Promoter Hypermethylation Patterns of p16, O6-Methylguanine-DNA-methyltransferase, and Death-associated Protein Kinase in Tumors and Saliva of Head and Neck Cancer Patients

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Abstract

Aberrant promoter hypermethylation is common in head and neck cancer and may be useful as a marker for cancer cells. We examined whether cells with tumor-specific aberrant DNA-methylation might be found in the saliva of affected patients. We tested 30 patients with primary head and neck tumors using methylation-specific PCR searching for promoter hypermethylation of the tumor suppressor gene p16 (CDKN2A), the DNA repair gene O6-methylguanine-DNA-methyltransferase (MGMT) and the putative metastasis suppressor gene death-associated protein kinase (DAP-K). Aberrant methylation of at least one of these genes was detected in 17 (56%) of 30 head and neck primary tumors; 14 (47%) of 30 at p16, 10 (33%) of 30 at Dap-K and 7 (23%) of 30 at MGMT. In 11 (65%) of 17 methylated primary tumors abnormal methylated DNA was detected in the matched saliva samples. Abnormal promoter methylation in saliva DNA was found in all tumor stages and more frequently in tumors located in the oral cavity. Moreover, none of the saliva from patients with methylation-negative tumors displayed methylation of any marker. Of 30 saliva samples from healthy control subjects (15 smokers and 15 nonsmokers), only one sample from a smoking patient was positive for DNA methylation at two target genes. Detection of aberrant promoter hypermethylation patterns of cancer-related genes in saliva of head and cancer patients is feasible and may be potentially useful for detecting and monitoring disease recurrence. Long-term longitudinal studies are needed to evaluate this approach for early detection of head and neck cancer in at-risk populations.

Introduction

Head and neck cancer, among the 10 most frequent cancers in the world, is common in certain regions and in patients with high tobacco use and alcohol consumption. Although scientific discovery has given us new insights into the etiology of the disease, it is estimated that 30,200 new cases of head and neck cancer will be diagnosed, and that there will be 7,800 deaths from the disease in the United States this year (1). In addition, the overall survival of patients with head and neck cancers has not markedly improved over the past 30 years. It is, therefore, important to identify new diagnostic approaches and therapeutic targets for this deadly disease (2).

An increasingly important pathway of transcriptional inactivation for many tumor suppressor genes is CpG islands hypermethylation within gene promoter regions (3). One important example is the gene encoding cyclin-dependent kinase inhibitor, p16INK4a (also known as CDKN2A), which is critical in the cyclinD-Rb pathway for maintaining the retinoblastoma (Rb) protein in its active, nonphosphorylated state (4). Control of cell cycling by this pathway is lost in virtually all tumors, either through disrupted p16INK4a function or mutations of Rb, but generally not both (4). Loss of p16INK4a occurs commonly by deletion (5–6), promoter hypermethylation (7–8) and more rarely by point mutation (5). p16 inactivation by promoter hypermethylation or other genetic alterations is seen in almost all head and neck tumors (9–13).

In addition to inactivation of classic tumor suppressor genes, additional genes have been found to be down-regulated by promoter hypermethylation and are implicated in tumor progression. One such example is MGMT, a DNA repair gene for guanosine methyl adducts that is frequently inactivated in lymphomas and in colon, lung, and brain tumors (14). Another example is DAP-K, which is a novel serine/threonine kinase whose expression is required for IFN-γ-induced apoptosis. It has been shown that DAP-K is also a potential metastasis inhibitor and is frequently altered in B-cell lymphomas and in lung cancer (15).

Previous studies have shown that senescent tumor cells may release DNA into the circulation, which is subsequently carried by and enriched in serum or plasma (16, 17). These studies have shown that it is possible to identify tumor-specific alterations, such as loss of heterozygosity (LOH) and microsatellite instability (MI) in the plasma and serum DNA of patients with head and neck carcinoma (10) and small cell lung carcinoma (18). Recent publications have demonstrated the presence of promoter hypermethylation in the serum DNA of lung (19), liver (20), breast (21), and head and neck cancer patients (11). In addition, genetic changes identified in tumors can also be detected in bodily fluids in direct contact with the neoplastic tissue, including the urine of bladder cancer patients (22).

Saliva is a readily obtained body fluid that contains cells shed from the mucosal lining of the mouth and throat. We undertook to study whether tumor-specific alterations could also be detected in the saliva of head and neck cancer patients. We studied the promoter hypermethylation pattern of the p16, MGMT, and DAP-K genes in tumor DNA of 30 head and neck primary tumors and paired saliva samples. We found promoter hypermethylation of one or more target genes in primary tumors and matched saliva DNA in many cases. Among 30 controls without cancer, only one smoking patient manifested any of these molecular markers in saliva.

