QUANTITATIVE HUMAN PAPILLOMAVIRUS 16 AND 18 LEVELS IN INCIDENT INFECTIONS AND CERVICAL LESION DEVELOPMENT

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Abstract

Human papillomavirus (HPV) RNA levels may be a more sensitive early indicator of predisposition to carcinogenesis than DNA levels. We evaluated whether levels of HPV-16 and HPV-18 DNA and messenger RNA (mRNA) in newly detected infections are associated with cervical lesion development. Female university students were recruited from 1990-2004. Cervical samples for HPV DNA, HPV mRNA, and Papanicolaou testing were collected tri-annually, and women were referred for colposcopically-directed biopsy when indicated. Quantitative real-time polymerase chain reaction of L1 and E7 DNA and E7 mRNA was performed on samples from women with HPV-16 and HPV-18 infections that were incidently detected by consensus PCR. Adjusting for other HPV types, increasing E7 cervical HPV-16 mRNA levels at the time of incident HPV-16 DNA detection were associated with an increased risk of cervical intraepithelial neoplasia grade 2 to 3 (HR per 1 $\log_{10}$ increase in mRNA=6.36,95%CI=2.00-20.23). Increasing HPV-16 mRNA levels were also associated with an increased risk of cervical squamous intraepithelial lesions; the risk was highest at the incident positive visit and decreased over time. Neither HPV-16 E7 DNA levels nor HPV-18 E7 DNA nor mRNA levels were significantly associated with cervical lesion development. Report of >1 new partner in the past 8 months (relative to no new partners) was associated with increased HPV mRNA (viral level ratio [VLR]=10.05,95%CI=1.09-92.56) and increased HPV DNA (VLR=16.80,95%CI=1.46-193.01). In newly detected HPV-16 infections, increasing levels of E7 mRNA appear to be associated with an increased risk of developing cervical pre-cancer.

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Keywords

HPV; viral load; mRNA; cervical pre-cancer

INTRODUCTION

Genital human papillomavirus (HPV) infections are highly prevalent in sexually active young women [Ho et al., 1998b; Moscicki et al., 2001; Winer et al., 2003; Woodman et al., 2001], and the role of certain HPV types (primarily 16 and 18) as the etiologic agents of cervical cancer has been well established [Koutsky and Kiviat, 1999; Lowy and Schiller, 1998]. However, given that the majority of infections are asymptomatic [Mao et al., 2003] and only a small proportion progress to cervical cancer [Dillner et al., 1997; Wallin et al., 1999], it is important to identify factors in conjunction with HPV positivity that increase the risk of disease progression. High viral levels may increase the risk of carcinogenic progression, but studies to date have been inconclusive [Wang and Hildesheim, 2003]. Previous studies have tended to report positive associations between HPV viral load and presence/increasing grade of cervical squamous intraepithelial lesions (SIL) or cervical intraepithelial neoplasia (CIN), but most have been cross-sectional in design [Carcopino et al., 2006; Cricca et al., 2007; Fiander et al., 2007; Flores et al., 2006; Forslund et al., 1997; Gravitt et al., 2003; Healey et al., 2001; Hernandez-Hernandez et al., 2003; Hesselink et al., 2004; Ho et al., 2006; Ho et al., 1998a; Huang et al., 2007; Ikenberg et al., 1997; Lai et al., 2006; Lillo et al., 2005; Lo et al., 2005; Oikonomou et al., 2006; Rajeevan et al., 2005; Santos et al., 2003; Snijders et al., 2006; Sun et al., 2001; Sun et al., 2002; Swan et al., 1999; Tsai et al., 2005; Wang-Johanning et al., 2002; Wu et al., 2006; Zerbini et al., 2001] and/or measured viral load in ways that were only semiquantitative or not type-specific [Castle et al., 2002; Dalstein et al., 2003; Healey et al., 2001; Hernandez-Hernandez et al., 2003; Hesselink et al., 2004; Ho et al., 1998a; Huang et al., 2007; Ikenberg et al., 1997; Lillo et al., 2005; Santos et al., 2003; Schlecht et al., 2003; Sun et al., 2001; Sun et al., 2002; Tsai et al., 2005; Wu et al., 2006; Zerbini et al., 2001]. Several studies have reported cross-sectional associations between HPV-16 viral load (measured by fully quantitative methods) and increasing grade of CIN or high-grade SIL [Carcopino et al., 2006; Cricca et al., 2007; Fiander et al., 2007; Forslund et al., 1997; Gravitt et al., 2003; Ho et al., 2006; Lai et al., 2006; Lo et al., 2005; Oikonomou et al., 2006; Rajeevan et al., 2005; Snijders et al., 2006; Swan et al., 1999; Wang-Johanning et al., 2002]. Six of these studies evaluated additional high-risk HPV types [Carcopino et al., 2006; Gravitt et al., 2003; Ho et al., 2006; Lai et al., 2006; Snijders et al., 2006; Swan et al., 1999], three of which reported positive associations between viral loads of non-HPV-16 types and increasing grade of cervical abnormalities [Carcopino et al., 2006; Ho et al., 2006; Snijders et al., 2006]. Less frequently, type-specific associations between HPV viral load and cervical lesion development have been evaluated prospectively. Several nested case-control studies have reported positive associations between high HPV-16 viral loads and subsequent risk of developing high-grade CIN [van Duin et al., 2002], cervical carcinoma in situ [Josefsson et al., 2000; Moberg et al., 2004; Ylitalo et al., 2000], or invasive cervical cancer [Moberg et al., 2005]. (In two of these studies, positive but attenuated associations were also observed between grouped HPV-18/45 viral load and development of carcinoma in situ [Moberg et al., 2004] and cervical cancer [Moberg et al., 2005].) In addition, a recent study based on a large longitudinal cohort reported a positive association between baseline HPV-16 viral load (measured by semiquantitative polymerase chain reaction (PCR) and restricted to women with single-type infections) and subsequent development of CIN grade 2 [Gravitt et al., 2007]. To our knowledge, no study to date has been designed to look at the prospective relationships between type-specific viral load and clinical outcomes among women with incidently detected HPV infections.

