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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Abstract

Purpose: Recently, we reported 13 possible cervical cancer–specific methylated biomarkers identified by pharmacologic unmasking microarray in combination with large-genome computational screening. The 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: Five of the 13 gene promoters (C13ORF18, CCNA1, TFPI2, C1ORF166, and NPTX1) were found to be more frequently methylated in frozen cervical cancer compared with normal cervix specimens. Quantitative methylation analysis for these five markers revealed that both CCNA1 and C13ORF18 were methylated in 68 of 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 of 43 and 0 of 43 CIN 0 (no cervical intraepithelial neoplasia) and in 1 of 41 and 0 of 41 CIN I, respectively. Furthermore, 8 of 43 CIN II, 22 of 43 CIN III, and 3 of 3 microinvasive 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. (Cancer Epidemiol Biomarkers Prev 2009;18(11):3000–7)

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). Cytomorphologic examination of cervical smears is a widely applied, although not ideal, screening method for cervical cancer and its precursors [cervical intraepithelial neoplasia (CIN); refs. 3-5]. High-risk HPV (hr-HPV) testing has been suggested to improve cervical cancer screening (6, 7). However, the specificity of hr-HPV testing, especially in a young screening population, is relatively low (8, 9). Therefore, other objective biomarkers are needed to improve specificity for cervical cancer screening (10). Promoter hypermethylation analysis (11-13) might represent such markers.

Promoter hypermethylation of tumor suppressor genes is a common feature of human cancers mostly resulting to silencing of gene expression (14). 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 (MSP) techniques. In fact, one would like to have a similar methylation marker as has been reported for GST-P1 in prostate cancer, in which promoter methylation is present in 95% of the adenocarcinomas, whereas the normal prostate tissue is negative (15). Promoter hypermethylation of tumor suppressor genes in general has been reported to be an early event in cervical carcinogenesis (16). 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 seemed to be feasible (10-13, 17-23). In these studies, a variety of gene promoters have 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 to obtain a high specificity. Recently, an editorial emphasized the need for greater...
standardization of current approaches and suggested that large-scale, nontargeted studies are necessary to further characterize DNA methylation biomarkers in cervical cancer (24).

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 reexpression on microarray analysis have indeed revealed such new candidate cancer-specific methylated genes (19, 25, 26). Using this approach in combination with a novel relaxation ranking methodology, we recently showed that genes were significantly enriched toward methylation in cervical cancer (27). 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 (28). 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 bisulfite sequencing PCR on 2 normal cervixes and 10 cervical cancers (28).

The aims of the present study were (a) to perform an in-depth analysis of the methylation patterns of these 13 candidate genes in cervical cancer and normal tissue specimens and (b) 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 (28), the following strategy was used (see Fig. 1). First, MSP for 13 genes was performed on DNA isolated from 20 normal cervixes 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 that from cervical scrapings, thereby allowing multiple MSFs. Genes were ranked on \( P \) value, and genes significantly more frequently methylated in cervical cancers compared with normal cervixes were selected for further evaluation in a second step by quantitative MSP (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 with 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). Concordance between the assays was very high as determined during optimization of QMSP, when the same samples used for MSP were analyzed (data not shown). 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 1 to III, or cervical cancer.

**Patients.** All patients referred from 2001 to 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. Gynecologic examination under general anesthesia was done in all cervical cancer patients for staging in accordance with the International Federation of Gynecology and Obstetrics (FIGO) criteria (29). 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 cervixes were obtained from patients without a history of abnormal Pap smears or any form of cancer who were planning to undergo a hysterectomy for nonmalignant 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer</th>
<th>Normal</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13orf18</td>
<td>13/20</td>
<td>1/20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>CCNA1</td>
<td>17/20</td>
<td>7/20</td>
<td>0.001</td>
</tr>
<tr>
<td>TFPI</td>
<td>19/20</td>
<td>9/20</td>
<td>0.001</td>
</tr>
<tr>
<td>C1orf166</td>
<td>8/20</td>
<td>1/20</td>
<td>0.02</td>
</tr>
<tr>
<td>Nptxi1</td>
<td>4/20</td>
<td>0/20</td>
<td>0.106</td>
</tr>
<tr>
<td>Gdapfl1</td>
<td>10/20</td>
<td>6/20</td>
<td>0.197</td>
</tr>
<tr>
<td>Ptg2/cox2</td>
<td>2/20</td>
<td>0/20</td>
<td>0.487</td>
</tr>
<tr>
<td>AsmItL</td>
<td>0/20</td>
<td>2/20</td>
<td>0.487</td>
</tr>
<tr>
<td>OgdhL</td>
<td>17/20</td>
<td>19/20</td>
<td>0.605</td>
</tr>
<tr>
<td>ArmC7</td>
<td>3/20</td>
<td>1/20</td>
<td>0.605</td>
</tr>
<tr>
<td>Hcpi</td>
<td>2/20</td>
<td>4/20</td>
<td>0.01</td>
</tr>
<tr>
<td>C9orf19</td>
<td>0/20</td>
<td>0/20</td>
<td>†</td>
</tr>
<tr>
<td>Dll4</td>
<td>0/20</td>
<td>0/20</td>
<td>†</td>
</tr>
</tbody>
</table>

