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Methylation of HPV16 genome CpG sites is associated with cervix precancer and cancer

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Abstract

Objective—Invasive cervix cancer (ICC) is the second most common malignant tumor in women. Human papillomavirus 16 (HPV16) causes more than 50% of all ICC and is a major cause of cervix intraepithelial neoplasia (CIN). DNA methylation is a covalent modification predominantly occurring at CpG dinucleotides. Such epigenetic modifications are associated with changes in DNA-protein interactions and gene activation. This study examined the association of viral and host genomic methylation patterns and cervix neoplasia.

Methods—Exfoliated cervical lavage samples positive for HPV16 from women with and without cytomorphic changes of infection (n=46), CIN2 (n=12), and CIN3+ (n=27) were used to interrogate the methylation patterns of the HPV16 L1 gene and upstream regulatory region (URR), five host nuclear genes (*TERT*, *RARB*, *DAPK1*, *MAL*, and *CADM1*), and mitochondrial DNA (mtDNA). DNA isolated from exfoliated cervicovaginal cells was treated with bisulfite, specific regions of the viral and host genome were PCR amplified and CpG methylation was quantified using EpiTYPER and pyrosequencing.

Results—Methylation at 14 of the tested CpG sites within the HPV16 L1 region were significantly higher in CIN3+ compared to HPV16 genomes from women without CIN3+. In contrast, only 2 out of 16 CpG sites in HPV16 URR, 5/5 in *TERT*, 1/4 in *DAPK1* and 1/3 mtDNA, and 2/5 in *RARB* were associated with increased methylation in CIN3+.

Conclusions—These results indicate that increased methylation of CpG sites in the HPV16 L1 ORF is associated with CIN3+ and thus, may constitute a potential biomarker for precancerous and cancerous cervix disease.

Keywords

cervical cancer; human papillomavirus; methylation

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Conflict of interest statement

The authors declare they have no conflicts of interest.

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Introduction

Invasive cervical cancer (ICC) is the second most common malignant tumor in women and is essentially always associated with persistent infection of oncogenic human papillomavirus (HPV), especially types 16 and 18, which account for ~50% and ~20% of ICC, respectively [1,2]. However, oncogenic HPV infections do not directly result in ICC. Instead, the natural history of oncogenic HPV and ICC consists of a set of transitions from HPV exposure and infection to persistence and precancer- high-grade cervical intraepithelial neoplasia (CIN) development and a final state of invasive carcinoma and metastatic disease [1]. Nevertheless, most HPV16 infections do not progress to cancer and there is a clinical need to identify additional markers associated with or predictive of the HPV16 infections that will progress to high-grade disease/cancer and require therapeutic intervention. Traditional screening modalities have relied on cytologic screening of Pap smears, which has a relatively low sensitivity [1]; accumulating data indicates that HPV testing is more sensitive although, not as specific (for review see [3]). Thus, additional biomarkers are needed to distinguish women infected with oncogenic HPV with or at significant risk to develop high-grade squamous intraepithelial lesions and/or early cancer. This will allow early treatment prior to the development of lethal invasive cervix cancer and would have significant benefits for physicians following women with a prevalently detected oncogenic HPV infection. Knowledge of the natural history of oncogenic HPV infections indicates that women with clearly documented incident new infections are not in the same risk category as those with prevalent infections [4,5].

A potential biomarker of high-grade CIN and cervix cancer is epigenetic changes (e.g., DNA methylation) to the genome of the cancer causing oncogenic HPV. DNA methylation is a covalent modification and mainly occurs at CpG dinucleotide sites. Recent studies indicate that patterns of HPV16 DNA methylation differ in cervix cancer and precancerous CIN lesions compared to HPV16 infected cervix tissue without high-grade disease, especially within the L1 open reading frame (ORF) and upstream regulatory region (URR) (for review see [6]). In addition, a set of host cellular genes have also been reported to vary in methylation status in high-grade CIN and cancer compared to normal cervix tissue (for review see [7]); taken together, methylation of viral and/or host genes may constitute a potential biomarker for precancer/cancer of the cervix. However, methylation of CpG sites is often not an all or nothing phenomenon given the cellular heterogeneity of screening cytologic and diagnostic tissue samples. Nevertheless, most previous studies have relied upon qualitative assessment of HPV methylation, such as methylation-dependent enzyme cleavage and sequencing bisulfite treated cloned DNA molecules. These early methods might have limited the identification of CpG sites associated with cervix precancer and cancer or biased the actual extent of methylation due to low number of sequenced clones. Moreover, most previous studies surveyed the HPV genome and nuclear genes separately, not allowing direct comparison of the state of methylation of virus and host in the same clinical samples. In addition to the HPV and host nuclear genomes, the mitochondrial DNA (mtDNA) genome represents another host genetic system susceptible to epigenetic changes that might influence the risk of cancer development [8]. However, the methylation of the mtDNA and its correlation with cervix precancer and cancer has not been investigated.

