Genome-wide DNA methylation profiling reveals methylation markers associated with 3q gain for detection of cervical pre-cancer and cancer

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Running title:

Methylation markers at 3q for cervical (pre)cancer detection

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Translational relevance

Implementation of primary high-risk HPV (hrHPV) DNA testing in cervical screening programs in the coming years results in better protection against cervical (pre)cancer. However, its specificity for clinically meaningful disease is lower compared to current cytology-based screening. Furthermore, current methods are unable to determine which precursor lesions will progress or regress, leading to over-referral and overtreatment. This study aimed to identify biologically relevant DNA methylation changes that enable identification of hrHPV-positive women with clinically relevant cervical lesions who are in need of referral. Following an unbiased genome-wide approach, we identified 3 novel biologically relevant methylation markers (*GHSR*, *SST* and *ZIC1*, all located on chromosome 3q), that provide most promising molecular triage markers for detection of cervical (pre)cancer in cervical scrapes. These methylation markers mainly detect advanced precursor lesions with a high likelihood of short-term progression risk to cancer, leading to detection of clinically relevant precursor lesions. These findings could greatly advance the management of hrHPV-positive women.

Abstract

Purpose

Epigenetic host cell changes involved in cervical cancer development following a persistent high-risk human papillomavirus (hrHPV) infection, provide promising markers for the management of hrHPV-positive women. In particular, markers based on DNA methylation of tumour suppressor gene promoters are valuable. These markers ideally identify hrHPV-positive women with pre-cancer (CIN2/3) in need of treatment. Here, we set out to identify biologically relevant methylation markers by genome-wide methylation analysis of both hrHPV-transformed cell lines and cervical tissue specimens.

Experimental Design and Results

Genome-wide discovery by next generation sequencing (NGS) of Methyl Binding Domain enriched DNA (MBD-Seq) yielded 20 candidate methylation target genes. Further verification and validation by multiplex targeted bisulphite NGS and (quantitative) methylation-specific PCR (MSP) resulted in 3 genes (*GHSR*, *SST* and *ZIC1*) that showed a significant increase in methylation with severity of disease in both tissue specimens and cervical scrapes ($p < 0.005$). The area under the ROC curve for CIN3 or worse varied between 0.86-0.89. Within the group of CIN2/3, methylation levels of all 3 genes increased with duration of lesion existence ($p < 0.0005$), characterized by duration of preceding hrHPV infection, and were significantly higher in the presence of a 3q gain ($p < 0.05$) in the corresponding tissue biopsy.

Conclusion

By unbiased genome-wide DNA methylation profiling and comprehensive step-wise verification and validation studies using *in vitro* and patient-derived samples, we identified 3 promising methylation markers (*GHSR*, *SST* and *ZIC1*) associated with a 3q gain for the detection of cervical (pre)cancer.

Introduction

Cervical cancer is caused by a persistent high-risk human papillomavirus (hrHPV) infection, with HPV16 and HPV18 being the most predominant types(1). Cervical squamous cell carcinoma (SCC) develops through well-defined precursor lesions (cervical intraepithelial neoplasia, classically graded 1 to 3 (CIN1-3)), of which CIN3 is considered the most advanced precursor lesion of cervical cancer(2–4). Common practice is the ablation of CIN2 and CIN3 tissue to prevent cervical cancer development. However, many CIN2 and some CIN3 lesions will regress spontaneously if left untreated(2,3,5). Current CIN classification is not able to identify which lesions will progress or regress, leading to considerable over-referral and overtreatment with associated cervical morbidity. The progression of CIN2/3 to SCC results from the accumulation of genetic and epigenetic alterations in the host cell over a time period of 15 to 30 years(2–4). Given their variable duration of existence, CIN2/3 represents a heterogeneous disease, with various risk of progression to cancer. Recent molecular studies on CIN2/3 indicate that a subset of these lesions display a cancer-like profile including hypermethylation of tumour suppressor genes and specific chromosomal alterations(6–8). These so-called advanced CIN2/3 lesions, characterized by a longer duration (≥ 5 years) of preceding hrHPV infection (PHI), are most likely to have a high short-term progression risk to cancer and are in need of treatment(2). DNA methylation-based assays that can be used as a biomarker for the detection of advanced CIN2/3 lesions would greatly advance the management of hrHPV-positive women by reducing overdiagnosis and overtreatment.

