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GENOMIC VARIATION OF HUMAN PAPILLOMAVIRUS TYPE 16 IN RELATION TO RISK FOR HIGH GRADE CERVICAL AND ANAL INTRAEPITHELIAL NEOPLASIA

by

Long Fu Xi

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

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Approved by *Laura Koutsky*
Chairperson of Supervisory Committee

Program Authorized to Offer Degree *Epidemiology*

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Doctoral Dissertation

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Abstract

GENOMIC VARIATION OF HUMAN PAPILLOMAVIRUS TYPE 16 IN RELATION TO RISK FOR HIGH GRADE CERVICAL AND ANAL INTRAEPITHELIAL NEOPLASIA

by Long Fu Xi

Chairperson of the Supervisory Committee: Associate Professor Laura A. Koutsky
Department of Epidemiology

Prospective studies were conducted among women attending a University and women presenting to a STD clinic to evaluate an association between risk of cervical intraepithelial neoplasia grade 2-3 (CIN 2-3) and human papillomavirus type 16 (HPV16) variants. CIN 2-3 was histologically confirmed in 9 of 57 HPV16 positive women attending the University and in 10 of 66 HPV16 positive women presenting to the STD clinic. Among university students, those with HPV16 non-prototype-like (NPL) variants were 6.5 (95% CI, 1.6-27.2) times more likely to develop CIN 2-3 than those with prototype-like (PL) variants. A similar association was observed among women presenting to the STD clinic (RR = 4.5; 95% CI, 0.9-23.8).

An association between risk of anal intraepithelial neoplasia grade 3 or carcinoma in situ (AIN3/CIS) and HPV16 variants was examined in a cohort of bisexual or homosexual men. Of 589 men, 37% were positive for HPV16, including 33% with PL variants and 4% with NPL variants. AIN3/CIS was histologically confirmed in 22 cases. Among men with HPV16 infection, those with NPL variants were 4.3 times (95% CI = 1.6-11.6) more likely to develop AIN3/CIS than those with PL variants. Additional adjustments for HIV

status and CD4 count or the level of HPV16 DNA did not alter risk estimates substantially. Neither a high level of viral DNA nor a prolonged period of DNA detection was associated with HPV16 NPL variants.

To verify whether HPV16 variants change over time, sequence variation of variants was examined in consecutive specimens from individuals. Seventy subjects who were repeatedly HPV16 DNA positive, over 2-8 four-monthly visits, showed an identical polymorphism at every visit. Sequencing many clones from each specimen confirmed that one major variant seemed to predominate over time, whereas minor variants appeared more transient.

While the data suggest that HPV16 NPL as compared to PL variants are associated with an increased risk of high grade lesions, the biological mechanism relating to this excess risk remains undetermined. In view of the relatively short time followed, it would not be appropriate to generalize our data beyond the observed time period nor to the risk of invasive cancer.

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INTRODUCTION

Human papillomaviruses (HPVs) play an important role on the etiology of neoplasia and invasive cancer (1-7). Different types of HPVs vary in tissue affinity and pathogenicity (8). Studies have demonstrated the presence of phylogenetically distinct variants of specific HPV types (9-12). Recent interest has focused on possible differences in the biologic characteristics of different variants.

A report of a case series (13) showed a possible link between risk of recurrent laryngeal papillomatosis and a deletion in the L1 region of HPV6a. Analysis of sequence variation in the E2 region of HPV18 from 20 clinical specimens (representing a spectrum of pathology from low grade cervical intraepithelial neoplasia [CIN] to invasive cervical cancer) suggested the presence of an HPV18 subtype with decreased oncogenic potential (14). Experimental studies have shown that nucleotide alterations of HPV16 affect the potential for oncogenic transformation in vitro (15), some alterations enhance promoter activities which drive transcription of oncoproteins E6 and E7 (16), and some alterations affect abilities to alter keratinocyte differentiation and to induce p53 degradation (17). It is well known that HPV16 is the most common cancer-associated HPV type. However, it is also the most common type of HPV in the cytologically normal population (18), and only a minority of individuals with HPV16 infection develop invasive cancer (19). These

observations prompted a cohort study of the relationship between natural variants of HPV16 and risk of biopsy-confirmed cervical intraepithelial neoplasia grade 2 or 3. This study is described in Chapter 1.

It has been postulated that anal intraepithelial neoplasia (AIN) and cervical intraepithelial neoplasia are multifocal disease processes (20). Pathologic studies have suggested a number of similarities between AIN and CIN, including morphologic and histologic features (21) and potential for progression to invasive cancer (22). HPVs are strongly associated with both anal and cervical neoplasia and even a pattern of an association between types of HPV and histopathological severity of the anal lesions (23-24) is similar to cervical lesions (25-26). Consequently, the association between HPV16 variants and risk of high grade AIN would be expected to resemble the association between HPV16 variants and risk of high grade CIN. This was the motivation for performing the cohort study presented in chapter 2. The goal of the study presented in chapter 2 was to examine the relationship between HPV16 natural variants and risk of biopsy-confirmed anal intraepithelial neoplasia grade 3 or carcinoma in situ, the most important precancerous lesion of anus (26-28).

An important technical issue underlying the above cohort studies is whether HPV16 variants change over time. Analyses of cohort data could be difficult to construct and interpret if transient infection by multiple variants was a frequent occurrence. It would be

necessary to consider the impact of both previous and current variants on risk of high grade lesions. The need for clarifying this issue led to the experimental study described in Chapter 3. This study sought to characterize HPV16 variants in cohort populations and to track sequence variation over time. Although this study did not directly address the associations between HPV16 variants and risk of high grade lesions it provided a solid foundation for studying these associations.

Data presented here will provide valuable information useful to the designing of cervical cancer screening and HPV vaccination programs.

CHAPTER 1: GENOMIC VARIATION OF HUMAN PAPILLOMAVIRUS TYPE 16 AND RISK FOR HIGH GRADE CERVICAL INTRAEPITHELIAL NEOPLASIA

To date, more than 80 types of human papillomaviruses (HPVs) have been identified and characterized. New HPV types are defined by less than 90% homology with other types, when comparing the DNA sequence of the complete L1 coding region; subtypes differ by between 2%-10%; and variants differ by less than 2% (29). Different types of HPVs vary in host specificity, tissue affinity, and pathogenicity (8). The possibility that the differences in biological characteristics might also be present among intratypic HPV variants deserves consideration.

Epidemiological studies have demonstrated strong and consistent associations between detection of HPV16 DNA and risk for cervical intraepithelial neoplasia (CIN) and cervical cancer (25, 30-32). However, HPV16 is also the most common type of HPV in the cytologically normal population (18), and only a minority of women with HPV16 infection develop cervical cancer (19). Experimental studies have shown that nucleotide alterations of HPV16 affect the potential for oncogenic transformation in vitro (15) and some alterations enhance promoter activities that drive transcription of oncoproteins E6 and E7 (16). Studies of genomic heterogeneity of HPV16 demonstrate the presence of multiple

variants in all populations examined to date (9-12). These observations suggest that natural variants of HPV16 in a population may not have the same biological behavior.

The aim of this study was to evaluate the relationship between natural variants of HPV16 and risk of biopsy-confirmed cervical intraepithelial neoplasia grade 2 or 3 (CIN 2-3); the most important precancerous lesion of uterine cervix (1, 3).

METHODS

STUDY POPULATION AND STUDY DESIGN

Study subjects were from two cohorts: female students enrolled in a university (1990-1995) and women presenting to a sexually transmitted disease (STD) clinic (1989-1995). All subjects provided written informed consent to participate in a protocol that had been approved by the University of Washington Institutional Review Board. At the time of the enrollment visit, university students were all between 18 and 20 years of age and STD clinic patients were between 16 and 47 years of age. The original cohorts and study design have been described (30, 33). Face to face, structured interviews were conducted to elicit information on demographic characteristics, sexual behaviors, and history of STDs. Vulvar, vaginal, and cervical swabs for HPV typing were collected at enrollment and approximately every 4 months thereafter. Samples were screened by a consensus primer polymerase chain reaction (PCR) amplification assay (34).

Eligible subjects for the current study were required to be without CIN 2-3 at entry and to have at least one HPV16 positive visit either at entry or during follow up. A total of 123 women who met these criteria were included in the analysis. The women included were followed from date of first HPV16 positive visit to date of biopsy-confirmed CIN 2-3, loss-to-follow up, or the end of 1995, whichever was earliest. Thirty-seven women, including 12 university students and 25 STD clinic patients, were lost to further follow up after an average of 4.8 visits (range 1-14) and therefore censored at the date of their last visit. Reasons for loss-to-follow up included: moved out of state (n = 11), unable to locate after 3 or more attempts (n = 13), refusal (n = 12), and major surgery (n = 1). The number of visits for each subject since the initial detection of HPV16 DNA ranged from 1 to 12 with a mean of 5.7 for women attending the University and from 1 to 16 with a mean of 7.0 for women presenting to the STD clinic. During follow up, subjects found to have lesions that were colposcopically or cytologically suggestive of CIN 2-3 were referred for biopsy. The preparation of biopsy specimens and staining of slides was standardized (39-40). Histologic diagnoses were assigned as negative, mildly atypical, or consistent with cervical intraepithelial neoplasia grade 1, 2, or 3 (40). None of the women had cytological, colposcopic, or histologic evidence of invasive cervical cancer.

CHARACTERIZATION HPV16 VARIANTS

Specimens positive for HPV16 in screening were further assayed by PCR based single stranded conformation polymorphism (SSCP) analysis to identify variants. The assay was conducted as previously described (35). Briefly, DNA amplification was completed in a Perkin-Elmer 9600 Thermal Cycler (Perkin-Elmer Cetus, Norwalk Conn) with 35 cycles. Each cycle consisted of denaturation (94° C, 25 s), annealing (62° C, 25 s) and extension (72° C, 50 s). [α-^{33}P] dATP (Du Pont NEN, Research Products, Boston, MA) was incorporated into PCR products during the amplification with a pair of type specific primers C and D (35), targeting 682 bp from nucleotides 7445 to 222 in the HPV16 noncoding region (NCR). PCR products were cleaved into three fragments, 318, 166, and 198 bp from 5' to 3', by restriction endonuclease digestion with DdeI, and electrophoresed in a 5% polyacrylamide gel with 10% glycerol.

The SSCP patterns of three fragments in the NCR were compared individually with the reference patterns from prototype plasmid HPV16 (pHPV16) DNA (36) and Caski cellular DNA (37) and with the patterns from other specimens. The designation of SSCP patterns was the same as that previously described (35). HPV16 isolates were regarded as different variants as long as any one of the 3 fragments showed different polymorphisms. On the basis of previous findings of the correlation between SSCP patterns and sequence variation (12), all HPV16 variants were classified into two groups. The prototype-like (PL) group included variants that displayed the reference SSCP pattern and variants with

one or two fragments that had only one or two nucleotide alterations. The non-prototype-like (NPL) group included variants that displayed non-reference SSCP patterns in all 3 fragments and that had many nucleotide alterations when compared to the HPV16 prototype. The determination and classification of variants was performed without knowledge of the clinical data.

Direct sequencing of PCR products has been described previously (35). In brief, PCR products of the entire NCR were generated with a pair of primers A and D. DNAs were purified with QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA). The purified templates (70-100 ng) were sequenced using ABI PRISM™ Dye Terminator Cycle Sequencing kit (Perkin-Elmer Cetus) based on the protocol recommended by the manufacturer. The sequencing reactions were run on an Applied Biosystem Model 373 A DNA sequencing system (Applied Biosystems Inc.). Sequences from nucleotide position 7469 to 191 were determined from both directions with two pairs of primers: primers C and F, or primers D and E (35). Sequences were analyzed with Sequencher™ (Gene Codes, Corp., Ann Arbor, MI).

A method designated lineage-specific hybridization (LSH) (38) has been developed to rapidly assign HPV16 variants to the 5 previously identified phylogenetic lineages of HPV16 (9). Briefly, HPV16 DNAs from 50 specimens from university women were amplified by nested PCR using E6 primers. The amplified products were hybridized to a

set of 23 E6 probes that target sequence variations at nucleotide positions 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532. The sequences of the primers and probes used and the conditions of PCR and hybridization were previously described (38).

STATISTICAL ANALYSES

Cox proportional hazard model (41) using EGRET (Statistics and Epidemiology Research Corp., Seattle, Wash) statistical software was used to estimate relative risks (RRs) and confidence intervals (CIs) for CIN 2-3 associated with HPV16 variants, while adjusting for age (≤20, ≥21 years), lifetime number of sexual partners (≤5, ≥6 partners), HPV16 status at entry (positive, negative), ethnic group (white, non-white), and number of visits positive for HPV16 (1-3, ≥4 visits). Kaplan-Meier product limit estimates were used to calculate the cumulative proportion of women in whom CIN 2-3 developed. The mean duration at risk per-person was examined by student's t test. Kappa value was used to assess a concordance of the classification of HPV16 variants by two detection systems.

RESULTS

DISTRIBUTION OF HPV16 VARIANTS

Overall, 176 of 482 visits from 57 women attending the University and 207 of 585 visits from 66 women presenting to the STD clinic were positive for HPV16. PL variants accounted for most of the HPV16 detected (79% in women attending the University and

86% in women presenting to the STD clinic). Women with HPV16 PL variants had a similar mean duration of follow up compared to those with an NPL variant (25.5 versus 18.4 months in university students, P = 0.17; 27.1 versus 23.4 months in STD clinic patients, P = 0.62). The distribution of HPV16 variant groups did not vary significantly by age, lifetime number of sexual partners, ethnicity, HPV16 status at entry, or number of visits positive for HPV16 (data not shown).

RISK OF CIN 2-3 IN RELATION TO HPV16 VARIANTS

During follow up, biopsy-confirmed CIN 2-3 developed in 9 (15.8%) of 57 HPV16 positive university students and 10 (15.2%) of 66 HPV16 positive STD clinic patients. Factors including age, lifetime number of sexual partners, HPV16 status at entry, race, and number of visits positive for HPV16 were not significantly associated with risk of biopsy-confirmed CIN 2-3 in either of the two cohorts (table 1). Among women attending the University, a 6.5-times greater risk (95% CI, 1.6-27.2) of biopsy-confirmed CIN 2-3 was associated with HPV16 NPL variants as compared to PL variants (table 2). A similar tendency of increased risk associated with HPV16 NPL variants was observed among women presenting to the STD clinic (RR = 4.5; 95% CI, 0.9-23.8). With the exclusion of women who were positive for HPV16 at entry, risk of CIN 2-3 remained elevated among university students with incident HPV16 NPL variants relative to those with an incident

PL variant infection (RR = 10.1; 95% CI, 1.8-55.8). Risk estimates based on incident HPV16 infection were not available for the STD clinic cohort.