In the present study, we used two quantitative methods, EpiTYPER and pyrosequencing, to assess the extent and patterns of CpG methylation within the HPV16 L1 and URR and a set of candidate host genes including- five different nuclear genes and mtDNA in clinical specimens previously identified to contain HPV16 DNA from different stages of neoplastic progression. This study identified multiple CpG sites undergoing methylation that are associated with high-grade cervix disease, thus providing further evidence for an association of HPV16 L1 methylation status and cervix precancer and cancer. The implication is that

methylation of the HPV16 genome could mechanistically be involved in the development of cancer and/or serve as a marker of, or predisposition to cervix precancer/cancer.

Material and Methods

Cervicovaginal Samples, DNA isolation, and HPV16 detection

For the current analyses, we included participants that had both a cervicovaginal lavage sample with HPV16 DNA detected and had cervical cytology and/or histopathology results at or within 3 months of the time the lavage was obtained. Samples from participants meeting these criteria from two previous studies (n=26 [9], and n=59 [10]) conducted by the laboratory and using the same sampling and HPV detection methods were utilized for the current analyses. Briefly, DNA was released from cervicovaginal cells and tested for the presence of HPV16 by MY09/MY11 PCR and type specific dot blot hybridization as previously described [9,10].

Classification of cervical pathology

Since all samples contained HPV16 DNA, we combined women with no detectable cervical neoplastic lesions and those with low-grade squamous intraepithelial lesion (LSIL) and/or CIN1. These diagnostic categories are considered to be cytopathic manifestations of HPV infection and thus equivalent to women with HPV infection and no detectable cytopathic abnormalities [1]. Subjects were categorized into the following groups: group 1 (no significant pathology/cytology or LSIL/CIN1, n = 46), group 2 (CIN2, n = 12), and group 3 (CIN3 and squamous cell cancer, n = 27).

Gene selection and assay designs

We selected two regions within the HPV16 genome previously suggested to be associated with cervix precancer and cancer: the HPV16 L1 ORF and URR region [6,11–13]; five nuclear gene promoter regions (i.e., telomerase reverse transcriptase, *TERT*; death-associated protein kinase 1, *DAPK1*; retinoic acid receptor, beta, *RARB*; cell adhesion molecule 1, *CADMI*; and mal, T-cell differentiation protein, *MAL*), all of which have been suggested to show increasing methylation with cervix cancer [7]; and, one region in the mtDNA hypervariable segment (HVS). Primers for PCR were designed using MethPrimer [14] (<http://www.urogene.org/methprimer/index1.html>) or EpiDesigner (<http://www.epidesigner.com/>). Oligonucleotide primers and target CpG sites are listed in Table S1. All primers were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, IA).

Bisulfite modification, amplification, and methylation quantification

The extent of CpG methylation was determined by EpiTYPER (Sequenom Inc., San Diego, CA) [15] or pyrosequencing (Qiagen, Germany) [16,17] according to the manufacturers' recommendation. In brief, DNA samples from cervicovaginal samples obtained by lavage and documented to contain HPV16 DNA were treated with freshly prepared bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the recommended protocol. Bisulfite treated DNA results in unmethylated C's being converted into U's, which are then converted to T's by Taq polymerase during PCR amplification; methylated C's remain unmodified. All segments were amplified by Platinum *Taq* high fidelity DNA polymerase (Invitrogen, Carlsbad, CA). For EpiTYPER, the PCR was performed using a 5' primer with a T7 promoter tag and the PCR product was *in vitro* transcribed into RNA, which results in the conversion of A's, C's, G's and T's into U's, G's, C's and A's, respectively. The transcribed RNA was then enzymatically cleaved at the C or U positions to generate short segments and the mass for each segment was determined and distinguished by

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS, Sequenom). The ratio between the segments with G's and A's is proportional to and thus can be used to represent the extent of methylation in the original DNA [15]. This later approach has the advantage of being able to survey CpG sites in a relatively large DNA segment, but has a relative low resolution for clustered CpG sites. For pyrosequencing, after bisulfite treatment, the DNA was amplified with a primer containing biotin, captured by streptavidin coated beads, and annealed to a sequencing primer. Sequence extension was performed by DNA polymerase with sequential addition of each dNTP and the released pyrophosphate was catalyzed by ATP sulfurylase, luciferase, and apyrase sequentially in order to generate light signals, which are detected by a charge-coupled device (CCD) camera [18] on a PSQ96 system (Qiagen). Since the bioluminometric response is linear for the sequential addition of dNTPs, the signal intensity reflects the original methylation ratio [16]. This technology is rapid and economic for clustered CpG sites, but has a relatively short read length. Combining these two approaches provides a means to efficiently interrogate the maximum number of methylation sites. Using these methods, 16 out of 19 CpG sites in the HPV16 L1 ORF, all 16 CpG sites in the HPV16 URR, 20 sites in five cellular gene promoters, and 3 sites in mtDNA could be evaluated.

