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Alterations of $p16^{INK4a}$ and $p14^{ARF}$ in Patients with Severe Oral Epithelial Dysplasia[1]

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ABSTRACT

A number of genetic aberrations have been reported in end-stage squamous cell carcinoma of the head and neck, including $p16^{INK4a}$ and $p14^{ARF}$ (*INK4a/ARF*) inactivation rates of 70–85%. Still, the cell cycle-regulatory genes $p16^{INK4a}$ and $p14^{ARF}$ remain poorly understood in oral cavity premalignant lesions. This study evaluated *INK4a/ARF* locus alterations in 26 patients (28 samples) deemed to be at increased risk for malignant transformation to squamous cell carcinoma due to the diagnosis of severe oral epithelial dysplasia. Microscopically confirmed dysplastic oral epithelium and matching normal tissue were laser capture-microdissected from paraffin sections, DNA was isolated, and molecular techniques were used to evaluate $p16^{INK4a}$ and $p14^{ARF}$ gene deletion, mutation, loss of heterozygosity (LOH), and hypermethylation events. Deletion of exon 1β, 1α, or 2 was detected in 3.8%, 11.5%, and 7.7% of patients, respectively. *INK4a* and *ARF* mutations were detected in 15.4% and 11.5% of patients with severe dysplasia of the oral epithelium. All identified mutations occurred in the *INK4a/ARF* conserved exon 2. Allelic imbalance was assessed using three markers previously reported to show high LOH rates in head and neck tumors. LOH was found in 42.1%, 35.0%, and 82.4% of patients for the markers *IFNα*, *D9S1748*, and *D9S171*, respectively. Hypermethylation of $p16^{INK4a}$ and $p14^{ARF}$ was detected in 57.7% and 3.8% of patients, respectively, using nested, two-stage methylation-specific PCR. The highest rates of $p16^{INK4a}$ hypermethylation occurred in lesions of the tongue and floor of the mouth. In addition, $p16^{INK4a}$ hypermethylation was significantly linked to LOH in two or more markers. These data support that *INK4a/ARF* locus alterations are frequent events preceding the development of oral cancer and that $p16^{INK4a}$ inactivation occurs to a greater extent in oral dysplasia than does $p14^{ARF}$ inactivation.

INTRODUCTION

Molecular alterations in a number of cell cycle-regulatory genes have been identified in end-stage SCC[3] of the head and neck, including *INK4a/ARF* inactivation rates of 70–85% (1–4). The *INK4a/ARF* locus is located on chromosome 9p21 and has the unique distinction of encoding two cell cycle-regulatory genes, $p16^{INK4a}$ and $p14^{ARF}$ (reviewed in Refs. 5–8). Briefly, alternative splicing of the first exon and common downstream exons permits one gene to encode two different products, which function via two distinct pathways to inhibit cell cycle progression. The tumor-suppressive activity of $p16^{INK4a}$ is ascribed to its ability to bind both cdk4 and cdk6. This in turn inhibits the catalytic activity of the cdk4/6-cyclin D complex, blocks retinoblastoma phosphorylation, and ultimately prevents cell cycle progression (5). In contrast, $p14^{ARF}$ interacts with the oncogenic protein MDM2, inducing stabilization of p53 and enhancing p53-related functions (9). Codeletion of $p16^{INK4a}$ and $p14^{ARF}$ has been described in a number of tumor types, including those of the head and neck (6). Thus, a single alteration in the *INK4a/ARF* locus can potentially disrupt the *p16-Rb* and *p14-p53* tumor suppressor pathways and facilitate cancer development. Moreover, a recent study by Bates *et al.* (10) reported the existence of cross-talk between the pathways, illustrating the complicated nature of these two gene products.

Numerous studies recognize the prominent tumor suppressor function of $p16^{INK4a}$; however, *INK4a/ARF* locus alterations in premalignant oral disease remain incompletely investigated and poorly understood. In this study, we sought to comprehensively determine the mode and incidence of $p16^{INK4a}$ and $p14^{ARF}$ alterations in patients diagnosed with severe oral epithelial dysplasia and to assess relationships between *INK4a/ARF* alterations and various patient characteristics. The risk of malignant transformation among patients with severe oral epithelial dysplasia has been reported to be 36% over a period 8 years (11). Genetic alterations in the 9p21 chromosomal region have been linked to malignant progression, and there is limited evidence suggesting a role for $p16^{INK4a}$ in cancer recurrence (4, 12). The main modes of $p16^{INK4a}$ inactivation in SCC of the head and neck are known to include homozygous deletions, mutations, and gene hypermethylation events (5). A limited number of studies have assessed $p16^{INK4a}$ inactivation in premalignant lesions of the head and neck and support a role for it in the development of precancerous lesions (13–15). However, in the populations previously assessed for $p16^{INK4a}$ inactivation, the pathologic diagnoses often varied, and not all modes of inactivation were evaluated within a single population. Thus, the current study was undertaken to characterize inactivation of $p16^{INK4a}$ and the lesser understood $p14^{ARF}$ in patients diagnosed with severely dysplastic oral lesions. Dysplastic areas were laser capture-microdissected in an attempt to obtain a pure population of cells for assessment of deletion, mutation, LOH, and hypermethylation events in a high-risk population with morphologically similar lesions. The fact that the lesions are all histologically severe dysplasia controls for pathological grade as a potential confounder and may foster improved understanding of a molecular profile predictive of risk for progression or cancer recurrence in years to come.

