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Introduction

Chromosomes were first identified in the mid-nineteenth century, but it took almost 75 years to count them accurately—it was not until 1956 that Tjio and Levan [1] reported their seminal observation that the human chromosome number was 46, not 48, as previously believed; Ford and Hamerton confirmed this finding later that year [2]. This serendipitous discovery (due to a laboratory error, hypotonic rather than isotonic solution was used during cell harvesting, which improved chromosome spreading) laid the foundation for further advances in cytogenetics (for reviews of the history of cytogenetics, see [3–7]). Continued improvements in cell culture and harvesting techniques permitted the identification of numerical abnormalities (e.g., Turner and Klinefelter syndromes and trisomies 13, 18, and 21) and major structural chromosomal abnormalities. Despite being able to identify chromosomes at that time only by size and centromere position, Peter Nowell and David Hungerford in 1960 noted that patients with chronic myelogenous leukemia (CML) had a small acrocentric chromosome that appeared deleted; this abnormal chromosome became known as the Philadelphia chromosome

after the city of its discovery [8, 9]. With the advent of banding techniques, however, Janet Rowley was able to recognize that the Philadelphia chromosome arose not from a deletion but rather from a reciprocal translocation between the long arms of a chromosome 9 and a chromosome 22 [10]. Later advances in molecular techniques enabled researchers to discover that the 9;22 translocation fused the *ABL1* gene in 9q34 to the *BCR* gene in 22q11.2 [11–14].

Such gene discoveries led to the next major advance in cytogenetic technology: molecular cytogenetics, specifically fluorescence in situ hybridization (FISH). Whereas conventional G-banded chromosomal analysis allows the entire genome to be analyzed, FISH evaluates specific genes and thus is a technique of much greater resolution and sensitivity. Although chromosomal banding and FISH may have been overshadowed in recent years by the tremendous advances made by highly complex technologies such as array-based comparative genomic hybridization and next-generation sequencing, still, the clinical utility of these two reliable techniques is undeniable. Not only is the demonstration by G-banding or FISH of specific chromosomal abnormalities and gene rearrangements a necessary component in the diagnosis of numerous malignancies, this information can often be obtained in less than 24 h and even, in some cases, the same day.

Since the discovery of the Philadelphia chromosome ushered in the era of genomic medicine, there has been extremely rapid growth in the

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understanding of the genetic basis of neoplasia—there are now almost 63,000 cases of chromosome abnormalities and over 1,500 gene fusions that have been reported in cancer [15]. Further advances in technology, and in the bioinformatics needed to analyze the massive amounts of data these technologies yield, will no doubt transform the field of cytogenomics as profoundly as did the discovery of the number of human chromosomes.

Conventional Cytogenetics

Conventional cytogenetic analysis is performed on metaphase (dividing) cells and provides information about the entire chromosome complement. A variety of tissue types can be cultured to yield metaphase cells for analysis, including peripheral blood, chorionic villi, amniotic fluid, bone marrow, lymph nodes, and solid tumors. Analyses of peripheral blood, chorionic villi, and amniotic fluid are typically performed to identify and characterize constitutional abnormalities (i.e., those present at birth and, barring mosaicism, found in every cell). These specimen types involve somewhat different culture conditions than do those for neoplastic conditions and thus lie outside the scope of this chapter. At a basic level, however, culture procedures have the same goal, namely, optimizing the conditions of cell culture media, temperature, pH, and sterility to stimulate cells to proceed through the cell cycle to mitosis (various culture protocols are described in [3, 16–18]). Cells from the submitted specimens are first isolated, either through centrifugation (for liquid specimens) or disaggregation (for solid tissue specimens), and then placed in tissue culture media. Culture conditions are typically optimized in each laboratory for particular specimen types and include the type of culture (suspension vs. *in situ*), variations on the length of time in culture, additives such as mitogens, and exposure time to a spindle fiber poison such as colcemid. The cultured cells are then harvested after exposure to a hypotonic solution and placed in fixative, typically a 3:1 methanol to acetic acid mixture (Carnoy's fixative). The resulting cell

suspension is “dropped” via pipette onto glass slides, an often idiosyncratic process driven by ambient conditions (e.g., temperature and humidity) and specimen cellularity as well as technologist experience.

