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Human Papillomavirus DNA methylation as a biomarker for cervical precancer: Consistency across 12 genotypes and potential impact on management of HPV-positive women

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Abstract

Purpose—Human papillomavirus (HPV) DNA methylation testing is a promising triage option for women testing HPV-positive during cervical cancer screening. However, the extent to which methylation indicates precancer for all 12 carcinogenic HPV types has not been evaluated.

Methods—In this nested case-control study, we tested up to 30 cases of precancer (CIN3/AIS) and 30 normal controls for each carcinogenic type (single infections with 16/18/31/33/35/39/45/51/52/56/58/59). Next-generation bisulfite sequencing was performed on CpG sites within the L1 and L2 genes. We calculated differences in methylation, odds ratios, and areas under the curve (AUC). Using a fixed sensitivity of 80%, we evaluated the specificity and the risk of CIN3/AIS for best performing CpG sites, and compared the performance of an explorative multi-type methylation assay with current triage strategies.

Results—Methylation was positively associated with CIN3/AIS across all 12 types. AUCs for the top sites ranged from 0.71 (HPV51 and HPV56) to 0.86 (HPV18). A combined 12-type methylation assay had the highest Youden's index (0.46), compared with cytology (0.31) and a 5-type methylation assay including only previously described types (0.26). The 12-type methylation

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assay had higher sensitivity (80% vs. 76.6%) and lower test positivity compared to cytology (38.5% vs. 48.7%). The risk of CIN3/AIS was highest for methylation positives and lowest for cytology- or HPV16/18-positives.

Conclusions—HPV DNA methylation is a general phenomenon marking the transition from HPV infection to precancer for all 12 carcinogenic types. Development of a combined multi-type methylation assay may serve as a triage test for HPV-positive women.

Keywords

Human papillomavirus; methylation; cervical cancer; screening; triage

INTRODUCTION

While vaccination against human papillomavirus (HPV) holds great long-term promise for the global prevention of cervical cancer, screening programs will remain the mainstay of cervical cancer prevention for decades to come. Worldwide, HPV DNA testing is gradually replacing cytology as the primary method for cervical cancer screening, based on its demonstrated superior sensitivity for detecting the main target of screening, i.e., cervical precancer, particularly cervical intraepithelial neoplasia grade 3 (CIN3).(1) Several features of HPV DNA testing make it particularly suitable for primary screening in both high-and low-resource settings, including its high reproducibility, sensitivity, cost-effectiveness, and the feasibility of testing in self-collected samples.(2) Moreover, a negative HPV test result provides significant long-term reassurance against the risk of developing CIN3/adenocarcinoma in situ (AIS) or cancer, allowing for extended screening intervals.(3) However, because most HPV infections are controlled successfully by the immune system and do not lead to cervical precancer or cancer, a major challenge is how to best manage women who test HPV-positive in primary screening.(4) In order to avoid unnecessary harms associated with excessive colposcopy referral and overtreatment, secondary triage tests are needed to distinguish benign, transient HPV infections from those that cause cervical precancers.(4,5) Current screening algorithms include the use of HPV16/18 genotyping and/or cytology for triage of HPV-positive women.(6) Other tests currently being evaluated include immunostaining for $p16^{INK4a}$/Ki-67(7), and automated cytology with or without partial HPV genotyping.(8) Quantitative assessment of DNA methylation of host and/or viral genes has also shown promise as a potential option for triage of HPV-positive women.(9,10) Unlike cytology and other morphological-based tests, an integrated, multi-type HPV DNA methylation assay would not require cytology infrastructure (i.e., no slide showing intact cervical cells would be needed), thus more amenable in self-collected specimens(11,12), and can be potentially applied as a reflex test to primary HPV screening.

We and others have previously shown an association of increased HPV DNA methylation in the late viral capsid genes, L1 and L2, with precancer in five important carcinogenic types including, HPV16, 18, 31, 33, and 45.(13–21) However, the extent to which DNA methylation is associated with cervical precancer for all other carcinogenic types has not been evaluated. We conducted a nested case-control study to evaluate the association of HPV DNA methylation in all 12 carcinogenic HPV types with cervical precancer and evaluated

the performance of an explorative, multi-type HPV DNA methylation assay for triage of HPV-positive women.

MATERIALS AND METHODS

Study Population

The HPV Persistence and Progression (PaP) Cohort is a repository of residual cervical specimens stored in specimen transport medium (STM; Qiagen, Valencia, CA), from women tested with Hybrid Capture 2 (HC2; Qiagen) during routine screening with co-testing (BD SurePath cytology and HC2) or HPV triage of Atypical Squamous Cells of Undetermined Significance (ASC-US) cytology at Kaiser Permanente Northern California (KPNC). Briefly, the PaP cohort consists of approximately 55,000 women 21 years of age and older who were screened between January 2007 to January 2011 and who had not opted out from having their residual specimen banked and tested for HPV-genotypes and related biomarkers. This study was conducted according to ethical guidelines and the principles embodied by the Declaration of Helsinki and has been approved yearly by the Institutional Review Board at KPNC and by the Special Studies Institutional Review Board at the National Cancer Institute.

De-identified age data and follow-up cytologic and histologic results were obtained from linked electronic health records, with outcomes available through 2014. Cytology results were reported using the Bethesda nomenclature(22), and categorized as Negative for Intraepithelial Lesion or Malignancy (NILM) or ASC-US or worse (ASC-US+).