Statistical analyses

The agreement between different methods to measure methylation was evaluated using Kappa statistics. The mean methylation value at specific CpG sites between disease groups was compared by Kruskal-Wallis or the Wilcoxon test. All analyses were conducted in Stata 11.0 (StataCorp LP, College Station, TX) and the null hypothesis was rejected when $P < 0.05$.

Results

Assay validation

The accurate quantification of methylation is predominantly dependent on the complete bisulfite conversion of DNA samples. Since there were multiple non-CpG cytosines existing in our target regions, the pyrosequencing result for these sites constituted an ideal internal control for bisulfite conversion. In nearly all of the non-CpG cytosines (> 98.0%), the converted C accounted for > 95.0% of the total nucleotides detected at the specific sites (data not shown); this indicated that use of fresh bisulfite resulted in effective conversion of cytosines as anticipated.

Since two different methods were used in this study, we evaluated the agreement between methods. To this end, we designed and performed an additional EpiTYPER assay for CpG sites at 5611 and 5617 in the HPV16 L1 ORF and compared the results with those obtained by pyrosequencing. For these sites, kappa values of 1.0 and 0.85 were obtained, indicating good agreement. To compare the quantitation of methylation using C and T cleavage by EpiTYPER, methylation at the CpG site 5709 in the L1 ORF was evaluated and had a kappa of 0.88, verifying that the quantification was independent of the site of cleavage using EpiTYPER. We also evaluated whether quantification of methylation was independent of bisulfite treatment and DNA amplification. To test these possibilities, we performed separate bisulfite conversions on eleven samples and repeated PCR with pyrosequencing quantification at HPV16 URR positions 31, 37, 43, 52, and 58. Comparison of repeat tests of CpG methylation at these five CpG sites yielded kappa values from 0.79 to 1.0.

Analysis of the cervix cancer cell line SiHa that contains an integrated HPV16 genome [19] disclosed a distinct difference between HPV16 L1 and URR CpG site methylation. Within the L1 ORF, the 16 CpG sites analyzed were all hypermethylated ranging from 84% to

100%. In contrast, all 16 CpG sites in URR showed < 5% methylation per site, consistent with previous reports [11,20]. These data indicate the assays used were reproducible and yielded the expected results for the SiHa cancer cell line.

Differential CpG methylation of DNA from exfoliated cervicovaginal cells from women with cervix neoplasia

The average percent methylation at each measured CpG site in viral and host DNA from exfoliated cervical cells of women with < CIN2 (group 1), CIN2 (group 2), and CIN3+ (group 3) is shown in Figures 1 and 2. Within the HPV16 L1 gene (Fig 1A), most CpG sites from all 3 groups of women showed a moderate level of methylation (range from 5% to 30%), which is consistent with other studies [12, 13, 21–23]. In contrast, essentially all of the CpG sites in the HPV16 URR showed a lack of methylation with the average percent methylation < 5% (Fig 1B). As for host nuclear genes, a distinct pattern was observed that was dependent on the gene tested. For the *TERT* gene, all five CpG sites showed reasonably high-levels of methylation that ranged from 50% to 70% (Fig 2); whereas, the 15 CpG sites in the *RARB*, *DAPK1*, *MAL* and *CADMI* host genes and 3 sites in mtDNA showed very low levels of methylation all < 1% (Fig 2).

To examine whether there was a difference in the percent methylation at each CpG site between different grades of disease and HPV16 infection, we further categorized individuals by their cervix lesion grade and compared the results. Out of 16 CpG sites in the HPV16 L1 ORF, 14 showed significant differences (Fig 1A). In contrast, only 2 sites in the HPV16 URR showed a significant difference (Fig 1B); albeit, the overall levels of methylation within the URR were 5 – 10 times lower than within the L1 ORF. Examination of methylation sites within specific cellular genes indicated that only CpG sites in the *TERT* promoter showed a consistent increase in the percent methylation associated with cervix precancer/cancer (Fig 2). Methylation at CpG sites within the *RARB*, *DAPK1*, *MAL* and *CADMI* host genes and mtDNA was uniformly low, although a few sites were noted to have differences in CpG methylation by disease state (Fig 2).

Discussion

In the present study, we used two approaches, EpiTYPER and pyrosequencing, to quantify the methylation at specific CpG sites within HPV16 genomic regions, select host nuclear genes, and a small region of mtDNA, to determine their association with high-grade cervix lesions and cancer. Our data indicate that multiple CpG sites showed differential methylation and were associated with CIN3+ lesions of the cervix compared to HPV16 infections without high-grade disease.

