Genome-wide DNA methylation assay reveals novel candidate biomarker genes in cervical cancer

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Abbreviations: CGI, CpG island; CIN3, cervical intraepithelial neoplasia grade; HPV, human papilloma virus; PCA, principal component analysis; SCC, squamous cell carcinoma; T-DMR, tissue-specific differentially methylated regions

Conflict of interest

The authors have declared that no competing interests exist.

Abstract

The oncogenic human papilloma viruses (HPVs) are associated with precancerous cervical lesions and development of cervical cancer. The DNA methylation signatures of host genome in normal, precancerous and cervical cancer tissue may indicate tissue-specific perturbation in carcinogenesis. The aim of this study was to identify new candidate genes that are differentially methylated in squamous cell carcinoma compared to the DNA samples from cervical intraepithelial neoplasia grade 3 (CIN3) and normal cervical scrapes. The Illumina Infinium Human Methylation 450 K BeadChip method identifies genome-wide DNA methylation changes in CpG islands, CpG shores and shelves. Our findings showed an extensive differential methylation signature in cervical cancer compared to the CIN3 or normal cervical tissues. The identified candidate biomarker genes for cervical cancer represent several types of mechanisms in the cellular machinery that are epigenetically deregulated by hypermethylation, such as membrane receptors, intracellular signaling and gene transcription. The results also confirm the extensive hypomethylation in cancer cells of genes in the immune system. These insights into the functional role of DNA methylome alterations in cervical cancer could be clinically applicable in diagnostics and prognostics, and may guide the development of new epigenetic therapies.

Introduction

Cervical cancer is the third most frequent cancer among women world-wide.[1] The causative agent is a persistent infection with one of fifteen oncogenic human papilloma viruses (HPVs) that leads to cervical precancerous lesions which may progress to cancer.[2] There are more than 100 identified HPV genotypes, 40 of them are considered to infect the genital tract and the types 16, 18 and 45 are found to be the most common in cervical cancer.[3]

The majority of the viral load is cleared by the cell-mediated immunity, but if it is not cleared the persistent infection affects the host cell apoptosis and cell cycle control, cell adhesion and DNA repair mechanisms.[4] It may also activate inflammatory pathways suggested to be critical for the tumor development.[5]

The integration of the HPV virus in the host genome is often located in the transcribed genomic region suggested as a mechanism adapted by the virus to improve the expression of some viral products, notably E6 and E7 viral oncogenes.[6, 7] In addition, the integration of HPV type 16 was strongly associated with progression of precancerous lesions, i.e. cervical intraepithelial neoplasia grade 1 to 3 (CIN1-CIN3).[8]

Genomic instability and other DNA alterations including epigenetic ones, causing changed gene expression, are common features in many cancers.[9] Regions differentially methylated in cancers often co-localize with tissue-specific DNA methylated regions (T-DMR).[10,11] In cervical cancer, the DNA methylation can occur on the integrated viral DNA but also induce aberrant DNA methylation in the host cell genome.[12] Increased methylation of HPV type 16 has been correlated with a more severe cancer progression.[13] Aberrant methylation status of several host genes related to cell cycle, apoptosis, development, cell adhesion and cellular signalling have been analyzed in clinical studies by Szalmaz et al. and Wentzen et al.[14, 15] These meta-studies also showed that there is a large variation of the DNA methylation frequency, implying that new biomarkers are needed for early detection and progression of cervical cancer.

In this study, we employed the Illumina Infinium Human Methylation 450 K BeadChip array to identify novel biomarker genes that are differentially methylated in cervical squamous cell carcinoma (SCC) DNA samples compared to DNA from normal cervical scrapes and CIN3 cervical tissue. This approach enabled us to acquire a comprehensive epigenetic signature between the different cervical tissues in terms of global methylation, regional methylation in CpG islands (CGI), shores, gene related regions (TSS, 5’UTR, gene-body, 3’UTR), and also methylation of distinct CpG sites that discriminates between these tissues.

