Gene promoter hypermethylation in leukoplakia of the oral mucosa

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Abstract: To examine whether aberrant DNA methylation in the promoter region might occur earlier in tumorigenesis, particularly in premalignant lesions, we examined biopsies from 111 participants in a chemoprevention trial aimed at reversal of oral leukoplakia, using methylation-specific polymerase chain reaction for the promoter regions of the tumor suppressor gene CDKN2A (p16), the putative metastasis suppressor gene DAP-K, the DNA repair gene O6-methylguanine-DNA-methyltransferase (MGMT), and the detoxification gene glutathione S-transferase p1 (GSTP1). p16 promoter hypermethylation was detected in 21 of 82 (25.6%), DAP-K hypermethylation in 28 of 87 (32.2%), and MGMT hypermethylation in 32 of 106 (30.2%) oral leukoplakia lesions analyzed. No aberrant methylation was found at the GSTP1 gene in 110 lesions examined. Among 68 biopsies analyzed for all three genes (p16, DAP-K, MGMT), 17 biopsies were detected with an abnormal methylation pattern at only one gene, 15 at two genes, and 8 at all three genes. Among clinical characteristics and their correlation with methylation, only alcohol consumption was correlated with DAP-K methylation (P = 0.027), while MGMT methylation was more frequent in females (P = 0.003) and nonsmokers (P = 0.0005). A significant correlation was found between p16 and DAP-K hypermethylation; p16 promoter was methylated in 14 (56%) of 25 lesions with DAP-K methylation, and only 5 (11.1%) of 45 DAP-K methylation-negative lesions (P = 0.0001). DAP-K aberrant methylation was also significantly correlated with MGMT methylation (16 of 31 in MGMT methylation-positive lesions versus 12 of 52 MGMT methylation-negative lesions, P = 0.0016).

Our results suggest that epigenetic mechanisms of inactivation, such as aberrant methylation of p16, DAP-K, and MGMT genes, occur early in head and neck tumorigenesis, and might play a role in the progression of these lesions.

Keywords: p16, DAP-K, MGMT, GSTP1 genes, methylation, leukoplakia

Introduction

Methylation of normally unmethylated CpG islands in gene promoter regions is an increasingly recognized epigenetic mechanism of transcriptional inactivation of tumor suppressor genes or DNA repair genes.1 Recent publications have reported that promoter hypermethylation of key genes in critical pathways is common in head and neck squamous cell cancers (HNSCC), as well as in serum and saliva of patients with such cancers.2,3 In the present study, to address whether aberrant methylation occurs earlier in carcinogenesis (in premalignant lesions), we analyzed the promoter hypermethylation pattern of the p16, DAP-K, MGMT, and GSTP1 genes using methylation-specific polymerase chain reaction (PCR) in 111 patients who presented with oral leukoplakia. p16INK4A is critical at the G1-S transition of the cell cycle, being responsible for maintaining Rb protein in its nonphosphorylated state,4 and it is frequently
lost in head and neck cancer through deletion, promoter hypermethylation, and rarely by mutations. MGMT is a DNA repair gene that is frequently inactivated in colon, lung, and brain tumors. DAP-K is a novel serine/threonine kinase required for interferon gamma-induced apoptotic cell death, and it is frequently methylated in lung cancer. The detoxification gene GSTP1 encodes a detoxifying enzyme and can be characteristically silenced in prostate, breast, and kidney tumors. GSTP1, in particular, is aberrantly methylated in approximately 90% of prostate carcinomas, and may be an ideal marker for this tumor type.

Materials and methods

Premalignant lesions and head and neck tumor samples
Baseline biopsies were obtained from patients who participated in a prospective, oral premalignancy chemoprevention trial in the years 1999–1999 at The University of Texas MD Anderson Cancer Center. Paraffin-embedded tissue blocks from biopsy samples at the leukoplakia site from a total of 111 patients were available in the institution’s tumor archive. The patient characteristics are presented in Table 1. Tissue sections from 10 patients with HNSCC during the same period were also available for this study. Four to five sections (4 µm) from each sample were stained with hematoxylin and eosin. One of these was reviewed by a pathologist for histologic features and the remaining sections were microdissected for methylation analyses. The institutional review boards approved the study to allow us to obtain tissue blocks.

Microdissection and DNA extraction
The epithelial part of each lesion was microdissected from three to four serial sections of each biopsy, as described previously. The samples were digested in 100 µL of 50 mM Tris-HCl (pH 8.0), 1% dodecyl sulfate, and proteinase K (0.5 mg/mL) at 42°C for 12–24 hours. Digested products were boiled for eight minutes to inactivate enzymes, and purified using phenol chloroform. DNA was precipitated by the ethanol precipitation method in the presence of glycogen (Boehringer Mannheim Biochemicals, Indianapolis, IN) and resuspended in distilled water.

