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Patterns of Cellular and HPV 16 Methylation as Biomarkers for Cervical Neoplasia

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Abstract

Aberrant promoter methylation of biologically relevant genes in cervical cancer and uneven CpG distribution within the human papillomavirus 16 (HPV16) enhancer region have been reported. Cervical samples and questionnaires from 151 women screened for cervical cancer in Appalachian Ohio were analyzed. Methylation was measured by bisulfite sequencing in candidate gene sites in *ESR1*, *DCC*, *p16*, and *LINE1* elements. Among 89 HPV16-positive women, CpG sites in the E6 promoter and enhancer regions and the L1 region of the HPV16 genome were measured. Methylation levels were compared by cervical cytology and HPV16 status. HPV methylation was low regardless of cytology status, however E6 methylation was significantly higher in women with normal cytology. *ESR1* and *DCC* methylation were significantly higher in HPV16-positive women. Increased methylation at sites in the E6 promoter region was associated with lower odds of abnormal cytology. Increased methylation in candidate genes was associated with higher odds of abnormal cytology, particularly *DCC* region 2.4, *DCC* region 2.6, *ESR1* region 3.2, and *LINE1* site 1.2. HPV16 genome CpG methylation was low except for the L1 region. In general, lower HPV16 methylation and higher candidate gene methylation levels were associated with higher odds of abnormal cytology.

Keywords

cervical cancer; screening; human papillomavirus; HPV; methylation; carcinogenesis

1. Introduction

Despite the success of cervical cancer control in the developed world, cervical cancer remains the second most common cancer among women worldwide and ranks first in many developing countries, with an estimated 529,828 new cases and 275,128 deaths occurring worldwide in 2008 (Ferlay et al., 2010). While marked declines in incidence have occurred in areas with organized cytology-based screening programs, the sensitivity of any abnormality on a single, conventional Papanicolaou (Pap) test for detecting high-grade cervical lesions is 55% to 80% (Benoit et al., 1984), and specificity ranges from 86–100% (Nanda et al., 2000). Indeed, the success of cervical cancer screening at reducing the incidence of invasive disease is attributed to the usual slow progression from cervical intraepithelial lesions to invasive cervical cancer, and use of regular, repeated Pap testing.

Persistent infection with a subgroup of 10–15 high-risk human papillomavirus (HPV) types is established as a necessary, but insufficient, cause of cervical cancer. HPV DNA has been detected in up to 99.7% of all cervical cancers, and infection with one of four HPV genotypes (16, 18, 31, or 45) is present in nearly 75% of all cervical cancers (Goldie et al., 2004). Eight HPV genotypes (16, 18, 45, 33, 31, 52, 58, and 35) contribute to over 90% of invasive cervical cancers worldwide (de Sanjose et al., 2010). Recognition of the causal role of HPV in cervical carcinogenesis, and the known limitations of the Pap test, has led to incorporation of HPV testing in early detection. However, due to the high prevalence of HPV infection, HPV testing is unlikely to achieve the specificity required for an effective screening test (Unger et al., 2004). The suboptimal specificity of current HPV-based cervical cancer screening strategies and lack of biomarkers to identify individuals at increased risk of progression leads to unnecessary referrals for colposcopic evaluation and over treatment of

cervical precancerous lesions (Schiffman et al., 2005). Even in light of evidence demonstrating that most low-grade squamous intraepithelial lesions and even a substantial proportion of high-grade squamous epithelial lesions will not progress to invasive cervical cancer (Montz et al., 1992; Ostor, 1993; Syrjanen et al., 1992), these lesions are often treated by excision or ablation due to an inability to distinguish features predictive of neoplastic progression. Thus, active research focuses on the identification of additional biomarkers of cervical neoplasia that improve the specificity of screening and diagnosis. Translation of these biomarkers to clinical settings could help predict cervical cancer risk, improve management strategies and reduce health care costs. Even in the era of effective HPV vaccines, these markers could improve screening for women with previous HPV infections or new infections with HPV types not covered by current prophylactic vaccines.