As figure 1 (a) shows, the overall cumulative proportion of university students who developed CIN 2-3 was higher among those with HPV16 NPL variants than that among those with PL variants (P = 0.01). The 30-month cumulative proportion of university students who developed CIN 2-3 from the time of the first visit positive for HPV16 was 57% for those with NPL variants, as compared to only 13% for those with a PL variant. Among women presenting to the STD clinic, the 30-month cumulative proportion of women with CIN 2-3 was 39% for those with NPL variants and 16% for those with PL variants (log rank test, P = 0.10) (figure 1 b). Of 19 CIN 2-3 cases from two cohorts, 17 (89%) developed CIN 2-3 within 30 months of the initial detection of HPV16. Two women who presented to the STD clinic developed CIN 2-3 approximately 40 months after their first positive HPV16 DNA test. Both of these women had a HPV16 PL variant.

CHARACTERIZATION OF PL AND NPL VARIANTS

The correspondence between the various genotyping schemes, including SSCP, sequencing, and LSH, and the resultant nomenclature is depicted in figure 2. DNA sequencing of HPV16 from nucleotide position 7469 to position 191 was performed in 19 specimens selected to be representative of all variants collected in this investigation. No

DNA sequence changes occurred that were not reflected as alterations in the SSCP patterns except for a T-to-G transition at nucleotide 42 in specimen HHH245. The SSCP pattern of the 198-bp fragment for this specimen could not be distinguished from an A-to-G alteration at nucleotide 131 observed in specimens HHH103 and HHH228 and in the Caski cell line. All fragments, which had the same nucleotide alterations, displayed the identical SSCP patterns. As compared to pHPV16, variants in group NPL carried more nucleotide alterations than variants in group PL.

Sequence variation in HPV16 E6 region for 50 specimens from university students was further assessed using a LSH assay. Of 10 specimens with HPV16 NPL variants, six were classified as Asian-American (AA) variants; two as African (Af) variants; one as European (E) variant, and one as Asian (As) variant, a subclass of European lineage (9, 11). All remaining 40 specimens with HPV16 PL variants were classified as European variants. The concordance of the classification of variants between LSH and SSCP analysis was high (96% exact agreement, Kappa value = 0.86). Women with HPV16 non-European variants were 4.5 times more likely to develop biopsy-confirmed CIN 2-3 compared to women with European variants (95% CI, 1.2-16.8).

DISCUSSION

Consistent with previous studies (18, 30-31), we observed a higher risk for CIN 2-3 among women with HPV16 infection than among those without HPV16 DNA detected (data not shown). Risk of biopsy-confirmed CIN 2-3 was associated with the infecting HPV16 variant, with NPL variants associated with the higher risk. One possible explanation for the greater risk of CIN 2-3 associated with HPV16 NPL variants is that women infected with HPV16 PL variants were more likely to be lost during follow up. However, length of follow-up was similar for those with PL and NPL variants. In addition, women followed until study end point closely resembled those lost to follow up before reaching a study end point with respect to age at entry, ethnicity, and lifetime number of sexual partners (data not shown).

Alternatively, the increased risk of CIN 2-3 associated with HPV16 NPL variants may represent a difference in the biological behavior of variants. In our data, the excess risk for CIN 2-3 associated with HPV16 NPL variants was not explained by factors implicated in previous studies (42-44), including high lifetime number of sexual partners, more HPV16 positive visits, and non-white ethnicity. The consistency of the association between risk of CIN 2-3 and HPV16 NPL variants across the two demographically different populations supports the hypothesis that biologic potential for HPV16 variants is not the same. In

addition, a significantly increased risk for anal intraepithelial neoplasia grade 2-3 was associated with HPV16 NPL variants in a similar cohort study of homosexual men presenting to the Seattle-King County AIDS Prevention Project (personal communication). Furthermore, the difference of risk for CIN 2-3 associated with HPV16 variants was consistently observed when the variants were classified using different testing systems based on regions other than the NCR.

It should be pointed out that both prototype HPV16 and the HPV16 PL variants in the Caski cell line originated from cancer patients. In view of the fact that the increased risk of CIN 2-3 was associated with HPV16 NPL variants rather than PL variants, one explanation could be that the lesions associated with NPL variants are not necessarily precursors of cervical cancer, but are more likely to be transient lesions resulting from productive HPV16 infection. NPL variants might have an intrinsic growth advantage over PL variants.

It is also possible that HPV16 variants might differ in cellular tropism. The type of cells may be important in determining the natural history of infection with specific variants through two possible ways. First, some types of cells might be more permissive for viral replication resulting in an over-growth of particular variants. The observation of an increased rate of nucleic acid synthesis in reserve cells as compared to those of basal cells of squamous portio epithelium (45) suggests that cellular factors in different types of cells

might stimulate the production of virions differently. Again, the lesions could be the direct result of a large amount of virus. Second, lesions derived from different types of cells, although showing similar morphological abnormalities, might have a variable biological behavior resulting in different potentials for progression to cervical cancer. It is well known that only a portion of women with CIN 2-3, if left untreated, would develop cervical cancer. Thus, it is likely that the histological classification of CIN 2-3 does not completely reflect biological potential.

The variable neoplastic potential of HPV16 variants is suggested, in part, by in vitro studies, indicating that nucleotide alterations in the NCR of HPV16 enhance promoter activity (16) or alter oncogenic potential in the presence of ras oncogene and hormone (15) and that a change in the E6 region may affect cytotoxic T lymphocyte responses (46). Some common nucleotide alterations in variants from our population were located in nuclear protein binding sites (47). However, it is unknown which of the reported nucleotide alterations modify biologic function leading to a change in oncogenicity in vivo. Furthermore, it has been reported that nucleotide alterations in one region of HPV16 often connect to some changes in other regions (10-11, 48). Thus, the sequence variation identified in the NCR may reflect a co-segregation of genetic alterations in other regions, including those not examined here. This linkage makes it feasible to tag HPV16 variants based on a partial sequence rather than whole genome. However, this linkage also

complicates an interpretation in targeting a relationship between a particular nucleotide alteration and the manifestation of phenotype.

Several limitations of the study should be noted. The number of study subjects was small and thus the confidence intervals were wide. Due to a limited sample size, we grouped individual variants, based on polymorphisms, into two categories. Thus, it is impossible to detect any differences of risk by individual variants within group. Nonetheless, this classification would not be expected to give an overestimate, given the assumption that HPV16 NPL variants might represent a group of variants with an increased risk for CIN 2-3. As stated in the method section, women who were positive for HPV16 at entry were included in the study. The initial HPV16 positive time for those women was unknown. Bias could be introduced if HPV16 status at entry was associated with a particular variant group. However, when the analysis was restricted to women who had incident HPV16 infection (i.e., HPV16 negative at entry) the association between NPL variants and CIN 2-3 remained strong. Finally, the data do not rule out the existence of other factors that might be associated with both HPV16 variant group and with risk for CIN 2-3.

In conclusion, the data from the present study suggest that the risk for CIN 2-3 is not the same for all variants of HPV16 and that NPL variants confer a greater risk compared to PL variants. The underlying important genomic differences remain to be determined.

Table 1 Characteristics of University Students and of STD Clinic Patients in Relation to Biopsy-Confirmed Cervical Intraepithelial Neoplasia Grade 2-3

Variables	University students (n=57)				STD clinic patients (n=66)			
	No. of subjects	No. with CIN 2-3	RR*	95% CI†	No. of subjects	No. with CIN 2-3	RR*	95% CI†
Age at entry								
≤20	57	9	-		17	4	1.0	
≥21	0	0			49	6	0.6	0.2-2.1
Racial group								
white	46	6	1.0		44	8	1.0	
non-white	11	3	2.3	0.6-9.1	12	2	1.3	0.3-6.1
Lifetime No. of sexual partners								
at entry ≤5	41	6	1.0		19	2	1.0	
≥6	16	3	0.9	0.2-3.7	47	8	1.8	0.4-8.3
HPV16 status at entry								
negative	33	6	1.0		32	7	1.0	
positive	24	3	0.5	0.1-2.2	34	3	0.4	0.1-1.7
No of visits positive by PCR								
1-3	37	6	1.0		41	4	1.0	
≥4	20	3	0.6	0.1-2.2	25	6	1.4	0.4-5.1

- not applicable; RR* estimates based on Cox regression; † 95% confidence interval

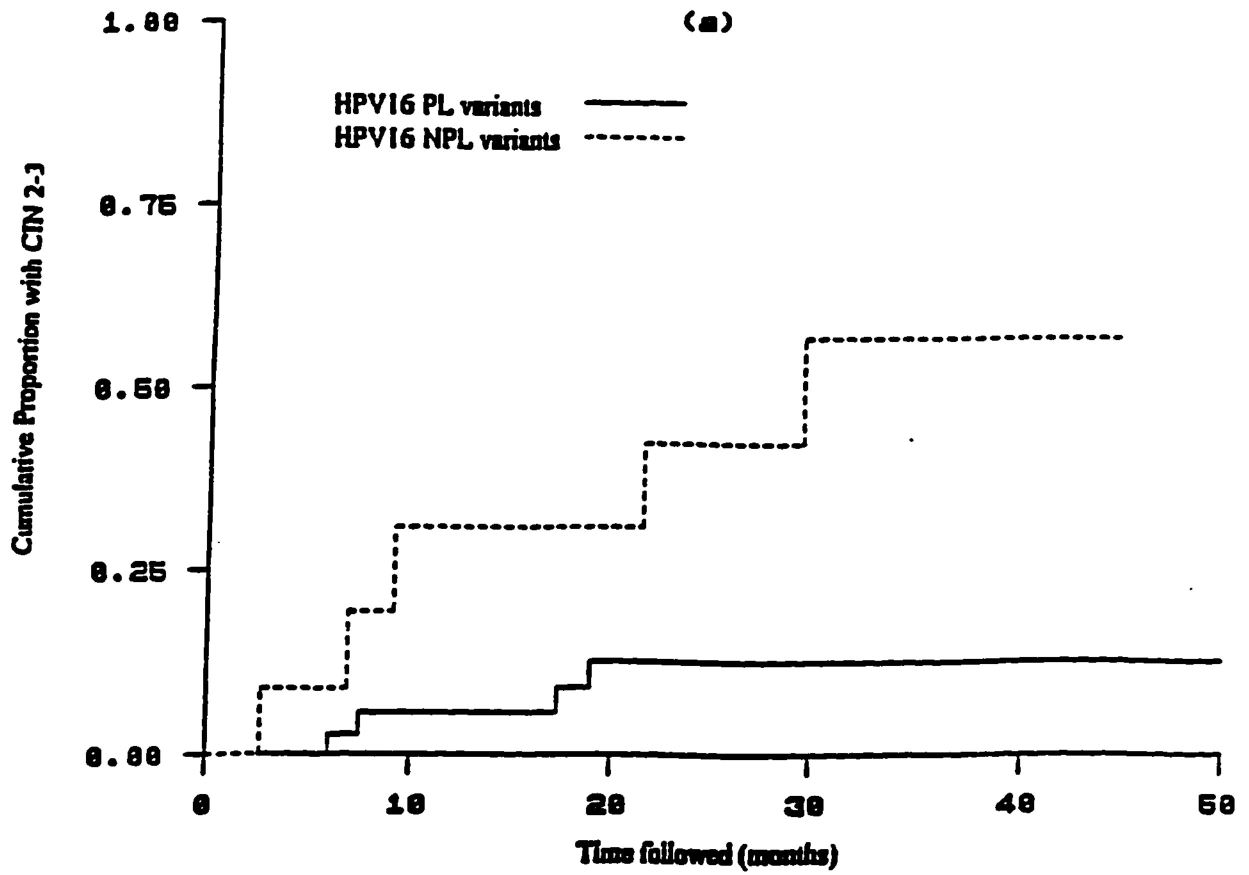
Table 2 Risk for Biopsy-Confirmed Cervical Intraepithelial Neoplasia (CIN) Grade 2-3 Associated with HPV16 Variants among University Students and among STD Clinic Patients

Cohorts	HPV16 variant group	No. (%) of CIN 2-3	No. of subjects	RR*	95% CI
University students	PL	4 (8.9)	45	1.0	
	NPL	5 (41.7)	12	6.5	1.6-27.2
STD clinic patients	PL	7 (12.3)	57	1.0	
	NPL	3 (33.3)	9	4.5	0.9-23.8

* RR estimates adjusted for age (≤20, ≥21 years), lifetime number of sexual partners (≤5, ≥6 partners), HPV16 status at entry (positive, negative), ethnic group (white, non-white), and number of visits positive for HPV16 (1-3, ≥4 visits).
† 95% confidence interval.

Figure 1 Cumulative Proportion with Cervical Intraepithelial Neoplasia Grade 2-3, by HPV16 Variant Group, among 57 HPV16 Positive University Students (a) and among 66 HPV16 Positive STD Clinic Patients (b), from the Time of the First Detection of HPV16 DNA

Overall log rank tests: P = 0.01, among women attending the University; P = 0.10, among women presenting to the STD clinic.


