# Chapter 3

## Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC

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Renée M Overmeer
Florianne E Henken
Peter JF Snijders
Debbie Claassen-Kramer
Johannes Berkhof
Theo JM Helmerhorst
Daniëlle AM Heideman
Saskia M Wilting
Yoshinori Murakami
Akihiko Ito
Chris JLM Meijer
Renske DM Steenbergen

**Abstract**

We previously showed that silencing of TSLC1, recently renamed CADM1, is functionally involved in high-risk HPV-mediated cervical carcinogenesis. CADM1 silencing often results from promoter methylation. Here, we determined the extent of CADM1 promoter methylation in cervical (pre)malignant lesions and its relation to anchorage independent growth and gene silencing to select a CADM1-based methylation marker for identification of women at risk of cervical cancer.

Methylation-specific PCRs targeting three regions within CADM1 promoter were performed on high-risk HPV-containing cell lines, PBMCs, normal cervical smears and (pre)malignant lesions. CADM1 protein expression in cervical tissues was analysed by immunohistochemistry. All statistical tests were two-sided.

Density of methylation was associated with the degree of anchorage independent growth and CADM1 gene silencing *in vitro*. In cervical squamous lesions methylation frequency and density increased with severity of disease. Dense methylation (defined as ≥2 methylated regions) increased from 5% in normal cervical samples to 30% in CIN3 lesions and 83% in squamous cell carcinomas (SCC) and was significantly associated with decreased CADM1 protein expression ($p<0.00005$). The frequency of dense methylation was significantly higher in ≥CIN3 compared with ≤CIN1 ($p=0.005$), as well as in SCC compared with adenocarcinomas (83% versus 23%; $p=0.002$).

Dense methylation of the CADM1 promoter associated with decreased protein expression may provide a valuable diagnostic marker for the triage of high-risk HPV-positive women at risk of ≥CIN3.

## Introduction

Cervical cancer is the second major cause of cancer-related mortality amongst women worldwide (1). Persistent infection with high-risk human papillomavirus (hrHPV) types is causally involved in both cervical squamous cell (SCCs; 80% of cases) and adenocarcinomas (AdCAs; 20% of cases). These tumours evolve from hrHPV positive non-invasive precursor stages, i.e. cervical intraepithelial neoplasia (CIN) and adenocarcinoma in situ (ACIS), respectively (2-6). hrHPV testing has been found to have additive value for management of women with equivocal cervical cytology and for cervical screening programs (7-10). However, in addition to hrHPV (epi)genetic alterations are indispensable for cancer development. Insight into these alterations may yield molecular biomarkers able to distinguish hrHPV positive women with clinically irrelevant hrHPV infections from those with persistent infections having a high-risk of cervical cancer.

Recent studies have shown that methylation of tumor suppressor gene promoters may provide powerful biomarkers for early cancer detection (11, 12). We previously showed that the tumor suppressor in lung cancer 1 (TSLC1) gene, recently renamed Cell Adhesion Molecule 1 (CADM1), is functionally involved in cervical carcinogenesis (13). CADM1 encodes an immunoglobulin-like cell surface protein involved in intracellular adhesion through homophilic and heterophilic trans-interactions (14-16).

By bisulfite sequencing of a 93bp promoter region, methylation of CADM1 was demonstrated in most cervical carcinomas and a subset of high-grade CIN lesions (13). Moreover, in most cervical cancer cell lines methylation of this region was correlated to CADM1 mRNA silencing. However, some exceptions existed showing either methylation within this region but still detectable CADM1 expression, or absence of methylation although CADM1 expression was strongly reduced. Based on these findings it can be hypothesized that methylation of additional and/or other CADM1 promoter regions are pivotal for gene silencing and the phenotypic consequence thereof and that methylation analysis of these region(s) provides the clinically most valuable CADM1-based methylation marker.

In order to test this hypothesis, we herein performed a comprehensive methylation analysis of three promoter regions in cervical samples and cell lines and correlated methylation patterns to severity of (pre)malignant disease,

reduced CADM1 protein expression, and anchorage independent growth.

## Materials and methods

### Cell culture

Primary foreskin keratinocytes (EK), HPV-immortalized cell lines FK16B and FK18B and SiHa cells were cultured as described previously (17). Anchorage-independent cell growth and raft cultures were performed as described before (13, 17).

### Clinical specimens

Normal cervical scrapes (n=39) were obtained from women participating in the population-based screening program. Scrapes were used as normal controls, as in our previous study and a pilot comparative study by MSP no methylation discrepancies were found between Pap1 scrapes and normal cervical biopsies (13). Tissue specimens of cervical normal glandular and squamous epithelium (n=2), CIN1 lesions (n=32), CIN3 lesions (n=37) and cervical AdCAs (n=31) and frozen tissue specimens of cervical SCCs (n=30) were collected during routine clinical practice from patients who underwent biopsy or surgery. Endocervical origin of AdCAs was verified by staining for carcinoembryonic antigen (CEA) and vimentin (18). Peripheral blood mononuclear cells (PBMCs) were derived from 12 healthy donors. This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center.