Epigenetic events, including hyper- or hypo-methylation of tumor-relevant genes, are recognized as major events in the origin of cancer (reviewed in (Herman and Baylin, 2003)). Silencing of tumor suppressor genes by hypermethylation of the promoter region of the gene is a common feature of human cancer. Though technological advances have made it possible to measure DNA methylation rapidly and reliably in the promoter regions of some genes, its predictive value and specific role in cervical carcinogenesis is not well understood. Multiple studies have noted aberrant methylation in the promoter regions of biologically relevant genes in cervical cancer (Duenas-Gonzalez et al., 2005; Henken et al., 2007; Huang et al., 2010; Kim et al., 2010a; Lai et al., 2010; Muller et al., 2004; Unger et al., 2004; Wang et al., 2008; Wisman et al., 2006) and in cervical squamous intraepithelial lesions (Apostolidou et al., 2009; Gustafson et al., 2004; Huang et al., 2010; Kahn et al., 2008; Kim et al., 2010a; Kim et al., 2010b; Lai et al., 2010; Lim et al., 2010; Wentzensen et al., 2009). In addition to host cellular gene methylation, studies have reported uneven distribution and clustering of the cytosine-guanine dinucleotide (CpG) pairs within the enhancer region of HPV 16 and HPV 18 genomes (Burnett and Sleeman, 1984; Piyathilake et al., 2011; Rosl et al., 1993). Epigenetic regulation of this region has been recognized for some time, and may play a role in the carcinogenic process by modifying the virulence resulting in increased risk of progression of HPV infection to high-grade pre-invasive cervical lesions (Badal et al., 2003; Kalantari et al., 2004; Rosl et al., 1993; Unger et al., 2004). However, this has not been evaluated in a large, population-based study. Additionally, no study has evaluated host and viral methylation simultaneously in the context of cervical carcinogenesis.

The present study examines DNA methylation patterns in biologically relevant host genes and HPV viral control regions, using banked samples and epidemiologic data collected as part of a large case-control study conducted in 17 clinics in Appalachian Ohio. The prevalence of DNA methylation was measured in the promoter region of a panel of selected host cellular genes and in the enhancer region of the HPV genome using Pyrosequencing technology that allows for measurement of methylation at each individual CpG. Analyses focused on three host genes which have been shown to be methylated in multiple cancers, including cervical cancer in some studies, and represent biologically plausible targets for epigenetic regulation (Duenas-Gonzalez et al., 2005). Methylation at *LINE1* elements, a marker of global DNA methylation, (Yang et al., 2004) was also measured. Methylation levels of the selected gene sites were then compared by cervical cytology and HPV 16 status, adjusting for HPV cofactors. Examining whether associations observed previously in cancers are also apparent in pre-invasive cervical samples will shed light on clinical opportunities for early detection and management of cervical disease. Because methylation status may be reversible, understanding the role of methylation in HPV-induced cervical carcinogenesis offers an attractive mechanism for disease prevention.

2. Material and Methods

2.1 Study population

This study took advantage of a well-annotated repository of biological samples, clinical data and questionnaires collected as part of the Community Awareness, Resources and Education (CARE) Project. The goal of the larger CARE Project was to investigate and characterize the environmental, societal, behavioral, and biological mechanisms of cervical abnormalities among women living in Appalachian Ohio. Appalachia has the highest rate of cervical cancer mortality among white women in the United States (Centers for Disease Control and Prevention, 2002; Hall et al., 2000). Between 1976 and 1996, Appalachian women had higher age-adjusted cervical cancer mortality rates compared to other U.S. white women (Hall et al., 2000).

Women scheduled for a routine Pap test on a day that a study nurse was in one of the 17 clinics located throughout Appalachian Ohio were asked to participate in the study. Eligible participants included females ages 18 and older residing in the 29 Appalachian Ohio counties, with an intact cervix and corpus, not currently pregnant, and no history of cervical cancer. The Institutional Review Boards of the Ohio State University, the University of Michigan and the Centers for Disease Control (CDC) approved this study.

2.2 Data collection

On the day of their Pap test, women signed a written informed consent, completed a short questionnaire prior to undergoing cervical cancer screening, and provided blood and saliva samples. The self-administered questionnaire assessed sociodemographic characteristics; medical, gynecologic and obstetric history; sexual behaviors; physical activity; and alcohol and tobacco use. During the scheduled exam, an additional cytology sample was taken and the physician obtained a sample for HPV typing.

2.3 Sample collection and processing

Cervical samples were collected in Standard Transport Media (Qiagen, Valencia, CA), frozen at -70°C and shipped on dry ice to the CDC. For HPV typing analyses, the samples were thawed, and 150 μl extracted using the MagNA Pure System[®] (Roche Diagnostics Corporation, Indianapolis, IN) with DNA III extraction kit yielding 100 μl extract. The residual sample was refrozen at -70°C . After completion of HPV testing, residual extracts and additional unextracted samples were shipped to the University of Michigan for methylation assays. For methylation analyses, DNA was extracted from cervical samples after minimal freeze-thaw cycles with the DNeasy mini kit (Qiagen, Valencia, CA).

2.4 HPV typing

HPV detection and typing was performed using the Research Use Only Linear Array (LA) genotyping assay (Roche Diagnostics, Indianapolis, IN) with manufacturer's protocol modified to use a 10 μl aliquot of each DNA extract in the 100 μl PCR and automation of hybridization and detection (Bee Robotics, Caernarfon, U.K.). Samples equivocal for HPV 52 (positive XR probe and also positive for HPV33, 35 or 58) were tested in a quantitative HPV52 PCR assay using an ABI 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) with type-specific primers and a FAM-labeled TaqMan probe together with β -globin-specific primers and probe (Onyekwuluje et al., 2012).

