Frequent Alterations of $p16^{INK4a}$ and $p14^{ARF}$ in Oral Proliferative Verrucous Leukoplakia

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Abstract

Proliferative verrucous leukoplakia (PVL) represents a rare but highly aggressive form of oral leukoplakia with >70% progressing to malignancy. Yet, PVL remains biologically and genetically poorly understood. This study evaluated the cell cycle regulatory genes, $p16^{INK4a}$ and $p14^{ARF}$, for homozygous deletion, loss of heterozygosity, and mutation events in 20 PVL cases. Deletion of exon 1, $1\alpha$, or 2 was detected in 40%, 35%, and 0% of patients, respectively. Deletions of exons $1\alpha$ and $1\beta$ markedly exceeded levels reported in non-PVL dysplasias and approximate or exceed levels reported in oral squamous cell carcinomas. Allelic imbalance was assessed for markers reported to be highly polymorphic in squamous cell carcinomas and in oral dysplasias. Loss of heterozygosity was detected in 35.3%, 26.3%, and 45.5% of PVLs for the markers IFN$\alpha$, D9S1748, and D9S171, respectively. INK4a and ARF sequence alterations were detected in 20% and 10% of PVL lesions, accordingly. These data show, for the first time, that both $p16^{INK4a}$ and $p14^{ARF}$ aberrations are common in oral verrucous leukoplakia; however, the mode and incidence of inactivation events differ considerably from those reported in non-PVL oral premalignancy. Specifically, concomitant loss of $p16^{INK4a}$ and $p14^{ARF}$ occurred in 45% of PVL patients greatly exceeding loss reported in non-PVL dysplastic oral epithelium (15%). In addition, $p14^{ARF}$ exon $1\beta$ deletions were highly elevated in PVLs compared with non-PVL dysplasias. These data illustrate that molecular alterations, even within a specific genetic region, are associated with distinct histologic types of oral premalignancy, which may affect disease progression, treatment strategies, and ultimately patient prognosis. (Cancer Epidemiol Biomarkers Prev 2008;17(11):3179–87)

Introduction

Proliferative verrucous leukoplakia (PVL) is a distinct form of oral leukoplakia characterized by high malignant transformation rates (60-100%), frequent recurrences (87-100%), resistance to standard treatment modalities, and high mortality rates (30-50%; refs. 1-6). Furthermore, early diagnosis of PVL is complicated by the fact that many of the morphologic hallmarks associated with progression to malignancy are absent in these lesions. PVL begins rather deceptively as simple hyperkeratosis without many of the morphologic hallmarks associated with progression to malignancy are absent in these lesions. Over time, these leukoplakias slowly grow and progress into persistent, multifocal, and ultimately irreversible lesions (1). In late PVL, the keratoses can become exophytic and verrucous, with frequent transformation to verrucous or squamous cell carcinoma (SCC; refs. 1-7).

The etiology of PVL remains unclear. The limited body of data on the topic, however, suggests that the pathogenesis of PVL-associated carcinomas is distinctive from non-PVL-associated cancers. Overall, twice as much oral cancer occurs in men compared with women. Yet, two of the largest studies of PVL patients reported a predilection for this lesion in elderly women, with a ratio as high as 4:1 for women to men (1-3). A national survey of head and neck verrucous carcinomas also reported oral verrucous carcinoma to be more common in elderly females compared with males (7). The reasons for the potential gender difference are not clear, but gender- and age-related effects on immune competence have been postulated (8). Further contrast lies in the finding that PVL and its progression to carcinoma are not significantly linked to tobacco use, a known risk factor for SCC development (1-4). Other cofactors postulated in the etiology of PVL include human papillomavirus (HPV) or Candida infection. Silverman et al. reported 68% of PVL patients to be positive for Candida albicans but did not find the fungal infection to be linked PVL occurrence or progression to carcinoma (2). To date, several studies in relatively small cohorts have investigated the role of HPV in verrucous lesions reporting between 0% and 89% of PVL lesions to be HPV positive (3, 5-11). Conflicting results with respect to HPV have also been reported for SCCs and verrucous carcinomas (12, 13); however, one of the largest studies evaluating HPV in verrucous and

Received 6/20/08; accepted 8/12/08.

