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Human papillomavirus type 16 L1/L2 DNA methylation shows weak association with cervical disease grade in young women

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ABSTRACT

Background: Persistent infection with human papillomavirus (HPV) type 16 causes the majority of cervical cancers. Genital HPV infection is very common, but neoplastic progression is uncommon. There is an urgent need for biomarkers associated with cervical neoplasia, to enable triage of women who test positive for HPV.
Objectives: To assess the ability of quantitative measurement of HPV16 DNA methylation to separate samples of different cytological and histological grades from young women, among whom rates of HPV infection are high.
Study design: DNA methylation was quantified by pyrosequencing of bisulphite converted DNA from liquid based cytology samples from 234 women (mean age 20.6 years) who tested positive for HPV16 and showed varying degrees of neoplasia. Methylation was assessed at CpGs in the HPV E2 and L1/L2 regions.
Results: The performance of methylation-based classifiers was assessed by ROC curve analyses. The best combination of CpGs (5600 and 5609) achieved AUCs of 0.656 (95% CI = 0.520–0.792) for separation of cytologically normal and severely dyskaryotic samples, and 0.639 (95% CI = 0.547–0.731) for separation of samples with or without high-grade neoplasia (CIN2+/−).
Conclusions: The data are consistent with HPV L1/L2 methylation being a marker of the duration of infection in a specific host. Assessment of HPV DNA methylation is hence a promising biomarker to triage HPV-positive cytology samples, but may have limited utility in young women. Future studies assessing the likely utility of HPV DNA methylation as a potential triage biomarker must take account of women's age.

1. Background

DNA methylation is the covalent addition of a methyl group to the 5′ position of cytosine or adenine, and occurs primarily at CpG dinucleotides. Dynamic methylation of DNA is a fundamental epigenetic mechanism that facilitates interaction between genotype and environment; it also plays an important role in development of many cancers [1]. Cervical cancer is caused by persistent infection with high-risk human papillomavirus (hrHPV). During carcinogenesis, substantial changes in methylation are observed in both the host cell and HPV genomes. Quantification of these changes may facilitate both diagnosis and prognostication [2,3].

Due to its high sensitivity and potential applicability to self-collected samples, testing for hrHPV is likely to replace cytology as the primary screening method to prevent cervical cancer [4,5]. Co-testing using assessment of HPV and cytology is now recommended for women aged 30–64 in the United States [6]. However, HPV testing has significantly lower specificity than cytology, and therefore, triage testing is required to prevent excessive referrals for colposcopy. This is a particular concern in relation to younger women among whom rates of transient, clinically irrelevant, HPV infection are high. Cytology is likely to be used to triage HPV-positive

Abbreviations: HPV, human papillomavirus; LBC, liquid based cytology; CIN, cervical intraepithelial neoplasia; LCR, long control region; AUROCC, area under receiver operating characteristic curve.

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samples [7], but a molecular test that would allow reflex testing of HPV-positive samples would streamline workflows and increase efficiency. A molecular test could also potentially be applicable to self-collected samples.

Quantitative measurement of HPV DNA methylation shows significant promise as a simple test for triage of HPV-positive women. Prospective data shows that in women infected with HPV16, the level of methylation of specific CpGs increases gradually with viral persistence and is highest in women with high grade neoplasia [3]. Cross-sectional studies have also demonstrated a trend for increased methylation with disease progression, but the results of these studies vary depending on the specific CpGs investigated, the assay used, and the type of material examined [8–11]. There is, therefore, a need to assess the utility of quantification of HPV DNA methylation in additional settings, and to investigate the effects of important variables such as age [12].

In a previous study, we have demonstrated good discrimination between normal and dyskaryotic cytology samples by assessment of DNA methylation at the HPV16 L1/L2 CpGs at nucleotide positions 5600 and 5609 (area under receiver operator characteristic curve (AUROCC) = 0.900, 95% CI = 0.793–1) [13].The aim of the current study was to determine whether assessment of the same CpGs would discriminate between women with normal cytology and those diagnosed with high grade cervical neoplasia, in a sample of young women (20–22 years) attending for their first cervical screen, among whom prevalence rates of HPV are high (hrHPV types present in 26.6%) [14].

2. Objectives

The primary aim was to assess the ability of quantitative measurement of HPV16 DNA methylation at L1/L2 CpGs 5600 and 5609 to separate samples from young women with and without high grade CIN.