As noted above, different studies (constitutional or neoplastic) and different tissues require culture modifications to increase the likelihood of obtaining metaphases from the cells of interest. This is critical in cancer studies because, unlike constitutional abnormalities that are present in every cell of the body, chromosome aberrations associated with malignancies are present only in the involved tissue or even, in the case of leukemias, only one particular cell line. Thus, in neoplasms of mature cells (e.g., mature B cells and plasma cells), the malignant cells may not be actively dividing and may require the addition of a mitogen to stimulate those cells to enter the cell cycle. Studies have shown that the addition of CpG motif-containing oligonucleotides such as DSP30 together with interleukin-2 increases the yield of chromosomal aberrations in mature B-cell neoplasms by G-banding analysis [19, 20]. After the slides have been dropped and aged by heating them in an oven for several hours, they are treated with a proteolytic enzyme such as trypsin or pancreatin and stained with a Giemsa/buffer solution, resulting in the series of alternating light and dark bands (G-bands) characteristic of each of the 22 pairs of autosomes and two sex chromosomes.

The cytogenetic technologist then analyzes (by comparing the two homologues of a chromosome pair band-for-band along their entire lengths) at least 20 metaphase cells, taking care not to skip cells with poor chromosome morphology, because these may be the malignant cells. G-banding enables detection of both numerical (gain or loss of a chromosome) and structural (e.g., translocation, deletion, inversion, etc.) abnormalities throughout the entire genome. These cells are photographed with a digital camera affixed to the microscope, and the technologist interacts with the resulting images via specialized image analysis software. At least two karyograms, images in which the chromosome pairs have been aligned and placed in order, are prepared. G-banding analysis at diagnosis

provides critical information about the types of abnormalities present, and whether or not they can be evaluated by FISH (see below); follow-up studies are compared with the diagnostic study to document therapeutic response. Periodic monitoring can detect cytogenetic evolution, which may even precede morphologic evidence of disease progression.

Even in the early days of cytogenetics, it was recognized that a uniform nomenclature was needed to describe and communicate findings accurately. In 1960, a group of cytogeneticists collaborated on a project to develop a system by which even complex numerical and/or structural abnormalities could be succinctly described. The resulting book would eventually come to be known as the International System for Human Cytogenetic Nomenclature (ISCN). Since its initial publication, the ISCN has been updated and revised several times (most recently in 2013) [21] to keep pace with the findings resulting from FISH and genomic microarray testing. The ISCN provides diagrammatic representations (ideograms) of each chromosome and its banding pattern at various levels of resolution; these ideograms permit cytogeneticists to identify breakpoints, the bands involved in structural rearrangements. The ISCN can be considered both a dictionary and a grammar book: the former, because it describes the abbreviations used for the various types of chromosomal abnormalities and defines basic concepts such as clones, and the latter, because it provides the rules for organizing nomenclature strings to describe the chromosomal complement. Below is an example of a nomenclature string that might be found in a case of CML (see also Fig. 2.1):

Because G-banding analysis can be performed on such a wide range of specimen types, it has been the principal means by which numerical and structural abnormalities have been identified in numerous neoplastic conditions. Because it provides a whole-genome view of these conditions, genome complexity can also be identified and investigated, which might otherwise be missed by more targeted approaches such as FISH. As will be described in a subsequent chapter, other whole-genome approaches such as array-based comparative genomic hybridization are now being commonly used, particularly in the evaluation of constitutional abnormalities and also in neoplastic conditions. Still, G-banding analysis has proved its utility since the discovery of the Philadelphia chromosome and for the foreseeable future will retain its important role in the diagnosis and treatment of malignant disorders.

Molecular Cytogenetics

As exemplified by the advances in the diagnosis and treatment of CML, conventional G-banding analyses often provide the initial clues as to which genes are involved in malignancies. Even if well-documented translocations are identified by G-banding, however, resolution is insufficient (each band can have 5–10 Mb of DNA) to determine if the characteristic gene rearrangement is present. Fluorescently labeled probes, typically several hundred Kb long and complementary to known genomic sequences, can be used to enumerate specific loci and to identify various structural rearrangements such as translocations and inversions. Such probes can also detect

46,XY,t(9;22)(q34;q11.2)[3/20]/48,sl,+8,+19[5/20]/49,sdl1,i(17)(q10),+der(22)t(9;22)[2/20]/46,XX[10/20]

↑

Clone 1 (stemline, sl): 15% of metaphases have a male karyotype with a reciprocal 9;22 translocation with breakpoints at 9q34 and 22q11.2

↑

Clone 2 (sideline [sdl] 1): In addition to the t(9;22) found in Clone 1 (stemline), 25% of metaphases have gain of one extra copy each of chromosomes 8 and 19