Results

DNA methylation profile in cervical cancer or CIN3 compared to the normal cervical tissue

The probe call rate was > 99% for all of the samples and 454, 215 CpG sites out of 485,577 were included in the analysis. The PCA analysis showed that all samples were close to each other. The majority of the cancer samples tended to hold together, followed by the cluster of CIN3 samples and other cluster of the normal cervical samples (Supplementary Figure 1). The frequency

distribution of the Δβ-values showed that cervical cancer tissue, compared to CIN3 and normal cervical tissue harbored hypermethylation at specific regions (data not shown). The frequency distribution of differentially methylated CpG sites across chromosomes showed that cancer tissue compared to the CIN3 and normal tissues had most differentially methylated CpG sites on chromosomes 1, 6 and 19 (Supplementary Figure 2).

The unsupervised hierarchical clustering analysis distinguished normal cervical tissues from tissues with CIN3 lesions and cancer tissues (Figure 1). In this heatmap, sixteen of the 25 statistically significant CpG sites belong to an annotated gene. The first ten CpG sites, located in different genes, are mostly hypomethylated in the cancer tissues as well as in the tissues with CIN3 lesions compared to the normal tissues (Figure 1). In contrast, the remaining 15 CpG sites in specific genes are mostly hypermethylated both in cancer and tissues with CIN3 lesion compared to the normal tissues. The mean β-values for all the 25 CpG sites are shown in Table 1.

Differentially methylated CpG loci and gene regions

We analyzed the location of the hypo- and hypermethylated CpG loci in relation to the CpG islands and gene context. Most of the differential methylation occurred in the context of a gene when comparing cancer tissue to precancerous and normal tissue (Figure 2). A large fraction of the hypermethylated CpG loci were located in the CGI in contrast to the hypomethylated ones that were mostly located in the CGI shore or further away (Figure 2).

In Supplementary Table 1 an overview of the number of differentially methylated regions and the range of the differences are shown comparing the three diagnostic groups. Most of the differentially methylated regions are found when comparing cancer tissue and normal cervical tissue. The majority of the events occur in the traditional gene promoter region (TSS1500, TSS200, 5'UTR, and 1st exon) and in the CpG islands. The comparison between cancer tissue and CIN3 tissues follow the same pattern. There are no differentially methylated regions reaching the criterion of Δβ of $\geq|0.2|$ when comparing the CIN3 tissues and normal cervical tissues.

Analysis of the differentially methylated regions in the vicinity of the traditional promoter region (TSS1500, TSS200, 5'UTR and 1st exon) in cancer tissues compared to the normal tissues showed that many of the hypomethylated genes in cancer were found to be involved in the immune system response (Table 2). The hypermethylated genes are mostly receptors and transcription factors involved in cell development (Table 3).

Identification of functionally-related gene groups

A total number of 74 gene ontology term clusters were generated when analyzing the hypomethylated CpG sites in cancer compared to normal cervical tissues. The GO_BP cluster with the highest enrichment score of 6.42 was related to the immune system (Supplementary Table 2). The GO_FAT terms included several KEGG pathways involving the immune system, such as antigen processing and presentation, autoimmune thyroid disease and asthma.

The gene ontology analysis of the hypermethylated CpG sites created three clusters, and GO_BP had the highest enrichment score of 36.52 (Supplementary Table 3). These gene ontology terms belong to the categories of cell development and cell differentiation. The GO_FAT and KEGG classification included categories of transcriptional regulation and DNA binding with the highest enrichment score of 24.41.

Candidate biomarker genes

The selection of candidate biomarker genes was based on both differentially methylated CpG sites generated with unsupervised hierarchical clustering and differentially methylated gene regions.

