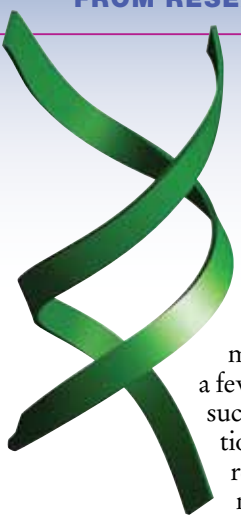


The Cancer Genome

Clinical characterizations by FISH and CGH

by AnneMarie W. Block, PhD; Sheila N. Sait, PhD; Norma J. Nowak, PhD; Daniel L. Stoler, PhD; and Garth R. Anderson, PhD

ILLUSTRATION/BIGSTOCKPHOTO

Some hematologic malignancies arise from a few genomic events such as simple translocations (i.e., breakage and removal of a large segment of DNA from one chromosome and attachment of the segment to a different chromosome). Solid tumors, however, reflect the consequences of accumulated genetic and epigenetic (i.e., changes "on" the gene, for example coupling to the DNA, rather than changes to the DNA sequence itself) events arising over a period of many years. In solid tumors, this process is facilitated by genomic instability resulting from environmental agents, such as smoking, and from defects in genes whose role involves the maintenance of genomic integrity.[1]

During the past several years, new technologies have dramatically increased our understanding of cancer at its fundamental genomic level. For the most part such technologies have revealed both point mutations and ever smaller and ever more abundant amplifications (increases in the number of copies of any particular piece of DNA), deletions (loss of pieces of DNA from chromosomes), and translocations, along with genomic heterogeneity at the cellular level.[2,3] Genomic sampling approaches first detected numerous events. This knowledge has been dramatically enhanced and augmented since 2006 by direct DNA sequencing of entire cancer genomes by The Cancer Genome Anatomy Project in the United States and by The Cancer Genome Project in the United Kingdom.[4,5] These studies have revealed genomic alterations far more extensive than long believed, and have conclusively established genomic heterogeneity within and among tumors.[2,6]

Moving from research into practice, clinical utilization of such genomic data becomes a question of *what do physicians need to know about a particular case*—and not *physicians must know everything they can possibly learn*. The cost-benefit ratio associated with obtaining genomic data also must be considered. In other words, while a multitude of genomic events have educated researchers about the enormous diversity and complexity of cancer, only a finite number of specific events currently have been found to be clinically valuable. These include the diagnostic *BCR-ABL* translocation in chronic myelogenous leukemia that underlies responsiveness to Gleevec® and amplification of *ERBB2* in breast cancer that is associated with responsiveness to Herceptin®.

