

Acquisition and Natural History of Human Papillomavirus Type 16 Variant Infection among a Cohort of Female University Students¹

Long Fu Xi, Joseph J. Carter, Denise A. Galloway, Jane Kuypers, James P. Hughes, Shu Kuang Lee, Diane E. Adam, Nancy B. Kiviat, and Laura A. Koutsky²

Department of Pathology [L. F. X., J. K., N. B. K.], School of Medicine and Departments of Epidemiology [S. K. L., D. E. A., L. A. K.] and Biostatistics [J. P. H.], School of Public Health, University of Washington and Fred Hutchinson Cancer Research Center [J. J. C., D. A. G.], Seattle, Washington 98103

Abstract

Analogous to the epidemiology of different types of human papillomaviruses (HPVs), variants of a single type such as HPV16 may differ in the natural course of infection. A prospective study was conducted in a cohort of female university students who did not have HPV16 DNA or antibody at enrollment. Subjects were followed every 4 months with Pap smear and colposcopic examinations and tests for HPV DNA and antibody. Of 528 women, 62 acquired HPV16 infection during follow-up. The 5-year cumulative incidence was 12.6% for infection with HPV16 prototype-like variants and 3.1% for infection with non-prototype-like (NPL) variants. Among those with incident HPV16 infection, the adjusted odds ratio of detecting NPL variants was 6.0 [95% confidence interval (CI), 1.3–27.5] for nonwhite women or white women who had had nonwhite sex partners compared with white women who had not had nonwhite sex partners. As compared with women who had not used hormonal contraceptives in the 8 months prior to the first HPV16-positive detection, the adjusted odds ratio of detecting NPL variants increased from 1.6 (95% CI, 0.3–9.1) for those who had used hormonal contraceptives for 1–4 months to 5.4 (95% CI, 1.0–28.3) for those who had used hormonal contraceptives for 5–8 months (P for trend = 0.04). There was no difference in HPV16 seroconversion rates between women with prototype-like variants and women with NPL variants. The increased risk for biopsy-confirmed cervical intraepithelial neoplasia grades 2 or 3 associated with detection of incident infection by NPL variants was not explained by differences in persistence between the two variant groups, indicating that biological mechanisms other than viral persistence may be responsible for the observed

difference in risk for cervical intraepithelial neoplasia grades 2 or 3.

Introduction

Different types of genital HPVs³ vary in incidence and in the natural course of infection; HPV16 is the individual type most commonly detected in genital infections, persists the longest, and is the most commonly detected virus in invasive cervical cancers (1, 2). Women with HPV16 infection, as compared with those without HPV16 infection, are at an increased risk for development of cervical neoplasia (3, 4). Recent reports (5–11) have noted an even greater risk associated with certain variants of HPV16 compared with others. It has been proposed that, analogous to the types of HPV, variants of HPV16 may differ in incidence and outcome of infection.

Several studies have shown that HPV16 variants segregate geographically (12–14), and in some cases there is a correlation between the variants and certain host HLA genotypes (15, 16). However, these studies addressed prevalent infections. To date, little is known about the incidence of variants in a defined population or about risk factors that may predispose women to infection by certain variants. In contrast to irrefutable evidence showing differences between HPV types in length of viral DNA persistence (1, 17–19), little is known about the differences between HPV16 variants, and only a few studies of prevalent infections have been reported (5, 20, 21). Studies on antibody responses by HPV16 variants are rare, and the reported findings are inconsistent (22, 23). Detection of certain HPV16 variants has been shown to be associated with concomitant diagnosis of cervical neoplasia in several studies (5–8), but not all studies (20, 24, 25). Although the initial report from our ongoing cohort showed a temporal relationship between HPV16 variants and development of cervical neoplasia (9), it was not possible to assess the role of viral persistence because both prevalent and incident infections were included.

The present study sought to determine the risk of and risk factors for acquisition of cervicovaginal infections with HPV16 variants in a cohort of female university students who did not have detectable HPV16 DNA at the time of enrollment. We also evaluated the persistence of viral DNA, antibody response, and risk for development of cervical lesions in relation to certain HPV16 variants among women who acquired incident HPV16 infection during follow-up.

Materials and Methods

Enrollment, Follow-up, and Clinical Examination. Study subjects were female students attending the University of

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² To whom requests for reprints should be addressed, at University of Washington, HPV Research Group, 1914 North 34th Street, Suite 300, Seattle, WA 98103. Phone: (206) 616-9784; Fax: (206) 616-9788; E-mail: kouts@u.washington.edu.

³ The abbreviations used are: HPV, human papillomavirus; SIL, squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia; SSCP, single-strand conformational polymorphism; PL, prototype-like; NPL, non-prototype-like; OR, odds ratio; RR, relative risk; CI, confidence interval.

Washington, who were recruited into a longitudinal study of the acquisition and natural history of genital HPV infection (9, 26). At the time of enrollment, all participants were required to be first- or second-year students, to be 18–20 years old, to be residents of the state of Washington, and to provide a written informed consent according to a protocol approved by the University of Washington Institutional Review Board. Between July 1990 and August 1997, a total of 603 eligible subjects were recruited. A trained nurse-practitioner (D. E. A.) interviewed study subjects in-person with a structured questionnaire that elicited information on demographic characteristics, gynecological and obstetric history, hygiene and menstrual factors, sexual behavior, birth control methods, alcohol consumption, cigarette smoking, and history of sexually transmitted diseases.

