Hypermethylation of MGMT and DAPK gene promoters is associated with tumorigenesis and metastasis in oral squamous cell carcinoma

Yong-Kie Wong1,2, Li-Tsu Lee1,2, Chung-Ji Liu2,3,4*

1 Department of Oral and Maxillofacial Surgery, Taichung Veterans General Hospital, Taichung, Taiwan
2 Dental School of National Yang Ming University, Taipei, Taiwan
3 Department of Oral and Maxillofacial Surgery, MacKay Memorial Hospital, Taipei, Taiwan
4 MacKay Medicine, Nursing and Management College, Taipei, Taiwan

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Abstract  Background/purpose: Oral squamous cell carcinoma (OSCC) is the fourth major cause of mortality among males in Asia. The tumorigenesis of OSCC is a multi-step process characterized by sequential morphological changes. The extent of lymph node metastasis is a major determinant in the prognosis of cancer. Hypermethylation is an important pathway for repression of gene transcription in cancer cells and a promising marker for cancer detection. It is also found in early metastatic cancer patients.

Materials and methods: Sixty-four histologically confirmed OSCC tissues and corresponding non-tumorous tissues were enrolled in this study. DNA was extracted. The promoter methylation status of the p16, death-associated protein kinase (DAPK), MGMT, and glutathione S-transferase genes in OSCC tissues was evaluated by a methylation-specific polymerase chain reaction analysis.

Results: Frequencies of promoter hypermethylation of p16, DAPK, and MGMT in OSCC tissue were 67.2%, 45.3%, and 31.3%, respectively. No methylation was found in normal oral mucosa. Methylation rates of MGMT (50%) and DAPK (55.6%) in metastasized OSCC were higher than those of MGMT (23.9%) and DAPK (41.3%) in nonmetastasized OSCC. No glutathione S-transferase P methylation was found in any tissue samples.

Conclusions: Our study supports the hypothesis that hypermethylation of p16 gene promoters may indicate a high risk of oral cancer, and hypermethylation of the MGMT and DAPK genes may be a major indicator of early OSCC metastasis.

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Introduction

Oral squamous cell carcinoma (OSCC) is the fourth major cause of mortality among men in Taiwan and its incidence rates continue to rise annually.\(^1\) OSCC is closely associated with tobacco consumption and the chewing of betel nut (Areca catechu), which is widely available on the island. Two-thirds of OSCC patients in Taiwan seek medical treatment at an advanced stage.\(^2\) Despite some progress in OSCC treatments (surgery is still the primary treatment), the overall 5-year survival rate for OSCC patients is 50%, which has not significantly changed over the past 2 decades.\(^3\) It was estimated that 20–50% of patients without clinically evident cervical lymph node metastases do in fact have microscopic metastases, which lead to poor prognoses.\(^4\)

It is, therefore, important to identify new diagnostic approaches and therapeutic targets for this deadly disease. Efforts to identify novel molecular predictors of behavior and therapeutic targets for OSCC have reported close relationships between several genetic and epigenetic alterations and the progressive development of OSCC.\(^5\)–\(^8\) Aberrant methylation of CpG islands in gene promoters is a common mechanism for suppressing gene expression in cancer cells. A process known as cytosine methylation can turn off the expressions of tumor-suppressor genes, just like a light switch, leading to oral cancer.\(^9\)–\(^14\)

Silencing of cancer-associated genes by hypermethylation of CpG islands within the promoter and/or 5’-regions of many genes is a common feature of human cancer and is often associated with a transcriptional block and loss of the relevant protein, which is analogous to genetic loss-of-function mutations, such as point mutations and deletions.\(^15\),\(^16\) Hypermethylation of the promoters of cancer-related genes is often associated with tumorigenesis and may also be involved in metastasis.\(^17\)–\(^21\) Previous studies of OSCC reported the hypermethylation of several genes involved in DNA repair [O6-methylguanine-DNA-methyltransferase (MGMT)], tumor suppression (p16), detoxification of xenobiotics [glutathione S-transferase P (GSTP1)], and apoptosis-related gene [death-associated protein kinase (DAPK)].\(^22\)–\(^24\)

