Detection of DNA hypermethylation as a diagnostic tool in cervical neoplasia
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Methylation markers for CCNA1 and C13ORF18 are strongly associated with high-grade cervical intraepithelial neoplasia and cervical cancer in cervical scrapings

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* These authors contributed equally

Abstract

Purpose: Recently, we reported 13 possible cervical cancer specific methylated biomarkers identified by pharmacological unmasking microarray in combination with large-genome computational screening. Aim of the present study was to perform an in-depth analysis of the methylation patterns of these 13 candidate genes in cervical neoplasia and to determine their diagnostic relevance.

Experimental design and results: 5 of 13 gene promoters (C13ORF18, CCNA1, TFPI2, C1ORF166 and NPTX1) were found to be more frequently methylated in frozen cervical cancer compared to normal cervix specimens. Quantitative methylation analysis for these 5 markers revealed that CCNA1 and C13ORF18 methylation were both present in 68/97 cervical scrapings from cervical cancer patients and in only 5 and 3 scrapings, respectively, from 103 healthy controls (p<0.0005). In cervical scrapings from patients referred with an abnormal PAP smear, CCNA1 and C13ORF18 were methylated in 2/43 and 0/43 CIN 0 and in 1/41 and 0/41 CIN I, respectively. Furthermore, 8/43 CIN II, 22/43 CIN III and 3/3 micro-invasive cancer patients were positive for both markers. Although sensitivity for CIN II or higher (for both markers 37%) was low, specificity (96% and 100%, respectively) and positive predictive value (92% and 100%, respectively) were high.

Conclusion: Methylation of CCNA1 and C13ORF18 in cervical scrapings is strongly associated with CIN II or higher grade lesions. Therefore, these markers might be used for direct referral to gynecologists for patients with a methylation positive scraping.
Introduction
Cervical cancer is an important cause of death in women worldwide(1). Cervical carcinogenesis is strongly associated with (high-risk) human papillomavirus (HPV) infections(2). Cytomorphological examination of cervical smears is a widely applied, though not ideal screening method for cervical cancer and its precursors (cervical intraepithelial neoplasia (CIN)(1;3;4). High risk HPV (hr-HPV) testing has been suggested to improve cervical cancer screening (5;6). However the specificity of hr-HPV testing, especially in a young screening population is relatively low(7;8). Therefore, other objective biomarkers are needed to improve specificity for cervical cancer screening(9). Promoter hypermethylation analysis(10-12) might represent such markers.

Promoter hypermethylation of tumor suppressor genes is a common feature of human cancers mostly resulting in silencing of gene expression(13). 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 (MSP). In fact, one would like to have a similar methylation marker as has been reported for \textit{GST-P1} in prostate cancer, in which promoter methylation is present in 95% of the adenocarcinomas, whereas the normal prostate tissue is negative(14). Promoter hypermethylation of tumor suppressor genes in general has been reported to be an early event in cervical carcinogenesis(15) and consequently hypermethylation analysis might be relevant especially for the early detection of cervical neoplasia.

Over the past years, assessment of methylation markers in cervical scrapings for the detection of cervical cancer and CIN appeared to be feasible(9-12;16-22). In these studies a variety of gene promoters has been investigated, mainly chosen due to their previously reported methylation status in cervical cancer tissue or in other tumor types. However, still only few of these methylation markers have been reported to be cervical cancer specific, i.e. most cancers/high-grade CIN are methylated and simultaneously no false positive results in scrapings of women with no or low-grade CIN. However, for most of these markers a threshold was set in order to obtain a high specificity. Recently, an editorial emphasized the need for greater standardization of current approaches and suggested that large-scale, non-targeted studies are necessary to further characterize DNA methylation biomarkers in cervical cancer(23).

