' gestation. Both types of procedure 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. 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- to 12- and 13-week gestation (0.6% in both cases vs 0% in CVS). Pseudomosaicism was seen in 1.2% of EA 11- to 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- to 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/pre-eclampsia 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 1914 CVS procedures, 34 had cytogenetic abnormalities, 2 were lost to follow-up, and 1878 were cytogenetically normal. Thirty-nine, or 2.1%, were lost or delivered before 28 weeks. This compares

Table 9
Volume of Amniotic Fluid (mL) Calculated Using All of 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

Source: Data from ref. *63*.

to 1861 EA procedures, of whom 38 had cytogenetic abnormalities, 3 were lost to follow-up, and 1820 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- to 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 *(81)*

Specimen Requirements

The volume of amniotic fluid obtained for prenatal diagnosis varies with the stage of gestation, with 15–20 mL conventionally removed by mid-trimester amniocentesis practitioners. In one report, data from several small studies was pooled and the volume of amniotic fluid for weeks 10–20 was calculated *(63)* (see **Table 9**). 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 *(82)*, whereas others withdrew 5–8 mL in pregnancies of 10 weeks, 4 days to 13 weeks, 6 days for an overall culture success rate of 99.7% *(79)*. 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 *(73)*. This probably explains the comparable culture success rate of EA compared to mid-trimester amniocentesis.

Chorionic Villus Sampling

Associated Risks, Limitations, Benefits, Turn-Around Time

Risks associated with CVS have extensively been studied. Perhaps the issue receiving the most attention in the past few years was raised by Boyd et al. involving 1 case *(83)* and then more extensively by Firth et al. *(84)*, who reported 5 babies with severe limb abnormalities out of 289 pregnancies in which TA CVS had been performed at 56–66 days' gestation. Four of these had oromandibular-limb hypogenesis syndrome. They hypothesized that CVS undertaken up to 66 days' gestation might 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 and Hsieh

(85,86). Monni et al. *(87)* suggested that the incidence and severity of limb defects was related to the gauge of the needle, because they used a 20-gauge needle, whereas Firth used an 18-gauge needle. In a series of 525 CVS procedures done before 66 days gestation, no severe limb defects were seen, and only 2 mild defects were seen in 2227 procedures that were done later *(87)*. Mahoney *(88)* then reported on two multicenter studies that compared transcervical CVS with amniocentesis, and another comparing TA CVS with transcervical CVS. Of 9588 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 *(89)*. 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, 2367 were done at 56–66 days, and 1 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 the result of their larger experience base.

In 1993, Jahoda et al. reported on 4300 consecutive transabdominal and transcervical CVS cases for which newborn follow-up information was obtained *(90)*. Of the 3973 infants born in this group, 3 (0.075%) had a terminal transverse limb defect. Two of these occurred in the transcervical CVS group sampled before 11 weeks' gestation (1389 patients), and the other one was in the transabdominal CVS group, sampled after 11 weeks (2584 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 *(91)*. The conclusions, based on a review of the literature, were mixed; some concluded that exposure to CVS appeared to cause limb defects, whereas 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 postfertilization (9 weeks after 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 *(92)*. Of 3430 pregnancies in which CVS had been performed, outcome information was available for 2759. Of these, 3 had transverse limb reduction defects, 2 among 804 CVS procedures performed at 9 weeks, and 1 among 1204 CVS procedures performed at 10 weeks. There were no limb reduction defects noted in 2192 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. *(93)* surveyed 165 obstetric units in Taiwan regarding the incidence of limb defects with and without CVS. 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. *(84)*. The 25 limb abnormalities in the non-CVS group involved syndactyly or polydactyly. In addition, oromandibular-limb hypogenesis was seen in 4 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 multistate case-controlled study comprising the years 1988–1992 *(94)*. 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 older 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 the absolute risk for transverse digital deficiency in babies after CVS was 1 per 2900 births (0.03%).

Froster and Jackson reported on outcome data in a World Health Organization (WHO) study on limb defects and CVS in 1996 *(95)*. 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. *(96)*, 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. 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 provided no evidence for any risk for congenital malformation caused by CVS.

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. *(97)* in a study from Victoria, Australia. A congenital malformations registry maintained there was reviewed by a medical geneticist, who classified all cases using the *International Classification of Diseases,* 9th edition *(98)*. All babies born with limb defects in 1990–1991 were identified, and the number of those whose mothers had 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 *(95)*, 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.

A subsequent study found no maternal age confounding effect in interpretation of CVS/transverse limb deficiency data *(99)*. 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 *(100,101)*, in large part the result of the concern regarding limb deficiencies. Its use is picking up again as more studies are published showing the safety of the procedure in the right hands at the right stage of pregnancy.

Fetal Loss in CVS

In the first large controlled study of the safety of CVS, Rhoads et al. *(102)* reported on 7 centers' experience with transcervical CVS in 2235 women compared to that of 651 women who had amniocentesis at 16 weeks' gestation. 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 *(103)*. Silver's group found that the number of placental passes and increased sample weight/aspiration attempt ratio might 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 *(104)*. Outcome information was available for 1609 singleton pregnancies in the CVS group and 1592 in the amniocentesis group. Thirty-one centers participated, and the numbers of cases submitted ranged from 4 to 1709. 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: 86/1528 in the successfully sampled CVS group and 25/1467 of the successfully sampled amniocentesis group (rate difference 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 *(105)*.

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% *(96)*. This risk was deemed comparable to that of amniocentesis.

Transabdominal Versus Transcervical CVS

Efficacy and risks associated with transcervical CVS (TC CVS) and transabdominal CVS (TA CVS) have been studied at several centers *(104,106–108)* (see **Fig. 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 placentae more easily. In their pilot study in 1988, Smidt-Jensen and Hahnemann *(108)* reported on 100 TA CVS cases at 8–12 weeks' gestation followed to term, compared to 200 amniocentesis cases. 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. *(106)* reported on efficiency and risk factors in 2411 patients, 1501 of whom had TC CVS and 910 of whom had TA CVS. 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 CVS and TC CVS *(107)*. 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 that "transabdominal and transcervical CVS appear equally effective, and by and large the choice might 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 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 *(109)* reported on numerical discrepancies between the karyotypes of fetal and placental cells, either full trisomies or mosaic aneuploidies, and similar reports followed *(110)*. 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 might not be representative of the fetus. Their

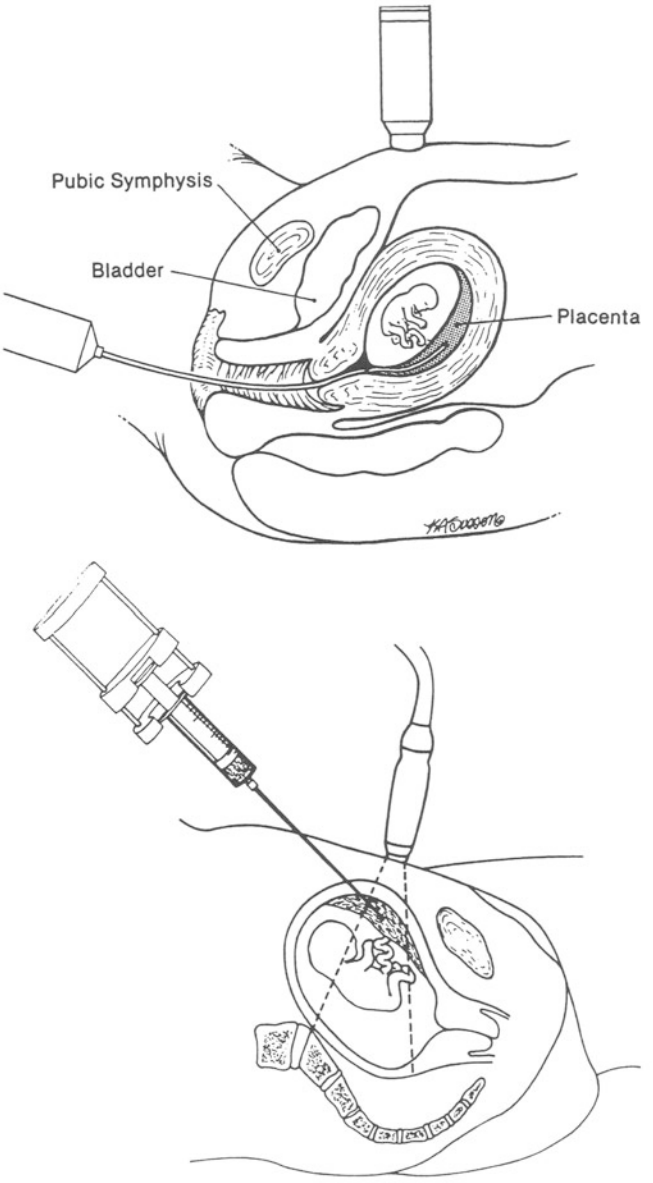

Fig. 1. Illustration of transcervical and transabdominal CVS. **Upper:** Transcervical CVS. A flexible catheter is introduced into the chorionic villi, or future placenta. **Lower:** transabdominal CVS. A spinal needle is inserted through the abdominal wall for sampling. (From ref. *281*, reprinted with permission.)

figures, although small, were similar to the 2% incidence of this phenomenon as previously reported *(111)*. Since then, others have found CPM to occur in 0.8–2% of viable pregnancies studied by CVS at 9–11 weeks' gestation *(112–119)* and in 0.1% or less in amniocentesis specimens *(75,114)*.

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. *(120)* found 6 cases of IUGR among 17 gestations with CVS-detected CPM, 5 in liveborns and 1 associated with intrauterine death. They noted that others had found a 22%

Table 10
Distribution of Specific Single Autosomal Trisomies in Each of the Groups of Mosaicism/Discrepancy in Chorionic Villus Tissue

Trisomy	CPM[a] (no. of case[s])	True fetal mosaicism (no. 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	
Total	192	20

[a] Includes all types of confined placental mosaicism, including direct-only, long-term culture-only, and both.

Source: Data from ref. *121*.

fetal loss rate among pregnancies with CPM. Wolstenhome et al. found 73 cases of CPM in 8004 CVS specimens from women referred for advanced maternal age, previous child with aneuploidy, or family history thereof *(119)*. 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, 7 were shown to have CPM involving the following chromosomes: 2 and 15 (1), 9 (1), 16 (3), del(13) (1) and 22 (1).

Hahnemann and Vejerslev *(121)* evaluated cytogenetic outcomes of 92,246 successfully karyotyped CVS specimens from 79 laboratories from 1986 to 1994. CVS mosaicism or nonmosaic fetoplacental discrepancy was found in 1415 (1.5%) of the specimens. **Table 10** shows the mosaic and nonmosaic chromosome findings. Their 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.

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 on 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 (UPD) (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 as a result of maternal disomy 15 *(119)*. 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 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 Chapter 19.

Interphase FISH in Confined Placental Mosaicism

Interphase FISH (see Chapter 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. *(112)* 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. 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 might be necessary to facilitate the prolonged survival of trisomy 18 conceptuses.

Schuring-Blom et al. *(122)* 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 were found to be chromosomally normal at amniocentesis. In all three cases, FISH showed the presence of the mosaic cell line confined to one part of the placenta.

Henderson et al. *(123)* 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. 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 CPM is diagnosed, 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 *(115,117,118)*. These studies documented false-negative and false-positive results using direct and cultured preparations, whereas 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 *(115)*, 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. In a study by Los et al. of 1829 consecutive CVS procedures with direct and long-term cultures, 1 conclusion was that using both modalities decreased the necessity for follow-up amniocentesis by 35% compared to that of long-term culture alone *(124.)*. In part at least, the finding that both techniques add to the diagnostic accuracy appeared to be related to the nonrandom findings of some trisomies in direct vs long-term cultured tissues. Trisomy 2 is seen more in cultured cells, and trisomy 3 is more often seen in direct preparations *(118,119)*. 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 *(125,126)*.

Maternal cell contamination (MCC) in CVS is generally the result of the lack of complete separation of chorionic villi from maternal decidua, and it is reported in an estimated 1.0–1.8% of cases *(115,117,118)*. The MCC reported in these studies is about half of the above figures, 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 *(115)*, 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%).

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. *(127)* illustrates the fact that a true low-level tissue-specific mosaicism can exist. 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 percutaneous umbilical blood sampling (PUBS) as follow-up studies because of possible CPM observed in CVS, one needs to weigh 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.

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; 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

Risks, Limitations, and Benefits

Percutaneous umbilical blood sampling is also known as periumbilical blood sampling, fetal blood sampling, or cordocentesis. The largest series in the literature regarding risks of PUBS *(128)* included outcomes of 1260 diagnostic cordocenteses among 3 fetal diagnosis centers and 25 practitioners. A fixed-needle guide was used in this study, and prospective data were 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 ±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. *(129)* in 1990, who pointed out that although 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. 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 0.2–2.0 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 1400 pregnancy outcomes after PUBS, the overall incidence of recognizable fetal bradycardia was estimated at 5% *(130)*. It was significantly more likely to occur when the umbilical artery was punctured. Boulot et al. *(131)* performed 322 PUBSs and noted fetal bradycardia, usually transitory, in 7.5% of their cases. Fetal bradycardia occurred in 2.5% of cases with normal outcome and in 12.5% of cases of fetal loss in one study *(129)*, and in another, 11 of 12 fetal losses were associated with prolonged fetal bradycardia *(130)*.

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. *(132)* compared the loss rates within 2 weeks of the procedure with the indications. Of 94 patients having prenatal diagnosis with normal ultrasound findings, 1 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" *(129)*. As a means of fetal karyotyping, it has the advantage of generating results in 2–4 days, compared to 6–14 days 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 *(133–136)* and thereby assist the couple in their decision-making.

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 *(137)*. For example, fetal blood karyotyping is not useful for the evaluation of mosaic or pseudomosaic trisomy 20. For further discussion of mosaicism, see the section Special Issues below and Chapter 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 might 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 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 Chapter 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 *(138)* (see **Fig. 2**).

Women 31 and Older with Twin Pregnancies

A 31-year-old woman 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

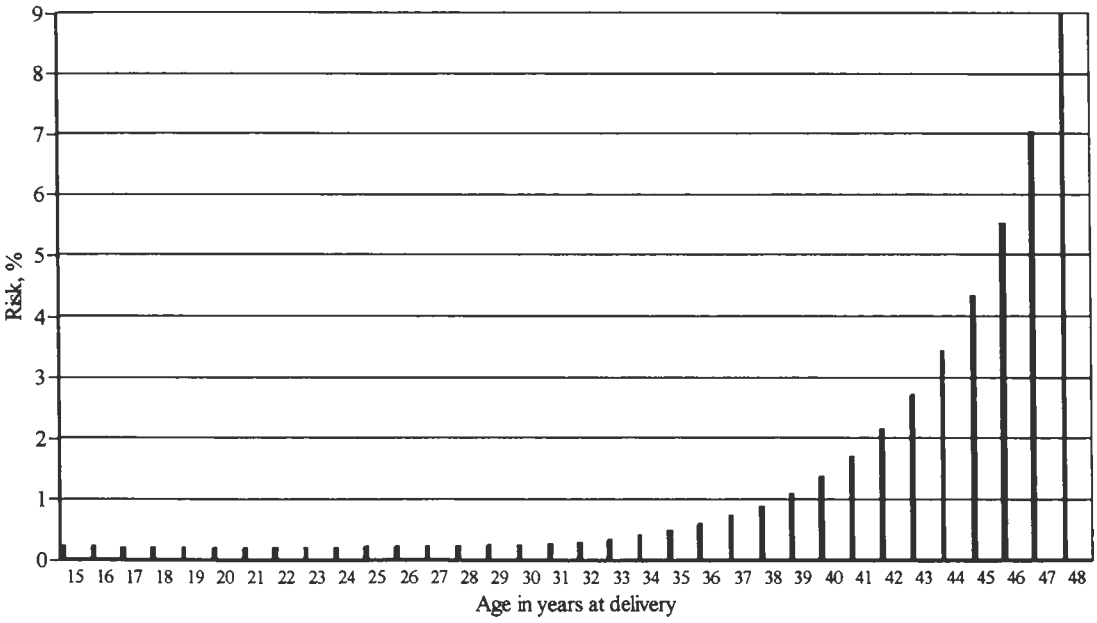

Fig. 2. Risk of chromosomally normal women to deliver chromosomally abnormal offspring. (From ref. *138*.)

has a chromosome abnormality is 5/3 × 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.

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

Seven 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 might 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 as a result of 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 as a result of impaired fetal movement in fetuses with neurologic disorders such as fetal akinesia.
- Congenital infection, acting through anemia or cardiac dysfunction *(139)*.

The fluid collects in the posterior neck fold, causing the appearance of a nuchal membrane separation on ultrasound examination (see **Fig. 3**). With resolution of the fluid collection, a nuchal fold or thickening develops.

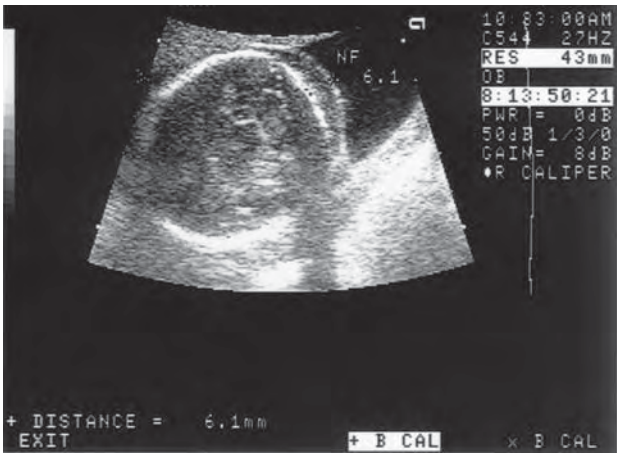

Fig. 3. Ultrasound image of increased nuchal fold (NF) measuring 6.1 mm (indicated by +) in a second-trimester fetus. (Courtesy of Greggory DeVore, M.D.

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 *(139–141)*. Nicolaides, a pioneer of first-trimester nuchal thickness ultrasound scans, cites a detection rate of 90% for chromosome abnormalities when 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% *(139)*.

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 *(142)*. Based on 22 other studies plus their own data, Landwehr et al. *(142)* found that 32% of 1649 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, 3308 fetuses of 14–24 weeks' gestation were evaluated *(143)*. 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. *(144)* in a study of 71 fetuses of 10–15 weeks' gestation, of which 63 were karyotyped. 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. *(145)* 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. Multiple logistic regression analysis was used to take into account data modifiers such as gestational age and maternal age. The authors exam-

ined the septated vs 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 *(142)*.

In 1015 fetuses of 10 to 14 weeks' gestation with nuchal fold thicknesses of 3 mm, 4 mm, 5 mm, 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 *(146)*. 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% *(147)*. Szabó et al. evaluated 2100 women under 35 years of age by ultrasound at 9–12 weeks' gestation *(148)*. 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.

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 *(149)*. In a review of ultrasound diagnosis of fetal abnormalities in the first trimester, Dugoff *(150)* 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 1000 for translucency 2.5–3.4 mm to 233 per 1000 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, in addition to close scrutiny of cardiac anatomy in the first trimester *(150)*.

Langford 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 2683 women screened, 8 cases of trisomy 21 were detected, all of which had a positive nuchal screen result. Serum screening of 1057 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 *(151)*.

See the subsection on nasal bone ultrasound findings in combination with nuchal thickening.

Cystic Hygroma and Cytogenetic Evaluation of Cystic Hygroma Fluid

Women whose second- or third-trimester fetuses have large cystic hygromas might not have an easily accessible fluid pocket in which to perform an amniocentesis. In such cases, paracentesis of the hygroma might yield a cytogenetic result, and at fetal demise or delivery, chorionic villous or placental cell cultures might 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 *(152)*.

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 1000 live births have a structural cardiac abnormality *(153)*. Prenatal data indicate that up to 32–48% of fetuses with cardiac abnormalities are chromosomally

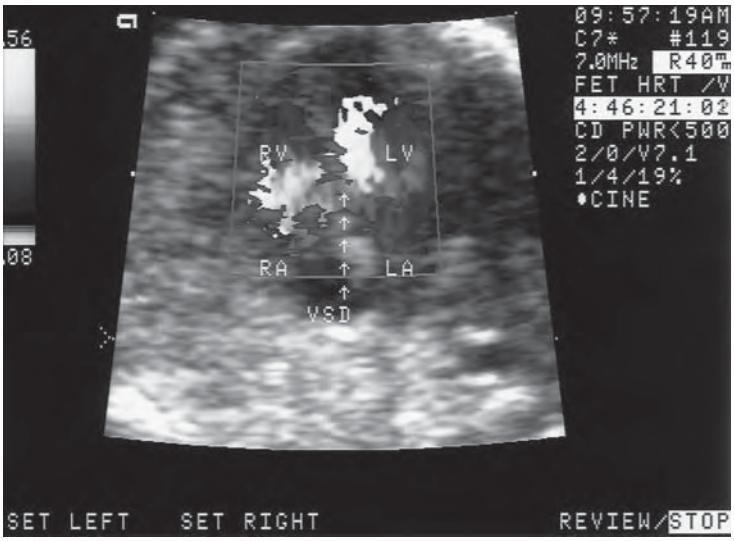

Fig. 4. Ultrasound image of a ventricular septal defect (indicated as VSD by arrows) in a 17-week-gestation fetus. RV = right ventricle; LV = left ventricle; RA = right atrium; LA = left atrium. (Courtesy of Greggory DeVore, M.D.)

abnormal *(153–155)*. 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 (VSD) (see **Fig. 4**), followed by tetralogy of Fallot (TOF), right or left hypoplastic heart, and transposition of the great arteries. Many investigators use the four-chamber view to evaluate the fetal heart, with an 80–92% sensitivity claimed by this method *(154)*. However, the four-chamber view alone will not detect TOF or transposition of the great arteries and only detects approximately 59% of heart abnormalities.

Extracardiac abnormalities are seen, depending on the gestational ages at which the ultrasound evaluations are performed and what is considered an abnormality, in 36–71% of fetuses with heart abnormalities *(153–155)*. 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 *(156)*.

A population-based study of the 22q11.2 deletion was undertaken by a group from Atlanta, GA. 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 5950 births *(157)*. Thirty-five of the children had

Table 11
Cardiovascular Abnormalities in Children wth 22q11.2 Deletion in Atlanta Study, 1994–1999

Finding	Total		% of
	No.	%[a]	total[b]
Cardiac abnormalities[c]	35	100	81
Interrupted aortic arch type B	8	23	19
Truncus arteriosus	4	11	19
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	1	3	2
Ventricular septal defect	7	20	16
Vascular abnormalities	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

Abbreviations: VSD = ventricular septal defect; ASD = atrial septal defect.
[a] Percentage among children with 22q11.2 deletion and cardiovascular findings *(n* = 35).
[b] Percentage among all children with 22q11.2 deletion *(n* = 43).
[c] One child had interrupted aortic arch and truncus arteriosus.
Source: Data from ref. *157*.

Table 12
Clinical Findings Amenable to Ultrasound Detection That Are Consistent with 22q11.2 Deletion

Finding	No.	%	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
Brainstem 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 cysts	1	2.3	43

Source: Data from ref. *(157)*.

heart abnormalities, as shown in **Table 11**. 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 TOF in the population were the result of the deletion. See **Tables 11** and **12** for a listing of the data from this study.

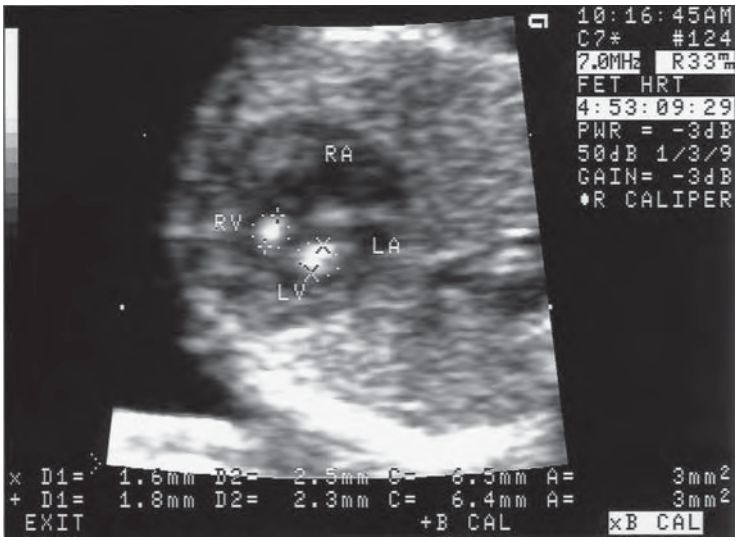

Fig. 5. Ultrasound image of intracardiac echogenic foci (indicated by +'s, ×'s, and circles of dots) in a 16-wk-gestation fetus with trisomy 13. No other abnormalities are detected. RV = right ventricle; LV = left ventricle; RA = right atrium; LA = left atrium. (Courtesy of Greggory DeVore, M.D.)

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 *(158)* (see **Fig. 5**). 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% *(159)*.

The association between left-ventricular echogenic foci and chromosome abnormalities was noted in a study of 2080 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 *(160)*.

The natural history of intracardiac echogenic foci was studied in a cohort of 1139 patients *(161)*. Echogenic foci were seen in 41 of 1139 fetuses, or 3.6%. In 38, the foci were in the left ventricle; in 2, they were in the right ventricle; and in 1, 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 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 *(162)*. 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 GM_1 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 intracardiac echogenic findings might not be.

Two subsequent publications, in contrast, found a significantly increased risk of trisomy 21 in fetuses with an intracardiac echogenic focus. In a study by Bromley et al. *(163)* of 1334 high-risk second-trimester patients, 66 (4.9%) had an echogenic intracardiac focus. Four of 22 (18%) trisomy

21 fetuses had an echogenic focus, compared with 62 (4.7%) of 1312 fetuses without trisomy 21. The presence of this finding increased the risk of trisomy 21 fourfold. 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 5 of 21 (24%) trisomy 21 fetuses compared to 4 of 75 (5%) controls, yielding an odds ratio (OR) for trisomy 21 of 5.5 ($1.12 < OR < 28.4$) when an echogenic focus is seen *(164)*.

The variations in reported incidences of intracardiac echogenic foci probably reflect the differences in definition of echogenic foci and in ultrasound machines. Ranzini et al. *(165)* noted 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 *(165)*. 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, $p = 0.005$) *(166)*. That some centers report an association between the foci and an increased incidence of trisomy 21 and other chromosome abnormalities whereas others do not might reflect differences in the populations studied—whether small or large, whether high risk or not. Overall, it is advisable to discuss the finding with the patient and counsel her of the probable increased risk of trisomy 21 and possibly other chromosome abnormalities even if no other abnormal ultrasound findings are present.

Nuchal Translucency and Intracardiac Echogenic 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 7686 normal singleton fetuses who had a nuchal translucency scan and either a subsequent normal follow-up scan at 18–23 weeks (n = 7447) or isolated intracardiac foci (n = 239) *(167)*. They found that the prevalence of intracardiac echogenic foci in fetuses with normal nuchal translucency was 2.9% versus 8.1% in the fetuses with abnormal nuchal translucency. The adjusted OR was 2.92. The authors concluded that an association exists between first-trimester nuchal translucency and second-trimester intracardiac echogenic foci, so they should not be used independently in risk calculations.

Nasal Bone

Hypoplasia or "absence" of the nasal bone has become the most rewarding recent ultrasound finding that appears to improve the detection of fetuses with trisomy 21 *(168–172)*. See **Fig. 6** for images of normal and hypoplastic nasal bones. 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 are 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%, 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% *(173)*.

Another study by Cicero et al. *(171)* attempted to answer the question concerning the association between "absence" of the nasal bone at 11–14 weeks and chromosome abnormalities. In this study, 3829 fetuses were studied. Maternal ethnic origin was recorded. The fetal profile was successfully recorded in 98.9% of cases. In 3358 of 3788 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.

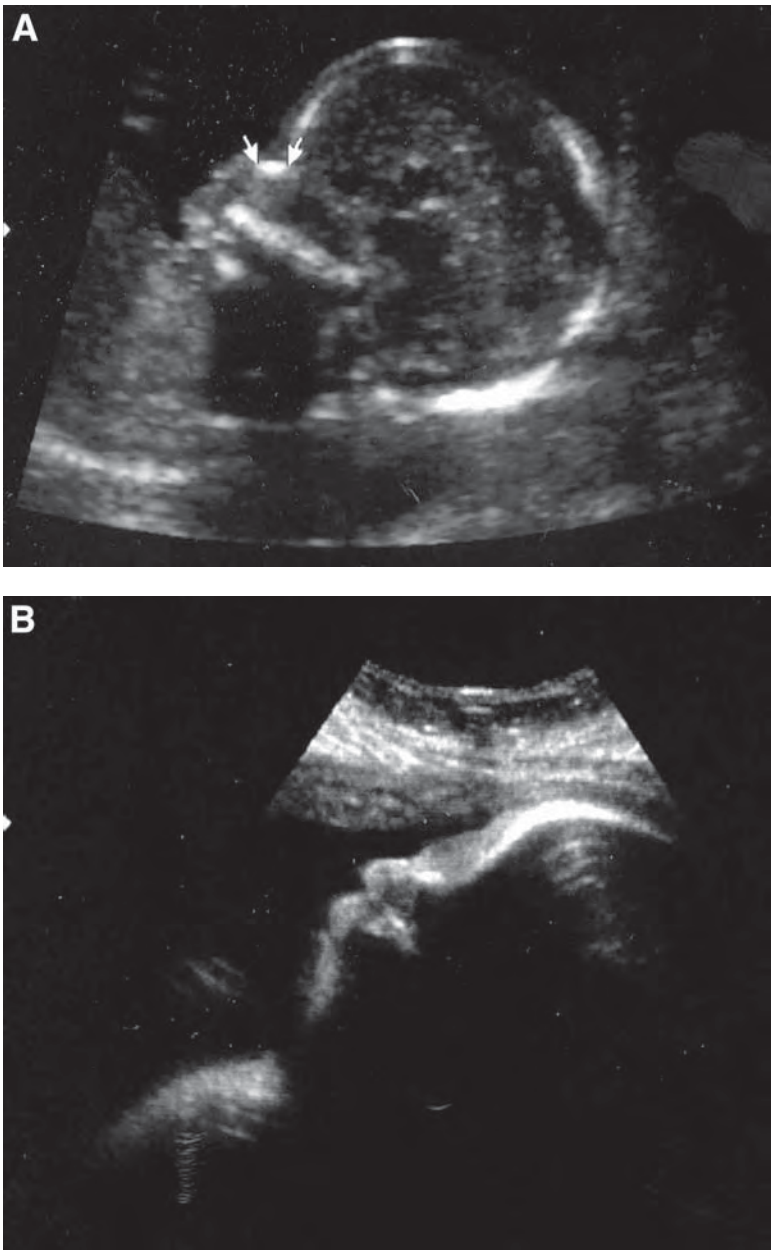

Fig. 6. Detection of nasal bone abnormalities with ultrasound. **(A)** A normal nasal bone is shown by arrows in a 27-week-gestation fetus; **(B)** a hypoplastic nasal bone is shown in a fetus at 32 weeks gestation. This fetus also has a thickened nuchal fold and the mother had a positive triple marker screen but refused amniocentesis. (Courtesy of David A. Miller, M.D.)

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 *(174)* 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. 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, two out of three Turner syndrome fetuses, and in 0.2% of fetuses with normal karyotypes *(170)*.

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. *(175)* performed ultrasound examinations on 1923 consecutive singleton pregnancies at 16–24 weeks and noted that nasal bone length increased as a function of gestational age, showing a linear relationship. Screening for trisomy 21 using the 5th percentile as a cut-off value resulted in a sensitivity of 59.1% for a 5.1% screen-positive rate. The likelihood ratio was 11.6.

A national collaborative study under the direction of Cicero is underway to evaluate this potentially important ultrasound finding more thoroughly.

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 *(176)*. This led to other studies with conflicting results. In 1996, Wickstrom et al. *(177)* published a prospective study of 7481 patients referred for prenatal ultrasound evaluation. Of these, 121 (1.6%) had isolated fetal pyelectasis (defined as 4 mm before 33 w eeks gestation and 7 mm at 33 w eeks 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 another 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. *(178)* studied 5944 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. *(177)*. Pyelectasis was seen in 4 of 23 (17.4%) of trisomy 21 fetuses and in 120 of 5876 (2%) of 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 *(179)* found that isolated pyelectasis was not associated with an increased risk for trisomy 21 unless other markers were present, such as those noted earlier *(178)*.

Degani et al. *(180)* evaluated the recurrence rate of fetal pyelectasis in subsequent pregnancies. They studied 420 women with 2 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. *(181)* studied 56 pregnant women with fetal pyelectasis or cystic lesions identified from 7500 ultrasound examinations. They found that none of 50 kidneys 15 mm or smaller in anteroposterior diameter had obstruction, and 11 of 14 (79%) of 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 could 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 (CPCs) has become recognized, along with several other fetal ultrasound findings, because of improvements in ultrasound imaging. CPCs were first described in 1984 *(182)*. The choroid plexuses are round or oval anechoid structures within the choroid plexus of the lateral ventricle derived from neuroepithelial folds. CPCs are seen in 0.18–2.3% of pregnancies *(183)*. These cysts usually disappear in the second trimester in normal pregnancies but could also disappear in chromosomally abnormal pregnancies *(184)*.

The first association between CPCs and fetal trisomy 18 was published in 1986 by Nicolaides et al. *(185)*. In the intervening years, many publications on the association between CPCs and chromosome abnormalities have appeared. Consensus has been reached as to the positive association between CPCs 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 *(186–190)* or whether the risk is not high enough to routinely recommend amniocentesis unless other risk factors are present *(183,191–193)*. Gross et al. *(192)* prospectively studied patients at their institution and reviewed literature to include a meta-analysis of other studies prospectively done with more than 10 cases of CPCs. From these data, they estimated the risk of trisomy 18 in fetuses with isolated CPCs to be 1 in 374. From the incidence of trisomy 18 and of isolated CPCs, plus these data, they estimated the positive predictive value of CPCs with trisomy 18 in the general prenatal population to be 1 in 390 *(192)*.

Nyberg et al. reviewed 47 consecutive cases of trisomy 18 and found that 12 of 47 fetuses (25%) had CPCs, 2 of which had no other ultrasound abnormality *(194)*. Although trisomy 18 is the chromosome abnormality most often associated with CPCs, seen in about three-fourths of aneuploid fetuses with CPCs *(191)*, trisomy 21, mosaic trisomy 9 *(192)*, triploidy *(183,186)*, 47,XXY and 45,X/46,XX *(186)*, trisomy 13 (183), unbalanced (3;13) translocation *(189)*, and cri-du-chat syndrome [del(5p)] *(195)* have also been seen in fetuses with CPCs.

Shields et al. *(186)* included mention of two issues in CPCs, namely size and unilaterality versus bilaterality. They concluded, 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 because of near-field artifact on ultrasound examination. These conclusions were also reached by Meyer et al. *(196)* in a retrospective review of 119 pregnancies with CPCs.

Demasio et al. performed a meta-analysis of 8 prospective trials of 106,732 women under 35 years of age with pregnancies affected by isolated choroid plexus cysts *(197)*. 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 1235 fetuses had CPCs for an incidence of 1.2%. None had chromosome abnormalities. The authors contended 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 CPCs *(198)*. 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, it was 1.87. The authors concluded that their data support offering women amniocentesis to evaluate trisomy 18 when maternal age is 36 or greater or when the risk for trisomy 18 detected by serum marker screening is greater than or equal to 1 in 3000. In another study by Ghidini et al., a likelihood ratio for trisomy 18 for isolated CPCs 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 *(199)*.

On balance, counseling regarding isolated CPCs clearly cannot be undertaken in a vacuum. A young woman with a negative triple marker screen for trisomies 18 and 21 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, CPCs are 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 CPCs are also reassuring; no effect on development was found as measured by the Denver II Developmental Screening Test *(200)*. Periodic cranial ultrasound should be performed after birth until the cysts have resolved.

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 could 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 *(201)*. Tables of risk for trisomy 21 for maternal age and maternal serum screening positivite 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 might not be recommended to women under age 40. Others have not found femur length to be reliable in ultrasound screening of trisomy 21 *(202,203)*, although humerus length does appear to be associated *(203)*. The positive predictive value for trisomy 21 in women with risks of 1 in 500 and 1 in 1000 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 6000 fetuses, and the conclusion was that the reference charts derived should be used in all Asian fetuses *(204)*. Thus, use of fetal biometric measures should be cautiously interpreted with racial factors in mind.

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 *(205–208)*. 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 *(205,206,208–216)*. The presence of coexisting elevated maternal serum α-fetoprotein increases the risk of adverse outcome, particularly fetal IUGR and demise *(206,216)*. See **Table 13**. The above-referenced studies 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 *(217)*.

The incidence of HEB in second-trimester fetuses with trisomy 21 is 7% *(218)*. The relative risk of adverse outcome in isolated HEB is 6.5 *(216)*.

Part of the reported variation in outcome of HEB is the result of different degrees of brightness of the finding and also to intermachine and interobserver variability (see **Fig. 7**). Grades of echogenicity, from 0 (isoechoic) to 3 (bonelike density) have been used *(206,215)*, 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 *(216)* commented on the decreased microvillar enzymes in amniotic fluid in pregnancies affected by trisomy 21, trisomy 18, and CF. It was thought

Table 13
Clinical Outcome of Second-Trimester Finding of Isolated Bright Hyperechoic Bowel

	Scioscia[a] *(205)*	Nyberg[b] *(206)*	Bromley[a] *(208)*	Slotnick[b] *(215)*	Muller[a] *(210)*	MacGregor[b] *(211)*
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 abnor. (%)	2[c] (11)	7[d] (11)	0	5[e] (4.9)	8 (4.5)	0/16 (0)
4. No. of cases with cystic fibrosis mutations (%)	0[f]/17 (0)	NT[b]	NT[g]	7/65 (11)	10/116[h] (8.6)	2/15 (13)
5. No. of cases with infections (%)	NT[g]	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[i]
7. No. of cases with nonelective demise (%)	2 (11)	3 (4.7)	15 (36)	—	24/104 (23)	3/45 (6.7)

Note: Excludes fetuses with ultrasound abnormalities other than isolated HEB.
[a] Retrospective study.
[b] Prospective study.
[c] Both trisomy 21.
[d] Five trisomy 21, one 47,XXX, one trisomy 13.
[e] All trisomy 21.
[f] Seven CF mutations tested.
[g] Not tested.
[h] One ΔF508 homozygote, nine heterozygotes; seven of the nine were unaffected, and the other two had no follow-up information. One to eight mutations tested.
[j] Not reported.

that the low levels in CF might be the result of delayed passage of meconium, and in trisomy 18 and 21, the delayed passage might be the result of decreased bowel motility or abnormal meconium. Fetuses with intra-amniotic bleeding have a to sevenfold increase in HEB *(208,218)*. 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 14**. Clearly, some ultrasound markers in isolation indicate a significant risk of chromosome abnormality, and others might not achieve significance unless other ultrasound abnormalities or other maternal risk factors are present.

In the past 15 years, medicine in the United States has evolved from recommending amniocentesis to women 35 and older to refining risks based on 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, as some women 35 and older now have their *a priori* risks altered downward and choose not to have amniocentesis as a result. Several scoring indices have been developed to provide individualized risk assessments *(203,219–223)*. The fact is that anyone with an ultrasound machine in the office can 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.

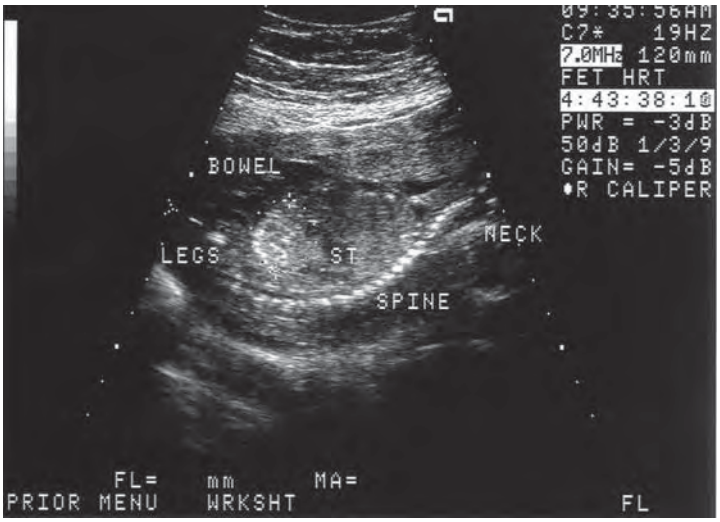

Fig. 7. Ultrasound image of moderately hyperechoic bowel (indicated by +'s and circle of dots) in a 17-wk-gestation fetus. ST = stippling, referring to pattern of hyperechogenicity. (Courtesy of Greggory DeVore, M.D.)

Positive Maternal Serum Marker Screen

High Maternal Serum α-Fetoprotein

The association between an elevated level (2.0 or 2.5 multiples of the median) of maternal serum α-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 1000 amniocenteses *(224)*. 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 greater than or equal to 1 in 1000 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 *(225)*, and in 1987, the association between high maternal serum human chorionic gonadotropin (hCG) *(226)* and low unconjugated estriol *(227)* and fetal Down syndrome was established. These three substances, or markers, are combined now in what is commonly known as triple marker screening (TMS). Hundreds of thousands of women in the United States have TMS in the second trimester of pregnancy, with a resultant increase in detection of trisomy 21 before age 35 and what appears to be a decrease in the incidence of Down syndrome births because of abortion of affected fetuses. The overall detection of trisomy 21 with TMS is about 65% with a mid-trimester risk cutoff of one 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) *(228)*.

Triple marker screening detects 60% of trisomy 18 fetuses as well, when a midtrimester risk cutoff of 1 in 100 is used *(229)*.

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 *(230)*. This is important to keep in mind when counseling patients.

Table 14
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% *(153–155)*	50–71% *(153–155)*	
Intracardiac echogenic foci	Not increased *(160)*; fourfold to fivefold baseline risk *(163, 164)*	1.6-fold baseline risk *(160)*	Isolated left ventricular foci appear more likely to be benign than multiple or right-sided foci *(162)*
Renal pyelectasis (>4mm before 33 wk and >7mm at 33 wk)	Not increased *(179)*; 3.3-fold increase over baseline *(177)*; 1 in 340 *(178)*	One in 90 *(178)*	In ref. *178*, no adjustment made for maternal age.
Choroid plexus cyst(s)	Not increased *(183, 191–193)*; 1 in 374 for trisomy 18 *(191)*; 1–2% *(186)*; 0.6% *(187)*; 2.4% *(196)*; 3.1% *(188)*; 4% *(189)*; 1 in 82 *(190)*; 1 in 150 *(191)*	82% *(186)*; 3.5% *(187)*; 5% *(106)*; 9.5%*188*; 1 in 3 *(101)*	No adjustment for maternal age in ref. *190*.
Septated nuchal membrane, 9–20 wk; >3mm, <15 wk and >5mm, 15–20 wk	56–60% *(142)*	Not studied	Ref. *142* is a retrospective database analysis. All pregnancies included in study had isolated nuchal finding and karyotype.
Simple nuchal membrane, 9–20 wk; >3mm, <15 wk; >5mm, 15–20 wk	10–25% *(142)*	Not studied	
Nuchal fold, >5mm, 15–20 wk	19–33% *(142)*	Not studied	
Nuchal thickening, 10–15 wk, >4mm	46% *(144)*	84% if hydrops and/or septations are present *(144)*	Best outcome was in nonseptated, nonhydropic; worst was in septated/ hydropic fetuses
Nuchal thickening, 9–15.5 wk, >3mm			
Septated	80% *(145)*	Other abnormalities only reported for chromosomally normal fetuses	
Simple	19% *(145)*; 27-fold risk for 34-year-old women and ninefold risk for women 35 and older		

(continued)

Table 14 *(continued)*

Finding	Risk(s) of aneuploidy if isolated finding	Risk(s) of aneuploidy if other ultrasound abnormalities are present	Comment
Short humerus, femur	Positive predictive value for trisomy 21 in women with risks of 1 in 500 and 1 in 1000 = 2.3% and 1.2%, respectively, for short humerus *(201)*. For short femur, some studies found very little increased risk *(202,203)*	Increased to variable degrees	If all long bones are of normal length and other ultrasound findings normal, some feel amniocentesis is not indicated in women under 40 *(201)*. Racial factors should be considered in any long bone measurement *(204)*.
Absent nasal bone	Absent or hypoplastic in 67–80% trisomy 21 fetuses compared to 1–2% chromosomally normal fetuses *(168–173)*	Appears to be independent finding from nuchal translucency, so can be used as independent markers in multiple marker algorithm *(173)*	Also seen more frequently in other chromosome abnormalities *(171)*

Other maternal serum markers have been studied, but none is used as commonly as TMS *(231)*. This will probably change in the next 10 years in favor of first-trimester screening alone or integrated screening strategies including data from first and second trimesters. The goal is to maximize detection while minimizing screen positive rates. See the discussion of integrated screening below.

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 in some centers 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 7063 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 *(232)*. 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. The positive predictive rate was 1 in 51. Women who were true positives had very high risks—median risk 1 in 22—compared to risks in women with false-positive results—median risk 1 in 111 *(233)*. 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 1–5% 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" *(234)*. 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 on 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 TMS alone *(235)*. Nevertheless, second-trimester quadruple screening has gained in popularity in recent years and this can be expected to continue.

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) *(236,237)*, were reported. Several more articles 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. Because of the nuchal translucency ultrasound technique being developed in England and some of the early studies having originated from there, Chasen et al. *(238)* studied a US population of 2131 pregnancies in 2003 in New York. 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 and Fetal Nuchal Translucency Screening—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 8514 patients with singleton pregnancies. 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 *(239)*.

Integrated and Combined Screening

The concept of integrated and combined screening is to examine whether first- and second-trimester screening can be used 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 above-mentioned second-trimester biochemical markers. This methodology was used to increase the detection of trisomy 18 *(240)* to detect 90% of trisomy 18 cases with a screen positive rate of 0.1%. A multicenter study called the First and Second Trimester Evaluation of Risk (FASTER) study is to have published its findings in early 2004 regarding its comparison of first- and second-trimester screening modalities.

Previous Pregnancy or Child with a Chromosome Abnormality

Having a previous pregnancy or child with certain chromosome abnormalities produces an increased risk of a future fetal chromosome abnormality *(241)*. The reasons for this are not known. There is some evidence that suggests that one or more recessive genes could predispose couples to nondisjunction.

Chromosome abnormalities known to increase the future risk of aneuploidy include all nonmosaic trisomies, 47,XXY, structural rearrangements, and marker chromosomes. Genetic counseling is suggested for couples who have had a pregnancy or child with any such karyotype, and ultrasound plus amniocentesis or CVS are recommended for consideration in future pregnancies.

Not known to be associated with an increased recurrence risk are 47,XYY, triploidy, tetraploidy, and 45,X. However, couples who have undergone the experience of having a pregnancy with one of these findings might wish to have genetic counseling, ultrasound, and prenatal chromosome analysis because of anxiety.

Mosaicism presents complicated counseling issues. It is prudent to apprise the couple of this and offer them the opportunity for prenatal diagnosis, as the risk of recurrence might be increased. Mosaicism is discussed below.

Other Indications for Prenatal Diagnosis

Pregnancy at Increased Risk for an X-Linked Disorder

For a growing list of X-linked conditions, prenatal diagnosis is available through linkage analysis, direct DNA studies, or enzymatic analysis of amniocytes. Patients are strongly advised to consult a genetics professional to inquire about availability of testing for a given disorder, given the rapidity of advances in the field. A website is also available (www.GeneTests.org) that lists molecular tests for

genetic disorders, along with listings of genetics centers, a glossary and descriptions of different genetic tests, and educational write-ups of many genetic disorders.

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 1000 Hispanic women and 0.66 per 1000 Asian women, with non-Hispanic Caucasians falling in between *(91)*. 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% *(242)*, so the increased fortification of grains with 1.4 mg folate per pound of enriched cereal-grain products by the Food and Drug Administration was announced in 1997. Having a previous affected pregnancy or child merits offering genetic counseling, ultrasound, and amniocentesis. Such women are advised to take 4 mg folate periconceptionally. All women of childbearing age, particularly those at increased risk for NTDs, should receive information about folate supplementation.

Chromosome abnormalities are associated with spina bifida and encephalocele *(243)* but do not appear to any significant degree to be associated with isolated anencephaly.

Chromosome Rearrangement in Either Member of a Couple

Some balanced structural rearrangements (see Chapter 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% *(244)*.

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 if the translocation is paternal *(244)* (see **Table 15**).

Most pericentric inversions (see Chapter 9), except the population variant inv(9), are associated with an increased risk of unbalanced offspring resulting from deletions/duplications, and individuals with such inversions should be offered amniocentesis. The risk of unbalanced offspring depends on the length of the inversion segments *(245)* (see **Table 16**). 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 might 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 *(246)*.

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.

Men with 47,XYY karyotypes usually have normal fertility and could 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 *(247)*.

A widely debated indication for prenatal diagnosis is a low level of mosacisim (3% or less) for 45,X/46,XX in the mother. This chromosome finding is quite common in unselected populations, and the bulk of evidence suggests that the 45,X cell line might be an artifact of culture or an age-related phenomenon. Data do not support an increased risk of chromosomally abnormal offspring in this population.

Pregnancy Exposed to Valproic Acid or Carbamazepine

These two anticonvulsant agents are known to be associated with an increased risk of NTDs *(248–250)*, so amniocentesis for measurement of amniotic fluid AFP and acetylcholinesterase is generally offered

Table 15
Prenatal Results for Robertsonian Translocations Involving Chromosomes 13, 14, or 15 and 21

Robertsonian translocationn type	Maternal carrier				Paternal carrier				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

Source: Data from ref. *244*.

Table 16
Prenatal Results for Pericentric Inversions (n = 173)

Method of ascertainment	Maternal carrier				Paternal carrier				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 (2.9%)	14	4	2	0	6	20
Other	63	4	2	99	68	3	0	71	140
Total	79	9	3 (3.3%)	91	74	8	0	82	173

Source: Data from ref. *244*.

to women who took these medications 3 months before and 1 month after conception. There is no known increased risk for chromosome abnormalities, but if amniocentesis is being performed, it is prudent to perform cytogenetic analysis on the specimen.

Women with Gestational Diabetes or Insulin-Dependent Diabetes Mellitus

It was thought in the past that women with gestational diabetes have a minimally increased risk of having offspring with malformations. It is now known that some women classified as having gestational diabetes are probably unrecognized insulin-dependent diabetics *(251)*. Rosenn et al. *(252)* and Schaefer et al. *(253)* evaluated glycemic thresholds as predictors for congenital malformations. Both found that a fasting first-trimester blood glucose concentration of less than 120 mg/dL was associated with no increased risk in malformations. Specifically, the risks for NTDs range from a 10-fold *(254)* to a 20-fold *(255)* increased risk in infants of diabetic mothers. Because some centers offer amniocentesis for detection of NTDs for this indication, cytogenetic analysis of the specimen would also be prudent.

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 *(256–259)*. Advanced paternal age is not associated with fetal chromosome abnormalities. It is, however, associated with a linearly increased risk of some autosomal dominant new mutations in the offspring *(260)*. In a policy statement on the subject, the American College of Medical Genetics points out the fourfold to fivefold risk in offspring of men in their forties versus those of men in their twenties. 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, Waardenberg 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 Chapter 18), hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), Duchenne muscular dystrophy, incontinentia pigmenti, Hunter syndrome, Bruton agammaglobulinemia, and retinitis pigmentosa *(261)*. The American College of Medical Genetics acknowledges the risk but states that ultrasound examination is usually of little benefit. Genetic counseling is indicated so the expectant couple can understand the issues, and it is prudent to offer detailed fetal ultrasound examination in pregnancies involving men 40–45 years and older *(261)*.

Special Issues

True Mosaicism and Pseudomosaicism

Mosaicism, or the presence of two or more cell lines in culture, 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 *(262–264)*.

The origin of the mosaic cell line cannot be determined without molecular studies. In general, however, it appears that the majority of 45,X/46,XX cases occur after a normal disomic fertilization, most mosaic trisomies are the result of postzygotic loss of the trisomic chromosome, and for trisomy 8, most cases are the result of somatic gain of the third chromosome 8 postzygotically *(265)*.

Table 17
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

[a]IUGR = intrauterine growth restriction
Source: Data from ref. *278*.

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 *(266,267)*. Even one cell with trisomy 8 could 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 could 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 *(30)*. See **Table 17** for incidences of mosaicism for specific chromosomes.

Schuring-Blom et al. *(268)* evaluated first-trimester cytotrophoblast 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. 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 as follows:

- 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. *(269)* 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. The risk of abnormal outcome in pregnancies with less than 50% trisomic cells and greater than 50% tri-

somic 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 an 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 were 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 *(270)* showed a total of 13 reported cases in which trisomy 12 mosaicism was observed in amniocytes. 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 was 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, tracheo-esophageal, 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 could be the result of unrecognized abnormalities in second-trimester fetuses, or there could have been a bias toward reporting live births with congenital abnormalities.

Outcomes of 144 cases of trisomy 20 mosaicism *(30)* indicate that 112 of 123 cases (91%) were associated with a normal phenotype; 18 of these were abortuses. In most cases, the cells with trisomy 20 are extraembryonic or largely confined to the placenta. Of the 11 abnormal outcomes, 3 were in liveborns and 8 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 1–2 years, all were normal except for 2 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, they should be done on kidney, skin, and placental tissues. Finally, true mosaic trisomy 20 could 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, whereas 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 *(271)*.

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 resulted 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 *(272)*. 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 *(273)*. 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 *(274)*.

Mosaic trisomy 16 is commonly reported in CVS cultures *(275,276)* 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. When CVS detection of mosaic trisomy 16 occurred, in 1 series of continued pregnancies, 13/63 resulted in fetal death, with 3 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 6, and the abnormalities were isolated to a single organ in 7. Of the continuing pregnancies, only 17 of 60, or 28%, appeared to be full-term, normal pregnancy outcomes *(276)*.

Finding mosaic trisomy 16 at amniocentesis appears to be associated with an elevated maternal serum AFP (MSAFP; see above). Hsu et al. *(277)* reported on a series of 11 cases diagnosed via amniocentesis ascertained after an elevated MSAFP. 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 gonadotropins.

In another series of 29 amniocentesis-diagnosed cases of trisomy 16 mosaicism not referred because of abnormal CVS results, the indication was elevated MSAFP in 12; in only 3 was the indication 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 2 other babies. Only four appeared to have a totally normal outcome *(276)*. 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 *(276)*.

Is there is a phenotype associated with trisomy 16 mosaicism? Some abnormalities have occurred more than once in affected fetuses and newborns (viz. VSD, complex heart disease, hypospadias, imperforate anus, inguinal hernia, club foot, and IUGR). The combination of an elevated maternal serum hCG or AFP, plus IUGR and one or more of the above-listed structural abnormalities could raise the clinical suspicion of mosaic trisomy 16 *(276)*.

Other Mosaic Trisomies and Monosomies

Trisomy 22 mosaicism was reported in a collection of 11 cases *(30)*. 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.

In a study of chromosome mosaicism of chromosomes other than 13, 18, 20, and 21, 1 to 25 cases each of mosaic trisomies 2–9, 11, 12, 14, 15, 16, 17, 19, and 22 were reported in 1 series *(278)*. 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 18** for more information on these mosaic trisomies. Hsu reported on 13 cases of autosomal monosomy mosaicism that had been prenatally diagnosed *(30)*. 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 reported to be phenotypically normal. If autosomal mosaic monosomy is detected, particularly of chromosomes 21 or 22, further workup, such as PUBS and ultrasound examination, is indicated.

Table 18
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	No. 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

Note: Very high = >60% risk; high = 40–59% risk; moderately high = 20–39% risk; moderate = up to 19% risk; low-risk, no abnormalities seen; undetermined-no cases for evaluation.
Source: From ref. *278*.

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 *(30)*. 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 Chapter 6). Cell culture failure is more likely to occur in advanced-gestation amniocentesis specimens, because 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 7852 (0.4%) amniocentesis specimens classified as unexplained growth failures, and, in this group, 10 women did not repeat the procedure and 22 did *(279)*. Of the 10 who did not, a fetal bladder-outlet obstruction, two stillbirths, and one acardiac twin resulted. Of the 22 who repeated, 18 had normal fetal karyotypes, but 4 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 is rarely found. Maternal cells were detected in 0.17% of 44,170 cultured amniotic fluid samples in one study *(280)*. Because one would expect to detect this in only 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 maternal cell contamination (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 *(281)*. Maternal cell contamination of less than 20% was seen in 19 specimens in which the placenta was posterior, and in 2 others, which were bloody specimens in 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% was seen 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.

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Cytogenetics of Spontaneous Abortion

Solveig M.V. Pflueger, PhD, MD

INTRODUCTION

Pregnancy loss is quite common, with 15–20% of recognized pregnancies resulting in failure. The majority of these occur early in gestation, although 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 can be raised regarding the loss, including the following: 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 provided could ultimately impact family planning and management of any future pregnancies the couple might 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 might be uncomfortable with discussing the loss, if they are even aware of it, and so might avoid the issue altogether. She might 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 might open dialogue with the patient and her partner about their specific concerns for the future. This, in turn, could 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 gonadotrophin (hCG) levels *(7–9)*. A four-year follow-up of 3084 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%

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Table 1
Intrauterine Mortality per 100 Ova Exposed to Fertilization

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

Source: Ref. *12*.

(11), a level that is quite low compared with most other mammalian species. Leridon *(12)* provides a useful summary table of pregnancy survival from fertilization to term, with only 31 survivors among 100 ova exposed to fertilization (see **Table 1**). 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.

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 1205 pregnancy losses of varying gestational ages submitted to the author's laboratory between 1992 and 1996 revealed 539 (45%) cases with identified cytogenetic abnormalities *(13)*. 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, because 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 *(14)* found that approximately two-thirds of losses under 8 weeks and nearly one-fourth of those between 8 and 12 weeks had abnormal karyotypes (see **Table 2**).

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 and that there will be an abnormal karyotype (see **Table 3**).

Gestational Age

Examination of induced abortuses confirms the greater incidence of karyotypic abnormalities earlier in pregnancy *(15)* (see **Table 4**). A total of 1197 pregnancies were examined. The rate of chromosomal abnormality varied with gestational age; 9.3% of cases were abnormal at 3–4 weeks, falling to 5.4% at 9–10 weeks.

Table 2
Chromosomal Abnormalities and Gestational Age

Gestational age (weeks)	No. 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%
Total weeks 2–7	1097	724	66.0%
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%
Total weeks 8–12	108	25	23%
Total	1205	749	62.2%

Source: Adapted from ref. *14*.

Table 3
Abnormal Development and Gestational Age

	4 weeks or less		5–8 weeks		9–12 weeks	
Study	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%)

Source: Adapted from ref. *14*.

Table 4
Chromosomal Abnormalities in Induced Abortuses

Developmental age	No. of cases	Abnormal cases (%)
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%)

Source: Ref. *15*.

The likelihood of detecting congenital anomalies in therapeutic terminations is variable and might be a reflection of the thoroughness of the examination and the skill of the examiner. However, identification of anomalous development could have considerable impact on future reproduction, and it is the opinion of the author that careful anatomic evaluation of aborted products of conception should be considered regardless of the whether the pregnancy is aborted spontaneously or induced.

Table 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%

Source: Adapted from ref. *18*.

In the second trimester, ascending infection becomes more frequent as a cause of spontaneous pregnancy loss. Abnormal karyotypes become less prevalent as pregnancy progresses because many of the less viable abnormal gestations have already undergone growth arrest and miscarriage. Gaillard et al. *(16)* studied 422 consecutive second-trimester losses. 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 the result of 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 1 in 200 live newborns exhibit 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 a balanced rearrangements *(17)*. 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 *(18)* examined the likelihood of loss for various chromosomal abnormalities, confirming a virtual 100% loss for autosomal monosomies and tetraploids. 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 (see **Table 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 *(19)* compared the frequency of chromosomal anomalies of spontaneous abortions and live births (see **Table 6**).

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 on the particular cytogenetic abnormality involved.

Table 6
Percent of Chromosomal Anomalies Among Spontaneous Abortions and Live Births

Anomaly	Spontaneous abortions	Live births
Autosomal trisomies		
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%
Total trisomies	21.33%	1.34%
Monosomy X	8.35%	0.01%
Sex chromosome trisomies	0.33%	0.15%
Triploids	5.79%	0.00%
Tetraploids	2.39%	0.00%
Total abnormal	41.52%	0.60%
Number karyotyped	3,353	31,521

Source: Adapted from ref. *19*.

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 on the type of chromosomal error.

TYPES OF ERROR 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 *(20,21)* revealed 921 abnormal karyotypes (61.5%). 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 10 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 might 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 1205 products of conception of varying gestational ages received in our 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 *(13)*. 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 as a result of nonchromosomal etiologies. The distribution of types of abnormality 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 as a result of the formation of a gamete with fewer or more than the usual number of chromosomes (see Chapter 2). With the exception of monosomy X, complete, apparently nonmosaic monosomies are almost invariably lethal early in gestation and are not usually identified in recognized pregnancies. Gene dosage effects or imprinting failure could 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 *(21–23)*. All autosomal trisomies have been reported in multiple studies with the unique exception of chromosome 1, the trisomy for which 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 might 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 (Edward syndrome) and trisomy 13 (Patau syndrome) are seen in approximately 1 in 6000–8000 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 Chapter 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 spontaneous abortions. Trisomy 21 is third most frequent, accounting for 10.5% of trisomies and 2.11% of spontaneous abortions *(18,23)* (see **Table 7**).

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 Down syndrome *(14)*.

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% *(24,25)*. Verp and Simpson *(26)* combined data from several smaller studies to suggest that the risk for an aneuploid liveborn following a trisomic abortus might also approximately 1%. 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 *(27)*. This raises the issue as to whether prenatal diagnosis should be offered to couples who have experienced a previous trisomic abortus.

There might be increased susceptibility to trisomic conceptuses 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. Our 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 might benefit from genetic counseling following a trisomy or any other chromosomally abnormal pregnancy.

Table 7
Distribution of Individual Trisomies Among Trisomic Spontaneous Abortions

Chromosome no.	% 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

Source: Data from refs. *18* and *23*.

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 on the chromosome involved. Of 436 informative cases reviewed by Hassold and colleagues, 407 trisomies were maternal in origin *(28)*. All cases of trisomy 16 and trisomy 22 were also maternal in origin, 19% of trisomies involving chromosomes 2–12 were paternal in origin, and 27% of trisomies of chromosomes 13–15 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 oöcytes reveals a significant percentage of cytogenetic abnormalities. Kamiguchi and colleagues found abnormal chromosomal complements in 24.3% of unfertilized oöcytes *(29)*. Aneuploidy was most commonly observed, followed by diploidy and structural abnormalities. A review of 1559 published cases revealed chromosomal abnormalities in 24% of mature oöcyte karyotypes *(30)*. The majority were aneuploid (22.8%); fewer had structural aberrations (1.2%). The particular chromosomes involved showed an unequal distribution with an excess of "D" and "G" group aneuploidies and less than expected "A" and "C" group examples (see Chapter 3 for a description of chromosome groups). It is of interest to note that only one oöcyte 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 might 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 resulting from nondisjunctional events and 10% had structural abnormalities *(31)*. More recently, fluorescence *in situ* hybridization (FISH) techniques have been used, allowing for examination of far greater numbers of sperm. FISH does have

Table 8
Effect of Maternal Age

Maternal age (years)	No. karyotyped	% abnormal	% trisomic	% nontrisomic
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

Source: Adapted from ref. *36*.

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 *(32)*. Disomy for chromosomes 1, 16, X, and Y ranged from 0.34% to 0.84% in infertile subjects and from 0.32% to 0.61% in fertile subjects. Guttenbach and colleagues examined 16,127 sperm from 8 healthy donors for disomy of chromosome 18 and found a range of 0.25–0.5% *(33)*. Examination of 76,253 sperm from 7 donors revealed a range of 0.31–0.34% of disomy for chromosomes 3, 7, 10, 11, 17, and X *(34)*. 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 *(19,35,36)* (see **Table 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 effect on the smaller chromosomes, with mean maternal age increasing with decreasing size of the trisomic chromosome *(22)*. Susceptibility to nondisjunction might not be the same for all chromosomes, and recurrence risks might be dependent on 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 *(18,37)*. The incidence of Turner syndrome in surviving pregnancies is approximately 1 in 1000 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 1000 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, might cause the patient to

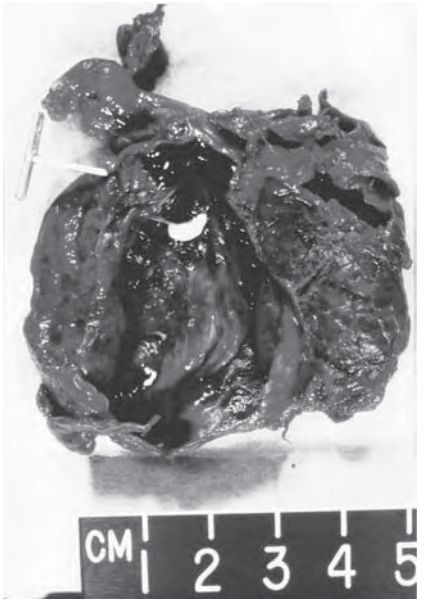

Fig. 1. Gestational sac with very small embryo, consistent with an underlying cytogenetic abnormality, often a nonviable trisomy or, as in this case, monosomy X.

present for evaluation. Some affected individuals might 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 *(23)*.

Monosomy X gestations vary considerably in phenotype and might 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 (see **Fig. 1**). A lesser number survive into the second trimester, at which time the phenotype is often that of an hydropic fetus with massive cystic hygroma (see **Fig. 2**). Renal and cardiac anomalies are frequently seen as well. During the third trimester, the appearance might 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 might 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 *(38,39)*. Rather, survival of the early pregnancy could be dependent on presence, in some tissues, of a second sex chromosome, either another X or a Y. The nonmosaic 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 *(39,40)*. This second cell line could be absent from many tissues and is often difficult to detect with routine cytogenetic studies, but could sometimes be identified using multiple sampling sites or FISH techniques. Although extensive efforts at identification of a second cell line might 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.