↑

Clone 3 (sideline [sdl] 2): In addition to the abnormalities found in Clone 2 (sideline 1), 10% of metaphases have an isochromosome for the long arm of a chromosome 17 and gain of a second Philadelphia chromosome

↑

This specimen shows chimerism for recipient (XY) and donor (XX) cells; 50% of metaphases are karyotypically normal female donor cells (listed after "/")

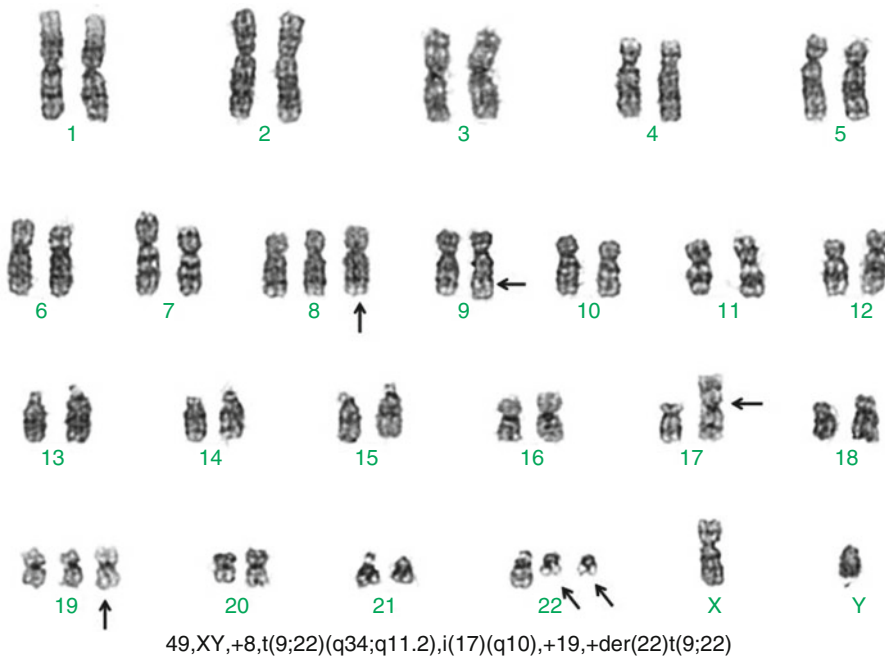


Fig. 2.1 Karyogram of a cell from a patient with chronic myelogenous leukemia. In addition to the t(9;22) resulting in the formation of the Philadelphia chromosome (the derivative chromosome 22), there are gains of one extra copy each of chromosomes 8 and 19, an isochromosome

composed of the long arms of a chromosome 17 joined in mirror image at the centromere (resulting in net loss of 17p and net gain of 17q), and gain of an extra copy of the Philadelphia chromosome

abnormalities that are cryptic (i.e., undetectable by G-banding) due either to the lower resolution of G-banding or to the exchange of regions with similar banding characteristics. Although FISH can be performed on metaphase cells, in cancer cases it is most frequently performed on interphase (nondividing) cells, allowing a large number of cells to be examined quickly, resulting in greater sensitivity and a more rapid turnaround time than G-banding analysis. Interphase cells are often obtained after culturing specimens for concomitant G-banding analysis, but as FISH does not require dividing cells, it can also be performed on a variety of other substrates, including smears prepared from peripheral blood or bone marrow; touch imprints; cytologic preparations; formalin-fixed, paraffin-embedded (FFPE) tissues; or enriched cell populations (e.g., after processing by flow cytometry or magnetic bead separation based on surface antigens). The benefits and limitations of each are outlined in Table 2.1.

Probes to clinically relevant genes are readily available from commercial vendors and can also be developed in-house. With the exception of some probes that have been approved by the US Food and Drug Administration (FDA), most are sold as analyte-specific reagents, which require laboratory validation and verification of test performance before clinical use; guidelines for validation procedures have been published [22–24].

