Journal of Cellular Biochemistry 25S:57–62 (1996)

Detection of Chromosome Instability of Tissue Fields at Risk: In Situ Hybridization

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Abstract Many human tumors are thought to develop along a multistep pathway in tissues that have encountered long periods of carcinogen exposure and thus have accumulated genetic hits in functional targets relevant to tumor evolution. The cumulative degree of genetic change is dependent on both exogenous (e.g., degree of carcinogen exposure) and endogenous factors (e.g., metabolism of procarcinogens, repair or misrepair capacity, proliferation properties of the tissue, capability of damaged cells to survive). Thus one approach to risk estimation is to measure the accumulated amount of genetic damage in a target tissue at risk for tumor development. Since one cannot predict the exact site of the future tumor, the risk assay must detect a generalized ongoing process of genetic instability from small, random biopsies. The technique of chromosome in situ hybridization involves the use of chromosome- or region-specific probes and provides an ability to directly visualize genetic change (e.g., random or clonal chromosome polysomy and monosomy) on thin tissue sections (where tissue architecture is maintained) or exfoliated cells. Analyses of normal and premalignant lesions adjacent to tumors (e.g., head and neck, lung, bladder, cervix, breast) have demonstrated that chromosome instability can be detected in the field of the tumor (i.e., in normal and premalignant cells in a tissue at 100% risk of tumor development) and the degree of chromosome instability increases with the degree of histologic progression toward cancer. Analyses of premalignant lesions (e.g., oral leukoplakia and erythroplakia from individuals at risk for aerodigestive tract cancer) by chromosome in situ hybridization have uncovered varying degrees of chromosome instability. However, approximately half of those individuals who showed a high degree of chromosome instability in biopsies subsequently developed aerodigestive tract cancer. Of interest, half of these tumors have developed away from the biopsied site, suggesting that the detection of a chromosome instability process in one aspect of the tissue might yield risk information for the total tissue field. These studies also suggest that chromosome in situ hybridization might be useful for identifying individuals with high tumor risk who might benefit from chemopreventive intervention. J. Cell. Biochem. 25S:57–62.

Key words: cancer risk; genetic instability; in situ hybridization

Most human tumors exhibit genetic changes involving regions of the genome whose alteration is associated with functional dysregulation of the cell. These genetic changes can take a number of forms including point mutations, deletion, amplification, changes in copy number, and rearrangements which alter the regulation of gene expression. These genetic changes are also frequently accompanied by changes in chromosome copy number and structure. Thus the visualization of chromosome events in tumors has provided unique insight into the particular genetic alterations important for the tumor phenotype [1].

GENOMIC INSTABILITY FOR RISK ASSESSMENT: RATIONALE

The development of many human epithelial tumors is believed to evolve over a long period of time and is associated with an accumulation

Contract grant sponsor: NIH, contract grant number CA-5205h, contract grant number CA 16672, contract grant number CA-68437, contract grant number CA-68089.

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Received 16 January 1996; Accepted 22 February 1996

of genetic hits over time in a target tissue. Theoretically, while genetic alterations may be occurring throughout a carcinogen-exposed tissue, those specific genetic hits important for tumor development may be selectively retained in clonal outgrowths which evolve toward cancer. The rate and degree of accumulation of genetic hits in a target tissue likely reflects the culmination of a variety of processes including the degree of exogenous carcinogen exposure and a variety of endogenous mitigating factors including the metabolism of exogenous and endogenous procarcinogens, the repair or misrepair capacities of the injured cells, the capability of damaged cells to survive, and the proliferation properties of the tissue [2]. Since many carcinogens induce chromosome aberrations, one approach for tumor risk assessment is to quantitate the rate and degree of accumulation of chromosome changes in the target tissue. Hypothetically, those individuals harboring the greatest degree of genetic change would be at the highest risk for developing cancer.