### Isolation of nucleic acids, HPV typing and RT-PCR analysis

DNA and RNA was isolated from cells using the Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands) and RNAzolB reagent (Tel-Test, Friendswood, TX), respectively. From tissues DNA was isolated according to standard procedures (19, 20). HPV DNA presence was determined, as described before (21, 22). HPV X are hrHPV types not reacting with RLB probes. CADM1 mRNA was quantified as described previously, using PBGD as reference (13).

### DNA modification and methylation-specific PCR analysis

DNA modification was performed using the EZ DNA Methylation Kit$^{TM}$ (Zymo Research, Orange, CA). MSPs targeting three regions within the CADM1 promoter (M1:-696 to -582, M5:-415 to -258 and M9:-61 to +39 relative to the

ATG) were performed using primers described in Table 1. PCR mixtures contained 25ng modified DNA, 0.5μM primers, each dNTP at 200μM, FastStart Taq PCR buffer (Roche Diagnostics), 1.5mM $MgCl_2$, and 1.25U FastStart Taq DNApolymerase (Roche Diagnostics). All U-targeted primers/probes were specific for unmethylated DNA, while M-targeted primers/probes only detected methylated DNA. $H_2O$, unmodified DNA, unmethylated DNA (EK) and SiHa were included as controls. Antisense primers were biotinylated for reverse line blot (RLB) detection. Analytical sensitivity was determined on SiHa DNA (100-50-10-5-1-0.5-0.1-0.05-0.01-0%) diluted in EK DNA.

PCR-products were visualized using RLB detection (22), in which denatured PCR-products were hybridized to unmethylated- and methylated-specific oligoprobes (Table 1). All MSP-RLB reactions were performed in duplicate. Upon discrepant results (<15% of cases) a third MSP-RLB assay was performed which was conclusive.

Table 1. Primers and probe sequences used for MSP analysis and RLB hybridization

| Region | Forward primer 5'-3' primer | Reverse primer 5'-3' primer | Annealing T |
|---|---|---|---|
| M1 | GAAAATTTTAGAATTCGATTTTACG | AAAATACATACGTACTTTACACG | 58 |
| U1 | GAAAATTTTAGAATTTGATTTTATG | AAAAAAATACATACATACTTTACACA | 57 |
| M5 | AAGGGAGATTTTTTAGTCGTC | CGAATTTTACTTTCCCCGAA | 50 |
| U5 | AAGGGAGATTTTTTAGTTGTTG | AATTCAAATTTTACTTTCCCCAAA | 58 |
| M9 | TTAGTTGTTCGTTCGGGTTTCGG | CGCACACTAAAATCCGCTCGA | 62 |
| U9 | TTAGTTGTTTGTTTGGGTTTTGGAGG | CACCACACACTAAAATCCACTCAA | 60 |
| **Region** | **RLB 5'-3' probe 1** | **RLB 5'-3' probe 2** | |
| M1 | TCGAGTTTATCGTTAGGTTG | CGGGTTTTTTCGTTTCGTC | |
| U1 | TTGAGTTTATTGTTAGGTTGTT | AGTGGGTTTTTTTGTTTTGTT | |
| M5 | CGTTTTTTGGAGTTCGAGT | GTACGTTAGGCGTTCGG | |
| U5 | TGTTTTTTGGAGTTTGAGTTT | GTATGTTAGGTGTTTGGGA | |
| M9 | TAGTTAACGTCGTTAGTTTGA | TAGGTGTTCGATATGGCGA | |
| U9 | ATGTTGTTAGTTTGAGGTAG | TAGGTGTTTGATATGGTTGA | |

Immunohistochemistry

Following antigen retrieval in citrate buffer (pH6.0; 120°C) and permeabilization with 0.1% Triton X-100/PBS (Merck, Darmstadt, Germany) slides were incubated overnight with CC2, a polyclonal antibody against C-terminus of CADM1 (15, 23) (1:400; PBS/ 1% normal goat serum/ 0.1% Triton X-100/ 4°C). The DAKO EnVision™+ System (Dako Netherlands B.V.) was used for visualization. Scoring for CADM1 positivity, by two pathologists, was graded as <10%, 10-70% and >70% positive epithelial cells (24). Normal cervical epithelial samples were included as positive controls. Nervous branches served as internal positive controls (25, 26).

Western blot analysis

Nuclear and cytoplasmic fractions of protein lysates were prepared according to standard procedures (27). Twenty microgram of protein was fractionated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were incubated with CC2 antibody (1:1000) or 3E1 antibody (1:1000; Anti-SynCAM/TSLC1, MBL International Corporation, Woburn, Ma, USA), a chicken monoclonal antibody directed against the outer membrane part of CADM1. Antibody binding was visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology Inc., Rockford, IL).