2.5 Sample selection

Determination of disease status was based on the results of clinical cytology review from a certified laboratory. Cytology results were classified according to the 2001 Bethesda System

for Reporting Pap Smear Results (Solomon et al., 2002). Women with an abnormal cytology (atypical squamous cells of undetermined significance, low grade squamous intraepithelial lesion, high grade squamous intraepithelial lesion, or atypical glandular cells) were considered cases, and controls were sampled from recruited patients who had normal cytology results. For each case, three controls from the same clinic as the case were selected randomly from those with normal results within a three-month time frame of the case cytology. For the present study, 156 samples were selected to include approximately equal numbers of cases (n=74) and controls (n=82) with adequate representation of HPV 16 positivity among both cases (67.6%) and controls (47.6%). Of the 156 selected samples, five did not yield enough DNA for methylation studies. Of the 151 samples with adequate DNA, there were 74 cases (67.6% were HPV 16 positive) and 77 controls (50.7% were HPV 16 positive). The 74 cases were comprised of 36 (48.7%) with atypical squamous cells of undetermined significance, 24 (32.4%) with low grade squamous intraepithelial lesions, 12 (16.2%) with high grade squamous intraepithelial lesions and 2 (2.7%) with atypical glandular cells.

2.6 Methylation assays

Bisulfite sequencing using Pyrosequencing technology (Biotage, Uppsala, Sweden) was used to quantify methylation in the promoter regions of three well-described candidate genes, *LINE1* methylation, and 10 CpG sites in the L1 3' and long control region (LCR) of the HPV genome. Analyses focused on three tumor suppressor genes, *ESR1* (estrogen receptor 1), *p16* (cyclin-dependent kinase inhibitor 2A) and *DCC* (deleted in colorectal carcinoma), based on reported associations of hypermethylation of these genes with either cervical cancer or cancers at other sites (Archer et al., 2010; Carvalho et al., 2006; Furtado et al., 2010; Heldring et al., 2007; Kim et al., 2010a; Stephen et al., 2009; Wisman et al., 2006). Specifically, the following candidate gene sites were examined in all participants for whom adequate DNA was available (n=151): 3 CpG sites in the *ESR1* promoter region, 5 in an intronic region of *DCC*, and 4 in the promoter region of *p16*. The following HPV genome sites were examined in 89 women who were positive for HPV 16: 6 CpG sites in the E6 promoter region, 3 in the E6 enhancer region, and 1 in the L1 region of HPV16.

Pyrosequencing provides a quantitative, site-specific quantification of methylation at individual CpG sites in a reliable and rapid manner. Briefly, approximately 250 nanograms of DNA was bisulfite converted using the EpiTect DNA bisulfite conversion kit (Qiagen, Valencia, CA). Bisulfite conversion of DNA changes non-5' methyl cytosines (C) to uracils (U); methylated Cs are protected from bisulfite conversion and are unchanged, leading to methylation-dependent sequence changes following PCR. The region of interest in the promoter region of the selected candidate genes and HPV genome were amplified using primers specific for bisulfite converted DNA and HotStarTaq master mix (Qiagen, Valencia, CA). Pyrosequencing was carried out on a PyroMarkMD using the manufacturer's standard protocol. Forward, reverse and sequencing primers for the 10 CpG sites in the HPV 16 promoter, enhancer and L1 regions (Rajeevan et al., 2006) as well as for 5 CpG sites in *DCC*, 3 CpG sites in *ESR1*, 4 CpG sites in *p16*, and 4 CpG sites in *LINE1* are listed in Supplementary Table 1.

2.7 Statistical Analysis

Methylation levels of selected HPV 16 genome sites were described only among HPV 16 positive women (n=89), while methylation levels of the selected candidate host genes were described among the total study population (n=151). Cytology status was categorized as normal and abnormal, with atypical squamous cells of undetermined significance, low grade squamous intraepithelial lesions, high grade squamous intraepithelial lesions, and atypical glandular cells categorized as abnormal cytology. Distributions of methylation levels at each

of the selected sites were compared by cytology and HPV 16 status using the non-parametric Wilcoxon-Mann-Whitney U test. Logistic regression models were used to estimate the odds of abnormal cytology for a 1 percentage increase in methylation level (continuous), adjusted for HPV cofactors. Logistic regression models were run separately for each DNA methylation site and were adjusted for age (continuous), lifetime cigarette smoking history (ever versus never), number of live births (continuous), age at first sexual intercourse (continuous), lifetime number of sex partners (continuous), and history of oral contraceptive use (ever versus never). P-values less than 0.05 were considered statistically significant. Analyses were conducted using SAS version 9.2 (SAS Institute, Inc., Cary, NC) (The SAS Institute, 2008).

