Detection of DNA hypermethylation as a diagnostic tool in cervical neoplasia in cervical neoplasia
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Detection of cervical cancer by Quantitative Methylation Specific PCR on cervico-vaginal samples obtained by a novel self-sampling device, a feasibility study

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Submitted
Abstract

Objective: Recently a self-sampling device for cervico-vaginal lavages has been introduced that allows sensitive high-risk human papillomavirus (hr-HPV) testing in population based screening for cervical neoplasia. For hr-HPV testing self-obtained lavages have been shown to be representative for detection of current HPV infections and cervical neoplasia, while in contrast cytomorphological assessment of these lavages appeared to be not representative for the underlying cervical neoplasia. In this study, we tested the feasibility for the detection of DNA hypermethylation by quantitative methylation specific PCR (QMSP) in cervico-vaginal samples.

Methods: Cervico-vaginal samples obtained by a self-sampling device and paired cervical scrapings were obtained from 20 cervical cancer patients. In addition to liquid based cytology (LBC) and hr-HPV DNA detection by Hybrid Capture II (HC-II), all samples were analyzed by QMSP for 3 gene promoters known to be frequently methylated in cervical cancer. Concordance between cervical scraping and lavage was measured by Cohen’s Kappa (k).

Results: Concordance between cervical scrapings and lavages was for QMSP of C13ORF18 $k=0.600 \ (p=0.006)$, CADM1 $k=0.583 \ (p=0.004)$ and CCNA1 $k=0.479 \ (p=0.024)$. Comparable concordance was observed for hr-HPV testing between cervical scraping and lavage ($k=0.565 \ (p=0.013)$). Concordance for cytomorphology between cervical scraping and lavage was low ($k=0.273 \ (p=0.076)$).

Conclusions: Concordance between DNA methylation testing in cervical scrapings and lavages was high, indicating a high potential of detection of (pre)malignant cervical cells using QMSP in cervico-vaginal samples obtained by a novel self-sampling device.
Methylation detection in self-obtained lavages

Introduction
Current population based screening programs for detection of (pre)malignant cervical lesions are based on cytomorphological assessment of cervical scrapings. Cytological screening is not an ideal method since its sensitivity varies from 30% to 87% and its specificity from 86% to 100% (1). Cytological screening is also less effective for the detection of adenocarcinoma (2;3), while the incidence of this malignant disease of the cervix is relatively increasing (4). Cervical carcinogenesis is highly associated with high-risk human papillomavirus (hr-HPV) infection and hr-HPV is detected in almost all high-grade intraepithelial lesions (HSIL) and cervical cancers (5;6). Hr-HPV DNA testing of cervical scrapings has been shown to improve sensitivity of cervical screening (7), but is also associated with low specificity (8;9), especially for young women (10).

More recently the detection of gene promoter hypermethylation of several cervical cancer specific genes by Quantitative Methylation Specific PCR (QMSP) has been suggested as an alternative diagnostic tool for early detection of (pre)malignant cervical lesions (11;12) and various methylated gene promoters in cervical neoplasia have been identified (13-20). Moreover, we and others have shown that the methylation status of a cervical scraping appears to be representative for the methylation status of the underlying lesion (11;14).

Despite improving screening tests technically, a major problem in current population based screening programs for cervical neoplasia is the participation rate. In the Netherlands, the total non-responders group is around 30%, which is comparable with other countries. Unfortunately, half of the cervical cancers is diagnosed in this group of women (21-25). A self-sampling method could increase the participation rate of a non-responder group with 30% (26). In countries currently without a population based screening program for cervical neoplasia, self-sampling might also be a practical alternative. For hr-HPV testing self-obtained lavages have been shown to be representative for detection of current HPV infections and cervical neoplasia, while in contrast cytomorphological assessment of these lavages appeared to be not representative for the underlying cervical neoplasia (27). Therefore, without existing data on detection of methylation in cervico-vaginal lavages and for improving applicability of self-sampling for population based screening, testing QMSP in cervico-vaginal lavages is required.

In the present study, we evaluated the detection of cervical cancer by QMSP of 3 cervical cancer specific biomarkers in cervico-vaginal samples obtained by a novel self-sampling device and compared these results with QMSP on cervical scrapings collected from the same patients.