MATERIALS AND METHODS

Patient Specimens. Twenty-eight biopsies from 26 consecutive patients with histologically confirmed severe oral epithelial dysplasia were obtained from the archives of the College of Dentistry, Department of Oral Surgery and Pathology at The Ohio State University from 1997 to 2000. One patient provided three sequential biopsies over the course of 3 years. Each case was reviewed by two board-certified oral pathologists (one of whom was S. R. M.). All biopsies were fixed in neutral buffered formalin, routinely processed, and embedded in paraffin. Severe epithelial dysplasia was defined as full thickness epithelial changes without invasion of the basement membrane.

Microdissection and DNA Isolation. Up to six (8-μm) sections of each case were cut, mounted on noncharged glass slides, dewaxed in xylene, rehydrated in graded alcohols, and briefly stained with H&E (15 s each).

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[3] The abbreviations used are: SCC, squamous cell carcinoma; LOH, loss of heterozygosity; CIS, carcinoma *in situ*; MSP, methylation-specific PCR; *INK4a/ARF*, $p16^{INK4a}$ and $p14^{ARF}$; SSCP, single-stranded conformational polymorphism; cdk, cyclin-dependent kinase.

Severely dysplastic oral epithelium was identified via bright-field microscopy and microdissected using the PixCell-II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA). On average, 2200 laser hits were fired using a 15-μm spot size to obtain sufficient material. Histologically normal connective tissue was microdissected from each case to serve as a control for molecular analysis. Captured cells were digested overnight at 55°C in proteinase K lysis buffer [0.5 mg/ml proteinase K, 50 mM Tris, 1 mM EDTA, and 0.5% Tween 20 (pH 8.5)]. Samples were heated for 10 min at 95°C to inactivate proteinase K and centrifuged, and the supernatant was used for PCR.

PCR Amplification and Analysis. Exons 1α, 1β, and 2 of the *INK4a/ARF* locus were amplified as described previously (16). Briefly, intron-based primers were used for the amplification of exons 1α and 1β. Exon 2 was amplified in two fragments to meet the size limitation associated with SSCP analysis (300 bp). In addition, one primer in each set for exon 2 amplification was intron-based to exclude potential coamplification of a similar gene family member or pseudogenes. Exon 3 was not analyzed because it contains only a small portion of the coding sequence for $p16^{INK4a}$ and none of the coding sequence for $p14^{ARF}$. The 20-μl PCR mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphate, 0.5 μM of each primer, 1× Invitrogen enhancer, 1 unit of Taq polymerase, and 3 μl of genomic DNA. Amplification was performed in a MJ Research Peltier Thermal Cycler (Waterton, ME) with PCR conditions of 95°C for 2 min followed by 40 cycles of 95°C for 50 s, 60°C for 20 s, 72°C for 50 s, and a final elongation step of 72°C for 7 min.

Mutation Analysis of $p16^{INK4a}$ and $p14^{ARF}$. *CDKN2A* alterations were detected by a nonradioactive SSCP technique and DNA sequencing as described previously (16). Positive SSCP results were confirmed by analysis of the corresponding replicate PCR sample, isolation of the mutant band, reamplification of the shifted band, and reexamination by SSCP and DNA sequencing. Experimental times and primer information were as reported previously by our laboratory (16).

Detection of Homozygous Deletions. The $p16^{INK4a}$ and $p14^{ARF}$ genes were processed as described under PCR amplification and analysis, except that multiplex PCR was performed using *HPRT* as an internal control gene. *HPRT* was coamplified with the specific exons of interest and electrophoresed through a 20% polyacrylamide Tris-borate EDTA minigel (Invitrogen, San Diego, CA) for deletion assessment.