* \( P \) value was calculated by \( \chi^2 \). If groups were too small, Fisher’s exact test was applied.
† No statistics could be computed as methylation mark is a constant.
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 (15%), 2 FIGO stage IIIB (10%), and 2 FIGO stage IV (10%)], as were frozen tissue samples of 20 normal cervix as controls. The median age of cervical cancer patients was 55 y [interquartile (IQ) range, 24-86 y], whereas that of healthy controls 50 y (IQ range, 38-66 y). 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 IIAB (11%), 16 FIGO stage IIB (16%), 2 FIGO stage IIIA (2%), 9 FIGO stage IIIB (9%), 2 FIGO stage IV A (2%), and 1 FIGO stage IV B (1%)].

**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 increased 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 increased with the severity of the lesion (both \(P < 0.0005\)).
Only hr-HPV

<table>
<thead>
<tr>
<th>Gene/HPV</th>
<th>CIN 0</th>
<th>CIN 0/I</th>
<th>HSIL/cancer</th>
<th>CIN 0</th>
<th>CIN 0/I</th>
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<tbody>
<tr>
<td>CCNA1</td>
<td>33/89 (37%)</td>
<td>19/21 (90%)</td>
<td>45/48 (94%)</td>
<td>30/30 (100%)</td>
<td>43/83 (50%)</td>
</tr>
<tr>
<td>C13ORF18</td>
<td>29/76 (38%)</td>
<td>21/21 (100%)</td>
<td>48/48 (100%)</td>
<td>21/22 (95%)</td>
<td>48/84 (56%)</td>
</tr>
<tr>
<td>CCNA1/C13ORF18</td>
<td>38/76 (50%)</td>
<td>21/21 (100%)</td>
<td>45/48 (94%)</td>
<td>30/30 (100%)</td>
<td>43/84 (50%)</td>
</tr>
</tbody>
</table>

Only hr-HPV-positive patients

CCNA1

C13ORF18

<table>
<thead>
<tr>
<th>Gene/HPV</th>
<th>CIN 0</th>
<th>CIN 0/I</th>
<th>HSIL/cancer</th>
<th>CIN 0</th>
<th>CIN 0/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNA1</td>
<td>33/33 (100%)</td>
<td>10/10 (100%)</td>
<td>20/20 (100%)</td>
<td>10/10 (100%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>C13ORF18</td>
<td>30/30 (100%)</td>
<td>10/10 (100%)</td>
<td>20/20 (100%)</td>
<td>10/10 (100%)</td>
<td>20/20 (100%)</td>
</tr>
</tbody>
</table>

Methylation-Sensitive PCR. MSP was performed after bisulfite treatment on denatured genomic DNA as previously reported (32). Bisulfite treatment was performed with the EZ DNA methylation kit according to the manufacturer’s protocol (Zymogen, BaseClear). For PCR, 50 ng of DNA were used. Primer pairs are listed in Supplementary Table S1. 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.) and a negative control, a pool of leukocyte DNA from healthy women, were used in each experiment.

