Methylation Analysis of the FAM19A4 Gene in Cervical Scrapes Is Highly Efficient in Detecting Cervical Carcinomas and Advanced CIN2/3 Lesions


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Methylation analysis of the **FAM19A4** gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions

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**Running title**

FAM19A4 methylation analysis of cervical scrapes

**Keywords**

Cervical cancer, cervical intraepithelial neoplasia, DNA methylation, HPV, triage

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ABSTRACT
Primary testing for human papillomavirus (HPV) in cervical screening requires triage to differentiate women with transient infection from those with persistent infection who require more intensive management given their risk for cervical (pre)cancer. In this study, the clinical performance of a novel methylation marker FAM19A4 for the triage of high-risk (hr)HPV-positive women was evaluated. Using a training-validation set approach, we analyzed a FAM19A4 quantitative methylation-specific PCR (qMSP). The validation set comprised hrHPV-positive cervical scrapes of 43 women with cervical intraepithelial neoplasia grade 3 or worse (CIN3+) and 135 women with ≤CIN1. The validation set comprised hrHPV-positive cervical scrapes of 52 women with CIN2+, including 33 CIN3+, 19 CIN2, and 166 women with ≤CIN1. The methylation threshold of FAM19A4 qMSP that gave rise to CIN3+ specificity of 70% in the validation set was applied in the validation set. This resulted in CIN3+ sensitivity of 75.8% [95% confidence interval (CI), 61.1-90.4] at 67.0% (95% CI, 60.3-73.8) specificity. Next, the validated qMSP was applied to an independent series of hrHPV-positive cervical scrapes of 22 women with cervical cancer, 29 with advanced CIN2/3 [i.e., women with a known preceding hrHPV infection (PHI) lasting ≥5 years as proxy of longer duration of lesion existence], and 19 with early CIN2/3 (i.e., PHI <5 years). All carcinomas (22/22) and advanced CIN2/3 lesions (29/29) were FAM19A4 methylation-positive, compared with 42.1% (8/19; 95% CI, 19.9-64.3) of early CIN2/3 lesions. In conclusion, FAM19A4 is an attractive triage marker for hrHPV-positive women, with a high reassurance for the detection of cervical carcinoma and advanced CIN2/3 lesions.
INTRODUCTION
An infection with a high-risk type of human papillomavirus (hrHPV) is necessary; however, it is not sufficient for the development of cervical cancer (1). Following a persistent hrHPV infection, genetic and epigenetic changes in the host and/or viral genome are acknowledged to be involved in progression toward invasive cervical cancer (2,3). Functional studies have shown that gene silencing by promoter hypermethylation of some tumor suppressor genes is a contributing factor to cervical carcinogenesis (4–8). Gene promoter hypermethylation can be easily assessed by sensitive, (quantitative) methylation-specific PCR (MSP)-based methods on cervical scrapes and even self-collected cervico-vaginal samples. This has led to the idea that methylation analysis can provide an attractive early-detection biomarker, amongst others, to be used as triage method for hrHPV-positive women in cervical screening (3,9). Indeed, promising results have been obtained (10–15) with sensitivities for CIN2+ [i.e., cervical intraepithelial neoplasia (CIN) grade 2 (CIN2), CIN3 and cervical cancer] and CIN3+ (i.e., CIN3 and cervical cancer) similar to those of cytology analysis on cervical scrapes (11,12), the latter currently being the most widely suggested triage tool. Of interest, recent work has revealed that methylation levels of several genes are particularly high in cervical scrapes of women with cervical cancer and advanced high-grade CIN lesions, the latter characterized by a longer duration (≥5 years) of a preceding hrHPV infection (PHI; (3,16)). As a consequence, methylation analysis could be particularly effective in detecting advanced precursor lesions (with likely a high short-term progression risk) and cervical cancers (3), and can serve as a complementary tool for cytology to gain a higher reassurance of not missing advanced lesions (17).

