Detecting Cervical Cancer by Quantitative Promoter Hypermethylation Assay on Cervical Scrapings: A Feasibility Study


Abstract
Current morphology-based cervical cancer screening is associated with significant false-positive and false-negative results. Tumor suppressor gene hypermethylation is frequently present in cervical cancer. It is unknown whether a cervical scraping reflects the methylation status of the underlying epithelium, and it is therefore unclear whether quantitative hypermethylation specific PCR (QMSP) on cervical scrapings could be used as a future screening method augmenting the current approach. Cervical scrapings and paired fresh frozen cervical tissue samples were obtained from 53 cervical cancer patients and 45 controls. All scrapings were morphologically scored and analyzed with QMSP for the genes APC, DAPK, MGMT, and GSTP1. To adjust for DNA input, hypermethylation ratios were calculated against DNA levels of a reference gene. Hypermethylation ratios of paired fresh frozen tissue samples and scrapings of cervical cancer patients and controls were strongly related (Spearman correlation coefficient, 0.80 for APC and 0.98 for DAPK, and 0.83 for MGMT; P < 0.001). More cervical cancer patients than controls were DAPK positive (P < 0.001). When cutoff levels for ratios were defined to be above the highest ratio observed in controls, QMSP in cervical scrapings identified 32 (67%) of 48 cervical cancer patients. This feasibility study demonstrates that QMSP on cervical scrapings holds promise as a new diagnostic tool for cervical cancer. The addition of more genes specifically methylated in cervical cancer will further improve the assay. (Mol Cancer Res 2004;2(5):289–95)

Introduction
Cervical cancer is an important cause of death in women worldwide (1). There is a strong association between certain subtypes (high risk) of human papilloma virus and cervical cancer (2). However, other factors are also involved in cervical carcinogenesis, because the majority of patients infected with human papilloma virus will not develop invasive cervical cancer (3).

Cytomorphologic examination of cervical smears is the most widely applied screening method for cervical cancer and its precursors. The Papanicolaou (Pap) smear has false-negatives rates of 2% to 40% due to a combination of sampling error, processing artifacts, and the nature of subjective interpretation (1, 4, 5). False-negative cytology may lead to a delay in the diagnosis of cervical cancer and can be found in about 50% of cases when previous negative smears are reviewed from the small proportion of screened women who develop invasive cancer (5). Moreover, as many as 20% of all Pap smears are interpreted as atypical squamous cells of undetermined significance or borderline dyskaryotic, leading to increased surveillance frequency and more invasive tests in many of these patients (4, 6). Although it has been suggested that high-risk human papilloma virus testing may well improve cervical cancer screening (7, 8), the specificity for high-grade cervical neoplasia of high-risk human papilloma virus testing is relatively low (9). Therefore, new objective diagnostic methods are needed.

Silencing of tumor suppressor–associated or other cancer-associated genes by methylation of CpG islands, located in the promoter and/or 5′ regions of many genes, is a common feature of human cancer (10). CpG island methylation is often associated with a transcriptional block and loss of the relevant protein (10). In addition to the functional implications of gene inactivation in tumor development, these aberrant methylation patterns represent excellent targets for novel diagnostic approaches based on methylation-sensitive PCR techniques. Recently, Dong et al. (11) showed that promoter hypermethylation of at least one of the genes P16, DAPK, MGMT, APC, HIC-1, and E-cadherin occurred in 79% of cervical cancer tissues and in none of normal cervical tissues from 24 hysterectomy specimens. Virmani et al. (12) detected aberrant methylation of at least one of the genes P16, RARβ, FHIT, GSTP1, MGMT, and hMLH1 in 14 of 19 cervical cancer tissue samples. These experiments were carried out using conventional methylation specific PCR (MSP). An advancement of this technique is real-time quantitative MSP (QMSP), which permits reliable quantification of methylated DNA. This
method is based on the continuous optical monitoring of a fluorogenic PCR. This PCR approach is more sensitive and more specific than conventional PCR and can therefore detect aberrant methylation patterns in human samples in the presence of normal DNA in a ratio of 1:10,000 (13).

