Chapter 3

Promoter methylation analysis of WNT/β-catenin signaling pathway regulators to detect adenocarcinoma or its precursor lesion of the cervix
Abstract

Cervical adenocarcinoma (AdCA) and adenocarcinoma in situ (ACIS) are frequently missed in cytology-based screening programs. Testing for high-risk human papillomavirus (hrHPV) improves their detection, but novel ACIS/AdCA specific biomarkers are needed to increase specificity for these lesions. Novel markers may be deduced from the WNT/β-catenin signaling pathway, which is aberrantly activated during cervical carcinogenesis. 

Promoter methylation of ten WNT-antagonists (APC, AXIN2, DKK3, SFRP2, SFRP4, SFRP5, SOX7, SOX17, WIF1 and WNTSA) was evaluated by methylation-specific PCR (MSP) on a small series of cervical tissue specimens, including AdCA and SCC. To estimate the diagnostic potential of the genes most frequently methylated in AdCA an extended series of ACIS, AdCA, CIN3, SCC, and normal cervical tissue specimens (n=131) as well as 49 hrHPV-positive scrapings were analysed by quantitative MSP (qMSP).

The frequency of DKK3 and SFRP2 methylation was significantly higher in AdCA compared to SCC, i.e. 82% vs. 18% (p<0.01) and 84% vs. 39% (p<0.01), respectively, while SOX17 methylation frequency was significantly higher in SCC than AdCA, i.e. 89% vs. 62% (p=0.05). Methylation frequencies ranged from 4% to 55% in precursor lesions and from 0% to 5% in normal biopsies. When tested on HPV-positive cervical scrapings, qMSP of the best ACIS/AdCA discriminator genes, i.e. DKK3 and SFRP2, detected all women with underlying ACIS/AdCA, compared to 3% of controls. DKK3 and SFRP2 promoter methylation is highly indicative for the presence of ACIS/AdCA, thereby providing promising triage markers for HPV-positive women at risk of ACIS/AdCA.

Introduction

Worldwide, the incidence of cervical adenocarcinoma (AdCA) has remained the same or even increased, despite cytology-based cervical screening programs. Cervical adenocarcinoma in situ (ACIS) and AdCA are frequently missed in cervical scrapings, probably due to their localization higher in the endocervical canal and thereby less prone to be represented in a sample of exfoliated cells. Infection with high-risk types of human papillomavirus (hrHPV) is the primary cause of cervical cancer and the hrHPV types 16, 18 and 45 are responsible for >95% of the ACIS and AdCA. Since, in contrast to cytology, cervical screening by primary hrHPV testing does not require intact endocervical cells, HPV testing is more sensitive for the detection of ACIS and AdCA than cytology. Recent studies showed that hrHPV-positive women with normal cytology, especially those testing HPV16, 18 or 45 positive, have an increased risk of ACIS/AdCA. These women would require follow-up by colposcopy and, if colposcopy is negative, endocervical curettage. However, since only a small fraction of hrHPV women will have or develop ACIS/AdCA, further stratification of this risk group by additional biomarkers is needed to enable the gynaecologist to decide on endocervical curettage or other diagnostic tools, such as a diagnostic cone biopsy.