The identification of cancer-specific methylated markers should provide new targets for diagnostic and therapeutic intervention. Methods for identifying such markers based on pharmacologic unmasking of the promoter region and detection of re-expression on microarray analysis have indeed revealed such new candidate cancer specific methylated genes(18;24;25). Using this approach in combination with a novel relaxation ranking methodology, we recently showed that genes were significantly enriched towards methylation in cervical cancer(26). In addition, we reported on the modification and improvement of the selection of candidate markers based on a promoter structure algorithm and microarray data generated from 20 cancer cell lines of 5 major cancer types(27). Regarding cervical cancer, our initial large-genome computational screening approach identified 45 cervical cancer specific putatively methylated
biomarkers. Preliminary screening indicated 13 potential cervical cancer specific genes, using BSP on 2 normal cervices and 10 cervical cancers(27).

Aim of the present study was 1) to perform an in-depth analysis of the methylation patterns of these 13 candidate genes in cervical cancer and normal tissue specimens and 2) to evaluate their possible relevance for detection of cervical neoplasia in a large series of scrapings from patients with cervical cancer, low- and high-grade CIN and from otherwise healthy women.

**Patients and Methods**

**General strategy**

For our in depth analysis of the methylation patterns of the 13 putative cervical cancer specific genes, previously identified by us(27), the following strategy was used (see figure 1). First MSP for 13 genes was performed on DNA isolated from 20 normal cervices and 20 cervical cancers. In this first step, macrodissected frozen tissue sections were used, as the amount of DNA isolated from frozen tissue sections is much larger than from cervical scrapings, thereby allowing multiple MSPs. Genes selected in the first step were further evaluated in a second step by QMSP in cervical scrapings from a large series of cervical cancer patients (n=97) and healthy age-matched controls (n=103). This second step enabled us to investigate the discriminative power of methylation analysis for cervical cancers compared to normal scrapings and to analyze if methylation is related to stage or histology (in the cervical cancer group) or to age (in the group of controls). The potential as a diagnostic tool of QMSP for the genes selected in the second step was finally evaluated in a third step in a large series of scrapings (n=185) from selected patients, referred to our department with an abnormal Pap smear and with no CIN (i.e. CIN 0), CIN grade I-III or cervical cancer.

**Patients**

All patients referred between 2001 and 2007 because of cervical cancer or an abnormal Pap smear were asked to participate in our study during their initial visit to the outpatient clinic of the University Medical Center Groningen. Gynecological examination under general anaesthesia was performed in all cervical cancer patients for staging in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria(28). Cervical scrapings were collected during the initial visit to the outpatient department or at gynecologic examination under general anesthesia. In patients referred for an abnormal Pap smear CIN diagnosis was always based on histology, from either a biopsy or large loop excision specimen. As healthy controls, scrapings and tissue of normal cervices were obtained from patients without a history of abnormal Pap smears or any form of cancer who were planned to undergo a hysterectomy for non-malignant reasons during the same period. Indications for hysterectomy were fibroids, prolaps uteri, adenomyosis, hypermenorrhea or a combination of these. All cervical tissues were judged as histopathologically normal.

For MSP analysis (step 1), frozen tissue samples of 20 squamous cell cervical cancer patients were selected (7 FIGO stage IB (35%), 6 FIGO stage IIA (30%), 3 FIGO stage IIB
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(15%), 2 FIGO stage IIIB (10%) and 2 FIGO stage IV (10%) and as controls frozen tissue samples of 20 normal cervices. Median age of cervical cancer patients was 55 years (IQ range 24 – 86) and of healthy controls 50 years (IQ range 38 – 66). For QMSP analysis (step 2), scrapings were selected randomly from our larger database (n = 411), including 97 cervical cancer patients (45 FIGO stage IB (46%), 11 FIGO stage IIA (11%), 11 FIGO stage IIA=B (11%), 16 FIGO stage IIB (16%), 2 FIGO stage IIIA (2%), 9 FIGO stage IIIB (9%), 2 FIGO stage IVA (2%) and 1 FIGO stage IVB (1%); 82 squamous cell carcinomas (85%) and 15 adenocarcinomas (15%)) and 103 patients with a normal cervix. Median age of both cervical cancer patients (median: 48 yrs, 23-88) and controls (median: 48 yrs, 30-77) was the same. For evaluation of the diagnostic performance of QMSP analysis for selected genes (step 3), we collected from our large database comprising all patients referred with an abnormal Pap smear (n=808), five groups, based on their histological diagnosis: CIN 0 (n=45, median age=38 yrs.), CIN I (n=44, median age=35 yrs.), CIN II (n=45, median age=35 yrs.), CIN III (n=47, median age=33 yrs.) and (micro)invasive cervical cancer (n=4, median age=42.5 yrs.). Informed consent was obtained from all patients and controls participating in this study. This study was approved by the Institutional Review Board of the UMCG.

