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Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study ☆

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ABSTRACT

Objective. To explore the feasibility of DNA methylation analysis for the detection of cervical neoplasia in self-obtained cervico-vaginal lavages.

Methods. Lavages collected by a self-sampling device and paired cervical scrapings were obtained from 20 cervical cancer patients and 23 patients referred with an abnormal cervical smear (15 with high-grade cervical intraepithelial neoplasia (CIN2+) and 8 without CIN). All lavages and scrapings were analyzed by liquid based cytology (LBC), Hybrid Capture II (HC-II) for hr-HPV DNA detection and by DNA methylation analysis (*JAM3*, *TERT*, *EPB41L3* and *C13ORF18*). Concordance between lavages and scrapings was measured by Cohen's Kappa (k).

Results. In lavages and scrapings from cervical cancer patients ($n=20$), methylation analysis was positive in 19 (95%) and 19 (95%), HC-II in 16 (80%) and 15 (75%) and LBC in 15 (75%) and 19 (95%), respectively. In lavages and scrapings from CIN2+ patients ($n=15$), methylation analysis was positive in 10 (67%) and 12 (80%), HC-II in 15 (100%) and 15 (100%) and LBC in 11 (73%) and 12 (80%), respectively. Concordance between cervical scrapings and lavages ($n=43$) was for LBC $k=0.522$ ($p<0.001$), hr-HPV testing $k=0.551$ ($p<0.001$) and DNA methylation analysis $k=0.653$ ($p<0.001$).

Conclusions. DNA methylation analysis in cervico-vaginal lavages obtained by a self-sampling device is feasible and its diagnostic performance appears to be at least comparable to the detection of cervical neoplasia by cytomorphology and hr-HPV. Our pilot study suggests that detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages warrants exploration of its use in large prospective studies.

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 with sensitivity for CIN2+ of 55% [1]. Cervical carcinogenesis is highly associated with high-risk human papillomavirus (hr-HPV) infection and hr-HPV is detected in almost all high-grade cervical intraepithelial neoplasia (CIN2+) and cervical cancers [2,3]. Hr-HPV testing of cervical scrapings has been shown to improve sensitivity of cervical screening [4]. However, one of the major problems of hr-HPV testing is the low specificity, especially for young women [5], resulting in a high false-positive rate.

DNA promoter methylation of tumor suppressor genes has been reported to be an early event in cervical carcinogenesis [6]. Therefore, a test based on methylation markers could be relevant for the early detection of cervical neoplasia especially using markers that are not methylated in normal cells. Various methylated gene promoters have been identified, although none of these markers have a sufficiently high sensitivity and/or specificity to be used as primary screening tool in population-based screening [7]. Recently, in our search for cervical cancer specific methylation markers with a specificity of ~100% and the highest sensitivity (>80%) [8], we identified 4 markers (*JAM3*, *TERT*, *EPB41L3* and *C13ORF18*) out of

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213 cervical cancer specific methylation markers selected from literature in combination fulfilling these criteria [9,10]. Since, in our laboratory, these 4 markers currently form the most optimal methylation marker panel available, we used this 4-gene panel in the present study.

Apart from 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 to other countries with population-based screening programs. Unfortunately, half of the cervical cancers are diagnosed in this group of women [11–15]. Introduction of a self-sampling method resulted in an increase of the participation rate of a non-responder group up to 30% [16,17]. In countries currently without a population-based screening program for cervical neoplasia, self-sampling might be also a practical alternative.

A recent study showed that hr-HPV testing in self-obtained lavages is representative for detection of current HPV infections, while in contrast cytomorphological assessment, liquid based cytology (LBC), of these lavages appeared to be not representative for the underlying cervical neoplasia [18]. Until now, no data on DNA methylation analysis in cervico-vaginal lavages are available.