Silencing of tumor suppressor genes by DNA methylation is well-described in human cancers, and may yield biomarkers with great diagnostic and prognostic potential. DNA methylation alterations detected in cervical cancers are of particular interest, as recent studies indicate that DNA methylation can be easily detected in cervical scrapings. So far, most epigenetic studies on cervical cancer focused predominantly on SCC. It is unclear to what extent the results obtained on cervical SCC can be translated to AdCA, since both histotypes have been demonstrated to display partially distinct epigenetic profiles. To define methylation markers enabling an improved detection of ACIS and AdCA, further insight in the biology of these lesions is needed. One of the well-studied signaling pathways involved in AdCA development, such as colorectal, breast and pancreatic cancer, is the developmental WNT/β-catenin signaling pathway. A hallmark of active WNT/β-catenin signaling are elevated levels of β-catenin, often resulting from mutations in the adenomatous polyposis coli (APC) gene encoding a β-catenin inhibitor, and/or the β-catenin gene. Elevated cytoplasmic and nuclear β-catenin levels have been found in cervical carcinomas and to a lesser extent in precursor lesions. In addition, there is functional evidence for WNT/β-catenin pathway deregulation during hrHPV-mediated transformation in vitro. So far, no mutations in the APC and β-catenin gene have been detected in cervical cancer. Hence, other mechanisms like overexpression of oncogenic WNT ligands or functional loss of negative WNT regulators by DNA methylation...
may contribute to pathway activation in cervical cancer. Interestingly, previous studies have reported DNA methylation of the WNT inhibitors APC, members of the DICKKOPF (DKK) and secreted frizzled-related protein (SFRP) family in cervical cancer. To the best of our knowledge, no comprehensive study on promoter methylation of multiple WNT inhibitors in cervical cancer and its potential implication for improved detection of cervical ACIS and AdCA has been performed. In this study, ten genes encoding negative WNT regulators (Figure 1), including cytosolic WNT antagonists (APC, AXIN2), extracellular WNT inhibitors (DKK3, SFRP2, SFRP4, SFRP5, WNT inhibitory factor-1 (WIF1)), nuclear proteins (sex-determining region Y-box 7 (SOX7) and 17 (SOX17)), and one non-transforming WNT ligand (wingless-type MMTV integration site family member 5A (WNT5A)) were assayed for promoter methylation in cervical AdCA and ACIS relative to SCC and cervical intraepithelial neoplasia grade 3 (CIN3). Subsequently, the diagnostic potential of the two most frequently methylated genes in ACIS/AdCA, to detect AdCA and ACIS was assayed in HPV-positive cervical scrapes.

**MATERIALS AND METHODS**

**CELL CULTURES**

Primary human foreskin keratinocytes (EKs) and the cervical cancer cell lines SiHa, CaSki and HeLa were cultured as described previously.

**CERVICAL TISSUE SAMPLES**

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens of normal cervix (n=20), ACIS (n=11), AdCA (n=45), CIN3 (n=27) and SCC (n=28) were collected during routine clinical practice and stored at the Department of Pathology at the VU University Medical Center (Amsterdam, The Netherlands) and Erasmus Medical Center (Rotterdam, The Netherlands). Normal specimens were obtained from non-cancer patients undergoing hysterectomy. Per histological subgroup the women had the following mean ages: normal 48 years (range 34-70); ACIS 37 years (range 23-55); AdCA 43 years (range 28-79); CIN3 35 years (range 28-45); SCC 47 years (range 30-74). The mean age of women with any cervical (pre)malignant disease was not significantly higher than that of women with normal histology.

Cervical scrapings were obtained from the population-based cervical screening trial POBASCAM, registered as ISRCTN20781131. We randomly selected 31 cervical scrapings of GP5+/6+-PCR hrHPV-positive women with normal cytology without evidence of ACIS/AdCA/CIN or SCC up to the next screening round after 5 years (controls), 8 scrapings classified as moderate dyskaryosis or worse of hrHPV-positive women with ACIS/AdCA diagnosed within 18 months of follow-up and 10 scrapings classified as normal cytology of hrHPV-positive women diagnosed with high grade squamous disease (CIN2 or worse) during follow up. The median age of the controls and women with ACIS/AdCA was 37 (range 30-60) and 34 (range 30-40) years, respectively.

This study was approved by the Institutional Review Boards of both the VU University Medical Center in Amsterdam and the Erasmus Medical Center in Rotterdam.

**DNA EXTRACTION, BISULFITE MODIFICATION AND HPV TYPING**

Genomic DNA from tissue samples was extracted using the High Pure PCR Template Preparation kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer’s recommendations. Genomic DNA from cell cultures was isolated with
UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (Invitrogen Life Science Ltd, Carlsbad, CA USA).