Methylation-specific polymerase chain reaction
DNA from oral premalignant lesions and tumor tissues were subjected to bisulfite treatment, as described previously. Briefly, 200 ng of DNA was denatured by NaOH and treated with sodium bisulfite (Sigma Chemical Co., St Louis, MO) by incubation at 55°C for 16–18 hours. DNA samples were then purified using Wizard DNA purification resin (Promega Corp., Madison, WI), treated with NaOH once more, precipitated by ethanol, and resuspended in distilled water. Bisulfite-modified DNA was amplified by PCR using two primer sets specific for methylated and two primer sets specific for unmethylated sequence of each gene. Primer sequences for the p16, DAP-K, MGMT, and GSTP1 genes were as described previously with minor modifications (Table 2). Hot start PCR was used. Human placental DNA treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA) was used as a positive control for methylated alleles of p16, DAP-K, MGMT, and GSTP1. DNA from normal lymphocytes was used as the control for unmethylated alleles of each gene, and water was substituted for DNA for each set of PCR as the negative control. Each PCR product (12.5 µL) was directly loaded onto 3% agarose gel, electrophoresed, and visualized under ultraviolet illumination after staining with ethidium bromide. PCR was repeated at least twice to ensure reproducibility of results.

Table 1 Clinical characteristics in 111 subjects with oral premalignant lesions

<table>
<thead>
<tr>
<th>Age median years (range)</th>
<th>56 (24–90)</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
<td>52</td>
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<tr>
<td>Histology</td>
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<tr>
<td>Dysplasia</td>
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<tr>
<td>Mild</td>
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</tr>
<tr>
<td>Moderate</td>
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<tr>
<td>Severe</td>
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<tr>
<td>Site</td>
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<tr>
<td>Oral cavity/buccal mucosa</td>
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<tr>
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<tr>
<td>Palate/retromolar trigone</td>
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<td>Lip</td>
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<tr>
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Results

Gene promoter hypermethylation profiles in leukoplakia

Of the 111 biopsy samples, an interpretable methylation pattern for p16, DAP-K, MGMT, and GSTP1 was obtained in 82, 87, 106, and 110 biopsies, respectively. The p16 tumor suppressor gene demonstrated promoter hypermethylation in 21 of 82 (25.6%), the DAP-K gene in 28 of 87 (32.2%), and the MGMT gene in 32 of 106 (30.2%) lesions. No aberrant methylation was found for the GSTP1 gene in 110 leukoplakia lesions analyzed. Representative results are shown in Figures 1–4. For comparison, we also show the methylation status of the above four genes in several head and neck tumor samples, respectively, in Figures 1–4.

Gene promoter hypermethylation and clinicopathologic characteristics

We examined the correlation of hypermethylation with gender, histology, site, smoking status, and alcohol consumption status, and the data are summarized in Table 3. Females (20 of 48) were more likely to demonstrate MGMT methylation than males (12 of 58, $P = 0.03$, Fisher’s exact test), but gender did not affect methylation status for the p16 and DAP-K genes. Methylation status of the three genes was not correlated with histology and site. The frequency of MGMT methylation was significantly higher in

never smokers (12 of 19 never, 7 of 48 former, and 12 of 38 current smokers, $P < 0.0005$, Fisher’s exact test), and a similar trend was seen for individuals who never consumed alcohol (12 of 30 versus 1 of 13 former and 18 of 62 current alcohol drinkers, $P = 0.09$, Fisher’s exact test). No significant correlation between smoking status and DAP-K methylation was seen. Although p16 methylation was more frequent in current smokers (9 of 30 versus 9 of 34 in former and 3 of 18 in never smokers, respectively) the difference was not significant. p16 and DAP-K methylation frequency was also higher, but not significantly so, in current alcohol drinkers (14 of 46 and 21 of 53, respectively, versus 1 of 9 and 0 of 11 former, respectively, and 6 of 27 and 7 of 22 never drinkers).

Association of hypermethylation in p16, DAP-K, and MGMT

In this study, methylation status for the p16, DAP kinase, and MGMT genes was simultaneously determined in 68 biopsy specimens. Forty of 68 (58.8%) had an abnormal methylation pattern in at least one gene. Among them, 17 (25.0%) had methylation at only one gene, 23 (33.8%) had methylation at two genes, and eight of 68 (11.8%) shared methylation for all three genes examined.

