Promoter Hypermethylation of Multiple Genes in Carcinoma of the Uterine Cervix

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ABSTRACT

Promoter hypermethylation is an important pathway for the repression of gene transcription in cancer. We investigated promoter hypermethylation of six genes, p16, APC, HIC-1, death-associated protein kinase (DAPK), O6-methylguanine-DNA-methyltransferase (MGMT), and E-cadherin, in uterine cervical carcinoma from 53 patients including 31 cases of squamous cell carcinoma (SCC) and 22 cases of adenocarcinoma (AC). Aberrant methylation of at least one of these genes was detected in 79% (42 of 53) of cases including 71% (22 of 31) of SCC and 91% (20 of 22) of AC cases. No aberrant methylation was detected in normal cervical tissue from 24 control hysterectomy specimens. There was no correlation between promoter hypermethylation at any gene and the presence of human papillomavirus-16 or -18 E7 DNA. In AC cases, promoter hypermethylation of the APC and HIC-1 genes was detected at a statistically significant higher frequency than in the SCC cases (APC, 60% versus 13%, P < 0.001; HIC-1, 63% versus 32%, P < 0.03). Conversely, promoter hypermethylation of p16 and DAPK was more common in SCC cases than in AC cases. Our results suggest that promoter hypermethylation is a frequent epigenetic event in cervical carcinoma. The pattern of gene promoter hypermethylation is distinctly different between AC and SCC. The absence of these epigenetic alterations in normal cervical tissue suggests that they may also be valuable as cancer markers.

INTRODUCTION

Cancer of the cervix is the second most common cancer in women worldwide (1). Multiple epidemiological studies indicate that HPV infection is frequently detected in cervical intraepithelial neoplasia and invasive cervical carcinoma (2). There is a strong association between certain subtypes of the HPV and cervical carcinoma (3). The E6 and E7 genes of oncogenic HPV-16 and -18 are known to play an important role in the early stages of malignant transformation and immortalization of cervical epithelial cells (3–5). Inactivation of the p53 and Rb gene products by the E6 and E7 proteins, respectively, is an important process in maintaining abnormal cellular proliferation by inactivating normal cell cycle checkpoints (5–7). In addition to HPV infection, it is clear that other factors are also involved in cervical carcinogenesis because the majority of patients with HPV-associated lesions do not progress to invasive cancer. Therefore, it is important to identify other genetic and epigenetic events in cervical carcinogenesis.

In the development of cancer, tumor suppressor genes are inactivated by a number of processes including point mutation and chromosomal deletion. Numerous studies have reported loss of heterozygosity in different chromosomal regions, suggesting the common inactivation of tumor suppressor genes in cervical carcinoma (8–11). In addition to chromosomal deletions, epigenetic silencing of tumor suppressor genes by promoter hypermethylation is also commonly seen in human cancer (12). Aberrant methylation of CpG islands within the promoter regions of tumor suppressor genes such as p16, VHIL, E-cadherin, APC, and hMLH1 has been identified in association with loss of protein expression in cancer cells (13–17). Several tumor types have also shown aberrant methylation at CpG islands in other genes, including the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT; Ref. 18), the potential metastasis inhibitor gene death-associated kinase (DAPK; Ref. 19), and the candidate tumor suppressor HIC-1 (20).

On the basis of these observations, we examined the promoter methylation status of the p16, DAPK, MGMT, APC, HIC-1, and E-cadherin genes in uterine cervical carcinoma and normal cervical tissues from patients without cancer using the MSP approach. We found frequent promoter hypermethylation of these genes related to tumor histology, but we did not find an association of methylation status with HPV infection.

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3 The abbreviations used are: HPV, human papillomavirus; SCC, squamous cell carcinoma; AC, adenocarcinoma; MSP, methylation-specific PCR.
MATERIALS AND METHODS

Sample Collection and DNA Extraction. Fifty-three tumor samples were collected from cases of invasive cervical carcinoma resected at the Samsung Cheil Hospital (Seoul, Korea) with institutional review board approval. There were 31 SCCs and 22 ACs. Twenty-four control samples of normal cervical hysterectomy specimens from patients without cancer at this institution were also analyzed. The histological type and grade of tumors were classified according to the WHO criteria. The stage of each cancer was established according to the International Federation of Gynecology and Obstetrics criteria. Paraffin-embedded primary tumor tissue and control samples were prepared from H&E-stained 10-μm-section slides. Tumor tissue was selected from an area with >75% malignant cells. DNA was purified by phenol-chloroform extraction and ethanol precipitation and dissolved in 50 μl of distilled water as described previously (21).

