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Genetic Heterogeneity in Saliva from Patients with Oral Squamous Carcinomas

Implications in Molecular Diagnosis and Screening

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We performed microsatellite analysis at chromosomal regions frequently altered in head and neck squamous carcinoma on matched saliva and tumor samples from 37 patients who had oral squamous carcinoma. The results were correlated with the cytologic findings and traditional clinicopathologic factors to assess the diagnostic and biological potential of these markers. Our data showed that 18 (49%) of the saliva samples and 32 (86%) of the tumors had loss of heterozygosity (LOH) in at least one of the 25 markers studied. In saliva, the combination of markers D3S1234, D9S156, and D17S799 identified 13 (72.2%) of the 18 patients with LOH in saliva ($P < 0.001$). For tumors, markers D3S1234, D8S254, and D9S171 together identified 27 (84.3%) of the 32 tumors with LOH at any of the loci tested ($P < 0.001$). Eleven (55%) of the 20 saliva samples with cytologic atypia and seven (35%) of the 17 specimens without atypia had LOH. Significant correlation between LOH in tumor at certain markers and smoking and alcohol use was found. Our results indicate that: 1) epithelial cells in saliva from patients with head and neck squamous tumorigenesis provide suitable material for genetic analysis; 2) combined application of certain markers improves the detection of genetic alteration in these patients; 3) clonal heterogeneity between saliva and matching tumor supports genetic instability of the mucosal field in some of these patients; and 4) LOH at certain chromosomal loci appears to be associated with smoking and alcohol consumption. ***(J Mol Diag 2001, 3:164–170)***

The oral cavity is an ideal site for screening individuals at high risk of developing head and neck squamous carcinoma (HNSC) because of the availability of cells shed in saliva and the convenience of visualizing and sampling lesions at these locations.[1–3] However, little progress has been made in the management of patients with oral squamous carcinoma due mainly to the nonspecific symptoms, minimal physical finding inpatients with early-stage cancer, and the lack of biological predictors of progression.[4] Identifying novel and reliable biogenetic markers for the biological assessment of squamous lesions may assist in early diagnosis and treatment of head and neck squamous tumorigenesis. Microsatellite DNA motifs consisting of highly polymorphic short tandem repeat sequences distributed throughout the genome have been widely and successfully used as markers for molecular analysis of tumorigenesis in head and neck and other neoplasms.[1,5–7]

Studies using microsatellite markers from different chromosomal arms in HNSC have shown that alterations at certain regions on chromosomes 3p, 9p, 17p and 18q to be associated with the development of these tumors.[1,2,6,8–12] Although the timing and the order of these alterations are currently unknown, different studies have shown high incidence of loss of heterozygosity (LOH) in noninvasive lesions indicating an early association with tumorigenesis.[1,6,7] Analysis of selected microsatellite markers at these regions on epithelial cells from patients' saliva is a convenient and non-invasive approach for molecular screening and early detection of this disease.[13–24] In this study, we evaluated the diagnostic and biological implications of the alterations at several of the above mentioned microsatellite markers in prospectively collected saliva and tumor specimens from patients who had oral squamous cell carcinoma.

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Materials and Methods

Forty matched histologically normal squamous mucosa and tumor tissues and saliva collected freshly from patients with primary untreated HNSC were prospectively acquired between 1996 and 1998 by the Department of Pathology at The University of Texas M. D. Anderson Cancer Center after obtaining patient consent. Ten saliva samples from normal individuals with negative history of cancer were also collected and used as a biological control. Histologically normal squamous mucosa from each cancer specimen was stripped from the farthest margin after frozen section evaluation and used as putative controls. Ficoll-Hypaque isolated lymphocytes from heparinized peripheral blood from all patients and 10 normal volunteer individuals were also obtained and immediately frozen; lymphocytes were used as control for saliva analysis from normal volunteers. Lymphocytes were used only if histologically matched mucosa was suspected to harbor alterations.

For all patients, tobacco and alcohol consumption histories were obtained from the epidemiology database. Patients' demographic, pathological, and clinical information was collected retrospectively from pathology reports and patient records. The 10 normal saliva samples from healthy individuals were obtained from five nonsmokers and five current smokers.