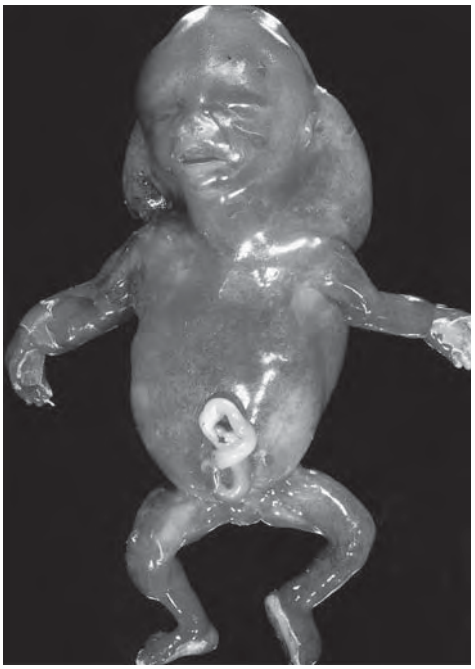

Fig. 2. 45,X spontaneous loss at mid-gestation. Note marked cystic hygroma and generalized edema.

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 *(14)*. The evidence that many cases of monosomy X are the result of postzygotic nondisjunction might possibly explain the difference in maternal age between aneuploid pregnancies with monosomy X and those with autosomal trisomies. Mitotic nondisjunction during embryogenesis appears to be a different process, which might 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 postzygotic/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 might be better tolerated by the developing conceptus than complete aneuploidy, and there is evidence that survival of a trisomic fetus to term might 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 Chapter 12). Early nondisjunction 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 could present a dilemma in interpretation of amniotic fluid karyotype, yet might not pose a problem for the fetus *(41)*. Within

the placental chorionic villous tissue, there might 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 *(42)* and with increased risk for adverse pregnancy outcome, including growth retardation and fetal demise *(43)*. Confined placental mosaicism could 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 *(43)*. 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 might be especially high in growth-disorganized embryos *(44)*. The cytogenetic contribution to human pregnancy failure might thus be even higher than estimates based on early series, because those cases were often examined using only a single tissue source, and some morphologically aberrant conceptuses classified as euploid might 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 postzygotic duplication of one of the chromosomes in an originally euploid cell line. The likelihood of one or the other mechanism might 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 *(45)*. Mosaic trisomy 8, on the other hand, might be more likely to survive when the aneuploid line is derived later, as a result of a postzygotic error in mitosis in a conceptus that was originally chromosomally normal.

Mosaicism in the placenta could 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 *(46,47)*. Mosaicism does not appear to be necessary for survival in trisomy 21, possible due to a less deleterious effect of this trisomy on placental function (46).

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 the potential loss of heterozygosity with expression of recessive traits only carried by one parent or because of effects of inappropriate imprinting (see Chapter 19). 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 could 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. Postzygotic 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 could also explain a 45,X/69,XXY fetus described by Betts and colleagues *(48)*. A number of

diploid/triploid mosaics have also been reported *(49)*. 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 *(50)*.

Errors in Fertilization

Errors in fertilization can lead to pregnancies with an extra complete set of chromosomes (triploidy) (see Chapter 8), and 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 Chapter 19) and the role imprinting plays in fetal development and carcinogenesis. Imprinting may have functions not only in gene expression early in embryogenesis but could also play a significant role in surveillance for chromosome loss later in life and thus help reduce the risk of cancer *(51)*.

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 from either 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 *(52)*. 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 oöcyte, have also been postulated *(53)*. 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 *(54,55)*.

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 (see **Fig. 3**). 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 (see **Fig. 4**). 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. 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 *(56)*. 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 *(57)*. 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

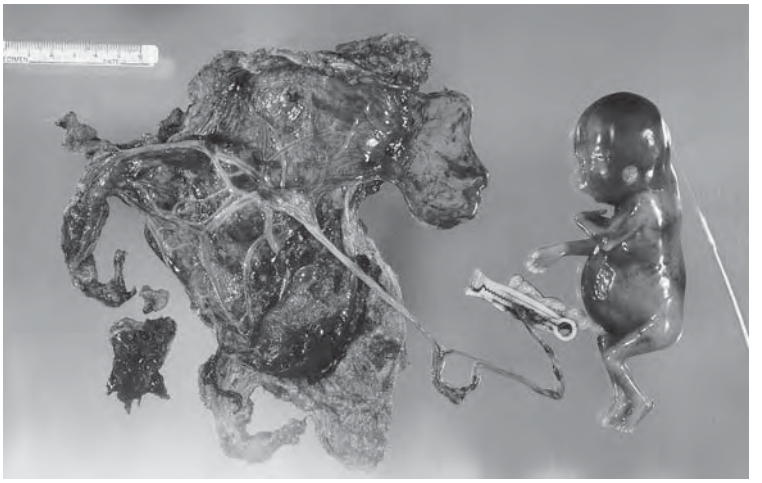

Fig. 3. Paternal triploid, 69,XXY karyotype. Patient presented with markedly elevated β-hCG at 16 weeks. Note the very large placenta in relation to the size of the fetus.

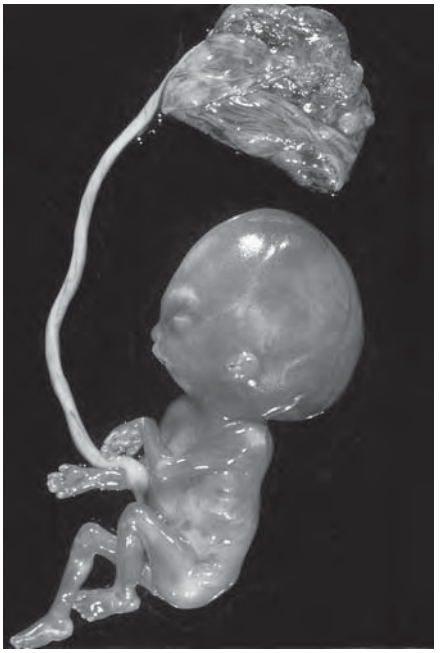

Fig. 4. Maternal triploid. Note the very small placenta in relation to fetal size, and fetus with micrognathia, syndactyly, and disproportionately large cranium in relation to body.

might 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 *(55)*.

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 *(58,59)*. The mechanism for malignant transformation in the complete mole appears to be relaxation of imprinting with expression of genes that would normally

be repressed *(60,61)*. Imprinting has also been suggested as an explanation for the difference in phenotype between the maternal and paternal triploids *(62)*.

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 1205 pregnancies following a complete molar pregnancy and found no increase in risk for stillbirth, prematurity, ectopic gestations, malformations, or spontaneous abortion *(63)*. However, there appears to be a recurrence risk of about 1–2% for a future mole following a molar pregnancy *(63–66)*. 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 *(63)*. Several pedigrees suggesting familial predisposition to molar pregnancies have also been reported *(67–70)*, although the significance of family history on risk has not yet been established with certainty.

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 5726 karyotyped spontaneous abortions, revealing 0.28% balanced and 1.54% unbalanced rearrangements *(71)*. Balanced rearrangements include Robertsonian translocations, reciprocal translocations, and inversions (see Chapter 9). A survey of the literature by Dewald and Michels revealed translocations in 2.1% of couples with recurrent miscarriage *(72)*. Translocations were found in 1.7% of male patients and 2.6% of female patients. This compares with an incidence of 1 in 500 (0.2%) in newborns *(73)*. The frequency of balanced rearrangements in spontaneous abortions is not markedly increased over that seen in live births. This is not unexpected, because 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 Chapters 3 and 9). These might occur *de novo* or be familial in origin. The incidence of unbalanced Robertsonian translocations is much higher in spontaneous abortuses that 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 *(74)*. 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 are *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. Because 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 might be more likely in those who have experienced both miscarriages and live births than in those with only miscarriages *(75,78)*.

Campana and colleagues note that the chromosomes and breakpoints involved in structural rearrangements do not appear to be random *(76)*. Survival of pregnancies with unbalanced chromosomal complements appears to be dependent on 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 *(77,78)*. 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 on 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 might not be applicable in all cases and more specific risks figures based on the particular chromosomes involved might be beneficial in evaluating reproductive options for a family in which a balanced translocation has been identified *(79)*.

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 Chapter 9; see also ref. *25*.

Chromosomally Normal Pregnancy Loss

Identification of the cytogenetically normal spontaneous abortion might be more important clinically than identification of the aberrant gestation. The risk of repeat miscarriage is higher when the prior loss is chromosomally normal *(80)*. 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 *(14)*. 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 *(81)*. 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 *(82,83)*.

Women with recurrent pregnancy losses and normal fetal karyotypes might be more likely to have underlying uterine abnormalities or endocrine dysfunction (see Chapter 11). Menstrual irregularities and elevated luteinizing hormone levels are more common in women with normal fetal karyotypes than in women with abnormal fetal karyotypes *(84)*.

Immunological disorders have also been linked with recurrent normal pregnancy loss *(85)*. Systemic lupus erythematosus is perhaps the best known, but other autoimmune conditions have also been implicated *(86)*. Because 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 *(87–89)*. 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 *(90)* and administration of intravenous immunoglobulin, have been suggested *(91)*.

Mutations that are lethal in the embryo are known from animal models and could also be a factor in recurrent euploid abortion in man *(92)*. Mutations in genes responsible for early organization of the embryo can have devastating effects on embryogenesis, with resultant pregnancy failure. Parental sharing of human leukocyte antigens (HLAs) might also increase risk for spontaneous abortion, although the mechanism is not yet clearly understood *(93)*. 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 could 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 *(80)*. 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 could offer a greater chance of success *(94)*. Placental tissue, on the other hand, often remains viable for a much longer period of time, because 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 *(95,96)*. 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 might be helpful in decreasing the likelihood of maternal cell contamination *(97)*.

Fluorescence *in situ* hybridization (see Chapter 17) using either tissue sections or disaggregated cells can be used in cases in which the tissue was accidentally fixed in formalin prior to receipt in the cytogenetics laboratory because it does not require dividing cells *(98)*. 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 *(99)*. This can be especially useful for products of conception with hydropic changes seen on histology, 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 *(100)*.

A newer methodology that has been proven useful for diagnosis of unbalanced karyotypes in cases for which dividing cells are not available is comparative genomic hybridization (CGH) *(101–103)*. This method is dependent on DNA extraction but does not require viable or intact cells and thus can be used for formalin-fixed frozen or paraffin-embedded tissues as well as fresh samples. Using different fluorochromes, test specimen and reference DNA samples are hybridized to normal metaphase chromosomes. The intensities between the test and reference samples are compared, enabling identification of gains or losses of individual chromosomes or chromosomal regions (see Chapter 17). 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 because the ratio of intensity between the sample being tested and the control would be constant across the genome, but flow cytometry could be used as an adjunct if abnormal ploidy were suspected *(104)*. Likewise, a balanced translocation or inversion would not be recognized using this technique.

Peripheral blood cytogenetic studies should 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 could 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 might 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 it 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 because 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, might be associated with significant recurrence risks yet might have normal karyotypes. Specific anomalies could 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 might 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 not indicated, as in a first loss with no other risk factors, or are not possible, such as 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 *(36)*. The results are summarized in **Table 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

Table 9
Phenotype of Abortus and Incidence of Abnormal Karyotypes

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

Source: Ref. *36*.

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 *(82,83,106)*.

Chromosome studies are especially useful for stillbirths suspected of having cytogenetic abnormalities, such as infants with congenital anomalies or intrauterine growth retardation. There might 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 might 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 the result of karyotypic abnormalities might 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 *(80)*. This would save approximately $2000 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 might 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 could indeed be justification for initiating further evaluation after the second failed pregnancy. Coulam compared 214 couples with a history of one 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-five 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 (8208 women and 7834 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 might 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 might 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 might be a consideration for some patients undergoing in vitro fertilization (IVF) 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 *(14)*. Our understanding 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 might also decrease the efficiency of this control process.

Although our understanding of pregnancy loss is limited and we cannot fully predict risks, we can attempt 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. We can also make prenatal diagnosis 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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14
Chromosome Instability

Xiao-Xiang Zhang, MD, PhD

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 once again 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, as well as cell cycle checkpoint genes has been implicated in the formation of aneuploidy, hypodiploidy, and polyploidy. 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)*.

The acquisition of chromosome abnormalities by target cells is a central event that contributes to malignant transformation and tumor development (see Chapter 16). In this chapter, we will focus on the 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 (see **Fig. 1**). All fragile sites are part of the chromosome structure and are inherited as Mendelian codominant traits.

Fragile sites are grouped into two classes: rare and common, based on their frequency of occurrence and means of induction. The 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

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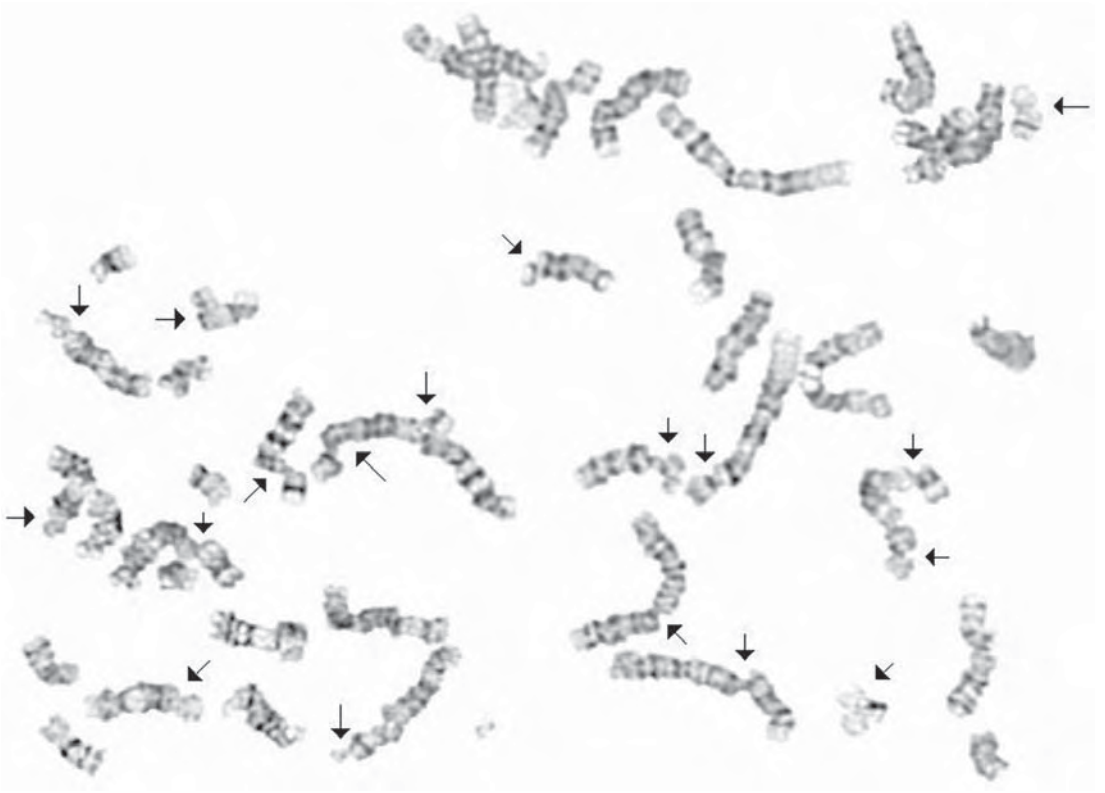

Fig. 1. An example of fragile sites on human chromosomes (arrows) in response to folate/thymidylate depletion using FUdR. See the text for details.

(FRA3B), 16q23 (FRA16D), 6q26 (FRA6E), 7q32 (FRA7H), and Xp22 (FRAXB) *(3)*. 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, whereas 1p21.3 (FRA1M) represents the rarest one, 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 according to the culture conditions required for expression. More than 150 fragile sites were recorded according to the criteria formulated at a session known as the Chromosome Coordinating Meeting *(4,5)*. Fragile sites have been found on every chromosome with the exception of chromosome 21. **Table 1** shows the classification and a list of the common and the rare fragile sites (*6,7*).

It is interesting to note that the locations of many common fragile sites were found to be highly conserved in human, gorilla, chimpanzee, and orangutan *(8)*, whereas none of the rare folate-sensitive fragile sites have been identified in species other than humans *(9)*.

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 our understanding of 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 repeat expansion has been found at any of the common fragile sites that have been studied. Sequence analysis of FRA3B, FRA7G, and FRA7H shows no striking molecular structure that could explain the fragility in these regions. It has been

Table 1
Common and Rare Fragile Sites

Common fragile sites					Rare fragile sites	
Mode of induction					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 refs. *6* and *7*.

proposed that delayed DNA replication underlies expression of fragile sites and that cytogenetic manifestation of these fragile sites is the result of incomplete DNA replication, which leads to a failure of chromatin compaction *(10)*. This becomes more obvious when DNA replication is perturbed by aphidicolin or folate induction.

Clinical Significance

The discovery of the fragile X syndrome (see Chapter 18) 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 could be mechanistically involved in constitutional 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 *(11)*, no other rare fragile site has, to date, been shown to predispose to any heritable chromosome abnormality or malignancy. In a study of 10,492

cases available from the literature, no statistical association between fragile sites and constitutional breakpoints was noted *(12)*. The occurrence of folate-sensitive autosomal rare fragile sites (ARFSs) was compared in populations of mentally retarded, mentally subnormal, and mentally normal children, and among the patients studied, the frequencies did not differ significantly *(13)*.

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 site might play a role in tumor development was initially proposed by Yunis and Soreng soon after fragile sites were discovered *(14)*. It has been well recognized that 50–70% of common fragile sites colocalize with oncogenes, tumor suppressor genes, and breakpoints in cancer rearrangements *(15)*. Subsequently, experimental evidence has revealed that fragile sites appear to be preferential targets for viral integration *(16)*. 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 *(17,18)*. 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 those in healthy control subjects *(19)*, and they suggested that the high expression of fra(3)(p14) in these patients and their relatives could be a valid marker for genetic predisposition to lung cancer.

However, arguments downplaying the role of common fragile sites in the tumorigenic process have come from the fact that these sites are virtually present in 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. Because 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. *(20)*

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 might 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, kidney, and anomalies of the skeleton and limbs show considerable overlaps with some clinical syndromes, such as VATER, TAR, and Holt–Oram syn-

dromes. Bone marrow failure leading to progressive pancytopenia and predisposition to cancers, especially acute myeloblastic leukemia (AML), is the major causes of death in FA patients. Auerbach *(21)* 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.

The disease has a worldwide prevalence of 1–5 per 10^6 and is found in all races and ethnic groups, with an estimated carrier frequency of 0.3–1% *(22)*. Recent studies indicate that there are at least eight genetically distinct complementation groups (A, B, C, D1, D2, E, F, and G). Most FA patients (60–80%) are assigned to group A, followed by groups C (8–14%) and G. Studies of FA patients from various subtypes did not demonstrate a strong influence of complementation group on clinical phenotype *(23)*. Six genes (*FANCA*, *C*, *D2*, *E*, *F*, and *G*) have been identified so far and they do not seem to share any common features. An exception is *FANCD2*, which exhibits a high degree of homology in lower organisms, indicating that this protein is evolutionary conserved, whereas the other FA genes have been superimposed later in evolution. Increasing evidence indicates that the multiple FA proteins cooperate in a biochemical pathway involved in cell cycle regulation and response to DNA damage. The interaction of *BRCA1* with the 8–FA protein pathway is likely to play a critical role as a caretaker of genomic integrity. Wijker et al. *(24)* screened the *FANCA* gene for mutations in a panel of 90 patients identified by the European FA research group. No hot spots were found, and the mutations were scattered throughout the gene. Most were predicted to result in the absence of the FANCA protein.

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 *(25)*.

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 (see **Fig. 2**). A quantitative fluorescence *in situ* hybridization (FISH) analysis showed an accelerated telomere shortening in both arms of FA chromosomes; this could explain a 10-fold increase in chromosome end fusions observed in FA cells *(26)*. 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 crosslinking agents such as mitomycin C (MMC) and diepoxybutane (DEB). The extreme variation of the phenotypes of FA makes the clinical diagnosis difficult and unreliable. 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. The best treatment is currently 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 ataxia cerebral palsy before the appearance of oculocutaneous telangiectases. Serum IgG2 or IgA levels are diminished or absent in 80% and 60% of patients, respectively *(27)*. An elevated α-fetoprotein (AFP) level is observed in majority of A-T patients, who have a strong predisposition to develop lymphacytic 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 United States Caucasian population *(28,29)*. The A-T heterozygote frequency is approximately 2.8%. The responsible gene, *ATM* (for Ataxia Telangiectasia Mutated), encodes a large protein kinase with a phosphatidylinositol 3-kinase-like

Fig. 2. Metaphase from a Fanconi anemia patient, observed in a clastogen-exposed lymphocyte culture. Note the chromosome breakage and radial formations. (Courtesy of Dr. Susan Olson.)

domain and was cloned in 1995. *ATM* is thought to play a central role in a signal transduction network that regulates cell cycle checkpoints, genetic recombination, apoptosis, and other cellular responses to DNA damage. 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 the 200 different mutations in the *ATM* gene *(30)*. However, a single mutation was observed in A-T patients of Jewish Moroccan or Tunisian origin *(31)*.

Greater than 70% of mutations are predicted to lead to protein truncation. Heterozygous carriers of an *ATM* mutation have a 6.1-fold relative risk of developing cancer, the most common form being breast cancer. It is important to realize that such carriers might account for 5% of all cancer patients in the United States *(32)*.

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

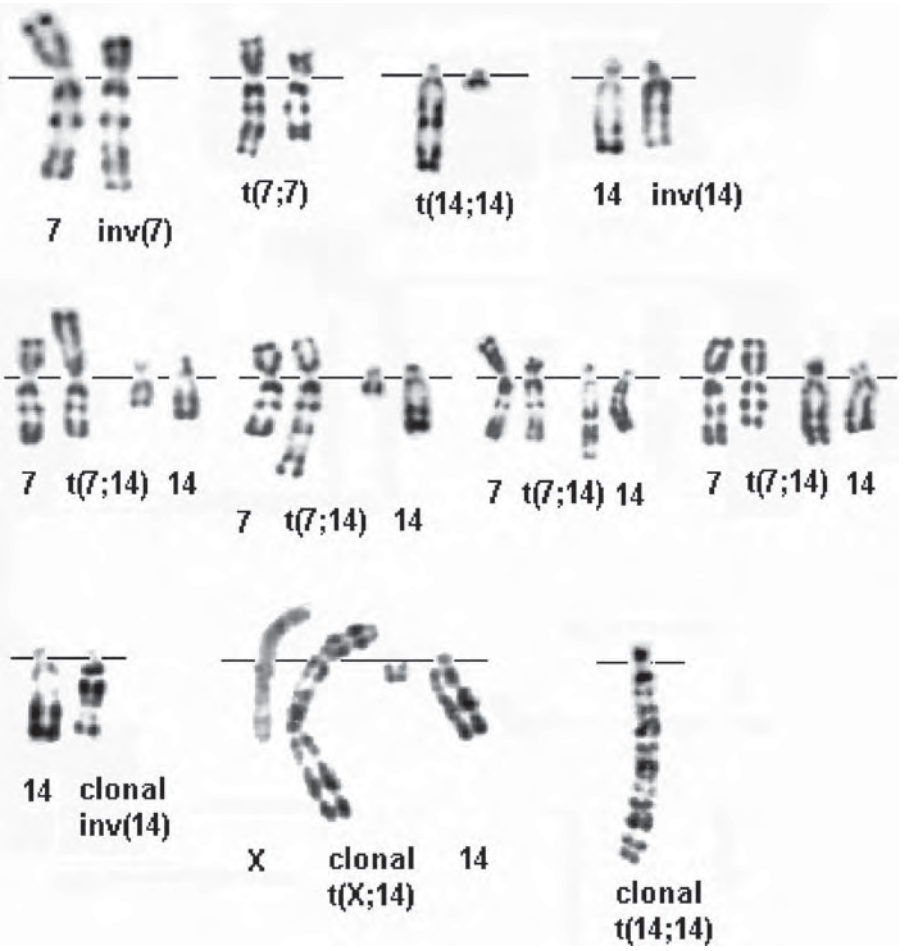

Fig. 3. Sporadic (rows 1 and 2) and clonal (row 3) rearrangements in ataxia telangiectasia (R-banding). Row 1, from left to right: inv(7)(p14q35), t(7;7)(p14;q35), t(14;14)(q11;q32), inv(14)(q11q32); row 2, from left to right: t(7;14)(p14;q11), t(7;14)(q35;q11), t(7;14)(p14;q32), t(7;14)(q35;q32); row 3, from left to right: inv(14)(q11;q32), t(X;14)(q28;q11) (note the late replicating X on the left), t(14;14)(q11;q32). (Courtesy of Alain Aurias and the Atlas of Genetics and Cytogenetics in Oncology and Haematology; modified figure reprinted from ref. *33* with permission of the publisher, EDK.)

(see **Fig. 3**) are often seen in A-T lymphocytes. 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 line than in normal cells, but extrachromosomal recombination frequencies were near normal *(34)*. Therefore, the defects in ataxia telangiectasia seem to be related primarily to the processes of DNA recombination, and increased recombination could contribute to the high cancer risk seen in A-T patients. Repair deficiencies after ionizing irradiation are secondary byproducts 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 telangiectasias. The large size of the *ATM* gene, together with the diversity and broad distributions 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.

Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is a rare disorder characterized by microcephaly, a "bird-like" face, growth retardation, lack of secondary sex development in females, immunodeficiency, and cancer predisposition. Because cells from NBS patients share cytogenetic features with A-T, such as spontaneous chromosome instability, clonal rearrangements (preferentially involving chromosomes 7 and 14), and hypersensitivity to ionizing radiation, NBS was originally thought to be a variant of A-T *(35)*. 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 (*NBS1*; Nibrin, or p95 protein of the NBS1/Mre11/Rad50 complex) *(36,37)* have proven that A-T and NBS are related but separate entities.

Several lines of evidence have suggested that ATM and NBS1 functionally interact in response to DNA damage induced by ionizing radiation (IR). Zhao et al. showed that NBS1 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 *(38)*. This observation links ATM and NBS1 in a common signaling pathway and provides an explanation for the phenotypic similarities between these two disorders.

A 5-bp truncating deletion (657Del5) has been identified in 90% of NBS patients. Recently a German group found a high carrier frequency (1/177) of 657Del5 mutations in three Slavic populations *(39,40)*.

ICF Syndrome

ICF (Immunodeficiency, Centromere instability, and Facial anomalies) syndrome is a recessive disorder resulting from mutations in the DNA methyltransferase 3B (*DNMT3B*) gene *(41)*. ICF syndrome is the only genetic disorder known to involve constitutive abnormalities of genomic methylation patterns.

ICF patients present with variable reductions in serum immunoglobulin levels. Mild facial dysmorphic features include hypertelorism, low-set ears, epicanthal folds, and macroglosia. Cytogenetic analysis of peripheral blood lymphocytes reveals multiradial configurations and a stretching of the pericentromeric heterochromatin of chromosomes 1, 9, and 16 (see **Fig. 4**). An increase in formation of micronuclei is also noted in ICF patients. Using FISH studies probes specific for alphoid satellite DNA (centromere), and classical satellite II DNA (paracentric heterochromatin), Sumner et al. *(42)* showed that it is always the paracentromeric heterochromatin of chromosomes 1, 9, and 16 that becomes decondensed and fused to form multriradial configurations. 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. Studies of the molecular phenotype of ICF cells revealed that extensive hypomethylation is associated with advanced replication time, nuclease hypersensitivity, and variable escape from silencing for genes on the Y and inactive X chromosomes *(43)*.

Robert Syndrome

Robert syndrome (RS) is characterized by craniofacial anomalies, limb defects, and prenatal 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. Despite the heterogeneous clinical presentation, complementation studies of cells derived from RS patients defined a single complementation group in RS (*44*). Premature centromere separation, centromere splitting, and puffing of heterochromatic regions near centromeres,

Fig. 4. Chromosome 1 multiradial configuration from a patient with ICF syndrome. Some stretching of the pericentromeric heterochromatin can also be seen. (Courtesy of Dr. Jeffrey Sawyer.)

particularly of chromosomes 1, 9, and 16, are commonly seen in metaphases of most (80%) of RS patients (see **Fig. 5**). Other cytogenetic abnormalities such as aneuploidy with random chromosome loss, micronuclei, and abnormal nuclear morphology are also observed. Barbosa et al. *(45)* demonstrated asynchronous replication of homologous α-satellite DNA, more evident in chromosomes 1, 9, and 16 in RS cells. This asynchrony, in turn, prevents the establishment of proper cohesion between sister chromatid heterochromatin, leading to chromosome lag and aneuploidy. RS has been interpreted as a human mitotic mutation syndrome, which leads to secondary developmental defects. Cytogenetic analysis by solid staining or C-banding has been used in the diagnosis of RS.

Werner Syndrome

Werner syndrome (WS) is a human premature aging syndrome manifested by sclerodermalike skin changes, especially in the extremities. Wizened and prematurely aged faces are often observed in individuals affected with WS (see **Fig. 6**). The most consistent feature of WS is cataracts. Variable features include diabetes mellitus, hypogonadism, osteoporosis, atherosclerosis, and an increased incidence of neoplasia. Malignant sarcomas, meningiomas, and carcinomas are seen in approximately 10% of WS patients. Cancer is the leading cause of death of WS patients. The prevalence of carriers is reported to be as high as 1/150 to 1/200 (*47*).

The frequency of spontaneous chromosome damage in WS is not as striking as it is in other chromosome instability syndromes. A variety of somatic chromosome rearrangements was noted in cultured skin fibroblasts from WS patients. Variegated translocation mosaicism (VTM) has been used to designate the pattern of pseudodiploidy with multiple, variable, and stable chromosome aberrations noted in WS cells.

Skin fibroblast lines established from WS 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 an extended cellular life-span. Telomerase activity and telomere extension is sufficient to prevent accelerated cell aging in WS fibroblast cultures (*48*).

The gene responsible for WS (*WRN*, with a total of 35 exons), was identified by positional cloning *(49)*. *WRN* is a DNA helicase belonging to the RecQ family and is an exonuclease that participates in

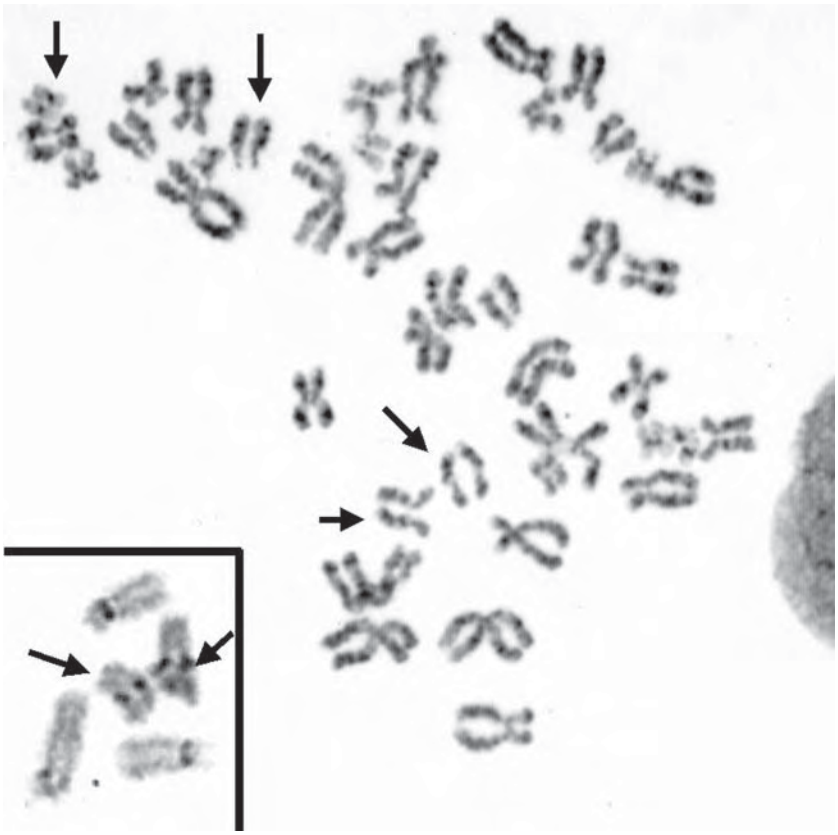

Fig. 5. G-Banded and C-banded (inset) image of cells from a patient with Robert syndrome, demonstrating premature centromere separation (arrows). (Courtesy of Dr. Mazin Qumsiyeh.)

the pathways of DNA repair, recombination, transcription, and replication. Loss of *WSN* function could promote genetic instability and disease via recombination-initiated mitotic arrest, cell death, or gene rearrangement. Mutations have been identified in the *WRN* gene in all WS-affected individuals studied, and these mutations were located at different sites across the coding region. All *WRN* mutations found to date either create stop codons or cause frame shifts that lead to premature termination. Not a single missense mutation had been identified. WS is the result of complete loss of function of the *WRN* gene product. One splice-junction mutation is found in 50–60% of Japanese WS patients (*50*). No genotype–phenotype correlation has been observed.

Bloom Syndrome

Bloom syndrome (BS) is a rare genetic disorder characterized clinically by growth retardation, proportionately short stature, sun sensitivity, erythematous facial skin lesions, immunodeficiency, and increased predisposition to cancer. Although BS occurs in many ethnic groups, Ashkenazi Jews have a significantly higher incidence. The gene frequency in this population is estimated to be 1/110.

Genomic instability is manifested by formations of quadriradial configurations of symmetric shape with centromeres in opposite arms. This rearrangement occurs before mitosis and is 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 (approximately 10-fold) level of sister chromatid exchange (SCE) in various cell types, such as lymphocytes,

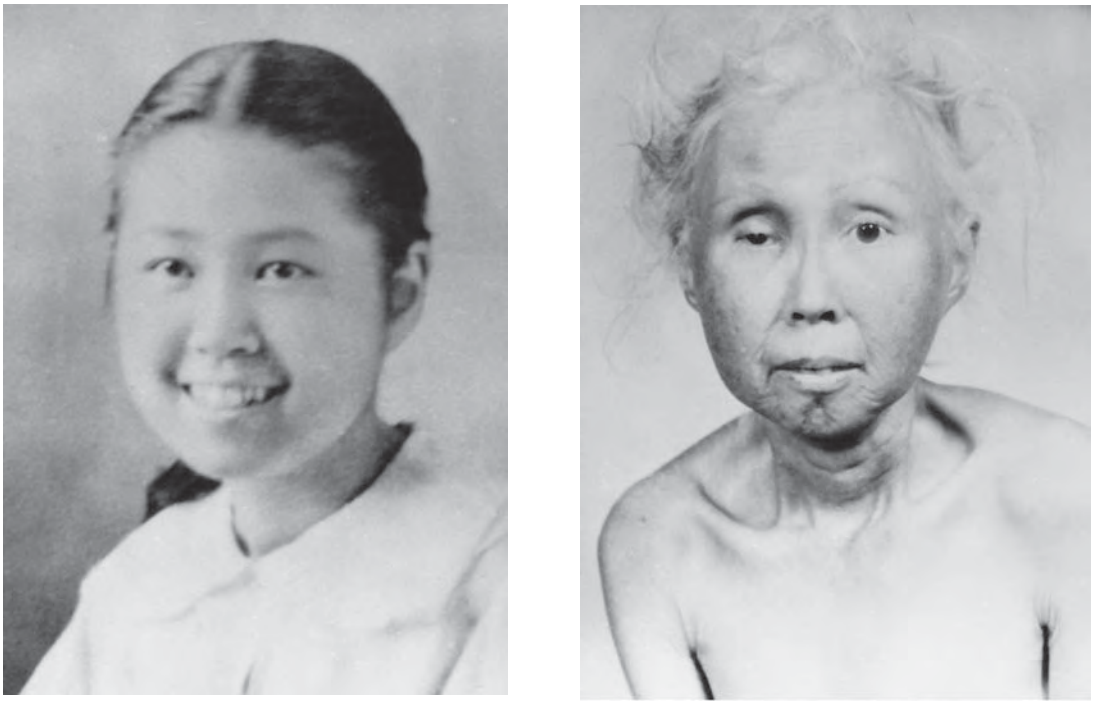

Fig. 6. A Werner syndrome patient at ages 15 and 48 years of age. (From ref. *46*, with permission. Courtesy of Nancy Hanson.)

fibroblasts and bone marrow cells in affected individuals *(51)* (see **Fig. 7**). A single complementation group was established to exist among patients of diverse ethnic origin.

Bloom syndrome arises from mutations in *BLM*, a gene encoding a protein with RecQ helicase function. Hyperrecombination 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 *(53)*. Gruber et al. demonstrated that carriers of a *BLM* mutation are at increased risk for colorectal cancer *(54)*. Mutation analysis indicates that the cause of most BSs 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 2281 of *BLM* was identified in 98% patients of Ashkenazic Jewish origin *(55)*.

A polymerase chain reaction (PCR)-based molecular diagnostic test is available for the Ashkenazic Jewish population. SCE analysis still represents the most distinctive cytogenetic diagnostic marker for BS.

Xeroderma Pigmentosum

Sensitivity to sunlight and the tendency to develop skin cancer at an early age are the key features of xeroderma pigmentosum (XP). Freckling in exposed areas occurs by 2 years of age in most patients. The neoplasms are predominantly basal cell or squamous cell carcinomas and malignant melanomas. Approximately 90% of the squmaous and basal cell carcinomas appear in the regions 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 2000 times higher than in the general population under 20 years of age (see **Fig. 8**).

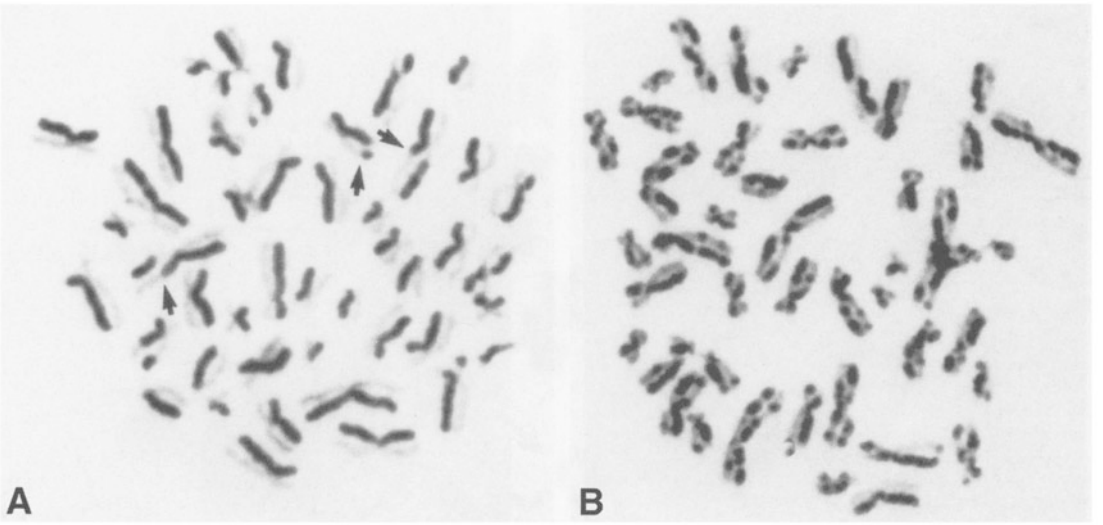

Fig. 7. Sister chromatid exchange: **(a)** two SCEs in a normal cell (arrows); **(b)** multiple SCEs in a cell from a patient with Bloom syndrome. (From ref. *52*, used by permission of Oxford University Press, Inc.)

Although the disorder is transmitted in an autosomal recessive manner, heterozygous carriers could be predisposed to skin cancers. Swift et al. reported that in 31 families of XP patients, blood relatives have a significantly higher frequency of nonmelanoma skin cancer than their spouses *(57)*.

Xeroderma pigmentosum has been found in all races. The frequency is approximately 1 in 1 10^6 in the United States and is considerably higher in Japan and North Africa. Consanguinity has been reported in nearly 30% of cases. Genetically, seven complementation groups (XP-A to XP-G) have been identified in one class of XP patients with defective excision of pyrimidine dimers (excision-deficient XP). A xeroderma pigmentosum variant (XP-V) with impaired replication of damaged DNA has also been identified. Groups A and C are the most common forms worldwide, XP-D and XP-F have intermediate frequency, and group F has exclusively been described in Japan. XP-A, XP-B, XP-D, and XP-G 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 could explain the clinical severity involving both 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 cases of XP. However, an increased rate of sister chromatid exchange and chromosome aberrations after exposure to ultraviolet (UV) light and chemical carcinogens has been reported *(58)*.

Ultraviolet 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 XP-V do not show a significant increase of UV sensitivity under standard test conditions, but a dramatically increased sensitivity is seen when XP-V 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 have been characterized.

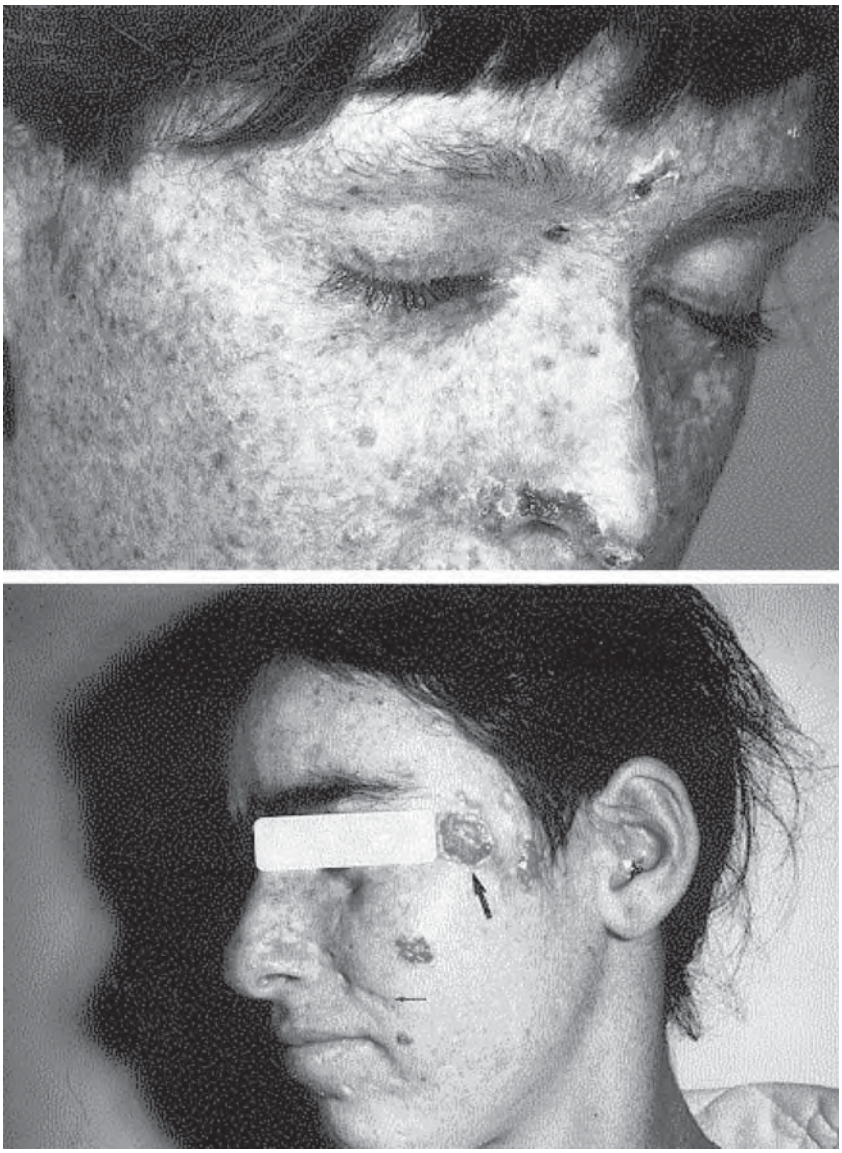

Fig. 8. Top: lesions of the face in an XP patient. Note multiple scars of carcinomas and an aged aspect of the skin. **Bottom:** multiple basocellular carcinomas on the face of an XP patient. The thick arrow points to a recent lesion and the thin arrow points to a scar of an old lesion. (Reprinted from ref. *56*; Image courtesy of Daniel Wallach, and used with permission of the *Atlas of Genetics and Cytogenetics* in *Oncology and Haematology*.)

All XP genes have been cloned. With the exception of XP-V, 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 *(59)*. Cockayne syndrome and the photosensitive form of trichothiodystrophy, two other NER-deficiency syndromes, should be considered in differential diagnosis because of the common feature of extreme sensitive to sunlight shared by these disorders.

Successful treatment for XP using a topical DNA repair enzyme has recently been reported *(60)*.

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IV Cancer Cytogenetics

15

Cytogenetics of Hematologic Neoplasms

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INTRODUCTION

Cancer is a genetic disease that could develop either from a predisposing mutation followed by acquired somatic mutations or from an accumulation of somatic mutations that develop into a cancer phenotype. Many different types of DNA alteration have been reported in cancer, with some of the recognized forms being as follows:

- Subtle DNA or RNA alterations
- DNA methylation
- Changes in chromosome number (aneuploidy)
- Loss of heterozygosity
- Chromosome translocations in somatic cells rather than in germ cells
- Gene amplification
- Incorporation of exogenous sequences

Oncogenes and tumor suppressor genes control cellular proliferation by cell death or cell birth, whereas caretaker genes control the rate of mutation. Cells with defective caretaker genes might acquire mutations in all genes, including oncogenes and tumor suppressor genes.

Chromosomal translocation plays a major role in the development of hematologic malignancies. About 50% of hematopoietic neoplasms somatically acquire chromosomal translocations, which activate proto-oncogenes in most cases. This could, in turn, disrupt the critical balance of cell proliferation, cell maturation, and cell death. In many cases, these chromosomal translocations fuse sequences of a transcription factor or tyrosine kinase (TK) receptor gene to unrelated genes, resulting in a chimeric protein with oncogenic properties. Most chromosomal translocation-induced hematopoietic neoplasms are restricted to a single lineage and, depending on the acquisition of the mutation, are arrested at a particular developmental stage of maturation. Occasionally, more than one lineage or developmental stage is affected, as can occur, for example, in *MLL* gene-related malignancies, suggesting involvement of genes at the pluripotent stem cell stage.

Historically, hematological malignancies have been classified according to morphological phenotype using what is known as the French–American–British (FAB) classification. Although this was a valuable tool for many years, advancing knowledge of chromosomal and gene-specific rearrangements, plus minimal correlation among morphologic categories, treatment responses, and prognosis, has rendered the FAB classification less useful. Over time, immunophenotypic, cytogenetic, and molecular genetic information has become essential for prognosis and treatment. There was a need to develop a multifaceted approach to the diagnosis and classification of these disorders. Since 1995, the European Association for Haematopatholgy and the Society for Haematopathology, in collaboration with many subspecialties, have developed the World Health Organization (WHO) classification.

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One important objective was to take advantage of recent genetic data and integrate clinical and pathological information in order to define hematological malignancies according to the cell of origin. Therefore, in this classification, the groups objectively incorporated information from cytogenetics and molecular genetics and developed a list and definitions of disease entities in collaboration with an International Clinical Advisory Committee to make this classification more useful in clinical practice. The new WHO classification of hematologic malignancies stratifies neoplasms primarily according to lineage (e.g., myeloid, lymphoid, histiocytic/dendritic cell, and mast cell). Within each category, neoplasms are further defined by a combination of morphology, immunophenotyping, genetic, and clinical information. The "cell of origin" in this classification is defined as the presenting cell phenotype, because in many cases, particularly in lymphoid disorders, the cell in which the initial transformation occurs is not known. The WHO classification defines tumors as deriving from myeloid and lymphoid tissues *(1)*.

The goal of this chapter is to describe hematologic malignancies according to the WHO classification, extract pertinent information for physicians and cytogeneticists, and incorporate current cytogenetic and molecular genetic information where available. Cross-referencing with FAB classification is occasionally listed, where possible, for the convenience of those readers who might be more familiar with it. As more and more genetic data become available, it is clear that genetic changes will continue to make a considerable impact on our understanding of disease biology and will result in improved diagnosis, treatment, and prognosis. This will allow a better understanding of the clinical correlation and significance of specific genetic changes and will clarify risk stratification and mechanisms of genomic alterations according to the WHO classification.

Historical Perspective

The cytogenetic contribution to our understanding of cancer has evolved over four decades and can be divided into three eras. We are moving into the fourth, or genomic, era.

The first era began with successful in vitro chromosome culture and identification of the correct human chromosome number. Next came the banding era, which included the classification of chromosomes using high-resolution banding. The third era has existed for over a decade, as fluorescence *in situ* hybridization (FISH) in its many diverse forms has revolutionized our understanding of cancer genetics. We are now entering a genomic era of high-resolution genomic microarrays and comparative genomic hybridization (BAC CGH and cDNA CGH). In the near future, integration of genomic changes to microarray expression data will be critical in identifying molecular targets for specific gene alteration.

The first era was started by Tijo and Levan in 1956, when in vitro cell culture and hypotonic treatment of dividing cells resulted in enough separation of the chromosomes to correctly identify the human chromosome complement as 46 *(2)*. In 1960, Nowell described that use of phytohemagglutinin (PHA) exposure to human lymphocytes could radically increase T-lymphocyte proliferation within 48–72 hours *(3)*. This transformed cytogenetics from a cumbersome research tool to a mainstream diagnostic test. That same year, Nowell and Hungerford *(4)* described the first consistent chromosomal aberration, an apparent deletion, in leukemia cells from patients with chronic myeloid leukemia (CML). This was the start of the identification of numerous consistent chromosome changes in many types of cancer *(4)*. The abnormal chromosome 22, referred to as the "Philadelphia chromosome," is now known to result from a balanced translocation between chromosomes 9 and 22, involving the *ABL* and *BCR* genes. The introduction of chromosome banding in the 1960s and 1970s, and more recently high-resolution chromosome banding and various FISH approaches have transformed cytogenetics into an integral part of patient work-up in cases of suspected malignancies. For more on these historical developments, see Chapter 1.

The discoveries of consistent translocations have improved our understanding of the genetic mechanisms and pathways involved in leukemogenesis *(5)*. In leukemias and other solid tumors, the presence of specifically translocated hybrid messenger RNA (mRNA) sequences becomes a marker

or specific probe for disease monitoring and helps in identifying patients who could not be diagnosed by conventional means. Recurring chromosomal translocations are continuing to be identified, and the importance of this cannot be underestimated as scientists continue using these translocations to clone genes and find molecular targets for treatment options. In recent years, we have seen numerous approaches using microarray CGH or BAC CGH, cDNA, and expression array platforms to better define cancer and understand the biology of disease by looking at genomic and gene expression data.

Yet, even after so much progress in this field, the cause of chromosome translocations that result in cancer remains one of the essential unanswered questions. For some translocations, in lymphoid tumors for example, the involvement of a recombinase enzyme seems fairly clear. For myeloid disorders, however, there is little evidence that recombinase has a role, and thus the focus is on other DNA sequences that might predispose to breaks, such as ALU and other repeat sequences, translin, and topoisomerase II (topo II) sites. The challenge for the future is to match our molecular genetic understanding with a functional understanding of the genes involved in translocations, the other oncogenes and tumor suppressor genes in normal cells, the genes that regulate them, and their downstream targets. This will provide a far more complete and robust understanding of the role that these genes play in growth and differentiation in normal and malignant cells *(6)*.

In the past, malignant leukemias and lymphomas were classified using various approaches: according to the clinical course, acute versus chronic, according to the primary site, and according to the phenotype by FAB classification. In 2001, WHO published its integrated classification, which is becoming a standard of classification throughout the world. In the WHO classifications, leukemias are primarily stratified into lineage specific types and then further characterized into clinically significant subgroups *(7)*. The major disease categories according to the WHO classification are listed in **Table 1**. This chapter does not cover every disorder classified by WHO, but, rather, focuses on those for which at least some cytogenetic data is available.

CHRONIC MYELOPROLIFERATIVE DISEASES (MPDs)

There are many common and consistent, nonspecific chromosomal aberrations in this group of disorders. Correlation with morphology, flow cytometry, and other laboratory and clinical data is imperative to make the correct diagnosis. This group of patients needs immediate attention and aggressive treatment, and if untreated, could die within months of presentation. If properly diagnosed and treated, patients can survive for many years depending on the disease subtype. So far, aside from CML, no other category in this group of disorders has shown any specific genetic alteration; however, activation of tyrosine kinase signal transduction pathways is frequently implicated in their pathogenesis *(8–10)*.

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is the paradigm of this category and is an excellent example of how genetic information and advancement in technology have contributed to the diagnosis, follow-up after treatment, and, finally, to the development of tailored medicine to treat genomic targets. This disorder is characterized by abnormal but effective hematopoiesis, resulting in the proliferation of mature cells, with high peripheral blood levels of one or more cell lines. Patients with CML often present with hepatosplenomegaly, which probably results from the high rate of sequestration of mature cells in these organs. The marrow is usually hypercellular, with mature cells and without dysplasia. The percentage of blasts is either normal or slightly increased (10%). Importantly, fibrosis is not a primary occurrence and is probably the result of abnormal production and release of cytokines and growth factors *(7,8)*.

Chronic myelogenous leukemia is defined as a qualitative disorder originating from two or more cell types with a multilineage phenotype. CML alone accounts for about 15–20% of all cases of leukemia. The disease can occur at any age, but the most common age of presentation is between the

Table 1
Disease Categories According to WHO Classification

- Chronic Myeloproliferative Diseases (MPDs)
  - Chronic myelogeneous leukemia
  - Chronic neutrophilic leukemia
  - Chronic eosinophilic leukemia and hypereosinophilic syndrome
  - Polycythemia vera
  - Chronic idiopathic myelofibrosis
  - Essential thrombocythemia
  - Myeloproliferative disease, unclassifiable
- Myelodysplastic/Myeloproliferative Diseases
  - Chronic myelomonocytic leukemia (CMML)
  - Atypical chronic myeloid leukemia (aCML)
  - Juvenile myelomonocytic leukemia (JMML)
  - Myelodysplastic/myeloproliferative diseases, unclassifiable
- Myelodysplastic Syndromes (MDSs)
  - Refractory anemia (RA)
  - Refractory anemia with ringed sideroblasts (RARS)
  - Refractory cytopenia with multilineage dysplasia (RCMD)
  - Refractory anemia (MDS) with excess blasts (RAEB)
  - Myelodysplastic syndrome associated with isolated del(5q) chromosome abnormality ("5q– syndrome")
  - Myelodysplastic syndrome, unclassifiable
- Acute Myeloid Leukemia (AML)
  - Acute myeloid leukemia with recurrent cytogenetic abnormalities
    - AML with t(8;21)(q22;q22) *AML1(CBFa)/ETO*) (FAB M2)
    - AML with inv(16)(p13q22) or t(16;16)(p13;q22), *(CBF*β*/MYH11)*
    - AML with t(15;17)(q22;q21) (*PML/RARa* and variants thereof) (FAB M3)
    - AML with 11q23 *(MLL)* abnormalities
  - Acute myeloid leukemia with multilineage dysplasia
    - With prior myelodysplastic syndrome
    - Without prior myelodysplastic syndrome
  - Acute myeloid leukemia and myelodysplastic syndrome, therapy-related
    - Alkylating agent related
    - Topoisomerase II inhibitor related
  - Acute myeloid leukemia not otherwise categorized
    - AML, minimally differentiated
    - AML without maturation
    - AML with maturation
    - Acute myelomonocytic leukemia (FAB M4)
    - Acute monoblastic and monocytic leukemia (FAB M5)
    - Acute erythroid leukemia (FAB M6)
    - Acute megakaryoblastic leukemia (FAB M7)
    - Acute basophilic leukemia
    - Acute panmyelosis with myelofibrosis
    - Myeloid sarcoma
  - Acute leukemia of ambiguous lineage
- Precursor B- and T-Cell Neoplasms
  - Precursor B-lymphoblastic leukemia/lymphoma
  - Precursor T-lymphoblastic leukemia/lymphoma
- Mature B-Cell Neoplasms
  - Chronic lymphocytic leukemia/small lymphocytic lymphoma
  - B-Cell prolymphocytic leukemia
  - Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia

Table 1 (continued)

- Splenic marginal zone lymphoma
- Hairy cell leukemia
- Plasma cell neoplasms
  - Plasma cell myeloma
  - Plasmacytoma
  - Solitary plasmacytoma of bone
  - Monoclonal immunoglobulin deposition diseases
  - Heavy-chain diseases
- Extranodal marginal zone B-cell lymphoma (MALT lymphoma)
- Nodal marginal zone B-cell lymphoma
- Follicular lymphoma
- Mantle cell lymphoma
- Diffuse large B-cell lymphoma
- Mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma
- Primary effusion lymphoma
- Burkitt lymphoma/leukemia
- Lymphomatoid granulomatosis

Mature T-Cell and NK-Cell Neoplasms
- T-CelI prolymphocytic leukemia
- T-Cell large granular lymphocytic leukemia
- Aggressive NK-cell leukemia
- Adult T-cell leukemia/lymphoma
- Extranodal NK/T-cell lymphoma, nasal type
- Enteropathy-type T-cell lymphoma
- Hepatosplenic T-cell lymphoma
- Subcutaneous panniculitis-like T-cell lymphoma
- Blastic NK-cell lymphoma
- Mycosis fungoides/Sézary syndrome
- Primary cutaneous CD-30 positive T-cell lymphoproliferative disorders
  - Primary cutaneous anaplastic large cell lymphoma (C-ALCL)
  - Lymphomatoid papulosis
  - Borderline lesions
- Angioimmunoblastic T-cell lymphoma
- Peripheral T-cell lymphoma, unspecified
- Anaplastic large cell lymphoma

Hodgkin’s Lymphoma
- Nodular lymphocyte predominant Hodgkin’s lymphoma
- Classical Hodgkin'slymphoma
  - Nodular sclerosis Hodgkin'slymphoma
  - Mixed cellular Hodgkin'slymphoma
  - Lymphocyte-rich classical Hodgkin'slymphoma
  - Lymphocyte-depleted Hodgkin'slymphoma

Immunodeficiency-Associated Lymphoproliferative Disorders
- Lymphoproliferative diseases associated with primary immune disorders
- Human immunodeficiency virus-related lymphomas
- Posttransplant lymphoproliferative disorders
- Methotrexate-associated lymphoproliferative disorders

Histiocytic and Dendritic Cell Neoplasms
- Histiocytic sarcoma
- Langerhans cell histiocytosis

(continued)

Table 1 (continued)

Langerhans cell sarcoma
Interdigitating dendritic cell sarcoma/tumor
Follicular dendritic cell sarcoma/tumor
Follicular dendritic cell sarcoma/tumor
Dendritic cell sarcoma, not otherwise specified
Mastocytosis
Cutaneous mastocytosis
Systemic mastocytis
Mast cell sarcoma
Extracutaneous mastocytoma

ages of 50 and 59 years *(8)*. In most cases, it is a triphasic disorder, starting with the chronic phase that, if left untreated, can proceed to a CML-accelerated phase and CML with blast crisis. This disorder is mainly of hematopoietic tissue in origin, involving primarily the blood, bone marrow, spleen, and liver, but during blast crisis, extramedullary tissues, including lymph nodes, skin, soft tissue, and sometimes the central nervous system, can be involved. The most common presenting features of CML are very mild to high white blood cell counts, fatigue, night sweats, and/or splenomegaly.

In the WHO classification, the diagnostic criterion for CML is the unequivocal presence of a "Philadelphia" (Ph) rearrangement [t(9;22)(q34;q11.2), see **Fig. 1t**], involving the Breakpoint Cluster Region and Ableson oncogenes (*BCR* and *ABL) (4,12)*. See also Fig. 9 of Chapter 17. Approximately 90–95% of CML patients present with a Philadelphia rearrangement at the time of initial diagnosis. The presence of this rearrangement has been seen in all lineages of maturing cells; therefore, this is a true multilineage disease. The remaining 5–10% of CML cases without a classic Philadelphia rearrangement present in varying forms, either involving other chromosomes (a complex rearrangement) or, in some cases, a cryptic translocation involving *BCR* and *ABL*. Therefore, the diagnostic criterion for CML is the unambiguous presence of the *BCR*/*ABL* translocation transcript *(13)*. Variant translocations could implicate a third or fourth chromosome. Although the involvement of chromosome 9 or chromosome 22 might be hidden and at times the karyotype appears normal ("Ph-negative CML"), the hybrid *BCR*/*ABL* gene is always present (otherwise, the disease is not CML). One of the proposed mechanisms of CML leukemogenesis is that "accidents" arise in a bone marrow stem cell during mitosis, producing the translocation between chromosomes 9 and 22, resulting in the *BCR*/*ABL* fusion gene.

The normal *ABL* gene is transcribed into an mRNA of 6–7 kb, which produces a 145-kDa protein with tyrosine kinase activity. The hybrid *BCR*/*ABL* gene is transcribed into an mRNA of 8.5 kb, which produces a protein of 210 kDa, with a subsequent increase in protein kinase activity and half-life. In CML, the breakpoints in the *BCR* gene are almost always in the major breakpoint cluster region or M-BCR, involving exons 12–16. This is also known as a B1/B5 translocation and results in an abnormal chimeric protein known as p210, with increased tyrosine kinase activity. In a minority of cases, the breakpoint in the *BCR* gene can occur in a minor region (m-BCR), in exons 17–20. This translocation has also been known as translocation C1/C4, and in this case, an even larger chimeric protein, p230, is produced. Interestingly, patients with the chimeric protein p230 usually demonstrate prominent neutrophilic maturation. Another chimeric protein is noted in the minor breakpoint cluster regions, which results from the translocation involving *BCR* exons 1 and 2 and produces a shorter fusion protein, p190. This chimeric protein is most frequently associated with "Philadelphia-positive" acute lymphoblastic leukemia. A small number of p190 chimeric proteins or gene products can be detected in CML, which represents an alternative splicing mechanism in this disorder *(14–16)*.

This fusion product or chimeric protein permanently activates tyrosine kinase that is freed from normal regulation of its parent *ABL* kinase. The *BCR*/*ABL* fusion protein begins to excessively phosphorylate multiple cellular proteins, resulting in an altered expression profile of the stem cell.

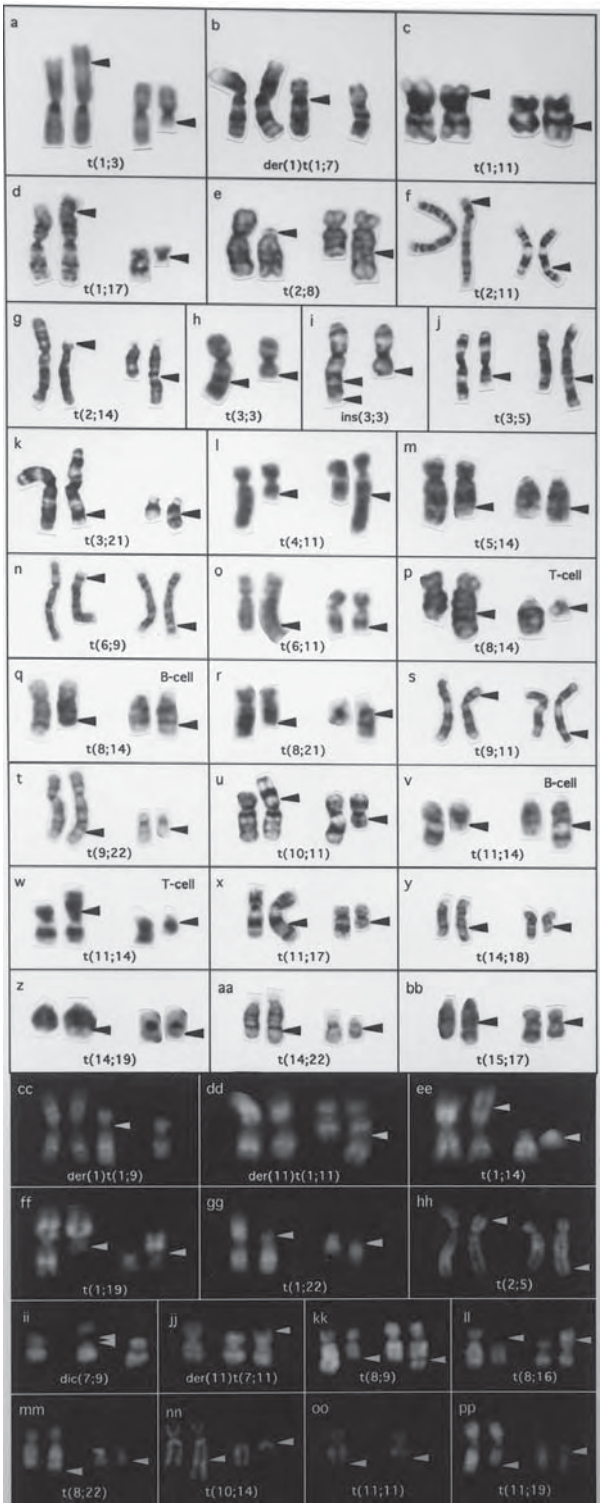

Fig. 1. Representative examples of translocations seen in hematologic disorders. (From *ref. 282*, with permission.)

Cytogenetics plays an imperative role in the analysis of CML patients. In addition to establishing diagnosis, it can predict clinical transformation of the chronic phase into accelerated phase or blast crisis. Some of the common cytogenetic changes that can occur preclinically when patients transform from chronic phase are trisomy of chromosome 8 and formation of an isochromosome 17q. There are many genes reported to have mutations in transformed stages, including *TP53*, *RB1*, *CDKN2A*, *INK4a*, *MINK*, *AML1*, and, *EVL1*, but their role in transformation, if any, is currently unknown *(17,18)*.

Recently, it has also been shown that in some cases of CML, there is a genomic deletion of chromosomes 9 and 22 sequences around the translocation breakpoints on the derivative chromosomes, and this might be a cause for poor or worse prognoses in this subgroup of CML patients *(19,20)*. Sometimes, these deletions are large enough to be detected by fluorescence *in situ* hybridization (FISH) analysis (see Chapter 17, Fig. 10). They are more commonly seen in patients with variant translocations, which account for about 5–10% of all CML cases. There has also been a suggestion that these deletions occur as a result of mitotic recombination errors when the cells are approaching accelerated or blast crisis phase. The prognostic value of these additional changes at the time of diagnosis is significant. In one study, about 9.8% of patients had additional changes at the time of diagnosis, and this was associated with a shorter median survival of 28 month compared to 48 month for patients with the Philadelphia rearrangement alone *(21)*.

