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Is human papillomavirus viral load a clinically useful predictive marker: a longitudinal study

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Abstract

Background—It has been suggested that in women who test positive for high-risk HPV types, viral load can distinguish women who are at increased risk of cervical neoplasia, from those who are not.

Methods—Quantitative PCR (qPCR) was used to measure HPV copy-number in serial samples taken from 60 and 58 young women previously found to have incident cervical HPV16 or HPV18 infections, respectively, using GP5+/GP6+ primers; women provided at least three samples for qPCR-testing, at least one of which was positive.

Results—A ten-fold increase in HPV16 or HPV18 copy-number was associated with a modestly increased risk of acquiring a cytological abnormality (HPV16: hazards ratio=1.76 (95% CI 1.38-2.25); HPV18: hazards ratio=1.59 (1.25-2.03)). However, in most women, copy-number increased during follow-up, before falling again. In women with a HPV16 infection, the median copy-number per 1,000 cells was 7.7 in their first qPCR-HPV-positive sample, 1,237 in the sample yielding the maximum copy-number and 7.8 in their last qPCR-HPV-positive sample; corresponding copy-numbers for women with HPV18 infection were 2.3, 87 and 2.4. Maximum HPV16 and HPV18 copy-number did not differ significantly between women acquiring an incident cervical cytological abnormality and those who did not.

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Authors and Contributions

Christothea Constandinou-Williams designed the study, analysed and interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published.

Stuart I Collins designed the study, analysed and interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published.

Sally Roberts was involved in the interpretation of the data, revised the article critically for important intellectual content, and approved the final version to be published.

Lawrence S Young conceived the study, interpreted the data, revised the article critically for important intellectual content, and approved the final version to be published.

Ciaran BJ Woodman conceived and designed the study, interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published. Ciaran BJ Woodman is the guarantor of this work.

Paul G Murray designed the study, interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published.

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Conclusion—While large relative increases in copy-number are associated with an increased risk of abnormality, a single measurement of viral load made at an indeterminate point during the natural history of HPV infection, does not reliably predict the risk of acquiring cervical neoplasia.

Impact—A single measure of HPV viral load cannot be considered a clinically useful biomarker.

Keywords

Cervix; Cohort study; Human papillomavirus; Viral load; Cytological abnormality

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BACKGROUND

The identification of high-risk (HR) human papillomavirus (HPV) types as a necessary cause of cervical cancer offers not only the prospect of effective primary prevention but also the possibility that testing for the presence of HR HPV types could improve the efficiency of cervical screening programmes. However, it is now clear that there is a compelling need for additional biomarkers which allow us to distinguish between those women who test positive for HR HPV and who are likely to acquire cervical neoplasia, and those women who test positive for HR HPV and who are at no increased risk (1). HPV viral load is an attractive candidate. Among women who test positive for HR HPV types, cytological abnormality is consistently found to be more common in those with a high-viral load than in those with a low-viral load (reviewed in reference 2). However, it is now clear that the relationship between the viral load of cervical HPV infections and cervical neoplasia is more complex than was previously thought. When viral load is measured using real-time quantitative PCR (qPCR) and normalised for specimen cellularity, longitudinal studies fail to reveal a consistent association between the viral load of cervical HPV infections and the risk of acquiring an epithelial abnormality of the cervix (1-4). Programme trials have concluded that a measurement of viral load provides little or no additional useful clinical information beyond that provided by cytological examination, or testing for the presence of HR HPV (1-5). However, in almost all of these studies, viral load is measured at a single point in time. The impact of changes in viral load after this time on the risk of acquiring cervical neoplasia remains undefined. We have addressed this issue by measuring the viral load of HPV16 and HPV18, the types most frequently detected in cervical cancers, in serial samples taken during the follow-up of a cohort of young women who were recruited soon after they first had sexual intercourse.