For this nested case-control study, cases were defined as having cervical intraepithelial grade 3 (CIN3) or adenocarcinoma in situ (AIS) histology from colposcopy biopsies (mean follow-up=0.66 years) and controls did not have histologic abnormalities (less than CIN2 histology and benign, ASC-US or low-grade cytology, LSIL) as of the last follow-up (mean follow-up=0.65 years). All samples selected for both cases and controls were positive for a single carcinogenic HPV type (16/18/31/33/35/39/45/51/52/56/58/59); however, we did not require samples to be negative for non-carcinogenic types tested in HC2. Multiple samples were collected from women in PaP. For cases, we selected the sample immediately preceding the CIN3/AIS diagnosis when available. We focused on risk of CIN3/AIS rather than CIN2 because CIN2 may not accurately reflect true cervical precancer and is unreliably diagnosed by pathologists.(23–25) For a small subset (n=12) that did not have samples available at the time of diagnosis, we selected samples from a visit prior to the CIN3/AIS attributed screening visit. Control samples were matched for HPV type and selected from the last available screening visit. For most types, we identified 30 cases and 30 controls; however, numbers of CIN3/AIS cases with single type infections were limited for the following types: HPV35 (n=8), HPV39 (n=25), HPV56 (n=17), and HPV59 (n=9).

HPV Detection and Typing

HC2 testing was conducted as part of routine cervical cancer screening at KPNC per the manufacturer’s instructions. DNA was extracted from the banked STM specimens as previously described.(26) Typing was performed in a variety of laboratories over the course

of several investigations. Assays used included Cobas, Linear Array, Onclarity, and MY09/11 L1 degenerate primer PCR (MY09/11 PCR) with type-specific dot-blot hybridization.(26–28)

Bisulfite Conversion

DNA was isolated from cervical cells and treated with bisulfite using the Qiagen EpiTect Bisulfite Kit (Qiagen) as recommended by the manufacturer. Negative and positive controls, DNase-free water and SiHa (ATCC® HTB-35™) and HeLa (ATCC® CCL-2™) DNA, respectively, were processed as described for the clinical samples. Cells were maintained for 20 passages in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, tested negative by e-Myco™ Mycoplasma PCR Detection kit 2.0 (iNtRON Biotechnology, Kyungki-Do, North Korea) March 2017, and DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen). Following bisulfite treatment and subsequent PCR, unmethylated cytosines (C's) are converted to uridine (U's) and then thymidine (T's), whereas methylated C's remain unmodified.(9)

HPV DNA methylation assays

Several regions containing CpG sites within the L1 and L2 genes of the viral genome of 12 carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) were selected for quantitative site-specific CpG methylation using next-generation sequencing (NGS) assays. Methylation-specific primers (Integrated DNA Technologies IDT, Coralville, IA) for bisulfite converted DNA were designed using MethPrimer (http://www.urogene.org/methprimer/index1.html).(29) Primers were designed to amplify fragments comprising at least 3 CpG sites per selected region. Each forward primer contained a unique 12 base pair (bp) Golay barcode attached to the 5' end of the forward primer sequence(10) (see Bioinformatics section below). Reverse primers were also labeled with a 12 bp Golay barcode to distinguish each sample in downstream bioinformatics analyses while increasing the number of multiplexing combinations.

PCR was performed using the Qiagen PyroMark PCR Kit. Each PCR reaction contained 1 μL of bisulfite converted DNA (DNA concentration ranged from 1.5 ng/μl to 4.8 ng/μl), 2.5 mM $MgCl_2$, 1 × PyroMark Master Mix, 5 μM of forward and reverse primers in a total volume of 25 μL. The conditions and annealing temperatures for each assay were as follows: initial denaturation step at 95° C for 15 min followed by 50 cycles: 30 sec at 94° C; 30 sec at the optimized primer-specific annealing temperature (56° C to 58° C); 30 sec at 72° C, and final extension for 10 min at 72° C.

PCR products were evaluated by 3% gel electrophoresis stained with ethidium bromide to confirm the expected amplicon fragment size (range = 111–292 bp). Five μL of each amplified PCR product were pooled (i.e., independent pools per each PCR assay, n=21 assays), then purified by gel excision using Qiagen QIAquick Gel Extraction Kit according to manufacturer's guidelines, and used to create libraries for NGS.

Libraries were constructed using KAPA LTP Library Preparation Kit for Illumina Platforms (Kapa Biosystems, Boston, MA) according to manufacturer's instructions. Libraries were sequenced using 250 bp paired-end reads on an Illumina HiSeq2500 at the Albert Einstein

College of Medicine Epigenomics Core Facility (Bronx, NY). Overall, percent methylation of 113 CpG sites throughout all the selected HR-HPV types was tested in single reaction tubes.(13,16,17)

Bioinformatic Analysis

Illumina sequencing data files were first filtered for low quality reads using Trim Galore v0.4.1 (Babraham Bioinformatics; http://www.bioinformatics.babraham.ac.uk) for a minimum average Phred score of 20. (30) Trim Galore was used for trimming low-quality base calls at the 3′ end of the target amplicon, adapter removal, and reporting sequencing quality using FastQC software. After pre-processing of the Illumina sequencing files, short sequences with less than 20 bp were also filtered out. An in-house pipeline was used to extract CpG methylation information from the Illumina sequencing files by demultiplexing the reads using a novobarcode computer package (Novocraft Technologies Sdn Bhd) based on the 12 bp Golay forward and reverse barcodes uniquely assigned to each sample as previously described, and removal of extended primer sequences from the demultiplexed reads using Prinseq v0.20.4. (31) Determination of CpG methylation status was performed using Bismark v0.16.3 and Bowtie v2.2.3 (32,33) with samtools v1.2. Custom R v3.2.1(34) scripts were incorporated into the pipeline to process the Bismark output by: i) producing a methylation pattern for every sequenced molecule, indicating whether cytosines were methylated (+), unmethylated (−), missing (o) or in disagreement for paired-end reads (x); ii) generating counts of each unique pattern per sample (i.e., methyl-haplotype); and iii) calculating methylation percentages for each assayed cytosine by comparing the ratio of methylated C's to the total number of methylated and unmethylated (C + T). The ratio of "*C/(C+T)*" indicates the proportion of methylated cytosines at each CpG site for the assayed sample.

