Patterns of \textit{CDKN2A} Gene Loss in Sequential Oral Epithelial Dysplasias and Carcinomas


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Abstract

The \textit{CDKN2A} gene locus encodes two different proteins derived from alternative splicing. p16 (exons 1\(\alpha\), 2, and 3) acts as a G\(_1\) cell cycle regulator, and p14\textsuperscript{ARF} (exons 1\(\beta\), 2, and 3) acts to modulate MDM2-mediated degradation of p53. Inactivation of p16 is a common finding in many cancers; however, there is little data on \textit{CDKN2A} gene abnormalities in oral precancer. In this longitudinal study, we examined changes in the \textit{CDKN2A} gene locus in sequential epithelial dysplasias and oral carcinomas from 11 patients. Genomic DNA was extracted from laser-micromanipulated lesional tissue, and exons 1\(\alpha\), 1\(\beta\), and 2 were analyzed by duplex PCR. Immunochemistry was done to identify p16 and p14\textsuperscript{ARF} protein expression. Two adjacent polymorphic microsatellite markers were used for allelotyping. Homozygous deletion of exon 1\(\alpha\) was identified in 2 of 17 (12\%) precancerous lesions. Loss of either exon 1\(\alpha\), exon 2, or both was seen in seven of nine (78\%) carcinomas. In five of these carcinomas, there was loss of only exon 1\(\alpha\). No case showed deletion of exon 1\(\beta\). In 5 of 11 patients, microsatellite markers showed differing patterns of allelic imbalance in the precancerous lesions and the subsequent carcinoma, suggesting a complex genetic pattern of progression from dysplasia to carcinoma. We conclude that during oral carcinogenesis homozygous deletion of exon 1\(\alpha\) of the \textit{CDKN2A} gene is common but that deletion of exon 2 and 1\(\beta\) is less frequent. Moreover, our results suggest that the progression from oral precancer to cancer, in some cases, is more complex genetically than predicted by linear models of carcinogenesis.

Introduction

The \textit{CDKN2A} gene locus on chromosome 9p21 encodes two different transcripts derived from alternative splicing of upstream exons (1\(\alpha\) and 1\(\beta\)) with a common splice acceptor site. The protein product of the 1\(\alpha\) transcript (p16) functions as a cell-cycle regulator by inhibiting cyclin D-associated CDK-4 and -6. Alterations of p16 are common in a wide range and large proportion of human cancers (1); e.g., homozygous deletions and intragenic mutations of the p16 gene are common findings in many cell lines including melanoma, esophageal, bladder, breast, ovarian, and astrocytoma (2). In some cell lines, the frequency of inactivation approaches 85%, supporting the concept that p16 inactivation provides a selective growth advantage to many human cancer cells (3). The 1\(\beta\) exon is spliced to the same second and third exons encoding a ubiquitously expressed M\(_r\) 13,800 protein termed p14\textsuperscript{ARF} (4). Overexpression of this protein induces G\(_1\)- and G\(_2\)-cell cycle arrest by a mechanism that is independent of CDKs (5). Increasingly, p14\textsuperscript{ARF} alterations are being recognized in human cancers. In some tumors, deletions of the \textit{CDKN2A} gene targets coding exons for both p16 and p14\textsuperscript{ARF} suggesting loss of both favors tumor development (6).

In HNSCC, cytogenetic abnormalities have been identified frequently involving chromosome 9p (7). Polymorphic microsatellite markers have shown that allelic imbalance of a region on chromosome 9p21 is one of the most frequent genetic abnormalities in HNSCC (8, 9). The \textit{CDKN2A} gene locus is contained within this region. Inactivation of p16 by gene deletion, mutation, or promoter methylation has been reported in over 80% of HNSCC (10). Homozygous deletion of the p16 gene accounts for 50% of all of the genetic inactivation events, with mutation and gene methylation accounting for 10 and 20% of events, respectively (1).