3. Results

3.1 Population Characteristics

Among 151 study participants, 89 (58.9%) were HPV 16 positive and 74 (49.0%) had an abnormal cytology, reflecting the sampling strategy based on HPV 16 and cytology status. The median age of HPV 16 positive participants was 23 years (range: 18–83) and the median age of participants with an abnormal cytology was 24 years (range: 18–60). Sociodemographic characteristics and HPV cofactors of the overall study population are described in Table 1. The median age of participants was 25 years (range: 18–83). Most participants were Caucasian (95.4%), never married (44.4%), had completed some college or less (93.4%), and had an annual household income of \$25,000 or less (52.3%). One-fourth (24.5%) of participants self-identified as Appalachian. With respect to HPV cofactors, the median age at first sexual intercourse was 16 years (range: 12–27) and the median number of lifetime sex partners was five (range: 0–33). Most (84.8%) participants reported a history of oral contraceptive use. Participants reported a median number of two live births (range: 0–8). A large proportion (70.9%) of participants reported a history of smoking at least 100 cigarettes in their lifetime, with 90 (59.6%) reporting current cigarette smoking.

3.2 Methylation Levels by Cytology Status

Among 89 HPV 16 positive women, HPV methylation levels were generally low regardless of cytology status (Table 2). Methylation levels were significantly higher in the E6 promoter region for subjects with normal cytology, although the differences by cytology status were small. The range of HPV methylation was much wider in normal subjects compared to those with abnormal cytology. Methylation levels for the E6 enhancer and L1 regions were similar regardless of cytology status. Among all 151 subjects, methylation of candidate host genes was also low. However, methylation levels tended to be higher in those with an abnormal cytology, with a tendency towards wider ranges of methylation. These differences were statistically significant in certain sites located in *DCC* and *ESR1*. Interestingly, only subjects with an abnormal cytology had high (>16%) mean levels of *DCC* methylation across the five sites analyzed (Figure 1), although this was true only for six of those with abnormal cytology (range: 17.0–70.2%), all HPV 16 positive. Of these six subjects with high average *DCC* methylation, 1 had atypical squamous cells of undetermined significance, 2 had low grade squamous intraepithelial lesions, 2 had high grade squamous intraepithelial lesions and 1 had atypical glandular cells cytology. While *LINE1* methylation was similar between the two cytology groups, all *LINE1* sites examined were higher in those subjects with an abnormal cytology, with the difference in site 2 bordering on statistical significance (82.03% vs. 80.87%, $p=0.06$).

3.3 Methylation Levels by HPV 16 Status

Similar trends were noted when stratifying by HPV 16 status (Table 3). *ESR1* methylation, notably, was statistically significantly higher in HPV 16 positive subjects compared to HPV 16 negative subjects for all three sites examined. *p16* methylation was also significantly higher in HPV 16 positive subjects, although the methylation levels overall were quite low. Two of the DCC sites examined were methylated at significantly higher levels among HPV 16 positive subjects compared to those who were HPV 16 negative.

3.4 Odds of Abnormal Cytology by Methylation Level

The odds of abnormal cytology by methylation level were modeled for each methylation site, adjusting for HPV cofactors (age, lifetime cigarette smoking history, number of live births, age at first sexual intercourse, lifetime sex partners and history of oral contraceptive use) (Table 4). Among 89 women who were HPV 16 positive, increased methylation in the E6 promoter region of the HPV 16 genome was significantly associated with lower odds of abnormal cytology, with an approximately 20% decrease in odds associated with the individual sites. There were no significant associations between methylation levels in the E6 enhancer or L1 regions of the HPV 16 genome and cytology status. Conversely, among the overall study population, increased methylation in candidate genes was associated with higher odds of abnormal cytology. In particular, *DCC* region 2.4 (OR = 1.27, 95% CI: 1.09–1.48), *DCC* region 2.6 (OR=1.11, 95% CI: 1.02–1.22), *ESR1* region 3.2 (OR = 1.22, 95% CI: 1.04–1.42) and *LINE1* site 1.2 (OR = 1.24, 95% CI: 1.02–1.52) were significantly associated with increased odds of abnormal cytology.