Procedures for setting up FISH are less time- and labor-intensive than those for G-banding analyses and, with some modifications depending on the type of substrate (suspension, smears, touch imprints, FFPE tissues), are essentially the same for all tissue types [16, 18, 25–27]. Briefly, an aliquot of the appropriate probe/buffer mixture is placed on a glass slide that has been etched to delimit an area with an appropriate concentration of nuclei. Cell concentration is important in that only nonoverlapping nuclei should be evaluated; thus, when making touch imprints, a gentle

Table 2.1 Suitability of various specimen types for FISH analysis

Substrate	Advantages	Disadvantages
Harvested cell suspensions	<ul style="list-style-type: none"> • Ability to correlate with G-banding findings • Typically yields uniform results 	<ul style="list-style-type: none"> • Lineage of mononuclear cells cannot be readily identified • Need to wait until after harvest to obtain cells
Smears (blood, bone marrow)	<ul style="list-style-type: none"> • Readily available • Can be set up same day (no need to wait for cell culture) 	<ul style="list-style-type: none"> • Red blood cells can sometimes obscure signals
Touch imprints	<ul style="list-style-type: none"> • Easy to prepare and store • FISH can be set up same day • Typically yield strong signals with little artifact 	<ul style="list-style-type: none"> • Tissue architecture not preserved • If too thick, cell clumping precludes analysis
FFPE tissues	<ul style="list-style-type: none"> • Readily available • Tissue architecture preserved • Can be performed on archived specimens 	<ul style="list-style-type: none"> • Signal strength can be affected by multiple factors (e.g., fixation time, type of fixative, decalcification) • Nuclear truncation due to cutting block during slide preparation results in artifactual loss of signals • Longer preparation time, same-day turnaround not possible
Isolated/separated cell populations	<ul style="list-style-type: none"> • Cell enrichment increases assay sensitivity 	<ul style="list-style-type: none"> • Isolation process time- and labor-intensive • May yield weaker signal intensity

touch typically yields better results. A coverslip is placed over the probe and its edges sealed with rubber cement. Both the probe and the specimen DNA are heat denatured, typically using an automated instrument analogous to a thermocycler. After denaturation (2–5 min depending on the specimen type), the instrument cools to 37 °C, where the slide remains for approximately 6–14 h; this hybridization process permits binding of the probe to the target sequence. After a wash step to remove residual probe and the addition of a nuclear counterstain such as 4',6-diamidino-2-phenylindole (DAPI), the cells can be evaluated under a fluorescence microscope. Interphase cells are evaluated according to criteria validated by each laboratory and according to manufacturer's recommendations and published criteria [22, 23]. It is important to have defined normal control ranges (cutoff values) to avoid false-positive results; laboratories also should have established criteria for how many cells are evaluated at diagnosis and for monitoring to rule out residual disease. Among the criteria evaluated when scoring are the sizes, intensity, and relative positions of

the signals as well as their number. Scoring at least some of the cells on single-pass filters (which allow only one color to be visualized and thus yield brighter signals) permits the detection of very small signals that may, in situations such as gene insertions, overlap but be masked by the signal of the partner gene. Unusual or unexpected patterns must also be evaluated (e.g., gain instead of rearrangement of a locus).

Several of the commonly used probe types are described below (see Fig. 2.2). As for conventional cytogenetics, ISCN designations allow the signal patterns identified to be conveyed succinctly. Examples of ISCN nomenclature for these FISH findings are provided in the legends accompanying these images.

Enumeration Probes (Fig. 2.2a)

- Directed against the centromeric or pericentromeric regions of each chromosome; these repetitive-sequence probes yield large, bright signals.

