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# Gene promoter methylation patterns throughout the process of cervical carcinogenesis

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**Abstract.** *Objectives*: To determine methylation status of nine genes, previously described to be frequently methylated in cervical cancer, in squamous intraepithelial lesions (SIL).

*Methods*: QMSP was performed in normal cervix, low-grade (L)SIL, high-grade (H)SIL, adenocarcinomas and squamous cell cervical cancers, and in corresponding cervical scrapings.

*Results*: Only *CCNA1* was never methylated in normal cervices and rarely in LSILs. All other genes showed methylation in normal cervices, with *CALCA*, *SPARC* and *RAR-*$\beta_2$ at high levels. Methylation frequency of 6 genes (*DAPK, APC, TFPI2, SPARC, CCNA1* and *CADM1*) increased with severity of the underlying cervical lesion. *DAPK* showed the highest increase in methylation frequency between LSIL and HSIL (10% vs. 40%, $p < 0.05$), while *CCNA1* and *TFPI2* were most prominently methylated in cervical cancers compared to HSILs (25% vs. 52%, $p < 0.05$, 30% vs. 58%, $p < 0.05$). *CADM1* methylation in cervical cancers was related to depth of invasion ($p < 0.05$) and lymph vascular space involvement ($p < 0.01$), suggesting a role in invasive potential of cervical cancers. Methylation ratios in scrapings reflected methylation status of the underlying lesions ($p < 0.05$).

*Conclusion*: Methylation of previously reported cervical cancer specific genes frequently occurs in normal epithelium. However, frequency of methylation increases during cervical carcinogenesis, with *CCNA1* and *DAPK* as the best markers to distinguish normal/LSIL from HSIL/cancer lesions.

Keywords: Methylation, cervical (intraepithelial) neoplasia, *DAPK*, *CCNA1*, *CADM1*

## 1. Introduction

Cervical cancer is the second most frequent gynecological malignancy among women worldwide, with highest incidence rates in developing countries [25]. Worldwide it is estimated that 490,000 new cervical cancer cases are diagnosed and 270,000 deaths will occur annually [16]. The majority of cervical cancer is squamous cell cancers and develops from precursor lesions, known as squamous intra-epithelial lesions (SIL). Low-grade SIL (LSIL) regresses in most cases, while high-grade SIL (HSIL) will progress to cervical cancer in 20–50% of cases when left untreated [21, 26]. Progression from LSIL to cervical cancer generally takes 10–15 years [22]. Although distinction between low and high-grade SIL is subjective, based on morphological criteria and not clearly discriminating between progressive and/or regressing lesions many clinicians feel inclined to treat HSIL, while LSIL is often managed by surveillance. Currently, apart from morphology, no prognostic markers with respect to spontaneous regression or progressive lesions exist, although many have been proposed such as oncogenic

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HPV DNA, telomerase activity and DNA methylation [8,27,29,40,41].

For the past decade, abnormal patterns of DNA methylation have been recognized as frequent molecular changes in neoplasia [14]. CpG islands in promoter regions of genes are targets for methylation, and if this occurs abundantly, transcription may be blocked. Methylation of tumor suppressor genes contributes to an immortalized phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth. It is known to be an early event in carcinogenesis of many different tumor types [3]. Several gene promoters were identified as being aberrantly methylated in cervical cancer [6,13,23,24,31]. Some studies also included precursor lesions and demonstrated that more samples are methylated with increasing severity of the underlying lesion [8,10,15,18,19,30,34,36,39], but positivity for HSIL analyzed in scrapings as well as in tissue is rather heterogeneous between studies [8,10,15, 18,19,30,34,36,38,39]. Methylation was mainly analyzed using non-quantitative methylation specific PCR (MSP). An advancement of conventional MSP is quantitative MSP (QMSP), which permits reliable quantification of methylated DNA [7]. The quantitative nature of the assay enables to set a cut-off at a certain level of methylation, for instance, above the level of the highest methylated normal cervices or LSIL cases, creating a specificity of 100%. We previously demonstrated in cervical scrapings a sensitivity of 89% to detect cervical cancer using methylation of a four gene panel (*DAPK, CALCA, ESR1* and *APC*), equivalent to Hr-HPV (90%) and cytomorphology (89%) [41]. Aim of the present study was to determine methylation status of previously reported cervical cancer specific methylated markers [18,33,34,41] (*CALCA, DAPK, ESR1*, *APC*, *RAR-*$\beta_2$, *SPARC*, *TFPI2*, *CCNA1* and *CADM1* (previously known as *TSLC1*)) in the course of cervical carcinogenesis using QMSP. To evaluate whether gene promoter methylation can be used to distinguish LSIL from HSIL, DNA of paraffin embedded tissues from normal cervix ($n = 20$), LSIL ($n = 20$), HSIL ($n = 20$), adenocarcinomas (AC) ($n = 20$) and squamous cell cervical cancers (SCC) ($n = 40$) was studied first because histology of the tissue is still considered as the golden standard in The Netherlands. In addition, in cervical cancer patients we correlated promoter methylation with clinicopathological characteristics. Finally, we performed QMSP of the same 9 genes to determine whether the methylation status of the underlying lesion was reflected in (55 available) corresponding cervical scrapings.

