Genome-wide DNA Methylation Profiling Reveals Methylation Markers Associated with 3q Gain for Detection of Cervical Precancer and Cancer

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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 tumor suppressor gene promoters are valuable. These markers ideally identify hrHPV-positive women with precancer (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 bisulfite 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 and 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.

Conclusions: By unbiased genome-wide DNA methylation profiling and comprehensive stepwise 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. *Clin Cancer Res; 23(14); 3813–22. ©2017 AACR.*

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 tumor 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

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Implementation of primary high-risk HPV (hrHPV) DNA testing in cervical screening programs in the coming years will result in better protection against cervical (pre)cancer. However, its specificity for clinically meaningful disease is lower compared with 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 the detection of clinically relevant precursor lesions. These findings could greatly advance the management of hrHPV-positive women.

domain–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 morphologic 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. In addition, stepwise verification and validation studies were performed using multiplex-targeted bisulfite 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 analyzed 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, repre-

senting the preimmortal 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 histologic 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 bisulfite NGS was performed on a subset of hrHPV-transformed cell lines and frozen tissue specimens used for discovery with sufficient material left, that is, 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 previously (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 hybridized to 105K arrays (Agilent Technologies; ref. 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 (ATCC) were cultured according to specifications (20). Cell lines were recently authenticated using the PowerPlex 16 System (Promega) 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 gynecologic outpatient populations. This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center and University Medical Center Groningen (Groningen, the Netherlands).

DNA isolation, bisulfite 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) using manufacturer's protocol. DNA from cervical scrapes was isolated using the Nucleo-Mag 96 Tissue kit (Macherey-Nagel) and a Microlab Star robotic system (Hamilton) according to the manufacturer's protocol. For multiplex-targeted bisulfite NGS, MSP, and qMSP analyses, DNA was bisulfite-converted using the EZ DNA Methylation kit (Zymo Research). HrHPV DNA detection was performed by GP5^{+}/6^{+} PCR enzyme immunoassay or HPV-Risk assay (Self-screen BV; refs. 22, 23).

MBD-Seq

Genome-wide DNA methylation profiling was performed by MBD-Seq, using the MethylCap kit v2 (Diagenode) with 500 ng input DNA combined with Illumina Genome Analyzer IIx 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 (−1,000 bp to +500 bp relative to the transcription start site). The read counts of all regions in cervical cancer (SiHa, SCC) were compared with the read counts in controls (HFK, CIN1). These regions were ranked on the basis of the difference in read counts to select the hypermethylated regions. Next, we analyzed 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 bisulfite NGS

For multiplex-targeted bisulfite 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). 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). 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) to increase the diversity and was loaded on a MiSeq Personal Sequencer (Illumina) using 5 pmol/L. A MiSeq Reagent Kit v2 (300 cycles; Illumina) was used in a paired-end 150 run according to manufacturer's protocol. All bisulfite 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 bisulfite-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 bisulfite-converted DNA as input material (28). MSP primers were designed to specifically amplify methylated bisulfite-treated DNA. *In vitro* methylated DNA (IVD) was used as positive control and unmodified SiHa DNA and H$_2$O served as negative controls. The housekeeping gene β-actin (*ACTB*) verified sufficient DNA quality and successful bisulfite conversion of all samples.