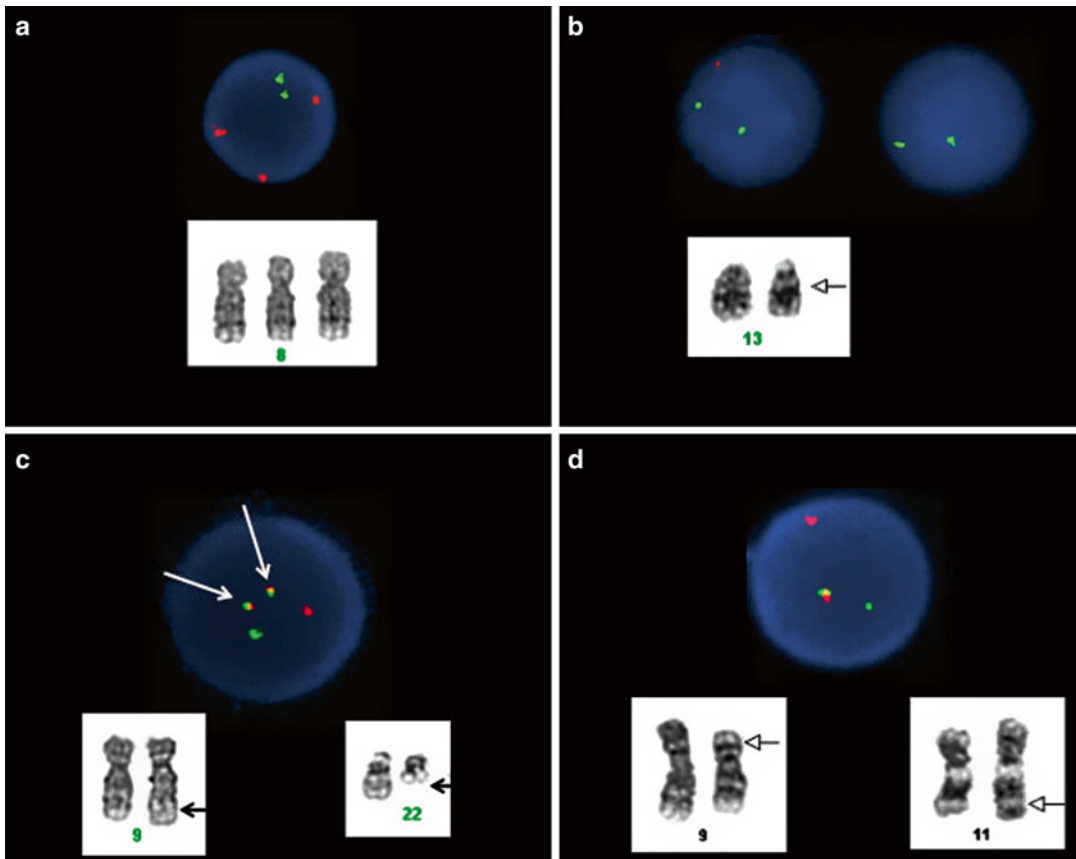


Fig. 2.2 (a) Three D8Z2 (centromere 8, *red*) signals and two D6Z1 (centromere 6, *green*) signals. ISCN designation: nuc ish(D8Z2x3,D6Z1x2). *Inset*: three copies of chromosome 8 by G-banding. (b) Left cell: one D13S319 (13q14, *red*) signal and two LAMP1 (13q34, *green*) signals, representing monoallelic loss of D13S319. ISCN designation: nuc ish(D13S319x1,LAMP1x2). *Inset*: G-banded chromosome 13 pair, one of which has an interstitial deletion involving 13q12-q14 (*arrow*). Right cell: no D13S319 (13q14, *red*) signal and two LAMP1 (13q34, *green*) signals, representing biallelic loss of D13S319. ISCN designation: nuc ish(D13S319x0,LAMP1x2). (c)

Three signals each for *ABL1* (*red*) and *BCR* (*green*), two of which are juxtaposed (“con”) to form yellow fusion signals (*arrows*). ISCN designation: nuc ish(*ABL1*,*BCR*) x3(*ABL1* con *BCR*x2). *Inset*: derivative chromosome 9 and derivative chromosome 22 resulting from a t(9;22) (q34;q11.2) (*arrows*). (d) Two *MLL* signals are present, one of which is intact (yellow fusion signal) and the other of which is separated (“sep”) into its component 5’ (*green*) and 3’ (*red*) signals. ISCN designation: nuc ish(*MLL*x2) (5’*MLL* sep 3’*MLL*x1). *Inset*: derivative chromosome 9 and derivative chromosome 11 resulting from a t(9;11) (p22;q23) (*arrows*)

- One signal per chromosome; used to count the number of copies of the chromosome in each cell.
- Clinical uses include evaluation of monosomies or trisomies, often seen in hematologic malignancies such as myeloid neoplasms (monosomy 7, trisomy 8) and chronic lymphocytic leukemia (trisomy 12).

Locus Specific (Unique Sequence) (Fig. 2.2b)

- Directed against specific genes or loci.
- One signal per chromosome; used primarily to evaluate gain or loss of the gene/locus.
- Clinical uses include evaluation of losses of genes/loci such as *ATM* and 13q14 (chronic

lymphocytic leukemia) and loci on 5q and 7q (myeloid neoplasms).

Dual Fusion (Fig. 2.2c)

- Each gene involved in the translocation is labeled in a different color.
- Juxtaposition of the genes due to the translocation results in a fusion signal on each of the chromosome partners (derivative chromosomes).
- Clinical uses include evaluation of recurring translocations seen in leukemias and lymphomas (e.g., *BCR-ABL1* in CML, *IGH-CCND1* in mantle cell lymphoma and plasma cell dyscrasias, *IGH-BCL2* in follicular and diffuse large B-cell lymphoma, *IGH-MYC* in Burkitt and double-hit lymphomas).