(a)
HPV16 PL variants
HPV16 NPL variants
Cumulative Proportion with CIN 2-3
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
Time followed (months)

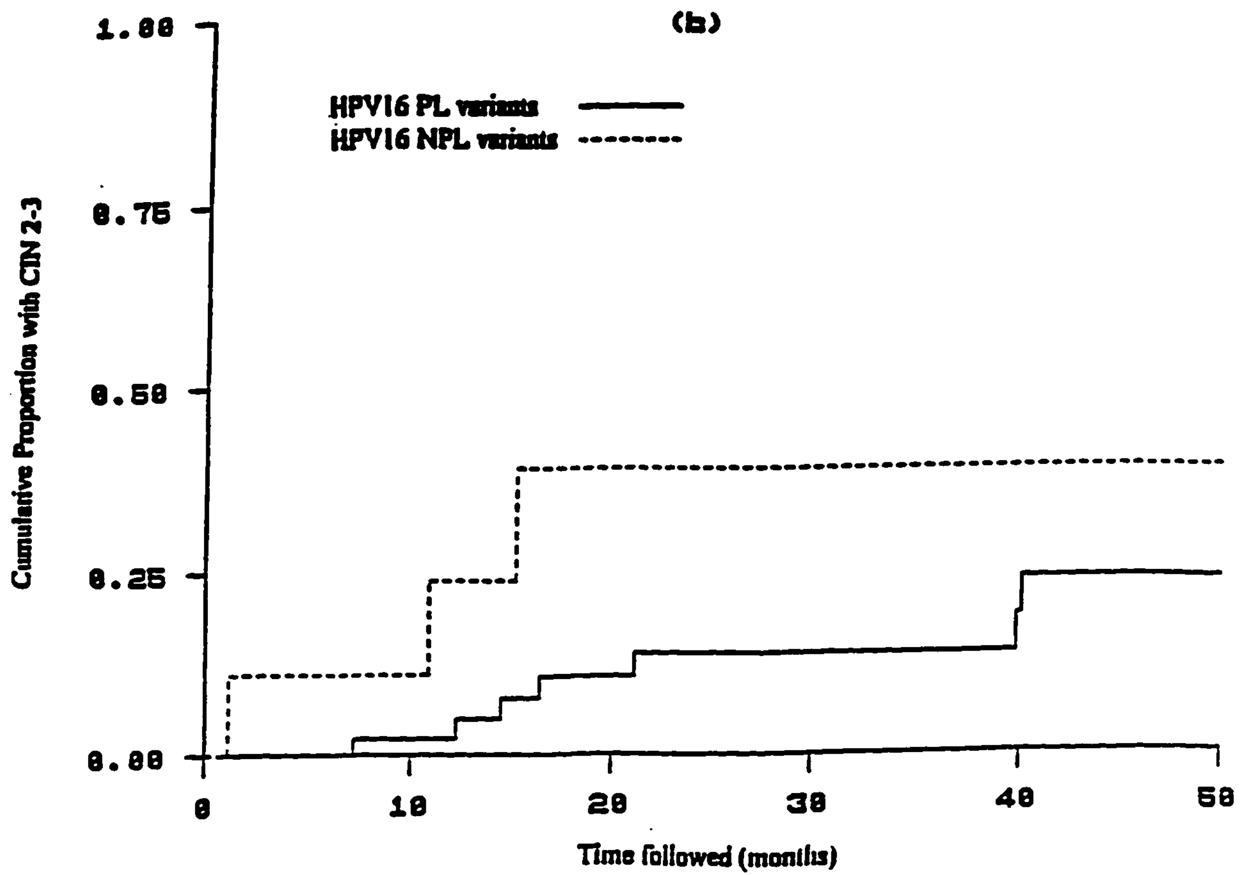

(b)
HPV16 PL variants
HPV16 NPL variants
Cumulative Proportion with CIN 2-3
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
Time followed (months)

Figure 2 DNA Sequence Analysis of HPV16 Variants from Nucleotide Position 7469 to 191 by Direct Sequencing of PCR Products and in E6 Region by Lineage-Specific Hybridization

The nucleotide positions are numbered according to those documented in Human Papillomavirus 1995 Compendium (26), corresponding to that of the original HPV16 sequence (20) with two greater than the original sequence from position 7434 to the end of NCR and same as the original sequence from position 1 to 3905. SSCP patterns of 318-, 166-, and 198-bp fragments are indicated from left to right, P, identical to the prototype pHPV16; C, identical to Caski; N, a novel pattern, different from pHPV16 or Caski (Unique patterns are individually indicated by numeric designations (subscripts)), and ##, lost one DdeI restriction enzyme digestion site. For N patterns which were not compared with other N patters on the same gel, no numeric designations were assigned. The lineage of major HPV16 variants (on the right) determined by LSH is given as the first letter assigned. A letter immediately following a dash and preceding a nucleotide position number represents the variation at the specific nucleotide designated compared to the reference sequence. Ultimate letters represent nucleotide variations at position 350 in addition to the specific variation. For each variant sequence, positions that are identical to the sequence of pHPV16 are marked with a dash in the alignment. The symbol "~" indicates no change from reference sequence determined by DNA sequencing but negative by LSH for both variant and reference probes at this position. The symbol ":" denotes an insertion at this position for two variants as compared to reference sequence.

ID	SSCP pattern	Variant group	HPV16 NCR: 318-bp fragment at nucleotide	HPV16 NCR: 166-bp fragment at nucleotide	HPV16 E6 region: 198-bp fragment at nucleotide	Lineage
			7 7 7 7 7 7 7 7 7 7 7 7 7 7	7 7 7 7 7 7 7 7 7 7		
			4 4 4 4 4 5 6 6 6 6 7 7 7 7	7 8 8 8 8 8 8 8 8 8	1 1 1 1 1 1 2 2 3 3 4 5	
			8 8 9 9 9 2 0 3 6 8 2 3 4 6	8 2 3 3 3 4 6 7 7 8 1 2	3 4 4 8 8 0 3 3 4 4 7 8 8 3 5 0 3	
			5 9 3 6 7 1 6 6 9 9 9 0 3 4	6 6 4 7 9 2 8 6 7 6 2 4	1 2 3 0 3 9 1 2 3 5 8 6 9 5 0 3 2	
pHPV16	PPP		A G T T : G T A C C A A T C	C G G A A G G C A C T C	C T C T A T A G C G T T A C T A A	
Caski	CCC		- - - - - A - - - - - - - -	- - - - - - - - - - C -	- - - - - - - G - - - -	
HHH60	PPP	PL	- - - - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	E-350T
HHH66	CPP	PL	- - - - - A - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - G - -	E-350G
HHH103	CCC	PL	- - - - - A - - - - - - - -	- - - - - - - - - - C -	- - - - - - - G - - - - - - - - -	E-G131T
HHH245	PPC	PL	- - - - - - - - - - - - - -	- - - - - - - - - - - -	- G - - - - - - - - - - - - - - -	E-350T
HHH101	N_1PP	PL	- - - - - A G - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - G - -	E-350G
HHH257	N_1PP	PL	- - - - - A G - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - G - -	E-350G
HHH165	N_2PP	PL	- - - C - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	E-350T
HHH260	N_3PP	PL	C - - - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	E-350T
HHH104	CPN_1	PL	- - - - - A - - - - - - - -	- - - - - - - - - - - -	- - - - - C - - - - - - - - G - -	E-C109G
HHH170	PPN	PL	- - - - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - C - - - - - - - - - - -	E-C109T
HHH105	NNP	PL	- - G - - - - - - - - - - -	- - - - - - - - T - - -	- - - - - - - - - - - - - - - - -	E-350T
HHH7	N_5PN_2	PL	- - - - T A - - - - - - - -	- - - - - - - - - - - -	- - - A - - - - - - - - - - - - -	E-350T
HHH218	N_5PN_2	PL	- - - - T A - - - - - - - -	- - - - - - - - - - - -	- - - A - - - - - - - - - - - - -	E-350T
HHH228	CN_1C	PL	- - - - - A - - - - - - - -	- - - - - - A - - - C -	- - - - - - - G - - - - - - G - -	E-G131G
HHH81	N_4##	NPL	- - - - - A - - - - - C - -	- - - - - A - - - - - T	- - - - - - - - - - - G - - - - -	As
HHH5	$N_6N_4N_4$	NPL	- A - - - A - - A - - T	T - T - - - - A - - - -	T - - - C - - C G T - A G T - - -	Af-1
HHH208	$N_7N_3N_3$	NPL	- A - - - A - - T A C - - T	T - - - - - - - - G - -	- - - - - - - - - T - A G T G - G	AA
HHH301	NNN	NPL	C A - - - A - - T A C - G T	T - - - - - - - G - -	- - - - - - - - - T - A G T G - G	AA
HHH198	$N_8N_5N_5$	NPL	C A - - - A - G T - - - - T	T A T C G - - - - - - -	T - T - - C - T G T - A G T - G -	Af2

CHAPTER 2: RISK OF ANAL INTRAEPITHELIAL NEOPLASIA GRADE 3 OR CARCINOMA IN SITU IN RELATION TO HUMAN PAPILLOMAVIRUS TYPE 16 VARIANTS

Current studies have demonstrated that infection with human papillomavirus type 16 (HPV16) is associated with markedly increased risk of development of cervical intraepithelial neoplasia grade 2-3 (CIN 2-3) (30, 49-53). We have reported a similar association between infection with HPV16 and risk of anal intraepithelial neoplasia grade 2-3 (AIN 2-3) (54). In addition, it has been found that, among those with HPV16 infection, repeated detection of HPV16 and detection of high levels of HPV16 DNA were associated with a further increase in risk of development of high grade cervical (30) or anal disease (54). Furthermore, it has been found that infection with human immunodeficiency virus (HIV) conferred an increased risk of AIN 2-3 even after the presence of HPV16 was taken into account (54).

Most recently, several studies have suggested that infection with different variants for a given HPV type might be associated with different clinical outcomes. Infection with HPV6a characterized by a deletion in the L1 region, has been reported to be associated with a risk of recurrent laryngeal papillomatosis (13) and a sequence variation in the E2 region of HPV18 has been found to be associated with decreased oncogenic potential (14). Our previous cohort study has suggested that non-prototype-like (NPL) variants of

HPV16, as compared to HPV16 prototype-like (PL) variants, appeared to confer an increased risk of biopsy-confirmed CIN 2-3 (55).

The present study sought to examine the relationship between risk of development of biopsy proven anal intraepithelial neoplasia grade 3 or carcinoma in situ (AIN3/CIS) and infection with specific variants of HPV16. Specifically, we wished to determine whether NPL variants of HPV16 confer a greater risk of AIN3/CIS than do PL variants, and if so, whether the excess risk is due to a prolonged persistence or a high level of viral DNA.

METHODS

STUDY POPULATION AND STUDY DESIGN

Between October 1989 and December 1995 all bisexual or homosexual men between 16 and 65 years of age, presenting to the AIDS Prevention Project of Seattle-King County Department of Public Health for HIV testing and counseling, were invited to participate in a cohort study of HIV, anal HPV infection, and risk of high grade squamous intraepithelial neoplasia. The original cohort and study design have been previously described (54, 56). Briefly, Eligible subjects were enrolled after giving written informed consent according to procedures approved by the Human Subject Review Committee of the University of Washington. A standardized interview concerning demographic characteristics, sexual behavior, and past and current medical history was administered to each subject. The

subjects enrolled were asked to return every 4-6 months for an interview, detailed genital examination, and collection of specimens. Blood was drawn at enrollment and each return visit for detection of antibody to HIV and for lymphocyte subset analysis. Anal swab specimens for cytologic screening and for HPV typing were collected at each visit.

For the present study, only subjects whose initial cytologic diagnoses were less than AIN3/CIS were eligible and visits since the initial HPV testing and cytologic screening were included. Of 943 eligible subjects, 354 were excluded due to lack of demographic information, HPV results, and/or cytologic diagnosis at all visits (n=114); no follow up visits (n=156); or specimens positive for HPV16 in initial polymerase chain reaction (PCR) screening but insufficient for single stranded conformation polymorphism (SSCP) analysis (n = 84). Overall, 3329 visits from 589 (62.5%) subjects were included. The number of visits for each subject ranged from 2 to 25 with a mean of 5.7. Follow up was terminated at the time of the last return visit, the development of biopsy-confirmed AIN3/CIS, or the end of 1995, whichever came first.

ASSESSMENT OF HPV16 VARIANTS AND DNA LEVEL

Anal samples were screened by a PCR based dot filter hybridization using a consensus primer amplification system and type specific oligonucleotide probes (34). Specimens positive for HPV16 by PCR were further assayed by PCR based SSCP analysis. The SSCP

assay was conducted as previously described (55). Briefly, DNA amplification was completed in a Perkin-Elmer 9600 Thermal Cycler (Perkin-Elmer Cetus, Norwalk Conn) with 35 cycles. Each cycle consisted of denaturation (94° C, 25 s), annealing (62° C, 25 s) and extension (72° C, 50 s). [α-^{33}P] dATP (Du Pont NEN, Research Products, Boston, MA) was incorporated into PCR products during the amplification with a pair of type specific primers C and D (55), targeting 682 bp from nucleotide position 7445 to 222 in the HPV16 noncoding region (NCR). PCR products were cleaved into three fragments, 318, 166, and 198 bp from 5' to 3', by restriction endonuclease digestion with DdeI, and electrophoresed in a 5% polyacrylamide gel with 10% glycerol.

The designation of SSCP patterns and classification of variant groups were the same as those previously described (55). In brief, the patterns from prototype plasmid HPV16 (pHPV16) (36) and HPV16 in Caski cell line (37) were used as the reference. HPV16 isolates were regarded as different variants as long as any one of the 3 fragments displayed different polymorphisms. The prototype-like (PL) group included variants that displayed the reference SSCP patterns and variants with one or two fragments that showed non-reference patterns. The non-prototype-like (NPL) group included variants that displayed non-reference SSCP patterns in all 3 fragments. The determination of SSCP patterns and classification of variant groups were performed without knowledge of the clinical data.

Specimens from 181 subjects were further assayed by lineage-specific hybridization (LSH) (38). The approach is based upon hybridization of PCR-amplified fragments of the E6 and L1 regions of HPV16 variants to a collection of 23 E6 and 12 L1 signature oligonucleotide probes that had been determined previously (11) to allow assignment of variants to one of 5 previously identified phylogenetic lineages. In this study, HPV16 DNAs were amplified by nested PCR using E6 primers. The amplified products were hybridized to a set of 23 E6 probes that target sequence variations at nucleotide positions 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532. The sequences of the primers and probes and the conditions of PCR and hybridization have been previously described (38).

Specimens which were positive for HPVs by PCR, were further analyzed by hybrid capture (HC) or southern transfer hybridization (STH) with three groups of probes targeting low risk, intermediate risk, and high risk HPV types, defined on the bases of strength of association with invasive cervical cancer. The probes for high risk HPVs included those targeting types 16, 18, and 45. The HC assay was performed based on the protocol recommended by the manufacturer (Vira type Plus, Digen Diagnostics, Beltsville MD). The STH assay was done as previously described (34). Since HC and STH assays were less sensitive than PCR in detection of HPV DNA, specimens which were positive for HPV16 by PCR and for high risk HPVs by HC or STH, were considered to have a

high level of HPV16 DNA, while those which were positive for HPV16 by PCR alone, were considered to have a low level of HPV16 DNA (56).

ASSESSMENT OF BIOPSY-CONFIRMED AIN3/CIS

During follow up, subjects found to have lesions suggested by colposcopic or cytologic examinations according to the Bethesda recommendations for cervical lesions (57) were referred for biopsy. Visits with biopsy results were considered to be non-refusal, while those recorded as refusal, referred, or delayed but without biopsy results at that visit nor the next following visit were defined as refusal. Methods for collection of biopsy specimens and histologic examination have been previously described (54). Histologic diagnoses were assigned as negative, mildly atypical, or consistent with anal intraepithelial neoplasia grade 1, 2, or 3, or anal carcinoma in situ. None of the men had cytologic, colposcopic, or histologic evidence of invasive cancer. Neither the decisions of referral for biopsy by clinicians and refusal of biopsy by study subjects nor the histologic diagnoses were made with a knowledge of HPV status.

STATISTICAL ANALYSES

Cox proportional hazard regression analyses (41) with an extension of counting process notation (58) using the statistical software package S-plus (MathSoft, Inc., Seattle, Wash) was used to estimate relative risks (RRs) and confidence intervals (CIs) for the association

between AIN3/CIS and HPV16 variants. Each subject was represented by a set of observations: s_{ij}, t_{ij}, δ_{ij}, and x_{ij}, $j = 1, \cdots\cdots, n_i$, where $(s_{ij}, t_{ij}]$ was an interval of risk, $\delta_{ij} = 1$ if the subject had initial biopsy-confirmed AIN3/CIS at time t_{ij}, and x_{ij} was the covariate vector over the time interval. The static covariates such as age at entry (≤ 30, >30 years), lifetime number of sexual partners (≤ 50, >50 partners), and history of drug use (never versus ever) were repeated over the multiple rows for a given subject. The time dependent covariates such as detection of HPV16 DNA (positive versus negative), HIV status (seropositive versus negative), and CD4 count ($\leq 500 \times 10^6/l$, $>500 \times 10^6/l$) were step functions with jump at the measurement time point s_{ij}. Once HPV16 DNA was initially detected, the following visits stayed in positive category. Visits, at which the number of CD4 was not counted, were filled in with the number of the most recent measurement. The level of HPV16 DNA was treated as either time fixed or time dependent covariate in the modeling. For time fixed, as long as one visit was positive at a high level, all visits since the initial detection of HPV16 were treated as having high level of viral DNA, while for time dependent, the level of viral DNA was based on each visits.