Grant support: National Cancer Institute, Grant T32 CA09338 (to LAK) and National Institute for Dental and Craniofacial Research, Grant R01-DE11943 (to CMW).

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Cancer Epidemiol Biomarkers Prev 2008;17(11). November 2008

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conventional leukoplasias reported HPV DNA rates of 24.1% and 25.5%, respectively (3). These data support that HPV does not play a differential role in the development of PVL lesions relative to non-PVL oral lesions. Alterations in several cell cycle regulatory genes, including p16INK4a and p14ARF, have frequently been reported in end-stage oral cancer and in non-PVL oral premalignancy (14-18). In contrast, the molecular profile of oral PVL is largely unknown. A study by Poh et al. reported that oral verrucous hyperplasias and verrucous carcinomas showed early loss of heterozygosity (LOH) at multiple arms, including chromosome 9p, which encodes p16INK4a and p14ARF (19). In addition, immunohistochemical analysis has shown overexpression of p16INK4a in oral verrucous carcinomas compared with SCCs, dysplasias, or normal epithelia (20), suggesting that this cell cycle regulatory gene may play a differential role in verrucous carcinoma. Thus, the current study was undertaken to investigate the mode and incidence of p16INK4a and p14ARF alterations in patients with PVL. A single alteration of the INK4a/ARF locus can potentially disrupt two distinct and important tumor suppressive pathways, p16-Rb and p14-p53 (reviewed in refs. 14, 21). Considering previous reports of aberrant p53 accumulation in PVL lesions (9, 22) and the linkage between p53 mutations and decreased survival in patients with SCC (23), we also included an immunohistochemical assessment of p53 in the PVL cases under study. Advancing our molecular knowledge of these aggressive premalignant lesions may facilitate improved diagnostic, preventive, and treatment strategies.

Materials and Methods

Patient Specimens. Biopsies from 20 patients with PVL were obtained from the archives of the College of Dentistry, Department of Oral Surgery and Pathology, The Ohio State University. Each case was reviewed by two board-certified oral pathologists (inclusive of S.R.M.). All biopsies were routinely fixed, processed, and paraffin embedded. Both clinical and microscopic features were used to diagnose PVL based on earlier criteria described by Hansen et al. (1). Clinically, all lesions had distinctive margins, with appearances ranging from corrugated plaques to granular papillary lesions. H&E-stained photomicrographs of representative PVL lesions are pictured in Fig. 1. Consistent with early lesions, histopathologic appearances ranged from epithelial atrophy, atypia, and hyperkeratosis to mild epithelial dysplasia.

Microdissection and DNA Isolation. The techniques have been described in detail in an earlier report (17). Briefly, up to six (8 μm) sections of each case were H&E stained, and the area of PVL, as well as patient matched normal tissue, was microdissected using the PixCell-II Laser Capture Microdissection System (Arcturus Engineering). Captured cells were digested overnight at 55°C in proteinase K lysis buffer [0.5 mg/mL proteinase K, 50 mmol/L Tris, 1 mmol/L EDTA, 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 (15, 17). Intron-based primers were used for the amplification of exons 1α and 1β, whereas exon 2 was amplified in two fragments to meet the size limitation associated with single-strand conformational polymorphism analysis (300 bp). Exon 3 was not analyzed for it contains only a small portion of the coding sequence for p16INK4a and none for p14ARF.

Mutation Analysis of p16INK4a and p14ARF. Sequence alterations were detected by using PCR followed by a nonradioactive single-strand conformational polymorphism technique and DNA sequencing (15). Positive single-strand conformational polymorphism results were confirmed by analysis of the corresponding replicate PCR sample, again isolating and reamplifying the shifted band, followed with reexamination by single-strand conformational polymorphism and sequencing.

Detection of Homozygous Deletions. The p16INK4a and p14ARF genes were processed as reported previously (17). Briefly, PCR was employed with HPRT, an internal control gene, coamplified with the specific exons of interest and electrophoresed through a 20% polyacrylamide TBE minigel (Invitrogen) for deletion assessment.