Multiplex qMSP for 3 targets and *ACTB* was designed as described previously (29), and performed using 50 ng of bisulfite-converted DNA and 200–300 nmol/L of each primer and fluorescent dye-labeled probe, on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The methylation values of the targets were normalized 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 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 Fig. 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 (Fig. 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).
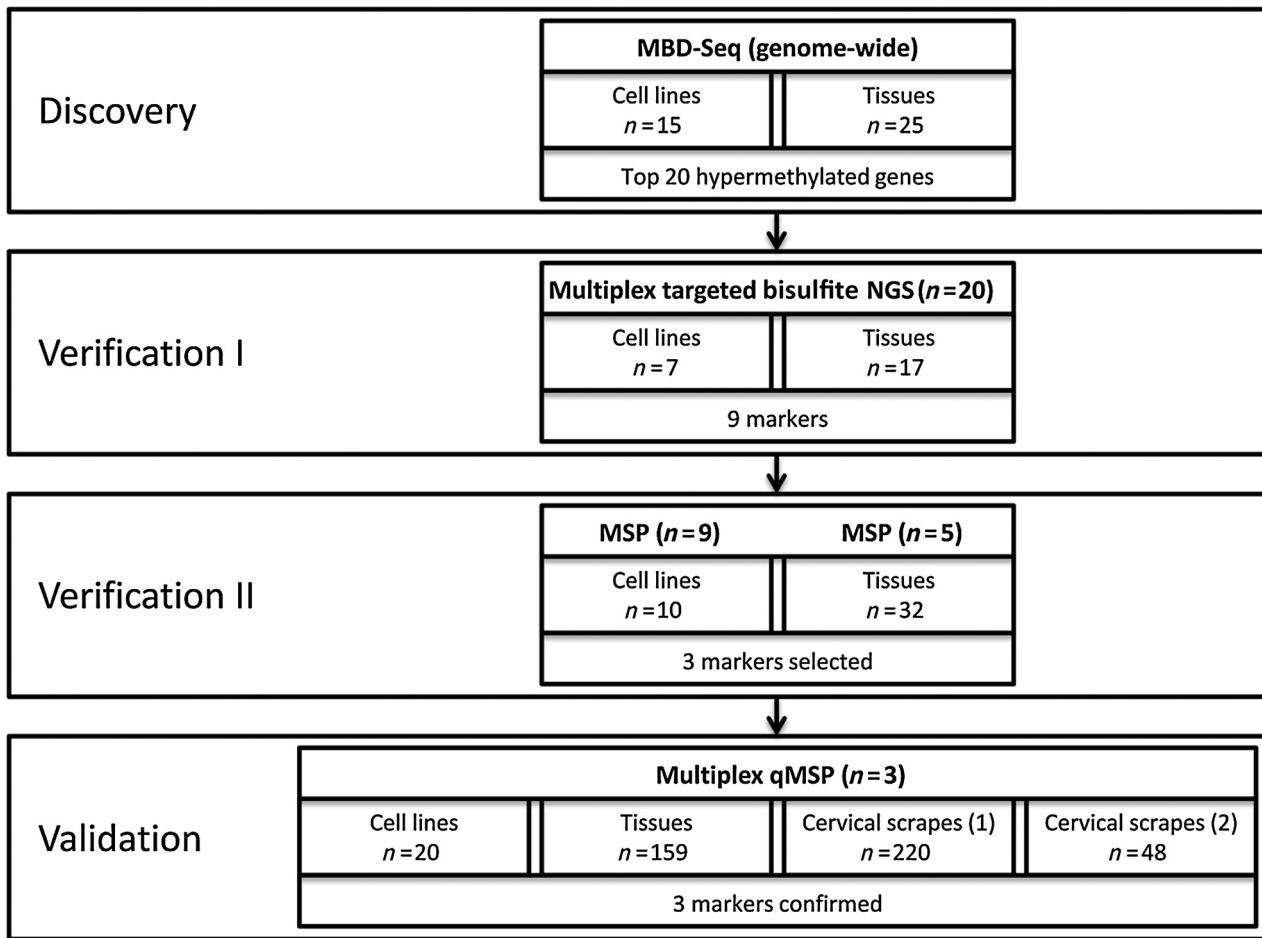
Figure 1.
Experimental setup of the study. qMSP, quantitative MSP; (1) a cross-sectional series of hrHPV-positive cervical scrapes; (2) a series of hrHPV-positive cervical scrapes from women with an underlying CIN2/3 lesion and with a known duration of preceding hrHPV infection.