Bisulfite Modification. One μg of genomic DNA was denatured with NaOH. The bisulfite treatment (during which methylated DNA is protected, and unmethylated cytosine is converted to uracil) was carried out for 16 h at 50°C on denatured genomic DNA as described by Herman et al. (22). DNA samples were then purified using the Wizard DNA Clean-Up System (Promega, Madison, WI), treated again with NaOH, ethanol-purified, and resuspended in water.

MSP and Direct Sequencing. The modified DNA was used as a template for MSP using primers specific for either the methylated or the modified unmethylated sequences (22). Appropriate negative and positive controls were included in each PCR reaction. Primer sequences for the p16 (22), MGMT (18), DAPK (19), and E-cadherin (22) genes were described previously. The primer sequences of HIC-1 gene promoter for the unmethylated reaction were 5′-TTGGTTTGTGTTTTTGTGTTTTTG-3′ (sense) and 5′-CACCCTAACACCACTTAAC-3′ (antisense), which amplify a 118-bp product. The primer sequences of HIC-1 gene promoter for the methylated reaction were 5′-TCGGTTTTCGCGTTTTGTTCGT-3′ (sense) and 5′-AACCGAAAATCTAACCCTTG-3′ (antisense), which amplify a 95-bp product. The 5′ position of the sense unmethylated and methylated primers corresponds to bp 20 and 26 of GenBank accession number L41919, respectively, and −617 and −611 relative to the HIC-1 major transcription start site, respectively. The primer sequences of APC gene promoter 1A for the unmethylated reaction were 5′-ATTATTTGTGATGTGGATTAGGT-3′ (sense) and 5′-ACCTCATATCATCTACATACACATACA-3′ (antisense), which amplify a 89-bp product, and the primer sequences of APC gene promoter 1A for the methylated reaction were 5′-CGTTGATGCTGCCAGTGAGTCCGGCC-3′ (sense) and 5′-CTCTATCGATCATGTGACG-3′ (antisense), which amplify a 84-bp product. The 5′ position of the sense unmethylated and methylated primers corresponds to bp 748 and 751 of GenBank accession number U02509, respectively, and −118 and −115 relative to the APC major transcription start site, respectively.

Step-down PCR reactions were performed in a 25 μl reaction volume, using previously published conditions (22) and 1.25 units of Ampli Taq polymerase (Perkin-Elmer, Branchburg, NJ) complexed with Taq start antibody (Clontech, Palo Alto, CA). Reactions were hot-started at 95°C for 5 min. This was followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and concluded at 72°C for 5 min. Reactions were analyzed on Spredex EL 400 gels in the SEA 2000 Apparatus System (Elchrom Scientific, Charm, Switzerland), stained with SYBR Gold (Molecular Probes, Eugene, OR), and visualized under UV illumination.

After detection of a PCR product with methylated primers for APC and HIC-1, direct PCR sequencing reactions were performed on four samples using the Amplicycle Sequencing Kit (Perkin-Elmer). We used two cases of AC and two cases of SCC. For the direct sequence analysis, in four of four cases, all of the CpG sites (11 of APC and 15 of HIC-1) in each amplified segment were found to be methylated (data not shown).

HPV E7 Type-specific PCR. Purified genomic DNA was amplified by PCR for the HPV-16 and HPV-18 E7 genes as well as for an internal reference gene, β-globin. Oligonucleotide primers were as follows: (a) HPV-16 E7, forward primer 5′-ATTATTAATGACAGCTCAGAGGA-3′ and reverse primer 5′-GCTTTGTACGCACAACTGAAACG-3′; (b) HPV-18 E7, forward primer 5′-AAGAAACGATGAAATAGTGGAA-3′ and reverse primer 5′-GGCTTCACACATCAACACA-3′; and (c) β-globin, forward primer 5′-GAAGGCGAAGGACAGG-TAC-3′ and reverse primer 5′-CAACTTCATACCGTTACC-3′. DNA made from confluent culture of the human cervical carcinoma cell lines CaSk (HPV-16) and HeLa (HPV-18; American Type culture Collection, Manassas, VA) was used as a positive control.

