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Early Genetic Changes During Upper Aerodigestive Tract Tumorigenesis

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Abstract Upper aerodigestive tract tumorigenesis has been hypothesized to represent a field cancerization process with multistep events based on its association with known carcinogens, its frequent associated premalignant lesions, and its multifocal clinical manifestation. To further explore this working hypothesis, we have examined normal tissue and premalignant lesions in the field of tumors for evidence of genetic change. Paraffin sections of head and neck tumors harboring neighboring premalignant lesions were explored for the presence of chromosome polysomies using *in situ* hybridization and chromosome-specific centromeric probes. Cells exhibiting random polysomy were observed in the premalignant regions near the tumors. The frequency of polysomy in the tumor field increased as the tissue progressed from normal morphology (33%), to hyperplasia (67%), to dysplasia (95%), and to squamous cell carcinoma (96%). These results support the notions of field cancerization and multistep tumorigenesis in the aerodigestive tract. To determine whether the degree of accumulated genetic alterations might serve as a biomarker for risk of developing malignancy, a set of biopsies of oral premalignant lesions (leukoplakia, erythroplakia) were retrospectively chosen for polysomy analysis from two groups of individuals: one group who subsequently developed oral cancer and one group who did not develop oral cancer. Three of the five individuals who showed significant chromosome polysomies in their biopsies subsequently developed oral cancer, whereas only one of eight individuals with little evidence of polysomy subsequently progressed to oral cancer. These results suggest that evidence of generalized genetic change or instability might be useful as a genetic biomarker for risk assessment.

Key words: head and neck cancer, leukoplakia, *in situ* hybridization, field cancerization, multistep carcinogenesis, polysomy

The goal of this research is to better understand tumorigenesis from a cellular point of view and then to identify potential mechanistic targets for chemopreventive intervention. In the upper aerodigestive tract, three general premises guide these studies. First, tumors arise in a field of cancerization. A whole tissue region exposed to carcinogens (*e.g.*, tobacco, alcohol) is at increased risk for tumor formation at distinct

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single or multiple sites. Second, tumorigenesis is a multistep process. An accumulation of genetic events results in phenotypic changes in the tissue, characterized by dysregulation of proliferation, differentiation, and cell loss. Third, effective chemopreventive intervention will reduce the rate of genetic damage accumulation, eliminate abnormal clones, and/or stabilize or reverse the phenotypic changes associated with malignant progression.

Evidence for the field cancerization premise comes from several sources. The risk of lung or head and neck tumor development has been correlated with the degree of carcinogenic exposure. Second and multiple primaries are common occurrences in the aerodigestive tract [1,2]. Genetic damage (*e.g.*, micronuclei) can be detected throughout the field at risk, not only at the tumor site [3]. Premature chromosome condensation analysis of histologically normal lung tissue of individuals with lung tumors shows significant levels of random and specific karyotypic changes [4]. These findings support the notion that carcinogen-induced genetic changes can occur throughout the upper aerodigestive tract. However, tumors arise only in those sites where there is an accumulation of specific genetic hits important for tumor development.

Evidence for the multistep tumorigenesis premise comes from a variety of animal carcinogenesis models [5]. Also, tumors of the human upper aerodigestive tract are often preceded or accompanied by premalignant lesions in the field [6]. In fact, it is not uncommon to observe an apparent succession of events from normal histology through hyperplasia and dysplasia to tumor histology, all within one tissue section of head and neck tumors.

VISUALIZATION OF EARLY GENETIC CHANGES ACCOMPANYING TUMORIGENESIS—*IN SITU* HYBRIDIZATION STUDIES

Conventional cytogenetic analysis of premalignant lesions or even normal epithelial tissue in the cancerization field is hampered by two problems. The frequency of mitotic figures is very low, and prolonged tissue culture would be required to obtain sufficient numbers of cells for analysis. Also, single cell suspensions are required, destroying tissue architecture prior to karyotypic evaluation. In order to directly visualize genetic changes in intact tissue, we have turned to the technique of chromosome *in situ* hybridization whereby chromosome-specific DNA probes are labeled, hybridized to tissue sections and the hybridization sites visualized by immunocytochemical techniques [7–10]. Using probes directed at repeat sequences near the centromeric regions of individual chromosomes, this technique detects gains and losses of chromosomes in individual nuclei in intact tissues. Using single copy DNA probes, this technique detects gene deletions or amplifications.

To better understand field cancerization and multistep tumorigenesis in the head and neck region, we have applied the technique of *in situ* hybridization to paraffin sections of 27 head and neck tumors exhibiting premalignant lesions directly adjacent to the primary tumor. This allowed us to visualize genetic changes directly on the tissue sections and to correlate the succession of genetic events with the transition from normal tissue morphology through hyperplasia and dysplasia to tumor, all within one tissue section. Mucosal tissue sections obtained from individuals without cancer and without tobacco exposure served as normal controls. DNA probes for the α-satellite regions of chromosomes 7 and 17 were used in this study to detect the occurrence of chromosomal polysomy.

