Risk of Anal Carcinoma in Situ in Relation to Human Papillomavirus
Type 16 Variants

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ABSTRACT

Infection with human papillomavirus (HPV), especially HPV16, is central to the development of squamous anogenital cancers and their precursor lesions, termed “squamous intraepithelial neoplasias.” Men who have sex with men, particularly those who are infected with HIV, are at a high risk for anal infection with HPV16 and for low-grade anal neoplasia; however, only a subset of these men develop anal invasive cancer or its immediate precursor lesion, anal carcinoma in situ (CIS). To examine the hypothesis that certain variants of HPV16 are most strongly associated with development of anal CIS, we followed 589 men who have sex with men whose initial anal cytological smears did not show anal CIS. Anoscopy, anal cytology, and PCR-based assays for detection and classification of HPV types were performed every 4–6 months, with HPV16 further classified by single-stranded conformation polymorphism analysis as being a prototype-like (PL) or non-prototype-like (NPL) variant. Anal CIS was histologically confirmed in 6 of 384 (1.6%) consistently HPV16-negative men, in 12 of 183 (6.6%) men with HPV16 PL variants, and in 4 of 22 (18.2%) men with HPV16 NPL variants. After adjustment for anal cytological diagnoses at study entry, HIV status and CD4 count, and detection of HPV types other than type 16, men with HPV16 NPL variants were 3.2 times (95% confidence interval, 1.0–10.3) more likely to develop anal CIS than were those with PL variants. Neither detection of HPV 16 DNA at high levels nor detection of HPV16 DNA for a prolonged period, factors that we previously demonstrated to be associated with risk of high-grade anal squamous intraepithelial neoplasia, was significantly associated with HPV16 NPL variants. The biological mechanism relating to this excess risk remains undetermined.

INTRODUCTION

It is now well established that some types of HPV play a central role in the pathogenesis of squamous anogenital cancers and their precursor lesions, termed “squamous intraepithelial neoplasias.” Several studies have demonstrated that, compared to the general population, MSM, especially those who are infected with HIV, are at markedly increased risk for anal neoplasia (1–8). However, although HPV16 infection, the HPV type most frequently associated with anogenital tract squamous cell cancers, is exceedingly common in the anal canal epithelium of MSM, only a subset of such infections are associated with subsequent development of anal invasive cancer or its immediate precursor lesion, anal CIS (6).

HPVs are classified into “types,” “subtypes,” or “variants,” based on the extent of DNA homology, and are referred to as high- or low-risk types, based upon the frequency with which that type has been identified in invasive cancers. Support for the idea that HPV variants might differ with respect to cancer risk comes from a number of in vitro studies (9–11), a report of an HPV18 variant with decreased oncogenic potential in vivo (12), and our recent observation that PL and NPL variants of HPV16 are associated with differing risks for the development of cervical intraepithelial neoplasia grades 2–3 (13). We undertook this cohort study among HIV-seropositive and HIV-seronegative men to determine whether different HPV16 variants vary in their association with development of anal CIS and with the amount of viral DNA and the length of viral persistence, factors that we previously demonstrated to be associated with risk of high-grade anal squamous intraepithelial neoplasia (6).

MATERIALS AND METHODS

Study Population and Study Design. Between October 1989 and December 1995, MSM presenting for HIV testing and counseling at the AIDS Prevention Project of the Seattle-King County Department of Public Health were invited to participate in a cohort study evaluating the risk of high-grade squamous intraepithelial neoplasia associated with HIV and anal HPV infection. The original cohort and study design have been described previously (5, 6). Briefly, participants were required to be at least 18 years of age and to provide 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. Enrolled subjects were asked to return every 4–6 months for an interview, detailed genital examination, and collection of specimens. Blood was drawn at enrollment and at each return visit for lymphocyte subset analysis and for detection of antibody to HIV. Anal swab specimens for cytological screening and for HPV typing were collected at each visit.

For this study, only subjects with entry-visit cytological diagnoses that were less severe than anal CIS were eligible. Of 943 eligible subjects, 354 (37.5%) were excluded due to missing demographic information, HPV results, and/or cytological diagnosis (n = 114); failure to return for follow-up after initial enrollment (n = 156); or inability to evaluate HPV16 positive-specimens by SSCP analysis due to lack of sufficient material (n = 84). Overall, 3329 visits following the initial HPV testing and cytological screening from 589 subjects were included. The number of visits for each subject ranged from 2 to 25, with a mean of 5.7.