Break Apart (Fig. 2.2d)

- 5' and 3' portions of a gene are labeled in different colors.
- Separation of fusion signal into separate red and green signals represents gene rearrangement.
- Clinical uses include evaluation of rearrangements involving promiscuous genes such as *MLL* that have multiple translocation partners or involving loci (e.g., *CBFB*, *MECOM*) associated with different types of rearrangements such as inversions and translocations.
- Easier to evaluate than dual-fusion probes in FFPE tissues in which nuclear overlap can cause false-positive fusion signals, thus, often used for solid tumors such as sarcomas (e.g., *EWSR1* in Ewing sarcoma, *SS18* in synovial sarcoma) for which only FFPE tissue may be available.

Paint (Fig. 2.3)

- Probe mixture hybridizes to the chromosome of interest along its entire length.
- Used on metaphase cells to further characterize complex rearrangements and chromosomes of unknown origin (e.g., marker chromosomes).

Because of its flexibility, FISH can be adapted to situations other than those mentioned above. For example, because it does not require dividing cells, it can be performed on uncultured cells to evaluate for the presence of diagnostic abnormalities (e.g., *PML-RARA* in acute promyelocytic leukemia) for which rapid turnaround time is crucial. FISH can also be performed on previously G-banded slides to further characterize G-banding findings in specific cells. Although these require a longer hybridization time (typically 36–48 h), abnormal metaphases can be located on the fluorescence microscope and the signal pattern evaluated. Many of the types of aberrations detectable by the FISH probes described above will be discussed in the relevant chapters on specific disease processes. However, several important findings that can be readily detected by FISH warrant mention here.

Gene amplification has important prognostic and therapeutic consequences in a variety of diseases, one of the most common of which is neuroblastoma. Although it can be suspected by G-banding, gene amplification requires confirmation by locus-specific (unique sequence) probes. Amplification of the *MYCN* locus in neuroblastoma is associated with aggressive disease and is a critical component in risk stratification and therapeutic regimens [28–31]. Although *MYCN* amplification can be evaluated in FFPE tissue, touch imprints are the preferred substrate due to ease of preparation, strength of signals, and more rapid turnaround time. Just as the American Society of Clinical Oncology/College of American Pathology (ASCO/CAP) Guidelines define the criteria for *HER2* amplification (see below), the International Neuroblastoma Risk Group Biology Committee has also issued criteria for *MYCN* amplification [28, 29]. Cases with *MYCN* amplification are often very highly amplified, precluding accurate enumeration of the signals (Fig. 2.4a). These signals are frequently scattered throughout the cell and reflect gene amplification on double minute chromosomes. In contrast, cases of acute lymphoblastic leukemia with *RUNX1* amplification often show much lower levels of amplification, with as few as 5–6 *RUNX1* signals per cell. These signals are often clustered together in interphase cells, representing amplification occurring within an abnormal chromosome 21 (Fig. 2.4b, c).