The molecular consequences of the cytogenetic changes that occur in CML evolution are still not well understood. Perhaps most of the molecular alterations responsible for disease progression are not detectable by cytogenetic analysis alone. Common "major route" cytogenetic changes in CML are trisomy 8, isochromosome 17q (see **Fig. 2rr**), an additional derivative chromosome 22 ("Philadelphia chromosome"), and trisomy 19. Less common "minor route" changes include trisomy 21, loss of the Y chromosome in men, monosomy 7, monosomy or trisomy 17, and a (3;21) translocation (see **Fig. 1k**). Patients often present with unique or "patient-specific" secondary changes. These all have some role to play in transformation to blast crisis and in prognosis. See **Table 2**.

Recent development of the drug imatinib mesylate (STI571, Gleevec™) has considerably changed the treatment protocol for patients with CML. Gleevec induces remission of CML as fast as any other therapy and achieves rates of cytogenetic remission far exceeding those induced by interferon α. It has a toxicity profile as favorable as that of hydroxyurea and far superior to that of interferon-α *(22)*. The drug rapidly reduces peripheral white cell counts and normalizes marrow appearance in CML. It also been shown to produce a complete cytogenetic response in a large number of patients. This treatment approach represents a new class of drugs that attack genomic targets and serves as a model for new treatment modalities in many other malignancies. Because this is a recent treatment protocol for CML, there is little correlation with the many different cytogenetic changes, and there are limited data on the long-term effect of imatinib. A certain percentage of patients also develop "resistance" to the drug and relapse. Therefore, we will have to wait to see if this therapy truly fulfills its promise.

A small proportion of patients with chronic myeloproliferative diseases have constitutive activation of the gene for platelet-derived growth factor-β receptor *(PDGFR*β*)*, which encodes a receptor tyrosine kinase. The *PDGFR*β gene is located on chromosome 5q33, and the activation is usually caused by a translocation, t(5;12)(q33;p13), associated with an *ETV6*/*PDGFR*β fusion. Imatinib mesylate also inhibits *PDGFR*β and *KIT* kinases and also has impressive clinical efficacy in patients with rearrangements involving these genes *(29,30)*.

It has been suggested that diagnosis of CML must always be confirmed by proving the presence of a *BCR*/*ABL* translocation either by cytogenetics, FISH, or polymerase chain reaction (PCR). Cases that morphologically suggest CML but are "Philadelphia negative" by routine cytogenetics should be aggressively pursued by other molecular methods to demonstrate cryptic *BCR*/*ABL* translocations. It has also been suggested that molecular confirmation or exclusion of CML in suspected cases is critical, as that would allow tailored therapy using imatinib. Misdiagnosing CML prevents patients from getting potential curative therapy, and it has been shown that this therapy is more effective when used

Table 2
Major Routes of Cytogenetic Evolution in CML Blast Crisis

Additional change	Frequency (%)
+Ph[a]	15
i(17q)	12
+8	11
+Ph, +8	8
+8,i(17q)	7
+Ph,+8,+19	5
+Ph,+19	4
+8,+19	2
+Ph,+8,i(17q)	2
+19	1
i(17q),+Ph	1
+8,i(17q),+19	1
+Ph,+8,i(17q), +19	1
i(17q),+19	>1
i(17q),+19, +Ph	>1

[a] +der(22)t(9;22)(q34.1;q11.2).

in the early course of the disease. In conclusion, cytogenetic and molecular evaluation of any cases of suspected CML are critical in the diagnosis and classification of this neoplasm.

Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia (CNL) is a rare myeloproliferative disease characterized by hepatosplenomegaly and sustained peripheral blood neutrophilia with bone marrow hypercellularity as a result of a neutrophilic granular site proliferation. The lack of a Philadelphia rearrangement is used as a diagnostic criterion for CNL. Whenever any evidence of a *BCR/ABL* translocation is seen, even in chimeric or cryptic form, the diagnosis of CNL should not be made and the case should be considered to be CML.

Cytogenetic changes in CNL are rarely seen and only about 10% of patients show nonspecific chromosome abnormalities. The most commonly seen abnormalities are trisomy of chromosome 8 and 9 and deletion of chromosome 22q and 11q, and, as indicated previously, a Philadelphia rearrangement is not present in any form *(23–27)*.

Chronic Eosinophilic Leukemia

Chronic eosinophilic leukemia (CEL) is characterized by trisomy of chromosome 8 and a translocation between chromosomes 5 and 12 at bands q33 and p13, respectively [t(5;12)(q33;q13)], involving the *TEL* and *PDGFR*β genes *(28–30)*. In addition, a dicentric (1;7) translocation, along with aberrations involving 8p11 at the *FGFR1* locus have been reported *(28–30)*.

Other Chronic Myeloproliferative Diseases

Cytogenetic abnormalities commonly seen in polycythemia vera (PV) are an extra copy of chromosome 8 and/or 9 and deletions of 13q, 20q (see **Fig. 2z,aa,w,x**) and 1p11. Almost all cases transforming into myelodysplastic syndrome show cytogenetic abnormalities, including the ones seen in therapy-related MDS and AML (discussed later) (*31–33)*.

In chronic idiopathic myelofibrosis (CIMF), an extra copy of chromosome 8, deletion of chromosome 20, and loss or deletion of chromosomes 7, 11q, and 13q have been seen in about 35% of cases *(34,35)*. In essential thrombocythemia (ET), trisomies of chromosomes 8 and 9, deletions of 13q

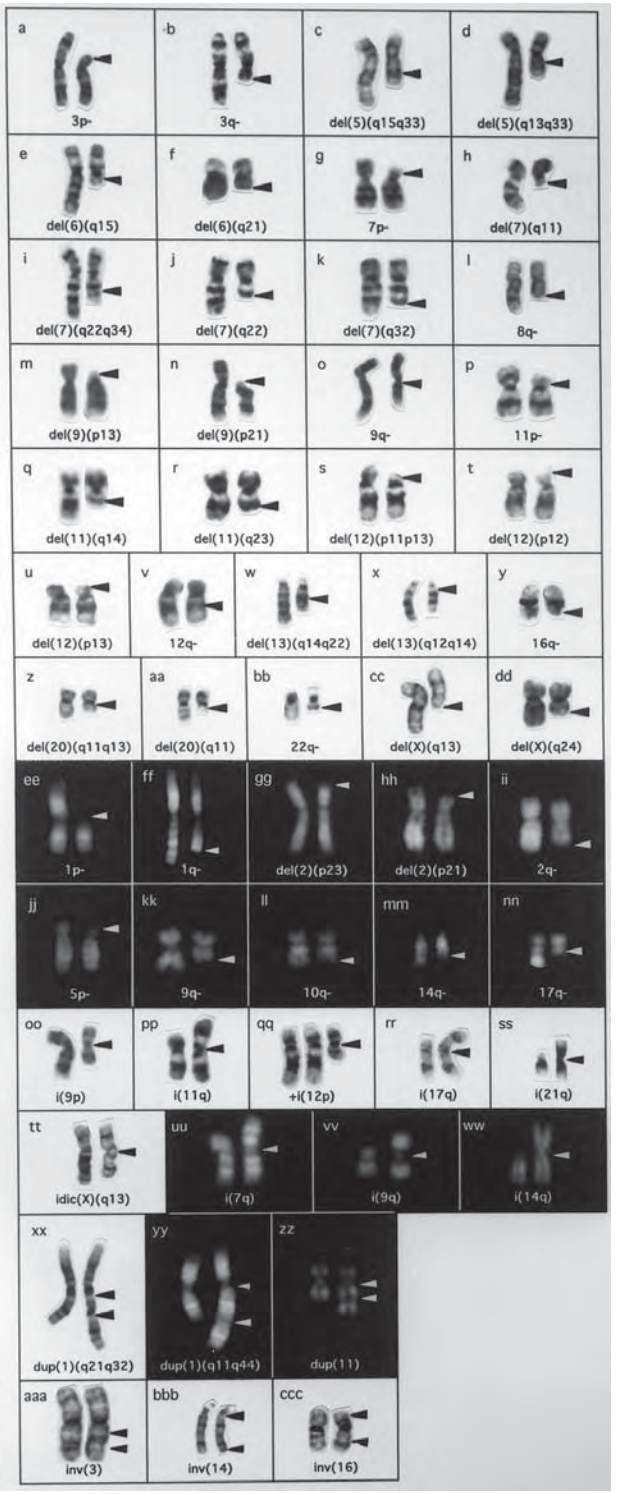

Fig. 2. Representative examples of inversions, isochromosomes, duplications and deletions seen in hematologic disorders. (From ref. *282*, with permission.)

and 5q, and translocations and inversions of chromosome 3 involving bands q21 and q26.2 (see **Fig. 2aaa**) have been reported *(39,40)*. As ET is often a diagnosis of exclusion, the absence of a Philadelphia rearrangement is necessary, as is the case with CNL.

MYELODYSPLASTIC/MYELOPROLIFERATIVE DISEASES

The myelodysplastic/myeloproliferative diseases (MDS/MPD) include chronic myelomonocytic leukemia, atypical chronic myeloid leukemia, juvenile myelomonocytic leukemia and "myelodysplastic/myeloproliferative disease, unclassified."

As the name indicates, this group includes cases with both dysplastic and proliferative morphology at the time of presentation. These cases are difficult to assign to either the myelodysplastic or myeloproliferative group. This category will be a focus of study of the molecular pathways involved in the control of proliferation and abnormal maturation, and dysplasia. Most of the clinical and pathological presentation is a result of the abnormal regulation of myeloid pathways or cellular proliferation. These are multilineage disorders with mild clinical symptoms, because of the complication of both cytopenia and nonfunctioning dysplastic cells.

At present, there have been no genetic defects identified that are specific to any of these entities. There are some recurring chromosomal defects, but they are nonspecific and are seen in many similar disorders. A high frequency of *NRAS* mutations have been reported in many MDS/MPD disorders, suggesting deregulation of the *RAS* pathway *(38)*. In chronic myelomonocytic leukemia (CMML) and atypical CML (aCML), translocations [t(5;12)(q33;p13) and t(5;10)(q33;q22)] have been reported, which result in fusions that enhance the tyrosine kinase activity of the receptor *PDGFR*β. This, in turn, leads to abnormal activation of the *RAS* pathway and other signal transduction pathways *(29,38)*. However, it is clear that this mechanism is not unique to these disorders.

Chronic Myelomonocytic Leukemia

Chronic myelomonocytic leukemia (CMML) is a clonal disorder of bone marrow stem cells characterized by persistent monocytosis, less then 20% blasts, dysplasia, and the absence of a Philadelphia rearrangement. In about 20–40% of cases, nonspecific clonal cytogenetic abnormalities are found. The most frequently recurring abnormalities include trisomy 8, monosomy 7 or deletions of 7q (see **Fig. 2h,i,j,k**), and structural abnormalities involving chromosome 12p *(39–43)*. The WHO classification suggests that isolated cases of i(17)(q10) are a unique group within the MDS/MPD, but many of these cases can be classified as CMML (see **Fig. 2rr**). In addition, as many as 40% of cases show point mutations of *RAS* at the time of the diagnosis or during the course of the disease *(44)*.

Atypical Chronic Myeloid Leukemia

Atypical chronic myeloid leukemia (aCML) is a leukemic disorder that demonstrates both myeloproliferative and dysplastic features at the time of initial presentation, hence it is included here. In most cases, the leukocytosis predominantly involves immature and mature neutrophils with a definitively dysplastic phenotype. In addition, multilineage dysplasia is commonly seen, thereby suggesting the stem cell origin of this disorder.

One of the diagnostic features of this condition is the absolute absence of a Philadelphia rearrangement or any evidence of a *BCR/ABL* fusion gene transcript. Clinically, most of the patients present with anemia or sometimes with thrombocytopenia. The chief complaint is often splenomegaly *(45–47)*. In most cases, the white blood cell (WBC) count is variable, with values ranging from 35×10^9 to 96×10^9 cells/L. Blasts usually account for more than 5% and less than 20% of the peripheral blood white cells. There is minimal or no absolute basophilia and less than 2% of the WBCs are basophils. There is minimal or no absolute monocytosis and monocytes represent less than 10% of WBCs. Bone marrow biopsy is usually hypercellular, with evidence of granulocytic

proliferation and granulocytic dysplasia. These cases could present with or without dysplasia of the erythroid and megakaryocytic lineages.

About 80% of atypical CML patients have chromosomal abnormalities *(46–48)*. Cytogenetic abnormalities in this disorder include trisomy of chromosomes 8 and 13, deletion of chromosome 20q and 12p, and isochromosome 17q (see F**ig. 2z,aa,s,t,u,rr**). As there are no specific chromosomal translocations, the diagnosis is usually made by morphology exam with the help of cytogenetics and other findings. A single case of atypical CML with a translocation involving chromosomes 5 and 10 [t(5;10)(q33;q21)] has been reported. This results in an abnormal fusion of the platelet-derived growth factor-β receptor gene, *PDGFR*β on 5q with the gene *H4*(*D10S170*) on 10q *(49,50)*.

Juvenile Myelomonocytic Leukemia

Juvenile myelomonocytic leukemia (JMML) is a clonal hematopoietic disorder that presents in childhood and is characterized by a definitive proliferation of granulocytic and monocytic lineages. In addition, erythroid and megakaryocytic abnormalities are frequently present. These findings are in concordance with the evidence that JMML arises from bone marrow stem cells with multilineage potential in the myeloid series *(51–53)*. JMML accounts for about 2–3% of all leukemias and 20–30% of all cases of myelodysplastic and myeloproliferative disease in patients less than 14 years of age *(54–56)*.

Morphologically, blood and bone marrow always demonstrate myelomonocytic proliferation. Hepatic and spleen infiltration are often seen. Clinically, most patients present with constitutional symptoms including malaise, pallor, and fever or evidence of an infection *(56–58)*. Diagnostic criteria for JMML include peripheral monocytosis of more than 10^9 cells/L, blasts, including promonocytes, of less than 20%, absence of the Philadelphia rearrangement, and no evidence of the *BCR*/*ABL* fusion gene transcript.

The majority of cases show evidence of clonal chromosome abnormalities. At present, no cytogenetic abnormalities or genetic lesions are specific for JMML, but they aid in diagnosis and are used as markers to follow a patient's progress *(58–60)*. In one way, cytogenetic analysis is essential, as the absence of the Philadelphia rearrangement or *BCR*/*ABL* fusion gene is a diagnostic criterion for this disorder. The most common cytogenetic abnormality is monosomy 7, which is seen in about 30–40% of patients. In a subgroup of JMML with neurofibromatosis (NF), loss of heterozygosity (of the normal NF allele) has been reported. This genetic alteration leads to loss of neurofibromin, a protein that plays an important role in the regulation of the *RAS* family of oncogenes *(57,60)*. Point mutations in the *RAS* genes are reported in the leukemic cells of about 20% of these patients *(61)*.

Overall, prognosis in JMML is poor to intermediate; if untreated, approximately 30% of the patients have rapid progression and die within 1 year of diagnosis *(58)*. Most patients die of organ failure resulting from leukemic infiltration. A small percentage of patients, perhaps 10–20%, evolve to acute leukemia. Although chemotherapy might benefit some patients, the overall survival is unusually poor, and, currently, bone marrow transplantation is the only therapy that has been demonstrated to clearly improve survival *(58)*.

MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes (MDSs) are a heterogeneous group of disorders. Patients usually present with some evidence of bone marrow failure and dysplasia in one or more of the myeloid cell lineages. This category includes refractory anemia, refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia, refractory anemia with excess blasts, myelodysplastic syndrome associated with isolated deletion 5q chromosome abnormality, and "myelodysplastic syndrome unclassified." MDS could evolve into acute myeloid leukemia (AML, see later) or patients could die as a result of consequences of marrow failure resulting from MDS alone. A subgroup of MDS with isolated 5q deletions (see **Fig. 2c,d**) is characterized by a collection of cytogenetic and morphologic features that are predictive of prognosis.

Table 3
Peripheral Blood and Bone Marrow Findings in Myelodysplastic Syndromes

Disease	Blood findings	Bone marrow findings
Refractory anemia (RA)	Anemia No or rare blasts	Erythroid dysplasia only <5% blasts <15% ringed sideroblasts
Refractory anemia with ringed sideroblasts (RARS)	Anemia No blasts	>15% ringed sideroblasts Erythroid dysplasia only <5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods $<1\times10^9$/L monocytes	Dysplasia in 10% of the cells of two or more myeloid cell lines <5% blasts in marrow No Auer rods <15% ringed sideroblasts
Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods $<1\times10^9$/L monocytes	Dysplasia in 10% of the cells in two or more myeloid cell lines 15% ringed sideroblasts <5% blasts No Auer rods
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenias <5% blasts No Auer rods 1×10^9/L monocytes	Unilineage or multilineage dysplasia 5–9% blasts No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenias 5–19% blasts Auer rods ± $<1\times10^9$/L monocytes	Unilineage or multilineage dysplasia 10–19% blasts Auer rods ±
Myelodysplastic syndrome—unclassified (MDS-U)	Cytopenias No or rare blasts No Auer rods	Unilineage dysplasia: one myeloid cell line <5%blasts No Auer rods
MDS associated with isolated del(5q)	Anemia Usually normal or increased platelet count 5% blasts	Normal to increased megakaryocytes with hypolobated nuclei <5% blasts Isolated del(5q) cytogenetic abnormality No Auer rods

Source: Data from ref. *65*.

Cytogenetic characterization of MDS is critical, as the type of MDS and the cytogenetic findings determine the therapeutic approach. Treatment ranges from observation only to chemotherapy and/or bone marrow transplantation. Cytogenetic and molecular studies play a major role in the evaluation of patients with myelodysplastic syndrome with regard to prognoses and selection of chemotherapy *(62)*; the reported frequency of MDS patients who present with abnormal karyotypes ranges from 33% to as high as 73%. Peripheral blood and bone marrow findings in myelodysplastic syndromes are well documented *(62)* (see **Table 3***)*, and a summary of chromosome abnormalities can be found in **Table 4**.

Table 4
Recurrent Primary Chromosome Aberrations in Myelodysplastic Syndromes

del(1)(p22)	+9
t(1;3)(p36;q21)	t(9;11)(p22;q23)
der(1;7)(q10;p10)	t(9;22)(q34;q11.2)
t(2;11)(p21–22;q23)	+11
t(3;21)(q26;q22)	del(11q)[c]
3q26 abnormalities*a*	del(12)(p11–13)
–5	del(13q)[d]
del(5q)*a*	16q22 abnormalities
t(5;12)(q33;p13)	i(17q)
+6	+19
t(6;9)(p23;q34)	del(20q)[e]
–7	+21
del(7q)	–X
+8	–Y
t(8;21)(q22;q22)	

[a] Includes inv(3)(q21q26), t(3;3)(q21;q26), ins(3;3)(q21q26).
[b] del(5)(q13q33) observed in "5q–" syndrome.
[c] Includes breakpoints at 11q14 and 11q23.
[d] Includes breakpoints 13q11.2-22.
[e] Includes breakpoints 20q11.2-13.

Refractory Anemia

Cytogenetic abnormalities can be observed in up to 25% of cases of refractory anemia (RA). Several different acquired clonal chromosomal abnormalities have been identified, and although useful for helping to establish a diagnosis of refractory anemia and for acting as a marker to follow a patient's progress, they are not specific. These abnormalities include deletion of chromosome 20, trisomy of chromosome 8, and abnormalities of chromosome 5 and/or 7 (see **Fig. 2z,aa,c,d,h,i,j,k**).

Refractory Anemia with Ringed Sideroblasts

Clonal chromosomal abnormalities are seen in fewer than 10% of cases of refractory anemia with ringed sideroblasts (RARS). If clonal cytogenetic abnormalities develop during the course of the disease, the case should be evaluated and appropriately reclassified, as this is evidence of another form of MDS or development of AML.

Refractory Cytopenia with Multilineage Dysplasia

The clonal chromosomal abnormalities seen in refractory cytopenia with multilineage dysplasia (RCMD) include trisomy 8, monosomy 7, deletion of 7q, monosomy 5, deletion of 5q, and deletion of 20q (see **Fig. 2c,d,h,i,j,k,z,aa**). Complex karyotypes might be found in up to 50% of patients with RCMD and RCMD with ringed sideroblasts (RCMD-RS).

Refractory Anemia with Excess of Blasts

About 30–50% of cases of refractory anemia with excess of blasts (RAEB) have clonal cytogenetic abnormalities. These include trisomy 8, deletion of 5q, monosomy 5, monosomy 7, deletion of 7q, and deletion of 20q (see **Fig. 2c,d,h,i,j,k,z,aa**). Complex karyotypes might also be observed in small percentage of cases. The number of blasts in bone marrow and blood is usually less than 5% *(63)*.

The del(5q) Syndrome

A major category in this subgroup is the group of patients with isolated 5q deletion syndrome [del(5q), formerly referred to as 5q–]. This syndrome primarily occurs in women and is characterized by megakaryocytes with hypolobated nuclei and refractory microcytic anemia, normal or increased platelet count, and a favorable clinical course. Symptoms are usually related to refractory anemia, which is often severe. The platelet count is generally normal to elevated and occasional blasts, usually less than 5%, are seen. Erythroid precursor cells show dysplastic features of varying degrees. Cytogenetically, this is a very special subgroup, as the sole cytogenetic abnormality involves a deletion of chromosome 5 with breakpoints in the long arm from q31 to q33 (see **Fig. 2c,d**). The size of the deletion and exact breakpoints are variable from case to case, and most deletions are interstitial. In the WHO classification, this syndrome has been recognized as a specific subtype of MDS because of the significance of isolated deletion 5q and relatively good prognosis. The significance of more than 5% marrow blasts in patients with an associated deletion of chromosome 5 is not clear. Some reports indicate that these patients have a worse prognosis than those with fewer than 5% blasts *(64)*.

Karyotypic evolution is uncommon. In more complex cases, in addition to the del(5q), other cytogenetic abnormalities including deletion of 17p with *TP53* mutation and more complex chromosomal changes are seen. When present, these are generally associated with an unfavorable clinical course. Other cytogenetic abnormalities, such as deletion of 2q, are associated with involvement of erythroid cells and megakaryocytes. Abnormalities of chromosome 3 are associated with MDS and AML with increased megakaryocytes. Additional cytogenetic abnormalities are associated with evolution to AML or a higher-grade myelodysplastic process. However, if any additional chromosome abnormalities are present, the case should not be placed in the category of isolated 5q deletion syndrome.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. The leukemia cells or blasts represent progenitors that are arrested in differentiation at a very early stage of myelopoiesis. AML is characterized by an accumulation of granulocyte or monocyte precursors in the bone marrow and blood. There is an increasing recognition of the importance of genetic events in the classification and therapy of AML, making it an excellent model for studying genetic regulation of differentiation and cancer progression.

Worldwide, the overall incidence of acute leukemia is approximately 4 per 100,000 per year, with 70% of these cases being acute myeloid leukemia. The vast majority of cases of AML occur in adults, and the median age is about 60 years with an incidence of 10 per 100,000 population per year in individuals 60 years and older. The possible etiological factors associated with leukemia and myelodysplastic syndrome include viruses, ionizing radiation, cytotoxic chemotherapy, and benzene.

Cytogenetically, AML is a very heterogeneous disease, with more then 160 recurrent structural chromosomal abnormalities having been reported *(65,66)*. Molecular dissection of many reciprocal translocations and inversions has resulted in cloning of the chimeric genes involved in tumorigenesis. The significance of cytogenetic studies of AML is twofold. First, it has significantly increased our knowledge of basic genetic mechanisms involved in tumorigenesis, thus contributing to our understanding of the remarkable histopathological, immunophenotypic, and clinical heterogeneity of AML. In the process of characterizing these specific chromosomal changes, which include translocations, inversions, deletions, and duplications, scientists have cloned many known genes involved in leukemogenesis. Second, chromosomal aberrations, irrespective of whether they are cloned molecularly, help as tumor markers for diagnostic and prognostic purposes. The incidence of karyotypes with clonal chromosomal aberrations in children with gene rearrangements is reported to be between 68% and 85% and has generally been higher than that in adults.

The WHO classification incorporates morphological, immunophenotypic, genetic, and clinical information to categorize AML. The threshold blast percentage is 20%, compared with 30% in the FAB classification. The WHO classification is as follows:

- Acute myeloid leukemia with recurrent genetic abnormalities
  - AML with t(8;21)(q22;q22) (*AML1* or *CBFα/ETO*) (Often FAB M2); see **Fig. 1r**
  - AML with abnormal eosinophils; inv(16)(p13q22) or t(16;16)(p13;q22) (*CBFβ/MYH11*) (often FAB $M4_{EO}$); see **Fig. 2ccc**
  - Acute promyelocytic leukemia ([AML with t(15;17)(q22;q12)(*PML/RARα*) and variants] (FAB M3); see **Fig. 1bb**.
  - AML with 11q23 (*MLL*) abnormalities
- Acute myeloid leukemia with multilineage dysplasia
- Acute myeloid leukemia and myelodysplastic syndrome or therapy-related AML
- Acute myeloid leukemia not otherwise characterized

Because of its heterogeneity, classification of AML into its various biologic entities is necessary in order to understand its pathogenesis and develop specific treatment approaches. Furthermore, the presence of chimeric transcripts in leukemic blasts has been shown to be among the most important independent prognostic parameters in AML. In the WHO classification, genetically defined subgroups of AML are classified and treatment decisions are often based on these disease-specific genetic defects. The incidence of abnormal karyotypes in AML is reported to be about 55–78% in adults and 77–85% in children. However, substantial portions of patients with AML show no chromosomal abnormalities. Recent studies show that cytogenetically normal patients often display submicroscopic gene alternations that can only be detected by a molecular method. For instance, approximately 6% of the adult AML patients with normal karyotypes have a partial tandem duplication within the *MLL* gene. In addition, over 20% of patients with such intragenic *MLL* abnormalities demonstrate a mutation/deletion involving *FLT3* (*CD135*), but this only occurs in 10% of AML cases with *MLL* translocations and only in 5% of adult AML with normal *MLL* status *(67)*.

Cytogenetic abnormalities in AML can be classified into primary and secondary abnormalities. The primary chromosomal abnormalities are frequently found as the sole karyotypic abnormality and are often specifically associated with a particular AML subtype. On average, about 55% of AML patients with a karyotypic abnormality have only one recognizable rearrangement. These primary chromosomal abnormalities are assumed to play an essential role in the early stages of tumorigenesis. On the other hand, secondary chromosomal abnormalities seem to play an important role in the progression of the disease, but they are rarely found alone. Many of the primary chromosomal abnormalities in AML are balanced translocations between two different chromosomes or inversions within a single chromosome; t(8;21), t(15;17) and inv(16) are examples. These balanced rearrangements disrupt critical genes involved in normal hematopoiesis, resulting in an abnormal process. On the other hand, many unbalanced abnormalities, including deletions or gains and losses of entire chromosomes, are thought to be primary abnormalities, but the pathogenic mechanism has not been resolved in any of these because of the large number of genes involved. The most common unbalanced abnormalities are deletions of 5q, monosomy 7, deletion of 7q, trisomy 8, deletion of 9q, trisomy 11, trisomy 13, and trisomy 1.

About 10–20% of AML patients show so-called complex or aberrant karyotypes, which are associated with a very poor prognosis. The definition of a complex karyotype varies among study groups. It is most commonly defined as the presence of at least three cytogenetic abnormalities.

In approximately 45% of AML patients with aberrant karyotypes that have two or more karyotypic abnormalities, one or more of these represents a secondary change. For example, in about 70–80% of patients with t(8;21), additional aberrations are observed, the most common being the loss of a sex chromosome. In addition, about 30–50% of patients with inv(16) show additional chromosome aberrations, with trisomies of chromosomes 8, 21, or 22 being most frequently observed. The frequency

of secondary chromosome aberration in patients with a t(15;17) is about 30–40%, with trisomy 8 and isochromosome of the derivative chromosome 17 being most common.

Acute Myeloid Leukemia with Recurrent Cytogenetic Abnormalities

Acute Myeloid Leukemia with t(8;21)(q22;q22) [AML1 (CBFα)/ETO]

Acute myeloid leukemia with t(8;21) (see **Fig. 1r**) generally shows maturation arrest in the neutrophilic lineage. The (8;21) translocation is the most common structural abnormality in AML and results in the fusion of *AML1* (also known as core-binding factor-α [*CBFα*]) at 21q22 and the *ETO* gene, a transcription factor, at 8q22 *(70)*. The *AML1/ETO* fusion protein is predominantly located in the nucleus of the leukemic cell and inhibits transcription. t(8;21) is found in about 5–20% of AML cases and in one-third of the karyotypically abnormal cases of AML with maturation. It occurs predominantly in younger patients. Bone marrow morphology shows large blasts with a blended basophilic cytoplasm, often containing numerous azurophilic granules. In some cases, a few blasts might show very large granules. Auer rods are frequently found and appear as single, long, sharp rods with tapered ends. Promyelocytes, myelocytes, and mature neutrophils with variable dysplasia are present in the bone marrow *(7)*. The postulated cell of origin in t(8;21) AML is the myeloid stem cell, with predominantly neutrophilic differentiation.

The presence of t(8;21) is prognostically significant. This karyotype is usually associated with a good prognosis when patients are treated with chemotherapy, and high complete remission rates with long-term survival are seen when they are treated with high-dose cytarabine in the consolidation phase. However, the presence of additional secondary changes, including expression of CD5, appears to adversely affect survival *(68–70)*.

Acute Myeloid Leukemia with inv(16) (p13q22) or t(16;16) (p13q22) (CBFβ/MYH11)

Acute myeloid leukemia with inv(16)(p13q22) (see **Fig. 2ccc**) or t(16;16)(p13;q22) is an acute leukemia with myelocytic and granulocytic differentiation and is characterized by the presence of an abnormal eosinophilia component in the marrow *(71,72)*. The combination of acute myelomonocytic leukemia with abnormal azurophilia is also referred to as acute myelomonocytic leukemia with eosinophilia ($AMML_{EO}$).

inv(16) or t(16;16) are found in approximately 10% of all AML cases. Both aberrations result in the fusion of *CBFβ* at 16q22 with the smooth muscle myosin heavy chain (*MYH11*) at 16p13 *(66)*. Initially, cytogenetic detection of these chromosomal abnormalities was poor because of their cryptic nature; they might be difficult to visualize in poor chromosome preparations. Development and use of new methodologies to diagnose these aberrations have significantly improved the detection of these abnormalities.

The bone marrow morphology shows features of acute myelomonocytic leukemia with a variable number of eosinophils, sometimes more than 5%, at all stages of maturation without significant maturation arrest. The most striking abnormalities involve the immature eosinophilic granules, mainly evident at the promyelocytic and myelocytic stages. Although the majority of cases of inv(16) have been identified as $AMML_{EO}$, occasionally cases of this neoplasm have been reported to lack eosinophilia. Another significant feature of this leukemia is that the blast percentage is occasionally below the WHO threshold level of 20%. [This is similar to cases with t(8;21) that also have less than 20% bone marrow blasts. Nevertheless, cases with these characteristic cytogenetic abnormalities should be diagnosed as acute myeloid leukemia.]

The possible cell of origin is of granulocytic and monocytic lineage or a hematopoietic stem cell with the potential to differentiate into granulocytic and monocytic lineages. Patients with this inversion or translocation achieve higher complete remission rates when treated with high doses of cytarabine in the consolidation phase *(73)*. Many cases with secondary chromosomal changes in addition to inv(16) or t(16;16) have been reported, but the prognostic significance does not seem to change.

Molecular studies are highly sensitive in monitoring disease during and after therapy. However, the value of molecular monitoring is limited, as the *CBFβ/MYH11* transcript can still be detected in long-term remission patients. FISH with appropriate probes for the 16p13 region detects deletions at the site proximal to the 5' breakpoint in around 20% of the patients with inv(16). The deleted segment extends further than the 5' breakpoint and *MYH11* gene and inevitably includes the *MPR* gene. Some patients show mosaicism of deleted and nondeleted metaphases, suggesting that the deletion is a secondary event during disease progression. No significant difference is observed in the survival of patients with or without 16p deletions associated with inv(16) or t(16;16).

*Acute Promyelocytic Leukemia, AML with t(15;17) (q22;q12) (*PML/RARα *and Variants)*

Acute promyelocytic leukemia (APL) is an acute myeloid leukemia in which abnormal promyelocytes predominate. Two types of morphology, hypergranular or (typical AML) and microgranular types, are seen. APL comprises about 5–8% of AML cases *(80)*. The disease can occur at any age, but most patients are adults in midlife. The disease is characterized by a t(15;17)(q22;q12) (see **Fig. 1bb**). See also Fig. 9 of Chapter 17. The translocation breakpoints involve the retinoic acid receptor α (*RARα*) locus on 17q (reported to be either at band q12 or band q21) and the promyelocytic leukemia (*PML*) locus on 15q. The rearrangement gives rise to a *PML/RARα* fusion gene. Both the *PML/RARα* and *RARα/PML* fusion products might be present in the leukemic cells *(66,75,76)*. Secondary chromosomal changes in addition to t(15;17) have been reported in about 25–40% of APL and could involve chromosomes 6–9, 12, 16, 17, and 21. Changes involving chromosome 8 are the most frequent and those involving chromosome 12 are the most rare.

In addition to the diagnostic value of t(15;17), its finding has therapeutic importance. The proposed cell of origin in APL is the myeloid stem cell with potential to differentiate to the granulocytic lineage. Acute promyelocytic leukemia has a particular sensitivity to treatment with all-trans retinoic acid (ATRA), which acts as a differentiating agent. The prognosis of APL treated optimally with ATRA and anthracycline is relatively favorable, very similar to AML with t(8;21) or inv(16) *(77–79)*.

There are a few variant translocations involving the *RARα* gene. In one, *RARα* fuses with the promyelocytic leukemia zinc-finger gene (*PLZF*) at chromosome 11q23.1 (see **Fig. 1x**), and in another, it fuses with the nuclear plasmin gene on chromosome 5. There is also a variant translocation [t(11;17)(q13;q12)] in which the nuclear mitotic associated apparatus gene (*NUMA*1) on chromosome 11 fuses with *RARα (76,80,81)*. These are of significant importance, as classical treatment with retinoic acid might not be effective for these variant translocations *(76)*.

Acute Myeloid Leukemia with 11q23 Abnormalities

Acute myeloid leukemia with 11q23 abnormalities is usually associated with monocytic features. Abnormalities of chromosome 11q23 are found in about 5–6% of AML cases, more commonly in children. The *MLL* ("mixed lineage leukemia" or "myeloid/lymphoid leukemia") gene at 11q23, also called *ALL1*, *HRX,* and *HTRX-1*, is the human homolog of the Drosophila trithorax gene. It encodes a putative DNA-binding protein that is involved in control of embryonic development and is characterized by multiple zinc-finger DNA-binding domains. Murine models suggest that *MLL* plays a major role in hematopoietic differentiation and regulates the transcriptional activities of other genes, namely *HOX* genes. It has been shown that in cases with a normal karyotype or with trisomy 11, the *MLL* gene sometimes shows internal tandem duplication. Molecular studies have demonstrated that this partially duplicated *MLL* is transcribed into mRNA that is capable of encoding a partially duplicated protein.

The possible cell of origin in this leukemia is the hematopoietic stem cell with multilineage potential. Morphologically, the predominant cell types are monoblasts and promonocytes. Monoblasts are large cells with abundant cytoplasm, which can be basophilic and show pseudopod formation. The promonocytes have an irregular and delicately convoluted nuclear concentration. The cytoplasm of promonocytes is usually less basophilic and sometimes more obviously granulated with occasional large azurophilic granules and vacuoles.

The *MLL* gene at 11q23 is involved in a number of translocations with different partner chromosomes. The more common translocations observed in childhood AML are t(9;11)(p21;q23) (see **Fig. 1s**) and t(11;19)(q23;p13) (see **Fig. 1pp**). Other observed translocations of 11q23 involve approximately 20 different partner chromosomes *(65)*.

Acute myeloid leukemia with associated abnormalities of 11q23 has an intermediate survival.

Acute Myeloid Leukemia with Multilineage Dysplasia

Acute myeloid leukemia with multilineage dysplasia characteristically shows more than 20% blasts in blood or marrow with visible dysplasia in two or more myeloid cell lineages, generally including megakaryocytes. By definition, dysplasia must be present in about 50% of the cells of at least two lines *(82)*, and these features must be present in pretreatment specimens. This entity could occur *de novo* or follow MDS or myelodysplastic qualitative disorders, in which, according to WHO, cell counts are close to normal but morphology is not.

Chromosome abnormalities in this subtype are similar to those found in myelodysplastic syndrome and often involve gain or loss of major segments of certain chromosomes. Some of the common changes are loss of chromosome 7 and 18, deletion of 17q, loss of chromosome 5 or deletion of 5q, gain of chromosomes 8, 9, 11, 19, and 21, deletion of chromosomes 11, 12p, and 20q, and less often specific translocations, like t(2;11)(p21;q23) (see **Fig. 1f**), t(1;7)(p10;q10) (usually unbalanced; see **Fig. 1b**), and a translocation involving chromosome regions 3q21 and 3q26. Abnormalities in the 3q26 region such as inv(3)(q21q26) (see **Fig. 2aaa**), t(3;3)(q21;q26) (see **Fig. 1h**), or ins(3;3)(q21;q26) (see **Fig. 1i**) are associated with multilineage AML and MDS with increased platelet production. inv(3)(q21q26) is also seen in other types of AML and myeloid qualitative syndrome associated with thrombocytosis and increased bone marrow megakaryocytes *(83–86)*. t(3;21)(q21;q26) is usually therapy-related or associated with myeloid leukemia as a secondary event at blasts crisis, whereas t(3;5)(q25;q34) (see **Fig. 1j**) is associated with multilineage dysplasia without thrombocytosis. In this subtype of leukemia, multilineage dysplasia has an adverse effect on the probability of achieving complete remission *(84–86)*.

Acute Myeloid Leukemia and Myelodysplastic Syndrome, Therapy Related

Therapy-related or secondary AML and myelodysplastic syndrome (t-AML/t-MDS) arise as a result of cytotoxic chemotherapy and/or radiation therapy. Two major types are recognized based on the causative agent: those that are alkylating agent/radiation related and those that are topoisomerase II inhibitor related *(87–90)*. These types of AML and MDS can also be classified, if appropriate, by the specific morphology originally seen with the qualifying term "therapy-related."

The process of acute leukemia frequently presents initially as a myelodysplastic syndrome, with evidence of bone marrow failure with isolated cytopenia or pancytopenia and associated myelodysplastic changes. Frank dysplastic features in multiple cell lineages usually follow this stage, during which the blast percentage in marrow is usually less than 5%.

Alkylating agent/radiation-related therapy-related AML, either presenting as AML or evolving from MDS, usually involves all myeloid cell lines. High incidences of clonal cytogenetic abnormalities are seen in this subgroup of leukemia. These abnormalities are similar to those seen in AML with multilineage dysplasias, MDS, refractory cytopenia with multilineage dysplasia, or refractory anemia. The common aberrations are unbalanced translocations or deletions involving chromosome 5 and/or 7, with the loss of all or part of the long arm of these chromosomes (see **Fig. 2c,d,h,i,j,k**). The deletion of the long arm of chromosome 5 usually includes bands q22-q23 *(87)*. Other chromosomes frequently involved in a nonrandom manner include chromosomes 1, 4, 12, 14, and 18. Complex nonspecific chromosomal abnormalities are the most common finding. Therapy-related leukemia with multiple cytogenetic abnormalities is refractory to any leukemia therapy and is associated with short or poor survival.

Topoisomerase II inhibitor-related AML characteristically has a significant monocytic component. Most cases fall in the category of acute monoblastic or myelomonocytic leukemia. The predominant

cytogenetic finding is a balanced translocation involving 11q23 (the *MLL* region). These are primarily t(9;11)(p22;q23) (see **Fig. 1s**), t(11;19)(q23;p13) (see **Fig. 1pp**), and t(6;11)(q27;q23) (see **Fig. 1o**) *(91)*. Other observed abnormalities include t(8;21)(q22;q22) (see **Fig. 1r**), t(3;21)(q26;q22) (see **Fig. 1k**), inv(16)(p13q22) (**Fig. 2ccc**), t(8;16)(p11.2;p13), and t(6;9)(p23;q34) (see **Fig. 1n**). Cases of APL with atypical t(15;17) have also been seen. Most of these therapy-related acute leukemias are multidrug-resistant and patients have short survival *(92)*.

Acute Myeloid Leukemia Not Otherwise Categorized

This category of AML encompasses those cases that do not fulfill criteria for inclusion in one of the previously described groups. The primary basis for sub classification within this category is the morphologic and cytochemical features of the leukemic cells and the degree of maturation.

Acute Myeloblastic Leukemia Minimally Differentiated

No unique chromosome abnormalities have been identified in this subtype of AML with minimal differentiation. The most common nonspecific abnormalities are complex karyotypes involving trisomy 4, trisomy 8, trisomy 13, and monosomy 7 *(93)*.

Acute Myeloblastic Leukemia with Maturation

Acute myeloblastic leukemia with maturation is characterized by the presence of 20% blasts in the bone marrow and evidence of relatively more neutrophilic maturation, usually more than 10%. Monocytes usually comprise less than 20% of marrow cells. In this group, there are notably some specific translocations, associated with the characteristic phenotype, that are very useful for the confirmation of diagnosis and follow-up of therapy. Deletion and translocation involving 12p11.2-p13 are associated with increased bone marrow basophilic cells. t(6;9)(p23;q34) results in the formation of a chimeric fusion gene, *DEK/CAN*. Rare cases with t(8;16)(p11.2;p13) with hemocytosis, particularly erythrocytosis are also seen *(83,94)*.

Acute Myelomonocytic Leukemia

Acute myelomonocytic leukemia is characterized by the proliferation of both neutrophil and monocyte precursors. The bone marrow shows more than 20% blasts, plus neutrophils, monocytes, and their precursors. Cytogenetically, only nonspecific abnormalities are present in majority of cases. Some cases have abnormalities involving chromosome 11q23, others have inv(16)(p13q22) (see **Fig. 2ccc**).

Acute Monoblastic Leukemia and Acute Monocytic Leukemia

Acute monoblastic leukemia and acute monocytic leukemia is a myeloid lineage leukemia in which 80% or more of the leukemic cells are of monocytic lineage, including monoblasts, promonocytes, and monocytes. A minor neutrophil component, less than 20%, might be present. This leukemia presents with bleeding disorders, extramedullary masses with cutaneous infiltration, and, often, central nervous system (CNS) involvement. Cytogenetically, there is a strong association between acute monoblastic leukemia and deletion and translocations involving 11q23, as described earlier.

Acute Erythroleukemia

Acute erythroleukemias are acute leukemias characterized by a predominant erythroid population. Two subtypes are recognized based on presence or absence of a significant myeloid component: erythroid leukemia with erythroid myeloid differentiation, and pure erythroleukemia. There are no specific chromosomal abnormalities described in this type of AML. Complex karyotypes with multiple structural abnormalities are common, with chromosomes 5 and 7 being the most frequently involved.

Acute Megakaryoblastic Leukemia

Acute megakaryoblastic leukemia is characterized by more than 50% blasts of megakaryocytic lineage. Patients with acute megakaryoblastic leukemia present with cytopenias, often thrombocytopenia,

although some could have thrombocytosis. Dysplastic features in neutrophils and platelets could be present. Organomegaly and/or hepatosplenomegaly are infrequent, except in children, particularly infants under 1 year of age, who often present with prominent abdominal masses and acute megakaryoblastic leukemia associated with t(1;22)(p13;q13) *(95)*. Cytogenetically, there is no unique chromosome abnormality associated with acute megakaryoblastic leukemia in adults. Some cases show inv(3)(q21q26) (see **Fig. 2aaa**), but this abnormality is also found in many other types of acute myeloid leukemia.

Summary

In recent years, scientific studies of AML, facilitated by cytogenetics and FISH, have focused on the identification of consistent chromosomal translocations and fusion proteins. A number of studies have pointed to the dominant role of lineage-specific transcription factors in normal hematopoietic differentiation. These studies predicted that the function of these transcription factor pathways would be disrupted in AML. Recent studies have confirmed this hypothesis, demonstrating that a number of AML cases are not associated with consistent chromosomal translocations, but, rather, have small mutations in the coding region of these lineage-specific transcription factors. In addition, in many cases of AML that are associated with a common chromosome translocation, the resulting translocation product disrupts the expression and function of the same lineage-specific factors. These results support the view that disruption of normal differentiation is the key component in the development of certain leukemias. Characterization of these transcription factor abnormalities has already affected classification schemes based on patient outcome. These transcription factor pathways represent important targets for therapeutic intervention of chimeric proteins and transcription factors.

In addition to the known translocations in AML, trisomies and monosomies also play an important role. Primary trisomies in AML include chromosomes 4, 8, 11, 13, 16, 19, 21, and 22. AML with trisomy 4 is believed to originate from an early myeloid precursor, as is MDS with a high blast component. This trisomy is consistently found in cases with double minutes. Double minutes are believed to represent gene amplification (see **Fig. 3**); in this case, the amplified gene is *MYC*, but its exact role in the neoplastic process is not known. Gene amplifications (e.g., *MLL* amplification) have also been described in various AML subtypes and in therapy-related AML/MDS, especially if alkylating agents were used. These patients are generally elderly with poor prognoses. Other gene mutations commonly seen in AML involve *FLT3*, *KIT*, *CEBPA*, *WT1*, *AML1*, *NRAS,* and *KRAS*.

For a listing of many cytogenetic abnormalities seen in AML, refer to **Table 5**.

PRECURSOR B-CELL AND T-CELL NEOPLASMS

Precursor B-Cell Acute Lymphoblastic Leukemia/Lymphoma

B-cell ALL (B-ALL) is a neoplasm of lymphoblasts committed to a B-cell lineage. It is characteristically composed of small to medium-sized blasts with scant cytoplasm, moderately condensed to dispersed chromatin, and prominent nuclei.

Blood and bone marrow are the most common sites of involvement. ALL is primarily a disease of children, as 75% of cases occur in children under 6 years of age. Approximately 80–85% are of a precursor B-cell phenotype.

When patients present primarily with involvement of nodal or extranodal sites, the disease is called B-cell lymphoblastic lymphoma. Therefore, if the patient presents with a mass lesion and 25% or fewer lymphoblasts in the marrow, the designation of lymphoma is preferred *(96)*. Precursor B-cell lymphoblastic lymphoma is uncommon, comprising approximately 10% of all cases of lymphoblastic lymphoma *(97–99)*. The most frequent sites of involvement of precursor B-cell lymphoma are the skin, bone marrow, soft tissue, and lymph nodes.

Most patients with B-cell ALL present with thrombocytopenia, and/or anemia, and/or neutropenia. Lymphadenopathy, hepatomegaly, and splenomegaly are also frequent. The degree of differentiation of the precursor B-cell lineage lymphoblasts has clinical and genetic correlates.

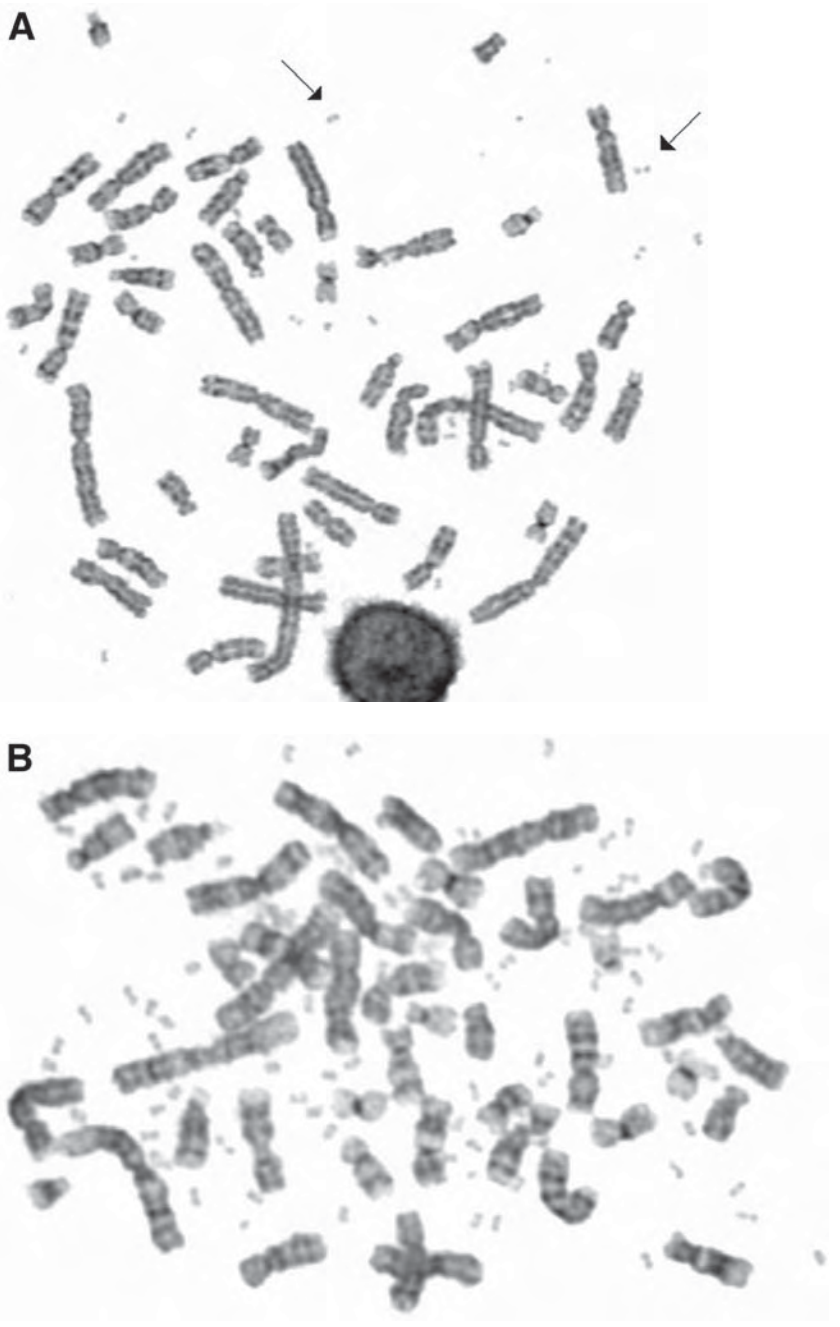

Fig. 3. Double minutes: **(A)** a cell with approximately 20 double minutes, 2 of which are arrowed; **(B)** a cell with many double minutes. Double minutes, which represent gene amplification (see text), are so named because of the typical structure. (Courtesy of Alma Ganezer and Dr. Paula Berry.)

In the early stages, so-called early precursor B-ALL, the blasts express CD19, cytoplasmic CD79a, cytoplasmic CD22, and nuclear tDt. In the intermediate stage, the blasts express CD10. In the most mature precursor B-cell differentiation stage, they express (cyt-μ) *(100)*.

Table 5
Association of Recurrent Primary Chromosome Aberrations in AML with Protein, Molecular Abnormality, Clinical Characteristics and Prognosis

Cytogenetic abnormality	Gene(s) involved	Protein	Clinical characteristics/prognosis
+1			
del(1)(q21)			
i(1)(q10)			
t(1;3)(p36;q21)			Preceded by MDS; dysmegakaryocytopoiesis
t(1;11)(p32;q23)[b]	*AF1P* (1p32)	Murine eps 15 homolog	
	MLL (11q23)	*Drosophilia* trithorax homolog	
t(1;11)(q21;q23)[b]	*AF1Q* (1q21)	No homolog to any known protein	
	MLL (11q23)	*Drosophilia* trithorax homolog	
t(1;17)(q36;q21)			
t(1;22)(p13;q13)			Thrombocytopenia, hepatosplenomegally, bone marrow fibrosis; 2° assoc. with der(1)t(1;22)
del(2)(p21)			
del(2)(p23)			
t(2;11)(p21;q23)			
t(2;3)(p23;q25)			
+3			
del(3)(q21)			
inv(3)(q21q26)[a,d]	Ribophorin (3q21)	RER transmembrane glycoprotein	Poor prognosis
	EVI1 (3q26)	Multiple zinc fingers	
t(3;3)(q21;q26)[a,d,e]	Ribophorin (3q21)	RER transmembrane glycoprotein	
	EVI1 (3q26)	Multiple zinc fingers	
t(3;5)(q25;q34)			Megakaryocytosis; young age at diagnosis; Sweet's syndrome
t(3;11)(q21;q13)			
t(3;12)(q26;p13)			
t(3;21)(p14;q22)[d]			
t(3;21)(q26;q22)[b,d]	*EVI1* (3q26)	Multiple zinc fingers	Genotoxic exposure
	AML1 (21q22)	CBFα, Drosophilia runt homolog	
t(3;21)(q26;q22)[b]	*EAP* (3q26)	Ribosomal protein L22	
	AML1 (21q22)	*Drosophilia* runt homolog	
t(3;21)(q26;q22)[b,d]	*MDS1* (3q26)	40% homolog to RIZ	
	AML1 (21q22)	CBFα, *Drosophilia* runt homolog	

(continued)

Table 5 *(continued)*

Cytogenetic abnormality	Gene(s) involved	Protein	Clinical characteristics/prognosis
+4			
del(4)(p15)			
t(4;12)(q11–13;p13)			
+5			
–5			Genotoxic exposure; poor prognosis
del(5)(q13q33)[f]			Genotoxic exposure; 2° assoc. with t(1;3), inv(3), –7/del(7q), poor prognosis
t(5;16)(q33;q22)			
+6			
del(6)(q21;22)			
del(6)(q15)			
i(6)(q10)			
t(6;9)(p23;q34)*b*	*DEK* (6p23)	Nuclear protein	Preceded by MDS; bone marrow basophilia; young age at diagnosis; Auer rods
	CAN (9q34)	nucleoporin	
t(6;11)(q27;q23)[b,d]	*AF6* (6q27)	GLGF motif	Localized infections
	MLL (11q23)	*Drosophilia* trithorax homolog	2° assoc. with t(1;22)
–7			Genotoxic exposure; 2° assoc. with inv(3); hepatosplenomegaly; poor prognosis
del(7)(p12p21)			
del(7)(q22)[g]			Genotoxic exposure; poor prognosis
der(1;7)(q10;p10)			Preceded by MDS; poor prognosis; genotoxic exposure
i(7)(q10)			
t(7;11)(p15;p15)[b,d]	*HOXA9* (7p15)	Class 1 homeobox	Auer rods
	NUP98 (11p15)	Nucleoporin	
t(7;11)(p13;p15)			
t(7;12)(q21;q13)			
t(7;21)(q31;q22)			
+8[e]			Preceded by MDS; 2° assoc. with der(1;7), t(6;9), t(9;11), t(9;22), t(15;17), inv(16); sole change in older patients; leukemia cutis; intermediate prognosis

del(8)(q22)			
t(8;16)(p11.2;p13)			Mainly infants/children; Erythrophagocytosis; DIC; poor prognosis
t(8;21)(q22;q22)[b,d]	*ETO* (8q22)	Zinc finger	Auer rods and eosinophilia; older children and adults; granulocytic sarcomas; intermediate prognosis
	AML1 (21q22)	CBFα, *Drosophilia* runt homolog	
t(8;22)(p11;q13)			
+9			
–9			
del(9)(p21)			
del(9)(q22)[h]			2° assoc. with t(8;21); agranular blasts; Auer rods
t(9;11)(p22;q23)[b]	*AF9* (9p22)	Nuclear protein, ENL homology	
	ALL1 (11q23)	*Drosophilia* trithorax homolog	
t(9;22)(q34;q11.2)[b]	*ABL* (9q34)	Tyrosine kinase	2° assoc. with der(22)t(9;22)
	BCR (22q11.2)	Serine kinase	
+10			
ins(10;11)(p11;q23q24)			
t(10;11)(p11-15;q13-23)[b]	*AF10* (p12)	Leucine zipper, zinc finger	
	MLL (11q23)	*Drosophilia* trithorax homolog	
t(10;17)(p13;q12–21)			
+11[b,c]	*MLL* (11q23)	*Drosophilia* trithorax homolog	Sole change in older patients; Auer rods
del(11)(p11-12p14-15)			
del(11)(q14-q23)			
del(11)(q23q25)			
i(11)(q10)			
t(11;11)(q23q25)			
t(11;15)(q23;q14–15)			
t(11;17)(q23;q21)[b]	*MLL* (11q23)	*Drosophilia* trithorax homolog	
	AF17 (17q21)	Leucine zipper, zinc finger	
t(11;17)(q23;q21)[b]	*PLZF* (11q23)	Zinc finger	Poor prognosis
	RARα (17q21)	Retinoic acid receptor α	
t(11;17)(q23;q23)			
t(11;17)(q23;q25)			
t(11;19)(q23;p13.3)[b]	*MLL* (11q23)	*Drosophilia* trithorax homolog	Mixed myeloid/lymphoid phenotype; mostly in male infants
	ENL (19p13.3)	Transcription factor	
t(11;19)(q23;p13.1)	*MLL* (11q23)	*Drosophilia* trithorax homolog	

(continued)

Table 5 *(continued)*

Cytogenetic abnormality	Gene(s) involved	Protein	Clinical characteristics/prognosis
	ELL (19p13.3)	Transcription factor	
t(11;20)(p15;q11)			
t(11;22)(q23;q11)			
+12			
del(12)(p12)[i]			2° assoc. with –7; genotoxic exposure 2° AML; poor prognosis
+i(12)(p10)			Primarily in males; mediastinal germ cell tumors
t(12;13)(p13;q14)			
t(12;14)(q13;q32)			
t(12;14)(q24;q32)			
t(12;17)(q13;p11-12)			
t(12;19)(q13;q13.3)			
t(12;22)(p12-13;q11-13)[b,d]	*TEL* (12p13)	ETS-related transcription factor	
	MN1 (22q11)	Nuclear protein	
+13			2° assoc. with t(6;9); seen as a sole abnormality in males; sole change in older patients; undifferentiated/biphenotypic
–13			
del(13)(q12q14)[j]			
i(13)(q10)			
+14			Sole change in older patients
–14			
i(14)(q10)			
+15			
t(15;17)(q22;q11–12)[b]	*PML* (15q21)	Zinc finger	
	RARα (17q21)	Retinoic acid receptor α	Older children or adults; absence of extramedullary leukemia; DIC; Auer rods; good prognosis
–16			
del(16)(q22)			Lymphadenopathy, hepatomegaly
der(16)(t(1;16)(q21;p13)[k]			
inv(16)(p13q22)[b]	*MYH11* (16p13)	Smooth muscle myosin heavy chain	Lymphadenopathy, hepatomegaly; good prognosis
	CBFβ (16q22)	Heterodimerizes with AML1	
t(16;16)(p13;q22)[b]	*MYH11* (16p13)	Smooth muscle myosin heavy chain	Lymphadenopathy, hepatomegaly; good prognosis
	CBFβ (16q22)	Heterodimerizes with AML1	
t(16;21)(p11.2;q22)[b]	*FUS* (16p11.2)	RNA-binding protein	
	ERG (21q22)	ETS-related transcription factor	Lymphadenopathy, hepatomegaly

+17	
–17	2° assoc. with +21
i(17)(q10)	MDS features; seen in older patients; poor prognosis
del(17)(q22)	
+18	
–18	2° assoc. with t(10;11)
+19	2° assoc. with t(1;22), t(9;22); seen as sole change in young patients
–19	
+20	
–20	
del(20)(q11.2q13)	
+21	MDS features
–21	
i(21)(q10)	
+22	MDS features; 2° assoc. with inv(16)
–22	2° assoc. with t(1;22)
del(22)(q11-13)	
–X	2° assoc. with t(8;22)
del(X)(q24)	
idic(X)(q13)	Found exclusively in women; seen in older patients
t(X;11)(q13;q23)	
t(X;11)(q24-25;q23)	
+X	2° assoc. with der(1;17)
–Y	Age related phenomenon in elderly patients; 2° assoc. with t(8;21)

[a]Gene activation.
[b]Gene fusion.
[c]Partial tandem gene duplication.
[d]Also observed in MDS.
[e]Also interpreted as ins(3;3)(q21;q21q26).
[f]Other breakpoints (between bands 5q11 and 5q35) also reported.
[g]Other breakpoints (between bands 7q11 and 7q36) also reported.
[h]Other breakpoints (between bands 9q11 and 9q34) also reported.
[i]Other breakpoints in 12p11 and 12q13) also reported.
[j]Other breakpoints (between bands 13q11 and 13q34) also reported.
[k]Also observed in ALL, solid tumors.
[l]Also observed in solid tumors.

As lymphocytes differentiate and mature, the structural changes they undergo are accompanied by functional changes. The principal change occurring in the maturation of immature lymphoblasts to lymphocytes is the somatic recombination of the immunoglobulin genes. Cytogenetic abnormalities play an important role in diagnosis, as well as in defining the treatment and in evaluating prognosis and risk factors. The cytogenetic abnormalities in precursor B-cell lymphoblastic leukemia/lymphoma fall into several groups as follows:

- Hypodiploid
- Hyperdiploid with fewer than 50 chromosomes
- Hyperdiploid with more than 50 chromosomes
- Translocations
  t(9;22)(q34;q11.2); *BCR/ABL*
  t(variable;11q23); *MLL* rearranged
  t(12;21)(p13;q22); *TEL/AML1*
  t(1;19)(q23;p13.3); *PBX/E2A*
- Pseudodiploid

With current treatment protocols, cases that are hyperdiploid with chromosome numbers between 51 and 65 carry a good prognosis. The karyotypes of patients with more than 50 chromosomes have certain features in common, such as extra copies of chromosome X, 4, 6, 10, 14, 17, 18, 20, and 21, duplication of 1q, and i(17q). Gain of chromosome 6 and the combination of trisomies 4 and 10 have been strongly associated with favorable outcomes. About 3% of cases show triploidy; this is more frequent in adult than in childhood ALL.

A favorable prognosis is also associated with a (12;21) translocation, which results in the fusion of the *TEL* gene at 12p13 with the transcription factor encoding gene *AML1*, at 21q22, resulting in compromised *AML1* transcriptional activity. The chimeric fusion gene is associated with superior treatment outcomes, with relapse-free survival seen in about 90% of cases. The prognostic strength of the *TEL* rearrangement is independent of other factors. Although this is the most common translocation in childhood ALL, FISH or an reverse-transcription–polymerase chain reaction (RT-PCR) assay is required to identify the gene fusion or chimeric transcript, as the translocation is not detectable with standard cytogenetic analysis. Because of the nature of the chromosome bands involved, this translocation was not reported until molecular evidence identified it.

In childhood cases, over 50% of patients with hyperdiploid karyotypes or t(12;21) have good prognoses *(101–103)*.

Several cytogenetic findings are associated with poor prognoses using current treatment protocols. These include t(9;22), t(4;11), and t(1;19), hypodiploidy, and near-haploidy

As described earlier, t(9;22) (see **Fig. 1t**) results in fusion of the *BCR* gene at 22q11.2 with *ABL*, a cytoplasmic tyrosine kinase gene at 9q34. In ALL, this translocation is more frequently seen in adults and is found in approximately 25% of adult patients. In most childhood cases of ALL a variant t(9;22) fusion protein, p190, is seen. t(4;11) (see **Fig. 1l**) results in fusion of the *MLL* gene at 11q23, which encodes a putative DNA-binding protein, and the transcription activator *AF4* at 4q21. (ALL with 11q23 abnormalities can also occur as therapy-related leukemia.) t(1;19), found in 25% of childhood B-ALL with cytoplasmic μ expression, fuses the transcription factor produced by *E2A* at 19p13.3 with *PBX1* at 1q23.

Most hypodiploid cases have a model number of 45 chromosomes and arise from loss of whole chromosomes, unbalanced translocations, or the formation of dicentric chromosomes. Hypodiploidy with 30–39 chromosomes is seen in about 2% of adult ALL.

The main clone in near-haploid cases contains at least one copy of each chromosome, with two sex chromosomes and two copies of chromosome 21 in most cases. In many near-haploid cases, there is a second abnormal cell line with a hyperdiploid karyotype. This hyperdiploid line usually contains exactly two copies of all chromosomes in the near-haploid cell line.

Table 6
Prognostic Implications of Genetic Alterations in Childhood Precursor B Lymphoblastic Leukemia

Cytogenetic finding	Genetic alteration	Frequency	Prognosis
t(9;22)(q34;q11.2)	*BCR/ABL*	3–4%	Unfavorable
t(4;11)(q21:q23)[a]	*AF4/MLL*	2–3%	Unfavorable
t(1;19)(q23;p13.3)	*PBX1* (*PBX1/E2A*)	6% (25% of pre B-all)	Unfavorable[b]
t(12;21)(p13;q22)[c]	*TEL/AML1*	16–29%	Favorable
Hyperdiploid >50		20–25%	Favorable
Hypodiploidy		5%	Unfavorable

Source: Data from ref. *7*.
[a] Prototype 11q23 translocation in precursor B ALL; other translocations might involve the MLL gene.
[b] Not uniformly unfavorable with all therapeutic regimens.
[c] Detected with molecular studies.

Other abnormalities, including deletions of 6q, deletions of 9p, deletions of 12p, hyperdiploidy with less than 51 chromosomes, near-triploidy, and near-tetraploidy, are associated with an intermediate prognosis.

Clonal karyotypic evolution has been reported to occur in almost 50% of ALL cases. The most frequent secondary chromosomal changes are an extra copy of chromosome 8, an extra copy of chromosome 21, loss of chromosome 7, and gains of chromosome X and 4. The most common structural rearrangements are deletions of 22q, i(7q), duplications of 1q, and deletions of chromosome 19 (see **Table 6**).

Precursor T-Lymphoblastic Leukemia/Lymphoblastic Lymphoma

Precursor T-lymphoblastic leukemia (T-ALL)/lymphoblastic lymphoma (T-LBL) are neoplasms of lymphoblasts committed to a T-cell lineage. They are typically composed of small to medium-sized blasts with scant cytoplasm, moderately condensed to dispersed chromatin, and inconspicuous nuclei.

