Risk of Cervical Intraepithelial Neoplasia Grade 2 or 3 after Loop Electrosurgical Excision Procedure Associated with Human Papillomavirus Type 16 Variants

Long Fu Xi,1,2 Nancy B. Kiviat,1 Cosette M. Wheeler,2 Aimee Kreimer,4 Jesse Ho,1 and Laura A. Koutsky4

1Department of Pathology, School of Medicine, and 2Department of Epidemiology, School of Public Health and Community Medicine, University of Washington, Seattle; 3Departments of Microbiology and Molecular Genetics, School of Medicine, University of New Mexico, Albuquerque; 4Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland

Identification of factors associated with risk of relapse after treatment for high-grade cervical intraepithelial neoplasia (CIN) has important clinical implications. Study subjects were women participating in the Atypical Squamous Cells of Undetermined Significance and Low-Grade Squamous Intraepithelial Lesion Triage Study who were treated for CIN3 by loop electrosurgical excision procedure (LEEP) and who had a baseline infection with human papillomavirus type 16 (HPV16). These women were followed every 6 months for 2 years. Post-LEEP CIN2-3 was found in 20 (10.0%) of the 201 women. An adjusted relative risk of 3.1 (95% confidence interval, 1.1–8.9) was associated with HPV16 non-European, compared with European, variants, a finding that is consistent with the variant-related risk of prevalent/incident high-grade CIN.

A conservative treatment by either excision or ablation of the transformation zone is commonly recommended for women with cervical intraepithelial neoplasia grade 2 or 3 (CIN2-3) [1]. Although such an intervention remarkably reduces the incidence of invasive cervical cancer (ICC), women after treatment remain at ~5-times greater risk for ICC than the general population [2]. Although whether a particular lesion will progress to cancer remains undetermined, it can no longer be in doubt that women with recurrent lesions are at higher risk of ICC. Identification of factors associated with posttreatment CIN2-3 is therefore of great importance in cervical cancer prevention.

Presence of oncogenic human papillomavirus (HPV) types after treatment is thought to be a risk factor for posttreatment CIN2-3 [3]. Type-specific correlations between the initial and recurrent lesions suggest that the viruses may not be completely removed by treatment [4]. A recent study further demonstrated that women with HPV16, detected 6 months after treatment, compared with those with other HPV types or without HPV infection, are at significantly higher risk of posttreatment CIN2-3 [5]. It is possible that among those with HPV16 infections, risk of posttreatment CIN2-3 may vary, because as shown in previous studies [6–8], the variants of HPV16 differ in risk of prevalent and incident CIN2-3.

To address this issue, we evaluated the risk of CIN2-3 in HPV16-positive women who had been treated for CIN3 by loop electrosurgical excision procedure (LEEP), the modality commonly used in the United States.

Subjects and methods. Study subjects were women who participated in the Atypical Squamous Cells of Undetermined Significance (ASCUS) and Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS). Details on the ALTS design have been described elsewhere [5]. Briefly, participants who were referred with cytologic diagnoses of ASCUS or LSIL were randomly assigned into 1 of 3 arms (i.e., immediate colposcopy, HPV triage, or conservative management). At enrollment, the following groups of women were referred for a colposcopy with colposcopic-directed biopsy of any visible lesions: all women in the immediate colposcopy arm, women who had a cytologic diagnosis of high-grade SIL (HSIL) in the conservative arm, and women who were positive for oncogenic HPV types in the HPV arm. All participants, regardless of study arms, were followed with cytologic and HPV testing every 6 months for 2 years. During follow-up, women were re-referred for colposcopy and biopsy if HSIL was found. At exit, participants underwent a procedure including cytologic testing, HPV testing, and colposcopic examination, with biopsy of any visible lesions. Cervical cytologic and histologic diagnoses were initially made by clinical center pathologists and then were reviewed by a panel of expert pathologists. A treatment by LEEP was...
Figure 1. Kaplan-Meier estimates of the likelihood of having cervical intraepithelial neoplasia grade 2 or 3 (CIN2-3) after a loop electrosurgical excision procedure for CIN3, comparing women with baseline infections with human papillomavirus type 16 European (solid line) and non-European (dashed line) variants. The plot was truncated at month 24 after treatment, because few women were followed beyond that time point. \( P = .07 \), log-rank test.

Cox proportional hazard regression analysis was used to examine the risk of post-LEEP CIN2-3 associated with HPV16 variants. Time to an event was defined as the duration between date of treatment and the first onset of CIN2-3. For women who did not have post-LEEP CIN2-3, the time was censored at the last visit date. Considering that the variants detected at baseline might no longer be relevant to the post-LEEP risk if they became undetectable, we performed an additional analysis, in which the time was censored at the first negative visit subsequent to the last HPV16-positive test. The 2-year cumulative proportion of post-LEEP CIN2-3 by HPV16 variants was estimated using the Kaplan-Meier plot. A Fisher’s exact test was used to compare incidence of post-LEEP CIN2-3 by HPV16 variants, stratified by post-LEEP HPV16 status. The mean age and mean length of follow-up between women with different variants were examined using Student’s \( t \) test. All statistical tests were 2-sided at the 5% significance level.

