Advances in Brief

Genetic Progression and Clonal Relationship of Recurrent Premalignant Head and Neck Lesions

Joseph Califano, William H. Westra, Glenn Meininger, Russel Corio, Wayne M. Koch, and David Sidransky

Abstract

We constructed a preliminary genetic progression model for head and neck squamous cell carcinoma (HNSC) based on the frequency of genetic alterations in preneoplastic and neoplastic lesions from single biopsy specimens. To firmly establish the temporal order of established genetic events in HNSC, we sampled serial biopsies from five patients with recurrent premalignant lesions at a single anatomic site over a period of time (1 month to 144 months). These lesions were examined by microsatellite analysis of the minimal regions of loss on the 10 most frequently lost chromosomal arms in HNSC. Each set of serial biopsies from all five patients demonstrated LOH (loss of heterozygosity) of identical alleles at multiple loci with identical boundaries between areas of LOH and retention of heterozygosity, indicating a common clonal origin for each set. Three patients demonstrated genetic progression (new regions of LOH) over time correlating with histopathological progression, one patient demonstrated lack of genetic progression associated with unchanged histopathological morphology, and one patient demonstrated histopathological progression without detection of a corresponding genetic progression event. For one of these patients with a laryngeal tumor, at least four separate steps in progression to malignancy could be determined, accompanied by spatial expansion of an increasingly altered clonal population from the ipsilateral to the contralateral side, ultimately resulting in a malignancy. Microsatellite-based genetic analysis of recurrent premalignant lesions indicates that these lesions arise from a common clonal progenitor, followed by outgrowth of clonal populations associated with progressive genetic alterations and phenotypic progression to malignancy.

Introduction

It is now generally accepted that most sporadic solid tumors arise from a multistep process of accumulated genetic alterations. A colorectal cancer model for the initiation and progression of malignancy has become a paradigm for other human solid tumors. Like colorectal cancer, HNSC is thought to progress through a series of well-defined clinical and histopathological stages. Recently, we described a preliminary genetic progression model for HNSC. This model is based on genetic alterations present in premalignant head and neck lesions and genetic progression found in adjacent, clonally related, but histopathologically distinct, areas within biopsy specimens. These studies have demonstrated that genetic progression of HNSC correlates with histopathological phenotype among a series of patients and between adjacent histopathologically distinct areas in a single patient. However, demonstration of genetic progression over time has not been reported in HNSC.

PCR based microsatellite marker analysis enables a determination of specific genetic changes that occur in lesions with diverse histopathology. This study correlates genetic changes and histopathological progression within recurrent premalignant and malignant head and neck lesions in individual patients. These results help to further establish the temporal order of genetic events in HNSC progression and provide insight into the underlying genetic changes and biological behavior that accompany clinical phenotypic progression.

Materials and Methods

Subject Selection Criteria. Subjects were selected on the basis of review of diagnoses of archival biopsy specimens from the Johns Hopkins Hospital Department of Pathology and review of the Johns Hopkins Head and Neck Tumor Registry. To be included, sequential biopsy specimens with a diagnosis of dysplasia or carcinoma in situ from the upper aerodigestive tract, with adequate tissue for analysis, were required.

Selection of Loci for Microsatellite Analysis. Ten chromosomal arms were chosen for this analysis on the basis of the following criteria: (a) they identify a minimal area of loss at a putative tumor suppressor gene locus; (b) they identify a proto-oncogene amplicon detectable by microsatellite analysis; or (c) they have displayed a substantial proportion of LOH (>40%) in invasive lesions by allelotype analysis. The 9p21 chromosomal band contains the p16 (MTS1) gene, a cyclin/cyclin-dependent kinase inhibitor involved in cell cycle regulation and corresponds to an area of genetic loss common to many solid tumors. At this time, this locus constitutes the region with the most frequent LOH in HNSC, and the p16 protein is not expressed in >80% of HNSC. The chromosomal band 11q13 includes the bcl-1/int-2 locus, an amplicon carrying the proto-oncogene cyclin D1, one of the few proto-oncogenes implicated in HNSC. LOH in this region actually represents allelic imbalance via amplification of cyclin D1, as confirmed by studies using fluorescence in situ hybridization. The tumor
suppressor gene p53 is commonly mutated in HNSC (9). The p53 gene is found on chromosomal arm 17p13, which also corresponds to an area of frequent LOH in HNSC (5). Chromosomal arm 3p has been shown to contain at least three putative HNSCC tumor suppressor loci (10, 11). Chromosomal band 13q21 contains an area with frequent LOH near the Rb locus that is now thought to include a second, novel tumor suppressor gene locus (12). Chromosomes 6p and 8 contain loci thought to contain putative tumor suppressor genes that have not been precisely mapped and are as yet unidentified but are included