Statistical Analysis

Using Pearson Chi-Square testing, the association between CADM1 protein expression and methylation status was analysed. Logistic regression analysis with age adjustment was used to examine whether pathology of the specimens could be predicted by the number of methylated regions. Separate analyses were performed for histological thresholds ≥CIN1 (normal cervical samples versus lesions of any grade), ≥CIN3 (≤CIN1 versus ≥CIN3) and SCC versus AdCA. Differences in the individual effect of each methylation location separately (i.e. M1, M5 and M9) on pathology (≤CIN1 versus ≥CIN3) were assessed by likelihood ratio testing. Statistical significance was ascertained at two-sided $P<0.05$.

## Results

Specificity and sensitivity of CADM1 MSP-RLB

For extensive analysis of CADM1 promoter methylation, three MSPs (M1, M5 and M9) were designed. Since MSP analysis is solely based on the detection of methylated CpGs at the primer regions, we included a reverse line blot detection analysis which at least in part overcomes these limitations of the MSP assay. For each MSP two probes were designed in order to increase the specificity and sensitivity of the detection of methylated CpGs. Together primers and probes recognize all 10 CpGs within both the M1 region and M5 region and 9 out of 10 CpGs within the M9 region.

The specificity of all MSP-RLB assays was proven using unmodified DNA (always negative) and bisulfite-treated DNA of primary keratinocytes (EK) and SiHa, being methylation negative and positive, respectively, at all regions. Previous bisulfite sequencing analysis showed no CADM1 promoter

methylation in primary keratinocytes, yet nearly all CpGs were methylated in SiHa cells (data not shown; (28). The analytical sensitivity of each MSP-RLB assay, determined on serial dilutions of SiHa DNA in EK DNA was 1%(M1), 5%(M5) and 0.5%(M9) (Figure 1).

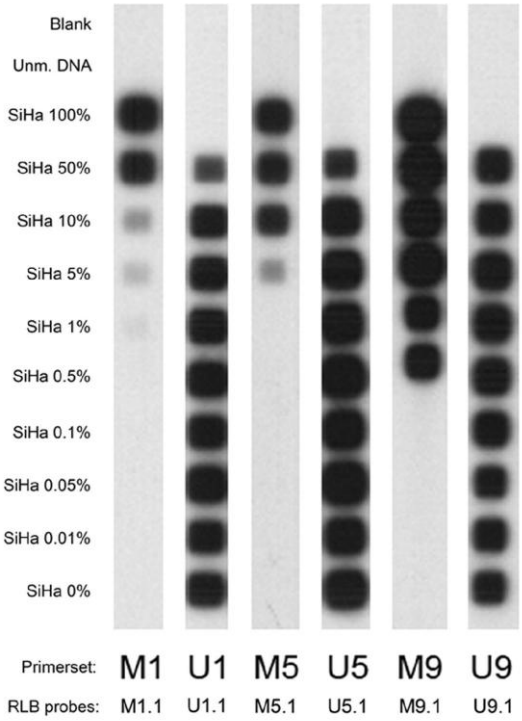


Figure 1. Sensitivity of MSP-RLB assays as assessed on a dilution range of SiHa DNA (100-50-10-5-1-0.5-0.1-0.05-0.01-0%) in a background of primary keratinocyte DNA. Unm. is unmodified SiHa DNA (e.g. without bisulfite treatment). M1, M5 and M9 indicate RLB signals obtained by methylation specific primers; U1, U5 and U9 indicate RLB signals obtained by primers specific for unmethylated DNA.

Progression to anchorage independence is associated with increased CADM1 promoter methylation

As our previous study showed a suppression of anchorage independent growth upon CADM1 overexpression in SiHa cells (13), we determined the correlation between CADM1 methylation at the three promoter regions and growth in soft agarose (Figure 2). Primary keratinocytes revealed no methylation and did not form colonies in soft agarose, whereas SiHa cells showing methylation at all three regions formed colonies at a high efficiency (>1600 colonies/5000 cells).

Interestingly, two passages of an HPV 18-immortalized keratinocytes cell line (FK18B) revealed a strong increase in colony formation with passaging. Methylation analysis showed no methylation in early passage cells, while both M1 and M9 regions were methylation positive in late passage cells. The increase in methylation from no region in early passage FK18B cells, to 2 regions in late passage FK18B cells and 3 regions in SiHa cells was associated with gradual silencing of CADM1 mRNA expression. The reduction in CADM1 mRNA expression in early passage FK18B cells showing no methylation is most likely resulting from the presence of an LOH at 11q22-23 in these cells (17). These data show that density of methylation is associated with the degree of anchorage independent growth and CADM1 gene silencing *in vitro*.

