Methylation of twelve CpGs in human papillomavirus type 16 [HPV16] as an informative biomarker for the triage of women positive for HPV16 infection

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Abstract

An accurate biomarker for the follow-up of women positive for human papillomavirus type 16 [HPV16] DNA may improve the efficiency of cervical cancer prevention. Previously we analyzed all 113 HPV16 CpGs in cervical cytology samples and discovered differential methylation at different stages of premalignancy. In the current study, we identified a methylation biomarker consisting of a panel of twelve HPV16 CpG sites in the E5, L2 and L1 open reading frames [ORFs], and tested whether it fulfilled three necessary conditions of a prospective biomarker. A total of 33 cytology samples from North American and West West African women with all grades of cervical intraepithelial neoplasia [CIN] and invasive cervical cancer [ICC] were analyzed by using DNA bisulfite-sequencing. The results showed [1] a highly significant trend for increasing HPV16 biomarker methylation with increasing histologic severity [P<0.0001], [2] 100% sensitivity for invasive cervical cancer [ICC] over a wide range of methylation cutoff scores; 80% detection of CIN3 at cutoff scores up to 39% methylation, and [3] substantially lower detection of CIN2, from 0% to 71%, depending on the cutoff score. Our results support the prognostic potential of the HPV16 methylation biomarker for the triage to colposcopy of women with HPV16-positive screening tests and, eventually, for the management of women with HPV16-positive CIN2.
Introduction

Over the last 50 years there has been a dramatic decrease in the incidence of invasive cervical cancer in the developed world. This decrease is largely thought to be the result of routine cytology-based screening for, and treatment of, women with lesions considered the immediate precursors of invasive cervical cancer [ICC], termed cervical intraepithelial neoplasia grade 2 or 3 [CIN2,3]. It is now widely agreed that infection with one or more of the 13 high-risk [HR] human papillomaviruses [HPVs] is central [and necessary] to the pathogenesis of ICC and its precursor lesions. Recently developed prophylactic vaccines, which prevent infection with the HPV types responsible for the majority of ICCs - HPV16 and 18, will clearly be important for cervical cancer control in the future (1). However, given that the currently available vaccines are not therapeutic for pre-existing HPV16 or HPV18 infections (2), they will not benefit the millions of women who are already infected.

The shortcomings of cytology-based screening are well described. They include the lack of reproducibility and the low sensitivity of a single cytologic smear, ranging from 30% to 60%, for the detection of CIN2,3+ (3-6). Prior to the widespread use of HPV DNA-based triage of atypical squamous cells of uncertain significance [ASCUS], women with any abnormal Pap findings [ASCUS or Low or High Grade Squamous intraepithelial lesions - LSIL or HSIL] were required to undergo further testing by repeat cytology and/or colposcopy and biopsy. It was deemed necessary to refer women with ASCUS to colposcopy because 7-20% of such women had been found to have underlying CIN2,3. Once it became clear that high-risk [HR] HPV infection was central to the development of all ICC, HR-HPV-based triage of ASCUS was integrated into cytology-based screening to decrease the number of women unnecessarily referred to colposcopy.

The increased sensitivity for invasive cervical cancer and high-grade CIN conferred by HR HPV DNA-based testing (7) led to its incorporation into primary cervical cancer screening. The American Society for Colposcopy and Cytopathology [ASCCP], American Society for Clinical Pathology [ASCP] and American Cancer Society [ACS] recently revised the cervical cancer screening guidelines and included genotyping for...
HPV16/18 (8). The guidelines stipulate that women between the ages of 30 and 65 should have both a Pap test and an HPV DNA test every 5 years. Those who are Pap-negative and HPV DNA-positive should be further screened for HPV16 and HPV18 DNA [or re-screened by cytology in 12 months]. In women between the ages of 21 and 29, an initial cytology result of ASCUS necessitates HPV DNA testing, which can include HPV16 and HPV18 [HPV16/18] genotyping (8). All women with HPV16/18-positive tests should be referred for immediate colposcopy (8). These practices can be expected to increase the colposcopy load considerably. They also can be expected to increase the false-negative rate of colposcopy, since most women with HPV16/18 are at low risk of CIN3/ICC (9-13). CIN2 is thought to be a mixture of benign CIN1 and premalignant CIN3 lesions (14), but because the CIN2 subtypes are histologically indistinguishable, most women with CIN2 are treated, like women with CIN3. Unnecessary colposcopy can lead to CIN overdiagnosis [misclassification] and overtreatment (7), which is not inconsequential because therapeutic intervention increases the risk of pre-term delivery in subsequent pregnancies (15, 16).