From the list of genes generated with unsupervised hierarchical clustering (Table 1) the selection criteria were 1) Δβ-value > 0.2 between the cancer and normal tissues, and 2) a mean baseline (normal tissue) β-value < 0.2 of a CpG site hypomethylated in cancer or a baseline (normal tissue) β-value > 0.4 for a CpG site hypermethylated in cancer. This approach led to the selection of six genes, the best candidate biomarkers: *RGS7, LHX8, STGALNAC5, TBX20, KCNA3,* and *ZSCAN18* (Table 1 and Table 4), all with ≥ 9 significant CpG sites.

From Table 2 and Table 3, displaying differentially methylated gene regions, we selected genes that were differentially methylated with a Δβ-value >|0.4|. The gene regions were then sorted according to the adjusted *p*-value and the top 20 regions were selected as candidates corresponding to 18 genes: *ACAN, AJAP1, BARHL2, BOLL, C1orf114, FBXL7, GALR1, GYPC, KIF19, MIR663, PTGDR, S1PR4, SORCS1, TRIM58, TTYH1, VSTM2B, ZIK1,* and *ZNF582*. The enlarged list of candidate biomarker genes is presented in Table 5. With exception of *S1PR4*, the candidate genes were hypermethylated in cancer compared to CIN3 and normal cervical tissues. As illustrative examples, the mean methylated fractions of all interrogated CpG sites in three selected genes, *S1PR4, TBX20*, and *ZNF582* are shown in Supplementary Figure 3. There is a stepwise increment in methylation (β-value) when comparing normal tissue, CIN3 tissues, and cervical cancer. We further validated the differential methylation of four selected genes, *TBX20*, *RGS7*, *KCNA3*, and *S1PR4,* with the Pyrosequencing assay technology. Supplementary Figure 4 shows the percentage methylation of the analyzed CpG sites in cervical cancer and normal tissues. The results are in very good agreement with our array data. The mean methylation of the cervical cancer tissues was higher compared to the normal cervical tissues in the *TBX20, RGS7*, and *KCNA3* genes, while the *S1PR4* gene was hypomethylated in cervical cancer tissues. We found a higher variation in the methylated fraction within the cervical cancer group compared to the normal cervical tissues, as detected also by the array technique (Table 1).

External validation

External validation of our candidate biomarker genes was done using the mRNA expression dataset from Peyon et al.[16], Scotto et al.[17], and Zhai et al.[18] which fulfilled quality criteria of tissue samples and employed high quality whole-genome expression arrays. Peyon et al.[16] reported probe sets for 21 of our 24 genes of interest whereas the two other sets reported for 14 of the genes. There was a good agreement with our DNA methylation data and the gene expression array; the fold

changes ranged between -1.09 and -2.20 for all the hypermethylated genes, see Table 4. The hypomethylated *S1PR4* gene was as expected up-regulated in cervical cancer in one gene expression dataset but it was down-regulated in the two others.

Discussion

The aim of this study was to acquire a comprehensive epigenetic signature of cervical cancer, and to identify new potential biomarker genes that are differentially methylated in cervical SCC compared to CIN3 or the normal cervical tissue. Our results showed that different cellular pathways are hypo- and hypermethylated in cancer tissue. A short list of 24 novel candidate biomarker genes emerged, which was consistent with the external validation sets and could ultimately be clinically applicable.

Fifteen of the 24 potential biomarker genes (*ACAN, Clorf114, FBXL7, GYPC, KCNA3, KIF19, LHX8, MIR663, RGS7, S1PR4, SORCS1, TBX20, TRIM58, TTYH1,* and *VSTM2B*) have not yet been correlated to any cancer type, while eight (*AJAP1, BARHL2, BOLL, GALR1, PTGDR, ST6GALNAC5, ZIK1,* and *ZSCAN18*) have been implicated in cancers other than cervical cancer (Table 4). Nevertheless, all of these genes are included in crucial cellular functions, such as cell signaling, gene transcription, immunity, glycosylation, membrane activity and ubiquitination. Therefore, the hypermethylation of genes involved in such processes may contribute to the accumulation of damages that can progress to cervical cancer. By external validation using three independent datasets, the gene expression of twenty three potential biomarkers was found to be down-regulated. This correlated with their hypermethylated state, confirming a link between DNA methylation and mRNA expression.