Most studies performed so far have used panels of methylation markers to reach sufficiently high sensitivities for high-grade CIN and cervical cancer (11,13,18,19). In search for novel methylation markers, we recently have performed methylation-specific digital karyotyping of different passages of HPV16E6E7-transduced primary human foreskin keratinocytes (20). This study resulted in the identification of novel DNA methylation events, including some directly following HPV16E6E7 expression, and others associated with the acquisition of an immortal phenotype (i.e., representing disease progression). The latter involved FAM19A4, LHX1, NKX2-8, PHACTR3 and PRDM14 genes. Pilot studies identified FAM19A4 as a promising candidate methylation triage marker for hrHPV-positive women. FAM19A4 family with sequence similarity 19 (chemokine (C-C motif)-like), member A4 is a member of the TAF family of five highly homologous genes that encode small secreted proteins. These proteins contain conserved cysteine residues at fixed positions, and are distantly related to MIP-1α, a member of C-C chemokine family that can serve as immunoregulator and chemokine (21).

The present study describes the verification and validation of the clinical performance of FAM19A4 methylation analysis by qMSP in a large series of hrHPV-positive cervical scrapes derived from a screening population. The validated FAM19A4 qMSP assay was additionally evaluated in an independent series of hrHPV-positive cervical scrapes in relation to severity and duration of the underlying lesion. For this purpose, scrapes from women with cervical cancer, and women with CIN2/3 with a PHI of <5year or ≥5 year, were used. PHI was used as proxy of lesion duration, and accordingly these CIN2/3 lesions were assigned as early and advanced disease stages (16), respectively.

MATERIALS AND METHODS

Study population

Cervical scrapes for training and validation

Independent training and validation sets of hrHPV-positive cervical scrapes (n=178 and n=218, respectively) were used. For the training set, baseline cervical scrapes of hrHPV-positive women who participated in the intervention arm of a population-based cervical screening trial (POBASCAM; (22,23)) were used. Scrapes in the validation set were from women who participated in population-based screening using the same screening and referral algorithm as in the intervention arm of the POBASCAM trial (11,24). For all women, cotesting for hrHPV and cytology on the cervical scrapes at baseline was performed. Cytology was scored using the CISOE-A (Composition, Inflammation, Squamous epithelium, Other abnormalities and endometrium and Endocervical columnar epithelium - Adequacy of the smear) classification, which is standard in the Netherlands and can be translated into the Bethesda classification (25). In this classification, borderline or mild dyskaryosis (BMD) equals low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells of undetermined significance (ASCUS) or atypical squamous cells not excluding high-grade squamous intraepithelial lesions (ASC-H). Moderate or worse dyskaryosis (>BMD) equals high-grade squamous intraepithelial
lesions (HSIL). All women with baseline >BMD cytology were directly referred for colposcopy, independent of hrHPV status. All hrHPV-positive women with baseline BMD cytology were advised to repeat cytology and hrHPV testing 6 and 18 months later. They were referred for colposcopy at 6 months, in case of >BMD cytology, or BMD cytology combined with a hrHPV-positive test result, and at 18 months in case of >BMD cytology and/or a hrHPV-positive test result. Women with a positive hrHPV test result and normal cytology at baseline were referred to repeat cytology and hrHPV testing at 6 and 18 months. They were referred to colposcopy at 6 months in case of >BMD cytology, and at 18 months in case of >BMD cytology and/or a positive hrHPV test result. At colposcopy visit, biopsies were taken for histology according to standard procedures in the Netherlands (26).

The training set comprised hrHPV-positive scrapes of 178 women. Of these, 43 were of women that were histologically diagnosed with a CIN3+ lesion within 36 months of follow-up [including 4 squamous cell carcinoma (SCC) and 1 adenocarcinoma (AdCa)]. These women had a median age of 31 years (range, 25-55) and 15 women had normal and 28 abnormal (i.e., BMD or worse) cytology at baseline. The remaining 135 scrapes were of women without evidence of CIN2+ further referred to as ≤CIN1 (including 27 CIN1 and 9 histologically confirmed absence of CIN) up to the next screening round (5 years later) and had a median age of 34 years (range,17-61). Of these, 99 women had normal and 36 abnormal cytology at baseline. The training set did not include any CIN2 lesions as CIN3+ is a better endpoint for (pre)cancer (11,27).