The over-diagnosis of clinically non-relevant CIN2 and CIN3 is now the major limitation in implementing HPV DNA-based screening, particularly among women between the ages of 25 and 35 years. While a number of potential biomarkers have high sensitivity for CIN2 and CIN3 (17), including HPV DNA methylation biomarkers (18), none thus far appears to distinguish HPV16/18-positive lesions at high vs. minimal of short-term risk of malignant progression. The development of a prognostic biomarker for this purpose would therefore be of great interest (19). DNA methylation biomarkers offer tremendous potential for the early detection of precancer and cancer. DNA methylation is involved in the epigenetic regulation of development and differentiation. During carcinogenesis, the process is progressively dysregulated, beginning at the earliest stages of neoplasia. Many HPV DNA methylation studies have focused on the viral long control region [LCR] and 3’ terminus of the L1 open reading frame [ORF] and have generally reported that the LCR sites were minimally methylated in most lesions, while the L1 sites were methylated more frequently in invasive cervical cancer than premalignant lesions (20-31). Other studies have reported an inverse relationship between elevated methylation in the E6 ORF and high-grade CIN (32, 33).

Previously, we mapped the methylation status of all 113 CpGs in HPV16 genomes in cellular samples from all grades of premalignant cervical lesions and discovered multiple CpGs that were methylated
at higher frequency in high-grade lesions (34). In the same year, another group mapped full-length HPV16 and HPV18 methylomes and reported elevated CpG methylation in the L1 and L2 [L1/L2] ORFs in invasive cervical cancer relative to premalignant lesions; that study did not however identify specific CpGs with differential methylation (35). More recently, additional studies have confirmed the association between elevated methylation at specific HPV16 CpGs in the L1/L2 ORFs in women with ≥CIN2 relative to women with lower-grade lesions or infections that cleared (18, 36-38).

Given the number of the differentially methylated HPV16 CpGs in our original study (34) and subsequent studies (18, 36-38), we hypothesized that a clinically useful biomarker could be developed based on fewer CpGs. In this proof-of-principal study we identified, through further analysis of the original data, a candidate biomarker consisting of twelve CpGs located in the HPV16 E5, L2 and L1 ORFs. The aim of the study was to evaluate whether this biomarker fulfilled three necessary conditions of a prognostic biomarker: [1] the level of positivity must correlate with the histologic severity, [2] the sensitivity for ICC must be virtually 100%, and [3] the detection rate for CIN2 must be substantially lower than 100%. The results show that our HPV16 methylation biomarker fulfilled all three conditions.

Materials and Methods

Clinical cytology samples and sources

The human institutional review boards [IRB] at Yale University, the University of Washington and the University of Dakar, Senegal, approved the protocols for this study. The Yale IRB waived written consent for this study because it presented no more than minimal risk or harm and involved no procedures for which written consent is normally required outside of the research context. Written consent was obtained from all West African patients in the study conducted jointly by the University of Washington and University of Dakar.

The eligibility criteria for the study were an HPV16 positive DNA test and a corresponding biopsy-confirmed diagnosis. From two populations of women, residual cervical cytology samples were obtained. The first population consisted primarily Caucasian women of middle to upper socioeconomic status, who were being routinely screened for cervical cancer by private gynecologists in the greater New Haven area, by the
Cytology Service of the Department of Pathology at Yale University [n=21]. The second population consisted of West African women who had never been screened for cervical cancer and who were attending a community health clinic in the greater area of Dakar, Senegal, West West Africa, for reasons unrelated to cervical cancer [n=12]. Those samples had been collected for prior studies of HPV and cervical cancer by one of the authors [NBK]. Biopsies from the American women were obtained within three months of the cytology sample, same day biopsies were obtained from the West African women. The mean age of the North American patients was 34 years [range, 23 to 65 years] and the West African patients, 44 years [range, 27 to 60 years].