Saliva Collection

After the mouth of each patient and normal subject was washed with sterile water, saliva specimens were collected in a sterile cap and transported immediately to the laboratory to be centrifuged at 1200 g for 5 minutes. The supernatants were decanted and the cell pellets were frozen at −80° in the same tubes. Three cases were eliminated for lack of sufficient DNA from saliva samples and the remaining 37 cases formed the cohort of materials for the analysis.

DNA Extraction

Extraction of DNA was performed using DNAzol (Molecular Research Center, Cincinnati, OH). Cells were lysed in DNAzol using a Tissue Tearor (Biospec Products, Bartlesville, OK) for fresh tissue, and a vortex homogenizer for saliva. The liberated DNA was precipitated with ethanol and resuspended in 10 mmol/L Tris, 1 mmol/L EDTA.

Microsatellite Analysis

Aliquots of DNA were subjected to standard polymerase chain reaction (PCR) analysis using primers for the following loci: D3S656, D3S1293, D3S1234, D3S1217, D3S1261, D8S254, D8S261, LPL-tet, D8S298, D8S283, D9S104, D9S156, D9S168, D9S171, D9S199, D17S513, TP53, D17S799, CHRNB1, D17S122, D18S46, D18S363, D18S35, D18S39, and D18S41 (Research Genetics, Huntsville, AL). The loci were chosen based on the frequency of their alteration in head and neck tumors. Microsatellite location on the chromosomal arms was determined based on the latest map in the Genetic Location Database of the University of Southampton, U. K.

One primer was end-labeled using γ^{32}P-ATP and T4 polynucleotide kinase. PCR was performed in a 20 μl volume with 10 ng of genomic DNA, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.001% gelatin, 0.5 μmol/L of each unlabeled primer, 0.01 μmol/L labeled primer, 0.2 mmol/L dNTPs, 5% dimethyl sulfoxide (Sigma Chemical, St. Louis, MO), and 0.5 units of Amplitaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT).

The amplification process consisted of: (a) an initial 10-minute denaturation step at 94°; (b) 35 cycles of denaturation at 94° for 30 seconds, annealing at 55–60° for 1 minute, and elongation at 72° for 1 minute; and (c) a final elongation at 72° for 5 minutes. Sequencing stop buffer was added to the reactions. PCR products were then denatured at 94° for 5 minutes and quickly chilled, and 4–6 μl was loaded on 7% acrylamide-urea sequencing gels containing 32% formamide. Electrophoresis was performed at 80 W for 2–4 hours, depending on the fragment size. The gels were dried and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL).

Evaluations were conducted by two independent observers who visually scored the pattern and the band intensity between normal tissue, tumor, and saliva specimens from each case before the identification of the patients. LOH was defined by the presence of an allelic band difference between nonmalignant epithelium and tumor or saliva of more than 50%. Visually suspicious cases were subjected to densitometry, and a difference of >30% was scored as LOH. Instability was defined as the appearance of a novel band that was not seen in the normal control.

Acridine Orange Flow Cytometry

Disaggregated cells were adjusted to 1.0×10^6 cells/ml. A cytospin preparation was evaluated for quality and cellular integrity. Cells were subsequently stained with acridine orange according to the two-step procedure of Traganos et al.[25] Ploidy status was defined by the DNA index, which represents the ratio of the relative G_0/G_1 stemline portion of the tumor samples to that of the normal peripheral blood lymphocytes. Diploid DNA is defined by a single G_0/G_1 peak with a DNA index of 1.0, and DNA aneuploidy is defined by the presence of one or more additional stemlines to the right (hyperdiploid, DI > 1.0) or the left (hypodiploid, <1.0) of the G_0/G_1 diploid peak. A near diploid (hypodiploid or hyperdiploid) DI was determined after mixing the test sample with lymphocyte controls. The coefficient of variation (CV) of DNA diploid and aneuploid stemline ranged from 2.1 to 5.4 with a mean of 3.6 ± 1.2 and 2.8 to 6.1 with a mean of 4.9 ± 1.6, respectively.

Statistical Methods

All correlations were performed using a two-tailed Fisher's exact test.