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3The abbreviations used are: MGMT, O6-methylguanine-DNA methyltransferase; DAP-K, death-associated protein kinase; MSP, methylation-specific PCR.
**Materials and Methods**

**Sample Collection and DNA Extraction.** Thirty primary tumors were collected from patients with diagnosed head and neck tumors between the years 1997 and 1999 at the Johns Hopkins University School of Medicine. Fresh tumors were obtained from surgical resection of the head and neck cancer patients. Saliva samples were collected by rinsing/gargling with normal saline from the same patients at diagnosis and stored in −80°C. Saliva from 30 healthy control subjects including 15 nonsmokers and 15 smokers were also collected. Tumor DNA was prepared as described previously (10). Saliva samples were subjected to centrifugation at 2500 rpm for 15 min. The supernatant was discarded, and the cell pellet was retained and placed in 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 72 h. Digested sample was subjected to phenol-chlorophorm extraction and ethanol precipitation (10). The Johns Hopkins Joint Committee or Clinical Investigation approved the study, and informed consent was obtained from all subjects.

**Bisulfite Treatment.** DNA from tumor and saliva specimens was subjected to bisulfite treatment, as described previously (23). Briefly, 1 μg of genomic DNA was resuspended in 50 μl of water and denatured in 0.2 M NaOH for 10 min at 37°C. The denatured DNA was then diluted in 550 μl of a freshly prepared solution containing 10 mM hydroquinone (Sigma) and 3 M sodium bisulfite (Sigma; pH 5.0). The DNA solution was covered with mineral oil and incubated for 16 h at 50°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System, Promega), treated with 0.3 M NaOH for 5 min at room temperature and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 28 μl of H₂O and used immediately or stored at −70°C.

**MSP.** The modified DNA was used as a template for PCR amplification using primers specific for either methylated or the modified unmethylated DNA. Because the bisulfite treatment converts unmethylated cytosines to uracil but leaves methylated cytosines intact, specific primers were designed to accommodate these changes. The primer sequences for p16 (23), DAP-K (19) and MGMT (14) were described previously. The primers used for the unmethylated reaction were: for p16, 5'-TATATAGGGTGGTGATGTGTTTTGG (sense) and 5'-CAACCAAGACCAAGCAACATC (antisense); for DAP-K, 5'-GGAGGATGATGGTTAGTATGTTTTGG (sense) and 5'-CAATCCATTCCAAACACCA-3' (antisense); and for MGMT, 5'-TTTAGTTTGGTTAGTTTGTAGTTTTGTGTT (sense) and 5'-AATCTCCACACCTTCTTCCAAAACAACAAA-3' (antisense). The primers for the methylated reaction were: for p16, 5'-TATATAGGGTGGGGCGGATCGC (sense) and 5'-TGTTTTGATGTTTGTAGGTTTTTGT (antisense); for DAP-K, 5'-GGAGGATGATGGTTAGTATGTTTTGG (sense) and 5'-TTTAGTTTGGTTAGTTTGTAGTTTTGTGTT (antisense); and for MGMT, 5'-TTTAGTTTGGTTAGTTTGTAGTTTTGTGTT (sense) and 5'-AATCTCCACACCTTCTTCCAAAACAACAAA-3' (antisense).

For PCR amplification, 2–3 μl of bisulfite-modified DNA was added in a final volume of 50 μl PCR mix containing 1X PCR buffer (16.6 mM ammonium sulfate-67 mM Tris (pH 8.8)/6.7 mM MgCl₂/10 mM 2-mercaptoethanol), and visualized under UV illumination.

**Statistical Analysis.** Statistical analyses were performed using the SigmaStat 1.02. The association between the discrete variables was assessed using Fisher’s exact test. Differences were considered statistically significant for P < 0.05.

**Results**

Thirty head and neck cancer patients and 30 controls without cancer (15 smokers and 15 nonsmokers) were entered into the study. We found that 56% (17 of 30) of head and neck primary tumors exhibited aberrant promoter hypermethylation in at least one of the genes studied (Table 1), a similar percentage to that previously described in head and neck cancer (11). The tumor suppressor gene p16 was hypermethylated in 47% (14 of 30) of the tumors and was found in all of the stages. The incidence of DAP-K promoter hypermethylation was 33% (10 of 30 tumors) and MGMT promoter hypermethylation was present in 23% (7 of 30) of the tumors. The incidence of hypermethylation of each gene was consistent with previous work in head and neck primary tumors (11, 12).