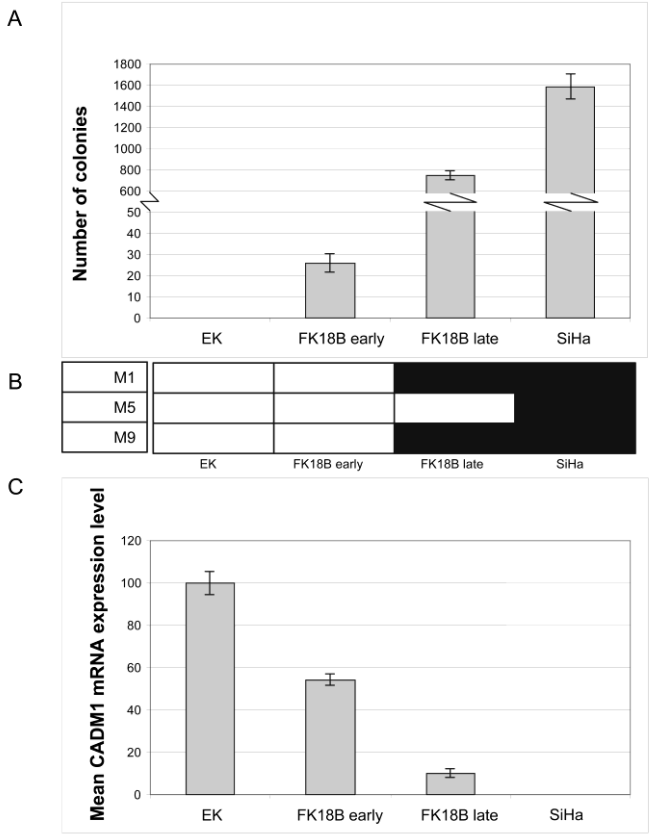


Figure 2. A: Anchorage independent growth, i.e. number of colonies per 5000 cells , B: CADM1 promoter methylation at M1, M5 and M9. DNA methylation is depicted in black, white boxes indicate unmethylated DNA. C: Relative CADM1 mRNA expression in primary keratinocytes (EK), an early and a late passage of FK18B cells and SiHa cells.

Frequency and density of CADM1 promoter methylation increase with the severity of squamous lesions

Next CADM1 MSP-RLB assays were applied to PBMCs (n=12), cytologically normal cervical scrapings (n=39), CIN1 lesions (n=32), CIN3 lesions (n=37), cervical SCCs (n=30) and cervical AdCAs (n=31). A summary of MSP-RLB results is shown in Figure 3 and Table 2.

Normal cervical scrapes were HPV negative. Sixteen percent (5/32) of CIN1 lesions, 95% (35/37) of CIN3 lesions, all SCCs (30/30) and 97% (30/31) of AdCA were hrHPV positive (see Figure 3). Representative examples of the MSP-RLB results are shown in Figure 4.

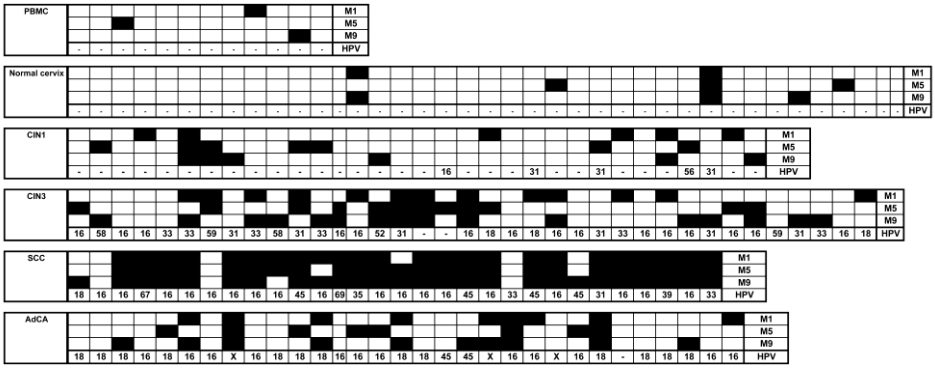


Figure 3. Summary of MSP-RLB data on peripheral blood mononuclear cells (PBMCs) and cervical specimens. DNA methylation is depicted in black, white boxes indicate unmethylated DNA; HPV status of specimens is depicted as specific HPV type present or negative (-).

Except from AdCAs, the methylation frequency (any methylation, Table 2) increased with severity of cervical disease, ranging from 13% in normals to 47% in CIN1, 73% in CIN3 lesions and 90% in SCCs. PBMCs displayed 25% of methylation at any location and AdCAs 55%. No significant relationship between the location of methylation and disease severity was observed (Likelihood ratio Chi-Square (2 degrees of freedom) = 0.04, $p>0.9$). Furthermore, HPV-positive samples included specimens without any methylation, indicating that methylation cannot solely be attributed to HPV presence.

