Sequential Loss of Heterozygosity at Microsatellite Motifs in Preinvasive and Invasive Head and Neck Squamous Carcinoma

Adel K. El-Naggar, Kenneth Hurr, John G. Batsakis, Mario A. Luna, Helmuth Goepfert, and Vicki Huff

Department of Pathology [A. E. N., K. H., J. G. B., M. A. L.,], Head and Neck Surgery [H. G.], Experimental Pediatrics [V. H.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Studies of sequential molecular alterations in noninvasive and invasive head and neck squamous carcinoma are few in number. Consequently, the genetic changes associated with the neoplastic transformation of these carcinomas have not been defined. To identify chromosomal alterations in preinvasive and invasive head and neck squamous carcinoma, we analyzed DNA from microdissected normal squamous epithelium, severe dysplasia, and invasive carcinoma samples from 20 patients for loss of heterozygosity (LOH) at microsatellite loci by multiplex PCR. Twenty-five microsatellite repeats on chromosomes 3p, 5q, 8p, 9p, and 9q, 11q, 17p, 17q, and 18p and 18q regions were used. In informative cases, LOH in noninvasive lesions was observed in 9p (28%), 9q and 18q (10%), 11q and 17p (7%), and 3p and 18p (5%). A high incidence of LOH in invasive carcinoma was observed at 3p (72%), 8p (53%), 3p (47%), 9q (35%), and 11q (33%). LOH was also associated with DNA aneuploidy, high tumor stage, and poor histological differentiation. Our results indicate that: (a) the high incidence of LOH at loci on chromosomes 9p, 8p, 3p, 9q, and 11q implicate these regions in head and neck squamous carcinoma tumorigenesis; (b) 9p loci alterations are manifested in the early development of these tumors; (c) LOH is correlated with poor prognostic clinicopathological factors; and (d) LOH at 8p loci appears to be associated with the tumor’s aggressive features.

INTRODUCTION

Sequential molecular analysis of pathologically defined premalignant and malignant lesions should allow for determining the nature of the abnormality and the stage at which genomic alterations develop (1). Functional loss of tumor suppressor genes is one of the most common genetic alterations in human solid neoplasms. Defining chromosomal regions that harbor biologically important suppressor genes may, therefore, have broad practical implications on the diagnosis and management of these tumors (2, 3).

The superficial and limited nature of premalignant lesions and the difficulties in obtaining sufficient fresh samples have precluded extensive sequential molecular genetic analysis of these tumors (4, 5). However, the development of the widely distributed and highly polymorphic microsatellite repeat motifs and their suitability for PCR amplification now allow rapid and extensive genomic analysis of small DNA samples from such limited lesions (6–9).

Alterations of microsatellites have been reported in several solid neoplasms and have led to the identification of loci involved in early and late development of certain cancers (10–15). To better define the genomic changes associated with HNSC3 tumorigenesis, fresh, microdissected normal squamous epithelium, and preinvasive and invasive squamous carcinoma specimens from 20 patients were analyzed using microsatellite markers of the commonly altered chromosomal regions reported in these tumors.

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2 To whom requests for reprints should be addressed, at The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 85, Houston, TX 77030.
3 The abbreviations used are: HNSC, head and neck squamous carcinoma; LOH, loss of heterozygosity.

MATERIALS AND METHODS

Normal-appearing squamous epithelium, severely dysplastic/carcinoma in situ mucosa, and invasive carcinoma from 20 patients with HNSC, prospectively accessioned in the Frozen Section Unit of the Department of Pathology, University of Texas M. D. Anderson Cancer Center from 1991 to 1993, formed the materials for this study. Peripheral blood lymphocytes from each patient were also collected. Normal squamous epithelium and epithelium with severe dysplasia/carcinoma in situ, exclusive of invasive carcinoma, were microdissected off the underlying submucosa after frozen section verification and localization. Histologically, normal squamous epithelium was obtained after frozen section verification from the farthest mucosal margin of the resected specimen. In most specimens at least 3 cm separated the edge of the carcinoma from this microdissected margin. Since precise grading of frozen dysplastic lesions is often difficult due to freezing artifacts, full-thickness disorganization, cellular atypia, and mitosis of the squamous epithelium were used as criteria for severe dysplasia/carcinoma in situ. Tissue samples from each specimen were immediately snap frozen and kept at −80°C until used.

DNA Extraction. For preparation of high molecular weight DNA, frozen tissues were ground in a lysis buffer containing 0.2 M Tris-Cl, 1% SDS, 0.25 M NaCl, and 25 mM EDTA (pH 8.5). Protease K was added to a final concentration of 200 μg/ml. DNA was then purified by extraction with phenol/chloroform and precipitated with ethanol.

Microsatellite Analysis. The microsatellites were selected to include those which have been involved in LOH of HNSC. DNA samples were amplified by PCR using primers (Research Genetics, Huntsville, AL) for the microsatellite motifs at chromosome 3p (D3S1307, GATA6F06, D3S1284), chromosome 5q (GATA3F03, GATA6E05, D5S408), chromosome 8p (LPL, D8S264), chromosome 9p (D9S168, D9S199), chromosome 9q (D9S301, D9S302, D9S158), chromosome 11q (D11S968), chromosome 17p (D17S799), chromosome 17q (D17S397, D17S795), chromosome 18p (D18S59, GATA11A06), and chromosome 18q (GATA2A12, D18S870).