In cervical carcinogenesis, both global DNA hypomethylation and hypermethylation of specific genes promoters has been suggested to occur.[19, 20] We found here that most of the analyzed CpG loci were equally methylated (94%) in cervical cancer cells and normal cervical cells (Supplementary Table 4). However, the genome-wide DNA methylation array confirmed that hypermethylation in cervical cancer really occurs at specific genes (24,199 CpG sites; Supplementary Table 4). Even though we included more CIN3 cases precisely to increase the power to detect differences between CIN3 and normal tissues, the differences between the methylome of cervical cancer vs. normal tissues was greatest in all analyses (*p*- and β-values) compared to the methylome of CIN3 vs. normal tissues, therefore our report focuses on these differences.

Distinctly different cellular pathways appear to be hypo- and hypermethylated. In cervical cancer, the hypomethylated genes were related to the immune system response (Supplementary Table 2); whereas the hypermethylated genes were involved in the biological processes of cell development, cell differentiation, and transcriptional regulation (Supplementary Table 3). Shen et al.[21] interrogated a smaller number of CpG sites (Infinium Human Methylation 27 BeadChip) in colon, kidney, stomach, lung and breast cancers as well as the corresponding normal tissue; they reported results consistent with our methylation data performed on cervical cancer.[21]

Aberrant DNA methylation signatures in cervical cancer tissue, compared to the normal cervical tissue, are located in different regions depending on the methylation status. Hypermethylated CpG sites are predominantly located in the CGIs and hypomethylated CpG sites in the CGI shores (Figure 2). In the gene context, most of the changes occurred in the TSS200 region

(Supplementary Table 1). Aberrant DNA methylation has been mostly studied in promoters with a CpG island. A few years back, CpG island shores were identified as potentially important regions harboring DNA methylation changes in colorectal cancer.[11] CpG island shore methylation was statistically significantly correlated with clinical characteristics in breast cancer when we analyzed the *SLC25A43* gene.[22] Feber et al.[23] analyzed pooled DNA from benign and malignant nerve sheath tumors with methylated DNA immunoprecipitation and next generation sequencing (MeDIP-seq), and suggested that both hypo- and hypermethylated differentially methylated regions were located in the CGI shore regions.[23] The MeDIP-seq methodology yields a semi-quantitative methylation percentage and differs from the Illumina Infinium Human Methylation 450 K BeadChip that detects selected CpG sites at a single-nucleotide resolution in virtually all genes.[24, 25]

Furthermore, methylation changes that are tumor driving were suggested to be present in premalignant lesions and to occur early in the field of carcinogenesis.[26] From a clinical perspective, methylation biomarkers for cervical cancer detection and prognosis are highly needed.[15] Lendvai et al.[19] recently used MeDIP-seq to perform a genome-wide analysis of high-grade CIN3 lesions compared with normal cervical epithelium, and identified 80 differentially methylated regions. They suggested *COL25A1* and *KATNAL2* genes as potential biomarkers for early detection of high-grade cervical intraepithelial neoplasia.[19] Our study also identified these two genes as differentially methylated in cervical cancer tissue compared to CIN3 or normal cervical tissue, but not when comparing CIN3 tissues and the normal cervical tissues (data not shown), indicating that these two genes might be late biomarkers, however they did not qualify as biomarkers according to our selection criteria. Our data extends the study of Lendivai et al.[19] by comparing the methylome of cervical cancer tissues compared to the CIN3 lesions and the normal tissues. By this approach we generated a list of 24 novel potential biomarker genes (Table 4).