## 2. Patients and methods

### 2.1. Patients

For the present study, we selected normal cervices from 20 patients, who underwent a hysterectomy for a non-malignant condition. Patients had no history of abnormal Pap smears or any form of cancer, and all cervical specimens were judged as benign by histopathological examination. Median age for these patients was 48 years (IQ range 47–51 years). Furthermore, we randomly selected biopsy specimens from (1) 20 patients with histologically confirmed LSIL who had undergone colposcopy with cervical biopsies because of an abnormal Pap smear; (2) 20 patients with HSIL on histological examination treated by large loop excision of the transformation zone. Median age for SIL patients was 40 years (IQ range 34–46 years); and (3) 60 cervical cancer patients (20 with adenocarcinoma (AC) and 40 with squamous cell carcinoma (SCC)); specimens were taken prior to treatment with (chemo)radiation or from radical hysterectomy specimens. FIGO stages were FIGO IA ($1/60 = 2\%$), FIGO IB ($26/60 = 43\%$), FIGO IIA ($5/60 = 8\%$), FIGO IIB ($18/60 = 30\%$), FIGO IIIB ($4/60 = 7\%$) and FIGO IV ($6/60 = 10\%$). Median age of cervical cancer patients was 46 years (IQ range 38–57 years). There were no differences between AC and SCC cases regarding FIGO stage or age. There was no difference in age between the cancers and normal cervices. However, SIL patients were significantly younger than patients with normal cervices and cervical cancers ($p < 0.0005$). All patients were treated in our hospital between March 1996 and December 2005. Tissue specimens were formalin fixed and paraffin embedded and retrieved from the Pathology archives of the University Medical Center Groningen (UMCG). All patients gave written informed consent to participate in a cervical cancer related translational research study in our hospital. Cervical scrapings from corresponding patients were available from 55 cases, including normal cervix ($n = 9$), LSIL ($n = 8$), HSIL ($n = 18$), AC ($n = 4$) and SCC ($n = 16$). Cervical scrapings were collected at initial visit to our outpatient department (SIL patients, 1 week before treatment) or at examination under anaesthesia (women with a normal cervix and cervical cancer patients). This study was approved by the Institutional Review Board of the UMCG.

### *2.2. Sample collection procedure and DNA isolation*

Sections (10 µm) were cut from tissue blocks. Parallel slides were stained with H&E in order to check for presence of specific tissue (i.e., normal epithelium, LSIL, HSIL, AC or SCC). Tissue slides were deparaffinized using 100% xylene followed by 100% ethanol. Pellet was resuspended in a buffer containing SDS-proteinase K, and DNA was extracted with phenol-chloroform followed by ethanol precipitation [9].

Cervical scrapings were collected using an Ayre's spatula and endocervical brush, as described [41]. Cytospins for cytomorphological assessment were made (1/5 Volume) and the rest was centrifuged, washed, snap-frozen in liquid nitrogen and stored at $-80$°C. DNA was extracted using standard salt-chloroform extraction and ethanol precipitation [41].

For quality control, genomic DNA was amplified in a multiplex PCR according to the BIOMED-2 protocol [35] and only DNA samples with PCR products of minimal 300 bp in size were included in this study.

### *2.3. HPV detection and typing*

Presence of high risk HPV was analyzed by PCR using HPV16 and HPV18 specific primers on DNA of the paraffin embedded tissue [2,37]. On all HPV16- or HPV18-negative cases, general primer-mediated PCR was performed using two HPV consensus primer sets, CPI/CPIIG and GP5+/6+, with subsequent nucleotide sequence analysis, as described previously [41].

### *2.4. Real-time quantitative methylation specific PCR (QMSP)*

QMSP was performed with bisulfite treated DNA as previously reported [7,27,41]. Bisulfite treatment was performed with the EZ DNA methylation kit according to manufacturer's protocol (Zymogen, BaseClear, Leiden, The Netherlands). Primer pairs, amplicon size and Genbank accession number of QMSP primers and probes are listed in Table 1. The housekeeping gene *$\beta$-actin* was chosen as reference for total DNA input measurement and DNA input was at least 225 pg *$\beta$-actin* (equivalent to a $Ct$-value of 34). QMSP was carried out in a total volume of 20 µl in 384 well plates in an Applied Biosystems 7900 Sequence Detector (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Each sample was analyzed in triplicate. Final reaction mixture consisted of 300 nM of each primer, 200 nM probe, 1X QuantiTect Probe PCR Kit (Qiagen, Venlo, The Netherlands) and 50 ng of bisulfite-converted genomic DNA. As positive control, serial dilutions of *in vitro* methylated genomic leucocyte DNA with Sss I (CpG) methyltransferase (New England Biolabs. Inc., Beverly, MA, USA) were used in each experiment, while genomic leucocyte DNA served as a negative control. All amplification curves were visualized and scored without knowledge of the clinical data. 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 *$\beta$-actin*) $\times$ 10,000) [7,27,41]. A DNA sample was considered methylated if at least 2 of 3 triplicates showed exponential curves with $Ct$-value below 50.

### *2.5. Statistical analysis*

All analyses were carried out using SPSS software package (SPSS 14.0, Chicago, IL, USA). Methylation ratios between groups were compared using Mann–Whitney U test (2 groups) or Kruskal–Wallis test ($>2$ groups). Associations between numerical parameters were analyzed using $\chi^2$ test and Fisher's exact test for small numbers. Correlations between methylation ratios were calculated using Spearman rank test. Observed differences with $p$-value $< 0.05$ were considered statistically significant.