We used multiplex PCR where two loci were amplified simultaneously in one reaction tube. One primer from each pair was end labeled using T4 polynucleotide kinase (USB, Cleveland, OH) and γ-32P-ATP (10 mCi/ml; DuPont New England Nuclear, Boston, MA). PCR was performed in a final volume of 25 μl containing 200 ng genomic DNA, 0.0125 μM labeled primer, 0.5 μM of each unlabeled primer, 250 μM deoxynucleotide triphosphate, 6.25% DMSo, 0.25 mM spermidine (Sigma Chemical Co., St. Louis, MO), 10 mM Tris-Cl (pH 8.4), 40 mM NaCl, 1.5 mM MgCl2, and 0.5 units Taq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT). Twenty-five cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min were performed using a initial denaturation step and final elongation step of 94°C for 5 min and 72°C for 3 min, respectively. After addition of 10 μl loading buffer, the PCR products were heat denatured and electroforesed on 7% urea-formamide-polyacrylamide gels at 80 W for 3 h, depending on the fragment size. The gels were exposed to Kodak X-Omat-AR film (Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens.

Acridine Orange Flow Cytometry. Single-cell suspensions from solid tissues were prepared by mechanically mincing fresh tissue in RPMI 1640 media (Irvine Scientific, Santa Ana, CA). Acridine orange staining was performed using a two-step method previously described (16).

RESULTS

Table 1 presents the clinicopathological characteristics and the flow cytometric findings of our study. Ten females and 10 males, who ranged in age between 36 and 74 (mean, 70.0) years, were the study population. Tumor sites included mobile tongue (10 specimens),
Tumor stage was distributed as follows: eight, stage II; six, stage III; and six, stage IV tumors. Flow cytometric DNA content analysis was performed on 17 tumors. Eleven tumors (62.5%) were diploid and six (37.5%) were DNA aneuploid. The proliferative fraction of these specimens ranged from 1 to 4%, with a mean of 2.2%. The proliferative fraction of 10 normal epithelium from surgical and fresh autopsy specimens ranged from 3 to 23%, with a mean of 14.9%. The proliferative fraction of 10 normal epithelium from surgical and fresh autopsy specimens ranged from 1 to 4%, with a mean of 2.2%.

Fig. 1 presents the chromosomal regions and the microsatellite loci chosen for the analysis. LOH was evaluated by visual comparison of the allelic density of the normal, dysplastic, and tumor samples. From complete loss and >50% reduction in allelic intensity were considered an LOH (Fig. 2).

No LOH at the informative loci of the different chromosomal regions was observed in any of the normal squamous epithelium samples when compared to autologous blood DNA. Of the dysplastic specimens, eight specimens (40%) showed LOH at different loci on chromosomes 3p, 8p, 9p, 9q, 17p, 18p, and 18q. The most frequent abnormality (28%) was noted on 9p21 and 9p23. In all of these instances the corresponding carcinoma samples manifested the same abnormality. Of the carcinoma samples, 16 (80.0%) showed LOH in at least one of the microsatellites markers examined. The most frequent loss was observed at loci on 9p (72%), 8p (53%), and 3p (47%). LOH at the remaining chromosomal regions ranged from 17 to 35% (Fig. 3).

Table 2 presents the correlation between tumors with >2 and ≤2 LOH loci, and the clinicopathological and DNA content parameters. All tumors with aneuploid DNA content showed >2 loci with LOH; 6 of the 11 diploid tumors had 2 or less loci with LOH, and 5 showed >2 loci with LOH (P = 0.01). Similarly, all 12 stage III and IV tumors showed >2 loci with LOH, while 6 of the 8 stage II disease had ≤2 loci with LOH (P = >0.001). All poorly differentiated tumors (7 specimens) showed >2 loci with LOH, and 6 of 13 moderately differentiated tumors had ≤2 loci with LOH (P = 0.05). Although the mean proliferative index of tumors with >2 loci with LOH was higher than those with ≤2 loci with LOH, this was not statistically significant (P = 0.17). Of the regions with high LOH, only alterations at 8p loci were more frequent in high stage (8 in stages III and IV versus 2 in stage II) and had DNA aneuploidy (7 in stages III and IV versus 3 in stage II).

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### Table 1: Clinicopathological factors, DNA flow cytometric data, and LOH in primary HNSC

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<th>Race</th>
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<th>Stage</th>
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<td>PD</td>
<td>1.00</td>
<td>23</td>
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* W, white; H, Hispanic; B, black; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; BOT, base of tongue; FOM, floor of mouth.