Statistical Methods

Reproducibility of intraplate duplicate PCR-positive samples for each assay (6% of samples per assay) was evaluated based on Pearson and Spearman correlation coefficients. Negative controls (DNase-free water) were processed along with regular samples to assess NGS "noise", ranging from 69.0 to 2,395.0 reads per plate. Any positive sample included for further analysis had at least 5x more reads than the plate-respective negative control (data not shown). Median site-specific CpG methylation levels in cases and controls were compared using the Mann-Whitney Rank Sum test for each HPV type. We used Spearman correlation coefficients to evaluate the relationship between age and methylation within types and to estimate the correlation of site-specific CpG methylation values within each type. We calculated the median Spearman rank coefficients within L1 and L2, respectively for each type. Methylation levels were categorized into tertiles for each site, using the distribution of methylation levels in control subjects. To determine the magnitude of association between site-specific CpG methylation levels and case status, odds ratio (ORs) with 95% confidence intervals (CI) were computed by dichotomizing the highest tertile versus the combined middle and low tertiles (i.e., T3 *versus* T2 + T1). Receiver operating characteristics (ROC) curves, and area under the curve (AUC) values were calculated for distributions of methylation levels in cases *versus* controls. We generated 1,000 bootstrap samples to estimate 95% CI's for AUC values. To account for multiple testing, p-values

within type were corrected using the Benjamini-Hochberg method, controlling for a false discovery rate at α=0.1. For cases with sufficient numbers, we also performed separate analyses by histology (AIS versus CIN3). Next, we calculated the specificity of DNA methylation testing at a fixed sensitivity of 80% for the best performing ("top") CpG site for each type, based on the highest AUC values. If multiple sites had the same AUC, we preferentially selected sites that have already been established as candidates in the literature, or sites that are homologous to other established candidates. To simulate the performance of a HPV DNA methylation assay in a screening population, we applied type-specific sensitivity and specificity estimates derived from the case-control sets to the approximately 30,000 women in the PaP cohort undergoing routine cervical screening. For this analysis, we assumed our sensitivity and specificity estimates from single-type infections would apply equally among the screening cohort of women with a combination of single- and multiple-type infections. We evaluated the risk of disease in methylation-positive women (i.e., the positive predictive value, PPV) and the risk of disease in women testing methylation-negative (i.e., the complement of the negative predictive value, cNPV). For comparison, we also calculated the type-specific 2-year risk of CIN3/AIS based on hierarchical type attribution in the PaP cohort, assuming our sensitivity and specificity estimates from single-type infections would apply equally among the screening cohort of women with a combination of single- and multiple-type infections. We compared these estimates to the 2-year risks of CIN3/AIS among the 30,000 women in PaP according to current management.(35) In HPV-positive women with ASC-US, a 2-year risk of 6.7% for CIN3+ was used as a benchmark for colposcopy referral and in HPV-positive women with NILM cytology, a risk of 3.9% is used was estimated as a benchmark for a one-year return.(4,35,36) We also simulated the potential performance of a 12-type combined HPV DNA methylation assay by fixing the overall sensitivity at 80% and within type, using the corresponding type-specific specificity to calculate the total number of true negatives across all types, which resulted in an overall specificity of 65.6%. We compared the performance of this combined methylation assay to Pap cytology (ASC-US+ versus NILM) and combined cytology and HPV16/18 genotyping (either ASC-US+ or HPV16/18 positive) in the population of 30,000 women. We also evaluated the performance of a combined methylation assay for the 10 most carcinogenic types, excluding HPV51 and HPV56. Additionally, as a validation step, we evaluated the performance of a combined methylation assay including only the 5 types that have been previously described in the literature (HPV16, HPV18, HPV31, HPV33, and HPV45). As a second validation analysis, we evaluated the 5-type combined methylation assay replacing our top sites with the top sites reported in the literature for which we had methylation data (HPV16 CpG#5611(16); HPV18 CpG#7041(13); HPV31 CpG#5524 (13); HPV33 CpG#6986 (15); HPV45 CpG#7135 (13)). For comparisons, Youden's index was calculated as sensitivity + specificity − 1. Analyses were performed in Stata version 14 (StataCorp, College Station, TX), all tests were two-sided with a significance level of $p < 0.05$.

RESULTS

A total of 299 cases and 360 controls were included in this analysis. The mean age of these HPV positive women was 41.4 years (range 21–72 years) and did not significantly differ by

case/control status, overall and within type (Table 1). Similarly, there was no significant correlation between age and CpG site methylation across any of the 12 HPV types, both overall and among cases and controls, respectively (data not shown). Methylation levels were not significantly associated with cytology, among cases and controls, respectively. The majority of controls had NILM cytology (65.9%), whereas the majority of the CIN3/AIS cases had ASC-US+ cytology (88.5%) (Table 1).