Currently, no data are available on whether cervical scrapings reflect the methylation status of the underlying cervical epithelium, and it is unknown whether QMSP on cervical scrapings could be used as a future screening method. In the present study, we examined the promoter hypermethylation status of the tumor suppressor genes APC, DAPK, GSTP1, and MGMT in cervical scrapings and paired fresh frozen tissue samples obtained from cervical cancer patients and controls.

Results

Patient Population

From March 2001 to August 2003, 108 patients met our inclusion criteria. Four patients refused to participate in the study; in two patients, no cervical scraping was taken; in three patients, the scraping taken was lost for analysis; and one control patient was excluded because of complex atypical hyperplasia of the endometrium. The specimens of 98 patients, 53 squamous cell cervical cancer patients and 45 controls, were used for further analysis. Fédération Internationale des Gynécologues et Obstétristes stage IA cervical cancer was diagnosed in 5 (9.4%), stage IB1 in 20 (38%), stage IB2 in 3 (5.7%), stage IIA in 5 (9.4%), stage IIB in 14 (26%), stage III in 5 (10%), and stage IV in 1 (1.9%) of the 53 cervical cancer patients. Indications for hysterectomy were uterine myomas in 20 (44%), uterine prolapse in 20 (44%), menometrorrhagia in 4 (8.9%), and severe dysmenorrhea in 1 (2.2%) of the 45 controls. The cervical cancer patients had a median age of 46 years (interquartile range, 37 to 59) and the controls had a median age of 49 years (interquartile range, 44 to 60).

Adequacy of Cervical Scrapings to Represent the Hypermethylation Status of the Underlying Cervical Epithelium

To assess whether cervical scrapings reflect the hypermethylation status of the underlying cervical epithelium, we performed QMSP analysis on cervical scrapings and paired fresh frozen tissue samples. DNA quality was sufficient to perform QMSP in 89 (48 cervical cancer scrapings and 41 control scrapings) of the 98 available scrapings. Paired fresh frozen tissue samples with sufficient DNA quality were available of 39 patients (21 cervical cancer and 18 control samples); in 23 control cervices, the epithelial orientation was insufficient to dissect epithelium from surrounding stromal tissue; in 21 cervical cancer patients, no fresh frozen tissue sample was taken because of possible interference in the diagnostic process; and in 13 patients, an insufficient percentage (<75%) of cancer cells was present. The observed ratios between methylated DNA and reference DNA of cervical scrapings were compared with the ratios observed in fresh frozen tissue samples. In a total number of 156 (39 samples × 4 different genes) paired QMSP results, the QMSP result of scraping and paired tissue corresponded in 152 (97%) analyzed pairs (Table 1). All three noncorresponding positive scrapings were found in controls, and in two of these three scrapings, β-actin DNA was much higher for the scraping than for the corresponding tissue sample (25 ng more β-actin DNA in the scraping than in the tissue sample in one sample and 11 ng more in the other sample). Spearman correlation coefficients were calculated for the genes with positive results in the 39 paired samples. High correlation coefficients were observed [0.80 (P < 0.001) for APC, 0.98 (P < 0.001) for DAPK, and 0.83 (P < 0.001) for MGMT], indicating that high hypermethylation ratios were present in scrapings from patients also harboring tumors with high hypermethylation ratios as illustrated for APC and DAPK in Fig. 1. Comparable correlation coefficients were obtained when cervical cancer samples and control samples were analyzed separately. It was concluded that the hypermethylation status of cervical epithelium is well represented by cervical scrapings.

Clinical Use of QMSP in Cervical Scrapings for Cervical Cancer Detection

To assess the possible clinical use of QMSP in cervical scrapings for cervical cancer detection, we performed QMSP on cervical scrapings and compared results obtained in cancer patients with results obtained in controls. All available 89 scrapings with sufficient DNA quality were included in further analysis because cervical scrapings represent the hypermethylation status of the underlying epithelium well. Methylated APC was detected in 26 (54%), DAPK in 35 (73%), MGMT in 5 (10%), and GSTP1 in 1 (2%) of 48 cervical cancer scrapings. In 41 control scrapings, methylated APC was amplified in 16 (39%), DAPK in 2 (4.9%; P < 0.001), MGMT in 6 (15%), and GSTP1 in none. Methylation ratios are illustrated in Fig. 2 for APC and DAPK.