In the clinical setting, the assessment of cancer risk in an individual by genetic assessment of the target tissue is limited by various considerations. First, it is difficult to obtain large tissue samples from individuals at risk. Thus, risk assessments must be performed on small tissue biopsies or cells exfoliated from the target tissue. Second, it is frequently difficult to visualize chromosome changes in fresh tissues by conventional mitotic analysis. Thus chromosome changes must be detectable on nondividing, interphase cells. Third, while one may be able to identify the tissue field at risk for cancer development, it is nearly impossible to predict the future site of the evolving tumor. Even when premalignant lesions are visible (e.g., leukoplakia, erythroplakia), it is not uncommon for tumors to develop in a site of the exposed field away from the identified lesion [3]. Thus, biopsies used for risk assessment should be considered random and the genetic assessments must reflect an ongoing process in the tissue that is associated with high risk in addition to an assessment of the specific degree of evolution of the particular lesion being examined.

This last notion is difficult to appreciate in the setting of our present concepts of tumor development. The elegant work of Vogelstein and colleagues and others have demonstrated that most tumors undergo a multistep set of genetic events during their evolution [4]. Some specific genetic events were found to be present at a higher frequency in early premalignant lesions while other specific genetic hits were preferentially found in more advanced premalignant lesions. Thus an early working hypothesis for the strategy of genetic risk assessment was that one could biopsy a tissue lesion and determine how many of the relevant genetic events had already occurred. Those lesions exhibiting a high percentage of the necessary changes would then be predicted to be at high risk for subsequent tumor development, and those lesions with only a small number of specific changes might be considered to be at low risk. However, this strategy needs to be reconsidered if the random biopsy is not obtained from the exact site of the evolving premalignant lesion.

An alternative strategy is to develop genetic assays that reflect the extent of an ongoing process of genetic instability in a risk tissue. The working assumption would then be that if genetic change is ongoing at a high rate throughout the tissue, there would be an increased likelihood that the specific genetic changes necessary for tumor development might be occurring somewhere in the tissue field. Thus, a genetic assessment on a randomly obtained biopsy might yield important risk information for the whole tissue field at risk, providing a field of cancerization exists.

There is considerable evidence that some epithelial tumors arise in a field of carcinogenesis such that the tumorigenesis process can be detected throughout the risk tissue [5,6]. From a clinical view, the occurrence of a first primary tumor (e.g., head and neck, lung, bladder, breast) places that individual at significant risk for the development of a second primary (genetically distinct from the first tumor) in that same tissue field. Similarly, these tumors are often preceded by the occurrence of premalignant or early lesions in the field at risk (but not necessarily at the site of the future tumor). From a histopathologic view, changes can be observed throughout the tissue field of the tumor as clearly demonstrated by Auerbach and colleagues through careful dissection of resected lungs [7]. The idea that these histologic changes are associated with the accumulation of genetic alterations is supported by our observations and those of others where chromosome changes

were detected in non-tumor lung tissue from individuals with lung cancer [8–10].

CHROMOSOME IN SITU HYBRIDIZATION (CISH)

One useful approach to the detection of genetic changes in small tissue samples is the use of chromosome in situ hybridization (CISH) whereby chromosome region-specific DNA probes are hybridized to either tissue sections or exfoliated cells [11]. The sites of hybridization can be visualized by fluorescence or immunohistochemical procedures and then evaluated under the fluorescence or light microscope. For interphase nuclei, the probes need to recognize small chromosome regions. For example, DNA probes which recognize repetitive DNA satellite sequences near the centromere regions of chromosomes are particularly useful because they allow the enumeration of chromosome copy numbers in individual cells. In the unperturbed situation, each somatic cell should show two chromosome signals for each pair of autosomal chromosomes. In the setting of genetic instability, dividing cells will improperly distribute chromosomes to daughter cells and this will result in some cells exhibiting abnormal chromosome copy numbers (0, 1, 3, 4, etc. copies) (Fig. 1). Thus the fraction of cells with abnormal chromosome copy numbers can reflect the level of a genetic instability process in a cell population. In this situation, it would not matter which chromosome was used for evaluation since the pattern of instability would be random. On the other hand, if the loss or gain of a particular chromosome were important for dysregulated cell growth or cell loss in a tissue (early steps in tumorigenesis), this would result in the outgrowth of cells exhibiting monosomy or polysomy for that chromosome. Thus, CISH can be useful for monitoring two important parameters of tumor development, i.e., genomic instability and clonal outgrowth.