Real-time QMSP. QMSP was performed with bisulfite-treated DNA as previously reported (13, 32, 33). Primer pairs and probes are listed in Supplementary Table S1. 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). Each sample was analyzed in triplicate. The final reaction mixture consisted of 300 nmol/L of each primer, 200 nmol/L probe, 1× QuantiTect Probe PCR Kit (Qiagen), and 50 ng of bisulfite-converted genomic DNA. As a positive control, serial dilutions of in vitro methylated genomic leukocyte DNA with Sss I (CpG) methyltransferase (New England Biolabs, Inc.) 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 two of three triplicates showed exponential curves with a Ct value of ≤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] × 10,000; refs. 13, 32, 33).
Statistics. All analyses were carried out using the SPSS software package (SPSS 14.0). 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’s t test. Observed differences with a P value of <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 ranked on tissue specimens from 20 normal cervices and 20 cervical cancers. In sum, of the initial 13 markers, the first five gene promoters (C13ORF18, CCNA1, TFPI2, 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 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, the first five gene promoters (C13ORF18, CCNA1, TFPI2, C1ORF166, and NPTX1) were selected for further evaluation in cervical scrapings by QMSP. NPTX1, the fifth marker in the list, was also included for further analysis, because this gene showed no methylation in any of the 20 normal cervices whereas it was methylated in 4 of 20 cervical cancers.

QMSP Analysis in Scrapings from Cervical Cancer Patients and Controls. QMSP for five gene promoters (C13ORF18, CCNA1, TFPI2, C1ORF166, and NPTX1) was performed on scrapings of normal cervices (n = 103) and cervical cancers (n = 97; Fig. 2). Both the level and frequency of methylation of all five gene promoters were higher in the cancer samples compared with the normal cervices (P < 0.0005). CCNA1 and C13ORF18 showed almost no methylation in the normal cervices (5% and 3%, respectively), whereas for both genes 71% of cancers were methylation positive. C1ORF166 was never positive in the normal cervices, but only 34% of cervical cancers were positive and this positivity was not additive to CCNA1 and/or C13ORF13 positivity. The other two 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 five analyzed genes was not related to age, indicating that methylation is not due to aging in the scrapings of the cancer group (data not shown).

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 (Fig. 3) revealed that levels and positivity of both gene promoters were increased with the severity of the underlying histologic lesion (P < 0.0005). Almost all scrapings from CIN 0 and CIN I patients were unmethylated for CCNA1 and C13ORF18, whereas 25% of scrapings from CIN II, 51% of CIN III, and all (n = 3) microinvasive cancers showed methylation (see Fig. 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 all 3 microinvasive 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, HPV70, and HPV90 were found in two CIN 0, three CIN I, and one 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 three 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 with methylation analysis; that is, 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 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 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 with 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 (28). Our present strategy using both cervical tissue specimens as well as cervical scrapings allows for a straightforward in-depth analysis of the methylation status 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 PPV (100% and 92%, respectively), whereas other studies searched for novel methylation markers (19, 22, 37). One of these studies (19) described a similar approach to our study, in which the pharmacologic unmasking microarray approach was used, resulting in the identification of six gene promoters (SPARC, TFPI2, RRAD, SFRP1, MT1G, and NME1) specifically methylated in cervical cancer. TFPI2 was the only gene promoter similarly identified from their study and ours. In the present study, TFPI2 was evaluated but 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. SPARC showed more frequent methylation in cervical cancers, but also many normal cervices were positive (13 of 20). Although SPARC and TFPI2 were the most promising gene promoters in the study of Sova et al. (19), 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 ~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 (38). 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. For instance, patients diagnosed with CIN II/III, preferably based on as small biopsies as possible (39), should be asked to participate in a wait-and-see study. Long-term follow-up of these patients by colposcopy and cytology

should allow us to analyze a possible relation between methylation status and regression/progression of the lesions. However, such studies are hard to carry out and easily flawed by different types of methodologic biases.

In this study, we showed that five genes were more frequently methylated in cervical cancer compared with normal cervical specimens. CCNA1, TP53, and NPTX1 were previously described to be frequently methylated in cervical cancer (19, 27, 40). Available data for CCNA1 and TP53 are in line with our present data. However, we found more TP53 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 Mull1 or Mullan, is a RING finger 3 ubiquitin ligase, anchored to mitochondria and implicated in the regulation of mitochondrial dynamics. Because C1orf166 has been reported to activate the NF-κB pathway (41, 42), its inactivation due to hypermethylation implies a functional role for NF-κB during progression of cervical cancer. However, this needs further study. Finally, C13orf18 represents a gene with an unknown function. Sequence comparisons suggest a role as phosphatase inhibitor 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 the 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.

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Disclosure of Potential Conflicts of Interest
A.G.J. van der Zee is a paid consultant for OncoMethylose Sciences S.A., Liège, Belgium.

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