Aberrant Methylation during Cervical Carcinogenesis

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ABSTRACT

We studied the pattern of aberrant methylation during the multistage pathogenesis of cervical cancers. We analyzed a total of 73 patient samples and 10 cervical cancer cell lines. In addition, tissue samples [peripheral blood lymphocytes (n = 10) and buccal epithelial cells (n = 12)] were obtained from 22 healthy volunteers. On the basis of the results of preliminary analysis, the cervical samples were grouped into three categories: (a) nondysplasia/low-grade cervical intraepithelial neoplasia (CIN; n = 37); (b) high-grade CIN (n = 17); and (c) invasive cancer (n = 19). The methylation status of six genes was determined (p16, RARβ, FHIT, GSTP1, MGMT, and hMLH1). Our main findings are as follows: (a) methylation was completely absent in control tissues; (b) the frequencies of methylation for all of the genes except hMLH1 were >20% in cervical cancers; (c) aberrant methylation commenced early during multistage pathogenesis and methylation of at least one gene was noted in 30% of the nondysplasia/low-grade CIN group; (d) an increasing trend for methylation was seen with increasing pathological change; (e) methylation of RARβ and GSTP1 were early events, p16 and MGMT methylation were intermediate events, and FHIT methylation was a late, tumor-associated event; and (f) methylation occurred independently of other risk factors including papillomavirus infection, smoking history, or hormone use. Although our findings need to be extended to a larger series, they suggest that the pattern of aberrant methylation in women with or without dysplasia may help identify subgroups at increased risk for histological progression or cancer development.

INTRODUCTION

Carcinoma of the cervix is the leading gynecological malignancy worldwide and the third most common gynecological malignancy in the United States (1). HPV3 is the major causative agent, and >90% of cervical squamous carcinomas and 50% of adenocarcinomas contain HPV DNA (2, 3). However, it is estimated that 20–30 million Americans harbor HPV DNA, and only a small subset will develop cervical cancers (2). As with other epithelial malignancies, cervical cancer is preceded by a number of histopathological changes (4). Precursor lesions are divided into CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia), and CIN 3 (severe dysplasia and carcinoma in situ; Ref. 4). Initially it was believed that all of the types of precursor lesions of squamous cell carcinoma represented a single disease progressive process, termed CIN (5). More recently it has been proposed that the terminology be changed to reflect better the underlying biological processes and that CIN 1 be called low-grade CIN (which represents a productive HPV viral infection) and that CIN 2 and 3 be called high-grade CIN (which represents a true neoplastic process confined to the epithelium; Ref. 4). Studies on the natural history of CIN, which affects millions of women annually, suggest that the majority of lesions will remain stable or spontaneously regress over time (6). However, there are no diagnostic or prognostic criteria that will identify those dysplasias that are destined toward malignant progression.

DNA methylation is a frequent epigenetic event in many human cancers (7, 8). A growing number of cancer-related genes are being recognized that harbor dense methylation of cytosine in normally unmethylated CpG-rich sequences, called CpG islands, located within the 5′ gene promoter regions (9). Several studies have indicated that methylation is essential for normal development (10), X chromosome inactivation (11), and imprinting (12, 13). Aberrant promoter methylation is an important mechanism for loss of gene function in tumors and may be more frequent than mutations in coding regions (14–17).

Loss of expression of p16 gene, often associated with aberrant methylation, is a frequent alteration in many types of human neoplasia (18, 19). In cancers of the lung and cervix it has been shown by in situ hybridization that aberrant methylation of the p16 gene occurs early within tumor cell populations in both in situ and invasive tumors (20). Methylation of p16 may be present in preneoplastic lesions of the bronchial epithelium and can be detected in sputum specimens from such subjects (21). Morton et al. (22) have reported aberrant methylation at D17S5 on chromosome 17p13.3 in benign prostatic hyperplasia. Progressive increase in de novo methylation of CpG islands also occurs in bladder cancer (23). Aberrant methylation of genes

3 The abbreviations used are: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; MSP, methylation-specific PCR; MI, methylation index; GST, glutathione S-transferase; MGMT, O6-methylguanine-DNA-methyltransferase.
may thus represent a promising avenue for studying preneoplasia and for risk assessment.

