Analysis of Aberrant DNA Methylation and Human Papillomavirus DNA in Cervicovaginal Specimens to Detect Invasive Cervical Cancer and Its Precursors

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ABSTRACT

Purpose: Cancer of the uterine cervix is an important cause of death in women worldwide. Pap smears as a tool for screening decreased the incidence and mortality of cervical cancer dramatically. This proof of principle study aimed to develop a potential tool for cervical screening using a test that can be applied by patients without visiting a physician and to increase the coverage rate, especially of the high-risk population with low socioeconomic status.

Experimental Design: Human papillomavirus (HPV) DNA testing and methylation analysis of DNA obtained from cervicovaginal specimens of 13, 31, and 11 patients with no dysplasia/low-grade squamous intraepithelial lesion (SIL), high-grade SIL, and invasive cervical cancer, respectively, collected on a tampon, was performed using PCR-based methods to detect invasive cervical cancer and study whether these changes are already present in the precursor lesions.

Results: High-risk HPV DNA was present in 68 and 82% of patients with high-grade SIL and invasive cervical cancer. DNA methylation of the 11 genes tested increased with severity of the cervical lesion. Unsupervised hierarchical cluster analysis using solely information on DNA methylation of the 11 genes was able to predict the presence of invasive cervical cancers: one of the two clusters formed contained 9 of 11 invasive cervical cancers, as well as two high-grade SILs.

Conclusions: HPV DNA and DNA methylation analyzed in cervicovaginal specimens are able to predict invasive cervical cancers. To detect all high-grade SILs when applying this test, genes that become methylated earlier throughout cervical carcinogenesis have to be defined.

INTRODUCTION

Cancer of the uterine cervix is an important cause of death in women worldwide (1). Since the introduction of Pap smears in screening programs, the incidence and mortality of cervical cancer have decreased dramatically (2). However, successful screening strongly depends on the coverage rate of the population and the sensitivity and specificity of the screening test. A meta-analysis of studies investigating the Pap test for the detection of cervical cancer and its precursors revealed a sensitivity ranging from 30 to 87% and a specificity ranging from 86 to 100% (3). Converging evidence from epidemiological and molecular studies suggests that infection with genital human papillomavirus (HPV) is causally linked to the development of cervical cancer (4). Therefore, testing for HPV DNA has been evaluated to improve cervical cancer screening. Numerous studies showed a high sensitivity for the HPV test in detecting cervical cancer and its precursors, whereas specificity was usually lower in comparison to cytology (5). To reduce the inconvenience and cost of repeated clinical visits, it has been proposed that women collect cervicovaginal specimens themselves, hopefully increasing the coverage of screening programs.

Several studies investigated HPV DNA detection rates between self-collected and physician-collected samples with varying concordance between the two different collection methods (6–8).

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. Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias (9). Recently, an aberrant methylation pattern was found during the multistage pathogenesis of cervical cancer with a trend to increasing methylation with increasing pathological changes (10). This study analyzed the methylation status of a panel of genes that are frequently methylated in cervical cancer or other malignancies.

The aim of this proof of principle study was to examine whether HPV DNA testing and methylation analysis of DNA obtained from cervicovaginal specimens, collected on a tampon, are able to detect invasive cervical cancer and whether these changes are already present in the precursor lesions.
MATERIALS AND METHODS

Patients and Samples. A total of 34 patients with cervical intraepithelial neoplasia (cervical intraepithelial neoplasia 1: 3 cases, cervical intraepithelial neoplasia 2: 18 cases, and cervical intraepithelial neoplasia 3: 13 cases) and 11 patients with invasive cervical cancer were included in this study. Patients were referred to our hospital for additional treatment because of abnormal Pap smear or obvious cervical lesion. All Pap smears were taken within 3 months before tampon collection. Pap smears were classified according to the Munich Cytological Classification. The correlation between the Munich Cytological Classification and the Bethesda System are shown in Supplemental Data. Patients underwent cervical conization or, in the case of obvious carcinomatous lesion, cervical biopsy to obtain a histological diagnosis. The level of dysplasia was staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. Five patients had a FIGO stage I, 3 had FIGO stage II, 2 had FIGO stage III, and 1 had FIGO stage IV. Additionally, 10 patients with normal cervical cytology and without cervical dysplasia in the histological examination who underwent hysterectomy because of fibroids were investigated. All patients, except 6 patients with invasive cervical cancer, were taken from a prior study performed to determine whether it is possible to detect endometrial cancer by analyzing methylated DNA in cervicovaginal specimens collected by tampon (11). Aberrant DNA methylation of 5 genes, namely CDH13, HSPA2, MLH1, RASSF1A, and SOCS2 was investigated in that study.