Results. Of the 201 samples obtained at enrollment from women treated for CIN3, 166 (82.6%) were positive for HPV16 European variants, 5 (2.5%) for African-1 variants, 11 (5.5%) for African-2 variants, 17 (8.5%) for Asian-American variants, and 2 (1.0%) for Asian variants. During follow-up, 20 (10.0%) [95% confidence interval \( \{CI\}, 6.2\%–14.9\%\)] of the 201 women developed histologically confirmed CIN2-3 after LEEP, including 13 (9.1%) of 143, 1 (14.3%) of 7, and 3 (20.0%) of 15 white women with European, African-2, and Asian-American variants, respectively; and 1 (5.9%) of 17 and 2 (40.0%) of 5 African American women with European and African-1

offered to women with histologically confirmed CIN2 or worse at any time during the trial. The study protocol was approved by the institutional human subject review board of the University of Washington.

A woman was eligible for the study if she had CIN3 histologically confirmed by the panel of expert pathologists within the first year of the trial and HPV16 DNA detected in her enrollment cervical sample by polymerase chain reaction (PCR)-based reverse-line hybridization. Of the 242 eligible women with CIN3, 37 were excluded: 9 without treatment records, 11 with no follow-up after treatment, 6 who were treated by modalities other than LEEP, and 11 without data on final disease ascertainment. Additionally excluded were 4 women whose samples were unavailable or negative for variant characterization, leaving 201 in the analyses. The end point was the first episode of post-LEEP CIN2-3 histologically confirmed by the panel of expert pathologists. This end point subsumes recurrence, persistence, or multifocal presence of the primary CIN3 lesions or possibly incident diseases.

HPV16-positive cervical samples at enrollment were assayed, as described elsewhere [9], by PCR-based DNA sequencing. A viral isolate was classified as a distinct variant if 1 or more nucleotide alterations were detected in the region from nucleotide positions 7723–567 (corresponding to the 3’ part of the long control region and the entire E6 region). According to the lineages categorized previously [10], HPV16 variants were classified as European, Asian, Asian-American, African-1, and African-2 variants.
Table 1. Cumulative events of cervical intraepithelial neoplasia grade 2 or 3 in women with human papillomavirus type 16 European and non-European variants, by time points after treatment.

<table>
<thead>
<tr>
<th>Variant, measurement</th>
<th>Time point after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 months</td>
</tr>
<tr>
<td>European (n = 166)</td>
<td></td>
</tr>
<tr>
<td>At risk at the beginning of the time point</td>
<td>158</td>
</tr>
<tr>
<td>Cumulative events</td>
<td>4</td>
</tr>
<tr>
<td>Cumulative rate, % (95% CI)</td>
<td>2.4 (0.1–4.8)</td>
</tr>
<tr>
<td>Non-European (n = 35)</td>
<td></td>
</tr>
<tr>
<td>At risk at the beginning of the time point</td>
<td>33</td>
</tr>
<tr>
<td>Cumulative events</td>
<td>2</td>
</tr>
<tr>
<td>Cumulative rate, % (95% CI)</td>
<td>5.7 (0.0–13.4)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of samples unless otherwise indicated. CI, confidence interval.

variants, respectively. Post-LEEP CIN2-3 was not found in 6 African American women with African-2 (n = 4) or Asian-American variants (n = 2), 6 Asian/Pacific Islander or American Indian/Alaskan women with European (n = 4) or Asian variants (n = 2), and 2 women with European variants who did not provide race information. Because of the small number of infections with variants other than European lineage, the African-1, African-2, Asian-American, and Asian variants were grouped together and designated as non-European variants in following analyses.

The mean duration (SD) of post-LEEP follow-up was 19.9 (5.1) months for women with European variants and 18.6 (5.8) months for those with non-European variants (P = .17). As shown in figure 1 and table 1, the cumulative proportion of post-LEEP CIN2-3 was somewhat higher in women with non-European variants than in those with European variants (P = .07, log-rank test). The mean age (SD) was 25.5 (5.3) years for women with European variants and 23.6 (4.6) years for those with non-European variants (P = .06). Compared with a proportion of 12.8% in 164 white women, non-European variants accounted for 37.1% of the infections in 35 nonwhite women (P = .002; 2 without race information). There were no appreciable differences in distribution of HPV16 variants by lifetime number of sex partners, number of Pap tests in the past 5 years, use of hormonal contraceptives, referral with cytologic diagnoses, or management arms (data not shown). After adjusting for age at enrollment and self-reported race, women with non-European, compared with European, variants were 3 times (95% CI, 1.1–8.9) more likely to have a post-LEEP CIN2-3 (table 2).