Allelic Imbalance. Genomic DNA from PVL lesions and patient matched normal control tissue was amplified using fluorescent PCR methods (17) and the microsatellite markers D9S1748, D9S171, and the IFN-α cluster (IFNs; Invitrogen). The 5’ forward amplification primer of each of the PCR primer pairs was synthesized with FAM, TET, or HEX fluorescent labels. An ABI 377 automated sequencer and GeneScan and Genotyper software were used for data collection and to quantify normal versus premalignant ampiclon patterns for each fluorescent marker. Allelic imbalance was determined by comparing the ratio of the two allele pairs in the PVL DNA with the two allele pairs in the corresponding patient-matched normal tissue with a shift of ≥40% considered LOH positive.

Structural and Biochemical Characterization of P16 Mutant Proteins. Based on results from our mutational analysis, A68T, P114L, and A148T mutants were constructed using Stratagene QuickChange method with p1G-p16 serving as template and expressed as glutathione S-transferase fusion proteins as described previously (15, 24). After affinity purification using reduced glutathione-agar resin (Sigma), the glutathione S-transferase tag was removed by thrombin digestion, and the free p16 proteins were further purified on a S-100 gel filtration column (Sigma). To evaluate the mutagenic effect on structure, p16 proteins were dissolved into 4 mmol/L HEPES, 1 μmol/L DTT, and 5 μmol/L EDTA (pH 7.5) in 100% H2O at the concentration of 0.2 to 0.4 mmol/L and analyzed on a Bruker DMX-600 nuclear magnetic resonance spectrometer at 20°C (15). To evaluate the mutagenic effect on cyclin-dependent kinase (CDK) 4-inhibitory activity, various amounts p16 mutant proteins were included in reaction mixtures containing 3 units CDK4/cyclin D2 holoenzyme, 100 ng recombinant Rb 790 to 927 protein, and 5 μCi [32P]γ-ATP (23). Reactions were incubated at 25°C for 15 min and stopped, and proteins in the reaction mixtures were separated using 10% SDS-PAGE. The incorporation of [32P] was quantitat-
ed using a PhosphorImager and the IC₅₀ values were determined as the concentrations required for 50% of maximum CDK4 inhibition.

**P53 Immunohistochemical Staining and Analysis.** Each PVL case was stained for p53 (clone DO7; Lab Vision) using standard avidin-biotin peroxidase immunohistochemical procedures as described previously (9) with minor modifications. In brief, PVL tissue sections (4 µm) were deparaffinized, rehydrated in graded alcohols, antigen retrieved for 6 min in citrate antigen unmasking solution (BioGenex), peroxide blocked for 20 min, protein blocked for 20 min, avidin and biotin blocked for 30 min, incubated for 1 h with primary antibody, and link and label applied for 20 min each followed by diaminobenzidine for 6 min and counterstaining with hematoxylin for 30 s. The percent positive labeling index was calculated for each PVL lesion stained using Image-Pro Plus (Media Cybernetics) analysis.

Figure 1. Representative photomicrographs of H&E-stained PVL lesions. **A** and **B.** In sections from patient 3, mild hyperkeratosis is evident, as is the characteristic basket-weave keratin appearance common in PVL lesions. This lesion also displays areas of mild dysplasia as evidenced by increases in the nuclear to cytoplasmic ratio, the presence of dyskeratotic nuclei, and hair-on-end nuclear morphology. There are also increased mitosis and a pronounced inflammatory infiltrate underlying the basal cells. **C** and **D.** Sections from patient 6 display marked hyperorthokeratosis and mild cellular atypia. **E** and **F.** Sections from patient 18 nicely illustrate the transition from normal epithelium (arrow) to the area of PVL. Orthokeratosis is again present, but the cells themselves are remarkably normal, with low mitotic activity and no atypia. This latter type of PVL is common and worrisome for it lacks many of the hallmarks associated with progression to malignancy. **Circled areas** in **A (×100), C (×40), and E (×40)** are shown at higher power in **B (×200), D (×100), and F (×100), respectively,** to permit greater visualization of the cellular detail.
Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD (y)</td>
<td>61 ± 13</td>
</tr>
<tr>
<td>Age, range (y)</td>
<td>33-84</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>8 (40.0)</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>12 (60.0)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td>15 (75.0)</td>
</tr>
<tr>
<td>African American</td>
<td>3 (15.0)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>12 (60.0)</td>
</tr>
<tr>
<td>Site of PVL, n (%)</td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>Vestibular mucosa</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>Gingival/alveolar process</td>
<td>4 (20.0)</td>
</tr>
<tr>
<td>Palate</td>
<td>4 (20.0)</td>
</tr>
<tr>
<td>Tongue</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>PVL with atypia and atrophy, n (%)</td>
<td>7 (35.0)</td>
</tr>
<tr>
<td>PVL with mild dysplasia, n (%)</td>
<td>12 (60.0)</td>
</tr>
</tbody>
</table>