Table 2. CADM1 promoter methylation frequencies in PBMCs and cervical specimens

| | single methylation | | | | double methylation | | | |
|---|---|---|---|---|---|---|---|---|
| | M1 (%) | M5 (%) | M9 (%) | overall | M1/M5 (%) | M1/M9(%) | M5/M9 (%) | overall |
| PBMCs (n=12) | 1 (8%) | 1 (8%) | 1 (8%) | 3 (25%) | - | - | - | - |
| Normal cervix (n=39) | - | 2 (5%) | 1 (3%) | 3 (8%) | - | 1 (3%) | - | 1 (3%) |
| CIN1 (n=32) | 4 (13%) | 5 (16%) | 3 (9%) | 12 (38%) | - | 1 (3%) | 1 (3%) | 2 (6%) |
| CIN3 (n=37) | 5 (14%) | 4 (11%) | 7 (19%) | 16 (43%) | 2 (5%) | 4 (11%) | 3 (8%) | 9 (24%) |
| SCC (n=30) | - | - | 2 (7%) | 2 (7%) | 1 (3%) | 1 (3%) | 1 (3%) | 3 (10%) |
| AdCA (n=31) | 2 (7%) | 5 (16%) | 3 (10%) | 10 (32%) | - | 4 (13%) | - | 4 (13%) |

| | triple methylation M1/M5/M9 (%) | dense methylation ≥2 regions (%) | any methylation (%) |
|---|---|---|---|
| PBMCs (n=12) | - | - | 3 (25%) |
| Normal cervix (n=39) | 1 (3%) | 2 (5%) | 5 (13%) |
| CIN1 (n=32) | 1 (3%) | 3 (9%) | 15 (47%) |
| CIN3 (n=37) | 2 (5%) | 11 (30%) | 27 (73%) |
| SCC (n=30) | 22 (73%) | 25 (83%) | 27 (90%) |
| AdCA (n=31) | 3 (10%) | 7 (23%) | 17 (55%) |

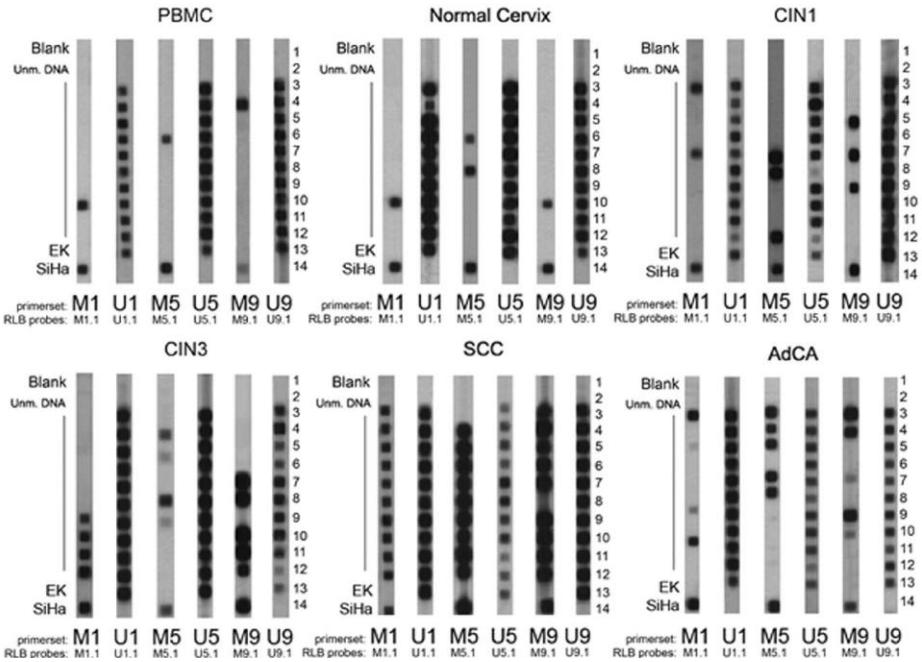


Figure 4. Representative examples of MSP-RLB results on PBMCs and cervical specimens. M1, M5 and M9 depict RLB signal obtained by methylation specific primers; U1, U5 and U9 depict RLB signal obtained by primers that are specific for unmethylated DNA. Sample 2 (Unm.) is unmodified SiHa DNA (e.g. without bisulfite treatment). Samples 3 to 12 are representative DNA isolates of the respective cells or tissue specimens analyzed; sample 13 is a control for unmethylated DNA (primary keratinocytes-EK); Sample 14 is a control for methylated DNA (SiHa).

Localization of CADM1 is epithelium-type dependent

In order to examine the correlation between the various methylation patterns and gene silencing, we assessed CADM1 protein expression in cervical (pre)malignant tissues. We first performed immunohistochemical stainings using CC2 antibody on normal glandular and squamous epithelium of the cervix (n=2) to reveal staining patterns.

In normal cervical epithelium CADM1 localization appeared dependent on epithelial origin. Glandular epithelium stained consistently membraneously (Figure 5A and B), whereas squamous epithelium showed nuclear staining (Figure 5C).