Squamous cell carcinoma of the cervix is a suitable tumor model to study genetic and epigenetic alterations in carcinogenesis because invasive carcinomas arise via a series of progressive intraepithelial lesions, which may be detected and monitored by relatively simple noninvasive procedures. Although there are reports of progressive global hypomethylation in cervical and ovarian cancers (24, 25), studies of promoter methylation have been limited to analyses of the p16 gene in cervical cancer (20, 26).

In this study we used the MSP method (27) to analyze the methylation status of a panel of genes that are frequently methylated in other tumor types. We analyzed the frequency and extent of methylation (by determining the methylation index) for six genes and correlated DNA methylation with histopathological changes that occur during the development of cervical cancer.

MATERIALS AND METHODS

Clinical Samples. All cervical specimens were collected from patients within the Parkland Health and Hospital System, Dallas, Texas, after Institutional Review Board approval, and signed consent was obtained from all of the subjects. The cases evaluated were randomly selected from a prospective collection of samples stored in our tissue banks. Gene promoter methylation was analyzed in a total of 73 samples from patients ranging in age from 16 to 60 years (mean age, 32 years) and from 10 cervical cancer cell lines. In addition, tissue samples [peripheral blood lymphocytes (n = 10) and buccal epithelial cells (n = 12)] were obtained from 22 healthy volunteers. Cervical samples (other than invasive cancer) were collected using the Thin Prep Cytology Collection System (CYTYC Corporation, Marlborough, MA) according to the manufacturer’s protocol. The sample vials were stored at ambient temperature and used within 30 months from the collection date. Thirty samples represented cytology-proven, dysplasia-negative (nondysplasia) cases from women without a history of prior dysplasia. Cytology- and histology-proven dysplasia samples were obtained immediately preceding clinically indicated cervical conization. The level of dysplasia assigned to the sample was determined by the highest grade of dysplasia seen within the conization specimen. Seven cases were classified as low-grade CIN (CIN 1) and 17 cases of high-grade CIN were represented by CIN 2 (n = 10) and CIN 3 (n = 7). Invasive cervical cancer tissues (n = 19) were obtained at the time of surgical evaluation by radical hysterectomy or examination under anesthesia. Random transvaginal tumor biopsies were obtained and flash-frozen in liquid nitrogen and stored at −150°C. Tumors were staged according to Federation International Gynecological Oncologists classification (28). HPV infection status was determined by PCR analysis using GP5+ and GP6+ consensus primers (29).

Cell Lines. Ten cervical cancer cell lines were studied, of which nine (C33A, SiHa, HeLa, MS751, C4I, C4II, HT-3, Caski, and ME180) were obtained from the American Type Culture Collection (Manassas, VA). Cell line HCC229 was established by us.

DNA Extraction. Genomic DNA was isolated from cells spun down from 5 ml of Thin Prep Cytology Collections and frozen tumor tissue by digestion with 100 μg/ml proteinase K followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation.

MSP. Methylation status of p16, RARB, FHIT, GSTP1, MGMT, and hMLH1 genes was determined using the MSP method (27).Primers and conditions for MSP assays were derived from Refs. 27 and 30–34 and are available from the authors.

Briefly, 1 μg of genomic DNA was modified by treatment with sodium bisulfite, which converts all of the unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. The modified DNA was purified using the Wizard DNA purification kit (Promega, Madison, WI), desulfonated with NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at −70°C until used. Specific primers were used to amplify regions of interest: One pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). PCR amplification was done with treated DNA as template as previously described using specific primer sequences for the methylated and unmethylated forms of the gene. DNA from control tissues of 22 healthy subjects along with water blanks were used as negative controls for the methylated genes. DNA from lymphocytes of healthy volunteers treated with Sss1 methyltransferase (New England BioLabs) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. PCR products were analyzed on 2% agarose gels containing ethidium bromide. When sufficient DNA was available, the bisulfite reaction was repeated for analysis.