Furthermore, while certain behavioral risk factors for HPV acquisition (including numbers of new and recent sex partners) have been well-documented [Ho et al., 1998b; Moscicki et al., 2001; Winer et al., 2003], data linking other risk factors (including smoking, oral contraceptive use, and frequency of intercourse) to HPV acquisition have been inconclusive [Winer and Koutsky, 2004]. It is possible that these types of risk factors may be more important in relation to viral levels at the time of acquisition than to acquisition per se. For example, increased viral exposure (e.g. through more frequent intercourse, intercourse without condoms, or intercourse with recently HPV-infected partners) or influences on host immune responses or viral replication (e.g. with smoking or oral contraceptive use) may lead to infections with higher viral levels. To our knowledge, no study to date has specifically investigated relationships between behavioral risk factors and HPV viral loads in incidently detected infections.

The goals of the present study were to quantify levels of L1 and E7 DNA, as well as E7 messenger RNA (mRNA) (a potentially more sensitive early indicator of predisposition to high-grade dysplasia or cancer than DNA [Wang-Johanning et al., 2002]), among women with incidently detected HPV-16 and HPV-18 infections by consensus PCR; to explore the relationships between type-specific viral levels and subsequent risk of developing HPV-related genital lesions; and to characterize behavioral risk factors for high viral levels.

METHODS

Study populations

Between 1990 and 1997 (Phase 1) and 2000 and 2004 (Phase 2), two cohorts of 18 to 22 year old female students at the University of Washington were recruited to participate in longitudinal studies of genital HPV infection. Recruitment strategies have been described previously [Winer et al., 2006; Winer et al., 2003]. The protocol was approved by the University of Washington Institutional Review Board.