The ratio cutoff value for each individual gene to be called “screen positive” was arbitrarily defined to be above the highest ratio observed in controls. APC ratios were “screen positive” in 6 (13%) patients, DAPK ratios in 26 (54%) patients, MGMT in 2 (4.2%) patients, and GSTP1 in 1 (2.1%) patient. Three patients were “screen positive” for more than one gene, two patients for DAPK and APC and one patient for DAPK and MGMT. Overall, 32 (67%) of 48 cervical cancer patients were “screen positive” for at least one gene. When cutoff values for all genes were based on the 95th percentile observed in controls, 5 (12%) controls and 35 (73%) of the cervical cancer patients would be “screen positive” for at least

Table 1. Correspondence of QMSP Results in Cervical Scrapings With QMSP Results in Paired Fresh Frozen Tissue Samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of Corresponding Positives</th>
<th>Number of Corresponding Negatives</th>
<th>Number of Noncorresponding Positives</th>
<th>Number of Noncorresponding Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>9</td>
<td>27</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>DAPK</td>
<td>14</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MGMT</td>
<td>3</td>
<td>35</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GSTP1</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Mol Cancer Res 2004;2(5). May 2004

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one gene. When a fixed cutoff value of 100 [average DNA quantity of methylated gene of interest/average DNA quantity for internal reference gene β-actin] \times 10,000 = 100] for each gene was chosen, positives were only observed for \textit{APC} \([n = 4 (8.3\%)]\) and \textit{DAPK} \([n = 12 (25\%)]\) and 15 (31\%) patients were “screen positive” for at least one of these two genes.

\textbf{Morphologic Pap Smear Classification and Real-Time QMSP}

For comparison with cytomorphologic screening, we used the cutoff values as defined above the highest control, because this appeared to be the optimal balance between sensitivity and specificity.

\textit{Controls.} For the 45 controls, a morphologic Pap smear classification was not obtained for 9 controls because the cervical scrapings of 5 controls were not available and another 4 scrapings were inadequate for cytomorphic assessment. All four inadequate scrapings had sufficient DNA input to perform QMSP. Although the cervical epithelium of all controls was histologically diagnosed to be normal, six scrapings were cytomorphologically classified with borderline dyskaryosis (comparable with atypical squamous cells of undetermined significance; Table 2). In five of these six scrapings, DNA input was sufficient to perform QMSP and all these scrapings were negative (partly due to our definition). Three of the four scrapings with insufficient input DNA for QMSP were taken from control patients operated on because of prolapse uteri and one was taken from a control patient with myomas.

\textit{Cancer Patients.} In the 53 cancer patients, a morphologic Pap smear classification was not obtained for 10 patients because the cervical scrapings of 2 cancer patients were not available for classification and another 8 scrapings were inadequate for cytomorphic assessment. Of these eight cervical cancer scrapings, seven had sufficient DNA input for QMSP of which three showed “screen-positive” ratios for at least one of the four genes analyzed. Four cervical cancer scrapings yielded insufficient DNA input, although the sample was adequate for cytomorphic assessment. In three of these, very few dysplastic cells were seen, and in the other two scrapings, many dysplastic cells accounted for the dysplastic morphologic result. One cervical cancer patient was underdiagnosed by cytomorphic assessment, which showed only borderline dyskaryosis (Table 3). In this borderline dyskaryotic scraping, DNA quality was sufficient for QMSP; however, no “screen-positive” ratio was observed for one of the four evaluated genes, although \textit{DAPK} amplification was present in the scraping. Of this patient, no tissue sample was available for analysis.

\textbf{Discussion}

Important drawbacks of conventional screening for cervical cancer have spurred the search for continued improvement of diagnostic accuracy of the Pap test. In this feasibility study, we show that cervical scrapings can be used to detect hypermethylation of tumor suppressor genes in cervical cancer because the hypermethylation status of the tumor was well represented by cervical scrapings and 67\% of the cervical cancer patients could be identified by QMSP for \textit{APC}, \textit{DAPK}, \textit{MGMT}, and \textit{GSTP1}.