At present, a number of methylated host cell genes have been described by us and others as promising markers for the management of hrHPV-positive women, such as *CADM1*, *MAL*, *miR124-2*, *FAM19A4* and *EPB41L3*(7,9–12). Here, we describe an unbiased genome-wide based approach, comprised of next generation sequencing (NGS) of Methyl Binding Domain (MBD) enriched DNA (MBD-Seq), to identify biologically relevant methylation-based markers within patient-derived and *in vitro* samples representing the multistep process of hrHPV-induced carcinogenesis. Our longitudinal *in vitro* model of primary keratinocytes immortalized by hrHPV types closely mimics *in vivo* carcinogenic stages, both with respect to molecular as well as morphological changes(13). This model system allows us to study the sequential order of accumulating epigenetic host cell changes associated with hrHPV-mediated carcinogenesis(14–16). Combined with the DNA methylation profiles of various stages of cervical (pre)cancer *in vivo*, this offers a rewarding strategy to identify biologically relevant methylation markers. Additionally, step-wise verification and validation studies were performed using multiplex targeted bisulphite NGS, methylation-specific PCR (MSP) and multiplex quantitative MSP (qMSP) on large clinical series of both cervical tissues and scrapes, comprising all stages of cervical carcinogenesis. To further understand the relation of DNA methylation levels in cervical scrapes to the severity and duration of the underlying cervical lesion, samples from women with CIN2/3 with a known duration of PHI, as a proxy of lesion duration, were included(6). Furthermore, to link methylation findings to other critical host cell alterations such as DNA copy number aberrations, we analysed previously obtained chromosomal profiles of these samples(6). Altogether, our comprehensive approach yielded 3 novel promising methylation markers associated with 3q gain for the detection of cervical (pre)cancer.

Materials and Methods

Cell lines and clinical specimens

Discovery

For genome-wide DNA methylation profiling by MBD-Seq, various cell lines and frozen cervical tissue specimens were used. Cell lines consisted of (i) primary human foreskin keratinocytes (HFK) from two donors, (ii) HFK from one donor transduced with HPV16E6E7 at passage 7 and passage 30, representing the pre-immortal and immortal stage, respectively(17), (iii) consecutive passages of HPV16- (FK16A and FK16B) and HPV18- (FK18A and FK18B) immortalized keratinocytes, including early (passages 11-30), middle (passages 30-70) and late (>passage 70) immortal passages, and (iv) cervical cancer cell line SiHa. The tissue specimens comprised CIN1 (n=3), CIN2/3 (n=12) and SCC (n=10). Given limitations in accurate histological grading of high-grade CIN on frozen tissue sections, CIN2 and CIN3 were grouped together in this frozen tissue sample series.

Verification I

Multiplex targeted bisulphite NGS was performed on a subset of hrHPV-transformed cell lines and frozen tissue specimens used for discovery with sufficient material left, i.e., HFKs from two donors, early and late immortal passages of FK16B and FK18B, SiHa, CIN2/3 lesions (n=8) and SCCs (n=7). In addition, 2 pooled control specimens comprising pooled DNAs of 3 normal cervical formalin-fixed paraffin-embedded (FFPE) tissue specimens or 6 scrapes from women without cervical disease, respectively, were used.

Verification II

For verification by MSP, HFKs from four different donors, late immortal passages of FK16A, FK16B and FK18A, cervical cancer cell lines SiHa, HeLa and CaSki, and FFPE tissue specimens including normal cervical tissue (n=11), CIN3 (n=11) and SCC (n=10) were used.

Validation

For validation by multiplex qMSP, HFKs from five different donors, middle and late immortal passages of FK16A, FK16B, FK18A and FK18B, and cervical cancer cell lines SiHa, HeLa and CaSki were used. In addition, an independent series of FFPE tissue specimens comprising normal cervical tissue (n=41), CIN1 (n=32), CIN2 (n=23), CIN3 (n=33) and SCC (n=30), as well as a series of scrapes comprising specimens from hrHPV-positive women with normal cytology and/or without evidence of CIN2+ (n=97) or from women who were histologically diagnosed with CIN2 (n=21), CIN3 (n=75) or SCC (n=27), were used. Another 48 cervical scrapes from women with a CIN2 or CIN3 lesion in the second screening round (interval 5 years) of the POBASCAM trial(18) were included. Details of this additional series have been described before(6–8). In brief, the 5-year history of hrHPV infection of these women was known and used as surrogate for duration of lesion existence. Women with a PHI <5 years were considered to have early CIN2/3 lesions (n=19) and women with a PHI ≥5 years were considered to have advanced CIN2/3 lesions (n=29). DNA copy number data were obtained during a previous study in which tissue specimens corresponding to this series of cervical scrapes were microdissected to enrich for the dysplastic areas and isolated DNA was hybridised to 105K arrays (Agilent Technologies, Palo Alto, USA)(6). DNA copy number data are available from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/projects/geo/>), through series accession number GSE30155.