Validation of Methylation for HPV Types 16, 18, 31, 33, and 45

We confirmed significant associations of methylation levels with CIN3/AIS for nearly all CpG sites within HPV16, HPV18, HPV31, HPV33, and HPV45 (Supplementary Table 1). Similar to what has been previously reported, associations were strongest for HPV16, HPV18, and HPV45. For HPV16, all 9 CpG sites had AUC values ≥0.7, with the top site in the L1 gene reaching 0.84 (CpG#5602). All 13 of the CpG sites in HPV18 had AUC values ≥0.75, with the top four sites in the L1 gene reaching 0.86 (CpG#7038, #7041, #7116, and #7122) and 12 of the 15 CpG sites in HPV45 had AUC values ≥0.7, with the top site in the L1 gene reaching up to 0.82 (CpG#7088). For HPV31, methylation values were higher in cases compared with controls (MW p-value <0.05) for most sites; however, only 5 out of the 13 CpG sites had AUC values ≥0.7, with the top site in the L1 gene reaching 0.75 (CpG#6363). Methylation levels tended to be lower in both cases and controls for HPV33, and only 2 out of the 9 CpG sites had AUC values ≥0.7, with the top site in the L1 gene reaching 0.73 (CpG#7034). Correlation of methylation levels within HPV type is shown in Supplementary Table 2. Methylation levels were highly correlated within HPV16, HPV31, HPV18, and HPV45 for both L1 and L2. In contrast, we observed lower correlations between sites within HPV33, particularly within the L1 gene.

A total of 27 cases (9.6%) were diagnosed with AIS and were positive for the following HPV types: HPV18 (n=16), HPV45 (n=6), HPV16 (n=5). Among HPV16-positive cases, methylation levels were significantly higher at all CpG sites in AIS compared with CIN3 (Figure 1), and the AUC value for the top performing site (CpG#5602) was 0.99 compared with 0.82 for CIN3. Among HPV18-positive cases, methylation levels were higher in AIS compared with CIN3, though only site #7062 was statistically significantly different (Figure 1). The AUC value for the top performing site in HPV18 (CpG#7041) was 0.88 for AIS compared with 0.83 for CIN3. Finally, among HPV45-positive cases, methylation levels were significantly higher in AIS compared with CIN3 for all CpG sites within L1 ($p<0.01$) (Figure 1), and the AUC for the top performing site in HPV45 (CpG#7088) was 1.0 for AIS compared with 0.77 for CIN3.

Methylation of HPV35, 39, 51, 52, 56, 58 and 59

We observed strong associations of methylation levels with CIN3/AIS for the majority of sites within HPV35, HPV39, HPV52, HPV58, and HPV59 (Supplementary Table 1), the 7 carcinogenic HPV types that have not been previously evaluated. Among these types, the strongest associations were observed for HPV39 and HPV59 (alpha-7 HPV types), with all sites having AUC values ≥0.8, respectively, with the top site in the L1 gene in HPV39 reaching 0.85 (CpG#5731) and the top two sites in the L1 gene in HPV59 reaching 0.91 (CpG#5584 and #5618; Table 2). For HPV35, all but one of the 15 CpG sites had AUC

values ≥0.7, with the top site in the L2 gene reaching 0.81 (CpG#4244), and of the 8 sites analyzed for HPV52, all but one had AUC values ≥0.7, with the top three sites (2 in L2, 1 in L1) reaching 0.77 (CpG#4258, #4274, and #7098). For HPV58, methylation levels were significantly higher in cases compared with controls for most sites; however, only 4 out of the 10 CpG sites had AUC values ≥0.7, with the top site in the L1 gene reaching 0.74 (CpG#6446). By comparison, we observed weaker associations of methylation levels with CIN3/AIS for HPV51 and HPV56. For HPV51, only 1 out of the 5 sites had significantly higher methylation in cases compared with controls, and had an AUC of 0.71 (CpG#5533). Similarly, for HPV56, 3 out of 7 sites had significantly higher methylation in cases compared with controls, and one CpG site had an AUC of 0.71 (CpG#5570). Correlation of methylation levels within HPV type is shown in Supplementary Table 2. For HPV35, methylation levels were more strongly correlated within L1 compared with L2, whereas for HPV52 and HPV58, methylation levels were more strongly correlated within L2. Among types with only one gene assayed, methylation levels were highly correlated within the L1 genes of HPV39 and moderately correlated within the L2 genes of HPV51 and HPV56.

Performance of Type-Specific HPV DNA Methylation for Detection of CIN3/AIS

We evaluated the potential performance of DNA methylation in the top CpG sites for each HR-HPV type by applying type-specific sensitivity and specificity estimates to the full screening cohort of 30,000 HPV-positive women. In the full PaP cohort, the HPV type-specific risks of CIN3/AIS ranged from 1.1% for HPV59 to 19.8% for HPV16 (Figure 2). The risks of CIN3/AIS in DNA methylation positives were higher than the type-specific risks, particularly HPV16 and HPV18; however, we observed the most clinically useful risk-stratification for HPV52, HPV39, HPV45, and HPV59, with DNA methylation positivity increasing the type-specific risks above the HPV+/ASCUS risk threshold for colposcopy referral. Among women below the cutoff for HPV methylation positivity (i.e., “methylation negatives”), the risk of CIN3/AIS was below the threshold for a one-year return (HPV+/NILM) for all types, with the exception of HPV16 and HPV33 (Figure 2). Methylation did not provide clinically meaningful risk-stratification for HPV51 and HPV56.