Sample collection procedure and DNA isolation
From the frozen tissue samples, sections (10 \( \mu \)m) were cut and in case of normal cervices macrodissection was performed to enrich for epithelial cells. The percentage of either normal or tumor cells present in the frozen sections used for DNA isolation was determined by staining parallel sections with hematoxylin and eosin. Cervical scrapings were collected using an Ayre's spatula and endocervical brush. The spatula and brush with the collected cells were then suspended in 5 ml of phosphate buffered saline (PBS: 6.4 mM Na2HPO4; 1.5 mM KH2PO4; 0.14 M NaCl; 2.7 mM KCl (pH 7.2)) and kept on ice until further processing. Of this cell suspension, 1 ml was used for cytomorphic assessment. Cytospins were Pap stained and routinely classified by two independent pathologists without knowledge of the molecular and clinical data according to a modified Papanicolaou system(29). The remaining part (4 ml) was pelleted, snap-frozen in liquid nitrogen and stored at -80°C as described previously(12). DNA was extracted using standard salt-chloroform extraction and ethanol precipitation for high molecular DNA and dissolved in 150 \( \mu \)l TE-4 buffer (10 mM Tris; 1 mM EDTA (pH 8.0))(12). For quality control, genomic DNA was amplified in a multiplex PCR according to the BIOMED-2 protocol(30).

HPV detection and typing
In all samples, presence of high risk HPV was analyzed by PCR using HPV16 and HPV18 specific primers. On all HPV16- or HPV18- negative cases, a general primer-mediated PCR was performed using two HPV consensus primer sets, CPI/CPIIG and GP5+/6+, with subsequent nucleotide sequence analysis as described previously(12). As a control for the specificity and sensitivity of each HPV-PCR, a serial dilution of DNA extracted from HPV16-positive CaSki and HPV18-positive HeLa cell lines.
Methylation specific PCR (MSP)

MSP was performed after bisulfite treatment on denatured genomic DNA as previously reported (31). Bisulfite treatment was performed with the EZ DNA methylation kit according to manufacturer’s protocol (Zymogen, BaseClear, Leiden, the Netherlands). For PCR, 50 ng of DNA was used. Primer pairs are available upon request. A sample was considered methylation positive when a PCR product of the right size was visible after 40 cycles of PCR. As a positive control, in vitro methylated genomic DNA with Sss I (CpG) methyltransferase (New England Biolabs. Inc., Beverly, MA) and a negative control, a pool of leukocyte DNA from healthy women were used in each experiment.

Real time quantitative methylation specific PCR (QMSP)

QMSP was performed with bisulfite treated DNA as previously reported (12;31;32). Primer pairs and probes are available upon request. The housekeeping gene β-actin was chosen as reference for total DNA input measurement. QMSP was carried out in a total volume of 20 μl in 384 well plates in an Applied Biosystems 7900 Sequence Detector (Applied Biosystems, Nieuwerkerk a/d IJsel, the Netherlands). Each sample was analyzed 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 in vitro methylated genomic leucocyte DNA with Sss I (CpG) methyltransferase (New England Biolabs. Inc., Beverly, MA) were used in each experiment. All amplification curves were visualized and scored without knowledge of the clinical data. A DNA sample was considered methylated if at least 2 of 3 triplicates showed exponential curves with a Ct-value below 50 and DNA input was at least 225 pg β-actin (equivalent to a Ct-value of 34). 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 β-actin) x 10000)(12;31;32).