software as described previously with the modification that P53-positive staining in the range of 10% to 24.9%, 25% to 39.9%, and ≥40% were designated +, ++, and ++++, respectively. Histologically normal oral epithelial sections were stained for P53 and used as negative controls as were PVL sections that were not incubated with the primary antibody.

Statistical Analysis. Associations between patient characteristics (nominal and ordinal scales) and gene inactivation events were analyzed using χ² contingency tables or Fisher’s exact test, where appropriate. 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. Patient characteristics are summarized in Table 1 with detailed individual-level patient information as it relates to INK4a/ARF locus alterations contained in Table 2. The study population was composed of 8 male and 12 female patients. Three patients were African American and 15 were Caucasian; for 2 patients, race information was unavailable. Age at biopsy ranged from 33 to 84 years, with an overall mean patient age of 61 years. In contrast, the mean age among the three Black patients was 53 years. Interestingly, Goodwin et al. recently reported that head and neck cancer incidence and mortality remains greater in Black Americans compared with Whites and peaks at an earlier age (25). Several reasons have been postulated to contribute to the incidence, mortality, survival, and age disparity between Blacks and Whites in terms of head and neck cancer (reviewed in ref. 25), but information is scarce pertaining to genetic or epigenetic differences, which may contribute in part to the unequal burden of oral cancer among Blacks. Herein, we report that deletion

Table 2. Summary of INK4a/ARF locus alterations in patients with PVL of the oral cavity

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender/race</th>
<th>Lesion site</th>
<th>Age</th>
<th>Mutation</th>
<th>Deletion</th>
<th>LOH</th>
<th>P53**</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p16</td>
<td>p14</td>
<td>1α</td>
<td>1β</td>
</tr>
<tr>
<td>1</td>
<td>Male/C</td>
<td>Vestibule</td>
<td>55</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Male/C</td>
<td>Vestibule</td>
<td>64</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>Female/C</td>
<td>Buccal mucosa</td>
<td>70</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>Female/C</td>
<td>Buccal mucosa</td>
<td>77</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>Female/AA</td>
<td>GA process</td>
<td>49</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>Female/C</td>
<td>Palate</td>
<td>46</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Male/NA</td>
<td>Vestibule</td>
<td>65</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>Male/C</td>
<td>Buccal mucosa</td>
<td>55</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>Female/C</td>
<td>Palate</td>
<td>33</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>Female/C</td>
<td>Palate</td>
<td>66</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>Female/AA</td>
<td>Buccal mucosa</td>
<td>60</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Female/C</td>
<td>GA process</td>
<td>62</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>13</td>
<td>Male/C</td>
<td>Vestibule</td>
<td>82</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NI</td>
</tr>
<tr>
<td>14</td>
<td>Male/C</td>
<td>GA process</td>
<td>58</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Female/C</td>
<td>GA process</td>
<td>73</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>Female/AA</td>
<td>Palate</td>
<td>51</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>Female/NA</td>
<td>Floor of mouth</td>
<td>84</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>Male/C</td>
<td>Buccal mucosa</td>
<td>57</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>Female/C</td>
<td>Tongue</td>
<td>63</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>20</td>
<td>Male/C</td>
<td>Vestibule</td>
<td>50</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Gender is significantly associated with the site of PVL (P = 0.019). The majority of lesions in males (62.5%) are located on the vestibular mucosa; in contrast, females presented with lesions on the palate (33.3%) followed by the buccal mucosa (25%) and gingival/alveolar process (25%).