One study has examined SCCs from the oral cavity for changes in p16 using allelic imbalance analysis, methylation, and immunohistochemistry, providing evidence that up to 87% of oral cancers show inactivation of p16 and that homozygous deletion is the most common mechanism of inactivation (11). Although inactivation of p16 appears to be common in many oral cancers, there is little direct data on \textit{CDKN2A} gene abnormalities in oral precancer. Moreover, interpreting genetic changes in oral precancerous lesions in a cross-sectional manner (pooled lesions from differing patients where it is not known which lesions progress to cancer and which do not) is problematic because long-term studies have shown that the overall risk of malignant transformation of all of the grades of epithelial dysplasia is only 16% (12). Therefore, assessment of the relative frequency and timing of specific genetic alterations may not be accurate because results may be diluted when lesions with differing risks of neoplastic transformation are pooled. The aim of this longitudinal (multiple lesions from patients with known outcomes are compared) study was to characterize deletional changes in the \textit{CDKN2A} gene locus in sequential epithelial dysplasias and carcinomas from the oral cavity.

Materials and Methods

Case Selection. Sequential biopsies were obtained from 11 patients attending the Department of Stomatology, University of California San Francisco. All of the biopsies were excisional and came from the same intraoral site showing progressive development of hyperkeratosis, epithelial dysplasia, carcinoma \textit{in situ}, or SCC. All of the cases were reviewed (by J. A. R., G. B., and R. C. K. J.) to confirm the histological diagnoses based on established historical criteria (13). There were 26 biopsies in total, comprised of 1 hyperkeratosis, 13 dysplasias, 3 carcinomas \textit{in situ}, and 9 carcinomas. Seven patients provided two sequential biopsies, and four patients provided three biopsies. All of the biopsies had been routinely fixed in formalin and processed to paraffin. Some of these biopsies had been used for an earlier study (14).

Microdissection and Genomic DNA Preparation. Three 10-μm sections of each case were micromanipulated, placed on glass slides, de-waxed in xylene, and rehydrated in graded ethanol solutions. Sections were then stained with 0.2% Evans’s blue and then air-dried. Lesional epithelium was microdissected using the PixCell-1 LCM system (Arcutus Engineering, Mountain View, CA; Ref. 15). For each specimen, at least 200 laser hits were used to obtain...
approximately $4 \times 10^5 \mu m^3$ of tissue (1200 cells). To minimize contamination from normal cells that may potentially mask gene deletions, care was taken to harvest only dysplastic epithelium or carcinoma identified by routine light microscopy at the time of dissection, avoiding morphologically normal epithelium or connective tissues. The captured cells were then incubated for 48 h in proteinase K lysis buffer (20 μg/μl solution proteinase K, 10 mM Tris, 100 mM EDTA, and 1% Tween 20 (pH 8.0]) at 55°C. The samples were then heated at 95°C for 15 min to inactivate the proteinase K, and PCR was performed on the solubilized DNA recovered in the supernatant.

**CDKN2A Homozygous Deletion Analysis.** The suitability of the extracted DNA for PCR was confirmed by amplification of a 208-bp fragment of the β-actin gene. For homozygous deletion analysis, a duplex PCR amplification was used to identify deletions of specific exons by simultaneously amplifying genomic DNA using two sets of primers, one for the target exon under study and the other designed to amplify a 181-bp fragment of the dystrophin gene (16). Primers for exons 1α, 1β, and 2 were designed to detect fragments less than 300 bp in size. For each primer set, the 5′ primer was conjugated with staining in normal cells were not scored. Only scored if there was nuclear staining in normal cells (lymphocytes and G. B., N. T.) as described previously (11). In all of the instances, cases were defined as a loss of one of the alleles (loss of heterozygosity) in the same section, and defined as a shift in size of one or both alleles (microsatellite instability; Ref. 17).

**PCR Product Analysis.** For analysis, 1 μl of each reaction product was mixed with 1.5 μl of loading dye, size-separated by gel electrophoresis in a 6% polyacrylamide gel, and analyzed using the MicroGene Blaster Fragment Tool software (Visible Genetics Inc., Toronto, Ontario, Canada). In each case, DNA from lesional tissue was compared with DNA amplified from connective tissue from the same biopsy. Homozygous loss was determined when there was no product for the target gene in lesional tissue but amplification of the dystrophin gene in both lesional tissue and connective tissue. In addition, connective tissue from the reference case must have also showed amplification of the target and control genes. All of the experiments were conducted at least in quadruplicate.