MSP. Aberrant methylation of the *INK4a/ARF* locus was determined by a nested, two-stage MSP method developed by Palmisano *et al.* (17). Procedural modifications included direct utilization of 1 μl of stage 1 PCR products before stage 2 PCR and changes in PCR conditions permitting optimal amplification with newly designed primers. Briefly, DNAs were bisulfate-modified using the CpGenome DNA modification kit (Intergen, Purchase, NY), and PCR was conducted to amplify a 208- and 259-bp fragment of the $p16^{INK4a}$ and $p14^{ARF}$ genes, respectively. Stage 2 MSP using unmethylated and methylation-specific primers required 1 μl of stage 1 product for the second PCR. The primer sequences used to amplify $p16^{INK4a}$ were 5′-GGAGAGGGGGAGAGTAGGT-3′ (outside forward), 5′-CTACAAACCCTCTACCCACCT-3′ (outside reverse), 5′-TGGGGAGTAGTATGGAGTTGGTGGT-3′ (unmethylated forward), 5′-CAACCCCAAACCACAACCATAA-3′ (unmethylated reverse), 5′-CGGGGAGTAGTATGGAGTCGGCGGC-3′ (methylated forward), and 5′-GACCCCGAACCGCGACCGTAA-3′ (methylated reverse). Four previously described sequences were used to evaluate *p14* methylation status (9), as were two newly designed methylation-specific forward primers. The outside $p14^{ARF}$ primers were 5′-TGGGTTTTAGTTTGTAGTTAA-3′ (forward) and 5′-CTCCTCAATAACATCAACAC-3′ (reverse). Methylation-specific $p14^{ARF}$ primers were 5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′ (unmethylated forward), 5′-ACCACACACACACCAAATCCA-3′ (unmethylated reverse), 5′-GTGTTAAAGGGCGGCGTAGC-3′ (methylated forward), and 5′-CGCGACGAACCGCACGCGCG-3′ (methylated reverse). Amplification conditions for the first PCR included 95°C for 2 min, denaturing at 95°C for 50 s, annealing at 60°C (*p16*) and 54°C (*p14)* for 50 s, extension at 72°C for 50 s for 40 cycles, and a final 10-min extension step. Conditions for the second PCR using methylation-specific primers were the same, except that the annealing temperatures were 64°C and 62°C, respectively, for $p16^{INK4a}$ and $p14^{ARF}$, and the number of cycles was lowered to 25. Placental DNA and methylation-positive cell lines (HS4, *p16*; HeLa, *p14*) served as negative and positive controls.

Allelic Imbalance. Genomic DNA from premalignant and patient-matched normal control tissues was amplified using fluorescent PCR methods and the microsatellite markers *D9S1748*, *D9S171*, and the *IFN*α cluster (Research Genetics, Huntsville, AL). The 5′ forward amplification primer of each of the PCR primer pairs was synthesized with fluorescent labels. Amplification was conducted as described above, except that the final PCR volume was 25 μl. Individual amplification reactions were pooled per patient for both normal and premalignant tissues. Next, 3 μl of the respective pooled panel were added to 3 μl of loading buffer and the TAMRA-500 molecular weight marker. Reactions were denatured and placed on ice, and 1.5 μl of each panel were loaded onto a 4.25% denaturing polyacrylamide Tris-borate EDTA gel. The data were collected on an ABI 377 automated sequencer, and GeneScan and Genotyper software was used to quantify normal *versus* premalignant amplicon patterns for each fluorescent marker. LOH markers were chosen based on their location on 9p and the fact that they previously showed high levels of LOH in head and neck tumors. Allele loss was calculated by the method of Canzian *et al.* (18), which determines allelic imbalance by calculating the ratio of the two allele pairs in normal tissue to that of the premalignant tissue, with a shift of 40% or more considered LOH positive.

Statistical Analysis. Associations between patient characteristics (nominal and ordinal scale) and gene inactivation events were analyzed using χ^2 contingency tables or Fisher's exact test. Relationships between patient age and gene inactivation were assessed using the unpaired *t* test. All statistical tests were two-sided, with $P < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Patient Characteristics. A total of 28 biopsies showing severe epithelial dysplasia, from 26 patients, were evaluated for alterations in the *INK4a/ARF* locus. Age at biopsy ranged from 26 to 87 years, with a mean patient age of 65 years. The study population was comprised of 15 male and 11 female patients. The most common sites of dysplasia in this population were the tongue (32%), floor of the mouth (25%), and buccal mucosa (25%). In this study, no significant differences emerged with regard to gender and site of dysplasia. However, among female participants, dysplasia most commonly occurred on the tongue (54%), followed by the floor of the mouth (18.2%). Similarly, recent data from the National Cancer Institute Surveillance, Epidemiology, and End Results database reported the most common site for head and neck cancer among females to be the tongue, followed by the floor of the mouth (19, 20). In contrast, male patients most commonly presented with dysplasia on the floor of the mouth (33%), followed by the buccal mucosa (26.7%) and tongue (20%). According to Surveillance, Epidemiology, and End Results data, the greatest number of head and neck cancers among males occurred on the tongue, followed by the lip and floor of the mouth (20).