The presence of HPV16 was confirmed in all 33 samples by PCR, as described previously (34). The HPV16 methylation data for the 33 new cases were obtained and initially analyzed without knowledge of the clinical data. The cytopathology and histopathology of the North American samples were reviewed by authors MH and AL, and the West African samples, by author NBK. The cytology was reported as negative, atypical squamous cells of undetermined significance [ASCUS], low-grade squamous intrap epithelial lesion [LSIL], high-grade SIL [HSIL], invasive cervical cancer [ICC], or unsatisfactory for evaluation [Unsat]. The histology was diagnosed as cervical intraepithelial neoplasia grade 1 [CIN1], grade 2 [CIN2], grade 3 [CIN3] or invasive cervical carcinoma [ICC].

**DNA bisulfite-sequencing and DNA sequence analysis**

The laboratory personnel were blinded to the clinical data. Sample DNAs were purified using the MasterPure™ DNA Purification kit [Epicentre Biotechnologies, Madison, WI] and bisulfite-converted using the EZ DNA Methylation-Gold Kit™ [Zymo Research, Irvine, CA]. The bisulfite-converted DNAs were PCR-amplified in a 30 ul reaction containing 1x AmpliTaq Gold® buffer 1, 0.25mM MgCl₂, 0.25 mM each dNTP, 0.1 uM each primer, 3 units AmpliTaq Gold® DNA polymerase [Invitrogen, Carlsbad, CA] and 0.3 units Pfu DNA polymerase [Promega, Madison, WI]. The twelve CpG sites were amplified in four bisulfite-PCR reactions using the primers listed in Table 1. The PCR profile was: 95°C for 10 mins, followed by five cycles of 95°C for 1 mins, 55°C for 2 mins, 72°C for 3 mins, followed by 35 cycles of 95°C for 1 mins, 60°C for 2 mins, 72°C for 3mins, and then 4°C; the 72°C steps were carried out at a ramp speed of 25%.
Each target sequence was amplified in at least one PCR reaction, and each amplicon was purified and sequenced in both directions by Beckman Coulter Genomics [Danvers MA]. Positive control PCRs were performed using in vitro methylated and bisulfite-converted HPV16 plasmid DNA. The DNA sequence reads were aligned against the HPV16 W12E isolate [GenBank accession no. AF125673] using the multiple sequence alignment program Clustal Omega [http://www.ebi.ac.uk/Tools/msa/clustalo/]. Virtually all CpGs in the positive control showed 100% methylation. For statistical analysis, we calculated the methylation score for each sample as follows: First, we counted each CpG with "C" in at least one DNA sequence read was counted as methylated; second, we estimated the percentage of HPV16 genomes with methylation at each CpG, by averaging all DNA sequence reads and multiplying by 100; finally, we averaged all CpG values per sample.

Statistical analysis

The primary goal of the present study was to assess whether a select subset of HPV16 CpGs had the potential to serve as a prognostic biomarker for cervical lesions at high vs. minimal short-term risk of malignant progression, using the different grades of CIN and ICC as surrogate markers of the risk of progression. First we re-analyzed the data from our previous study (34) to identify HPV16 CpGs that were methylated significantly more frequently in CIN2 or CIN3 than CIN1, using the Mann-Whitney test (39). To establish whether there was a relationship between HPV16 CpG methylation and histologic severity, we evaluated the methylation score across histologic severity of lesions using the Jonckheere-Terpstra test (40), which is a non-parametric test for ordinal outcomes such as histologic diagnoses of increasing severity. The Jonckheere-Terpstra test was then used to reexamine the original data after stratification into three groups, with CIN1 or CIN2 or CIN3.

The sensitivity of a clinical biomarker for cancer is commonly assessed by the balance between its true-positive and false-positive rates at all possible cutoff points, using receiver operator characteristic [ROC] curves. We used the ROC curve to evaluate the sensitivity of the biomarker for ICC. We also used ROC curves to evaluate the detection rates ["sensitivities"] for CIN3 and CIN2, which were expected to be lower and substantially lower, respectively, than the sensitivity for ICC.
Analyses were conducted using SAS 9.2 [Cary, NC] and R-statistical package (41). Statistical significance was established at alpha ≤0.05.

Results

Selection and analysis of a panel of HPV16 CpGs as a methylation biomarker for cytology-based evaluation of histologic severity

To select a small number of HPV16 CpGs as a candidate biomarker for analysis in this study, we reanalyzed our original data for the lesions with biopsy-confirmed diagnoses (34). This analysis identified multiple CpGs with differential methylation, from which we selected a panel of twelve CpGs that were methylated significantly more frequently in high-grade CIN2 or CIN3 [CIN2/CIN3] than in low-grade CIN1 [P=0.0007] [Figure 1A]. These CpGs are located in the E5, L2 and L1 ORFs.