Eligible women were followed at regular intervals, with visits scheduled every 4 months. Information regarding changes in recent sexual activities, birth control methods, and other lifestyle factors since the previous visit was obtained at each return visit. At enrollment and each subsequent return visit, subjects underwent standardized colposcopic inspection and Pap smear. Cytological abnormalities were classified according to the Bethesda system as atypical squamous cells of undetermined significance, atypical glandular cells of undetermined significance, or low- or high-grade SIL (27). During follow-up, those found to have cervical lesions that were suggestive of high-grade (e.g., a single high-grade SIL, 2 low-grade SILs, or ≥ 3 consecutive atypical squamous cells of undetermined significance or atypical glandular cells of undetermined significance Pap smears) were referred for biopsy. Histological diagnoses were assigned as negative, mildly atypical, or consistent with CIN grade 1, 2, or 3 (28). All of the diagnoses were made without the knowledge of HPV results.

For the current study, we excluded women who failed to return for follow-up after initial enrollment ($n = 46$), who did not provide any specimens for HPV assay ($n = 2$), or who had HPV16 DNA detected at study entry ($n = 27$). Of the 528 women included, 367 (69.5%) have completed follow-up (either seen for nine more visits or followed for >36 months), 30 (5.7%) were continuing follow-up (had not yet completed follow-up but remained in the cohort), and 131 (24.8%) were lost to follow-up after an average of 17.6 ± 10.8 (SD) months of follow-up (had not completed follow-up and had made no return visits since January 1999). A total of 4902 visits from the 528 women were available for the current study. Reasons for loss to follow-up included the following: (a) graduation ($n = 3$); (b) moved out of state ($n = 51$); (c) unable to locate after three or more attempts ($n = 32$); and (d) refusal ($n = 45$). Women who did not provide return visits or who were lost to follow-up did not differ significantly from those who completed follow-up or remained in the cohort with respect to race, history of hormonal contraceptive use, lifetime number of sex partners at study entry, and infection with HPV types other than HPV16 (data not shown).

Characterization of HPV16 Variants. Cervical and vulvovaginal swab specimens for detection and typing of HPV DNA were collected at enrollment and each return visit. Specimens were assayed by PCR amplification and dot blot hybridization as described previously (29). Briefly, DNA samples were PCR-amplified using HPV L1 consensus primers MY09, MY11, and HMB01 and human β -globin primers PC04 and GH20 in a standard procedure. The success of PCR amplification was indicated by the presence of the β -globin fragments on ethidium bromide-stained polyacrylamide gels. PCR products were dotted onto nylon filters and

probed with a biotin-labeled generic probe and with seven mixtures of individual type-specific oligonucleotide probes targeting HPV6 and HPV11; HPV16; HPV18; HPV31, HPV33, HPV35, and HPV39; HPV45 and HPV56; HPV40, HPV42, HPV53, and HPV54; and HPV51, HPV52, HPV55, and HPV58. Specimens that were positive for generic probe but not for any of the type-specific probes were considered to be positive for unclassified genital HPV types.

To characterize nucleotide alterations of HPV16 variants, HPV16-positive specimens collected before January 1999 were assayed by PCR-based SSCP analysis (30), and a subset of these samples that showed representative SSCP patterns was additionally tested by DNA sequencing. HPV16-positive samples collected after January 1999 were assayed by PCR-based direct DNA sequencing. In brief, DNA amplification was conducted with a pair of HPV16-specific primers, C and D, which targeted 682 bp from nucleotide position 7445 to 222, corresponding to the part of the noncoding and E6 regions (30). During the amplification, [α - 33 P]dATP (Du Pont New England Nuclear Research Products, Boston, MA) was incorporated into PCR products. The amplified products were cleaved into three fragments (318, 166, and 198 bp) from 5' to 3' by restriction endonuclease digestion with *DdeI* and then electrophoresed in a 5% polyacrylamide gel with 10% glycerol. The SSCP patterns of three fragments from each specimen were compared individually with the reference patterns from prototype plasmid HPV16 (pHPV16) DNA (31) and Caski cellular DNA (32) and with the patterns from other specimens.

Primers C and D were also used to generate PCR products for DNA sequencing. The PCR products were isolated by agarose gel electrophoresis and then purified with a QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA). The DNA templates were sequenced using the BigDye Terminator Cycle Sequencing kit according to the protocol recommended by the manufacturer (PE Applied Biosystems, Foster City, CA). Sequencing reaction products were column-purified and then run on an Applied Biosystem Model 377 DNA sequencing system. Sequences were determined from both directions and analyzed using sequencer package (Gene Codes Corp., Ann Arbor, MI).

As described previously (9), HPV16 variants were categorized into two groups. The PL group included variants that displayed the reference SSCP patterns and variants with one or two fragments that showed nonreference patterns. The NPL group included variants that displayed nonreference SSCP patterns in all three fragments. For the specimens tested by direct DNA sequencing, the variant classification was based on the magnitude of nucleotide alterations. As reported previously (9), NPL variants, as compared with the HPV16 prototype, have 5–10 more nucleotide alterations in the region analyzed than do PL variants. The characterization of HPV16 variants was done without knowledge of the clinical and epidemiological information.