The validation set comprised a consecutive series of 250 hrHPV-positive scrapes. Of 218 samples, sufficient material was left for qMSP validation and valid qMSP results were obtained. This series comprised 52 women with a CIN2+ lesion within 36 months of follow-up [2 SCCs and 1 adenocarcinoma in situ (ACIS), 30 CIN3, 19 CIN2] with a median age of 34.5 years (range, 24-58). Of these, 19 women had normal and 33 abnormal cytology at baseline. The remaining 166 scrapes were of women who had no evidence of CIN2+ (including 11 CIN1 and 8 histologically confirmed absence of CIN) within the same follow-up time. Of these, 139 had normal and 27 had abnormal cytology at baseline. The median age of this group was 39 years (range, 19-62).

**Cervical scrapes of women with cervical carcinoma or CIN2/3 with a known duration of preceding hrHPV infection**

Separate series of hrHPV-positive cervical scrapes were used to quantitatively evaluate FAM19A4 methylation in relation to severity and duration of the underlying lesion. This series comprised (i) 22 hrHPV-positive cervical scrapes of women diagnosed with cervical cancer during population-based screening or whilst visiting a gynecological outpatient clinic (19 SCC, 1 adenosquamous carcinoma and 2 AdCa). Of these scrapes, 19 had abnormal and 3 normal cytology. The median age of women was 38 years (range, 30-85); and (ii) 48 hrHPV-positive cervical scrapes of women diagnosed with a CIN2/3 in the second round of the control arm of the POBASCAM trial (blind HPV testing), and accordingly have a known 5-year history of hrHPV infection. The Scrapes evaluated preceded the CIN2/3 biopsy. The duration of prior hrHPV infection was considered a proxy for duration of CIN2/3 existence (16,28). Women with same hrHPV-type in both screening rounds were considered to have a PHI of ≥5 years, and their CIN2/3 lesions were considered advanced CIN2/3 lesions (n=29). Women who acquired the hrHPV infection after study entrance (PHI<5 years) were considered to have early CIN2/3 lesions (n=19). Of the 29 hrHPV-positive scrapes of women with advanced CIN2/3, 5 had normal cytology and 24 had abnormal cytology. The median age of these women was 40 years (range, 34-56). Of the 19 hrHPV-positive scrapes of women with early CIN2/3, 7 had normal cytology and 12 had abnormal cytology. The median age of these women was 40 years (range, 39-50). This study followed the ethical guidelines of the Institutional Review Board of the Medical Center.

**DNA isolation, bisulphite treatment, and qMSP methylation analysis**

DNA from cervical scrapes was isolated using the Nucleo-Spin 96 Tissue kit (Macherey-Nagel) and a Microlab Star robotic system (Hamilton, Germany) according to manufacturers’ protocol (11).

Extracted DNA was subjected to bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research, USA) as described previously (7,8). Bisulphite-converted DNA was used as template for DNA methylation analysis. DNA methylation analysis of FAM19A4 was performed by qMSP using housekeeping gene β-actin (ACTB) as a reference gene (20). A multiplex qMSP assay was developed according to criteria described by Snellenberg and colleagues. (29). Specificity of each primer pair for bisulphite-converted methylated DNA was confirmed by absence of amplification of unmodified DNA to ensure that no amplification would occur in case of incomplete bisulphite conversion. Quantification Cycle (Cq) values were measured at a fixed fluorescence threshold. Samples with a Cq >40 for FAM19A4 were considered to represent a negative test result. All samples had a Cq value for ACTB.
<32 to assure good sample quality. All analyses were performed on an ABI 7500 real-time PCR-system (Applied Biosystems, USA). The FAM19A4 result of a sample was expressed in Cq ratio, calculated by the following formula: $2^{-[\text{Cq (ACTB)} - \text{Cq (FAM19A4)}]} \times 100$.