Each PCR reaction was performed under standard conditions in a total volume of 20 μl containing 5 μl of template DNA, 2.5 μM each primer, 50 μM deoxynucleotide triphosphate, 3.75 mM MgCl2, 1.25 units of Ampli Taq Gold (Perkin-Elmer), and 2 μl of 10× PCR Gold Buffer (Perkin-Elmer). Reactions were denatured for 10 min at 95°C and incubated for 40 cycles (95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C). The reaction product was analyzed using the same system used in MSP.

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

RESULTS

We searched for the presence of promoter hypermethylation at six genes (p16, APC, HIC-1, DAPK, MGMT, and E-cadherin) in cervical cancer and normal tissue (Fig. 1). Abrupt promoter hypermethylation at any of these genes studied was detected in 79% (42 of 53) of cervical carcinoma cases. These alterations were detected in 91% (20 of 22) of AC cases and 71% (22 of 31) of SCC cases (Table 1). Promoter hypermethylation of p16 was detected in 30% (16 of 53) of all cases, and DAPK promoter hypermethylation was very common in SCC [61% (19 of 31)]. APC promoter hypermethylation was detected in 32% (17 of 53) of all cases including 13 of 22 (59%) AC cases. HIC-1 promoter hypermethylation was present in 45% (24 of 53) of all cases and was also very frequent [64% (14 of 22)] in AC cases. MGMT promoter hypermethylation and E-cadherin promoter hypermethylation were less common (Table
1. Promoter hypermethylation of the \textit{p16} and \textit{DAPK} genes was detected more frequently in SCC cases than in AC cases (\textit{p16}, 39\% versus 18\%; \textit{DAPK}, 61\% versus 36\%; \textit{P} < 0.05). In AC cases, promoter hypermethylation of the \textit{APC} and \textit{HIC-1} genes was detected at a significantly higher frequency than in the SCC cases (\textit{APC}, 60\% versus 13\% (\textit{P} < 0.001); \textit{HIC-1}, 63\% versus 32\% (\textit{P} < 0.03)). None of the 24 normal cervical tissues demonstrated methylation at any gene.

Table 1  Methylation of multiple genes in uterine cervical carcinoma

<table>
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<tr>
<th>Characteristics</th>
<th>\textit{n}\textsuperscript{a}</th>
<th>\textit{DAPK}</th>
<th>\textit{HIC-1}</th>
<th>\textit{APC}</th>
<th>\textit{p16}</th>
<th>\textit{E-cadherin}</th>
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<tr>
<td>SCC</td>
<td>31</td>
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<td>60%\textsuperscript{d}</td>
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<td>45%</td>
<td>36%</td>
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<td>45%</td>
<td>32%</td>
<td>30%</td>
<td>28%</td>
<td>8%</td>
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\textsuperscript{a} n, number of cases examined.
\textsuperscript{b} P < 0.05, Fisher’s exact test.
\textsuperscript{c} P < 0.03, Fisher’s exact test.
\textsuperscript{d} P < 0.001, Fisher’s exact test.

The 53 cases of cervical carcinoma were also analyzed for the presence of oncogenic HPV DNA using a PCR-based method with type-specific (HPV-16 or -18) oligonucleotide PCR primers. HPV E7 DNA was detected in 91\% (48 of 53) of cases: HPV-16 E7 DNA was detected in 64\% (34 of 53) of cases, and HPV-18 E7 DNA was detected in 26\% (14 of 53) of cases. We found significant differences in the frequency and subtype of HPV infection between SCC and AC (Table 2). In SCC, HPV-16 E7 DNA was detected in 25 of 31 (81\%) tumors, but in AC, HPV-16 E7 DNA was detected in only 9 of 22 (41\%) cases (\textit{P} < 0.004). HPV-18 E7 DNA was detected in only 1 of 31 (3\%) SCC cases and 13 of 22 (59\%) AC tumors (\textit{P} < 0.001), a percentage similar to that described previously in cervical cancer (23). There was no association between methylation status of any gene (or group of genes), HPV infection, or age.

DISCUSSION

Even with extensive screening programs, cervical cancer is still among the five most common causes of cancer deaths in women (1, 24). Epidemiological and laboratory data suggest that the presence of specific HPV subtypes and the integration of their HPV DNA into the genome are important factors in the development of cervical neoplasia (25–27). The discovery and molecular cloning of HPV-16 and -18 from cervical cancers and the subsequent demonstration of these viral DNAs in cervical carcinomas suggest an etiological role of these viruses in carcinogenesis (28). However, because the majority of patients with HPV infection do not develop invasive lesions, HPV infection alone is probably insufficient for complete neoplastic transformation of cervical cells, suggesting the involvement of
other genetic and epigenetic events in cervical carcinogenesis. It is well known that accumulation of molecular changes leads to malignant transformation of normal cells through dysplasia and carcinoma in situ to invasive tumors (29). However, the sequence of molecular events responsible for cervical carcinogenesis has not yet been elucidated.