For the new set of cytology samples, DNA methylation was determined by bisulfite-sequencing using four PCR primer pairs [Table 1]. For each sample, a biomarker methylation score, estimating the percentage of HPV16 genomes with methylation across the twelve CpGs, was calculated as described in the Methods section. All amplicons were successfully amplified from each sample DNA, and readable DNA sequence reads were obtained for 386 of the 396 HPV16 CpGs in the 33 samples [97.5% complete data].

Trend for increasing HPV16 biomarker methylation with increasing histologic severity

To evaluate whether the percent methylation of the biomarker increased with the histologic severity of cervical neoplasia, we stratified the methylation data by the histologic diagnoses. Statistical analysis of the stratified data showed that the HPV16 methylation score increased progressively and markedly with the histologic severity, from a mean of 6.6% in low-grade CIN1, to 20.1% in CIN2, 54.2% in CIN3 and 88.2% in invasive cervical cancer [ICC] [P<0.0001] [Table 2]. Thus the level of biomarker methylation correlated with the histologic severity of all grades of CIN and ICC, one of the necessary conditions of a prognostic biomarker. These results together with the demographic diversity of the clinical samples, from American to African women, indicates that the trend for increasing biomarker methylation to parallel increasing histologic severity is a general feature of cervical HPV16 infections.
In light of these results, we reexamined the data from our original study, this time stratifying the cases into three groups, with CIN1 or CIN2 or CIN3. As shown in Figure 1B, the frequency of methylation was highest for CIN3, followed by CIN2 and, finally, CIN1. Thus, without consciously selecting the 12 HPV16 CpGs for their ability to distinguish all three grades of CIN, this analysis showed that they did so.

**Receiver operator characteristic curves for ICC, CIN3 and CIN2**

To evaluate the biomarker's sensitivity for ICC and its rates of detection of CIN2 and CIN3, we measured its ability to discriminate among the different states of neoplasia at all possible cutoff values, using receiver operator characteristic [ROC] curves. The disease states were ICC, CIN3 and CIN2. The specificity reference was CIN1, which is benign. The cutoff values were the methylation scores. As shown in Figure 2, the ROC curves for ICC, CIN3 and CIN2 exhibited decreasing strengths of discrimination, with areas under the curve [AUCs] of 1.00, 0.82 and 0.72, respectively. These results reinforced the trend results reported above.

**Cutoff scores for a positive HPV16 methylation biomarker test**

If a prognostic HPV16 methylation biomarker assay is to be developed for clinical translation, a cutoff score with optimal ability to discriminate between neoplastic lesions at high vs. minimal short-term risk of progression will be chosen. Since the actual clinical endpoints in this cross-sectional study were not known, the grades of CIN and ICC were used as surrogate markers of the risk of progression. At a cutoff score of 32% methylation, the biomarker detected 100% of ICC, 80% of CIN3 and 29% of CIN2 lesions [Table 3]. The assay's stringency could also be altered. For example, lowering the cutoff score to 10% methylation increased the detection rate for CIN2 to 71%. This change also reduced the assay's specificity, which nonetheless remained quite high, at 75% [Table 3]. Conversely, raising the score to 42% methylation reduced the detection rate for CIN2 to 0%. Importantly, the cutoff score could be raised to as high as 75% methylation without affecting the 100% sensitivity for ICC.

**Implications for colposcopy triage**
If used for the immediate follow-up of women with HPV16 DNA-positive screening tests, an HPV16 DNA methylation assay would triage to colposcopy the subset of women with biomarker methylation above a chosen cutoff score. At the 32% methylation cutoff, the HPV16 methylation biomarker would triage 39% of the women in our study for colposcopy, while detecting 100% of ICCs and 80% of CIN3 lesions. Even at the low cutoff of 10% methylation, the assay would refer just 55% of the women.

