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Cytogenetic Profiling Using Fluorescence *In Situ* Hybridization (FISH) and Comparative Genomic Hybridization (CGH)

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Abstract Fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) allow cytogenetic analyses of primary tumors without culture. CGH allows detection and mapping of allelic imbalance by simultaneous *in situ* hybridization of differentially labeled tumor (green fluorescing) and normal DNA (red fluorescing) to a normal human metaphase spread. Regions of increased or decreased copy number in the tumor are mapped onto the normal metaphase chromosomes as increases or decreases in the green to red fluorescence ratio. This technique gives a comprehensive assessment of gene dosage imbalance throughout the tumor. However, it is limited, at present, to fairly large tumors containing few normal cells. FISH, on the other hand, allows analysis of DNA sequence copy number at specific loci in single nuclei. A wide variety of DNA probes is available for FISH, including chromosome-specific probes which hybridize to alpha-satellite pericentromeric DNA regions (to detect changes in specific chromosome copy number and overall ploidy) and specific locus probes targeting 20–150 kilobase sequences (to detect specific amplifications, deletions, breakpoints, or rearrangements). FISH using these probes has been applied to interphase nuclei in touch preparations, smears from fine needle aspirates, and thin (<6 µm) and thick (>20 µm) sections cut from formalin-fixed, paraffin-embedded tissue. Analysis of thick sections allows accurate actual signal enumeration within the histological context. This approach may allow analysis of subtle premalignant, early malignant, and infiltrating tumors in which malignant cells must be differentiated from nonmalignant cells. These capabilities suggest a strategy of tumor analysis, beginning with CGH analysis of advanced tumors to identify regions of common gene dosage imbalance, followed by FISH with specific probes to these regions to study their presence in earlier stage lesions. © 1993 Wiley-Liss, Inc.

Key words: Comparative genomic hybridization, confocal microscopy, DNA amplification, DNA deletion, DNA probe, interphase cytogenetics, formalin, paraffin, ploidy, repetitive sequence

Solid tumor cytogenetic research has advanced in recent years beyond karyotypic demonstrations of recurrent ploidy alterations, translocations, and deletions in malignant cell lines. As a result, several cellular oncogenes and tumor suppressor genes have been identified that may play a role in tumorigenesis and/or that result in aberrant cellular physiological processes favoring tumor progression. Understanding such aberrations may assist in the search for new chemotherapeutic agents since these agents may be directed toward the aberrant gene products or metabolic pathways that result from the genetic aberrations. Evaluation of possible chemopreventive agents may be facilitated by identification of early genetic events (*e.g.*, genomic instability or

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subpopulations carrying specific aberrations) that may be eliminated or reversed by effective agents. Use of these endpoints in chemopreventive agent testing could significantly reduce the time and expense required to test each agent both in animal models and humans. The complement of fluorescence *in situ* hybridization (FISH) techniques provides a set of tools that may facilitate development of these genetic endpoints.

One approach to identifying genomic regions important in premalignant and early neoplastic events is to first identify regions of recurrent alteration in advanced cancers and then to determine which alterations also occur early. Comparative genomic hybridization (CGH) makes this possible by scanning the entire genome for relative increases and decreases of DNA sequence number to characterize gene dosage abnormalities [1]. As illustrated in Figure 1 [2], CGH is based on two-color FISH to normal metaphase spreads using differentially labeled total genomic tumor DNA and normal (or other) reference DNA. DNA sequence copy number differences throughout the genome are identified through measurements of the ratio of the hybridization intensity of the tumor DNA to that of the reference DNA. In general, tumor DNA is detected using a green fluorescing agent and reference