DNAs, including in vitro methylated DNA (IVD) and CpGenome Universal Unmethylated DNA (Millipore™, Billerica, MA, USA), were bisulfite treated using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA). HPV detection and genotyping was performed using the GP5+/6+-PCR with an enzyme immunoassay (EIA) readout followed by reverse-line-blot analysis of EIA positive cases.

Methylation Specific PCR (MSP)
MSP primers of 10 selected candidate markers are described in Table 1. β-actin (ACTB) was included as a control for sample integrity and bisulfite conversion. MSP, using 50 ng of modified DNA and FastStart Taq DNA polymerase (Roche Diagnostics, Woerden, The Netherlands) was performed as described previously.

Quantitative MSP
DNA methylation of DKK3, SFRP2, SOX17 and WIFI was quantified using hydrolysis probes (Table 1). QMSP reaction were performed as described before. All samples were tested in duplicate. Methylation values were normalised to the reference gene ACTB and 100% real-time PCR efficiencies (>90%) with high linearity (Pearson correlation coefficient R>0.994). The lower detection limit (determined by the lowest consistently detected concentration of DNA) for reliable quantification was at least 0.05 ng methylated DNA per reaction for DKK3, SFRP2, SOX17 and WIFI, with a coefficient of variation of 0.61, 0.73, 1.23, and 0.39, respectively. All unmethylated samples had a ACTB Ct <32, indicating sufficient DNA quality. The 99% confidence interval of normal DNA spiked with methylated CaSki DNA. QMSPs showed high real-time PCR efficiencies (>90%) with high linearity (Pearson correlation coefficient R=0.994). The lower detection limit (determined by the lowest consistently detected concentration of DNA) for reliable quantification was at least 0.05 ng methylated DNA per reaction for DKK3, SFRP2, SOX17 and WIFI, with a coefficient of variation of 0.61, 0.73, 1.93, and 0.48%, respectively.

Table 1. Primers and probes sequences.

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<th>Antisense (5’ – 3’)</th>
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<th>Sequence accession number</th>
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QMSP Probes*

* qMSP probes were combined with the same primers as used in the MSP
** The DKK3 probe was conjugated to a minor-groove-binder-replacement (XS probe) at the 3’-end

CpG sites are presented in bold

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Promoter methylation of WNT/β-catenin modulators in cervical AdCA

A small tissue panel consisting of normal cervical samples (n=8), AdCA (n=12) and SCC (n=9) was subsequently tested to discriminate those markers that would most likely differentiate between AdCA and normal samples, and between AdCA and SCC (Figure 2). In normal specimens occasional, sometimes very weak, methylation positivity was seen for SFRP2, SFRP4, SFRP5, SOX17 and WIF1, while APC, AXIN2 and WNT5A tested negative. DKK3 showed weak signals in virtually all normal samples and in subsequent analysis only samples showing increased signal intensity compared to the normal samples were scored positive for DKK3 methylation. AdCA displayed high methylation frequencies (83% to 100%) for DKK3, SFRP2, SOX17 and WIF1, intermediate frequencies (33% to 50%) for APC, SFRP4, SFRP5 and WNT5A and no methylation for AXIN2.

In SCC, WIF1 and SOX17 showed high methylation frequencies (82% and 91%, respectively); SFRP2, SFRP4, SFRP5 and WIF1 revealed intermediate methylation frequencies (33%-67%), whereas no or infrequent methylation (0% to 22%) was found for APC, AXIN2, DKK3 and WNT5A. The four markers with the highest methylation frequencies in AdCA, i.e. DKK3, SFRP2, SOX17 and WIF1, were selected for further quantitative MSP (qMSP) analysis on a larger set of tissue specimens.

QMSP analysis of DKK3, SFRP2, SOX17 and WIF1 in cervical tissue specimens

HrHPV genotyping and qMSP for the four selected genes were performed on 20 normal cervical specimens, 11 ACIS, 45 AdCA, 27 CIN3 and 28 SCC (Figure 3). All normal specimens were hrHPV negative, whereas all lesions with the exception of one CIN3 were hrHPV positive.