Discussion

Previously we discovered multiple specific HPV16 CpGs that were methylated at increasing frequency in premalignant cervical lesions of increasing pathologic severity (34). Here, we further analyzed the original data and selected a subset of twelve HPV16 CpGs as a candidate prognostic biomarker for improved cervical cancer prevention. New samples of exfoliated cervical cells from 33 North American and West African women with biopsy-confirmed diagnoses were analyzed by DNA bisulfite-sequencing. Using a cross-sectional design, we evaluated three necessary conditions of a prognostic biomarker: [1] strong correlation between the level of biomarker positivity and histologic severity, [2] virtually 100% sensitivity for ICC, and [3] a rate of detection of CIN2 substantially below 100%.

We found a highly significant trend between increasing HPV16 biomarker methylation and increasing histologic severity. The methylation score rose from 6.6% to 20.1% to 54.2% to 88.2%, respectively, as the histopathology rose from CIN1 to CIN2 to CIN3 to ICC [P<0.0001]. Thus the biomarker that we identified by analyzing premalignant cervical lesions (34) was methylated at even higher frequency in ICCs. An analogous trend was found by analyzing ROC curves, using a different statistical approach. The demographic diversity of the women from whom the samples were obtained, from North American to West African, together with the consistency of the results, indicate that the correlation between increasing methylation and increasing histologic severity are universal characteristics of cervical HPV16 infections. Moreover, they show that the HPV16 methylation biomarker fulfilled the first condition required of a prognostic biomarker.

The ROC curves allowed us to evaluate the biomarker's sensitivity for ICC and its rates of detection for CIN2 and CIN3 at all possible cutoff scores. Importantly, at cutoff scores as high as 75% methylation, the biomarker sustained 100% sensitivity for ICC, the second condition required of a prognostic biomarker. The
detection of CIN2, in contrast, varied from 0% to 71% at cutoff scores ranging from 42% to 10% methylation, respectively. Because the biomarker detected substantially fewer than 100% of CIN2 cases, it also fulfilled the third condition required of a prognostic biomarker.

CIN3 is generally more likely to progress to malignancy than CIN2, but not all CIN3 lesions would progress if not treated (12). At cutoff scores up to 39% methylation, the biomarker consistently detected 80% of CIN3 lesions. The only CIN3 lesion that it “missed” showed no methylation. Based on the natural history of CIN3 (11, 42-44), this lesion may have been at minimal short-term risk of malignant progression. This interpretation is further supported by a recent report that even among HPV16 infections that were destined to progress, malignant conversion was preceded by a latency period of 21 to 27 years (45). The prognostic potential of our HPV16 methylation biomarker is consistent with what has been reported for certain host gene DNA methylation differences between pre/cancer cells and normal cells at other body sites [e.g. (46-49)].

The sustained 100% sensitivity for ICC, the consistent detection of 80% of CIN3 lesions, and the variable detection of CIN2 over a wide range of cutoff scores indicates the robustness of the biomarker. Given the clinical heterogeneity of all grades of CIN, the most clinically relevant cutoff score awaits future determination in prospective clinical trials. The robustness of the biomarker means that significant lesions would be detected even with a subset of missing data. In this study, none of our cases had to be eliminated due to missing data, although data were missing for three CpGs sites in two cases, and a single CpG site in four other cases. The satisfactory outcomes reflect both the choice of CpG sites and their number. Because proximal and distal DNA sequence variations, including single nucleotide polymorphisms [SNPs], influence DNA methylation (50-52), an HPV16 biomarker with twelve CpGs, compared to a much smaller number, is also more likely to minimize false-negative results in women infected with variant HPV16 genomes (53).

If a DNA methylation assay based on the twelve HPV16 CpG-containing biomarker was used in a setting in which biopsy confirmation was deemed necessary before treatment, the number of HPV16 DNA-positive women referred for additional testing could be substantially reduced compared to other strategies. In this study, a cutoff score as low as 10% methylation would have referred just 55% of the HPV16-positive patients for colposcopy, a substantial reduction over the current referral rate of 100% for all HPV16-positive women (8). At higher cutoff scores, the referral rate would be lower. In a general screening population, where CIN3 and
ICC are uncommon, an HPV16 DNA methylation-based assay would significantly reduce the rate of colposcopy referral.