Data collection

Clinical visits were scheduled at 4-month intervals. At each visit, a nurse practitioner administered a face-to-face interview (to collect medical and sexual history information) and performed a standardized pelvic examination. In addition, women in Phase 2 completed a web-based sexual behavior diary every two weeks that was used to supplement the information collected in the face-to-face interviews [Baer et al., 2002; Winer et al., 2006]. During each pelvic examination, cervical Dacron-tipped swab samples were collected into specimen-transport medium (Digene Corporation, Gaithersburg, MD) for HPV DNA testing. Beginning in November 1995, an additional cervical swab sample was collected into 1 ml of Ultraspec™ (Biotecx Laboratories, Inc., Houston, TX) for mRNA testing using a quantitative real-time reverse transcriptase PCR assay for HPV E7 mRNA. Pelvic exams also included colposcopic examination of the cervix and collection of cervical cytologic specimens, with referral to colposopically-directed biopsy when indicated [Winer et al., 2003]. DNA samples were stored at −20° and RNA samples at −70° C prior to testing.

HPV DNA testing by consensus PCR

Cervical swab specimens were tested for HPV DNA using PCR amplification and dot-blot hybridization methods that have been described previously [Winer et al., 2006; Winer et al., 2003]. Briefly, in Phase 1, one fiftieth of each sample was amplified with consensus primers and three microliters of PCR products were dotted onto filters and probed with a biotin-labeled generic probe. Positive samples were then tested for types 6, 11, 16, 18, 31, 45, 56 and the following type mixtures: 31/33/35/39, 40/42/53/54, and 51/52/55/58 [Winer et al., 2003]. In Phase 2, one two-hundred-fiftieth of each sample was amplified with consensus

primers and ten microliters of PCR products were dotted onto nylon filters and probed with a biotin-labeled generic probe. Positive samples were then tested for 38 individual types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, CP6108, and IS39 [Winer et al., 2006].

Selection of samples for type-specific quantitative DNA and mRNA testing

Samples from women with HPV-16 and HPV-18 infections (identified by consensus PCR) were selected for type-specific quantitative DNA and mRNA testing, including the visit at which HPV-16 or HPV-18 infection was first detected by consensus PCR. Quantitative DNA testing was performed in 1999 (by Merck Research Laboratories [West Point, PA]) on samples from women in Phase 1. After quantitative DNA testing was completed, additional funding was obtained to perform quantitative mRNA testing (in 2005) on samples from women in Phases 1 and 2. In this manuscript, we included only quantitative results from the follow-up visit at which HPV-16 or HPV-18 infection was incidently detected by consensus PCR (excluding HPV-16 or HPV-18 infections that were first detected at the enrollment visit).

Quantitative HPV DNA testing

A multiplex real-time PCR assay was used to quantify copy numbers of HPV-16 and HPV-18 L1 and E7 genes and human beta globin gene. The amplification reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), and 4 µl of extracted DNA was amplified in each reaction. The assay utilized the 5'exonuclease activity of Taq polymerase and dual-labeled fluorescent probes for real-time detection of amplified products. Type- and gene-specific primers were designed to amplify small regions of the L1 and E7 genes for both HPV-16 and HPV-18. The primer and probe sequences are shown in Table I. A standard curve was generated by the addition of known amounts of plasmid DNA for the particular gene being detected. The standard curve was generated from 10^7 copies/µl plasmid stocks diluted 1:50 in carrier *E. Coli* DNA to give 2×10^5 copies/µl. Serial ten-fold dilutions in carrier DNA were then made to generate a range from 2×10^5 copies/µl down to 2 copies/µl. The quantitative results were corrected for cellularity using a measure of human genome equivalents and reported as the amount of HPV DNA per 1000 cells. DNA copy numbers were $\log_{10}$-transformed. For the purpose of conducting statistical analyses, samples without detectable viral DNA by the quantitative assay were assigned a $\log_{10}$-transformed copy number of zero. All samples were positive for beta globin.

Quantitative HPV mRNA testing

Aliquots of 0.5 ml were purified using the QIAamp mRNA mini kit (Qiagen Inc., Valencia, CA), suspended in 50 µl ultra high quality DEPC-treated water, and stored at −70° C for further assay. Any residue DNA was removed through incubation with 1 U of amplification grade DNAse I at room temperature for 15 minutes, and a 1 µl aliquot of each mRNA sample was assayed by real-time PCR with primers and probe targeting the beta actin gene in parallel without the reverse transcriptase procedure to confirm a lack of DNA contamination. In separate reactions, levels of HPV-16 E7, HPV-18 E7, and beta actin mRNA were measured by one-step reverse transcriptase multiplex real-time PCR. ABI EZ-RT-PCR kit (Applied Biosystems, Foster City, CA), a reagent specifically designed to provide optimal performance, was used according to the manufacturer's instructions to ensure a robust reaction. Briefly, the amplification reactions were performed in triplicate using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) in a 20µl final volume. Primers and probe for HPV-16 and HPV-18 E7 mRNA (shown in Table I) were designed using ABI Primer Express software. Reverse transcription and