HFKs were obtained and cultured as described before(19). Cervical cancer cell lines SiHa, HeLa and CaSki (American Type Culture Collection) were cultured according to specifications(20). Cell lines were recently authenticated using the PowerPlex 16 System (Promega, Madison, WI, USA) and were negative for mycoplasma. The establishment and culturing of HFKs transduced with HPV16E6E7 as well as FK16A, FK16B, FK18A and FK18B have been described previously(17,19). All biopsies were

collected during the course of routine clinical practice at the Department of Obstetrics and Gynaecology at VU University Medical Center, Amsterdam, The Netherlands. Cervical scrapes were obtained from screening or gynaecologic outpatient populations. This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center and University Medical Center Groningen.

DNA isolation, bisulphite treatment and hrHPV testing

Isolation of DNA from cell lines was performed by proteinase K digestion followed by standard phenol-chloroform extraction as described previously(21). Isolation of DNA from tissue specimens was performed by High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland) using manufacturer's protocol. DNA from cervical scrapes was isolated using the NucleoMag 96 Tissue kit (Macherey-Nagel, Düren, Germany) and a Microlab Star robotic system (Hamilton, Martinsried, Germany) according to manufacturer's protocol. For multiplex targeted bisulphite NGS, MSP and qMSP analyses, DNA was bisulphite-converted using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). HrHPV DNA detection was performed by GP5+/6+ PCR enzyme immunoassay or HPV-Risk assay (Self-screen BV, Amsterdam, The Netherlands)(22,23).

MBD-Seq

Genome-wide DNA methylation profiling was performed by MBD-Seq, using the MethylCap kit v2 (Diagenode, Liège, Belgium) with 500 ng input DNA combined with Illumina Genome Analyzer Ix paired-end sequencing as described before(24,25). Next, Bowtie 1.0.0 was used to map the obtained paired-end reads from fastq-files to the human reference genome GRCh37(26). These mapped sequencing results were visualized in the H2G2 genome browser (<http://h2g2.ugent.be/biobix.html>). From this browser, we extracted the read counts of all predefined methylated regions located in promoters (-1000 bp to +500 bp relative to the transcription start site). The read counts of all regions in cervical cancer (SiHa, SCC) were compared to the read counts in controls (HFK, CIN1). These regions were ranked based on the difference in read counts in order to select the hypermethylated regions. Next, we analysed hypermethylation of these regions in the precursors (HFKs transduced with HPV16E6E7, FK16A, FK16B, FK18A, FK18B and CIN2/3). From this list, we selected the top 20 most hypermethylated promoters.

Multiplex targeted bisulphite NGS

For multiplex targeted bisulphite NGS, a total of 62 primer pairs were designed to amplify the 20 selected methylated regions by PCR with on average 3 amplicons per target. Each forward and reverse primer contained a universal extension. The amplicons were pooled together per sample and purified from primer dimers using the Gel Extraction kit (Qiagen, Hilden, Germany). A second PCR was performed on the pool of amplicons using primers annealing to the universal extension to add the flow cell binding sites p5 and p7 and a unique barcode to every sample. The concentration of every sample library was measured using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Up to 12 uniquely barcoded sample libraries were equimolarly pooled prior to sequencing. This final pool contained 10% spike-in of PhiX Control v3 (Illumina, San Diego, CA, USA) to increase the diversity and was loaded on a MiSeq Personal Sequencer (Illumina) using 5 pM. A MiSeq Reagent Kit v2 (300 cycles; Illumina) was used in a paired-end 150 run according to manufacturer's protocol. All bisulphite sequencing data described in this study were processed as follows: Quality was evaluated using FastQC Version 0.11.2, the reads were aligned on the bisulphite treated version of the human genome and methylation calls were performed in Bismark version 0.12.9(27). Reads were combined to represent a DNA methylation profile for each sample.

MSP and multiplex qMSP

MSP was performed as previously described using 50 ng of bisulphite-converted DNA as input material(28). MSP primers were designed to specifically amplify methylated bisulphite treated DNA. *In vitro* methylated DNA (IVD) was used as positive control and unmodified SiHa DNA and H₂O served

as negative controls. The housekeeping gene β -actin (*ACTB*) verified sufficient DNA quality and successful bisulphite conversion of all samples.

Multiplex qMSP for 3 targets and *ACTB* was designed as described before(29), and performed using 50 ng of bisulphite-converted DNA and 200-300 nM of each primer and fluorescent dye-labelled probe, on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The methylation values of the targets were normalised to reference gene *ACTB* using the comparative Cq method ($2^{-\Delta Cq} \times 100$) to obtain Cq ratios. All samples in qMSP analysis had an *ACTB* Cq value <32, indicating suitability for DNA methylation analysis.