Comparison of DNA Methylation Performance to Established Triage Algorithms

We compared the performance of DNA methylation to that of standalone cytology and combined cytology and HPV 16/18 genotyping by weighting back to the full PaP cohort. For this analysis, we simulated the performance of a combined 12-type methylation assay with an overall sensitivity of 80% and specificity of 65.6%. We also evaluated a combined 10-type methylation assay, excluding HPV51 and HPV56 based on their lower risk, and a combined 5-type methylation assay including only the 5 HPV types that have been previously described in the literature. As shown in Table 3, the risk of CIN3/AIS was highest in women testing positive for DNA methylation and lowest among those positive for either ASC-US+ cytology or HPV16/18. Compared with cytology alone, DNA methylation had lower test positivity at a slightly higher sensitivity. Although combined cytology and HPV16/18 genotyping showed somewhat higher sensitivity, nearly twice as many women tested positive and would be sent to colposcopy compared with DNA methylation at that threshold. The risk of CIN3/AIS was lowest among women testing negative for both cytology and HPV16/18 followed by methylation (12- and 10-type combined assays), and

was below the colposcopy referral threshold for all tests. Based on the Youden's index, which gives equal weight to sensitivity and specificity, the 12-type methylation assay ranked highest (Youden's index of 0.46), followed by the 10-type methylation assay (Youden's index of 0.43), cytology (Youden's index of 0.31) and the 5-type methylation assay (Youden's index of 0.26). Results from our validation analysis showed similar performance when we replaced our top CpG sites with top sites reported in the literature.

DISCUSSION

Successful implementation of primary HPV screening requires triage tests that can identify HPV-positive women with increased risk of cancer, while limiting colposcopy referral and overtreatment among those at lower risk. We observed a strong association of increased methylation with CIN3/AIS across all 12 carcinogenic HPV types, suggesting that DNA methylation is a general phenomenon in the transition from HPV infection to cervical precancer, although HR-HPV types do show some variability. Strong associations were particularly observed for alpha-7 genotypes, compared with alpha-9 types and in AIS compared with CIN3. The higher methylation observed for alpha-7 compared to alpha-9 types may be related to biologic differences between these clades, whereas the higher levels of methylation in glandular lesions may be due to a delayed detection of precancers higher up in the cervical canal that manifest clinically with bigger lesion size and greater levels of methylated HPV DNA. Type-specific DNA methylation showed good risk stratification for all HPV-positive women and improved performance compared with HPV16/18 genotyping and Pap cytology, suggesting that HPV DNA methylation has potential for clinical use as a triage test for HPV-positive women.

Our results provide independent validation of the association of elevated methylation in the L1 and L2 regions with risk of CIN3/AIS for HPV16, HPV18, HPV31, HPV33, and HPV45. We replicated associations for most CpG sites in HPV16 (16,18,21), HPV18 (13,15), HPV31 (13,15,18), and HPV45 (13), and some sites within HPV33 (15). Observed differences may be due to the use of different methylation assays (e.g., pyrosequencing versus next-generation bisulfite sequencing), study population characteristics, and/or case definitions (e.g., CIN2+ versus CIN3 in our study). Although the top performing sites in our current study did not always completely overlap with the best performing sites in prior studies, we observed similar results in our validation analysis using the top sites reported in the literature for the performance a combined methylation assay. This is supported by the relatively high correlation observed between sites within L1 and L2 within HPV type.

In an analysis weighted back to the entire PaP cohort of HPV-positive women, methylation testing demonstrated clinically useful risk stratification for CIN3/AIS for nearly all the carcinogenic types. In this population, the risk of CIN3/AIS in methylation-positive women far exceeded the HPV+/ASC-US risk threshold for all alpha-9 and alpha-7 HPV types. Moreover, for alpha-7 HPV types 39, 45, and 59, methylation testing provided additional risk stratification among women testing methylation-positive, whose risk exceeded the threshold for colposcopy referral, whereas the type-specific risks alone did not exceed this threshold. Among methylation-negative women, the risk of CIN3/AIS was lower than the risk for HPV+/NILM (one-year return) for alpha-9 types HPV35, 52, and 58, and all alpha-7

types. Because of the low risk associated with HPV51 and HPV56 infection (i.e., common in CIN3 lesions, but rarely progress to cancer) and the limited discrimination provided by methylation testing for these types, we did not observe clinically useful risk stratification for HPV51 and HPV56.

We simulated the performance of a combined DNA methylation assay covering 12, 10, and 5 carcinogenic types and compared these with currently recommended triage tests for HPV-positive women, namely HPV16/18 genotyping and Pap cytology. In our study, each combined methylation assay had a lower positivity and higher specificity compared with cytology and combined cytology and HPV16/18 genotyping, with correspondingly higher risk in methylation positives compared with cytology and combined cytology and HPV16/18 genotyping, and lower risk in methylation negatives for a combined 12-and 10-type assay compared with cytology alone.

Introduction of primary HPV screening offers the possibility of testing in self-collected samples; however, triage remains a critical issue, particularly in low-resource settings where infrastructure for screening and treatment are limited.(2) Of all the triage tests currently under consideration, only methylation has demonstrated feasibility in self-collected specimens.(5) While the current literature has focused on host gene methylation(11,12,37–39), it is reasonable to assume that viral DNA methylation would have similar performance. Collectively, our findings support the development of a combined methylation assay that pools individual CpG sites across multiple high-risk HPV types into one integrated test. A multi-type HPV DNA methylation assay could potentially serve as a point-of-care test, providing integrated HPV test results, genotyping, and methylation information all derived from the same specimen.(10) As a critical next step, we plan to develop and evaluate a multi-type HPV methylation assay for the clinical management of HPV-positive women in a large screening population. Development of such an assay will require important considerations regarding the selection of a parsimonious set of discriminatory sites and optimization of methylation cutpoints, as well as a better understanding of the long-term predictive value of HPV methylation in prospective studies. It will also be important to determine whether the addition of candidate host methylation markers can improve risk stratification by providing independent information beyond HPV DNA methylation.