1C, Caucasian; AA, African American.

2PVL with mild dysplasia; GA process, gingival/alveolar process.

3All mutations detected were in exon 2.

4African American race correlates significantly to concomitant deletion of exons 1α and 1β (P = 0.012) as well as deletion of 1α (P = 0.043) but not for deletion of 1β (P = 0.069) as determined by Fisher’s exact test.

5LOH indicates the number of positive LOH markers detected per patient. NA, not applicable because inadequate normal sample remained to compare the test values to; NI, noninformative for the marker.

6P53-positive staining was categorized as + (10-24.9%), ++ (25-39.9%), and +++ (≥40%).

7Percentage refers to the percentage of patients whose tissue contained the genetic aberration or P53-positive immunohistochemical staining of ≥10%.

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of exon 1α as well as concomitant deletion of exons 1α and 1β is significantly correlated with African American race as shown in Table 2. This is a relationship that merits further investigation in a larger cohort.

PVL lesions evaluated in this study occurred most frequently on the buccal mucosa (25%) or vestibular mucosa (25%) followed by the gingival/alveolar process (20%) or palate (20%), with only a single PVL lesion located on the tongue or floor of the mouth. In contrast, nonverruccous oral premalignant lesions occur most frequently in anatomic pooling areas, such as the tongue and floor of the mouth (26). In addition, Bagan et al. have reported differences in the location of cancers progressing from PVL precursor lesions compared with non-PVL lesions (27, 28). In our study cohort, gender was significantly associated with the site of PVL (P = 0.019). PVL most frequently occurred on the vestibular mucosa in male patients (62.5%), whereas no females presented with lesions at this location. In contrast, females most commonly presented with lesions of the palate (n = 4; 33.3%) followed by the buccal mucosa (n = 3; 25%) or gingival/alveolar process (n = 3; 25%). Oddly, none of the male subjects presented with lesions on the palate. The reason for differences in the location of PVL lesions between the male and the female subjects is unknown but may be due to differences in exposures to causative factors between men and women. A study by Silverman et al. also noted that PVL sites differed based on gender. In a study of 43 women and 11 men with PVL, Silverman et al. reported the most common site to be the buccal mucosa in women and the tongue in men (2).

Sixty percent of the PVL biopsies exhibited areas of focal low-grade dysplasia; however, the presence of dysplasia did not significantly correlate with any patient characteristics or gene inactivation events. It is generally accepted that leukoplakia accompanied with dysplasia imparts increased risk for malignant transformation, especially high-grade dysplasia. Curiously, although PVLs rarely contain areas of high-grade dysplasia, progression to carcinoma is reported to occur in 70% to 90% of PVL cases (1-3). This compares to a transformation rate of 36% over a period of 8 years for other dysplastic leukoplasias (29), supporting the concept that PVL lesions are biologically and phenotypically unique premalignant lesions.

LOH on Chromosome Arm 9p. Chromosomal loss in the region 9p21 was assessed using three markers reported to be highly polymorphic in head and neck tumors, oral dysplasias, and oral verruocous lesions (16, 17, 19, 30-33). Consideration to the markers relative location on chromosome 9p was also given. INF1 is located telomeric to exon 3, D9S1748 is located between exons 1α and 1β, and D9S1717 is located centromeric to exon 1β. Allelic loss at 9p has been associated with histologic progression to cancer, cancer recurrence, and responsiveness to treatment (3-34).