Statistical analyses

For each methylated gene, Wilcoxon rank test was applied to test the difference in methylation levels between disease categories with a significance level of 0.05 (two-sided). For logistic regression analysis in cervical scrapes, samples from women with CIN3 or SCC (CIN3+) were categorized as cases and those from hrHPV-positive women with normal cytology and/or without evidence of CIN2+ were categorized as controls. To assess the relationship between the outcome of the samples (case or control) and the methylation markers, univariable logistic regression analysis was performed on the square root transformed Cq ratios of *GHSR*, *SST* and *ZIC1*. The performance of the logistic regression model was visualized by receiver operating characteristics (ROC) curve, and was evaluated by sensitivity, specificity and area under the curve (AUC). Predicted probabilities, representing the risk for an underlying CIN3+, were calculated using the univariable logistic regression models of *GHSR*, *SST* and *ZIC1*. Samples with predicted probabilities above the optimum threshold (*GHSR*: 0.396; *SST*: 0.358; *ZIC1*: 0.354) were scored methylation-positive for the respective marker.

Results

An outline of the study is given in Figure 1 and Supplementary Table S1.

Genome-wide DNA methylation profiling by MBD-Seq

For discovery of DNA methylation profiles associated with hrHPV-induced carcinogenesis, genome-wide MBD-Seq was performed on primary keratinocytes, hrHPV-transformed cell lines and cervical tissue specimens (Figure 1; Discovery). Differentially methylated promoter regions were identified as those with a difference in cervical cancer samples (SiHa, SCC) over normal/low-grade samples (HFK, CIN1), and precursors (hrHPV-transformed cell lines and CIN2/3 lesions) over normal/low-grade samples. Differentially methylated promoters were ranked to select the top 20 candidate hypermethylation target genes (Table 1).

Verification analyses and selection of candidate methylation markers

Verification of the top 20 candidates by multiplex targeted bisulphite NGS was performed on a subset of samples used in the MBD-Seq screen (Figure 1; Verification I). Increased methylation of 9 genes, *GALR1*, *GHSR*, *IRX1*, *NEUROG3*, *POU4F3*, *PROX1*, *SIM2*, *SST* and *ZIC1*, was verified in hrHPV-immortalized keratinocytes, cervical cancer cells, CIN2/3 lesions and SCCs compared to controls (Table 1). The remaining 11 genes were excluded from further studies due to the detection of methylation in controls (*ANKRD2*, *CLDN18*, *GRK7*, *HIPK4*, *SLC45A2*, *TBX19* and *TFDP3*) or technical issues (*MYCL2*, *PDE4DIP*, *RXFP3* and *SOX14*) (Table 1).

Subsequent MSP analysis confirmed methylation of all 9 genes in late immortal passages of hrHPV-immortalized keratinocytes and cervical cancer cell lines, while HFKs were mainly methylation-negative (Figure 1; Verification II; Table 1, Supplementary Fig. S1A). *SIM2* was clearly methylation-positive in 1 out of 4 HFKs. *GALR1*, *IRX1* and *NEUROG3* were ambiguously methylation-positive in some late immortal passages of hrHPV-immortalized keratinocytes. The remaining five genes (*GHSR*, *POU4F3*, *PROX1*, *SST* and *ZIC1*) were further evaluated by MSP in a series of cervical tissue specimens (Figure 1; Verification II) confirming increased methylation frequency with increasing disease severity (Table 1, Supplementary Fig. S1B and Fig. S2). Two genes, *POU4F3* and *PROX1*, were methylation-negative in 1 out of 10 SCCs leaving 3 genes (*GHSR*, *SST* and *ZIC1*) for a comprehensive validation analysis.

Validation of candidate markers in cell lines and cervical tissue specimens by qMSP analysis

Based on the verification findings, *GHSR*, *SST* and *ZIC1*, were further analysed by multiplex qMSP on cell lines and independent large series of cervical tissue specimens (Figure 1; Validation). No *SST* and *ZIC1* methylation was detected in any of the HFKs, whereas *GHSR* showed very low levels of methylation in 3 out of 5 HFKs. Methylation of all 3 genes significantly increased with passaging of hrHPV-immortalized keratinocytes and was highest in cervical cancer cell lines ($p < 0.05$; Figure 2A-C). For all 3 genes, a gradual increase in methylation was observed with increasing severity of cervical disease. Methylation levels were significantly increased in CIN2 compared to normal cervical tissues ($p < 0.005$), in CIN3 compared to CIN2 ($p < 0.05$), as well as in SCCs compared to CIN3 ($p < 0.0005$; Figure 2D-F).