One of our candidate biomarker gene, *ZNF582*, was previously shown to be hypermethylated in cervical cancer suggesting silencing of the *ZNF582* gene in cervical neoplasms,[27](Suplementary Figure 3). Although the biological function of *ZNF582* is not yet well characterized, it could be a tumor suppressor gene as well as potential candidate gene for molecular cervical cancer screening.[27] Another highly interesting gene is *GALR1,* which inhibits a key regulatory enzyme adenylyl cyclase. This gene was suggested to be hypermethylated in a subset of head and neck cancer caused by oncogenic HPV types, notably HPV 16.[28] Thus, frequent promoter hypermethylation of *GALR1* and cellular growth suppression after re-expression of the gene supports the hypothesis that *GALR1* could be a tumor suppressor gene in head and neck cancer,[28] as well as in cervical cancer (our study).

Our findings of hypomethylated genes in the immune system provide strong evidence that the immune response is under control of hypermethylation in normal cells, while in cancer cells, these genes become active by losing the methylation mark in the gene promoter region which represses the gene expression. One example is the hypomethylated *AIM2* gene, whose functional protein forms an inflammasome, resulting in caspase activation in inflammatory cells. This induction of inflammatory cell response could occur in cervical cancer cells due to the hypomethylation of genes such as the *AIM2* gene. Furthermore, AIM2 protein has been found overexpressed in a wide variety of tumor types, including neuroectodermal tumors,[29] pancreatic cancer,[30] and in leukemias.[31]

Another interesting hypomethylated gene is *HLA-DPB1*, whose gene product belongs to the HLA class II molecules, which plays a central role in the immune system by presenting peptides

derived from extracellular proteins. There is evidence that HLA-DP participates in the functional T-cell responses against HPV in cervical cancer.[32] The host immune response to HPV is generally at low-level because HPV is shielded in the basal epithelial cells from the circulating immune cells during initial stages of the infection. Furthermore, HPV employs several mechanisms to down-regulate innate and cell-mediated immunity.[33] This is confined to the early stages of infection since it is known that functionally active immune responses are generated at later stages of the HPV infection.[34] Thus, our findings in cervical cancer are consistent with an immune response against HPV, which is activated through hypomethylation of specific genes included in the immune response and inflammatory reactions.

The immune system, upon activation by hypomethylation, can prevent tumor development by suppressing the viral infection, prevent an inflammatory environment leading to tumorgenesis, and identify tumor cells based on their antigen expression and eliminate them.[35] Inflammatory cells are suggested to affect the tumor microenvironment, which in turn can promote or repress the tumor growth.[36] The theoretical model of aberrant DNA methylation induced by chronic inflammation involves cytokine production TNFα, IL-1β, and oxidative stress in epithelial cells.[36, 37] Thus, without adequate immune responses the transformation to cancer might be quicker. This is the case of the adenovirus 12, which has the capability to turn off the immune system genes which may lead to tumor progression.[38] Tumor tissue may contain increased amounts of leukocytes of various types, which could partly account for the observed hypomethylation of immune-system-related genes. The recent introduction into clinical practice of liquid-based cervical cytology may offer opportunities to address this issue in future studies.

In order to determine if DNA methylation changes occur early or late in cervical carcinogenesis the genome-wide DNA methylation studies should extend to lower CIN grades, CIN1 and CIN2 as well as atypical squamous cells of undetermined significance (ASCUS). However, our study clearly indicates that DNA methylation profile of specific genes in samples with high-grade cervical lesions (CIN3) is located between samples with normal diagnosis and samples with invasive cervical cancer (Supplementary Figure 1), but closer to cluster of normal samples, as exemplified by specific CpG sites in the *ZNF582*, *TBX20* and *S1PR4* genes (Supplementary Figure 3).

We have validated our array results with an independent method analyzing the methylated fraction of four genes (Supplementary Figure 4). Our results suggest as previously reported by Cachill et al.[39] and Deneberg et al.[40], that the Illumina Human DNA methylation 450K array measures differential DNA methylation comparable to the golden standard Pyrosequencing assay technology.