## 3. Results

### *3.1. Gene promoter methylation and HPV typing throughout cervical carcinogenesis*

Figure 1A shows methylation ratios and the proportion of methylation positive samples per tissue category for the nine gene promoters. Only *CCNA1* was not methylated in normal cervices (0/20) and rarely in LSIL (1/20). All the other gene promoters were methylated in normal cervices for *CALCA* (16/20), *SPARC* (13/20), *APC* (8/20) and *RAR-$\beta_2$* (17/20) at high levels. For all gene promoters, median level of methylation increased significantly ($p < 0.05$, Fig. 1A) with severity of the underlying lesion. This relation was independent of the percentage of dysplastic cells in the specimens (data not shown). For most gene promoters (except for *CALCA*, *ESR1* and *RAR-$\beta_2$*) also proportion of methylated cases increased

Table 1

Primers and probe sequences

| Gene | Forward 5′-3′ primer | 6-FAM 5′-3′TAMRA probe | Reverse 5′-3′ primer | Genbank no. | Amplicon size |
|---|---|---|---|---|---|
| *ACTB* | TGGTGATGGAGGAGGTTTAGTAAGT | ACCACCACCCAACACACAATAACAAACACA | AACCAATAAAACCTACTCCTCCCTTAA | Y00474 | 133 bp; 390–522 |
| *APC* | GAACCAAAACGCTCCCCAT | CCCGTCGAAAACCCGCCGATTA (antisense) | TTATATGTCGGTTACGTGCGTTTATAT | U02509 | 74 bp; 761–834 |
| *CALCA* | GTTTTGGAAGTATGAGGGTGACG | ATTCCGCCAATACACAACAACCAATAAACG | TTCCCGCCGCTATAAATCG | X15943 | 101 bp; 1706–1806 |
| *DAPK* | GGATAGTCGGATCGAGTTAACGTC | TTCGGTAATTCGTAGCGGTAGGGTTTGG | CCCTCCCAAACGCCGA | X76104 | 98 bp; 5–102 |
| *ESR1* | GGCGTTCGTTTTGGGATTG | CGATAAAACCGAACGACCCGACGA | GCCGACACGCGAACTCTAA | X62462 | 101 bp; 2784–2884 |
| *RAR-*$\beta_2$ | GGGATTAGAATTTTTTATGCGAGTTGT | TGTCGAGAACGCGAGCGATTCG | TACCCCGACGATACCCAAAC | NM_000965 | 92 bp; 63–154 |
| *SPARC* | TTTCGCGGTTTTTTAGATTGTTC | CGACAAACAAAACGCGCTCTCCG | CATACCTCAATAACAAACAAACAAACG | NM_003118 | 70 bp; 28–97 |
| *TFPI2* | GCGGTTTTTTGTTTTAGGC | CCCCGCATAAAACGAACACCCGAA | GACGAAAATCGACCGAACGC | NM_006528 | 68 bp; −57–10 |
| *CADM1* | GAAATTTGTAACGTTTGGTTCG | AGGTTAGATGTATTCGGTGTGCGGGA | CGCTATATCAAACCGACG | NM_014333 | 99 bp; −348–250 |
| *CCNA1* | GTTATGGCGATGCGGTTTC | TTTCCATACCGACCGCGACAAACG | CCAACCTAAAAAACGACCGA | NM_003914 | 152 bp; −317–166 |

with severity of the underlying lesion (Fig. 1A). The highest increase in proportion of methylation between LSIL and HSIL was observed for *DAPK* (10% vs. 40%, $p < 0.05$), while *CCNA1* and *TFPI2* were most prominently methylated in cervical cancers compared to HSIL (25% vs. 52%, $p < 0.05$ and 30% vs. 58%, $p < 0.05$). Although not significant, *CADM1* became mainly methylated in the progression of LSIL–HSIL (30% vs. 50%, $p = 0.197$), while *ESR1* became mainly methylated in cervical cancers (10% vs. 30%, $p = 0.074$).

Proportion of Hr-HPV positive samples also increased with the severity of the underlying lesion (Table 2). Although Hr-HPV was related to methylation of *DAPK* ($p = 0.024$), *TFPI2*, *SPARC*, *CCNA1* and *CADM1* (each $p < 0.001$), this association was lost when corrected for the severity of the underlying lesion (data not shown).

### *3.2. Methylation status in relation with clinicopathological characteristics in cervical cancer*

Table 3 summarizes methylation status of the gene promoters in relation to clinicopathological characteristics of the 60 cervical cancer patients. Positive methylation of *DAPK, CADM1* or *CCNA1* was related to squamous cell histiotype. Tumors methylated for *ESR1* or *RAR-*$\beta_2$ were more often early stage tumors (FIGO IB/IIA), while tumors methylated for *TFPI2* were often of a higher stage. *CADM1* positive tumors had deeper stromal invasion and LVSI, suggesting a role in the invasive potential of cervical cancers.

### *3.3. Gene promoter methylation as a diagnostic tool*

From $55/120$ patients, corresponding scrapings were available to evaluate whether the methylation status in the scraping was similar to that of the underlying tissue. For all gene promoters (except for *APC*) methylation levels of scrapings were strongly related to methylation levels of corresponding tissue (all $p < 0.004$) (Fig. 2), indicating that the methylation status determined in scrapings is reflecting the methylation status in the tissue. However, discrepancies were observed, with most of discrepant samples depicted in SIL patients.

For *DAPK*, *ESR1*, *TFPI2* and *CCNA1*, frequency of positive scrapings with methylation increased with severity of the underlying lesion (Fig. 1B). *CCNA1* was the best marker since it was methylated in only few normal cervices (11.1%) and LSIL (25%), while frequent methylation was observed in scrapings of HSIL (55.6%) and mostly in cancers (80%) ($p < 0.0005$, Fig. 1B).

## 4. Discussion

The present study demonstrates that proportion of methylated samples increases with severity of the underlying premalignant cervical lesion for most of the previously reported cervical cancer specific methylation markers. However, for many gene promoters, previously presented to be cancer specific, normal cervices already showed median methylation ratios higher than that of SIL lesions, indicating that methylation of these gene promoters in normal tissue is more common than generally assumed. This observation has important impact on the interpretation of studies on cancer specific methylation markers that did not use proper normal cervical controls. We conclude that those genes that are already frequently methylated in normal cervices (*RAR-*$\beta_2$, *SPARC*, *CALCA* and *APC*) are not cervical cancer specific and as such not useful as markers for detection of cervical cancer or its precursors.