Marker potential of *GHSR*, *SST* and *ZIC1* in cervical scrapes

Next, validation analysis included multiplex qMSP on a total of 220 hrHPV-positive cervical scrapes to evaluate the potential of *GHSR*, *SST* and *ZIC1* methylation analysis to detect cervical (pre)cancer (Figure 1; Validation). For all 3 genes, fold changes in methylation levels compared to the reference group (hrHPV-positive controls) increased with severity of underlying disease (Table 2A). Largest fold changes were seen for *ZIC1*, showing a 10.1-fold increase in scrapes from women with CIN2, elevating to a 58.7-fold change in scrapes from women with CIN3, and a 921.1 fold-change in scrapes from women with SCC. Differences were significant for all 3 genes in hrHPV-positive scrapes from

women with CIN3+ compared to hrHPV-positive controls ($p<0.0005$) and hrHPV-positive scrapes from women with CIN2 ($p<0.005$; Supplementary Fig. S3). Logistic regression analysis showed a similar clinical performance for CIN3+ detection in hrHPV-positive cervical scrapes for *GHSR*, *SST* and *ZIC1*, with an AUC of 0.87, 0.86 and 0.89, respectively (Figure 3A; Table 3). *ZIC1* presented the highest AUC, which corresponds at a certain threshold to a sensitivity of 86.3% and a specificity of 80.4% (Table 3). All cervical cancers were detected in scrapes by *GHSR*, whereas *SST* and *ZIC1* detected identical 26 of 27 (96.3%) cervical cancers.

Methylation levels of *GHSR*, *SST* and *ZIC1* in cervical scrapes from women with CIN2/3 lesions with different duration of existence and known DNA copy number profile

To evaluate marker potential in relation to advanced CIN2/3, cervical scrapes from women with an underlying CIN2/3 lesion with a known duration of PHI, reflecting duration of lesion existence (19 early CIN2/3 lesions: <5 year PHI; 29 advanced CIN2/3 lesions: ≥ 5 year PHI) were analysed by multiplex qMSP (Figure 1; Validation). Methylation levels of *GHSR*, *SST* and *ZIC1* increased with duration of PHI. Methylation levels of *GHSR*, *SST* and *ZIC1* were significantly increased in cervical scrapes from women with advanced CIN2/3 compared to scrapes from women with early CIN2/3 ($p<0.0005$; Figure 3B-D), showing a 13.3-, 9.7- and 47.1-fold increase, respectively, over the reference (Table 2B), with methylation levels in scrapes from women with early CIN2/3 being comparable to the reference group. Using the above described logistic regression model, most cervical scrapes from women with advanced CIN2/3 scored methylation-positive by *GHSR* (20/29; 69%), *SST* (17/29; 58.6%) and *ZIC1* (20/29; 69%), whereas all (*SST* and *ZIC1*) or majority (*GHSR*; 18/19; 94.7%) of scrapes from women with early CIN2/3 were methylation-negative. A combination of *GHSR* and *ZIC1*, showing methylation-positivity for at least one of the markers, increased the detection of advanced CIN2/3 (22/29; 75.9%).

As *GHSR*, *SST* and *ZIC1* are all located on chromosome 3q, a region that is gained in 55% of cervical SCC cases(30), we additionally evaluated a potential association of methylation levels with gain of the gene locus. For this purpose, copy number data obtained during previous studies on the underlying advanced and early CIN2/3 lesions were used(6). Methylation levels of all 3 genes in the cervical scrapes were significantly higher in the presence of a 3q gain in the corresponding CIN2/3 lesions compared to the absence of a 3q gain ($p<0.05$; Figure 3E-G), which was also reflected by their fold changes over the reference group (i.e. 50.9-153 in presence of 3q gain versus 2.30-29.6 in absence of 3q gain; Table 2B). For comparison, previously obtained methylation levels of *FAM19A4* (chr. 3p14.1), *CADM1* (chr. 11q23.2), *MAL* (chr. 2q11.1) and *miR124-2* (chr. 8q12.3)(7,8) were not significantly different between scrapes from women with advanced CIN2/3 with and without a 3q gain (Supplementary Fig. S4).