Statistics

All analyses were carried out using the SPSS software package (SPSS 14.0, Chicago, IL, USA). Methylation ratios between groups were compared using the Mann-Whitney U test (2 groups) or the Kruskal-Wallis test (>2 groups). Associations between numerical parameters were analyzed using the χ² test with Fisher’s exact test for small numbers when appropriate. Associations between positive methylation and age were analyzed with Student T-test. Observed differences with a p-value <0.05 were considered statistically significant.

Results

MSP analysis in normal cervices and cervical cancer tissues

Table 1 summarizes MSP analysis in frozen tissue specimens from 20 normal cervices and 20 cervical cancer patients. C13orf18, CCNA1, TFPI, C1orf166 and NPTX1 were more frequently methylated in cancers versus controls. OGDHL was methylated in almost all cancers and normal cervices and GDAP1L1 was methylated in half of the cancers and half of the normal
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cervices. PTGS2, ASMTL, ARMC7, HCP1, C9ORF19 and DLL4 showed no or less methylation in cancers versus normal cervices. In sum, of the initial 13 markers five gene promoters (C13orf18, CCNA1, TFPI2, C1orf166 and NPTX1) showed significantly more methylation in cervical cancers compared to normal cervices and were therefore selected for further evaluation in cervical scrapings.

**QMSP analysis in scrapings from cervical cancer patients and controls**
QMSP for 5 gene promoters (C13orf18, CCNA1, TFPI2, C1orf166 and NPTX1) was performed on scrapings of normal cervices (n=103) and cervical cancers (n=97) (figure 2). Both the level and frequency of methylation of all 5 gene promoters were higher in the cancer samples compared to the normal cervices (p<0.0005). CCNA1 and C13orf18 showed almost no methylation in the normal cervices (5% and 3%, respectively), while for both genes 71% of cancers were methylation positive. C1orf166 was never positive in the normal cervices, however, only 34% of cervical cancers were positive and this positivity was not additive to CCNA1 and/or C13orf13 positivity. The other 2 gene promoters, TFPI2 and NPTX1, frequently showed methylation in the normal cervical scrapings. Therefore, CCNA1 and C13orf18 were selected for further validation of their diagnostic performance. In the control group, methylation positivity of the 5 analyzed genes was not related to age indicating that methylation is not due to aging in the scrapings of the cancer group.

**QMSP and hr-HPV analysis in scrapings from patients with an abnormal Pap smear**
QMSP for CCNA1 and C13orf18 was performed on scrapings from 173 patients referred to our department with an abnormal Pap smear, as DNA input was too low for 12 patients. QMSP analysis for CCNA1 and C13orf18 (figure 3) revealed that levels and positivity of both gene promoters were increased with the severity of the underlying histological lesion (p<0.0005). Almost all scrapings from CIN 0 and CIN I patients were unmethylated for CCNA1 and C13orf18, while 25% of scrapings from CIN II, 51% of CIN III and all (n=3) micro-invasive cancers showed methylation (see figure 3).

Hr-HPV was detected in 20 of 43 CIN 0, 27 of 41 CIN I, 32 of 43 CIN II, 41 of 43 CIN III and 3 of micro-invasive cancers. Hr-HPV was related with the severity of the underlying lesion (p<0.0005). The following high-risk types were found: 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59 and 66. In addition, HPV6, 70 and 90 were found in 2 CIN 0, 3 CIN I and 1 CIN II, however these were not depicted as hr-HPV.

**Diagnostic performance of hr-HPV and QMSP for CCNA1 and C13orf18**
Table 2 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for CIN II or higher of the different assays. Specificity for CIN II or higher of CCNA1 and C13orf18 promoter methylation analysis alone or combined was high (>93%) with a low sensitivity (37%). Interestingly, all 3 cancers were depicted by QMSP and 51% of the CIN III patients. Especially, the PPVs for CIN II or higher of promoter methylation analysis for CCNA1 and C13orf18 were high (92% and 100%, respectively).
Hr-HPV analysis showed the opposite result compared to methylation analysis i.e. high sensitivity (85%), but low specificity (53%-44%) for detection of CIN II or higher, with a moderate PPV and NPV.

