Allelic Loss and Replication Errors at Microsatellite Loci on Chromosome 11p in Head and Neck Squamous Carcinoma: Association with Aggressive Biological Features

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ABSTRACT

The frequent loss of heterozygosity (LOH) demonstrated at chromosome 11p regions in several sporadic malignancies has suggested the presence of tumor suppressor genes at these locations. To obtain detailed mapped incidence of the microsatellite alterations at these regions and to investigate the possible correlation between the genotype and the pathobiological characteristics of head and neck squamous carcinoma, we analyzed paired DNA samples from normal mucosa and primary tumor specimens from 56 patients with these tumors. Our results show that 50.9% of the tumors had microsatellite alterations at one or more of these loci. LOH was manifested in 45.5% and instability in 5.5% of the tumors. 11p15 loci showed more frequent LOH (39.6%) than those of 11p13 (29.3%) and 11p11–12 (18.8%); the *D11S988* (11p15) marker showed the highest single locus incidence of LOH (29.7%). Eight tumors (22.2%) demonstrated simultaneous LOH at both the 11p15 and 11p13 regions. LOH was significantly associated with poor histological differentiation, DNA aneuploidy, and high proliferative activity in these neoplasms. Our study extends the involvement of the 11p13 and 11p15 regions to head and neck squamous tumorigenesis and indicates that the terminal loci of 11p may harbor a tumor suppressor gene(s) associated with the progression of these tumors.

INTRODUCTION

Characteristic molecular events associated with HNSC[2] initiation and progression, similar to that proposed for colon carcinoma (1, 2) have yet to be developed. Efforts to achieve this goal have been hampered by the lack of definitive familiar risk, a multitude of environmental factors, and the paucity and/or complexity of cytogenetic information on tumors from these patients (3–6). Advances in molecular genetic techniques and availability of markers for short tandem repeat polymorphism (microsatellites) have allowed for rapid screening of chromosomal alterations harboring putative tumor suppressor genes associated with HNSC (7, 8).

Studies of several human neoplasms have shown frequent LOH at specific regions on the short arm of chromosome 11 (9–15). The results of these investigations have implied that 11p13 and 11p15 loci harbor a putative tumor suppressor gene(s) associated with the development and/or progression of these tumors (16–20). More recently, the human 11p11–12 region has been shown to suppress tumorigenicity in rat liver tumor, suggesting the presence of another suppressor gene at this location (21). Only two molecular genetic studies of primary HNSC have included 11p markers in a LOH screening (7, 22). The limited number of loci tested and the widely varied difference in the incidence of genetic alterations in these studies render the assessment of the 11p regions involvement in HNSC difficult.

The objectives of this study were (*a*) to obtain a more detailed estimate of *11p* loci involvement in HNSC and (*b*) to correlate the genotype with clinicopathological features.

MATERIALS AND METHODS

Paired normal mucosa and invasive squamous carcinoma samples from 56 resected specimens from patients with HNSC, accessioned between March 1993 and October 1994 at the Surgical Pathology Frozen Section Unit of the Department of Pathology, University of Texas M. D. Anderson Cancer Center, formed the materials for this study. After frozen section verification, normal squamous mucosa was microdissected off the submucosa from the most distant margin of the specimens. Tumor samples were carefully selected from the most invasive part and verified to contain >80% neoplastic cells by frozen section evaluation. Tumor specimens were divided into three equal parts for flow cytometry, formalin fixation and paraffin-embedding, and snap freezing. Peripheral blood lymphocytes were collected from each patient in EDTA. Lymphocytes were harvested after Ficoll-Hypaque centrifugation and immediately snap frozen in liquid nitrogen and kept at −80°C along with the tissue samples until used.

DNA Extraction. For preparation of high molecular weight DNA, frozen tissues measuring 0.3–1.0 g were ground in a lysing buffer containing 0.2 M Tris-Cl, 1% SDS, 0.25 M NaCl, and 25 mM EDTA (pH 9.5). Proteinase K enzyme with a final concentration of 200 μg/ml was added to the tissues and incubated for 24 h at 42°C. DNA was then purified by phenol/chloroform extraction and precipitated with ethanol. DNA from peripheral blood lymphocyte was also extracted using the same method. The concentration of DNA was obtained by measuring the optical density at 260 and 280 nm (Ultraspec III; Pharmacia) and ranged from 200 to 1200 μg/ml in a total volume of 0.3 ml.