4. Discussion

The goal of this study was to determine if there was measurable methylation in cervical cancer screening samples, and if so, whether specific CpG sites in the selected candidate genes or HPV 16 genome were especially informative for differentiating cytology or HPV 16 status. Methylation of the HPV 16 genome tended to be higher in women with normal cytology than in women with abnormal cytology. Although the mean HPV 16 methylation levels were low, increased methylation at several sites in the E6 promoter region was significantly associated with a decreased risk of abnormal cytology after adjustment for HPV cofactors. Using pyrosequencing technology and a similar epidemiological approach as the present study, Piyathilake et al. reported that increased methylation of HPV 16 E6 enhancer and promoter sites was significantly associated with lower odds of being diagnosed with high-grade cervical intraepithelial lesions, after adjustment for relevant sociodemographic characteristics, lifestyle factors and established cervical cancer risk factors (Piyathilake et al., 2011). Though the mechanisms require further elucidation, methylation of the HPV genome could be a host defense mechanism for suppressing transcription of foreign DNA or a strategy used by the virus to maintain persistent infection, or both (Ahuja and Issa, 2000). Conversely, methylation levels of the candidate gene sites tended to be higher in women with abnormal cytology than in women with normal cytology, and a few sites were significantly associated with abnormal cytology after adjustment for HPV cofactors. For both HPV genome and candidate gene methylation, the range of methylation could be informative. The range of HPV 16 methylation was much wider among women with normal cytology compared to those with abnormal cytology. Patterns of methylation of HPV genes may vary with the viral life cycle. Methylation levels among HPV 16 positive subjects with normal cytology could reflect an incident HPV infection or one that is clearing; however, longitudinal study designs are required to clarify the time course of HPV infection and cervical disease in relation to methylation levels.

Three candidate genes were chosen for this study: *p16*, *DCC*, and *ESR1*. *p16* methylation has been identified as an important early event for squamous cell neoplasias (Nuovo et al., 1999). In cervical cancer, methylation of *p16* is not thought to be an underlying pathologic mechanism; rather, p16 inactivation occurs through interaction with HPV oncogenes (Nehls et al., 2008). Multiple studies have evaluated *p16* methylation in cervical screening samples or cancers, with inconsistent results, (Furtado et al., 2010; Kim et al., 2010a) but in general find the prevalence of p16 methylation ranging from 19% to 61%. In this study, mean *p16* methylation levels were even lower (ranged from 0.8–3.1% for the four sites examined) and were not predictive of abnormal cytology. Kim et al. (2010a) found no statistically significant trend of increasing *p16* methylation with increasing severity of cervical squamous lesions. In contrast, using a MSP-based method, Furtado et al. found a significantly higher prevalence of *p16* promoter methylation in patients with HSIL (55.6%) compared to those with normal cytology (20%) (Furtado et al., 2010). They did not find a statistically significant association between *p16* promoter methylation and HPV 16 status, regardless of cytology status (Furtado et al., 2010). In the present study, significant differences in *p16* methylation between HPV 16-positive and -negative women were observed, though the mean levels were very low. Of note, a few high *p16* methylation measurements among HPV 16 positive subjects were observed. Additional, appropriately designed studies are needed to evaluate whether these high levels may be indicative of risk for persistent HPV infection or cancer progression.

DCC methylation has been identified as biologically important in head and neck squamous cell carcinoma (HNSCC) (Carvalho et al., 2006). Additionally, members of this group have identified *DCC* and *ESR1* differentially methylated in HPV-positive HNSCC cell lines compared to HPV-negative HNSCC cell lines (Sartor et al., 2011). In the present study, *DCC* had the highest mean methylation levels, and in particular region 2.4 was associated with both cytology and HPV 16 status. In addition, *DCC* region 2.5 was significantly associated with HPV 16 status and *DCC* region 2.6 was significantly associated with abnormal cytology. *DCC* methylation was highly variable, and there were six subjects with notably high mean levels of *DCC* methylation and corresponding abnormal cytology (of which 1 was atypical squamous cells of undetermined significance, 2 were low grade squamous intraepithelial lesions, 2 were high grade squamous intraepithelial lesions and 1 was atypical glandular cells), indicating that *DCC* may be another promising marker of abnormal cytology.

ESR1 was chosen due to its significance and differential promoter methylation in other cancers (Archer et al., 2010; Heldring et al., 2007; Stephen et al., 2009). Additionally, Wisman et al. identified more *ESR1* promoter hypermethylation in cervical scrapings from cancer patients compared to normal subjects (64% versus 5.3% hypermethylated; $p < 0.0005$) (Wisman et al., 2006). Similar to the observations for *DCC* methylation, higher *ESR1* methylation at specific sites was associated both with abnormal cytology and HPV 16 positivity. *ESR1* methylation was significantly higher in HPV 16 positive subjects compared to HPV 16 negative subjects for all three sites examined. After adjusting for HPV cofactors, *ESR1* region 3.2 was significantly associated with higher odds of abnormal cytology, suggesting potential application for early cervical cancer detection.

Long interspersed nuclear element-1 (*LINE1* or L1) sequences are highly repeated and widely interspersed human retrotransposon sequences (Kazazian and Moran, 1998) and constitute about 17% of the human genome (Lander et al., 2001). Decreased methylation of CpG sites in *LINE1* elements is thought to be a general indication of genomic instability. In the present study, *LINE1* methylation did not differ significantly between women with and without an abnormal cytology, although increased methylation at *LINE1* site 2 was associated with abnormal cytology after adjustment for cofactors. Interestingly, women with

an abnormal cytology were more likely to have higher methylation at site 2. Work in progress by members of this group suggests that, when comparing HPV positive and HPV negative HNSCC tumors, those that are HPV positive have higher *LINE1* methylation than HPV negative samples. One study found increasing levels of *LINE1* methylation with cervical cancer progression in a small number (Shuangshoti et al., 2007). However, results of the present study indicate that mechanisms other than global demethylation may be important in the genomic instability thought to be an initiating event in cervical carcinogenesis.