In precursor T-lymphoblastic leukemia, the primary site of involvement is bone marrow and blood. Other possible sites of involvement include peripheral lymph nodes, skin, liver, spleen, central nervous system, and gonads. In pediatric cases, these neoplasms are usually treated as high-risk diseases.

With a primary involvement of nodal or extranodal sites, the designation is T-lymphoblastic lymphoma. The malignant process is confined to mass lesions, with minimal or no evidence of blood and bone marrow involvement.

Precursor T-ALL comprises about 12–15% of childhood ALL. It is more common in adults than younger children and more common in males than females. Patients present with high leukocyte counts and often with large mediastinal or other tissue masses. Approximately 50% of T-lymphoblastic lymphomas present with such masses. These often exhibit rapid growth with pleural effusions.

T-ALL is typically associated with a high white cell count, greater age at presentation, poor prognosis, and has clinical, biochemical, immunologic, and chromosomal features that are distinct from those of B-lineage ALL.

Bone marrow morphology of lymphoblasts in T-ALL and T-LBL is similar to precursor B-lymphoblasts. In a smear, the cells are of medium size with high nuclear cytoplasmic ratio, but there might be a considerable size range from small lymphoblasts with very condensed nuclear chromatin and no evident nuclei, to larger blasts with finely dispersed chromatin and relatively prominent nuclei.

By immunophenotype analysis, lymphoblasts in T-ALL/LBL are TdD+ and have variably expressed CD1a, CD2, CD3, CD4, CD5, CD7, and CD8. Of these, CD7 and cytoplasmic CD3 are most often positive and only CD3 is considered lineage specific *(104)*.

Cytogenetic abnormalities in T-ALL and T-LBL are observed less frequently than in B-ALL. In contrast to B-ALL, for the T-ALL/LBL, such changes are not useful at this point for risk assessment or prognosis. In about one-third of T-ALL/LBL, chromosomal translocations involve the T-cell receptor α-locus at 14q11.2 [often via inv(14)(q11.2q32); see **Fig. 2bbb**], the β-locus at 7q35, and the γ-locus at 7p14–15, with a variety of partner genes *(113)*. These include the transcription factor *MYC* at 8q24.1 (see **Fig. 1p**), *TAL1* at 1p32, *RBTN1* at 11p15, *RBTN2* at 11pl3, *HOX11* at 10q24, and the cytoplasmic tyrosine kinase *LCK* at 1p34.3–35. In most cases, these translocations lead to the dysregulation of transcription of the partner genes by juxtaposition with a regulatory region of one of T-cell receptor loci. In about 25% of cases of T-cell ALL, the *TAL1* locus is dysregulated by microscopic deletions in its 5' regulatory region rather than by translocation. Loss of heterozygosity of the tumor suppressor gene *CDKN2A* (an inhibitor of cyclin-dependent kinase 4 [*CDK4*]) at 9p21 occurs more frequently than visible deletions; only about 30% of these cases are cytogenetically abnormal.

Prior to the advent of current therapeutic protocols, the prognosis of childhood T-ALL/LBL was unfavorable, but with current treatments regimes, survival is compatible to B-ALL. A recent oncology group study described the 5-year event-free survivals by karyotype group: 51% of those with an abnormal karyotype versus 62% of those with a normal karyotype (4% statistical error). These data should be viewed as promising, but require confirmation from another series before the associations are considered definitive *(105,106)*.

MATURE B-CELL NEOPLASMS

Mature B-cell neoplasms comprise about 90% of lymphoid neoplasms. The two most common types are large-cell lymphoma and follicular lymphoma, which comprise about 50% of all non-Hodgkin's lymphomas. They represent approximately 4% of new cancers each year around the world *(107,108)*.

Mature B-cell neoplasms resemble normal stages of B-cell differentiation and typically have distinctive morphology and immunophenotypes that allows them to be readily classified according to their cells of origin. The major known risk factor for mature B-cell neoplasia appears to be an abnormality of the immune system, either an immunodeficiency or autoimmune disease. Infectious agents have also been shown to contribute in the development of several types of mature B-cell lymphomas *(109)*.

In the WHO classification, the mature B-cell lymphomas are listed according their major clinical presentations. These are predominately disseminated leukemic types, primary extranodal lymphomas, and predominately nodal lymphomas, which might involve extranodal sites as well.

Several mature B-cell neoplasms have characteristic genetic abnormalities that are important in determining their biologic features and are very useful in differential diagnoses. These aberrations include t(11;14)(q13;q32) in mantle cell lymphoma (see **Fig. 1v**), t(14;18)(q32;q21) in follicular lymphoma (see **Fig. 1y**), t(8;14)(q24;q32) in Burkitt lymphoma (see **Fig. 1q**), and t(11;18)(q21;q21) in MALT lymphoma. The first three translocations place cell locus oncogenes under the control of the immunoglobulin heavy-chain gene on the long arm of chromosome 14, resulting in constitutive activation of the oncogenes, whereas the (11;18) translocation results in a chimeric fusion protein involving *BIRC3* (*API2*) on chromosome 11 and *MALT1* on chromosome 18. In follicular lymphoma and MALT lymphoma, these translocations result in overexpression of an apoptosis inhibitor gene (*BCL2* or *API2*, respectively), whereas in Burkitt lymphoma and mantle cell lymphoma, the translocations results in overexpression of genes associated with proliferation (*CCND*1, also called *BCL1*, and *MYC*, respectively).

In general, these neoplasms are extremely heterogeneous, and knowledge of the correct diagnosis is essential to predict the outcome and direct therapy. More precise subclassifications of these neoplasms have led to more innovative therapies, including localized radiation therapy for eradication of MALT lymphoma and humanized anti-CD20 as an adjunct to therapy for all types of CD20-positive B-cell lymphomas.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma is a neoplasm of monomorphic small round B-lymphocytes in the peripheral blood, bone marrow, and lymph nodes. These cells are mixed with prolymphocytes and paraimmunoblasts (pseudofollicles), and usually express CD5 and CD23. The term "small cell lymphoma" (SCL) is consistent with CLL that is restricted to cases where the tissue morphology and immunophenotype are that of CLL, but that are nonleukemic and present in nodal and extranodal sites.

There are many names for this group of neoplasms, including well-differentiated lymphocytic diffuse leukemia, CLL, immunocytoma, lymphoplasmacytoid type, small lymphocyte B-CLL, small lymphocytic leukemia, consistent with CLL, and B-cell chronic lymphocytic leukemia.

Chronic lymphocytic leukemia comprises about 90% of chronic lymphoid leukemias in the United States and Europe. According to a recent study, it constitutes about 6.7% of non-Hodgkin's lymphoma *(107)*. The majority of patients are greater than 50 years old and the median age is 65, with male-to-female ratio of 2 : 1.

Patients with CLL are usually asymptomatic at presentation; however, some show fatigue, autoimmune hemolytic anemia, infections, splenomegaly, hepatomegaly, lymphadenopathy, or extranodal infiltrates *(110,111)*. They might show involvement of bone marrow and peripheral blood at the time of diagnosis, with a total lymphocyte count in excess of 10^{10} cells/L. Lymph nodes, liver, and spleen are typically infiltrated with leukemic cells. Lymph node morphology shows nodal enlargement and invasion of the architecture with a pseudofollicular pattern of regularly distributed pale areas containing larger cells in a dark background of small cells *(112,113)*. Mitotic activity is typically very low. Pseudofollicles, also known as proliferation centers or growth centers, with a continuum of small, medium, and large cells, are present. Bone marrow morphology shows small lymphocytes with clumped chromatin, and scant, clear to lightly basophilic cytoplasm and with a regular outline are seen. Smudge or basket cells are typically seen in blood smears.

In many cases of CLL, Ig heavy- and light-chains genes are rearranged. There is recent evidence that there are two distinct types of CLL, defined by somatic mutational analysis of immunoglobulin genes. Forty to fifty percent of patients show no somatic mutation of the variable region gene, consistent with naïve B-cells, whereas 50–60% have somatic mutations consistent with a derivation from both germinal center and B-lineage cells *(114)*.

About 80% of cases exhibit abnormalities when examined by cytogenetics and FISH analysis *(115)*. Trisomy 12 is present in about 20% of patients, and deletion of chromosome 13q14 is seen in up to 50% of cases *(115,116)*. Cases with trisomy 12 predominately have nonmutated immunoglobulin variable-region genes, whereas those with 13q14 abnormalities more often have mutations in this region.

Deletions of 11q22–23 are found in about 20% of cases, and somatic mutations have also been found in the homologous allele in this group of cases *(117)*. Deletions of 6q21 or 17p13 (the *P53* locus) are seen in 5% and 10% of cases, respectively *(118)*. t(11;14) and other *BCL1* gene rearrangements have been reported, but most of these cases might be examples of leukemic mantle cell lymphoma *(115)*.

Cytogenetic analysis of bone marrow from CLL patients has always been a difficult task. Many laboratories use B-cell mitogens, which results in proliferation of B-cells. Trisomy 12 has been reported at much higher rates in such cases, as compared to cases without B-cell stimulation. Whether this represents true in vivo status or is a tissue culture artifact is not yet known.

Recent advances in molecular cytogenetics (FISH) have changed the cytogenetic look of CLL (see Chapter 17, **Fig. 11**). Deletions of 13q, involving the *RB1* gene, have been found in about 50% of cases studied by FISH. Interestingly, these deletions are sometimes also present in a homozygous state, in which both copies of chromosome 13 are deleted (see **Fig. 2w,x**).

Deletion and mutation of *P53* have been associated with the resistance to treatment and represent an independent marker for poor survival. Deletions of 11q have been associated with extensive nodal

involvement, rapid disease progression, and short survival time *(118)*. In one study, deletion of chromosome bands 11q22–23 was seen by cytogenetics in 6% of cases, but when the same cases were studied with FISH utilizing specific probes, the incidence of 11q deletions was found to be about 20% and was the second most frequent aberration following deletion of 13q14. Interestingly, 13q and 11q deletions are more frequent than the highly recognized trisomy 12.

A study by the first International World Congress on Cytogenetics of CLL (IWCCLL) showed a correlation between karyotypic finding and overall survival in 391 patients with B-CLL. Patients whose leukemic cells had a normal karyotype had a better survival (median 15 years) than those whose cells had clonal cytogenetic aberrations (median: 7.7 years). In addition, patients with complex chromosomal abnormalities had a worse outcome than those with single aberrations *(119)*.

Chronic lymphocytic leukemia represents a good example of how the use of molecular cytogenetic and molecular genetic techniques have led to the identification of two new independent prognostic markers, deletion of the *TP53* tumor suppressor gene, and deletion of genomic region 11q22.3 to q23.1. This should be evaluated further prospectively in large clinical trials.

The clinical course in CLL is indolent, but the disease is not usually considered to be curable with available therapy. The overall 5-year actual survival of CLL patients in recent studies was 51%, with a failure-free survival (no markers of relapse) rate of 25% *(107)*. Trisomy 12 correlates with atypical morphology and an aggressive clinical course *(115,120)*, whereas abnormalities of chromosome 13q14 are associated with long-term survival and good prognosis. Patients whose tumors have mutations in Ig gene variable regions have a better prognosis than those with germline VH region mutations. In addition, patients with tumor cells that express CD38 appear to have a worse prognosis than those that do not express it *(114,121)*. Cases with 13q22–23 deletions have extensive lymphadenopathy and poor survival. Cases with *TP53* abnormalities have also been associated with a poor prognosis.

Transformation of CLL to high-grade lymphoma occurs in approximately 3.5% of cases. These are usually diffuse large B-cell lymphomas (see below).

Splenic Marginal Zone Lymphoma

Splenic marginal zone lymphoma (SMZL) is a B-cell neoplasm comprised of small lymphocytes that surround and replace the splenic white bulk germinal centers. The lymphoma cells can be found in peripheral blood as villous lymphocytes. This is a rare disorder, representing less than 1% of lymphoid neoplasms, but it might account for most cases of otherwise unclassified chronic lymphoid leukemia that are CD5 negative. The tumor mainly involves the spleen and the splenic hyler lymph nodes, bone marrow, and, often, peripheral blood. The usual presentation is splenomegaly, occasionally accompanied by autoimmune thrombocytopenia or anemia, and variable presence of peripheral blood villous lymphocytes *(122,123)*.

Tumor cells have surface IgM and IgD and are CD20 positive, CD79a positive, CD5 negative, CD10 negative, CD23 negative, CD43 negative, and CD103 negative *(124,125)*. The absence of CD5 and CD43 is useful in excluding CLL and mantle cell lymphoma, the absence of CD103 excludes hairy cell leukemia, and the absence of CD10 excludes follicular lymphoma. Immunoglobulin heavy and light genes are rearranged in SMZL, and most cases have somatic mutations. In addition, intraclonal variations have been detected, suggesting ongoing mutation *(126)*.

Cytogenetically, allelic loss of chromosome 7q21-q32 has been described in up to 40% of small marginal zone lymphomas *(127)*. Dysregulation of the *CDK6* gene located at 7q21, resulting from translocations involving this chromosomal region, has been reported in several cases of splenic lymphoma with villous lymphocytes (SLVL) *(128)*. Trisomy 3 and t(11;18), common in extranodal marginal zone lymphoma, are not uncommon in SMZL, where trisomy 3 has been described in 17% of cases *(129)*. *BCL2* gene rearrangements, notably t(14;18)(q32;q21), have not been described in this category.

The clinical course of SMZL is indolent, even with bone marrow involvement *(130)*. Response to chemotherapy of the type that is typically effective in other chronic lymphoid leukemias is often

poor, but patients typically have hematologic response to splenectomy, with long-term survival. As in other indolent B-cell lymphomas, transformation to large B-cell lymphoma (see below) might occur.

Plasma Cell Neoplasms

These amino-secretory disorders result from the expansion of a single clone of immunoglobulin secreting, terminally differentiated, end-stage B-cells. The distinction of an M (monoclonal) component in the serum and urine by protein electrophoresis has led to various designations for these disorders, including monoclonal gammopathies, dysproteinemias, and paraproteinemias.

Plasma Cell Myeloma

Plasma cell myeloma (multiple myeloma) is a bone-marrow-based multifocal plasma cell neoplasm characterized by a serum monoclonal protein, skeletal destruction with osteolytic lesions, bone pain, hypocalcemia, and anemia *(131)*. The disease spans the spectrum from localized to indolent to aggressive disseminated forms, with plasma cell infiltration of various organs. In the United States, plasma cell myeloma is the most common lymphoid malignancy in African-Americans and the second most common in Caucasians, and it represents 15% of all hematologic disorders.

A constellation of etiologic, clinical, laboratory, and pathological findings are combined to provide diagnostic criteria for plasma cell myeloma. Three clinical variants are described: nonsecretory myeloma, indolent myeloma, and plasma cell leukemia.

Molecular studies of immunoglobulin genes commonly reveal clonal rearrangements. In addition, an immunoglobulin gene deletion is sometimes found in patients with light-chain-only disease or dense proteinuria *(132)*.

Cytogenetic analysis in plasma cell myeloma is always difficult because of a low proliferation fraction in most cases *(133,134)*. Recent studies using cytokine-stimulated bone marrow cultures and *in situ* hybridization have increased the proportion of informative cases. Structural and numerical chromosomal abnormalities are described in 20–60% of newly diagnosed patients, with a mean of 30–40%, and in 60–70% of patients with progressive disease, indicating an ascending scale of chromosomal aberrations in pathogenesis *(134–136)*. Complex karyotypes with multichromosomal gains and losses are the most frequent changes, but translocations, deletions, and mutations are all reported. Gains of chromosomes 3, 5, 7, 9, 11, 15, and 19 and losses of chromosomes X, 8, 13, and 14 are most common. Common structural abnormalities involve chromosomes 1, 11, and 14, with translocations involving rearrangement of the *BCL1* locus on 11q the most common *(134–137)*. This translocation juxtaposes the cyclin D1 gene (*CCND1*, *BCL1*, *PRAD1*) with an Ig γ switch region, resulting in overexpression of cyclin D1 *(138,139)*. Altered expression of the *PAX5* gene on chromosome 9 is thought to result in a loss of CD19, heralding the transition from normal CD19-positive plasma cells to CD19-negative myeloma cells *(140)*. Deletions of 17p13, reported in about 25% of cases, are associated with allelic loss of *TP53* and might predict a poor outcome. Deletion of the long arm of chromosome 7 has been related to alteration of the multidrug-resistance gene, confirming an increased clinical drug-resistance phenotype *(141)*.

Plasma cell myeloma is usually incurable. Median survival is 3 years, with 10% survival at 10 years *(132,142)*.

Extranodal Marginal Zone B-Cell Lymphoma of Mucosa-Associated Lymphoid Tissue

Mucosa-associated lymphoid tissue (MALT) lymphoma is an extranodal lymphoma comprised morphologically of heterogeneous small B-cells, including marginal zone cells, cells resembling monocytoid cells with small lymphocytes and scattered immunoblasts, and centroblastlike cells. Plasma cell differentiation is also present in a proportion of cells. Other synonyms for this disorder are well-differentiated lymphocytic lymphoma, plasmacytoid lymphocytic lymphoma, poorly differentiated lymphocytic lymphoma, immunocytoma, lymphocytic, plasmacytic–lymphocytic, small

cleaved cell lymphoma, small lymphocytic lymphoplasmacytoid lymphoma, and diffuse small-cleaved cell lymphoma.

Approximately 7–8% of all B-cell lymphomas and up to 50% of primary gastric lymphomas are of the MALT type *(143,144)*. This is an adult lymphoma with the gastrointestinal tract as the most common site. About 85% occur in the stomach. Patients with Sjögren syndrome and Hashimoto's thyroiditis are at increased risk of developing MALT lymphoma *(145,146)*. Bone marrow involvement is seen in about 20% of patients, but the frequency seems to vary among sites, being lower for gastric cases and higher for primary ocular or pulmonary cases. Hussell and colleagues have shown that continued proliferation of gastric MALT lymphoma cells from patients infected with *Helicobacter pylori* depends on the presence of T-cells specifically activated by *H. pylori* antigens *(147)*. The importance of this has been clearly demonstrated by the induction of remission in mild gastric lymphoma with antibiotic therapy to eradicate *H. pylori*.

Morphological lymph node examination demonstrates that lymphoma cells infiltrate around reactive B-cell follicles, which they ultimately overrun *(148,149)*. The differential diagnosis includes reactive processes and other small cell lymphomas.

Immunoglobulin genes are rearranged and show somatic mutation of the variable regions *(150)*. In borderline cases, demonstration of B-cell clonality via molecular analysis might be necessary to establish or exclude MALT lymphoma.

Cytogenetically, trisomy 3 is seen in about 60% of cases and t(11;18)(q21;q21) is seen in 25–50% of cases (*151–153)*. As described earlier, this translocation results in fusion of the apoptosis-inhibitor gene *API2* to a novel gene (*MALT1*) at 18q21 *(154)*. Importantly t(11;18) is not found in large B-cell gastric lymphoma, one of the differential diagnoses.

Most of these lymphomas are indolent and respond well to the radiation therapy. *H. pylori*-associated lymphomas are treated with antibiotics. Cases with t(11;18) seem to be resistant to anti-*H. pylori* treatment.

Follicular Lymphoma

Follicular lymphoma (FL) is a neoplasm of follicular center B-cells (centrocytes/cleaved follicle center cells [FCC] and centroblasts/noncleaved FCC) that has at least a partially follicular pattern. Other synonyms of FL are nodular poorly differentiated lymphocytic lymphoma, mixed lymphocytic hystiocytic lymphoma, hystiocytic or undifferentiated lymphoma by the Rappaport classification; centroblastic/centrocytic follicular lymphoma, follicular and diffuse lymphoma, centroblastic lymphoma, follicular lymphoma (by the Kyle classification), small cleaved large cell, small noncleaved, or large noncleaved follicular center cell lymphoma (by the Lukes–Collins classification), follicular small cleaved, mixed large, or small noncleaved cell lymphoma (by the Working Formulation), and follicular center lymphoma (by the REAL [Revised European American Classification of Lymphoid Neoplasms] classification).

Follicular lymphoma comprises about 35% of adult non-Hodgkin's lymphoma in the United States, and 22% worldwide *(107)*. It affects predominately adults, with a median age of 59 years and a male-to-female ratio of 1 : 1.7. Follicular lymphoma rarely occurs in individuals under the age 20. FL predominately involves lymph nodes, but could also involve the spleen, bone marrow, and peripheral blood. Most patients have widespread disease at diagnosis, including involvement of peripheral and central lymph nodes and the spleen. The bone marrow is involved in 40% of cases. Lymph node morphology is predominately associated with follicular patterns. Neoplastic follicles are closely packed, often poorly defined, and are surrounded by small lymphocytes that might resemble the mantle of a normal follicle.

Immunohistochemical stains for the bcl-2 protein is useful in distinguishing neoplastic follicles from reactive ones (neoplastic follicles are bcl-2 positive), but are not useful in distinguishing reactive follicles from other types of low-grade B-cell lymphoma. For example, cutaneous follicular lymphoma is frequently bcl-2 negative.

Immunoglobulin heavy chains and light chains are rearranged in follicular lymphoma. Variable region genes show extensive somatic mutations, with intraclonal heterogeneity consistent with a derivation from follicular center cells *(155,156)*.

Almost all cases of follicular lymphoma have cytogenetic anomalies *(157)*. The most common is t(14;18)(q32;q21) (see **Fig. 1y**), in which the *BCL2* gene is juxtaposed with the Ig heavy-chain locus and is seen in about 70–95% of cases *(158,159)*. This translocation is not associated with either a better or worse prognosis. Also, a rarely seen translocation [t(2;18)(p12;q21)] fuses *BCL2* with the Ig κ light-chain gene on chromosome 2. Other cytogenetic abnormalities include gain of chromosome 7, gain of chromosome 18, and involvement of chromosomes 3q27-q28, 6q23-q36, and 17p. In addition to the *BCL2* gene rearrangement, *BCL6* mutations are also seen. Deletions and other alteration of chromosome 9p involving the *CDKN2B* and *CDKN2A* gene loci have been reported in cases of follicular lymphoma that transforms to diffuse large B-cell lymphoma (DLBCL or B-cell DLCL; see below).

Mantle Cell Lymphoma

This is a neoplasm of B-cell origin; cell morphology is composed of monomorphic small- to medium-sized lymphoid cells with asymmetrical nuclei. These cells morphologically closely resemble centrocytes/cleaved follicular center cells (FCCs; see the subsection Follicular Lymphoma) but frequently have slightly less irregular nuclear contours *(160,161)*. Mantle cell lymphoma (MCL) comprises approximately 3–10% of non-Hodgkin's lymphoma *(107)*. It occurs in middle-aged to older individuals, with a median age of about 60. Lymph nodes are the most commonly involved site. Spleen and bone marrow, with or without blood involvement, are other frequent sites of disease presentation *(162,163)*.

Under the microscope, MCL demonstrates architectural obliteration via monomorphic lymphoid proliferation, with a vaguely nodular, diffuse growth pattern *(164,165)*. A true follicular node pattern is very rarely present. The majority of cases have immunoglobulin heavy-chain and light-chain gene rearrangements, but variable-region genes are not mutated in most cases. This is consistent with a pregerminal center B-cell origin, but a small portion of cases also shows somatic mutations suggestive of postfollicular phenotype *(166–168)*.

Conventional cytogenetic analysis demonstrates a translocation between the immunoglobulin heavy-chain and cyclin D1 (*CCND1*, *BCL1*, *PRAD1*) genes, t(11;14)(q13;q32) (see **Fig. 1v**) in 70–75% of cases *(169–171)*. However, almost all cases demonstrate this gene rearrangement using FISH probes specific to these regions *(172,173)*. In addition, almost all cases show overexpression of cyclin D1 mRNA by Northern blot techniques *(174)*. A minority of cases, especially those of blastoid and other more aggressive types, have additional mutations, deletions, or other abnormalities in genes for negative cell-cycle-regulating proteins such as *TP53*, *P16* (*CDKN2A*), and *P18* (*CDKN2C*) *(175,176)*. *BCL2* and *MYC* rearrangements are usually absent. Many cases have point mutation and/or deletion of the *ATM* (ataxia telangiectasia-mutated) gene at 11q22.3-q23.1 *(164)*. A recent study using oligonucleotide microarray analysis reported 12 of 28 MCL patients (43%) with such mutations *(177)*.

In addition to t(11;14), there are other relatively frequent cytogenetic abnormalities seen in MCL, some of which are also common in CLL. These include 13q14 deletions, total or partial trisomy 12, and 17p deletions, among others. The pleomorphic blastoid variant of MCL has a high incidence of tetraploidy.

Mantle cell lymphoma has a median survival of 3–5 years, but the vast majority of patients cannot be cured. Adverse prognostic indicators include trisomy 12, unbalanced cytogenetic abnormalities, and complex karyotype. *TP53* mutation/overexpression and a variety of other clinical parameters are also poor prognostic indicators. The presence of a karyotype with t(11;14) as the sole anomaly predicts an intermediate clinical outcome, whereas cases with normal karyotypes are associated with a better prognosis *(178)*.

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is characterized by diffuse proliferation of large neoplastic B-lymphoid cells with large nuclei, sized equal to or exceeding normal macrophage nuclei, or more than twice the size of a normal lymphocyte. The cytological features differ among different variants described with this disorder.

Diffuse large B-cell lymphoma constitutes about 30–40% of adult non-Hodgkin's lymphoma in developed countries. In developing countries, it constitutes an even higher proportion of lymphoma cases. The median age is the seventh decade, but the range is broad and these tumors can even be seen in children. The typical presentation is a rapidly enlarging, soft, symptomatic mass or single nodule at an external site *(107,108)*. The etiology of DLBCL remains unknown at present.

Morphological exam shows large B-cell lymphoma typically replacing normal architectural underlying lymph node or extranodal tissue in a diffuse pattern. So far, immunophenotypic and phenotypic parameters have not helped to delineate distinctive morphologic subtypes, with rare exceptions. The morphological variants of this disorder are centroblastic, immunoblastic, T-cell/histiocytic rich, and anaplastic.

Immunoglobulin heavy- and light-chain genes are rearranged in most cases, in addition to somatic mutations, especially in the variable regions. A translocation involving *BCL2*, the hallmark of follicular lymphoma, occurs in 20–30% of cases of DLBCL *(179,180)*.

Up to 30% of cases demonstrate recurrent abnormalities of the chromosome 3q27 region, involving the candidate proto-oncogene *BCL6 (181)*. The t(8;14) that results in deregulation of *MYC* occurs in DLBCL as well as in Burkitt lymphoma (see next subsection), and it differentiates DLBCL from other phenotypically similar lymphomas. Many cases with an aggressive disease process show complex, nonspecific cytogenetic abnormalities. The most frequent chromosomal aberrations in these cases are losses of material on chromosome 6q and gains of parts of various chromosomes *(182)*.

Diffuse large B-cell lymphoma is an aggressive disease, but usually responds well to multiagent chemotherapy. A high proliferative rate has been associated with a worse survival rate, and *TP53* overexpression in the majority of malignant cells is another adverse prognostic indicator *(183)*.

BCL2 expression has been associated with disease-free survival, and in some case studies, a translocation involving *BCL6* has been reported to be associated with a better overall prognosis *(184)*.

Burkitt Lymphoma

Burkitt lymphoma (BL) is a particularly aggressive lymphoma, often presenting at external sites or even as an acute leukemia, composed of pleomorphic medium-sized B-cells with basophilic cytoplasm and plentiful mitotic figures *(185–187)*. Burkitt lymphoma is relatively common in children and accounts for approximately 30–50% of all childhood lymphomas. Other names for Burkitt lymphoma in past literature are undifferentiated lymphoma, Burkitt-type lymphoma, small noncleaved follicular center cell lymphoma, small noncleaved cell lymphoma, and Burkitt lymphoma with intracytoplasmic immunoglobulin.

Burkitt lymphoma cells show clonal rearrangements of immunoglobulin heavy-chain and light-chain genes; translocation involving *MYC* is a constant genetic feature and is now considered as a definitive diagnostic criterion *(187,188)*. Somatic mutations of the Ig genes are also found, consistent with a germinal center stage of differentiation *(189,190)*. All cases have a translocation of the *MYC* oncogene at 8q24.1 to either the immunoglobulin heavy-chain region (*IGH*) on 14q32 [t(8;14)(q24.1;q32); see **Fig. 1q**], the Ig κ light-chain region (*IGK*) on 2p12 [t(2;8)(p12;q24.1); see **Fig. 1e**], or the Ig light-chain region (*IGL*) on 22q11.2 [t(8;22)(q24.1;q11.2); see **Fig. 1mm**]. As a result of these translocations, *MYC* is constitutively expressed because of the influence of the Ig gene promoters.

The deregulation of *MYC* plays a decisive role in lymphoma genesis by driving cells through the cell cycle *(191,192)*. *MYC* also activates target genes specifically involved in apoptosis. Mutations in *MYC* could further enhance its tumorigenicity *(193)*.

Other genetic lesions in BL include inactivation of *TP53* secondary to mutation in up to 30% of sporadic and endemic cases. It should be noted that *MYC* translocations are not entirely specific for BL. For example, a *MYC* translocation has been reported in secondary precursor B-lymphoblastic leukemia/lymphoma following follicular lymphoma.

Three clinical variants of BL are recognized, each manifesting its differences in clinical presentation, morphology, and biology. The clinical presentation varies according to the epidemiology, subtype, and site of involvement:

- *Endemic Burkitt lymphoma.* This lymphoma occurs in equatorial Africa, representing the most common malignancy of childhood in this area, with an incidence peak at 4–7 years and a male to female ratio of 2 : 1. Epstein-Barr virus (EBV) plays an important role in endemic BL.
- *Sporadic Burkitt lymphoma.* This variant is seen throughout the world, mainly in children and young adults. The incidence is low, representing 1–2% of all lymphoma in western Europe and the United States.
- *Immunodeficiency-associated Burkitt lymphoma.* This variant is seen primarily in association with human immunodeficiency virus (HIV) infection, occurring often as the initial manifestation of acquired immunodeficiency syndrome. Most patients present with advanced disease, often with a high tumor burden because of the short doubling time of the tumor.

In endemic cases, the breakpoints on chromosome 14 involve the heavy-chain gene adjoining region, whereas in sporadic cases, the translocation involves the Ig switch region.

The classical morphology of BL is observed in the endemic type and seen in a high percentage of sporadic BL cases, particularly in children. The medium-sized cells show a diffuse, monotonous pattern of infiltration.

The endemic and sporadic BLs are highly aggressive, but potentially curable. The treatment should begin as early as possible because of short doubling time of the tumor. The outcome for childhood Burkitt and Burkitt-like lymphomas has recently improved with the use of short and intensive B-cell non-Hodgkin's lymphoma-directed therapy *(194)*.

Variants of BL include Burkitt lymphoma with plasmacytoid differentiation and atypical Burkitt/Burkitt-like lymphoma.

MATURE T-CELL AND NK-CELL NEOPLASMS

Mature T-cell and natural killer (NK) cell neoplasms are relatively uncommon, but show a significant difference in incidence throughout different parts of the world. For example, they are more common in Asians than in other races *(107)*. Association of Epstein–Barr virus with NK-/T-cell lymphomas and NK-cell leukemias is well known. Clinically, these are among the most aggressive of all hematopoietic and lymphoid neoplasms. They usually present at an advanced stage of clinical course and are in general resistant to chemotherapy. The WHO definition of T- and NK-cell neoplasms is based on morphological, immunophenotypic, and genetics features. The WHO histological classification of mature T-cell and NK-cell neoplasms includes the following:

- Leukemic/disseminated type
  - T-Cell prolymphocytic leukemia
  - T-Cell large granular lymphocytic leukemia
  - Aggressive NK-cell leukemia
  - Adult T-cell leukemia lymphoma
- Cutaneous type
  - Blastic N cell lymphoma
  - Mycosis fungoides/Sézary syndrome
  - Primary cutaneous anaplastic lymphoma
  - Lymphoid papulosis

- Other extranodal type
  - Extranodal NK-/T-cell lymphoma nasal type
  - Enteropathy type T-cell lymphoma
  - Hepatosplenic lymphoma
  - Subcutaneous panniculitis-like T-cell lymphoma
- Nodal type
  - Angioimmunoblastic T-cell lymphoma
  - Proliferative T-cell lymphoma, unspecific
  - Anaplastic large cell lymphoma
- Neoplasm of uncertain lineage and stage of differentiation

As a group, these lymphomas are derived from mature or postthymic T-cells. As NK-cells are closely related and share some of the immunophenotype and functional properties of T-cells, these two classes of neoplasms in the new WHO classification are considered together and account for about 12% of all non-Hodgkin's lymphomas.

The most common subtypes of mature T-cell lymphomas are prolymphocytic lymphomas, unspecified and anaplastic large cell lymphomas.

T-cell Prolymphocytic Leukemia

T-Cell prolymphocytic leukemia (T-PLL) is an aggressive T-cell leukemia characterized by the proliferation of small to medium-sized polymorphocytes (polymorphonuclear lymphocytes) with a premature postthymic T-cell phenotype. The disease usually involves blood, bone marrow, lymph nodes, liver, spleen, and skin. This pathology has also been known as the "knobby" type of T-cell leukemia, T-prolymphocytic leukemia, and T-cell chronic lymphocytic leukemia.

At the time of presentation, most patients have hepatosplenomegaly and generalized lymphadenopathy. A skin infiltration is seen in 20% of these patients, with serous effusion in some cases *(195)*. Anemia and thrombocytopenia are common and the lymphocyte count is typically high, usually greater than 10^{11} cells/L. The diagnosis is usually made on blood films, which show a predominance of small to medium-sized lymphoid cells with nongranular basophilic cytoplasm, round to oval, with markedly irregular nuclei and a visible nucleolus *(196)*.

Chromosome abnormalities in T-PLL result in the genes for the α-, β-, γ-, and δ-chains of the T-cell receptor being clonally rearranged. The most frequent involves inversion of chromosome 14, with breakpoints in the long arm at bands q11.2 and q32 [inv(14)(q11.2q32); see **Fig. 2bbb**], seen in about 80% of patients. In 10% of cases, there is a reciprocal translocation involving both chromosomes 14 [t(14;14)(q11.2;q32)] *(197,198)*. These rearrangements juxtapose the *TCR*α/δ locus with oncogenes *TCL1A* and *TCL1B* at 14q32.1, resulting in their activation *(199,200)*.

t(11;14)(p13;q11.2) (see **Fig. 1w**) is the most frequently occurring translocation in childhood T-ALL, juxtaposing *RBTN2* on 11p with *TCR*α/δ. The translocation t(X;14)(q28;q11.2) is less common, but it also involves the *TCR*α/δ locus on chromosome 14q11.2, this time with the *MTCP1* gene, which is homologous to *TCL1A*, at Xq28 *(201)*. Both *TCL1A* and *MTCP1* have oncogenic properties, as both can induce T-cell leukemia. Other T-cell receptor chain gene-related aberrations are t(10;14)(q24;q11.2), involving *HOX11* on 10q and rearrangements involving *TCR*β at 7q35 or *TCR*γ at 7p15 *(106)*.

Recent molecular and FISH studies also demonstrate deletions at 11q23, the locus for the ataxia telangiectasia-mutated (*ATM*) gene, and mutational analysis has shown a clustering of mutation at the *ATM* locus in T-PLL *(202,203)*.

In addition, abnormalities of chromosome 8, including an isodicentric chromosome [idic(8)(p11.2); see Chapter 3], t(8;8)(p11.2;q12), and trisomy 8q are seen in 70–80% of cases *(204)*. Deletions of 12p13 are also a feature of T-PLL, detectable with molecular analysis *(205)*.

Aggressive NK-Cell Leukemia

Aggressive NK-cell leukemia is characterized by a systemic involvement, via proliferation of NK-cells, and a short and aggressive clinical course *(206–208)* and is also known as large granular lymphocyte leukemia, NK-cell type, or aggressive NK-cell leukemia/lymphoma. This is rare form of leukemia/lymphoma, which is more prevalent among Asians than among Caucasians. It is considered to be a disease of middle age, but actually has a bimodal distribution, also affecting teenagers and young adults *(206,207)*. This disease has an aggressive clinical course, resulting in a fatal outcome in 1–2 years. In fact, many patients die within days to weeks of initial presentation *(208)*. This leukemia needs to be differentiated from an indolent NK-cell lymphoproliferative disorder that occurs mostly in adults, in which most patients are asymptomatic, although they might have vasculitis syndrome.

The most common sites of presentation are the peripheral blood, bone marrow, liver, and spleen, but any organ can show involvement. Patients present with few constitutional symptoms and a leukemic blood picture. The Epstein–Barr virus (EBV) has been suggested to play a possible etiologic role, as the great majority of these cases harbor EBV in a clonal episomal form. However, some consider this association to be controversial, as EBV-negative patients have been seen.

Morphologically, leukemic cells are slightly larger than normal with granular cytoplasm and irregular hypochromatic nuclei. The T-cell receptor genes are, in most cases, in germline configuration and this of diagnostic significance. Therefore, clonality has to be established by other methods, such as cytogenetic studies and/or X-chromosome inactivation in female patients.

Many cytogenetic abnormalities have been reported; most common is a deletion of chromosome 6q21-q25 *(209)*. Cytogenetic and molecular analysis has also shown DNA losses at chromosomes 11q, 13q, and 17p to be recurrent aberrations in NK-cell malignancies. Frequent DNA gains are also found involving chromosomes 1p, 6p, 11q, 12q, 17q, 19p, 20q, and Xp *(210)*.

Extranodal NK-/T-Cell Lymphoma, Nasal Type

This is predominately an external lymphoma characterized by a broad morphologic spectrum, also known as angiocentric T-cell lymphoma, malignant midline reticulosis, polymorphic reticulosis, midline granuloma, and/or angiocentric immunoproliferative lesion. The infiltrate is often angiocentric (affecting blood vessels), with prominent necrosis and vascular destruction. It is designated NK-/T-cell rather than NK-cell lymphoma because although most cases appear to be NK-cell neoplasms (EBV-positive and CD56-positive), rare cases show an EBV positive and CD56 negative T-cell phenotype. The nasal cavity is the most common and typical site of involvement, hence the name. However, identical neoplasms could occur in extranodal organs.

Epstein–Barr virus involvement, irrespective of the ethnicity of the patient, suggests a probable pathologic role of the virus *(211–213)*. Interestingly, T-cell receptor and immunoglobulin genes are in germline configuration in the majority of the cases.

A variety of cytogenetic aberrations have been reported, but, so far, no specific translocations have been identified. Aberrations involving chromosome 6q are frequent, with deletions of the chromosome at the q21-q25 region or isochromosome 6 [i(6)(p10); see Chapter 3] being the most common recurrent chromosomal abnormalities, but it is currently unclear whether they represents primary aberrations or progression of an associated event *(209,214)*. Other nonrandom abnormalities include +X, i(1q), i(7q), +8, del(13q), del(17p), i(17q), and 11q23 rearrangements.

Chromosomal analysis via comparative genomic hybridization (CGH) (see Chapter 17) has revealed frequent DNA losses at 1p, 17p, and 12q and gains at 2q, 13q, and 10q, suggesting that these regions could be targets for further molecular genetic analysis to investigate tumor suppressor genes or oncogenes associated with tumorigenesis of NK-/T-cell lymphoma *(215)*. Notably, this study contradicts the finding of frequent 6q deletion described in earlier reports.

Prognosis is usually poor in extranodal NK-/T-cell nasal lymphoma. Lymphoma that has spread outside the nasal cavity is highly aggressive, with short survival time and poor response to therapy *(207,208)*.

Mycosis Fungoides and Sézary Syndrome

Mycosis fungoides (MF) is an indolent T-cell lymphoma that is distinguished from other lymphomas by its initial appearance on the skin. The histological diagnosis of MF might be difficult because there is significant overlap with features of neoplastic T-cell infiltrates and inflammatory dermatoses. MF generally occurs in a mixed, reactive background and can show only a slight degree of cytologic atypia, also rendering histological diagnosis difficult.

Mycosis fungoides is a mature T-cell lymphoma presenting in the skin with patches and flakes characterized by epidermal and dermal infiltration of small to medium-sized T-cells with cerebriform nuclei. It is also known as cerebriform T-cell lymphoma and small cell cerebriform lymphoma. The disease, as a rule, is limited to skin for a prolonged number of years *(216)*. Extracutaneous spreading can occur in the advanced stage, mainly to lymph node, liver, spleen, lungs, and blood. When large numbers of tumor cells are found in the blood, the condition is called Sézary syndrome (SS).

The complete pathogenesis of this disease process is unknown at present. HTLV or a related virus has been implicated in some studies, which have shown that truncated provirus sequences, similar to tax and/or pol, could be detected by PCR in 30–90% of patients *(217,218)*. With immunophenotypic studies, a lack of CD7 expression is frequent in all stages of the disease. However, this feature is of limited value from a diagnostic point of view, because the lack of CD7 could also be seen in a benign, cutaneous lymphoid lesion *(219)*.

T-Cell receptor genes are clonally rearranged in most cases of MF *(220)*. Inactivation of *CDKN2A/P16* and *PTEN* have been reported *(221)* and could be associated with disease progression.

Limited cytogenetic studies have shown complex nonspecific karyotypes in many SS patients, particularly in advanced stages. Recently, FISH analysis showed chromosome 1p and 17q rearrangements in 5 of 15 SS cases and chromosome 10 abnormalities in 4 SS cases, consistent with both the G-banded karyotype and the CGH results *(222)*. In addition, allelotyping analysis of 33 MF patients using chromosome 1 markers suggested minimal regions of deletion at D1S228 (1p36), D1S2766 (1p22), and D1S397 (1q25).

In a recent study, Karenko et al. *(223)* studied correlations between cytogenetic abnormalities and disease progress in patients with MM or SS. This small study has revealed that the rate of chromosomal aberrations is associated with the activity of the disease and has a prognostic significance. Aberrations of chromosomes 1, 6, and 11, although increasing with progression of the disorder, seem to be a hallmark of existing disease, detectable even in remission. Aberrations of chromosomes 8 and 17 are associated with active or progressive disease *(223)*. Both SS and late stages of MF showed a similar pattern of chromosomal abnormalities, but no chromosomal changes were found in patients with early-stage MF *(222)*.

Angioimmunoblastic T-Cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AILT) is a peripheral T-cell disorder always associated with systemic disease. Evidence of polymorphic infiltrate involving lymph nodes, with a prominent proliferation of high endothelial venules and follicular dendritic cells, is present.

Angioimmunoblastic T-cell lymphoma is a rare subtype of lymphoma, making up only 1–2% of non-Hodgkin's lymphomas; however, it accounts for a major subset of peripheral T-cell lymphomas. It has clinical and pathologic features that set it apart from other B- and T-cell lymphomas. In past literature, this disease was referred to as immunoblastic lymphadenopathy; AILT was initially felt to be an atypical reactive process, angioimmunoblastic lymphadenopathy, with an increased risk of progression to lymphoma. Currently, it is believed that AILT is a proliferative T-cell lymphoma. However, some argue that atypical or clonal proliferation could precede the development of this lymphoma and believe that angioimmunoblastic lymphadenopathy could be, in some cases, a preneoplastic process. Most patients exhibit immunodeficiency, but the immune abnormalities appear secondary to the neoplastic process rather than preceding it. Cells positive for EBV are found in the majority of cases.

Angioimmunoblastic T-cell lymphoma usually presents with advanced stage disease, including systemic symptoms and polyclonal proliferation *(224)*. With morphological examination, the lymph node architecture is regressed and follicles are often evident. Lymphocytes show minimal cytologic atypia, and this form of lymphoma might be difficult to distinguish from atypical T zone (interfollicular area) hyperplasia.

T-cell receptor genes are rearranged in about 75% of cases *(225–227)*. Immunoglobulin gene rearrangement might be found in only about 10% of cases, and most correlate with expanded EBV-positive B-cell clones *(228)*. The most frequent cytogenetic abnormalities are trisomies of chromosome X, 3, and 5 *(229,230)*.

The clinical course is medium to aggressive in most cases, with an average survival rate of less than 3 years. Patients often succumb to infectious complications, which make the delivery of aggressive chemotherapy difficult *(224,231)*.

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is a T-cell disorder consisting of lymphoid cells that are usually large and pleomorphic with plentiful cytoplasm, often with horseshoe- or kidney-shaped nuclei. Multiple nuclei can occur in a wreath formation and can give rise to cells resembling Reed–Sternberg cells (see the section Hodgkin's lymphoma). ALCL cells are CD30 (Ki-1) positive, and in most cases express cytotoxic granule-associated proteins *(232)*. The majority of cases, they stain positive for the protein anaplastic lymphoma kinase (ALK). ALK-positive cases normally involve lymph nodes as well as some extranodal sites, frequently skin, bone, soft tissue, lung, and liver. At the time of presentation, the majority of patients are already at the advanced stage of the disease *(233,234)*, which includes para- or intra-abdominal lymphadenopathy, often associated with extranodal infiltrates, with involvement of the bone marrow in the majority of cases.

Other terms used in literature include T-cell immunoblastic sarcoma, large cell anaplastic lymphoma, diffuse large cell immunoblastic lymphoma, anaplastic large cell lymphoma T-/null-cell type, malignant histiocytosis, sinusoidal large cell lymphoma, and Ki-1 lymphoma representing atypical histiocytosis

Anaplastic large cell lymphoma accounts for about 3% of adult non-Hodgkin's lymphoma and is more common in the pediatric population, where it represents about 10–30% of lymphoma cases *(233)*.

Primary systemic anaplastic large cell lymphoma must be distinguished from primary cutaneous anaplastic large cell lymphoma (C-ALCL) and from other subtypes of T- or B-cell lymphoma with anaplastic features and/or CD30 expression.

Differential diagnosis in some cases might be difficult because of morphological similarities between ALCL and Hodgkin lymphoma, as occasional cases of ALCL with partial nodal involvement might have an indistinct nodular pattern and be associated with sclerosis. Many cases of Hodgkin lymphomas, particularly the nodular scleroising subtype (see a later subsection), could contain confluent sheaths of Reed–Sternberg cell variants and resemble ALCL. Also, expression of CD30 is associated with Hodgkin lymphoma. However, because Hodgkin lymphoma in the vast majority of cases is a B-cell neoplasm and ALCL is a T-cell neoplasm, cytogenetic and immunophenotypic characterization can easily distinguish these categories. In the WHO classification, the category of Hodgkin's-like or Hodgkin's-related ALCL no longer exists.

Molecular genetic studies of ALCL have revealed that approximately 90% of patients exhibit clonally rearranged T-cell receptor genes, irrespective of whether they express T-cell antigens *(235)*. EBV sequences are consistently absent, and this feature is helpful in distinguishing ALCL from CD30 positive EBV-expressing B-cell lymphomas that had previously been misinterpreted as ALCL.

Expression of ALK protein in ALCL is the result of the genetic alterations of the *ALK* locus on chromosome 2p25. Several different cytogenetic aberrations have been characterized, but the most frequent one is t(2;5)(p23;q35) between the *ALK* gene on chromosome 2 and the nucleophosmin gene (*NPM1*) on chromosome 5 *(236,237)*.

ALK encodes a tyrosine kinase receptor belonging to the insulin receptor superfamily, which is normally silent in lymphoid cells. With t(2;5), the nucleophosmin housekeeping gene fuses with *ALK* to produce a chimeric protein *(238,239)*, in which the N-terminal portion from *NPM1* is linked to the intracytoplasmic portion of *ALK*. The particular cytoplasmic and nuclear staining seen in these cases can be explained by the formation of dimers between wild-type nucleophosmin and the *NPM1/ALK* fusion protein.

Many variant translocations involving *ALK* and other partner genes on chromosomes X, 1, 2, 3, 17, 19, and 22 have also been reported. Some ALK-positive ALCLs are associated with the presence of t(1;2)(q25;p23). This translocation involves *TPM3* gene on chromosome 1, which encodes a non-muscular tropomyosin α-chain. In cases with t(1;2) that express the *TPM3/ALK* fusion protein, ALK staining is restricted to the cytoplasm of malignant cells and in virtually all cases is strongest near the cell membrane.

Other genes can fuse with *ALK*; examples include two variant rearrangements; t(2;3)(p23;q35) and inv(2)(p23q35). Two different fusion proteins, *TFG/ALK* short and *TFG/ALK* long, are associated with the rare t(2;3)(p23;q35), which involves the *TFG* (tropomyosin receptor kinase-fused) gene on chromosome 3. inv(2)(p23q25) involves the *ATIC* gene (also known as *PURH*) on 2q, which encodes 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase *(240,241)*. This gene plays a key role in *de novo* purine biosynthesis *(242)*.

For prognosis, ALK expression correlates with the expression of other markers, such as epithelial membrane antigen (EMA) and a cytotoxic phenotype and is strongly associated with younger age groups, lower international prognostic index (IPI) risk groups, and a good prognosis. ALK-negative ALCL, however, shows a more heterogeneous immunophenotype and clinical behavior. Genetic studies of ALK-negative cases have not been performed in detail, but might be of future use in determining whether ALK-positive and ALK-negative ALCL are part of the same disease entity.

RT-PCR is one of the methods commonly use for detecting the (2;5) translocation, but cases with variant translocations will not be amplified/identified by standard RT-PCR using primers that are specific for *ALK* and *NPM1*. Therefore, at present, cytogenetic studies should always be part of the workup. Also, the (2;5) translocation leads to positive staining for ALK in both the nucleus and cytoplasm, but with the variant translocations, often only cytoplasmic staining will be observed. Therefore, immunohistochemistry has largely supplemented molecular analysis for the diagnosis of ALCL.

Recent microarray-based CGH analysis (see Chapter 17) of a few cases revealed genomic imbalances (GI) in all cases studied. This includes oncogene copy number gains of *FGFR1* (8p11.1-p11.2) in three cases and *NRAS* (1p13.2), *MYCN* (2p24.1), *RAF1* (3p25), *CTSB* (8p22), *FES* (15q26.1), and *CBFA2* (21q22.3) in two cases. Real-time PCR analysis of nine DNA samples from eight cases with cytogenetic and genomic imbalances detected amplifications of *CTSB* and *RAF1* in seven cases (88%), of *REL* (2p12p-13) and *JUNB* (19p13.2) in six cases (75%), and of *MYCN* and *YES1* (18p11.3) in four cases (50%) *(243)*. Prognostic parameters associated with such changes are still not very well defined and are definitely needed to determine treatment strategies in individual patients *(244)*.

HODGKIN LYMPHOMA

Thomas Hodgkin is widely attributed with the first description of human lymphoma, originally known as Hodgkin's disease. The disease was also referred to as lymphogranulomatosis, but this term is no longer used. This disorder accounts for approximately 30% of all lymphomas.

With minor modifications, the Revised European American Lymphoma (REAL) classification has been adopted by the World Health Organization, resulting in the REAL/WHO classification, now the most widely used system for classification of Hodgkin lymphoma (HL). HL is comprised of two distinct entities, nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) and classical Hodgkin lymphoma. The latter is further divided into four subtypes: lymphocyte rich, nodular sclerosing, mixed cellularity, and lymphocyte depleted

The differences between these two entities involve the clinical features and behavior of the disease and is based largely on the types and proportions of noncancerous cells.

Some of the common findings in both types of HL include the following:

- The disease usually arises in a lymph node, virtually in the cellular-rich region, and involves contiguous rather than disseminated nodes.
- The majority of deaths occur in young adults.
- Neoplastic tissues usually contain a small number of scattered large mononuclear and multinucleated tumor cells known as Hodgkin's Reed–Sternberg (H-RS) cells. HL is a unique form of cancer, as affected nodes generally contain a few cancer (H-RS) cells surrounded by many more noncancerous cells. This ratio might be as high as 1000 to 1.
- T-lymphocytes usually ring the tumor cells in a rosette like manner.

Nodular Lymphocyte Predominant Hodgkin Lymphoma

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) represents approximately 5% of all Hodgkin lymphomas. It is a B-cell neoplasm with monoclonal proliferation and is characterized by a nodular or a nodular and diffuse polymorphous proliferation of scattered, large, neoplastic cells known as popcorn cells or lymphocytic and/or histiocytic (L&H) Reed–Sternberg cell variants. The usual sites of involvement at the time of presentation are cervical, axillary, or inguinal lymph nodes. Patients typically present with a localized proliferative lymphadenopathy.

With morphological exam, lymph node architecture is totally or partially replaced by a nodular or nodular end diffused infiltrate, predominately consisting of small lymphocytes, histiocytes, epithelioid cells, and intermingled L&H cells. Notably, neutrophils and eosinophils are absent in both nodular and diffuse regions. With immunophenotyping, L&H cells are positive for CD20, CD79a, BCL6, and CD45 in nearly all cases *(245–247)*. In most instances, they are also Ig J chain and CD75 positive *(255)*.

Organic cation transported 2 (*OCT2*) is a transcription factor that induces immunoglobulin synthesis by activating the promoter of immunoglobulin genes. Immunolabeling for *OCT2* selectively highlights L&H cells and might become a useful means of identifying them *(248)* and in differenting between NLPHL and classical Hodgkin lymphoma *(249)*.

The L&H cells, in any given case, have identical monoclonally rearranged immunoglobulin genes *(250–252)*. These rearrangements are usually not detectable in whole-tissue DNA, but only in the DNA of an isolated single islet. The variable region of the Ig heavy chain shows a significantly high rate of somatic mutations and also shows evidence of ongoing mutations. Germinal center B-cells might be at the blastic stage and are possible cells of disease origin. The prognosis of patients with early-stage disease is very good, with 10-year survival in more than 80% of cases *(253)*.

Classical Hodgkin Lymphoma

Classical Hodgkin lymphoma (CHL) is defined as a monoclonal B-cell neoplasm. The cell morphology is defined as the presence of mononuclear and multinucleated Hodgkin's Reed–Sternberg cells in an infiltrate containing a variable mixture of non-neoplastic small lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, fibroblasts, and collagen fibers. The immunophenotypic and genetic features of the mononuclear and multinucleated cells are indistinguishable, but the clinical features and association with EBV show differences *(254)*.

Classical Hodgkin lymphoma accounts for the majority (95%) of all HLs and shows a bimodal age group peak at 15 and 35 years of age. Primary involvement in this lymphoma is extranodal. At initial presentation, about half of the patients have stage I or stage II (localized) disease. The introduction of modern radiation and chemotherapy has made CHL curable in the majority of cases.

Approximately 60% of patients, the majority of them with the nodular sclerosing subtype, usually have mediastinal infiltrations. Lymph node architecture is replaced by a variable number of H-RS cells mixed with an inflammatory background. Classical diagnostic Reed–Sternberg cells are large,

have plentiful slightly basophilic cytoplasm, and have at least two nuclear lobes. With immunophenotyping, H-RS cells are positive for CD30 in nearly all cases and for CD15 in the majority of cases *(232,255–257)*, are usually negative for CD45, and are consistently negative for Ig J chain, CD75, and macrophage-specific markers such as the PG-M1 epitope of the CD68 molecule *(258)*. Classical HL is associated with overexpression and an abnormal pattern of cytokines and chemokines and/or receptors in the H-RS cells, which contain monoclonal immunoglobulin gene rearrangements in greater than 98% of cases and monoclonal T-cell receptor gene rearrangements in rare cases *(259–261)*.

Somatic mutations in the variable region of the Ig heavy-chain genes are seen frequently. These findings indicate a derivation of H-RS cells from germinal center B-cells or their progeny. Typically, B-cells that have lost the capacity to express immunoglobulin rapidly undergo apoptosis. However, H-RS cells that are incapable of producing immunoglobulins do not die, as the apoptotic pathway is blocked in these cells.

Classical HL cases with abundant neoplastic cells might bear a resemblance to an anaplastic large cell lymphoma (see above) and, in fact, many cases previously diagnosed as the lymphocyte-depleted subtype have been shown to actually represent this type of non-Hodgkin lymphoma, prompting some to doubt whether lymphocyte depleted HL is a true category at all.

Conventional cytogenetic and FISH studies show ploidy and hyperploidy consistent with multinuclearity of the neoplastic cells. However, these techniques fail to demonstrate recurrent and specific chromosomal changes in classical HL *(262,263)*. Comparative genomic hybridization, however, revealed recurrent gain of chromosomal subregions 2p, 9p, and 12q and distinct high-level amplification on chromosome bands 4p16, 4q23-q24, and 9p23-p24 *(264)*. t(14;18) and t(2;5) are absent from H-RS cells and this can be of diagnostic significance *(265,266)*.

IMMUNODEFICIENCY-ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS

The World Health Organization classifies this group of disorders into four major clinical subtypes:

- Primary immunodefiency syndromes and other primary immune disorders
- Infection with the human immunodefiency virus (HIV)
- Iatrogenic immunosuppression in patients with solid-organ or bone marrow allograft
- Iatrogenic immunosuppression associated with methotrexate treatment

Lymphoproliferative disorders (LPDs) associated with immunodefiency are a heterogenous group and the nature of the immune defect is highly variable. For example, in cases of X-linked LPD, the defect is in the immune surveillance, and in other cases it can be due to a defective DNA repair system or defective apoptosis. Therefore, each primary immune disorder should be considered separately.

Lymphomas Associated with Infection by Human Immunodeficiency Virus

These heterogeneous disorders are predominately aggressive B-cell lymphomas in patients who are immunocompromised; patients with HIV infections are prone to develop these lymphomas.

Lymphomas diagnosed in HIV-positive patients are monoclonal B-cell neoplasms, with evidence of clonal proliferation of immunoglobulin genes detected by the Southern blot or PCR techniques *(267–269)*. Most cases also show somatic mutations involving immunoglobulin genes *(270)*. There are also T-cell cases, which have clonal rearrangement of T-cell genes *(271,272)*. Cases of HIV-associated Burkitt lymphoma, like other cases of Burkitt lymphoma, have genetic abnormalities affecting 8q24.1, the chromosomal location of the *MYC* oncogene. In these cases, the typical t(8;14),(q24;q23.2) or its variants affecting the light-chain genes at 2p11.2 and 22q11.2 have been described. In addition to truncation within or around the *MYC* locus, point mutations in the first intron/exon regulatory region are also present. Translocations of 8q24.1 are also detected in about 20% of diffuse large B-cell lymphomas (see above). In addition, a rearrangement of *BCL6* (a proto-

oncogene located at band 3q27 that belongs to family of transcription factors containing zinc-finger domains) is confined to diffuse large B-cell lymphoma. Frequent mutation of the 5' noncoding region of *BCL6* occurring independently of *BCL6* rearrangements is detected in Burkitt lymphoma and DLBCL and presents the most common genetic alteration in the HIV-related lymphomas *(273–275)*. In a small number of cases, mutations of the *RAS* family oncogenes are also present *(273)*.

HISTIOCYTIC AND DENDRITIC CELL NEOPLASMS

These are two of the most rare disorders of hematopoietic and lymphoid lineage. Histiocytes and accessory cells play a major role in the processing and presentation of antigen to both T- and B-cells. The WHO classification of histiocytic and dendritic cell neoplasms includes histiocytic sarcoma, Langerhans cell sarcoma, interdigitating dendritic cell sarcoma/tumor, follicular dendritic cell sarcoma/tumor, and dendritic cell sarcoma, not otherwise specified.

Interestingly, historical literature describes the possibility of antigen receptor gene rearrangement in histiocytic sarcoma, but recent data indicate that precise definition of histiocytic sarcoma requires the absence of clonal immunoglobulin and T-cell receptor genes *(258)*.

No definitive cytogenetic abnormalities have been reported, probably the result of the poor growth potential of these cells in culture.

MASTOCYTOSIS

As the name implies, mastocytosis is the proliferation of mast cells and their accumulation in one of the organ systems. It is a diverse and heterogeneous group of disorders, with clinical features ranging from indolent skin lesions to highly aggressive systemic manifestations. WHO classifies mastocytosis into cutaneous mastocytosis (CM), indolent systemic mastocytosis (ISM), systemic mastocytosis with associated clonal, hematological non-mast-cell lineage disease (SM-AHNMD), aggressive systemic mastocytosis (ASM), mast cell sarcoma (MCS), and extracutaneous mastocytosis.

Mastocytosis can occur at any age, but CM is more common in children. The diagnosis of mastocytosis usually made via microscopic analysis of a biopsy of the lesion. The presence of mast cells in aggregates with giemsa or toluidine blue staining is diagnostic.

Point mutations of *KIT*, a proto-oncogene that encodes a stem cell factor receptor tyrosine kinase, have been shown to be a recurrent event. The most frequent mutation is the substitution of Val for Asp at codon 816 that spontaneously activates the *KIT* oncogene *(276–279)*.

There are conflicting reports regarding the presence of trisomy 8 and 9 in mastocytosis *(280)*. A reported association between myeloproliferation disorders and systemic mast cell disease could be explained by the finding that trisomies 8 and 9 are common in both disorders. Recently, Swolin et al. reported no evidence of trisomies 8 or 9 in this disorder *(281)*. With this very limited and conflicting data, cytogenetics is of very limited clinical significance at this time and is not recommended for evaluation.

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16

Cytogenetics of Solid Tumors

Jonathan A. Fletcher, MD

INTRODUCTION

The field of cytogenetics has had a great impact on clinical and basic sciences in hematology and oncology, and both karyotyping and fluorescence *in situ* hybridization (FISH) assays are of growing relevance in solid tumor oncology. Although most of the cancer cytogenetic work in clinical laboratories is directed to hematological disorders, it is increasingly evident that cytogenetic assays are essential in providing diagnostic or prognostic information for various solid tumors (see **Tables 1** and **2**).

However, several aspects of the cytogenetic approach present unique challenges in solid tumors. Whereas hematological neoplasms can be sampled by minimally invasive methods, such as bone marrow aspiration or, sometimes, even phlebotomy, solid tumors are generally karyotyped using specimens obtained by open biopsy. Therefore, solid-tumor cytogenetic analyses are typically performed at the time of initial diagnosis or when the tumor is rebiopsied at the time of clinical progression, but they are not performed routinely to monitor treatment response in a given patient. In contrast, hematologic cytogenetic analyses are often repeated at regular intervals in a given patient, so as to monitor disease activity. Another difference between hematological and solid-tumor cytogenetics is that solid-tumor karyotypes are often extremely complex, particularly those in highly malignant solid tumors. A single metaphase cell might contain dozens of clonal and nonclonal chromosomal aberrations, and in such tumors, it is impractical to characterize the exact mechanisms of rearrangement responsible for each chromosomal aberration, particularly in the course of a routine clinical analysis. A final difference is that the solid-tumor sample generally must be disaggregated by mechanical and enzymatic methods before the cells are placed in tissue culture.

Although solid tumors are less readily accessible to biopsy compared to hematological neoplasms, it is increasingly common to sample solid tumors by fine-needle percutaneous approaches. Needle sampling is often performed under ultrasound or computed tomography (CT) guidance and can involve taking a fine-needle aspirate or needle core biopsy from the tumor. Solid tumor samples obtained by these methods can be karyotyped successfully *(1–4)*, but the small amount of starting material is a constraint in that fewer cultures can be established. FISH analyses, on the other hand, are straightforward in fine-needle specimens *(5,6)*.

Since the mid-1980s, various advances in tumor cell culture, including the use of collagenase for cell disaggregation *(7)*, have enabled more routine cytogenetic analysis of solid tumors. Nonrandom chromosomal abnormalities have been described in many varieties of solid tumors, and many of these are diagnostically or prognostically relevant (see **Tables 1** and **2**). Increasingly, the methods used for these analyses have become standardized between different clinical laboratories, although there remain many variations of the basic methods. Irrespective of the particular methods used, there are many general considerations that influence the success of tumor cytogenetic analyses.