LOH was the most frequent molecular alteration detected in the PVL lesions investigated. Allelic loss at 9p21 was detected in at least one microsatellite marker in 63.2% of PVL patients. Approximately 47% of cases presented with LOH in a single marker, 10.5% in two markers, and 5.3% in all three markers located. Similarly, Poh et al. evaluated verruocous carcinomas and verruocous hyperplasias for LOH at 9p21 and reported LOH events in 77% and 46%, respectively (19). As summarized in Table 2, the highest LOH rates in PVL patients occurred for the marker D9S171 (45.5%) followed by INF1 (35.3%) and D9S1748 (26.3%). We found this same trend in severely dysplastic non-PVL lesions, but interestingly the level of LOH for D9S171 was markedly higher at 82.4% in the severely dysplastic non-PVL lesions (17), supporting that these two premalignant populations differ in the incidence of LOH for specific markers in the 9p21 chromosomal region.

\[ p16^{INK4a} \text{ and } p14^{ARF} \text{ Homozygous Deletion.} \]

INK4a/ARF inactivation events in patients with PVL are summarized in Table 2. Homozygous deletion of the CDKN2A locus has not been assessed previously in PVLs and only low rates of exon 1β deletion have been found in non-PVL dysplastic oral cavity lesions (16, 17). Strikingly, homozgyous deletion of exon 1β of the \( p14^{ARF} \) gene was prevalent in 40.0% (8 of 20) of PVL lesions. In comparison, exon 1β deletions have been reported in the range of 24% to 28% in oral SCC (35, 36). In addition, several studies report that exon 1β deletions are frequently accompanied by deletions of exons 2 and 1α, indicating complete loss of the INK4a/ARF locus in end-stage oral cancer (35, 36). Over 60% (5 of 8) of PVL patients with exon 1β deletion had concomitant loss of exon 1α, whereas none showed homozygous deletion of shared exon 2. Three of the five patients, with concomitant loss, had allelic loss for the marker D9S1748, located between exons 1α and 1β, suggesting a rather large loss in this chromosomal region. Only one patient had an exclusive deletion in \( p14^{ARF} \), and two patients had \( p16^{INK4a} \)-specific deletions, both located in exon 1α. Overall, homozgyous deletion of exon 1α occurred in 35% of PVL lesions, nearing levels reported in end-stage oral carcinomas (37). In non-PVL oral dysplasias, exon 1α deletion rates have been found to be much lower (~12%; refs. 21, 38). Surprisingly, deletion of exon 1α as well as concomitant deletion of exons 1α and 1β correlated significantly with the African American race. The reason for this association is unclear and merits investigation in a larger cohort in which INK4a/ARF inactivation events are prevalent. Frequent loss of \( p16 \) protein expression has been reported in Black Africans with malignant melanomas; however, similar elevated rates of \( p16 \) loss has also been reported among Caucasian patients (39). Furthermore, a study assessing \( p16 \) protein loss in gastric carcinomas found a strong trend for African American patients to have fewer \( p16 \)-negative tumors compared with non-Hispanic Whites or Hispanic patients (40).

Our deletion results suggest that \( p14^{ARF} \) plays a more salient role in the development of PVL lesions compared with other oral premalignant lesions, which may contribute to PVLs aggressiveness and high rates of malignant transformation. High rates of genetic aberrations in the \( p14^{ARF} \) gene have recently been linked to recurrence of head and neck SCCs, but the ARF alterations showed no relationship to p53 mutations (41). In addition, an immunohistochemical evaluation by Saito et al. reported low levels of p53 staining in verruocous carcinomas (18.6%) relative to levels in severe oral dysplasias (29.3%) and SCCs (50.9%; ref. 20). Further studies are needed to determine whether \( p14^{ARF} \) alterations are independent of p53 status in oral PVLs.
\textbf{p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$ Mutation Analysis.} A recent article by Szklarczyk et al. recently pointed out that “in various cancers, 206 nucleotides in the overlap between the two reading frames carry combinations of 117 point mutations, of which 40, 15, and 62 affect INK4a, ARF, or both proteins, respectively” (38, 41). Herein, we present for the first time the specific INK4a/ARF mutations detected in PVL lesions. Table 3 summarizes the INK4a/ARF sequence alterations in oral PVLs and predicts resultant changes of p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$ transcripts and proteins. Single-strand conformational polymorphism analysis of the INK4a/ARF locus revealed abnormal band shifts in 20% (4 of 20) of PVL samples. These findings indicate that mutational events occur less frequently than LOH or deletional events and suggest that exon 2 is the primary target for mutational events in PVL lesions. As discussed above, there seemed to be a predilection for homozygous deletion of exon 1b, closely followed by exon 1a, and no homozygous deletions in exon 2. Overall, four patients had sequence alterations that were