Methylation status was available for both DAP-K and p16, for DAP-K and MGMT, and for p16 and MGMT in 70, 83, and 79 matched biopsies, respectively. There was

<table>
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<th>Table 2 Primers for methylation-specific polymerase chain reaction</th>
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<td><strong>Primer set</strong></td>
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<tr>
<td>p16-M</td>
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<tr>
<td>p16-U</td>
</tr>
<tr>
<td>DAP-M</td>
</tr>
<tr>
<td>DAP-U</td>
</tr>
<tr>
<td>MGMT-M</td>
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<tr>
<td>MGMT-U</td>
</tr>
<tr>
<td>GSTP1-M</td>
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<tr>
<td>GSTP1-U</td>
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</table>

**Figure 1** Examination of methylation of the p16 gene in seven leukoplakia and six head and neck tumor samples. M (size marker), indicates the polymerase chain reaction product produced by the primer sets specific for the methylated, and U indicates that produced by primer sets specific for the unmethylated. Four leukoplakia samples (1B, 4B, 7B, 13B) show methylation, and three samples (2B, 6B, 9B) are unmethylated. Three head and neck tumor samples (1, 2, 15) show methylation, and two samples (4, 7) are unmethylated. P, SssI-treated DNA as a positive control; N, peripheral blood DNA as a negative control.
a positive correlation between presence of p16 and DAP-K hypermethylation; p16 hypermethylation was found in 14 (56%) of 25 DAP-K methylation-positive lesions and five (11.1%) of 45 DAP-K methylation-negative lesions (P = 0.0001, Fisher’s exact test). A significant correlation was also found for the concomitant presence of DAP-K and MGMT aberrant methylation. DAP-K methylation was detected in 16 (51.6%) of 31 MGMT methylation-positive lesions and only 12 (23.1%) of 52 MGMT methylation-negative lesions (P = 0.016, Fisher’s exact test). A trend was seen for correlation of methylation of p16 and MGMT but this did not reach statistical significance; 10 (37.0%) of 27 MGMT methylation-positive lesions showed p16 hypermethylation, compared with 11 (21.2%) of 52 MGMT methylation-negative lesions.

Discussion
An increasingly important pathway of transcriptional inactivation for many tumor suppressor and DNA repair genes in CpG island hypermethylation within the gene promoter region has been reported recently. It is now generally accepted that promoter hypermethylation plays an important role in the inactivation of tumor suppressor genes in established cancer. Sanchez-Cespedes et al and Rosas et al have examined large series of head and neck tumors and have found frequent promoter hypermethylation of the tumor suppressor genes p16, DAP-K, and MGMT in tumor specimens, and serum and saliva of head and neck cancer patients. Consistent with these reports, several other groups have also investigated the methylation profile of genes p16, DAPK, and MGMT in HNSCC (including oral squamous cell carcinoma), which is a promising biomarker for the follow-up and early detection of head and neck cancer recurrence. Steinmann et al showed that methylation of the tumor-related genes was significantly more frequent in squamous cell carcinomas of the head and neck than normal tissues and early premalignant lesions. Our data suggest that multiple epigenetic abnormalities have already occurred in oral premalignant lesions similar to early genetic alterations. We found promoter hypermethylation at p16 in 21 of 82 (25.6%), DAP-K in 28 of 87 (32.2%), and MGMT in 32 of 106 (30.2%) of premalignant oral lesions.

Interestingly, there was no difference between the incidence of p16, DAP-K, and MGMT gene methylation in premalignant lesions and those previously reported in HNSCC. Although the frequency of DAP-K hypermethylation was reported as 18% in the first series of 111 HNSCC patients, it was 30% in the second series, which is very similar to the frequency observed in oral premalignant lesions in our study. These findings indicate that the methylation of these genes occurred very early in the process of head and neck carcinogenesis and possibly additional genetic and epigenetic alterations occurred later to drive the tumorigenesis process.

Figure 2 Examination of methylation of the DAP-K gene in five leukoplakia and four head and neck tumor samples. M (size marker) indicates the polymerase chain reaction product produced by the primer sets specific for the methylated, and U indicates that produced by primer sets specific for the unmethylated. Three leukoplakia samples (1B, 4B, 12B) show methylation, and two samples (12B, 14B) are unmethylated. Two head and neck tumor samples (1, 2) show methylation, and two samples (2, 12) are unmethylated. P, SssI-treated DNA as a positive control; N, peripheral blood DNA as a negative control.