Assessment of HPV16 Variants and DNA Level. Anal samples were screened by a PCR-based dot-filter hybridization using a consensus primer amplification system (14). The PCR products were probed with a biotin-labeled generic HPV probe and with mixtures of biotin-labeled type-specific oligonucleotide probes for HPV6 and 11, HPV16, HPV18, HPV45, and HPV31, HPV 33, 35, and 39. Specimens hybridizing with the generic probe but not with any of the type-specific probes were said to contain unclassified HPV's. Specimens that were positive for HPV16 by PCR were further assayed by PCR-based SSCP analysis as described previously (15). Briefly, DNA amplification was completed in a Perkin-Elmer 9600 Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT) for 35 cycles. Amplification products were screened on a 1% agarose gel and then detected by autoradiography. DNA samples from 25 type-specific positive controls were run on each gel. Specimens were considered positive for HPV16 if they failed to PCR amplify and were positive for HPV16 type-specific bands.

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2 To whom requests for reprints should be addressed, at Harborview Medical Center, 325 9th Avenue, Seattle, WA 98004.
3 The abbreviations used are: HPV, human papillomavirus; MSM, men who have sex with men; CIS, carcinoma in situ; PL, prototype-like; NPL, non-prototype-like; SSCP, single-stranded conformation polymorphism; RR, relative risk; CI, confidence interval.

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polycrylamide gel with 10% glycerol. The SSCP patterns from the prototype plasmid HPV16 (16) and HPV16 in Caski cell line (17) were used as the reference. The determination of SSCP patterns and classification of variant groups were performed without knowledge of the clinical data. HPV16 isolates were regarded as different variants as long as any one of the three fragments displayed a polymorphism differing from any other isolates. All HPV16 variants were classified into two groups. The PL group included variants that displayed the reference SSCP patterns and variants with one or two fragments that showed nonreference patterns. The NPL group included variants that displayed nonreference SSCP patterns in all three fragments. As compared to the HPV16 prototype, NPL variants usually carry 5–10 more nucleotide alterations in the region analyzed than do PL variants (13).

Specimens from 176 HPV16-positive men were further assayed by lineage-specific hybridization (18) to allow assignment of HPV16 variants to one of five previously identified phylogenetic lineages (19). Briefly, 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 for PCR and hybridization have been described previously (18).

Specimens that were positive for HPV DNA by PCR were further analyzed by hybrid capture assay or Southern transfer hybridization with three groups of probes targeting HPV6 or 11, HPV31, 33, 35, or 39, and HPV16, 18, or 45. The hybrid capture assay was performed based on the protocol recommended by the manufacturer (Digene Diagnostics, Silver Spring, MD). The Southern transfer hybridization assay was done as described previously (14). A sub-analysis based on specimens positive by PCR for HPV16 DNA but not for HPV18 and/or 45 was performed. Specimens that were positive by these less sensitive nonamplified methods were considered to have a low level of HPV16 DNA. Specimens that were positive for HPV16 by PCR alone were considered to have a high level of HPV16 DNA (5).

Assessment of Biopsy-confirmed Anal CIS. During follow-up, any subject with changes suggestive of severe dysplasia on colposcopic or cytological examination (according to the WHO or the Bethesda recommendations for cervical lesions; Ref. 20) was referred for biopsy. Methods for collection of biopsy specimens and histological examination have been described previously (6). Histological diagnoses were assigned as negative, mildly atypical, or consistent with anal intraepithelial neoplasia grade 1, 2, or 3 anal CIS. None of the men had colposcopic, colposcopic, or histological evidence of invasive cancer. As is well established in the diagnoses of cervical lesions, it is difficult for pathologists to distinguish reproducibly between intraepithelial neoplasia grade 3 and CIS. We included the diagnosis of anal intraepithelial neoplasia grade 3 in anal CIS. Decisions to refer for biopsy, refusal of biopsy by study subjects, and the histological diagnoses were made without knowledge of HPV status.