Sample Collection. Samples and clinical data were collected after informed consent was obtained. To ensure standardized sample collection, a tampon (o.b. comfort mini tampons; Johnson & Johnson GmbH, Hallein, Austria) was inserted in the vagina by a physician after speculum examination before surgery (the day before) and retained intravaginal for 30 min. Preparation of the samples was described previously (11). Briefly, the tampon was transferred to a 50-ml tube after removal, and 1.2 ml of PBS buffer were added to the tampon. Centrifugation at 1000 × g for 10 min produced supernatant and a pellet. Aliquots (0.2 ml) of the supernatant were each mixed with 0.2 ml from the working solution of the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) and stored at −30°C until DNA isolation.

DNA Isolation and Methylation Analysis. Genomic DNA from samples was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics) according to the manufacturer’s protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA. Eleven genes known to be methylated in cervical cancer were selected for methylation analysis (10, 12–14). After sodium bisulfite conversion, the 11 genes (SOCS1, CDH1, TIMP3, GSTP1, DAPK, hTERT, CDH13, HSPA2, MLH1, RASSF1A, and SOCS2) underwent methylation analysis by means of the fluorescence-based, real-time PCR MethyLight assay as described previously (15, 16). Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β-actin (ACTB) to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using 5ss (New England Biolabs)-treated human WBC DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the Gene:Actb ratio of a sample by the Gene:Actb ratio of 5ss-treated DNA and multiplying by 100. The result is given as percentage of fully methylated reference. A gene was deemed methylated if the percentage of fully methylated reference value was >0. Primers and probes used for MethyLight reactions are listed in Table 1.
HPV DNA Analysis. HPV PCR enzyme immunoassay was done as described previously (17). Briefly, 10 μl of purified total cellular DNA were used for PCR using consensus primers GP5+/bioGP6+. The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate, 1 unit of thermostable DNA polymerase (TaqDNA Polymerase; Roche, Vienna, Austria), and 50 pmol each of the GP5+/bioGP6+ primers (MWG-Biotech, Ebersberg, Germany). A 4-min denaturation step at 94°C was followed by 40 cycles of amplification in a PCR cycler (Gene Amp PCR System 9600; Perkin-Elmer, Norwalk, CT). To determine the HPV type, the PCR product was subjected to an enzyme immunoassay recognizing 14 different high-risk HPV types as described by Jacobs et al. (17).

Statistical Analysis. Associations between categorical variables were tested with Pearson’s χ² test (or Fisher’s exact test) and the Mantel-Haenzel Test. Correlations between ordinal variables were evaluated using the Spearman Rank Correlation Coefficient. For unsupervised hierarchical clustering of clinical cases and genes, we used the complete linkage aggregation method and the Manhattan distance function.

All statistical calculations were performed using SPSS, version 11.0, for Windows and Gene Expression Similarity Suite.³

RESULTS

This proof of principle study investigated cervicovaginal specimens sampled by intravaginal tampon application in patients without cervical dysplasia, patients with low-grade and high-grade squamous intraepithelial lesion (SIL), and patients with invasive cervical cancer for HPV DNA and aberrant methylation of 11 genes. Pap smear revealed normal cytology (Pap II) for all patients without dysplasia. The three cases with low-grade SIL had Pap III, Pap IIID, and Pap IV, and all patients with high-grade SIL had abnormal cytology (Fig. 1). From patients with invasive cervical cancer, Pap smear was available in 4 of 11 cases, and all 4 cases had abnormal cytology (Fig. 1). In 7 cases, a carcinomatous cervical lesion was obvious, and a cervical biopsy was taken instead of a Pap smear. High-risk HPV DNA was detected in 2 of 3, 21 of 31, and 9 of 11 in low-grade SIL, high-grade SIL, and invasive cervical cancer, respectively (Table 2). Patients without cervical dysplasia showed no HPV infection. HPV-positive cervical cancers were large cell squamous cancers and one adeno cancer, whereas both HPV-negative cases were small cell cervical cancers. Differences in methylation of the investigated genes between the nondysplasia group (n = 10) and the low-grade SIL group (n = 3) were not statistically significant. Therefore, the results from these two groups were combined and compared with the high-grade SIL and invasive cancer groups. An overview of the frequency of methylated genes is given in Table 2. All investigated genes, except GSTP1 and SOCS2, were significantly more frequently methylated in high-grade SIL and/or invasive cancer in comparison to the nondysplasia/low-grade SIL group. CDH1 and SOCS2 were found to be methylated in nearly a quarter of the nondysplasia/low-grade SIL patients, whereas hTERT was methylated exclusively in specimens obtained from cervical