thermal cycling conditions were 30 minutes at 60° C followed by 5 minutes at 95° C and then 40 cycles of 20 seconds at 94° C and 1 minute at 62° C. SiHa (HPV-16) and HeLa (HPV-18) mRNAs were used as positive controls to monitor the variability of the assay, and three negative controls were included in each run. Standard curves for HPV mRNA were generated by plotting the Ct values against the known log copy numbers (10^6 to 10^2 copies in a 10-fold dilution series), and inferring the copy numbers for unknown samples from the generated regression line. Levels of E7 mRNA were normalized according to the input of beta actin (derived from the standard curve ranging from 1 to 81 picograms of cellular RNA in a 3-fold dilution series) and expressed as the number of copies per picogram cellular RNA. The mRNA copy numbers were log_{10}-transformed, and the means of the triplicate results for each sample were calculated. For the purpose of conducting statistical analyses, samples with undetectable mRNA were assigned a log_{10}-transformed copy number of zero. Samples negative for beta actin (2 percent of all samples) were excluded.

Cytologic and histologic testing

Pap smears were reviewed by a cytotechnologist and all smears showing abnormalities and all biopsies were reviewed by the study pathologist. Pap smear findings were classified according to the Bethesda system [Kurman et al., 1994] as normal, atypical squamous cells, low-grade SIL or high-grade SIL. Biopsy tissue was diagnosed as showing CIN grade 1, 2, or 3. None of the women developed cytologic or histologic evidence of invasive cervical cancer.

Statistical analyses

All analyses were restricted to women who had a quantitative test result at the same visit as the incident consensus positive test result. Pearson correlation coefficients were used to evaluate pair-wise correlations between levels of L1 DNA and E7 DNA. Levels of HPV-16 L1 and E7 DNA were highly correlated (r=0.99, p<0.0001) (similar correlations were observed for HPV-18); therefore, all of the analyses described below that refer to quantitative DNA are based on the E7 gene.

Cox proportional hazards methods were used to examine the associations between levels of HPV DNA and mRNA in the cervix and the subsequent risk of developing cervical SIL or CIN grade 2 to 3 (CIN 2-3). At-risk time was calculated from the time of the incident consensus positive to the date of lesion detection (including the incident consensus positive visit). Separate type-specific analyses were conducted for SIL and CIN 2-3 for quantitative HPV-16 E7 DNA, HPV-16 E7 mRNA, HPV-18 E7 DNA, and HPV-18 E7 mRNA. Log_{10} levels of HPV-16 or HPV-18 E7 DNA or E7 mRNA at the time of the incidently detected infection (a fixed continuous variable) was the main predictor of interest. Detection of other HPV types (yes/no; based on consensus PCR) was included as a time-dependent covariate for SIL analyses. Qualitative detection of other high-risk HPV types [Winer et al., 2006] (yes/no; based on consensus PCR) was included as a time-dependent covariate for CIN 2-3 analyses. Scaled Schoenfeld residuals [Therneau and Grambsch, 2000] were used to test for proportionality of hazards over time; when the proportionality assumption of the Cox model was violated, a viral level by time interaction term was included in the model.

Linear regression was used to estimate the effects of partner and partnership characteristics on levels of cervical mRNA at the time of incident detection. In this analysis, log_{10} mRNA level was considered the dependent variable. Because we did not anticipate the relationships between behavioral risk factors and quantitative viral levels to vary by HPV type, quantitative levels of HPV-16 and HPV-18 were included in the same model. Generalized estimating equations with robust variance estimates and a working independent correlation structure were used to account for correlation between repeated visits within subjects. All