Data Analysis. Statistical analyses were performed using χ² tests. The trend of increasing methylation with invasiveness was determined using the Mantel-Haenszel χ² test. To compare the extent of methylation for the panel of genes examined, we calculated the MI for each case as follows and then determined the mean for the different groups.

\[ MI = \frac{\text{Total no. of genes methylated}}{\text{Total no. of genes analyzed}} \]

Differences of MI between the different groups were analyzed using the Wilcoxon/Kruskal-Wallis one-way nonparametric test. For all of the tests, P < 0.05 was regarded as statistically significant.

RESULTS

The methylation status of six gene promoter regions was determined in cervical samples. By mixing experiments, we determined that the sensitivity of MSP for the genes used was 1 positive cell in between 1000–10,000 negative cells (data not shown). Thus, even a small number of methylation-positive cells in routinely collected Thin Prep samples would be sufficient for detection by MSP.

Differences between the nondysplasia group and low-grade CIN were not statistically significant. Low-grade CIN samples showed a very low incidence of methylation. Although 6/7
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Fig. 1. MSP in cervical DNA samples. p16U, unmethylated form of p16 gene; p16, M, RARβ, MGMT, and GSTP1, methylated forms of the respective genes; M, size marker. Top three panels, 100-bp DNA ladder; bottom two panels, 25-bp DNA ladder. Lanes 1–3, 4–6, and 7–9, cases from nondysplasia/low-grade CIN, high-grade CIN, and tumor cases, respectively. Presence of a band in each panel indicates amplification for the corresponding gene analyzed. Positive control (P) for the unmethylated form is normal lymphocyte DNA and for the methylated form is normal lymphocyte DNA treated with SssI methyl transferase before bisulfite modification. Blank Lane, negative PCR control (no template DNA).

Table 1 Frequency of aberrant gene methylation during cervical carcinogenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Controls (n = 22)*</th>
<th>Nondysplasia/low-grade CIN (n = 37)</th>
<th>High grade CIN (n = 17)</th>
<th>Invasive cancer (n = 19)</th>
<th>Cervical cancer cell lines (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (24)*</td>
<td>8 (42)*</td>
<td>2 (20)</td>
</tr>
<tr>
<td>RARβ</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>FHIT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>GSTP1</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>6 (32)*</td>
<td>1 (10)</td>
</tr>
<tr>
<td>MGMT</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>3 (18)</td>
<td>5 (26)*</td>
<td>3 (30)</td>
</tr>
<tr>
<td>hMLH1</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>5 (29)*</td>
<td>5 (26)*</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Any gene</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>6 (32)*</td>
<td>5 (26)*</td>
<td>7 (70)</td>
</tr>
</tbody>
</table>

* Controls consisted of buccal smears and peripheral blood mononuclear cells from healthy subjects.
* Significant differences between nondysplasia/low-grade CIN group and high-grade CIN were detected for p16 (P = 0.01), MGMT (P = 0.004), and any gene (P = 0.005).
* Significant differences between nondysplasia/low grade CIN group and tumors were detected for p16 (P = 0.001), FHIT (P = 0.002), MGMT (P = 0.006), and any gene (P = 0.002).

low-grade CIN samples showed absence of methylation for any gene, only one sample was methylated for hMLH1. Thus, the results from these two groups were combined and compared with the high-grade CIN and cancer groups.

Figure 1 shows representative examples of the MSP products analyzed on agarose gel for the p16, RARβ, MGMT, and GSTP1 genes. Table 1 summarizes the incidence of methylation of the specific genes in the following groups: nondysplasia/low-grade CIN (CIN 1), high-grade dysplasia (CIN 2 + CIN 3), invasive cervical tumors, and cervical carcinoma cell lines.

