Understanding the heterogeneity of high-grade CIN lesions
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Chapter 6

CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease

Submitted for publication

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DNA methylation in hrHPV-positive cervical scrapes

Abstract

Combined detection of CADM1 and MAL promoter methylation in cervical scrapes is a promising triage strategy for hrHPV-positive women. Here, CADM1 and MAL DNA methylation levels were analysed in cervical scrapes of hrHPV-positive women from a screening cohort and cancer patients and related to severity of cervical disease as assessed by histology either or not combined with cytology. For a separate subset of cervical intraepithelial neoplasia grade 2/3 (CIN2/3), methylation levels were related to duration of preceding hrHPV infection (PHI; < and ≥5 years).

The cross-sectional series included 167 women with ≤CIN1, 54 with CIN2/3 and 44 with carcinoma. In addition, 19 women with CIN2/3<5yrPHI and 29 with CIN2/3≥5yrPHI were analysed. Methylation levels were determined by quantitative methylation-specific PCR and normal cytology scrapes of women with ≤CIN1 served as a reference.

CADM1 and MAL methylation levels increased proportional to the severity of the underlying lesion, showing an increase of 5.3- and 6.2-fold in CIN2/3, respectively, and 143.5- and 454.9-fold in carcinomas, respectively, compared to the reference. Methylation levels were also elevated in CIN2/3 with a longer duration of PHI (i.e. 11.5- and 13.6-fold, respectively). Moreover, per histological category, methylation levels were higher in abnormal versus normal cytology. In fact, methylation levels in CIN2/3 with normal cytology were similar to ≤CIN1 with abnormal cytology and CIN2/3 with <5yrPHI, all approximately 3-fold increased.

To conclude, CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes are related to the degree and duration of underlying cervical disease and markedly increased in cervical cancer.
Introduction

Persistent infection with high-risk human papillomavirus (hrHPV) has been causally related to the development of cervical cancer.\(^1\) The majority of cervical cancers are squamous cell carcinoma (SCC), which are preceded by non-invasive lesions referred to as cervical intraepithelial neoplasia (CIN).\(^2\) Histologically, these lesions can be divided into mild (CIN1), moderate (CIN2), or severe (CIN3, including carcinoma \textit{in situ}) lesions depending on the replacement of the epithelial lining by atypical cells. CIN1 (i.e. low-grade CIN) and part of CIN2 are mostly a consequence of productive hrHPV infections, while CIN3 and the remaining part of CIN2 are associated with transforming hrHPV infections with the potential of malignant progression towards invasive carcinoma.\(^3\) The category of CIN2/3 (i.e. high-grade CIN) reflects a heterogeneous disease, both in terms of progression risk to invasive cancer, a process which generally lasts up to 10 to 30 years\(^{3-5}\) and in terms of the number of chromosomal aberrations.\(^6\) We recently have demonstrated that the heterogeneity at the chromosomal level of CIN3 was related to the duration of preceding hrHPV infection (further referred to as PHI)\(^7\), whereby a significantly higher number of chromosomal aberrations was detected in lesions of women with a long-term (i.e. \(\geq 5\) years) PHI. These data support the notion that the number of chromosomal aberrations increases with progression of high-grade CIN disease. In addition to these chromosomal aberrations, epigenetic alterations, including DNA methylation of host cell genes involved in cervical carcinogenesis, are frequently detected in cervical carcinoma and a subset of CIN2/3 (reviewed by Wentzensen\(^8\)). In fact, combined detection of promoter methylation of two such genes, CADM1 and MAL, in cervical scrapes of hrHPV-positive women was equally discriminatory for CIN3 or worse (CIN3+) as cytology or cytology combined with HPV16/18 genotyping, dependent on the threshold setting of the quantitative methylation-specific PCR (qMSP) assays.\(^9\) To what extent CIN2/3 of women with methylation-negative cervical scrapes reflect earlier stages of disease compared to CIN2/3 that are scored methylation-positive is a matter of debate.

Here, we aimed to test the hypothesis that promoter methylation levels of such host cell genes increase with severity and duration of cervical disease. For this purpose, levels of CADM1 and MAL promoter methylation in a screening cohort of hrHPV-positive women were related to parameters that previously have been related to severity of CIN disease, including histological outcome in combination with cytological score.\(^{10}\) Additionally, CADM1 and MAL methylation levels were examined in hrHPV-positive cervical scrapes of women with CIN2/3 following a short-term (<5 years) and long-term (\(\geq 5\) years) PHI, respectively.\(^7\)
Materials and Methods

Cervical scrapes of women from a cross-sectional screening cohort
Quantitative methylation-specific PCR (qMSP) data of 221 consecutive hrHPV-positive women, participating in the population-based screening study Amsterdam (POBASCAM), were used. Included were 54 women with CIN2/3, 19 of whom had scrapes with normal cytology. The median age of this group was 34 years (range 24-49). In addition, 167 women were included without evidence of CIN2/3 or cervical cancer (CIN2+), hereafter referred to as ≤CIN1; 140 of these women had scrapes with normal cytology and an HPV and cytology double negative cervical scrape in follow-up. Their median age was 39 years (range 19-62).

