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 for death-associated protein kinase (*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. *p16*^{INK4A} 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

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lost in head and neck cancer through deletion,⁵ promoter hypermethylation,⁶ and rarely by mutations.⁷ *MGMT* is a DNA repair gene that is frequently inactivated in colon, lung, and brain tumors.⁸ *DAP-K* is a novel serine/threonine kinase required for interferon gamma-induced apoptotic cell death, and it is frequently methylated in lung cancer.⁹ The detoxification gene *GSTP1* encodes a detoxifying enzyme and can be characteristically silenced in prostate, breast, and kidney tumors.^{10,11} *GSTP1*, in particular, is aberrantly methylated in approximately 90% of prostate carcinomas, and may be an ideal marker for this tumor type.¹²

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.¹³ 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.⁹

Table 1 Clinical characteristics in 111 subjects with oral premalignant lesions

Age median years (range)	56 (24–90)
Gender	
Male	59
Female	52
Histology	43
Hyperkeratosis without hyperplasia	30
Hyperplasia	47
Dysplasia	
Mild	27
Moderate	6
Severe	1
Site	
Oral cavity/buccal mucosa	44
Tongue/floor of mouth	44
Palate/retromolar trigone	15
Lip	8
Smoking status	
Never	21
Former	49
Current	40
Missing	1
Alcohol consumption	
Never	32
Former	14
Current	64
Missing	1

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).^{9,12,14,15} 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 2 Primers for methylation-specific polymerase chain reaction

Primer set	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (base pairs)	Annealing temperature (°C)
<i>p16</i> -M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACC GCGACCGTAA	150	64
<i>p16</i> -U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
<i>DAP</i> -M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAACGCCGA	98	56
<i>DAP</i> -U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	106	61
<i>MGMT</i> -M	GTATGGATTTTAGCGTCGGTC	TAAACTCGACGAACGCCG	110	59
<i>MGMT</i> -U	ATGGTATGGATTTTAGTGTGGTT	ACTATACCTAAACTCAACAAACACC	130	59
<i>GSTPI</i> -M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	91	57
<i>GSTPI</i> -U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAAACA	97	57

Results

Gene promoter hypermethylation profiles in leukoplakia

Of the 111 biopsy samples, an interpretable methylation pattern for *p16*, *DAP-K*, *MGMT*, and *GSTPI* 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 *GSTPI* 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

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.

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, 22B) show methylation, and two samples (12B, 14B) are unmethylated. Two head and neck tumor samples (1, 20) 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.

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.¹⁶ 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 al³ and Rosas et al² 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

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.

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.^{17–21} Steinmann et al showed that methylation of the tumor-related genes was significantly more frequent in squamous cell carcinomas of the head and neck (42%) compared with normal samples (23%, $P < 0.05$),²² although caution must be exercised using these markers for diagnosis because some of them appear to be age-related.²³ However, little is known about the epigenetic alteration in premalignant lesions, although recently several reports indicated the correlation between some tumor-related genes, such as *CDKN2A/p16*, *DAP-K*, and *MGMT* and premalignancy in endometrial, endobronchial, and cervical lesions.^{24–26} In this study, we investigated the methylation status of *p16*, *DAP-K*, *MGMT*, and *GSTP1* genes in leukoplakia patients, in an effort to characterize the timing of these epigenetic alterations during head and neck carcinogenesis.

Our data suggest that multiple epigenetic abnormalities have already occurred in oral premalignant lesions similar to early genetic alterations.^{27,28} 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.^{2,3} Although the frequency of *DAP-K* hypermethylation was reported as 18% in the first series of 111 HNSCC patients,³ it was 30% in the second series,² 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 tumorigenesis and possibly additional genetic and epigenetic alterations occurred later to drive the tumorigenesis process.

Table 3

	<i>p16</i> methylation		<i>P</i>	<i>DAP-K</i> methylation		<i>P</i>	<i>MGMT</i> methylation		<i>P</i>
	Absent	Present		Absent	Present		Absent	Present	
Gender			0.62			0.82			0.03
Male	31	9		32	14		46	12	
Female	30	12		27	14		28	20	
Histology			0.27			0.21			0.26
Early	38	15		40	14		51	19	
Dysplasia	21	4		17	12		20	13	
Smoking			0.62			0.44			0.0005
Never	15	3		12	7		7	12	
Former	25	9		27	9		41	7	
Current	21	9		19	12		26	12	
Alcohol			0.47			0.027			0.09
Never	21	6		15	7		18	12	
Former	8	1		11	0		12	1	
Current	32	14		32	21		44	18	
Site			0.75			0.39			0.44
Oral cavity	27	7		22	15		29	15	
Tongue	24	10		27	10		30	14	
Others	10	4		10	3		15	3	

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.¹⁴ 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,²⁹ pancreatic adenocarcinoma,³⁰ and colorectal cancers,³¹ 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.^{32,33} *p16*^{INK4a} is an inhibitor of cyclin-dependent kinase CDK4 and CDK6 and regulates the cell cycle negatively. Methylation of *p16*^{INK4a}^{34,35} in cancer and premalignancy and *DAP-K* at least in cancers^{3,9,12} 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 remove alkyl adducts from the O⁶ position of guanine, thereby protecting cells against lethal crosslinks caused by alkylating agents and their carcinogenic and cytotoxic effects.³⁶ 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.^{37,38}

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*.^{39,40} Interestingly, a smoking-related increase in *MGMT* expression has been described in human lung carcinomas.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,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.

Aberrant 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.

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