analyses included a main-effect term for HPV type (HPV-16 or HPV-18). Variables considered as potential risk factors for levels of E7 mRNA included number of sex acts per week reported at the visit at which incident type-specific HPV detection occurred (continuous), current smoking (yes/no), current use of hormonal contraceptives (yes/no), number of new partners in the past 8 months (0/1/>1), and condom use reported over the past 8 months (restricted to women who were sexually active in the past 8 months) (always or sometimes/never). Based on our previous observation that the majority of new HPV infections associated with a first sex partner were detected up to 8 months after first intercourse, we chose 8 months as a relevant time period for assessing sexual behavior variables [Winer et al., 2006]. The linear regression beta coefficients were back-transformed (by taking the antilogarithm) to represent the ratio increase or decrease in mRNA for a one unit change in the covariate. We refer to the back-transformed beta coefficients as "viral level ratios." Similar methods were used to examine associations between behavioral risk factors and cervical HPV E7 DNA levels. Number of sex acts per week was not evaluated as a risk factor for quantitative levels of E7 DNA, however, due to a high proportion of missing data for this variable prior to 1996.

RESULTS

Quantitative DNA testing was performed on 46 cervical samples from women with incident consensus positive results (HPV-16: 33; HPV-18: 13) and quantitative mRNA testing was performed on 53 cervical samples from women with incident consensus positive results (HPV-16: 42; HPV-18: 11). These 99 samples represent 79 incidently detected infections (60 HPV-16 and 19 HPV-18) in 72 women; 20 incidently detected infections were tested for both quantitative DNA and quantitative mRNA, and 7 women contributed both an HPV-16 and an HPV-18 infection. At enrollment, the mean age of these 72 women was 19.3 (standard deviation 0.5) years, and women were followed for an average of 27.1 (standard deviation 14.3) months after incidently detected infection. The corresponding cytologic diagnosis and behavioral variables assessed at the time of incidently detected infection are shown in Table II. The majority of samples collected from women with incidently detected HPV-16 or HPV-18 infections by consensus PCR were concurrently positive for quantitative E7 DNA or mRNA (Table III).

In longitudinal analyses of HPV viral levels in the cervix and cervical SIL, HPV-16 E7 mRNA levels at the time of incidently detected HPV-16 were associated with an increased risk of developing cervical SIL and histologically-confirmed CIN 2-3 (Table IV). The association was stronger when restricting to CIN 3 (hazard ratio (HR) per 1 $\log_{10}$ increase in viral load =48.94, 95% CI=5.37 to 445.66, adjusted for concurrent detection of other high-risk HPV types). A time-dependent association between mRNA level and risk of cervical SIL was observed, whereby the risk was strongest at the time of incidently detected infection and decreased over time (Table IV). Proportional hazards testing suggested a similar trend for CIN 2-3, but because there were too few events to fit a model with a viral level by time interaction term, only one overall hazard ratio is reported (Table IV). Neither HPV-16 E7 DNA level at time of incidently detected HPV-16, nor HPV-18 E7 mRNA nor E7 DNA level at time of incidently detected HPV-18 was statistically significantly associated with the development of either cervical SIL or CIN 2-3 (Table IV).

In univariate analyses adjusted for type (HPV-16 versus HPV-18), report of >1 new partner in the past 8 months at the time of incidently detected HPV-16 or HPV-18 (relative to no new partners) was associated with increased HPV E7 mRNA (viral level ratio (VLR)=10.05, 95% CI=1.09-92.56) and increased HPV E7 DNA (VLR=16.80, 95% CI=1.46-193.01). Other variables evaluated were not statistically significantly associated with viral levels (data not shown).

DISCUSSION

We used real-time PCR assays to obtain type-specific, fully quantitative measurements of HPV-16 and HPV-18 L1 and E7 DNA and E7 mRNA levels in newly detected infections of the cervix. Real-time PCR is considered to be the most accurate and controlled method of estimating quantitative HPV because it allows for type-specific measurements and adjustment for the amount of virus per cell, is designed to keep contamination to a minimum, and is reproducible and accurate over a large copy number range [Cubie et al., 2001; Tucker et al., 2001; Wang-Johanning et al., 2002].