As HPV DNA testing has also been suggested as a primary screening tool in population-based cervical screening, we also determined sensitivity, specificity, PPV and NPV of hypermethylation detection for CCNA1 and C13ORF18 in hr-HPV positive patients. This analysis indicates that the test performance of our methylation markers in the hr-HPV positive patients is equal to the test performance in the whole group of patients (see table 2).

Finally, we also analyzed the performance of hr-HPV DNA testing and methylation analysis as triage tests in patients referred with a Pap smear comprising atypical squamous cells of unknown significance (ASCUS) or low-grade dysplasia. The test performance of hr-HPV in this specific group was moderate (41%-80%). The specificity and PPV for CIN II or higher of methylation analysis was high (>93%) and equal when compared to the whole group.

**Discussion**

Using a promoter structure algorithm and microarray expression data, we previously identified 13 potential gene promoters specifically methylated in cervical cancer(27). Our present strategy using both cervical tissue specimens as well as cervical scrapings allows for a straightforward in-depth analysis of the methylation patterns of these gene promoters and their possible diagnostic relevance in cervical neoplasia. Our study indicates that in cervical scrapings hypermethylation analysis for two markers (CCNA1 and C13ORF18) has a high specificity (96% and 100%, respectively) and high positive predictive value (PPV) (100% and 92%, respectively), but low sensitivity for CIN II or higher (36% and 38%, respectively).

The current approach for early detection of cervical neoplasia in population based screening programs is still cytomorphological assessment of cervical scrapings, despite its low sensitivity(33). Therefore, new approaches for detection of cervical neoplasia need to be developed. In this respect, population based screening with hr-HPV DNA testing has been extensively studied by many different investigators and appears to be on the brink of (Western) worldwide introduction. Our study however shows again the low specificity of hr-HPV testing and it is clear that especially in younger populations this approach for population based screening needs a triage test to prevent massive referrals to gynecologists(34). Examples of such triage tests are cytology or methylation tests. Different characteristics of a triage test after a positive hr-HPV test can be envisioned. Our methylation test has a high positive predictive value for CIN II or higher and therefore, hr-HPV positive patients with a positive methylation test could be referred to a gynecologist directly for colposcopy and treatment in the same procedure. An important advantage of a methylation test in this respect is, that such a test can be performed on the already available specimen, thus avoiding extra visits to general practitioners or gynecologists. Besides using our methylation test as a triage test in primary hr-HPV screening, this test might also be applied in a triage setting after primary cytological screening for patients referred with a smear showing ASCUS or mild dysplasia. In case of a positive test, again a more efficient see and treat policy could be followed instead of taking colposcopically guided biopsies.
Methylation markers in cervical cancer screening

It is obvious that cost-effectiveness of the introduction of our methylation test as a triage test should be further explored in prospective large-scale trials in women participating in population-based screening programs.

Figure 4 shows possible scenarios for applications of our current methylation test. However, due to the low NPV of our current methylation test, massive referrals to gynecologists after a positive hr-HPV DNA and negative methylation test are still not prevented and therefore another triage test such as cytology will still be needed.

In order to become integrated in primary screening for cervical neoplasia, better or additional methylation markers should be added in order to increase the sensitivity, without loosing the specificity for CIN II or higher grade lesions. Many studies have been conducted to analyze specific gene promoter methylation in cervical scrapings(10-12;17;20;35), while others studies searched for novel methylation markers(18;21;36). One of these studies(18) described a similar approach to our study, in which the pharmacological unmasking microarray approach was used resulting in the identification of 6 gene promoters (SPARC, TFPI2, RRAD, SFRP1, MT1G, and NMES1) specifically methylated in cervical cancer. TFPI2 was the only gene promoter similarly identified from their and our study. In the present study, TFPI2 was evaluated although not further analyzed in the cross-sectional study as 26% of the normal cervical scrapings showed methylation. We evaluated methylation of SPARC previously in both paraffin tissues and matched scrapings of histologically proven (pre)malignant cervical lesions (Yang et al., paper submitted for publication). SPARC showed more frequent methylation in cervical cancers, but also many normal cervixes were positive (13/20). Although SPARC and TFPI2 were the most promising gene promoters in the study of Sova et al.(18), our evaluation in paraffin tissues and scrapings showed a low specificity.