The aim of the present pilot study was to explore the feasibility of DNA methylation analysis for detection of cervical neoplasia in self-obtained cervico-vaginal lavages. In that respect we compared 1) results from DNA methylation analysis in cervico-vaginal lavages obtained by a self-sampling device to DNA methylation analysis on cervical scrapings collected from the same patients and 2) the detection of CIN2+ by DNA methylation analysis to a currently available methodology such as HC-II and LBC.

Patients and methods

Patients

Patients referred for cervical cancer or with an abnormal cervical smear were asked to participate in this study during their initial visit to the outpatient clinic of the University Medical Center Groningen. For all cervical cancer patients, an examination under general anesthesia was planned for staging in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria. During this examination, samples were taken by a gynecologist. For patients referred with an abnormal cervical smear, samples were taken during routine gynecologic examination at the first outpatient clinic visit. In all patients, cervico-vaginal cells were collected first with a self-sampling device (Delphi Screener®, Delphi Bioscience B.V., Scherpenzeel, The Netherlands), followed by a cervical scraping. Twenty consecutive cervical cancer patients (in the period of November 2007–March 2008) were included in this study and twenty-three consecutive patients referred with an abnormal cervical smear (October 2008–May 2009). Histological classification of cervical cancer patients revealed 15 with squamous cell carcinoma (75%), 4 with adenocarcinoma (20%) and 1 with adenosquamous carcinoma (5%). These patients were divided into 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 and 2 (10%) FIGO stage IIIB. The median age of the cervical cancer patients was 45 years (range 22–85 years). Histological classification of patients referred with an abnormal cervical smear revealed 3 micro-invasive carcinoma, 8 CIN3, 4 CIN2 (15 = CIN2+) and 1 CIN1 and 7 no dysplasia (CIN0) (8 = CIN0/1). Median age of patients referred with an abnormal cervical smear was 35 years (range 22–61 years). This study was approved and followed the ethical guidelines of the Institutional Review Board of the University Medical Center Groningen. All patients gave written informed consent.

Sample collection and DNA extraction

Cervico-vaginal cells were collected using a self-sampling device as described previously [18]. 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 (2 ml), Hybrid Capture II HPV testing (2 ml) and DNA isolation (6 ml). 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 milliliters was stored for DNA isolation. One milliliter was resuspended in one milliliter carbowax for cytomorphology and one milliliter in one milliliter 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. LBC was performed on cytospins (from lavages and cervical scrapings) that 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 [19].

Quantitative methylation specific PCR (QMSP)

QMSP was performed as we described previously [9,20]. In short, bisulfite treatment on denatured genomic DNA was performed with the EZ DNA methylation kit according to manufacturer's protocol (Zymogen, BaseClear, Leiden, The Netherlands). To correct for total DNA input, QMSP of 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 7900HT Fast Real-Time PCR System (Applied Biosystems, Nieuwekerk a/d IJsel, The Netherlands). The final reaction mixture consisted of 600 nM of each primer, 200 nM probe, 1× 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 methylated with SssI (CpG) methyltransferase (New England Biolabs. Inc., Beverly, MA), were used in each experiment. DNA methylation analysis was performed for four genes (*JAM3*, *TERT*, *EPB41L3* and *C13ORF18*) in triplicate. The QMSP primer and probe sequences used in this study are given in Table 1. DNA methylation analysis was scored positive when one of the genes showed any DNA methylation.

HPV detection

For detection of the presence of hr-HPV, standard Digene Hybrid Capture II (HC-II) DNA testing was used according to manufacturer's protocol (http://www.qiagen.com).

Statistical analysis

To determine the detection rate, CIN2+ was taken as a cut off value for the three tests in cervical scrapings and lavages. Concordance between cervical scrapings and lavages was measured by Cohen's Kappa. In cervical cancer patients, visible tumor cells in both samples were taken as cut off value to measure concordance for LBC. In patients referred with an abnormal cervical smear, moderate dysplasia was taken as cut off value to measure concordance for LBC. Statistical significance was assumed if the p value was <0.05.