Detection of Antibodies to HPV16 Capsids. The sera collected over time were tested by ELISA as described by Carter *et al.* (26). Briefly, H16.V5 monoclonal antibodies, which recognize a type-specific conformational epitope on the surface of HPV16 L1 capsids, were coated as a capture antibody on the 96-well microplate. Nonspecific binding was blocked by the addition of PBS with 5% goat serum and 0.05% Tween 20 after the capture antibody attachment. Capsids produced using recombinant vaccinia viruses expressing HPV16 L1 strain 114K were then added into the wells at an optimum dilution. Human sera that were diluted 1:100 in blocking buffer were tested in triplicate with and without capsids. After incubation at 37°C for

1 h, plates were washed, antihuman IgG conjugated to alkaline phosphatase was added (Roche, Indianapolis, IN), and the plates were again incubated at 37°C for 1 h. Alkaline phosphatase was detected with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) for 30 min. The ELISA value was calculated as the difference in natural logarithm of the median absorbance between the wells with and without capsids. The cutoff point was calculated as 2 SDs above the mean ELISA value of a set of 76 serum samples from women who had no PCR-detectable HPV DNA and reported no previous sex partners at the time of serum collection.

Statistical Analyses. Kaplan-Meier product limit estimates were used to estimate the cumulative proportion of women becoming positive for HPV16 PL or NPL variants among women who did not have detectable HPV16 at study entry and of women becoming HPV16 negative among women who had incident infection with HPV16 PL or NPL variants during follow-up. A log-rank test (33) was used to assess the overall differences in these proportions between variants. Because HPV16 status was assessed only at visits that were scheduled every 4 months, the onset of event (becoming positive or reverting to negative) was assumed to be at the midpoint between visits.

Women who entered the study without detectable HPV16 DNA but became positive at a subsequent visit were considered to have incident infection. To identify variant-specific risk factors, an unconditional logistic regression (34) was used to estimate ORs and 95% CIs. In this analysis, a length of 1–4 months was assigned to women who reported use of hormonal contraceptives at either the initial HPV16-positive visit or the previous visit, and a length of 5–8 months was assigned to those who reported use at both visits. The race of male sex partners was characterized as either white or nonwhite (including African, Asian, Hispanic, Native American, or others). If more than one partner or race was reported, nonwhite had precedence over white. Because we did not collect information on the race of all past sex partners at the study entry, the racial groups of sex partners were based only on the partners identified from 1 month before study entry through the initial HPV16-positive visits. However, we found that incident HPV infection was not associated with the number of sex partners before study entry (35). The ORs for racial groups were adjusted for time of recent use of hormonal contraceptives, whereas the ORs for use of hormonal contraceptives were adjusted for racial groups of the women and their male sex partners.

To determine whether women with HPV16 NPL variants used hormonal contraceptives more regularly than did those with PL variants, we compared the frequency of visits at which the use of hormonal contraceptives was reported during the time from study entry through the initial HPV16-positive visit. Because the use of hormonal contraceptives at consecutive visits for an individual might be correlated, the ORs and 95% CIs were estimated using a logistic regression model with a generalized estimating equation approach (36), which accounted for the additional variance introduced by the intraindividual correlation.

Among women with incident HPV16 infection, Cox proportional hazard regression analysis (37) was used to examine associations between HPV16 variants and risks for incident SIL and for biopsy-confirmed CIN grade 2–3. The starting date was defined as the midpoint between the initial HPV16-positive visit and the previous visit. The end point was the date at which the cervical lesion (SIL or CIN grade 2–3, depending on which

was being evaluated) was initially diagnosed or the last visit date for those who did not develop cervical lesions. The RRs and 95% CIs were adjusted for race and current use of hormonal contraceptives.

The median time to seroconversion after first detection of HPV16 DNA was also estimated using the Kaplan-Meier plot. The time variable was defined as the date of initial HPV16 infection (the midpoint between the first HPV16-positive date and one previous visit) until the date of seroconversion (the midpoint between the seroconversion date and one previous visit) or, for women who did not seroconvert, the last visit date. If there was no serum result at the visit right before the date of seroconversion, the conversion was assumed to occur at the midpoint between the observed seroconversion and the latest seronegative date. If the women seroconverted at the first HPV16-positive visit, a quarter of the interval between the date of seroconversion and one visit before was used as the time to seroconversion.

Fisher's exact test was used to compare the proportions of women with a single HPV-16-positive visit and the proportions of seroconverted women between those with PL and with NPL variants. All reported *P*s were two-sided.