Statistical Analyses. Cox proportional hazard regression analysis (21) with an extension of counting process notation (22) using the statistical software package S-plus (MathSoft, Inc., Seattle, WA) was used to estimate RR and 95% CI associated with developing anal CIS. For the entire cohort, the starting point was the time of study entry. For the subset analyses comparing the risk of anal CIS between men with HPV16 PL and those with NPL variants, the date of initial detection of HPV16 DNA was used as the starting point. The ending point was the date at which biopsy-confirmed anal CIS was first diagnosed, the visit at which the referral for biopsy was refused, or the last visit date for those who did not develop anal CIS or refuse biopsy. Covariates for multivariate analyses included: anal cytological diagnoses at study entry; detection of unclassified HPVs, HPV6 or 11, HPV18, 31, 33, 35, 39 or 45, HPV16 variants; and a factored variable combining HIV status and CD4 count (HIV-seronegative, HIV-seropositive with CD4 count of >500 x 10^6/liter, and HIV-seropositive with CD4 count of ≤500 x 10^6/liter). Visits at which the number of CD4 cells was not counted were filled in with the most recent measurement. Visits following the one at which a specific HP type was initially detected were treated as positive for that HP type. Additional time-dependent covariates used in subset analyses among HPV16 positive men were HPV16 DNA level (high or low) and cumulative number of HPV16-positive visits.

Kaplan-Meier product limit estimates were used to calculate the cumulative proportion of men in whom initial anal CIS developed and the proportion of men in whom HPV16 DNA remained positive. The overall differences in these proportions between men with HPV16 PL and those with NPL variants were assessed using a log-rank test (23). In analyses estimating the proportion developing anal CIS, the starting point was defined as the date of the initial HPV16-positive visit and the ending point, the date at which biopsy-confirmed anal CIS was first diagnosed, the visit at which the referral for biopsy was refused, or the last visit date. In analyses estimating the proportion of patients remaining HPV16 positive, the starting point was defined as before and the ending point was defined as the date at which HPV16 DNA initially reverted to negative status or the date of the last visit date for those not becoming negative.

A relationship between HPV16 DNA level and variants among visits positive for HPV16 by PCR was examined using generalized estimating equations (24) specifying a logit link function, which models log odds as a linear function, between the outcome (detecting HPV16 DNA at a high level) and the covariate (HPV16 PL versus NPL variants). Concordance between the classification of HPV16 variants by lineage-specific hybridization and by SSCP analysis was assessed using a κ statistic. A χ^2 test was used to compare distributions of subject characteristics by HPV16 variants. The mean length of follow-up for men with HPV16 PL or NPL variants was examined by Student's t test.

RESULTS

Characteristics of the Study Population by HPV16 Status at Entry. At study entry, 160 of 589 (27.2%) study participants were positive for HPV16 DNA by PCR. NPL variants accounted for 12.5% (20 of 160) of the HPV16 infections detected. Presented in Table 1 are selected characteristics of study participants by HPV16 status and variants. Compared to those who were positive for HPV16 PL variants, men infected with NPL variants were somewhat more likely to report a history of illicit drug use (P = 0.05) and to present with anal smears showing mild or moderate dysplasia at study entry (P = 0.15)
but did not differ substantially with respect to age at study entry, race, age of first anal intercourse, a self-report of >50 lifetime sex partners, or a history of sexually transmitted diseases.

At study entry, 313 of 589 (53.1%) men were HIV seropositive. HPV16 DNA was detected among 117 of the 313 (37.4%) men who were HIV seropositive and 43 of the 276 (15.6%) men who were HIV seronegative (P < 0.01). Sixteen of the 117 (13.7%) HIV-seropositive and HPV16-positive men and 4 of the 43 (9.3%) HIV-seronegative and HPV16-positive men had NPL variants present. NPL variants accounted for 19.2% of all HPV16 infections among HPV16-positive men who were HIV seropositive with entry CD4 counts of ≤500 × 10^6/liter. In contrast, only 8.2% of all HPV16 infections among HPV16-positive men who were HIV-seropositive with CD4 counts of >500 × 10^6/liter and 9.3% of all HPV16 infections within HIV-seronegative men were NPL variants. These differences were, however, not statistically significant (P = 0.16).