³ Internet address: http://genome.tugraz.at/Software/Genesis/Genesis.html.
Table 2  Methylation status of genes and high-risk human papillomavirus DNA in patients with no dysplasia/low-grade squamous intraepithelial lesion (SIL), high-grade SIL, and invasive cervical cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>No dysplasia/low-grade SIL (%)</th>
<th>High-grade SIL (%)</th>
<th>Invasive Cancer (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 31)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>SOCS1 methylated</td>
<td>0</td>
<td>7</td>
<td>50</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CDH1 methylated</td>
<td>23</td>
<td>39</td>
<td>91</td>
<td>0.002*</td>
</tr>
<tr>
<td>TMPI methylated</td>
<td>0</td>
<td>16</td>
<td>100</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>GSTP1 methylated</td>
<td>0</td>
<td>7</td>
<td>18</td>
<td>0.224</td>
</tr>
<tr>
<td>DAPK methylated</td>
<td>8</td>
<td>23</td>
<td>82</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>hTERT methylated</td>
<td>8</td>
<td>13</td>
<td>82</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CDH13 methylated</td>
<td>8</td>
<td>13</td>
<td>82</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HSPA2 methylated</td>
<td>0</td>
<td>3</td>
<td>73</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MLH1 methylated</td>
<td>0</td>
<td>3</td>
<td>36</td>
<td>0.002*</td>
</tr>
<tr>
<td>RASSF1A methylated</td>
<td>8</td>
<td>0</td>
<td>46</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>SOCS2 methylated</td>
<td>23</td>
<td>45</td>
<td>64</td>
<td>0.132</td>
</tr>
<tr>
<td>At least one gene methylated</td>
<td>39</td>
<td>58</td>
<td>100</td>
<td>0.007*</td>
</tr>
<tr>
<td>Three or more genes methylated</td>
<td>8</td>
<td>23</td>
<td>100</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>High-risk human papillomavirus DNA positive</td>
<td>15</td>
<td>68</td>
<td>82</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*a* Additionally presence of significant linear association (Mantel-Haenzel, *P* < 0.01).

...other content...

**DISCUSSION**

Up to 60% of women diagnosed as having invasive cervical cancer were not screened despite health maintenance organization enrollment (18). It has been shown that HPV is causally linked to the development of cervical cancer (4), and numerous studies have revealed a high sensitivity for the HPV test in detecting cervical cancer and its precursors, whereas specificity was usually lower in comparison to cytology (5). To reduce the inconvenience and cost of repeated clinical visits, it has been proposed that women collect cervicovaginal specimens themselves, thus hopefully increasing the coverage of screening programs. Previous studies investigating HPV DNA detection rates between self-collected and physician-collected samples described varying concordance between the two different collection methods (6–8). Our study detected high-risk HPV DNA obtained in 68% of high-grade SIL and in 82% of invasive cervical cancer patients. Recently, it was shown that clinician-directed swabs detect up to 28% more HPV-positive women in comparison to tampon-collected specimens (7). In light of the fact that numerous studies have revealed a ~100% sensitivity in detecting SIL and cervical cancer by HPV DNA testing of physician-collected samples (5), specimen collection by tampon seems not to be a feasible method for HPV DNA detection.

It has been proposed that in addition to HPV infection, genetic or epigenetic alterations may be required to maintain a malignant phenotype. Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias (9). Recently, it was suggested that aberrant methylation may play a role in cervical carcinogenesis (10). Therefore, we additionally investigated the methylation status of genes known to be methylated in cervical cancer (10, 12–14, 19) and in various malignancies (20–25). hTERT was never found to be methylated in cervical intraepithelial neoplasias, whereas 82% of the specimens obtained from cervical cancer patients were methylated. This finding suggests that methylation of hTERT is a late event in cervical carcinogenesis. SOCS2 and CDH1 were methylated in nearly a quarter of patients from the nondysplasia/low-grade SIL groups, indicating that methylation of these genes is an early event in cervical carcinogenesis. Our results clearly show that an increasing percentage of methylation-positive samples is associated with increasing pathological changes of the cervix uteri (Fig. 2), suggesting that methylation, in addition to HPV infection, is an important factor in cervical carcinogenesis. Comparison between aberrant hypermethylation and abnormal Pap smear revealed no significant correlation. This comparison is of limited value because Pap smear was not available in 7 of 11 cervical cancer patients. Additionally, no significant correlation between HPV positivity and aberrant hypermethylation was observed. It can be speculated that aberrant methylation in women with or without HPV infection may help identify subgroups at increased risk for histological progression or cancer development.

Our proof of principle study shows that it is possible to detect HPV and aberrant hypermethylation of various genes in DNA from tampon-collected samples. We were able to identify 9 of 11 invasive cervical cancers by unsupervised hierarchical cluster analysis using solely information on DNA methylation of the 11 genes. Both methods combined detect a high percentage of high-grade cervical lesions, although sensitivity and...
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The specificity of this test for high-grade SIL are too low to be applicable as a screening test. Because the selection of the 11 genes used for analysis was based mainly on the fact that they have been described as methylated in invasive cancers, additional studies need to define genes that become methylated at an earlier time in cervical carcinogenesis. Once that is the case, this simple approach may become useful for the early detection of SIL and invasive cervical cancers by means of DNA methylation analysis in cervicovaginal specimens isolated from tampons inserted by women and sent to a laboratory for testing.

REFERENCES

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