Verification analyses and selection of candidate methylation markers

Verification of the top 20 candidates by multiplex-targeted bisulfite NGS was performed on a subset of samples used in the MBD-Seq screen (Fig. 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 with 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 (Fig. 1; Verification II; Table 1; Supplementary Fig. S1A). *SIM2* was clearly methylation-positive in 1 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 (Fig. 1; Verification II) confirming increased methylation frequency with increasing disease severity (Table 1; Supplementary Figs. S1B and S2). Two genes, *POU4F3* and *PROX1*, were methylation-negative in 1 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

On the basis of the verification findings, *GHSR*, *SST*, and *ZIC1*, were further analyzed by multiplex qMSP on cell lines and independent large series of cervical tissue specimens (Fig. 1; Validation). No *SST* and *ZIC1* methylation was detected in any of the HFKs, whereas *GHSR* showed very low levels of methylation in 3 of 5 HFKs. Methylation of all 3 genes significantly increased with passaging of hrHPV-immortalized keratinocytes and was highest in cervical cancer cell lines ($P < 0.05$; Fig. 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 with normal cervical tissues ($P < 0.005$), in CIN3 compared with CIN2 ($P < 0.05$), as well as in SCCs compared with CIN3 ($P < 0.0005$; Fig. 2D–F).

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

Gene	Verification by targeted bisulfite NGS		Verification by MSP	
	Cell lines	Tissues	Cell lines	Tissues
ANKRD2	−	−		
CLDN18	−	−		
GALR1	+	+	+	
GHSR	+	+	+	+
GRK7	−	−		
HIPK4	−	−		
IRX1	+	+	+	
MYCL2	na	na		
NEUROG3	+	+	+	
PDE4DIP	+	na		