Discussion

By unbiased genome-wide DNA methylation profiling and subsequent comprehensive step-wise verification and validation studies using *in vitro* and patient-derived samples representing the multistep process of hrHPV-induced carcinogenesis, we identified 3 new promising methylation markers (*GHSR*, *SST* and *ZIC1*) associated with 3q gain for the detection of cervical (pre)cancer. Methylation levels of *GHSR*, *SST* and *ZIC1* showed a significant increase with severity of disease, reaching high levels in advanced CIN2/3 and SCC, both when analysing tissue specimens as well as cervical scrapes. Within the group of women with CIN2/3 lesions, methylation levels of all 3 genes in cervical scrapes increased with duration of lesion existence ($p < 0.0005$) and were significantly higher in the presence of a 3q gain ($p < 0.05$) in the corresponding tissue biopsy. Logistic regression analysis of *GHSR*, *SST* and *ZIC1* in hrHPV-positive cervical scrapes for CIN3+ detection resulted in an AUC of 0.87, 0.86 and 0.89, respectively, showing a similar and good clinical performance of all 3 genes, supporting the potential of these methylation markers for the management of hrHPV-positive women. In contrast to cytology-based triage tests, such as p16/Ki-67 dual-stained cytology, methylation marker analysis has an improved reliability and it is applicable on both cervical scrapes and self-collected cervical-vaginal specimens. Methylation marker analysis may also be combined with HPV16/18 genotyping, and has been found complementary in detection of CIN2/3 and cancer. DNA methylation is especially sensitive for detection of cancer and advanced CIN2/3, whereas most early CIN2/3 are detected by HPV16/18 genotyping(31). Additionally, these 3 markers could potentially also be useful for early detection of other HPV-associated cancer types, such as a subset of head and neck cancers and other anogenital cancers.

The advantages of our study are the evaluation of large, independent series of different *in vitro* and patient-derived sample types and the use of a systematic discovery-verification-validation approach. MBD-Seq is an effective discovery methodology for biologically relevant methylation markers as it enriches for methylation targets characterized by methylation of multiple consecutive CpGs, which is a characteristic of gene silencing when affecting promoter regions, and an advantage over commonly used techniques detecting only methylation of single CpGs(32,33). Furthermore, using MBD-Seq we could also detect methylated HPV DNA by analysis of the non-aligned reads(24). In the current study, both *in vitro* and patient-obtained samples comprising all stages of cervical carcinogenesis were used, which allowed us to evaluate the succession of methylation events and the selection of methylation targets which are biologically relevant and associated with hrHPV-mediated carcinogenesis. Furthermore, by using both sample types during step-wise verification and validation, we could exclude candidates showing methylation very early or relatively late during carcinogenesis, and accordingly would result in unfavorable clinical specificity or sensitivity, respectively.

High-grade CIN lesions (CIN2/3) represent a heterogeneous stage of disease, only a subset of which may progress to cancer over a period of 15 to 30 years(2,3). Previous data have shown that the heterogeneity in duration of lesion existence is reflected by their molecular profile, with significant increased copy number alterations and methylation levels in advanced CIN2/3 lesions compared to early CIN2/3 lesions(6–8). In line with previous findings(7,8), present data show that the methylation levels of *GHSR*, *SST* and *ZIC1* are significantly increased in cervical scrapes from women with advanced CIN2/3 lesions compared to scrapes from women with early CIN2/3 lesions, which is also reflected in their frequency of methylation-positivity. Of note and in addition to previous findings, the current study shows that methylation of all 3 genes, which are located at chromosome 3q, i.e., *GHSR* (3q26), *SST* (3q28) and *ZIC1* (3q24), is related to the presence of a 3q gain. Gain at 3q, which is the most frequently gained region in cervical cancer(6,30), as well as amplification of the human telomerase gene (*TERC*; 3q26), have been suggested as a marker for progression to cancer(34,35). The observation that methylation levels of genes at other chromosomal locations were not significantly increased in cervical scrapes from women with advanced CIN2/3 with a 3q gain, suggests

that DNA methylation of *GHSR*, *SST* and *ZIC1* may be biologically important to compensate for increased gene dosage during cervical carcinogenesis.

At present, there are only a few studies which investigated a relation between DNA methylation and DNA copy number profiles in cervical cancer, and those were mostly focussed on allelic losses, such as for chromosomes 11q and 3p(20). In lung cancer, a correlation between DNA hypermethylation and gene amplification has been described for *SHOX2*, which is also located on chromosome 3q(36). Another study described a significant association between DNA methylation of the *HOXA* locus and a gain of chromosome 7 in glioblastoma(37). MBD-Seq on cervical specimens by Boers et al., reported 15 candidate methylation targets(38), part of which were also located on known copy number gains, i.e. *ST6GALNAC5* (1p), *LHX8* (1p), *CDH6* (5p) and *IRX1* (5p). Yet, it is unclear whether DNA hypermethylation is a response reaction to copy number alterations or a cause of copy number alterations due to destabilization of the genome. Further studies are warranted to investigate the co-evolution of DNA methylation and copy number alterations, in particular copy number gains, using integrated copy number and methylation analysis. In the current study, we were unfortunately unable to acquire information on copy numbers from our sequencing data due to varying read-counts between samples using MBD-Seq analysis.