**Allelic Imbalance.** Genomic DNA from the precancerous lesions and carcinomas was analyzed using the polymorphic microsatellite markers D9S1748, which lies between exons 1β and 1α, and D9S1749, which flanks exon 3 (see Table 1). PCR was conducted under standardized conditions that have been described previously (17), and products were size-separated on an automated DNA sequencer (ABI 3300). Allelic imbalance was detected by comparison with normal control tissue (connective tissue), obtained from the same section, and defined as a loss of one of the alleles (loss of heterozygosity) or a shift in size of one or both alleles (microsatellite instability; Ref. 17).

**Homozygous Deletion Analysis.** Immunohistochemistry was performed as described previously (14) to determine the expression levels of p16 and p14 ARF allele and the other designed to amplify a 181-bp fragment of the dystrophin gene (16). Primers for exons 1α, 1β, and 2 were designed to detect fragments less than 300 bp in size. For each primer set, the 5′ primer was conjugated with staining in normal cells were not scored. Only scored if there was nuclear staining in normal cells (lymphocytes and G. B., N. T.) as described previously (11). In all of the instances, cases were defined as a loss of one of the alleles (loss of heterozygosity) in the same section, and defined as a shift in size of one or both alleles (microsatellite instability; Ref. 17).

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**Quantification of Immunohistochemistry.** Immunohistochemical staining for p16 and p14 ARF proteins was scored by three examiners (R. C. K.J., G. B., N. T.) as described previously (11). In all of the instances, cases were only scored if there was nuclear staining in normal cells (lymphocytes and fibroblasts) in the adjacent connective tissues. Cases where there was no staining in normal cells were not scored.

**Results**

There were 26 sequential biopsies from 11 patients with oral dysplasia and carcinoma. There were six men and five women in the series. In nine patients, there was progression of dysplasia to carcinoma over a mean period of 2.2 years (range, 1–8 years). Two patients with diagnoses of dysplasia developed carcinoma in situ but did not develop cancer.

Amplification of a 208-bp fragment of the β-actin gene was seen in

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**Table 1 Allelegram, gene, and protein status**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Lesion</th>
<th>Exon 2</th>
<th>Exon 1α</th>
<th>Exon 1β</th>
<th>p14ARF</th>
<th>p16</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mild dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Moderate dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Carcinoma in situ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mild dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Severe dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mild dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Moderate dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Hyperkeratosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Moderate dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Mild dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Moderate dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Patient numbers are listed first with sequential biopsies grouped and listed vertically. CDKN2A gene status: +, no deletion; –, exonic loss; D9S1748 and D9S1749, retention of heterozygosity; +, microsatellite instability; ■, loss of heterozygosity; □, noninformative; Immunohistochemistry: +, nuclear expression in tumor cells; –, loss of expression in tumor cells (≤5% +); X, indeterminate because of lack of expression in nuclei of stromal (normal) cells.
all of the cases, confirming the suitability of microdissected DNA for PCR amplification in each case. Results of the deletion analysis and allelic imbalance are shown in Table 1. Loss of exon 1α (Fig. 1A) was identified in 2 of 17 (12%) precancerous lesions, both mild dysplasias. None of the precancerous lesions showed loss of exon 2. Deletion of exon 1β was not identified in any of the precancerous lesions or carcinomas. Loss of either exon 1α, exon 2, or both was seen in 7 of 9 (72%) carcinomas (Fig. 1B). In all of the cases, there was loss of exon 1α either alone or combined with the loss of exon 2.

Of the nine patients who progressed from oral precancer to cancer, loss of CDKN2A-coding exons was seen in the precancerous lesions from two patients (Table 1, patient numbers 4 and 7). These two cases also showed loss in the subsequent carcinomas. Two patients (numbers 10 and 11) had retention of exons 1α and 2 in the precancerous lesions but loss of both exons in the subsequent carcinomas.