POU4F3	+	+	+	+
PROX1	+	+	+	+
RXFP3	na	+		
SIM2	+	+	+	
SLC45A2	−	−		
SOX14	na	na		
SST	+	+	+	+
TBX19	−	−		
TFDP3	−	−		
ZIC1	+	+	+	+

NOTE: +, 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.

Abbreviation: na, not assessable.

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 (Fig. 1; Validation). For all 3 genes, fold changes in methylation levels compared with 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 with 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 (Fig. 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 durations of lesion existence (19 early CIN2/3 lesions: <5-year PHI; 29 advanced CIN2/3 lesions: ≥5-year PHI), were analyzed by multiplex qMSP (Fig. 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 with scrapes from women with early CIN2/3 ($P < 0.0005$; Fig. 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 with 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 with the absence of a 3q gain ($P < 0.05$; Fig. 3E–G), which was also reflected by their fold changes over the reference group (i.e., 50.9–153.0 in presence of 3q gain versus 2.3–29.6 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; refs. 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 stepwise 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 analyzing 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). In
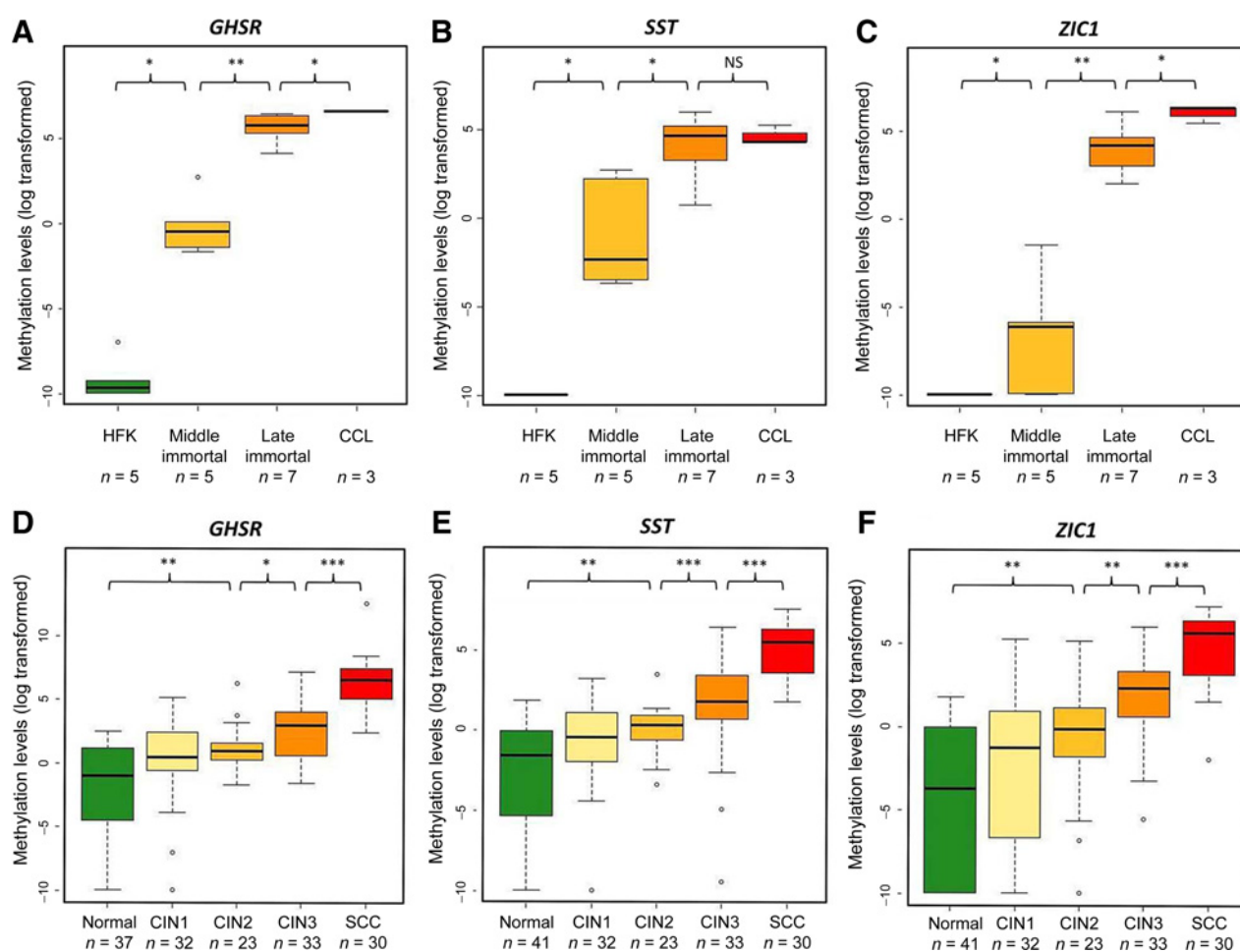
Figure 2.
Validation of *GHSR*, *SST*, and *ZIC1* by qMSP in *in vitro* model and in cervical tissue specimens. Methylation levels of *GHSR* (**A**), *SST* (**B**), and *ZIC1* (**C**) in *in vitro* model represented by the log2-transformed Cq ratios (*y*-axis) in the different groups of cell lines (*x*-axis). 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.