Patients and Methods

Patients
Patients referred for cervical cancer were asked to participate in this study during their initial visit to the outpatient clinic of the University Medical Center Groningen in the time period November 2007 to March 2008. For all patients an examination under general anesthesia was planned for staging in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria. After obtaining written informed consent, first cervico-vaginal cells
were collected with a self-sampling device (Panterhei® Screener, Pantarhei Devices B.V., Zeist, The Netherlands), followed by a cervical scraping and a cervical biopsy to confirm diagnosis. All samples were taken during examination under general anesthesia by a gynecologic oncologist. Twenty consecutive cervical cancer patients were included in this study. Histological classification revealed: 15 with squamous cell carcinoma (75%), 4 with adenocarcinoma (20%) and 1 with aden-squamous carcinoma (5%). These patients were divided in 9 (45%) FIGO stage IB1, 3 (15%) FIGO stage IB2, 1 (5%) FIGO stage IIA, 4 (20%) FIGO stage IIB, 1 (5%) FIGO stage IIIA, 2 (10%) FIGO stage IIIB. The median age of the cervical cancer patients was 45 years, with a minimum age of 22 years and a maximum age of 85 years. This study was approved and followed the ethical guidelines of the Institutional Review Board of the University Medical Center Groningen.

Sample collection and DNA extraction
Cervico-vaginal cells were collected using a self-sampling device as described previously (27). In brief, the instrument is filled with 5 ml buffered saline and after release of the buffered saline into the vagina the buffered saline is aspirated back automatically by releasing the plunger. The solution containing cervico-vaginal cells was collected in ethanol-carbowax (2% polyethylene glycol, 50% ethanol). A total volume of 10 ml containing cervico-vaginal cells was divided into 3 fractions for cytomorphological assessment (1/5), Hybrid Capture II HPV testing (1/5) and DNA isolation (3/5). The cervical scrapings were collected using the Cervex-Brush® Combi Sterile (Rovers Medical Devices B.V., Oss, The Netherlands) and cells were resuspended in 5 ml PBS. Three ml was stored for DNA isolation (3/5). One fraction was resuspended in 1 ml carbowax for cytomorphology (1/5) and one fraction was resuspended in 1 ml carbowax for Hybrid Capture II HPV testing. Samples for Hybrid Capture II HPV testing were stored at 4°C and samples for DNA isolation were stored at -80°C. Cytospins (from lavage and cervical scraping) were Pap-stained and routinely classified by two cytologists and a pathologist without knowledge of the molecular and clinical data. DNA isolation was performed using standard salt-chloroform extraction and isopropanol precipitation. Precipitated DNA was resuspended in 150 µl of Tris-EDTA buffer. Genomic DNA was amplified in a multiplex PCR according to the BIOMED-2 protocol, to check the DNA quality (28).

Quantitative Methylation Specific PCR (QMSP)
QMSP was performed after bisulfite treatment on denatured genomic DNA as previously reported (12). Bisulfite treatment was performed with the EZ DNA methylation kit according to manufacturer's protocol (Zymogen, BaseClear, Leiden, The Netherlands). To correct for total DNA input, the housekeeping gene β-actin was used as a reference. QMSP was carried out in a total volume of 20 µl in 384 well plates in an Applied Biosystems 7900 Sequence Detector (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Each sample was analysed in triplicate. The final reaction mixture consisted of 300 nM of each primer, 200 nM probe, 1x QuantiTect Probe PCR Kit (Qiagen, Venlo, The Netherlands) and 50 ng of bisulfite converted genomic DNA. As a positive control, serial dilutions of genomic leukocyte DNA, in vitro
Methylation detection in self-obtained lavages

methylated with SssI (CpG) methyltransferase (New England Biolabs. Inc., Beverly, MA), were used in each experiment. A DNA sample was considered to be methylated if at least 2 or 3 triplicates showed exponential curves with a Ct-value below 50 and DNA input was at least 225 pg β-actin. All amplification curves were visualised and scored without knowledge of the clinical data. Three genes (C13ORF18, CADM1 and CCNA1) all known to be frequently methylated in cervical cancer were tested with QMSP (18;19).

HPV detection and typing
For detection of the presence of hr-HPV, liquid based cervical cells were used for Digene Hybrid Capture II (HC-II) DNA testing according to manufacturing protocol (www.qiagen.com). To confirm and specify HPV status of all samples, a specific HPV 16 PCR and a general primer-mediated PCR was performed using primer set GP5+/6+ as described previously (29). On all HPV 16 negative cases a subsequent nucleotide sequence analysis as described previously was performed (30).

Statistical Analysis
Comparison in detection rate for all tests between cervical scraping and cervico-vaginal lavage was analysed by Chi-square testing. Concordance between cervical scraping and lavage was measured by Cohen's Kappa. Statistical significance was assumed if the P value was < 0.05.

Results
In this study 20 patients referred for cervical cancer were included and in all cases cervical cancer was confirmed by histology. LBC revealed tumour cells in 19/20 cervical scrapings and in 15/20 lavages. The cervical scraping containing no tumour cells was classified as normal and the lavage of this patient did also not contain tumour cells. The four other lavages without visible tumour cells were classified as normal (n=2) and as containing atypical cells (n=2). Concordance between cervical scraping and lavage for LBC was $k=0.273$ (p=0.076) (table 1).