Our results are in agreement with other recent studies of HPV16 DNA methylation. Sun et al. reported significantly increasing methylation in women with ≤CIN1, CIN2 and CIN3/ICC, at fourteen L1 CpGs, including six in our biomarker, at nucleotides [nts] 5600, 5606, 5707, 5724, 6365 and 6579 [referred to as 5602, 5608, 5709, 5726, 63657 and 6581] (36). Mirabello et al. reported elevated HPV16 methylation in women with CIN3 or persistent HPV16, relative to women whose infections cleared, at multiple CpGs (37). Prior to correcting for multiple comparisons, two of the E5 CpGs and four of the L1/L2 CpGs in our biomarker showed significantly elevated methylation; they were at nts 3937, 3941, 5171, 5600, 5606 and 5724 [referred to as 5173, 5602, 5608, 5726] (37). In a subsequent study, Mirabello et al. reported increasing HPV16 methylation in women with CIN2 or CIN3 or ICC, relative to women whose infections cleared, at multiple CpGs, including nine in our biomarker, at nts 5126, 5171, 5600, 5606, 5707, 5724, 6365, 6387 and 6579 [referred to as 5128, 5173, 5602, 5608, 5709, 5726, 6367, 6389, 6581] (38). Elevated methylation at five of these CpGs was also found in samples collected five months before a diagnosis of CIN2, CIN3 or ICC, indicating their prognostic potential. These five were at nts 5171, 5126, 6365, 6387 and 6579 (38). Lorincz et al. recently reported a different candidate HPV16 methylation biomarker for the follow-up of women with HPV16 DNA-positive screening tests (18). That biomarker contained two of the L1 CpGs in our biomarker, at nts 6365 and 6387 [referred to as 6367 and 6389], plus five L2 CpGs that we did not analyze in the current study. It showed significantly elevated methylation in samples from women diagnosed with either CIN2 or CIN3 six to twelve months after a low-grade or equivocal cytology report (18). While the biomarker proposed by Lorincz et al. had high sensitivity for CIN2 [and CIN3] lesions, our HPV16 methylation biomarker detected only a subset of CIN2 lesions. Based on the natural history of CIN, our undetected CIN2 cases were likely at minimal short-term risk of progression. A biomarker that could distinguish cervical lesions at high vs. minimal risk of short-term progression would have a distinct advantage over biomarkers that detected virtually all lesions with CIN2 or worse.

In conclusion, this study confirms our original findings (34) and extends them to a specific subset of HPV16 CpGs as well as to invasive cervical cancer. Moreover, it shows that the 12 CpG-containing biomarker fulfilled all three conditions required of a prospective biomarker. With further validation in prospective clinical
trials, a clinical assay based on our biomarker could expedite the diagnosis [and treatment] of significant cervical lesions; reduce large numbers of unnecessary cervical colposcopy procedures; and reduce unnecessary therapeutic intervention for a subset of women with CIN2. Finally, it could greatly reduce the high annual cost of cervical cancer prevention, which is $6 billion in the United States.

**Acknowledgements**

We thank Jose Costa for helpful discussions.
Figure Legends

Figure 1. Differential methylation at twelve selected HPV16 CpGs in cervical cells from premalignant lesions with biopsy-confirmed diagnoses from our original study (34). The markers indicate cases diagnosed as CIN1 [open triangles], CIN2 or CIN3 [filled circles] [panel A], and CIN2 [filled diamonds] and CIN3 [filled squares] [panel B]. A case was counted as methylated if it had any methylation at a given CpG. The methylation difference across the twelve CpGs was significant at P=0.0007 [Panel A, Mann-Whitney test] and P<0.0001 [Panel B, Jonckheere-Terpstra test].

Figure 2. ROC curves for the twelve CpG-based HPV16 methylation biomarker. The curves represent the discrimination between ICC vs. CIN1 [A], CIN3 vs. CIN1 [B] and CIN2 vs. CIN1 [C]. Also shown are the areas under the curve [AUCs] and 95% confidence intervals [in parentheses].
References