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Table 1
Typical Cytogenetic Aberrations in Soft Tissue and Bone Tumors

Histologic findings	Characteristic cytogenetic events	Molecular events	Frequency	Diagnostic utility?
Alveolar soft part sarcoma	t(X;17)(p11.2;q25)	*ASPL-TFE3* fusion	>90%	Yes
Aneurysmal bone cyst (extraosseous)	16q22 and 17p13 rearrangements		>50%	Yes
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11.2)	*FUS-ATF1* fusion		
Chondromyoxid fibroma	Deletion of 6q		>75%	Yes
Chondrosarcoma				
Skeletal	Complex*		>75%	?
Extraskeletal myxoid	t(9;22)(q22;q12)	*EWS-NR4A3* fusion	>75%	Yes
	t(9;17)(q22;q11)	*TAF2N-NR4A3* fusion	<10%	Yes
	t(9;15)(q22;q21)	*TCF12-NR4A3* fusion	<10%	Yes
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS-ATF1* fusion	>75%	Yes
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS-WT1* fusion	>75%	Yes
Dermatofibrosarcoma protuberans	Ring form of chromosomes 17 and 22	*COL1A1-PDGFB* fusion	>75%	Yes
	t(17;22)(q21;q13)	*COL1A1-PDGFB* fusion	10%	Yes
Endometrial stromal tumor	t(7;17)(p15;q21)	*JAZF1-JJAZ1*	30%	Yes
Ewing's sarcoma	t(11;22)(q24;q12)	*EWS-FLI1* fusion	>80%	Yes
	t(21;22)(q12;q12)	*EWS-ERG* fusion	5–10%	Yes
	t(2;22)(q33;q12)	*EWS-FEV* fusion	<5%	Yes
	t(7;22)(p22;q12)	*EWS-ETV1* fusion	<5%	Yes
	t(17;22)(q12;q12)	*EWS-E1AF* fusion	<5%	Yes
	inv*(22)*(q12q12)	*EWS-ZSG*	<5%	Yes
Fibrosarcoma, infantile	t(12;15)(p13;q26)	*ETV6-NTRK3* fusion	>75%	Yes
	Trisomies 8, 11, 17, and 20		>75%	Yes
Gastrointestinal stromal tumor	Monosomies 14 and 22		>75%	Yes
	Deletion of 1p		>25%	No
		KIT mutation	>90%	Yes
Giant cell tumor	Telomeric associations		>50%	?
Hibernoma	11q13 rearrangement		>50%	Yes
Inflammatory myofibroblastic tumor	2p23 rearrangement	*ALK* fusion genes	50%	Yes
Leiomyoma				
Uterine	t(12;14)(q15;q24) or deletion of 7q	*HMGIC* rearrangement	40%	Yes
Extrauterine	Deletion of 1p		?	?
Leiomyosarcoma	Deletion of 1p		>50%	No
Lipoblastoma	8q12 rearrangement or polysomy 8	*PLAG1* oncogenes	>80%	Yes
Lipoma				
Typical	12q15 rearrangement	*HMGIC* rearrangement	60%	Yes
Spindle cell or pleomorphic	Deletion of 13q or 16q		>75%	Yes

Aytpical (see well-differentiated liposarcoma)				
Chondroid	t(11;16)(q13;p12–13)		?	Yes
Liposarcoma				
Well differentiated	Ring form of chromosome 12		>75%	Yes
Myxoid/round cell	t(12;16)(q13;p11.2)	*TLS-CHOP* fusion	>75%	Yes
	t(12;22)(q13;q12)	*EWS-CHOP* fusion	<5%	Yes
Pleomorphic	Complex*		90%	No
Malignant fibrous histiocytoma				
Myxoid	Ring form of chromosome 12		?	?
High grade	Complex*		>90%	No
Myxofibrosarcoma	*See* Malignant fibrous histiocytoma			
Malignant peripheral nerve sheath tumor	*See* Schwannoma			
Mesothelioma	Deletion of 1p	*?BCL10* inactivation	>50%	Yes
	Deletion of 9p	*CDKN2A, CDKN2B,* and *CDKN2D* inactivation	>75%	Yes
	Deletion of 22q	*NF2* inactivation	>50%	Yes
	Deletions of 3p and 6q		>50%	Yes
Neuroblastoma				
Good prognosis	Hyperdiploid, no 1p deletion		40%	Yes
Poor prognosis	1p deletion		40%	Yes
	Double minute chromosomes	*MYCN* amplification	>25%	Yes
Osteochondroma	Deletion of 8q	*EXT1* inactivation	>25%	?
Osteosarcoma				
Low grade	Ring chromosomes		>50%	Yes
High grade	Complex*	*RB1* and *TP53* inactivation	>80%	?
Pigmented villonodular synovitis	Trisomies 5 and 7		>25%	?
Primitive neuroectodermal tumor	*See* Ewing's sarcoma			
Rhabdoid tumor	Deletion of 22q	*INI1* inactivation	>90%	Yes
Rhabdomyosarcoma				
Alveolar	t(2;13)(q35;q14)	*PAX3-FKHR* fusion	>75%	Yes
	t(1;13)(p36;q14), double minutes	*PAX7-FKHR* fusion	10–20%	Yes
Embryonal	Trisomies 2q, 8 and 20		>75%	Yes
		Loss of heterozygosity at 11p15	>75%	Yes
Schwannoma				
Benign	Deletion of 22q	*NF2* inactivation	>80%	Yes
Malignant, low grade	None			
Malignant, high grade	Complex*		>90%	No
Synovial sarcoma				
Monophasic	t(X;18)(p11.2;q11.2)	*SYT-SSX1* or *SYT-SSX2* fusion	>90%	Yes
Biphasic	t(X;18)(p11.2;q11.2)	*SYT-SSX1* fusion	>90%	Yes

*Consistent finding of extremely complex karyotypes containing muliple numerical and structural chromosomal aberrations.

Table 2
Typical Cytogenetic Aberrations in Nonmesenchymal Solid Tumors

Histologic findings	Characteristic cytogenetic events	Molecular events	Frequency	Diagnostic utility?
Adenoid cystic carcinoma	6q translocations and deletions		>50%	Yes
Germ cell tumor	Isochromosome 12p		>75%	Yes
Hepatoblastoma	Trisomies 2q and 20		>75%	Yes
Medulloblastoma	Isochromosome 17q		>25%	Yes
Meningioma	Monosomy 22		90%	Yes
	1p deletion		25%	Yes
Midline lethal carcinoma	t(15;19)(q14;p13)	*BRD4-NUT* fusion	>75%	Yes
Oligodendroglioma	Deletion of 1p and 19q		50%	No
Pleomorphic adenoma (salivary gland)	8q12 rearrangement	*PLAG1* fusion oncogenes	>50%	Yes
	12q15 rearrangement	*HMGIC* oncogenes	<20%	Yes
Renal carcinoma				
Clear cell	Deletion of 3p		>90%	Yes
Papillary adult	Trisomies 3, 7, 16, 17, and 20		>90%	Yes
Papillarylike, young adults	Xp11 rearrangement	*TFE3* fusion	>50%	Yes
	6p21 rearrangement	*TFEB* fusion	>50%	Yes
Oncocytoma	Monosomy 1 with loss of X or Y		>25%	Yes
	11q13 rearrangement		>25%	Yes
Chromophobe	Monosomies 1, 2, 3, 6, 10, 13, 17, and 21		>75%	Yes
Thyroid carcinoma				
Papillary	10q11.2 rearrangement	*RET* fusion oncogenes	>30%	Yes
	1q21 rearrangement	*NTRK1* fusion oncogenes	>10%	Yes
Follicular	t(2;3)(q13;p25)	*PAX8-PPARG* fusion	>40%	Yes
Mucoepidermoid carcinoma	t(11;19)(q21;p13)	*MECT1-MAML2* fusion	>50%	Yes

Solid-Tumor Cytogenetics: Art or Science?

Various factors determine the success of solid-tumor cytogenetics, not least of which being the experience of a given laboratory in processing and analyzing such specimens. Four of the main considerations are as follows:

1. *Unpredictable growth of the neoplastic cells in tissue culture.* Benign solid tumors generally contain few mitotic cells, and one must often wait for a week or more before such specimens begin to proliferate actively in tissue culture. In the meantime, the neoplastic cells might be overgrown by non-neoplastic cells (see point 2). Surprisingly, there are many highly malignant solid tumors that also grow poorly in tissue culture, despite the fact that they were growing rapidly in the patient. Such tumors can sometimes be stimulated in culture by use of relevant culture media and growth factors, but it is impractical in the clinical cytogenetics laboratory to stock the various different growth factors and media that are optimal for many varieties of solid tumors. Therefore, in practice, it is challenging to culture certain types of solid tumors in the clinical laboratory. A notable example of this is prostate cancer, which, although a very common type of cancer, requires extremely specialized methods for successful tissue culture. It is also important to recognize that only a minority of the overall neoplastic population in a given sample might be capable of growing under a particular set of tissue culture conditions. Therefore, one cannot assume that a clonally abnormal karyotype is representative of the overall neoplastic process. For example, in a given tumor, the final karyotype might be representative of the components of the tumor that were either more or less clinically aggressive, depending on which component was best suited to growing under the particular tissue culture conditions used for that case.
2. *Overgrowth of neoplastic cells by "reactive" non-neoplastic cells.* All solid-tumor biopsies contain mixtures of neoplastic and non-neoplastic cells. The non-neoplastic elements can include fibroblasts, normal epithelial cells, endothelial cells, or glial cells (in the case of brain tumors), depending on the type and location of the tumor. Any of these reactive cell types, in a given specimen, can grow more successfully that the neoplastic cells in culture. Therefore, culture overgrowth by reactive cells is the most common explanation for a normal diploid karyotype in solid-tumor cytogenetics. For this reason, it is crucial to learn the morphology of the common sorts of reactive cells, which can be distinguished from the neoplastic cells by daily evaluation of the cultures via phase-contrast inverted microscopy. Metaphase cells should be harvested at the first signs of reactive cell overgrowth, even in cases where the neoplastic population has not yet begun to grow actively. Otherwise, the finding of a spurious normal karyotype, deriving from the overgrowth of reactive cells, will be the predictable end result of the cytogenetic analysis.
3. *Destruction of tumor cultures by bacterial or fungal infection.* Loss of solid-tumor cytogenetic cultures to infection should be an uncommon event, providing that the specimen transport and culture media contain broad-spectrum antibiotics (e.g., penicillin/streptomycin) and antifungals (e.g., amphotericin). Infectious contamination generally occurs when specimens have been crudely handled in the pathology department, either by cutting a sample with a previously used blade or by placing the sample directly on a dirty cutting surface. Infectious contamination can be unavoidable in the case of specimens from body regions that are extensively colonized by bacteria (e.g., in the case of a colorectal carcinoma whose surface is within the intestinal lumen).
4. *Failure of tumor cultures to grow because of nonviable tumor.* Many solid tumors, particularly those that are highly malignant, are largely composed of nonviable regions, or regions with few neoplastic cells. Such regions can be extensively necrotic, because the tumor cells have died, having outstripped their blood supply. Other regions of a tumor mass can be composed largely of blood (hemorrhage) or scarred tissue (fibrosis). Therefore, it is crucial that the pathologist select a maximally viable tumor region for the solid-tumor cytogenetic analysis.

Molecular Cytogenetics

Whereas conventional cytogenetic analyses are performed using various staining techniques that highlight chromosome bands, the various molecular cytogenetics methods involve evaluation of relevant chromosome regions using DNA probes *(8,9)*. Most molecular cytogenetic methods are based on ISH (*in situ* hybridization); that is, the DNA probes are hybridized and evaluated in the cellular, *in situ*, context. ISH assays can be performed with fluorescence or enzymatic detection, which are referred to as FISH (fluorescence *in situ* hybridization) (see Chapter 17) and CISH (chromogenic *in situ* hybridization), respectively. There are pros and cons to both of these methods. FISH assays are simpler

and more sensitive than CISH, but CISH is visualized on an ordinary brightfield microscope, whereas FISH requires a high-quality fluorescence microscope and appropriate imaging software (see Chapter 7). The CISH detection reactions, generally performed using peroxidase or alkaline phosphatase strategies *(9–11)*, are also extremely stable and can, therefore, be archived for many years.

The FISH/CISH analyses are most conveniently performed using cytogenetic preparations, but they are increasingly being applied to paraffin sections and other archival pathology preparations as well. One substantial advantage in the use of paraffin sections is that the well-preserved cell morphology can be used as a guide to enable evaluation of the chromosomal events in only the relevant cell populations. An example is the evaluation of *ERBB2* (HER-2/*neu*) gene amplification in breast cancer, where hybridization against paraffin sections permits scoring of the *ERBB2* gene signals precisely in the invasive carcinoma components of the tumor biopsy (12,13; see also Chapter 17, Fig. 14). A drawback in the use of paraffin tissue sections is that the nuclei are generally incomplete, having been sliced during preparation of the sections, which are typically no more than 4 μm in thickness *(14)*. ISH can also be carried out against nuclei disaggregated from thick (50–60 μm) paraffin sections *(15)*, but the author has found that this often results in substantial damage to the nuclei. Alternate methods, including disaggregation of cells from thin core biopsies of the paraffin block, could circumvent these limitations *(16)*.

Most FISH studies performed in clinical cytogenetics laboratories address focused questions, such as whether there are deletions, rearrangements, or amplifications of particular gene loci or of particular chromosomes. However, various molecular cytogenetic methods have expanded the capabilities of solid-tumor molecular cytogenetics by enabling fluorescence evaluation of the entire karyotype or of the entire genome. Examples include comparative genomic hybridization (CGH) *(17,18)* and multiplex FISH (M-FISH) *(19)*, both of which permit genomewide evaluation of chromosomal aberrations (see Chapter 17).

Comparative genomic hybridization is performed by extracting total genomic DNAs from a tumor of interest and from a non-neoplastic control cell population. These DNAs are differentially labeled (e.g., tumor DNA with fluorescein and control DNA with rhodamine), and are then cohybridized against normal metaphase cells (metaphase CGH) or against arrays of genomic or cDNA clones (array CGH). Chromosomal regions that are overrepresented (amplified) or underrepresented (deleted) in the tumor DNA will be manifested as color shifts when the metaphase cells, or arrays, are visualized under fluorescence. An advantage of CGH, compared to conventional karyotyping, is that the tumor DNA can be isolated from frozen or paraffin specimens, without need for cell culture. In addition, the array CGH methods can detect very small deletions, which would be overlooked by traditional cytogenetic banding assays. However, CGH does not detect balanced chromosomal rearrangements (e.g., balanced translocations that are diagnostic markers in many soft tissue tumors and in some carcinomas) (see Chapter 17, Figs. 16 and 17).

Genomewide molecular cytogenetics can also be performed using M-FISH, in which panels of DNA probes are cohybridized against tumor metaphase cells *(19)*. Whereas conventional FISH techniques involve hybridization of one or two fluorescence-tagged probes, M-FISH can utilize probes for each chromosome or chromosome arm (24 or more probes). Each probe is detected combinatorally, using different ratios of fluorescence markers. By varying the ratio of each fluor, each chromosome can be given a unique color. Thus, M-FISH enables a comprehensive ISH screen of the entire tumor cell karyotype. M-FISH is a powerful research tool in solid-tumor cytogenetics and has been particularly useful in elucidating complex karyotypes and in identifying recurring deletion or amplification regions within those karyotypes *(20,21)*. However, it has not been adopted widely for routine clinical applications, given that it requires specialized equipment and is relatively time-consuming and costly (see Chapter 17, Fig. 18).

CYTOGENETIC MECHANISMS IN SOLID TUMORS

The cytogenetic aberrations in solid tumors vary from extremely simple, involving loss or rearrangement of a single chromosome, to highly complex. Complex abnormal karyotypes, which typi-

cally contain numerous clonal and nonclonal chromosomal aberrations, are most often found in highly malignant solid tumors. On the other hand, noncomplex karyotypes can be found in either benign or malignant tumors. Therefore, the absence of cytogenetic complexity is not, in itself, a reassuring finding. Although increased cytogenetic complexity generally correlates with increasing histological grade, there is also considerable variability in cytogenetic complexity between different types of solid tumor. Among the epithelial tumors, for example, breast carcinomas invariably have complex karyotypes, whereas renal carcinomas generally have noncomplex karyotypes. Among the mesenchymal tumors, osteosarcomas invariably have complex karyotypes, whereas equally malignant Ewing's sarcomas have noncomplex karyotypes.

The chromosome aberrations in solid tumors result in translocation, deletion, or amplification of target genes. Translocations are particularly frequent in sarcomas, where they usually create fusions of genes at the breakpoints of the participant chromosomes *(22,23)*. Deletions are frequent in carcinomas, where they likely result in loss of tumor suppressor genes. Amplifications, which are manifest as intrachromosomal homogeneously staining regions or as extrachromosomal double minutes (see also Chapter 15, Fig. 3) are seen occasionally in solid tumors of all types and can be of both prognostic and therapeutic relevance *(13,24)*.

DIAGNOSTIC AND PROGNOSTIC APPLICATIONS

Cytogenetic analyses have given extraordinary insights into the biology and pathogenesis of solid tumors and, in some cases, these insights have then provided the basis for more accurate assessment of diagnosis and prognosis. However, cytogenetic methods are not used routinely in the clinical setting for all solid-tumor types. In some solid tumors, particularly those that are clinically benign, there is no need for cytogenetic adjuncts, as the diagnosis and prognosis (with expectant cure after adequate surgery) are straightforward. Other solid tumors, of which prostate cancer is a good example, do not grow well in conventional tissue culture, and therefore routine karyotyping is not an option. Widespread application of cytogenetics in such tumors awaits the identification of key genetic predictors, which might be identified in tumor interphase cells by FISH methods. Still other solid tumors have extremely complex karyotypes, and there has been little clinical advantage in cytogenetic analysis of these, given the formidable task of describing the many abnormal chromosomes and given the questionable clinical relevance of the individual chromosomal perturbations. The following sections will highlight the applications of cytogenetics in mesenchymal and renal tumors, where the technical challenges of the cytogenetic assays are surmountable and where the cytogenetic findings often provide important diagnostic information.

MESENCYMAL TUMORS (SOFT TISSUE AND BONE TUMORS)

Ewing's Sarcoma

Ewing's sarcomas are highly aggressive bone and soft tissue tumors, in which the neoplastic cells are generally of the small round cell type. Most Ewing's sarcomas contain chromosome translocations involving the Ewing's sarcoma gene (*EWS*), which is located on the long arm of chromosome 22. These translocations involve a number of partner genes (see **Table 1**); the most common rearrangement is t(11;22)(q24;q12) (see **Fig. 1**), which results in oncogenic fusion of the *FLI1* gene on chromosome 11 with the *EWS* gene *(25–28)*. *FLI1* encodes a transcription factor belonging to the ETS family of transcription factors, and the oncogenic *EWS-FLI1* fusion gene encodes an activated version of this transcription factor. Other Ewing's sacomas have variant translocations in which the *EWS* gene is fused with other ETS family transcription factor genes (see **Table 1**) *(29–33)*. The Ewing's gene translocations are apparently essential, because they are found in virtually all Ewing's sarcomas. These translocations are detected readily by conventional cytogenetic methods, even using needle biopsy material, because Ewing's sarcoma cells grow well in culture *(2)*. The translocations

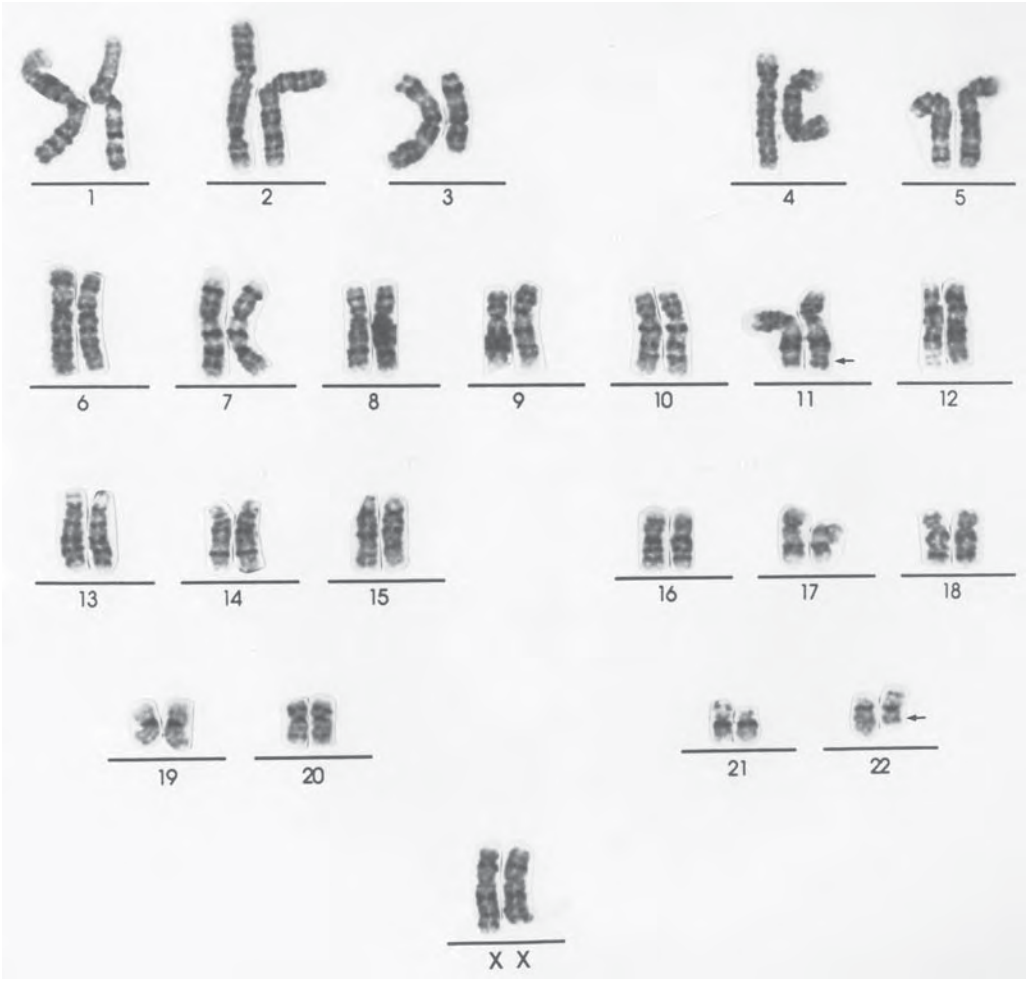

Fig. 1. Ewing's sarcoma karyotype showing translocation involving chromosomes 11 and 22. Arrows indicate the translocation breakpoints on the rearranged chromosomes. Deletion of 3q was another clonal aberration in this tumor.

can also be detected by FISH, using probes to the *EWS* and *FLI1* regions *(34)*, or by reverse transcriptase–polymerase chain reaction (RT-PCR) *(35)*. Advantages of PCR include superior sensitivity and identification of breakpoint locations within the translocated genes. Several studies indicate that *EWS-FLI1* breakpoint locations might be prognostic in Ewing's sarcoma *(36–38)*, although this information is not used routinely to guide therapeutic decisions. Alternately, a more universal FISH strategy, for detection of all *EWS* translocations, can be performed using dual-color probes flanking each side of the *EWS* locus *(39)*.

Rhabdomyosarcoma

Rhabdomyosarcomas are tumors featuring skeletal muscle differentiation, which include several distinct histological subtypes. The most common varieties are the embryonal and alveolar forms of rhabdomyosarcoma, and although these two forms have different natural histories (the alveolar subtype being more aggressive), they can be difficult to distinguish histologically. Cytogenetic studies have affirmed the distinct natures of these rhabdomyosarcoma subtypes, in that alveolar rhab-

domyosarcomas are characterized by reciprocal chromosome translocations involving the *FKHR* (Forkhead transcription factor) gene on chromosome 13, whereas embryonal rhabdomyosarcomas lack such translocations. Most alveolar rhabdomyosarcomas have fusion of the *FKHR* gene with the *PAX3* gene on chromosome 2 *(40–42)*, but a smaller number contain fusions of *FKHR* with the *PAX7* gene on chromosome 1 *(43)*. Notably, the *PAX7-FKHR* fusion is often highly amplified—in the form of double minute chromosomes—whereas the more common *PAX3-FKHR* fusions are not. This difference appears to reflect the lower intrinsic expression of *PAX7-FKHR*, relative to that of *PAX3-FKHR*, with genomic amplification therefore required to provide a comparable level of oncogene transcript *(44)*. The normal *FKHR, PAX3,* and *PAX7* genes encode transcription factors, and the *PAX3-FKHR* and *PAX7-FKHR* fusion oncogenes encode activated forms of those transcription factors *(45,46)*. Embryonal rhabdomyosarcomas typically lack *FKHR* translocations and have a distinctive cytogenetic profile including extra copies of chromosomes 2, 8, and 20. Deletions of chromosome 11p have been studied extensively in embryonal rhabdomyosarcoma, and for some time, they were viewed as the cardinal cytogenetic aberrations in these tumors. However, the 11p deletions are infrequent compared to the chromosomal polysomies.

Synovial Sarcoma

Synovial sarcomas can be either biphasic (in which the tumor contains both spindle cell and epithelioid elements) or monophasic (in which the tumor is predominantly spindle cell), and both of these subtypes feature a reciprocal translocation involving the X chromosome and chromosome 18, t(X;18)(p11.2;q11.2) *(47,48)*. The (X;18) translocation is found in more than 90% of synovial sarcomas, but not in histologic mimics such as hemangiopericytoma, mesothelioma, leiomyosarcoma, or malignant peripheral nerve sheath tumor. The molecular underpinnings of the (X;18) translocation are complex in that the oncogene on chromosome 18 (*SYT* or *SS18*) can be fused with either of two nearly identical genes (*SSX1* or *SSX2*) on the X chromosome *(49)*. *SSX1* and *SSX2* are neighboring genes, and given their close proximity, it is impossible to distinguish *SYT-SSX1* and *SYT-SSX2* translocations using conventional chromosomal banding methods. However, the alternate SSX fusions can be demonstrated by FISH (see Chapter 17, Fig. 13) or RT-PCR *(50,51)*. Notably, synovial sarcomas with the *SYT-SSX1* fusion are invariably biphasic, whereas those with *SYT-SSX2* can be either biphasic or monophasic and have better metastasis-free survival compared to those with *SYT-SSX1* fusions *(52)*.

Adipose Tumors

Adipose tumors present a paradigm in solid-tumor cytogenetics, in that virtually all histological subtypes, whether benign or malignant, contain distinctive chromosomal aberrations (see **Table 1**). Useful diagnostic markers include 12q rearrangement in lipoma, ring chromosomes in well-differentiated and dedifferentiated liposarcoma, t(12;16) translocations in mxyoid/round cell liposarcoma, and cytogenetic complexity in pleomorphic liposarcomas.

Benign lipomas can be grouped into three general cytogenetic categories: (1) those with rearrangements of the mid-portion of the long arm of chromosome 12 (band 12q15); (2) those with clonal aberrations not involving 12q15; and (3) those with normal karyotypes *(53–55)*. The chromosome 12q15 rearrangements target the *HMGIC* (high-mobility group IC) transcriptional regulatory gene *(56)*, with formation of an *HMGIC* fusion oncogene, usually resulting from an inversion or translocation with various partner chromosomes. Other nonrandom aberrations include rearrangement of the short arm of chromosome 6 and deletion of the long arm of chromosome 13, which are each seen in fewer than 10% of lipomas *(54)*. Lipomas with deletions of the long arm of chromosome 16, often accompanied by deletions of the long arm of chromosome 13, generally have spindle cell or pleomorphic histology *(55,57)*.

Characteristic cytogenetic aberrations are found in several other benign adipose tumors. Lipoblastomas are pediatric adipose tumors containing variable numbers of primitive cells (lipoblasts), and these tumors generally contain translocations involving the long arm of chromosome 8 at bands 8q11-q12, which result

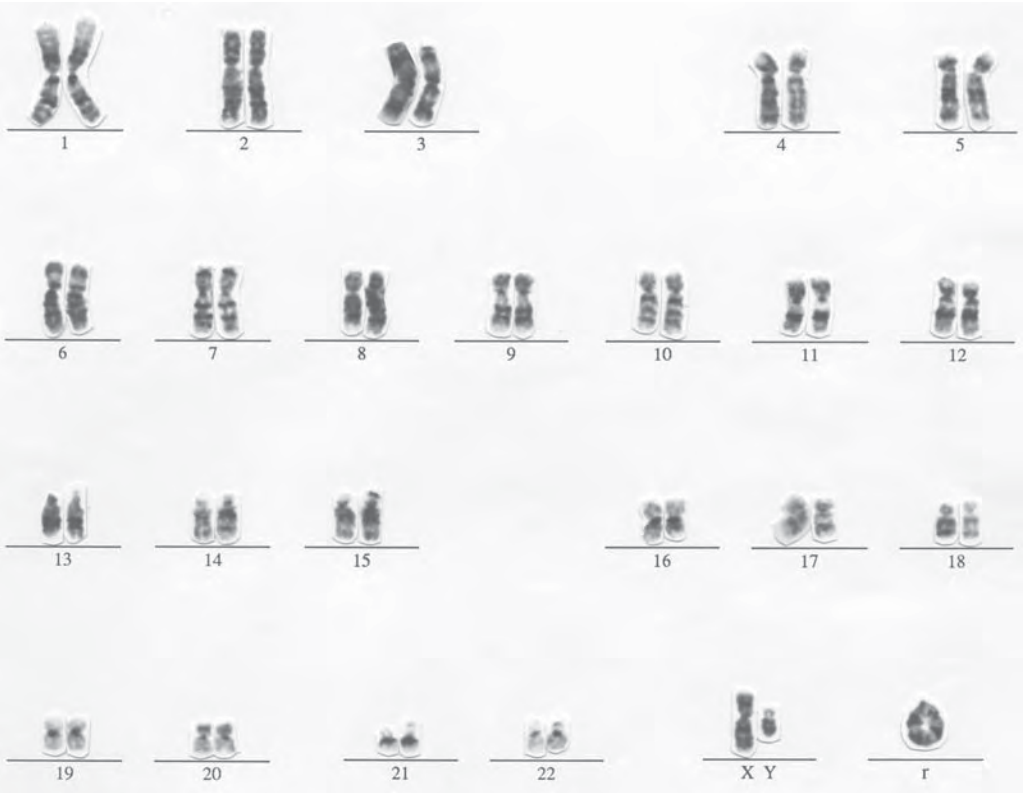

Fig. 2. Typical noncomplex karyotype in a dermatofibrosarcoma protuberans, showing a supernumerary ring chromosome as the only cytogenetic aberration.

in rearrangement of the *PLAG1* oncogene *(58)*. Hibernomas, which contain adipose cells with a "brown fat" phenotype, generally have rearrangements of the chromosome 11 long arm *(59)*.

The most diagnostically useful aberration in malignant adipose tumors is a translocation between chromosomes 12 and 16, t(12;16)(q13;p11.2). This translocation is found in myxoid liposarcomas *(55,60,61)* and is retained in cases that acquire round cell features *(62,63)*. The (12;16) translocation results in fusion of the *CHOP* gene on chromosome 12 with the *TLS* gene on chromosome 16 *(64,65)*, and the resultant fusion oncoprotein is an activated transcription factor. t(12;16) has not been found in other subtypes of liposarcoma or in other types of myxoid soft tissue tumors *(66,67)*.

Well-differentiated liposarcomas (atypical lipomas) contain large "giant marker" chromosomes and ring chromosomes (see **Fig. 2**). These chromosomes are generally comprised of chromosome 12 material, often admixed with components of several other chromosomes *(55,68)*. The ring and "giant marker" chromosomes contain various amplified genes, but the essential gene amplification targets have not been pinpointed. Notably, these amplifications are retained when well-differentiated liposarcomas progress to dedifferentiated liposarcomas, which are substantially more aggressive than the well-differentiated phase of the tumor.

Clear Cell Sarcoma (Malignant Melanoma of Soft Parts)

Clear cell sarcomas of soft tissues resemble cutaneous malignant melanomas phenotypically and therefore have been referred to as "melanomas of the soft parts." Despite their histologic similarities,

clear cell sarcoma and true melanoma are quite different clinically. Whereas most melanomas are of cutaneous origin, clear cell sarcomas generally present as isolated masses in deep soft tissues, without apparent origin from skin. More than 75% of clear cell sarcomas contain a chromosomal rearrangement, t(12;22)(q13;q12), that has never been reported in cutaneous melanoma and that hence serves as a reliable marker in distinguishing these two tumor types. The (12;22) translocation fuses the *ATF1* gene on chromosome 12 with the *EWS* gene on chromosome 22 *(69,70)*. *ATF1* encodes a transcription factor, and the biological implications of the translocation are probably similar to those in Ewing's sarcoma translocations, as discussed earlier.

Desmoplastic Small Round Cell Tumor

Desmoplastic small round cell tumors are aggressive and chemotherapy-resistant neoplasms that arise usually from intraabdominal soft tissues *(71)*. They are composed of undifferentiated malignant small round cells within a striking desmoplastic reaction *(71)*, and virtually all cases express an *EWS-WT1* fusion oncogene *(72,73)*. The *EWS-WT1* oncogene results from translocation between the short arm of chromosome 11 and the long arm of chromosome 22, fusing the *WT1* (Wilms tumor) and *EWS* (Ewing's) genes *(74,75)*. The *EWS-WT1* oncoprotein is a transcriptional regulator that upregulates expression of platelet derived-growth factor-α (*PDGFA*) *(76)*. *PDGFA* activates potent mitogenic signaling pathways in fibroblasts *(77)*, thereby likely contributing to the prominent desmoplastic reaction.

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) are low-grade spindle cell tumors that can occasionally progress to a more aggressive "fibrosarcomatous" phase. Most DFSP contain ring chromosomes (see **Fig. 3**) comprised of sequences from chromosomes 17 and 22 *(78,79)*. The ring chromosomes contain multiple copies of a fusion gene, *COL1A1-PDGFB*, in which *COL1A1* (a collagen gene) is contributed by chromosome 17 and *PDGFB* (platelet derived growth factor beta gene) by chromosome 22 *(80,81)*. Occasional dermatofibrosarcoma protuberans have balanced (17;22) translocations, which result in a single copy of the *COL1A1-PDGFB* fusion gene. The *COL1A1-PDGFB* oncogene results in overexpression of PDGFB, which is a growth factor that activates platelet-derived growth factor-β receptor (PDGFRB) and platelet derived growth factor receptor alpha (PDGFRA). This cytogenetic observation suggested the possibility that patients with inoperable DFSP might benefit from treatment with PDGFR inhibitors (e.g., imatinib mesylate) (see Chapter 15), and this hypothesis has been confirmed by impressive clinical responses in several patients *(82,83)*.

Desmoid Tumors

Desmoid tumors, which are also known as deep fibromatoses, contain various cytogenetic or molecular aberrations, including *APC* (adenomatous polyposis coli) and β-catenin mutations *(84,85)*, and trisomies for chromosomes 8 or 20 *(86)*. Cytogenetic deletions of the long arm of chromosome 5 are seen in occasional desmoids, resulting in loss of the *APC* tumor suppressor gene *(87,88)*. However, the most common mutations, particularly in nonfamilial desmoid tumors, are those that activate β-catenin, and they are found in approximately 50% of cases *(84,85)*. These mutations result in stabilization, and resultant overexpression of the β-catenin protein. It is likely that the cytogenetic aberrations in desmoid tumors, particularly trisomies 8 and 20, are mechanisms of progression, and are acquired subsequent to the APC or β-catenin mutations.

Infantile Fibrosarcoma

Infantile fibrosaromas are congenital tumors comprised of fibroblastlike cells and that often show high mitotic activity. These tumors are distinguished by trisomies of chromosomes 8, 11, 17, and 20 *(89)*. This same group of trisomies is found in the histologically similar pediatric renal tumor, mesoblastic

Fig. 3. Metaphase cell from a well-differentiated liposarcoma showing two ring chromosomes (arrows), along with giant marker chromosomes.

nephroma *(90)*. Most infantile fibrosarcomas and cellular mesoblastic nephromas also contain a diagnostic chromosome translocation, t(12;15)(p13;q26), which is cryptic when studied by traditional cytogenetic banding methods *(91–93)*. t(12;15)(p13;q26) results in fusion of the *ETV6* gene on chromosome 12 with the *NTRK3* gene on chromosome 15. Although challenging to detect by banding methods, the (12;15) translocation is demonstrated readily by FISH or RT-PCR *(91–93)*.

Inflammatory Myofibroblastic Tumor

Inflammatory myofibroblastic tumor (also known as "inflammatory pseudotumor") is composed of myofibroblastic cells admixed with a prominent inflammatory infiltrate of lymphocytes and plasma cells. The inflammatory component of these tumors is non-neoplastic (and therefore lacks cytogenetic aberrations), whereas the myofibroblastic cells contain clonal chromosome aberrations *(94–96)*. A subset of inflammatory myofibroblastic tumors have cytogenetic rearrangements that activate the *ALK* receptor tyrosine kinase gene on chromosome 2 *(97)*. *ALK* is also activated, typically by fusion with the *NPM* gene on chromosome 5, in many anaplastic large cell lymphomas (see Chapter 15). Cytogenetic or FISH analyses for *ALK* rearrangement are useful in distinguishing inflammatory myofibroblastic tumors from histologically similar spindle cell proliferations.

Fig. 4. Noncomplex karyotype in a gastrointestinal stromal tumor with loss of chromosome 14 as an isolated chromosomal abnormality.

Gastrointestinal Stromal Tumors

Most gastrointestinal stromal tumors (GISTs) contain activating mutations of the *KIT* or *PDGFRA* oncogenes *(98–100)*. These mutations have been targeted with spectacular success using the KIT inhibitor, imatinib mesylate (STI571, Gleevec) *(101,102)*. In addition, germline (inherited) *KIT* mutations are responsible for rare syndromes of familial, multifocal GISTs *(103)*. Both the germline and somatic *KIT* aberrations are point mutations that are not evident at the cytogenetic level of resolution. However, most GISTs also have one or more chromosomal deletions *(100,104)*, and the cytogenetic profile in GISTs is different from that in histological mimics such as leiomyoma and leiomyosarcoma (see **Table 1**). The cytogenetic aberrations in GISTs appear to be secondary events and it is likely that *KIT* mutations initiate the neoplastic process in many GISTs, whereas cytogenetic aberrations are important in the biological and clinical progression of those tumors. Benign GISTs can have normal karyotypes or isolated losses of 14q and 22q. Borderline malignant GISTs invariably have loss of 14q, which is often accompanied by loss of 1p, 9p, 11p, or 22q (see **Fig. 4**). Highly malignant GISTs usually contain at least three of the above-mentioned chromosomal deletions, although their karyotypes are nonetheless simpler than those in most cancers that are histological GIST mimics. Molecular cytogenetic screening by comparative genomic hybridization (CGH) (see Chapter 17) has also revealed correlations between increasing numbers of chromosomal aberrations and aggressive clinicopathological behavior. El-Rifai et al. reported such correlations in 95 GISTs, including 24 benign, 36 malignant primary, and 35 metastatic tumors *(105)*. The mean number of demonstrable chromosomal aberrations were 2.6, 7.5, and 9.0, respectively, in the benign, malignant primary, and metastatic GISTs. Deletions of chromosome arms 1p, 14q, and 22q were found in both benign and

malignant GISTs. However, other aberrations, including 9p deletion, 8q amplification, and 17q amplification, were found almost exclusively in malignant GISTs *(105)*.

The 9p deletions in GIST appear to target the *p16* (*CDKN2A*) gene *(106)*, but the other genes involved in GIST cytogenetic aberrations have not yet been identified. Notably, the cytogenetics literature for GISTs is somewhat confusing, because karyotypes published before 1995 were described as being from gastrointestinal leiomyomas and leiomyosarcomas. It is interesting, in retrospect, that various investigators had reported a distinctive "subgroup" of gastrointestinal leiomyosarcomas with noncomplex karyotypes and deletions of chromosomes 14, 22, and 1p. These are the tumors that are now known to be GISTs.

Malignant Peripheral Nerve Sheath Tumors

Benign and malignant peripheral nerve sheath tumors are seen with increased frequency in patients with the hereditary neurofibromatosis syndromes. These are the most common tumor predisposition syndromes, affecting 1 in 3500 individuals worldwide. Neurofibromas and malignant peripheral nerve sheath tumors are common in individuals with neurofibromatosis type 1, whereas benign schwannomas are associated with neurofibromatosis type 2 (central neurofibromatosis). Characterization of the neurofibromatosis syndrome genes has shed substantial light on the pathogenesis of peripheral nerve sheath tumors. The neurofibromatosis type 1 and type 2 genes (*NF1* and *NF2*) are located on chromosomes 17 and 22, respectively, and both of these genes encode tumor suppressor proteins that normally constrain cell proliferation *(107–112)*. Malignant peripheral nerve sheath tumors (MPNSTs) often have deletions of the *NF1* gene, which can be demonstrated by FISH assays. These *NF1* gene aberrations are accompanied by a generally complex karyotype, suggesting that genetic instability plays a prominent role in the development of MPNSTs. Notably, *NF1* gene deletions can also be shown in the Schwann cell component of neurofibromas *(113)*. This observation supports the view that neurofibromas are clonal schwannian neoplasms, whereas the other admixed cell lineages, including fibroblasts, mast cells, and perineural cells, are reactive.

Mesothelioma

Mesotheliomas are highly lethal tumors arising from pleural, pericardial, or peritoneal surfaces. These tumors arise from exposure to asbestos fibers, and although rare they are a major public health concern. Mesotheliomas can be composed of either epithelial-type or spindled cells or of a mixture of these two cell types. Therefore, they have varied histologies, which creates diagnostic challenges. In particular, epithelial-type pleural mesothelioma can be difficult to distinguish from adenocarcinoma of the lung, and malignant mesothelioma in pleural or ascites fluid specimens can be difficult to distinguish from non-neoplastic, reactive, mesothelial cells.

Cytogenetic studies have revealed a characteristic profile of chromosomal deletions in mesotheliomas, and these deletions are found in all histologic subtypes of mesothelioma, implicating loss of several tumor suppressor genes as critical events in mesothelioma pathogenesis. Notably, mesothelioma karyotypes are less complex than those in most lung carcinomas. In particular, epithelial-type mesothelioma karyotypes often have fewer than five chromosomal abnormalities per cell (see **Fig. 5**), whereas bronchogenic adenocarcinomas typically have more than 20 chromosomal abnormalities per cell. However, the karyotypes in sarcomatoid (spindle cell) mesotheliomas can be more complex than those in epithelial-type cases. The deletional hot spots are within the long arms of chromosomes 6 and 22 and within the short arms of chromosomes 1, 3, and 9 (see **Fig. 5**) *(114)*. Notably, the pathogenesis of some mesothelioma chromosome deletions might be directly attributable to asbestos-mediated chromosome damage *(115,116)*.

Neuroblastoma

Most neuroblastomas can be assigned to one of two cytogenetic and clinicopathologic groups. The first group includes neuroblastomas with a clinically favorable biology. These tumors respond well

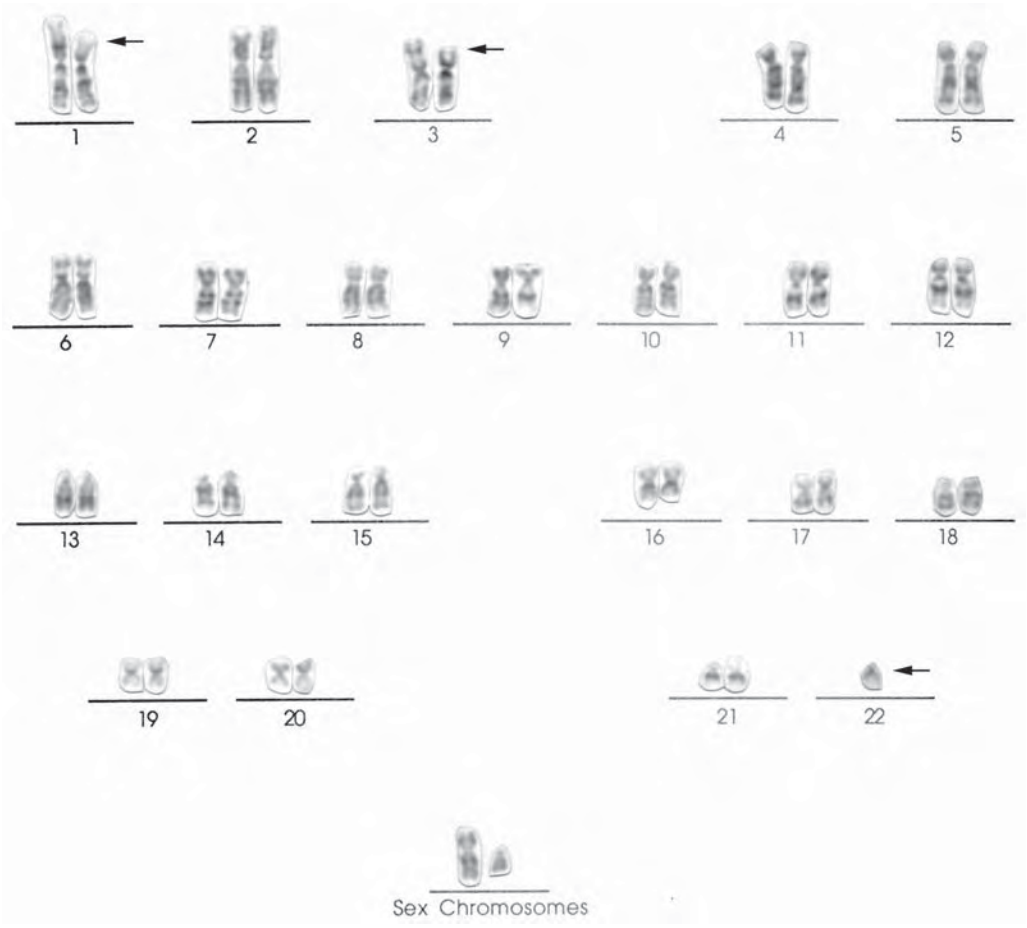

Fig. 5. Karyotype of a malignant mesothelioma in which arrows indicate rearrangements resulting in loss of material from the short arms of chromosomes 1 and 3 and from the long arm of chromosome 22.

to chemotherapy and can even undergo spontaneous regression, and from a cytogenetic standpoint, they have near-triploid karyotypes without 1p deletion or *N-MYC* amplification *(117)*. The second group includes tumors with near-diploid or near-tetraploid karyotypes, 1p deletion, and, in many cases, *N-MYC* amplification (see **Fig. 6**) *(118)*. *N-MYC* amplification, typically manifested as double minute chromosomes, is arguably the most ominous of the adverse prognostic markers. In children, *N-MYC* amplified neuroblastomas are rarely curable, although complete remissions can sometimes be obtained using intensive myeloablative chemotherapy. Therefore, genetic analyses can be useful adjuncts in determining appropriate intensity of therapy, particularly for those children whose prognoses are not clear based on clinical parameters *(118)*. Cytogenetic analyses of neuroblastoma have been difficult, however, because the tumor cells from most favorable prognosis neuroblastomas fail to divide in culture *(118)*. On the other hand, *N-myc* amplification and chromosome 1 deletions can be demonstrated in interphase cells by FISH *(119,120)*.

Rhabdoid Tumors

Most malignant rhabdoid tumors, whether arising in soft tissues, kidney, or the central nervous system, have deletions of the long arm of chromosome 22. The chromosome 22 deletions target a

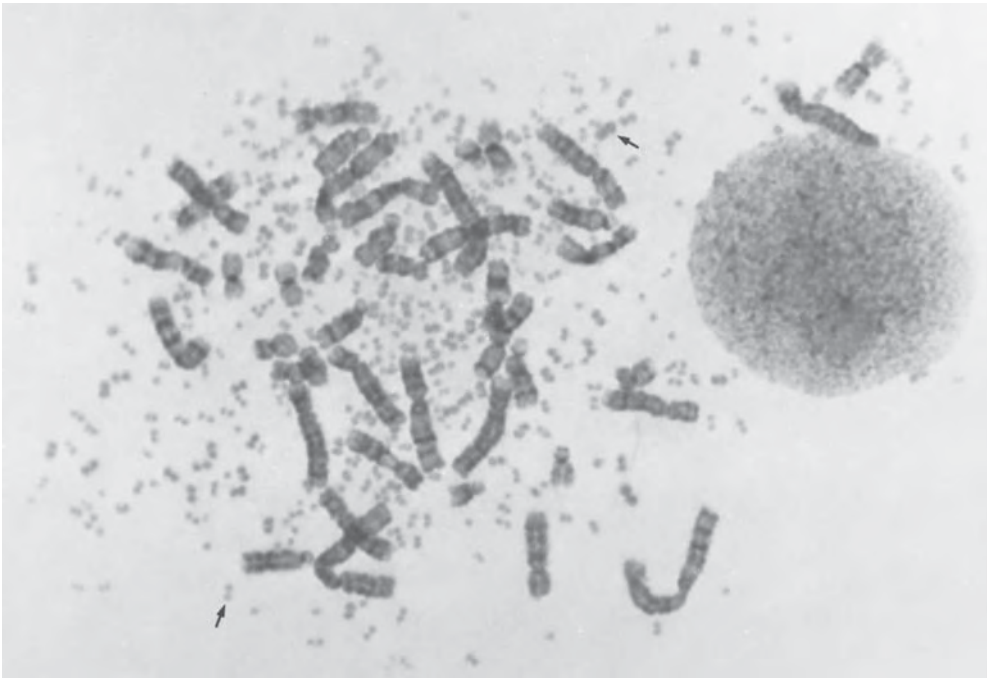

Fig. 6. Neuroblastoma metaphase cell with numerous double minute chromosomes of varying sizes (arrows).

tumor suppressor gene, *INI1* (also known as *SNF5* or *SMARCB1*), which encodes a protein involved in chromatin remodeling *(121,122)*. The rhabdoid tumor karyotypic profile is quite characteristic, inasmuch as the chromosome 22 deletion is often the only detectable cytogenetic aberration, suggesting that *INI1* inactivation is a relatively early event in rhabdoid tumorigenesis. Additional evidence of an essential tumorigenic role includes the finding of germline *INI1* mutations in some individuals with rhabdoid tumors *(122,123)* and the development of rhabdoid tumors in mice with inactivating *INI1* mutations *(124)*.

Smooth Muscle Tumors

Malignant smooth muscle tumors (i.e., leiomyosarcomas) generally have complex karyotypes, but the most consistent finding has been deletion of the short arm of chromosome 1 *(125,126)* (see **Table 1**). The 1p deletion is not helpful diagnostically, because similar deletions are found in many of the leiomyosarcoma mimics, including malignant fibrous histiocytoma, malignant peripheral nerve sheath tumors, and gastrointestinal stromal tumors *(126)*. The cytogenetic complexity in leiomyosarcomas can be striking even in low-grade specimens (see **Fig. 7**) *(127)*. Benign smooth muscle tumors (leiomyomas), particularly those of uterine origin, contain various translocations and deletions, but generally in the context of a simple karyotype. Approximately 50% of benign leiomyomas lack evident cytogenetic aberrations.

Deletions of the long arm of chromosome 7 are found in 15–25% of uterine leiomyomas, whereas trisomy 12 and rearrangements of the short arm of chromosome 6 are each found in approximately 10–15% of cases *(128–130)*. However, the most distinctive cytogenetic abnormality in leiomyoma is a translocation involving chromosomes 12 and 14 that is found in approximately 20% of uterine cases. This translocation appears to induce overexpression of the *HMGIC* (high-mobility group IC) gene on chromosome 12 by virtue of juxtaposition with the *RAD51* (recombinational repair) gene on chromosome 14 *(131)*.

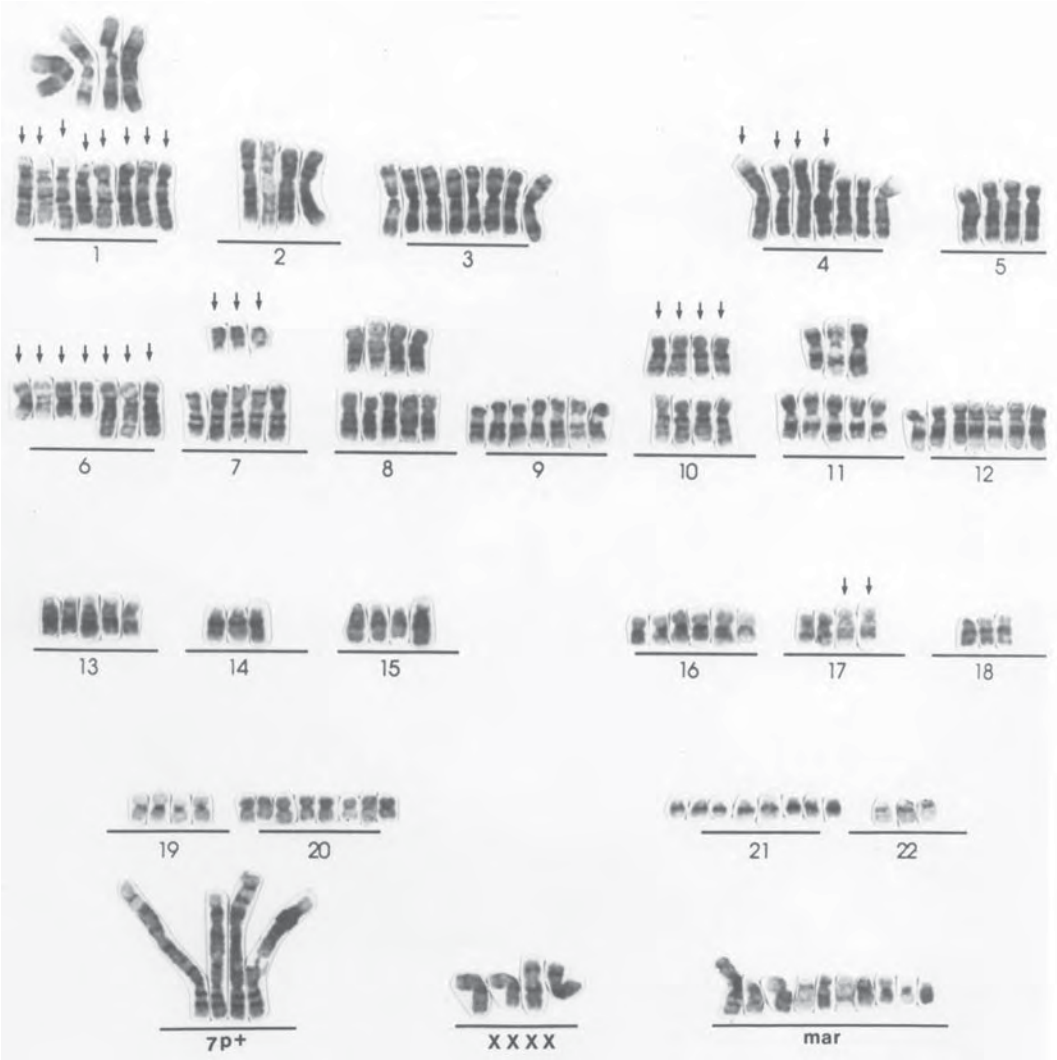

Fig. 7. Karyotype of a low-grade leiomyosarcoma. Arrows indicate clonal chromosome rearrangements that were found in all cells analyzed from this tumor. "mar" is an abbreviation for marker, which indicates an abnormal chromosome of uncertain origin. Chromosome rearrangements not designated by arrows (e.g., the bizarre chromosome 7 rearrangements at lower left) were not present consistently and reflect the genetic heterogeneity in this tumor.

EPITHELIAL TUMORS

Renal Tumors

The clinical relevance of chromosome aberrations in renal tumors is in many respects a paradigm for the potential uses of cytogenetics in other types of solid tumors, such as sarcomas (see above). Consequently, these are among the more common solid-tumor types analyzed in clinical cytogenetic laboratories, and the cytogenetic associations in these tumors will be discussed in detail here.

Characteristic cytogenetic aberrations have been identified in virtually all types of renal cancer (see **Table 2**) and include the ubiquitous deletion of chromosome 3 short-arm material in nonpapillary

clear cell and granular carcinomas *(132–134)* and trisomies of various chromosomes in papillary carcinomas *(135)*. Likewise, most pediatric renal tumors contain characteristic cytogenetic aberrations. Deletion of 11p is a well-known aberration in Wilms tumors, but several other aberrations, including additional copies of chromosome 12, are more frequent and have apparent prognostic relevance *(136,137)*. Another pediatric renal tumor with extremely consistent cytogenetic aberrations is mesoblastic nephroma, in which various chromosomal trisomies are associated with oncogenic fusion of the *TEL* (*ETV6*) and *NTRK3* genes on chromosomes 12 and 15, respectively *(92,93)*.

Renal Carcinomas

Cytogenetic analyses have proven extremely useful in the diagnostic evaluation of various histological subtypes of renal cancer, notably renal carcinomas. These differ from carcinomas in most other organs in that they have relatively noncomplex karyotypes and have distinctive cytogenetic profiles that correlate well with the different renal carcinoma histologies. Indeed, the architecture of renal cell carcinomas is quite variable. Most are composed of sheets of cells, having an abundant, clear, cytoplasm, which are arranged in trabecular or tubular patterns. However, a minority of cases are predominantly papillary, and smaller numbers of renal carcinomas (about 5–15% of the total) have granular, sarcomatoid, or chromophobe histologies *(138)*. Both nonpapillary and papillary renal cell carcinomas are associated with distinctive genetic aberrations, and the following discussion addresses the various histologic subtypes separately.

Clear Cell and Granular Renal Cell Carcinomas

Chromosome 3p deletion is the most frequent cytogenetic aberration in clear cell and granular nonpapillary renal cell carcinomas. This deletion was found in 70–90% of nonpapillary tumors in some series *(132,134)*, and loss of heterozygosity has been confirmed for several regions of 3p in a similar percentage of cases *(139)*. One target of the 3p deletions is the von Hippel–Lindau tumor suppressor gene (*VHL*), located near the telomeric aspect of 3p *(140)*. Von Hippel–Lindau syndrome results from inheritance of a defective *VHL* allele, which predisposes to development of bilateral and multifocal renal cell carcinomas. Furthermore, the *VHL* gene is mutated in at least 50% of sporadic nonpapillary renal cell carcinomas *(141,142)*. Another potential target of the 3p deletions in sporadic renal cell carcinomas is the more centromeric *FHIT* tumor suppressor locus in band 3p14 *(143)*. This gene is interrupted, and presumably inactivated, by a germline chromosomal translocation that is associated with development of renal cell carcinoma in several kindreds *(144)*. Diagnostic evaluation of 3p deletions is helpful in the distinction between papillary and nonpapillary renal cell carcinomas. More than 80% of clear cell and granular nonpapillary renal cell carcinomas have 3p deletions, whereas fewer than 10% of papillary renal cell carcinomas have such deletions. The various 3p tumor suppressor genes are of potential therapeutic relevance, because most nonpapillary renal cell carcinomas have mutations that inactivate genes in this region.

Although deletion of 3p is the most frequent cytogenetic aberration in clear cell and granular nonpapillary renal cell carcinoma, several other nonrandom cytogenetic aberrations participate in the genesis of these tumors. Nonrandom cytogenetic aberrations found in at least 10% of cases include extra copies of 5q, trisomy 7, deletion of 17p, and loss of the Y chromosome *(134)*. The relevance of isolated trisomy 7, or loss of the Y chromosome, has been a matter of contention, because these same aberrations can be demonstrated, at low levels, in non-neoplastic kidney tissues *(145–147)*.

Chromophobe Carcinoma

Chromophobe carcinomas account for approximately 5% of renal cell carcinomas and are characterized by pale reticular cytoplasm, positive reaction with Hale's acid iron colloid, and ultrastructural presence of cytoplasmic microvesicles and dysmorphic mitochondria *(148)*. Most chromophobe carcinomas have extremely hypodiploid karyotypes, containing 31–37 chromosomes and typically including monosomies of chromosomes 1, 2, 6, 10, 13, 17, and 21 *(149)*. Some chromophobe carci-

nomas are difficult to distinguish from oncocytomas and clear cell/granular renal cell carcinomas, and in such cases, demonstration of the typical hypodiploid karyotype can be diagnostic. Extremely hypodiploid karyotypes, particularly those with chromosome counts less than 40, are uncommon in solid tumors generally, and the characteristic group of monosomies found in chromophobe cell carcinomas has not been described in other renal cell cancer histologies.

Papillary Renal Carcinomas

Approximately 10% of all renal carcinomas are papillary, and the cytogenetic profiles for papillary renal cell carcinomas are distinctive. The differential diagnosis of papillary renal carcinoma can be challenging, particularly with respect to distinction from benign renal adenoma or from clear cell carcinoma. Benign renal adenomas are common tumors that are often discovered incidentally by radiography, angiography, or at autopsy. It has been proposed that papillary renal neoplasms smaller than 3 cm be classified as benign adenomas, whereas those larger than 3 cm should be classified as carcinomas. However, size alone does not permit accurate estimation of malignant potential. Distinction between adenoma and carcinoma is further confounded by the observation that kidneys involved by papillary renal cell carcinoma often contain papillary renal adenomas, and the suspicion that papillary renal cell carcinomas arise from the adenomas has been reinforced by cytogenetic studies. Renal adenomas and papillary carcinomas contain a similar core group of chromosome aberrations (–Y, +7, +17), but these are generally the *only* cytogenetic aberrations seen in the adenomas *(150–152)*, whereas papillary carcinomas are apt to have acquired several additional aberrations *(153)*. Hence, cytogenetic studies indicate a continuum of chromosome aberrations in papillary renal cell tumors, with gain of chromosomes 7 and 17 contributing to the early phases of nonmalignant neoplastic progression. Kovacs et al. recommend that renal papillary tumors with isolated trisomies 7 and 17 (with or without loss of a sex chromosome) be classified as papillary renal cell adenomas, irrespective of size *(150)*. The same investigators recommend that papillary renal cell tumors with complex karyotypic aberrations be classified as carcinomas, even when small in size. However, published studies provide little or no clinical follow-up data for these cytogenetic subgroups, and it is not yet known whether karyotypic complexity predicts patient outcome.

The cytogenetic profile can be useful in resolving a differential diagnosis of papillary versus clear cell/granular renal cell carcinoma. Most papillary renal cell carcinomas contain some nonpapillary components, and arbitrary cutoffs are used to define the minimal percentage of papillary components required for diagnosis as true papillary renal cell carcinoma. Reliable distinction between papillary and nonpapillary renal cell carcinoma is important clinically because papillary carcinomas appear to have a better prognosis, stage for stage, than do nonpapillary carcinomas *(154)*. As discussed earlier, the most frequent cytogenetic aberrations in papillary renal cell carcinomas include trisomies of chromosomes 7, 16, and 17, and loss of the Y chromosome; each of these aberrations is found in at least 50% of papillary renal cell carcinomas (see **Fig. 8**) *(155,156)*. Additional nonrandom chromosome aberrations, which are found in 10–50% of papillary renal cell carcinomas, include trisomies 3, 8, 12, and 20 *(150)*. In contrast, clear cell/granular carcinomas rarely have trisomies 16 or 17, but virtually always have a cytogenetic deletion of 3p14–21, which is found in fewer than 10% of papillary renal cell carcinomas *(132,134)*.

Pediatric Renal Cell Carcinomas with Xp11.2 or 6p21 Translocations

Pediatric renal cell carcinomas are uncommon, and they differ from adult cases (which most often have clear cell/granular histology) in that they very often have papillary components. Unlike the adult papillary renal carcinomas, with their distinctive profile of chromosomal trisomies, the pediatric renal carcinomas most often have translocations involving the X chromosome and chromosome 6. These translocations are not restricted to pediatric renal carcinomas, but are also found occasionally in adult papillary carcinomas *(157)*. The unifying theme for the pediatric carcinoma translocations is that they target various members of the MiT family of transcription factors. The most frequent translocation is

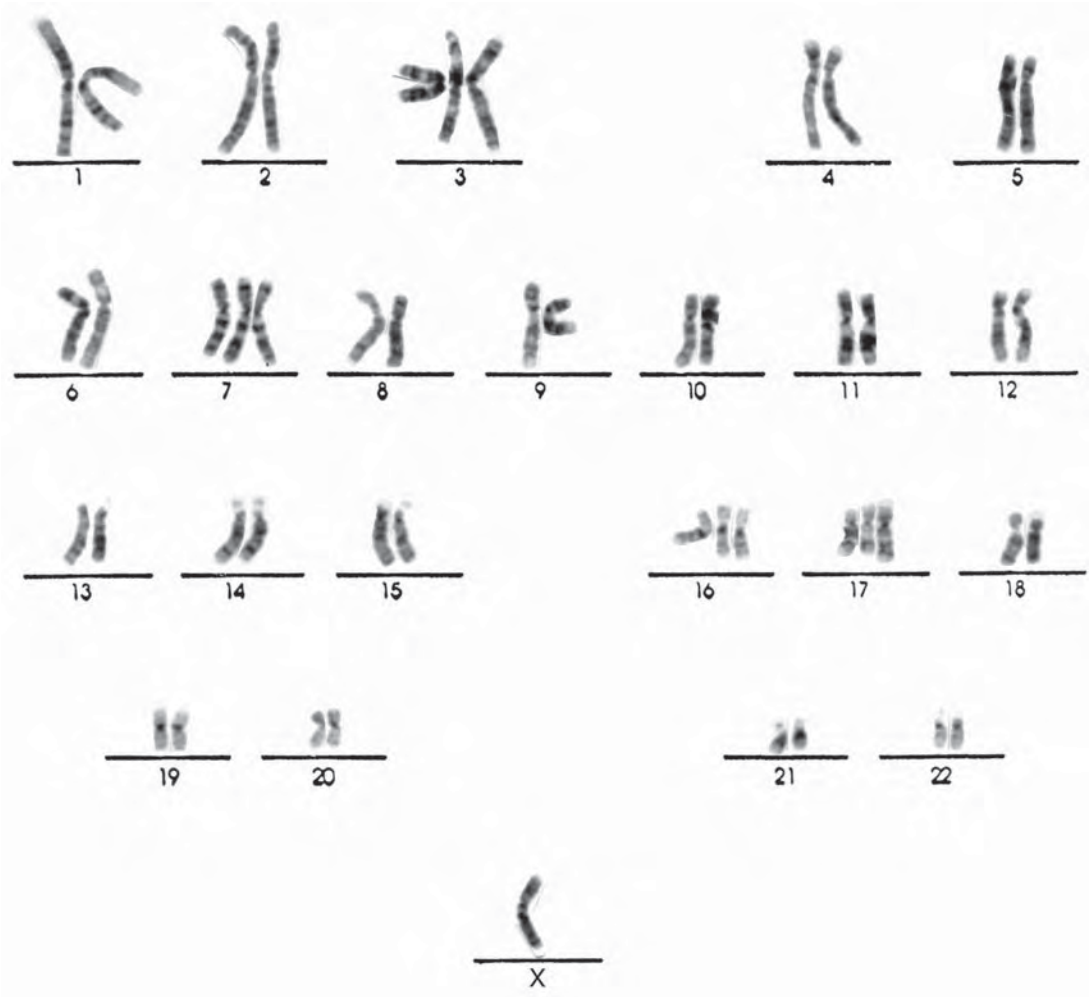

Fig. 8. Karyotype of a papillary renal cell carcinoma, showing typical trisomies for chromosomes 3, 7, 16, and 17.

t(X;1)(p11.2;q21.2), resulting in fusion of *PRCC* (at chromosome band 1q21.2) and the MiT family member *TFE3* (at chromosome band Xp11.2). There are variants of this translocation, including a t(X;17)(p11.2;q25) in which *TFE3* is fused with the *ASPL* gene at chromosome band 17q25 *(158)*. Another pediatric translocation, t(6;11)(p21;q13) fuses the MiT family member *TFEB* (at chromosome band 6p21) with the *alpha* gene (at chromosome band 11q13) *(159)*.

Oncocytoma

Oncocytomas are epithelial neoplasms composed of large cells that are typically eosinophilic because of the presence of abundant mitochondria. These neoplasms are regarded by most as benign and are invariably cured after surgical resection. However, rare cases attain a large size (> 10 cm) and have substantial histologic overlap with "oncocytic" renal cell carcinomas. In such cases, cytogenetics can be useful in securing the diagnosis.

Oncocytomas have distinctive cytogenetic features and they lack the various cytogenetic aberrations found in other types of renal neoplasia. Approximately 30–50% of oncocytomas have loss of an entire chromosome 1 and a sex chromosome (either X or Y) or have translocations involving

chromosome band 11q13 *(160–164)*. Further, oncocytomas lack the 3p deletions seen in clear cell/granular or chromophobe renal cell carcinomas, which are the histologies generally considered in their differential diagnosis.