Fluorescent *in situ* Hybridization (FISH) and Cytogenetic Analysis

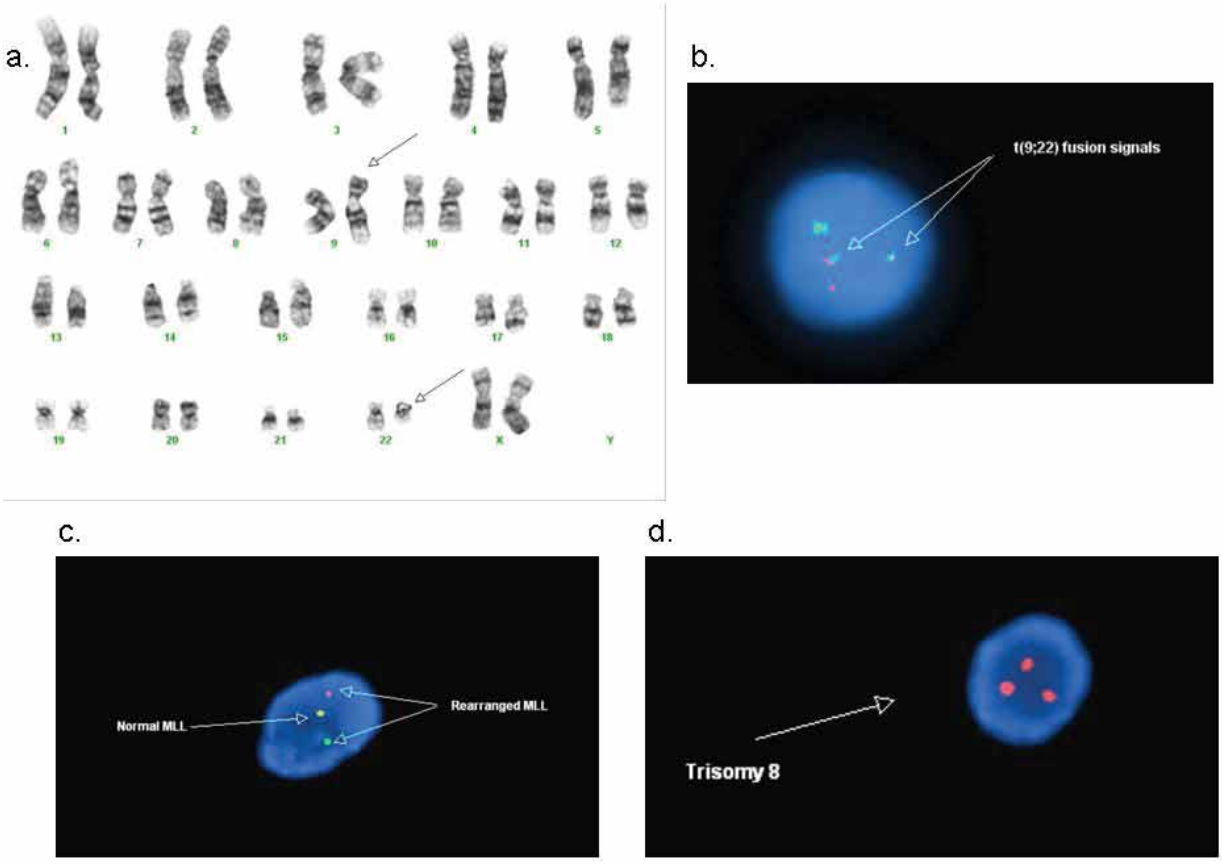
Conventional cytogenetic analysis first afforded a low-cost, practical window into the human genome. The significance of chromosome abnormalities observed in cancer cells and their relevance to the process of tumorigenesis dates to the late 19th and early 20th century when advances in optics, stains, and tissue manipulation led to the first descriptions of malignant tumors. Cytogenetics may be the only laboratory discipline that requires actively dividing cells to successfully complete analysis, since microscopic examination is performed on cells in the metaphase stage of mitosis. These preparations are enhanced by the use of spindle fiber inhibitors and hypotonic processing to aid in the "spreading" of chromosomes. The characteristic "bar code" appearance of banded chromosomes is achieved by the use of a digesting enzyme and DNA-binding dyes such as Giemsa. Light and dark banding reflects the relative DNA base content of those regions, with the average chromosome band representing three million base pairs of DNA.

In 1960 Nowell and Hungerford first identified the presence of a small marker chromosome in preparations derived from patients with chronic myeloid leukemia (CML).[7] This Philadelphia (Ph) chromosome was later confirmed by Rowley and colleagues to be the result of a reciprocal rearrangement between chromosomes #9 and #22 (Figure 1a).[8] A cascade of documented cytogenetic abnormalities soon followed this observation. Paralleling this compilation was the completion of the Human Genome Project and its assignment of genes to specific chromosomal breakpoints, i.e., those sites where chromosomal translocations have occurred. In CML, molecular gene mapping localized the *ABL* oncogene to the 9q34 breakpoint and *BCR* to a limited breakpoint cluster region at 22q11.2.[9-11]

Chromosome abnormalities in neoplasia are restricted to the tumor tissue and are not present in other cells of the body, making them an ideal disease-specific marker. The distribution of abnormalities is highly non-random and clonal (originating in a single cell, and passed on through cell division to daughter cells) in nature. Both benign and malignant tumors have been shown to exhibit chromosome abnormalities. Patients who have received radiation and/or chemotherapy are at risk for developing a secondary myelodysplastic syndrome or leukemia characterized by a specific subset of often complex, balanced and unbalanced chromosome abnormalities reflecting the cells' inability to repair DNA damage.[12]

To date, more than 56,000 chromosome aberrations have been reported as individual cases in the Mitelman Database of Chromosome Aberrations in Cancer.[13] Structural chromosome rearrangements include balanced and unbalanced transloca-