To our knowledge, this is the first study to evaluate the performance of DNA methylation testing for the 12 most important carcinogenic HPV types. Our study was nested in a large population of HPV-positive women undergoing routine screening with cytology and HPV co-testing, enabling sufficient statistical power for our analyses. We carried out HR-HPV DNA methylation testing using highly reliable next-generation bisulfite sequencing assays with high reproducibility, and evaluated the risk of histologically confirmed CIN3/AIS, the most reproducible and clinically meaningful endpoint for cervical screening. We successfully replicated findings for 5 previously analyzed HPV genotypes. Some important limitations are also worth noting. First, despite being nested in a large screening cohort, our study was cross-sectional in design, and the number of cases with single-type infections was limited for certain types (e.g., HPV35 and HPV59). While this design does not permit an evaluation of the long-term risk prediction of these DNA methylation markers, it demonstrates the potential of DNA methylation as an immediate triage assay for CIN3/AIS

detection among HPV-positive women. Second, we did not conduct a formal replication study to validate associations for the seven new types. However, for all types, we selected only a limited number of CpG sites within the L1 and L2 genes informed by previous findings (18)). Thus, for all types, we had strong priors that these sites would be associated with disease status. Our analysis demonstrates a proof-of-principle that we intend to validate in a larger prospective study of HPV-positive women. Finally, while we restricted our analysis to single-type infections to limit the challenges of type attribution when multiple types are present(40), it is possible that a type-specific DNA methylation assay may perform differently in women with multiple infections. Previously, Wentzensen *et al.* evaluated methylation levels in cases and controls infected with multiple versus single carcinogenic HPV types and found that among women infected with multiple types, higher methylation may help to distinguish which of the infections is causal.(13) In line with these findings, Vasiljević et al., found that methylation in HPV18 and HPV31 is higher in cases with a single infection compared with multiple infections(15). It is possible that certain type-specific associations of methylation with case-control status may be attenuated in women with multiple infections if the causal type was misattributed. However, we expect a combined methylation assay to have similar performance irrespective of whether a woman is infected with one or multiple HPV types.

In conclusion, we evaluated the performance of HPV DNA methylation across 12 carcinogenic HPV types for the triage of HPV-positive women. Our findings suggest that a methylation assay targeting the most important carcinogenic HPV types has the potential to serve as a triage test for HPV-positive women in high-resource settings. If such an assay can be developed as a one-tube reaction, there could be important applications for screening and triage in low-resource settings as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIS	adenocarcinoma in situ
ASC-US	atypical squamous cells of undetermined significance
CIN	cervical intraepithelial neoplasia
HPV	human papillomavirus
NILM	negative for intraepithelial lesion or malignancy

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TRANSLATIONAL RELEVANCE

Human papillomavirus (HPV) testing is the most sensitive method for cervical screening, but its use is limited by lack of specificity, i.e., the high numbers of women testing positive. Previous studies have shown that cervical HPV DNA methylation testing is a promising triage option for women testing positive for certain types of HPV; however, the previous results were restricted to only a few HPV types. Our analysis demonstrates that the association of increased DNA methylation with cervical precancer is a general phenomenon, common to all 12 carcinogenic HPV types. HPV DNA methylation showed good risk stratification for precancer and improved performance compared to the most commonly recommended triage strategy (HPV16/18 genotyping and Pap cytology), suggesting that HPV DNA methylation has potential for clinical use as a triage test for HPV-positive women. Collectively, our findings support the development of a combined methylation assay that pools individual CpG sites across multiple high-risk HPV types into one integrated test.