Results

Incidence of Infection with HPV16 Variants. In a cohort of 528 women, the mean age \pm SD at enrollment was 18.7 \pm 1.0 years. These women entered into the cohort between July 1990 and August 1997, and all of them were negative for HPV16 DNA at the time of enrollment. By the end of 1999, the mean length \pm SD of follow-up was 39.2 \pm 16.1 months from the time of enrollment. Over the course of follow-up, 62 women acquired newly detected HPV16 infection, including 48 with PL variants and 14 with NPL variants. The cumulative incidence of infection with HPV16 PL variants was significantly higher than the cumulative incidence of HPV16 NPL variants (Fig. 1; log-rank test, *P* < 0.01). The annual incidence of infection with NPL variants tended to decrease over time after enrollment, with 1.6% in the first year as compared with 1.0% and 0.5% in the second and third year, respectively. A similar downward trend was observed for incident infection with PL variants, with 3.9% in the first year after enrollment down to 1.1% in the fifth year.

Factors Associated with Risk for Incident Infection with HPV16 PL or NPL Variants. To determine variant-specific risk factors, we confined the analyses to a subset of women who had incident HPV16 infection. As shown in Table 1, an increased risk for acquiring NPL variants appeared to be associated with nonwhite compared with white race (OR = 3.1; 95% CI, 0.8–12.6) and with nonwhite *versus* white sex partners (OR = 4.6; 95% CI, 1.0–20.8). In a further analysis, we took both the women's race and their partners' race into account, combining nonwhite women with white women having nonwhite partners. After adjusting for the time of recent hormonal contraceptive use, the OR of detecting HPV16 NPL variants was 6.0 (95% CI, 1.3–27.5) for nonwhites or whites with nonwhite sex partners as compared with whites who had not reported ever having had a nonwhite sex partner before and at the time of initial infection.

After adjusting for the racial groups of the women and their sex partners, an OR of 2.9 (95% CI, 0.7–11.8) was observed for the association between detection of NPL variants and history of hormonal contraceptive use at study entry (Table 1). Relative to women who had not used hormonal contraceptives in the 8 months before the first detection of HPV16 DNA,

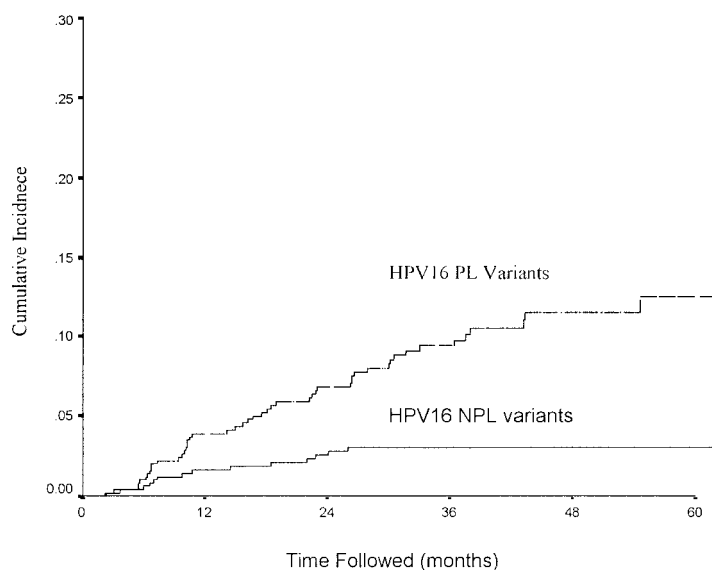


Fig. 1. Kaplan-Meier estimates of cumulative incident infection with HPV16 PL (dashed line) and NPL variants (solid line) among female students who did not have PCR-detectable HPV16 DNA at enrollment. Log-rank test, $P < 0.01$ for overall cumulative incidence of infection with HPV16 PL and NPL variants. The annual incidence was derived from cumulative incidence, which is not equal to the quotient of number of infections divided by number of women at risk due to the right censoring.

No. at risk at the beginning of the time Interval	528	448	399	256	137
No. with PL variant infection	19	13	10	5	1
No. with NPL variant infection	8	4	2	0	0
Annual PL incidence, %	3.9	2.9	2.6	2.1	1.1
Annual NPL incidence, %	1.6	1.0	0.5	0.0	0.0
Cumulative PL incidence, % (95% CI)	3.9 (2.5-6.0)	6.8 (4.9-9.5)	9.4 (7.0-12.5)	11.5 (8.7-15.2)	12.6 (9.2-16.9)
Cumulative NPL incidence, % (95% CI)	1.6 (0.8-3.2)	2.6 (1.5-4.5)	3.1 (1.8-5.1)	3.1 (1.8-5.1)	3.1 (1.8-5.1)

the OR of detecting NPL variants increased from 1.6 (95% CI, 0.3–9.1) for those who had used hormonal contraceptives for 1–4 months to 5.4 (95% CI, 1.0–28.3) for those who had used hormonal contraceptives for 5–8 months (P for trend = 0.04). In a further analysis that sought to examine whether women with NPL as compared with those with PL variants were more likely to have visits at which current use of hormonal contraceptives was reported, we included visits from study entry through the first HPV16-positive visit. After adjustment for racial groups of the women and their sex partners, the OR of reporting a current use of hormonal contraceptives, as estimated by the generalized estimating equation approach, was 1.9 (95% CI, 0.9–4.1) for women with NPL as compared with those with PL variants. Risk estimates were not materially altered by excluding those visits by women who reported no sexual intercourse since the last visit.