Figure 1. Cytogenetic Analyses and FISH



(a) Karyogram of a CML cell showing the characteristic rearrangement between chromosomes 9 and 22. Arrows indicate the abnormal chromosomes. (b) FISH demonstrating the same translocation as in (a). Arrows indicate the colocalization of the signals for BCR on chromosome 22 (orange) and ABL on chromosome 9 (green). (c) Split signal FISH assay showing rearrangement of the MLL gene. Green and orange labeled probes closely flanking the MLL genes produce a yellow signal. Arrows indicate the physically separated sequences resulting in isolated fluorochromes. (d) Interphase FISH demonstrating trisomy of chromosome 8.

tions, as well as inversions and insertions. These rearrangements may be early or initiating steps in tumorigenesis, as well as late events during progression or within the malignant tumor itself. Such events are readily revealed by fluorescent *in situ* hybridization (FISH) (Figure 1b,c).

The effects of chromosomal gains and losses are more complex since the regions involved may range from the submicroscopic to whole chromosomes. Tumor suppressor gene (a cell division regulating gene that normally limits the growth of tumors) deletion in multiple tumor types is common. While whole chromosome abnormalities are easily identified by conventional analysis, newer technologies such as array comparative genomic hybridization (aCGH) have been instrumental in identifying smaller regions of gain or loss. Amplifications of chromosomes may also be visualized by FISH (Figure 1d).

A disproportionate number of unique, disease-specific chromosomal rearrangements were initially reported in hematologic malignancies[14] and in tumors of mesenchymal (cells that develop into connective tissue, blood vessels, and lymphatic tissue) origin, probably due to the complexity and hetreogeneity of solid tumor genomes. Specific chromosomal rearrangements have been described in prostate cancer and non-small cell lung cancer using molecular methodology.[15,16]

In the clinical setting, cytogenetic analysis has proven to be an invaluable tool in the diagnosis, prognosis, and management of hematologic malignancies while aiding in the differential diagnosis between solid tumor types with common features.[17-22] The World Health Organization, in its classification of tumors of the hematopoietic and lymphoid tissues, now classifies some acute myeloid leukemias by recurrent genetic abnormality.[23]

Chromosomal abnormalities have also been used to identify a subset of patients whose genetic lesions are functional targets for therapy.[24] Therapies specifically targeting identifiable cytogenetic events have included:

- Imatinib, dasatinib, and

nilotinib, which target (t(9;22)(q34;q11.2) in chronic myeloid leukemia, acute lymphoblastic leukemia, and acute myeloid leukemia[25,26]
- All-*trans* retinoic acid and arsenic trioxide, which target (t(15;17)(q22;q12) in acute promyelocytic leukemia[27]
- Lenalidomide, which targets (deletion 5q32/*RPS14*) in myelodysplastic syndrome[28,29]
- Trastuzumab, which targets (17q21.1/*ERBB2* amplification) in breast cancer.[30,31]

Fluorescent *in situ* Hybridization

The advent of FISH marked a major improvement in the capabilities of cytogenetics and its roles in diagnostics and research. FISH analysis is based on tagging a probe DNA sequence with a fluorescent dye, then hybridizing the probe DNA to a target complementary sequence contained in any cytological material such as metaphase chromosomes or interphase cells. (Hybrization is the base pairing of two single strands of DNA or RNA.) Probes are labeled either directly by the incorporation of fluorescent nucleotides or indirectly by labeling with reporter molecules that are subsequently detected by fluorescent antibodies or other affinity molecules. The probes and target material are then visualized with fluorescence microscopy. This combined molecular and cytological approach has led to a several-fold increase in resolution relative to that possible with conventional cytogenetic technology.

Another development in the evolution of FISH technology was the increase in the number of differentially labeled probes that could be hybridized and imaged simultaneously. This increase resulted in a number of specific whole genome painting technologies, including multiplex-FISH (M-FISH), spectral karyotyping (SKY), and combined binary ratio labeling (COBRA). Target resolution also progressively improved from whole chromosomes to interphase nuclei to chromatin strands [chromatin is the complex of nucleic acids (DNA or RNA) and proteins that make up chromosomes].

Application of FISH technology, as with SKY and M-FISH, to metaphase chromosomes is now used to identify the origins of marker chromosomes that cannot be identified by conventional banding techniques. This technology has also been used to estimate the level of genomic instability in cells.[3]

RxFISH is a color-banding technique that takes advantage of cross-species homologies between human and ape to generate a banding pattern on human metaphase chromosomes. Used in combination with G-banding (G-banding is chromosomal profiling based on the microscopic visualization of chromosomal bands after staining with Giemsa stain), RxFISH can be used to provide detailed information on chromosomal breakpoints.