The four tumor suppressor genes analyzed in the present study were chosen because promoter hypermethylation and transcriptional repression of these genes might mediate tumorigenesis as demonstrated in cervical and other squamous cell cancers by conventional MSP (11, 12, 14). \textit{MGMT} is a DNA repair gene, \textit{GSTP1} is a detoxifying gene, \textit{DAPK} is a proapoptotic gene and potentially inhibits metastasis, and \textit{APC}...
mediates proliferative signals (15-19). Analyzing paraffin-embedded tissue samples by conventional MSP demonstrated hypermethylation of \( \text{APC} \), \( \text{DAPK} \), and \( \text{MGMT} \) in 31%, 61%, and 10% of squamous cell cervical cancers, respectively (11). These percentages correspond well with our observations by analyzing cervical scrapings with QMSP. Adenocarcinoma subtypes of cervical cancer have distinct hypermethylation patterns (11). \( \text{GSTP1} \) methylation was detected in 4 of 19 cervical cancer tissues of unknown histologic cell type (12), whereas in our series of 48 squamous cell cervical cancer patients, \( \text{GSTP1} \) hypermethylation was demonstrated in only 1 patient. \( \text{GSTP1} \) may be especially associated with the adenomatous cell type, as \( \text{GSTP1} \) is frequently present in prostate adenocarcinoma, while it was demonstrated in only 3 of 73 transitional cell carcinoma samples of bladder cancer patients (20, 21).

Our study shows that cervical scrapings represent the hypermethylation status of underlying cervical epithelium well, given the high correlation coefficients between hypermethylation ratios of scrapings and tissue samples for all genes analyzed. No other data are available on a direct comparison between paired cervical scrapings and underlying tissue samples. Chan et al. (21) showed frequent methylation of \( \text{RARb}, \text{DAPK}, \text{E-cadherin}, \text{P16}, \text{P15}, \text{GSTP1}, \) and \( \text{MGMT} \) in urinary bladder cancer and 21 paired voided urine samples. MSP analysis in voided urine resulted in no noncorresponding positives when compared with paraffin-embedded tumors. However, noncorresponding negative results were frequent [17 (19%) of 88 paired MSP analyses]. Still, MSP analysis for seven genes was more sensitive for detection of bladder cancer than morphologic assessment of urine. Jéronimo et al. (20) analyzed \( \text{GSTP1} \) hypermethylation in voided urine and frozen or paraffin-embedded tissue samples of patients with prostate cancer by

<table>
<thead>
<tr>
<th>Morphologic Classification</th>
<th>Number of Patients</th>
<th>Sufficient DNA Input</th>
<th>Screen Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No dyskaryosis</td>
<td>30</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Borderline dyskaryosis</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Inadequate</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Not assessed</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>41</td>
<td>0</td>
</tr>
</tbody>
</table>

*Adequate DNA input is defined as \( \beta\text{-actin} \) DNA above 225 pg.

**“Screen positive” for hypermethylation is defined as \( \geq 1 \) of the analyzed tumor suppressor genes positive above the defined cutoff value.

<table>
<thead>
<tr>
<th>Morphologic Classification</th>
<th>Number of Patients</th>
<th>Fédération Internationale des Gynécologues et Obstétristes Stage</th>
<th>Sufficient DNA Input</th>
<th>Screen Positive</th>
</tr>
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<tbody>
<tr>
<td>IA</td>
<td>1</td>
<td>IB/IIA</td>
<td>1</td>
<td>1</td>
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<tr>
<td>IB-IV</td>
<td></td>
<td></td>
<td>18</td>
<td>11</td>
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</table>

<table>
<thead>
<tr>
<th>Morcellation Classification</th>
<th>Number of Patients</th>
<th>Fédération Internationale des Gynécologues et Obstétristes Stage</th>
<th>Sufficient DNA Input</th>
<th>Screen Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borderline dyskaryosis</td>
<td>19</td>
<td>14</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Severe dyskaryosis/ carcinoma in situ</td>
<td>23</td>
<td>12</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>Squamous cell cancer</td>
<td></td>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Inadequate</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Not assessed</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>28</td>
<td>20</td>
<td>48</td>
</tr>
</tbody>
</table>

*Adequate DNA input is defined as \( \beta\text{-actin} \) DNA above 225 pg.