Conclusion

In this study, we selected candidate genes with CpG sites highly methylated in cervical cancer tissue and without methylation in normal cervical tissue. This combination provides an optimal condition for a successful diagnostic test. The results highlight the extensive differential methylation signature in HPV induced cervical cancer tissue compared to CIN3 or normal cervical tissues. The 24 candidate biomarkers that we identified represent several types of mechanisms in the cellular machinery that are epigenetically deregulated, such as membrane receptor function, intracellular signaling, and gene transcription. External validation confirmed a link between DNA methylation and mRNA levels for these genes. Considering the extensive hypomethylation of genes

involved in the immune system, epigenetic therapy and immune therapy might need to be combined when treating cervical cancer. Understanding of the functional role of DNA methylation alterations in cancer genomes may prove to be clinically applicable in disease diagnostics and prognostics, and may guide the development of new epigenetic therapies.

Materials and methods

Study group

The cervical specimens were collected in the Sisters of Mercy Hospital, Zagreb, Croatia during the period from 2004 to 2011. The clinical characteristics of the specimens are presented in Supplementary Table 5. The cytological diagnoses were obtained for normal cervical smears, high-grade squamous intraepithelial lesion (HSIL)/CIN3, while cervical SCC samples were defined by histopathology.[41] Subsequently, cervical specimens were tested and typed for HPV.[42]

The cervical sample collection is regulated through Laboratory service request forms that have to be signed and approved by the practicing physician. The extracted DNA from cervical specimens was processed without initial knowledge of patient data (age, diagnosis, HPV detection and typing result). The study was approved by the Ethical Board of the Rudjer Boskovic Institute, as well as the Ethical Board of Sisters of Mercy Hospital, and conforms to the Helsinki declaration (DoH/Oct2008). The Ethical Review Board, Uppsala, approved the Swedish participation in this study.

DNA preparation

DNA from cervical cell samples was isolated on the BioRobot EZ1 (Qiagen, Hilden, Germany) according to the manufacturer's instruction. After DNA extraction, the purified DNA was dissolved in 50–100 µl of tri-distillate sterile water and stored at –20°C until further analysis. Each DNA was analyzed by electrophoresis on 1% agarose gels and spectrophotometrically.[43]

HPV detection and typing

Three sets of consensus primers for HPV detection were used: PGMY09/PGMY11,[44] L1C1/L1C2-1/L1C2-2 [45] and GP5+/GP6+.[46] The quality of the isolated DNA was tested by amplification of the 268 bp sequence of the β-globin gene using PC04/GH20 primers[47] in a multiplex PCR with PGMY primers. Type-specific primers for HPV types 6/11, 16, 18, 31, 33, 45, 52 and 58 were used for HPV typing according to Milutin-Gasperov et al., 2007.[42] Aliquots of each PCR product (10 µl) were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. The amplified products were visualized by UV irradiation of the gels either by ImageMaster VDS (Pharmacia Biotech) or UVItec Cambridge (Alliance 4.7).

Methylation array

The Illumina Infinium Human Methylation 450 K BeadChip (Illumina, Sweden) includes 485,577 CpG sites located all over the genome.[24] The quality and integrity of the samples were evaluated with Nano Drop and gel electrophoresis; samples were included if the ratio $A_{260/280}$ was between 1.7-1.9, the ratio $A_{260/230}$ was > 1 and the samples resolved on gel electrophoresis showed a distinct DNA band > 10,000 bp. In this study 20 normal cervical samples (HPV negative), 18 samples with CIN3 lesions (HPV positive) and 6 cervical cancer tissues (HPV positive) were included. The DNA methylation assay was performed as follows. Briefly, approximately 500 ng of DNA from cancer, CIN3 lesions, and normal cervical tissues were bisulfite treated with Zymo EZ DNA Methylation Gold kit (Zymo Research, USA) according to manufacturer's procedure. After the bisulfite treatment, 200 ng DNA was subjected to the whole genome amplification (WGA) and enzymatic digestion with reagents provided within the Infinium Human Methylation 450K kit (Illumina, Sweden). The hybridization of the samples on the BeadChips and washing procedures followed the standard procedures obtained from Illumina. The iScan scanner (Illumina, Sweden) was used to read the BeadChips and the data collection was performed in the GenomeStudio software (version 1.0).