> While a multitude of **genomic events** have educated research about the complexity of cancer, ***only a few have been found to be clinically valuable.***

FISH and the Field of Oncology

The application of FISH technology to interphase cells has been particularly successful in the field of oncology. Interphase cytogenetics has been used to aid diagnosis as well as to follow patients and monitor minimal residual disease and micro metastasis. Since cells in division are not necessary, interphase FISH can be applied to a wide variety of targets including bone marrow and peripheral blood smears, paraffin-embedded tissue sections, and cytological material like urine and spinal fluid.

Well-characterized chromosomal rearrangements associated with particular diseases can be readily identified, such as the CML-associated Philadelphia chromosome translocation producing a fusion of *BCR* on chromosome 22 and *ABL* on chromosome 9. With this method, probes are made with DNA from the *BCR* and *ABL* loci using different fluorochromes (orange and green) to reveal the *BCR/ABL* fusion product (see Figure 1b). Thus a normal unaffected cell has two orange and two green signals, while a cell carrying the translocation has one orange and one green signal and two yellow fusion signals resulting from overlapping orange and green signals. This strategy has also been used for several additional translocations, particularly in hematologic malignancies.

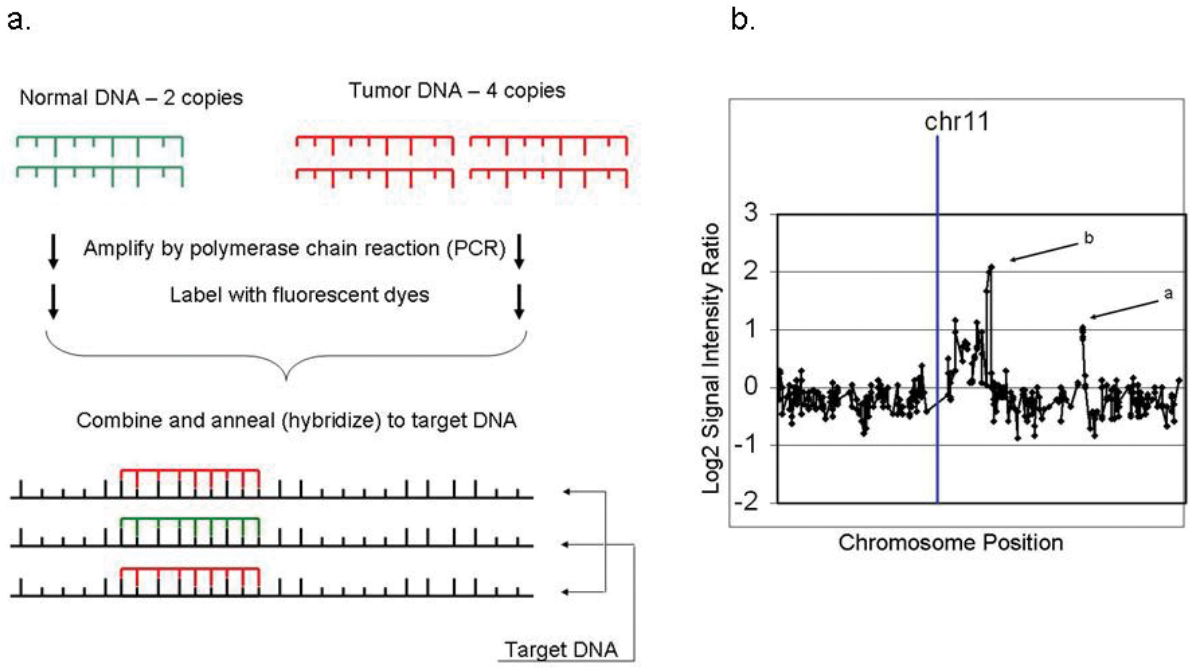
Another FISH application widely used in oncology is the split signal FISH. The split signal FISH assay is a dual-color assay that differentially labels flanking regions of a breakpoint locus. Thus, in normal cells the signals co-localize or overlap, but in affected cells the signals will separate, appearing as two different colors (Fig 1c). Interphase cell FISH is also used widely to assess ploidy (the number of sets of chromosomes in a cell). This assessment is most commonly done with centromere-specific probes (centromere is the constricted region near the center of a human chromosome), but locus-specific probes have also been used (Fig 1d). The best example of this is the use of FISH probes to detect the amplification of the HER2 gene in human breast cancers to identify patients who would benefit from trastuzumab (Herceptin) therapy.