Figure 3 summarizes at what stage during cervical carcinogenesis gene promoters become more prominently methylated. Recently, it has been described that DNA methyltransferase I (DNMT1) expression, involved in *de novo* methylation of gene promoters, is increased during cervical carcinogenesis [28]. In addition, HPV18 E7 can target DNMT1, resulting in upregulation of its expression [4]. In our present study 35% of LSIL and 90% of HSIL/cancer were positive for Hr-HPV, which is in agreement with other studies [12]. Such a high frequency of Hr-HPV might be important for early *de novo* promoter methylation of tumor suppressor genes during cervical carcinogenesis, as is also demonstrated for some genes in our study. However, all tissue categories were highly methylated for *RAR-*$\beta_2$, *SPARC*, *CALCA* and *APC*, including normal cervices. In these normal cervices, no Hr-HPV was detected, indicating that methylation in cervical epithelium also may occur without concurrent HPV infection. *DAPK* and *CADM1* became more often methylated in HSIL, while *CCNA, TFPI2* and *ESR1* were more often methylated in cancer lesions. Recently, sequential promoter methylation [11] and mRNA down-regulation [5] has been described during HPV-16 or HPV-18 mediated transformation of cultured human keratinocytes. *ESR1* became methylated in the early immortal stage (com-

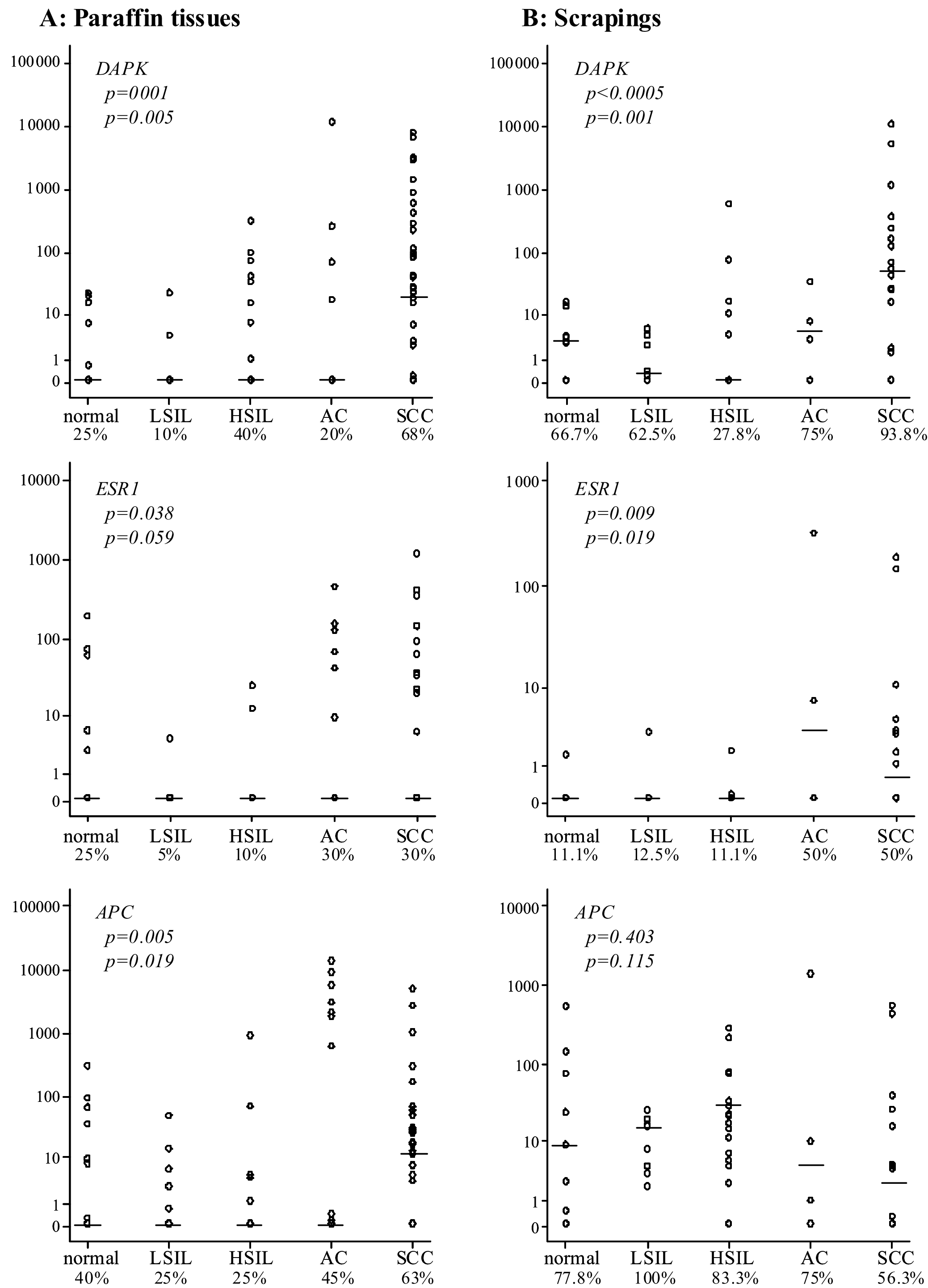


Fig. 1. Methylation ratio and frequency of positive methylation samples of nine gene promoters in normal cervices, LSIL, HSIL and cervical cancer lesions. On the left-hand side (A) methylation is shown determined in the paraffin embedded tissue of normal ($n = 20$), LSIL ($n = 20$), HSIL ($n = 20$), AC ($n = 20$) and SCC ($n = 40$) and on the right-hand side (B) the methylation is shown determined in the scrapings of normal ($n = 9$), LSIL ($n = 8$), HSIL ($n = 18$), AC ($n = 4$) and SCC ($n = 16$). The bars represent the median methylation ratio. $p$-values are shown for the calculation of the methylation ratio and methylation frequency in relation with severity of the lesion by respectively Kruskall–Wallis (upper) and $\chi^2$ (lower) statistics. [a]No statistics are computed because *CALCA* was methylated in all scrapings.