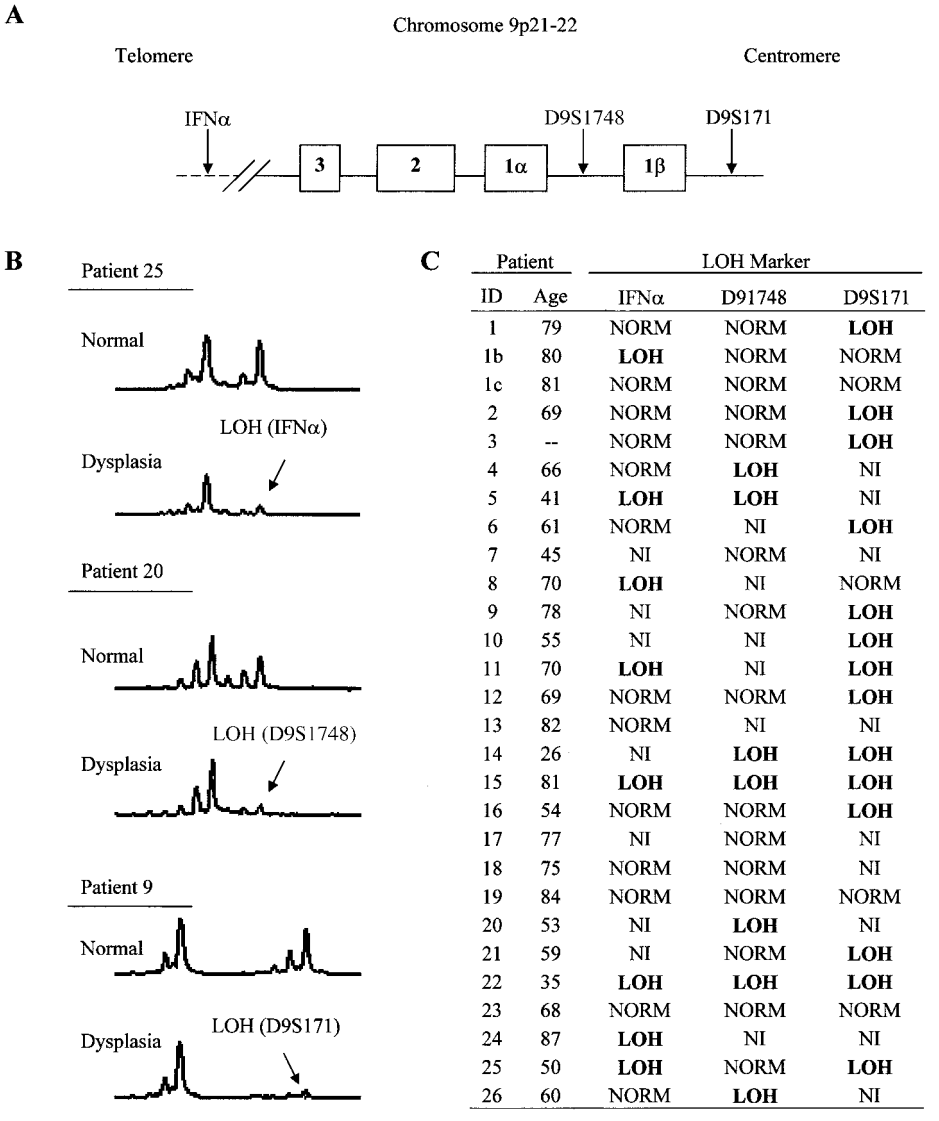
Twenty-five percent of patients exhibited dysplasia with oral leukoplakia, and one patient exhibited dysplastic erythroplakia. Leukoplakia exhibiting dysplastic changes has previously been reported to impart increased risk for malignant transformation to SCC (12, 21). A study by Silverman *et al.* (11) found 36% of dysplastic leukoplakias transformed to carcinoma over a period of 8 years. In our study, the clinical diagnosis of leukoplakia was not significantly linked to gender, lesional site, or inactivation of $p16^{INK4a}$ or $p14^{ARF}$; however, we urge caution because the number of patients with leukoplakia is small ($n = 7$), reducing the statistical power to detect meaningful differences. Pathology reports indicated that CIS or superficially invasive SCC could not be ruled out in >50% of patients under study. This ruling did not correlate with any patient characteristics or $p16^{INK4a}$ or $p14^{ARF}$ gene inactivation events. Thus, the fact that CIS or superficially invasive SCC could not be ruled out appeared to be more a function of biopsy sampling and biopsy size than linkage to individual tissue morphology.

Allelic Imbalance on Chromosome Arm 9p. LOH on the short arm of chromosome 9p is the most common defect reported in SCC of

the head and neck, ranging from 45% to 95% (22–24). A number of studies have also found high frequencies of LOH in oral premalignant lesions and found a positive correlation between LOH and histological progression, cancer development, and cancer recurrence (11, 14, 24, 25). In the current study, LOH was the most frequent molecular alteration detected. Allelic loss at chromosome 9p21 was assessed using three markers reported to be highly polymorphic in head and neck tumors or oral premalignant lesions (14, 15, 26, 27). The markers were also chosen based on their relative location on chromosome 9p. Details of LOH are presented in Fig. 1. Allelic loss at 9p21 was detected in at least one microsatellite marker in 76.9% of patients. As summarized in Fig. 1*C*, LOH occurred in 42.1%, 35.0%, and 82.4% of cases that were informative for the markers *IFNα*, *D9S1748*, and *D9S171*, respectively. Approximately, 58% of cases presented with LOH in a single marker, 15.4% of cases presented with LOH in two markers, and 7.7% of cases presented with LOH in all three markers.