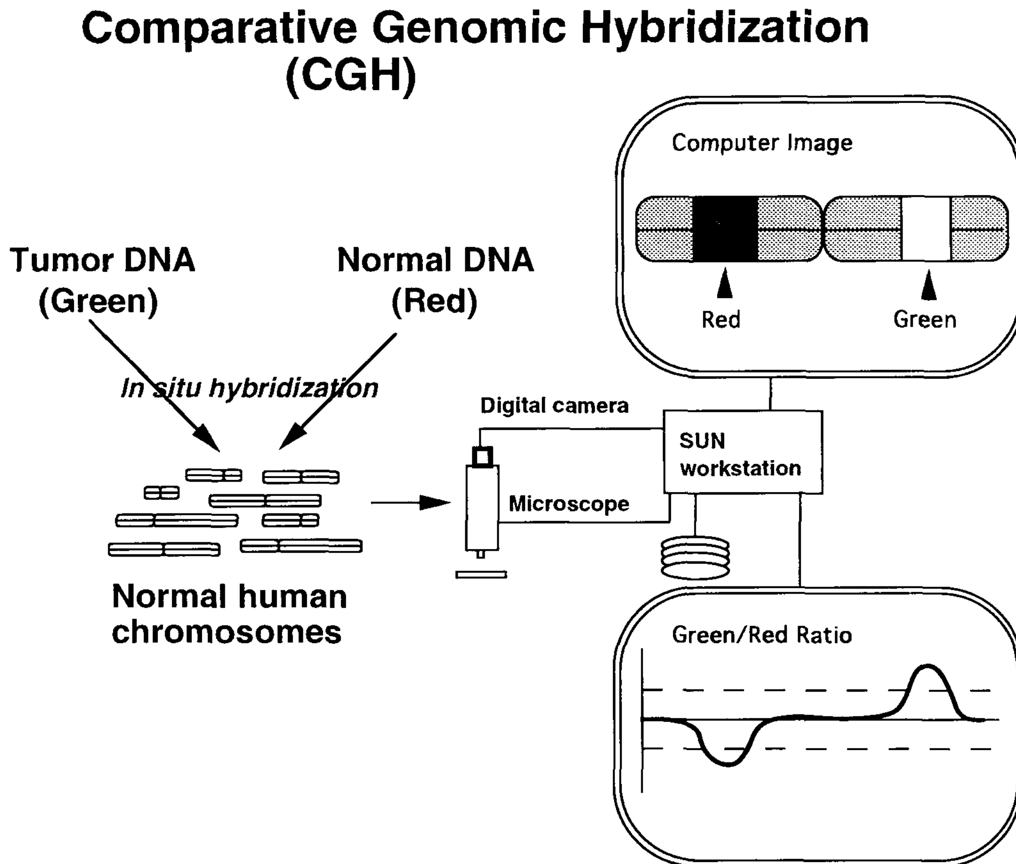

Fig. 1. Diagram illustrating the sequence of events in comparative genomic hybridization (CGH). Tumor genomic DNA probe (green) and normal reference genomic DNA probe (red) are hybridized to normal metaphase chromosomes. After hybridization, the metaphases are imaged with fluorescence microscopy, photographed and processed using imaging software. A computer-generated image and a histogram of the green/red fluorescence intensity ratio for each chromosome is generated. (Reproduced with permission from the publisher from "Cytogenetics of Cutaneous Malignant Melanoma."[2])

DNA is detected using a red fluorescing agent. Thus, chromosome regions in which tumor DNA sequences are overrepresented have a green/red fluorescence ratio of >1.0 and regions where the tumor DNA sequences are underrepresented (deleted) have a ratio of <1.0. Metaphases are analyzed using a digital imaging microscope equipped with filters that allow separate acquisition of images of blue, green, and red fluorescence. The blue fluorescence image shows the DAPI staining pattern used to identify each chromosome, and the green and red fluorescence images show the tumor and reference DNA probe hybridization patterns, respectively. CGH presently is possible only for more advanced tumors, from which a few micrograms of fairly pure tumor genomic DNA can be isolated.

CGH removes the need for *in vitro* tumor cell culture as required for traditional karyotyping and therefore reduces subpopulation selection. Additionally, information is obtained regarding the genomic origin of extrachromosomal DNA such as double minute DNA fragments. Extrachromosomal material is often ignored in karyotyping because the fragments are too small to be recognized by morphology or banding pattern. The sensitivity in detecting amplifications and deletions varies somewhat among experiments. In general, however, changes by one in the number of copies of a region of the genome can be detected when the involved region is larger than 10–20 Mb. Amplification of smaller regions can be detected when the level of amplification is 5- to 10-fold [3,4]. CGH identifies relative gene dosage abnormalities at the sites of several previously defined proto-oncogenes and tumor suppressor genes. However, advanced tumors usually reveal abnormalities at sites not previously implicated in tumor progression. CGH localization of these abnormalities may lead to identification of genes important in tumorigenesis and progression.