MATERIALS AND METHODS

Study Population

As previously described, a cohort of 2,011 women aged 15 to 19 years who visited one Birmingham Brook Advisory Centre (a family planning clinic) in Birmingham, UK, were recruited between 1988 and 1992, and asked to re-attend at intervals of six months: follow-up ended on 31st August 1997 (6). At each visit, one cervical sample was taken for cytological examination, following which a second sample was taken and stored for subsequent virological examination. All women in whom a cytological abnormality was identified were immediately referred to a dedicated research clinic, irrespective of the severity of that abnormality. In this clinic, a sample of any colposcopically abnormal epithelium was removed for histological examination. Colposcopic and cytological surveillance was maintained in these women, and treatment postponed, until there was histological evidence of high-grade CIN (CIN2 or CIN3), at which point women left the study. After all clinical follow-up had ended, cervical samples were tested for the presence of HPV DNA using a general primer (GP5+/GP6+) mediated polymerase chain reaction (PCR) and further PCR tests were done with type-specific primers on samples that were

HPV-positive (6); where appropriate, to avoid confusion we subsequently refer to this testing strategy as the "GP5+/GP6+ system". A 2μl aliquot was taken from the stored sample and DNA extracted using guanidinium thiocyanate acid; 100ng of sample DNA was then used in a 50μl PCR reaction, as previously described (7). The study was approved by the appropriate ethical committee and informed oral consent was obtained from all women. The study population for this analysis comprises the subset of all women who were cytologically normal and HPV-negative at study entry, and who first tested positive during follow-up for HPV16 or HPV18, or both, using the GP5+/GP6+ system.

Sample preparation and measurement of viral load

For this analysis, DNA was isolated from study samples using Proteinase K digestion and phenol/chloroform extraction, according to methods previously described (7). HPV viral load was measured using a modified singleplex qPCR assay (ABI 7700 Applied Biosystems, Warrington, UK). In brief, sequence-specific primers and Fluorescein-labelled probes (Eurofins MWG Operon, Ebersberg, Germany) were designed for GAPDH, HPV16 E6 and HPV18 E7 (supplemental information: table S1). Genomic DNA (50 ng) and standards (ten-fold plasmid dilutions between 10^8 and 10^2 copies of GAPDH, HPV16 and HPV18) were amplified using TaqMan® Universal PCR Master Mix (4304437: Applied Biosystems, Warrington, UK) with 0.4 μmol/L of appropriate primer mix. Amplifications were performed using the ABI 7700 sequence detection system and cycle conditions for GAPDH, HPV16 E6 and HPV18 E7 were: 50°C for 2 min, 95°C for 12 min, followed by 50 cycles of 95°C for 15 s and 55°C for 30 s. The HPV16-positive cervical carcinoma cell line SiHa, and the HPV18-positive cell line HeLa, were used as positive controls. Standard curves using HPV16 plasmids, or HPV18 plasmids, and GAPDH plasmids were used to generate measurements of viral load normalised for cellular DNA content.

Statistical analysis

Analyses of the association between viral load and the acquisition of cervical cytological abnormality were undertaken using methods appropriate for interval-censored time-to-event data. Time to acquisition of cytological abnormality was measured from the date of the first qPCR-evaluable sample until the interval between the date of the first detection of cytological abnormality and the date of the immediately preceding cytologically normal smear; censoring occurred on the earliest of the date of the last qPCR-evaluable sample, or the date of the last smear. Estimates of hazards ratios were obtained using a semi-parametric method for modelling interval-censored time-to-event data as a generalized linear model (8), with the logarithm-to-base 10 of the maximum viral load observed prior to and including a given point in time treated as a time-varying covariate: note that since the logarithm-to-base 10 of this estimate of viral load was entered into the model as a continuous variable, a change in value of 1 corresponds to a ten-fold increase in viral load. 95% confidence intervals were constructed from parameter estimates and their standard errors. Tests of hypotheses were undertaken using likelihood ratio tests. All tests of statistical significance were conducted at the 5% two-sided significance level.

Descriptive analysis of changes over time in viral load

Modelling viral load kinetics proved impossible because of the statistically intractable nature of the data (see supplementary figure S1 and supplementary tables S2 and S3). Therefore, a parsimonious approach was taken when reporting changes over time in viral load. A purely descriptive, quantitative, analysis of numerical changes was undertaken: numerical values were compared at face value and the change was categorised accordingly. Arbitrary fold changes were not imposed.