In our analysis of LOH and patient characteristics, the mean age of patients with LOH in one or fewer markers was statistically higher (69.2 years) than the age of patients (53.1 years) with LOH in two or three markers ($P = 0.020$). Furthermore, LOH in two or more markers was significantly associated with $p16^{INK4a}$ hypermethylation ($P = 0.007$). All cases with LOH in two or three markers were hypermethylated for $p16^{INK4a}$. An earlier study assessing prevalence of $p16^{INK4a}$ alterations in head and neck tumors reported that 65% of the cases with biallelic gene inactivation showed LOH and hypermethylation (25). In this study population, we found no microsatellite instability in any of the three markers evaluated. This contrasts with a recent study by Shahnavaz *et al.* (15) that found higher rates of microsatellite instability than LOH in oral precancerous lesions and subsequent SCC that developed in 11 patients. Our study findings may differ with respect to microsatellite instability for a number of reasons, including the fact that different microsatellite markers were assessed. The patient populations were also from different geographic areas and may have had other disparate characteristics impacting chromosomal defects. In addition, there were differences in the histological grade of the premalignant lesions in the two patient populations. All 26 patients in our study were diagnosed with severe dysplasia, whereas in the study by Shanavaz *et al.* (15), only 2 patients had severe dysplasia, and the others presented with mild or moderate dysplasia. Other groups have reported similar LOH rates in oral dysplasias or CIS in this chromosomal region (28).

In this study cohort, LOH in two or more markers was significantly linked to lesions on the floor of the mouth ($P = 0.023$). Although tobacco exposure information was not available for this patient cohort,



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Patient		LOH Marker		
ID	Age	IFNα	D91748	D9S171
1	79	NORM	NORM	**LOH**
1b	80	**LOH**	NORM	NORM
1c	81	NORM	NORM	NORM
2	69	NORM	NORM	**LOH**
3	--	NORM	NORM	**LOH**
4	66	NORM	**LOH**	NI
5	41	**LOH**	**LOH**	NI
6	61	NORM	NI	**LOH**
7	45	NI	NORM	NI
8	70	**LOH**	NI	NORM
9	78	NI	NORM	**LOH**
10	55	NI	NI	**LOH**
11	70	**LOH**	NI	**LOH**
12	69	NORM	NORM	**LOH**
13	82	NORM	NI	NI
14	26	NI	**LOH**	**LOH**
15	81	**LOH**	**LOH**	**LOH**
16	54	NORM	NORM	**LOH**
17	77	NI	NORM	NI
18	75	NORM	NORM	NI
19	84	NORM	NORM	NORM
20	53	NI	**LOH**	NI
21	59	NI	NORM	**LOH**
22	35	**LOH**	**LOH**	**LOH**
23	68	NORM	NORM	NORM
24	87	**LOH**	NI	NI
25	50	**LOH**	NORM	**LOH**
26	60	NORM	**LOH**	NI
Mean		42.1%	35.0%	82.4%

Fig. 1. LOH analysis in severe oral epithelial dysplasias. *A*, allelogram depicting the three exons of $p16^{INK4a}$ (exons 3, 2, and 1α) and $p14^{ARF}$ (shared exons 3 and 2, with alternatively spliced exon 1β) and the relative positions of the microsatellite markers *IFNα*, *D9S1748*, and *DS171*. *B*, traces of allelic loss at chromosome 9p21–22 in patients 25, 20, and 9, respectively. The normal tissue results are shown in the *top trace*, and patient-matched dysplastic tissue is depicted in the *bottom trace* for each patient and marker. The *top traces* (normal tissue) show amplification of two alleles, whereas the *bottom traces* of patient-matched dysplastic tissue show allelic loss (as indicated by the *arrow*). *C*, summary of LOH results by patient. Patient age at the time of biopsy is given in years. The mean LOH levels reported reflect the percentage of LOH-positive patients out of the total number of patients informative for each marker. *NORM*, normal or retention of both alleles; *NI*, noninformative for the marker. Over 75% percent of patients with severe dysplasia showed LOH in at least one marker, with the highest LOH reported for the centromeric marker *D9S171*.

Table 1 *Summary of INK4α/ARF locus alterations in patients with severe dysplasia of the oral cavity*