Table 1. Clinicopathological and Epidemiological Characteristics of Patients with Oral Squamous Carcinoma

Case no.*	Sex	Grade	Site	Size (cm)	Stage	Sm†	Alcohol HX	DI	PI
1	M	MD	Tongue	4.9	II	Yes	Yes	1	7
2	F	PD	FOM	n/a	U	No	No	1	10
3	M	MD	Palate	4	I	Yes	Yes	1	13
4	M	WD	Tongue	2.5	II	Quit	Yes	1.67	6
5	M	WD	Tongue	2.5	II	Quit	Quit	1.82	9
6	F	PD	Palate	5.5	U	Yes	None	1	3
7	M	MD	Tongue	2	II	Quit	None	1.82	17
8	F	MD	Tongue	0.9	III	Yes	Social	1.9	8
9	M	MD	Tongue	4	III	Yes	Social	1	5
11	M	MD	Tongue	n/a	III	Yes	Social	1	13
12	M	MD	Tongue	4.6	III	Yes	Quit	1	19
13	M	MD	FOM	2.2	III	Yes	Yes	1.71	27
14	M	MD	Tongue	4.5	II	Yes	Yes	1	22
15	M	MD	Tongue	1.5	U	Unknown	Social	1.35	17
16	F	WD	FOM	4.5	II	No	No	1	6
18	M	MD	Tongue	2	II	Yes	Yes	1.72	10
19	M	PD	Tongue	4.5	III	Yes	No	1	8
20	M	MD	Tongue	n/a	III	Yes	Quit	1.58	16
22	M	MD	FOM	4	U	Yes	Quit	n/a	n/a
23	M	PD	Tongue	1.7	U	Quit	Yes	1.73	24
24	M	PD	Tongue	0.7	I	Quit	Yes	n/a	n/a
25	M	PD	FOM	8	III	No	No	1	n/a
26	M	MD	Tongue	2	IV	No	Social	1	9
27	M	PD	FOM	n/a	III	Quit	Quit	n/a	n/a
28	M	MD	FOM	n/a	II	Yes	Yes	n/a	n/a
29	F	MD	FOM	n/a	III	Quit	Yes	n/a	n/a
30	F	MD	Tongue	n/a	II	No	Social	n/a	n/a
31	M	MD	Tongue	1.5	II	Yes	Social	n/a	n/a
32	M	MD	Tongue	1.8	III	Quit	Social	1	5
33	M	MD	Tongue	2.2	II	No	Social	1	14
34	F	MD	Tongue	n/a	II	No	No	n/a	n/a
35	M	PD	Gingiva	2.7	III	Quit	Unknown	1	4
36	F	MD	Tongue	1.5	II	Yes	Social	n/a	n/a
37	M	PD	Tongue	3.5	III	Yes	Quit	1.66	6
38	F	PD	Tongue	2	IV	Yes	Social	2.33	12
39	F	MD	Tongue	n/a	II	No	No	n/a	n/a
40	M	WD	FOM	n/a	I	Quit	Social	1	5

* Cases 10, 17, and 21 were dropped during the study for lack of sufficient DNA to complete the study.
† Quit: ceased smoking for at least one year and no current drinking; Yes: alcohol >7 drinks/week; smoking, at least 1 pack/day.
M, male; F, female; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; FOM, floor of mouth; U, unknown; DI, DNA index; PI, proliferative index; Sm, smoking; n/a, not available.

Results

Table 1 presents the clinicopathologic and epidemiological characteristics of the 37 study subjects. None of the histologically normal squamous mucosas used as a control showed any alterations at the microsatellite sites tested. Accordingly, no further analysis of the lymphocytes from these patients were performed. The incidence of LOH at individual markers and its occurrence per chromosomal arm for both tumor and corresponding saliva samples are presented in Table 2 and Figure 1. Chromosomes 9p, 3p, and 17p showed the highest incidence of LOH in both tumor and saliva. We also investigated the use of small combinations of markers to improve the detection of genetic alterations in both saliva and tumor samples as a likely approach for future clinical applications. Figure 2 illustrates LOH found in tumor and saliva (A), tumor alone (B), and saliva alone (C).