To the best of our knowledge, methylation of *GHSR* and *ZIC1* has not been described before in cervical cancer, whereas *SST* was identified previously by Ongenaert et al(39). *GHSR* is the growth hormone secretagogue receptor, or ghrelin receptor, which regulates growth hormone secretion and functions in energy homeostasis(40). Methylation of *GHSR* has been described before in lung cancer, breast cancer, glioblastoma and B cell chronic lymphocytic leukaemia(41). *ZIC1*, zinc finger of the cerebellum family member 1, functions as a transcription factor and has an important role in central nervous system, muscle and skeletal development(42). Methylation of *ZIC1* has been described previously for other tumour types, such as thyroid, ovarian, colon and gastric cancer(43–46). *SST* or somatostatin functions as an inhibitor with anti-secretory, anti-proliferative and anti-angiogenic effects(47) and is, in addition to cervical cancer, also found to be methylated in gastric, esophageal, colon and head and neck cancer(48–51). The biological function and expression regulation of these 3 genes in HPV-transformed cells remains to be determined. Based on our in-house and publically available expression profiling data(52) of cervical tissue specimens and cell lines no firm conclusions can be drawn on their expression regulation as expression levels were relatively low in control samples. Likewise, differential protein expression was not detected in the Human Protein Atlas (<http://www.proteinatlas.org/>), since both cervical cancer tissue and normal tissue showed low or non-detectable protein levels.

In conclusion, using an unbiased genome-wide DNA methylation profiling approach and subsequent step-wise verification and validation studies on hrHPV-transformed cell lines and clinical cervical specimens, we identified 3 methylation markers, *GHSR*, *SST* and *ZIC1*, associated with 3q gain, for detection of cervical (pre)cancer. Our findings warrant further clinical validation of *GHSR*, *SST* and *ZIC1* in a prospective study to verify their potential as biomarkers for the management of hrHPV-positive women.

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Conflict of interest:

P. Snijders, C. Meijer, R. Steenbergen and D. Heideman have minority stake in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam, which owns patents related to this work. P. Snijders has been on the speaker's bureau of Roche, Abbott, Gen-Probe, Qiagen and Seegene. He is consultant for Crucell Holland B.V. C. Meijer has participated in the sponsored speaker's bureau of Merck, GSK, Qiagen, Menarini, Seegene, and Roche, and served occasionally on the scientific advisory board of GSK, Qiagen, Merck, and Roche. C. Meijer has occasionally been a consultant for Qiagen and Gentcel and was a minority shareholder of Diassay B.V till March 2016 and of Delphi Biosciences till 2014. D. Heideman serves occasionally on the scientific advisory boards of Amgen and Pfizer. W. Van Criekinge has a relationship with MDxHealth, Irvine, CA, USA. G. Wisman holds patents related to the work. All other authors have no conflict of interest to declare.

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Tables

Table 1. The selected 20 novel candidate methylation markers and the summarized results of the verification analyses.

Gene	Verification by targeted bisulphite NGS		Verification by MSP	
	Cell lines	Tissues	Cell lines	Tissues
<i>ANKRD2</i>	-	-		
<i>CLDN18</i>	-	-		
<i>GALR1</i>	+	+	+	
<i>GHSR</i>	+	+	+	+
<i>GRK7</i>	-	-		
<i>HIPK4</i>	-	-		
<i>IRX1</i>	+	+	+	
<i>MYCL2</i>	na	na		
<i>NEUROG3</i>	+	+	+	
<i>PDE4DIP</i>	+	na		
<i>POU4F3</i>	+	+	+	+
<i>PROX1</i>	+	+	+	+
<i>RXFP3</i>	na	+		
<i>SIM2</i>	+	+	+	
<i>SLC45A2</i>	-	-		
<i>SOX14</i>	na	na		
<i>SST</i>	+	+	+	+
<i>TBX19</i>	-	-		
<i>TFDP3</i>	-	-		
<i>ZIC1</i>	+	+	+	+

+ verified differential methylation

- not verified differential methylation (i.e., detection of methylation in controls)

Highlighted in bold: 3 methylation markers selected for multiplex quantitative MSP

NGS: next generation sequencing; MSP: methylation-specific PCR; na: not assessable

Table 2. Methylation level fold changes of *GHSR*, *SST* and *ZIC1* per lesion category in cervical scrapes.