Data processing

The Infinium Human 450K Methylation Bead chip has several built in sample dependent and independent controls that measure the processing of the chip. They were analyzed in the Genome Studio software with the Dash-Board module, prior to further statistical analyzes. The probe call rate for a passed sample should be > 98%, and the detection *p*-value for the probes should be < 0.01, otherwise they were excluded. The β-value generated for each CpG locus measures the intensity of methylated ($\beta = 1$) and un-methylated probes ($\beta = 0$). It is calculated as $\beta = [M/(M+U+100)]$, where M = methylated allele and U = un-methylated allele. The study cohort contains only women; therefore the Y-chromosome was excluded from the analysis. The raw β-values were generated with the GenomeBead studio using the settings normalisation controls and background subtraction. This data has been deposited to the Gene Expression Omnibus database under the accession number GSE46306.

A CpG locus was considered differentially methylated if the Δβ-value was $\geq |0.2|$ and the adjusted *p*-value < 0.05. This cut-off value of $|0.2|$ represents the 99% confidence interval of the detection limit.[25]

Unsupervised clustering of CpG loci and DAVID analysis

Heat map showing clustering of the patient samples was performed based on *p*-value < 0.05 and Δβ-value ± 0.5 between CIN3 tissues and normal tissues. They were produced with the Illumina Methylation Analyzer (IMA) package version 2.1.1 (Illumina, Sweden). The Database for Annotation, Visualization and Integrated Discovery (DAVID) [48] was used to analyse biological features associated with genes hypo- or hypermethylated in cervical cancer compared with normal cervical tissue. The gene lists were obtained with the criteria *p*-value < 0.05 and a more stringent Δβ-value of

> |0.3| (between cancer and normal tissues) due to the input limit of genes to the DAVID tool. The gene ontology (GO) groups were clustered into functional annotation referring to biological process (GO_BP), cellular component (GO_CC) and molecular function (GO_MF). GO_FAT terms were also retrieved to filter the broadest GO categories. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify functional gene groups and ontology terms that are significantly overrepresented.

Differentially methylated CpG loci and gene regions

The CpG loci were categorised as either hypermethylated (positive $\Delta\beta$-value) or hypomethylated (negative $\Delta\beta$-value). The CpG loci were then categorized in the context of (I) CpG islands, CpG shores, CpG shelves or others and (II) genomic location of TSS1500 (1.5 kb within transcriptional start site), TSS200 (200 bp within transcriptional start site), 5’UTR (5' untranslated region) and 1st exon, gene body and 3’UTR (3' untranslated region) or others. To compare the regions (CpG island, CpG shore, CpG shelves, TSS1500, TSS200, 5’UTR, 1st exon, gene body and 3’UTR) between the different diagnostic groups, the median β-value was calculated including all the CpG sites in the analysed region. Each region contains on average 1.53 – 9.92 probes.[25]

External validation of the candidate biomarker genes

Three datasets GSE6791,[16] GSE9750,[17] and GSE7803[18] deposited to the Gene Expression Omnibus (GEO) database were used for external validation of mRNA expression of candidate biomarker genes. The datasets measured gene expression by the Affymetrix GeneChip Human genome U133 Plus 2.0 (GSE6791) array and Affymetrix HG-U133A array (GSE9750 and GSE7803). The GES6791dataset was composed of 20 cervical cancer samples (17 HPV+, 3 HPV-) and 8 normal cervical samples (HPV-); the GSE9750 dataset of 27 cervical cancers (HPV+) and 24 normal cervical tissues (HPV-); and GSE7803 dataset of 24 cervical cancer tissues (HPV+) and 10 normal cervical tissues (HPV-). Using the online tool GEO2R (www.ncbi.nlm.nih.-gov/geo/geo2r), the log fold change (FC) was calculated to estimate the differences in the gene expression in cervical cancer compared to the normal cervical samples. The unadjusted p-values < 0.05 were considered as statistically significant, since these analyses were hypothesis driven.