Saliva

Of all 37 saliva specimens, 18 samples (49%) manifested LOH, and 19 samples (51%) lacked any abnormalities. The most frequent LOH (21.6%) was found at marker D3S1234 (8 cases), but adding markers D17S799, and D9S156 (13 35%) of the 37 cases analyzed showed LOH which led to 100% specificity and 44% sensitivity of LOH detection. Eleven instances of instability were identified in saliva from three cases. None of the 10 informative saliva samples from healthy individuals showed any LOH at the markers used.

Tumor

Thirty-two (86%) tumors showed LOH in at least one marker, and five (14%) had no LOH. The two markers that exhibited LOH most frequently in tumor tissue were D9S171 and D9S156, which identified 17 (53%) inci-

Table 2. Incidence of LOH in Heterozygous Cases (%) of Saliva and Tumor Specimens of Patients with HNSC

Marker	Heterozygous (informative)	Tumor only	Saliva only	Tumor and Saliva
D3S656	24	10	0	2
D3S1293	32	5	2	2
D3S1234	23	7	3	5
D3S1217	33	11	2	2
D3S1261	33	12	2	1
D8S254	26	11	1	0
D8S261	23	9	0	0
LPL-tet	28	9	0	1
D8S298	26	7	0	0
D8S283	30	7	1	0
D9S104	31	12	1	1
D9S156	28	16	3	1
D9S168	23	11	2	1
D9S171	29	16	2	1
D9S199	32	13	0	1
D17S513	27	10	0	2
TP53	33	9	1	1
D17S799	29	9	1	2
CHRNB1	29	9	1	1
D17S122	26	7	0	1
D18S46	31	11	1	1
D18S363	20	7	0	0
D18S35	28	9	0	1
D18S39	22	10	0	0
D18S41	23	6	0	0

dences of LOH each. The combination of markers D9S171 + D3S1234 identified 24 (75%) of the 32 tumors with LOH ($P = 0.051$). Addition of marker D8S254 to the above combination identified three more instances of LOH for a total of 27 cases ($P = 0.009$). This marker combination gave 84% sensitivity and 100% specificity of LOH detection. Various other combinations of markers, especially those of D9S171, D3S1234, D8S254, and D9S156, identified similar incidence of tumors with LOH.

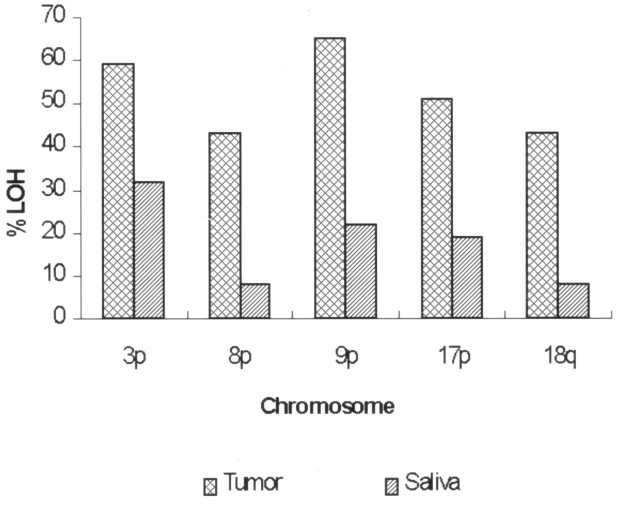

Figure 1. Frequency of LOH by chromosomal arm in tumor and saliva samples.

Four incidences of instability were also noted in tumors from four different patients.

Saliva versus *Tumor*

Sixteen tumors from the 18 patients with LOH in saliva samples (in one or more of the 25 markers) had LOH in corresponding tumors. Also, 16 tumors from the 19 patients with no LOH in saliva samples had LOH ($P = 1.00$, Fisher's exact test); the occurrence of LOH in saliva did not predict the LOH in corresponding tumor samples. One pair of markers (D3S1234 + D9S199) showed a statistically significant correlation in LOH between saliva and tumor ($P = 0.038$), as did many sets of three markers. The majority of these sets of three markers showed high agreement between saliva and tumor (LOH in both saliva and tumor or lack of LOH in both saliva and tumor). However, there was no significant correlation between LOH in saliva and in tumor for individual markers. In three instances, reciprocal LOH was noted between saliva and matching tumor specimens (Figure 2A). In these instances, the finding was considered discordant. No concomitant instability was found in any saliva and tumor samples manifesting this feature.