Promoter methylation frequency for DKK3 was 55% in ACIS, 82% in AdCA, 33% in CIN3 and 18% in SCC. For SFRP2 methylation rates were 45% in ACIS, 84% in AdCA, 4% in CIN3 and 39% in SCC. The methylation frequencies for SOX17 and WIF1 were as follows: ACIS: 55% and 27%, AdCA: 62% and 71%, CIN3: 67% and 33% and SCC: 89% and 54%, respectively. Five percent of normal samples revealed DKK3, SFRP2 and SOX17 methylation and none were WIF1 methylation positive.

Methylation frequencies for DKK3 and SFRP2 were significantly higher in AdCA compared to SCC and ACIS (p<0.01). Moreover, methylation frequencies for DKK3 and SFRP2 were significantly higher in AdCA than in SCC (p<0.01). SOX17 was more frequently methylated in SCC than AdCA (p<0.05). In ACIS SFRP2 methylation was significantly more frequent than in CIN3 (p<0.01). Additionally, the methylation levels of DKK3 and SFRP2 were significantly higher in AdCA compared to SCC and ACIS (p<0.01 and p<0.05, respectively) and vice versa for SOX17 (p<0.01) (Figure 4).

Results

Promoter methylation of ten WNT/β-catenin signaling regulators in cervical tissue specimens

Ten MSPs for the WNT inhibitors APC, AXIN2, DKK3, SFRP2, SFRP4, SFRP5, SOX7, SOX17, WNT5A and WIF1 were analytically validated, using universal unmethylated DNA (UUD-B), non-bisulfite-treated CaSki DNA and primary keratinocyte DNA (EK) as negative controls and in vitro methylated DNA (IVD) as positive control. Whereas all ten MSPs resulted in a clear amplicon with IVD, signals were absent or at maximum very weak with negative controls (Figure 2, left panel). Subsequent analysis of the cervical cancer cell lines SiHa, CaSki and HeLa revealed methylation of DKK3, SFRP2, SFRP4, SFRP5 and SOX17 in all three cell lines. In addition, CaSki cells revealed AXIN2, WNT5A and WIF1 methylation. SiHa and HeLa showed methylation of one additional gene, i.e. APC and AXIN2, respectively. Since none of the cervical cancer cell lines showed SOX7 methylation, this gene was excluded from further analysis.

Figure 2. Promoter methylation of WNT inhibitors is frequent in cervical cancer. The methylation status of nine WNT inhibitors was determined for 29 cervical tissue specimens (8 normals; 12 adenocarcinomas (AdCA) and 9 squamous cell carcinomas (SCC)). In vitro methylated DNA (IVD) and the cervical cancer cell line CaSki were used as positive control for methylation. Genomic DNA of CaSki, unmethylated DNA (UUD-B) and primary foreskin keratinocytes (EKs) were used as negative controls. The ACTB gene was included as a control for bisulfite treatment.
Figure 3. Summary of the qMSP results on cervical tissue specimens. DNA methylation is depicted in black; white boxes indicate unmethylated DNA; the HPV status of specimens is depicted as specific HPV type present or negative (-).