3. Study design

3.1. Samples and DNA extraction

Methylation of HPV DNA was investigated in 289 samples collected in BD Surepath liquid based cytology (LBC) media (Becton Dickinson Co., NJ, USA) during a pre-vaccination HPV prevalence study conducted at Cardiff University in collaboration with Cervical Screening Wales (CSW) [14]. Women aged 20–22 years, not offered the HPV vaccine and attending their first call for cervical screening between April 2009 and July 2010, were eligible for inclusion; samples were analysed for 13,306 women. Cervical cytology samples were processed by CSW according to the British Society of Clinical Cytology guidelines. For eligible women, residual LBC samples from the original cytology samples were flagged and transported to Cardiff University. Samples from all women who tested positive for HPV16 and were referred for colposcopy within the screening round were included in the current study, plus a further 22 samples from a random sample of HPV16-positive women with normal cytology. Patients referred for colposcopy were managed as recommended by NHS cervical screening guidelines. CSW provided clinical data (cytology and colposcopy results) for the current study based on a pseudo-anonymous case identifier.

DNA was extracted by overnight digestion in 50 mM Tris with 1 mg/ml Proteinase K followed by heat inactivation (100 °C, 5 min) and centrifugation. HPV typing was performed as previously described using GP5+/6+ PCR ELISA [15,16]. Samples that tested positive for HPV16 were included in the methylation analyses. Research ethics committee approval was obtained for use of all clinical material.

Table 1
Primer sequences and CpGs sequenced.

E2	
Sense primer	GTGAAATTATTAGGTAGTATTTGG
Antisense primer	BTN[b]-CAACAACTTAATAATATAACAAAAA
Sequencing primer	GTGAAATTATTAGGTAGTA
Sequenced CpGs	nt[a] 3411, 3414, 3416, 3432, 3435, 3447, 3461, 3472
Amplified DNA	148 bp (nt[a] 3378–3525)
L1/L2	
Sense primer	BTN[b]-TTATTGTTGATGTAGGTGATTT
Antisense primer	CCCAATAACCTCACTAAACAACC
Sequencing primer	TAACCTCACTAAACAACCAA
Sequenced CpGs	nt[a] 5600, 5606, 5609, 5615
Amplified DNA	118 bp (nt[a] 5551–5668)

[a] All nucleotide positions are relative to NC001526.1.
[b] BTN refers to the position of a biotin label.

4. Sodium bisulphite treatment and pyrosequencing

For each sample, sodium bisulphite treated DNA (500 ng) was prepared using the EZ DNA Methylation Kit (Zymo Research Corporation, CA, USA) according to the manufacturer's instructions. Regions of the E2 and L1/L2 overlap ORFs were amplified and pyrosequenced as previously described [13]. PCRs were performed using the primers described in Table 1 using ZymoTaq Premix hot start Taq (Zymo Research Corporation, CA, USA). The pyrosequencing reactions were performed using PyroMark Gold Q96 Reagents and PyroMark Q96 ID Instrument (Qiagen, Hilden, Germany).

5. Statistical analyses

All statistical analyses were performed using SPSS 18. ROC curves and AUC were calculated for mean values of combinations of CpGs. Kruskal–Wallis tests were performed to compare median methylation among disease groups for each HPV region, and each CpG tested. To account for multiple comparisons, *P*-values were corrected using the Benjamini–Hochberg FDR method to ensure that the expected proportion of falsely positive associations remained below 0.05 [17]. Receiver operating characteristic (ROC) analysis was used to examine the relationship between sensitivity and specificity. DNA methylation was quantified as mean methylation of specific CpGs. The performance of each combination of CpGs was assessed by comparing the area under the curve (AUC).

6. Results

In the original cohort of 13,306 samples, 3545 (26.6%) tested positive for at least one hrHPV type, and 1323 (9.9%) tested positive for HPV16 [14]. Of the women who tested positive for HPV16, 267 (20.2%) subsequently attended colposcopy and were included in the current study. Methylation assays were performed on 289 samples; 55 samples (19%) failed to produce adequate PCR prod-

Table 2
Cohort composition.

Cytology	*n*	Histology	*n*
Negative	22	No CIN	21
Borderline	63	CIN 1	34
Mild	54	CIN 2	51
Moderate	39	CIN 3	92
Severe	52	Adenocarcinoma in situ	2
Glandular neoplasia	2		
Missing	2		
Total	234	Total	200