Whole normal diploid cells contain two signals for each set of autosomal chromosomes. However, when analysis is performed on histological sections, parts of nuclei are truncated and thus cells may exhibit 0, 1, or 2 copies of each autosomal chromosome. Indeed, nuclei of epithelial cells from normal controls and from lymphocytes in the tumor sections averaged about one signal per nucleus. In this study, however, no nuclei from the normal control or from lymphocytes exhibited three or more copies of either chromosome 7 or 17. In contrast, cells with three or more copies of either chromosome were observed in about one-third of the cases in which morphologically normal epithelium adjacent to head and neck tumors was examined (Table I). The frequency of cases with evidence of polysomy increased to two-thirds in the tumor-associated hyperplastic tissue, and to 95% of cases where dysplastic tissue adjacent to

TABLE I. Percentage of Cases Exhibiting Three or More Copies of Chromosomes 7 or 17 in Premalignant Regions Near Head and Neck Tumors (n = 27)

Histologic Stage	Chromosome 7	Chromosome 17
Adjacent Normal	35%	40%
Hyperplasia	67%	47%
Dysplasia	95%	81%
Squamous Cell Carcinoma	96%	96%

Note: No cells with three or more signals were observed in lymphocytes in the tumor specimen or in epithelial oral mucosal cells from non-cancer individuals.

the tumor was examined [11]. In most cases, the presence of cells with three or more copies did not appear to be the result of a clonal outgrowth because the mean normalized chromosome index did not reach a value of 1.5, which would suggest outgrowth of a trisomic clone. Rather, the presence of nuclei with three or more copies might suggest a generalized genetic change, resulting from either carcinogenic insult or a process of intrinsic genetic instability. The finding of genetic changes extending into the premalignant field supports the notion of field cancerization. The finding that the degree of genetic change increases as the tissue passes from normal morphology through successive premalignant steps to tumor, supports the theory of a multistep process where genetic changes accumulate.

IMPLICATIONS OF VISUALIZING GENETIC CHANGES IN PREMALIGNANT LESIONS

One corollary to the theory of field cancerization and multistep tumorigenesis is that the degree of genetic change in a tissue field at risk might be related to the degree of risk for tumor development in that field. To examine this hypothesis, a set of biopsies of oral premalignant lesions (*i.e.*, leukoplakia, erythroplakia) were retrospectively chosen for chromosomal polysomy analysis from two groups of individuals, one group who subsequently developed oral cancer and one group who did not. Six of the seven dysplastic lesions examined showed polysomy in more than 2% of the cells examined, while only two of six hyperplastic lesions exhibited such polysomy. This result demonstrates that genetic damage can be visualized in premalignant lesions of individuals at risk for developing tumors. More strikingly, of the five individuals whose premalignant tissue biopsies showed polysomy in more than 5% of the cells examined, three have so far progressed to oral cancer. In contrast, only one of the remaining eight individuals who showed polysomy in less than 5% of the cells has progressed to cancer so far [12]. This result suggests that those individuals exhibiting higher levels of polysomy in premalignant lesions are at increased risk for tumor development in the tissue field. If such results are confirmed by more extensive studies, it will suggest that polysomy measurements by *in situ* hybridization may be useful in chemoprevention studies to identify individuals at high risk for developing malignancy.

One problem with the above polysomy analysis is that it does not distinguish between generalized genetic instability (*e.g.*, due to carcinogen exposure) and the outgrowth of a clone that has obtained proliferative advantage. To address this latter question, we have developed an image analysis program that records both the chromosome copy number in a cell (after *in situ* hybridization) and the location of that cell in the tissue section. This generates a genetic map of the premalignant lesion and makes it easy to see the outgrowth of clones in a tissue section (see cover illustration). Our program facilitates genotype/phenotype analyses in adjacent tissue sections, since different events can be correlated in two-dimensional space. Such studies are ongoing in premalignant and malignant lesions

of the upper aerodigestive tract as well as in other tissue sites where field cancerization is suspected.

CONCLUSION

The studies reported here indicate that the technique of *in situ* hybridization is useful for detecting genetic changes in premalignant tissues. The findings so far lend genetic support to the theories of field cancerization and multistep tumorigenesis in the upper aerodigestive tract. The findings also suggest that the detection of genetic change in these tissues might serve as a useful biomarker during chemoprevention studies, as well as a tool to better understand molecular and cellular events associated with tumorigenesis.

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