addition, 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

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

A.	Ct Ratio *GHSR*		CT ratio *SST*		CT ratio *ZIC1*		Fold changes over reference		
Category	Median	Range	Median	Range	Median	Range	*GHSR*	*SST*	*ZIC1*
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.	Ct ratio *GHSR*		CT ratio *SST*		CT ratio *ZIC1*		Fold changes over reference		
Category	Median	Range	Median	Range	Median	Range	*GHSR*	*SST*	*ZIC1*
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
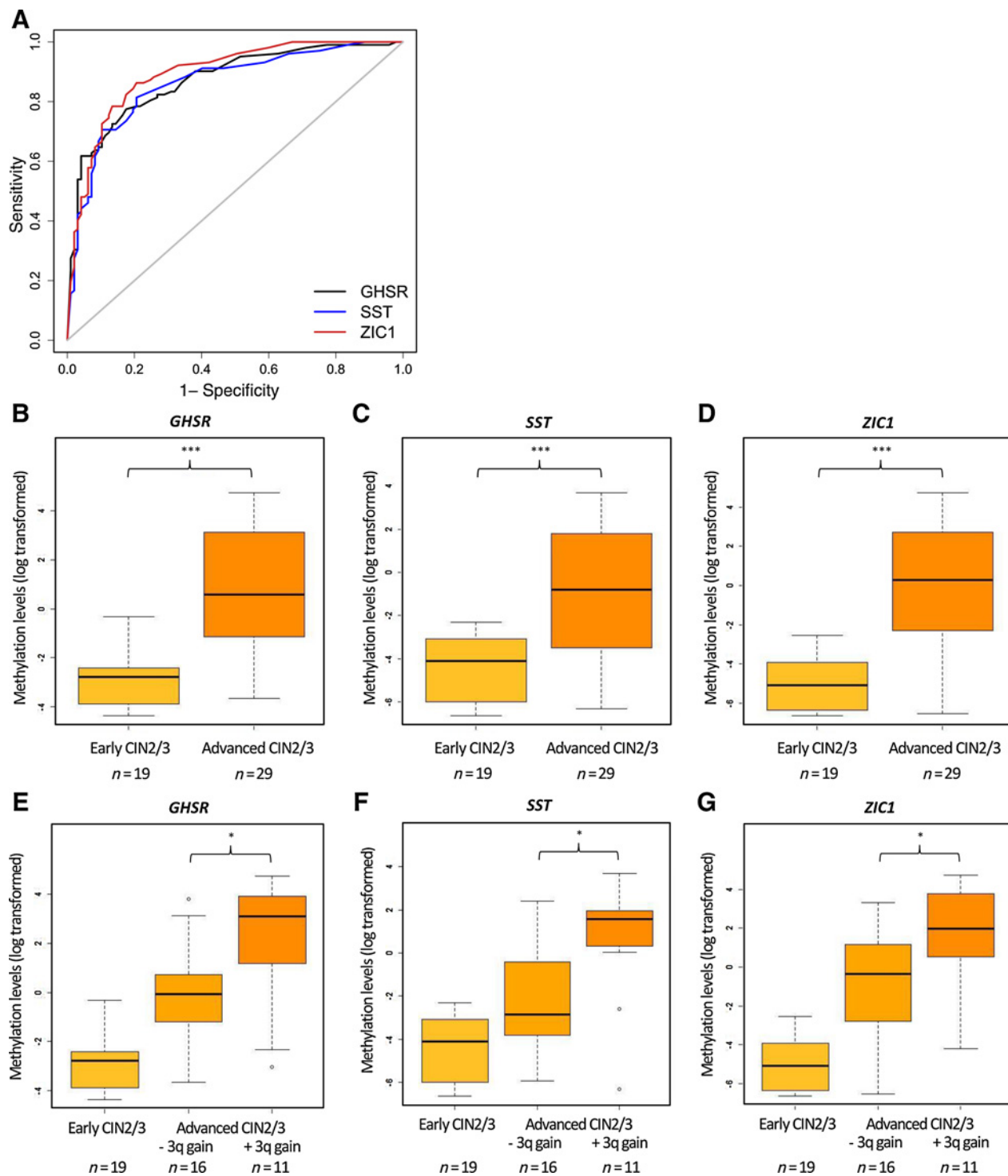
Figure 3.
Validation of *GHSR, SST*, and *ZIC1* in hrHPV-positive cervical scrapes and their relation to duration of lesion existence and DNA copy number aberrations. ROC curves of *GHSR, SST*, and *ZIC1* for CIN3+ detection in hrHPV-positive cervical scrapes (**A**). Methylation levels of *GHSR* (**B**), *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; *, *P* < 0.05; ***, *P* < 0.0005.

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

Methylation marker	AUC	Sensitivity (%)	Specificity (%)	P^a
GHSR	0.87	76.5	83.5	0.000
SST	0.86	81.4	79.4	0.000
ZIC1	0.89	86.3	80.4	0.000

[a]P value of the corresponding methylation marker in the univariable logistic regression model.

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 nonaligned 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 stepwise 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 with early CIN2/3 lesions (6–8). In line with previous findings (7, 8), current 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 with 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, that is, *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 focused 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 and colleagues, reported 15 candidate methylation targets (38), part of which were also located on known copy number gains, that is, *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 coevolution 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 and colleagues (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 leukemia (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 tumor types, such as thyroid, ovarian, colon, and gastric cancer (43–46). *SST* or somatostatin functions as an inhibitor with anti-secretory, antiproliferative, and antiangiogenic 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. On the basis of our in-house and publicly 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/), as 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 stepwise 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.