A. Category	Ct ratio <i>GHSR</i>		CT ratio <i>SST</i>		CT ratio <i>ZIC1</i>		Fold changes over reference		
	Median	Range	Median	Range	Median	Range	<i>GHSR</i>	<i>SST</i>	<i>ZIC1</i>
hrHPV+ control	0.11	0-38.63	0.06	0-19.09	0.03	0-40.53	1 (reference)	1 (reference)	1 (reference)
CIN2	0.28	0-8.33	0.06	0-3.19	0.26	0-7.65	2.50	1.09	10.08
CIN3	1.57	0.01-51.43	0.65	0.01-27.56	1.51	0.01-48.42	14.07	11.05	58.66
SCC	29.78	0.85-134.04	9.13	0.06-69.81	23.78	0.15-111.41	266.06	154.91	921.12
B. Category	Ct ratio <i>GHSR</i>		CT ratio <i>SST</i>		CT ratio <i>ZIC1</i>		Fold changes over reference		
	Median	Range	Median	Range	Median	Range	<i>GHSR</i>	<i>SST</i>	<i>ZIC1</i>
Early CIN2/3	0.14	0.04-0.78	0.05	0-0.19	0.02	0-0.16	1.22	0.81	0.74
Advanced CIN2/3	1.49	0.07-26.58	0.57	0-12.79	1.22	0-26.24	13.33	9.65	47.09
Without 3q gain	0.95	0.07-14.07	0.13	0.01-5.26	0.76	0-10.05	8.47	2.28	29.60
With 3q gain	8.56	0.11-26.58	3.00	0-12.79	3.95	0.04-26.24	76.42	50.90	153.04

hrHPV+: high-risk human papillomavirus-positive; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma

Table 3. AUC, sensitivity and specificity of *GHSR*, *SST* and *ZIC1* for endpoint CIN3+ in cervical scrapes.

Methylation marker	AUC	Sensitivity (%)	Specificity (%)	p value^a
<i>GHSR</i>	0.87	76.5	83.5	0.000
<i>SST</i>	0.86	81.4	79.4	0.000
<i>ZIC1</i>	0.89	86.3	80.4	0.000

^a p value of the corresponding methylation marker in the univariable logistic regression model.

AUC: area under the curve

Figures

Figure 1. Experimental setup of the study. MBD-Seq: methyl binding domain; NGS: next generation sequencing; MSP: methylation-specific PCR; qMSP: quantitative MSP; (1) a cross-sectional series of hrHPV-positive cervical scrapes; (2) a series of hrHPV-positive cervical scrapes from women with an underlying CIN2/3 lesion and with a known duration of preceding hrHPV infection.

Figure 2. Validation of *GHSR*, *SST* and *ZIC1* by qMSP in *in vitro* model and in cervical tissue specimens. Methylation levels of *GHSR* (A), *SST* (B) and *ZIC1* (C) in *in vitro* model represented by the log2-transformed Cq ratios (y-axis) in the different groups of cell lines (x-axis). Methylation levels of *GHSR* (D), *SST* (E) and *ZIC1* (F) in cervical tissue specimens represented by the log2-transformed Cq ratios (y-axis) in different histology groups (x-axis). HFK: human foreskin keratinocytes; middle immortal: FK16A/B and FK18A/B p30-p70; late immortal: FK16A/B and FK18A/B >p70; CCL: cancer cell lines; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma.

* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; NS: not significant.

Figure 3. Validation of *GHSR*, *SST* and *ZIC1* in hrHPV-positive cervical scrapes and their relation to duration of lesions existence and DNA copy number aberrations. Receiver operating characteristics (ROC) curves of *GHSR*, *SST* and *ZIC1* for CIN3+ detection in hrHPV-positive cervical scrapes (**A**). Methylation levels of *GHSR* (**B**), *SST* (**C**) and *ZIC1* (**D**) in hrHPV-positive cervical scrapes from women with CIN2/3 with a known duration of previous hrHPV infection (PHI), as a proxy for the duration of lesion existence; Early CIN2/3 corresponds to <5 year PHI; Advanced CIN2/3 corresponds to ≥ 5 year PHI. Methylation levels of *GHSR* (**E**), *SST* (**F**) and *ZIC1* (**G**) in hrHPV-positive cervical scrapes in relation to the presence of a chromosomal gain of respective chromosomal region (i.e., chr. 3q26, chr. 3q28 and chr. 3q24, respectively) in the underlying advanced CIN2/3 lesions. The x-axis displays the different groups (i.e., with (+3q gain) or without (- 3q gain) 3q gain) and the y-axis displays the log2-transformed Cq ratios representing methylation levels. CIN: cervical intraepithelial neoplasia; * $p < 0.05$; *** $p < 0.0005$.