\begin{table}[h]
\centering
\caption{Mutations of the INK4a/ARF locus in oral PVL and their effect on p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$ transcripts}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
ID & Site & Nucleotide change in INK4a & Codon & Coding change & Result & p14$^{\text{ARF}}$ \\
\hline
3 & Buccal mucosa & G→A & 68 & Ala→Thr & P & 82 & Arg→His & M \\
 & C→T & 71 & Asn→Asn & S & 86 & Leu→Leu & S \\
7 & Vestibule & C→T & 114 & Pro→Leu & M & 128 & Ala→Ala & S \\
10 & Palate & C→A & 147 & Ala→Ala & S & NA & NA \\
12 & GA process & G→A & 148 & Ala→Thr & P & NA & NA \\
\hline
\end{tabular}
\end{table}

Abbreviations: M, missense; P, polymorphism; S, silent; NA, not applicable.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Structural and biochemical characterization of P16 mutant proteins. A. Localization of P16 mutants in the structure of P16. The structure was reconstructed using coordinates from PDB 1DC2, and mutant residues are highlighted (yellow). B. Structural and biochemical comparisons between wild-type and mutant P16. P16 proteins were analyzed using one-dimensional nuclear magnetic resonance, and only spectra responsible for the aromatic residues are presented. IC$_{50}$ values represent the concentrations of P16 proteins required to achieve 50% of the maximum inhibition of CDK4 kinase activity, and only changes in the IC$_{50}$ values more than 2-fold compared with wild-type P16 are regarded as significant.}
\end{figure}
detected in codons 68, 71, 114, 147, and 148 of the p16\(^{INK4a}\) gene and codons 82, 86, and 128 of the p14\(^{ARF}\) gene. All detected band shifts were single-base changes located in conserved exon 2. Two patients with sequence alterations also had homozygous deletion of exon 1\(\beta\). Detected alterations of the p16\(^{INK4a}\) gene included one missense mutation, one silent mutation, and two previously reported polymorphisms in codons 68 and 148. All of the identified mutations involved a single codon with the exception of patient 3, who exhibited changes in codons 68 and 71. Alterations in each of these codons have been reported previously (reviewed in ref. 38); however, in codon 71, previous reports indicate a C→G or A, not the C→T, transversion detected in this patient. Overall, sequence alterations in the INK4a/ARF locus occur at rates similar to those found in severely dysplastic oral epithelium; however, the targeted codons differ and PVL mutations appear less likely to alter p14 proteins (17). Three of the detected changes are expected to result in altered p16\(^{INK4a}\) protein as is one of the alterations of the p14\(^{ARF}\) gene. Earlier work in our laboratory evaluating SCCs and dysplastic lesions also found the greatest number of INK4a/ARF mutations to occur in exon 2 (15, 17).

**Structural and Biochemical Characterization of P16 Mutant Proteins.** As shown in Fig. 2A and described in Table 4, A68T, P114L, and A148T mutants are located within the beginning of the second loop, the end of the second loop, and the flexible COOH-terminal stretch of P16 (25). As revealed in the crystal structure of the P16/CDK6 complex, main contacts between p16 and CDK6 are located within the second and third loops, and the second and third ankyrin repeats, where the flexible NH\(_2\) and COOH termini contribute little to the structure and function of p16 (42). Hence, it is assumed that mutations at residues A68 and P114, not A148, may bring about perturbations in the structure and function of P16. This notion was supported by our further nuclear magnetic resonance and biochemical studies. As shown in Fig. 2B, although the spectra of P16 A68T and A148T are very similar to those of wild-type P16, there are significant differences in nuclear magnetic resonance spectra between P16 P114L and the wild-type as most of the specific peaks in the spectra of P16 L116P disappear and the remaining peaks are broadened, indicating that the global structure of p16 P114L is significantly perturbed. In terms of the CDK4-inhibitory activities of these mutants, P16 P114L does not exhibit any detectable CDK4-inhibitory activity and the activity of p16 A68T is moderately decreased with a 2.5-fold increase in the IC\(_{50}\) value compared with the wild-type p16. In contrast, p16 A148T has an IC\(_{50}\) value almost identical to that of the wild-type, implying that the A148T mutation does not significantly affect the CDK4-inhibitory activity of P16. In addition, the mutation that brought about A68T of P16 also led to a R82H missense mutation in P14ARF. It has been reported that R82H mutation does not cause any detectable change in cellular distribution, HDM2 binding, or induction of P14ARF (43). Therefore, the G→A transversion in specimen 3 could only impair the p16/CDK4/Rb/E2F pathway.