By immunohistochemistry, we were only able to assess p16 protein loss in 11 of the 26 cases, because normal cells in the connective tissue did not show nuclear staining for the protein in the remaining 17 cases and, therefore, were not scored. We found loss of p16 protein expression in six of seven carcinomas. None of the three informative dysplasias showed loss. All of the cases where there was loss of p16 protein staining showed loss of exon 1α by gene analysis. Conversely, all of the assessible cases where we identified loss of exon 1α by gene analysis failed to express p16 protein.

All of the precancerous lesions and carcinomas showed high levels of nuclear p14ARF staining (>50% + cells) apart from two carcinomas where there was reduced nuclear expression in tumor cells (<5% + cells). One of these carcinomas (patient number 11) had a deletion of exon 2. The other carcinoma (patient number 7) had retention of exon 2 but loss of exon 1α. One other carcinoma (patient number 10) showed deletion of exon 2 but retention of exon 1α and showed high levels of p14ARF protein expression (Fig. 2).

Using the marker D9S1748, two precancerous lesions and one carcinoma were homozygous and, therefore, noninformative (Table 1). Of the remainder, microsatellite instability was identified in 6 of

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**Fig. 1.** A, PCR products of duplex PCR size-separated by gel electrophoresis and analyzed by the MicroGene Blaster Fragment Tool software. Lane 1, molecular size marker indicating 150 bp. Lane 2, connective tissue from SCC from patient number 4 showing amplification of a 122-bp fragment of exon 1α and a 181-bp fragment of the dystrophin gene. Lane 3, homozygous deletion of exon 1α (arrow) in the carcinoma from patient number 4. Dystrophin is amplified in this reaction as an internal control. B, PCR products of duplex PCR size-separated by gel electrophoresis and analyzed by MicroGene Blaster Fragment Tool software. Lane 1, molecular size marker indicating 150 bp. Lane 2, connective tissue from SCC from patient number 10 showing amplification of a 229-bp fragment of exon 2 and a 181-bp fragment of the dystrophin gene. Lane 3, homozygous deletion of exon 2 (arrow) in the carcinoma from patient number 10. Dystrophin is amplified in this reaction as an internal control.

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**Fig. 2.** A, immunohistochemistry showing loss of nuclear expression of p14ARF protein in an oral SCC. B, the dysplastic epithelium, overlying this tumor, shows strong nuclear expression of p14ARF protein. (×200)
13 precancerous lesions and in 3 of 8 carcinomas. Loss of heterozygosity was identified in three precancerous lesions and in two carcinomas. Using the marker D9S1749, microsatellite instability was identified in 4 of 13 informative precancerous lesions and in 1 of 6 informative carcinomas. Of the informative cases, loss of heterozygosity was seen in 2 of 13 precancerous lesions and in 2 of 6 carcinomas.

Discussion

Our results support the concept that inactivation of p16 by homozygous deletion is frequent in oral carcinomas and are similar to other studies examining p16 protein by immunohistochemistry showing inactivation in up to 87% of oral cancers (11). We also found that 2 of 17 (12%) precancerous lesions showed loss of exon 1α. These results differ from a cross-sectional study of p16 protein in oral precancer where 43% of 23 lesions showed no staining (18). Because we did not analyze promoter methylation or gene mutations, it is likely that the prevalence of p16 inactivation in our series of precancers and cancers may be higher because of these alternate mechanisms of inactivation. Our results suggest that homozygous gene deletion is the most common mechanism of p16 inactivation in oral cancers (Table 1). The high prevalence of CDKN2A homozygous gene deletions in our series of oral cancers likely reflects the use of laser microdissection to enrich the tumor cell population and eliminates connective tissue elements and normal epithelial cells that may mask p16 homozygous deletion (15).