Comparative Genomic Hybridization

CGH was derived from fluorescent *in situ* hybridization.[39,40] The key changes were that enzymatically generated fragmented copies of one entire genome, each labeled with a fluorescing dye, were mixed with fragmented copies of a different entire genome labeled with a different dye, and then the labeled fragments were simultaneously annealed to a template of unlabeled copies of the entire first genome.

Figure 2a shows a schematic illustration of this process. Normal DNA, containing two copies of a particular sequence, and tumor DNA with a

Figure 2. Comparative Genomic Hybridization: Schematic and Analysis



(a) Competitive hybridization. PCR amplified probes from normal and tumor DNA are labeled with different fluorescing dyes and hybridized to the arrayed target normal DNA. For the given sequence, additional copies are found in the tumor and a corresponding greater amount of tumor-derived probe will anneal to the target. The signal intensities for each dye are measured and the ratio plotted against the target's position in the genome, as shown in b.
(b) CGH profile for chromosome 11 from a head and neck squamous cell carcinoma. Position along the chromosome is shown on the x-axis, with the vertical line indicating the position of the centromere. The y-axis shows the ratio of tumor to normal signal intensity in log base2. Points with a ratio above 0 are amplifications, below are deletions, and points on the line are diploid (2 copies of the DNA sequence). For example, point a in the figure has a signal intensity ratio of 21, therefore there are 4 copies of the sequence in the tumor. The signal intensity ration at point b is 22, thus there are 8 copies of this DNA sequence in the tumor.

gain of two additional copies of that sequence, are separately amplified via the polymerase chain reaction, differentially labeled with fluorescing dyes and together annealed to the arrayed target DNA. For every normal-cell-derived sequence that anneals to the target sequence on the microarray, two tumor-derived sequences will anneal. The fluorescent intensity of each dye at each point on the target can be readily measured and plotted graphically to show the position on the chromosome where the copy number variation has occurred. Figure 2b shows an example of such data for a head and neck squamous cell carcinoma for chromosome 11, where two separate amplified regions can be seen.

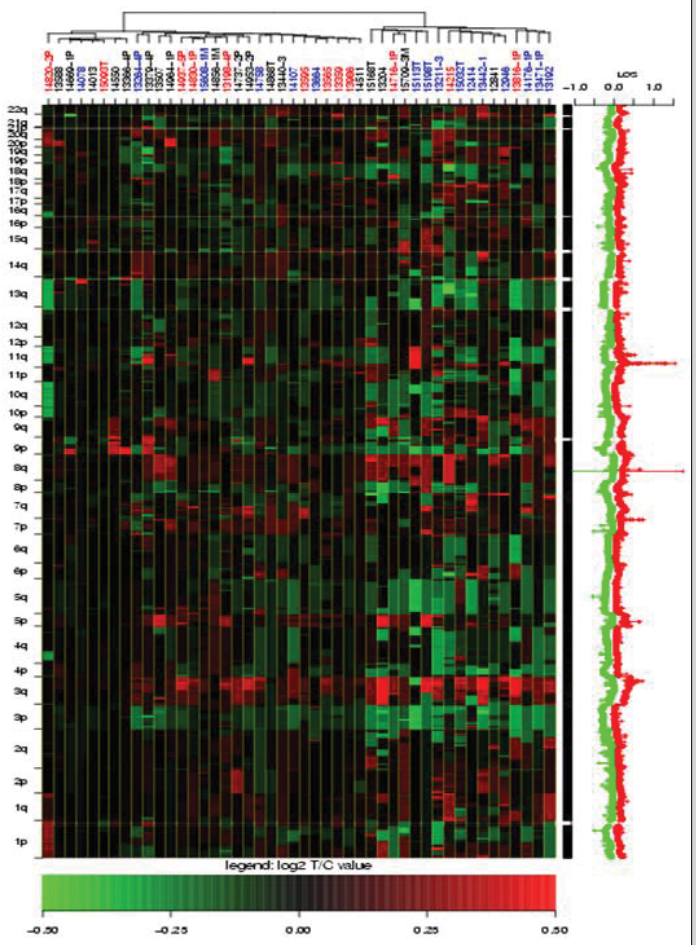
As this technique was initially developed, the annealing was done to entire metaphase chromosomes spread on a glass microscope slide. Higher resolutions were achieved as the annealing template was fragmented on microarrays of progressively smaller segments of the entire genome.[41,42] BACs, bacterial artificial chromosomes (large segments of DNA, 100,000 to 200,000 bases, from another species [human] cloned into bacteria), containing approximately 150 kilobase inserts of human DNA became invaluable as they were individually identified and mapped from BAC libraries, and assembled on defined microarrays.[43] Further improvements have been achieved as single nucleotide polymorphisms (SNPs) were identified through repeated sequencing of the human genome. (SNPs are common, but minute, variations that occur in human DNA at a frequency of about one every 1,000 bases.) SNPs have been used to generate commercial microarrays with resolutions better than 10 kilobases.[44] A detailed comparison of CGH technologies with currently available microarray platforms has been recently made (Hester et al., *Journal of Biomolecular Techniques*, in press).