**“Screen positive” for hypermethylation is defined as \( \geq 1 \) of the analyzed tumor suppressor genes positive above the defined cutoff value.
MSP and QMSP and showed that, in urine, no noncorresponding positives were observed but noncorresponding negatives were frequent [50 (73%) and 42 (61%) of 69 paired analyses with MSP and QMSP, respectively].

In our study on cervical scrapings, no noncorresponding negatives were observed for APC, DAPK, and GSTP1 and only one noncorresponding negative was observed for MGMT. Testing cervical scrapings for cancer by molecular changes—based assays will likely continue to cause false-negatives, especially due to sampling errors. Even with sensitive molecular assays, false-negatives will occur if no tumor cells are collected by scraping the cervix. However, our data show that only a very low number of dysplastic cells appear to be necessary for an adequate QMSP, because DNA input was sufficient for QMSP in seven of eight cervical cancer scrapings that were inadequate for cytomorphic assessment while three of these seven were “screen positive.”

For DAPK, no noncorresponding positives were observed in scrapings. MGMT and GSTP1 analysis hardly contributed to the identification of cervical cancer patients by QMSP on cervical scrapings, and for APC, three noncorresponding positives were observed. For two of these three, the explanation for the conflicting results may be the higher (>11 ng more β-actin DNA input) β-actin DNA levels in the scrapings than in the tissue samples. However, the three false-positive results could also have been the result of technical failure in the tissue samples, meaning that the tissue samples were APC hypermethylation positive although not detected by QMSP. Another explanation may be that APC hypermethylation was positive in noncervical cancer cells. APC methylation has been demonstrated in several non-neoplastic tissues, as has also been shown for DAPK (22-24). In the present study, DAPK gene was the most cervical cancer sensitive and specific, despite its detection in two controls who both had normal cervical epithelium as confirmed by final histopathologic review. Cervical scrapings, apart from cervical epithelial cells, may also contain vaginal epithelial cells, endometrial cells, and leukocytes. Although methylation in non-neoplastic cells may be the reason for some noncorresponding positive results or positive results in controls, we still expect consequences for the use of QMSP as a screening tool to be low. Malignant cells usually have significantly higher methylation levels when positive than methylated nonmalignant cells. By expressing methylation results as a ratio with a reference gene, QMSP takes advantage of this characteristic (25).

A key issue for molecular diagnosis is the ability to detect cancer cells missed by routine cytopathology. On direct comparison, cytomorphic assessment underestimated one case of stage IB disease in our study, which is not surprising because false-negative rates of the conventional Pap smear are reported to be between 2% and 40% (1, 4, 5). This morphologically false-negative smear was not “screen positive” for QMSP, because the DAPK/β-actin ratio was below cutoff and methylation for none of the three other genes was detected. However, when more genes would have been available for analysis, QMSP might have been able to identify this cervical cancer case because sample adequacy was sufficient for QMSP.

In our series of cervical cancer patients, the frequency of the cytomorphicologic results “severe dyskaryosis/carcinoma in situ” may seem high (37%) in comparison with other studies with conventional Pap smears. One of the consequences, however, of manufacturing Pap smears by cytospin, as was performed in our study, is that mucus and debris are largely washed away. In conventional Pap smears, a background of blood and tumor debris distinguishes a cancer smear from a carcinoma in situ smear. Furthermore, smears classified as severe dyskaryosis/carcinoma in situ were not considered by us to be underestimates because these results lead to immediate referral to a gynecologist.

Apart from the possible identification of cervical cancer patients, missed by cytology, QMSP on cervical scrapings may also have the advantage of hardly any false-positive results in women with normal cervical epithelium. Although all cervical epithelia of control patients were confirmed to be normal, 13% of the paired cervical smears were cytologically classified with borderline dyskaryosis. All controls were “screen negative” for QMSP. Because this 100% specificity was partly due to the definition we used to set cutoff values, future studies are warranted to assess whether the promise of few false-positives by QMSP in cervical cancer screening will hold, which of course will largely depend on how cutoff values will be defined.