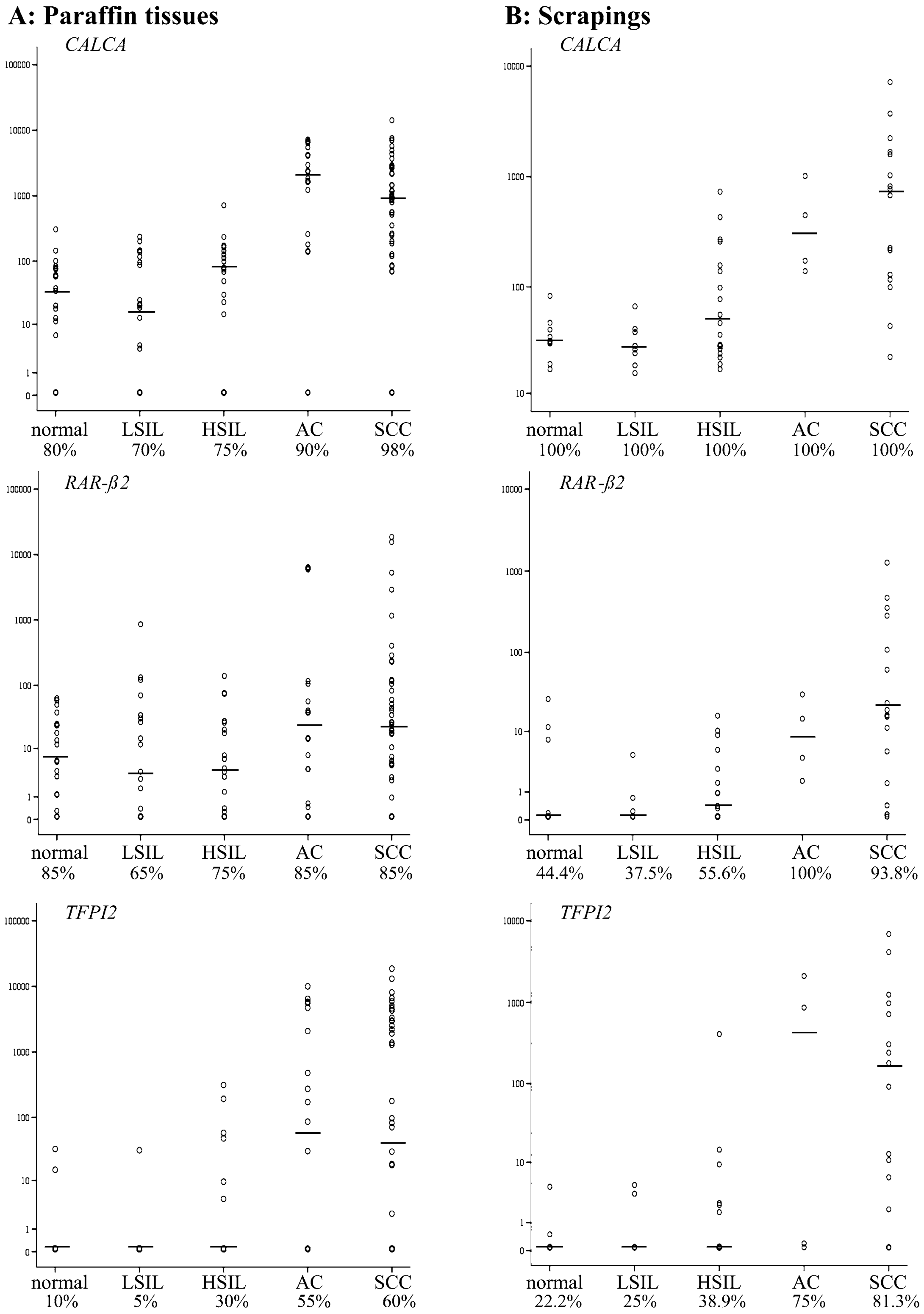


Fig. 1. (Continued.)