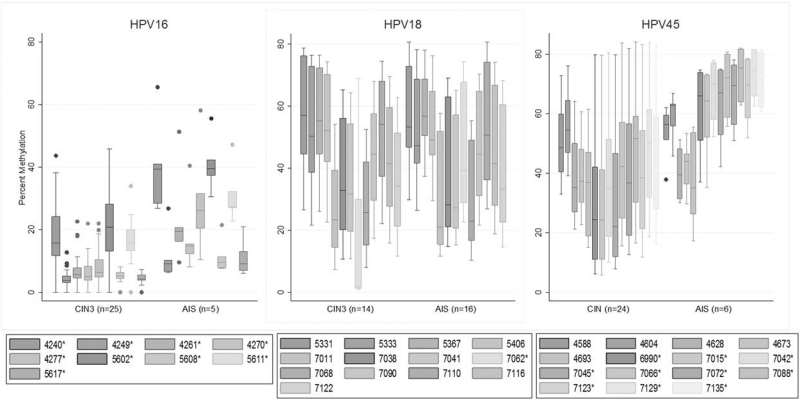
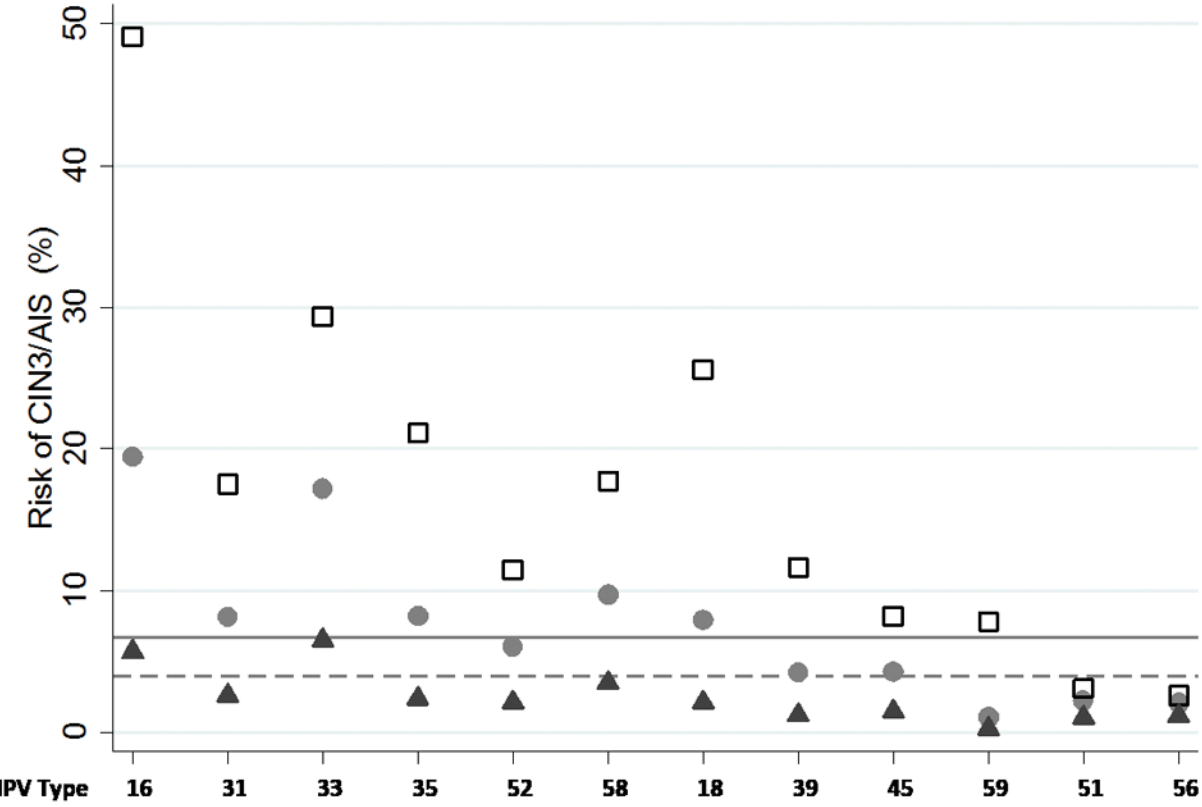


Figure 1. Plot of the distribution of methylation levels in CIN3 versus AIS in cases positive for HPV16, HPV18, or HPV45

Percent methylation is shown on the y-axis. Each colored box represents a CpG site position, with asterisks indicating Mann-Whitney p-value <0.05.

Abbreviations: HPV, human papillomavirus; CIN3, cervical intraepithelial neoplasia grade 3; AIS, adenocarcinoma in situ



Gene	L1	L1	L1	L2	L2	L1	L1	L1	L1	L1	L2	L1
CpG Site	5602	6363	7034	4244	4258	6446	7041	5731	7088	5584	5533	5570
Cases (n)	1,107	274	65	62	238	109	149	50	61	10	59	25
Controls (n)	4,592	3,098	314	691	3,686	1,012	1,732	1,131	1,359	916	2,628	1,207
Specificity	80.0%	66.7%	60.0%	73.3%	60.0%	60.0%	80.0%	73.3%	60.0%	90.0%	43.3%	36.%
AUC	0.84	0.75	0.73	0.81	0.77	0.74	0.86	0.85	0.82	0.91	0.71	0.71

Figure 2. Type-Specific Clinical Performance of DNA Methylation for Detection of CIN3/AIS

The risk of CIN3/AIS for each HPV type and top CpG site was calculated by weighting back to the full PaP cohort of HPV-positive women. Type specific risks were derived from the PaP cohort data. For methylation, sensitivity (fixed at 80%) and the corresponding type-specific specificity were used to estimate the risk of CIN3/AIS among women testing methylation positive at each site. Similarly, these estimates were used to calculate the risk of CIN3/AIS among women testing negative for DNA methylation. The solid line indicates the risk of CIN3/AIS for HPV+/ASC-US and the dashed line indicates the risk of CIN3/AIS for HPV+/NILM in the PaP cohort. Solid circles = HPV-type specific risk, hollow squares = type-specific risk in methylation positives, and solid triangles = type-specific risk in methylation negatives.

Abbreviations: HPV, human papillomavirus; AIS, adenocarcinoma in situ; AUC, area under the curve

Table 1

Distribution of Age and Cytology in Cases and Controls, by HPV Type

	Cases (n)	Controls(n)	Mean (SD) Age Cases	Mean (SD) AgeControls	CytologyCases, n (%)		CytologyControls, n (%)	
					NILM	ASC-US+	NILM	ASC-US+
HPV Type								
Alpha-9	158	180	41.0 (10.2)	41.9 (11.6)	18 (11.5)	139 (88.5)	118 (65.9)	61 (34.1)
16	30	30	41.6 (9.9)	37.2 (8.8)	3 (10.0)	27 (90.0)	21 (70.0)	9 (30.0)
31	30	30	41.5 (10.4)	44.6 (11.6)	6 (20.0)	24 (80.0)	17 (56.7)	13 (43.3)
33	30	30	41.2 (11.0)	44.7 (13.2)	4 (13.8)	25 (86.2)	18 (60.0)	12 (40.0)
35	8	30	47.0 (13.5)	44.5 (12.3)	1 (12.5)	7 (87.5)	21 (70.0)	9 (30.0)
52	30	30	39.4 (9.4)	37.5 (9.2)	2 (6.7)	28 (93.3)	21 (70.0)	9 (30.0)
58	30	30	39.5 (9.1)	42.7 (11.7)	2 (6.7)	28 (93.3)	20 (66.7)	10 (33.3)
Alpha-7	94	120	39.6 (8.7)	41.4 (11.7)	12 (12.8)	82 (87.2)	79 (68.1)	37 (31.9)
18	30	30	39.4 (10.5)	39.6 (11.3)	5 (16.7)	25 (83.3)	19 (65.6)	11 (44.4)
39	25	30	37.8 (8.3)	37.3 (7.8)	4 (16.0)	21 (84.0)	16 (57.1)	12 (42.9)
45	30	30	41.6 (7.4)	44.9 (12.9)	2 (6.7)	28 (93.3)	18 (60.0)	12 (40.0)
59	9	30	38.7 (7.4)	44.0 (13.0)	1 (11.1)	8 (88.9)	26 (86.7)	4 (13.3)
Alpha-5								
51	30	30	39.2 (12.3)	41.6 (10.4)	4 (13.8)	25 (86.2)	18 (60.0)	12 (40.0)
Alpha-6								
56	17	30	51.1 (12.7)	44.7 (12.7)	3 (17.7)	14 (82.3)	10 (35.7)	18 (64.3)