Although a consensus has emerged on hypermethylation, present studies disagree on methylation rates.\(^24\)–\(^26\) Thus, two important questions are in order: to what degree does CpG island hypermethylation contribute to the genomic abnormalities in OSCC in Taiwan; and do hypermethylation events accumulate as cells progress toward metastasis? To detect the presence of neoplastic DNA methylation, we used a real-time quantitative methylation-specific polymerase chain reaction (MS-PCR) to analyze promoter hypermethylation rates of the p16, DAPK, MGMT, and GSTP1 genes in 64 OSCC tumors and corresponding adjacent normal mucosa. Twenty normal gingival mucosal samples obtained from healthy persons, who did not smoke or chew betel nut, served as a control group.

Materials and methods

Subjects

This study was approved by the institutional review board of Taichung Veterans General Hospital, Taichung, Taiwan. Sixty-four histologically confirmed OSCC tissues and corresponding nontumorous tissues were randomly obtained from patients in 1997–2004 from the Oral Maxillofacial Surgery Section of Taichung Veterans General Hospital. The detailed clinical data, including site and differentiation of the tumor and patient habits were carefully documented. Twenty healthy oral mucosa samples of the third molar region were obtained from patients who received impacted wisdom-tooth extraction. All samples were collected in accordance with institutional guidelines for the protection of human subjects. Fresh tumors obtained from surgical resection were immediately stored in liquid nitrogen until DNA extraction.

DNA extraction and purification

A tissue pellet was placed in genomic DNA isolation reagent (GenoType, Nehren, Baden-Württemberg, Germany) with proteinase K (0.5 mg/mL) at 50°C for 24 hours. Digested samples were subjected to DNA extraction using the Wizard Genomic DNA kit according to the protocol of the manufacturer (Promega, Madison, WI, USA).

Bisulfite treatment

DNA from tissue specimens was subjected to bisulfite treatment, as described previously.\(^24\),\(^33\)–\(^35\) Briefly, 2 μg of genomic DNA was re-suspended in 50 μL of water and denatured in 0.2M NaOH for 10 minutes at 37°C. The denatured DNA was diluted in 550 μL of a freshly prepared solution containing 10 mM hydroquinone (Sigma, St Louis, MO, USA) and 3M sodium bisulfite (Sigma; pH 5.0). The DNA solution was covered with mineral oil and incubated for 16 hours at 50°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System, Promega), treated with 0.3M NaOH for 5 minutes at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 20 μL H₂O and used immediately or stored at –70°C.

Methylation-specific polymerase chain reaction (MS-PCR)

The modified DNA served as a template for PCR amplification using primers specific for either methylated or modified unmethylated DNA. Because bisulfite treatment converts unmethylated cytosine to uracil while leaving methylated cytosine intact, specific primers were designed to accommodate these changes (Table 1). For the PCR amplification, 2 μL of bisulfite-modified DNA was added to a final volume of 25 μL PCR mix containing 1 unit FastStart Taq polymerase (Roche, California,USA), 1x PCR buffer, 2mM MgCl₂, 0.2mM dNTPs, and a primer (5μM each per reaction). All PCR amplifications were performed in a Peltier Thermal cycler (MJ Research, Massachusetts, USA). Reactants were first incubated at 95°C for 5 minutes, followed by 35 cycles of 95°C for 50 seconds, the specific annealing temperature for each gene for 50 seconds, and 72°C for 50 seconds, followed by a final 10-min extension at 72°C.\(^25\) PCR products were directly loaded onto
of the detoxifier gene, GSTP1, was not present in OSCC of 64 corresponding nontumorous tissues. Methylation detected in 60 (94.0%) of 64 OSCC tissues and in 45 (70.3%) of 64 corresponding nontumorous tissues. Aberrant methylation of at least one of these genes was observed in 67.2% and 45.3% of the OSCC tissue and in 31.3% and 40.6% of the corresponding nontumorous tissues. This indicates a higher disparity in p16 methylation rates between carcinoma tissues and corresponding nontumorous tissue was statistically significant (Table 2A).