Kaplan-Meier product limit estimates were used to calculate the cumulative proportion of subjects in whom initial AIN3/CIS developed and to examine the proportion of those in whom HPV16 DNA reverted to negative status. The log-rank statistic was used to test differences in the proportion of men developing AIN3/CIS or of men reverting to HPV16

negativity among those with PL as compared to NPL variants. The starting point for these analyses was defined as the date of the initial HPV16 positive visit and the ending point the first date at which AIN3/CIS was histologically confirmed (for estimates of proportion developing AIN3/CIS) or the date at which HPV16 DNA initially reverted to negative status (for estimates of proportion with HPV16 DNA reversion) or the last visit date for those not attaining those endpoints. Since the date of initial HPV16 infection was unclear for subjects who were positive for HPV16 at entry, the date of entry was assumed to be the starting point. In computation of positive duration of HPV16, for subjects who had a negative interval between positive visits, the date of the initial negative visit was treated as ending point and the date of the next following positive visit as next starting point. The same rule was applied to subjects who had more than one negative interval.

A relationship between level of HPV16 DNA and variants among visits positive for HPV16 by PCR was examined using generalized estimating equations (GEE) (59) specifying a logit link function between the outcome (detection of high risk group of HPVs by STH or HC) and the covariates. Excluded were visits from which no sufficient DNAs were available for HC or STH assay. A concordance of the classification of HPV16 variants by lineage-specific hybridization and by SSCP analysis was assessed with Kappa value.

RESULTS

Of 589 men, 219 (37%) had at least one visit positive for HPV16, including 195 (33%) with PL variants and 24 (4%) with NPL variants. The mean time followed was 22.6 months (SD, ± 18.1) for men who were negative for HPV16 at all visits and 26.7 (SD, ± 19.4) for those with PL variants and 21.9 (SD, ± 20.4) for those with NPL variants since initial detection of HPV16 DNA. Compared to men without HPV16 infection, those with PL or NPL variants had more lifetime number of sexual partners, reported a history of drug use more frequently and were more likely to be HIV seropositive and immunosuppressed (table 3). Among HPV16 positive men, those with NPL variants were more likely than those with PL variants to have a late age at study entry and an early age of the first receptive anal intercourse; to be non-white; to engage in male sexual partners only; to report a history of drug use or other sexually transmitted diseases; to be HIV-seropositive and immunosuppressed; and to be positive for HPV16 at entry (table 3).

During follow up, 248 men were referred for biopsy. AIN3/CIS was histologically confirmed in 22 cases. The remaining 226 men had 348 referral visits. Of these 348 visits, 118 (34%) were refusal. The proportion of refusal was 32% (65/205), 38% (50/131), and 25% (3/12) for visits from men who were negative for HPV16, positive for PL variants, and positive for NPL variants respectively (P = 0.38). Of 22 AIN3/CIS cases, 16 were positive for HPV16 and all of them developed AIN3/CIS within 30 months of the initial

detection of HPV16. The 30-month cumulative proportion of men who developed AIN3/CIS from the date of the initial detection of HPV16 was 24% for those with NPL variants and 8% for those with PL variants (figure 3). The overall cumulative proportion with AIN3/CIS was significantly higher for men with HPV16 NPL variants than for those with PL variants (log rank test, P = 0.02).

Factors, including age at entry, race, marriage, sexual preference, age at the first anal receptive intercourse, lifetime number of sexual partners, and history of drug use or STDs were not significantly associated with risk of biopsy-confirmed AIN3/CIS in univariate analyses (data not shown). After adjusting for age at entry, lifetime number of sexual partners, and history of drug use, men with detectable HPV16 DNA had a relative risk of 7.1 (95% CI = 2.7-18.3) for developing biopsy-confirmed AIN3/CIS as compared to those without detectable HPV16 DNA. Relative to those who were negative for HPV16, the men who were positive for HPV16 at either low or high level of viral DNA or who had either PL or NPL variants had a significantly increased risk of AIN3/CIS (table 4). When analyses were confined to men who had at least one visit positive for HPV16, there was no significant increase in risk of AIN3/CIS for those with a high level of HPV16 DNA as compared to those with a low level. However, after adjusting for age at entry, lifetime number of sexual partners, and history of drug use, men with HPV16 NPL variants were 4.3-times (95% CI = 1.6-11.6) more likely to develop biopsy-confirmed AIN3/CIS than those with PL variants.

In an analysis that sought to examine the impact of HIV infection and immunosuppression on the association between risk of AIN3/CIS and HPV16 variants, data were split based on HIV status and CD4 counts. An elevated risk associated with HPV16 NPL variants relative to PL variants, although not significant, was consistently observed across men who were HIV seronegative (RR = 8.5), who were HIV seropositive but non-immunosuppressed (CD4 counts >500x10^{6}/l) (RR = 2.0), or who were HIV seropositive and immunosuppressed (CD4 counts ≤500x10^{6}/l) (RR = 2.9) (table 5). When all HPV16 positive men were included, further adjustment for HIV status and the number of CD4 counts, in addition to age at entry, lifetime number of sexual partners, and history of drug use, had little effect on the estimate of risk associated with NPL variants, (RR = 4.2; 95% CI = 1.4-12.0).

To determine whether NPL variants carried an intrinsic growth advantage over PL variants, we examined the relationship between HPV16 variants and the level and persistence of viral DNA. In total, 761 visits from 219 men were positive for HPV16 by PCR. Of these 761 visits, 723 were assayed by STH or HC and 474 were positive for HPV16 DNA at a high level (table 6). The likelihood of the frequency of visits with a high level of HPV16 DNA was similar between those with NPL variants (66.0%) and those with PL variants (63.7%) among men who did not develop AIN3/CIS (GEE modeling: OR = 1.0; 95% CI, 0.5-2.1). However, among men who developed AIN3/CIS, visits with

NPL variants were slightly less likely than those with PL variants to be positive for HPV16 DNA at a high level (73.1% versus 81.0%) (OR = 0.3; 95% CI, 0.1-1.3). The proportion of visits with high level of HPV16 DNA was slightly higher for men who developed AIN3/CIS than for those who did not, either with NPL variants (73.1% versus 66.0%) or with PL variants (81.0% versus 63.7%). After further adjusting for the level of HPV16 DNA, in addition to age at entry, lifetime number of sexual partners, and history of drug use, the magnitude of the association between risk of AIN3/CIS and HPV16 NPL variants did not vary substantially whatever the viral level was treated as time-fixed (RR = 4.3; 95% CI, 1.5-12.2) or as time-dependent covariate (RR = 5.0; 95% CI, 1.7-14.3). As shown in figure 4, the median duration from the time of the initial detection of HPV16 DNA to the first negative visit was 16.3 months for men with PL variants and 14.2 months for those with NPL variants. The overall likelihood of persistence was similar between PL variants and NPL variants (log rank test: P = 0.99). When the likelihood of persistence was assessed separately among men who were positive for HPV16 at entry and among those who were negative, no significant differences were observed between NPL and PL variants (data not shown). The exclusion of positive visits after the development of AIN3/CIS did not change the results (data not shown).

To verify the sequence variation identified by SSCP analysis, the E6 region of HPV16 from 181 specimens was further assayed using LSH. Of 20 specimens with HPV16 NPL variants, eight were classified as African (Af) variants; six as American-Asian (AA)

variants; four as Asian (As) variants, and two as European (E) variants. All 161 specimens with HPV16 PL variants were classified as European variants. The concordance of the classification of variants between LSH and SSCP analysis is shown in table 7 (99% exact agreement, Kappa value = 0.94). A relationship between risk of AIN3/CIS and HPV16 variants was re-evaluated based on classification of variants by LSH. After adjusting for age at entry, lifetime number of sexual partners, and history of drug use, men with HPV16 non-European variants including Af, AA, and As variants were 5.8 times more likely to develop biopsy-confirmed AIN3/CIS than those with European variants (95% CI, 2.2-15.4),.

DISCUSSION

Several limitations should be addressed. Subjects included selected themselves to participate and to come back for follow up. Thus, they are not representatives of the overall cohort nor of the population from which the cohort was derived, affecting the generalizability of the findings. In addition, we were able to include only 62.5% of eligible men. If men excluded differed from those included regarding the impact of HPV16 variants on risk for AIN3/CIS, our results may be biased. Although this potential bias cannot be assessed directly, the available data indicate that the distribution of age, ethnicity, sexual behavior, history of drug use and STDs, and HIV status at entry were

comparable between men included and those excluded, not taking those without demographic data into account (data not shown).

Loss-to-follow up is a special concern in a longitudinal study. The greater risk of AIN3/CIS associated with HPV16 NPL variants could result from a possibility that the time followed for men with PL variants might not be sufficient enough to show the development of AIN3/CIS. However, the fact that the mean duration followed since the initial detection of HPV16 DNA was slightly shorter for men with NPL variants than for those with PL variants strongly argues against this interpretation. In addition, it was found that men with PL variants were less likely than those with NPL variants (59% versus 77%) to be followed less than 30-months which covered a period from the time of the initial detection of HPV16 DNA to the date of all AIN3/CIS histologically confirmed. Unless the men with PL variants, who were censored early, had been more likely to develop AIN3/CIS in late time than those with NPL variants, our estimates might have been biased.

An additional source of bias could arise from outcome ascertainment, since not all men who had been referred for biopsy underwent biopsy. AIN3/CIS would have been confirmed histologically in a certain proportion of non-compliance men, had they undergone biopsy. We were not able to verify the reasons for refusal. If compliance for biopsy was related to HPV16 variants, our findings would be biased. While this potential

misclassification cannot be dismissed, the fact that both clinicians and referees were unaware of HPV16 status, specifically, the proportion of refusal was similar between men with NPL variants and those with PL variants makes it doubtful that the non-compliance had a substantial impact on the study results.

It should be noted that the time of the initial HPV16 infection for men who were positive at entry was unknown. The date of entry was arbitrarily assigned as starting time for HPV16 infection. This could potentially bias our estimates, if the positive duration of PL variants before the enrollment differed from that of NPL variants. We assumed that the length from the time of the initial HPV16 infection to the time of enrollment was independent on variants. The validity of our results is partially dependent on the appropriateness of this assumption. Given the similar medium and likelihood of persistence between PL and NPL variants either among men who were positive for HPV16 at entry or among those who were negative for HPV16 at entry (data not shown), the positive duration before the enrollment would not be expected to differ too much between PL and NPL variants. Consequently, this arbitrary assignment would not be expected to influence the results substantially.

In accord with our a priori hypothesis, we found that the risk of AIN3/CIS was associated with HPV16 infection and that men with NPL variants were more likely to develop AIN3/CIS than those with PL variants. A similar classification of variants was derived by

two different testing systems and based on two different regions. A higher risk of AIN3/CIS was also observed for men with HPV16 non-European variants, including Af, AA, and As variants, as compared to those with European variants, corresponding to PL variants. In addition, we found that the increased risk of AIN3/CIS associated with NPL variants was not explained by HIV infection nor immunosuppression. It has been reported that the prevalence of HPV infection and HPV related epithelial abnormalities is higher in HIV seropositive women than in HIV seronegative women (60). HIV infection may influence the pathogenesis of HPV associated lesions either directly through molecular interactions between HIV and HPV or indirectly through the effects of the immunosuppression, resulting in an increased HPV replication or an alteration of immune surveillance of HPV related neoplasia.

In the same population, previous findings indicates that HIV infection and immunosuppression are independently associated with the risk of AIN (56) and risk of high grade AIN (54). Thus, we paid particular attention to the possible impact of HIV infection and CD4 count on the association between risk of AIN3/CIS and HPV16 variants. We observed that the proportion of men who were HIV seropositive and with low CD4 count was higher among those with NPL variants than among those with PL variants. Conceivably, the increased risk of AIN3/CIS associated with NPL variants might result from the correlation between HIV infection and low CD4 count and NPL variants. However, arguing against this is the fact that an elevated risk, although not significant,

was consistently associated with NPL variants across men who did not have HIV infection and those who had HIV infection and were or were not immunosuppressed. In addition, further adjusting for HIV infection and CD4 count, in addition to age, lifetime number sexual partners, and history of drug use, yielded an equivalent result of the association between risk of AIN3/CIS and HPV16 variants.

Earlier studies indicate that the level of HPV DNA is an important factor for risk of genital lesions (25). High level of HPV16 DNA could result from either an increase in the amount of virus within each infected cell or a greater number of infected cells, which might directly result in more easily identified morphologic changes. Our results offered some support for this in that men with AIN3/CIS were more likely than those without AIN3/CIS to be positive for either HPV16 PL or NPL variants at a high level. However, there was no appreciable difference in risk associated with NPL variants with regard to whether the level of HPV16 DNA was adjusted or whether it was treated as a time-fixed or time-dependent covariate. In this regard, our results suggest that high level of viral DNA may be a risk factor but the increased risk associated with NPL variants could be beyond the explanation of the level of viral DNA, given that the approach used for estimating of the level of viral DNA is reasonable. In addition, in view of the fact that the proportion of visits with a high level of HPV16 DNA was similar between PL and NPL variants among men without AIN3/CIS but slightly higher for those with PL variants than for those with NPL variants among those with AIN3/CIS, the high level of viral DNA

might be more critical for PL variants than for NPL variants to be as a risk factor for AIN3/CIS.

Previous studies have suggested a positive relationship between risk of genital lesions and number of HPV positive visits (30, 54). However, subjects who have more positive visits might be followed longer, from the time of the initial HPV detection, than those who have less positive visits. Consequently, this could lead towards an overestimate of the risk associated with a higher number of positive visits. In present study, we attempted to clarify whether the increased risk associated with NPL variants could be explained by a prolonged positive duration. Given the statistical difficulties in evaluating the influence of a length of infection on risk of AIN3/CIS, we compared the median and likelihood of persistence between PL and NPL variants rather than included the number of positive visits in the model. NPL variants did not have a potential for a prolonged persistence compared to PL variants in either incident or prevalent HPV16 cases.