Besides the need to identify a methylation marker that is able to detect all CIN II and higher grade lesions, the biological process of de novo methylation of promoter regions of tumor suppressor genes in cervical carcinogenesis might be of interest. We show in our study that approximately 25% of CIN II scrapings and 50% of CIN III scrapings are positive for C13ORF18 and/or CCNA1. It is also generally assumed that approximately these percentages of CIN II/III will progress to cancer when left untreated(37). One could hypothesize that only methylation positive lesions are propelled to progress and therefore need treatment. However, it will be difficult to explore such a hypothesis. E.g. patients diagnosed with CIN II/III, preferably based on as small biopsies as possible(38), should be asked to participate in a wait-and-see study. Long-term follow-up of these patients by colposcopy should allow us to analyze possible relation between methylation status and regression/progression of the lesions. However, such studies are hard to perform and easily flawed by different types of methodological biases.

In this study, we showed that 5 genes were specifically methylated in cervical cancer compared to normal cervical specimens. CCNA1, TFPI2 and NPTX1 were previously described to be frequently methylated in cervical cancer(18;26;39). Available data for CCNA1 and TFPI2 is in line with our present data. However, we found more TFPI2 methylation in scrapings of normal cervixes and less CCNA1 methylation in scrapings of cervical cancers, which might be because of the small group size of the other studies. C13ORF18 and C1ORF166 have not previously
been described to be methylated in any type of cancer. C1ORF166, now known as MUL1 or MULAN, is a RING finger E3 ubiquitin ligase, anchored to mitochondria and implicated in the regulation of mitochondrial dynamics. Because C1OR166 has been reported to activate NF-kB pathway(40;41), its inactivation due to hypermethylation implies a functional role for NF-kB during progression of cervical cancer. However, this needs further studies. Finally, C13ORF18, represents a gene with unknown function. Sequence comparisons suggest a role as phosphatase inhibitor (www.genecards.com) that would fit with the function of a tumor suppressor gene inactivated in cancer by hypermethylation. The elucidation of the function of this gene is now subject of our future research.

In conclusion, two gene promoters, CCNA1 and C13ORF18 showed more methylation with increasing severity of the underlying lesion analyzed in cervical scrapings from patients with an abnormal smear. The PPV for our methylation test with these markers was high. Whether patients with a methylation positive scraping should be directly referred for treatment of their cervical neoplasia deserves further exploration in prospective studies on population-based screening for cervical cancer.

Acknowledgments
This study was supported by OncoMethylome Sciences S.A., Liège, Belgium and by the Dutch Cancer Society (NKB) (project-number RUG 2004-3161). N. Yang is a bursary of Bernouilli.


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(37) McCredie MR, Sharples KJ, Paul C, Baranyai J, Medley G, Jones RW, Skegg DC. Natural history of cervical neoplasia and risk of invasive cancer in women with


Table 1: Methylation positivity in frozen tissue obtained from patients with normal cervix or cervical cancer.