Disclosure of Potential Conflicts of Interest

P.J.F. Snijders has received speakers bureau honoraria from Roche, Qiagen, Gen-Probe, Abbott, and Seegene and has provided expert testimony for Self-screen BV. W. Van Criekinge is a chief scientific officer at MDxHealth and is a consultant/advisory board member for MDxealth. G.B.A. Wisman has ownership interest (including patents) in patents. C.J.L.M. Meijer is a chief executive officer at Self-Screen BV (off-spin from VUMC Amsterdam), has received speakers bureau honoraria from GSK, SP/MSD, Roche, Qiagen, Menarini, and Seegene, has ownership interest (including patents) in Self-screen BV (Self-screen holds patents on CADM1, miR124-2, Mal, and FAM19A4), Delphi Biosciences (till March 2014), Diassay BV (till March 2016), and is a consultant/advisory board member for Qiagen and Genticel. D.A.M. Heideman has ownership interest (including patents) in a minority stake in Self-screen BV (a spin-off company of the VU University Medical Center Amsterdam, which owns patents related to this work) and is a consultant/advisory board member for Pfizer and Amgen. R.D.M. Steenbergen has ownership interest (including patents) in Self-screen BV. No potential conflicts of interest were disclosed by the other authors.

References

1. Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12–9.
2. Steenbergen RDM, Snijders PJF, Heideman DAM, Meijer CJLM. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer 2014;14:395–405.
3. McCredie MRE, Sharples KJ, Paul C, Baranyai J, Medley G, Jones RW, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol 2008;9:425–34.
4. Vink MA, Bogaards JA, Van Kemenade FJ, De Melker HE, Meijer CJLM, Berkhof J. Clinical progression of high-grade cervical intraepithelial neoplasia: Estimating the time to preclinical cervical cancer from doubly censored national registry data. Am J Epidemiol 2013;178:1161–9.
5. Trimble CL, Piantadosi S, Gravitt P, Ronnett B, Pizer E, Elko A, et al. Spontaneous regression of high-grade cervical dysplasia: effects of human papillomavirus type and HLA phenotype. Clin Cancer Res 2005;11:4717–23.
6. Bierkens M, Wilting SM, van Wieringen WN, van Kemenade FJ, Bleeker MCG, Jordanova ES, et al. Chromosomal profiles of high-grade cervical intraepithelial neoplasia relate to duration of preceding high-risk human papillomavirus infection. Int J Cancer 2012;131:579–85.
7. De Strooper LMA, Meijer CJLM, Berkhof J, Hesselink AT, Snijders PJF, Steenbergen RDM, et al. Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. Cancer Prev Res 2014;7:1251–7.
8. Bierkens M, Hesselink AT, Meijer CJLM, Heideman DAM, Wisman GBA, Van Der Zee AGJ, et al. CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. Int J Cancer 2013;133:1293–300.
9. Eijsink JJH, Lendvai Á, Deregowski V, Klip HG, Verpooten G, Dehaspe L, et al. A four-gene methylation marker panel as triage test in high-risk human papillomavirus positive patients. Int J Cancer 2012;130:1861–9.
10. Lorincz AT, Brentnall AR, Scibior-bentkowska D, Reuter C, Banwait R, Cadman L, et al. Validation of a DNA methylation HPV triage classifier in a screening sample. Int J Cancer 2016;138:2745–51.