Wilms Tumor

Wilms tumors are the most common type of renal cancer in children. They typically contain primitive blastemal cells that differentiate into epithelial tubular and/or mesenchymal populations. The classic "triphasic" Wilms tumor contains an admixture of these blastemal, epithelial, and mesenchymal components, and all three cell types can contain the same clonal chromosome aberrations *(165)*. There is no one cytogenetic aberration found across the board in Wilms tumors, but various aberrations are found individually in at least 10% of cases. These include trisomies 6, 8, 12, and 18 and deletions of 11p13, 11p15, and 16q (see **Table 2**) *(137,166,167)*.

Deletion of 11p13 is the most extensively characterized cytogenetic aberration in Wilms tumors. This aberration became the focus of many studies after reports that individuals with the WAGR (Wilms tumor with aniridia, genitourinary malformations, and retardation) syndrome often had constitutional deletions at chromosome band 11p13. The Wilms tumor suppressor gene (*WT1*) is deleted in WAGR syndrome. This gene has been cloned and characterized *(168)* and appears to play a critical role in urogenital development *(168,169)*. However, the WAGR phenotype results from deletions of several genes, with the *WT1* gene deletion responsible for predisposition to Wilms tumors and deletion of a neighboring gene, *PAX6*, responsible for aniridia *(170)*. Although most or all WAGR-associated Wilms tumors have complete inactivation of *WT1*, such inactivation is found in fewer than 20% of sporadic Wilms tumors.

Various cytogenetic features are associated with anaplastic histologic features in Wilms tumor. Complex karyotypes with chromosome counts in the triploid to tetraploid range are generally found in tumors with either diffuse or focal anaplasia *(171)*. Similarly, Wilms tumors with *p53* tumor suppressor gene mutations generally have some degree of anaplasia *(172)*.

Mesoblastic Nephroma

Mesoblastic nephromas are the most common renal tumors diagnosed in neonates, but are rarely encountered in older children. The histology of mesoblastic nephroma is varied. Those composed of bland, benign-appearing, spindle cells are known as "classic" *(173)*, whereas those with more cellularity, mitoses, and necrosis are known as "cellular" mesoblastic nephroma *(174)*. Trisomy 11 is a consistent cytogenetic aberration in mesoblastic nephromas and is generally found only in the "cellular" histology cases *(90,175)*. Approximately 70% of cellular mesoblastic nephromas contain trisomy 11, often accompanied by trisomies for chromosomes 8 and 17 *(90)*. By contrast, clonal chromosome aberrations have not been identified in classic histology mesoblastic nephromas. These findings suggest that trisomy 11, with or without other clonal chromosome aberrations, is associated with progression from classic to cellular histology in mesoblastic nephroma. Mesoblastic nephromas with trisomy 11 generally also contain a balanced translocation, t(12;15)(p13;q26), resulting in oncogenic fusion of the *ETV6* and *NTRK3* genes. t(12;15)(p13;q26) is difficult to detect using standard chromosome banding methods, but it is readily demonstrable by FISH. These cytogenetic associations have been useful diagnostically, because Wilms tumors (which are the entity most often confused with mesoblastic nephroma) rarely have trisomy 11 and have not been found to have the (12;15) translocation *(166,167)*.

BREAST CANCER

Most breast cancers have complex karyotypes, and traditional cytogenetic analysis is not performed generally in the routine diagnostic evaluation of breast cancer. However, several cytogenetic amplifications, particularly those targeting the *MYC* and *ERBB2* (*HER-2/neu*) genes, have assumed prominence in the prognostic and therapeutic evaluation of breast cancer patients. Both *MYC* and

ERBB2 amplification are associated with poor prognosis, and *ERBB2* amplification has special significance in that it identifies a subgroup of patients who will likely benefit from Herceptin (anti-ERBB2) immunologic therapies. The *ERBB2* gene is localized to chromosome 17q and encodes a transmembrane tyrosine kinase receptor protein that is a member of the EGFR or HER family. This family of receptors is involved in cell–cell and cell–stroma communication through a process known as signal transduction, in which external growth factors activate the receptors, thereby resulting in phosphorylation and activation of various intracellular signaling intermediates, many of which possess enzymatic activity.

Although *ERBB2* can be evaluated conveniently by immunohistochemistry in breast cancer biopsies, the FISH technique has some advantages and is viewed by many as the "gold standard" in identifying patients most apt to benefit from Herceptin. FISH evaluations of *ERBB2* amplification have a built-in internal control consisting of the two *ERBB2* gene signals in the non-neoplastic cells in the specimen. Disadvantages of FISH testing include the higher cost of each test, the longer time required for slide scoring, requirement of a fluorescent microscope, the inability to preserve the slides for storage and review, and, occasionally, difficulty in identifying the invasive tumor cells. Two versions of the FISH assay are Food and Drug Administration (FDA)-approved; the Ventana Inform™ test that measures only *ERBB2* gene copies and the Abbott–Vysis PathVysion™ test that includes a chromosome 17 probe in a dual-color format (see Chapter 17, Fig. 14). Published studies indicate that the two assays are highly correlative *(176)*. Chromogenic *in situ* hybridization (CISH) features the advantages of both IHC (routine microscope, lower cost, familiarity) and FISH (built-in internal control, objective scoring, the more robust DNA target), but is not, to date, FDA-approved for selecting patient eligibility for Herceptin treatment *(177,178)*.

PROSTATE CANCER

Prostate cancers contain various recurrent cytogenetic aberrations, of which gain of 8q and deletions of 8p, 10q, and 16q are among the more frequently observed rearrangements *(179–182)*. Presence of 8p deletion is predictive of a poor prognosis, with reduced time to disease progression, and the combined presence of 8p and 16q deletions defines a group with even worse prognosis *(183,184)*. Evaluation of these deletions is performed by FISH, or by loss of heterozygosity (allelotyping) analyses, which can be performed on frozen or paraffin-embedded materials. Although molecular cytogenetics is not used presently in the routine diagnostic or clinical evaluations of prostate cancer, these data suggest that FISH has substantial potential for predictive studies in the future. Conventional karyotyping is seldom possible, because prostate carcinoma cells grow poorly in tissue culture. Therefore, the metaphases obtained from prostate cancer cultures are invariably diploid, having derived from reactive stromal or epithelial cells in the tumor specimen.

BLADDER CANCER

Bladder cancers are among the most frequent adult cancers, and there is strong evidence that many bladder cancers arise following exposure to carcinogens. The cytogenetic profiles in bladder cancer are less well defined than those in the different histologic types of renal cancer. However, trisomy 7 and deletions of several chromosome regions (e.g., 8p, 9q, 9p, and 17p) are found in substantial numbers of bladder tumors *(185–187)*. Genetic aberrations are associated with histologic progression in bladder cancer, and a particularly exciting development in bladder cancer cytogenetics is the use of multicolor FISH probes for detection of clonal chromosome aberrations in exfoliated cells obtained from urine specimens *(188,189)*. These FISH methods can be employed to detect small numbers of clonally aberrant cells in urine specimens, thereby providing the opportunity to screen patients for recurrence of superficial (low stage and low grade) transitional bladder carcinomas, where the neoplastic cells are shed freely into urine. One such assay (Vysis UroVysion™) utilizes FISH probes to score for gains of chromosomes 3, 7, and/or 17 and loss of 9p21 (see Chapter 17, Fig. 15). The need

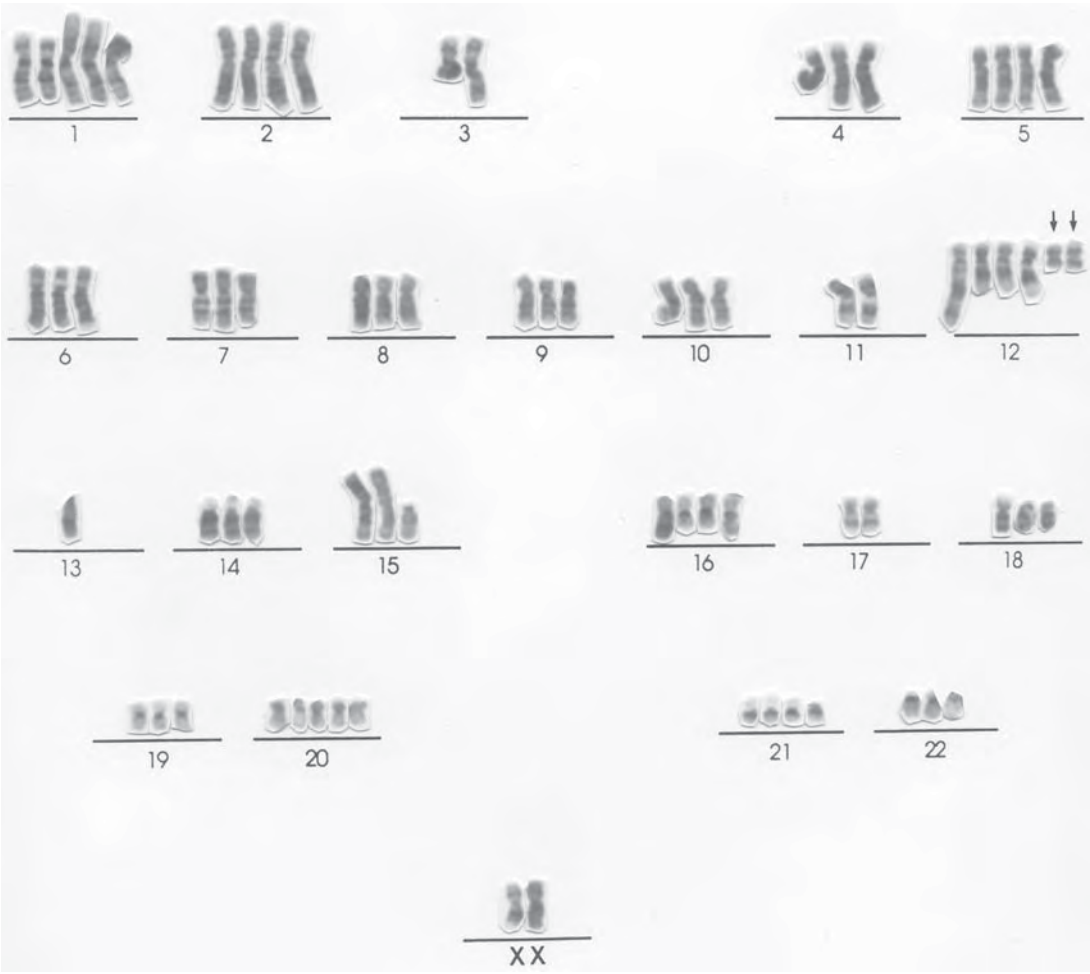

Fig. 9. Complex karyotype in a malignant germ cell tumor, including multiple copies of isochromosome 12p (arrows).

for such assays stems from the fact that neoplastic cells shed from low-grade lesions are often impossible to distinguish cytologically from non-neoplastic uroepithelial cells. Thus, FISH provides a useful adjunct to cytology that can spare patients the discomfort of invasive biopsy procedures.

The diagnostic applications of FISH in bladder carcinoma might not be limited to follow-up of patients with superficial carcinomas. Several bladder cancer risk groups can be identified based on environmental and occupational exposures to carcinogens such as cyclophosphamide, tobacco, and various chemicals used in synthesis of azo dyes and pigments *(190–192)*. Individuals with chronic bladder infection by *Schistosoma haematobium* are also at high risk for bladder cancer *(193)*. Such infections are endemic in Egypt, where bladder carcinoma is the most common malignant tumor, accounting for 20% of all cancers. FISH might a useful diagnostic adjunct in these high-risk groups, particularly in patients with potential signs or symptoms (e.g., unexplained hematuria) of bladder cancer.

BRAIN TUMORS

Distinctive cytogenetic aberrations are found in many of the major subtypes of brain tumor (see **Table 2**), but karyotyping of brain tumors is not performed routinely in most cytogenetics

laboratories. This is, in part, because many brain tumors do not grow well in culture, particularly when the biopsy material is limited in size or in viability. In addition, some of the clinically relevant cytogenetic aberrations are amenable to evaluation by FISH, which can be performed on frozen or paraffin-embedded material once the histologic diagnosis has been established. Examples include 1p and 19q deletions in oligodendroglioma, the presence of which portends a favorable response to multiagent chemotherapy regimens *(194,195)*. Other FISH response predictors have been identified in oligodendroglioma; these include amplification of *EGFR* (epidermal growth factor receptor) at 7p12 and homozygous deletion of *CDKN2A* at 9p21, which correlate with poor response to chemotherapy and reduced survival *(194)*. Molecular cytogenetic response predictors will likely be identified in other brain tumor subtypes. For example, FISH evaluation of *EGFR* amplification in glioblastoma multiforme might be useful in identifying patients who will benefit from small molecule or immunologic therapies that inhibit EGFR *(196)*.

GERM CELL TUMORS

Many germ cell tumors contain a characteristic cytogenetic marker, isochromosome 12p, which is often found in the context of a moderately complex karyotype, with clonal polysomies and rearrangements of various other chromosomes *(197)* (see **Fig. 9**). The isochromosome 12p is uncommon, albeit not unprecedented in carcinomas and sarcomas *(198)*. Therefore, demonstration of isochromosome 12p, particularly in any poorly differentiated cancer, should provoke strong suspicion of a germ cell origin, but should not be taken as *de facto* evidence of such origin. The diagnostic distinction between germ cell and non-germ-cell tumors is relevant clinically, because malignant germ cell tumors often respond well to cisplatin-based (and other) multiagent chemotherapy regimens.

CONCLUSIONS

Most malignant solid tumors have clonal chromosome aberrations that can be identified using cytogenetic methods. Accordingly, a normal karyotype in a malignant solid tumor usually signifies overgrowth by non-neoplastic, stromal, cells. Benign tumors, on the other hand, often have normal karyotypes. Cytogenetic profiles are often diagnostic in sarcomas and in renal cancers but are less useful in certain other solid tumors, particularly those with extremely complex karyotypes or in which the cells grow poorly in tissue culture. The major determinants of success in solid-tumor cytogenetics include: (1) viable starting material, (2) minimal presence of non-neoplastic cells in the cultures, and (3) culture conditions that support growth of the neoplastic cells. These cell culture hurdles can be overcome by performing the analyses on fresh, frozen, or paraffin-embedded tumor (e.g., by FISH).

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V Fluorescence *In Situ* Hybridization

17

Fluorescence *In Situ* Hybridization

Daynna J. Wolff, PhD and Stuart Schwartz, PhD

INTRODUCTION

Dr. Seuss's eloquent "One FISH, two FISH, red FISH, blue FISH" *(1)* could have been describing one of the most significant advancements in clinical cytogenetics, fluorescence *in situ* hybridization (FISH). The process, as described by Pinkel et al. in 1988 *(2)*, involved fluorescent detection of probe DNA hybridized to chromosomal target sequences. The overall hybridization was essentially the same one that had been in use with 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. Standardized nomenclature rules for FISH were published in The International System for Cytogenetic Nomenclature (ISCN 1995; see Chapter 3) and the American College of Medical Genetics (ACMG) has developed standards and guidelines for the use of FISH in clinical laboratory testing (www.acmg.net).

This chapter will focus on the current clinical applications of FISH technologies. Although not every situation can be covered, we have attempted to include tests that are used by the majority of clinical cytogenetic laboratories. Improvements in FISH technology and applications are evolving rapidly and we acknowledge that this chapter will eventually be outdated.

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 a high-temperature incubation in a formamide/salt solution. The probe is applied in great excess, so the kinetics ensure that the probe anneals to the target DNA. Probe detection is accomplished by ultraviolet (UV)-light excitement of a fluorochrome, such as fluorescein-5-thiocyanate (FITC) or rhodamine, which is directly attached to the probe DNA, or by incubation of a hapten (biotin or digoxygenin)-labeled probe with a fluorescent conjugate. (See **Fig. 1**.)

Probes

Given the abundance of sequence data available from the Human Genome Project, probes amenable for FISH procedures can 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

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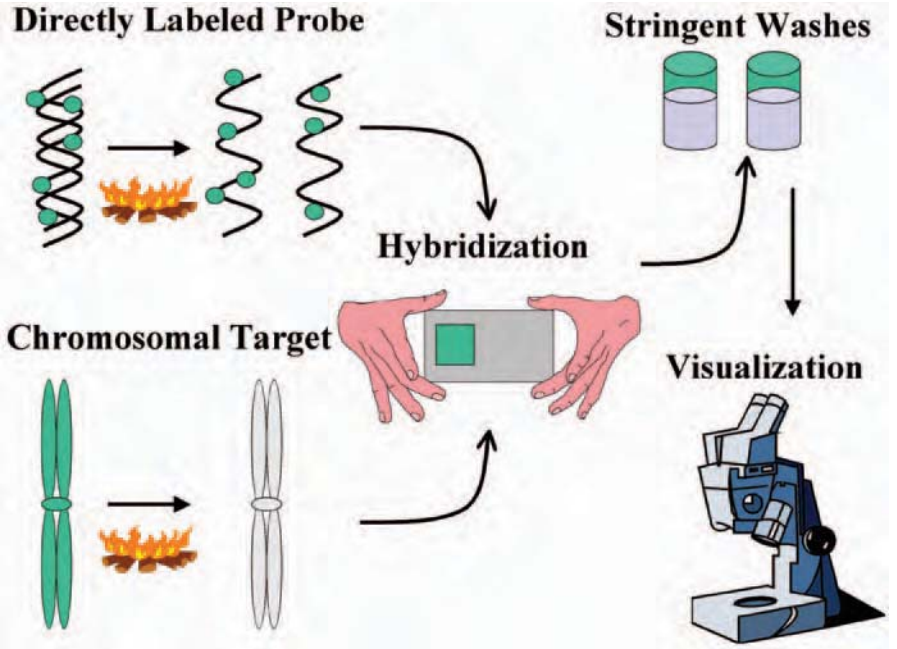

Fig. 1. Schematic representation of the basic steps of the FISH procedure. Both the probe and chromosomal target are heat denatured. Probe sequences hybridize to the complementary target sequences and nonspecific binding is eliminated via stringent washing. The probe hybridization is detected with fluorescence microscopy.

widely used repetitive sequence probes are for the α-satellite sequences located at the centromeres of human chromosomes. α-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 α-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. These probes are particularly useful for the detection of aneuploidy in both metaphase and interphase cells. In addition, α-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 Chapter 15). Other types of repetitive sequences for which probes have been developed include the β-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 they are valuable for the study of structural aberrations.

Whole chromosome probes (WCPs), 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 can 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 polymerase chain reaction (PCR) *(6)*. WCPs are commercially available for each human chromosome and are most frequently used for the study of structural aberrations. For example, WCPs can be used to

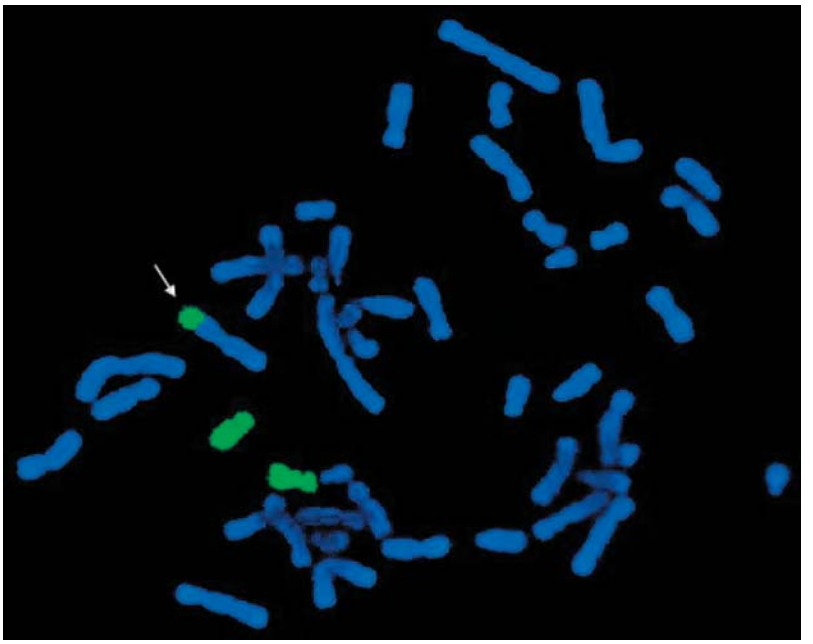

Fig. 2. Characterization of a structurally abnormal chromosome 7 in a patient with an unbalanced translocation. A chromosome 17 library ("painting" probe) was applied to peripheral blood metaphase cells. Both normal chromosomes 17 hybridized entirely, and the unidentifiable material attached to the short arm chromosome 7 (arrow) is also painted. The patient is, therefore, trisomic for most of chromosome 17.

identify the chromosomal origin of additional unknown material of derivative chromosomes and to confirm the cytogenetic interpretation of translocations (see **Fig. 2**).

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 generated using PCR with sequence-specific primers. For probes with extraneous repetitive sequences, Cot-1 DNA is added to the hybridization mixture to block nonspecific binding so that only the unique sequences are visualized. Unique sequence probes, which range in size from approximately 1 kilobase (kb) to >1 megabase (Mb), can 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 3' and 5' regions of the *MLL* gene, allow for detection of cryptic translocations involving this important cancer locus.

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 digoxygenin) 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–strepavidin or digoxygenin–antidigoxygenin. Fluorochromes, such as rhodamine, Texas Red, or fluorescein, are conjugated to the strepavidin or antidigoxygenin and detected on excitation with a fluorescence microscope. Alternatively, directly labeled probes, with the fluorochrome attached to the probe nucleotides, require no secondary detection and are directly visualized after fluorescent excitation.

Specimen Types

Fluorescence *in situ* hybridization (FISH) can be applied to a variety of specimen types depending on 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 nucleus 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 for translocation analysis). In addition to uncultured cells, interphase analysis can also be performed on slides prepared for routine chromosome analysis, paraffin block sections, disaggregated cells from paraffin blocks, and touch preparations of cells from lymph nodes or solid tumors. In cases for which metaphase chromosomes are limited, of poor quality, or unavailable, FISH provides a means for study rather than considering the analysis a failure. Analysis of interphase cells also allows for an increased number of cells to be assessed. However, given that interphase studies cannot be verified by visualization on *in situ* chromosomes, quality assurance is of the utmost importance to ensure correct interpretation of signal patterns.

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 definition of subtle deletions or duplications, identification of marker chromosomes, and characterization of other chromosomal rearrangements.

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 (see **Table 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, which both occur in approximately 1/10,000 individuals, are microdeletion syndromes involving the lack of expression of the maternal or paternal genes, respectively, in 15q11.2–15q13. Seventy percent of cases are the result of a deletion (see **Fig. 3a**). Others causes include uniparental disomy (UPD), imprinting mutations (see Chapter 19), and, for Angelman syndrome, mutations of the *UBE3A* gene. The deletions involve approximately 2–5 Mb of DNA and can 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 *(7,8)*. The reciprocal product of the unequal crossing-over event, resulting in duplications of 15q11-q13, has been associated with autism (see **Fig. 3b**).

Several disorders involving unequal crossing-over mediated by LCRs in the short arm of chromosome 17 are routinely studied by FISH analysis *(9)*. Miller–Dieker syndrome involves the loss of approximately 2 Mb of DNA in 17p13.3 including the *LIS1* (lissencephaly 1 gene) and other gene(s) responsible for the dysmorphic features *(10)*. FISH with a probe for the *LIS1* gene allows for the detection of the Miller–Dieker syndrome deletion and can also be useful for a proportion of cases with isolated lissencephaly. An LCR-mediated mechanism results in a different deletion in the short arm of chromosome 17 [del(17)(p11.2p11.2)], causing Smith–Magenis syndrome, and also in a syn-

Table 1
Microdeletion Syndromes

Syndrome	Deletion	Probe[a]	Phenotype
Angelman	15q11.2–15q13	SNRPN, D15S10	Severe mental retardation; hypotonia; ataxia; lack of speech; hypopigmentation; seizures; inappropriate laughter; dysmorphic features
DiGeorge	22q11.2	D22S75	Dysmorphic features, congenital heart disease; absence of thymus; growth failure; cognitive deficits
Miller–Dieker	17p13.3	LIS1	Severe mental retardation; lissencephaly; dysmorphic facial features
Prader–Willi	15q11.2–15q13	SNRPN	Mental retardation; hypotonia; feeding difficulty; genital hyperplasia; obesity; hyperphagia; dysmorphic features
Smith Magenis	17p11.2	SHMT1, TOP3, FLI1, LLGL1	Mental retardation; speech delay; bizarre behavior; peripheral neuropathy; dysmorphic facial features
Velocardiofacial	22q11.2	TUPLE1	Delayed development; pharyngeal deficiency; abnormal facies; palatal defects; congenital heart defects
Williams	7q11.2	ELN	Mental retardation, hypercalcemia; elfin facies; gregarious personality; congenital heart disease

[a] The FISH probes used to diagnose the syndrome are listed in this column and are all commercially available.

drome resulting from a duplication of this region [dup(17)(p11.2p11.2)] *(11)*, both of which can be diagnosed using FISH with probes from the critical region. Interphase FISH with a probe for a 1.4 Mb area of 17p12 is 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 1/2000 to 1/3000 individuals. Because of the frequency of this syndrome and its association with congenital heart disease, fetuses and newborns with a heart defect are routinely be studied for a 22q deletion. This syndrome, in contrast to other microdeletion syndromes, is inherited in about 10% of the cases. Therefore, FISH studies of parents of an affected individual are recommended. The deletions involve approximately 2 Mb of DNA and are easily detected by FISH with the TUPLE1 probe or a probe for the DNA segment D22S75. The majority of these deletions, which are also mediated by repetitive duplicated regions, occur at the same proximal and distal breakpoint *(12)*. Duplications of 22q11.2 are associated with dysmorphic features, growth failure, cognitive deficits, hearing loss, and velopharyngeal insufficiency (*13*).

Williams syndrome involves the loss of the 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 cannot be detected by G-banding, but it 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, dsymorphic facial features, a gregarious personality, premature aging of the skin, and a congenital heart disease (supravalvular aortic stenosis) *(14)*.

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. Translocations or insertions involving segments below this threshold may be visualized with M-FISH (see below). In particular, recognition

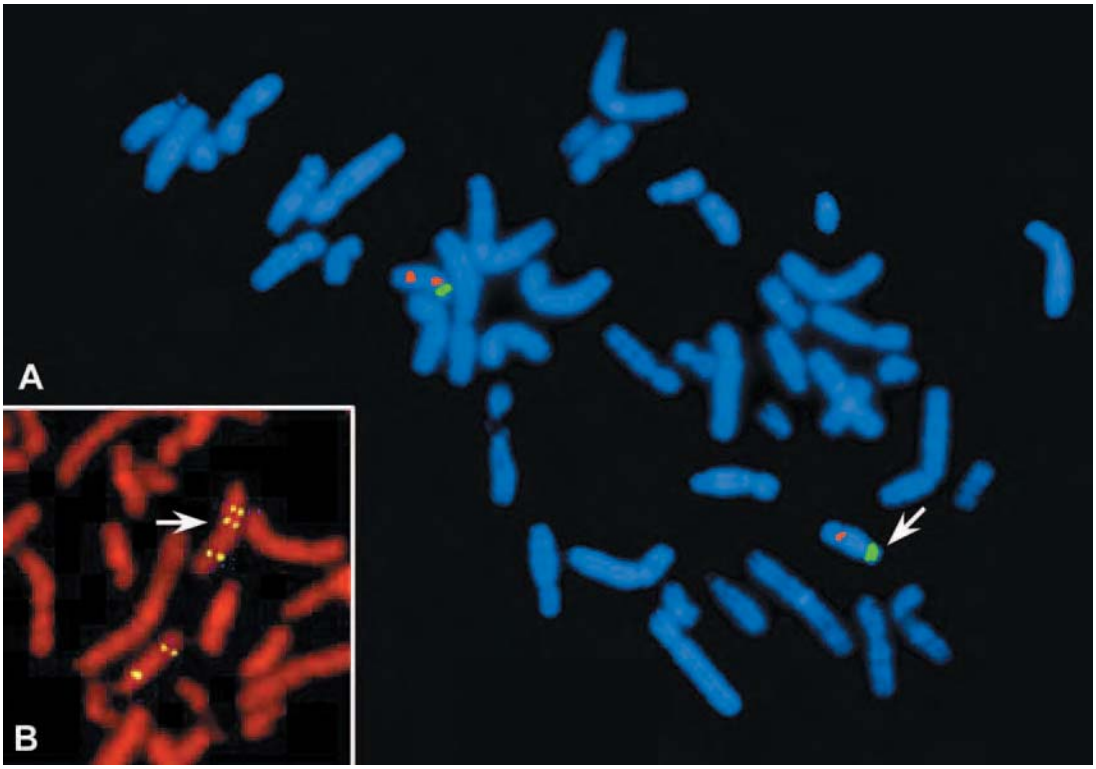

Fig. 3. Example of FISH to a single-copy target using a cosmid (SNRPN) to the Prader–Willi "critical region" localized to 15q11-13. **(A)** A metaphase in which 1 normal chromosome 15 has 3 hybridization signals from a centromeric control probe (green), a distal control probe (red), and a probe to the 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 control probe (both red) were utilized. 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.

of abnormalities in the telomeric regions that are not visualized well with G-banding and were historically studied with R- or T-banding, are difficult. Given that these regions are gene rich, they have particular relevance for clinical studies.

Located immediately proximal to the terminal telomeric repeated DNA segment is a telomere-associated repeat (TAR). Homologous recombination within both the telomeric regions and TAR can cause deletions and duplications of the adjacent unique DNA sequence (subtelomeric sequences). FISH probes consisting of unique sequences of DNA from the subtelomeric region approximately 100–300 kb from the end of each human chromosome have been developed, with a few exceptions; there are no probes for the individual acrocentric short arms and Xp and Yp share similar sequences, as do Xq and Yq. Subtelomeric probes are available individually or, as described by Knight et al. *(15)*, as part of a "multiprobe coverslip device." In this system, all of the probes are placed onto 24 squares on a device that is hybridized to cells on a single slide (see **Fig. 4**).

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%) *(16,17)* (see **Table 2**). The majority of studies of abnormalities in the subtelomeric regions

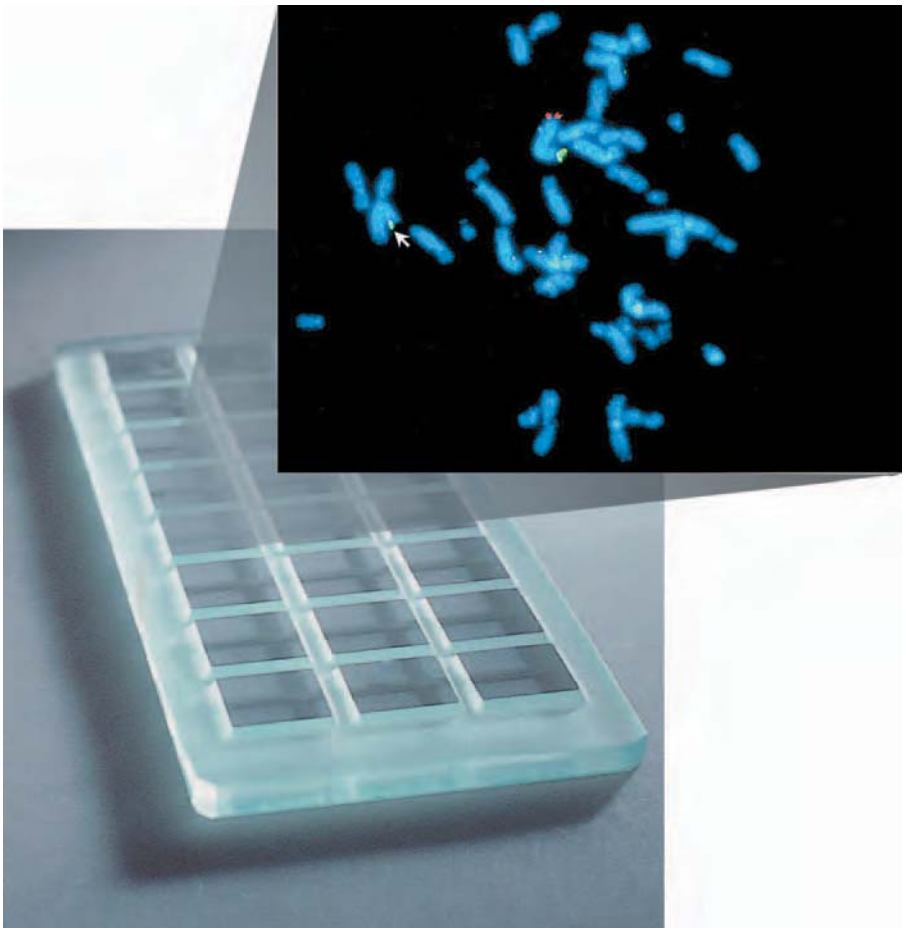

Fig. 4. The Cytocell Chromoprobe Multiprobe-T System contains lyophilized probes for each of the chromosome-specific subtelomeric regions on each of the 24 raised squares of the device. Hybridization of the device to metaphase spreads from a patient with a diagnosis of autism revealed two green signals at the 2p subtelomeres and a single red signal consistent with a subtelomeric deletion of 2q (arrow).

have been performed using FISH and, in general, these studies have confirmed the efficacy of using subtelomeric probes to study 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, telomeric regions involved in small terminal deletions detected cytogenetically are also commonly detected by subtelomeric FISH. These areas of involvement include 1p, 1q, 2q (see **Fig. 4**), 8p, 10q, and 22q *(18–20,25)*.

Even if the precise frequency of cryptic rearrangements has not been established, subtelomeric FISH studies are widely used in the cytogenetics laboratory. A five-item checklist has been developed to improve detection rates of subtelomeric aberrations *(26)*, with the most significant items being prenatal onset of growth retardation and a positive family history of mental retardation. In addition, patients with moderate to severe mental retardation have a higher frequency of subtelomeric abnormalities, as do patients that have dysmorphic features in addition to mental retardation.

Table 2
Subtelomeric Studies

Study (ref.)	No. studied	Ascertainment	Frequency
Knight et al. (1999) *(18)*	284	Moderate to severe retardation	7.4%
	182	Mild retardation	0.5%
Rossi et al. (2001) *(21)*	200	Idiopathic mental retardation	6.5%
Joyce et al. (2002) (16)	200	Unexplained developmental delay	0%
Clarkson et al. (2002) *(23)*	50	Idiopathic mental retardation	4%
Riesel et al. (2001) *(19)*	252	Mild to severe mental retardation	5.2%
Anderlid et al. (2002) *(23)*	50	Idiopathic mental retardation	9%
Jalal et al. (2003) *(20)*	372	Idiopathic mental retardation	6.8%
Baker et al. (2002) *(24)*	53	Idiopathic mental retardation (isolated)	1.9%
	197	Idiopathic MR (dysmorphic features)	4.1%
Popp et al. (2002) *(17)*	30	Unexplained developmental retardation	13.3%

Duplications and Marker Chromosomes

Characterization of *de novo* duplication and marker chromosomes has valuable implications with respect to phenotype/karyotype correlation. FISH is the optimal method for such studies.

Approximately 70% of chromosomal duplications are intrachromosomal, whereas 30% involve a nonhomologous chromosome *(27)*. Thus, for the majority of cases, FISH with a single chromosomal library or locus-specific probe from the chromosome with the abnormality will confirm the origin of the duplicated material. There are two basic ways to approach the identification of extra nonhomologous (interchromosomal) material: initial recognition by G-banding and subsequent confirmation with a chromosomal library or locus-specific probe, or multicolor FISH (M-FISH) (see the section Specialized and Evolving FISH Technologies, below).

With quality high-resolution chromosome studies, the suspected origin of the majority of interchromosomal duplications might be limited to a few chromosomal regions. Thus, duplications usually are confirmed with FISH for one to four chromosomal libraries (see **Fig. 5**). If there is no "best guess" based on the banding studies, M-FISH (see below) can be performed to identify the material's chromosomal origin. In many cases, once the chromosomal origin is determined by M-FISH, the cytogeneticist can then re-examine the G-banding pattern and determine the bands involved. However, additional probes might be needed to resolve the exact duplication. Alternatively, pan-subtelomeric probes may be used to ascertain the chromosomal arm from which the material originated.

Chromosomes that are unidentifiable by routine banding are termed "markers" (see Chapters 3 and 8). Marker chromosomes represent a heterogeneous group and are typically extra structurally abnormal chromosomes (ESACs). The most common types of marker, for which clinical phenotypes have been defined, can be fully characterized using FISH (see **Table 3**). Other types of markers can be partially defined by FISH, and the impact of these chromosomes on the clinical phenotype often cannot be reliably predicted.

In general, there are two basic methods for delineating the chromosomal origin of marker chromosomes. These include identification by using M-FISH or utilizing individual chromosomal libraries or α-satellite DNA probes. M-FISH can identify the chromosomal origin of many markers. However, this methodology will not always allow for an understating of the implication of the markers, as the exact chromosomal region of origin cannot be determined with these methods. An alternative approach is to initially use a pancentromeric probe to determine if α-satellite DNA is present in the marker and then to use chromosome-specific α-satellite probes to establish the origin. Although analphoid markers are rare, if there is not α-satellite DNA present as determined by the lack of signal using the

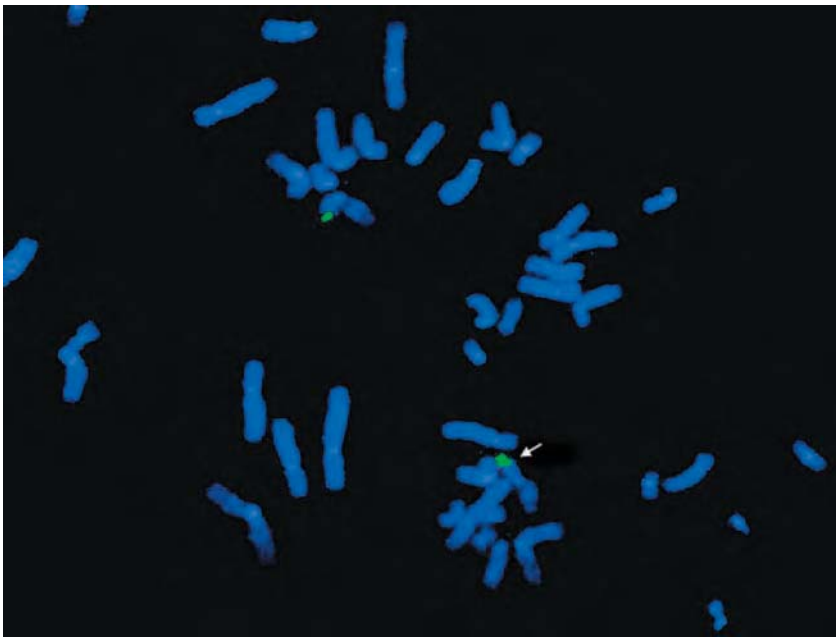

Fig. 5. Partial metaphase spread from a patient with a duplication involving chromosome 11. A BAC localized to chromosome 11p15.5 produced one signal on the normal chromosome 11 and a double signal on the duplicated chromosome 11 (arrow). The duplication in the short arm of chromosome 11 was detected in a newborn that was large-for-gestational age. The infant also had an omphalocele and was diagnosed with Beckwith–Wiedemann syndrome.

pancentromeric probe, there is a high chance for an abnormal phenotype. The identity of markers lacking α-satellite DNA can be determined using a combination of FISH with chromosomal libraries along with high-resolution G-banded analysis.

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 Chapter 3) and should be studied with α-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 not associated with an abnormal phenotype, whereas a monocentric, monosatellited chromosome could be. If a satellited marker is derived from a chromosome 15, *SNRPN* status should be determined (see **Fig. 6**). If *SNRPN* is present, the karyotype would be associated with an abnormal phenotype *(30)*.

Sex chromosome markers are usually found in an individual who has 46 chromosomes, with only 1 normal X and a marker chromosome in place of a second sex chromosome. These abnormal chromosomes should be initially studied with X and Y α-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 Chapter 10). If *XIST* is absent, the phenotype will likely be associated with mental retardation/developmental delay *(31)*. If the marker originates from a Y chromosome, it should be studied with a probe for *SRY* to better understand its effect on the phenotype.

The last category of markers is comprised of ring or marker chromosomes that cannot be placed into any of the other groups. M-FISH or FISH along with each α-satellite probe is useful for

Table 3
Marker Chromosome Assessment

Type of marker	FISH probe result	Associated syndrome/phenotype[a] (estimated risk for abnormality)*
ESAC	Pancentromeric, no α-satellite	High risk for abnormality; phenotype dependent on euchromatin present
Bisatellited/ monocentric	α-sat +: 13/21, 14/22, 15	General risk for bi-sat = 11%
idic(15)	SNRPN–positive	~0% risk
	SNRPN–negative	95%–MR
idic(22)	ATP6E–present	5%–MR (usually resulting from UPD) Cat-eye syndrome
Monosatellited	α-sat +: 13/21, 14/22	No general risk, dependent on whether euchromatic material present
Nonsatellited	α-Satellite present	General risk for nonsatellited = 11%
metacentric	α-Satellite present for 8, 9, 12, or 18 centromere	If metacentric, risk for MR = ~100%
Sex chromosome	DXZ1 (X centromere) +	
	XIST–positive	Turner syndrome only > 95%
	XIST–negative	Majority–MR
	DYZ3 (Y centromere)	
	SRY–positive	Male phenotype
	SRY–negative	Female phenotype

[a] Data from refs. *28* and *29*.

determining the chromosomal origin of such markers. However, this information does not usually allow for specific clinical risk estimations for genetic counseling (see Chapter 20). In a research setting, once the origin of the marker has been determined, single-copy probes in both the p and q pericentromeric regions can be studied to assist in karyotype/phenotype correlations.

Prenatal Studies

Fluorescence *in situ* hybridization has been widely used for the detection and analysis of prenatal chromosomal abnormalities (see Chapter 12). One major advantage of FISH technology is the ability to study uncultured material to produce a rapid result. In addition, FISH is useful for characterizing or detecting 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. 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 will ascertain numerical abnormalities for these chromosomes (see **Fig. 7**) and will also detect triploidy.

In 1992, Klinger et al. *(42)* first demonstrated the feasibility of detecting aneuploidy in uncultured amniocytes by using FISH in a prospective study. They constructed probes derived from specific subregions of chromosomes 13, 18, 21, X, and Y and analyzed 526 samples in a blind fashion. All 21 abnormal samples were correctly identified in this study. Since this initial work, a number of studies have validated this approach (see **Table 4**.)

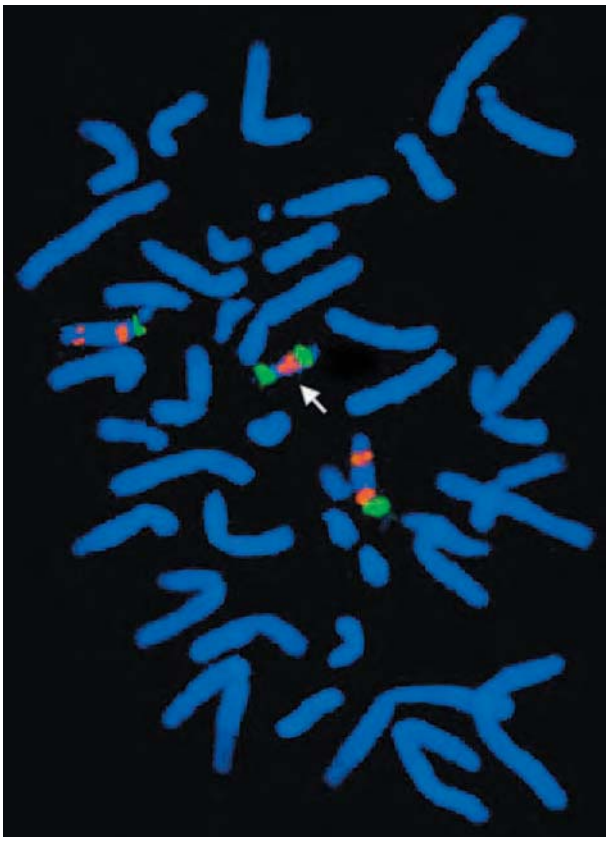

Fig. 6. A dicentric chromosome hybridized with dual-color chromosome 15 probes, including both an α-satellite DNA probe (green) along with a single-copy *SNRPN* probe (red). Signals from both probes are present on the normal chromosomes 15. The marker chromosome (arrow) has two α-satellite DNA signals, 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, not on the marker chromosome. This abnormality was ascertained in a 6-year-old female with hypotonia, behavior difficulties, learning problems, and autism.

A number of technological advances led to improvement of the initial prenatal FISH studies. These include a direct labeled probe set consisting of three repetitive DNA probes (18, X, and Y) and two single-copy DNA probes localized to the long arms of chromosome 13 and 21, and significantly improved methods for preparing uncultured cells for analysis.

Although this technology can provide answers within 24 hours of obtaining a sample, it is limited in that it can only detect aneuploidies for a limited number of chromosomes (13, 18, 21, X, and Y). In a 5-year collaborative study, a total of 146,128 amniocenteses were performed revealing a total of 4163 abnormalities; however, only 69.4% of these would have been detected using interphase analysis of uncultured amniotic fluid cells *(41)*. A similar number (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 detection rate 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. *(38)* reported on a 2-year multicenter retrospective analysis and review of literature of the AneuVysion assay (Vysis, Inc.). Of the 29,039 studies they were able to document, there was

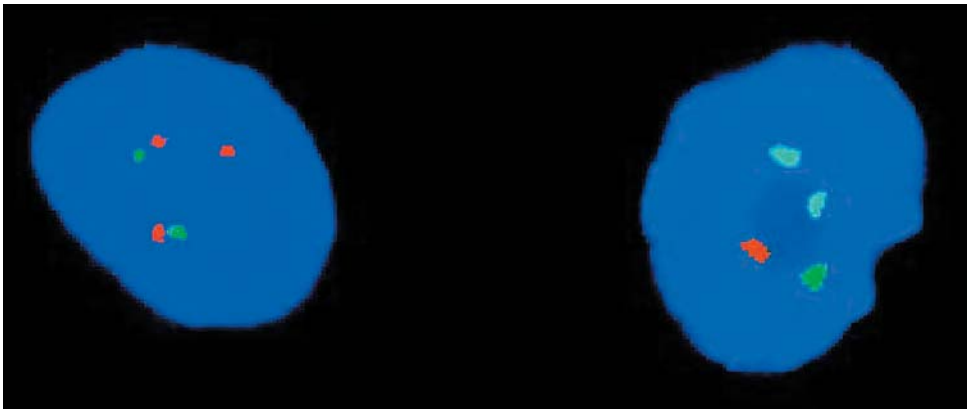

Fig. 7. Prenatal ploidy analysis utilizing Vysis AneuVysion analysis of uncultured amniotic fluid cells using unique copy probes for the long arms of chromosomes 13, 18, 21, X, and Y. The results in these interphase cells are consistent with a XY fetus with trisomy 21. **Left:** Probes for chromosomes 13 (two green signals) and 21 (three orange signals); **right:** Probes for chromosomes 18 (two aqua signals), X (green signal), and Y (orange signal). Nuclei are counterstained blue with DAPI.

Table 4
Prenatal Ploidy Analysis

Study	No.	False (+)	False (–)	Uninformative
Ward et al. (1993) *(32)*	4500	0.1%	0.2%	6.1%
Mercier et al. (1995) *(33)*	630	0	(1) 0.2%	
Bryndorf et al. (1997) *(34)*	2000	0	0	7%
Jalal et al. (1998) *(35)*	508	0	0	
Eiben et al. (1999) *(36)*	>3000	0	0	
Weremowicz et al. (2001) *(37)*	911	(1) 0.1%	(5) 0.5%	3.0%
Tepperberg et al. (2001) *(38)*	5197	(1) 0.003%	(7) 0.024%	
Sawa et al. (2001) *(39)*	2639	0	0	6.0%
Witters et al. (2002) *(40)*	5049	0	0	0.26%

only 1 false-positive (0.003%) and 7 false-negative (0.024%) results. They 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 because of maternal screening results or ultrasound findings. Because 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: (1) FISH results, (2) routine chromosome analysis, and (3) clinical information (e.g., ultrasound examination).

Although the vast majority of prenatal aneuploid analyses have been on amniotic fluid cells, other cell types have been utilized including chorionic fluid cells, in vitro fertilization (IVF) specimens, and fetal cells found in maternal serum. Studies on CVS cells have been few because of the ability to perform a 24-hour chromosome analysis (see Chapter 12), however, data from these limited studies provide results similar to the amniotic fluid studies.

Preimplantation/Embryo Studies

Preimplantation genetic diagnosis (PGD) is the early diagnosis of genetic disorders, prior to the onset of pregnancy. Embryos or oöcytes 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 can 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 *(43)*. As with prenatal diagnosis, the common probes used for ploidy assessment are for chromosomes 13, 18, 21, X, and Y. FISH with subtelomeric probes is useful for PGD of translocations when one of the parents is a known carrier.

Although FISH is the most widely used method for PGD for some genetic diagnoses, there are several limitations with this technology *(43)*. 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 to the study of five chromosomes because of the restricted number of fluorochromes and the need to eliminate technical artifacts (overlapping signals) in a single cell. However, for couples with a high risk of having a child with a genetic disease, PGD using FISH is valuable for 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 Chapter 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 (see **Fig 8**).

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 Chapters 15 and 16). Probes have been developed for the majority of recurrent aberrations found in hematologic malignancies, and the National Cancer Institute (NCI) has undertaken an endeavor to produce resources for the genetic study of solid tumors. Cancer-specific FISH probes and their characteristics are presented in **Table 5**. Several of these diseases and appropriate probes are discussed in detail below.

Acute Myelogenous Leukemia

Approximately 40–60% of acute myelogenous leukemia (AML) patients exhibit genetic aberrations that are easily detected by FISH, and in 2001, the World Health Organization (WHO) established an AML classification system that was based on recurrent genetic abnormalities *(45)* (see also Chapter 15). For each category, classical cytogenetics identifies the majority of aberrations; however, FISH can be used to detect cryptic abnormalities and variant rearrangements and to monitor disease states during and following treatment.

The t(8;21) juxtaposes the *AML1* gene on chromosome 21 and the *ETO* gene on chromosome 8. A dual-color, dual-fusion (DCDF) probe has been developed to detect the fusion products on the derivative 8 and the derivative 21 chromosomes (see **Fig. 9**). Similarly, a DCDF probe can be used for AML with t(15;17) in which there is a juxtaposition of the retinoic acid receptor-α (*RARα*) gene at 17q12 and the *PML* (promyelocytic leukemia) gene at 15q22. 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 hours) of the *PML/RARα* fusion is of utmost importance, so that patients can begin 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, because patients with variant translocations do not respond to ATRA treatment. The t(11;17) and other *RARα* variants can be identified with a *RARα* break-apart probe.

Acute myelogenous leukemia with inv(16)(p13q22) or t(16;16)(p13;q22) results from the fusion of the core-binding factor-β (*CBFβ*) gene at 16q22 to the muscle myosin heavy chain (*MYH11*) at

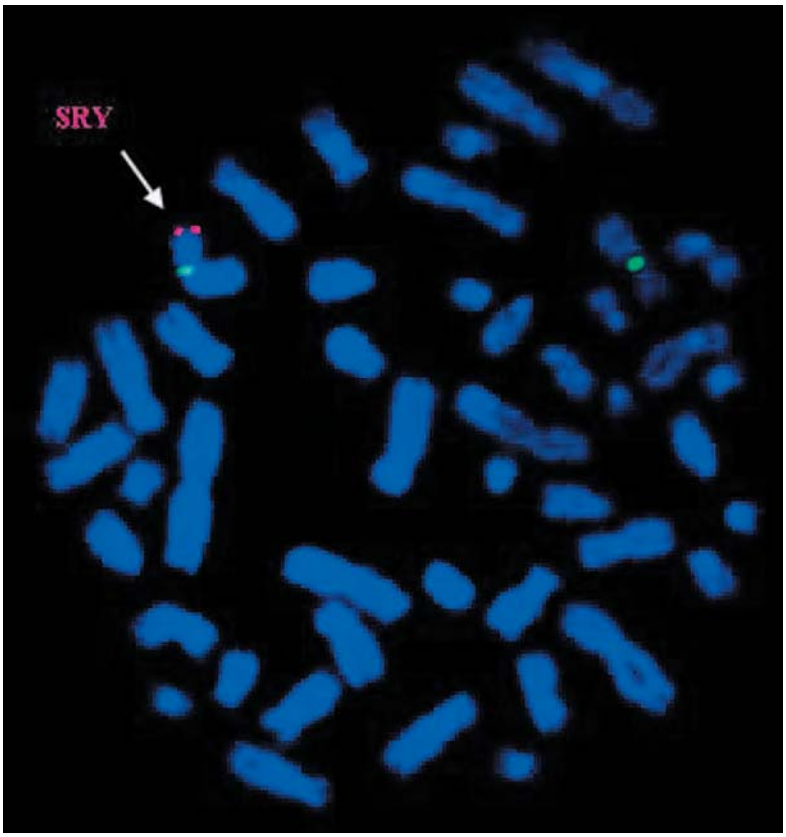

Fig. 8. Metaphases from an XX sex-reversed male were hybridized with probes for the X centromere (green) and a probe for the *SRY* gene (red). Results demonstrated a cryptic translocation in which *SRY* was present on the short arm of one X chromosome. Chromosomes were counterstained blue with DAPI.

p13. The fusion product interferes with the core-binding transcription pathway that is needed for normal hematopoiesis. Break-apart (BAP) FISH probes have been developed that bind to the 3' and 5' regions of the *CBFβ* 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. Over 30 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 (see **Fig. 9**). In addition, the BAP allows for the assessment of copy number of *MLL* to determine if deletions or duplications of the gene have occurred.

Table 5
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—*ABL* 22—*BCR*	CML, ALL, AML	DCDF, DCSF, DCES, FCDF
t(15;17)(q22;q21.1)	15—*PML* 17—*RARα*	AML	DCDF, DCSF, BAP
t(∗;11)(∗.∗; q23)	11—*MLL*	ALL, AML	BAP
t(8;21)(q22;q22)	8—*ETO* 21—*AML1*	AML	DCDF
inv(16)(p13q22) or t(16;16)(p13;q22)	16q22—*CBFβ*	AML	BAP
t(12;21)(p13;q22)	12—*TEL* 21—*AML1*	ALL	DCES
Trisomy 8	8—8cen	AML, CML	SC
t(8;14)(q24;q32)	8—*MYC* 14—*IGH*	ALL, NHL	DCDF
t(11;14)(q13;q34)	11—*CCND1* 14—*IGH*	NHL, MM	DCDF
t(14;18)(q32;q21)	14—*IGH* 18—*BCL2*	NHL	DCDF
t(∗;14)(∗.∗;q32)	14—*IGH*	NHL, MM	BAP
Del(13)(q14) or –13	Unknown tumor suppressor	CLL, MM	SC, PP
Trisomy 12	12—12cen unknown gene(s)	CLL	SC, PP
del(11)(q23)	11—*ATM*	CLL	SC, PP
del(17)(p13.1)	17—*TP53*	CLL, MM, NHL	SC, PP

[a] An asterisk (*) is used to delineate multiple loci or breakpoints.
[b] Abbreviations: ALL = acute lymphoid leukemia; AML = acute myeloid leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; NHL = non-Hodgkin's lymphoma; MM = multiple myeloma
[c] Abbreviations: 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 (see **Fig. 8**).

Chronic Myelogenous Leukemia

The t(9;22)(q34;q11.2) fuses the 5' region of the *BCR* (breakpoint cluster region) gene at 22q11.2 to the 3' region of the Abelson (*ABL*) oncogene at 9q34, producing a novel protein with tyrosine kinase activity. Multiple commercial FISH probe combinations are available to detect the *BCR/ABL* fusion *in situ*, including a dual-color, single-fusion (DCSF) probe set that detects *BCR/ABL* on the "Philadelphia chromosome" [der(22)] (see **Fig. 9**), a dual-color, single-fusion with an extra signal (DCES) probe set that detects the der(22) *BCR/ABL* fusion and a residual signal on the der(9) (see **Fig. 9**), a dual-color, dual-fusion probe set that detects the fusion products on both derivative chromosomes (see **Figs. 9** and **10**) and a four-color, dual-signal exchange probe set (F-FISH) that detects the translocation products on both derivative chromosomes and allows for identification of the derivatives in interphase cells (see **Fig. 11**) *(46)*. Each probe set is useful for identifying the *BCR/ABL* fusion event in diagnostic samples. However, the ES and both the two-color, dual-fusion and four-color, dual-signal exchange probe sets offer increased sensitivity for posttreatment residual disease detection because the abnormal signal patterns produced by the latter probes rarely occur by random chance. These are particularly useful for detection of the nearly 20% of patients with a t(9;22)(q34;q11.2)

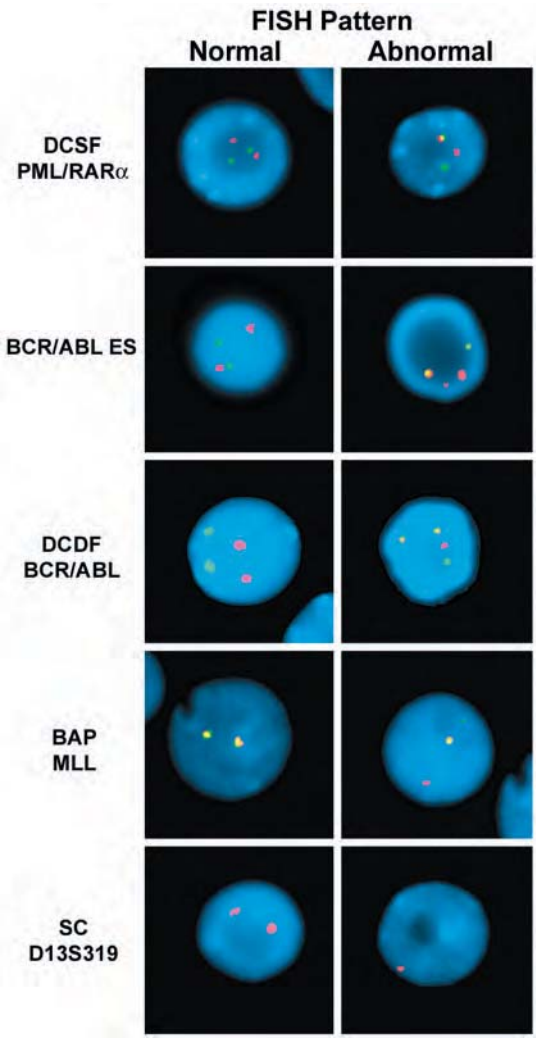

Fig. 9. Examples of normal (column A) and abnormal (column B) results for hematologic malignancies with various FISH probe types. The probe type and a chromosomal abnormality exemplifying typical results are given.

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 *ABL* 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) is associated with a relatively poor prognosis and reduced response to treatment *(48–50)*, with average long-term survival reduced from 88 months to 38 months. FISH is the optimal methodology for the detection of this clinically relevant deletion, as it is not visible with routine cytogenetics. An additional probe (for the argininosuccinate synthetase gene [*ASS*] at 9q34) is often utilized to eliminate the possibility of false positives (see **Fig. 10**) *(51)*. Alternatively, use of the four-color, dual-signal exchange probe set allows for definitive identification of deletions and other unusual rearrangements involving the derivative chromosomes (see **Fig. 11d, e**).

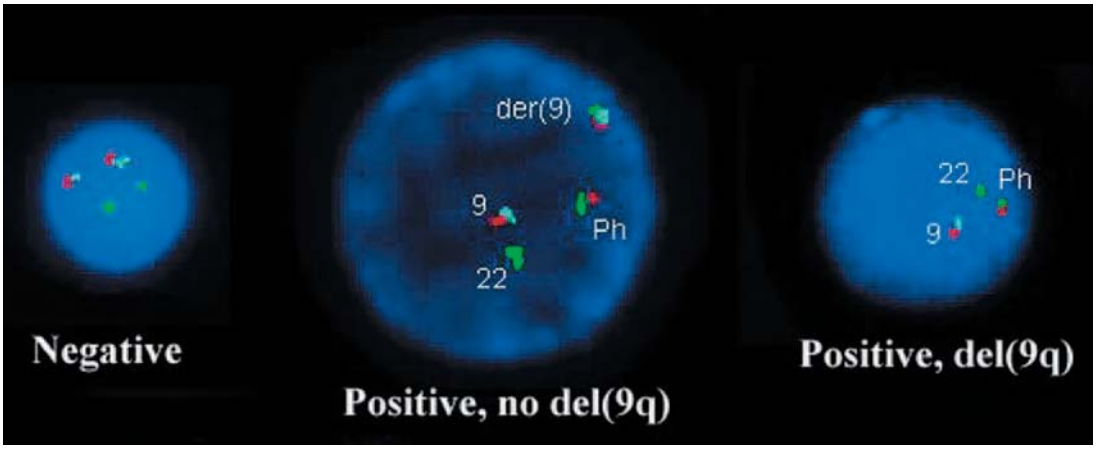

Fig. 10. Detection of the deleted der(9) associated with decreased long-term survival in chronic myelogenous leukemia, using dual-color, dual-fusion *BCR/ABL* probes along with a probe for the argininosuccinate synthetase gene (*ASS*) on 9q34 (Vysis, Inc.). The *ASS* probe is labeled with an aqua fluorophore and hybridizes adjacent to *ABL* on 9q. **Left:** An example of the hybridization pattern seen in a normal cell. Two green *BCR* signals and two red *ABL*/aqua *ASS* signals represent the two chromosomes 22 and 9, respectively. **Center:** A positive cell with no deletion of the der(9). The normal chromosomes 9 and 22 appear as in the left, the "Philadelphia" chromosome (Ph) results in a *BCR/ABL* fusion, and the derivative chromosome 9 produces all three signals. **Right:** A positive cell with a deletion of the der(9). In this case, the deletion is large enough to result in loss of all signals on the der(9). Without the additional probe, it would not be possible to determine which derivative chromosome was deleted. In other cases, loss of the aqua signal only or other signal combinations facilitate the same interpretation.

Acute Lymphocytic Leukemia

Routine cytogenetic studies for acute lymphocytic leukemia (ALL) often produce suboptimal preparations resulting in decreased abnormality detection rates. Therefore, FISH is a useful and necessary adjunct to routine testing. Many clinical laboratories offer an ALL FISH screening panel assessment that might include probes for t(9;22) (*BCR/ABL)*, *MLL*, t(12;21), *MYC*, and a common ALL-associated deletion in 9p21-22 *(52,53)*. The panel of probes will detect the majority of genetic aberrations associated with ALL, particularly because the screen is also useful for unmasking hidden hyperdiploidy when multiple signals are seen for various probes.

Similar to *MLL* (see above), the *MYC* gene at 8q24 is involved in several translocations that result in the dysregulation of the gene. A break-apart *MYC* probe is useful for detecting rearrangements that are often associated with juxtaposition of *MYC* and immunoglobulin genes on chromosomes 14, 2, and 22 for B-cell ALL or the T-cell receptor genes for T-cell ALL.

t(12;21) brings the *TEL* gene at 12p13 adjacent to the *AML1* gene at 21q22. This translocation is present in approximately 30% of childhood pre-B-ALL and 3–4% of adult ALL. t(12;21) cannot be detected by standard cytogenetics unless a more complex rearrangement is present. Therefore, FISH with probes for *TEL* and *AML1* provides a definitive diagnostic assay, as well as a means for treatment monitoring, for this subgroup of patients (see **Fig. 9**). Any patient diagnosed with pre-B-cell ALL should be studied with this probe set.

B-Cell Disorders

B-cell disorders have traditionally presented challenges to the cytogenetics laboratory. The mitotic index of the cells in question is usually quite low and the mitogens available to improve this can be expensive, toxic, and frequently marginally effective. The use of appropriately constructed panels of

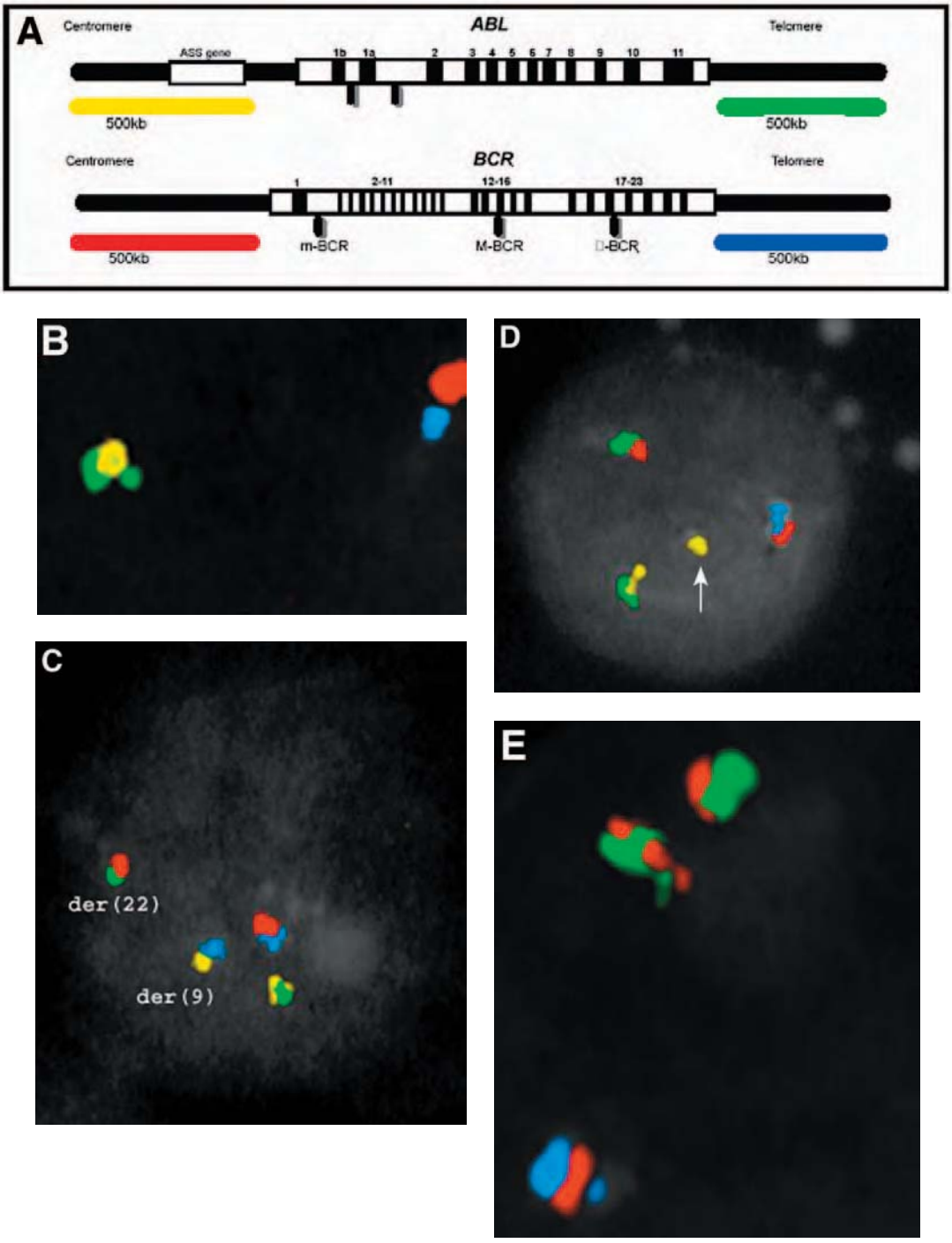

Fig. 11. Four-color FISH (F-FISH) for detection of *BCR/ABL* gene rearrangements. **(A)** Genomic organization of *BCR* and *ABL* genes. Colored bars indicate approximate positions of the probes. Shaded lines indicate different breakpoints in *BCR* and *ABL*. **(B)** Appearance of a normal cell using F-FISH. **(C)** Typical *BCR/ABL* gene rearrangement detected with F-FISH. **(D)** Signal pattern produced by a cell with a *BCR/ABL* gene

Table 6
Cytogenetic Aberrations in CLL

Cytogenetic aberration	Gene(s) involved	% cases detected by FISH[a]	Prognosis (median survival)[b]
del(13)(q14)	Unknown	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)	*ATM*	15–18%	Poor (79 months)
del(17)(p13)	*TP53*	7–8%	Poor (32 months)

[a] Data from refs. *54* and *55*.
[b] Data from ref. *54*.