**Statistical analysis**

Assessment of FAM19A4 methylation analysis was performed by a training-validation set approach using two independent series of cervical scrapes that were sufficiently large to ensure an unbiased assay analysis. In the training set, a Receiver Operating Characteristics (ROC) curve of the FAM19A4 qMSP assay was made for all ratio values and the area under the curve (AUC) was determined. The threshold value that gave rise to a CIN3+ specificity of 70% in the validation set was chosen to consider a specimen positive for FAM19A4 methylation. With this threshold, the biomarker test was converted into a categorical variable and subsequently evaluated in the independent validation set. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and referral rate were determined together with 95% Wald confidence intervals (95% CI), for outcome of CIN2+ and CIN3+. The threshold value for scoring cytology positive was ASCUS (i.e., BMD). For quantitative evaluation of FAM19A4 methylation, fold changes over a reference category (i.e., hrHPV-positive women with ≤CIN1 in the validation set) were determined. Differences in methylation levels between the different groups were analyzed by Mann–Whitney U test. P values below 0.05 were considered significant. All statistical analyses and computation of graphs were performed in IBM SPSS Statistics 20 and Excel.

**RESULTS**

**Clinical validation of FAM19A4 on physician-taken cervical scrapes of hrHPV-positive women**

In the training set of 178 hrHPV-positive cervical scrapes, FAM19A4 methylation analysis revealed a significant discrimination of women with CIN3+ from those with ≤CIN1 ($P = 0.004$). ROC curve analysis showed an AUC of 0.775 (Fig. 1). The methylation threshold of FAM19A4 that in the training set gave rise to a CIN3+ specificity of 70% was chosen for validation set analysis. In the validation set ($n=218$), application of this threshold resulted in a CIN3+ sensitivity of 75.8% (95% CI, 61.1-90.4) at a specificity of 67.0% (95% CI, 60.3-73.8), and a CIN2+ sensitivity of 69.2% (95% CI, 56.7-81.8) at 69.9% (95% CI, 62.9-76.9) specificity. In comparison, application of cytology (threshold ASCUS) in this set, reached a CIN3+ sensitivity of 63.6% (95% CI, 47.2-80.0) at a specificity of 78.9% (95% CI, 73.0-84.4), and a CIN2+ sensitivity of 63.5% (95% CI, 50.4-76.5) at 83.7% (95% CI, 79.1-89.3) specificity. Corresponding PPVs, NPVs, and referral rates for endpoints CIN2+ and CIN3+ are presented in Table 1. It was observed that FAM19A4 methylation analysis scored more carcinoma and CIN3 lesions positive than cytology [i.e., 3 carcinoma by FAM19A4 compared to 2 by cytology, and 73.3% CIN3 (95% CI: 57.5-89.2) by FAM19A4 compared to 63.3% CIN3 (95% CI: 46.1-80.6) by cytology]; while cytology was more often positive among CIN2 lesions [i.e., 57.9% (95% CI: 35.7-80.1) by FAM19A4 compared to 63.2% (95% CI: 41.1-84.4) by cytology].