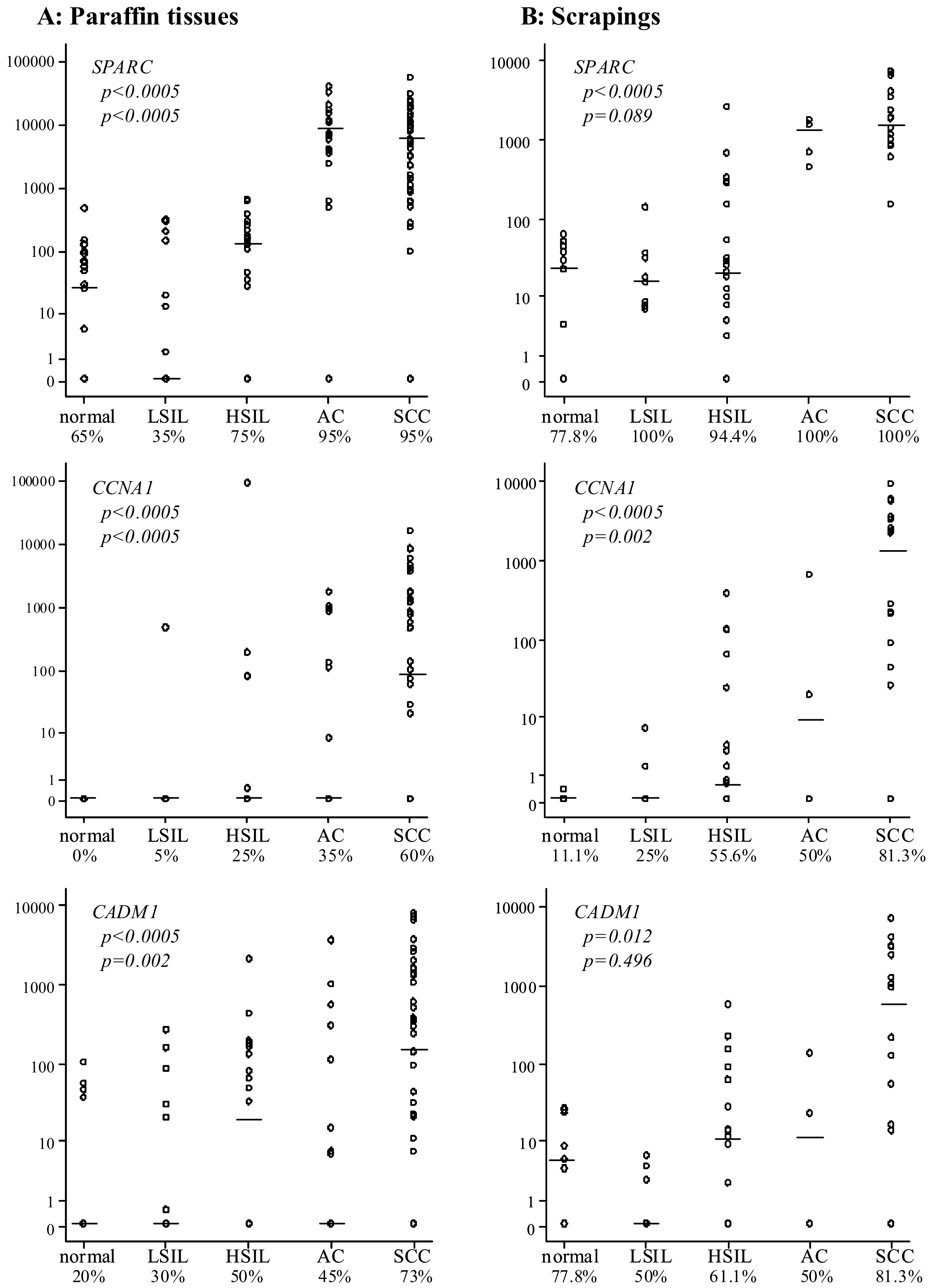


Fig. 1. (Continued.)

Table 2
Hr-HPV analysis in paraffin samples

| Hr-HPV | Normal | LSIL | HSIL | Cancer | AC | SCC |
|---|---|---|---|---|---|---|
| Cases | 20 | 20 | 20 | 60 | 20 | 40 |
| HPV16 | 0 | 3 | 12 | 41 | 14 | 27 |
| HPV18 | 0 | 1 | 2 | 9 | 5 | 4 |
| Other type | 0 | 3[a, b] | 5[c] | 9[d,e] | 1[e] | 8[d,e] |
| Total Hr-HPV (%) | 0 (0%) | 7 (35%) | 18 (90%) | 54 (90%) | 17 (85%) | 37 (93%) |

*Notes*: [a]2 cases with HPV6 were not included; [b]2 cases with HPV31 and 1 case with HPV 52; [c]2 cases with HPV31, 1 case with HPV45, 1 case with HPV51 and 1 case with HPV58; [d]1 case with HPV6 was not included; [e]2 cases (1 AC and 1 SCC) with HPV31, 2 cases with HPV33, 3 cases with HPV45, 1 case with HPV52 and 1 case with HPV73.

Table 3
The number of positive methylated gene promoters in relation with the clinicopathological characteristics of 60 cervical cancer cases

| Clinicopathological characteristics | | $n$ | *DAPK* | *CALCA* | *ESR1* | *APC* | *RAR-$\beta_2$* | *TFPI2* | *SPARC* | *CCNA1* | *CADM1* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Disease recurrence | No | 44 | 24 | 41 | 17 | 25 | 38 | 24 | 42 | 25 | 27 |
| | Yes | 10 | 4 | 8 | 1 | 6 | 8 | 8 | 10 | 4 | 7 |
| Death of disease | No | 44 | 22 | 40 | 17 | 25 | 37 | 24 | 42 | 23 | 27 |
| | Yes | 14 | 8 | 13 | 1 | 7 | 12 | 10 | 13 | 7 | 9 |
| | | | | | $p = 0.044$ | | | | | | |
| Stromal invasion | $<$10 mm | 14 | 7 | 12 | 6 | 10 | 13 | 8 | 13 | 8 | 6 |
| | $\geqslant$10 mm | 30 | 15 | 29 | 9 | 15 | 25 | 20 | 30 | 15 | 23 |
| | | | | | | | | | | | $p = 0.042$ |
| FIGO stage | IB/IIA | 34 | 17 | 32 | 14 | 21 | 32 | 16 | 33 | 18 | 21 |
| | IIB–IVB | 26 | 14 | 23 | 4 | 13 | 19 | 19 | 24 | 13 | 17 |
| | | | | | $p = 0.031$ | | $p = 0.032$ | $p = 0.043$ | | | |
| Histology | SCC | 40 | 27 | 37 | 12 | 25 | 34 | 24 | 38 | 24 | 29 |
| | AC | 20 | 4 | 18 | 6 | 9 | 17 | 11 | 19 | 7 | 9 |
| | | | $p = 0.001$ | | | | | | | $p = 0.068$ | $p = 0.037$ |
| Tumor volume | $<$4 cm | 28 | 13 | 26 | 11 | 17 | 26 | 13 | 27 | 14 | 17 |
| | $\geqslant$4 cm | 31 | 17 | 28 | 7 | 16 | 24 | 21 | 29 | 16 | 20 |
| Differentiation grade | I/II | 36 | 18 | 33 | 13 | 20 | 31 | 21 | 35 | 18 | 22 |
| | III | 23 | 13 | 21 | 5 | 13 | 20 | 14 | 21 | 13 | 16 |
| Pelvic lymph node metastasis (PLNM)* | No | 22 | 9 | 21 | 11 | 13 | 20 | 13 | 22 | 12 | 12 |
| | Yes | 13 | 8 | 12 | 3 | 8 | 12 | 8 | 12 | 8 | 10 |
| Lymph vascular space involvement (LVSI)* | No | 30 | 13 | 27 | 9 | 14 | 24 | 20 | 28 | 13 | 14 |
| | Yes | 22 | 13 | 20 | 7 | 15 | 19 | 12 | 21 | 12 | 18 |
| | | | | | | | | | | | $p = 0.01$ |