**PROOF-OF-PRINCIPLE ANALYSIS ON CERVICAL SCRAPINGS**

Since hypermethylation of DKK3 and SFRP2 appeared most discriminative for ACIS/AdCA, we determined the potential diagnostic value of DKK3 and SFRP2 methylation analysis for the detection of ACIS/AdCA in cervical scrapings. Hence, we performed a proof-of-principle analysis on 39 hrHPV-positive cervical scrapings collected during a population-based screening trial (POBASCAM) 31. Eight cervical scrapings were derived from women diagnosed with ACIS/AdCA, 31 scrapings from women with normal cytology without evidence of cervical premalignant or malignant disease after 5 year follow-up and 10 normal cytology samples of women diagnosed with high grade squamous lesions (CIN2 or worse) during follow up. Since ACIS and AdCA occur at a very low frequency in the general population, we did not have more samples of women with ACIS/AdCA available from this large screening trial. Of the hrHPV-positive scrapings of women with normal cytology, representing 3.6% of the screening population, 58% contained HPV16 or 18. All scrapings of women with ACIS/AdCA tested here were HPV16 or 18 positive. QMSP analysis showed methylation positivity for both markers in all 8 scrapings of women with ACIS/AdCA. None of the 31 normal cytology samples without cervical disease during follow-up was positive for DKK3 methylation, and only one (3%) was SFRP2 methylation positive. Similarly, only

Figure 4. Association between methylation, disease stage, and histotype. Scatter plots of the levels of DKK3 (A), SFRP2 (B), SOX17 (C), and WIF1 (D) methylation. On the y-axes levels of methylated DNA are presented; on the x-axes the samples are grouped for each disease stage and histotype. The grey dotted line indicates the cut off value.
one of 10 normal cytology samples of women diagnosed with squamous cervical disease during follow-up tested positive for DKK3 and SFRP2, indicating that this marker panel is highly specific for ACIS/AdCA. The value of these markers is further underlined by the fact that upon histological revision of the biopsy corresponding to the DKK3/SFRP2 methylation positive scraping, an ACIS was detected next to the originally diagnosed CIN2 lesion.

**DISCUSSION**

Epigenetic markers based on alterations in the WNT signaling pathway are currently highlighted as new promising markers for diagnosis and prognosis in many types of cancer. Here we evaluated their potential diagnostic value for the early detection of cervical cancer, with particular focus on AdCA and precursors (ACIS). DNA methylation analysis of ten negative modulators of WNT/β-catenin signaling pathway revealed that all except one (i.e. SOX7) gene promoters were methylated in one or more cervical cancer cell lines, as well as in a subset of cervical AdCA and SCC samples. For five gene promoters i.e. APC, AXIN2, SFRP4, SFRP5 and WNTTSAA methylation rates were relatively low (<56%) in both AdCA and SCC and therefore not analysed further. QMSP analyses of the remaining four genes, i.e. DKK3, SFRP2, SOX17 and WIFI1, showed that DKK3, SFRP2 and SOX17 were differentially methylated between AdCA and SCC, suggesting that histotype-specific alterations within the WNT signaling pathway exist. This is in concordance with previous studies, where APC and SFRP5 were found to be more frequently methylated in cervical AdCA compared to SCC.

One of the most frequently methylated regulators in cervical AdCA, as demonstrated in this study, is DKK3. A previous study found 31% of cervical cancer specimens methylated for DKK3, which is consistent with our study (SCCs and AdCAs combined), presuming that the specimens tested by Lee et al. represented a combination of SCC and AdCA. Present data indicate that DNA methylation of DKK3 is significantly more frequent in AdCA than SCC (82% vs. 18%, p<0.01, respectively). Moreover, DKK3 tested methylation positive in all hrHPV-positive scrapings of women with ACIS or AdCA versus none of the controls, addressing the potential value of DKK3 as biomarker for cervical ACIS/AdCA detection. Functional studies have shown that DKK3 possesses antiproliferative activity in cervical cancer cells, prostate and hepatoma cancer cell lines, though not in melanoma cell lines, supporting a tissue- and histotype-specific function of DKK3 in human tumors.