Patient no.	Gender	Lesion site[a]	LOH[b]	Mutation[c]		Deletion			Methylation[d]	
				p16	*p14*	2	1α	1β	*p16*	*p14*
1[e]	Male	Buccal mucosa	1	+	+	−	−	−	−	−
1b[e]	Male	Buccal mucosa	1	−	−	−	−	−	−	−
1c[e]	Male	Buccal mucosa	0	−	−	−	−	−	+	−
2	Male	Floor of mouth	1	+	+	−	−	−	+	−
3	Female	Palate	1	+	−	−	+	−	−	−
4	Male	Palate[a]	1	−	−	+	+	−	−	−
5	Male	Floor of mouth[a]	2	−	−	+	+	−	+	−
6	Female	Tongue	1	−	−	−	−	−	+	−
7	Male	Buccal mucosa	0	−	−	−	−	−	−	−
8	Male	Lip	1	−	−	−	−	−	−	−
9	Male	Palate[a]	1	−	−	−	−	−	−	−
10	Male	Buccal mucosa	1	−	−	−	−	−	−	+
11	Male	Floor of mouth	2	−	−	−	−	+	+	−
12	Male	Floor of mouth	2	−	−	−	−	−	+	−
13	Female	Lip	0	−	−	−	−	−	−	−
14	Male	Tongue	2	−	−	−	−	−	+	−
15	Female	Floor of mouth	3	−	−	−	−	−	+	−
16	Female	Tongue	1	−	−	−	−	−	+	−
17	Female	Tongue[a]	0	−	−	−	−	−	+	−
18	Female	Tongue	0	+	+	−	−	−	+	−
19	Male	Buccal mucosa	0	−	−	−	−	−	−	−
20	Female	Tongue[a]	1	−	−	−	−	−	−	−
21	Male	Tongue[a]	1	−	−	−	−	−	+	−
22	Male	Tongue	3	−	−	−	−	−	+	−
23	Female	Tongue	0	−	−	−	−	−	+	−
24	Female	Buccal mucosa	1	−	−	−	−	−	−	−
25	Female	Floor of mouth	2	−	−	−	−	−	+	−
26	Male	Floor of mouth[a]	1	−	−	−	−	−	−	−
Percentage[f]			76.9	15.4	11.5	7.7	11.5	3.8	57.7	3.8

[a] Dysplastic lesions with leukoplakia.
[b] LOH indicates the number of positive LOH markers detected per patient sample analyzed.
[c] All mutations detected were in exon 2.
[d] Hypermethylation of $p16^{INK4a}$ correlates with lesions present on the tongue and floor of the mouth and is significantly linked to the presence of LOH in two or more markers ($P \leq 0.01$), χ^2 test.
[e] Tissue was obtained from the same patient over 3 consecutive years.
[f] Percentage refers to the percentage of patients whose tissue contained the genetic aberration.

recent studies have emerged linking LOH to tobacco exposure among patients with upper aerodigestive tract cancers (29–32). Furthermore, studies support site specificity of head and neck SCC in both smokers and nonsmokers (27, 29, 30). Smoking-associated head and neck SCCs develop on the floor of the mouth; whereas nonsmokers present with oral cancers of the tongue, followed by the buccal and alveolar mucosa (30). These relationships merit further study in a larger cohort in which tobacco and alcohol exposure information is available.

***p16*^{INK4a} and *p14*^{ARF} Methylation Status.** The frequency of aberrant methylation for the genes $p16^{INK4a}$ and $p14^{ARF}$ was determined for all 26 patients and 28 dysplastic biopsy specimens. The results are summarized in Table 1, and a representative gel is pictured in Fig. 2. The detection of both methylated and unmethylated sequences in same lesion may be due to the fact that the population of cells analyzed, although histologically similar, remains genetically diverse and/or may indicate that methylation is not an all or nothing event in oral precancer. A total of 57.7% of patients (15 of 26) had methylation for the $p16^{INK4a}$ gene, whereas only a single patient (3.8%) was positive for $p14^{ARF}$ methylation. Similar rates of $p16^{INK4a}$ hypermethylation have been detected in SCCs of the head and neck (33, 34), and even higher levels of $p16^{INK4a}$ methylation (82%) were recently detected in severely dysplastic oral lesions (24). We attribute high levels of $p16^{INK4a}$ methylation detection to the fact that a sensitive two-stage nested MSP approach was used coupled with laser capture microdissection, permitting assessment of a rather pure population of severely dysplastic cells.

In addition to the association between $p16^{INK4a}$ methylation and increased LOH, methylation was significantly associated with the site of dysplasia ($P = 0.001$). In terms of the $p16^{INK4a}$-methylated samples, 53% were biopsies from the tongue, 40% were biopsies from the floor of the mouth, and 6.7% were biopsies from the buccal mucosa; there were no $p16^{INK4a}$-methylated samples from the palate or lip. Alternatively, 88% (8 of 9) of tongue lesions and 85% (6 of 7) of biopsies from the floor of the mouth were positive for $p16^{INK4a}$ methylation. In contrast, only 15% of buccal mucosa biopsies were $p16^{INK4a}$ methylation positive, and none of the biopsies from the palate or lip were hypermethylated. The floor of the mouth and ventrolateral tongue are considered sites with increased risk for malignant progression (31). A recent report by Koch *et al.* (30) found that floor of the mouth cancers rarely develop in nonsmokers. In addition, Soria *et al.* (34) recently evaluated aberrant methylation in bronchial brush samples from former smokers and found significantly higher $p16^{INK4a}$ methylation in former smokers with a history of previous cancer compared with former smokers without a history of cancer. Unfortunately, as mentioned previously, tobacco and alcohol exposure information was not available in this patient population. Thus, it is possible that the linkage between the site of dysplasia and methylation status is due in part to exposures for which we cannot account. Methylation did not appear to be statistically linked to any other clinicopathological features evaluated. Aging has been one feature previously reported to be linked to methylation events; however, in the population under study, we found no significant difference in mean age between $p16^{INK4a}$ methylation-positive and -negative patients. To the contrary, methylation-positive patients were on average 8.8 years younger than methylation-negative patients (61.1 years *versus* 69.9 years, respectively). To our knowledge, no other study has evaluated $p14^{ARF}$ methylation in oral premalignant lesions. Only one patient was positive for $p14^{ARF}$ methylation, supporting that $p16^{INK4a}$ methylation is more prevalent in patients with high-grade oral dysplasia and $p16^{INK4a}$ hypermethylation may be of greater importance in oral cancer development. Gene hypermethylation reportedly leads to chromosomal instability, which in turn supports further gene-inactivating events via LOH, mutation, or deletion and ultimately contributes to the transcriptional silencing of tumor suppressor genes. We did