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[2] The abbreviations used are: HNSC, head and neck squamous carcinoma; LOH, loss of heterozygosity.

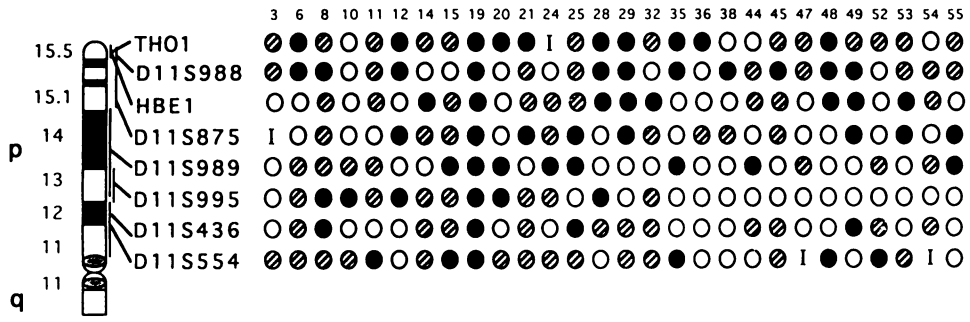

Fig. 1 Allelotyping of 11p microsatellite markers in the 29 cases with alterations. ●, LOH in tumor tissue; ○, homozygosity in normal and tumor tissue; ⊘, heterozygosity in normal and tumor tissue. *I*, instability.

Microsatellite Analysis. Eight microsatellite loci of chromosome 11p were used. The following primers for PCR amplification of microsatellite markers were obtained from Research Genetics (Huntsville, AL): 11p15.5 (*Thol*, *HBE1*), 11p15 (*D11S988*, *D11875*), 11p14–13 (*D11S989*), 11p13 (*D115995*), 11p11.22–11p12 (*D11S436*), and 11p11.2–12 (*D11S554*).

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 (United States Biochemical, Cleveland, OH) and [γ-^{32}P]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 label primer, 0.5 μM of each unlabeled primer, 250 μM of deoxynucleotide triphosphate, 6.25% DMSO, 0.25 mM spermidine (Sigma Chemical Co., St. Louis, MO), 10 mM Tris-HCl (pH 8.4), 40 mM NaCl, 1.5 mM $MgCl_2$, 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 an 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 electrophoresed on 7% urea-formamide-polyacrylamide gels at 80 W for 3 to 5 h depending on the fragment size. The gels were exposed to Kodak X-O-Mat-AR film (Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens. LOH was determined by visual comparison of the allelic band densities between normal and corresponding tumor samples. A reduction in band density of >50% was considered as LOH. The reduction was independently determined by two observers, and a third observer was consulted on the questionable and/or disputed samples. Microsatellite instability was scored if: (*a*) there were additional band(s) in tumor tissue that were not observed in the corresponding normal pattern or (*b*) there was a band shift in tumor samples that contrasted to those of corresponding normal bands.

To exclude the possibility of technical artifacts or contamination, all of the differences described were reproduced by independent PCR reactions and separate gel loadings.

Acridine Orange Flow Cytometry. Single-cell suspensions from carefully selected tumor samples after frozen section verification of tumor predominance were prepared by fine mincing followed by vigorous vortexing of fresh tissue in RPMI 1640 media (Irvine Scientific, Santa Ana, CA) and filtration through a 35-μm nylon filter. A Giemsa-stained cytospin preparation of each cell suspension was performed to evaluate tumor cell:host cell ratio and cell integrity, and the extent of cell clumping was reviewed. Samples with less than 75% single neoplastic elements (bare nuclei and intact cells) were excluded. Acridine orange staining was performed using a two-step method previously described by Traganos *et al.* (23). DNA/RNA analysis was performed with an EPICS Profile Cytometer (EPICS Division of Coulter Corp., Hialeah, FL) equipped with an argon ion laser emitting 488 nm (15 nW). A 525BP filter was used to collect green fluorescence (DNA), and a 610LP filter was used to collect red fluorescence (RNA). Peak *versus* integral signals were collected to discriminate doublets. Histograms were analyzed using the "Histogram Analysis" menu option of the Profile software.