Interestingly, also for SFRP2 a significantly increased methylation rate was found in AdCA in comparison with SCC (84% vs. 39%; p<0.01). In addition to cancer tissues, we also found methylation of SFRP2 in all cervical scrapings of hrHPV-positive women with ACIS/AdCA, whereas only 3% hrHPV-positive controls scrapings tested positive. A previous study of Chung et al. demonstrated a high DNA methylation frequency of SFRP2 in cervical scrapings of AdCA patients (83%) in comparison with SCC (81%) using a semi-quantitative MSP and qMSP analysis may show similar sensitivities, but the specificity of qMSP analysis may be higher as compared with MSP. Furthermore, frequencies of promoter methylation may vary among different ethnic groups, implying the need to optimize DNA methylation markers for individual target populations. In our study we found that SFRP2 methylation was significantly more frequent in ACIS (45%) than CIN3 (4%), supporting a predominant role for SFRP2 promoter methylation in AdCA development. Overexpression of SFRP2 has previously been demonstrated to suppress proliferation, colony formation and invasion of cervical cancer cells.

In contrast to DKK3 and SFRP2, SOX17 promoter methylation was more frequent in SCC than AdCA. So far, SOX17 promoter methylation and concomitant reduced SOX17 mRNA expression has been described in gastric, colorectal, and breast cancer. To the best of our knowledge this is the first study in which frequent methylation of SOX17 in cervical cancer is described. Interestingly, promoter methylation of DKK3, SFRP2 and SOX17 was associated with gene silencing in CaSki cells (data not shown), and therefore could have a mechanistic consequence. While, as described above, a mechanistic involvement of DKK3 and SFRP2 in cervical carcinogenesis has yet been demonstrated, the functional role of SOX17 remains to be established.

WIF1 was the only histotype-independent methylation marker showing frequent methylation in both SCC and AdCA and has also not been described before in cervical cancer. Other studies on DNA methylation-associated silencing of WIF1 have also demonstrated a tumor suppressive role in various types of cancers. Evaluation of cytology based organised screening programmes show that AdCA and ACIS are frequently missed, resulting in a stable incidence and mortality of cervical cancer, in contrast to SCC where significant reductions in incidence and mortality are seen. hrHPV testing is more sensitive than cytology to detect cervical lesions and expected to better detect ACIS/AdCA. However, since hrHPV testing also detects transient HPV infections resulting in a lower specificity for the detection of ACIS/AdCA, additional triage markers are needed to prevent overtreatment. Our proof-of-principle analysis on cervical scrapings indicates that further risk stratification of hrHPV positive women, particularly
women who are HPV 16, 18 and 45 positive, by testing for DKK3 and SFRP2 promoter methylation will strongly improve the specific detection of ACIS and AdCA. Further clinical validation studies on larger series of cervical scrapings collected during population-based screening studies evaluating primary hrHPV-testing, such as the POBASCAM, VUSA-screen, Swedescrreen or ARTISTIC trial 49,50, will reveal the diagnostic value of this marker panel for the detection of ACIS/AdCA in hrHPV-positive scrapings. Such a study has recently been conducted for the methylation markers CADM1 and MAL, which were found to be equally discriminatory for CIN3+ as cytology or cytology plus HPV16/18 genotyping 51. Implementation of methylation markers in the triage of hrHPV-positive women is expected to guide the decision making of the gynaecologist. hrHPV-positive women who test positive for DKK3 and/or SFRP2 methylation are suspected of ACIS/AdCA and require colposcopic inspection of the transformation zone. Biopsies should be taken from abnormal areas or a blind biopsy is needed in case of absence of abnormalities in the transformation zone. When both colposcopy and the biopsy are classified as normal an endocervical curettage is not recommended when no abnormalities in the transformation zone. When both colposcopy and the biopsy are classified as normal an endocervical curettage is not recommended when no abnormalities in the transformation zone. In conclusion, our data show that negative modulators of the WNT/β-catenin signaling pathway are frequently affected by promoter methylation during cervical carcinogenesis, with DKK3 and SFRP2 promoter methylation being most specifically associated with the development of AdCA. Moreover, both DKK3 and SFRP2 promoter methylation could also be specifically detected in scrapings of HPV-positive women with underlying ACIS or AdCA. Hence superior detection of ACIS and AdCA may be obtained by testing hrHPV positive women for these adenocarcinoma (in situ) specific markers.

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References
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