**Classification of HPV16 Variants over Time.** In addition to the 160 men who had HPV16 detected at study entry, 45 of those who were negative for HPV16 at entry had HPV16 DNA detected during follow-up. Of the 205 men who had HPV16 detected at some point during the study, 172 had anal HPV16 detected at more than one visit, and of these, 97 had adequate material available for SSCP analysis from at least two visits. A total of 306 samples from these 97 men (range, two to nine visits per subject) were tested by SSCP analysis to determine whether the HPV16 variant that was initially present remained constant over time. Eighty-seven men were classified as being infected with HPV16 PL variants, and 10 were classified as being infected with NPL variants. The initial HPV16 variant detected in each subject was consistently detected at all visits, as detected by SSCP analysis.

**Development of Anal CIS over Time.** The average length of follow-up for the entire cohort was 25.5 months (SD = 18.9 months). The mean length of follow-up (measured from the date of the initial detection of HPV16 DNA) did not differ substantially between men with PL variants (mean = 28.2 months; SD = 18.7) and those with NPL variants (mean = 23.9 months; SD = 20.1; P = 0.32). Over the course of follow-up, 200 of 589 men underwent biopsy; 48 were referred for biopsy but refused. Of the 200 men undergoing biopsy, 29 whose previous biopsy did not show anal CIS were subsequently referred for a second biopsy but refused. There was no association between refusal to be biopsied and infection with PL or NPL variants with 39 of 150 (26%) men without HPV16, 34 of 111 (31%) with HPV16 PL variants, and 4 of 16 (25%) with HPV16 NPL variants refusing biopsy. Overall, anal CIS was histologically confirmed in 22 of 589 study participants, including 7 of 270 (2.6%) HIV-seronegative versus 15 of 319 (4.7%) HIV-seropositive men (6 men HIV-seroconverted during follow-up) or 12 of 420 (2.9%) men presenting with anal smears showing normal cytology at study entry versus 10 of 169 (5.9%) men presenting with anal smears showing mild or moderate dysplasia. Characteristics such as age at study entry, race, age at first anal-receptive intercourse, lifetime number of sex partners, and self-report of a history of illicit drug use or of sexually transmitted diseases were not significantly associated with risk of biopsy-confirmed anal CIS (data not shown).

**Risk of Anal CIS in Relationship to Detection of Specific Types and Variants of Anal HPV.** Multivariate Cox regression analysis in which HPV types and HPV16 variants, in addition to anal cytological diagnoses at study entry and HIV status and CD4 count, were simultaneously included demonstrated greater risk for development of anal CIS associated with detecting HPV6 or 11, HPV18, 31, 33, 35, 39, or 45, HPV16 PL variants, and HPV16 NPL variants but not unclassified types of HPV (Table 2). Biopsy-confirmed anal CIS was diagnosed in 12 of 183 (6.6%) men positive for HPV16 PL variants, in 4 of 22 (18.2%) men positive for HPV16 NPL variants, and in 6 of 384 (1.6%) men who were always negative for HPV16.

Considering only those men who were infected with HPV16, the overall cumulative proportion of men developing anal CIS as estimated using Kaplan-Meier plots was significantly higher for those with HPV16 NPL variants (26.3%) than it was for those with PL variants (9.0%; log-rank test, P = 0.02; Fig. 1). All 16 HPV16-positive men who developed anal CIS, regardless of the variant of HPV16 present, did so within 30 months of the initial detection of HPV16 DNA. Relative to those infected with PL variants of HPV16, men infected with NPL variants were 3.2 times more likely to develop anal CIS (95% CI, 1.0–10.3) after adjustment for anal cytological diagnoses at study entry, HIV status and CD4 count, and detection of unclassified HPV variants, HPV6 or 11, and HPV18, 31, 33, 35, 39, or 45 (Table 3). Of the 16 men who developed anal CIS, 10 had HPV16 detected in a swab specimen on the day of biopsy, whereas 6 had HPV16 DNA detected in a swab specimen, prior to but not on the same day as the biopsy. HPV16 DNA was detected in biopsy specimens from 8 of the 10 cases with HPV16-positive swab specimens at the time of biopsy. The remaining two cases had biopsy specimens that were insufficient for PCR-assay, both of whom had PL variants detected in the swab specimens. Of the six cases whose swab specimens were negative for HPV16 on the day of biopsy, one had an HPV16 NPL variant and five had PL variants detected during visits prior to biopsy; these men were classified in the analyses on the basis of the swab results. Four of the six biopsy specimens from these men were positive for HPV types other than 16, one was negative for all HPV types, and one was inadequate for testing. In an analysis excluding these six men who were not confirmed to be HPV16 positive on the day of biopsy, the risk of anal CIS remained elevated among men with HPV16 NPL variants relative to those with PL variants (RR = 3.7; 95% CI, 1.0–14.7).