Factors such as positivity for other HPV types and greater lifetime number of sex partners, although related to HPV16 detection, were not variant specific, and additional adjustment for these factors did not substantially change the magnitude of risk estimates presented in Table 1 (data not shown). Additional factors that were not associated with risk for HPV16 PL or NPL variants included lower monthly income ($\leq \$1,000$ /family member), younger age at first intercourse (< 18 years), reporting a history of sexually transmitted diseases, shorter duration since last intercourse (≤ 3 days), more alcohol consumption (> 3 glasses/week), and percentage of the cervix showing cervical ectopy (data not shown).

Duration of Detectable HPV16 DNA by Variants. The mean length \pm SD of follow-up since the initial HPV16-positive visit was 23.5 ± 15.9 months for women with PL variants and 28.5 ± 14.3 months for those with NPL variants. HPV16 DNA

was detected during 184 visits (in cervical samples alone, 25 visits; in vulvovaginal samples alone, 60 visits; and in samples from both sites, 99 visits). NPL variants accounted for 21.0% of HPV16-positive cervical samples, a proportion similar to that of HPV16-positive vulvovaginal samples (20.8%). Of the 62 HPV16-positive women, 18 had only a single HPV16-positive visit (HPV16 was initially detected in 8 of these 18 women at the last visit). A single positive visit that was not the final visit was found in 14.6% of 41 women with PL and in 30.8% of 13 women with NPL variants (Fisher's exact test, $P = 0.23$). Among those who had more than one positive visit, the initial predominant variant continued to be detected at the following positive visits for all but one woman. This woman had a change of predominant variant after the fourth positive visit. Both variants, according our classification criteria, belonged to the PL group.

During follow-up, 30 (62.5%) of the 48 women with PL variants and 12 (85.7%) of the 14 women with NPL variants became negative. All but two women who had reverted to negativity remained negative. These two women, one with a PL variant and the other with an NPL variant, had the same variant detected after one negative visit. The median time (95% CI) to HPV16 DNA negative from time of initial positive visit was 17.2 months (range, 9.1–25.2 months) for women with PL variants and 14.3 months (range, 7.5–21.1 months) for those with NPL variants. As shown in Fig. 2, the overall likelihood of becoming negative was similar between women with PL and women with NPL variants (log-rank test, $P = 0.85$). Women infected with NPL variants underwent biopsy somewhat more frequently than did those with PL variants. Whereas these women were treated as being censored at the time of initial

Table 1 Association between HPV16 NPL variants and ethnicity and use of hormonal contraceptives among female students with incident HPV16 infection

	No. with HPV16 PL variants (<i>n</i> = 48)	No. with HPV16 NPL variants (<i>n</i> = 14)	OR (95% CI) ^a	<i>P</i> ^b
Racial group of study subjects				
White	39	9	1.0	
Nonwhite ^c	9	5	3.1 (0.8–12.6)	
Racial group of their sex partners				
White	32	5	1.0	
Nonwhite ^c	12	6	4.6 (1.0–20.8)	
Unknown ^d	4	3		
Racial group of study subjects and their sex partners				
White only	29	4	1.0	
Mixed or nonwhite only ^e	16	8	6.0 (1.3–27.5)	
Unknown ^f	3	2		
Reporting a history of hormonal contraceptive use at entry				
No	23	5	1.0	
Yes	25	9	2.9 (0.7–11.8)	
Time (mo.) of hormonal contraceptive use in 8 months prior to initial detection				
None	21	4	1.0	0.04
1–4	13	3	1.6 (0.3–9.1)	
5–8	14	7	5.4 (1.0–28.3)	

^a Adjusted for time of hormonal contraceptive use and ethnicity of study subjects and their sex partners (when appropriate).

^b Testing for linear trend.

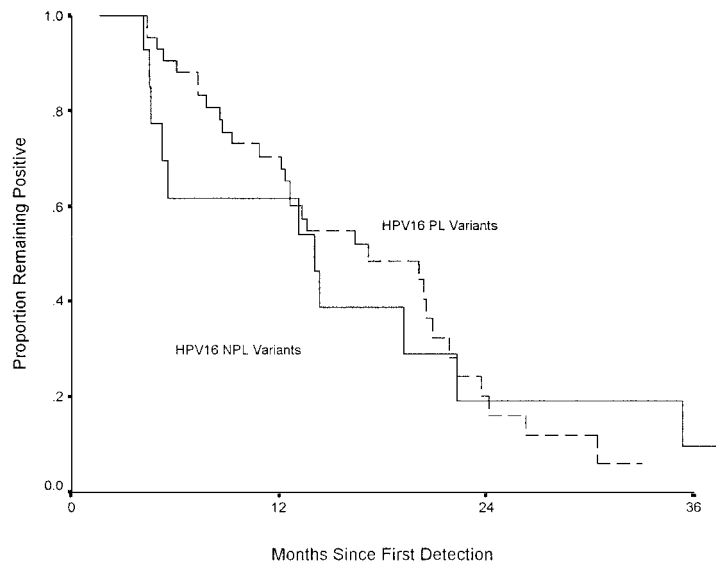
^c Including African, Asian, Hispanic, Native American, and others.

^d Not available for seven women (five whites and two nonwhites) who did not provide racial information on their male sex partners.