**Correlation with clinicopathological data**

According to the clinical data we obtained, frequencies of p16, DAPK, and MGMT gene promoter hypermethylation did not differ based on the tumor site (P > 0.05). Methylation rates of the p16, DAPK, and MGMT promoter genes in OSCC tissues were 75%, 43.8%, and 25% for the buccal mucosa and 62.5%, 62.5%, and 37.5% for the tongue, respectively. Tumors that were nonmetastatic had higher percentages of MGMT and DAPK methylation than metastatic primary tumors. The percentages for the latter were 50.0 and 55.6, whereas those of the former were only 23.9 and 41.3. Hypermethylated p16 promoters were found in 63% of nonmetastasized tumors and in 77.8% of metastatic tumors (but was not statistically significant).

**Discussion**

Methylation is the main epigenetic modification in humans and changes in methylation patterns play important roles in tumorigenesis and metastasis. The MS-PCR technique enables precise mapping of methylation patterns in CpG islands of genomic DNA. This study found higher frequencies of overall hypermethylation in three genes (p16, MGMT, and DAPK) in primary tumors of oral cancer tissues.
than in adjacent tissues (70.3%). This result is similar to that of a study on Indian subjects, which found 87% of overall hypermethylation in the three genes of oral cancer tissues and 77% in adjacent tissues. Furthermore, our data showed that p16, DAPK, and MGMT hypermethylation rates in OSCC had CpG region frequencies of 67.2%, 45.3%, and 31.3% respectively, whereas p16, DAPK, and MGMT hypermethylation rates in adjacent tissues were 31.3%, 40.6%, and 25.0% respectively. The adjacent tissues represent clinically normal tissues with a high frequency of hypermethylation, which may indicate a molecular pathology, i.e., epigenetic aberrations and a relatively higher risk of progression toward malignant changes.

p16 is a tumor-suppressor gene that inhibits cyclin-dependent kinase 4 and 6 activities and arrests the cell cycle in the G1 phase. Aberrant methylation and mutation of p16/MTS1 in OSCC of patients was found in our previous study. Moreover, the frequency of hypermethylation of p16 from the present study was 67.2%. This frequency is much higher than that in other countries such as the US and Japan, the frequencies of which are in the range of 21–47%. However, the hypermethylation frequency we

Table 2 Aberrant methylation patterns in different tissues.

<table>
<thead>
<tr>
<th>Tissue specimens</th>
<th>Gene</th>
<th>p16</th>
<th>DAPK</th>
<th>MGMT</th>
<th>GSTP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td>0% (0/20)</td>
<td>0% (0/20)</td>
<td>0% (0/20)</td>
</tr>
<tr>
<td>Adjacent normal tissue</td>
<td>31.3% (20/64)</td>
<td>40.6% (26/64)</td>
<td>25% (16/64)</td>
<td>0% (0/64)</td>
<td></td>
</tr>
<tr>
<td>OSCC</td>
<td>67.2% (43/64)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.3% (29/64)</td>
<td>31.3% (20/64)</td>
<td>0% (0/64)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSCC (nonmetastatic)</td>
<td>63.0% (29/46)</td>
<td>41.3% (19/46)</td>
<td>23.9% (11/46)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OSCC (metastatic)</td>
<td>77.8% (14/18)</td>
<td>55.6% (10/18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50% (9/18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The methylation rate of carcinoma tissues and corresponding nontumorous tissue is statistically significant P < 0.05.

<sup>b</sup> The methylation rate of carcinoma tissues with metastasis and nonmetastasis is statistically significant P < 0.05.