Fig. 1 Allelograms of biopsy specimens from patients 1–4. Part a for patient 1 indicates biopsy of dysplastic lesion with negative histopathological margins, and rebiopsy 1 year later (b) showed dysplastic epithelium. Identical boundaries between loss and retention are shown on chromosomal arm 3p. Loss of identical alleles for loci demonstrating LOH were found and are shown. Patient 2 received an initial biopsy of carcinoma in situ (a), demonstrating margins free of dysplastic epithelium. Subsequent biopsy 1 month later (b) showed epithelial dysplasia. Note identical pattern of allelic loss at three loci with identical boundaries between loss and retention on chromosomal arms 3p and 11q13 but additional loss at two loci (17p13 and 13q21) in the earlier, higher grade lesion. Patient 3 also demonstrated an identical pattern of allelic loss at one locus, 9p21, with identical boundaries between loss and retention but additional loss at two loci (17p13 and 13q21) in the earlier, higher grade lesion biopsied 1 year earlier. Patient 4 shows a matching pattern of LOH at five loci with an identical breakpoint at chromosomal arm 9p. Two additional loci, located at 8q and 13q21, display additional LOH in the biopsy taken 1 month later, indicating that additional genetic alterations have been acquired. Allelograms from patient 5 are provided, with detailed explanation in the text. [Dys, dysplasia.]
because these chromosomal arms are lost in a high percentage of preinvasive and invasive lesions (2, 5). Microsatellite markers included in this study are: D3S1067, D3S1284, D3S1038, D3S1007 (chromosomal arm 3p), D6S265, TCTE, D6S105 (chromosomal arm 6p), D8S261, D8S262, D8S257, D8S167, D8S273 (chromosomal arm 8), IFN-α, D9S736, D9S171 (chromosomal arm 9p21), D11S873, INT-2, PYGM (chromosomal arm 11q13), D13S170, D13S133 (chromosomal arm 13q21), TP-53, and CHRNB-1 (chromosomal arm 17p13).

**Tissue and DNA Extraction.** Tissue was obtained from archival, paraffin-embedded blocks from the Johns Hopkins Hospital Department of Pathology or from freshly frozen tissue, obtained with consent from Johns Hopkins Hospital patients. Representative sections from tissue used for DNA extraction were stained with H&E, and the diagnosis was confirmed for each lesion by a pathologist (W. H. W.). Freshly frozen tissue from a biopsy specimen was meticulously dissected on a cryostat to ensure that the specimen contained at least 75% epithelial cells from the mucosal lesion. Areas of homogeneous histopathological appearance were dissected separately if more than one distinct area of histopathological alteration existed. A control section was stained before microdissection and after each interval of 12 sections to ensure an adequate proportion of epithelial cells from the mucosal lesion of interest. Approximately 12–35 12-μm sections, depending on the adequacy of the specimen, were then collected and placed in 1% SDS/proteinase K (0.5 mg/ml) at 58°C for 24 h. Freshly frozen tissue from a biopsy specimen was meticulously dissected on a cryostat to ensure that the specimen contained at least 75% epithelial cells from the mucosal lesion. Areas of homogeneous histopathological appearance were dissected separately if more than one distinct area of histopathological alteration existed. A control section was stained before microdissection and after each interval of 12 sections to ensure an adequate proportion of epithelial cells from the mucosal lesion of interest. Approximately 12–35 12-μm sections, depending on the adequacy of the specimen, were then collected and placed in 1% SDS/proteinase K (0.5 mg/ml) at 58°C for 24 h. Paraffin-embedded tissue from directed mucosal biopsies was sectioned into 25 14-μm sections. Each individual section was placed on a glass slide and individually microdissected using a dissecting microscope to obtain >75% epithelial cells. The samples were placed in xylene overnight to remove the paraffin, pelleted in 70% ethanol, dried, and incubated in SDS/proteinase K at 58°C for 72 h.
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Digested tissue from both sources was then subjected to phenolchloroform extraction and ethanol precipitation as described previously (12). Normal control DNA was obtained either by: (a) venipuncture and isolation of lymphocyte DNA as described previously (12); (b) microdissection of nonepithelial normal tissue in the previously mentioned archival, paraffin-embedded biopsy specimens; or if necessary, (c) isolation of DNA from nonepithelial, paraffin-embedded tissue from archival paraffin blocks other than the biopsy specimen blocks in the manner described above.