Measurement of viral load as a time-varying covariate

Each woman had repeated measurements of viral load made at intervals of approximately six months. For illustrative purposes only, these repeat measurements can be considered as a series of consecutive pairs of visits. For any given pair of consecutive visits there are two viral load measurements available, that taken in the baseline sample (the first sample of the pair) and that taken in the follow-up sample (the second sample). The current viral load for a woman will be that measured in the second sample of the relevant pair; viral load status "lagged" by one measurement will be that measured in the first sample of the relevant pair. The sample containing the first detection of cytological abnormality will always be the last follow-up sample for a woman (because observations are terminated at this point). Therefore, when analysing the association between viral load and the acquisition of cytological abnormality, an analysis based on the first sample is preferred to that based on the second sample, because the former allows us to describe more precisely the temporal relationship between the detection of HPV DNA and the first acquisition of cytological abnormality. In this manuscript, we analysed the association between the maximum viral load observed prior to a given point in time and the risk of acquiring an incident cervical cytological abnormality. This was a longitudinal analysis in which different women were compared against each other, rather than making within-woman comparisons. Note that in this case, although HPV viral load is measured as a time-varying covariate, only a *single* measurement of viral load is used as a risk factor. For example, consider two women, woman A and woman B, who are both cytologically normal in their cervical sample taken at study entry. Suppose that these women both made visits at time zero (study entry), and then at six, 12 and 18 months after study entry (this is an artificial example since visits were made in continuous time, so it is highly unlikely that two women would have *exactly* the same visit pattern, despite the study design). Suppose further that for both women, the first detection of a cervical cytological abnormality occurred at the 18 month visit. For woman A, suppose that the observed measurements of HPV16 viral load were 0, 10,202, 8,529 and 568 copies per 1,000 cells at times zero, six, 12 and 18 months, respectively (i.e. an increase in the magnitude of HPV16 viral load over time, followed by a decrease). For woman B, suppose that the observed measurements of HPV16 viral load were 0, 435, 1,095 and 1,872 copies per 1,000 cells at times zero, six, 12 and 18 months, respectively (i.e. an increasing HPV16 viral load over time for the first 18 months of follow-up). Examination of supplementary figure S1 reveals that study numbers 69 (for HPV16) and 240 (HPV16 and HPV18) have similar patterns in viral load of the relevant type(s) over varying periods of time for woman A. Similarly for study numbers 147 (HPV16) and 1367 (HPV18) for woman B. When the first detection of a cervical cytological abnormality occurs at the 18 month visit, the maximum viral load observed prior to and including that point in time (18 months) is 10,202 copies per 1,000 cells for woman A and 1,095 copies per 1,000 cells for woman B (compared to woman B, woman A has a 10,202 / 1,095 = 9.3-fold greater HPV16 viral load).

RESULTS

Before investigating the relationship between viral load and the risk of acquiring an incident cervical cytological abnormality, we first describe how cervical HPV viral load changes over time in women infected with HPV16 or HPV18 (illustrative examples are provided in supplementary material figure S1).

Viral load waxes and wanes during follow-up

For this analysis, the study population was restricted to 60 and 58 women who had had an incident HPV16 or HPV18 infection, respectively, detected using the GP5+/GP6+ system;

and who provided at least three evaluable samples for qPCR-testing during follow-up, at least one of which was positive.

Sixty women were tested for HPV16 using qPCR in three or more samples: 41 tested positive for HPV16 in three or more samples; 10 in two; and nine in one. Fifty-eight women were tested for HPV18 using qPCR in three or more samples: 39 tested positive for HPV18 in three or more samples; five in two; and 14 in one. In 60 women with a HPV16 infection, the median copy-number (viral load) per 1,000 cells was 7.7 in their first qPCR-HPV-positive sample, 1,237 in the sample yielding the maximum viral load, and 7.8 in their last HPV-positive sample; the corresponding copy-numbers for the 58 women with HPV18 infection were 2.3, 87 and 2.4 per 1,000 cells.