Consistent with a previous report [Wang-Johanning et al., 2002], we detected type-specific E7 mRNA in the majority of cervical swab samples testing positive for HPV-16 or HPV-18 by consensus PCR. Cross-sectional associations between presence [Ho et al., 1994; Lamarcq et al., 2002] or high levels [Rajeevan et al., 2005; Scheurer et al., 2005; Wang-Johanning et al., 2002] of HPV E7 mRNA and increasing grade of cervical lesions have been reported previously. In the present study, increasing HPV-16 E7 mRNA level (but not DNA level) at the time of incident HPV-16 detection was associated with an increased risk of developing cervical lesions (both cytologically-diagnosed and histologically-confirmed). Not surprisingly, the relationship between mRNA levels and SIL was highest at the time of incident HPV detection and decreased over time.

It should be noted that most HPV mRNAs are bicistronic or polycistronic. Therefore, although our primers and probe for viral mRNA were designed to target the E7 gene, they likely picked up a group of early mRNAs, including spliced and unspliced mRNAs that contain E7 open reading frames. These mRNAs may or may not encode E7 proteins, encode E7 proteins in varying efficiencies, or encode viral proteins other than E7, depending on the splicing patterns [Tang et al., 2006], extreme degree of leaky scanning [Stacey et al., 2000], and cellular translation machinery [Oh et al., 2006]. Because of the polycistronic nature of HPV mRNA and the posttranscriptional restrictions on gene expression, it would not be appropriate to infer that a specific viral protein is expressed by measuring the level of mRNAs which contain the open reading frame. Consequently, a high level of E7 mRNA detected in a particular sample does not necessarily mean that this sample has a high level of E7 oncoprotein. Nevertheless, our findings that HPV-16 E7 mRNA levels, but not E7 DNA levels, were associated with cervical lesions suggest that gene expression at the transcription level (mRNA) may be a more sensitive early marker of progression than viral load at the replication level (DNA). While the underlying mechanism for this is not clear, it may involve more productive viral transcription.

Several prospective [Gravitt et al., 2007] and nested case-control studies [Josefsson et al., 2000; Moberg et al., 2004; van Duin et al., 2002; Ylitalo et al., 2000] have noted that higher HPV-16 DNA viral loads in archived cervical smears were related to subsequent development of CIN 2-3 or greater. In contrast, we did not observe any statistically significant associations between incidently detected HPV-16 DNA levels and cervical lesion development. The difference between our findings and those reported previously could be attributable to differences in the predictive value of incident versus prevalent HPV-16 DNA levels, or age differences between study populations (our study population was younger than previous studies evaluating viral levels in prevalent HPV infections). Alternatively, given our small sample size, we cannot rule out the possibility that HPV-16 DNA levels in incidently detected infections are predictive of cervical lesion development.

No statistically significant associations were observed between HPV-18 E7 mRNA or DNA levels and lesion development. Given the small numbers of women with incident HPV-18 infections in our sample, it is difficult to evaluate whether the null associations we observed

could be reflective of differences in the early natural history of HPV-18 versus HPV-16 infections. Since HPV-18 integrates into the host genome more frequently than HPV-16 during malignant transformation [Badaracco et al., 2002], levels of HPV-18 virus detected by routine cervical sampling may be less indicative of dysplastic progression. Whether this difference would be reflected in early lesions, however, is unclear. Alternatively, since HPV-18 has an affinity for endocervical glandular cells (which are less accessible to sampling) [Rohan, 2004], cervical swabs may be less effective for detecting levels of HPV-18 virus.

When we examined proximal behavioral risk factors in relation to cervical HPV E7 viral levels (both DNA and mRNA) at the time of incident HPV infection, report of more than one new sex partner (relative to no new partners) in the past 8 months was the only variable that was significantly associated with increased viral levels. New partners may represent more recent or frequent viral exposure, either of which might manifest in higher-level infections. On the contrary, incident infections detected among women reporting no new partners in the past 8 months may be more representative of infections with longer incubation periods between exposure and detection. While none of the other variables evaluated (including number of sex acts, condom use, smoking, and hormonal contraceptive use) was associated with viral levels, given our small sample, such associations are plausible and further evaluation is warranted. Given the observed associations between viral levels and cervical lesion development, reducing behavioral risk factors for high HPV viral levels could be an effective strategy for preventing pre-cancerous cervical lesions. Furthermore, higher viral loads are associated with an increased risk of HPV transmission [Bleeker et al., 2005].