^e Nonwhite women with white or nonwhite sex partners or white women with nonwhite sex partners; additionally including two nonwhite women who did not provide racial information on their male sex partners.

^f Not available for five white women who did not provide racial information on their male sex partners.

Fig. 2. Kaplan-Meier estimates of proportion of women remaining HPV16-positive from the time of the first detection of HPV16 DNA among women who had incident infection with PL (dashed line) and NPL (solid line) variants. Log-rank test, *P* = 0.85 for the overall likelihood of remaining HPV16-positive between women with PL variants and those with NPL variants. The plot was truncated at the time point of 36 months after the first detection of HPV16 DNA because only a few women reached that time point and only one woman with NPL variants became negative after that time point.



No. becoming negative during the interval/ No. remaining positive at the beginning of the interval	PL	NPL	PL	NPL	PL	NPL
	12/48	5/14	15/27	5/8	3/5	1/2

biopsy, the likelihood of becoming negative between those with PL and with NPL variants remained similar (data not shown).

Risk for HPV16-positive Cervical Lesions by Variants. Among 62 women with incident HPV16 infection, cervical SIL was initially diagnosed by cytology in 11 women at visits coincident with initial HPV16-positive detection and in 19 women at visits subsequent to initial HPV16-positive detection. Two cases with SIL diagnosed at the first HPV16-positive visit were excluded from the analysis because they had also had a

diagnosis of SIL at the previous visit. There was no indication that the risks for incident cervical SIL (Table 2) or vaginal SIL (data not shown) varied with infection by different HPV16 variants.

Overall, 23 of 62 women who had incident HPV16 infection were referred for biopsy, and none of them refused. CIN grade 2–3 was histologically confirmed in 6 of 48 (12.5%) women with HPV16 PL variants and in 6 of 14 (42.9%) women with NPL variants. There were no significant differences in the

Table 2 RRs for incident SIL detected by cytology and for incident biopsy-confirmed CIN grade 2–3 in relation to HPV16 variants among female students with incident HPV16 infection

	No. with SIL/no. at risk ^a	Crude RR (95% CI)	Adjusted RR ^b (95% CI)	No. with CIN 2–3/No. at risk	Crude RR (95% CI)	Adjusted RR ^b (95% CI)
HPV16 PL variants	21/47	1.0	1.0	6/48	1.0	1.0
HPV16 NPL variants	7/13	1.2 (0.5–2.8)	1.0 (0.4–2.4)	6/14	3.5 (1.1–10.9)	3.5 (1.0–11.8)

^a Excluding two women who had low-grade SIL at both the initial HPV16-positive visit and the previous visit.

^b Adjusting for ethnicity and current use of hormonal contraceptives.

Table 3 HPV16 seroconversion among female students with incident infection by HPV16 PL or NPL variants^a

	Infected with HPV16 PL variants (<i>n</i> = 46)	Infected with HPV16 NPL variants (<i>n</i> = 13)
Total no. (%) seroconverted	30 (65.2)	8 (61.5)
No. (%) seroconverted within the first 6 months ^b	8 (20.0)	4 (33.3)
Median months (95% CI) to seroconversion	8.2 (5.9–10.5)	6.2 (2.1–10.3)

^a Excluding three women who did not provide any samples for serology at the time of initial HPV16 detection and thereafter.

^b Excluding seven women, one with NPL and six with PL variants, who had a length of follow-up of <6 months after the first detection of HPV16 DNA.

severity, involvement of the cervical canal, or vascular patterns of the lesions between CIN grade 2–3 cases associated with PL variants and those associated with NPL variants (data not shown). After adjusting for race and current use of hormonal contraceptives, the RR of 3.5 (95% CI, 1.0–11.8) for CIN grade 2–3 was associated with HPV16 NPL variants as compared with PL variants (Table 2). The risk estimate was not substantially affected by further adjustment for cumulative lifetime number of sex partners, having new sex partners, or infection with HPV types other than HPV16 (data not shown). All but two CIN grade 2–3 cases were diagnosed at the visits coincident with HPV16-positive detection. With an exclusion of the two cases whose CIN grade 2–3 was diagnosed at the visit after the last HPV16-positive detection, the RR of CIN grade 2–3 was 5.2 (95% CI, 1.5–18.4) for women with HPV16 NPL variants as compared with those with PL variants. There were seven women from whom HPV16 DNA was detected only in vulvovaginal samples. In a further analysis in which HPV16 status was determined according to the testing results of cervical samples alone, the increased risk of CIN grade 2–3 remained associated with HPV16 NPL variant status (RR = 3.3; 95% CI, 1.0–10.8).

Detection of HPV16 Antibody Responses among Women with Incident Infection of PL or NPL Variants. Among women with incident HPV16 infection from whom serum samples were available since the initial detection of HPV16 DNA, the overall seroconversion rate was 65.2% for those with PL variants and 61.5% for those with NPL variants (Table 3). All but three seropositive women (one with NPL variants and two with PL variants) had antibodies against HPV16 initially detected within 20 months after the first detection of HPV16 DNA. Among those who had at least 6 months of follow-up after the initial HPV16-positive visit, 4 of 12 (33.3%) with NPL variants as compared with 8 of 40 (20.0%) with PL variants seroconverted within the first 6 months (Fisher exact test, $P = 0.44$). The median time to seroconversion from the visit at which the HPV16 DNA was initially detected was 8.2 months (95% CI, 5.9–10.5 months) for those with PL variants and 6.2 months (95% CI, 2.1–10.3 months) for those with NPL variants. Of the 35 women who provided at least one follow-up visit after the initial seropositive visit, the majority remained seropositive within the study period, even for those who had been

followed for more than 36 months after seroconversion (data not shown).