Viral load appeared to wax and wane during follow-up: supplementary tables S2 and S3 present the viral loads of consecutive samples tested for HPV16 and HPV18 by qPCR, respectively. The maximum viral load observed during follow-up was greater than that detected in the first qPCR-positive sample in 43 women with a HPV16 infection and in 35 with a HPV18 infection; and was greater than that detected in the last qPCR-positive sample in 37 of these women with a HPV16 infection, and in 32 with a HPV18 infection (table 1). A similar trend was seen when the median viral load for the first and last qPCR-positive samples, and for the sample containing the maximum viral load, were compared.

Increasing viral load is associated with an increased risk of acquiring an incident cervical cytological abnormality

When analysing the association between viral load and the acquisition of cervical cytological abnormality, the only restriction placed on the study population was that viral load had to have been measured at study entry. Of the 62 women with a HPV16 infection contributing to this analysis, 37 first had an abnormal smear during follow-up; this was reported as containing borderline nuclear abnormalities (BNA) in 19, and mild dyskaryosis (MD) in 18. Of 56 women with a HPV18 infection who contributed to this analysis, 32 first had an abnormal smear during follow-up: this was reported as BNA in 19, and MD in 13.

Neither the maximum HPV16 viral load, nor the maximum HPV18 viral load observed during follow-up, differed significantly between women who subsequently acquired an incident cytological abnormality and those who did not (Wilcoxon rank-sum test with continuity correction: W=426; p=0.60 for HPV16, and W=432; p=0.42 for HPV18). In contrast, when viral load was modelled as a $\log_{10}$-transformed continuous covariate, controlling for whether or not a woman had ever tested positive for the relevant type using qPCR, a ten-fold increase in either HPV16 or HPV18 viral load was associated with a significantly increased risk of acquiring a cervical cytological abnormality (hazards ratio=1.76 (95% CI 1.38 to 2.25) for HPV16; hazards ratio=1.59 (1.25 to 2.03)) for HPV18) (table 2). Further controlling for the detection of HPV types other than 16 and 18, using the GP5+/GP6+ system, had a negligible impact on the estimated hazards ratios (data not shown).

DISCUSSION

We have shown that a ten-fold increase in HPV viral load is associated with a significantly increased risk of acquiring an incident cervical cytological abnormality in women with cervical HPV16 or HPV18 infections, or both, during follow-up. It is important to emphasise that the change in viral load contributing to this increased risk was observed in the period *before* cytological abnormality was diagnosed. Our use of serial sampling allowed us to overcome some of the methodological limitations of previous studies which have reported an association between changes in HPV viral load and the acquisition or

progression of cervical neoplasia. In these studies, viral load was measured at only two time-points, with the sample taken at the time of diagnosis of cervical neoplasia used to provide the second of the two samples necessary to define the change in viral load (1,9). Observations on viral load made at, or after, the time of diagnosis of an event are uninformative with respect to determining the change in viral load necessary for that event to occur: an outcome cannot be attributed to a given level of exposure until that period of exposure has been completed (2).

However, our observations also help to explain why the measurement of HPV viral load might be less informative than was once hoped. We have shown that the HPV viral load in cervical samples waxes and wanes during the course of an infection. Therefore, it is impossible to predict from a single measurement, made at an indeterminate point during the natural history of that infection, what the viral load kinetics of that infection will be: an initially low viral load may be followed by a high viral load, and *vice versa*. This may explain, in part, the discrepant results reported by longitudinal studies which relate the acquisition, or progression, of cervical neoplasia to a single measurement of viral load made in a baseline sample (1-4). Although our repeated observations allowed us to define the maximum HPV viral load attained during an episode of cervical HPV infection, even this single measurement was insufficient to distinguish those women who acquired an incident cervical cytological abnormality from those who did not. The substantial overlap in the range of maximum HPV viral loads for these two groups, one that is reprised in other studies, illustrates why a clinically useful cut-off for HPV viral load has yet to be defined (10).