Figure 3 Examination of methylation of the MGMT gene in five leukoplakia and four head and neck tumor samples. M (size marker) indicates the polymerase chain reaction product produced by the primer sets specific for the methylated, and U indicates that produced by primer sets specific for the unmethylated. Two leukoplakia samples (6B, 15B) show methylation, and three samples (1B, 7B, 10B) are unmethylated. Two head and neck tumor samples (6, 7) show methylation, and two samples (1, 8) are unmethylated. P, SssI-treated DNA as a positive control; N, peripheral blood DNA as a negative control.

Figure 4 Examination of methylation of the GSTP1 gene in two head and neck tumor samples. M (size marker) indicates the polymerase chain reaction product produced by the primer sets specific for the methylated, and U indicates that produced by primer sets specific for the unmethylated. One head and neck tumor sample (1) shows methylation and another sample (10) is unmethylated. P, SssI-treated DNA as a positive control; N, peripheral blood DNA as a negative control; and water as a negative PCR control.
GSTP1 was not methylated in any of the oral premalignant lesions examined, suggesting that the gene is not an important determinant of head and neck tumorigenesis as opposed to other tumors, such as breast, prostate, and renal cancers. We found that only one tumor sample showed GSTP1 methylation, as seen in Figure 4. It appears that GSTP1 methylation would not be a frequent event in head and neck cancer although we are unable to explain the initiation of the methylation. Previous studies have shown that hypermethylation is not limited to a single gene, but affects multiple genes concurrently. The presence of such a hypermethylation phenotype was confirmed in several human neoplasms such as gastric cancers, pancreatic adenocarcinoma, and colorectal cancers, among others. In this study, we also found that the p16, DAP-K, and MGMT genes were simultaneously methylated in some of the precancer lesions. Twenty-three of 68 (33.8%) methylation-positive premalignant lesions showed epigenetic changes at more than one gene, and eight of 68 (11.8%) were methylated for all the three genes examined simultaneously, thereby implying a general deregulation of CpG island methylation in precancerous lesions, similar to that in cancers. In particular, the p16 gene was inactivated more frequently in oral premalignant lesions exhibiting DAP-K gene hypermethylation than those that did not (56% versus 11.1%); the difference was significant. The MGMT gene was inactivated more frequently in oral premalignant lesions harboring MGMT gene hypermethylation than those that did not (51.6% versus 23.1%); this difference was also significant. The fact that several genes are frequently methylated in oral premalignant lesions implies that the mechanism that normally protects GpG islands from methylation is defective in premalignant cells.

It is unclear whether the positive correlation between p16 and DAP-K methylation and MGMT and DAP-K methylation has any biologic significance, but it is possible that these alterations contribute through different pathways and mechanisms to the tumorigenesis process. DAP-K is a Ca²⁺/calmodulin-dependent serine/threonine kinase gene that contains ankyrin repeats and a death domain. It was recently found to display strong tumor suppressive activities, coupling control of apoptosis with metastasis. p16INK4a is an inhibitor of cyclin-dependent kinase CDK4 and CDK6 and regulates the cell cycle negatively. Methylation of p16INK4a in cancer and premalignancy and DAP-K at least in cancers are now recognized as widespread epigenetic alterations in a variety of human cancers.

One interesting observation in our study is that MGMT promoter methylation is observed more frequently in lesions from nonsmokers. MGMT is a DNA repair protein that removes alkyl adducts from the O6 position of guanine, thereby protecting cells against lethal crosslinks caused by alkylating agents and their carcinogenic and cytotoxic effects. The level of MGMT varies widely according to tumor type and among tumors of the same type. Although changes in MGMT expression are incompletely understood, p53 at physiologic and supraphysiologic levels has been previously found to curtail MGMT transcription.
Inactivation of MGMT by promoter hypermethylation is associated with G to A transition mutations in K-ras and G:C to A:T transition mutations in p53.\textsuperscript{39,40} Interestingly, a smoking-related increase in MGMT expression has been described in human lung carcinomas.\textsuperscript{41} The study of genetic and epigenetic alterations leading to cancer development in nonsmokers is of major interest, and the finding of MGMT hypermethylation in nonsmokers possibly provides a different mechanism of genetic instability through promotion of p53 transitional mutations, as opposed to the usual smoking-related transversions,\textsuperscript{42} and through targeting of other genes, such as K-ras. It is even possible that MGMT promoter methylation confers a special mutation phenotype characterized by numerous transition mutations affecting important genes. Further study of MGMT hypermethylation in the subset of nonsmokers is clearly warranted.

Aberant methylation in oral premalignant lesions was frequently detected in our study, but whether such epigenetic abnormalities are useful adjuncts to histopathologic evaluation of oral mucosal lesions for prediction of risk of malignant transformation remains to be determined through long-term follow-up of the population in this study.

**Disclosure**

The authors have no conflicts of interest to report in this work.

**References**