Discussion

In this cohort of female students attending the University of Washington, most of whom had recently (within the last year) initiated sexual activity, the first year incidence was 3.8% for HPV16 PL variants and 1.7% for NPL variants. The incidence of both variant groups decreased in subsequent years. Although the reason for decline in annual incidence is not understood, it may be due to the fact that genital HPV infection is acquired early after initiation of sexual activity (38, 39). PL variants accounted for a large proportion of HPV16 infections in this study population. Importantly, our data showed differences in risk factor profiles for acquisition of HPV16 variants, with nonwhite ethnicity and use of hormonal contraceptives being risk factors for NPL variants.

NPL variants as defined in this study were similar to those commonly detected in Africa, whereas the PL variant group includes those variants that are most prevalent in Europe and North America (13). The tendency of nonwhite women to be at an increased risk for NPL variants appears to go along with findings of the geographic distribution of HPV16 variants (12–14). Consistent with this, our results showed an association between detection of NPL variants and report of nonwhite male sex partners. These data suggest that NPL variants may be more prevalent in both male and female nonwhite young adult populations residing in North America. The underlying biological mechanism for the association between race and risk for HPV16 variants is presently unclear but may be mediated through certain host factors. One interpretation is that the association between race and HPV16 variants may simply reflect long-term sexual mixing patterns in the population, whereby sex more commonly occurs between members of the same race than between members of different races. It is also possible that certain HLA alleles may preferentially predispose women to establish and/or retain infection with particular HPV16 variants.

Use of hormonal contraceptives was the other risk factor for HPV16 NPL variants. Our data showed a trend of increase in risk for detecting NPL variants in women who frequently reported use of hormonal contraceptives and in women who had a relatively longer duration of use of hormonal contraceptive

before the first detection of HPV16 DNA. Given a strong correlation between use of hormonal contraceptives and sexual activity, we considered the possibility that the observed association might result from different patterns of sexual activity rather than a direct effect from hormonal contraceptives. However, risk estimates were not substantially affected when the analysis was confined to visits at which the women reported having had sexual intercourse since the last visit. It is therefore possible that small amounts of additional hormones may favor the acquisition of newly detectable NPL variants. Certain nucleotide alterations in NPL variants may result in altered responses to the hormonal environment in host. Support for this comes from experimental studies showing the presence of glucocorticoid response element sites in the noncoding region (40, 41). One recent study has further demonstrated a relationship between increased levels of hormonal-induced viral E6-E6 RNA and mutated glucocorticoid response element constructs (42).

The association between viral persistence and HPV16 variants has been reported previously (5, 20), but the findings have been inconsistent. In these studies, the variants were defined according to a single nucleotide alteration at position 350 in the E6 region, and HPV16 infections of unknown duration were detected at study entry. Although one recent study reported that relative to nononcogenic HPV types, the OR for viral persistence was somewhat high for non-European as compared with European variants, the difference between the two variant groups was not substantial (11). In addition, defining persistence in these studies was only dichotomized based on the presence or absence of HPV16 DNA in one (5) or more (11, 20) consecutive visits. Our data provided no evidence for a difference in persistent detection of PL and NPL variants. In our present study, we included incident infections alone and directly measured the median duration and probability of becoming PCR test negative.

It should be noted that in this study, HPV16 DNA detection was performed periodically with an interval of approximately 4 months. Therefore, infections with a duration of <4 months may have been missed. This could bias the duration estimates of viral persistence upwards. However, it most likely affected PL and NPL variants similarly because there was no evidence that the shortest period of persistence detected (*i.e.*, a single positive visit) was associated with variant status. We also considered that women infected with HPV16 NPL variants underwent biopsy somewhat more frequently than did those with PL variants and that biopsy procedure might change a potential for viral persistence by eliminating the focus of the infection. However, while treating women who underwent biopsy as being censored at the time of first biopsy, the analyses yielded equivalent results. Finally, we recognize that our data addressed only a likelihood of viral DNA changing from detectable to undetectable status. It is presently unclear whether return to DNA negativity reflects complete clearing of the viral variants or merely suppression of the viruses to a level below detection.