Microsatellite Analysis. Microsatellite markers suitable for PCR analysis were obtained from Research Genetics. Prior to amplification, 50 ng of one primer from each pair were end labeled with [γ-32P]ATP (20 M Ci; Amersham) and T4 kinase (New England Biolabs) in a total volume of 50 μl. PCR reactions were carried out in a total volume of 12.5 μl containing 10 ng of genomic DNA, 0.2 ng of labeled primer, and 15 ng of each unlabeled primer. The PCR buffer included 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, 1% DMSO to which were added 1.5 mM deoxynucleotide triphosphates and 1.0 unit of Taq DNA polymerase (Boehringer Mannheim). PCR amplifications of each primer set were performed for 30–35 cycles consisting of denaturation at 95°C for 30 s, annealing at 50–60°C for 60 s, and extension at 70°C for 60 s as described (12). One-third of the PCR product was separated on 8% urea-formamide-polyacrylamide gels and exposed to film from 4 to 48 h as described. For informative cases, allelic loss (or allelic imbalance in the case of the 11q13 locus) was scored if one allele was decreased in tumor DNA when compared with the same allele in the corresponding allele in Fig. 1 shows an identical pattern of allelic loss at three loci with identical boundaries between loss and retention on chromosomal arm 3p and 11q13 but additional loss at two loci (17p13 and 13q21) in the earlier, more aggressive lesion. Similarly, another patient (no. 3), initially biopsied for carcinoma in situ in a leukoplakic lesion on the left lateral tongue, was rebiopsied 1 year later for a dysplastic lesion at the same site. Once again, both lesions showed a common loss and identical breakpoint at 9p21 but additional loss at 17p13 and 13q21 in the initial biopsy. In each case, identical loss/retention boundaries were identified, and the same alleles (either paternal or maternal) were lost, confirming the interpretation that the lesions are derived from the same progenitor clone. It is notable that neither lesion from this patient or from the patient shown in Fig. 1 displayed any evidence of dysplastic cells in the epithelial margins of the initial biopsy of carcinoma in situ. The subsequent biopsy showed only dysplasia in both cases, and the more malignant appearance of the earlier lesions corresponded with chromosomal loss at two additional loci. In both of these examples, there is no known mechanism by which the cells in the later lesions may have regained previously lost chromosomal fragments. Instead, it is likely that subsequent lesions represent persistence of a more benign subclone along the progression pathway that was not excised during the initial biopsy but one that shared identical, early genetic events with the initially excised lesion. In addition, identical patterns of LOH were observed at 9p21, 3p, and 17p13 in these sequential biopsy specimens, in agreement with their role as early, important events in tumor progression.

Another patient (no. 4) was rebiopsied over a 1-month interval for a leukoplakic lesion of the TVC, showing a matching pattern of loss at five loci with an identical breakpoint at 3p in lesions that were both diagnosed as dysplastic (Fig. 1). However, two loci, 8q and 13q21, displayed LOH in the second biopsy specimen, indicating that a subclone from a common progenitor cell developed additional genetic alterations and acquired a growth advantage, producing a persistent dysplastic lesion.