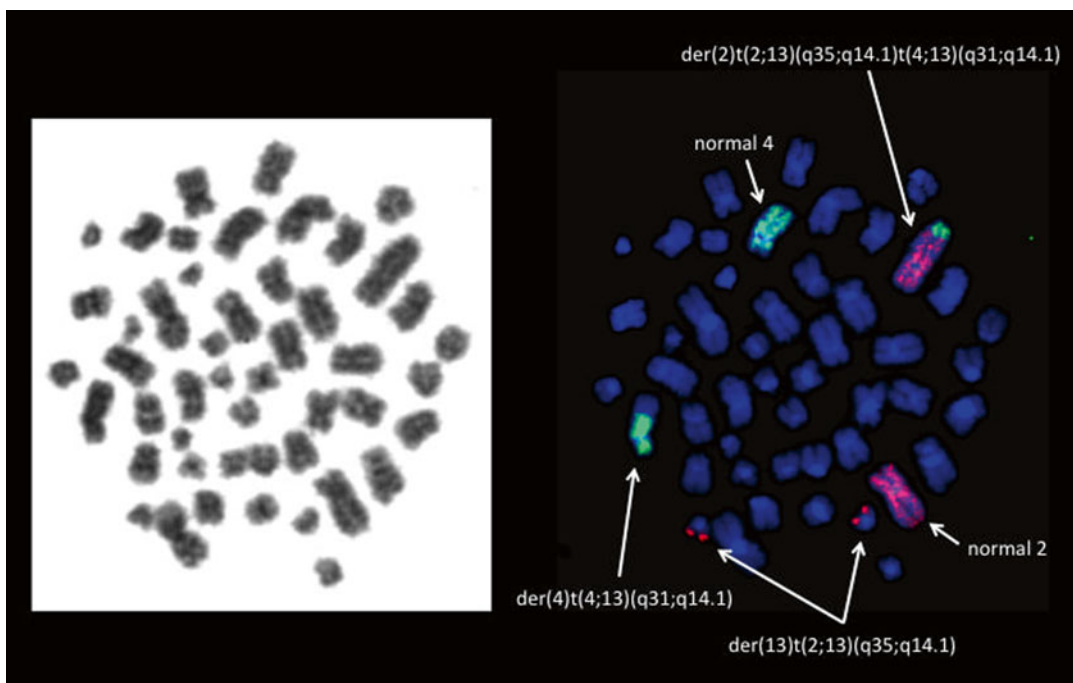


Fig. 2.3 Sequential FISH after G-banding using whole chromosome paint probes to chromosome 2 (red) and chromosome 4 (green). FISH confirmed that the complex rearrangements seen by G-banding resulted from a 2;13

translocation and a subsequent translocation of the derivative chromosome 2 and a chromosome 4. Two copies of the derivative chromosome 13 are present

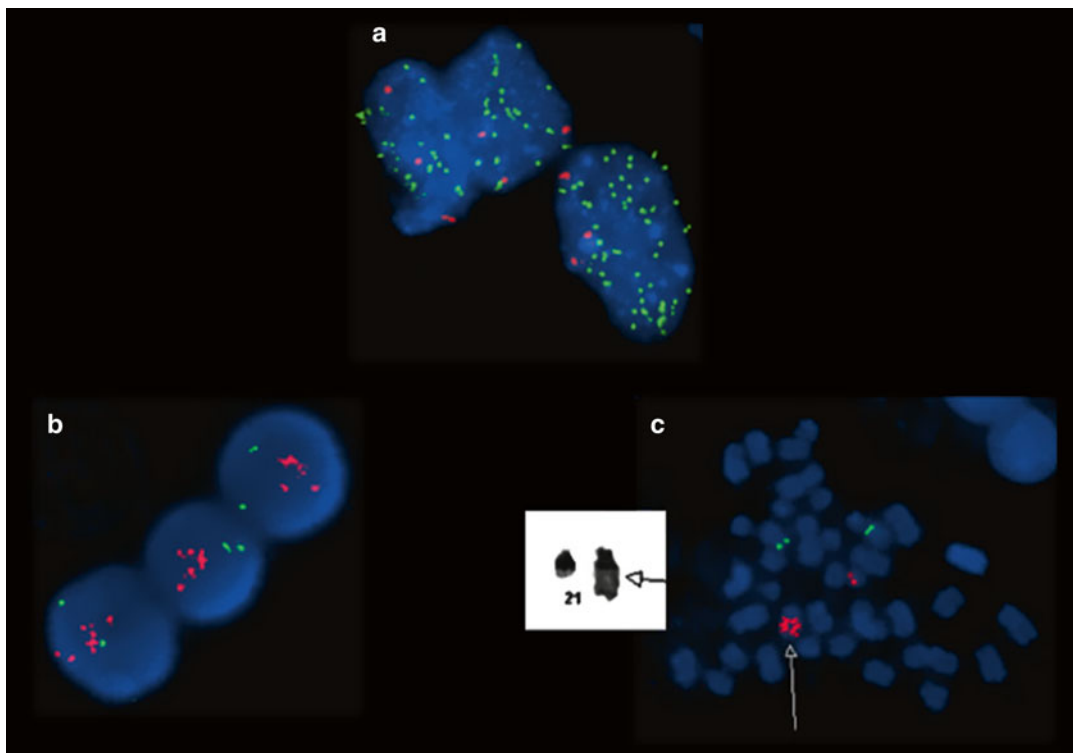


Fig. 2.4 (a) *MYCN* amplification: multiple *MYCN* (green) signals in the setting of three chromosome 2 centromere signals (red). (b) *RUNX1* amplification, interphase: multiple *RUNX1* (21q22, red) signals clustered;

two normal *ETV6* (12p13, green) signals. (c) *RUNX1* amplification, metaphase: multiple *RUNX1* (red) signals clustered along the length of one copy of chromosome 21 (arrow), seen by both FISH and G-banding (inset)