Cytologic Atypia and LOH in Saliva

No malignant cells were identified in any of the saliva samples by cytologic examination of giemsa-stained slide preparation. Twenty (54%) of the 37 saliva specimens had cytologic atypia. Nine (45%) of the 20 cases with cytologic atypia had no alterations, and 11 of the remaining (55%) had LOH; 7 (41%) of the 17 samples with cytologically normal epithelial cells had LOH in at least one marker ($P = 0.79$). Although no correlation between LOH at single marker and atypia was found, two markers showed the highest association with cytological atypia (D9S156 and D9S168, $P = 0.132$ and $P = 0.217$, respectively). Combining either of these markers with D3S1261 showed statistical correlation between LOH and atypia ($P = 0.02$) with 60% and 100% specificity ($P = 0.020$). The inclusion of a third marker did not improve the correlation.

LOH and Clinicopathological Factors

A correlation between certain marker combinations and smoking history at four markers alone or in various combinations (D3S1293, CHRNB1, D8S298, and D9S104) showed correlation with this feature. This and other quadruple combinations of markers identified LOH in 15 of the 18 smokers ($P = 0.0001$). Interestingly, patients who quit smoking showed an LOH frequency that was between that of smokers and nonsmokers: five of the 10 patients who quit smoking had LOH in at least one of these markers in their tumors. Multiple markers in tumors correlated with stage (e.g., D3S1261, D8S283, D9S156, D9S168, and TP53); the combination with the highest correlation was D3S1261, D9S156, and TP53 ($P = 0.002$). Similarly, the combination D18S363 and D8S283

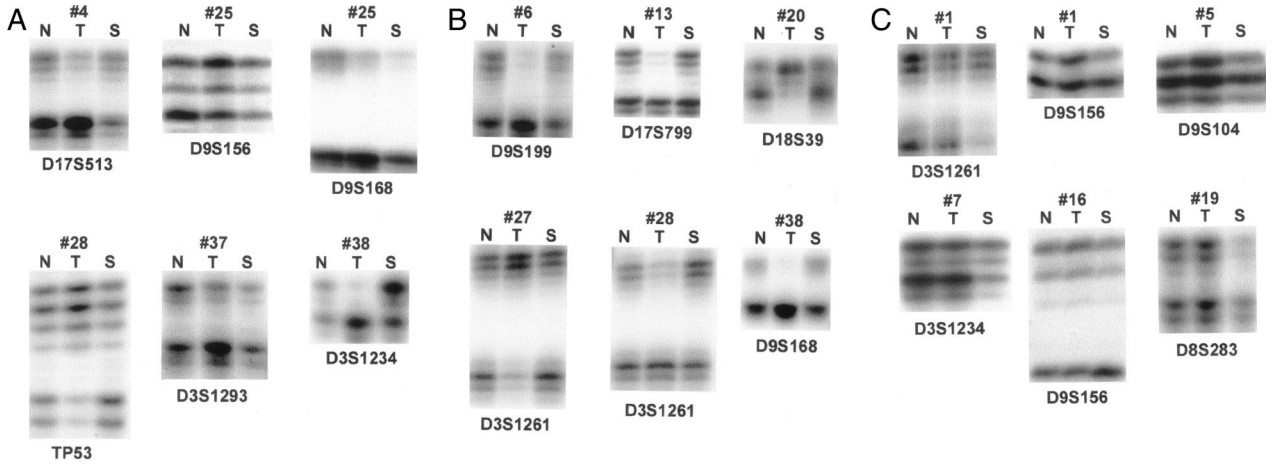

Figure 2. Representative illustrations of LOH in specimens. **A:** LOH in both tumor and saliva; **B:** LOH in tumor alone; **C:** LOH in saliva alone. N, normal; T, tumor; S, saliva.

was significantly correlated with the DNA index (P = 0.002). The only factor significantly correlated with LOH at marker D3S1248 in saliva was the DNA index (P = 0.014).