**Confirmation of the Association between Risk of Anal CIS and HPV16 Variants Using Lineage-specific Hybridization.** To verify the sequence variation identified by SSCP analysis and to confirm that sequence variation was associated with increased risk of development of anal CIS, we used an alternative method for identification and classification of HPV16 variants, termed lineage-specific hybridization, that has been developed to rapidly assign HPV16 variants to the five previously identified phylogenetic lineages of HPV16 (19). One hundred seventy-six samples that were sufficient for further assay were blindly analyzed as described previously (18). Of 18 specimens with HPV16 NPL variants, 16 were classified as non-European variants, including 7 African variants, 5 American-Asian variants, and 4 Asian variants, whereas 2 were classified as European variants. All

<table>
<thead>
<tr>
<th>HPV status</th>
<th>RR*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified HPVs</td>
<td>1.0</td>
<td>0.3–7.2</td>
</tr>
<tr>
<td>-</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>HPV6 or 11</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>2.7</td>
<td>1.0–7.1</td>
</tr>
<tr>
<td>HPV18, 31, 33, 35, 39, or 45</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>3.5</td>
<td>1.0–11.9</td>
</tr>
<tr>
<td>HPV16</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>3.3</td>
<td>1.1–9.4</td>
</tr>
<tr>
<td>PL variants</td>
<td>10.6</td>
<td>2.7–41.6</td>
</tr>
<tr>
<td>NPL variants</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* RR was estimated from Cox regression analysis with simultaneously adjustment for all variables listed in the table, in addition to anal cytological diagnoses at study entry, and HIV serological status, and CD4 count. ^ Specimens were positive for HPVs by generic probe but negative by type-specific probes.
158 specimens with HPV16 PL variants were classified as European variants. Non-European variants did not appear to be more frequently detected in nonwhite than in white men (data not shown). The classification of European versus non-European variants by lineage-specific hybridization was highly concordant with the classification of PL versus NPL variants by SSCP analysis ($\kappa = 0.93$). The relationship between risk of anal CIS and HPV16 variants was reevaluated according to the classification by lineage-specific hybridization. In Cox regression analysis, after adjustment for anal cytological diagnoses at study entry, HIV status and CD4 count, and detection of unclassified HPVs, HPV6 or 11, and HPV18, 31, 33, 35, 39, or 45, men with HPV16 non-European variants, compared to those with European variants, were 4.6 times more likely to develop biopsy-confirmed anal CIS (95% CI, 1.4-15.1).

HPV16 Variants Do Not Differ with Respect to Level of HPV16 DNA Present or Persistence of HPV DNA. Specimens from 431 visits that were positive for HPV16 by PCR (but negative for HPV18 and 45), were additionally assayed by either hybrid capture or Southern transfer hybridization to determine whether relatively “high” or “low” levels of HPV16 DNA were present. HPV16 DNA was detected at high levels (i.e., positive by hybrid capture or Southern transfer hybridization) among 214 (56.3%) of 380 visits from men with PL variants and 32 (62.7%) of 51 visits from those with NPL variants. Because the DNA level of HPV16 in specimens collected over time from an individual may be correlated, we examined the association between HPV16 DNA level and variants using a logistic regression model with a generalized estimating equation approach to take this intraperson correlation into account. Overall, men with HPV16 NPL versus those with PL variants were not more likely to have visits with HPV16 DNA detected at high levels (odds ratio = 1.4; 95% CI, 0.7-2.9).