In our setting, analyzing patients already referred because of cervical cancer and controls, the choice of a cutoff for all genes tested above the highest ratio observed in controls represented the optimal balance between sensitivity and specificity. The distributions of the calculated ratios (Fig. 2) show that, in the future, the choice for a “screen-positive” cutoff value may best be made separately for each gene. APC appears to be less sensitive and specific than DAPK; however, high (above 100) APC ratios may also be very specific for cancer. Defining the cutoff value for APC above 100 will improve specificity. However, sensitivity will be lower than of DAPK and the use of APC hypermethylation for the detection of cervical cancer remains questionable. For future population-based screening purposes, hypermethylation ratios should be determined by comparing large series of healthy women with series of cervical intraepithelial neoplastic lesions and cervical cancer patients and by constructing receiver operating characteristic curves in large preclinical populations.

Theoretically, QMSP appears to be very suitable as a cervical cancer detection technique, because the PCR reaction is amenable to high-throughput techniques, allowing the analysis of close to 400 samples in <2 h without requirement for gel electrophoresis. The identification of 67% of cervical cancer patients by QMSP for APC, DAPK, MGMT, and GSTP1 demonstrates that it will be necessary to identify other genes more specifically methylated in (cervical) cancer to compose a both sensitive and specific cervical cancer hypermethylation panel. The candidate tumor suppressor gene TSLC1 may be such an interesting candidate. Recently, Steenbergen et al. (26) showed that methylation of TSLC1 was present in 59% of 49 cancer tissues, 35% of 20 high-grade cervical intraepithelial neoplastic lesions, and 0% of 11 low-grade cervical intraepithelial neoplastic lesions.

QMSP on cervical scrapings is a promising new diagnostic tool for the detection of cervical cancer and will improve as more genes specifically methylated in cervical cancer are identified and added to the assay.
Patients and Methods

Patients

Cervical Cancer Patients. From March 2001 to August 2003, all patients referred because of cervical cancer or abnormal cervical cytology were asked to participate in our research program during their initial visit to the outpatient clinic of the University Hospital Groningen. To obtain a homogeneous population, we chose for the present study to only analyze those patients diagnosed with squamous cell cervical cancer, in whom cervical cancer had not been fully removed by exconization or loop excision before referral. Gynecologic examination under general anesthesia was performed in all cervical cancer patients for staging in accordance with the Federation Internationale des Gynaecologistes et Obstetrites criteria (27). During this procedure, lesion size (largest diameter) and tumor spread beyond the cervix were estimated routinely.

All cervical scrapings were collected during the initial visit or before bimanual examination under general anesthesia. All tissue samples used for the study were collected during bimanual examination or at surgery, which was chosen as primary treatment for patients with stage IB1/IIA (tumor size ≤ 4 cm) cervical cancer.

Controls

Controls served as patients without a history of abnormal Pap smears, who planned to undergo a hysterectomy because of non–(pre)malignant disease in the same study period. All samples used for the study were collected during surgery. Cervical epithelium of all control patients was confirmed to be normal on final histopathologic review.

The study was approved by the medical ethical committee of the University Hospital Groningen and all patients gave written informed consent.

Sample Collection

Cervical Scrapings. The cervix of both cervical cancer and control patients was scraped with the blunt or pointed end of an Ayre’s spatula and with an endocervical brush. The scraped cells were suspended in 5 mL ice-cold PBS and kept on ice until further processing. Of this cell suspension, 1 mL was used for further processing. Of this cell suspension, 1 mL was used for

Table 4. Primer and Probe Sequences Used for the QMSP and Reference Gene Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
<th>TaqMan Probe (6FAM5′→3′TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated APC</td>
<td>AAACAAAAACGCTCCCCAT</td>
<td>TTAATGCTGGTTAC GTGCCGTTTATAT</td>
<td>CCCCTCGAACAAGCCGCACATTAG</td>
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<td>Methylated GSTP1</td>
<td>GCGCCCAATACCTAACACCAACGAC</td>
<td>GTATTTTTTCGGGAGGGGAGGC</td>
<td>ATCTCCCGAGTACGACCCCTTACCG</td>
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<tr>
<td>Methylated MGMT</td>
<td>CGGATAGTGAGTCCTGCTAAGCTC</td>
<td>CCCCTCCAAACGGCGGTAACG</td>
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<td>Methylated DAPK</td>
<td>GGTGATGAGGAGGTGGTTCAGTTAAGT</td>
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<td>β-actin</td>
<td>GGCTGTTTTAAGGAGGGAGG</td>
<td>GTATTTTTTCGGGAGGGGAGGC</td>
<td>ATCTCCCGAGTACGACCCCTTACCG</td>
</tr>
</tbody>
</table>