DNA methylation is an epigenetic modification essential to mammalian development. However, aberrations in DNA methylation including widespread hypomethylation and more focal hypermethylation have been consistently detected in human tumor and transformed cell lines (30–32). Hypermethylation of normally unmethylated CpG islands in the promoter regions often occurs in important tumor suppressor genes such as VHL, hMLH1, E-cadherin, APC, and p16 (13–17). This study is among the first general surveys of promoter hypermethylation in cervical carcinoma. Wong et al. (33) evaluated 98 cervical carcinomas and detected methylation of p16 in 31% of tumors. In a study by Nuovo et al. (34), hypermethylation-induced inactivation of the p16 gene was reported as an early event in uterine cervical carcinoma. Recently, promoter hypermethylation has been identified and associated with loss of expression of many potentially interesting genes in cancer cells. These include the DNA repair gene MGMT, which is frequently inactivated in lymphoma and brain, colorectal, and lung cancers (19); the potential metastasis inhibitor DAPK gene altered in leukemia, and lung cancer (19, 35); and the candidate tumor suppressor HIC-1 gene inactivated in leukemia and breast cancer (36, 37).

Here, we analyzed the methylation pattern of promoter regions of six tumor-related genes in the same cohort of cervical carcinoma patients using MSP. Promoter hypermethylation was seen most commonly in DAPK and HIC-1 and less commonly in the other tested genes. Overall, 79% of cervical tumors showed promoter hypermethylation at one or more genes. Moreover, hypermethylation was not limited to a single target but was often found in multiple genes in each patient studied. Interestingly, the subset of genes methylated varied in each patient. In fact, 33 of 53 (62%) cervical cancers displayed concordant methylation in at least two genes, and 9 of 53 (17%) cervical cancers harbored hypermethylation status in only one gene. It is clear that aberrant hypermethylation of promoter regions is not confined to a single target gene in cervical carcinoma but rather occurs concurrently in many loci (including untested genes) in an individual patient.

By studying the methylation profile of several target genes for the AC and SCC subtypes of cervical carcinoma, we found that methylation of p16 and DAPK was detected in AC at a lower frequency than in SCC (Table 1), whereas promoter hypermethylation of APC and HIC-1 was present at a significantly higher frequency in AC than in SCC (Table 1). The pattern of promoter hypermethylation is distinctly different between AC and SCC of the cervix, suggesting a distinct pathway of progression for these different tumor types. Epigenetic silencing of p16 leads to absence of the protein and is analogous to homozygous deletion and/or point mutation providing the cell with a selective growth advantage (13). Because Rb and p16 inactivations are inversely correlated in most tumor types, it is tempting to speculate that HPV infection provides an additional growth advantage predominantly by inactivation of p53 through the E6 protein. Although we could not test for RNA expression directly in these paraffin-embedded tissues, it is likely that promoter hypermethylation of the other genes tested also led to inactivation of critical pathways as described previously in cell lines and fresh tumors (12–15, 18, 19, 35, 38). In this study, we have demonstrated that promoter hypermethylation (often with HPV infection) is a frequent epigenetic event in cervical carcinoma. The fact that multiple genes are frequently methylated suggests that the mechanism that normally protects CpG islands from methylation may be defective in cancer cells. The epigenetic alterations found in our cancer cases were absent in normal tissue, suggesting that they may also be useful as cancer-specific markers (19, 39–42).

### Table 2 Prevalence of HPV-16 and HPV-18 in uterine cervical carcinoma

<table>
<thead>
<tr>
<th>HPV Type</th>
<th>SCC</th>
<th>AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16 E7</td>
<td>81% (25/31)*</td>
<td>41% (9/22)</td>
</tr>
<tr>
<td>HPV-18 E7</td>
<td>3% (1/31)</td>
<td>59% (13/22)*</td>
</tr>
</tbody>
</table>

* P < 0.004, Fisher’s exact test, SCC versus AC.

* P < 0.001, Fisher’s exact test, SCC versus AC.

**REFERENCES**


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