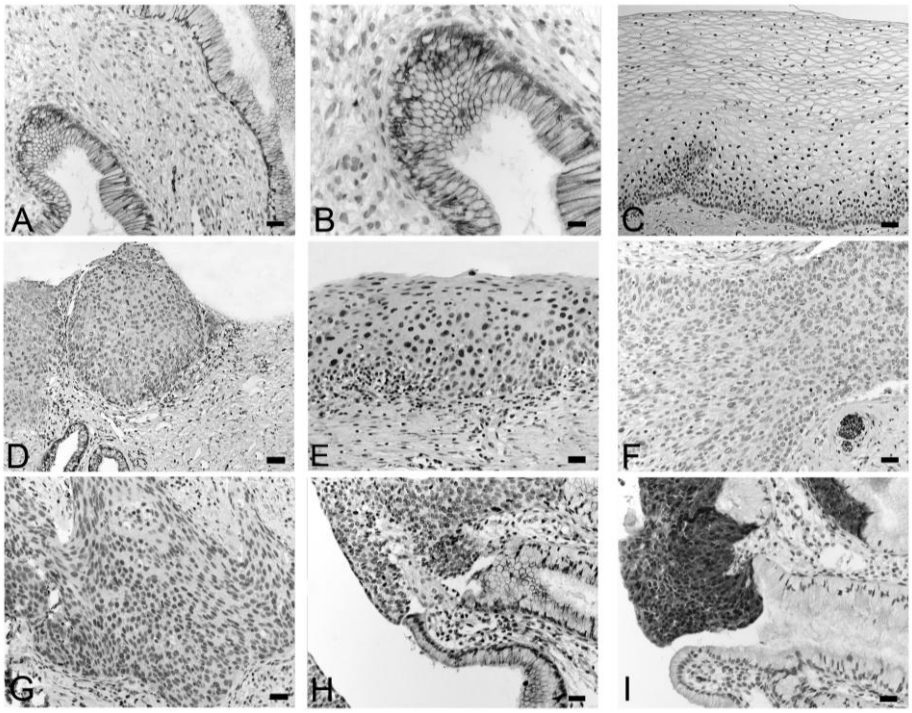


Figure 5. Representative immunohistochemical staining results for CADM1 (A-H) and $p16^{INK4A}$ (I). A and B: normal glandular epithelium of cervical glands; C: normal cervical squamous epithelium. D: CIN3 lesion with dense methylation; E: CIN2/3 lesion without methylation. F: SCC showing dense methylation; G: SCC without methylation. H: Transition normal cervical glandular epithelium to CIN3 lesion with methylation of a single promoter region; I: AdCA without methylation. In all photomicrographs except B , scale bar = 50 μm; in photomicrograph B, scale bar = 25 μm.

Nuclear CADM1 expression in squamous epithelial cells was confirmed in raft cultures of EK and FK18B cells (Fig 6A and B) as well as by Western blot analysis (Fig 6C). Both EK and FK18B showed a 100kD protein product in nuclear enriched lysates using CC2 antibody (directed against the cytoplasmic tail), suggestive of a full length protein (16). Application of a second antibody directed against the outer-membrane part (3E1) (29) showed a major 100 kD product in nuclear lysates, as well as a minor band of 75kD, most likely representing immature CADM1 or a soluble isoform (Fig 6D) (15, 30). 293 cells served as positive control (15), and showed CADM1 expression in both nuclear and cytoplasmic fractions. The nature of the 115 kD product detected in both nuclear and cytoplasmic lysates using CC2 and 3E1 remains to be elucidated.