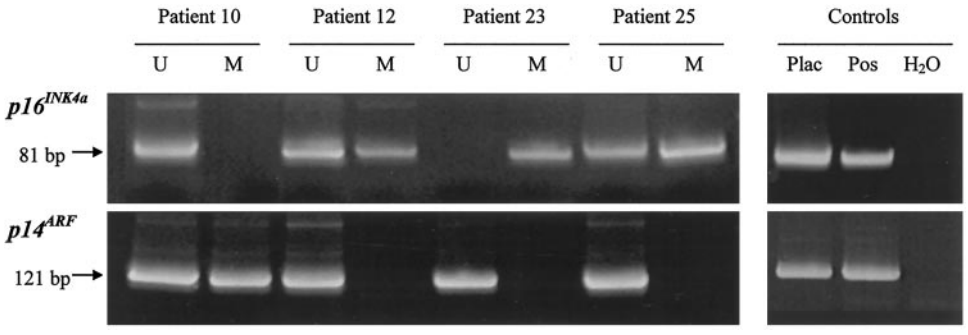

Fig. 2. Representative examples of MSP for $p16^{INK4a}$ and $p14^{ARF}$. The presence of a visible PCR product in *Lanes U* indicates the presence of unmethylated genes, and the presence of a visible PCR product in *Lanes M* indicates the presence of methylated genes. The results from four cases are shown for each gene. Placenta (*Plac*) served as an unmethylated control, and the HS4 and HeLa cell lines served as methylation-positive (*Pos*) controls for $p16^{INK4a}$ and $p14^{ARF}$, respectively. H_2O served as a negative control for the overall reaction.

not detect any significant correlation between *p16* or *p14* hypermethylation and *INK4a/ARF* mutation or deletion events; however, greater frequencies of mutation, deletion, and *p14* hypermethylation events would likely be required to accurately access such relationships, given the cohort size.

***p16*^{INK4a} and *p14*^{ARF} Homozygous Deletion.** A summary of *INK4a/ARF* inactivation events in patients with high-grade oral cavity dysplasia is presented in Table 1. Homozygous deletion of exon 1α was the most prevalent and was found in 11.5% (3 of 26) of patients. Our findings are similar in terms of deletion prevalence to those of another study reporting selective loss of exon 1α in 12% of patients with mild dysplasia of the oral epithelium (10). Exon 2 deletions were detected in two patients (7.7%), both of whom had a deletion of exon 1α. Concomitant loss of exons 1α and 2 has previously been reported in patients with oral SCC (35). Perhaps there are unique subpopulations that differ in their deletion profiles, or exon 2 deletions may be relatively late events, following other modes of inactivation. Only one patient (patient 11) was found to have an exon 1β deletion, and this same patient had LOH in the centromeric marker D9S171 as well as in the telomeric marker IFNα.

***p16*^{INK4a} and *p14*^{ARF} Mutation Analysis.** Fig. 3*A* summarizes the *INK4a/ARF* mutations detected in severely dysplastic lesions of the oral cavity and predicts resultant alterations of *p16*^{INK4a} and *p14*^{ARF} transcripts and proteins. SSCP analysis of the *INK4a/ARF* locus revealed abnormal band shifts in approximately 15% (4 of 26) of patients with high-grade dysplasia. This appears to be the first assessment of *INK4a/ARF* mutations in histologically severe oral epithelial dysplasias. A representative shift and subsequent sequence analysis are pictured in Fig. 3, *B* and *C*. These novel findings indicate that mutational events occur at about the same incidence as deletional events in oral premalignant lesions but that the exon targeted for mutational events may differ. All mutations were single-base changes located in conserved exon 2. As discussed above, exon 1α seemed to be the preferred target for deletion events, followed by exon 2. Earlier work in our laboratory evaluated 100 SCCs of the head and neck for *INK4a/ARF* alterations and found 27% of them to exhibit sequence alterations in this locus. In the head and neck tumors evaluated, 91% of mutations involved exon 2, and 9% of mutations involved exon 1α, whereas no mutations were found in exon 1β (16). In our current analysis of severe dysplasias of the oral cavity, we observed no mutations in exon 1β or 1α, implying that mutations in exon 2 are more prevalent in precancerous lesions as well as in SCCs.