11. Hesselink AT, Heideman DAM, Steenbergen RDM, Coupé VMH, Overmeer RM, Rijkaart D, et al. Combined promoter methylation analysis of CADM1 and MAL: An objective triage tool for high-risk human papillomavirus DNA-positive women. Clin Cancer Res 2011;17:2459–65.
12. Verhoef VMJ, Bosgraaf RP, Van Kemenade FJ, Rozendaal L, Heideman DAM, Hesselink AT, et al. Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): A randomised controlled non-inferiority trial. Lancet Oncol 2014;15:315–22.
13. Snijders PJF, Steenbergen RDM, Heideman DAM, Meijer CJLM. HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol 2006;208:152–64.
14. Henken FE, Wilting SM, Overmeer RM, van Rietschoten JGI, Nygren AOH, Errami A, et al. Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. Br J Cancer 2007;97:1457–64.
15. Schütze DM, Kooter JM, Wilting SM, Meijer CJ, Quint W, Snijders PJ, et al. Longitudinal assessment of DNA methylation changes during HPVE6E7-induced immortalization of primary keratinocytes. Epigenetics 2015;10:1–9.
16. Schutze DM, Krijgsman O, Snijders PJF, Ylstra B, Weischenfeldt J, Mardin BR, et al. Immortalization capacity of HPV types is inversely related to chromosomal instability. Oncotarget 2016;7:37608–21.
17. Steenbergen RDM, Ongenaert M, Snellenberg S, Trooskens G, Van Der Meide WF, Pandey D, et al. Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. J Pathol 2013;231:53–62.
18. Bulkmans NWJ, Berkhof J, Rozendaal L, van Kemenade FJ, Boeke a JP, Bulk S, et al. Human papillomavirus DNA testing for the detection of cervical intraepithelial neoplasia grade 3 and cancer: 5-year follow-up of a randomised controlled implementation trial. Lancet 2007;370:1764–72.
19. Steenbergen RD, Walboomers JM, Meijer CJLM, van der Raaij-Helmer EM, Parker JN, Chow LT, et al. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. Oncogene 1996;13:1249–57.
20. Steenbergen RDM, Kramer D, Braakhuis BJM, Stern PL, Verheijen RHM, Meijer CJLM, et al. TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. J Natl Cancer Inst 2004;96:294–305.
21. van Zeeburg HJT, Snijders PJF, Pals G, Hermsen M a J a, Rooimans M a, Bagby G, et al. Generation and molecular characterization of head and neck squamous cell lines of fanconi anemia patients. Cancer Res 2005;65:1271–6.
22. Jacobs M V, Snijders PJ, Brule AJC, Van Den, Helmerhorst TJ, Meijer CJLM, Walboomers JM. A general primer GP5+/GP6+-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. J Clin Microbiol 1997;35:791–5.
23. Hesselink AT, Berkhof J, van der Salm ML, van Splunter AP, Geelen TH, van Kemenade FJ, et al. Clinical validation of the HPV-risk assay, a novel real-time PCR assay for detection of high-risk human papillomavirus DNA by targeting the E7 region. J Clin Microbiol 2014;52:890–6.
24. Mensaert K, Van Criekinge W, Thas O, Schuuring E, Steenbergen RDM, Wisman GBA, et al. Mining for viral fragments in methylation enriched sequencing data. Front Genet 2015;6:16.
25. De Meyer T, Mampaey E, Vlemmix M, Denil S, Trooskens G, Renard J-P, et al. Quality evaluation of methyl binding domain based kits for enrichment DNA-methylation sequencing. PLoS One 2013;8:e59068.
26. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.