Sixty-five percent (11 of 17) of the patients showing hypermethylation in the tumor DNA also demonstrated abnormal methylation in saliva DNA (Table 1). Representative MSP analyses for p16, DAP-K, and MGMT in tumor and paired saliva are shown in Fig. 1. We found that the frequency of promoter hypermethylation detection in saliva DNA was similar for each gene: 79% (11 of 14) of cases for p16, 60% (6 of 10 cases) for DAP-K, and 50% (4 of 7 cases) for MGMT (Table 2). Each of the 11 patients who had abnormal promoter hypermethylation in saliva DNA harbored identical alterations in the primary tumor DNA (Table 1). Conversely, only six samples showed aberrant methylation in the tumors that was not detected in saliva (Table 1).

In the control group, we found normal promoter hypermethylation in only one of the 30 saliva samples. This patient was from the smoking group and displayed promoter hypermethylation at both p16 and MGMT, but had no evidence of oral cancer. After abnormal promoter hypermethylation analysis of all of the samples was completed, clinical data were correlated with the results. The presence of hypermethylation occurred in all of the stages. Detection of abnormal methylation in saliva DNA appeared to be related to the site of the primary tumor. We found a higher rate of abnormal hypermethylation in saliva DNA (8 of 10 patients) with oral cavity primary tumors compared with other sites (3 of 20; P < 0.001; Fisher’s exact test).

**Discussion**

Previous studies have identified several tumor-specific genetic alterations in the plasma and serum DNA of cancer patients. These...
genetic alterations have been successfully used as indicators of tumor burden in lung and head and neck cancer patients (11, 19). Recently, saliva has shown promise in the early diagnosis of dental caries and periodontal disease (24, 25). Tumor-specific p53 mutations and microsatellite alterations were previously identified in the saliva of head and neck patients (13, 26). However, the large number of individual p53 mutations limits the usefulness of oncogene mutation detection in cost-effective early-detection approaches. We thus decided to study whether epigenetic alterations, specifically abnormal promoter hypermethylation, could be easily detected in the saliva of head and neck cancer patients.

Methylation is the main epigenetic modification in humans and changes in methylation patterns play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands in the promoter regions of many tumor suppressor genes correlates with loss of expression (3). Recently, the development of the sensitive MSP technique (23) has simplified the study of genes inactivated by promoter hypermethylation in human cancer. The advantages of MSP are its simplicity, its specificity for each gene, and its high sensitivity, allowing detection of 1 methylated gene copy in 1000 unmethylated copies (23). In contrast to many other methods of mutation detection, this assay is cost-effective and is easy to carry out, allowing the study of multiple markers by rapid analysis. Moreover, the MSP method in our study demonstrated great specificity, because we did not find any abnormal methylation in saliva DNA if the same alteration was not present in the primary tumor (Table 1). Although we found abnormal promoter hypermethylation in the saliva of one smoker, we attribute this result to the fact that both genes can be altered early in carcinogenesis. This patient may thus harbor an abnormal clone of cells detected by the assay. Whether this clone will progress to preneoplasia or cancer is a matter of speculation. Our study demonstrates for the first time, that it is possible to detect abnormal promoter hypermethylation in saliva DNA from head and neck cancer patients using MSP. This assay allows sensitive and accurate detection of tumor DNA in saliva of circulating DNA and may be potentially useful for detecting and monitoring recurrence in patients with head and neck cancer. Interestingly, saliva detection appeared particularly useful for oral cavity tumors. The timing of aberrant hypermethylation needs to be studied in the natural progression from preneoplasia to cancer. Retrospective and longitudinal studies also need to be used to assess the value of this approach. Nevertheless, molecular analysis of saliva may one day offer a cost-effective approach for detection of head and neck cancer in at-risk populations.

Table 2 Gene promoter hypermethylation in primary head and neck tumors and saliva

<table>
<thead>
<tr>
<th>Gene aberrantly methylated</th>
<th>Tumor positive/ total number (%)</th>
<th>Saliva positive/ total methylated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>14/30 (47)</td>
<td>11/14 (79)</td>
</tr>
<tr>
<td>DAP-K</td>
<td>10/30 (33)</td>
<td>6/10 (60)</td>
</tr>
<tr>
<td>MGMT</td>
<td>7/30 (23)</td>
<td>4/7 (50)</td>
</tr>
<tr>
<td>Any</td>
<td>17/30 (56)</td>
<td>11/17 (65)</td>
</tr>
</tbody>
</table>

References


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