The initial report from this cohort has shown an increased risk for development of biopsy-confirmed CIN grade 2–3 associated with NPL variants among women who had either prevalent or incident HPV16 infection (9). This study extends the previous observation by examining risk for biopsy-confirmed CIN grade 2–3 among women with incident HPV16 infections. In this study, we did not observe appreciable difference in risk for incident SIL between women with HPV16 PL and women with NPL variants. This might be explained by the fact that many other factors such as infection with other HPV

types may greatly contribute to risk for SIL and therefore dilute the possible risk difference between the variants. Importantly, the increased risk for CIN grade 2–3 was found to be associated with NPL as compared with PL variants, a finding consistent with our previous report and with two recent studies, which used a variant classification system of European *versus* non-European variants that is equivalent to the one used in this study [*i.e.*, European is equivalent to PL variants, and non-European is equivalent to NPL variants (10, 11)]. The increased risk associated with NPL variants was not explained by factors previously shown to be related to risk for cervical neoplasia such as infection with other HPV types, greater number of sexual partners, and a prolonged viral persistence or by factors unique to risk for NPL variants. It is also unlikely that the risk difference was due to chances of being referred for biopsy because biopsy referral criteria were standardized for the cohort.

One explanation could be that the variants have slightly different tropisms, with NPL variants preferentially infecting the cervix rather than the vulva and vagina, or that the cervix could be more permissive for replication of NPL variants. Arguing against this is the fact that the proportion of NPL variants was similar between HPV16-positive cervical specimens and vulvovaginal specimens. In addition, the increased risk of CIN grade 2–3 associated with NPL variants remained when HPV16 status was determined by results of cervical samples alone. It is presently unclear whether the increased risk results from differential cellular tropisms, with some types of cells being more permissive for replication of NPL variants, or whether types of infected cervical cells vary in their potential to progress to advanced cervical lesions. It is also possible that the increased risk may result from differences in host factors. If certain factors such as HLA influence the ability of particular variants to become established or to replicate, perhaps due to immune surveillance mechanisms (15), it would be of interest to see whether these factors also play a role in the pathogenesis of variant-related cervical lesions. A third and highly plausible explanation for the increased risk associated with NPL variants is that nucleotide alterations affect the biological properties of variants, with some alterations resulting in an increased neoplastic potential. Supporting evidence has been provided by *in vitro* studies showing that certain nucleotide alterations in the HPV16 noncoding region affect promoter activity (43) and cell transformation (40) and that natural HPV16 mutants differ in transcriptional activities (44) and in abilities to induce p53 degradation and keratinocyte differentiation (45). These properties are believed to be relevant to the pathogenesis of cervical neoplasia. It will be important to elucidate what and how specific nucleotide alterations present in the NPL variants correlate individually or in combination to their biological properties *in vitro* and *in vivo*.

Studies of antibody responses to HPV16 variants are rare, with inconsistent findings reported. One study showed that women infected with a particular HPV16 variant were less likely to be antibody positive than women with the prototype (22), whereas another study demonstrated that lack of antibody response was independent of viral sequence variation (23). However, because both studies included serology results based on a single measurement, rate of seroconversion could not be estimated. In this prospective study, we observed a similar rate of seroconversion for women with incident HPV16 PL and NPL variants. There was no significant difference in the median time to seroconversion from the time of first HPV16 DNA detection. In a previous report from this population (46), the median time to seroconversion was estimated as 11.8 months,

which is longer than the current estimate. This is because the previous analysis excluded women with incomplete serological data, particularly those who had no serological results from the visit that corresponded to the first detection of HPV16 DNA but were seropositive at the subsequent visit. When the analysis was restricted to the same group of women used in the previous report (46), we still failed to find a significant difference in seroconversion between those with PL and with NPL variants. The follow-up time in this study was not long enough to examine the persistence of antibody responses by the variants. However, most of the women remained seropositive at the last visit, even those who had been followed for >36 months after seroconversion.

Although the current cohort included >500 female students with an average of 39 months of follow-up, only 62 incident HPV16 infections were identified. Secondly, approximately 25% of the cohort was lost before completing 3 years of follow-up. However, women who failed to return for follow-up did not differ significantly from those who continued to be followed with respect to race, a history of hormonal contraceptive use, sexual behavior, and infection with other HPV types at study entry. A third concern is the length of time followed after the initial HPV16-positive visit. Although women with NPL and PL variants were followed for an equivalent time period, this time may not have been sufficient to fully examine the late effects of these infections. At present, there are no data to suggest that women with PL variants as compared with those with NPL variants are more likely to develop high-grade cervical lesions after 3 years. Lastly, we noted incomplete information on the race of male sex partners. In the classification of racial groups of the women and their male sex partners, we did not take all past sex partners at study entry into consideration, only sex partner(s) reported in the month before study enrollment. However, there was no reason to suspect that the missing racial information differed by variant status, and we did not observe an association between incident HPV16 infection and the number of sex partners before study enrollment.

Despite the limitations presented, this study represents one of the most comprehensive and systematic attempts to address the acquisition and natural history of HPV16 variants. Our cohort data were not susceptible to selection biases that may arise in conventional case-control studies. Because data and specimens were prospectively collected at regular time intervals, and assessments of the variants and diagnoses of the cervical lesions were done without knowledge of other information, we minimized possible biases resulting from recall and ascertainment. Importantly, our data included only incident infections and therefore avoided bias introduced by the use of prevalent infections.

In summary, our data indicate that nonwhite race and use of hormonal contraceptives appear to be associated with an increased risk for acquisition of newly detectable HPV16 NPL variants. Although there was no difference in viral DNA persistence between the two groups of variants, NPL variants, as compared with PL variants, increased the risk for high-grade but not low-grade cervical lesions.

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