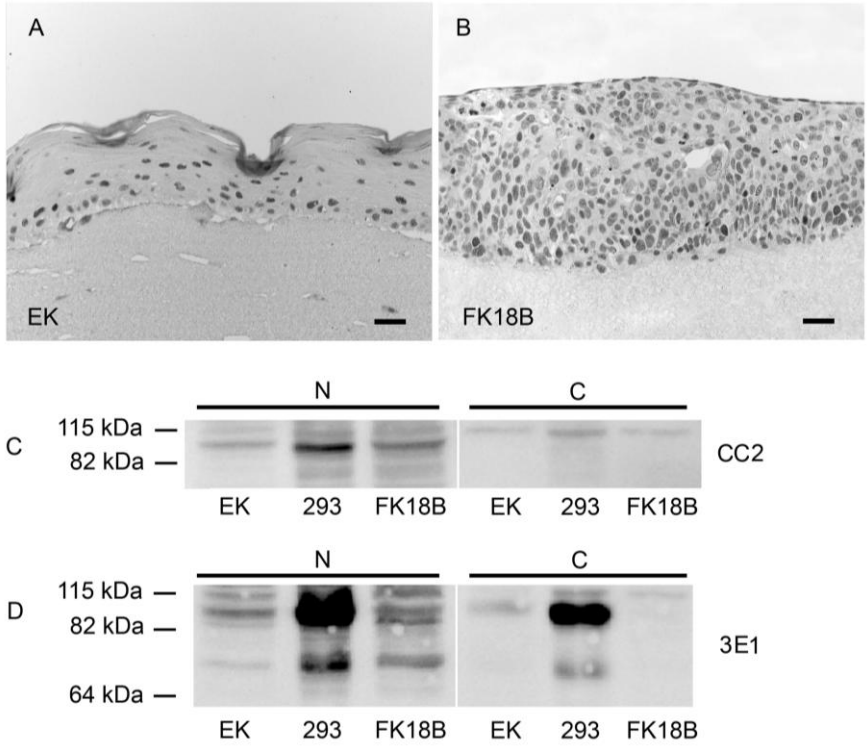


Figure 6. Representative immunohistochemical staining results for CADM1 protein expression on organotypic raft cultures and Western blot analysis. A: Organotypic raft culture of primary keratinocytes (EK); B: Organotypic raft culture of HPV18-immortalized primary keratinocytes (FK18B); C: Western blot analysis on nuclear (N) and cytoplasmic (C) protein fractions using CC2 antibody and D 3E1 antibody. In photomicrographs A and B , scale bar = 50 μm.

Reduced CADM1 protein expression is associated with dense methylation

Stainings were extended with a selected group of CIN3 lesions (n=18) and SCCs (n=13). Selection was based on an equal distribution of single, double and triple methylation frequencies and various combinations of methylated regions within the CADM1 promoter. Examples of CIN2/3 lesions with and without dense methylation and reduced CADM1 staining are represented in Figures 5D and E. Figure 5F shows a SCC with dense methylation and reduced CADM1 expression, while nuclear CADM1 staining in a SCC without methylation is shown in Figure 5G. Figure 5H demonstrates membranous CADM1 staining in normal glandular epithelium adjacent to a CIN3 lesion having a nuclear expression pattern. Internal positive controls, i.e. adjacent normal epithelium or nervous branches, were always positive. Comparison of methylation patterns to immunohistochemical expression revealed CADM1 expression in >70% of epithelial cells in all except one case with ≤1 methylated region (92%; 11/12) (Table 3). On the other hand, 42% (8/19) of cases with ≥2 methylated regions showed reduced expression (i.e. between 10-70% of epithelial cells staining positive) and another 47% (9/19) showed strongly reduced/no expression (i.e. <10% of cells staining positive). The association between methylation at ≥2 sites and reduced CADM1 protein expression (i.e. scores of <10% and 10-70% combined) was highly significant (Pearson chi-square-test; p<0.00005). Immunohistochemical analysis of six AdCAs showed reduced CADM1 expression in a single case. Analogous to normal glandular epithelium, CADM1 staining was restricted to the cell membranes (Figure 5I).

Table 3. Correlation between methylation patterns and reduced protein expression of CADM1 as determined in CIN3 lesions and cervical SCC. Immunohistochemical scoring was graded as expression in <10% of epithelial cells, 10-70% of epithelial cells and >70% of epithelial cells.

| | **staining** | | | **Total** | **p-value** |
|---|---|---|---|---|---|
| **methylation status** | <10% positive | 10%-70% positive | >70% positive | | |
| no/single | 1 | | 11 | 12 | |
| double | 4 | 6 | 1 | 11 | |
| triple | 5 | 2 | 1 | 8 | |
| total | 10 | 8 | 13 | 31 | <0.00005 |

Age-adjusted univariate logistic regression analysis of regional methylation

Since our data revealed that dense methylation (i.e ≥ 2 methylated regions) of the CADM1 promoter is associated with reduced protein expression of the CADM1 protein, we further explored the distribution of both occasional and dense methylation within the different groups of (pre)malignant cervical lesions. By age-adjusted univariate logistic regression analysis of regional methylation, methylation was significantly more common in squamous lesions of any grade than in normal cervical controls (Odd's ratio (OR)= 16.8, 95% CI= 5.7 – 48.9, p<0.00001). Increasing the histological threshold to high-grade lesions and cervical SCC (≥ CIN3) did not distort the association with methylation (OR 9.9, 95% CI= 4.4 – 22.0, p<0.00001). When comparing normal cervical samples with ≥CIN1, dense methylation was not related to the presence of a squamous lesion of any grade (OR = 2.3, 95% CI= 0.3 – 15.4, p=0.4). However, dense methylation was more common in ≥ CIN3 than in ≤CIN1 (OR 5.6, 95% CI= 1.7 – 18.5, p=0.005). When comparing cervical SCC to AdCA, dense methylation was more common in SCC (OR 17.2, 95% CI= 2.9 – 101.1, p=0.002).