To determine whether men infected with NPL variants compared to those with PL variants had HPV16 DNA detected for longer periods of time, we analyzed time to first HPV16-negative visit. During the course of follow-up, 73 of 205 (35.6%) HPV16-positive men became HPV16 DNA negative. Overall, the median time to the first HPV16 negative visit from initial detection of HPV16 DNA was 22.8 months (95% CI, 13.9-31.8). Although no significant difference was observed in the time to become HPV16 negative between men with HPV16 NPL and those with PL variants (log-rank test: $P = 0.28$), the analysis was limited due to lack of sufficient power. To control for the possible effects of level and length of detection of HPV16 DNA, we included, in a Cox model, HPV16 DNA level and cumulative number of positive visits as time-dependent covariates in addition to anal cytological diagnoses at study entry, HIV status and CD4 count, and detection of types and variants of HPV. Risk for anal CIS associated with HPV16 NPL relative to PL variants did not change substantially (RR = 3.4; 95% CI, 1.0-11.3).

### DISCUSSION

In this study we showed that, as compared to HPV16 PL variants, NPL variants were more strongly associated with development of anal CIS. We have recently reported that infection with HPV16 NPL variants was associated with an increased risk of development of cervical intraepithelial neoplasia grade 2-3 (13). This study extends this previous observation to anal neoplasia and to risk of development of CIS, the lesion thought to be the immediate precursor to invasive cancer. The majority of women in the previous study developed cervical intraepithelial neoplasia grade 2, a lesion thought to be less closely related to invasive cancer than is CIS. Our earlier studies and those of others indicate that the risk for high-grade lesions is related to HPV DNA amount (as reflected by the level of HPV detected) and to persistence of detectable HPV DNA (as reflected by the number of HPV-positive visits; Refs. 6, 25, and 26). In this study, however, the proportion of visits with high levels of HPV16 DNA detected was remarkably similar among those with NPL and PL variants. In addition, compared to men with HPV16 PL variants, those with NPL variants did not have HPV DNA detected for longer periods of time.

### Table 3

<table>
<thead>
<tr>
<th>HPV16 variant group</th>
<th>No. of subjects</th>
<th>No. with anal CIS (%)</th>
<th>RR*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>183</td>
<td>12 (6.6)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>NPL</td>
<td>22</td>
<td>4 (18.2)</td>
<td>3.2</td>
<td>1.0-10.3</td>
</tr>
</tbody>
</table>

* Risk of anal CIS for men infected with HPV16 NPL variants relative to those infected with HPV16 PL variants estimated from Cox regression analyses, adjusting for anal cytological diagnoses at study entry, HIV serological status and CD4 count, and detection of HPV6 or 11, HPV18, 31, 33, 35, 39, or 45, and unclassified HPVs.
Additional adjustment for the level of HPV16 DNA and cumulative number of positive visits did not appreciably change the estimate of risk of anal CIS associated with NPL relative to PL variants. Furthermore, the NPL variant-related excess risk was not explained by the presence of anal mild or moderate dysplasia at study entry nor by factors that we have previously reported to be associated with risk of development of anal neoplasia (5, 6), including HIV seropositivity and immunosuppression and infection with HPV types other than type 16.

Although this is the first study demonstrating that infection with different variants of HPV16 varies in their association with anal CIS, it is well established that risk of anal dysplasia varies significantly with different types of HPV (5, 6, 8). Given this and the fact that the regions of greatest intratypic variability are known to correspond to regions of greatest intertypic variability (27), nucleotide alterations that define different variants might well have biological properties associated with varying risk for anal CIS. A number of *in vitro* observations support our findings. These include studies demonstrating that a natural point mutation at a YY1 site in the noncoding region greatly enhances promoter activities that drive transcription of oncoproteins of E6 and E7 (9) and that nucleotide alterations at glucocorticoid responsive element sites in the noncoding region of HPV16 affect the transformation and alter the oncogenic potential in the presence of *ras* oncogene and hormone (10). Moreover, Conrad-Stoppler *et al.* (11) have shown that the natural variants of HPV16 E6 protein differed in their abilities to alter keratinocyte differentiation and induce p53 degradation. Taken together, these *in vitro* studies imply that the increased risk of anal CIS associated with HPV16 NPL relative to PL variants might be related to certain nucleotide alterations present in these variants. However, nucleotide alterations in one region of HPV16 often connect to changes in other regions (18, 19, 28, 29), and thus, it is not clear whether the areas we have targeted for examination in fact contain changes that confer important differences in biological behavior.