Statistics. Fisher's exact and χ^2 tests were used to determine the association between microsatellite alteration and different clinicopathological features.

RESULTS

The patients were 40 males and 16 females who ranged in age from 38 to 88 years, with a mean of 60.2 years. Histologically, there were 9 well-differentiated, 28 moderately differentiated, and 19 poorly differentiated carcinomas. Tumor locations were as follows: 21 oral tongue; 8 floor of mouth; 22 larynx; 4 nasal cavity, and 1 maxillary antrum. Of the 53 tumors with available staging information, 4 were stage I, 13 were stage II, 21 were stage III, and 15 were stage IV. Twenty-two (38.6%) neoplasms manifested diploid DNA, and 34 (60.7%) were DNA aneuploid. The proliferative fractions of the 54 analyzable specimens ranged from 3 to 31%, with a mean of 10.9%.

Fig. 1 presents the order of loci and microsatellite alterations in the study cohort (31, 32). Overall, 29 (51.8%) of the tumors had microsatellite alterations. LOH was manifested in 26 (46.4%), and instability was noted in 5 (8.9%) cases. Representative examples of the LOH and instability are shown in Fig. 2. In three cases (cases 3, 47, and 54), instability without LOH was noted. The incidence of LOH was 18.8% (9/48 informative tumors) at 11p11–12 regions, 29.3% (12/41 informative tumors) at 11p13 region, and 40.8% (20/49 informative tumors) at telomeric *11p15* loci. LOH at both the *11p13* and *11p15* loci was found in 8 (22.2%) of the combined 36 informative cases. Fig. 3 presents the frequency of LOH only at individual loci on the short arm of chromosome 11. The highest frequency of LOH was observed in 11 (29.7%) of 37 informative specimens at the *D11S988 (11p15)* locus.

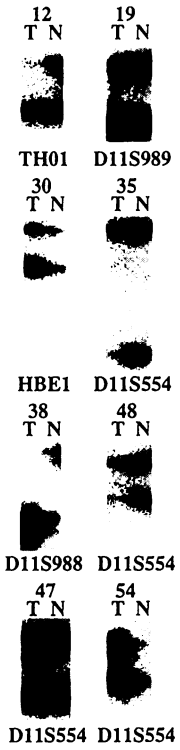

Fig. 2 Representative autoradiographs of LOH of microsatellite markers on paired normal *(N)* and tumor *(T)* tissue from HNSC. Loss of the upper allele in tumors 12 and 38 is observed. The reduction in the upper bands of tumors 19 and 30 is less than expected if a complete loss of the allele was present. Tumors 35 and 48 show loss of the lower allele in tumor tissue. Cases 47 and 54 represent autoradiograph microsatellite instability.

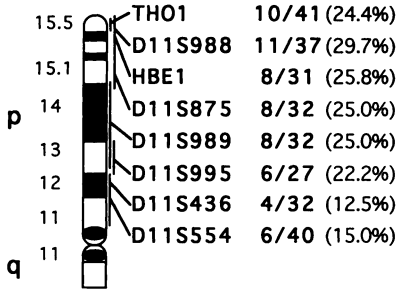

Fig. 3 Diagram of chromosome 11p band and markers indicating the percentage of frequency of LOH for each marker.

Table 1 presents the correlation between clinicopathological parameters and LOH at *11p* microsatellite loci. Significant statistical correlations between LOH and poor histological differentiation, DNA aneuploidy, and high proliferative activity were noted. No significant association among age, sex, stage, and LOH at 11p alterations was found. LOH at noncontiguous regions was observed in four tumors (7.1%). The remaining specimens manifested either contiguous losses or were noninformative at intervening loci.

Table 1 Correlation between LOH at chromosome 11p loci and clinicopathological factors in HNSC

Characteristics	LOH +	LOH −	*P*
Age			
Mean (yr)	59.6	61.1	NA[a]
Sex			
Male	18	22	0.99
Female	7	9	
Histopathological grade			
I and II	11	26	0.002
III	14	5	
DNA ploidy			
Diploid	4	18	0.002
Aneuploid	21	13	
Proliferative activity			
≥10	18	10	0.005
<10	6	19	
Stage			
I and II	6	10	0.55
III and IV	19	20	

[a] NA, not applicable.