To address the working hypothesis that CISH might be useful in the detection of a high-risk field, we chose first to examine normal and premalignant tissues in fields at 100% risk of developing a tumor, i.e., adjacent normal and premalignant lesions in the field of an existing tumor. Using paraffin-embedded tissue blocks of Head and Neck tumors that contained contiguous adjacent premalignant and normal epithelium, we demonstrated that CISH allowed the detection of a process of genomic instability that extended out to the histologically normal epithelium adjacent to the tumor [12]. In this case, the marker of instability was the presence of cells exhibiting three or more copies of a particular chromosome. This is an extremely rare event in unperturbed normal cell populations, especially when tissue sectioning causes nuclear truncation and an underrepresentation

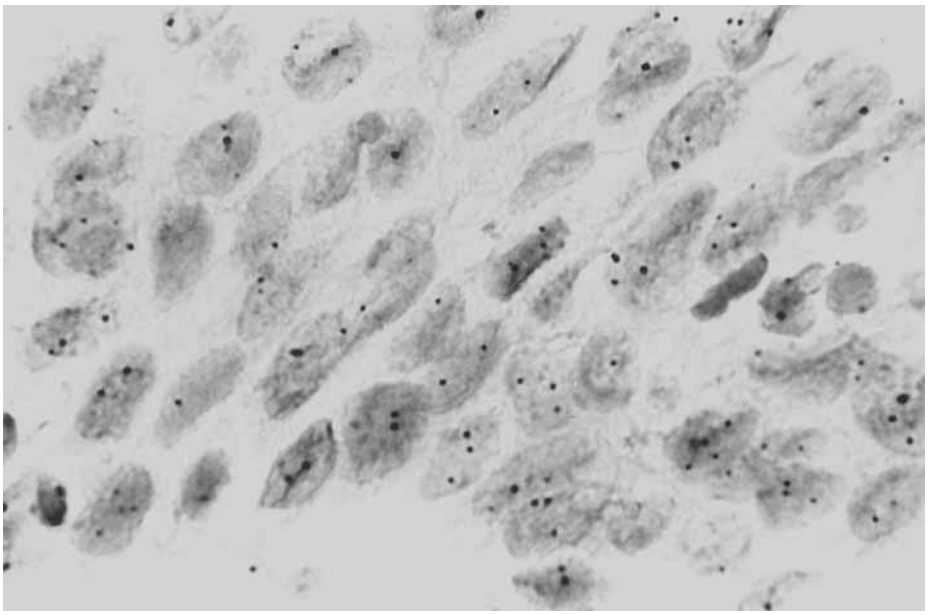

Fig. 1. Chromosome in situ hybridization (CISH) using a chromosome 7 probe on a tissue section of a hyperplastic lesion adjacent to a head and neck tumor. Note the presence of cells exhibiting chromosome polysomy, i.e., three chromosome copies per cell.

of chromosome copy number. Similar observations have been made by in situ hybridization in the examination of normal surrounding epithelium in other tumor sites [13–15].

USE OF CISH FOR RISK ASSESSMENT

If premalignant lesions in the field of a tumor (i.e., 100% risk lesions) exhibit evidence of an ongoing genetic instability process, the next question is whether premalignant lesions in tissues at lesser risk for tumor development also harbor evidence of genetic instability. If so, one might postulate that those individuals whose premalignant tissues show the highest degree of genetic instability would be at the highest cancer risk. To address both questions in a retrospective fashion, we carried out a pilot examination of 13 biopsies obtained from individuals with oral premalignancy (oral leukoplakia or erythroplakia) [16]. We purposely included some biopsies from individuals who subsequently developed head and neck cancer. While there was variability of the degree of genetic instability within histologic stages of premalignancy, dysplastic lesions in general showed a higher degree of chromosome instability than did nondysplastic lesions in accord with the notion that the finding of dysplasia is associated with a high cancer risk. However, of importance to the notion of risk quantitation by in situ hybridization, three of the five cases that subsequently developed tumors showed a relatively high degree of chromosome instability in their biopsies, including one case exhibiting only hyperplasia. In contrast, only one of the eight lesions in individuals who did not develop subsequent tumors exhibited a high degree of genomic instability (Table I).