One possible explanation for the increased risk of AIN3/CIS associated with HPV16 NPL variants is that variants may carry different biological properties. Previous findings indicates that, as compared to HPV16 prototype, NPL variants had more nucleotide alterations than PL variants (55). It is possible that certain nucleotide alterations would change the biological properties. This is supported, in part, by experimental studies, indicating that the L1 protein from 114K variant of HPV16 can efficiently assemble into

virus-like particles while L1 expressed from HPV16 prototype can not (61). The only difference in this region between two variants is a single amino acid change at residue 202. A study by May et al. reported that a natural point mutation at a YY1 site in the NCR of HPV16 enhanced promoter activities that drive transcription of oncoproteins of E6 and E7 (16). It is noteworthy that, in a rodent cell model, the sequence alterations at glucocorticoid responsive element sites in the NCR of HPV16 affect the transformation and alter the oncogenic potential in the presence of ras oncogene and hormone (15). In a study of the role of HPV16 E6 to stimulate p53 degradation on E6-induced immortalization, Dalal et al. (62) generated a series of HPV16 E6 mutants by using a PCR-based misincorporation and site directed mutagenesis and demonstrated significant changes of the efficiency of immortalization of mammary epithelial cells and of degradation of p53, resulting from nucleotide alterations at certain positions. Recent data have shown that natural variants of HPV16 E6 protein differ in their abilities to alter keratinocyte differentiation and to induce p53 degradation (17). Taken together, these experimental studies imply that some nucleotide alterations of HPV16 variants might greatly alter biologic characteristics. From another perspective, we were not able to determine which nucleotide changes might be critical. It would be inappropriated, in this observational study, to speculate a linkage between risk of AIN3/CIS and particular nucleotide alterations, since nucleotide alterations in one region of HPV16 often link to changes in other regions including those unexamined (9-11, 38). Nevertheless, the

sequence variation of NPL variants could be at least regarded as a biomarker, representing a group of variants with an increased risk of AIN3/CIS.

Alternatively, the increased risk associated with NPL variants could be due to an insufficient immune surveillance resulted from either nucleotide alterations of the variants or genetic characteristics of the host. HPV16 E6 and E7 proteins are often present in cervical tumors, implicating these proteins as potential target for immunological control by specific cytotoxic T lymphocytes (CTLs) (63). A study by Ellis et al. (47) demonstrated a significant association between HLA-B7 and HPV16 variant with a particular change at one of the three potential CTL epitopes in E6 region. Although the binding studies showed that the variant peptide bound to HLA-B7 in a similar manner to prototype, computer modeling predicted that the orientation of the variant peptide within the HLA-B7 binding groove would be altered relative to prototype peptide. This alteration was predicted to affect the amino acid residues exposed for interaction with T-cell receptor. They speculated that changes of this epitope might affect CTL responses and escape immune surveillance directed at prototype HPV16 E6 in HLA-B7 individuals. It is also possible that a certain immunogenetic characteristics may predispose to the progression of low grade to high grade lesions or to invasive cancer. Previous studies suggested that HLA-DQB1*0602-DRB1*1501 haplotype was associated with HPV16 positive cervical cancer patients (64) and that loss of HLA-B7/B40 expression was significantly correlated to metastatic spread of cervical neoplasia (65). In reality, HPV16 variants could be

associated with some specific MHC alleles which may be relevant to immune surveillance and the virally associated lesions.

The data from the present study suggest that risk of AIN3/CIS is associated with detection of HPV16 DNA and that NPL variants are associated with a even greater risk compared to PL variants. The excess risk associated with NPL variants could not be explained by a high level of viral DNA, a prolonged positive duration, nor the effects of HIV infection and immunosuppression. While we hypothesize that a difference in risk of AIN3/CIS may be a reflection of a difference in biologic characteristics between PL and NPL variants or a difference in host-virus interaction relating to immune surveillance and immunogenetics, the real mechanism remains undetermined. Given a relatively short period followed, a long-term impact of variants on risk of AIN3/CIS has not been fully evaluated. Thus, these results should not be generalized to the time window beyond observed nor the risk of invasive cancer.

Table 3 Characteristics of Cohort Men by HPV16 Status and Variants

Variables	No. (%) of those negative for HPV16 (n=370)	No. (%) of those with PL variants (n=195)	No. (%) of those with NPL variants (n=24)
>30 years of age at entry	223 (60.3)	120 (61.5)	17 (70.8)
Non-white†	43 (12.7)	19 (10.2)	4 (18.2)
Never married ‡	281 (84.1)	154 (83.2)	17 (77.3)
Male sexual partner only	253 (68.4)	128 (65.6)	18 (75.0)
≤20 years of age at first anal intercourse¥	198 (55.3)	104 (54.5)	16 (66.7)
>50 lifetime sexual partners at entry #	190 (51.9)	120 (62.5)	16 (66.7)
History of drug user ‡	207 (56.1)	121 (62.1)	20 (83.3)
History of other STDs	247 (66.8)	134 (68.7)	19 (79.2)
Initial CD4 count $\leq 500\times10^6/l$ *	79 (22.0)	56 (29.3)	12 (52.2)
HIV seropositive♣	168 (45.4)	131 (67.1)	20 (83.3)

Table 3 (continued)

Ever positive by STH or HC§	-	150 (78.1)	18 (75.0)
Positive for HPV16 by PCR at entry	-	139 (71.3)	19 (79.2)

† Missing data for 41 men (31 HPV16 negative, 8 with PL variants, and 2 with NPL variants).
‡ Missing data for 48 men (36 HPV16 negative, 10 with PL variants, and 2 with NPL variants).
¶ Missing data for 16 men (12 HPV16 negative and 4 with PL variants).
# Missing data for 7 men (4 HPV16 negative and 3 with PL variants).
‡ Missing data for one HPV16 negative man
* Missing data for 16 men (11 HPV16 negative, 4 with PL variants, and 1 with NPL variants).
§ Missing data for 3 men with PL variants; positive for HPV16 by PCR and for high risk group of HPVs by STH or HC.
- not applicable
♣ Eight men who were HIV-seropositive during follow up.
Percentages were calculated after excluding men with missing data. Sixty-one men who were positive for HPV16 during follow up.

Table 4 Risk of Biopsy-Confirmed Anal Intraepithelial Neoplasia Grade 3 (AIN3) or Carcinoma in Situ (CIS) in Relation to HPV16 Status, Level of Viral DNA, and Variants

	No of men	No. of those with AIN 3/CIS	RR† (95% CI)	RR‡ (95% CI)
HPV16 detected by PCR				
Never	370	6	1.0	
Ever	219	16	7.1 (2.7-18.3)	
Level of HPV16 DNA*				
Low level	48	3	4.1 (1.1-16.1)	1.0
High level	168	13	5.7 (2.1-15.3)	1.2 (0.4-3.8)
HPV16 variants				
PL	195	12	5.8 (2.2-15.6)	1.0
NPL	24	4	21.8 (6.5-73.4)	4.3 (1.6-11.6)

* Low level, visits positive for HPV16 by PCR alone; high level, at least one visit positive for HPV16 by PCR and positive for high risk group of HPVs by southern transfer hybridization or hybrid capture; missing data for 3 men with PL variants.
† Risk of AIN3/CIS for men with HPV16 detected by PCR, with low or high level of HPV16 DNA, or with PL or NPL variants relative to those without detectable HPV16 at any visits, adjusting for age, lifetime sexual partners, and history of drug use.
‡ Risk of AIN3/CIS for men with high level of HPV16 DNA relative to those with low level or for men with NPL variants relative to those with PL variants, adjusting for age; lifetime sexual partners, and history of drug use.

Table 5 Risk of Biopsy-Confirmed Anal Intraepithelial Neoplasia Grade 3 (AIN3) or Carcinoma in Situ (CIS) Associated with HPV16 Variants among HPV16 Positive Men Stratified by HIV Status and CD4 Count

HIV serology	CD4 count	HPV16	RR†	95% CI
Negative or positive	>500 or ≤500 ($x10^6/l$)	PL variants	1.0	
		NPL variants	4.2	1.4-12.0
Negative	>500 or ≤500 ($x10^6/l$)	PL variants	1.0	
		NPL variants	8.5	0.9-75.7
Positive	>500 ($x10^6/l$)	PL variants	1.0	
		NPL variants	2.0	0.3-15.0
Positive	≤500 ($x10^6/l$)	PL variants	1.0	
		NPL variants	2.9	0.6-14.1

† Risk of AIN3/CIS in relation to HPV16 variants among men who were HIV seronegative, seropositive but CD4 count >500 ($x10^6/l$), or seropositive and CD4 count ≤500 ($x10^6/l$); risk of AIN3/CIS in relation to HPV16 variants among all subjects, further adjusting for HIV status and CD4 count in addition to age, lifetime sexual partners, and history of drug use.

Table 6 Relationship between HPV16 DNA Level and Variants Stratified by Men with or without Anal Intraepithelial Neoplasia Grade 3 (AIN3) or Carcinoma in Situ (CIS)

	HPV16	No. of visits‡	% with high level of HPV16 DNA*	OR† (95% CI)
All subjects	PL variants	644	65.2	1.0
	NPL variants	79	68.3	1.1 (0.5-2.1)
Men without AIN3/CIS	PL variants	586	63.7	1.0
	NPL variants	53	66.0	1.0 (0.5-2.1)
Men with AIN3/CIS	PL variants	58	81.0	1.0
	NPL variants	26	73.1	0.3 (0.1-1.3)

* High level of HPV16 DNA, visit positive for HPV16 by PCR and positive for high risk group of HPVs by southern transfer hybridization (STH) or hybrid capture (HC).
‡ Excluded were 38 visits form which no specimens were available for STH or HC analysis.
† Adjusting for age, lifetime sexual partners, and history of drug use.

Table 7 Classification of HPV16 Variants by Single Stranded Conformation Polymorphism (SSCP) Analysis and by Lineage-Specific Hybridization (LSH)

		LSH	
		European variants†	Non-European variants‡
SSCP analysis	PL variants	161	0
	NPL variants	2	18

† European (E) variants, including E-350T, E-350G, E-G131T, E-G131G, E-C109G, and E-C109T variants. European variants are given as the first letter assigned. A letter immediately following a dash and preceding a nucleotide position number represents the variation at the specific nucleotide designated compared to the reference sequence. Ultimate letters represent nucleotide variations at position 350 in addition to the specific variation.

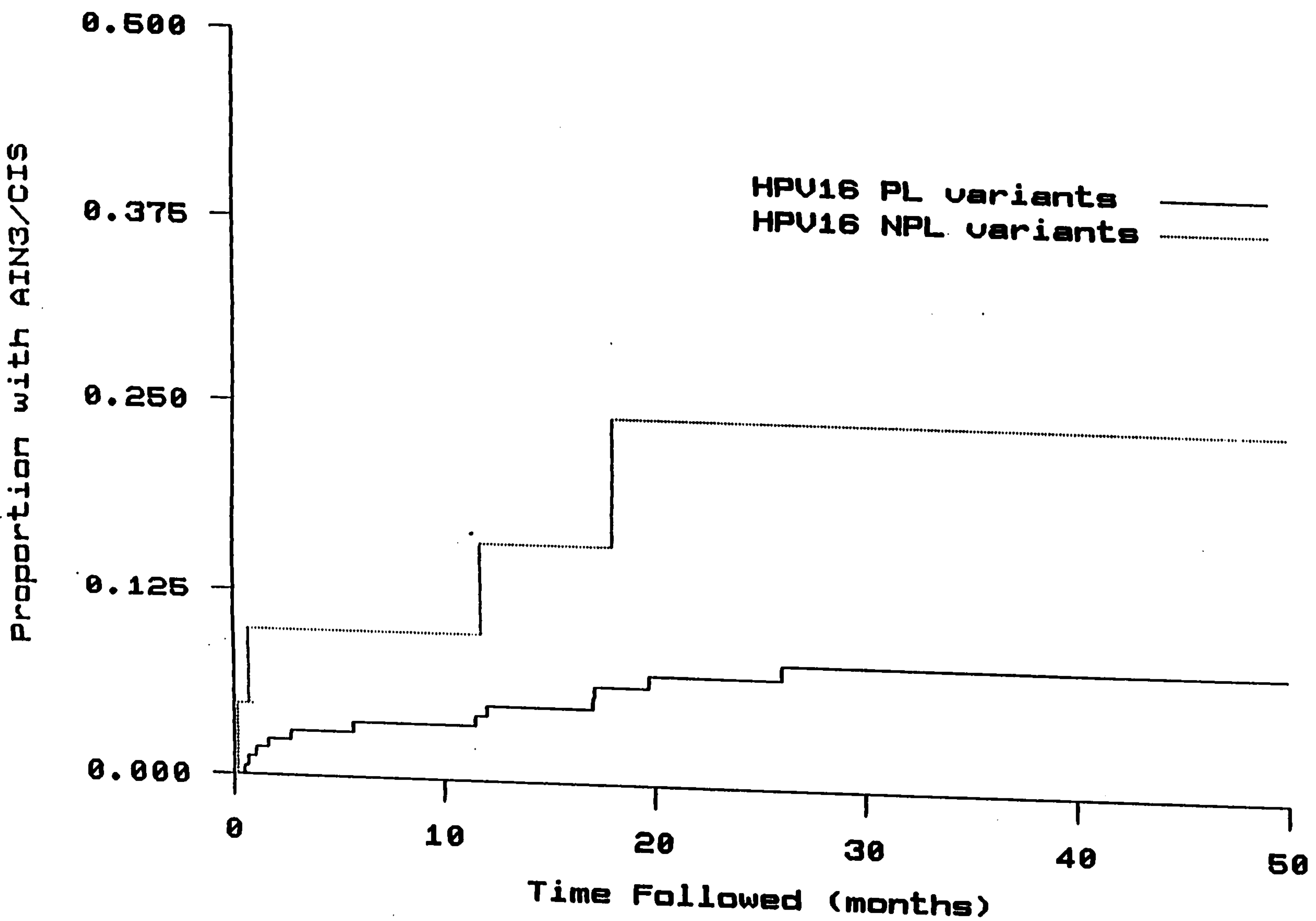
‡ Non-European variants, including Asian-American (AA), Asian (As), and African (Af1) variants.

% of agreement = 99.

Kappa value = 0.94.