FAM19A4 methylation in cervical scrapes of women with cervical cancer and CIN2/3 lesions with different duration of existence

We next evaluated the frequency of FAM19A4 methylation positivity in an independent series of hrHPV-positive cervical scrapes of women with different underlying disease severities [i.e., early CIN2/3 with PHI <5 years ($n = 19$), advanced CIN2/3 with PHI ≥5 years ($n = 29$) and cervical carcinoma ($n = 22$)]. FAM19A4 methylation was particularly associated with advanced disease, scoring 100% positive in samples of women with cervical cancer (22/22) and women with advanced CIN2/3 lesions (29/29), compared with 42.1% (8/19; 95% CI, 19.9-64.3) of women with early CIN2/3 lesions (Fig. 2). In the same series, cytology was abnormal (≥BMD) in 86.4% (19/22; 95% CI, 72.0-100) of women with cervical cancer, 82.8% (24/29; 95% CI, 69.0-76.5) of women with advanced CIN2/3 and 63.2% (12/19; 95% CI, 41.5-84.8) of women with early CIN2/3. Thus, FAM19A4 methylation analysis tended to be more competent than cytology in detecting cervical carcinomas and advanced CIN2/3, whereas cytology had a relatively higher preference for early CIN2/3 (Figure 2). When considering FAM19A4 methylation levels (expressed as Cq ratio; Table 2), an increase proportional to the duration of lesion existence was observed with both a significant increase between early and late CIN2/3 ($P < 0.001$), and between late CIN2/3 and cervical cancer ($P = 0.001$). A 3.1-fold increase in methylation levels is seen in early CIN2/3 compared to the reference (≤CIN1 validation set,
methylation analysis as triage marker for hrHPV-positive women. This would obviate the need of additional visits to the physician for cytology triage (12). Given recent studies on the basis of screening and allows the efficient detection of CIN2/3+ lesions (33–38). Since triage by previous findings of DNA methylation analysis being more sensitive over cytology for the detection of the most advanced lesions and cervical cancers (3,16,17). As shown in Fig. 2, our findings are in line with a recent hypothesis that both tests do not detect exactly the same lesions, with DNA methylation analysis having a preference for detecting the more advanced CIN3+ lesions, and cytology tending to also detect early CIN2/3 lesions (3,17). Our findings support that the FAM19A4 methylation marker can serve as an alternative or complementary tool for cytology (17) to gain a higher reassurance of not missing advanced lesions and cervical cancer (3). It should be noticed that our study was performed within the setting of well-organized screening in the Netherlands with a high quality standard of cytology reading (32). In countries without less organized cytology infrastructure, objective molecular triage testing by the FAM19A4 methylation marker might even have higher additive value in terms of reassurance. In line with Bierkens and colleagues, (16) reporting on CADM1/MAL methylation, methylation levels of FAM19A4 increased with increasing disease severity, being particularly high in cervical scrapes of women with cervical cancer. These data reflect that hrHPV-positive women with a positive FAM19A4 methylation test should be sent for immediate colposcopy given high risk of advanced lesions in need of treatment. FAM19A4 methylation-negative women could be offered a repeat test after 12–18 months instead of direct colposcopy referral (3) which could markedly reduce over referral and overtreatment. The effect on patient outcome of such strategy, nonetheless, requires confirmation in a prospective trial with intervention based on methylation status. We acknowledge that in such scenario part of CIN2/3 lesions may remain undetected. These lesions are most likely early-onset lesions with few chromosomal abnormalities (28), and are supposed to have low risk to progress to invasive cancer within the screening interval. Yet, to fully support this hypothesis, additional proof is needed, for example by demonstrating no or limited chromosomal aberrations in these lesions (28) or by active surveillance of women with FAM19A4 methylation-negative, colposcopically evaluable small CIN2. The high confidence of not having cervical cancer in case of absence of FAM19A4 methylation, is supported by a recent analysis of an extra series of hrHPV-positive cervical scrapes of women with cervical SCC with unknown cytology (n=35), all of which were FAM19A4 methylation-positive (data not shown). Previous studies have shown that self-sampling can increase the participation rate in population-based screening and allows the efficient detection of CIN2/3+ lesions (33–38). Since triage by methylation markers is possible on the same sample used for hrHPV-testing, this would obviate the need of additional visits to the physician for cytology triage (12). Given recent studies on the
application of methylation marker testing to self-collected specimens (10,12,15), further evaluation of FAM19A4 as a methylation triage test in hrHPV-positive self-samples is warranted. In conclusion, FAM19A4 is an attractive methylation triage marker for hrHPV-positive women that reaches an overall CIN3+ sensitivity of 75.8% at a specificity of 67.0%, with particularly a high reassurance for the detection of cervical carcinoma and advanced CIN2/3 lesions.