DAPK = death-associated protein kinase; MGMT = O6-methylguanine-DNA-methyltransferase; OSCC = oral squamous cell carcinoma.
found is strikingly similar to the p16 frequency of methylation of 66.7% among Indian subjects. These may be caused by ethnic and gender differences. The loss of p16 through p16 promoter methylation in our patients may have caused loss of function during tumor development. The hypermethylation of p16 of the primary tumor was also found to have slightly increased the metastasis rate in our cancer patients, but we could not establish statistical significance. This finding concurs with that of Maruya, who reported that p16 is less likely to be related to lymph node metastasis. We believe that promoter hypermethylation of the DAPK gene can cause potential metastasis in OSCC primary tumors.

Promoter hypermethylation of DAPK was also found in 45.3% of primary OSCC tissues in our study. This protein is a mediator of apoptosis induced by interferon-γ. Our finding is similar to that of Sanchez-Cespedes et al., who observed a positive correlation between the methylation of DAPK and the presence of lymph node metastases in patients with head and neck cancer. We believe that promoter hypermethylation of the DAPK gene can cause potential metastasis in OSCC primary tumors.

Hypermethylation of MGMT in tumors with metastasis was observed in our study. MGMT is a DNA repair protein that removes adducts from O(6)alkyl G in DNA. If not removed, adducts are mispaired with T during DNA replication, resulting in G-to-A mutations. Thus, loss of MGMT will cause increased mutation rates. Hypermethylation of MGMT is closely associated with point mutations of K-ras at codons 12 and 13, lymph node invasion, tumor stage, and disease-free survival. We found a significant correlation between the methylation of MGMT and the development of metastases.

The promoter region of the detoxifying gene, GSTP1, is commonly hypermethylated in the promoter region in prostate adenocarcinoma and hepatocellular carcinoma, but it was not hypermethylated in either oral cancer tissue, adjacent mucosa, or normal oral mucosa, as previously reported. The regulation of GSTP1 expression in OSCC might not occur through promoter methylation.

In summary, we concluded from our analysis that a significant statistical correlation exists of aberrant promoter hypermethylation patterns of one or more cancer-related genes in primary tumors of oral carcinogenesis and metastatic lesions. Our data support a number of recent studies that detected gene promoter hypermethylation in the serum and sputum of patients with head and neck tumors. Promoter hypermethylation may thus be useful as a tumor marker for early diagnosis and disease monitoring.

### Table 3

<table>
<thead>
<tr>
<th>Number of case (n)</th>
<th>Gene methylation</th>
<th>Sign.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p16 No. (%)</td>
<td>DAPK No. (%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58 (65.5)</td>
<td>25 (43.1)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (83.3)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>37 (67.6)</td>
<td>17 (45.9)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>27 (66.7)</td>
<td>10 (37.0)</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>32 (75.0)</td>
<td>14 (43.8)</td>
</tr>
<tr>
<td>Tongue</td>
<td>18 (62.5)</td>
<td>15 (62.5)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (62.5)</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>6 (66.7)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Well-Mod</td>
<td>18 (50.0)</td>
<td>6 (38.9)</td>
</tr>
<tr>
<td>Mod</td>
<td>34 (76.5)</td>
<td>17 (50.0)</td>
</tr>
<tr>
<td>Mod-poor, poor</td>
<td>6 (66.7)</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Betel nut</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>50 (68.0)</td>
<td>18 (36.0)</td>
</tr>
<tr>
<td>–</td>
<td>14 (71.4)</td>
<td>10 (71.4)</td>
</tr>
<tr>
<td>Smoke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>50 (66.0)</td>
<td>20 (40.0)</td>
</tr>
<tr>
<td>–</td>
<td>14 (71.4)</td>
<td>7 (50.0)</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>40 (67.5)</td>
<td>18 (45.0)</td>
</tr>
<tr>
<td>–</td>
<td>14 (71.4)</td>
<td>5 (35.7)</td>
</tr>
</tbody>
</table>

DAPK = death-associated protein kinase; MGMT = O6-methylguanine-DNA-methyltransferase; No. = number; NS = nonsignificant at <0.05 level of significance for each of the genes; Sign. = significance.
Acknowledgments

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References