We found that exon 1α was the most frequent target of exonic loss in the precancer and cancers. Exon 1α was lost in seven of nine carcinomas, and in two of these there was also loss of exon 2. No tumor showed loss of exon 2 alone. Similarly, in the precancerous lesions there was loss of only exon 1α in two lesions but no loss of exon 2. Our results are similar to one other study (19) that also showed selective loss of exon 1α but retention of exon 2 in oral carcinomas. Therefore, taken together, these findings suggest that selective loss of coding exons of CDKN2A in oral cancers and that the region of deletion may be smaller than thought previously (11).

Homozygous deletion of exon 1β was not seen in any of the 17 precancerous lesions or 9 carcinomas. No previous study has directly examined exon 1β in oral precancers or cancers. These results are novel and suggest that exon 1β is not a target of deletion in oral carcinomas. Loss of exon 2 should prevent the formation of p14ARF because this exon forms the COOH terminus of the protein. One carcinoma with loss of exon 2 showed a marked reduction in p14ARF protein staining by immunohistochemistry, but in another with exon 2 loss, there was strong staining similar to tumors where there was retention of all of the p14ARF coding exons. The antibody that we used in this study recognizes the NH2 terminus of the p14ARF protein, and it is conceivable that protein expression in this one case where there was exon 2 deletion may represent a truncated form of the protein. Recent studies (20) have suggested that the NH2-terminal domain of the protein coded by the exon 1α region of the gene is necessary and sufficient to induce cell cycle arrest. However, we were unable to perform Western blotting to determine the size of the p14ARF protein in these cases because our material was formalin fixed and paraffin embedded and, therefore, not suitable for this analysis.

We used two microsatellite markers located within and adjacent to the CDKN2A gene complex for allelotyping in these progressive lesions. Interestingly, we found that the frequency of allelic imbalance using these markers was higher than the frequency of homozygous deletion of components of the CDKN2A gene complex. Despite careful microdissection, it is conceivable that normal cells or lesional cells that retained CDKN2A-coding exons may have still contaminated the lesional tissues and, therefore, may account for the lower frequency of homozygous deletion than allelic imbalance. This may have been particularly true for the precancerous lesions and resulted in an underestimation of the number of cases showing homozygous loss of components of CDKN2A. Alternatively, these results may suggest the presence of another gene adjacent to CDKN2A that may also be a target for loss or mutation in oral carcinogenesis.

Interestingly, in some cases we identified differing patterns of allelic imbalance in the precancerous lesions and the subsequent carcinomas; e.g., patient number 2 showed dissimilar patterns of microsatellite instability using both markers in the two temporally distinct precancerous lesions (Fig. 3). Moreover, comparison of the patterns of microsatellite instability and loss of heterozygosity in the dysplasias and carcinoma suggested that the mild dysplasia could not have evolved into the temporally distinct moderate dysplasia and carcinoma. In 5 of the 11 patients (patient numbers 2, 4, 5, 8, and 11) studied, we noted patterns of allelic imbalance in the precancerous lesions that could not be reconciled with a model of linear evolution of normal epithelium through epithelial dysplasia of worsening severity to oral cancer. Therefore, our results suggest that the progression from normal oral mucosa to oral carcinoma, in some cases but not all, may involve the parallel evolution of multiple genetically distinct clones of aberrant epithelial cells at one site. This finding is similar to recent studies of metastatic lesions of the esophagus as they progress to carcinoma. Here, complex progression patterns are seen that are not consistent with linear progression models (21). These findings have implications for the clinical prediction of progression from dysplasia to carcinoma. Prediction of cancer development based on genetic analysis of a single precancerous lesion may be problematic based on assumptions of linear cancer progression. Clearly, a larger study of sequential biopsies of oral precancer and cancer is needed to assess this issue.

In conclusion, we have studied a series of oral premalignant lesions
that have shown progression to oral carcinoma and found frequent homozygous deletion of components of the CDKN2A gene locus in both precancerous lesions and cancer. We also found a complex pattern of allelic loss around the CDKN2A gene complex in the precancerous lesions and in the subsequent cancers, suggesting that the progression from oral precancer to oral cancer may not always occur in a linear fashion.

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References

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