## Discussion

Using hrHPV-transformed keratinocyte cell lines we showed that increased CADM1 promoter methylation and concomitant gene silencing is functionally related to anchorage independent growth. Extensive methylation analysis of the CADM1 promoter demonstrated that within the group of squamous cervical lesions both the frequency and density of methylation increased proportional to the severity of disease. In addition, dense methylation was significantly more frequent in high-grade CIN lesions and SCC (≥CIN3) compared with normal cervical epithelial samples and low-grade lesions (≤CIN1). Moreover, since methylation at any region was also evident in a significant proportion of PBMCs and lesions ≤CIN1, these data suggest that density of CADM1 promoter methylation rather than methylation at a single randomly chosen region within the CADM1 promoter provides a specific biomarker for ≥CIN3.
Also, we showed by immunohistochemistry that only dense methylation (≥2 methylated regions) rather than occasional methylation was associated with decreased protein expression of the CADM1 protein and as such of biological relevance. This is further exemplified by density of CADM1 methylation being proportional to the degree of anchorage independent growth and gene silencing in HPV-tranformed keratinocytes (13).

Interestingly, dense CADM1 promoter methylation was primarily associated with high-grade CIN and SCC and less prominent in AdCA. Analogous to the present observation, methylation of a number of other tumor suppressor genes as well as genetic alterations are also histotype dependent in cervical carcinomas (20, 31-36), suggesting that both histotypes may likely develop through distinct carcinogenic pathways.

In contrast to for example hMLH1 methylation in which methylation of a single 70bp promoter region was directly correlated to gene silencing in colorectal cell lines, none of the three CADM1 regions analysed showed such a correlation in cervical specimens (37). The high frequency of reduced CADM1 protein expression in cervical SCC reflected by the detection of dense methylation in 83% of cases, is supported by another study showing reduced CADM1 mRNA expression in 77% of cervical carcinomas (38). Since CADM1 plays an important role in both tumor invasion and immune evasion (13, 14), the reduction of CADM1 protein expression as a result of promoter methylation is plausible to provide a growth advantage during cervical carcinogenesis.

Surprisingly, it appeared that localization of the CADM1 protein as detected by immunohistochemistry was related to the epithelial origin. Whereas normal cervical glandular epithelium, both normal and adenocarcinomas showed a specific membranous staining, in cervical squamous epithelium a profound nuclear staining was seen. The same phenomenon was observed in lung tissue specimens (data not shown). Both immunohistochemistry on raft cultures and Western blot analysis on nuclear and cytoplasmic fractions of primary and HPV18-transformed keratinocytes confirmed the nuclear localization of CADM1 in squamous epithelial cells. The presence of a full length product in nuclear extract suggests that the complete protein is transported to the nucleus or nuclear envelope. In previous studies, by others and us, loss of CADM1 function has been related to reduced cell adhesion and suppression of tumorigenicity, invasion and anchorage independent growth (13, 39, 40). Moreover**,** CADM1 overexpression has been described to inhibit proliferation and induce apoptosis in epithelial cells (33,36). Both the FERM-binding and PDZ-interacting domain in the cytoplasmic tail of CADM1 appeared essential for CADM1 its tumor suppressive activity (41). The FERM-domain interacts with DAL-1, but DAL-1 independent tumor suppression by CADM1 has also been described (15). Whereas CADM1 and DAL-1 are associated at cell-cell interaction sites, it can be speculated that DAL-1 independent tumor suppressive activity is related to a yet unknown nuclear function of CADM1.

Similar to our findings on CADM1 also the transmembrane glycoprotein EGFR/ErbB1 has been detected in the nucleus of many tissues, where it may act as a transcriptional regulator (42). Likewise, other receptor proteins, including ErbB2, ErbB3 and ErbB4 can be localized in nuclei, having roles distinct from their functions as membrane proteins (43). Consequently, further biochemical studies are warranted to elucidate the function of CADM1 in the nucleus.

To determine ultimately the clinical value of dense CADM1 promoter methylation for the identification of the subset of hrHPV positive women at highest risk of invasive SCC longitudinal studies on exfoliated cells are needed. Two previously reported pilot studies on exfoliated cells support our finding that testing for CADM1 methylation will be a promising diagnostic tool for the triage of hrHPV-positive women. Gustafson and co-workers (44) showed that amongst 15 methylation markers tested in liquid-based cervical cytology samples, CADM1 appeared the best individual gene in distinguishing high-grade squamous intraepithelial lesions (HSIL/CIN2-3) from low-grade SIL (LSIL/CIN1) and normal samples. Moreover, we found that methylation of the promoter of CADM1 could be detected in archival cervical cytology smears up to 7 years prior to cervical cancer diagnosis (13). Nevertheless, next to CADM1 additional markers will be required to further improve the diagnostic performance of a methylation marker-based test for the detection of high grade CIN and cervical cancer.

In conclusion, dense CADM1 promoter methylation rather than occasional methylation is predictive of decreased protein expression of CADM1 and is significantly associated with the development of ≥CIN3. Hence, testing for dense CADM1 promoter methylation may provide a powerful diagnostic marker for the triage of hrHPV-positive women at risk of ≥CIN3.

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