Pyrosequencing, used to measure promoter methylation levels in the present study, is a high-throughput method to determine methylation at individual CpG sites and allows for integration of a bisulfite conversion control. Thus, every permutation of CpG methylation can be measured, in contrast to methylation-specific PCR (MSP) based methods, including MethyLight. In the present study, methylation levels in *DCC* region 2.4, for example, were significantly different between women with normal and abnormal cytology. However, MSP-based methods are much more sensitive, and have a high ability to detect the presence of methylated alleles. Cervical cytology samples are a heterogeneous mixture, with both abnormal and normal cells, and thus the low levels of methylation noted in these samples are not surprising.

This is the first study to examine methylation in both the HPV 16 and host cellular genomes in relation to cervical disease status, while adjusting for established HPV cofactors. This study provided a unique opportunity to characterize methylation patterns in screening samples from a medically underserved population enriched for cervical abnormalities, overcoming an important methodological limitation of other studies on this topic. Controls (women with normal cytology) were sampled from the same reference population as the cases. Because the epidemiologic data were collected via questionnaire on the same day as the cytology, prior to subjects' knowledge of their screening results, the potential for recall bias (differential recall of HPV cofactors between cases and controls) was minimized. However, some potential limitations of this study should be considered when interpreting the findings. Because this was a cross-sectional study, it was not possible to evaluate associations between methylation levels and long-term HPV persistence, which has been established as the primary risk factor for cervical cancer. Age-related methylation changes have been reported across several tissue types (Wojdacz and Hansen, 2006) and may be a predisposing factor for neoplasia (Ahuja and Issa, 2000). The observed wider HPV 16 methylation ranges for patients with normal cytology could be due to age differences in the study sample. While the influence of age on methylation levels could not be evaluated due to the cross-sectional nature of the study and the small sample sizes upon further stratification, logistic regression models predicting abnormal cytology included age as a covariate to adjust for the potential confounding effects of age. This study purposely oversampled for HPV 16 positivity among the cases and controls; thus, the 151 participants selected for the current study may not represent the larger CARE Project population (n=1131) with respect to certain epidemiologic characteristics. In the absence of colposcopy and biopsy results to determine cervical disease, clinical cytology results were used, so there may be some degree of misclassification of lesion severity. However, all abnormal cytology results (atypical squamous cells of undetermined significance, low grade squamous intraepithelial lesions, high grade squamous intraepithelial lesions, and atypical glandular cells) were grouped for analysis purposes. It was not possible to analyze the data with respect to severity of cytologic abnormality due to small numbers in each category upon further stratification by HPV status and HPV cofactors. This was further complicated by the heterogeneous cell mixture in the samples that may dilute the true methylation profile of the abnormal cells. Further understanding of the extent of both host cellular and viral gene

methylation – and at what stage methylation of these genes reflects a functionally important step – is warranted but will require longitudinal study designs.

5. Conclusions

This study provides some groundwork for future studies of the clinical utility of methylation markers for cervical cancer screening. Additional planned analyses will incorporate food frequency questionnaire data to explore a possible influence of nutritional status and micronutrients, particularly folate, on methylation levels and cervical disease status. In the future, identification of a panel composed of targets methylated frequently in cervical disease may permit the development of a clinical assay for distinguishing HPV infections that are likely to progress to cancer from those that are destined to regress. This biomarker panel could be combined with existing screening technologies to improve early detection of cervical cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Methylation levels in select host genes and HPV viral control regions were examined
- Methylation was compared by cytology and HPV16 status, adjusted for cofactors
- Higher methylation of candidate gene sites increased the odds of abnormal cytology
- Higher methylation of E6 promoter region lowered the odds of abnormal cytology
- Future work should clarify the role of methylation in cervical cancer screening

Figure 1.

DCC methylation at 5 sites by cytology status. Red lines indicate abnormal cytology, blue lines indicate normal cytology. Only subjects with an abnormal cytology had high (>16%) *mean* levels of *DCC* methylation across the five sites analyzed, though this was true only for six of those with abnormal cytology, all HPV 16 positive.