Figure 3 Cumulative Proportion with Anal Intraepithelial Neoplasia Grade 3 (AIN3) or Carcinoma in Situ (CIS) from the Time of the Initial Detection of HPV16 DNA Stratified by Variant Groups among Men Presenting to the AIDS Prevention Project

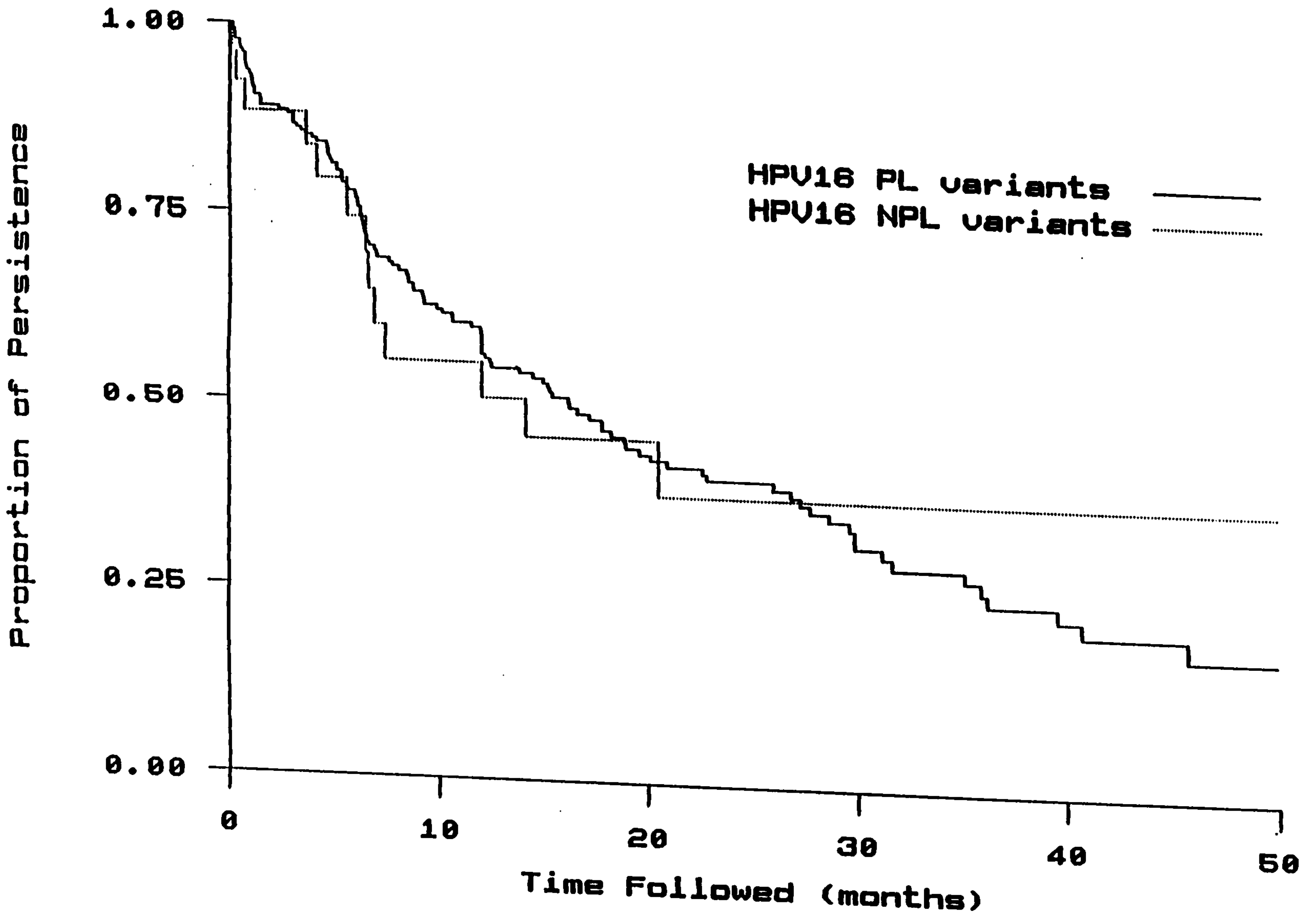
The curves were truncated at 50 months of follow up. Overall log rank tests: $P = 0.02$.



HPV16 PL variants
HPV16 NPL variants
0.500
0.375
0.250
0.125
0.000
Proportion with AIN3/CIS
0
10
20
30
40
50
Time Followed (months)

Figure 4 Proportion of Persistence from the Time of the Initial Detection of HPV16 DNA Stratified by Variant Groups among Men Presenting to the AIDS Prevention Project

The curves were truncated at 50 months of follow up. Overall log rank tests: $P = 0.99$.


HPV16 PL variants
HPV16 NPL variants
Proportion of Persistence
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
Time Followed (months)

CHAPTER 3: ANALYSIS OF HUMAN PAPILLOMAVIRUS TYPE 16 VARIANTS INDICATES ESTABLISHMENT OF PERSISTENT INFECTION

Over the past decade, convincing evidence has accumulated to support an association of human papillomaviruses (HPVs) with the development of cervical cancer and pre-cancerous cervical intraepithelial neoplasia (CIN) (1, 3, 18, 25, 31, 66-70). However, the natural history of HPV infections is less well understood. Regression or persistence of HPVs analyzed in longitudinal investigations showed a considerable flux in detectable HPV DNA; and repeated examinations increased the cumulative prevalence of HPV positivity considerably (71-72). Several studies have shown that the number of HPV DNA positive tests in the follow up interval is positively related to the risk for progression to CIN (30, 73). Since HPV DNA is often detected only intermittently when women are examined repeatedly over time (30, 72-75), and there are multiple HPV16 variants in the population (10, 76-77), it is of interest to determine whether repeated detection of HPV DNA over time represents persistence of initial infection, or repeated transient infections. Conventional HPV typing distinguishes persistence from re-acquisition when different types of HPV DNA are identified in consecutive specimens. However, when the same type of HPV is detected, then HPV typing alone can not distinguish whether the subsequent positive sample represents acquisition or emergence of a new variant, or persistence of the

original infection. Thus, it would be desirable to further characterize HPV DNA detected over time by identifying individual molecular variants.

New rules have emerged for classifying papillomaviruses into types, subtypes and variants (29). Types are defined as less than 90% homologous to other types based on the DNA sequence of the E6, E7 and L1 regions of an intact 7.9 kb genome; subtypes have between 2%-10% sequence variability; and variants have less than 2% sequence variation. The extent of variation may differ among types: for instance several subtypes of HPV 6 have been described, whereas studies on genomic heterogeneity of HPV16 have found a maximal sequence variation of less than 2% within this type (10, 76). Sequence diversity of HPV16 in specimens from cervical cancer patients resembles that found in specimens from homosexual men (35). Patterns of geographical clustering of variants of HPV16 have been demonstrated by sequencing a segment of the noncoding region (NCR) (10, 78). Individual variants were detected by sequencing cloned products of a PCR amplified segment of the NCR. Sequence variation of HPV16 has also been used for studying viral evolution (10) and viral transmission through sexual activity (79). More than one HPV16 variant has been observed in some individuals (10, 35, 76-77), a frequent occurrence in one study (10). However, these studies are primarily cross-sectional; long term variation within individuals or populations over time remain undetermined.

The present study was designed to detect and characterize HPV16 variants in three cohort populations, and to track sequence variation, over time. Variants were assessed both by single stranded conformational polymorphism (SSCP) analysis, and by DNA sequencing. Our data indicate that a predominant HPV16 variant persists in an individual over time, though co-infection with minor HPV16 variants also occurs.

METHODS

STUDY POPULATION AND STUDY DESIGN

The subjects were selected for analysis from three parallel ongoing cohorts, including a cohort of women (HDS) attending a clinic for sexually transmitted diseases (STD), a cohort of female college students enrolled at the University of Washington (HHH), and a cohort of pregnant women (HOB) at Harborview Medical Center. The original cohort study design and results from HPV typing have been described elsewhere (30, 33). Vulvar, vaginal and cervical swabs for HPV typing were collected from women in the HDS and HHH cohorts at enrollment and every 4-6 months thereafter. From women in the HOB cohort, swabs were collected at < 20 and 34-38 weeks of gestation and at 6 weeks and 12 months post partum. Samples were analyzed by the PCR method using a consensus primer amplification system and type specific oligonucleotide probes (34). A total of 404 HPV16 positive specimens were available from 110 women including 212 HPV16 positive specimens from 42 women attending a STD clinic; 127 from 40 college women; and 66

from 28 pregnant women. One specimen from a college woman was later excluded because the polymorphism of cellular DNA from this specimen differed from the rest of other specimens from the same woman (data not shown). The number of HPV16 positive visits for each woman ranged from 1 to 8 during the follow up from 1989 to 1993.

PCR BASED SINGLE STRANDED CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

The methods used for PCR-SSCP analysis have been described previously (35). Minor modifications were made for the present study. In brief, DNAs were extracted from swab scrapes in 50 uL specimen transport medium (Digene Diagnostics, Silver Spring, MD) and dissolved in 15 µL 10 m*M* TRIS-HCl (pH 7.6) with 1 m*M* EDTA. A PCR assay was performed in a total volume of 10 µL containing 3 µL of DNA extract. A total of 35 cycles were run, each cycle consisting of denaturation (94°C, 1 min.), annealing (62°C, 1 min.) and extension (72°C, 2 min.). [α-^{32}P] dCTP (Du Pont NEN, Boston, MA) was incorporated into PCR products in the process of amplification with a pair of primers C and D (35).

PCR products of 682 bp from nt. 7445 to 222 in the noncoding region were digested with DdeI (New England Biolabs, Beverly, MA). Electrophoresis was performed in a 5% polyacrylamide gel with 10% glycerol. Two positive controls, 0.1 pg. prototype plasmid HPV16 (pHPV16) DNA (36) and 40 pg. Caski cellular DNA (37), and one negative

control were included in each set of PCR-SSCP assays. The mobilities of the single stranded DNAs of HPV16 from specimens were compared with those from pHPV16, Caski and other specimens in a single non-denaturing gel. Specimens from a single woman were compared on the same gel and then representatives from each gel were compared together.

DNA SEQUENCING

PCR products of the whole NCR were generated with a pair of primers: primer G 5'-GGCGTCGACCTCTACAACTGCTAAACG (nt. 7117-7136), and primer H 5'-GGGAAGCTTGCAGTTCTCTTTTGGTGC (nt. 104-85), which contained SalI or HindIII sites at their 5' terminus, respectively. Target fragments were generated with Deep $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA) from 9 specimens and with Taq polymerase (BRL Life Tech Inc., Gaithersburg, MD) for 6 specimens. The thermal cycling program was: 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s with a total of 25 cycles. The PCR products were digested with SalI and HindIII (New England Biolabs, Beverly, MA), gel purified with Glas Pac^{TM} kit (National Scientific Supply Co, San Rafael, CA), and ligated into SalI-HindIII cleaved pBS vector (Stratagene, La Jolla, CA). The resulting ligation products were electroporated into the bacterial strain DH10b (BRL Life Tech Inc., Gaithersburg, MD). Clones containing target inserts were identified by digestion with appropriate restriction enzymes.

Plasmid DNAs were purified with QIAWell-8 Plasmid kit (Qiagen Inc., Chatsworth, CA). The purified plasmid DNA (1 μg) was sequenced using Taq Dye Deoxy™ Terminator Cycle Sequencing kit (Applied Biosystems Inc., Foster, CA) based on the protocol recommended by the manufacturer. Briefly, twenty five cycles at 96° C, 50° C, and 60° C for 30 s, 15 s, and 4 min., respectively, were run in a Thermal Cycler. Fluorescence labeled dideoxynucleotides were incorporated into the DNA as the terminating base. Centri-Sep spin columns (Princeton Separations Inc., Adelphia, NJ) were used to remove excess DyeDeoxy™ terminators from completed DNA sequencing reaction. Finally, the sequencing reactions were run on an Applied Biosystem Model 373 A DNA sequencing system (Applied Biosystems Inc.). Sequences were determined from both directions with two pairs of primers: primers C and F, or primers H and E. The sequence of primers E and F has been described previously (35).

Determination of HPV16 variants and multiple variant infection

Using primers C and D, then digesting the PCR products with DdeI generated 3 fragments: 318, 166, and 198 bp for each specimen. The patterns for each of the three fragments were designated as follows: P, identical to the prototype pHPV16; C, identical to Caski; or N1, N2, etc., a novel pattern, not the same as pHPV16 or Caski. Two isolates

were designated as different variants, if the mobility of any one of the three fragments showed a difference.

To evaluate the existence of multiple variants by sequencing, 10 colonies from each specimen were examined. Sequence variations that were found at least twice in the same sample were counted as variants, and those sequences found only once were counted as potential artifacts introduced by PCR, except when the sequence of the variant was identical to that of previously identified variants. The predominant variant is defined as the most intense banding pattern observed by SSCP and by the greatest proportion of colonies with a given sequence. In SSCP analysis, multiple variants in a single specimen were considered to be present when there were one or more bands in addition to the predominant SSCP pattern.

RESULTS

GENOMIC VARIABILITY OF HPV16

Sequence variation of HPV16 from nucleotide position 7445 to 222 was determined by SSCP. Overall, 10 variants were found among 212 specimens from 42 women attending a STD clinic, 16 among 127 specimens from 40 college women, and 11 among 66 specimens from 28 pregnant women (table 8). SSCP patterns of a 682 bp segment of the HPV16 NCR, representing 10 variants identified in women attending a STD clinic, are

depicted in figure 5. The most common pattern in two of the three cohorts was PPP (lane 5), identical to the pattern from pHPV16 (lane 3). The second most frequent pattern in those cohorts and the most common pattern among the college women was CPP (lane 7), with only one point mutation at nucleotide position 7519 as compared to the sequence of pHPV16 (35). Together these two patterns accounted for 50%-70% of all the HPV16 variants. Each of the other SSCP patterns was observed in only a few per cent of the subjects in each cohort.

EVIDENCE FOR INFECTION BY MULTIPLE VARIANTS BY SSCP ANALYSIS

The SSCP patterns as shown in Figure 5, and for greater than 90% of all specimens tested, were generally not complicated, i.e. all of the bands were of the mobility previously predicted (35) for single or double strands of the appropriate size fragment. These patterns suggested that most of the specimens contained either a single variant of HPV16 or a predominant variant with one or more minor variants. By mixing known variants at different ratios and then performing PCR amplification it was not possible to detect a second SSCP pattern unless the variant represented more than 20% of the HPV16 genomes (data not shown). A few specimens showed additional bands by SSCP analysis that were indicative of multiple variants. Figure 6 shows two examples. The basic patterns of PPP (lane 3) and CCC (lane 4) were seen in specimens from two subjects (lane 5 and lane 6, respectively). The arrows indicate extra bands regarded as likely to indicate the

presence of multiple variants. In total, SSCP analysis provided evidence for infection with more than one variant in 10 specimens from 5 of 42 women attending the STD clinic, in 12 specimens from 6 of 40 college women, and in one specimen from 1 of 28 pregnant women (table 9). The number of samples showing evidence of more than one variant was low, ranging from 1.5% in the pregnant women to 9.4% in the college women.

PREDOMINANT VARIANTS OVER TIME

Given the diversity of HPV16 variants within these cohorts, a different SSCP pattern might be expected in a portion of consecutive specimens, if the initial variant was replaced by another variant over time. Multiple specimens from women were collected at various intervals after the initial specimen. Women who experienced one or more HPV16 positive visit are presented in table 10. The mean numbers of HPV16 positive visits were 5.0, 3.0, and 2.4 with a range of 1-8, 1-6, and 1-3 among women attending the STD clinic, college women, and pregnant women, respectively. However, not all women had the same number of visits within each group, and the cohorts were followed at different intervals. Multiple positive specimens from an individual woman were compared in a single gel. Figure 7 shows an example of SSCP patterns from 16 specimens collected from two women (8 specimens each) in the HDS cohort. Identical SSCP patterns were observed at all 8 HPV16 positive visits for each of the women; PPP presented as a predominant pattern in subject HDS88 and $N_1N_1N_4$ in HDS81. Visit 3 of subject HDS81 (figure 7) is another

example of a specimen that is likely to have multiple variants. An extra band that runs between the 166 and 198 bp fragments is seen in addition to the predominant pattern and suggests the presence of more than one variant.