Real-time quantitative PCR, as used in this study, is now probably the method of choice when measuring viral load, in so far as it provides a type-specific assay which allows for normalisation for cellular content, and has, as we have found, a dynamic range, of at least seven logs (10^8 to 10^2 copies). However, comparisons made using this assay may still be confounded by differences in the proportion of infected and uninfected cells present in a given sample. Sampling variation, or heterogeneity within cervical lesions, may also distort comparisons: for example, viral load is reported to vary with the endocervical cell content of the cytological sample (11); and to be higher in women with high-grade CIN when low-grade CIN is also present than when it is not present (12). Of course, it is possible that serial measurements of viral load might yet be shown to provide clinically useful information. However, the interpretation of such changes in viral load over time may not be a simple matter because of our imperfect understanding of the natural history of cervical HPV infections. For example, counter-intuitively, a fall in HPV viral load may be associated with other aspects of viral infection which are themselves associated with disease progression. For example, we have shown using samples taken from this cohort, that disruption of the HPV E2 gene is a common and early event in the natural history of incident cervical HPV16 and HPV18 infections, and is associated with a substantial reduction in viral load (7). However, in no longitudinal survey linking viral load to a disease outcome, have changes in integration status over time been defined. Finally, it must be remembered that viral replication is not necessary for maintaining the malignant phenotype. *In situ* assays on cervical cancer cell lines have found that not all HPV copies are transcriptionally active; and in women with incident HPV16 infections, HPV E7 messenger RNA levels, but not viral load, are associated with an increased risk of developing squamous intra-epithelial lesions (13, 14). Robust measurements of type-specific viral load in samples in which integration status and the expression of viral oncogenes are also defined, will continue to provide useful insights into the pathogenesis of HPV-associated disease. However, unless and until other longitudinal studies report an increased risk of cervical neoplasia associated with high HPV viral load in women with normal cervical smears, the *clinical* value of a single measurement of HPV viral load must be considered unproven.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Changes in HPV viral load in cervical samples taken during follow-up according to HPV type[a]

The study population was restricted to women who provided at least three evaluable cervical samples for qPCR-testing for HPV16, or HPV18, or both, during follow-up, at least one of which was qPCR-positive.

HPV type	Number of women in the analysis	Number of samples in the analysis	Maximum viral load compared with viral load in the first qPCR-positive sample			Viral load in the last qPCR-positive sample compared with that in the sample containing the maximum viral load			Number of women
			Median (IQR[b]) viral load[c] in the first qPCR-positive sample	Median (IQR[b]) viral load[c] in the sample containing the maximum viral load		Median (IQR[b]) viral load[c] in the sample containing the maximum viral load	Median (IQR[b]) viral load[c] in the last qPCR-positive sample		
HPV16	60	429	7 (1 to 72)	25559 (748 to 337398)	Increased	25559 (748 to 337398)	3 (0.9 to 372)	Decreased	37
			4 (1 to 33)	1310 (279 to 69135)	Increased	1310 (279 to 69135)	1310 (279.1 to 69135)	Same	6
			371 (22 to 5924)	371 (22 to 5924)	Same	371 (22 to 5924)	1 (0.2 to 6)	Decreased	17
HPV18	58	386	2 (1 to 34)	758 (42 to 11749)	Increased	758 (42 to 11749)	3 (0.8 to 7)	Decreased	27
			0.2 (0.009 to 3)	1351 (0.2 to 24536)	Increased	1351 (0.2 to 24536)	1351 (0.2 to 24536)	Same	8
			659 (11 to 28588)	659 (11 to 28588)	Same	659 (11 to 28588)	1 (0.5 to 12)	Decreased	20
			1 (0.05 to 9)	1 (0.05 to 9)	Same	1 (0.05 to 9)	1 (0.05 to 9)	Same	3

[a] For ease of presentation, as few decimal places as possible were used to complete this table;

[b] inter-quartile range;

[c] copy number per 1,000 cells

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Table 2

The association between the logarithm-to-base 10 of the greatest cervical HPV viral load observed prior to the detection of cervical cytological abnormality and the risk of acquiring an incident cervical cytological abnormality

HPV type	Number of women in the analysis	Number with incident cervical cytological abnormalities	Hazards ratio per ten-fold increase in viral load	95% confidence interval	Likelihood ratio test[a]
HPV16	62	37	1.76	1.38 to 2.25	21.8;1;p<0.001
HPV18	56	32	1.59	1.25 to 2.03	12.1;1;p<0.001

[a]Chi-squared statistic; degrees of freedom; p-value