In addition to the small sample size, other limitations of our exploratory analysis should be noted. First, while we observed positive associations between increasing cervical HPV-16 E7 mRNA levels in incident infections and the subsequent risk of developing CIN 2-3, the clinical utility of this finding is limited as no clear threshold for identifying women likely to develop high-grade lesions (either CIN 2 or CIN 3) was identified. Second, type-specific HPV positivity was determined using genital swab samples; however, without tissue dissection, it is not possible to know with certainty whether the lesions detected after incident HPV-16 or HPV-18 infection were due to these or other HPV types. Third, given that primers for quantitative PCR were designed based on prototype sequences, it is possible that primer sequence mismatches between prototype and non-prototype HPV variants, if present, could have affected the quantitative results. We did not, however, observe any appreciable differences in DNA levels between HPV-16 prototype versus non-prototype variants (data not shown), and we previously reported no appreciable differences in viral DNA levels by HPV16 variants [Xi et al., 2008] or by HPV18 variants [Xi et al., in press] using the same primers and probe that were used for amplifying E7 mRNA. Finally, our results may not generalize to other populations of women, including those that are older or immunocompromised.

In conclusion, in this prospective study of newly detected HPV infections in young women, increasing levels of HPV-16 E7 mRNA were associated with an increased risk of developing cervical pre-cancer. More longitudinal studies using state-of-the-art techniques for real-time, type-specific quantitative PCR are needed to further explore the relationships between type-specific viral levels and dysplastic progression. While clinically-relevant cutoff levels could not be evaluated in this small, exploratory study, the information gained supports the need for further research on the potential utility of HPV viral level measurements in determining risk for HPV-related precancerous lesions. In particular, in the era of prophylactic HPV vaccines, mRNA levels may provide a useful indicator of the clinical importance of breakthrough infections.

Acknowledgments

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Table I

Primer and probe sequences for real-time PCR of HPV-16 and HPV-18 L1 and E7 DNA and HPV-16 and HPV-18 E7 mRNA

	Sequence (5'→3')	Nucleotide position
DNA		
HPV-16-E7-forward primer	AGAACCGGACAGAGCCCATTAC	696-717
HPV-16-E7-reverse primer	GCCCATTAACAGGTCTTCCAAAG	794-816
HPV-16-E7-probe	CGCACAACCGAAGCGTAGAGTCACACTT	739-766
HPV-16-L1-forward primer	TCCAGATACACACAGCGGCTGG	5971-5990
HPV-16-L1-reverse primer	AATAAAGGATGGCCACTAATGCCC	6040-6063
HPV-16-L1-probe	AATGGCTGACCACGACCTACCTCAACA	6007-6033
HPV-18-E7-forward primer	GAACCACAACGTCACACAATG	752-772
HPV-18-E7-reverse primer	CAGAAACAGCTGCTGGAATG	843-862
HPV-18-E7-probe	TCTGCTGAGCTTTCTACTACTAGCTCAATTCTG	799-831
HPV-18-L1-forward primer	TTGGTTCAGGCTGGATTGC	7020-7038
HPV-18-L1-reverse primer	GCTTTGGCAGGTTTAGAAGAC	7091-7110
HPV-18-L1 probe	CCTCGCAAAACGTTCTGCTCCATCTGCCAC	7059-7087
mRNA		
HPV-16-E7-forward primer	CCGGACAGAGCCCATTACAAT	700-720
HPV-16-E7-reverse primer	ACGTGTGTGCTTTGTACGCAC	782-762
HPV-16-E7-probe	TGTTGCAAGTGTGACTCTACGCTTCGGT	733-760
HPV-18-E7-forward primer	GACTCAGAGGAAGAAAACGATGAAA	686-710
HPV-18-E7-reverse primer	GTGACGTTGTGGTTCGGCT	766-748
HPV-18-E7-probe	TGGAGTTAATCATCAACATTTACCA	715-739

Table II

Cytologic diagnosis and behavioral variables at the time of incident HPV detection (n=79[a])

Variable	n(%)
Cytologic Diagnosis	
Normal	57 (72)
Atypical squamous cells	11 (14)
Low-grade SIL	9 (11)
High-grade SIL	2 (3)
Currently smoking	
Yes	23 (29)
No	56 (71)
Currently using hormonal contraceptives	
Yes	44 (56)
No	35 (44)
Number of new partners in the past 8 months	
1	32 (41)
>1	15 (19)
Condom use in the past 8 months[b]	
Always or sometimes	54 (73)
Never	20 (27)

[a]Quantitative DNA and mRNA testing was performed on 79 incidently detected HPV infections (60 HPV-16 infections and 19 HPV-18 infections) in 72 women.