Sequence alterations were identified in four patients, including changes in codons 58, 74, 82, 89, and 148 of *p16*^{INK4a} and codons 72, 89, 96, and 103 of *p14*^{ARF}. In terms of sequence changes affecting *p16*^{INK4a}, three were missense mutations, one was a silent mutation, and one was a polymorphism in codon 148, which has been previously reported in SCCs of the head and neck (8, 16, 36). In addition to earlier reports of a G→A transition at codon 148, a C→T transition in codon 58 has been identified as a frequent mutation resulting in a *p16* truncation (reviewed in Ref. 8). However, we found a G→A transition in codon 58, which results in a *p14*^{ARF} truncation. One patient (patient 1) exhibited changes in two different codons (codons 82 and 89), whereas all other mutations involved a single codon. We are unaware of other reports of mutations in codon 82. Four of the detected mutations are expected to result in altered p16 and p14 proteins.

To summarize, these data support that *p16*^{INK4a} is frequently inactivated in dysplastic oral epithelial lesions via LOH, hypermethylation, deletion, and mutation events, in descending order. Thus, oral biopsies that morphologically represent premalignant lesions already possess genetic aberrations commonly found in end-stage oral cancer.

A

Patient no.	Site	Nucleotide change in *INK4a*	*p16*^{INK4a} Transcript			*p14*^{ARF} Transcript		
			Codon	Coding Change	Result[a]	Codon	Coding Change	Result[a]
1	Buccal Mucosa	G → C	82	Val → Leu	M	96	Arg → Leu	M
		G → A	89	Gly → Ser	M	103	Gly → Gln	M
2	Floor of Mouth	C → T	74	Asp → Asp	S	89	Arg → Ser	M
3	Palate	G → A	148	Ala → Thr	P	NA		
18	Tongue	G → A	58	Arg → Gln	M	72	Pro → Stop	T

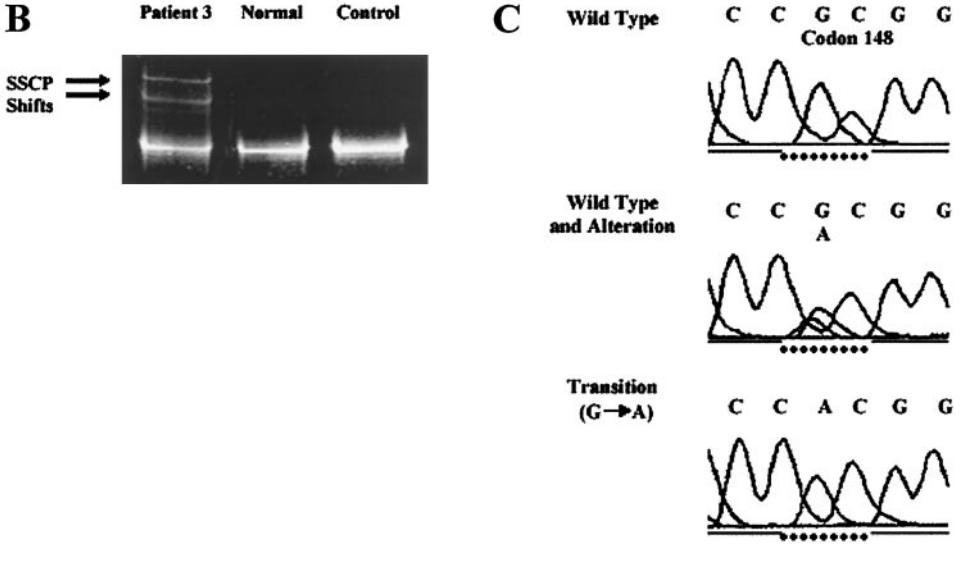

Fig. 3. Mutations of the *INK4a/ARF* locus in dysplastic oral cavity lesions and their effect on *p16*^{INK4a} and *p14*^{ARF} transcripts. *A*, details of *INK4a/ARF* locus alterations by patient and site. *M*, missense; *T*, truncation; *P*, polymorphism; *S*, silent; *NA*, not applicable. *B*, representative example of *p16*^{INK4a} mutation analysis as detected by cold SSCP. Dysplastic epithelium from patient 3 resulted in *p16*^{INK4a} exon 2 shifts, as compared with normal and placental control tissues. *C* shows the sequencing trace indicating a G→A transition in codon 148.

The temporality of *INK4a/ARF* alterations cannot be assessed in this cohort, given that only a single biopsy was available from all but one of the study participants. Interestingly, in the one patient contributing three biopsies over 3 years, divergent *p16* aberrations were found, supporting the observation that even within a specified genetic region, the clonal evolution of premalignant cells is not necessarily linear.

Although the sample size of 26 patients or 28 samples appears relatively small, this is the largest cohort of patients with premalignant lesions of the same histological category to be assessed for $p16^{INK4a}$ and $p14^{ARF}$ alterations. Characterization of *INK4a/ARF* inactivation events in populations at increased risk for malignant transformation may provide insight into which alterations or combinations of inactivation events are most critical for cancer development. To date, only one of the study patients has developed a SCC, but we will continue to follow these patients with the intent of improving our understanding of the role that $p16^{INK4a}$ and $p14^{ARF}$ plays in cancer progression.

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