Cervical scrapes of women with < and ≥5 years preceding hrHPV infection
To determine the influence of the duration of preceding hrHPV infection, a surrogate for the duration of existence of a lesion, on the methylation levels, hrHPV-positive cervical scrapes of 48 women with CIN2/3 detected in the second screening round (approximately 5 years later) of POBASCAM were included. All women had normal cytology at baseline of POBASCAM and had no intervention until the subsequent screening round. For 19 of these women, no hrHPV infection could be detected in the baseline sample and these women were classified as having PHI of <5 years (<5yrPHI). In the remaining 29 women, an hrHPV infection was detected in the baseline sample and these women were classified as having PHI of ≥5 years (≥5yrPHI). These latter women belonged to the control group in which referral at baseline was based on cytology only. It was ensured that in these cases the hrHPV type in the cervical scrape preceding the biopsy matched the type detected at baseline. The median age of women in this study group was 40 years (range 34-56).

Cervical scrapes of women with carcinoma
Cervical scrapes of 44 women with cervical cancer (i.e. 34 squamous cell carcinoma (SCC) and 6 adenocarcinoma, AdCa) from the outpatient clinics of the VU University medical center (n=18) and of the University Medical Center Groningen (n=26) were included for comparison. The median age of women with cervical carcinoma was 44 years (range 30-61 years).

This study was approved by the Institutional Review Boards of both the VU University medical center in Amsterdam and the University Medical Center Groningen.

Disease categorisation
All biopsies were graded according to the CIN-classification. Cytological scrapes were classified using the CISOE-A system and were ranked as either normal or abnormal cytology (i.e. borderline mild/moderate dyskaryosis or worse, ≥BMD, equalling ASCUS or worse according to the 2001 Bethesda system).
In an attempt to categorise women according to increasing severity of the underlying cervical lesion a first level of categorisation was based on histology (i.e. ≤CIN1, CIN2/3 and carcinoma), in which the ≤CIN1 group included women without evidence for clinically relevant disease because of an HPV and cytology double negative cervical scrape in follow-up. Within the CIN2/3 category, subgroups were further defined based on < and ≥5yrPHI, which were assigned a lower and higher disease stage, respectively. As duration of preceding hrHPV infection was unknown in the cross-sectional screening cohort, cytological classification was included as an alternative, though less well defined, proxy for disease staging. Cervical scrapes of 140 women with normal cytology and ≤CIN1, obtained from the cross-sectional screening cohort, served as a reference in all comparisons. We considered ≤CIN1 with abnormal cytology (further referred to as ≤CIN1-abnormal cytology) representative of either (1) relatively new lesions, (2) regressive lesions, or (3) potential lesions missed by colposcopy. Thus, this group was considered a more severe disease category than ≤CIN1 with normal cytology (the reference group). Irrespective the cytology status CIN2/3 was considered more severe than ≤CIN1. CIN2/3-normal cytology was considered to represent (1) relatively new lesions, (2) regressive lesions, (3) absence of intact abnormal indicator cells or (4) a cytological sampling error. This disease category was classified as less severe than CIN2/3-abnormal cytology.

The characteristics of the cervical scrapes included in the study, either or not stratified for cytology are summarised in Table 1.

**Table 1**: Overview of the hrHPV-positive cervical scrapes analysed in this study.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Fold-change</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>CADM1</td>
<td>MAL</td>
<td></td>
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<tr>
<td>≤CIN1</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>CIN2/3*</td>
<td>5.3</td>
<td>6.2</td>
<td></td>
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<tr>
<td>CIN2/3&lt;5yrPHI</td>
<td>3</td>
<td>3.6</td>
<td></td>
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<tr>
<td>CIN2/3≥5yrPHI</td>
<td>11.5</td>
<td>13.6</td>
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<tr>
<td>Carcinoma</td>
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<td>453.9</td>
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<table>
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<th>Sub-categories stratified by cytology</th>
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<td>≤CIN1-normal cytology</td>
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<td>1 (reference)</td>
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<td>≤CIN1-abnormal cytology</td>
<td>3</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>CIN2/3*-normal cytology</td>
<td>3.1</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>CIN2/3*-abnormal cytology</td>
<td>9.5</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

* cross sectional series
DNA methylation in hrHPV-positive cervical scrapes

DNA isolation, HPV testing, bisulphite treatment and quantitative methylation-specific PCR