Finally, patient 5 is represented in Figs. 1 and 2. This patient received serial bilateral biopsies for persistent laryngeal leukoplakia demonstrating dysplasia on six separate occasions over a 12-year period, culminating in diagnosis of a left true vocal cord HNSC. At least four separate genetic progression events can be discerned from this examination. We noted that identical alleles were lost in each of the six loci of interest when LOH occurred, indicating a common clonal origin for each lesion. We also noted that genetic alterations initially evident on biopsies taken from the left TVC subsequently became manifest on both vocal cords. The corresponding allelogram in Fig. 1 shows an identical pattern of allelic loss at three loci with identical boundaries between loss

Results

Four patients underwent repeat biopsies of persistent premalignant lesions at an identical anatomic site, with time intervals between biopsies ranging from 1 to 12 months. One patient underwent biopsies at six separate timepoints over a 12-year period. All five patients demonstrated LOH of identical alleles at multiple loci with identical boundaries between areas of loss and retention (“breakpoints”) for each set of serial biopsies, indicating a common clonal origin for each set. Fig. 1 shows an allelogram of patient 1, who was biopsied for a leukoplakic lesion in the identical location in the floor of the mouth twice over a 1-year interval. This allelogram shows an identical pattern of LOH at four separate chromosomal loci and an identical boundary between areas of loss and retention on chromosomal arm 3p. The corresponding histopathological appearance of moderate dysplasia did not change during this time interval. Histopathological analysis of the first biopsy demonstrated normal epithelium in all margins. Therefore, these results indicate that the recurrent lesion arose from a subclinical persistence of the original population. If genetic progression occurred, it was not detected by histopathological examination or by analysis of LOH.

Patient 2 was rebiopsied at the ventral tongue over a 1-month interval for a leukoplakic lesion with some erythroplasia. The initial lesion was diagnosed as a carcinoma in situ, but the second biopsy demonstrated moderate dysplasia. The corresponding allelogram in Fig. 1 shows an identical pattern of allelic loss at three loci with identical boundaries between loss and retention on chromosomal arm 3p and 11q13 but additional loss at two loci (17p13 and 13q21) in the earlier, more aggressive lesion. Similarly, another patient (no. 3), initially biopsied for carcinoma in situ in a leukoplakic lesion on the left lateral tongue, was rebiopsied 1 year later for a dysplastic lesion at the same site. Once again, both lesions showed a common loss and identical breakpoint at 9p21 but additional loss at 17p13 and 13q21 in the initial biopsy. In each case, identical loss/retention boundaries were identified, and the same alleles (either paternal or maternal) were lost, confirming the interpretation that the lesions are derived from the same progenitor clone. It is notable that neither lesion from this patient or from the patient shown in Fig. 1 displayed any evidence of dysplastic cells in the epithelial margins of the initial biopsy of carcinoma in situ. The subsequent biopsy showed only dysplasia in both cases, and the more malignant appearance of the earlier lesions corresponded with chromosomal loss at two additional loci. In both of these examples, there is no known mechanism by which the cells in the later lesions may have regained previously lost chromosomal fragments. Instead, it is likely that subsequent lesions represent persistence of a more benign subclone along the progression pathway that was not excised during the initial biopsy but one that shared identical, early genetic events with the initially excised lesion. In addition, identical patterns of LOH were observed at 9p21, 3p, and 17p13 in these sequential biopsy specimens, in agreement with their role as early, important events in tumor progression.

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This first biopsy specimen shows LOH on both chromosomes 13q11 and 17p13 on the left TVC, with LOH only of chromosome 17p13 on the right TVC. This implies that the right TVC specimen represents a clonal proliferation earlier on the progression pathway that may have originated on the left TVC. 13q11 LOH represents a subsequent genetic event resulting in further clonal outgrowth (Fig. 3). 17p13 loss is most likely the initial genetic event, and 13q11 is most likely the second genetic event. Subsequent genetic events seen in the left TVC in year 2 are LOH of chromosomes 3p, 8, and 9p21. This clonal out-
growth expands to the right TVC in year 4. A likely sequence of genetic alteration for this tumor would therefore be 17p13 LOH, followed by 13q11 LOH, followed by 9p21, 8, and 3p LOH in an undetermined order. Again, other losses at different chromosomal loci may have occurred during this time period but were not detectable by our analysis.