After LEEP, HPV16 DNA was detected in 1 or more visits in 47 (23.5%) of the 200 women who had HPV testing results; of them, non-European variants accounted for 19.1% (9/47) of the post-LEEP infections, a proportion similar to that detected at baseline. Compared with a proportion of 16.2% in 37 white women, non-European variants accounted for 33.3% of the post-LEEP infections in 9 African American women (1 without race information). The cumulative incidence of post-LEEP CIN2-3 was 28.9% (11/38) and 44.4% (4/9), respectively, in women with European and non-European variants who were positive for HPV16 DNA after LEEP (P = .44) and 2.4% (3/127) and 7.7% (2/26), respectively, in those who were negative for HPV16 DNA after LEEP (P = .20). When censoring women at the first negative visit after the last HPV16-positive test, the increased risk of post-LEEP CIN2-3 remained associated with non-European, compared with European, variants (adjusted relative risk, 2.7 [95% CI, 0.9–8.6]).

Discussion. In this study, CIN2-3 after LEEP for CIN3 was found in 10% of women with a baseline HPV16 infection. Infection with non-European, compared with European, var-

Table 2. Risk of cervical intraepithelial neoplasia grade 2 or 3 (CIN2-3) after a loop electrosurgical excision procedure (LEEP) for histologically confirmed CIN3 associated with human papillomavirus type 16 variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Subjects, no. (%)</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>With post-LEEP CIN2-3</td>
</tr>
<tr>
<td>European</td>
<td>166</td>
<td>14 (8.4)</td>
</tr>
<tr>
<td>Non-European</td>
<td>35</td>
<td>6 (17.1)</td>
</tr>
</tbody>
</table>

NOTE. CI, confidence interval; RR, relative risk.

a Adjusted for age at study entry and self-reported race.
variants was associated with a 3-fold higher risk of post-LEEP CIN2-3. The risk association was not explained by age and race. It is also unlikely that the association resulted from differential disease ascertainment, because women in ALTS were rigorously followed and examined according to the study protocol. Although this is a retrospective cohort analysis, potential exposure ascertainment bias is minimal because characterization of HPV16 variants was done without knowledge of any clinical information. In the present study, we performed variant analysis only on enrollment samples, because, as shown elsewhere [11], the predominant variant is a consequence of an initial infection and persists stably over time. One concern, however, is the therapy-related elimination of the virus. The variants detected at baseline may no longer play a role, if they become undetectable after LEEP. Biases could have occurred if viral clearance had been differentially related to the variants. Arguing against this is the fact that the proportion of non-European variants was very similar between the pre- and post-LEEP infections. Also, when the visits after the first HPV16-negative test were excluded from the analysis, a higher risk of post-LEEP CIN2-3 remained associated with non-European compared with European variants.

The increased risk associated with HPV16 non-European, relative to European, variants has been reported previously from studies of incidence and prevalence of high-grade CIN [6–8]. The present study lends further support to previous findings by showing a higher rate of post-LEEP CIN2-3 in women with non-European variants than in those with European variants. The consistency of associating different clinical end points with the variants strongly supports the hypothesis that these variants differ in their oncogenic potentials.

The underlying mechanism for differences in risk of relapse by HPV16 variants is presently unclear, but it may be attributable to polymorphisms of viral genome. As shown in studies in vitro [12, 13], HPV16 variants differ in their abilities to induce p53 degradation, keratinocyte differentiation, and E2-related transcription of E6/E7 oncogenes. It is plausible that some nucleotide alterations may directly alter the variants’ oncogenic potentials and may be responsible for the observed risk differences. It is also possible that the difference in risk of relapse by HPV16 variants results from the effectiveness of the host’s cellular immune response to these variants. A report by Sarkar et al. [14] has shown that presence of cellular immune responses directed against synthetic HPV16 E6 and E7 peptides was related to recurrence after treatment for HPV-associated CIN. Infection with non-European variants may be related to a decreased cellular immunity, perhaps through a polymorphism-related evasion of host immune surveillance. This hypothesis is supported by studies [15] showing significant associations of various HLA alleles with certain HPV16 variants. It is likely that specific HLA haplotypes are associated with inadequate immune presentation of epitopes encoded by particular HPV variants, thereby affecting the ability of these variants to lead to disease progression. Last, in the present study, we examined sequence variation in the E6 region and in part of the long control region. It is not known which polymorphisms could be responsible for difference in risk of post-LEEP CIN2-3 because those may be linked changes throughout the genome.

Several limitations of the study should be addressed. First, because of a limited number of post-LEEP CIN2-3 events, we were unable to assess the risk by individual non-European lineages. Second, we were unable to distinguish, as is inherent to these types of studies, recurrence after treatment from residual or possibly incident disease. From the view of clinical management of women treated for CIN3, however, the implication of these results is the same. Last, although almost all study subjects underwent the final disease ascertainment, we might have still missed some lesions that transiently occurred within the 6-month intervals. Presently, however, there is no evidence to suggest that this would be differentially related to the variants.

In summary, the present data indicate that among women with HPV16 infection who were treated for CIN3, the increased risk of post-LEEP CIN2-3 was associated with non-European, compared with European, variants, a finding consistent with the variant-related risk of prevalent/incident high-grade CIN.

Acknowledgments

We thank the Atypical Squamous Cells of Undetermined Significance and Low-Grade Squamous Intraepithelial Lesion Triage Study Group for providing the biological specimens and human papillomavirus typing results and Ms. Marsha Weese for help in manuscript preparation.

References