Gene amplification is also well documented in breast cancer. *HER2* (*ERBB2*) amplification, found in approximately 10–30 % of cases of invasive breast cancer, has been associated with aggressive disease and also with response to particular chemotherapeutic regimens (reviewed in [32]). Patients with *HER2* amplification may also benefit from targeted therapy with trastuzumab, a monoclonal antibody directed against *HER2*. Although *HER2* amplification can also be seen in in situ carcinoma, its prognostic significance is limited to cases in which it is found in invasive carcinoma. Because it is critical to evaluate only invasive carcinoma, it is necessary to perform this assay on FFPE specimens to preserve tissue architecture. Before evaluating, a pathologist marks the areas of invasive carcinoma on a hematoxylin- and eosin-stained slide, and the technologist uses that marked slide to identify the corresponding area of invasive carcinoma on the FISH slide.

Because of its importance clinically and also as a criterion for entry onto various clinical trials, it is critical to perform, analyze, and interpret *HER2* cases according to uniform criteria. With the goal of ensuring accuracy and uniformity among laboratories performing *HER2* testing, the ASCO/CAP Guidelines [33] and the subsequent Update [34] defined specific preanalytic, analytic, and postanalytic criteria that each laboratory performing *HER2* testing must follow. In cases in which a dual-color probe set is used (*HER2* and the chromosome 17 centromere as a control), *HER2* amplification is defined as a *HER2*/centromere 17 ratio per cell of ≥ 2.0 or ≥ 6.0 copies of *HER2* per cell. The Guidelines also define an equivocal category (*HER2*/centromere 17 ratio of < 2.0 and ≥ 4.0 and < 6.0 *HER2* signals per cell). These scoring criteria are specifically for breast cancer; *HER2* analyses performed on other tissue types (e.g., esophageal or gastric tissue) use slightly different criteria [35, 36]. Subsequent to the publication of the first ASCO/CAP document, an expert panel published guidelines for evaluating cases with intratumoral heterogeneity, a well-documented challenge when performing *HER2* and other FFPE FISH [37]. In situ hybridization techniques other than FISH have also been used to detect *HER2* amplification [38–40].

A number of disease processes have several commonly occurring genetic abnormalities that may occur individually or together. Because probes can be readily multiplexed, mixtures of FISH probes (“panels”) are often used to detect several of these abnormalities in a single assay. FISH panels to detect the most common abnormalities in voided urine [41, 42] and chronic lymphocytic leukemia [43] are commercially available (e.g., Abbott Molecular, Abbott Park, IL, USA; Cytocell, Cambridge, UK). These probe sets can also be used in other clinical situations; for example, the bladder cancer probe set (Abbott Molecular, Abbott Park, IL, USA) has also been used in cytologic specimens obtained from biliary tract brushings [42]. FISH is also playing an important role in therapy as well as diagnosis. As the documentation of genetic abnormalities is becoming increasingly important in determining responsiveness to therapeutic regimens, some guidelines mandate the use of FISH for the detection of certain abnormalities (e.g., *ALK* rearrangement in lung cancer) [44–47].

Although most clinical laboratories use commercially available probes for the more frequently occurring abnormalities, the ready availability of sequence data makes it possible for laboratories to design and label their own FISH probes. Reasons for doing this include but are not limited to lack of a commercially available probe for the region of interest or the need for smaller probes to detect abnormalities of very small genes. For example, the *TP53* gene is approximately 20 Kb, but commercially available probes may be severalfold larger; losses involving only the gene or with small flanking regions would be undetectable, as very small differences in signal size cannot be resolved at the microscope. Additionally, copy number abnormalities that are detected by other methods such as array-comparative genomic hybridization can be validated by FISH and used as a means to monitor the disease.

Conclusions

A number of new genomic techniques, such as array-based comparative genomic hybridization and next-generation sequencing, are entering

widespread clinical use and have enabled researchers to make significant contributions to elucidating the genetic basis of both constitutional and acquired disorders. However, reliable and less complex methods such as G-banding and FISH still play an important role in the diagnosis, prognosis, and therapy of many diseases. Not only can they be used in concert to provide a whole-genome view with the capability of targeting specific loci, they also are able to detect low-level mosaicism and balanced rearrangements that might be missed by other techniques. In light of the rapid progress that is being made both clinically and in the laboratory, it is imperative that clinicians and pathologists educate each other and collaborate to incorporate these advances into routine practice and to determine the most informative combination of testing methods to diagnose, treat, and monitor patients.

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