Table 1

Sociodemographic characteristics and HPV cofactors of the study population (n=151)

	n (%)
<i>Sociodemographic characteristics</i>	
Age (median, SD)	25 (13.1)
Race	
Caucasian	144 (95.4)
Other	6 (4.0)
Missing	1 (0.7)
Marital status	
Never married	67 (44.4)
Member of unmarried couple	18 (11.9)
Married	38 (25.2)
Divorced/Separated/Widowed	22 (14.6)
Missing/Refused	6 (4.0)
Education level	
High school graduate/GED	88 (58.3)
Some college/Technical or trade degree/Associates degree	53 (35.1)
Bachelor's degree:	8 (5.3)
Graduate degree	2 (1.3)
Full time employment	61 (40.4)
Annual household income	
\$25,000	79 (52.3)
>\$25,000	48 (31.8)
Missing/Unknown/Refused	24 (15.9)
Self-identify as Appalachian	37 (24.5)
<i>HPV cofactors</i>	
Age at first sexual intercourse (median, SD)	16 (2.6)
Number of lifetime sex partners (median, SD)	5 (6.5)
Number of live births (median, SD)	2 (1.2)
Ever used oral contraceptives	128 (84.8)
Smoked 100 cigarettes in lifetime	107 (70.9)
Currently smoke cigarettes	90 (59.6)

Abbreviations: SD, standard deviation

Table 2

DNA methylation levels at selected sites of the HPV 16 and human genomes by cytology status

	Abnormal Cytology % Methylation		Normal Cytology % Methylation		p-value ^d
	Mean (SD)	Range	Mean (SD)	Range	
HPV 16 genome (n=89) ^b					
HPV E6 promoter site 1	4.04 (4.49)	0–18.01	8.46 (8.88)	0–51.01	0.008
HPV E6 promoter site 2	4.93 (2.85)	0–11.96	7.10 (3.27)	0–17.33	0.0002
HPV E6 promoter site 3	4.76 (2.64)	1.87–14.93	7.60 (7.92)	2.74–51.72	0.0009
HPV E6 promoter site 4	3.44 (3.92)	0–14.05	7.86 (8.93)	0–56.74	<0.0001
HPV E6 promoter site 5	3.84 (3.03)	0–13.44	8.09 (9.06)	0–56.41	<0.0001
HPV E6 promoter site 6	4.33 (3.04)	0–14.36	9.21 (8.79)	0–56.23	<0.0001
HPV E6 enhancer site 1	3.33 (2.51)	0–7.91	4.49 (4.76)	0–22.52	0.41
HPV E6 enhancer site 2	3.37 (2.81)	0–13.12	3.08 (3.46)	0–12.10	0.50
HPV E6 enhancer site 3	5.73 (3.07)	2.68–16.25	8.01 (7.72)	0–39.59	0.14
HPV L1	24.88 (19.24)	0–100.0	25.73 (22.30)	0–96.93	0.98
Human genome (n=151)					
DCC-region 2.3	10.54 (9.16)	0–73.0	9.06 (2.37)	3.62–15.46	0.75
DCC-region 2.4	10.53 (9.41)	4.50–71.63	7.24 (2.31)	3.20–12.88	0.002
DCC-region 2.5	12.46 (8.61)	6.24–68.88	10.59 (2.37)	6.56–17.41	0.50
DCC-region 2.6	10.71 (8.71)	3.36–67.20	8.88 (3.65)	4.08–21.72	0.23
DCC-region 2.7	9.09 (9.20)	0–70.09	7.15 (4.43)	3.69–39.79	0.07
ESR1 region 3.1	6.81 (2.48)	2.64–14.78	6.83 (3.01)	0–17.97	0.81
ESR1 region 3.2	5.55 (2.59)	0–9.52	4.50 (2.48)	0–8.56	0.01
ESR1 region 3.3	7.30 (6.57)	0–55.59	6.96 (12.20)	0–100.0	0.01
p16region 1	2.74 (4.64)	0–32.89	2.49 (5.09)	0–32.14	0.16
p16region 2	3.69 (7.91)	0–56.84	2.43 (3.10)	0–22.30	0.07
p16region 3	0.88 (1.49)	0–9.49	0.73 (1.14)	0–8.27	0.83
p16region 4	3.39 (4.26)	1.17–20.92	2.20 (1.45)	1.32–8.63	0.34
LINE1 site 1	81.48 (3.10)	69.40–86.21	80.72 (4.40)	69.43–87.85	0.73
LINE1 site 2	82.03 (1.97)	77.08–89.80	80.87 (2.61)	73.76–84.55	0.06
LINE1 site 3	79.21 (2.80)	71.00–88.47	78.32 (3.42)	69.22–85.68	0.28

	Abnormal Cytology % Methylation		Normal Cytology % Methylation		p-value ^a
	Mean (SD)	Range	Mean (SD)	Range	
LINE1 site 4	76.56 (3.37)	62.24—87.71	76.47 (2.37)	69.04—86.32	0.70

^aWilcoxon-Mann-Whitney U test p-value for comparison of distribution of methylation levels at selected site by cytology status

^bHPV 16-positive women

Abbreviations: SD, standard deviation

Table 3

DNA methylation levels at selected candidate gene sites and LINE1 by HPV 16 status (n=151)