In total, 70 (64%) women (32 from HDS, 24 from HHH, and 14 from HOB cohorts) had SSCP data on more than one HPV16 positive visit; 364 specimens in all (table 10). No change in the predominant variant was detected in any of the women examined, regardless of whether the variant was common or rare. The longest period for which multiple HPV16 positive samples were available was 2.8 years, and during that interval the predominant HPV16 variant was unchanged.

MULTIPLE VARIANTS DETECTED BY DNA SEQUENCING

SSCP analysis reveals the HPV16 variant or variants that are the most abundant but may miss variants that are present as a minor proportion of the HPV16 genomes, or variants whose sequence alterations do not result in bands with altered mobility in this particular gel system. Similarly, direct sequencing of the amplified PCR product will also provide data about the most abundant variants in the sample. To further explore the possibility of infection with multiple variants, the PCR amplified HPV16 NCR from specimens of 8 women in the HDS cohort were sub-cloned and sequenced. Three of the women were selected because their SSCP patterns suggested that multiple variants were present in at

least one specimen; an additional 5 women were selected whose specimens showed a single predominant variant by SSCP analysis. Ten colonies from each specimen were examined. Sequencing analysis was performed from nucleotide position 7445 to 7830 in four women (table 11) and from 7748 to 104 in the other (table 12). Sequencing some isolates from nt. 7748 to 104 rather than all from nt. 7445 to 7830 attempted to identify the minor variant in some specimens (HDS84, 3/26/90 and 7/23/90; and HDS117, 4/9/92) or loss of one DdeI site (HDS106) that were suggested by the SSCP patterns. Twenty eight sequence alterations resulting from 60 colonies whose target inserts were generated with Taq DNA polymerase and 6 base changes from 90 colonies whose inserts were generated with Vent DNA polymerase were classified as probable PCR artifacts (data not shown) because the sequence was not confirmed by an identical sequence from another colony. One alteration, a C to T transition at nt. 24 in a variant from HDS106, changed the wild type sequence of CTAAG to TTAAG which led to loss of one DdeI site, thereby losing the 166 and 198 bp fragments and generating a 364 bp fragment (figure 5, lane 14). Nine point mutations were identified in variants from HDS109 and HDS81 which showed $N_1N_1N_4$ pattern (figure 5, lane 11). Of the 15 specimens from 8 women, more than one HPV16 variant was found in 9 specimens by sequencing. Of the 3 specimens in which the presence of more than one variant was indicated by SSCP analysis, all were confirmed to have multiple variants by sequencing (table 12). However, of 12 specimens in which only a single variant was detected by SSCP analysis, 6 were demonstrated to have multiple variants by sequencing.

MINOR VARIANTS OVER TIME

Twelve women were shown to have multiple variants by SSCP analysis (table 9), three of whom had only one positive visit. The remaining 9 women had more than one HPV16 DNA positive visit, ranging from 2 to 6. Three individuals showed consistent extra bands in all HPV16 positive specimens. In the remaining 6 women the predominant pattern was constant for each woman, however the extra bands did not persist over time (data not shown).

The HPV16 variants from five women were followed over time by sequencing (tables 4 and 5). One (HDS109) had a single variant initially and only that variant was detected four months later. Two variants were detected initially in subject HDS81, and only those two variants were detected at each of three subsequent visits spanning 28 months. HDS81 showed a $N_1N_1N_4$ SSCP pattern, and a variant having 9 point mutations represented the majority of colonies sequenced from all specimens except one collected on February 24, 1992; the other variant, identical to the sequence of that segment of the NCR from pHPV16, was a minor variant that was not detected by SSCP analysis and was found less frequently among the colonies that were sequenced. Specimens from three women, HDS106, HDS84, and HDS117, had multiple HPV16 variants at their initial visit, and the collection of minor variants changed over time with some variants failing to be detected at

subsequent visits and other newly detected variants appearing. For example, variant HDS106-b was present initially, was not detected 3 months later, and variant HDS106-c was seen at that time. Similarly, HDS84-b and HDS117-b were not detected at subsequent visits, and HDS84-d appeared. Although the minor variants were not always detectable, the predominant variants (HDS81-a, HDS106-a, HDS84-a, and HDS117-a) persisted over time in all women examined.

DISCUSSION

Sequence variation among HPV16 genomes has been analyzed initially in the NCR rather than in other regions, since genomic sequences usually diverge more in noncoding than in coding regions (80). Two types accounted for 50-70% of all HPV16 variants in the three cohorts. The remaining HPV16 variants displayed considerable genetic diversity. This intratypic variation in the HPV16 NCR forms the basis for a molecular epidemiological approach to study persistence and transmission of papillomavirus infections. It should be noted that the ratio of absolute number of HPV16 variants to the number of women examined presented here has only limited meaning since the number of variants found is related to the number of women examined, but will not increase proportionally with the increase of the number of women examined. The sequence alterations found in these three Seattle-based cohorts cluster to hot spots in the NCR and in some cases are located at

positions that have been shown by others to vary (10, 35, 76-77). Whether the plethora of HPV16 genotypes will have phenotypic differences is an interesting question.

This study demonstrates that the use of different methodologies will influence conclusions about the heterogeneity of HPV16 infections. Bernard and colleagues (10, 76) have studied the diversity of HPV16 by sequencing cloned PCR products from a 364 bp fragment of the NCR from many samples. That approach maximizes the detection of variants but unless a large number of clones from each specimen are sequenced, can miss the fact that one variant is more abundant than others. Potentially artifactual variants arise during the course of PCR amplification. The Taq polymerase has been estimated to have an error rate of 10^{-5} which means that almost half of the amplified products of a 500 bp fragment will have mutations(81-83). Our results confirmed that amplification with the Vent polymerase, with a reported error rate of 10^{-6}, introduced fewer mutations. The requirement that a sequence must be identified twice to be counted as a variant effectively eliminates the possibility of creating variants by PCR. The probability of identifying a minor variant was estimated based on the following formula (84):

$$P = 1 - \sum_{i=0}^{1} \binom{n}{i} p^{i} (1-p)^{n-i}$$

where P is the probability of identifying the minor variant; p, the proportion of DNA fragments from a minor variant in the PCR products; *i*, the number of colonies indicating

a minor variant; n, the number of clones that are sequenced and $\binom{n}{i}$, the number of ways of choosing i from n. Of 10 colonies examined, it was expected that the probability of identifying a minor variant at least twice would be 85%, given that no less than 30% of PCR products are generated from that variant. HDS106-c and HDS117-b, although represented by only one clone, have been defined as minor variants since the chance of obtaining the sequences of prototype HPV16 (HDS106-c) or the variant (HDS117-b) identified in other women by PCR error is too small to be possible. These calculations also point out that some minor variants will be missed if only 10 clones are sequenced. The ability to detect minor variants using SSCP analysis is quite limited in that the variant must represent a substantial proportion of the total number of HPV16 genomes to be detected. Six of 12 specimens, in which only a predominant variant was detected by SSCP analysis, were revealed to have multiple variants by sequencing. The proportion of the minor variant in these specimens could be too low to show a detectable banding pattern. A reconstruction experiment with mixing of two known variants indicated that the minor variant will be detectable only if that variant accounts for more than 20% of HPV16 genomes. One minor variant, from specimen of HDS81 on February 24, 1992 represented 50% of ten colonies sequenced, was missed by SSCP analysis. One possible explanation is sampling variation during picking colonies; the real proportion of that variant might be much less than 50%. Alternatively, some variants might have sequence changes that result in fragments whose mobility cannot be distinguished from the mobility of fragments of

some other variants. On the positive side, SSCP analysis did show that among individuals with multiple HPV16 variants, a single HPV variant was prominent and remained as the predominant variant at subsequent visits. Both methods contribute to a better understanding of the natural history of HPV infections.

The presence of more than one HPV16 variant in an individual woman was demonstrated by both SSCP analysis and sequencing, and has also been observed by others (76) and in our previous study (77). It has been postulated that spontaneous mutations occur so rarely in HPVs that a variant does not change during the life span of an infected women (10). If this is so, the minor variants present in the specimens most likely represent separate infections, rather than mutation of the initial variant. It is possible that a single exposure transmits multiple HPV16 variants, or that exposure to new partners results in multiple separate infections.

The most striking finding of this study is that identical predominant SSCP patterns were observed over time for each of the 70 women for whom more than one HPV16 positive sample was available. Sequencing of multiple cloned PCR products also substantiated the observation that a predominant variant remained over time. Several interpretations of this observation are possible.

One explanation could be that certain HPV variants have a biological advantage in certain hosts and that once the favored variant infects, it outgrows the other variants. Because it has not been possible to propagate HPVs in culture, the viral factors that influence the rate of replication are not known, apart from an understanding that both the E1 and E2 genes are essential for plasmid replication, along with a cis-acting origin of replication located within the NCR (85). Whether some variants would have an intrinsic growth advantage over other variants is unclear. Related to this is the possibility that variants might have slightly different tropisms; for example a particular variant might preferentially infect the cervix compared with the vulva or vagina, and the cervix could be more permissive for viral replication. Arguing against the notion that individual variants have distinct replication rates or tropisms is the finding that in some individuals a particular variant is the predominant variant (for instance PPP in many people), while in other individuals (like HDS81) the PPP variant is a minor species. That suggests that even the more frequently detected variants do not have an inherent growth advantage over other isolates since in some individuals they do not become the predominant variant. It is also possible that a variety of host factors could influence the ability of a particular variant to become established or to replicate. Genetic factors that influence the permissivity of viral infections have been identified in inbred strains of mice, though their role in humans has not been studied. Previous or concurrent exposure to other HPV types or other STDs could influence the replication of a particular HPV16 variant by altering the immune response, the effect of inflammatory cytokines, the number of permissive cells, etc. It is important to

note however, that sequencing of multiple cloned PCR products from an individual over time showed that the variant that was most abundant initially, remained most abundant at subsequent visits. In no case did a different variant with a growth advantage emerge at a later time. To determine whether the variant that establishes an initial infection becomes the predominant variant, it will be necessary to identify women who are experiencing primary HPV16 infection and characterize their variants in longitudinal studies. It is possible that minor variants also persist, but at a threshold below detection, perhaps becoming detectable intermittently due to a burst of replication of the variant rather than because of exogenous exposure. Many factors will influence the ability to detect variants, including the proportion of the genomes that they represent and the number of clones that are sequenced.

Another possibility to explain predominance of the same variant pattern in consecutive specimens is repeated re-acquisition of the same variant from external sources, perhaps by exposure to the same sexual partner. It is not unreasonable that a woman infected with a particular variant has a good chance to be re-infected with the same variant. However many women changed partners during the study, and were presumably exposed to other HPV16 variants. The fact that initial SSCP patterns persisted over time in all women examined, regardless of whether the pattern is common or rare, argues against repeated re-acquisition of predominant HPV16 variants from sex partners as the explanation for persistence of the same predominant variant over time. Further, the diversity of patterns

among women, contrasted with the identical patterns for specimens obtained over time from a single woman, supports the hypothesis that HPV16 establishes a persistent infection with a predominant variant in individual women, over the time frame encompassed by this study. Women infected with common variants such as PPP or CPP would have a high probability of being re-infected with the same variant by a new partner, but women infected with a rare NNN variant would also have a high probability of being infected by a common variant when exposed to a new partner. Thus the likelihood of women infected with rare variants only being re-exposed to the identical rare variant is low.

A third, and highly plausible explanation for the observation that 70 women harbored stable predominant HPV16 variants over time is that it is the initial HPV16 infection which results in the establishment of a persistent, apparently predominant infection. The finding that the predominant variant was always present in the first HPV16 positive specimen, particularly from women in the HHH cohort who generally had few sexual partners, supports this hypothesis. However it will be important to identify women that are experiencing primary HPV16 infection to address this hypothesis more directly. An important implication of this hypothesis is that initial infection results in protection that prevents or limits re-infection with variants of the same type. The basis of such protection is not known but could be immunological. Data in rabbits indicates that animals that spontaneously eradicate their papillomas cannot be subsequently infected experimentally

with cottontail rabbit papillomavirus (86). If it can be shown that immunological responses mediate protection from reinfection with the same type, then understanding the mechanism of protection could be important in designing prophylactic vaccines. The detection of minor variants, that over time do not displace the major variant may be the result of subsequent exposures to HPV16. It will be important to correlate the appearance of variants with information about new partners during the interval, or to identify other risk factors associated with the detection of minor variants. Among the women with multiple variants who were followed over time, there was a tendency for minor variants to disappear at subsequent visits. A larger study with longer follow-up is needed to more fully examine the hypothesis that the predominant variant is a consequence of an initial infection and persists stably, while subsequent infections with other HPV16 variants result in the more transient persistence of minor variants. It should be noted that our conclusions about persistence are based on women who were HPV16 positive at multiple visits over a relatively short time (4 months to 3 years). Additional studies are needed to examine persistence in individuals who rapidly become HPV16 DNA negative and may become positive years or decades later.

Table 8 HPV16 Variants Identified by Single-Stranded Conformational Polymorphism (SSCP) Analysis of Noncoding Region from 3 Cohorts of Women

Cohort	Variant	SSCP patterns* Length of fragment (bp)			Samples (n=212)		Subjects (n=42)	
		318	166	198	No.	%	No.	%
HDS	1	P	P	P	86	41	16	38
	2	C	P	P	68	32	14	32
	3	C	C	C	11	5	2	6
	4	C	P	N_1	4	2	1	2
	5	C	P	N_2	8	4	1	2
	6	P	P	N_3	2	1	1	2
	7	N_1	N_1	N_4	16	8	3	7
	8	N_2	N_2	N_5	7	3	1	2
	9	N_3	-**	-**	7	3	2	6
	10	N_4	-**	-**	3	1	1	2
HHH	1	C	P	P	38	30	11	28
	2	P	P	P	31	24	9	23
	3.	C	C	C	2	2	2	5
	4	P	P	C	3	2	1	2
	5	C	N_1	C	5	4	1	2
	6	C	P	N_1	4	3	2	5
	7	N_1	P	P	10	8	1	2
	8	N_2	P	P	1	1	1	2
	9	N_3	P	P	1	1	1	2

Table 8 (continued)

	10	N_4	P	N_2	9	7	1	2
	11	N_5	N_2	P	2	2	1	2
	12	N_6	-**	-**	3	2	1	2
	13	N_7	N_3	N_3	1	1	1	2
	14	N_8	N_4	N_4	12	9	5	12
	15	N_9	N_5	N_5	2	2	1	2
	16	N_{10}	N_3	N_6	3	3	1	2
HOB	1	P	P	P	20	30	8	29
	2	C	P	P	16	24	7	25
	3	C	C	C	1	2	1	4
	4	C	-**	-**	2	3	1	4
	5	N_1	N_1	N_1	1	2	1	4
	6	N_2	N_2	N_2	3	5	1	4
	7	N_3	N_3	N_3	16	24	5	18
	8	N_4	N_4	N_4	2	3	1	4
	9	N_5	N_5	N_5	3	5	1	4
	10	N_3	N_6	N_3	1	2	1	4
	11	N_6	N_7	N_6	1	2	1	4

*. P, identical to the prototype pHPV16; C, identical to Caski; or N1, N2, etc., a novel pattern, not the same as pHPV16 or Caski. **. -. lost one DdeI digestion site.