*Note*: * Lymph node status is only known for patients who underwent primary surgical treatment.

parable to HSIL [32]), *DAPK* and *RAR-$\beta_2$* in the late immortal stage (comparable to HSIL), while *CADM1* in tumorigenic cells (comparable to cancer). In combination with our data, it appears that this experimental model indeed mimics the situation *in vivo* regarding a role of HPV in regulating DNA methylation for at least some of the cervical cancer specific genes.

*CALCA, APC, ESR1, TFPI2* and *SPARC* methylation has not previously been analyzed in precursor cervical lesions. *ESR1* was shown to be a promising diagnostic marker in our previous study [41], because in scrapings it was methylated in only one normal cervix (5%) compared to 64% of cervical cancers. In the present study, we show comparable results for the scrapings ($1/9$ normal cervices, $1/8$ LSIL, $2/18$ HSIL and $10/20$ cervical cancers). In paraffin tissue, proportion of methylated samples was higher in normal cervices. For some gene promoters, amongst which *ESR1*, it is known that methylation occurs in normal tissue, and that level of methylation increases with age [1]. In

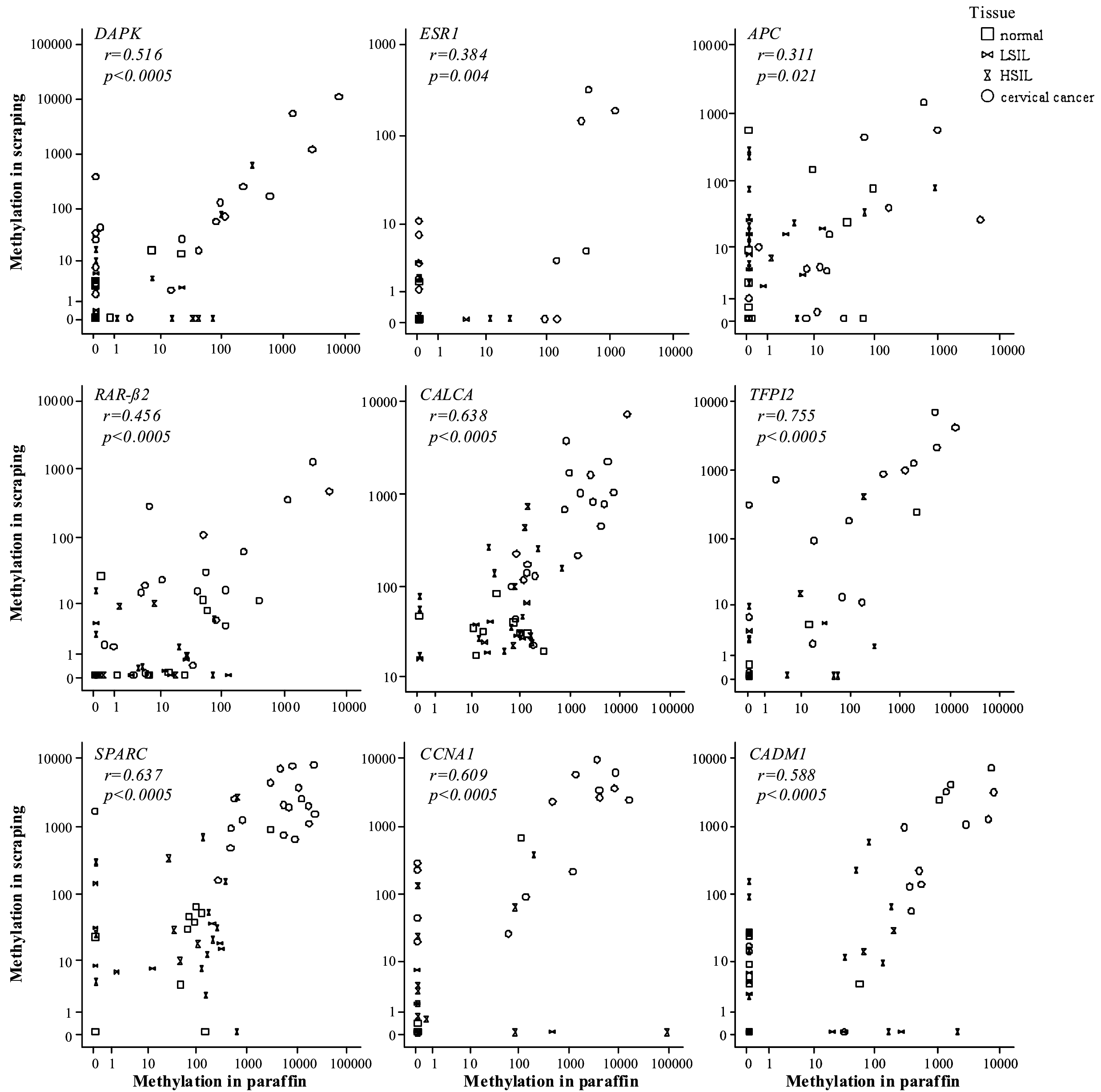


Fig. 2. Methylation ratios determined in paraffin tissue in comparison to the methylation ratio determined in the scraping of the same patient subdivided in the different tissue categories.

the present study, median age of women with a normal cervix was significant higher compared to the age of SIL patients. This might explain the higher level of methylation of normal cervices compared to SIL.