Because of these promising pilot studies, we carried out a prospective study of oral premalignancy biopsies obtained just prior to entry onto a chemopreventive intervention of limited duration [17]. The individuals were entered onto the study between the years 1988 and 1990, thus a median follow-up of ≥5 years is available. The duration of chemopreventive intervention on this study was 12 months and biopsies were taken at 0, 3, and 12 months of treatment. Of the 30 biopsies available so far from oral premalignant lesions obtained prior to entry onto the trial, fifteen lesions showed a high degree of chromosome instability (i.e., ≥3.5% of the cells showing ≥3 chromosome copies per cell). Seven of these 15 cases (47%) have so far developed a tumor in the aerodigestive tract [18]. In contrast, only one case of 15 (7%) with a low degree of chromosome instability subsequently developed a tumor (Table II).

Two additional important findings are emerging from this ongoing study. First, while dysplastic lesions in general showed greater genetic instability than hyperplastic lesions, some tumors did arise in individuals whose biopsy lesions showed a high degree of genetic instability despite a hyperplastic histology. Second, nearly half the tumors developed well away from the physical site of the biopsy. These findings support the possibility that randomly obtained biopsies from a tissue field at tumor risk might be informative for risk assessment for the field as a whole. It also supports the idea that CISH might be useful in the assessment of cancer risk in tissues undergoing a field cancerization process since it provides unique information beyond histology.

The above studies involving individuals with oral premalignant lesions represented a situation of intermediate cancer risk (i.e., approximately 30% risk). It is then of interest to determine if the chromosome in situ hybridization technique might be useful in situations of lower cancer risk. To examine this possibility, we examined endobronchial biopsies obtained from individuals with a significant smoking history (i.e., > 20 pack years) who displayed early lung

TABLE I. Association of Genomic Instability and Subsequent Head and Neck Tumor Development (Pilot Study)

Fraction of Cells with ≥ 3 copies*	Fraction of Patients Developing Head and Neck Tumors (%)
≥5%	3/5 (60)
<5%	1/8 (13)

*Measure by in situ hybridization using chromosome-specific probes (chromosome 7 and 17) on tissue sections of leukoplakia biopsies.

TABLE II. Association of Genomic Instability and Subsequent Head and Neck Tumor Development (Prospective Study)

Fraction of Cells with ≥ 3 copies*	Fraction of Patients Developing Head and Neck Tumors (%)
≥3.5%	8/15 (53.3)
<3.5%	0/15 (0)

*Measure by in situ hybridization using chromosome 9-specific probes on tissue sections of leukoplakia biopsies.

changes (i.e., bronchial metaplasia). Biopsies were obtained from six different sites in the lung, and tissue sections were examined by CISH. While the number of cases examined is too small and the time of follow-up too short for cancer risk interpretations, some early findings are of interest. First, evidence of chromosome instability was observed throughout the regions of the lung that were accessible for biopsy (Fig. 2). Second, the levels of instability observed in one biopsy site were generally similar to that observed in another biopsy site, suggesting a wide field of carcinogen exposure. Third, using an image analysis system to record the location and chromosome copy number of each cell scored in a biopsy section, we were able to probe the biopsies for localized regions of high chromosome instability or apparent clonal outgrowth of cells showing three or more chromosome copies. In terms of risk assessment, the development of clonal outgrowth might have risk implications and allow one to distinguish reactive changes in a carcinogen-exposed tissue from changes associated with further evolution to cancer. Moreover, the identification of a clone prior to chemopreventive treatment might provide a biomarker for interpreting the response to intervention in post-treatment biopsies.