Pyrosequencing validation of differential methylation

We developed assays for the following genes: *TBX20, RGS7, KCNA3*, and *S1PR4*. The PCR and sequencing primers were designed to assess the exactly same CpG sites analyzed by the array. All primers were purchased from www.biomers.net (Ulm/Donau, Germany). In the Supplementary Table 6, the sequences, amplicon sizes, and the optimal annealing temperatures are indicated.

The analysis was performed on 5 cervical normal and 5 cancer tissues, which were already tested by Illumina Infinium Human Methylation 450 K BeadChip array. Briefly, approximately 500 ng extracted DNA was used for the bisulfite treatment performed with the EZ DNA Methylation Gold kit

according to the instructions by the manufacturer and eluted in 20 µl elution buffer (Zymo Research, Orion Diagnostica, Sweden). The PCR reactions were performed in a total volume of 40 µl with the KAPPA2G Robust HotStarTaq DNA Polymerase Kit (Kapa Biosystems, Inc.), containing 0.10 µmol/L of each primer, 0.5 units of Taq polymerase, 1.5 mM $MgCl_2$, and 0.1 mM each of dGTP, dATP, dTTP, dCTP and 50 ng of bisulphite treated DNA was added as template. The PCR reaction for the *RGS7* gene contained also the 1X Enhancer solution provided in the kit. The PCR program was as follows: initial denaturation step of 1 min at 95°C, followed by 45 cycles of 30 sec denaturation at 95°C, specific annealing T_a for 30 sec and extension for 30 sec at 72°C and one cycle of final extension for 30 sec at 72°C. Sequencing was performed using a Pyromark Gold Q96 Reagent Kit and a PSQ 96ID system (Qiagen) as described previously by Farkas et al.[49] The nucleotide addition order was optimized by the Pyro Q-CpG software version 1.0.9 (Qiagen) and the results were automatically analyzed using the same software.

Statistical analysis

All statistical analyses were performed using the R software with the IMA package.[50] The raw β-values were arcsine square root transformed and the empirical Bayes moderated t-statistic was used to generate the *p*-values.[51] The Benjamini-Hochberg method was used to adjust the *p*-values for multiple testing.[52] For site-level analysis the mean Δβ-values were used and for the regional analysis median Δβ-values value was calculated including all CpG sites in one region. The principal component analysis (PCA) was performed to visualise the difference in DNA methylation between the cancer and normal tissue samples. In the DAVID analysis the statistic used was the modified Fishers exact *p*-value, adjusted for multiple testing with the Bonferroni method.[53]

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Legends to Figures

Figure 1. Unsupervised clustering analysis of the normal cervical tissue (N), CIN3 tissue (CIN3), and the cervical cancer tissue (CC).

The color gradient green to red displays the β-value and can range from 0 – 1.

Figure 2. Distribution of hypermethylated and hypomethylated CpG locus in CpG island (CGI), CpG island shore, CpG island shelves and other region.

Legends to Supplementary Figures

Supplementary Figure 1. Principal component analysis.

The grouping of cancer (green), precancerous (CIN3, blue) and normal cervical samples (red).

Supplementary Figure 2. Frequency distribution of hypo- and hypermethylated loci on different chromosomes.

The X chromosome is denoted as 23.

Supplementary Figure 3. Interrogated CpG sites in the *ZNF582, S1PR4*, and *TBX20* genes.

Supplementary Figure 4. Validation of the differential methylation with the Pyrosequencing assay technology of the *TBX20*, *RGS7*, *KCNA3*, and *S1PR4* genes in cervical cancer tissue (CC) and normal cervical tissue (NT). The horizontal line is displaying the mean methylated fraction of the group.