Regions of the genome associated with early tumorigenic events identified by CGH may be further characterized using FISH with DNA probes targeting these regions. FISH [5] can provide precise information about changes in chromosome copy number, DNA segment copy number, and genomic integrity (*e.g.*, translocations, inversions, *etc.*) [3,4,6–20] some of which are not detectable using standard karyotyping. Several different probes may be analyzed simultaneously using different fluorochromes to label each probe [21]. FISH complements CGH analysis by allowing assessment of genetic heterogeneity and detection of subpopulations carrying specific aberrations.

Though FISH may be applied to a variety of cell preparations (*e.g.*, touch preparations, fine needle aspirate smears, dispersed nuclei from fresh, frozen, or formalin-fixed tissue), tissue sections provide further information by establishing a bridge between cytogenetic and histologic analysis. This extension is important in attempting to analyze premalignant and early malignant lesions that may be small, clonal, and morphologically distinct. FISH to thin sections maintains the histology; however, most nuclei are cut so that accurate assessment of gene copy number is difficult. Statistical corrections to predict the actual number of FISH signals per cell may be employed [22,23]; however, subtle alterations and aberrant cells present at low frequency may be missed. FISH to thick sections (>20 μ) cut from paraffin blocks provides a layer of uncut cells in the center of the section for analysis by laser scanning confocal microscopy. DNA probes directly labeled with fluorochromes are employed to reduce the amount of nonspecific ic background fluorescence. Thick-section FISH enables greater use of formalin-fixed, paraffin-embedded tissue in tumor research, which resolves many of the problems associated with obtaining fresh tissue. Tissue from early neoplastic processes is often only available in this form, as it is submitted in its entirety for processing to provide a pathological diagnosis and information about the adequacy of excision. Early neoplastic processes, where the cells of interest are often few in number or admixed with other nonmalignant cells, may be analyzed in this manner. Tumor heterogeneity may also be accounted for during cytogenetic analysis by this technique; subpopulations within a tumor can be identified histologically. Correlation with traditional pathologic tumor grading systems, many of which are closely correlated with prognosis, is also possible.

Characterization of early neoplastic genetic events using these tools might not only elucidate the biology of cancer initiation, but may also identify surrogate genetic endpoints useful in chemopreventive agent testing. We suggest two different genetic endpoints that may be

measured to assess the utility of chemopreventive agents—genetic instability and specific aberrations that enable cancer progression. Genetic instability, identified by the frequency of cells carrying random genetic aberrations, has been identified as an early event in neoplasia and could be one surrogate genetic endpoint. Instability may be detected using FISH for analysis of the resulting gene copy number changes. If early genetic instability is epigenetic (*i.e.*, caused by nonheritable, environmental influences), one effect of a successful chemopreventive agent may be to reduce the frequency of cells carrying gene or chromosome copy number aberrations. Specific genetic aberrations involving a certain frequency of clonal subpopulations carrying the specific aberrations that enable cancer progression may be a second surrogate genetic endpoint. Tumorigenesis may begin with a single clonal genetic event that predisposes to genetic instability, increased proliferation, or other biological effects favoring tumor progression. Useful chemopreventive agents may reduce the frequency of clonal subpopulations carrying enabling aberrations identified using FISH with probes to the premalignant lesions (*e.g.*, by eliminating the specific subpopulation or by eliminating the proliferative advantage generated by the aberration). Such agents are likely to be effective only for specific aberrations. Certainly, the ultimate use of these genetic endpoints in chemopreventive agent testing is dependent upon characterization of early neoplastic genetic events which, at this time, are poorly characterized in most solid tumors.

In summary, the variety of FISH techniques may be used in concert to profile cancer progression. CGH provides a scan of the entire genome in advanced tumors or malignant cell lines for large DNA copy number amplifications and deletions and, in our experience, does reveal involvement of genomic regions not previously implicated in tumorigenesis. These regions may be further characterized using FISH with probes specific for regions of interest. Application to tissue sections of sufficient thickness to provide uncut nuclei for analysis allows analysis of cells from lesions at different stages of tumor progression. Analysis of premalignant lesions using FISH may reveal genetically aberrant subpopulations whose frequency is reduced by useful chemopreventive agents.

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