DISCUSSION

Frequent LOH at chromosome 11p loci have recently been demonstrated in several hereditary and sporadic pediatric (15) and adult solid neoplasms (10, 13, 14, 16, 18–25). These data suggest that loci at the 11p13 and 11p15 regions house tumor suppressor genes associated with tumorigenesis. Functional support for these data and the possible involvement of the 11p11–12 region have been drawn from experimental studies of somatic cell hybrids of HeLa, human fibroblast, and rat liver epithelial cell lines and microcell transfer of human chromosome 11 experiments (21, 26, 27).

Similar studies in HNSC are few in number and inconclusive. The only two molecular investigations using 11p markers in HNSC have reported a markedly disparate incidence of 11p loci alterations (7, 22). Such disagreement could be attributed to the number and the localization of markers used, interpretive, and/or patient population differences. We selected several microsatellite markers spanning these loci to determine the incidence, localization, and clinical implications of alterations in HNSC.

Our results showed that 51.8% of HNSC tumors manifest microsatellite alterations at 11p loci tested. This frequency is in agreement with Lydiatt *et al.*'s (22) primary HNSC study and Bradford *et al.*'s (6) study of HNSC cell lines and those of others in different solid neoplasms (9, 12, 17–19). Our findings therefore extend and implicate the presence of a tumor suppressor gene(s) at these regions in the oncogenesis of HNSC. In addition, our results indicate that the critical region appears to be distal to the *D11S989* marker and that *D11S988* constitutes the locus with the highest incidence of loss. This is supported by a recent study of human gliomas (28) and those with

detailed mapping data of 11p15 regions in other solid neoplasms (19, 25, 29).

In contrast to our findings, an earlier study of head and neck carcinoma by Nawroz *et al.* (7), using the *D11S988* marker, showed a low incidence of alteration. This discordance may be related to the relatively small number of carcinomas examined by these authors and/or to differences in patient and/or tumor characteristics. The effect of normal host cell contamination on false heterozygosity in tumor specimens (13), although cannot entirely be ruled out, may have been minimal since enriched tumor tissue was utilized in both of these investigations.

LOH was manifested in 12 (29.3%) and 20 (40.8%) of the informative cases at 11p13 and 11p15 loci, respectively; simultaneous LOH at both regions was noted in 8 (22.9%) of the combined informative specimens. These data suggest that 11p15 loci are more frequently associated with tumorigenesis, and a locus at 11p13 may independently be involved in a small subset of tumors. The latter is supported by previous cytogenetic analysis of squamous carcinoma cell lines (6). The implications of 11p15 *versus* 11p13 LOH with respect to tumor aggressiveness/clinical prognosis, however, are currently unknown and remain to be investigated. In our study, LOH at continuous loci was observed in two lesions. The molecular alterations in these two tumors may either have occurred independently in the same clone and/or at temporally separate points in different cell populations during tumor progression (30). The biological significance of this finding is currently unclear.

Our results also show that LOH at 11p loci was associated with aggressive biological characteristics in these neoplams. Significant correlations between 11p LOH and poor histological differentiation and DNA aneuploidy and high proliferative activity were found. A similar association has previously been reported in lung and ovarian carcinoma (9, 14, 31). No correlation between 11p alteration and the patients' age, gender, or tumor stage was observed. Others have also reported similar findings in lung and Wilm's tumors (12, 13). Our study suggests that alteration of an 11p locus probably plays a role in HNSC progression rather than initiation or early development. Consistent with this hypothesis is the finding that 11p LOH was observed in only a subset of tumors that showed aggressive characteristics. Studies of 11p loci in preinvasive lesions and metastatic tumors may shed more light on the sequential involvement of these loci and their association with clinical outcome.

Microsatellite instability was noticed in approximately 9% of the neoplasms, suggesting a minor role in the development of these tumors. Although this is generally in agreement with the majority of the investigated sporadic malignancies (32–35), it is considerably lower than those reported by others (36, 37). This may be related to differences in interpretive and technical factors, tumor types, and/or the stability of the loci tested.

The development of extensive and refined mapping of these regions should allow for future identification of these putative tumor suppressor genes (38).

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