Real-Time QMSP

QMSP for APC, DAPK, GSTP1, and MGMT was performed after bisulfite treatment on denatured genomic DNA (29), as reported previously for APC and GSTP1 (30, 31). β-actin was chosen as internal reference gene. The amplicon sizes for the QMSP were 74 bp for APC (position 761 to 834, Genbank accession no. U02509), 101 bp for DAPK (position 5 to 102, Genbank accession no. X76104), 122 bp for MGMT (position 1029 to 1150, Genbank accession no. X61657), and 140 bp for GSTP1 (position 1033 to 1172, Genbank accession no. M24485). The basis of primer design has been described previously (14, 30). For primer sequences, see Table 4. Amplifications were carried out in 384-well plates. As positive controls, serial dilutions of in vitro CpG methylated DNA with 5xS1 (CPg) methylase (New England Biolabs, Inc., Beverly, MA) were used by which a calibration curve was constructed for each plate. The calibration curve was used to set a plate specific threshold for positivity and to determine DNA equivalents for the results obtained. Multiple water blanks were included as negative controls. Dilution experiments showed linearity of amplification down to a dilution of 1:10,000 for methylated promoter DNA as well as for unmethylated β-actin DNA. All samples were analyzed at least in duplicate. For quality control, all amplification curves were visualized and scored without knowledge of the clinical data. Per analysis, the methylation result was considered positive when the QMSP amplification curve crossed the set threshold before 50 cycles. However, amplification above threshold without an exponential curve was considered to be the result of stochastic amplification and the results of such a single analysis were therefore disregarded. Samples that were only one time “positive” or “negative” in duplicate analyses after quality control were analyzed at least in quadruplicate. No samples had to be disregarded because of stochastic amplification because sufficient analyses were performed to obtain reproducible results. In further statistical analysis, for every single gene, only a sample with sufficient DNA input (at least 225 pg β-actin DNA because, in samples with DNA input below 225 pg, stochastic amplification was too frequent) that was
positive at least twice after multiple analyses was considered to be hypermethylation positive. A sample with sufficient DNA input that was negative at least twice and at most one time positive after multiple analyses was considered to be negative.

Cytomorphologic Examination

After scraping of the cervix, 1 mL of cell suspension was diluted with ethanol-carbowax (7% polyethylene glycol, 50% ethanol) and, after resuspending, centrifuged for 10 min at 1000 rpm. The cell pellet was resuspended in ethanol-carbowax until an appropriate cell concentration was obtained. Hettich cytopsins (Hettich centrifuge, Depex B.V., Veenendaal, the Netherlands) were made on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-treated slides by centrifugation for 10 min at 1000 rpm. Cytopsins were Pap stained and routinely classified by two independent pathologists without knowledge of the clinical data. In the Netherlands, cervical smears are classified according to a modified Pap system in which borderline dyskaryosis corresponds well with the Bethesda classification atypical squamous cells of undetermined significance, mild dyskaryosis with low-grade squamous intraepithelial lesion, and moderate and severe dyskaryosis/carcinoma in situ with high-grade squamous intraepithelial lesion (31, 32).

Statistical Analysis

QMSP values were adjusted for DNA input by expressing results as ratios between two absolute measurements (average DNA quantity of methylated gene of interest/average DNA quantity for internal reference gene $\beta$-actin) $\times 10,000$; more detailed information was described previously (13, 17). The correlation between methylation ratios as determined in cervical scrapings and fresh frozen tissue samples was tested for all genes separately by the Spearman test. Differences in the heights of ratios between cancer patients and controls were tested with the Mann-Whitney $U$ test for all genes. To evaluate the clinical value of QMSP in cervical scrapings, “screen-positive” cutoff values were chosen above the highest observed in controls for all genes separately.

Observed differences were considered to be significant when associated with $P = 0.05$. All analyses were carried out using the SPSS software package (SPSS 11.5, Chicago, IL).

Acknowledgments

We thank Drs. E.G.E. de Vries, E. Schuuring, and S. de Jong for critically reading and reviewing our manuscript.

References

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