*TFPI2* and *SPARC* were the two most promising gene promoters from Sova et al., performing QMSP on cervical scrapings from controls versus cervical cancers [33]. Both gene promoters showed hypermethylation in 20/22 (91%) cervical cancers compared to only 3/21 (14%) controls [33]. For *TFPI2* promoter methylation, our study in tissue samples as well as in scrapings confirms these data. For *SPARC*, we found high numbers of paraffin samples and scrapings to be methylated in cervical cancers and in HSIL cases. However, we also detected methylation in almost all normal cervices and LSIL, in contrast to Sova et al. [33] who described SPARC hypermethylation in only 5% of normal scrapings, using although the same

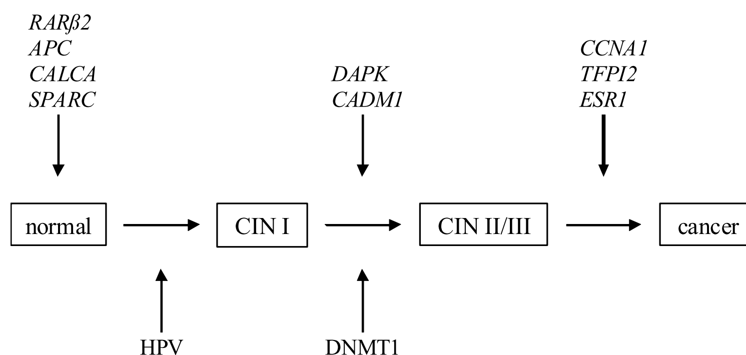


Fig. 3. Schematic model of the course of DNA promoter methylation in cervical carcinogenesis. The amount of dysplastic cells are depicted in gray. The arrows indicate at which stage of cervical carcinogenesis the gene promoters became mainly methylated or transfection with HPV.

primers. An explanation for this discrepancy is that Sova et al. defined a cut-off determined as the median value of histological normal samples. Cases with levels above this cut-off were regarded as hypermethylated [33]. If we would have used a similar approach, our analyses would reveal hypermethylation in 100% of cancers and 25% (5/20) of normal cervices. However, since levels of *SPARC* methylation are relatively high in most normal and LSIL tissues, *SPARC* does not suit for a cancer-specific methylation marker and therefore is not a good marker to discriminate between normal/LSIL and HSIL/cancer.

Whether or not a gene promoter should be included in QMSP analysis for detection of HSIL and cervical cancer depends on several factors. First, methylation detected in the scraping should reflect the methylation status of the underlying lesion, as we showed for most of the gene promoters analyzed in this study and which is in line with other studies [8,27]. Furthermore, sensitivity of QMSP for HSIL/cancer and specificity for normal cervices/LSIL should be high, which influences the choice of genes. We show in this study that *CCNA1* is the most promising gene promoter to detect 80% of cervical cancers and 56% of HSIL lesions, while most LSIL lesions were not detected. However, if we had chosen to set a cut-off above the highest normal cervix ("hypermethylated") as we also did in our previous studies [27,41], some gene promoters seem to be even more promising, such as *SPARC* with 20 of 20 cancers hypermethylated and 6 of 18 HSIL. However, for these gene promoters the cut-off is hard to draw, as already 7/9 normal cervices are positive for methylation and it might be that methylation level is increased when a large group of normal cervical scrapings will be analyzed. On the other hand, *CCNA1* is different as almost none of the normal cervices and LSIL were positive for methylation. *CCNA1* is therefore a promising gene promoter using QMSP to be analyzed in future studies with much larger series of scrapings from patients referred for an abnormal Pap smear.

The relatively large number of carcinomas in our study allowed us to analyze hypermethylation status in relation to clinicopathologic characteristics such as histology, stage, etc. For survival analysis, however, we regarded number of patients as too low and diversity with respect to stage and treatment modalities too large. Squamous cell cancers had a higher frequency of methylated *DAPK*, *CADM1* and *CCNA1* than adenocarcinomas, which is in agreement with our and other studies [11,17,41]. Interestingly, it has been reported that loss of *CADM1* expression, possibly due to hypermethylation, has been associated with metastasis [20], which is in agreement with our data, which show that *CADM1* methylation is related to stromal invasion and LVSI. Our data suggest a possible role in the invasive potential of cervical cancers by silencing of *CADM1* gene expression induced by its promoter methylation.

In conclusion, our study demonstrates that methylation of many previously reported cervical cancer specific genes (*RAR-*$\beta_2$, *SPARC*, *CALCA* and *APC*) frequently occurs in normal cervical epithelium at relatively high levels and consequently are not very useful markers to discriminate between normal/LSIL and HSIL/cancer. *CCNA1* and *DAPK* gene promoter methylation are the best methylation markers to distinguish normal/LSIL from HSIL/cancer. Array based approaches using precursor cervical tissue specimens should help to identify more appropriate cervical cancer specific gene promoters that might improve sensitivity and specificity of QMSP for detection of HSILs and cervical cancers in the future.

## Conflict of interest

Prof. A.G.J. van der Zee is a paid consultant for OncoMethylome Sciences S.A., Liège, Belgium. However, the company did not influence the study design, analysis and interpretation of data, in the writing of the report and in the decision to submit the report for publication.

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