Since the HPV16 URR plays an important role in regulation of viral gene expression, its methylation patterns and influence on viral gene expression have been studied by multiple groups [24] and this region has been consistently observed to be hypomethylated or not methylated [12,13,21,22], supporting the results of the current investigation. Nevertheless, some studies reported that this region is highly or moderately methylated [11,25,26]. The discrepancy for these observations is unknown, but could be related to differences in techniques and/or tissue sampling differences. For instance, methylation in tissue is not always uniform [13]. Therefore, when methylation-sensitive enzyme restriction cleavage was used [25,26], a low level of methylation, or incomplete enzyme cleavage can also yield a band that can be interpreted as active methylation. A study that used bisulfite treatment followed by cloning and sequencing [11] obtained a ~ four-fold higher methylation rate in the HPV16 URR (~10–20% for most sites) than the current study. Since only a limited number of clones was sequenced (usually 5) [11], it is possible that the methylated molecules were over represented in the total sample and thus the result does not reflect the

actual methylation pattern. Advances in DNA methylation methods, as used in the current study provide a higher resolution of CpG methylation status.

Multiple nuclear genes have been observed to show high and increasing methylation with cervix cancer development [7]. However, except for *TERT*, the four genes in our survey showed a very low percent of methylation. This discrepancy could be explained by the fact that only a small proportion of the exfoliated cells in cervicovaginal lavage samples are derived from the lesions and thus the majority of CpG dinucleotides are unmethylated. Therefore, the epigenetic alterations of high-grade lesions might be masked by the cellular composition of lavage samples. In cervical scraping or biopsies, the squamous cells might account for a larger portion of the total sample, thus yielding a higher percent of methylated CpG sites. In contrast, the HPV16 genomes are present only in infected tissues, derived directly from cells from the lesions. Therefore, the methylation signal for the HPV genome is relatively independent of tissue composition and thus enriched, but could be influenced by multiple lesions or regions of infection.

Mitochondrial (mt) DNA is an often overlooked genetic system in host cells, and its methylation pattern has not been investigated in high-grade cervix disease, to our knowledge. Similar to previous studies in gastrointestinal cancers [27], hypomethylation was observed in mtDNA in both cervical samples and the SiHa cell line (data not shown). This phenomenon might be attributed to the lack of methylase activity in mitochondria [27], the cell sampling bias in cervicovaginal lavage or a lack of association with cervix cancer. Further studies will be needed comparing cells directly obtained from cervix lesions through microdissection.

In summary, this study identified multiple CpG sites with diverse methylation patterns associated with CIN3+ lesions. Nevertheless, based on the overall low percent of CpG methylation in the HPV16 URR, nuclear genes, and mtDNA region most of these tested CpG sites were not predictive of disease state. However, CpG methylation sites within the HPV16 L1 ORF of exfoliated cervical cells from women with CIN3+ showed significantly higher levels of methylation. Thus, methylation status of the L1 provided additional information as to whether a woman with HPV16 has significant disease. Whether this molecular signature can serve as a predictive marker separating women at risk but not currently with high-grade disease will require prospective analyses. Moreover, whether host methylation of the HPV16 L1 gene is part of the host defense mechanism or is related to tissue differentiation requires further investigation, as does whether patterns of methylation will be found for other HPV type infections associated with the development of cervix cancer. Clearly, further work is required on this promising biomarker of cervix precancer and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The HPV16 methylation patterns in different disease groups for (A) HPV16 L1 ORF and (B) HPV16 URR. The height of each bar indicates the mean CpG methylation (percent methylation scale is shown on the left y-axis) at each CpG site and blue, red, and green denote group 1 (n=46, HPV16 infection +/- viral cytomorphic changes, all <CIN2); group 2 (n=12, HPV16 with histological diagnosis of CIN2); and, group 3 (n=27, HPV16 and CIN3+), respectively. The purple and blue squares indicate the calculated *P* values for Kruskal-Wallis or Wilcoxon test, respectively, corresponding to the scale shown on the right y-axis. The number below the figure denotes the HPV16 genome position of CpG sites.

Figure 2.

Methylation patterns of CpG sites within host nuclear and mitochondrial genes. Gene symbols: telomerase reverse transcriptase, *TERT*; death-associated protein kinase 1, *DAPK1*; retinoic acid receptor, beta, *RARB*; cell adhesion molecule 1, *CADMI*; and mal, T-cell differentiation protein, *MAL*. The height of the bar indicates the mean of CpG methylation (left y-axis) at each CpG site and blue, red, and green denote group 1, 2, and 3, respectively, as described in Figure 1. The purple and blue squares indicate the calculated *P* values for Kruskal-Wallis or Wilcoxon test, respectively, with the scale shown on the right y-axis.