**Table 1.** Bisulfite-PCR primers for amplification of the twelve CpGs in the HPV16 biomarker

<table>
<thead>
<tr>
<th>ORF</th>
<th>HPV16 CpGs</th>
<th>Primer</th>
<th>Sequence [5' to 3']</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>E5</td>
<td>3887, 3937, 3941</td>
<td>11F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATA GGA TAT AAT GTA AAA TAT AAA AGT GTA</td>
<td>284 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11R&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ATA TAT AAA CAC AAA CAA AAA CAA C</td>
<td>[320 bp]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15F&lt;sup&gt;d&lt;/sup&gt;</td>
<td>GTT TTA GAT TTT GAT TTT TTG GAT ATA GTT</td>
<td>139 bp</td>
</tr>
<tr>
<td>L2</td>
<td>5126, 5171</td>
<td>15R2</td>
<td>CCT TAA CAC CTA TAA ATT TTC CAC TAC</td>
<td>[175 bp]</td>
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<tr>
<td>L1/L2</td>
<td>5600, 5606, 5707,</td>
<td>16F&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TTG ATG TAG GTG ATT TTT ATT TAT ATT TTA</td>
<td>484 bp</td>
</tr>
<tr>
<td>L1</td>
<td>5724, 5925</td>
<td>16R2</td>
<td>AAA CAC TAA CAT TTT CTA TAT CAT CCA ATT</td>
<td>[520 bp]</td>
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<tr>
<td>L1</td>
<td>6365, 6387, 6579</td>
<td>17F&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TTT AGA TTA TAT TAA GGT GTT AGA ATT</td>
<td>299 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17R&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CAA ATA ATT AAT TAC CCC AAC AAA TAC</td>
<td>[335 bp]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide positions of the targeted cytosines, in the 16W12E isolate of HPV16 [GenBank *accession no. AF125673*].

<sup>b</sup> To the 5' end of each primer, an M13 sequence was added for DNA sequencing. The M13 sequences were TGTAAAACGACGGCCAGT [F] and CAGGAAACAGCTATGACC [R].

<sup>c</sup> Length in base pairs excluding [or including] the M13 sequence.

<sup>d</sup> Reported previously [6].
Table 2. Trend for increasing HPV16 biomarker methylation with increasing histologic severity

<table>
<thead>
<tr>
<th>Histologya</th>
<th>Nb</th>
<th>Median, %c</th>
<th>Mean, %d</th>
<th>P-valuee</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>16</td>
<td>3.1</td>
<td>6.6 ± 2.2</td>
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<tr>
<td>CIN 2</td>
<td>7</td>
<td>16.7</td>
<td>20.1 ± 6.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CIN 3</td>
<td>5</td>
<td>62.9</td>
<td>54.2 ± 15.8</td>
<td></td>
</tr>
<tr>
<td>ICC</td>
<td>5</td>
<td>90.9</td>
<td>88.2 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>All cases</td>
<td>33</td>
<td>15.3</td>
<td>29.1 ± 6.0</td>
<td></td>
</tr>
</tbody>
</table>

Note: CIN, cervical intraepithelial neoplasia. ICC, invasive cervical cancer (ICC).

Number of cases per diagnosis.

Median percent of HPV16 genomes with methylation per case.

Mean percent of HPV16 genomes with methylation per case ± standard error of the mean.

Trend for increasing methylation with increasing histologic severity, from CIN1 to CIN2 to CIN3 to ICC. P-value calculated using a two-tailed Jonckheere-Terpstra test.
Table 3. Performance of the HPV16 methylation biomarker and estimated colposcopy referrals

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>Sensitivity / Detection</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Colposcopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met score</td>
<td>ICC</td>
<td>CIN3</td>
<td>CIN2</td>
<td>ICC</td>
<td>CIN3</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>80</td>
<td>71.4</td>
<td>75</td>
<td>55.6</td>
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</table>

a Selected cutoff scores.

b Minimum biomarker methylation score [%] for a positive test.

c Sensitivity for ICC and detection rate for CIN3 and CIN2, relative to CIN1.

d Number of cases analyzed for each characteristic, e.g. n=21 for ICC includes the cases with ICC [n=5] plus the cases with CIN1 [n=16].

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e Specificity for CIN1 vs. CIN2+.

f Positive predictive value vs. CIN1.

g Negative predictive value for CIN1 vs. CIN2+.

h Estimated rate of colposcopy referrals based on positive tests.

i Not applicable, because no CIN2 cases were detected at a cutoff score of 42% methylation.
Figure 2

A  ICC
AUC = 1.00 (1.00, 1.00)

B  CIN3
AUC = 0.82 (0.46, 1.00)

C  CIN2
AUC = 0.72 (0.44, 1.00)
Cancer Prevention Research

Methylation of twelve CpGs in human papillomavirus type 16 (HPV16) as an informative biomarker for the triage of women positive for HPV16 infection

Janet L. Brandsma, Malini Harigopal, Nancy B. Kiviat, et al.

Cancer Prev Res  Published OnlineFirst February 20, 2014.

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