	HPV 16 Positive (n=89) % Methylation		HPV 16 Negative (n=62) % Methylation		p-value ^a
	Mean (SD)	Range	Mean (SD)	Range	
<i>DCC</i> region 2.3	10.44 (8.21)	3.62 – 73	8.85 (2.75)	0 – 15.46	0.52
<i>DCC</i> region 2.4	10.04 (8.54)	4.26 – 71.63	7.18 (2.4)	3.2 – 12.66	0.002
<i>DCC</i> region 2.5	12.54 (7.75)	6.63 – 68.88	10.06 (2.33)	6.24 – 17.41	0.002
<i>DCC</i> region 2.6	10.14 (8.16)	3.36 – 67.2	9.21 (3.12)	4.84 – 21.72	0.38
<i>DCC</i> region 2.7	8.71 (8.37)	0 – 70.09	7.15 (4.73)	3.69 – 39.79	0.12
<i>ESR1</i> region 3.1	7.88 (1.96)	0 – 14.78	5.21 (2.97)	1.16 – 17.97	<.001
<i>ESR1</i> region 3.2	6.28 (2.35)	0 – 9.52	3.11 (1.6)	0 – 7.05	<.001
<i>ESR1</i> region 3.3	8.03 (10.39)	0 – 100	5.66 (8.31)	0 – 55.59	<.001
<i>p16</i> region 1	3.43 (6.06)	0 – 32.89	1.45 (1.14)	0 – 8.85	<.0001
<i>p16</i> region 2	3.3 (7.4)	0 – 56.84	2.71 (3.2)	1.09 – 22.3	0.06
<i>p16</i> region 3	0.71 (1.39)	0 – 9.49	0.95 (1.18)	0 – 8.27	0.001
<i>p16</i> region 4	2.66 (2.34)	1.17 – 9.65	2.84 (3.65)	1.32 – 20.92	0.41
LINE1 site 1	81.36 (3.16)	67.27 – 85.89	80.52 (4.75)	69.4 – 87.85	0.48
LINE1 site 2	81.77 (1.79)	74.77 – 84.53	80.89 (2.96)	73.75 – 89.8	0.1
LINE1 site 3	79.15 (2.72)	70.66 – 85.68	78.22 (3.62)	69.22 – 88.47	0.14
LINE1 site 4	76.69 (2.49)	72.67 – 87.71	76.32 (3.23)	62.23 – 84.89	0.55

^aWilcoxon-Mann-Whitney U test p-value for comparison of distribution of methylation levels at selected site by HPV 16 status

Abbreviations: SD, standard deviation

Table 4

Odds of abnormal cytology by methylation level at selected sites of the HPV 16 and human genomes, adjusted for HPV cofactors

DNA methylation site	Abnormal cytology ^a OR (95% CI)
HPV 16 genome (n=89)^b	
HPV E6 promoter site 1	0.85 (0.76–0.96)
HPV E6 promoter site 2	0.78 (0.65–0.93)
HPV E6 promoter site 3	0.80 (0.67–0.95)
HPV E6 promoter site 4	0.79 (0.69–0.91)
HPV E6 promoter site 5	0.79 (0.67–0.92)
HPV E6 promoter site 6	0.74 (0.62–0.87)
HPV E6 enhancer site 1	0.93 (0.79–1.08)
HPV E6 enhancer site 2	1.09 (0.91–1.31)
HPV E6 enhancer site 3	0.97 (0.87–1.07)
HPV L1	0.98 (0.96–1.01)
Human genome (n=151)	
DCC region 2.3	1.07 (0.99–1.17)
DCC region 2.4	1.27 (1.09–1.48)
DCC region 2.5	1.13 (1.00–1.27)
DCC region 2.6	1.11 (1.02–1.22)
DCC region 2.7	1.09 (1.00–1.17)
ESR1 region 3.1	0.99 (0.87–1.13)
ESR1 region 3.2	1.22 (1.04–1.42)
ESR1 region 3.3	1.01 (0.97–1.04)
p16 region 1	1.00 (0.93–1.08)
p16 region 2	1.05 (0.96–1.15)
p16 region 3	1.07 (0.82–1.41)
p16 region 4	1.17 (0.86–1.59)
LINE1 site 1.1	1.04 (0.93–1.17)
LINE1 site 1.2	1.24 (1.02–1.52)
LINE1 site 1.3	1.08 (0.94–1.24)
LINE1 site 1.4	1.06 (0.91–1.25)

^aResults of logistic regression models predicting abnormal cytology for a 1 percentage increase in methylation level run separately for each selected DNA methylation site; Each model is adjusted for HPV cofactors (age, lifetime cigarette smoking history, number of live births, age at first sexual intercourse, lifetime sex partners, history of oral contraceptive use)

^bHPV 16-positive women

Abbreviations: OR, odds ratio; CI, confidence interval