HDS. women attending a STD clinic; HHH. college students; and HOB. pregnant women.

Variants were compared within a cohort rather than across cohorts.

Table 9 Evidence of Multiple Variants Identified by Single-Stranded Conformational Polymorphism Analysis

Population	Samples			Subjects		
	No.	More than one variant		No.	More than one variant	
		No.	%		No.	%
HDS	212	10	4.7	42	5	11.9
HHH	127	12	9.4	40	6	15.0
HOB	66	1	1.5	28	1	4.0

HDS. women attending a STD clinic.
HHH. college students.
HOB. pregnant women.

Table 10 Variability of Predominant Single-Stranded Conformational Polymorphism Pattern over Time

No. of positive visits[1]	Women attending a STD clinic		College women		pregnant women	
	No. of subjects	No. (%) with identical pattern	No. of subjects	No. (%) with identical pattern	No. of subjects	No. (%) with identical pattern
1	10	-	16	-	14	-
2	9	9 (100)	13	13 (100)	9	9 (100)
3	12	12 (100)	3	3 (100)	5	5 (100)
4	5	5 (100)	3	3 (100)		
5	1	1 (100)	3	3 (100)		
6	3	3 (100)	2	2 (100)		
8	2	2 (100)				
Total	42		40		28	

1. HPV16 positive at any visit.

Table 11 DNA Sequence Analysis of HPV16 Variants in the Noncoding Region from Nucleotide Position 7445 to 7830

ID	Date	Segments (bp)									# of colonies
		318						166			
		7483	7487	7519	7667	7687	7727	7741	7762	7784	
pHPV16		A	G	G	C	C	A	T	C	C	
HDS85	6/12/90	-	-	A	-	-	-	-	-	-	10
HDS69	10/16/89	-	-	-	-	-	-	-	-	-	10
HDS81-a	10/04/89*	C	A	A	T	A	C	G	T	T	7
HDS81-b		-	-	-	-	-	-	-	-	-	3
HDS81-a	11/07/90	C	A	A	T	A	C	G	T	T	8
HDS81-b		-	-	-	-	-	-	-	-	-	2
HDS81-a	06/19/91*	C	A	A	T	A	C	G	T	T	7
HDS81-b		-	-	-	-	-	-	-	-	-	3
HDS81-a	2/24/92*	C	A	A	T	A	C	G	T	T	5
HDS81-b		-	-	-	-	-	-	-	-	-	5
HDS109	7/25/90*	C	A	A	T	A	C	G	T	T	10
HDS109	11/30/90*	C	A	A	T	A	C	G	T	T	10

*. PCR products were generated with Vent polymerase.
a and b. HPV16 variants.

Table 12 DNA Sequence Analysis of HPV16 Variants in Noncoding Region from Nucleotide Position 7748 to 104

ID	Date	Segments (bp)					# of colonies
		166				198	
		7790	7840	7867	12	24	
pHPV16		C	G	G	T	C	
HDS138	5/01/91	-	-	-	-	-	10
HDS106-a	2/13/90	-	A	-	-	T	6
HDS106-b		-	-	A	C	-	4
HDS106-a	5/8/90*	-	A	-	-	T	9
HDS106-c		-	-	-	-	-	1
HDS84-a	3/26/90#	-	-	A	C	-	5
HDS84-b		T	-	A	C	-	2
HDS84-c		-	-	-	-	-	3
HDS84-a	7/23/90*#	-	-	A	C	-	3
HDS84-d		-	-	-	C	-	2
HDS84-c		-	-	-	-	-	5
HDS117-a	4/9/92*#	-	-	-	-	-	9
HDS117-b		-	-	A	C	-	1
HDS117-a	8/12/92*	-	-	-	-	-	10

*. PCR products were generated with Vent polymerase.
#. The presence of multiple variants was suggested by SSCP analysis.
a, b, c, and d. HPV16 variants.

Figure 5 Single-Stranded Conformational Polymorphism (SSCP) Analysis of HPV16 Variants from Nucleotide Position 7445 to 222 in Specimens from Women Attending a STD Clinic

Numbers on the left indicate the base pair of fragment. Using the mobility of ds DNAs as reference, the bands which migrated closest to the ds DNA band were determined to be the corresponding ss DNA. The SSCP patterns are defined as following: P, identical to the prototype pHPV16; C, identical to Caski; or N1, N2, etc., a novel pattern, not the same as pHPV16 or Caski.

The representative SSCP patterns of HPV16 variants from nt 7445 to 222 exhibited are from: 1, double stranded (ds) pHPV16; 2, ds Caski; 3, single stranded (ss) pHPV16; 4, ss Caski; 5, HDS102, PPP; 6, HDS205, CCC; 7, HDS210, CPP; 8, HDS82, CPN_2; 9, HDS5, PPN_3, 10, HDS198, CPN_1; 11, HDS12, $N_1N_1N_4$; 12, HDS135, $N_2N_2N_5$; 13, HDS34, N_3##; 14, HDS190, N_4##.

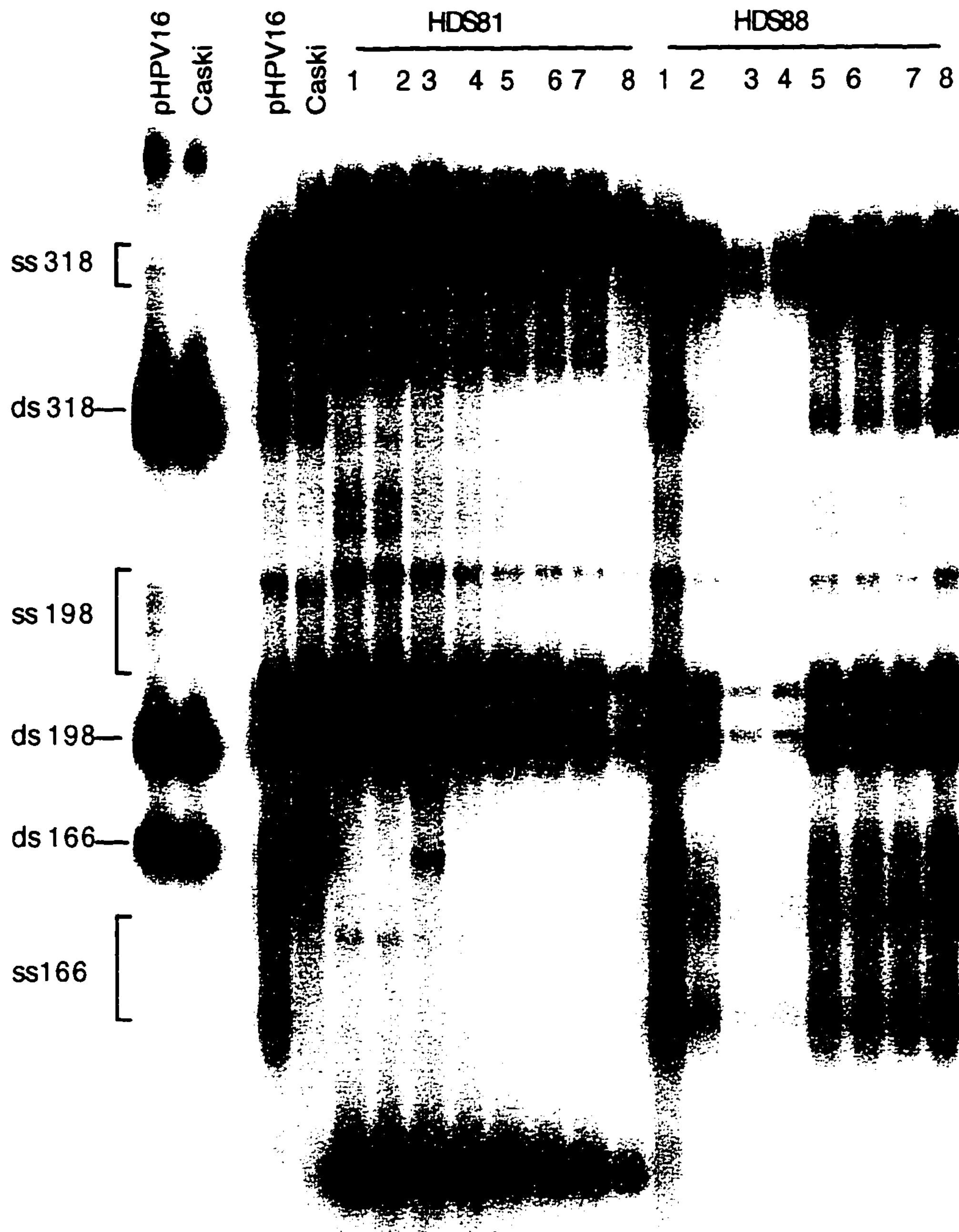

pHPV16
Caski
pHPV16
Caski
HDS81
HDS88
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
ss 318
ds 318
ss 198
ds 198
ds 166
ss166

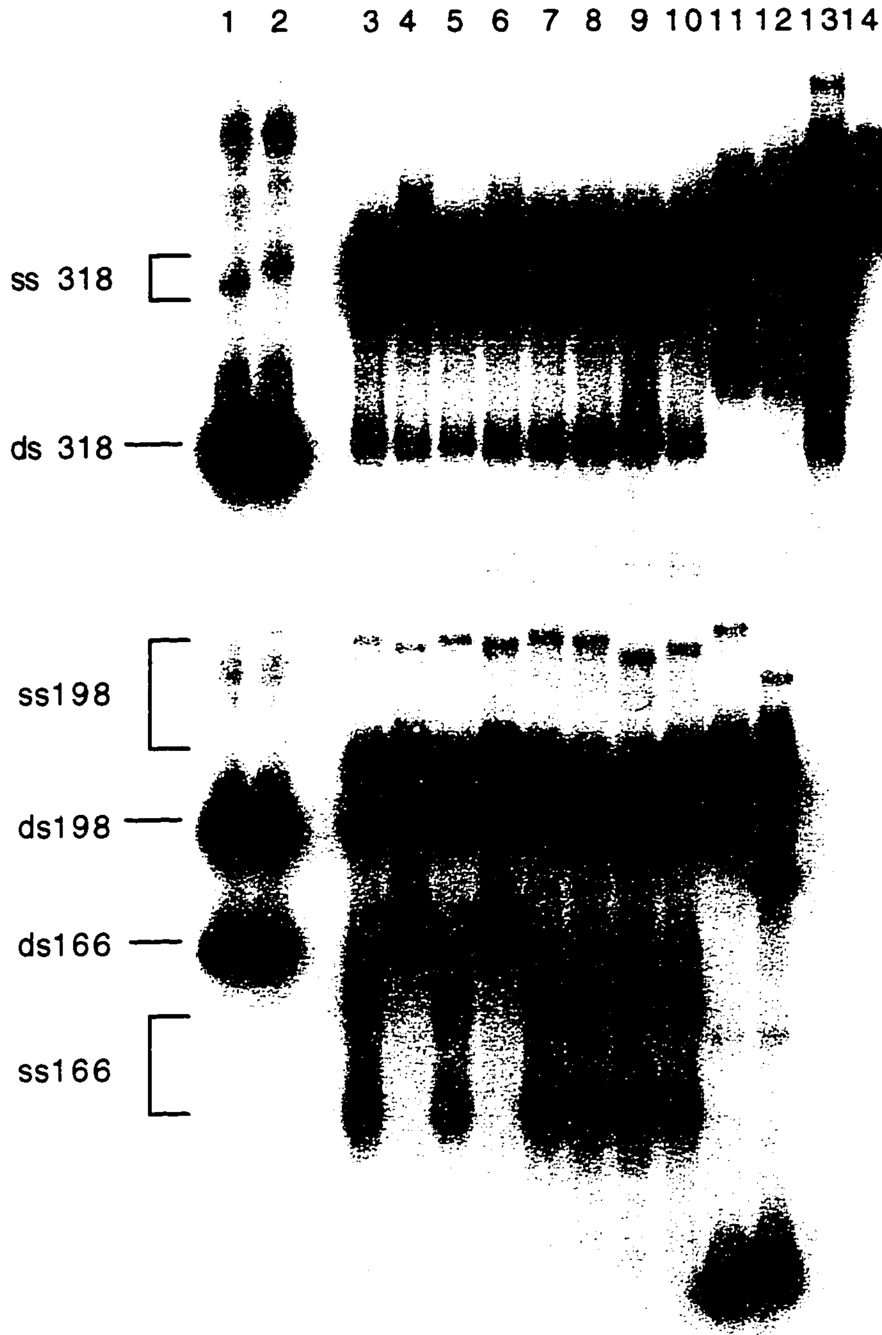
Figure 6 Single-Stranded Conformational Polymorphism (SSCP) Analysis Indicating the Presence of More Than One HPV16 Variant in a Single Specimen

Numbers on the left indicate the base pair of fragment. The SSCP patterns of HPV16 variants from nt 7445 to 222 are presented from samples: 1, double stranded (ds) pHPV16; 2, ds Caski; 3, single stranded (ss) pHPV16; 4, ss Caski; 5, HDS108; 6, HDS84. Arrows indicate the extra bands in addition to the basic patters; PPP, pattern identical to the prototype pHPV16, from HDS108 (lane 5); and CCC, pattern identical to Caski, from HDS84 (lane 6).

1
2
3
4
5
6
ss318
ds318
ss198
ds198
ds166
ss166

Figure 7 Single-Stranded Conformational Polymorphism (SSCP) Analysis of HPV16 Variants from Nucleotide Position 7445 to 222 in 8 Consecutive Specimens from 2 Women

Specimens from 8 positive visits, spanning 28 months, from each of the two women were analyzed. Numbers on the left indicate the base pair of fragment. ds is defined as double stranded DNA and ss single stranded DNA. Numbers on the top indicate the visit order. HPV16 variant from each woman showed an identical predominant SSCP pattern: PPP, pattern identical to the prototype pHPV16, in HDS88; and $N_1N_1N_4$, a novel pattern, not the same as pHPV16 or Caski, in HDS81. The extra band that runs between the 166 and 198 bp fragments from specimen at visit 3 of HDS81 suggests the presence of more than one variant. Specimens from visits 1, 4, 6 and 8 from HDS81 were further analyzed by sequencing.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
ss 318
ds 318
ss198
ds198
ds166
ss166

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ABSTRACTS

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