While the studies described here focused on biopsy material taken from tissue fields thought to be at increased risk for tumor development, CISH is also applicable to exfoliated cells from high-risk tissues. For example, Segers et al. [19] probed cervical smears from women with various grades of cervical intraepithelial neoplasia (CIN) and observed evidence of chromosome instability. Moreover, the degree of instability was found to increase with evidence of more advanced lesions. Nevertheless, there was a large variation in the degree of chromosome instability observed within CIN stages, suggesting that in situ hybridization studies might provide useful risk information in addition to histology. Similar approaches are also being tested by several laboratories for risk assessments in other organ sites such as bladder (examination of exfoliated cells in voided urine and bladder washings) and lung (examination of sputum samples).

SUMMARY AND CONCLUSIONS

In summary, the studies described here suggest that chromosome in situ hybridization might be a useful technique for risk assessment

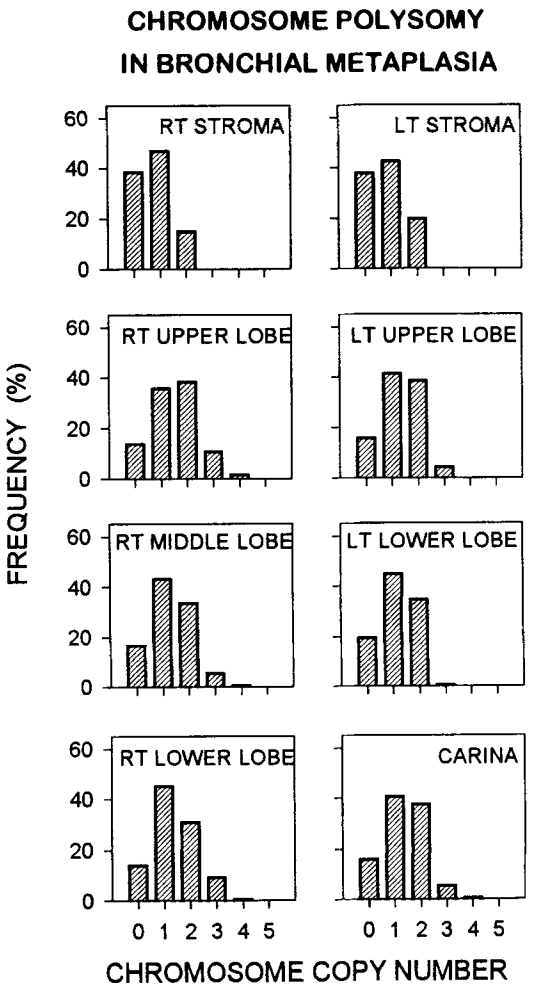

Fig. 2. CISH evidence for chromosome polysomy bronchial biopsy specimens obtained from throughout the lungs of a subject with a heavy smoking history. Note the presence of cells with three or more chromosome 9 copies in the epithelial (but not the stromal) cells in each biopsy.

in the setting of tissues where a process of field cancerization and multistep tumorigenesis is ongoing. While this technique is also useful for identifying specific genetic changes in tissues or lesions with tumor risk (e.g., the detection of loss of specific genetic regions or gene amplification), the focus of this paper has been placed on the detection of a process of generalized genetic instability in target tissues where one cannot positively identify the tissue site that will ultimately develop a tumor. The detection of genetic instability appears to provide additional information beyond histology and may be useful for the identification of individuals with a high risk of developing cancer.

The ability to assess individual risk will be important for future chemoprevention studies

for several reasons. First, by categorizing individuals according to their relative degree of risk, one can explore the possibility that different individuals might benefit from different chemopreventive strategies. For example, low risk individuals might benefit from long term intervention aimed at lowering the rate of accumulation of genetic hits (e.g., smoking cessation, dietary changes, antioxidants) and sequential in situ hybridization measurements might serve as intermediate endpoints of response. On the other hand, high-risk individuals might benefit from more aggressive chemopreventive intervention, and in situ hybridization studies might be useful for determining whether response is associated with extinction of aberrant clones or reversal of an aberrant phenotype. This latter distinction would be important in determining the duration of treatment. Finally, the combination of genetic and phenotypic studies on biopsies obtained during chemoprevention trials will provide unique insight into the tumorigenesis process and will facilitate the development of new preventive approaches.

ACKNOWLEDGMENTS

These studies were supported in part by NIH grants CA-52051, CA-16672, CA-68437, and CA-68089.

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