Other possible explanations of the increased risk conferred by NPL variants for anal CIS include the possibility of inadequate immune surveillance resulting from either the specific genetic characteristics of the host or nucleotide alterations of the variants. A study by Apple *et al.* (30) has shown that certain HLA haplotypes, such as DQB1*0602-DRB1*1501 and DQB1*0302-DRB1*0407, were significantly associated with HPV16-related cervical CIS and invasive cancer but not with cervical neoplasia related to other HPV types in a population of Hispanic women. This finding suggests a type-specific association between HLA haplotypes and cervical lesions. Ellis and coworkers (31) reported the HLA-B7 haplotype to be strongly associated with infection with an HPV16 variant (nt131G variant) carrying an amino acid change at one of the three potential CTSL epitopes in the E6 region, suggesting that inadequate T-cell surveillance of certain variants may exist in association with specific HLA haplotypes. A cross-sectional study of women with persistent low-grade cervical cytological abnormalities also indicated an association between HPV16 nt131G variant and risk of underlying high grade cervical intraepithelial neoplasia and relative lack of antibody response to virus-like particles (32). It is possible that the increased risk of anal CIS associated with NPL variants may result in part from an interaction between certain HPV variants and HLA molecules. Because HLA typing was not performed in our study, this hypothesis will require further investigation.

Several potential limitations of this study should be addressed. First, only 62.5% of subjects enrolled in the study returned for follow-up. This could potentially bias our estimates if the magnitude of the association between anal CIS and HPV16 variants among the men not included in our study varied significantly from that observed in our study. Given the similarities between those who did and did not return regarding demographic characteristics, sexual behavior, HIV status, and history of sexually transmitted diseases (6), it is unlikely that our findings are compromised by selection bias. Second, although differential loss to follow-up is a special concern in a longitudinal study, it was not observed in this study. Men with HPV16 PL variants and those with NPL variants were followed for an equivalent time period. Moreover, there are no data to suggest that men with PL versus NPL variants would have been more likely to develop anal CIS at a later date had they been followed for a longer period of time. Third, the proportion of men refusing biopsy (*n* = 77) who otherwise would have had anal CIS histologically confirmed is unknown. If compliance with biopsy-referral was positively associated with the presence of HPV16 PL variants, the risk associated with NPL variants could be overestimated. Arguing against this is the fact that both clinicians and referred subjects were unaware of HPV16 status and also that there was no relationship between biopsy refusal and infection with PL or NPL variants. Fourth, in our analyses, visits following the one at which a specific HPV type was initially detected were treated as positive for that HPV type. This is based on an assumption that HPVs play a role in the risk of development of anal CIS not only at the time when HPV DNA is detected but also during a certain time period following the initial positive visit. The risk of anal CIS associated with certain HPV types could be overestimated if those types tend not to persist and only affect the pathogenesis of neoplasia over a short time period. However, when we conducted analyses treating HPV status as a time-dependent covariate, based on the last visit, current visit, or both, NPL, compared to PL variants, were still associated with an increased risk of anal CIS (data not shown). Last, it should be noted that men who were positive for HPV16 at study entry (prevalent infection) were included in all analyses if they returned for follow-up. The duration of prior HPV16 positivity for these men is unknown. Thus, analyses comparing persistence of PL versus NPL variants may be subject to bias if duration of previous HPV16 positivity differed significantly between the two groups. Furthermore, inclusion of prevalent HPV16 infections could bias estimates of the risk of anal CIS associated with HPV16 variants, if the positive duration before study entry was differentially associated with type of variant. Although this cannot be dismissed, the observation from a cohort of university women showing a similar length of detecting HPV16 DNA between PL and NPL variants in both incident and prevalent infection* argues against the possibility of substantial bias in our risk estimates.

In conclusion, the data from this study suggest that HPV16 NPL as compared to PL variants are associated with an increased risk of development of anal CIS. Although we hypothesize that the increased risk of anal CIS associated with HPV16 NPL variants may be related to changes in the biological properties of the virus or reflect variant-related immune surveillance, the actual mechanism remains undetermined. Further studies are needed to examine whether such variants are associated with invasive cancers.

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**REFERENCES**


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Risk of Anal Carcinoma in Situ in Relation to Human Papillomavirus Type 16 Variants


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