Discussion

In the past several years, tumor progression models have been constructed for multiple tumor types, including HNSC, by correlating a particular genetic alteration with histopathologic progression (2). The initial description of genetic progression of solid tumors in the context of colon cancer noted: (a) that tumors progress via the inactivation of tumor suppressor genes and activation of proto-oncogenes; (b) specific events occur in a generalized order of progression; and (c) the temporal order of genetic alteration is not identical for each individual tumor, but the accumulation of genetic events is critical for determining tumor progression (1).

The data presented in this study reinforce the paradigm of a genetic progression model for HNSC as presented previously. Certain genetic events (9p21 LOH, 3p LOH, and 17p13 LOH) tend to occur earlier on the progression pathway, but other, usually late-occurring events (13q11, 8), may also occur early in the time course of progression. For all patients, the accumulation of genetic events was associated with histopathological progression.

In addition, the last patient presented (no. 5) demonstrated that progression to malignancy occurs over a period of years. During this progression, a significant number of genetic events occur early on the progression pathway, whereas clinical expression of a malignant phenotype (symptoms or gross morphological changes) occurs later. For this patient, all detectable genetic alterations at the loci we tested were described 9 years before the clinical appearance of malignancy. This is consistent with our previous observation that the incidence of genetic alteration in dysplastic, premalignant lesions is greater than half the rate of genetic alteration found in invasive HNSC (2). When coupled with the observation that the latency period between carcinogen exposure and appearance of malignancy may be as long as 25 years in HNSC, these data suggest that a significant amount of detectable genetic alteration may be present in affected mucosa years before an invasive phenotype is produced.

Finally, the last patient (no. 5) also afforded insight into the nature of clonal expansion for HNSC and human cancers in general. As successive genetic alterations were acquired by a dominant clonal population in this patient, a phenotypic progression to malignancy was seen, accompanied by a wave of clonal expansion to the contralateral TVC in the absence of an invasive phenotype. This demonstrates that clonal, epithelial populations, conferred with a significant growth advantage, may migrate to distances of several centimeters. Supporting evidence for this phenomenon is also provided by: (a) studies that indicate a clonal relationship between adjacent epithelial areas of diverse histopathological appearance (2, 13); (b) studies that indicate that a second primary HNSC is usually clonally related to the initial primary HNSC, despite an anatomically distant site of origin (14); and (c) studies that demonstrate patches of normal appearing epithelia in the upper aerodigestive tract that are clonally related to regional metastases in patients with HNSC of unknown primary origin (15).

These observations have several clinical implications. Clonal genetic alterations may precede the development of malignancy by a significant amount of time, at least several years. Clonal expansion and lateral migration of genetically altered clonal populations may involve a significant portion of the upper aerodigestive tract mucosa before the appearance of a malignant phenotype. Taken together, these observations indicate that there may be a prolonged latency period during which clonal genetic populations may be detected, but invasive progression has not yet taken place.

Characterization of genetic events in premalignant lesions may allow definition of those lesions that may display a more aggressive clinical behavior, perhaps warranting more aggressive treatment strategies. Genetic progression may therefore provide additional indications of clinical behavior when combined with histopathological appearance (16). Chemopreventive strategies could be targeted to these early clonal cell populations. Finally, these populations of clonally expanded, genetically altered cells would be the ideal targets of a strategy aimed at early detection of HNSC before development of an invasive phenotype. A large clonal population of the aerodigestive mucosa harboring early genetic changes should shed sufficient material to allow detection of genetic alterations in saliva. This hypothesis constitutes the basis of a novel approach for cancer detection in patients with HNSC (17).

References

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