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Conflict of interest
C. Meijer, P. Snijders, R. Steenbergen and D. Heideman are shareholders of Self-Screen BV, a spin-off company of VU University Medical Center. All other authors declare that they have no conflicts of interest.
REFERENCES


### Table 1: Sensitivity, specificity, PPV, NPV, and referral rates for colposcopy for endpoints CIN2+ and CIN3+ in the validation set of 218 hrHPV-positive cervical scrapes.

<table>
<thead>
<tr>
<th>Triage</th>
<th>Endpoint</th>
<th>Sensitivity [% (95% CI)]</th>
<th>Specificity [% (95% CI)]</th>
<th>PPV [% (95% CI)]</th>
<th>NPV [% (95% CI)]</th>
<th>Referral rate [% (95% CI)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM19A4</td>
<td>CIN2+</td>
<td>69.2 (56.7-81.8)</td>
<td>69.9 (62.9-76.9)</td>
<td>41.9 (31.4-52.3)</td>
<td>87.9 (82.3-93.4)</td>
<td>39.4 (33.0-45.9)</td>
</tr>
<tr>
<td></td>
<td>CIN3+</td>
<td>75.8 (61.1-90.4)</td>
<td>67.0 (60.3-73.8)</td>
<td>29.1 (19.5-38.7)</td>
<td>93.9 (89.9-98.0)</td>
<td>39.4 (33.0-45.9)</td>
</tr>
<tr>
<td>Cytology</td>
<td>CIN2+</td>
<td>63.5 (50.4-76.5)</td>
<td>83.7 (79.1-89.3)</td>
<td>55.0 (42.4-67.6)</td>
<td>88.0 (82.9-93.)</td>
<td>27.5 (21.6-33.5)</td>
</tr>
<tr>
<td></td>
<td>CIN3+</td>
<td>63.6 (47.2-80.0)</td>
<td>78.9 (73.0-84.4)</td>
<td>35.0 (22.9-47.1)</td>
<td>92.4 (88.3-96.5)</td>
<td>27.5 (21.6-33.5)</td>
</tr>
</tbody>
</table>

* at the threshold that gave rise to a 70% CIN3+ specificity in the validation set. ** threshold ASCUS

### Table 2: FAM19A4 methylation levels per lesion category

<table>
<thead>
<tr>
<th>Category</th>
<th>Cq ratio FAM19A4</th>
<th>Median</th>
<th>Range</th>
<th>Fold changes over reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ CIN1</td>
<td>0.123</td>
<td>0-41.94</td>
<td>1* (reference)</td>
<td></td>
</tr>
<tr>
<td>early CIN2/3</td>
<td>0.383</td>
<td>0-2.09</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>advanced CIN2/3</td>
<td>8.357</td>
<td>0.44-94.81</td>
<td>67.9</td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td>33.309</td>
<td>1.35-167.56</td>
<td>270.8</td>
<td></td>
</tr>
</tbody>
</table>

* ≤ CIN1 from validation set (n=166)
FIGURES
Figure 1: ROC curve of FAM19A4 methylation analysis in the validation set of 178 hrHPV-positive cervical scrapes.

The sensitivity (y-axis) in relation to 1-specificity (x-axis) of FAM19A4 methylation analysis is shown.
Figure 2: *FAM19A4* methylation analysis and cytology in relation to duration of CIN disease and cervical cancer.

The sensitivity (y-axis) of *FAM19A4* methylation analysis (black bars) and cytology (grey bars, stripes) in hrHPV-positive cervical scrapes in relation to duration of CIN disease and cervical cancer (x-axis) is shown.