27. Krueger F, Andrews SR. Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 2011;27:1571–2.
28. Overmeer RM, Henken FE, Snijders PJF, Claassen-Kramer D, Berkhof J, Helmerhorst TJM, et al. Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. J Pathol 2008;215:388–97.
29. Snellenberg S, De Strooper LMA, Hesselink AT, Meijer CJLM, Snijders PJF, Heideman DAM, et al. Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. BMC Cancer 2012;12:551.
30. Thomas LK, Bermego JL, Vinokurova S, Jensen K, Bierkens M, Steenbergen RDM, et al. Chromosomal gains and losses in human papillomavirus-associated neoplasia of the lower genital tract – A systematic review and meta-analysis. Eur J Cancer 2014;50:85–98.
31. Luttmer R, De Strooper LMA, Steenbergen RDM, Berkhof J, Snijders PJF, Heideman DA, et al. Management of high-risk HPV-positive women for detection of cervical (pre)cancer. Expert Rev Mol Diagn 2016;16:961–74.
32. De Meyer T, Bady P, Trooskens G, Kurscheid S, Bloch J, Kros JM, et al. Genome-wide DNA methylation detection by MethylCap-seq and Infinium HumanMethylation450 BeadChips: an independent large-scale comparison. Sci Rep 2015;5:15375.
33. Bock C, Tomazou EM, Brinkman AB, Müller F, Simmer F, Gu H, et al. Quantitative comparison of genome-wide DNA methylation mapping technologies. Nat Biotechnol 2010;28:1106–14.
34. Rodolakis A, Biliatis I, Symiakaki H, Kershnar E, Kilpatrick MW, Haidopoulos D, et al. Role of chromosome 3q26 gain in predicting progression of cervical dysplasia. Int J Gynecol Cancer 2012;22: 742–7.
35. Heselmeyer-Haddad K, Sommerfeld K, White NM, Chaudhri N, Morrison LE, Palanisamy N, et al. Genomic amplification of the human telomerase gene (TERC) in pap smears predicts the development of cervical cancer. Am J Pathol 2005;166:1229–38.
36. Schneider KU, Dietrich D, Fleischhacker M, Leschber G, Merk J, Schäper F, et al. Correlation of SHOX2 gene amplification and DNA methylation in lung cancer tumors. BMC Cancer 2011;11:102.
37. Kurscheid S, Bady P, Sciuscio D, Samarzija I, Shay T, Vassallo I, et al. Chromosome 7 gain and DNA hypermethylation at the HOXA10 locus are associated with expression of a stem cell related HOX-signature in glioblastoma. Genome Biol 2015;16:16.
38. Boers A, Wang R, van Leeuwen RW, Klip HG, Bock GH De, Hollema H, et al. Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. Clin Epigenetics 2016; 8:29.
39. Ongenaert M, Wisman GBA, Volders HH, Koning AJ, van der Zee AGJ, Van Criekinge W, et al. Discovery of DNA methylation markers in cervical cancer using relaxation ranking. BMC Med Genomics 2008;1:57.
40. Shi L, Du X, Jiang H, Xie J. Ghrelin and neurodegenerative disorders—a review. Mol Neurobiol 2017;54:1144–55.
41. Moskalev EA, Jandaghi P, Fallah M, Manoochehri M, Botla SK, Kolychev O V, et al. GHSR DNA hypermethylation is a common epigenetic alteration of high diagnostic value in a broad spectrum of cancers. Oncotarget 2014; 6:4418–27.
42. Grinberg I, Millen KJ. The ZIC gene family in development and disease. Clin Genet 2005;67:290–6.
43. Qiang W, Zhao Y, Yang Q, Liu W, Guan H, Lv S, et al. ZIC1 is a putative tumor suppressor in thyroid cancer by modulating major signaling pathways and transcription factor FOXO3a. J Clin Endocrinol Metab 2014;99:1163–72.
44. Huang RL, Gu F, Kirma NB, Ruan J, Chen CL, Wang HC, et al. Comprehensive methylome analysis of ovarian tumors reveals hedgehog signaling pathway regulators as prognostic DNA methylation biomarkers. Epigenetics 2013;8:624–34.
45. Gan L, Chen S, Zhong J, Wang X, Lam EKY, Liu X, et al. ZIC1 is downregulated through promoter hypermethylation, and functions as a tumor suppressor gene in colorectal cancer. PLoS One 2011;6:e16916.
46. Wang LJ, Jin HC, Wang X, Lam EKY, Bin Zhang J, Liu X, et al. ZIC1 is downregulated through promoter hypermethylation in gastric cancer. Biochem Biophys Res Commun 2009;379:959–63.
47. Rai U, Thrimawithana T, Valery C, Young S. Therapeutic uses of somatostatin and its analogues: current view and potential applications. Pharmacol Ther 2015;152:98–110.
48. Jackson K, Soutto M, Peng D, Hu T, Marshal D, El-Rifai W. Epigenetic silencing of somatostatin in gastric cancer. Dig Dis Sci 2011;56:125–30.
49. Jin Z, Mori Y, Hamilton JP, Olaru A, Sato F, Yang J, et al. Hypermethylation of the somatostatin promoter is a common, early event in human esophageal carcinogenesis. Cancer 2008;112:43–9.
50. Mori Y, Cai K, Cheng Y, Wang S, Paun B, Hamilton JP, et al. A genome-wide search identifies epigenetic silencing of somatostatin, tachykinin-1, and 5 other genes in colon cancer. Gastroenterology 2006;131:797–808.
51. Misawa K, Misawa Y, Kondo H, Mochizuki D, Imai A, Fukushima H, et al. Aberrant methylation inactivates somatostatin and somatostatin receptor type 1 in head and neck squamous cell carcinoma. PLoS One 2015;10: e0118588.
52. den Boon JA, Pyeon D, Wang SS, Horswill M, Schiffman M, Sherman M, et al. Molecular transitions from papillomavirus infection to cervical precancer and cancer: Role of stromal estrogen receptor signaling. Proc Natl Acad Sci U S A 2015;112:E3255–64.