To determine whether the detection of methylation was similar in paired cervical scrapings and lavages, we performed QMSP for three cervical cancer related methylated gene promoters (table 1). The cervical scraping containing no visible tumour cells was methylation negative for all gene promoters tested, while the corresponding lavage was methylation positive for all tested gene promoters also without containing visible tumour cells. Two other lavages containing no tumour cells (classified as normal and atypical cells) were methylation negative for all gene promoters tested. However, in these patients all tested gene promoters were also negative in cervical scrapings, despite the presence of tumour cells. In total, 3 patients did not show methylation in both cervical scraping and lavage, while 2 lavages did not contain tumour cells. There was no statistical significant difference in detection rate for all tested genes between cervical scrapings and lavages. Concordance between cervical scrapings and lavages for all genes tested was high ($k>0.479$) and statistically significant (table 1).

Detection and concordance for hr-HPV by HC-II in cervical scrapings and lavages is shown in table 1. All cervical scrapings of the three patients negative for hr-HPV contained
tumour cells. Furthermore, these three patients negative for hr-HPV, were positive for one or more methylation markers in the cervical scraping and/or lavage. Concordance between cervical scrapings and lavages for hr-HPV testing by HC-II was high ($k=0.565$) and statistically significant (table 1).

**Discussion**

Our study shows for the first time that detection of DNA methylation in cervico-vaginal samples obtained by a novel self-sampling device is feasible and appears to be representative for the methylation status of the underlying lesion. Concordance between methylated gene promoters for cervical scrapings and lavages was high, as was also the case for hr-HPV testing, but not for cytomorphology.

Cervico-vaginal samples contain many normal vaginal cells and therefore a relative few abnormal cells can be easily missed by cytomorphological assessment of these lavages. In contrast, the PCR based QMSP assay needs only a few neoplastic cells to detect gene promoter methylation and this might explain that three out of 5 lavages are positive for one or more methylated gene promoters, while no tumour cells were observed.

For this feasibility study, we selected three gene promoters that each showed hypermethylation in 70% of cervical cancers in our hands, which explains the three methylation negative patients (scrapings and lavages). Ongoing work in our laboratory is to identify new specific cervical neoplasia methylated gene promoters, especially those that are not methylated in normal tissue and low-grade squamous intraepithelial lesions (LSIL), but are methylated in high-grade squamous intraepithelial lesions (HSIL) or cervical cancer.

Cervico-vaginal samples have previously been shown to be representative for detection of current hr-HPV DNA (27). In the future, population based screening programs for cervical neoplasia might be based on hr-HPV testing, a very sensitive test, in combination with a triage test, such as QMSP for a panel of hypermethylated genes. Such a combination test should have both a high negative as well as positive predictive value, preventing massive referrals to gynaecologists for further examination. An additional important advantage of QMSP as triage test after hr-HPV testing is, that both tests can be performed on the same sample, thereby avoiding additional gynaecologic examination of the patients.

In conclusion, we show that detection of methylation in cervico-vaginal lavages is feasibly and appears to be representative for the methylation status of the underlying lesion. The value of QMSP as a triage test in hr-HPV positive women needs to be further explored.

**Acknowledgement**

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Reference List


Table 1. Detection and concordance: cervical scraping versus cervico-vaginal lavage.

<table>
<thead>
<tr>
<th>Test</th>
<th>Detection rate cervical scraping</th>
<th>Detection rate cervico-vaginal lavage</th>
<th>Kappa</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBC</td>
<td>95% (19/20)</td>
<td>75% (15/20)</td>
<td>0.273</td>
<td>0.076</td>
</tr>
<tr>
<td>HC-II*</td>
<td>75% (15/20)</td>
<td>80% (16/20)</td>
<td>0.565</td>
<td>0.013</td>
</tr>
<tr>
<td>C13ORF18</td>
<td>50% (10/20)</td>
<td>60% (12/20)</td>
<td>0.600</td>
<td>0.006</td>
</tr>
<tr>
<td>CADM1</td>
<td>70% (14/20)</td>
<td>85% (17/20)</td>
<td>0.583</td>
<td>0.004</td>
</tr>
<tr>
<td>CCNA1</td>
<td>70% (14/20)</td>
<td>55% (11/20)</td>
<td>0.479</td>
<td>0.024</td>
</tr>
</tbody>
</table>

*Hr-HPV was specified by HPV PCR resulting in: 12 HPV-16, 2 HPV-18, 1 HPV-31, 1 HPV-45, 1 HPV-59 and 3 negative for hr-HPV.