FISH probes, which 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. Such panels have demonstrated that, as with myeloid disorders, two or more abnormalities are frequently present.

Chronic Lymphocytic Leukemia/lymphoma

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 studies only detect genetic aberrations in approximately 40% of cases. Interphase FISH is a more sensitive assay and FISH has largely replaced conventional cytogenetics for the detection of genetic aberrations in CLL. FISH studies reveal that the most common abnormalities include deletions of 13q14, trisomy 12, deletions of 11q22.3-q23.1, deletions of 17p13, and deletions of 6q21-q23 (see **Table 6**). These genomic aberrations are important independent predictors of disease progression and survival, thus FISH analysis with a panel of probes for relevant aberrations is recommended for CLL patients *(54)*. A commercially available panel includes probes used to detect deletions of 13q14, 11q22–23, and 17p13 and trisomy for chromosome 12 (see **Fig. 12a**). Abnormalities of 13q14 are present in approximately 50–60% of CLL patients and this deletion is associated with a good prognosis *(54,55)*. The tumor suppressor gene in 13q14 of importance to CLL has not been identified; however, the relevant gene has been mapped distal to the retinoblastoma gene (*RB1*) *(56)*. 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 *(54,57)*. Trisomy 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, with some patients presenting with what appears to be advanced stage disease *(54)*. In roughly 10% of B-CLL patients, the *TP53* gene at 17p13 is deleted. This abnormality confers the worst prognosis for CLL patients and is associated with decreased survival and increased drug resistance *(58)*. Because it is hypothesized that CLL clones accumulate genetic aberrations as the disease advances, FISH is appropriate for initial and follow-up studies *(55)*.

Fig. 11. (*continued*) rearrangement and a deletion involving the derivative chromosome 9 (arrow). **(E)** Cell with a *BCR/ABL* gene rearrangement and an additional der(22). Note the definitive identification of the abnormalities present in **(D)** and **(E)** as a result of the 4-color strategy. Typical rearrangements or deletions are also readily and unequivocally interpretable using this method. (Courtesy of Dr. Nalla Palanisamy and Cancer Genetics.)

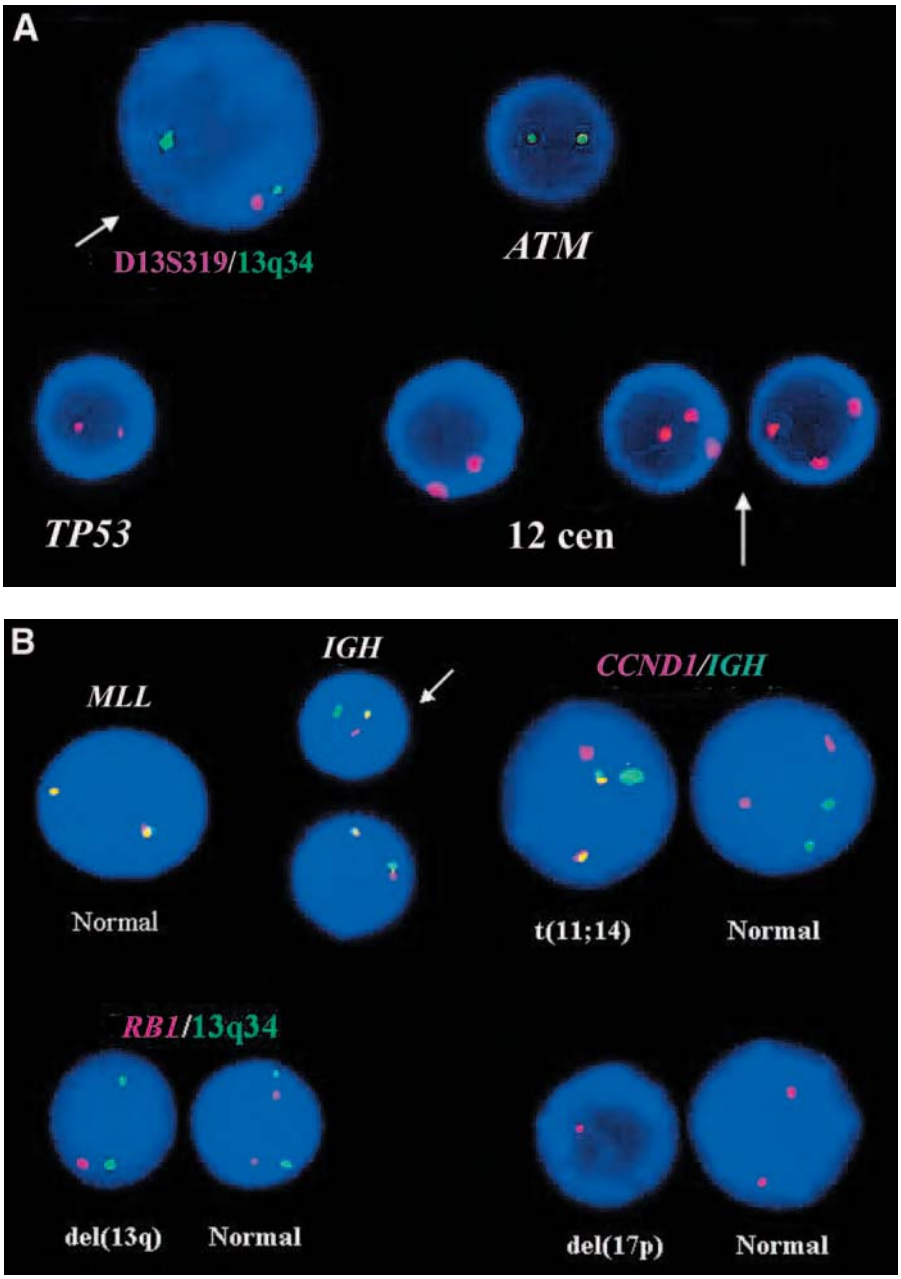

Fig. 12. FISH panels for B-cell disorders. **(A)** Results from a peripheral blood sample from a patient with CLL, hybridized with the Vysis CLL probe panel. **Top row:** A deletion of chromosome 13q is evident from the presence of two 13q34 control signals (green) and only one signal for the D13S319 probe (red). The ATM probe produced two signals, indicating no deletion. **Bottom row:** A normal signal pattern for *TP53*, and both normal and trisomy 12 cells revealed with a chromosome 12 centromere probe. B: A panel of probes hybridized to peripheral blood from a patient with plasma cell myeloma. An MLL break-apart probe produces two fusion signals, indicating no rearrangement involving this gene. An *IGH* break-apart probe, however, reveals both normal (*bottom*) and abnormal (*top*, arrow) cells in which a rearrangement involving *IGH* is evident by the separation of one red and one green signal. Subsequent analysis with probes for t(11;14)(q13.q32) demonstrates this rearrangement. A deletion of the *RB-1* locus on chromosome 13 is also present, as is a deletion of the *P53* locus on 17p.

Plasma Cell Myeloma (Multiple Myeloma)/Plasmocytoma

Chromosomal abnormalities have been reported in approximately 30–50% of disorders of plasma cells, and interphase FISH detects deletions and translocations in at least 90% of cases studied *(59)*. Identification of the cytogenetic aberrations has led to the identification of subgroups of plasma cell myeloma with unique clinical and biologic features *(60)*. Translocations involving the Ig heavy-chain locus (*IGH*) at 14q32 are frequent and appear to represent early genetic changes. The most common translocations include t(4;14)(p16.3;q32), t(11;14)(q13;q32) (Chapter 15, Fig. 1v), and t(14;16)(q13;q21). Patients with t(4;14) and t(14;16) fall within a poor prognosis subgroup, whereas those with t(11;14) have a good prognosis *(60)*. 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. Given that each of the translocations has prognostic implications, specific probes for each of the translocations are of great value. FISH-detected 13q14 deletions, using a probe for the locus D13S319, are found in approximately 40% of cases and loss of an as yet unidentified tumor suppressor gene is an independent adverse prognostic factor *(61)*. FISH is also useful for detecting deletions of *TP53* at 17p13 (see **Fig. 9**) that are associated with a poor prognosis *(62)*. As with B-CLL, a panel of probes can 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 *(60)*. (See **Fig. 12B**). It should be noted, however, that detection rates for FISH panels directed at diagnosing plasma cell myeloma are often lower in practice than suggested by the literature. This is due to many of the patients being assessed for a monoclonal gammopathy of unspecified significance (MGUS) that do not actually have myeloma. The results of a FISH panel in such cases will be negative.

Non-Hodgkin's Lymphoma

The genetic hallmarks of many non-Hodgkin's lymphoma (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 (*IGH*) gene at 14q32. Several translocations represent the primary event producing the initial disease state. t(14;18)(q32;q21) (see Chapter 15, Fig. 1y) that juxtaposes the *IGH* locus with the *BCL2* gene is virtually pathognomonic for follicular lymphoma and can also be seen in a percentage of diffuse large cell lymphomas. For mantle cell lymphoma, *IGH* is positioned next to the *BCL1* gene by a t(11;14)(q13;q32) (see Chapter 15, Fig. 1v). 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 *(59)*. Burkitt's lymphoma (BL), 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. Juxtaposition of IgH and the *MYC* gene (8q24) results in overexpression of the transcription factor c-myc (see Chapter 15, Fig. 1q,e). The utility of FISH with a DCDF probe for high grade lymphomas is in the rapid diagnosis of the (8;14) translocation, particularly because treatment strategies differ between BL and other high-grade lymphomas. A dual-color break-apart probe for the *ALK* gene at 2p23 can 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 bone marrow to assess for involvement of this tissue.

Sex-Mismatched Bone Marrow or Stem Cell Transplant

For many hematologic malignancies, bone marrow or stem cell transplantation can be a reasonable treatment and/or the only hope to cure the patient of disease. FISH is particularly useful for

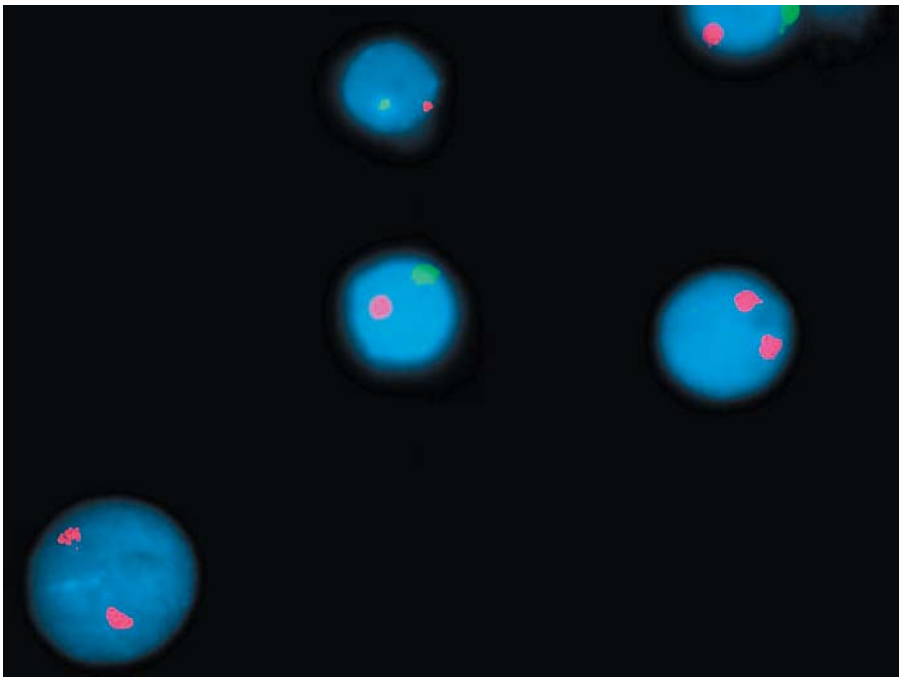

Fig. 13. FISH is the most sensitive assessment for opposite-sex bone marrow transplantation engraftment status studies. For this female patient who was transplanted with marrow from a male, both cell types (two red signals consistent with 2 X centromeres; one red signal and one green signal consistent with one X centromere and one Y heterochromatic region) were seen consistent with partial engraftment.

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) (see **Fig. 13**) are employed in the analysis of more than 200 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 might not be well defined by banding. FISH has proven to be a useful tool for detecting abnormalities that allow for proper diagnosis of tumors and/or 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 the section Specialized and Evolving FISH Technologies below) has proven particularly useful for characterizing the complex karyotypes associated with some solid tumors.

Fluorescence *in situ* hybridization is useful for detecting rearrangements associated with soft tissue masses that might be difficult to diagnose by morphology alone. For example, FISH has been used to detect t(11;22)(q24;q12) involving the *FL1* and *EWS* genes associated with Ewing's sarcoma *(63)*, t(X;18)(p11.2;q11.2) that juxtaposes the *SYT* and *SSX1* or *SSX2* genes in synovial sarcoma *(64)* (see **Fig. 14**), and t(2;13)(q35;q14) that fuses the *PAX3* and *FKHR* genes in rhabdomyosarcomas *(65)*. 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 *(66,67)*. As more

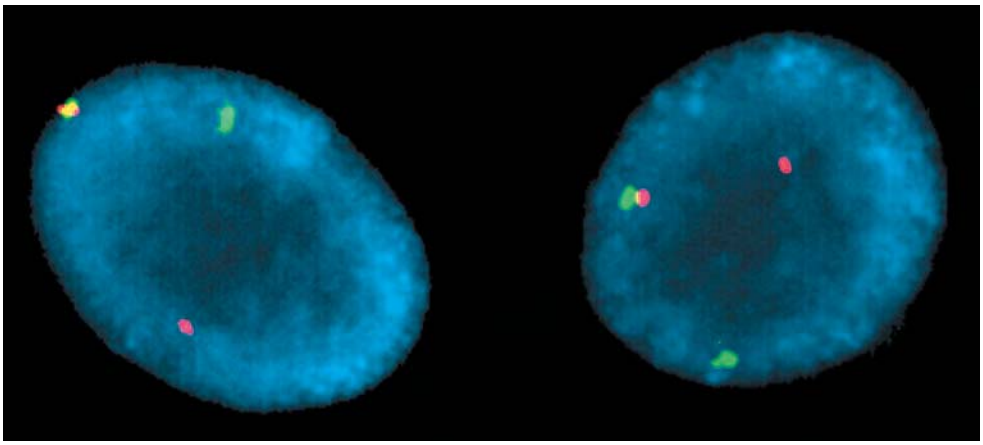

Fig. 14. FISH for rearrangement of the *SYT* locus in a synovial sarcoma. Probes on the telomeric and centromeric sides of *SYT* are detected with FITC (green) and Texas Red, respectively. One pair of green–red probe signals is split apart in each cell as a result of the rearrangement of the *SYT* gene.

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.

HER2 and Breast Cancer

Amplification of the *HER2* (Her-2/*neu*) gene 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 *(68,69)*. HER2 assessment is useful for prognosis, chemotherapy responsiveness, and selection for targeted monoclonal antibody therapy (Herceptin®) *(69)*. FISH is the most sensitive and specific Food and Drug Administration (FDA)-approved methodology for *HER2* detection *(70)*. FISH with a probe for the *HER2* gene (17q11.2) and, usually, an α-satellite probe for the centromere of chromosome 17 (in a second color) are hybridized to 4-micron sections of paraffin-embedded tumor samples that have been identified by a pathologist. The invasive component of the cancer is scored for the number of signals, and a *HER2* : 17 centomere ratio is calculated. A ratio of 2.0 indicates *HER2* gene amplification (see **Fig. 15**). These results are used in conjunction with clinical findings to guide treatment options for the patients.

Bladder Cancer Recurrence Screening

Bladder cancer is a relatively common cancer that has a greater than 70% chance of tumor recurrence *(71)*. A multi-target FISH assay has been developed for monitoring recurrence of bladder cancer in conjunction with cystoscopy (UroVysion, Vysis, Downers Grove, IL). A panel of probes, consisting of α-satellite probes for chromosomes 3, 7, and 17 and a locus-specific probe for 9p21 (see **Fig. 16**), are used to detect chromosomal aberrations that are commonly associated with bladder cancer *(72)*. 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 the detection of transitional cell carcinoma in cytologically equivocal and negative urine samples, often providing the earliest measure of bladder cancer recurrence (anticipatory positives) *(73)*. Clinical trials to determine the assay's utility for detection of early-stage disease are in progress.

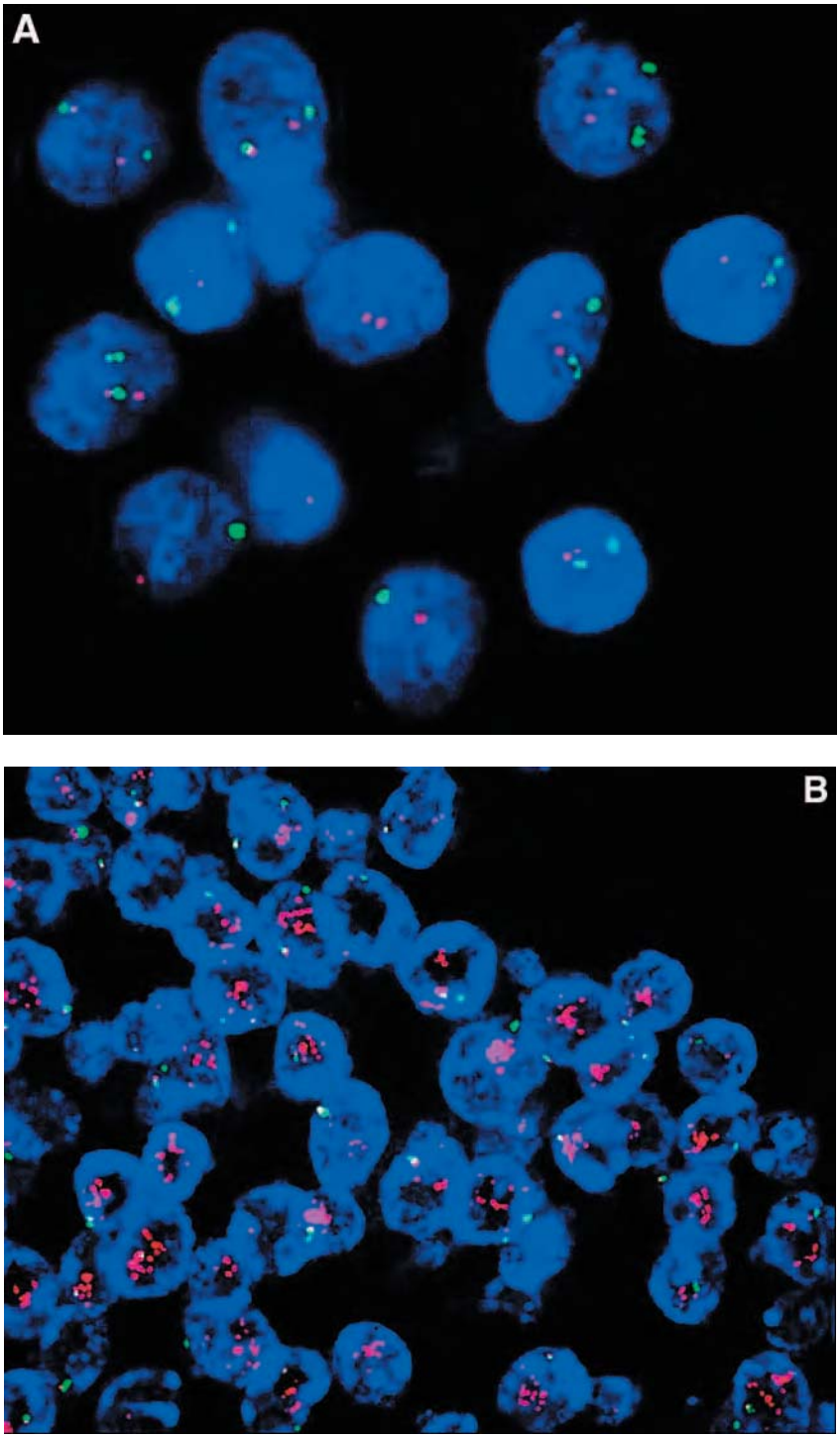

Fig. 15. *HER2* analysis for carcinoma of the breast. Green signals represent the chromosome 17 centromere probe, whereas the *HER2* probe signals are red. A *HER2* : 17cen ratio of 2.0 represents amplification of the *HER2* gene. See text for details. **(A)** Normal cells, with two red and two green signals; **(B)** *HER2* amplification.

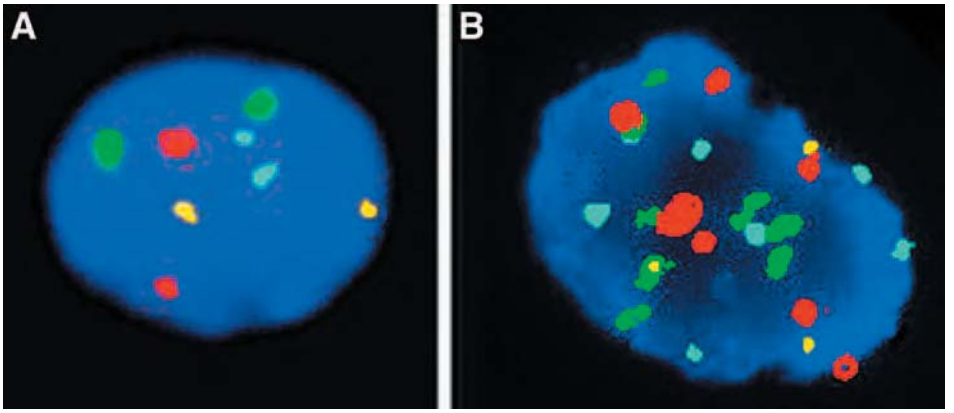

Fig. 16. Examples of normal **(A)** and abnormal **(B)** results for the Vysis UroVysion assay used to monitor for bladder cancer recurrence in urine or bladder wash samples. The normal signal pattern reveals 2 red signals for the chromosome 3 centromere, two green signals for the chromosome 7 centromere, 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.

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. According to the Standards and Guidelines for Clinical Genetics Laboratories from the American College of Medical Genetics (ACMG) *(5)*, prior to utilizing a probe for clinical purposes, probe validation must be performed. 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 percentage of cells with an apparent "abnormal" pattern that occur randomly. Thus, depending on the normal cutoff point, probes might or might 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 noncolorblind 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 1 image saved for documentation. A large number of nuclei (approximately 200) are generally scored for an interphase study, with at least 1 image documenting results. Many commercially available probe mixes contain internal control probes that identify the chromosome of interest. In addition, the normal homolog signal can 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 Chapter 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 FISH 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), CGH microarrays, multiplex-FISH (M-FISH), fiber-FISH, m-banding, primed labeling (PRINS), and reverse hybridization (see ref. *74* for a review). Many of these techniques are used for clinical analysis, but some are more regularly used on a research basis.

Comparative Genomic Hybridization (CGH)

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 growing cells. DNA is extracted from the cells of interest and labeled with a red fluorophore, whereas normal DNA is labeled with a green fluorophore. These are mixed together in equal proportions and hybridized to normal metaphase preparations. The red : green ratio generated by the two samples of DNA is analyzed by a computer software program that detects gains and/or losses of material from the test DNA. Where there is a normal amount of genetic material, the equal hybridization of red patient DNA and green control DNA will appear yellow. If there is a loss of genetic material, the segment will appear green and a gain of material will produce a red signal. This technology has been used successfully for clinical analysis *(75)*; however, it is limited in that its resolution is about 10–20 Mb. It is also not useful for detecting balanced rearrangements. (See **Fig. 17** and cover background image.)

CGH Microarrays

Comparative genomic hybridization to bacterial artificial chromosome (BAC)/P1 insert clone artificial chromosome (PAC) arrays has recently been used to obtain a higher-resolution analysis. The sample/control mix is hybridized to a BAC array rather than to normal metaphase chromosomes. Using a printer, the BAC DNA is spotted on a microscope slide (typically in multiple copies for each clone), and the hybridization takes place *in situ*. Ratios between the sample and control DNAs are determined with a scanner and interpreted with special software.

The resolution of this analysis is much greater than conventional CGH or G-banded chromosomes and is determined by the number and size of the clones on the array. Clones have been chosen from the entire genome to construct a 2400 BAC microarray *(76)*, which can be used for constitutional abnormalities (e.g., Down syndrome and Cri du Chat syndrome; see Chapter 8) resulting from the gain or loss of a chromosomal region *(77)*. More specialized arrays have also been developed for high-resolution analysis of a particular chromosome or chromosome region *(78)* or for the subtelomeric regions *(79)*. To date the vast majority of this work has involved the study of cancers, revealing putative oncogenes and tumor suppressor genes in a number of tumors *(80–83)*. It is expected that CGH used with BAC/PAC arrays (CGH microarrays or CGH karyotyping) will be routinely used in the diagnostic laboratory within the next 5 years. (See **Fig. 18**.)

M-FISH

Multiplex-FISH (M-FISH) (see **Fig. 19**) 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 to create a distinct computer-generated false color for each chromosome (*86*).

As described earlier, the use of different fluorophore colors (and the appropriate bandpass filters; see Chapter 7) allows one to examine 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 seven (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

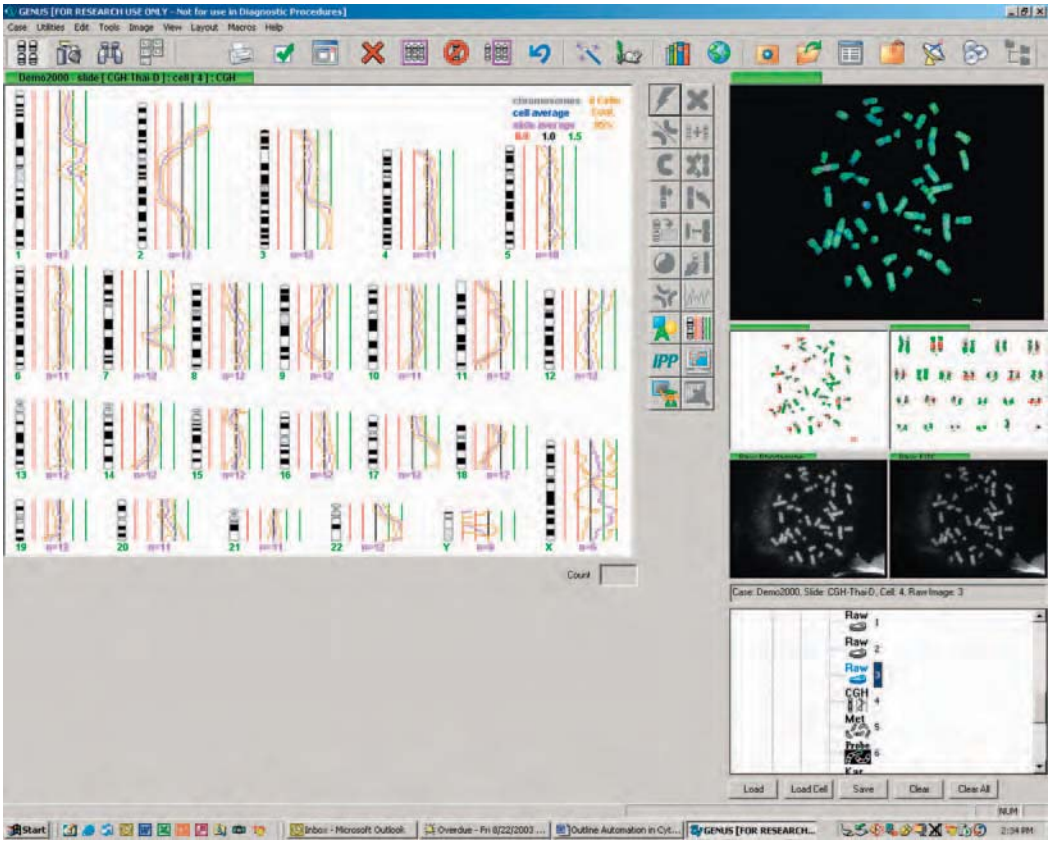

Fig. 17. Software interface of a CGH analysis system, showing the gain and loss profiles of the chromosomes. See text for details. (Courtesy of Applied Imaging.)

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 analyzed the acquired data 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 (see center cover image). As described earlier, this technology is also very useful for determining the origin of duplications and marker chromosomes, or for detecting cryptic rearrangements (see bottom cover image).

mBAND Analysis

Multicolor banding *(87)* uses chromosome specific mixtures of partial chromosome paints that are labeled with various fluorochromes. 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 (see **Fig. 20**) and can be particularly useful in preparations with shorter chromosomes.

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 *(88)*. The probes are applied and can be physically

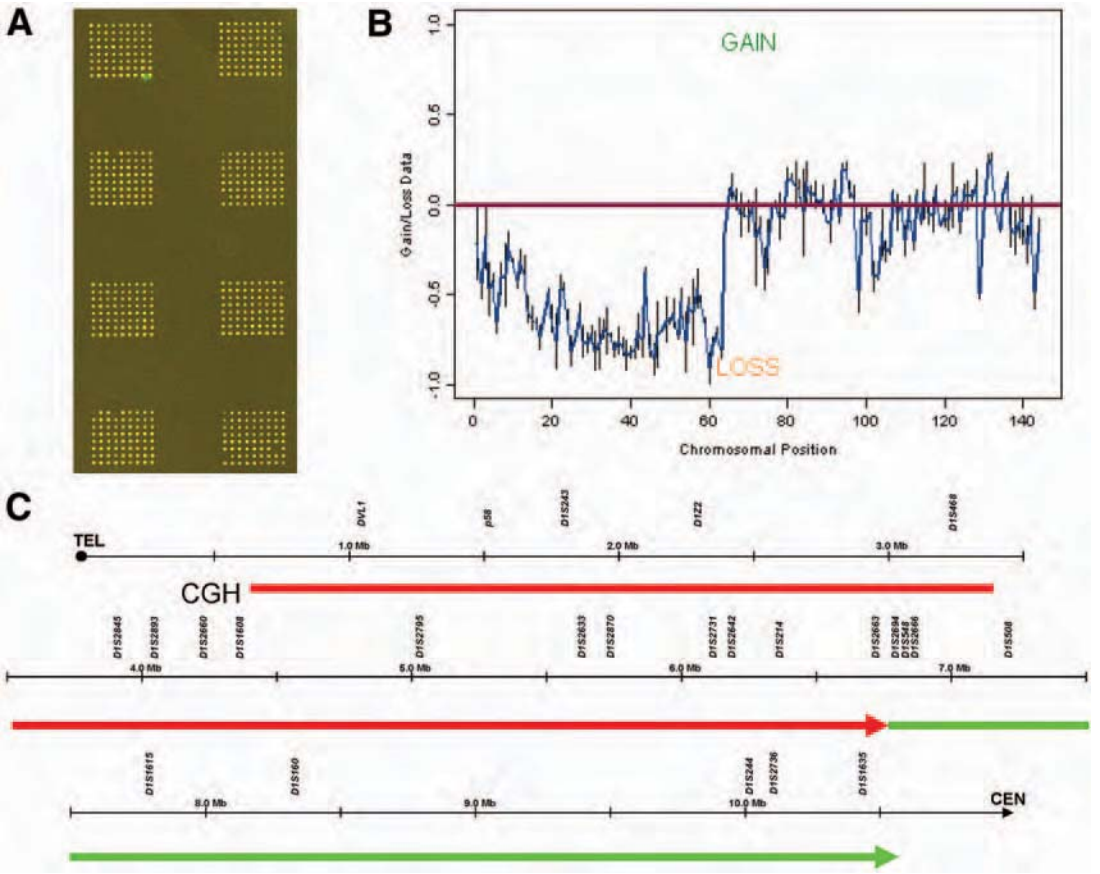

Fig. 18. Array CGH analysis of a subject with monosomy 1p36. **(A)** Subject DNA is compared to a control DNA sample using CGH. The microarray is constructed from 97 large insert clones from the most distal 10.5 Mb of 1p36 *(84)*. **(B)** Ratio of subject to control DNA is plotted for each large insert clone. Clones 1–97 *(left)* represent the 1p36 contig. Clones 98–144 *(right)* represent each telomere (41 sites), 3 clones for the X chromosome, and 3 clones for the Y chromosome. Deletion is indicated by those ratios that fall below –0.5. Gain of DNA copy number are those ratios above 0.5. Equal DNA copy numbers between subject and control are around zero *(85)*. Shown is subject 11 from ref. *85*. **(C)** Schematic of 1p36 showing location of genetic markers. The red line indicates deletion, whereas the green line indicates retention (normal) as determined by array CGH. This patient has a terminal deletion approx 6.75 Mb in size. (Courtesy of Dr. Shaffer and Dr. Yu, Washington State University, Spokane and Baylor College of Medicine, Houston.)

ordered on the fibers. This provides a much higher spatial resolution and allows for correct orientation precise mapping of the probes.

Primed In Situ *Labeling*

Primed *in situ* labeling (PRINS) is essentially PCR on a slide *(89)*. 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 nucleotides are incorporated during the reaction. This technology is utilized both clinically and for research purposes. It has been used successfully with both repetitive and single-copy probes. One of the more useful applications of this technologies is differentiation of the α-satellite sequences for chromosomes 13 and 21, something that cannot be accomplished with traditional FISH. (See **Fig. 21**.)

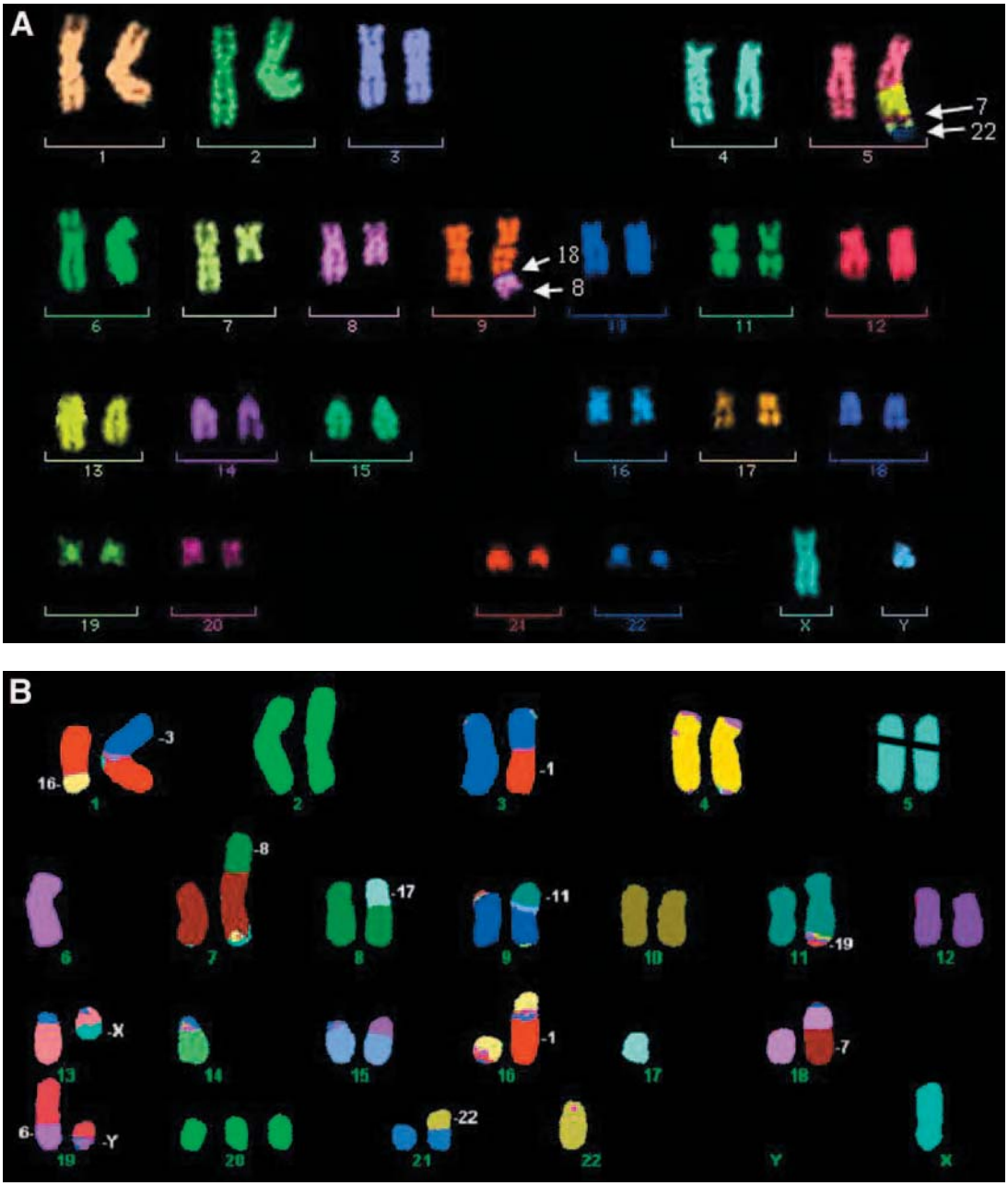

Fig. 19. Multiplex or multicolor FISH (M-FISH) analysis for cancer. **(A)** Metaphase from a leukemia patient with complex rearrangements; **(B)** analysis of a hypodiploid colon cancer line with multiple numerical and structural chromosome abnormalities. The multicolor approach is useful and successful for identifying both rearrangements and aneuploidies. The origin of the different chromosomes in the rearrangements is noted on the karyotypes.

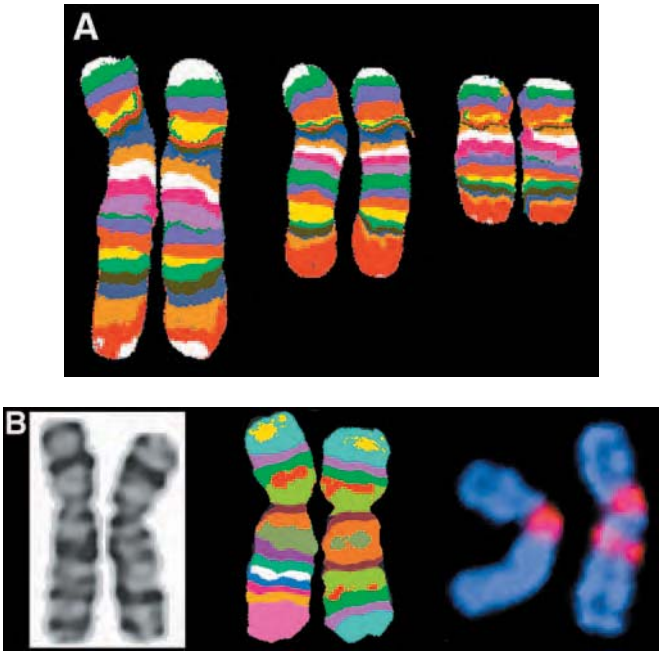

Fig. 20. Multicolor banding: **(A)** region-specific probes labeled with different partial chromosome paints (PCP) and computer false color (MetaSystems' mBAND) produces a definable number of colored bands per chromosome, regardless of chromosome length; **(B)** this example shows an abnormal X chromosome (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*). (Courtesy of MetaSystems Group, Inc.)

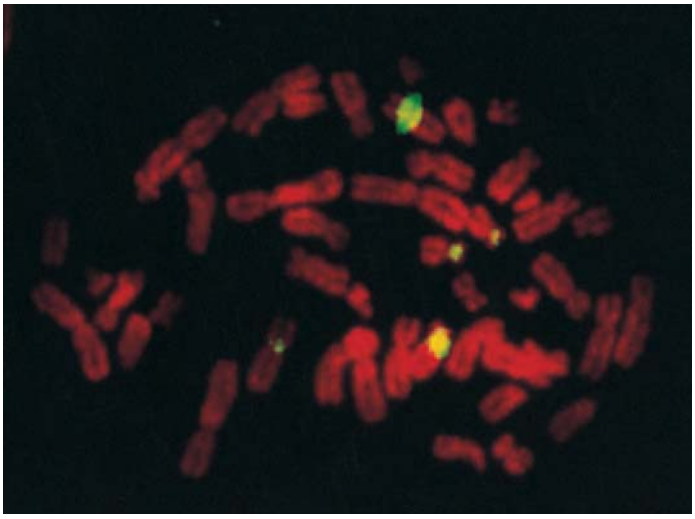

Fig. 21. Primed random *in situ* hybridization (PRINS). Metaphase chromosomes are subjected to PRINS with α-satellite oligonucleotides specific for chromosomes X, 11, and 17. Bright yellow fluorescein staining is seen at the centromeres of these chromosomes. See text for details. (Courtesy of Drs. Steen Kolvraa and Lars Bolund, Aarhus University, Sweden.)

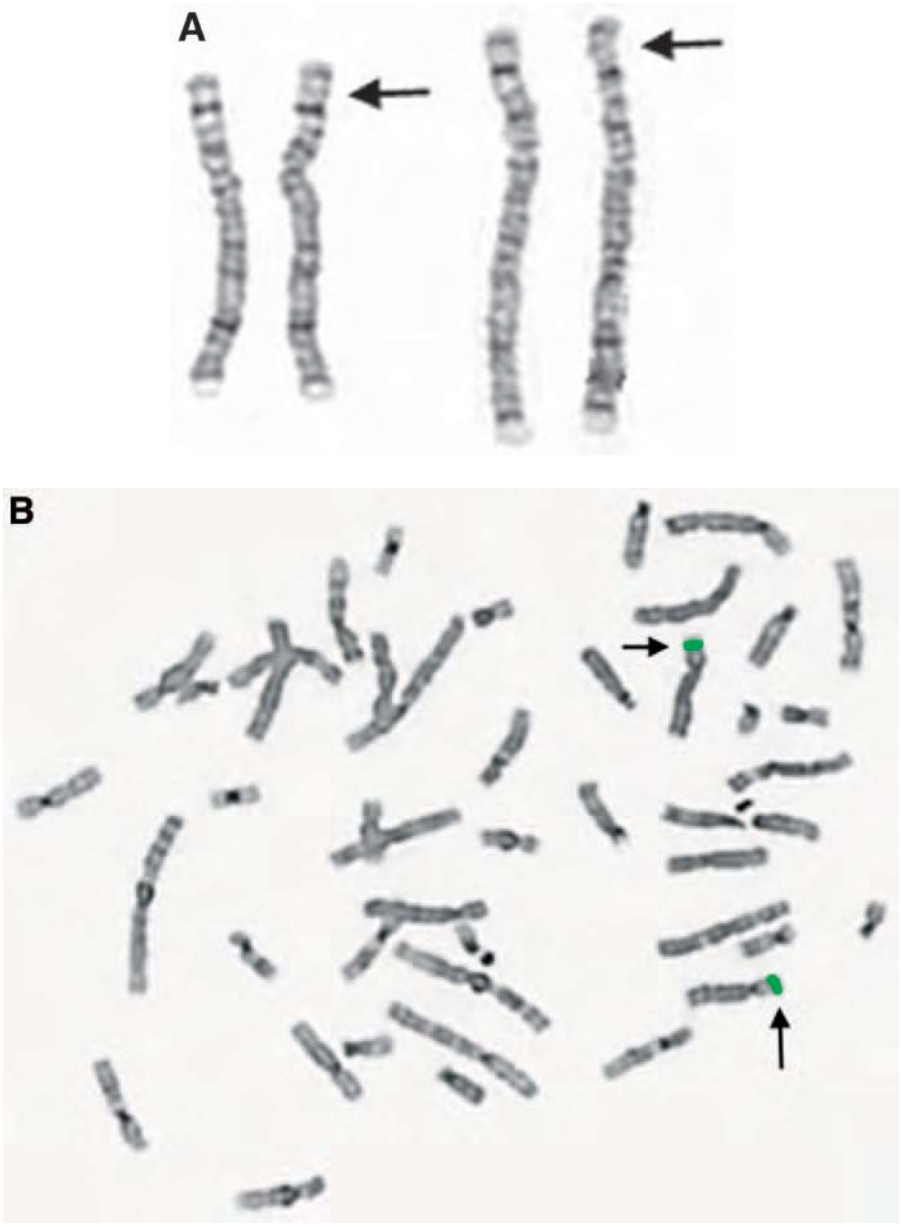

Fig. 22. Reverse FISH of a patient with an abnormal chromosome 8. G-banding **(A)** suggested a duplication of bands 8p23.1-p23.3. Two pairs of chromosomes 8 are shown; arrows indicate the additional band. This band was microdissected and the DNA was amplified, labeled, and used as a FISH probe. **(B,C)**: hybridization to normal chromosomes. The same metaphase is imaged with reverse DAPI to approximate G-banding patterns and identify the two chromosomes 8 **(B)** and with typical DAPI staining **(C)**. 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.)

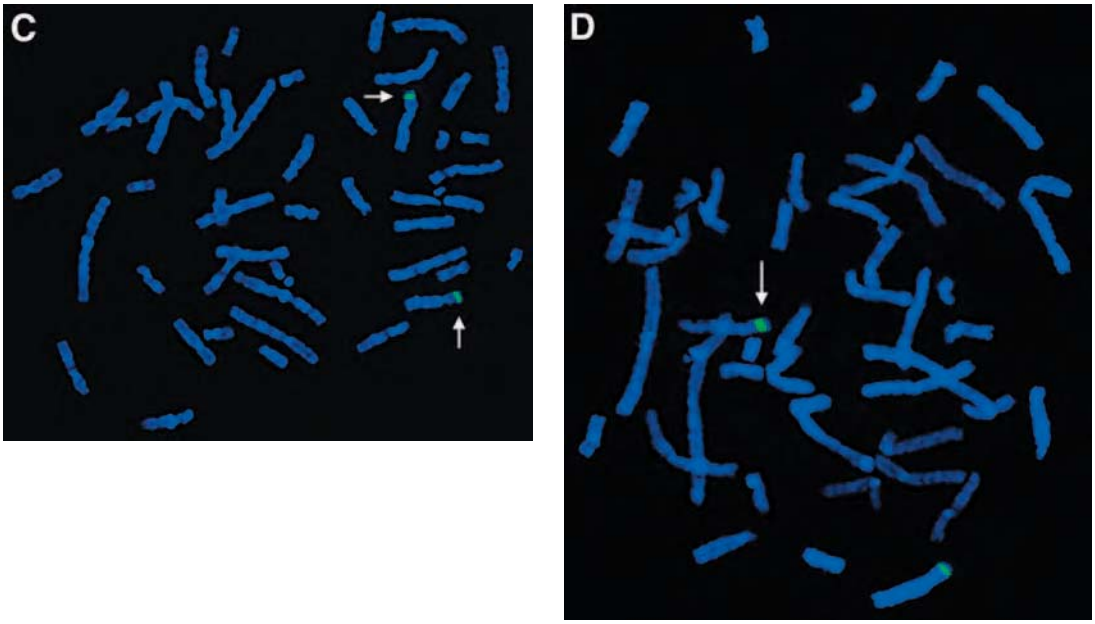

Fig. 22 (*continued*)

Reverse FISH

Reverse FISH is used to identify material of unknown origin *(90)*. Marker chromosomes, duplications or other unidentified material are flow sorted or microdissected off 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 to normal or patient metaphase chromosomes to identify the origin of the unknown material. This technology has been successfully used to identify a variety of chromosome abnormalities. (See **Fig. 22**.)

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VI Beyond Chromosomes

Editors' Foreword to Section VI

The progress that has been made in the clinical analysis of chromosomes in the five decades since Tjio and Levan is impressive. The resolution possible with the light microscope has become so good that to go any further almost requires analysis of the DNA itself. Indeed, given the current interdependency between chromosome analysis and FISH, the distinction between classical cytogenetics and molecular analysis is no longer as discrete as it once was. The same can be said for the actual definition of a chromosome abnormality, given what is now known about imprinting and uniparental disomy.

The information provided by the cytogenetics laboratory has become so complex that it often requires the expertise of a specialist who can assist both patient and health care provider with its interpretation. This is the role traditionally played by the genetic counselor.

In this section, we explore the continuum that is cytogenetics, molecular genetics, and imprinting/UPD, as well as the practical utilization of data via genetic counseling.

18

Fragile X

From Cytogenetics to Molecular Genetics

Dana C. Crawford, PhD and Patricia N. Howard-Peebles, PhD

INTRODUCTION TO HUMAN FRAGILE SITES

The first fragile site identified in humans was on chromosome 9q, as described by Dekaban in 1965 *(1)*. Fragile sites were an active area of cytogenetic research during the late 1970s and most of the 1980s stimulated by: a link between the fragile site at Xq27 and X-linked mental retardation, the discovery that fragile site expression was directly related to the tissue culture conditions used for cell preparations *(2)*, and a possible relationship between fragile sites and cancer/cancer cytogenetics. The application of molecular techniques to fragile sites began in the early 1990s with the discovery of a new mutation mechanism for fragile Xq27, which resulted in the identification of a new type of human disease.

In 1979, Sutherland defined a fragile site as a specific point on a chromosome, which appears as a nonstaining gap, usually on both arms or chromatids *(3)*. In a family, this site is always in the same location, is inherited as a Mendelian co-dominant, and results in chromosome fragility under appropriate tissue culturing conditions. Over 100 human fragile sites have been described, and they can be classified into two major categories: rare or common (see Chapter 14).

Clinical significance has been established for two fragile sites: FRAXA (Xq27.3) and FRAXE (Xq28). Both are rare, folate-sensitive fragile sites. FRAXA is the fragile X (fraX), which is associated with the fragile X syndrome, the most common form of familial mental retardation. FRAXE is associated with a mild form of X-linked mental retardation.

Fragile sites require an induction system for consistent cytogenetic expression. The majority of cytogenetic studies have been performed on phytohemagglutinin (PHA)-stimulated lymphocytes, due to induction problems in other tissue types. Tissue culture conditions and modifications for induction of all fragile sites are detailed by Sutherland and Hecht *(4,5)*.

The folate-sensitive fragile sites including fraX can be induced by multiple methods, as summarized in **Table 1**. Method 4 has an advantage of being somewhat less cytotoxic to the cells during culturing *(7)*. Numerous physical and chemical factors affect the ultimate level of expression of fragile sites. Sutherland and Hecht provided extensive details and documentation of requirements for fragile site expression *(5)*. Because of the stringent requirements for folate-sensitive fragile site expression, basic guidelines were developed to assure quality clinical testing for fraX *(8,9)*.

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 *(10)*. These observations were compatible with X-linked inheritance, and

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Table 1
Methods for Inducing Folate-Sensitive Fragile Sites

Number	Method	Additive[a]
1	Thymidine and folic acid deprivation[b]	—
2	Inhibiting folate metabolism	Aminopterin Methotrexate Trimethoprin
3	Inhibiting thymidylate synthetase	Fluorodeoxyuridine (FUdR) Fluorodeoxycytidine (FCdR)
4	Excess thymidine	300–600 mg/L
5	Combination of nos. 3 and 4[c]	FUdR + thymidine (300–600 mg/L)

[a] All additives applied for last 24 hours of culturing time.
[b] Requires serum supplements of 5%, pH 7.3, and sterility of cultures.
[c] See also reference *6*.

numerous reports appeared in the literature *(11)*. 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), and the heterogeneity of this nonspecific X-linked MR disorder became apparent *(12)*. Numerous disorders have been delineated from this original subgroup of MR males and, in a continuing effort, a total of at least 124 X-linked disorders involving MR have been described *(13,14)*. 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 fraX *(15)*. However, the popular name for this disorder became fragile X syndrome.

Inheritance of fraX

It became apparent soon after the cytogenetic test became available that the inheritance and penetrance of fragile X syndrome was unlike that 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. 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 *(16,17)*. Their observation, that the mothers of TMs are much less likely to have affected offspring than are the TMs unaffected daughters, became known as the Sherman paradox. Other unusual features of fragile X syndrome 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 that do unaffected carrier females.

Cytogenetic Expression of fraX

The fraX site is located in band Xq27.3, one of six fragile sites located on the X chromosome (see **Table 2**). It can be visualized in both solid stained and banded preparations (see **Figure 1**). However, banded preparations are required because other fragile sites and lesions can mimic fraX *(5,18,19)*. Three other fragile sites have been found in bands Xq27–28: FRAXD *(20)*, FRAXE *(21)*, and FRAXF *(22)*. The latter two sites (see **Table 2**) cannot be cytogenetically distinguished from fraX. The standard ISCN nomenclature (see Chapter 3) to cytogenetically designate fraX is 46,Y,fra(X)(q27.3) for an affected male and 46,X,fra(X)(q27.3) for an expressing female *(23)*.

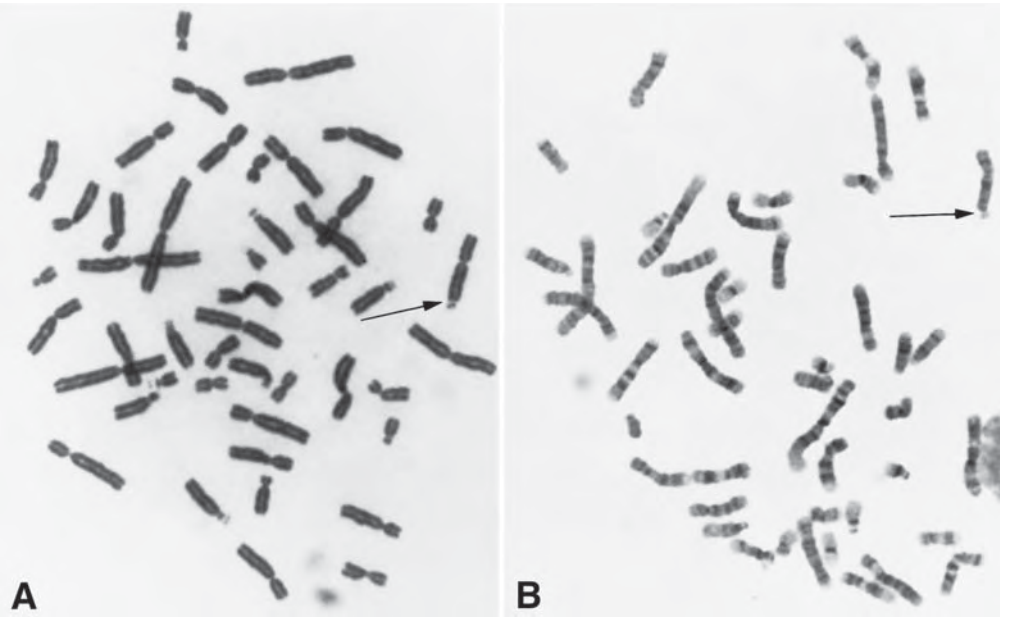

Fig. 1. Appearance of FRAXA: **(A)** conventional stain (Giemsa) and **(B)** GTG-banded. The arrow indicates the location of the fraX site.

Table 2
Fragile Sites on X Chromosome

Gene symbol	Location	Type	Subtype[a]
FRAXA	Xq27.3	Rare	Folate-sensitive
FRAXB	Xp22.31	Common	Aphidicolin inducible
FRAXC	Xq22.1	Common	Aphidicolin inducible
FRAXD	Xq27.2	Common	Aphidicolin inducible
FRAXE	Xq28	Rare	Folate-sensitive
FRAXF	Xq28	Rare	Folate-sensitive

[a] Subtypes are discussed in Chapter 14.

FraX is not a chromosome abnormality. It is a chromosomal "marker" that allowed us to diagnose fragile X syndrome in most cases while better techniques were being developed. Thus, the table developed for chromosomal mosaicism *(24)* does not apply.

Cytogenetic Expression in Affected Males and Carrier Females

In affected males, fraX expression varied from less than 4% to 50%, with the low-expressing males comprising a minority of the diagnosed cases. However, this group is the origin of the false-negative males diagnosable with molecular techniques. Why fraX does not express in more than 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 *(8,9)*.

Table 3
Classification of Trinucleotide Repeat Diseases

Class	*n*	Repeat	Position of repeat	Examples (locus)
1	3	CGG	5' Untranslated region	Fragile X syndrome (*FMR1*)
				FRAXE syndrome (*FMR2*)
	1	CAG		Spinocerebellar ataxia type 12 (SCA12)
2	2	CTG	3' Untranslated region	Myotonic dystrophy (DM)
3	8	CAG	Inside coding region	Huntington disease (HD)
				Spinocerebellar ataxia type 1 (SCA1)
				Kennedy disease (*AR*)
4	1	GAA	In first intron	Friedreich ataxia (*FRDA*)

Prenatal Diagnosis

Prenatal testing was available on an experimental basis beginning in 1981. 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, whereas chorionic villus sampling (CVS) was the standard in Europe and Australiasia. 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 below) exceeded 400 cases. The "state of the art" was summarized at the Fourth International Workshop on Fragile X and X-linked Mental Retardation *(25)*.

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 fragile X syndrome 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 fragile X syndrome gene, 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 get more reliable results than with chromosome analysis alone.

Trinucleotide Repeats—Classification

The early 1990s marked the discovery of a new type of genetic mutation among humans: the trinucleotide or dynamic repeat. The mechanism causing the fragile X syndrome mutation was first identified in 1991 *(26–28)* and revealed that the mutation results from the expansion of a trinucleotide repeat located in or near an expressed sequence. 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, at least 15 human diseases are associated with the expansion of a trinucleotide repeat *(29,30)*.

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. **Table 3** summarizes the four classes presently known *(29,30)*. One characteristic of these disorders, each generation showing an earlier age of onset

Table 4
Characteristics of the Cloned Folate-Sensitive Fragile Sites[a]

			Copy number		
Symbol	Location	Disease	Normal	Premutation	Full mutation
FRAXA	Xq27.3	Fragile X syndrome	6–54	61–200	230 to > 1000
FRAXE	Xq28	Fragile XE syndrome	6–25	?50–200	200 to > 800
FRAXF	Xq28	None	6–29	?	300 to 1000
FRA16A	16p13.1	None	16–50	?50–200	?1000–2000
FRA11B	11q23.3	Offspring predisposed to Jacobsen syndrome	11	85–100	100–1000

[a] *Source:* Adapted from reference *32*.

and increasing severity, is known as *anticipation*. All the disorders are either X-linked or autosomal dominant except Friedreich ataxia, which is autosomal recessive.

The CGG trinucleotide repeats (included in Class 1) are located at folate-sensitive fragile sites *(31)* and their characteristics are summarized in **Table 4**. Based on the trinucleotide repeat size in FRAXA and FRAXE, an individual can be classified as *normal, premutation,* or *full mutation*. An individual with a *normal* repeat size is characterized by stability of the repeat length and normal intelligence, whereas an individual with a *premutation* repeat size shows instability of the repeat length from generation to generation, but normal intelligence. In contrast, *full mutation* individuals have massive repeat sizes differing in lengths ("mosaic") in a pattern that is often conserved across tissues, resulting in fragile X syndrome. The values of these repeat lengths for fraX are listed in **Table 4**.

Instability of the CGG Repeat

Through observational studies of families with the fragile X syndrome, several factors involved in CGG repeat instability have been proposed, including sex of the transmitting parent, 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 germline, and the size of the resulting expansion is positively correlated with the maternal repeat size *(33–37)*. In contrast, when passed through a male germline, the premutation does not dramatically change in repeat size and often contracts or remains the same *(36–38)*.

In addition to the sex of the transmitting parent, the size and structure of the CGG repeat have been implicated in playing 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 the fragile X syndrome, premutation-sized repeats usually have one AGG at the proximal most end of the repeat, or none at all *(39–41)*. Transmission studies of families with premutation- or intermediate-sized repeats demonstrate that these are unstable if more than 34 repeats at the 3' end of the repeat structure are uninterrupted by an AGG *(36,39,41)*. 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. Although the role of the AGG interruption has only been minimally defined by experimental studies *(42)*, 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 *(43–45)*.

Despite the identification of these factors, it is clear that other yet-to-be identified factors are involved in the expansion process. These unknown factors could include both *cis*- and *trans*-acting factors. Two *cis*-acting factors proposed in the literature are chromosomal background *(44)* and the

origin of replication associated with the location of the CGG repeat *(46)*. No *trans*-acting factor has been identified; however, several have been suggested, most of which involve proteins from the DNA replication and repair systems *(45)*. A "familial" factor has been proposed from the observation that the size of the repeat expansion is more similar among siblings from the same family as compared with siblings across families *(36)*.

The Fragile X Gene and Its Product—FMR1 and FMRP

The fragile X syndrome mental retardation gene-1, or *FMR1*, was identified through positional cloning *(26–28)*. *FMR1* encompasses 38 kb of Xq27.3 and consists of 17 exons *(47)*. 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 does not change in size when passed from parent to offspring *(33)*. The most common form of the repeat size found in human populations studied is 28–30 CGG repeats *(48–51)*. Although the CGG repeat has no known function, it is found in all species of mammals investigated *(52,53)*. However, the repeat is found as a CCT in chickens *(54)* and is not found in invertebrates such as *Drosophila melanogaster (55)*.

The common CGG repeat sizes have not proven to be associated with a disease phenotype; however, the consequence of an expanded CGG repeat (>230 repeats) in *FMR1* is the fragile X syndrome. The hyperexpanded CGG repeat signals the hypermethlyation *(26,56)* and deacetylation *(57)* of the *FMR1* promoter, the CGG repeat, and a nearby CpG island, which transcriptionally silences the gene *(58,59)*. 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 *(60,61)*.

Because the fragile X syndrome 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 *(47,62)*. 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 *(63–65)*. High expression of *FMR1* in regions of the brain such as the neurons of the hippocampus and the granular layer of the cerebellum *(66,67)* is consistent with the mental retardation phenotype typical of the fragile X syndrome (see the section: Clinical Aspects of Fragile X 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 *(68,69)*. Analysis of mouse and human genomic sequences demonstrates similarities in gene structure among *FMR1*, *FXR1*, and *FXR2*, suggesting an ancestral gene is common to the three genes *(70)*. The function of *FXR1* and *FXR2* is 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 in function. Although there are similarities, significant differences have been noted *(71)*. Furthermore, *FXR1* and *FXR2* are not overexpressed in cells from persons with the fragile X syndrome, suggesting that neither gene product compensates for the loss of the *FMR1* gene product *(72,73)*.

The full-length protein product of *FMR1* is 69 kDa in size and is known as the fragile X mental retardation protein, or FMRP *(74)*. At the protein level, *FMR1* is highly conserved across humans *(27)*, mice *(62)*, *Xenopus laevis (75)*, and chickens *(54)*. Although not as highly conserved as among vertebrates, a homolog for the *FMR1* coding sequence has also been identified in *Drosophila melanogaster (76)*.

In the last 10 years, much has been accomplished in elucidating the function of FMRP and how its absence leads to the development of the fragile X syndrome 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 *(74,77)*. Second, FMRP contains both a nuclear localization signal and a nuclear export signal *(78)*. FMRP is primarily a cytosolic protein, but its presence in the nucleus has been reported by nuclear staining experiments *(66,79)*. Furthermore, FMRP has been detected in the nuclear pore *(80)*. Taken together, current evidence suggests that FMRP shuttles between the nucleus and the cytoplasm.

In addition, experimental evidence suggests that FMRP is involved in translational activities. FMRP forms complexes with messenger ribonuclear particles (mRNP) and is associated with translating ribosomes *(78,81,82)*. Because RNP particles 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 might play a role in regulation of translation for certain messages. Laggerbauer et al. *(83)* demonstrated that FMRP suppresses translation by preventing the assembly of the 80S subunit of the ribosome on the target RNAs. New evidence suggests that translational control might be mediated through the RNA interference (RNAi) and/or micro-RNA (miRNA) pathways *(84,85)*.

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 *(71)*. 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 *(74)*. Nearly a decade would pass before the identity of the specific mRNAs (other than the *FMR1* transcript) binding to FMRP would be identified *(86–88)*. These mRNAs contain a G-quartet structure, a specific nucleic acid structure that facilitates binding to FMRP. Recent work also shows that FMRP can be phosphorylated, a mechanism that possibly affects the binding of specific mRNAs *(89)*. Undoubtedly, research in the next decade will identify additional mRNA targets, whose roles will be crucial for not only the understanding of the fragile X syndrome but other human behavioral and cognitive disorders as well.

CLINICAL ASPECTS OF FRAGILE X SYNDROME

Full Mutation Phenotypes

Physical Phenotype

In males, the classic features of fragile X syndrome 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. 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. A small subgroup of males with a "Prader-Willi-like" phenotype has been described by Fryns et al. *(90)*. Heterozygous females express these same features of fragile X syndrome, with manifestations being more common in females with mental disability than in those of normal intelligence.