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**GENETIC INSTABILITY IN HEAD AND NECK CANCER**

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**Table 2**: Correlation between tumors with >2 and ≤2 LOH loci, and the clinicopathological and DNA content parameters. All tumors with aneuploid DNA content showed >2 loci with LOH; 6 of the 11 diploid tumors had 2 or less loci with LOH, and 5 showed >2 loci with LOH (P = 0.01). Similarly, all 12 stage III and IV tumors showed >2 loci with LOH, while 6 of the 8 stage II disease had ≤2 loci with LOH (P = >0.001). All poorly differentiated tumors (7 specimens) showed >2 loci with LOH, and 6 of 13 moderately differentiated tumors had ≤2 loci with LOH (P = 0.05). Although the mean proliferative index of tumors with >2 loci with LOH was higher than those with ≤2 loci with LOH, this was not statistically significant (P = 0.17). Of the regions with high LOH, only alterations at 8p loci were more frequent in high stage (8 in stages III and IV versus 2 in stage II) and had DNA aneuploidy (7 in stages III and IV versus 3 in stage II).

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**SPECIMEN TYPE**

![Fig. 1. Allelotyping results of all dysplastic (D) and carcinoma specimens (T) at different chromosomal loci.](cancerres.aacrjournals.org)
GENETIC INSTABILITY IN HEAD AND NECK CANCER

This, however, did not reach a significant statistical correlation (P = 0.17 and 0.37, respectively). No apparent association between LOH at 3p markers and aggressive tumor characteristics was noted.

DISCUSSION

Previous cytogenetic and molecular genetic studies of HNSC have shown certain chromosomal regions with recurring alterations (17–26). The majority of these investigations have been performed on advanced, invasive neoplasms in which multiple genetic changes and clonal heterogeneity confound the results (20, 26). Accordingly, characteristic temporal pattern and/or a stage at which alterations occur has not been defined. Since tumorigenesis is a sequential accumulation of genetic alterations (1, 27–29), analysis of early and late neoplastic lesions may define the genetic changes associated with the development and progression of HNSC.

In this study we microdissected freshly frozen autologous normal, severely dysplastic squamous epithelium, and invasive squamous carcinoma in patients with head and neck tumors. We found 9p loci to be most frequently reduced to homozygosity in invasive carcinoma (72%), in agreement with a previous study (25). Interestingly, 28% of noninvasive, dysplastic lesions also displayed LOH at 9p, indicating an early involvement of a gene(s) at this region in the genesis of these lesions. Of the 13 invasive tumors that showed 9p LOH, 38.4% of the autologous normal squamous epithelium and carcinoma tissue samples are better able to define the progressive genomic alterations of HNSC, since it is done in a series of tissues from the same individual.

This same 9p region has been frequently altered in several other solid tumors (30–38) and implicated in the early development of others (5, 35). Among potential candidate genes in this region, the MTS1/p16 and p15 genes are a likely target for mutation (39–42). This, however, has more recently been contested by several recent studies indicating a lack of mutations in several primary neoplasms (41–44). In contrast to previous investigations, our study of autologous uninvolved squamous epithelium, noninvasive, and invasive samples is better able to define the progressive genomic alterations of HNSC, since it is done in a series of tissues from the same individual.

Overall, one third of noninvasive squamous lesions displayed LOH at microsatellite loci examined, with up to 15.7% showing 18q alterations. In invasive carcinoma, LOH was detected more frequently at 8p, 3p, and 9q than at 5q, 11q, 17p, 17q, 18p, and 18q loci. Contrary to previous reports (25), we observed LOH at 9q in 35% of the invasive cancers using the same microsatellite markers. Our results are in accordance with previous studies showing high frequency of LOH at 3p (45–49) and 9q (23, 25, 26) loci. We, however, observed a lower frequency of 5q LOH than Ah-see et al. did (26). This discrepancy may be related to differences in the patient population.

Our results showed no detectable LOH in histologically normal squamous epithelium. This is contrary to the findings of Lee et al. (4) relating to LOH for 13q loci. It is not clear, however, if the "nonmalignant mucosa adjacent to tumor" in their study means the absence of mild and/or moderate dysplasia. The lack of alterations in normal squamous epithelium in the field of excision in our study and those of others (10) suggest that such genetic abnormalities correspond to histological changes.

Table 2. Correlation between LOH at microsatellite markers and clinicopathological factors and DNA content data

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<tr>
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<td>6</td>
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<tr>
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<td>7</td>
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<td>Proliferative index (mean)</td>
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</tr>
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</table>

Fig. 2. LOH in selected cases. N, normal squamous epithelium; D, severe dysplasia; T, invasive carcinoma.

Fig. 3. Summary of the frequencies of LOH at different chromosomal arms in dysplasia and carcinoma samples.

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Our data indicate that chromosomal aberrations at certain loci are present in preinvasive lesions and that additional abnormalities continue to develop during neoplastic transformation. The most likely region associated with early development is the 9p21 region and alterations at 8p loci may be associated with adverse biological features. In our study, tumors with marked LOH were preponderantly aneuploid, high stage, and poorly differentiated. These results corroborate similar studies in other tumors and indicate an association between the extent of LOH and tumor aggressiveness (14, 15, 49, 50). Additional studies to identify the timing at which this alteration occurs and to localize the putative suppressor genes at these locations are under way.

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REFERENCES

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