Techniques for high-resolution genome-wide analysis such as BAC-array CGH or SNP-array CGH detect chromosomal copy number aberrations in human tumors. SNP microarray CGH adds the capability of revealing losses of heterozygosity (loss of having two different alleles of the same gene), which does not necessarily require a change of copy

number as in cases where uniparental disomies (both copies of a chromosome from one parent) have arisen.[45] These CGH approaches still have their limitations in the analysis of tumor genomes though. Most solid tumors (and some leukemias) are not diploid (having the normal amount of DNA per cell, two sets of chromosomes), and the chromosome number varies among individual tumor cells. Such heterogeneity causes these array-based CGH approaches to reveal the average genomic composition for the many cells used in the isolation of the particular tumor DNA being assayed. With smaller and smaller samples being assayed, many heterogeneities within each tumor can be revealed, which in itself is informative.

These resources and their application in array-based technology have substantially advanced our genotyping and phenotyping ability towards comprehensive profiling of tumors. So far, many non-random, recurrent abnormalities have been identified through massive screening of specific tumor types, as is illustrated for head and neck carcinomas (Figure 3). In turn, this approach can be expected to allow us to identify disease subtypes within traditional pathological classifications that likely will be diagnostically and prognostically useful.

Figure 3. BAC-Microarray CGH Analysis of 46 Head and Neck HNSCC DNAs



Heatmap of log2 tumor/control values for samples (red: pharynx, black: oral cavity, and blue: larynx). The plot to the right of the heatmap contains the means of the upper 25% of the samples (red) and the lower 25% of the samples (green).

At this point, microarray CGH generates far more information for each sample than is applicable for clinical use, and at far greater cost than with FISH. However microarray CGH is exceedingly useful for research purposes, and additional future clinical applications can be expected. Not having to grow cells in culture is a significant time advantage for CGH, and the technologies of microarray CGH are clearly amenable to substantial reductions in cost per sample. In part these reductions will likely arise from reductions in the amount of information generated per sample to only that of value in the clinic, increasing the competitiveness of CGH methodology for clinical utilization.

Where Do We Go From Here?

For those tumors such as chronic myelogenous leukemia with relatively stable genomes with few alterations, FISH will remain ideal for the visible future. The situation becomes far more complex for the common solid tumors, as well as for several other leukemias.

FISH and CGH are but two current, practical approaches to characterize cancer cell genomes. For research applications, it may be ideal to have more and more detailed information down to the precise DNA sequence of each tumor genome. We can now sequence a tumor genome,[4,5] but it still does not reveal the sequence heterogeneities present within each individual tumor cell. So should we sequence the genome for every individual cell? This might be a worthy research project, although the genomic instability of cancer cells will generate new sequences in any remaining viable tumor cells.[46]

At some point there is a balance between how much genomic information can be obtained, how much information is needed, and at what cost.[47] Overlooked in the cataloging of numerous individual genomic alterations is the larger picture of the degree and forms of genomic instability in the tumor. For the tumor to somatically evolve more aggressive and metastatic tumor cells (which may be unknown to the clinician), the degree of genomic instability can provide an indication of the likelihood that such highly dangerous cells are present somewhere in the patient. Such information can be expected to become a significant element in the genomic data used clinically.

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