Behavioral Phenotype

The behavior of males with fraX, especially in childhood, is more consistent and diagnostic than the physical features. They are typically hyperactive and delayed speech. Other complicating features can include irritability, hypotonia, perseveration in speech and behavior, and autistic-like features such as hand flapping, hand biting, and poor eye contact. Social anxiety and avoidance are prominent features of fragile X syndrome in both sexes.

Recently, Hagerman *(91)* reviewed in detail the physical and behavioral phenotype of fragile X syndrome (see also reference *92*). The variability of expression makes clinical diagnosis difficult. Therefore, fragile X syndrome should be considered in the differential diagnosis of all mentally retarded individuals.

Cognitive Phenotype

In males, preliminary evidence suggests there are specific deficits in arithmetic, visual-motor skills, short-term auditory memory, and spatial skills. The IQ decreases with age, although the reason for this longitudinal decline is unclear *(93)*. Adult males with fraX function within the moderate to severe 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 *(94)*. In recent studies of protein expression, FMRP appears to be a good predictor of IQ in these males *(95)*.

In females, 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 (IQ = 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. Recent studies of protein expression show a strong correlation between FMRP and IQ *(95,96)*.

Other Clinical Aspects

A recent review explores the neurologic and pathologic findings in fragile X syndrome *(92)*. Medical follow-up, pharmacotherapy, treatment of emotional and behavioral problems, and intervention approaches for fragile X syndrome have also been reviewed *(97)*.

Premutation Carrier Phenotypes

Transmitting Male Carriers

Unlike the full mutation, the existence of a phenotypic consequence of the premutation in males is controversial. Among the few phenotypic studies of male premutation carriers, many are case reports of individuals ascertained in a clinic setting, including descriptions of boys with learning deficits who inherited the premutation *(98)* and a recently ascertained group of adult male premutation carriers with intention tremor, parkinsonism, and general brain atrophy *(99)*. These case reports are tantalizing; however, given the obvious problem of ascertainment bias, the premutation cannot be associated with the phenotype until a proper controlled study has been performed and replicated. Unfortunately, few proper studies are available for male premutation carriers and the results from these studies are conflicting *(100–102)*.

Although cognitive or behavioral deficits have not been definitively subscribed 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 *(58,66)*. Recent changes in technology, however, have made measurements of *FMR1* mRNA more sensitive and accurate. Using this technology, Tassone et al. re-examined 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 *(103)*, whereas carriers with 55–100 repeats had a twofold increase *(104)* compared with controls. Moreover, these high-end premutation carriers (100–200 repeats) had reduced levels of FMRP compared with controls *(103)*. Additional experiments suggest that the elevated level of *FMR1* mRNA is correlated with CGG repeat size *(105)* and is not simply a response to decreased levels of FMRP *(104)*.

Female Carriers

Many conflicting reports exist in the literature concerning cognitive, behavioral, and physical phenotypes among female premutation carriers. These reports have recently been reviewed *(106,107)*. For reports on cognitive ability, studies of varying designs have shown that the prevalence of mental retardation, the range of cognitive ability *(108)*, or the range of IQ scores among adult female premutation carriers did not differ compared with control groups *(102)*. 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 *(109)*. However, many of these suggested differences were not replicated in other studies. Finally, 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; however, two studies suggest otherwise *(106,107)*.

Although the existence of a cognitive, behavioral, or physical phenotype among premutation females remains controversial, one consequence is consistently associated with the premutation: premature ovarian failure. Premature ovarian failure (POF) 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 POF were anecdotally noted at The 1st (Inter)National Fragile X Conference (1987) in Denver, Colorado *(110)*. Schwartz et al. *(111)* were the first to report an association between the fragile X premutation and POF in a multicenter study. The relationship between the fragile X premutation and POF was eventually confirmed by a large, multi-center study that demonstrated that 16% of premutation carriers experienced POF, whereas only 0.4% of non-carriers and none of the full mutation carriers experienced POF *(112)*. Results from this collaborative effort conclusively demonstrated that the premutation form of the CGG repeat, not the full mutation, is associated with POF. Also, these data, combined with additional reports from other sites, suggest that the rate of POF among premutation carriers is 21% (95% confidence interval: 15–27%) *(113)*. Overall, approximately 14% of idiopathic familial POF and 2% of sporadic POF in the general population can be attributed to the premutation allele *(113)*.

The cause of POF among premutation carriers remains elusive and is the subject of intense research. Many models have been proposed to explain the role of the premutation allele (as opposed to the full mutation allele) in the development of POF 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 POF is one of the factors that can limit the usefulness of preimplantation genetic testing (PGT) as a reproductive option for carrier females *(114–116)*. In fact, recent hormonal studies suggest that female premutation carriers might unknowingly be experiencing ovarian dysfunction at an early age and be facing a poorer prognosis for future pregnancy much earlier than expected *(117)*. The objective of PGT for fragile X syndrome 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 POF, to have unaffected children *(118)*.

Intermediate Carriers

Intermediate alleles, also known as "gray-zone" alleles, range from approximately 40 to 60 CGG repeats and are classified differently than premutation or common alleles in that they might or might not be transmitted unstably from parent to offspring *(36)*. Intermediate alleles, like premutation alleles, do not cause hypermethlyation 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 *(50,119)*. The results from these data suggested, for the first time, that large CGG repeats not limited to permutations were somehow responsible for the child's placement in special education. Although an excess of intermediate and premutation alleles has not been observed in other special education populations *(120)*, new cognitive *(121)* and molecular data *(105)* warrant further research to identify and define a phenotypic consequence of high-repeat alleles of *FMR1*, if one exists.

Timing of Premutation Expansion

One of the yet unsolved questions is when, in development, the expansion from premutation to full mutation occurs. Expansion could occur during oögenesis (meiotic) or after fertilization (mitotic).

Reyniers et al. *(122)* showed that full mutation or mosaic full/premutation males only produce premutation sperm and, therefore, premutation daughters, because repeat expansion occurs only in females *(33)*. Testicular selection against full mutation sperm is unlikely since male *Fmr1* knockout mice show fertility *(123)*. These data support a model of expansion only in somatic cells and protection of the premutation in the germline cells. However, Malter et al. showed, in full mutation fetuses, that only full mutation alleles (in the unmethylated state) were found in oöcytes 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 *(124)*. 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 oögenesis or very early in embryogenesis, prior to general methylation. The answer requires analysis of oöcytes from premutation females.

CURRENT GENETIC ASPECTS OF FRAGILE X SYNDROME

Prevalence of Full Mutations and Premutations

Using the cytogenetic technique developed in the late 1970s described above *(2)*, Webb et al. *(125)* and Turner et al. *(126)* tested school-aged children with mental retardation from Coventry, England, and Sydney, Australia, respectively, for the fraX. In both studies, the investigators assumed that all males affected with the fragile X syndrome are mentally retarded and would be found among programs, schools, or institutions for children with special needs. Under this assumption, both groups tested the target population for fraX and extrapolated their findings to the general population, giving an estimate of 1 in 1000 males and 1 in 2610 males, respectively. Similarly designed studies in Sweden *(127)* and Finland *(128)* supported these estimates of the prevalence of the fragile X syndrome among males and firmly established the syndrome as the second most common cause of mental retardation.

As previously discussed, the cytogenetic test employed by these early prevalence studies proved to be inaccurate, missing 6–10% of affected males and at least 30% of the females *(100,129)*. The cytogenetic test also produced false positives, which is discussed in further detail below. Once *FMR1* was cloned in 1991, more accurate and sensitive techniques became available for diagnosis. Using the new DNA-based technology, the Coventry and Sydney groups revisited their original study populations and revised the prevalence of the full mutation as 1 in 4167 and 1 in 4348 males, respectively *(130)*. Since then, several large, population-based studies have established that the prevalence of the full mutation is probably between 1 in 6000 to 1 in 4000 males of northern European descent *(131)*. Although fraX has been identified in individuals with cytogenetic abnormalities such as XXY, XXX, XYY, and +21, as well as in those with other genetic disorders such as neurofibromatosis, these cases are likely coincidental as a result of the frequencies of both disorders in human populations. Despite the downward revision of prevalence, the fragile X syndrome remains the second most frequent known cause of mental impairment, surpassed only by Down syndrome.

Estimates for the prevalence of the full mutation among other racial/ethnic groups as well as females are generally lacking. To date, only two population-based studies in African-derived populations have been performed, and they suggest that the frequency of the full mutation is at least equal, if not higher, compared with European-derived populations *(131)*. For females, no population-based studies have been performed to date. Based on the fact that the gene responsible for the fragile X syndrome is on the X chromosome and the fact that only females can transmit the disease-causing mutation to their offspring, the prevalence of the full mutation among females is expected to equal the prevalence estimated for males. However, because of X-activation and possibly other factors, only 30–50% of females with the full mutation are mentally retarded (IQ <70) *(132)*, yielding an estimate of 1 in 13,333 to 1 in 8000 females affected by the fragile X-syndrome in the general population. Conversely, 1 in 8000 to 1 in 5714 females are expected to carry the full mutation, but have an IQ >70.

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 germline *(34)*. 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 *(133)*. Studies of premutations among families with a member affected by the fragile X syndrome suggest that the smallest premutation to expand to the full mutation in a single generation is 59 repeats *(133)*. However, small premutation alleles (approximately 50–65 repeats) ascertained from the general population have proven to be more stable than those ascertained from families with the fragile X syndrome *(133)*. Given the uncertainty in the lower boundary of the premutation, the prevalence of the premutation varies from study to study, depending on the range of CGG repeats that are considered premutations. The prevalence for the premutation among Caucasian females ranges from 1 in 116 *(134)* to 1 in 468 *(135)* females. For Caucasian males, studies suggest that the prevalence for the premutation ranges from 1 in 813 *(136)* to 1 in 1866 *(119)*. The prevalence of premutations among other racial/ethnic groups has not yet been determined.

Molecular Rules of Inheritance

DNA analysis of fraX can detect all stages of the trinucleotide repeat expansion. Reduced penetrance, the Sherman paradox, and other unusual characteristics of fragile X syndrome were explained by the silent premutation state. The rules of inheritance *(92)* as we now understand them include the following:

1. Every affected individual has a carrier mother with observable expansions. No new mutation has gone directly from normal to full. Full mutation males do not pass a full mutation to their daughters.
2. Carrier females could have a full mutation or a premutation. Affected females have full mutations and unaffected females could 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 99 become full mutations in subsequent offspring *(133)*.
4. Premutations appear to be inherited silently for many generations. No family has been found in which the normal allele to premutation allele has been documented. Thus, many present families could have the same ancestral premutation, but this cannot be traced reliably. Using polymorphism analysis, Smits et al. showed one family with five living fraX males who share an X chromosome to be related through their last common ancestor six or more generations in the past *(137)*.

DIAGNOSTIC LABORATORY TESTING FOR FRAGILE X SYNDROME

Cytogenetic Testing

From 1977 to 1992, the standard laboratory test for diagnosis of fragile X syndrome was cytogenetic scoring for expression of the fraX in metaphase cells. Compared to routine karyotype analysis, fraX testing had many technical difficulties as well as biological limitations. One significant advantage was that the test was combined with routine chromosome analysis, and as a result, chromosome abnormalities could be diagnosed as well.

Re-evaluation of Negative Results

Cytogenetic testing appears to have been over 90% effective in diagnosing males with fragile X syndrome *(129,138)*. However, premutations carriers usually do not express cytogenetically at all, and full mutation females frequently have lower expression levels than affected males. Thus, all potential carriers in fraX families who tested negative by the cytogenetic test should be retested with DNA methodology. The same applies to individuals with a strong fragile X syndrome phenotype.

Re-evaluation of Positive Results

It is now apparent that the false-positive rate for cytogenetic testing was significant in both affected and carrier individuals *(130,138)*. The other three fragile sites in the Xq27-28 region, one common site (FRAXD) and two rare sites (FRAXE and FRAXF), were the major contributors, because the rare sites cannot be cytogenetically distinguished from FRAXA. However, technical and interpretative problems in the laboratory were also factors. Any family with a cytogenetic diagnosis of fragile X syndrome should have one family member (affected or obligate carrier) tested with DNA methodology, especially prior to carrier testing in other family members via DNA technology. Normal females who were defined as carriers based on low-level cytogenetic expression should be retested as well *(138)*.

Molecular Testing

By the time DNA-based diagnosis of fraX became available, the problems with cytogenetic testing had become apparent *(139)*. First, fraX expression was variable (between 1% and 50%), with females usually having fewer positive cells than males, and obligate carriers often tested negative. Second, the presence of the other three fragile sites on Xq reduced the reliability of cytogenetic scoring. Finally, lower expression in cell types other than lymphocytes compromised prenatal diagnosis. DNA-based testing has solved all these problems and usually costs less as well. Thus, cytogenetic fraX testing should be retired, as it is less accurate and more expensive. In fact, the reimbursement (CPT) code for such testing has been deleted.

The objective of all DNA-based methods for fraX is to identify a piece of DNA containing the CGG repeat and determine its length by electrophoresis in order to classify it as normal, premutation or full mutation.

DNA-based Methods

The two DNA-based methods available for fraX 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. Southern blots are better for 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). According to a recent report by the Quality Assurance Subcommittee of the American College of Medical Genetics Laboratory Practice Committee, both DNA-based methods are considered diagnostic and are 99% sensitive and 100% specific *(140)*. Detailed descriptions of these techniques and illustrations are provided by Maddalena et al. *(92)*.

An important caveat for DNA-based methods is the fact that a small percentage (<1%) of patients with the fragile X syndrome have a normal CGG repeat size. To date, numerous deletions have been reported in the literature *(141)*. Also, two other types of mutation have been reported: (1) a 2-bp substitution that alters splicing and leads to altered levels of FMRP *(142)* and (2) a missense mutation that leads to dysfunctional FMRP *(143)*. Prior et al. *(144)* reported a case of germline mosaicism, an important issue when counseling deletion families.

Protein/mRNA-based Diagnosis

Monoclonal antibodies against FMRP have been used with success to diagnose affected males and some affected females *(145)*. This method cannot be used for premutation testing, but it is more rapid than DNA-based testing. It has been successfully utilized for prenatal diagnosis *(146)* and can also be used for a patient with the physical and mental features of the fragile X syndrome without evidence for an expanded CGG repeat. The protein test can be performed on a variety of samples, including blood and hair root *(147)*.

FRAXE SYDROME

Cytogenetically, FRAXE was described in 1992 *(21)*. The gene (*FMR2*) is located 600 kb distal to FRAXA, and the repeat sizes in normal, premutation, and full mutation individuals are similar (see

Table 4). FRAXE expansion can decrease or increase in both males and females, and two deletions have been identified *(148)*. No point mutations within *FMR2* 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 could further define this phenotype from that of FRAXA *(149,150)*. A knockout mouse model for *FMR2* exhibiting impaired learning and memory might also help to further define the mild phenotype of FRAXE observed in humans *(151)*. Preliminary work suggests that *FMR2*'s gene product acts as a transcription activator *(152)*, but its function in relation to the phenotype remains largely unknown. FRAXE expansions are not common in human populations (approximately 1 in 23,500 individuals) *(119)*, and, although available, DNA analysis for the FRAXE expansion in not widely utilized.

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 because 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

Prenatal DNA testing is indicated in families in which 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 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, but interpretation must be based on the size of the allele, not its methylation pattern. Maternal cell contamination, if present, can be seen by fetal to maternal comparison. This is most evident when using double digests and the CVS specimen is not methylated *(153)*.

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 it is based on the risk probabilities developed in family studies of such females.

Genetic Counseling

Genetic counseling is a vital part of a multi-disciplinary approach to helping families adjust to and cope with the stresses of fragile X syndrome and its impact on the family. [See the excellent review by Gane and Cronister *(154)*.] Genetic counseling covers a multitude of areas such as diagnosis, prognosis, recurrence risks, family planning options, management, psychosocial issues, to name a few. It provides the family 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 Chapter 20.

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Genomic Imprinting and Uniparental Disomy

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INTRODUCTION

Genomic imprinting refers to the process of differential modification and expression of parental alleles. As a result, the same gene can function 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 *(1)* 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 *(2)*.

Evidence for the existence of genomic imprinting is manifold. Initial experimental approaches include studies in mouse embryos using nuclear-transplantation techniques *(3–7)*. These experiments involve the removal and reintroduction of pronuclei into zygotes, thus creating embryos that have either only the maternal or paternal genome. In parthenogenetic eggs (i.e., eggs that contain two maternal pronuclei and no paternal pronucleus), fetal development is relatively good but extraembryonic tissue development is poor. In contrast, in androgenetic eggs (i.e., eggs containing two paternal pronuclei and no maternal pronucleus), the development of extraembryonic tissue is good, but fetal development is poor. In either case, the embryos fail 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, whereas 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 Chapter 8). Ovarian teratoma is an embryonal tumor that contains tissues predominantly derived from ectodermal germ layers, 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 retardation and abnormally small placentas are seen, whereas 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

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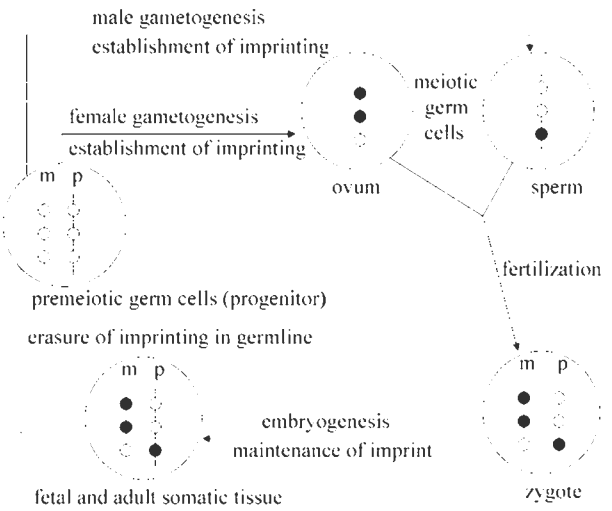

Fig. 1. Diagrammatic representation of the imprinting process. Open circles represent undermethylated genes; filled circles represent hypermethylated genes. m = maternally derived chromosome; p = paternally derived chromosome. See text for details.

mammals is, in general, random in somatic cells *(15)*, studies in interspecies crosses between marsupials reveal that the paternally derived X chromosome is preferentially inactivated in female kangaroo somatic tissues *(16)*. In extraembryonic tissues, the paternally derived X chromosome is preferentially inactivated in mice *(17–19)*. Data on the pattern of X inactivation in trophoblastic cells of human placenta are less uniform; both preferential inactivation of the paternal X *(20,21)* and random X inactivation *(22–24)* have been reported.

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 *(25)*. It is estimated that approximately 0.5% of mammalian genes are imprinted *(26)*. To date, approximately 25 imprinted genes have been identified in humans, and many more possibly imprinted genes are waiting to be identified and confirmed *(27)*.

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 *(28,29)* (see **Fig. 1**). Thus, stable and differential modification of chromatin is required. Differential methylation 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 *(30)*. Yen et al. showed that the human *HPRT* (hypoxanthine phosphoribosyltransferase) gene is hypomethylated on the active X chromosome relative to the inactive X *(31)*. 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 *(32)*. These observations suggest that DNA

methylation could 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 is 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 changes from generation to generation depending on the sex of the parent transmitting the transgene *(33–35)*. 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 is undermethylated in all tissues and is expressed only in the heart *(35)*. This observation suggests that methylation status alone does not determine the expression of a transgene and that undermethylation might 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 *(36)*. By comparing CpG methylation and nuclease sensitivity of chromatin in mouse embryos, Ferguson-Smith et al. *(37)* 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. This normally silent paternal *H19* allele is activated in DNA methyltransferase-deficient embryos *(38)*, providing in vivo evidence that a direct correlation is present between DNA methylation and gene activity.

Studies of the mouse *Igf2* gene showed that, contrary to *H19*, the paternal allele is expressed in embryos, whereas the maternal allele is silent, but both parental alleles are transcriptionally active in the choroid plexus and leptomeninges *(39)*. Therefore, imprinting of *Igf2* might also be tissue-specific. In addition, studies using mouse embryos with maternal duplication and paternal deficiency of the region of chromosome 7 that encompasses *Igf2* showed that the chromatin of the 5' region of the repressed maternal *Igf2* allele is potentially active for transcription, that is, it is hypomethylated and contains DNase I hypersensitive sites *(40)*. 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 (41,42)*. 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 *(43)*. The parental-origin-specific difference in methylation for this gene has been demonstrated in two distinct CpG islands *(44)*. 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-q13. These include the *ZNF127*/DN34 gene (D15S9) studied in PWS and AS patients *(45)* and in complete hydatidiform moles *(46)*, the small nuclear ribonucleoprotein polypeptide N *(SNRPN)* gene *(47,48)*, and the DNA sequence PW71 (D15S63) *(49)*. 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)

(50) and *IGF2* (paternal allele active) *(51,52)*, both located on the short arm of chromosome 11 at band 11p15. In the case of *IGF2*, although it is the paternal allele that is active, the maternal allele is hypomethylated and 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 *(53)*.

A difference in DNA replication timing of maternal and paternal alleles of imprinted genes has also been observed *(54–57)*. Cell cycle replication timing has been shown to correlate with gene activity: genes that are expressed generally replicate earlier *(58,59)*. Furthermore, most genes on homologous chromosomes replicate synchronously *(60)*. This is not the case for imprinted genes. Using fluorescence *in situ* hybridization (FISH) (see Chapter 17) on interphase nuclei and scoring for the stage of the two alleles in the S-phase, Kitsberg et al. *(54)* showed that the imprinted genes *H19, Igf2, Igf2r*, and *Snrpn* in mice and their corresponding positions in the human genome all replicate asynchronously, with the paternal allele replicating early. Studies of genes in the 15q11-q13 region in humans demonstrated that most show a paternal-early/maternal-late pattern, with some exhibiting the opposite pattern *(55,56)*. Therefore, it appears that imprinted genes are embedded in DNA domains with differential replication patterns, which might provide a structural imprint for parental identity *(55)*.

Thus, the process of genomic imprinting is very complex, and although DNA methylation plays a critical role in genomic imprinting, the process is much more complex than simply inactivating a gene by methylation. It could involve an interaction among DNA methylation, chromatin compaction *(61)*, DNA replication timing, and potentially other mechanisms *(62)*.

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 the phenotype depends on 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 situations arising from uniparental disomy (UPD). In addition, imprinted genes could 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 *(63–65)*.

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-q13 region *(66–68)*, but they involve different genes *(69,70)*. The etiologies of these disorders include (1) the absence of a parent-specific contribution of this region as a result of either deletion *(71–74)* or UPD *(75–79)*, (2) disruptions in the imprinting process *(80–84)*, and (3) mutations within the gene *(70,85)*.

The clinical phenotype of PWS has been well characterized *(86)*. Briefly, it includes hypotonia during infancy, obesity, hyperphagia, hypogonadism, characteristic facies, small hands and feet, hypopigmentation, and mental deficiency. Approximately 70% of cases have an interstitial deletion of a 4-Mb sequence at 15q11-q13 on the paternally derived chromosome 15 *(61)*. Approximately 25% of cases are the result of maternal UPD for chromosome 15 *(75,78)*, and 2% or so as a result of an abnormality of the imprinting process, causing a maternal methylation imprint on the paternal chromosome 15 *(82,83)*. Many paternally expressed transcripts have been identified in a cluster in the proximal part of the 15q11-q13 region. These include *ZNF127, NDN, MAGEL2 (NDNL1), SNURF/SNRPN, PAR-5, IPW, PAR-1, PWCR1,* and at lease seven additional transcripts (reviewed in ref. *87,88–90*). This clustering of paternally expressed transcripts suggests strong regional control of

the imprinting process *(90)*. It appears that PWS is a contiguous gene syndrome; no PWS patient has been reported who has a mutation of only one of the PWS region genes.

The clinical phenotype of AS patients is distinct from that of PWS *(91)*. Briefly, it includes microcephaly, ataxia, characteristic gait, inappropriate laughter, seizures, severe mental retardation, and hypopigmentation. Approximately 70% of AS patients have a deletion of the same 4-Mb sequence at 15q11-q13 on the maternally derived chromosome 15 *(72–74)*. From 2% to 5% are the result of paternal UPD for chromosome 15 *(76,77,79)*, 6–10% as a result of an abnormality of the imprinting process, causing a paternal methylation imprint on the maternal chromosome 15 *(80–82,84)*, and 4–6% as a result of a mutation within the AS gene (reviewed in ref. *92*; see also refs. *70* and *85)*. 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 *(70,85)*. The imprinting of *UBE3A* is tissue-specific, being restricted to the brain *(93–95)*. More recently, another imprinted gene, *ATP10C*, mapped within 200 Kb telomeric to *UBE3A*, has also been shown to be expressed only on the maternal allele *(96)*. It is speculated that *ATP10C* could be involved in phospholipid transport and could also contribute to the AS phenotype. Both *UBE3A* and *ATP10C* are located at the distal part of the 15q11-q13 region.

In both PWS and AS patients with abnormalities of the imprinting process, Buiting et al. identified inherited microdeletions in the 15q11-q13 region *(97)*. 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 *(98–100)*. 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; that is, 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, FISh to detect 15q11-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.

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), rhabdomyosarcoma, and hepatoblastoma *(101,102)* (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 the following:

1. Paternal UPD for the p15 region of chromosome 11 in approximately 20% of sporadic cases *(103,104)*.
2. Cytogenetic abnormalities involving 11p15, present in a small number (approximately 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 *(105,106)*, and maternally inherited balanced rearrangements involving 11p15 *(106,107)*.

3. Imprinting certer mutation in the gene cluster *IGF2*/*H19* or *KCNQ1*/*KCNQ1OT1 (108)*. In familial cases, the segregation appears to be autosomal dominant with incomplete penetrance *(102)*. Furthermore, penetrance appears to be more complete with maternal inheritance; that is, there is an excess of transmitting females *(109,110)*.
4. Mutation in the maternally active *CDKNIC* ($p57^{KIP2}$) gene *(111)*. *CDKNIC* , a cyclin-dependent kinase inhibitor, is a negative regulator of cell proliferation; its overexpression arrests cells in G1. Germline *CDKNIC* mutations have been found in 40% of familial and 5% of sporadic BWS cases *(112)*.

Linkage studies confirm that BWS maps to 11p15 *(113,114)*. Imprinted genes in this region have been shown to consist of two domains separated by nonimprinted genes *(115,116)*. The proximal centromeric domain contains *CDKNIC* ($p57^{KIP2}$) (maternal allele active) *(117,118)*, *KCNQ1* (maternal allele active), and *KCNQ1OT1 (LIT1)* (paternal allele active) *(108,119)*. The distal telomeric domain contains *H19* (maternal allele active) and *IGF2* (paternal allele active). The paternally expressed genes are growth promoter genes, whereas 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 allele from both parents, an altered pattern of allelic methylation of *H19* and *IGF2* has been reported *(104,120)*. In these patients, a paternal imprint pattern is seen on the maternal allele, which results in the nonexpression of *H19*, whereas *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 IC 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, thus preventing the resetting of imprinting in the maternal germline.

In addition to these abnormalities involving 11p15, other not yet well-defined mechanisms or genetic loci might 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 *CDKNIC* gene, and methylation study of *IGF2, H19,* and *KCNQ1OT1*. A recent study reported that by analyzing the methylation status of the *KCNQ1OT* and *H19* genes in leukocytes, over 70% of the 97 patients could be diagnosed *(121)*. Of all cases with abnormal methylation, 80% involved the promoter region of the *KCNQ1OT* gene and 20% the *H19* gene.

Cancer

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 *(122,123)*. Approximately 30% of cases are familial. Mutation in *SDHD*, a gene mapped to 11q23 that encodes a mitochondrial respiratory chain protein, has recently been reported in families with paraganglioma *(124–126)*. Inheritance of PGL is autosomal dominant, with both males and females affected. However, transmission is almost exclusively through the father *(122,127,128)*. Only male gene carriers will have affected offspring. The disease is not observed in the offspring of affected females until subsequent generations, when transmission of the gene through a male carrier has occurred. These observations suggest genomic imprinting. However, expression of *SDHD* is biallelic; that is, it is expressed from both maternal and paternal alleles, in all tissues studied to date (lymphoblastoid cell lines, adult brain, fetal brain and kidney) *(124)*. 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 might 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, whereas duplication of certain maternal alleles can inhibit cell proliferation.

In Wilms tumor and rhabdomyosarcoma, LOH involves chromosome 11 *(129–131)*. LOH does not involve markers for 11p13, the proposed Wilms tumor locus, but only markers on 11p15.5 *(130)*. Known imprinted genes in the 11p15.5 region include *H19*, *IGF2,* and *CDKNIC* ($p57^{KIP2}$) (see above). The expression of *CDKNIC* is reduced in Wilms tumor *(118)*. In addition, by using several overlapping subchromosomal transferable fragments from 11p15 distinct from *H19* and *IGF2*, Koi et al. *(132)* were able to obtain in vitro growth arrest of rhabdomyosarcoma cells. These observations suggest that *CDKNIC*, which is normally active on the maternal allele only, might be a candidate for a tumor suppressor gene. Loss of the active *CDKNIC* allele on the maternal chromosome results in tumor development. In addition to LOH, another possible mechanism, loss of imprinting LOI (see the subsection on BWS), has been proposed. Ogawa et al. *(133)* reported biallelic *IGF2* RNA synthesis in four of 30 Wilms tumors they studied. 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 *(134)*. Disruption of the imprinting mechanism (i.e., LOI), might 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 *(135)*. These studies indicate that although 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 (*RB*) on chromosome 13 at band q14 has been observed *(136)*. 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 *(137,138)*, consistent with the general observation that new germline mutations arise predominantly during spermatogenesis. 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 *(139)*, suggesting that genomic imprinting might be involved. Data are less clear in sporadic retinoblastomas. No predilection in the parental origin of the somatic allele loss was noted in some studies *(138,140)*, but a preferential loss of the maternal allele, which implies a preferential initial somatic mutation on the paternal allele, was reported in one study *(141)*. Thus, the role of genomic imprinting in retinoblastoma is unclear at this time.

Neuroblastoma

In neuroblastoma, deletions of chromosome 1p and amplification of the *MYCN* gene on chromosome 2p are frequently seen *(142)*. Preferential amplification of the paternal *MYCN* allele in neuroblastoma tumor tissues has been reported *(143)*. In tumors with *MYCN* amplification, loss of parental 1p alleles was found to be random *(143,144)*. In tumors without *MYCN* amplification, loss of 1p was previously reported to be preferentially maternal (16 of 17 cases) *(144)*, but random in a more recent study *(145)*. Thus, there is no clear evidence that the putative tumor suppressor gene mapped at 1p36.2-36.3 is imprinted, and the role of imprinting in neuroblastoma is not clear at the present time.

UNIPARENTAL DISOMY

The term uniparental disomy (UPD) was introduced by Engel in 1980 *(146)*. It describes a phenomenon in which both homologs or 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 *iso*disomy describes a situation 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 *hetero*disomy 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.

Uniparental disomy for an entire chromosome can occur as a result of gamete complementation, as suggested by Engel *(146)*. Because aneuploidy is relatively frequent in gametes, the chance union of two gametes, one hypohaploid, the other hyperhaploid for the same chromosome, will result in a diploid zygote with UPD for that chromosome. Structural rearrangements, such as Robertsonian or reciprocal translocations (see Chapter 9), increase the chance of meiotic malsegregation and thus could predispose to UPD. This is best illustrated by the case reported by Wang et al. *(147)*, 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 (see **Fig. 2** and Chapters 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" *(148)*. The vast majority of trisomic conceptuses are nonviable; they could 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 (see **Fig. 3**). Because the loss occurs postzygotically, mosaicism in such conceptuses is often observed, with the trisomic cell line sometimes confined to the placenta (see Chapter 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 by duplication of the single chromosome in monosomic conceptuses *(149)*. 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 *(149)* and later in many other patients with recessive disorders and UPD (see below). 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 might modify the phenotype.

The number of reported UPD cases has recently been increasing rapidly. Of the 47 possible types of UPD of whole chromosomes, 34 have been reported to date. Some provide clear evidence for imprinting and some seem to suggest no such effect, whereas others will require accumulation of additional data before their status in this regard can be determined.

upd(1)mat

At least five 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

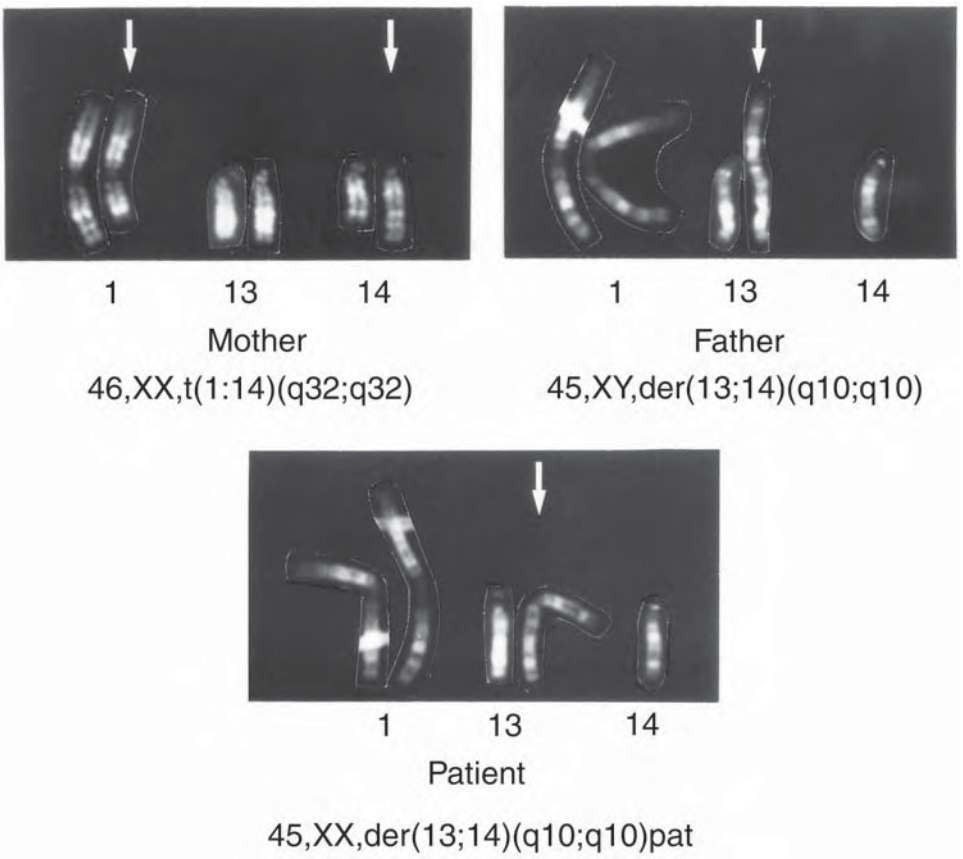

Fig. 2. An example of paternal UPD formation by gamete complementation. Malsegregation involving chromosome 14 occurred in both parents as the result of structural rearrangements [Mother: reciprocal translocation t(1;14)(q32;q32); father: Robertsonian translocation der(13;14)(q10;q10)]. The patient inherited 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.

nonsense mutation in the *LAMB3* gene on chromosome 1 *(150)*. 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 Chadiak–Higashi syndrome (CHS) was found to be homozygous for a nonsense mutation in the *LYST* gene for CHS on chromosome 1 *(151)*. The mother was a carrier of the mutation, whereas the father had two normal *LYST* alleles. Two additional unrelated patients had lethal trifunctional protein deficiency resulting from homozygous α-subunit mutations. In both patients, the mothers were heterozygous for the mutation and the fathers did not have the mutation *(152)*. Another case involved a patient with insulin-dependent diabetes mellitus. Maternal UPD for chromosome 1 was accidentally discovered during a family linkage study *(153)*. 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 five 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

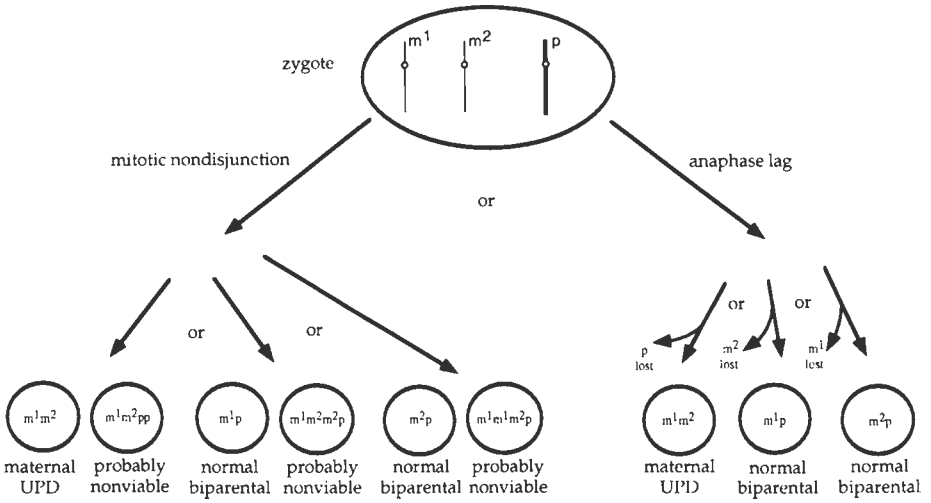

Fig. 3. A diagrammatic representation of maternal UPD formation by "trisomy rescue." A trisomic zygote resulting from maternal meiosis I nondisjunction is depicted here. Loss of one of the trisomic chromosomes through either mitotic nondisjunction or anaphase lag results in euploidy. Uniparental disomy occurs in one-third of these cases. m^1 and m^2: maternally derived chromosomes; p: paternally derived chromosome.

which the father was a heterozygote and the mother was normal *(154)*. The child was otherwise developmentally normal. Three additional patients, one with congenital insensitivity to pain with anhidrosis, one with Herlitz junctional epidermolysis bulosa, and one with Leber congenital amaurosis, were recently reported *(155–157)*. All three 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 Chapters 3 and 9] *(158)*. 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 seven cases. Four cases were associated with confined placental mosaicism (CPM) for trisomy 2 *(159–162)*, two cases resulted from *de novo* isochromosome formation of the short arm and long arm of chromosome 2 [i(2)(p10),i(2)(q10); see Chapters 3 and 9] *(163,164)*, and one case was discovered at age 3 during paternity testing *(165)*. In the latter case and one of the cases with isochromosomes, no phenotypic abnormalities were present *(161,165)*. A common phenotype was observed in the other five cases. This includes intrauterine growth retardation (IUGR), oligohydramnios, pulmonary hypoplasia, hypospadias (in two patients), and normal development in the four surviving patients at ages 6 months, 20 months, 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 cases with isochromosomes *(164)*, suggesting a possible imprinting effect of maternal UPD 2. 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 *(166)*. Therefore, it is still not clear whether maternal UPD 2 confers an imprinting effect.

upd(2)pat

One case of paternal isodisomy for the entire chromosome 2 was recently reported. A 34-year-old woman diagnosed with retinitis pigmentosa was found to have a homozygous *MERTK* mutation *(157)*. She was otherwise phenotypically normal. The patient's father was heterozygous for the mutation and the mother did not carry the mutation. Another case with isodisomy for paternal 2p as described in the subsection on upd(2)mat was phenotypically normal. Paternal UPD 2 therefore does not appear to have an imprinting effect.

upd(4)mat

A single 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 Chapters 3 and 9] was reported in an abstract *(167)*. Cytogenetic studies were performed because of multiple early miscarriages. The patient was otherwise phenotypically normal. Another case with confined placental mosaicism for trisomy 4 in a fetus with IUGR and oligohydramnios followed by intrauterine fetal death at 30 weeks of gestation was determined to have maternal UPD 4 *(168)*. 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 *(169)*. 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. Paternal UPD 5 is unlikely to have an imprinting effect.

upd(6)mat

Maternal uniparental isodisomy for chromosome 6 was first identified in a renal transplant patient in the process of human leukocyte antigen (HLA) typing *(170)*. Another patient with congenital adrenal hyperplasia resulting from unmasking of the maternally inherited mutation in the 21-hydroxylase gene had IUGR but good catch-up growth *(171)*. There is no clear evidence for an imprinting effect.

upd(6)pat

More than ten cases of paternal uniparental isodisomy for chromosome 6 have been reported (reviewed in ref. *172)*. All cases involve isodisomy, including two with segmental disomy *(173,174)*. This suggests that paternal UPD for chromosome 6 usually results from postzygotic error. Many patients had transient neonatal diabetes mellitus (TNDM) associated with very low birth weight. Recently, an imprinted cell cycle control gene *ZAC*/*PLAGL1* at 6q24 with differential methylation of parental alleles has been identified *(175,176)*. This gene is expressed only from the paternal allele and is a strong candidate gene 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 one-fifth of cases of TNDM *(176)*. Paternal UPD 6 clearly has an imprinting effect.

upd(7)mat

More than 20 patients with maternal UPD for chromosome 7 have been reported in the literature *(25,177–179)*. This was the first documented UPD in humans, identified in two individuals with cystic fibrosis and short stature *(149,180)*. Approximately 7–10% of patients with Silver–Russell syndrome (SRS) are noted to have maternal UPD 7 *(178,179,181,182)*. SRS is a heterogeneous disorder. The clinical phenotype includes intrauterine and persistent postnatal growth retardation, body asymmetry, triangular face, prominent forehead, decreased subcutaneous tissue, delayed bone age, and usually normal intelligence. Two regions on chromosome 7 have recently been shown to

cause SRS *(183)*. One region at 7p11.2-p13 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, a strong candidate gene for SRS *(184–186)*. A second region at 7q31-qter contains the two other imprinted genes on chromosome 7 identified to date: *PEG1/MEST* and γ2-*COP*. The role of these two genes in SRS is not yet clear *(179,187)*. Maternal UPD 7 clearly has an imprinting effect.

upd(7)pat

Two 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 *(188)*. The other 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 *(189)*. In addition, two patients had paternal isodisomy 7p and maternal isodisomy 7q *(190,191)*. These two patients had similar phenotypes that resembled that 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 *(192)*. 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 *(193)*. This $5^1/_2$-year-old girl had normal development and lipoprotein lipase (LPL) deficiency as a result of a mutation of the *LPL* gene. The patient was ascertained because of 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 might not exist.

upd(9)mat

Six 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 *(194)*. Two homozygotic female twins had Leigh syndrome as a result of inheriting two copies of the mutated *SBRF-1* gene from their mother *(195)*. 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 Chapter 12) for trisomy 9 *(196)*. Pathological examination of the abortus was not possible. 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 Chapters 3 and 9]. Molecular analysis demonstrated maternal isodisomy *(197)*. The available data indicate that maternal UPD 9 might not have an imprinting effect.

upd(10)mat

A single case of prenatally diagnosed maternal UPD for chromosome 10 associated with confined placental mosaicism (see Chapter 12) has been reported *(198)*. The infant was phenotypically and developmentally normal at 8 months of age. 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 three cases. One patient had hemihypertrophy, congenital adrenal carcinoma, and Wilms tumor *(199)*. The second had associated confined placental mosaicism (see Chapter 12) for trisomy 11 and intrauterine death occurred between 19 and 20 weeks gestation. This fetus had growth retardation, aberrant intestinal rotation, and hypospadias *(200)*. 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 *(201)*. In addition, many cases of paternal segmental UPD for distal 11p associated with BWS have been observed (see above). The existence of an imprinting effect resulting from paternal UPD 11 is clear.

upd(12)mat

A single case of maternal UPD for chromosome 12 was recently reported *(202)*. The infant had normal somatic and psychomotor development with no congenital anomalies or dysmorphic features at 6 weeks of age. Karyotype showed mosaicism with the presence in some cells of a small marker chromosome that consisted of chromosome 12 centromeric heterochromatin with no euchromatic material. This indicates that the mechanism for the occurrence of UPD in this case is by trisomy rescue. It appears that maternal UPD 12 might not have an imprinting effect.

upd(13)mat

At least two cases of maternal UPD for chromosome 13 have been reported *(203,204)*. 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 as a result of maternal UPD 13 is very unlikely.

upd(13)pat

Five cases of paternal UPD for chromosome 13 have been reported. One was the mother of one of the maternal UPD 13 patients described above *(203)*. 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 *(205)*. The other four cases all had *de novo* der(13;13)(q10;q10) translocations (see Chapters 3 and 9) *(206–208)*. Three of them were complete isodisomies, and one exhibited evidence of recombination with proximal isodisomy and distal heterodisomy. All four patients were phenotypically normal. Therefore, paternal UPD 13 does not appear to have an imprinting effect.

upd(14)mat

Maternal UPD for chromosome 14 has been reported in at least 20 cases (reviewed in refs. *209* and *210,211–213)*. A distinct clinical phenotype is present and consists of 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. Evidence for an imprinting effect resulting from maternal UPD 14 is clear.

upd(14)pat

Nine cases of paternal UPD for chromosome 14 have been reported *(147,214–218)*. 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 the only patient who was beyond 20 months of age at the time of reporting *(147)*. These observations indicate that an imprinting effect resulting from paternal UPD 14 exists.

Studies comparing maternal and paternal UPD cases with cases of partial trisomy *(219)* and partial monosomy *(220)* of various segments of 14q suggest that 14q23-q32 might be the region where the imprinted genes on chromosome 14 reside.

Human chromosome 14 has significant homology to mouse chromosomes 12 and 14 *(221)*. Mouse chromosome 12 is imprinted, and both maternal and paternal disomy cause early embryonic death *(222)*. 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 *(75,78,223,224)*. As discussed above, upd(15)mat accounts for approximately 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, whereas a higher frequency of hypopigmentation is seen in patients as a result of deletion of paternal 15q11-q13 *(223–225)*. 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 *P* gene (mouse homolog pink-eyed dilution *p* gene) located at 15q11-q13 *(226–228)*. The human *P* gene is not imprinted and both copies are functional in UPD patients. Hypopigmentation is therefore more prominent in PWS patients because of deletion. Differences in other clinical features between these two groups are less clear-cut. Although there might not be a significant difference in the overall severity, female UPD patients were found to be less severely affected than female deletion patients in one study *(223)*, and UPD patients were found to be less likely to have "typical" facial appearance than deletion patients in another study *(224)*. These differences can again be attributed, at least partially, to the presence of two copies of nonimprinted genes in UPD cases, whereas there is haploinsufficiency of these genes in the deletion cases.

upd(15)pat

At least 19 cases of paternal UPD for chromosome 15 associated with Angelman syndrome have been reported in the literature *(77,79,229–231)*. Paternal UPD 15 accounts for approximately 2–5% of AS patients. AS patients with paternal UPD could have a milder phenotype than those with a maternal deletion of 15q11-q13 *(79,229–231)*, although reports showing no difference in clinical severity in these two groups of patients are also available *(230)*. One possible mechanism for the milder phenotype in UPD patients might be the presence of many nonimprinted genes in the 15q11-q13 region in these patients, whereas these are absent in deletion patients. Alternatively, as proposed by Bottani et al. *(79)*, it might be the result of 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.

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 *(232–240)*, and potentially many more cases are not reported. 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 might result from the presence of trisomy 16 cells in the placenta *(232)*; however, no catch-up growth was observed in these patients. 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 atrioventricular (A-V) canal defect in one and atrial septal defect (ASD) and ventricular septal defect (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) might exist *(235,237,239)*. In addition, in a recent 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 *(241)*. Although not yet certain, the existence of an imprinting effect as a result of maternal UPD 16 is a distinct possibility.

upd(16)pat

A single case of paternal UPD for chromosome 16 has been reported *(242)*. This case was associated with confined placental mosaicism (see Chapter 12). Paternal isodisomy for chromosome 16 was prenatally diagnosed and confirmed after birth. IUGR was present with catch-up growth observed at 13 months of age. Minor physical abnormalities included bilateral pes calcaneus and an 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 single case of maternal UPD involving the entire chromosome 17 was reported in a 2-year-old boy with trisomy 17 confined placental mosaicism (see Chapter 12) *(243)*. His growth and psychomotor development was normal. There is no evidence that maternal UPD 17 confers an imprinting effect.

upd(20)mat

Three cases of maternal UPD 20 have been reported *(244–246)*. 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 Chapter 12) for trisomy 20. The common features in these three patients at ages 4 years, 35 months, and 17 months, respectively, are prenatal and postnatal growth retardation. Isolated findings included mild facial dysmorphism, strabismus, microcephaly, macrocephaly, developmental delay, and hyperactivity. It is not clear whether maternal UPD 20 has an imprinting effect.

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 *(247)*. 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 anotia, microcephaly, congenital heart disease, and Hirschsprung disease. However, this case was complicated by the presence of trisomy 20 cells in skin and the possibility of deletion of genes at the terminal rearrangement site. 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 two patients *(248,249)*. Both had a balanced *de novo* (21;21) Robertsonian translocation (see Chapters 3 and 9) and were phenotypically normal. Although maternal UPD 21 has been reported in early abortus specimens *(250)*, it has not been possible to clearly attribute embryonal death to UPD. Therefore, maternal UPD 21 might be considered at this time to have no imprinting effect.

upd(21)pat

Two cases of paternal UPD for chromosome 21 have been reported *(251,252)*. In both cases, UPD resulted from *de novo* formation of a Robertsonian translocation (see Chapters 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 *(253–255)*. All three phenotypically normal individuals were ascertained via history of multiple spontaneous abortions and were found to have balanced (22;22) Robertsonian translocations (see Chapters 3 and 9). Another prenatally diagnosed case with nonmosaic trisomy 22 in placental tissue and apparently nonmosaic normal 46,XY cells in newborn blood had severe IUGR, first-degree hypospadias, and other features attributed to prematurity *(256)*. 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 *(257)*. Again, it was observed in a phenotypically normal individual with a balanced (22;22) Robertsonian translocation (see Chapters 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 *(258,259)*. The first two cases were detected by screening a normal population of 117 individuals. The third patient had Duchenne muscular dystrophy resulting from homozygosity of a maternally inherited deletion of exon 50 of the dystrophin gene. These observations indicate that maternal UPD for the X chromosome might not have an imprinting effect.

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 Chapter 10) has been reported *(260)*. 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.

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 *(261)*. 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 might therefore not have an imprinting effect.

Summary

In summary, of 47 possible maternal and paternal UPDs for whole chromosomes in humans, 34 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), one might have an imprinting effect (2mat), 19 are unlikely to have imprinting effects [1mat, 1pat, 2pat, 4mat, 5pat, 6mat, 8pat, 9mat, 10mat, 12mat, 13mat, 13pat, 17mat, 21mat, 21pat, 22mat, 22pat, Xmat, and (XY)pat], and the status is not known for 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 the following:

- Confined placental mosaicism (CPM) (see Chapter 12) with a trisomic cell line for chromosomes 6, 7, 11, 14, or 15 (and possibly also 16) found in chorionic villus sampling 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 Chapters 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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20
Genetic Counseling

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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. The following is the definition of genetic counseling adopted in 1983 by the National Society of Genetic Counselors (NSGC), the professional membership organization for genetic counseling:

> 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 might 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

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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, 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 on 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* (see **Fig. 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, in obtaining the information for the pedigree, the genetic counselor gains useful information about the dynamics of the family in general and in relation to the condition in question *(5)*. In addition, the pedigree 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 *(6)*.

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 clini-

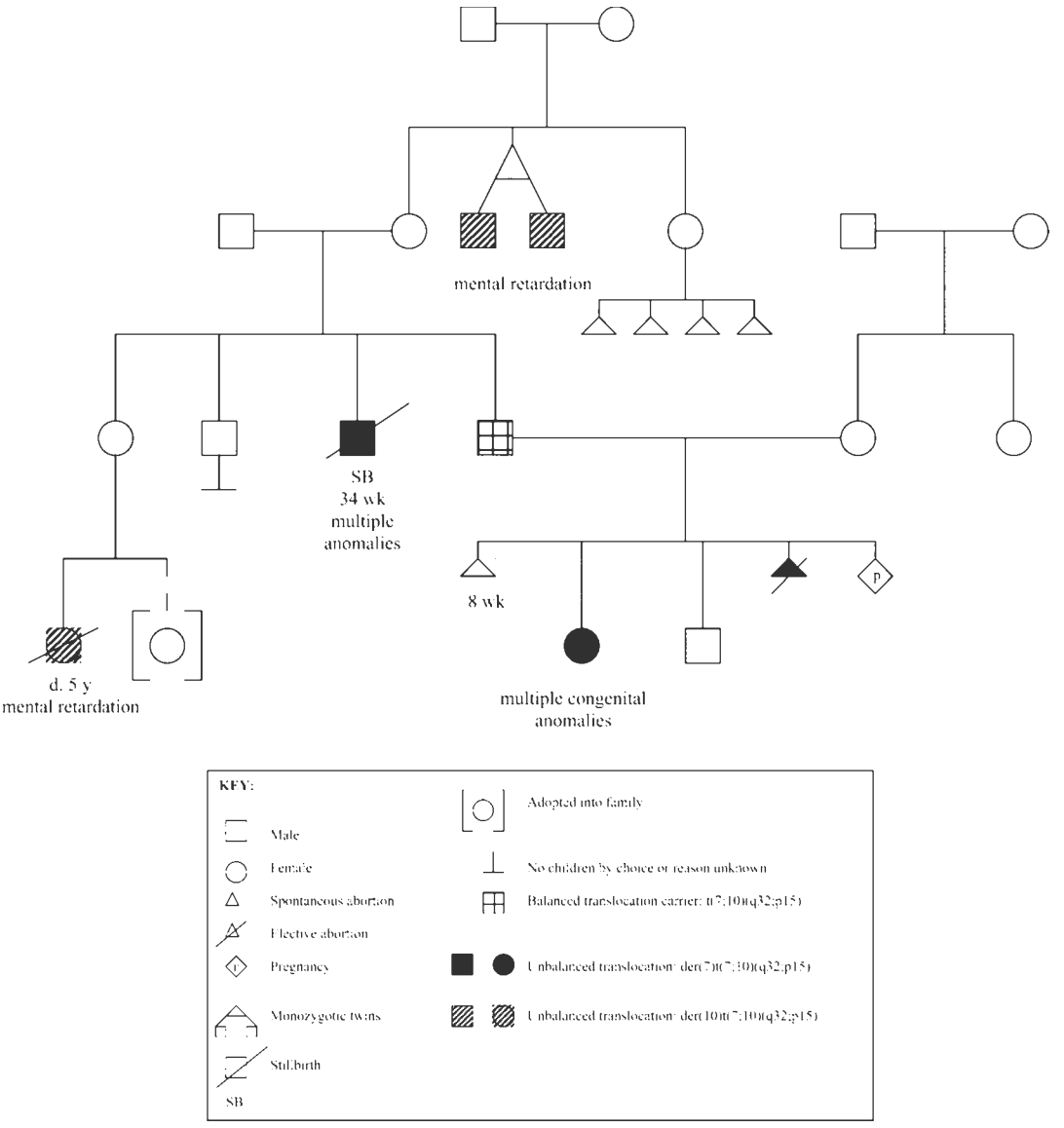

Fig. 1. Pedigree of a family carrying a balanced translocation involving the long arm of chromosome 7 and the short arm of chromosome 10. See key for interpretation of symbols.

cal 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 or 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 might 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 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 might 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 on 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 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 Chapter 18), and hemophilia, a bleeding disorder.

It should be briefly noted that, in the past, cytogenetic methods were routinely employed to test for fragile X syndrome. Using a folate-deprived culture medium, the fragile site on the long arm of the X chromosome associated with this condition could be expressed. Due to serious limitations of this methodology in detecting affected individuals and carriers, molecular diagnostic testing has replaced cytogenetic analysis for fragile X syndrome as the preferred testing method *(7–9)* (see Chapter 18).

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 *(10)*.

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 multi-organ 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 Chapter 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 *ABL*, is associated with chronic myelogenous leukemia (CML). Similarly, Burkitt's 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 *(10,11)*. Occasionally, when chromosome analysis is performed for the indication of a hematological abnormality, a chromosome abnormality that might 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 *(11)*.

Consanguinity

When both members of a couple share at least one common ancestor, they should 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 *(12)*. 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 to have a pregnancy or child affected with a chromosome abnormality increases with advancing maternal age (**Table 1**) *(13)*. Therefore, the current standard of care is to routinely offer prenatal diagnosis (see Chapter 12) to all pregnant women who will be 35 or older at their estimated date of delivery (EDD) *(14)*.

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 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 a 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 Chapter 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 to 15% of all recognized pregnancies end in miscarriage *(15)* (see Chapter 13). There are many possible causes of miscarriage, including a chromosomally abnormal conceptus. Approximately 50% of recognized first-trimester miscarriages are chromosomally abnormal (*10,15,16*). 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.

Table 1
Risks for Chromosome Abnormalities at Term by Maternal Age

Maternal Age at Term	Risk for Trisomy 21 *(49)*[a]	Risk for Any Chromosome Abnormality *(13)*[a,b]
15	1:1578	1:454
16	1:1572	1:475
17	1:1565	1:499
18	1:1556	1:515
19	1:1544	1:555
20	1:1528	1:525
21	1:1507	1:525
22	1:1481	1:499
23	1:1447	1:499
24	1:1404	1:475
25	1:1351	1:475
26	1:1286	1:475
27	1:1208	1:454
28	1:1119	1:434
29	1:1018	1:416
30	1:909	1:384
31	1:796	1:384
32	1:683	1:312
33	1:574	1:285
34	1.474	1:243
35	1:384	1:178
36	1:307	1:148
37	1:242	1:122
38	1:189	1:104
39	1:146	1:80
40	1:112	1:62
41	1:85	1:48
42	1:65	1:38
43	1:49	1:30
44	1:37	1:23
45	1:28	1:18
46	1:21	1:14
47	1:15	1:10
48	1:11	1:8
49	1:8	1:6
50	1:6	data not available

[a] Risks based on maternal age at term. Term risks do not include chromosomally abnormal fetuses spontaneously lost before term.

[b] Includes risk for trisomy 21. Does not include 47,XXX.

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 routine chromosome analysis, whereas others, such as microdeletion syndromes, require specialized analysis, such as fluorescence *in situ* hybridization (FISH) (see Chapter 17). 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 on 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 pregnancy might be an option, or during childhood, adolescence, or adulthood. As previously mentioned, during postnatal counseling for a chromosome abnormality, 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 usually caused by trisomy 21, is the most common human chromosome abnormality, affecting approximately 1 in 800 individuals (*10,17*). 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. Everyone with Down syndrome has some degree of mental retardation. The average IQ is in the low to moderate range (40–70), although a range of mental capability exists. Children with Down syndrome often benefit from early programs aimed at stimulation, developmental enrichment, and education (*17,18*).

Trisomy 13

Trisomy 13 results in severe mental retardation and multiple birth defects. The abnormalities most commonly noted in this condition involve the heart, brain, eyes, lip and palate (cleft lip and/or cleft palate), hands and feet (such as polydactyly or extra digits), genitalia, kidneys, and gastrointestinal system. This condition is frequently fatal early in infancy with only 5% of affected individuals surviving the first 6 months of life *(10,18)*.

Trisomy 18

Like trisomy 13, trisomy 18 results in severe mental retardation and birth defects. Congenital heart defects and abdominal wall defects are common, as is growth deficiency. Several other congenital anomalies, including those involving the kidneys, central nervous system, skeletal system, and genitalia are also associated with this condition. Approximately 5–10% of babies affected with trisomy 18 survive the first year of life (*10,18*).

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 one 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 Chapter 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. 1**; see also Chapter 9).

Microdeletion Syndromes

Microdeletion syndromes, as their name implies, are the result of relatively small chromosomal deletions that are usually undetectable via routine cytogenetic analysis. When a clinician suspects that an individual is affected with one of these conditions, FISH 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 DiGeorge syndrome when a heart defect is noted on prenatal ultrasound. In these cases, FISH 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 Chapters 12 and 17.

DiGeorge Syndrome/Velocardiofacial Syndrome (VCFS)

This syndrome results from a deletion involving the long arm of chromosome 22 [del(22)(q11.21q11.23)]. 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 can be sporadic, but it 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 problems, low muscle tone in infancy, hypernasal speech, low calcium levels, renal abnormalities, mental illness, and characteristic facial features *(18)*.

Prader–Willi Syndrome

Approximately 70% 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 Chapter 19). 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 *(18)*. See also Chapter 9.

Angelman Syndrome

Approximately 60–80% 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 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, inappropriate excessive fits of laughter, "jerky" limb movements, characteristic facial features, sleep abnormalities, and seizures *(18)*. Imprinting also plays a role in this disorder. See Chapter 9.

Williams Syndrome

Williams syndrome is the result of a microdeletion on chromosome 7 at the q11.23 locus and involves 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, renal 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 *(18)*. See Chapter 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 usually 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, poor sleep patterns after infancy, characteristic facial features, and behavioral problems. The behavioral problems often include self-injury, attention deficit, and temper tantrums *(15)*.

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 2 years of life *(18)*.

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 *(19)* and can be detected with FISH probes for the unique subtelomeric regions of most chromosomes (see Chapter 17). 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 on 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 *(20,21)*.

Chromosome Instability Syndromes

As discussed in Chapter 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 *(15)*.

Infertility

At times, when one of the members of a couple is a carrier of a structural chromosome rearrangement (see Chapter 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 Chapters 10 and 11.

Sex Chromosome Abnormalities

It has been estimated that, overall, approximately 1/400 infants have some form of sex chromosome aneuploidy *(22)*. A thorough discussion of sex chromosomes and sex chromosome abnormalities can be found in Chapter 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 might create for a young

adult a potentially unique and more delicate set of psychosocial issues, as this diagnosis could 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 1 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 might 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 *(10,18,22)*.

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 *(18,22)*.

Turner Syndrome

Turner syndrome, 45,X, is estimated to affect approximately 1 in 2000 liveborn 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 could 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 are widely spaced. Learning difficulties can be present, although most affected individuals have a normal IQ *(10,18,22)*.

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 *(10,18,22)*. 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 *(10)*.

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 overrepresented in prisons and mental hospitals. Fertility is usually normal *(10,18,22)*.

47,XXX

Females with 47,XXX could be of above average height and experience learning disabilities. Otherwise, there are no remarkable phenotypic features that are associated with this condition *(10,18,22)*.

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 *(16)*. 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 liveborn offspring with birth defects and/or mental retardation (see **Fig. 1**). The risk for an abnormal liveborn 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 *(15)*. 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 Chapter 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 are also discussed. Prenatal chromosome analysis is routinely performed via CVS or amniocentesis (see Chapter 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 or 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 is generally quoted as approximately 1/100 or 1%. 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 retardation 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 *(10,15,23,24)*.

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. Present in this sample are

fetal amniocytes, which are placed in culture and the chromosomes 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 is generally quoted as approximately 1/200 or 0.5% *(10,15)*.

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 Chapter 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 *(25–27)*. Although FISH can yield important information in a short period of time, it is not a substitute for routine cytogenetic analysis. Furthermore, a recommendation from the American College of Medical Genetics states that irreversible action should not be taken on the basis of a FISH result alone *(28)*. 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 can be performed on prenatal specimens for the detection of translocations involving the subtelomeres.

Abnormal Prenatal Screen

Although 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. Serum screening is generally offered to women who are under age 35 but can also be offered to women who are 35 or older and are undecided about pursuing invasive diagnostic testing for chromosome abnormalities. The patient or couple should be fully counseled about the limitations of screening, particularly if the mother is 35 or older. 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 could 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 such screening.

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. 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, these data are used to generate estimated risks for Down syndrome and trisomy 18 *(29–31)*. 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 *(32–35)*.

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 three or four pregnancy-related proteins and is often referred to as the triple screen or quad screen, depending on the number of proteins analyzed. In the triple screen, AFP, human chorionic gonadotropin (hCG), and unconjugated estriol (uE3) are analyzed. In the quad screen, dimeric inhibin A (DIA) is added. As with first-trimester screening, the levels of these analytes, combined with certain other information, yields a risk estimate for Down syndrome and trisomy 18. Unlike first-trimester screening, second-trimester maternal serum screening also screens for the presence of an open fetal defect, such as a neural tube or abdominal wall defect, through the analysis of the level of AFP present in the maternal serum *(10)*.

The relatively new *integrated screen* takes advantage of both of the aforementioned screening tests through the collection of both a first- and second-trimester maternal blood sample. The results of these analyses are combined to yield a second-trimester result. A nuchal translucency measurement, obtained via first-trimester ultrasound, can also be factored into the risk calculation. The integrated screen is expected to yield a higher detection rate and lower false-positive rate for Down syndrome than either first- or second-trimester screening alone *(36)*.

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 subsection First-Trimester Screening). The percentage of aneuploid pregnancies with a demonstrable abnormality on ultrasound depends on the particular chromosome abnormality and the experience of the sonographer. Some of the aneuploidy markers that are potentially detectable on prenatal ultrasound include cardiac malformations, altered fetal growth, duodenal atresia, and cystic hygroma, among others. 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 *(10,37,38)*. See Chapter 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 *(39)*. 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 *(10)*. The prenatal identification of a chromosome abnormality (or any anomaly or genetic condition for that matter) can be traumatic and heartbreaking 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 Chapter 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 might 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 *(40)*.

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. Hypospadius and other nongenital 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 *(41,42)*.

When an apparently balanced *chromosome rearrangement* is identified by CVS or amniocentesis, the first step is to perform chromosome analysis 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 Chapter 19), 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 *(15)*. 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 approx 6.1%. The estimated risks for abnormality associated with a *de novo* Robertsonian translocation or inversion are 3.7% and 9.4%, respectively *(43)*. 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 is 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 Chapter 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 *(15)*.

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 *(15)*. 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 *(15)*.

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 *(44–47)*.

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 1000 *(48)*. The identification of such a marker can create a frustrating situation for the parents, as there is a lack of substantial information about many of these markers. 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 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 *(48)*. 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* nonsatellited marker *(43)*.

Certain markers 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 varying features, ranging from mental retardation and clinical features of Prader–Willi/Angelman syndrome to a normal phenotype *(48)*. See Chapters 8 and 9.

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 in which 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. With the continuing development of new technologies in the field of genetics and the revealing of the genetic contributions to human life and disease, particularly in the realm of genetic predisposition to adult-onset conditions, the public, government, and scientific communities will surely face increasingly complex ethical dilemmas.

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