Abbreviations: HPV, human papillomavirus; SD, standard deviation; NILM, negative for intraepithelial lesion or malignancy; ASC-US+, atypical squamous cells of undetermined significance or worse

Table 2

Type-Specific Associations of HPV DNA Methylation with Cervical Precancer for the Top CpG Sites

HPV Type	Gene	CpG Site	Controls Median (IQR)	Cases Median (IQR)	OR (95% CI)	p-value	AUC	BS 95% CI[a]
16	L1	5602	13.37 (5.7)	27.89 (13.3)	13.00 (3.5 – 47.6)	0.0001	0.84	0.74–0.95
31	L1	6363	17.09 (13.2)	25.95 (11.6)	18.00 (4.4 – 74.0)	<0.0001	0.75	0.62–0.88
33	L1	7034	0.93 (0.5)	1.26 (0.7)	4.67 (1.6 – 13.9)	0.0056	0.73	0.60–0.86
35	L2	4244	2.19 (2.8)	6.61 (7.3)	14.00 (1.5 – 130.0)	0.0203	0.81	0.66–0.97
52	L2	4258	22.93 (17.0)	39.20 (20.0)	5.50 (1.8 – 16.7)	0.0026	0.77	0.66–0.89
58	L1	6446	12.20 (7.6)	16.52 (13.9)	5.50 (1.8 – 16.7)	0.0026	0.74	0.61–0.86
18	L1	7041	14.60 (5.3)	29.20 (34.8)	18.00 (4.4 – 74.0)	0.0001	0.86	0.76–0.96
39	L1	5731	22.24 (15.1)	34.92 (19.5)	23.00 (4.5 – 117.6)	0.0002	0.85	0.74–0.95
45	L1	7088	23.72 (16.5)	55.26 (28.7)	6.57 (2.1 – 20.5)	0.0012	0.82	0.71–0.93
59	L1	5584	4.69 (9.6)	26.56 (11.5)	16.00 (1.8 – 146.3)	0.0141	0.91	0.82–1.00
51	L2	5533	10.56 (5.6)	18.57 (16.5)	4.00 (1.4 – 11.7)	0.0114	0.71	0.56–0.84
56	L1	5570	8.92 (6.0)	17.80 (11.7)	3.67 (1.1 – 12.8)	0.0418	0.71	0.55–0.87

[a]We generated 1,000 bootstrap samples to estimate 95% CI's for AUC values

Abbreviations: AIS, adenocarcinoma in situ; ASC-US+, atypical squamous cells of undetermined significance or worse; AUC, area under the curve; BS, bootstrap; CI, confidence interval; CIN, cervical intraepithelial neoplasia (grade 2 or 3); HPV, human papillomavirus; IQR, interquartile range; MW, Mann-Whitney; NILM, Negative for Intraepithelial Lesion or Malignancy; OR, odds ratio

Table 3

Comparison of the Performance Established Triage Tests with a Combined 12-Type DNA Methylation Assay

	Test Positivity	Sensitivity	Specificity	Youden's Index	Risk in Positives	Risk in Negatives
Cytology	48.7%	76.6%	54.1%	0.31	14.3%	4.2%
Cytology OR HPV16/18	63.7%	89.9%	38.8%	0.29	12.8%	2.5%
Methylation 12 Types	38.5%	80.0%	65.6%	0.46	18.9%	3.0%
Methylation 10 Types	29.0%	77.0%	65.7%	0.43	23.9%	3.3%
Methylation 5 Types	17.5%	60.0%	65.7%	0.26	30.8%	5.7%
Methylation 5 Types with Validation Sites	20.5%	60.0%	62.4%	0.22	26.3%	6.0%

We calculated the overall specificity of a combined 12-type DNA methylation assay by fixing the sensitivity at 80% for each type, and used the resulting type-specific specificity (shown in Figure 2) to estimate the total number of true negatives across all types. Cytology positive is defined as ASC-US+, Cytology or HPV16/18 is positive if either ASC-US+ OR HPV16+ and/or HPV18+. As a sensitivity analysis, we also evaluated the performance of a 10-type HPV methylation assay (excluding HPV51 and HPV56) and a 5-type methylation assay (HPV16/18/31/33/45). As a validation step, we evaluated the top 5 sites reported in the literature (i.e., "validation sites", HPV16 CpG#5611; HPV18 CpG#7041; HPV31 CpG#5524; HPV33 CpG#76986; HPV45 CpG#7135).

Abbreviations: HPV, human papillomavirus