[b]Restricted to incidently detected infections in women who were sexually active during the past 8 months

Table III

HPV-16 and HPV-18 E7 mRNA and E7 DNA viral levels at the time of incident HPV-16 DNA and HPV-18 DNA detection by consensus PCR.

HPV-16

Quantitative measurement[a]	No. available samples	No. with detectable virus (%)	Mean (SD) log₁₀- copy number
Cervical E7 mRNA	42	35 (83.3)	2.96^{b} (0.71)
Cervical E7 DNA	33	29 (87.9)	3.55^{c} (1.68)

HPV-18

Quantitative measurement[a]	No. available samples	No. with detectable virus (%)	Mean (SD) log₁₀- copy number
Cervical E7 mRNA	11	10 (90.9)	2.33^{b} (1.02)
Cervical E7 DNA	13	12 (92.3)	3.71^{c} (1.47)

[a] among samples with incidently detected cervical HPV infection by consensus PCR

[b] per picogram beta actin among samples with detectable E7 mRNA

[c] per 1000 cells among samples with detectable E7 DNA

Table IV

Hazard ratios for the associations between $\log_{10}$ levels of HPV E7 mRNA[a] and E7 DNA[b] at time of incident HPV detection and risk of developing cervical SIL or CIN 2-3.

	Adjusted HR per 1 $\log_{10}$ increase in viral level (95% CI)	No. events/Person-years at-risk
E7mRNA		
HPV-16 (n=42)		
Cervical SIL[c]		20[d]/47.3
0 months:	7.73 (1.85-32.22)[e]	
6 months:	4.43 (1.46-13.42)[e]	
12 months:	2.54 (0.98-6.55)[e]	
18 months:	1.45 (0.52-4.04)[e]	
CIN 2-3[f]	6.36 (2.00-20.23)[g]	7[h]/71.9
HPV-18 (n=11)		
Cervical SIL	0.84 (0.43-1.65)[e]	8[i]/10.3
CIN 2-3	1.45 (0.38-5.50)[g]	2[j]/16.2
E7 DNA		
HPV-16 (n=33)		
Cervical SIL	1.36 (0.92-2.00)[e]	18[k]/41.2
CIN 2-3	1.18 (0.77-1.80)[g]	5[l]/70.5
HPV-18 (n=13)		
Cervical SIL	0.86 (0.57-1.32)[e]	9[m]/9.5
CIN 2-3	0.92 (0.54-1.56)[g]	2[n]/19.3

[a] per picogram cellular RNA

[b] per 1000 cells

[c] For this model, the hazard ratio was found to be time-dependent. Therefore, 4 hazard ratios are reported at 0, 6, 12, and 18 months.

[d] 14 low-grade SIL and 6 high-grade SIL

[e] adjusted for presence of other HPV type(s) at the visit that cervical SIL was assessed

[f] Proportional hazards testing suggested that the hazard ratio was time-dependent in this model. However, there were not enough endpoints to fit a model with a time-dependent hazard ratio. Therefore, only one hazard ratio is reported.

[g] adjusted for presence of other high-risk HPV type(s) at the visit immediately preceding the date of CIN 2-3 diagnosis

[h] 4 CIN 2 and 3 CIN 3

[i] 7 low-grade SIL and 1 high-grade SIL

[j] 1 CIN 2 and 1 CIN 3

[k] 17 low-grade SIL and 1 high-grade SIL

[l] 3 CIN 2 and 2 CIN 3

[m] 7 low-grade SIL and 2 high-grade SIL

[n] 1 CIN 2 and 1 CIN 3