Saliva from Smoking and Nonsmoking Normal Volunteers

All of the 10 normal saliva samples were informative for at least one of the markers used. None of the DNA extracted from the 10 saliva specimens from normal nonsmoking and smoking individuals manifested any LOH at the markers analyzed. Only one saliva specimen from a normal smoker showed instability at one marker on the short arm of chromosome 3.

Discussion

Molecular genetic studies of HNSC have demonstrated frequent genetic alterations at certain chromosomal regions in premalignant lesions and invasive tumors.[1–9] The studies also showed that certain regions on chromosomes 3p, 9p, 8p, and 17p are frequently altered in dysplastic lesions[1,6–8] and may constitute an early event in lesion development. Analysis of these markers in oral secretions and other accessible specimens may allow for rapid, inexpensive, and objective assessment of the genetic abnormalities at these sites for early detection, screening of individuals at high risk, and follow-up of patients with cancer.[2,3,26]

In this study, approximately 50% of the saliva samples and 86% of the tumor specimens manifested microsatellite LOH. The incidence of LOH in saliva, however, could have been higher had a method to enrich the epithelial cells in specimens been used.[27] The results, however, support those of previous studies and further underscore the early association of these markers with HNSC tumorigenesis.[1,2,6,7] The genetic heterogeneity between saliva negative for malignant cells and corresponding tumor specimens in certain cases, highlight the presence of genetic alterations in the squamous epithelial cells lining the oral cavity exclusive of the cancer site.[28,29,30–34]

We, however, found no LOH in any of the histologically normal squamous mucosas used as control and we attribute this to the relatively small contribution of the squamous epithelium relative to the subepithelial elements in the tissues used. Microdissection of epithelial cells may have led to the identification of LOH in these histologically nondysplastic squamous mucosa as reported in at high risk patients[7,34] studied by our group. Nonetheless, the finding of LOH in saliva samples lacking malignant cells on cytologic evaluation, lends further credence to the field cancerization hypothesis and the increased risk of a second primary cancer developing in these patients[7,26,35,36,37]. Although none of the patients in this study manifested evidence of recurrence or secondary tumors thus far, a longer follow-up period is required to substantiate this notion. Other studies, however, have shown a high concordance in matched secretion and tumor specimens, indicating common clonal derivation[16,17,20,23,24,38,39]. In these studies, however, malignant cells were identified and analyzed in the cellular sources used.

In our study, although no cytological evidence of malignancy in any of the saliva samples was identified, cytologic atypia was found in more than 50% of the specimens. This feature correlated significantly with LOH at two markers on the short arm of chromosomes 3 and 9. Our findings, along with those from previous studies of head and neck, lung, and bladder tumors indicate that combining molecular and cytologic analyses in secretions may provide additional information for better identification of high risk patients[5,7–9,20,39,40]. Our results also show that alterations at microsatellite markers in tumors correlated significantly with aggressive clinicopathologic factors in these patients. A similar correlation has been reported in other studies, indicating that alterations in certain chromosomal loci are associated with the pathobiologic characteristics of these tumors and may be used for the biological assessment of these tumors.[10,11] Of particular interest in our study is the finding of a signifi-

cant correlation between LOH at certain chromosomal regions in tumor and smoking and alcohol consumption. Additional investigations of these regions may lead to identifying the markers or genes that are associated with carcinogenesis. Previous studies have also reported an association between LOH in these same chromosomal loci and tumors in the upper respiratory tract.[41,42]

In conclusion, the results of our study indicate that, in patients with HNSC, saliva is a readily available source of DNA for genetic analysis.[42,43] Enriching for epithelial cells may increase the correlation obtained between saliva and tumor specimens. Alternatively, cells obtained by buccal brushing of multiple sites in the oral cavity may be used as a noninvasive substitute for molecular analysis.[26,44] Our results lend molecular evidence for field cancerization[45,46] and the use of selective genetic markers in early detection of HNSC in people at high risk.[24,43,47]

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