DNA was isolated as described previously.\textsuperscript{9,13} The presence of hrHPV DNA was determined using the GP5+/6+-PCR-EIA detection and typing method.\textsuperscript{16} For scrapes derived from the University Medical Center Groningen, DNA was isolated and HPV detection and typing was performed as described previously.\textsuperscript{17-19} Isolated DNA was subjected to bisulphite treatment and quantitative methylation-specific PCR (qMSP) analysis for CADM1 M18 and MAL M1 as described before.\textsuperscript{20,21} The β-actin gene was chosen as an internal reference.\textsuperscript{22} Cycle threshold ($C_T$) values were measured at a fixed fluorescence threshold of 0.01 and $C_T$-ratios were determined as described in Hesselink \textit{et al.}\textsuperscript{9} Raw qMSP values were already available for the screening cohort.\textsuperscript{9}

\textbf{Statistical analysis}

Overall methylation levels per disease category were examined and fold-changes over the reference (i.e. cervical scrapes of 140 women with normal cytology and ≤CIN1, obtained from the cross-sectional screening cohort) were calculated using the median methylation levels. The Mann-Whitney U test was used to determine whether any differences between the various groups were significant. Analyses were performed in SPSS (version 17). P-values below 0.05 were considered significant.

\textbf{Results}

\textit{Methylation levels in hrHPV-positive cervical scrapes increase proportional to degree of underlying cervical disease and peak in women with cervical cancer}

To test CADM1 and MAL methylation levels in hrHPV-positive cervical scrapes in relation to histological classification of underlying disease, hrHPV-positive scrapes of 54 women with CIN2/3 and 44 women with cervical cancer were analysed and resulting methylation levels compared to those of 140 hrHPV-positive women with ≤CIN1-normal cytology (the reference group). CADM1 and MAL methylation levels were 5.3- and 6.2-fold increased, respectively, in women with CIN2/3 compared to the reference group (both $p<0.0005$; Table 2A). Women with cervical carcinoma displayed the highest methylation levels, i.e. 143.5- and 453.9-fold increase for CADM1 and MAL, respectively.

\textit{Methylation levels in hrHPV-positive women with CIN2/3 increase with duration of preceding hrHPV infection}

In the CIN2/3 described in the previous paragraph no information on duration of PHI and therefore proxy of duration of lesion existence was known as these were obtained from the first screening round of POBASCAM. Therefore, cervical
scrapes of the second screening round were also examined and the duration of PHI (<5 years and ≥5 years) was used as a surrogate marker for the duration of existence of the lesion. The fold-changes of CIN2/3<5yrPHI and CIN2/3≥5yrPHI compared to the reference group are given in Table 2A. Methylation levels increased with duration of PHI; whereas methylation levels of CADM1 and MAL were 3.0- and 3.6-fold increased in the CIN2/3<5yrPHI group compared to the reference group, respectively, CIN2/3≥5yrPHI had further elevated methylation levels, being 11.5- and 13.6-fold increased compared to the reference group. The elevation of methylation levels for both CADM1 and MAL was significantly higher in CIN2/3≥5yrPHI compared to CIN2/3<5yrPHI (p=0.023 and p=0.005, respectively; Figure 1). Still, CADM1 and MAL methylation levels in the carcinoma group were significantly higher than in both CIN2/3<5yrPHI (both p<0.0005) and CIN2/3≥5yrPHI groups (p=0.002 and p<0.0005, respectively).

**Methylation levels increase in relation to both histology and cytology**

In order to further test the hypothesis that methylation levels increase with progression of CIN2/3, women from the cross-sectional cohort were further stratified by baseline cytology results, as shown in Table 2B. In case of ≤CIN1-abnormal cytology, methylation levels were increased 3.0- and 3.1-fold, respectively, compared to the ≤CIN1-normal cytology reference group (p=0.0005 and p=0.049, respectively). Methylation levels for CADM1 and MAL in CIN2/3-normal cytology were similar to levels of ≤CIN1-abnormal cytology, i.e. 3.1- and 2.5-fold higher, respectively, compared to the reference group (p=0.001 and p=0.154, respectively; Figure 2). CIN2/3-abnormal cytology showed even further elevated methylation levels for both markers, i.e. 9.5- and 7.8-fold increase,
DNA methylation in hrHPV-positive cervical scrapes

respectively. The increase in methylation levels in CIN2/3-abnormal cytology compared to ≤CIN1-abnormal cytology nearly reached significance for CADM1 and was significant for MAL (p=0.067 and p=0.031, respectively).

Methylation levels in women with CIN2/3-normal cytology and ≤CIN1-abnormal cytology were comparable to those of women with CIN2/3<5yrPHI (Table 2). Due

Figure 1: Methylation levels, in log_{10} scale, relative to β-actin for (A) CADM1 and (B) MAL in hrHPV-positive cervical scrapes of women with underlying CIN2/3 with < and ≥5 year preceding hrHPV infection.