The presence of an unmethylated p16 gene in all of the samples tested confirmed the integrity of the DNA in these samples. We found that all of the control samples from healthy volunteers were completely negative for methylation. A relatively low frequency of methylation (<30%) for some genes was seen in the nondysplasia/low-grade CIN group. Methylation for at least one gene in the panel analyzed was a frequent event in high-grade CIN (71%) and tumor (74%) samples, and these figures were statistically significant compared with the nondysplasia/low-grade CIN cases (P = 0.005 and P = 0.002, respectively). Furthermore, we found a difference in the pattern of methylation during cervical carcinogenesis for the six genes examined. Of the six genes examined, five genes had methylation frequencies of >20% in tumors. The p16 and MGMT genes were significantly more methylated both in high-grade lesions (CIN 2 and 3; P = 0.01 and P = 0.004, respectively) as well as in cervical tumors (P = 0.001 and P = 0.006, respectively) than in the nondysplasia/low-grade CIN group. The high-grade CIN and tumors showed a similar methylation pattern except for the FHIT gene. It was interesting to note that methylation of FHIT was absent in the preinvasive lesions but was frequent in invasive tumors (32%, P = 0.002). There were no significant differences in the frequencies of methylation in cervical cell lines compared with invasive tumors.

As shown in Fig. 2, there was a significant trend for increasing methylation with increasing degree of histopathological change (P = 0.001). The mean MI, an indication of the degree of overall methylation, was found to be higher in high-grade dysplasia and in tumors than in the nondysplasia/low-grade CIN group (P = 0.006 and P = 0.02, respectively).
Although the difference in mean MI between the high-grade dysplasias and tumor group was not significant, the mean MI of stage II tumors (0.35) was significantly higher ($P < 0.02$) than the mean of stage I tumors (0.17; Fig. 3).

There was no association of aberrant methylation with HPV, smoking, or hormone use.

**DISCUSSION**

Previous studies have described the importance of DNA methylation in human cancers and have focused on regions of the genome that might have functional significance resulting from the extinction of gene activity. Considerable variations in promoter methylation for individual genes exist in the profiles of different cancers (8, 35). There is virtually no published information on the methylation changes during the multistage pathogenesis of cervical cancer. This report describes the frequency and promoter methylation during the development of cervical cancers with reference to six tumor suppressor genes that are frequently methylated in other tumor types.

FHIT and $p16$ gene abnormalities are frequent in cervical carcinomas. Methylation of these genes was not detected in control tissues (lymphocytes and buccal epithelium) from healthy volunteers. The $p16$ ($p16^{INK4A}$) gene plays a vital role in controlling the cell cycle. Progressive methylation of the $p16$ gene has been reported in early lesions of squamous carcinomas of the lung (21) and in 31% of cervical cancers (26). We found a 42% incidence of methylation for $p16$ in cervical cancers, and in 24% of high-grade dysplasias. Methylation was rare (3%) in nondysplasia/low-grade CIN specimens.

Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by the retinoic acid receptor $\beta$ (RAR$\beta$) gene. Because receptor isoforms RAR$\beta$2 and RAR$\beta$4 are frequently repressed in human lung and breast cancers (36, 37), we investigated methylation of their promotor, P2. We found similar incidences of methylation of the P2 promotor of this gene in the tumors (26%) and in high-grade CIN (29%) and a somewhat lower rate in the nondysplasia/low-grade CIN cases (11%). The incidence present in the latter group was (along with GSTP1) the highest for any of the genes tested, indicating that methylation of these genes are very early changes during cervical cancer pathogenesis.

A variety of abnormalities of the FHIT gene, located within a common fragile site FRA3B at 3p14, are common in many forms of cancers (38). Aberrant methylation of the FHIT gene was seen in 32% of the cancers but in only a single intraepithelial lesion (3%). Allelic losses, homozygous deletions, and aberrant FHIT transcripts are common in cervical cancers but occur at much lower frequencies in preneoplastic lesions (39, 40). These results suggest that molecular alterations of this gene, including methylation, are a late event in tumorigenesis.