<table>
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<tr>
<th>gene</th>
<th>cancer</th>
<th>normal</th>
<th>p-value&lt;sup&gt;1&lt;/sup&gt;</th>
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<td>7/20</td>
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<td>9/20</td>
<td>0.001</td>
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<td>C1ORF166</td>
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<td>1/20</td>
<td>0.02</td>
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<tr>
<td>NPTX1</td>
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<td>0.106</td>
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<td>GDAP1L1</td>
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<td>6/20</td>
<td>0.197</td>
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<td>0/20</td>
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<td>2&lt;sup&gt;2&lt;/sup&gt;</td>
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</table>

<sup>1</sup>: p-value was calculated by chi-square. If groups were too small, the Fisher exact test was applied. <sup>2</sup>: No statistics could be computed as methylation mark is a constant.
Table 2: Sensitivity to detect CIN II/III (HSIL) or cancer, specificity for only CIN 0 or CIN 0/I, positive predictive value (PPV) for HSIL and cancer and negative predictive value (NPV) for CIN 0 or CIN 0/I of methylation positivity or hr-HPV in scrapings obtained from patients with an abnormal smear (n=173).

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<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
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<td>CIN 0/I</td>
<td>HSIL/ca</td>
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<tr>
<td><strong>CCNA1</strong></td>
<td>33/89 (37%)</td>
<td>41/43 (95%)</td>
<td>81/84 (96%)</td>
<td>33/36 (92%)</td>
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<tr>
<td><strong>C13ORF18</strong></td>
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<td><strong>CCNA1/C13ORF18</strong></td>
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<td>41/43 (95%)</td>
<td>81/84 (96%)</td>
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<td><strong>Hr-HPV</strong></td>
<td>76/89 (85%)</td>
<td>23/43 (53%)</td>
<td>37/84 (44%)</td>
<td>76/123 (62%)</td>
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Only Hr-HPV pos pts

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<th></th>
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<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
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<td>CIN 0/I</td>
<td>HSIL/ca</td>
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<td><strong>CCNA1</strong></td>
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<td>19/21 (90%)</td>
<td>45/48 (94%)</td>
<td>29/32 (91%)</td>
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<td><strong>C13ORF18</strong></td>
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<td>21/21 (100%)</td>
<td>48/48 (100%)</td>
<td>30/30 (100%)</td>
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<td><strong>CCNA1/C13ORF18</strong></td>
<td>38/76 (50%)</td>
<td>19/21 (90%)</td>
<td>45/48 (94%)</td>
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Only Pap II/IIIa pts

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<th>PPV</th>
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<tr>
<td></td>
<td>HSIL/ca</td>
<td>CIN 0</td>
<td>CIN 0/I</td>
<td>HSIL/ca</td>
</tr>
<tr>
<td><strong>CCNA1</strong></td>
<td>8/39 (21%)</td>
<td>40/42 (95%)</td>
<td>75/78 (96%)</td>
<td>8/11 (73%)</td>
</tr>
<tr>
<td><strong>C13ORF18</strong></td>
<td>5/39 (13%)</td>
<td>42/42 (100%)</td>
<td>78/78 (100%)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td><strong>CCNA1/C13ORF18</strong></td>
<td>10/39 (26%)</td>
<td>40/42 (95%)</td>
<td>75/78 (96%)</td>
<td>10/13 (77%)</td>
</tr>
<tr>
<td><strong>Hr-HPV</strong></td>
<td>30/39 (77%)</td>
<td>23/42 (55%)</td>
<td>35/78 (45%)</td>
<td>30/73 (41%)</td>
</tr>
</tbody>
</table>
Figure 1: Flow scheme for analysis of the diagnostic performance of 13 cervical cancer specific genes identified in our previous study [27].
Figure 2: Methylation ratio and frequency in cervical scrapings obtained from patients with normal cervix or cervical cancer (squamous cell cervical cancer (SCC) and adenocarcinomas (AC)). The level and frequency of methylation for all gene promoters are increasing with the severity of the lesion (all p<0.0005).
Figure 3: Methylation ratio and frequency in cervical scrapings obtained from patients referred with an abnormal smear (n=173). The final diagnosis was no CIN (CIN 0), CIN I, CIN II, CIN III or micro-invasive cancer ((m)CC). The level and frequency of methylation is increasing with the severity of the lesion (both p<0.0005).
Figure 4: Proposed scheme for the incorporation of a methylation test in cervical cancer screening if either hr-HPV or cytology will be used as primary screening.