Figure 2: Methylation levels, in log_{10} scale, relative to β-actin for (A) CADM1 and (B) MAL in cervical scrapes stratified for normal and abnormal cytology of women with either no underlying disease (≤CIN1) or with CIN2/3.
to the low numbers of normal cytology samples no cytology sub-categorisation could be made for CIN2/3 with a known duration of PHI and for carcinoma.

Discussion

Quantitative measurement of DNA methylation within the promoter regions of tumour suppressor genes CADM1 and MAL in hrHPV-positive cervical scrapes showed increased methylation levels proportional to degree and duration of underlying cervical disease. Both women with CIN2/3 and cervical cancer revealed significantly elevated methylation compared to the reference group (i.e., women with ≤CIN1-normal cytology). Moreover, methylation levels in women with CIN2/3≥5yrPHI had significantly higher methylation levels compared to those with CIN2/3<5yrPHI. Since duration of preceding hrHPV infection can be considered as a surrogate for lesion age, this suggests that methylation levels increase with lesion duration. With cytology added to further refine disease stage methylation levels were consistently lower for samples with normal cytology compared to abnormal cytology in each histology class. Moreover, methylation levels of both CADM1 and MAL peak in cervical scrapes of women with cervical cancer.

These data indicate that elevated methylation levels are suggestive of progressive CIN disease and may be a reflection of the size of the underlying CIN. More advanced and larger lesions are likely to exfoliate more aberrant cells, facilitating the detection of promoter methylation in cervical scrapes. Accordingly, Wentzensen et al. and Yang et al. recently demonstrated that high-grade squamous intraepithelial lesion (HSIL) cytology and CIN3 colposcopy and biopsy results indicate larger CIN3. The observation that scrapes of women with CIN2/3 and a short-term (<5 years) PHI revealed lower methylation levels compared to lesions with long-term infections indicates that part of the scrapes of women with a short-term hrHPV infection may be scored as ‘methylation-negative’ when choosing certain thresholds of the respective qMSPs for scoring methylation. Though not significant (Fisher exact test, p=0.18), the proportion of scrapes with normal cytology was higher in women with CIN2/3 and a short-term hrHPV infection, i.e. 37%, compared to 17% in women with CIN2/3 and a long-term hrHPV infection. This supports the notion that CIN2/3 with short-term hrHPV infection are early onset CIN2/3 having a low progression-risk for carcinoma. This is in line with our recent finding that CIN2/3 with a long-term and persistent hrHPV infection (≥5 years) had significantly more chromosomal aberrations compared to CIN2/3 of women with a short-term hrHPV infection.

Interestingly, methylation levels in women with CIN2/3-normal cytology and ≤CIN1-abnormal cytology were comparable to those detected in women with CIN2/3<5yrPHI. This is in agreement with low to intermediate levels of methylation being indicative of a relatively new CIN2/3, or a potentially regressive lesion, and do not point to a high short-term progression risk. In fact, methylation levels were higher in more advanced CIN2/3 (≥5yrPHI) and dramatically increased in
case of cervical cancer, indicating that cancers are unlikely to be missed by these methylation markers.

Remarkably, within the group of hrHPV positive women with normal cytology, a significant increase in CADM1 methylation and an increase in MAL methylation were seen in CIN2/3 compared to ≤CIN1. This implies that methylation analysis is capable of accurately detecting CIN2/3 that are missed by cytology, potentially due to absence of intact, abnormal indicator cells, or due to cytological sampling error. In fact, upon setting a certain threshold for methylation-positivity, as described in our previous study\(^9\), 75% (15/20) of CIN2+ and 85% (11/13) of CIN3+ could be detected at a specificity of 66% and 65%, respectively, in hrHPV-positive women with normal cytology in our population-based screening cohort. In comparison, HPV16/18 genotyping, a currently proposed triage strategy for hrHPV-positive women with normal cytology\(^25\), only reached a sensitivity of 50% for CIN2+ and 54% for CIN3+ at a similar specificity (69%) (data not shown).

Present data are in line with previous studies showing an increase in the number of methylation-positive samples, or an increase in methylation levels with either the degree of histological or cytological abnormality.\(^8,20,26-33\) However, to the best of our knowledge, this is the first study to demonstrate a relationship to duration of high-grade CIN disease.

Based on present findings it may be suggested that undetectable or very low methylation levels point to a low or negligible risk of clinically meaningful cervical disease. Intermediate levels indicate potential risk of underlying disease, warranting close surveillance. High methylation levels indicate the presence of clinically relevant disease requiring immediate referral to the gynaecologist.
Reference List


DNA methylation in hrHPV-positive cervical scrapes


DNA methylation in hrHPV-positive cervical scrapes