GSTs are a superfamily of enzymes that play an important role in protecting cells from cytotoxic and carcinogenic agents. Polymorphisms of the pi-class of GSTs have been associated with genetic susceptibility to many cancers (41). Recently, it has been reported that regulatory sequences near the GSTP1 gene, which encodes the human pi-class GST, are commonly methylated in prostate (83%), breast (31%), and renal cancers (20%), whereas methylation was absent in ovarian and endometrial tumors (31). We found methylation of GSTP1 in 21% of carcinomas and in 18% of high-grade CIN. The incidence in the nondysplasia/low-grade CIN group was somewhat lower (11%), although these differences were not significant.

MGMT is a DNA repair protein that removes mutagenic
and cytotoxic adducts from O6-guanine in DNA. Frequent methylation of MGMT associated with gene silencing occurs in gliomas and colon cancers (33, 42). MGMT was methylated at relatively high frequencies in high-grade CIN and in invasive carcinomas (26–29%), although methylation was rare in the nondysplasia/low-grade CIN group. Our findings suggest that methylation of MGMT, which modulates response to cytotoxic agents (43), may play an important role in cervical cancers.

A aberrant methylation of hMLH1 gene is recognized as the underlying mechanism for microsatellite instability in colon and endometrial cancers (44), and the gene is frequently methylated in endometrial carcinomas (45). In a small series, allelic loss at the gene location was found in 22% of cervical carcinomas (46). We found that methylation of hMLH1 in cervical cancers and their preneoplastic lesions are rare events (3–6%).

The MI between the various groups increased with increasing severity of histopathological change. Methylation commenced early during multistage carcinogenesis, and the MI of the nondysplasia/low-grade CIN group was 0.05. In the high-grade CIN group the MI was 0.18. The MIs of tumors and cell lines were similar (0.25 and 0.20, respectively). The MI has been determined with reference to the genes analyzed in this article and therefore is relevant only to the panel studied. Although the MIs of tumors and high-grade CIN were not significantly different and ~70% of the individual samples demonstrated methylation of at least one of the six genes tested, the overall pattern of the two groups demonstrated important differences. In tumors, frequent methylation (>20% frequency) was noted for five of the six genes tested (p16, RARβ, FHIT, GSTP1, and MGMT). In high-grade CIN, FHIT methylation was absent. The overall frequency of methylation for any gene in cell lines (70%) was similar to that in tumor tissue (74%). There were no statistically significant differences between the cell lines and tumor tissues for individual markers. A 30% overall rate for methylation of any gene in the nondysplasia/low-grade CIN group was unexpected and may represent early molecular events in the lower genital tract that occur because of the field cancerization effect. Alternatively, this observation could be attributable to unrecognized dysplastic cells remaining in the cellular suspension that were not seen in the monolayer slide preparation used for clinical diagnosis (i.e., false-negative PAP smear). In this group only the methylation frequencies of RARβ and GSTP1 exceeded 10%. These results suggest that methylation commences early during multistage pathogenesis and that gene methylation is not random but follows a defined sequence. Thus, methylation of RARβ and GSTP1 are early events, p16 and MGMT methylation are intermediate events, and FHIT methylation is a late, tumor-associated event. It is important to note that our nondysplasia cases were not from symptom-free subjects but from women attending a gynecological clinic, and most had been exposed to multiple risk factors, including papillomavirus, smoking, or oral contraceptive usage. Methylation was not correlated with exposure to other risk factors, including papillomavirus.

Although our findings need to be extended to a larger series, they suggest that the pattern of aberrant methylation in women with or without dysplasia may help identify subgroups at increased risk for histological progression or cancer development. These studies can be performed on routinely collected clinical samples after completion of cytological examination.

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