**P53 Immunohistochemical Staining.** Each PVL case was stained for P53 using standard immunohistochemical procedures. A previous report evaluating aberrant P53 accumulation in PVL lesions from patients from the same oral pathology service as our study cohort reported positive staining in 80% (8 of 10) of the cases as defined by a relative positive area of >23.25% and a mean labeling index of 31.9% (9). As indicated in Table 2, we noted similar rates of P53-positive staining in PVL cases. Overall, 85% (17 of 20) of the PVL lesions were p53 positive as defined by a relative positive area of ≥10%. Specifically, 25% (5 of 20) of patients expressed P53 positivity in the range of 10% to 24.9%, 55% (11 of 20) in the range of 25% to 39.9%, and 5% (1 of 20) stained >40% for P53. In addition, 15% (3 of 20) of PVL-positive patients were P53 negative (<10% P53 staining), as were the normal oral epithelial tissues used as staining controls. No clear association emerged between P53.

---

**Table 4. Association between structure-function relationship of the INK4a/ARF locus and mutation location in oral PVL**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location in P16 protein</th>
<th>Mutagenic effect on structure and function</th>
<th>Cancers containing similar mutations</th>
<th>Change in P14ARF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A68T</td>
<td>First residue in loop 2</td>
<td>No significant structural perturbation</td>
<td>A68V in melanoma</td>
<td>R82H, no detectable change in cellular localization, HDM2 binding, or induction (44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDK4-inhibitory activity moderately decreased (25, 44)</td>
<td>A68T in esophageal SCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A68L and A68T in melanoma (43)</td>
<td></td>
</tr>
<tr>
<td>P114L</td>
<td>Last residue in loop 3</td>
<td>CDK4-inhibitory activity significantly decreased due to global structural perturbation as well as aggregation (45)</td>
<td>P114S in esophageal SCC</td>
<td>None</td>
</tr>
<tr>
<td>A148T</td>
<td>Flexible, randomly coiled COOH terminus</td>
<td>No detectable structural or functional changes (23, 45)</td>
<td>P114L in melanoma and fibrosarcoma (43)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polymorphic in melanoma (43) and head and neck SCC (16)</td>
<td></td>
</tr>
</tbody>
</table>
protein staining and aberrations in INK4a/ARF in the PVL tissues assessed. p53 mutation analysis was not conducted in this study, as a previous assessment in 10 patients revealed no mutations (9).

In the present study, we report, for the first time, that the cell cycle regulatory genes p16INK4a and p14ARF are frequently altered in PVL. Furthermore, our findings indicate that there is a notable difference in the mode and incidence of INK4a/ARF inactivation events in oral PVL compared with non-PVL high-risk premalignant lesions. A deletion or mutation event involving both p16INK4a and p14ARF genes was detected in 45% of the PVL lesions assessed. This contrasts sharply with our earlier study in non-PVL oral dysplasias, which revealed a 15% incidence of concomitant alterations in p16INK4a and p14ARF. Thus, high rates of molecular alterations are occurring in key cell cycle regulatory pathways in PVL lesions, which are associated with distinct histologic and clinical subtypes of oral premalignancy. These findings have the potential to eventually influence oral premalignant diagnosis, management, and patient prognosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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Cancer Epidemiology, Biomarkers & Prevention

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