Short Communication

Semi-quantitative Human Papillomavirus Type 16 Viral Load and the Prospective Risk of Cervical Precancer and Cancer

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Abstract

We examined whether higher human papillomavirus type 16 (HPV16) viral load predicted risk of cervical intraepithelial neoplasia 3 (CIN3) or cancer (together termed ≥CIN3) within a cohort of 20,810 women followed for 10 years with cytologic screening. Semiquantitative viral load for HPV16 was measured on baseline cervicovaginal specimens using a type-specific hybridization probe test with signal amplification. An increased risk of ≥CIN3 associated with higher HPV16 viral load was found only among cytologically negative women in early follow-up, suggesting that these cases were related to the detection of prevalent lesions missed at baseline. Women with higher HPV16 viral load were more likely to undergo ablative treatment during follow-up than those with lower viral load ($P_{\text{trend}} = 0.008$), possibly diminishing any additional risk for ≥CIN3 attributable to higher HPV16 viral loads. (Cancer Epidemiol Biomarkers Prev 2005;14(5):1311–4)

Introduction

Cervical infection by a group of ~15 oncogenic human papillomavirus (HPV) types causes virtually all cervical cancers worldwide (1-3). HPV is a common sexually transmitted agent (4, 5) that typically clears within 1 to 2 years. Many HPV infections do not cause detectable cytologic abnormalities (6, 7); however, some infections persist and progress over several years to high-grade cervical intraepithelial neoplasia (CIN3). Left untreated, a substantial proportion of these precancers eventually develop into invasive cervical cancer (8).

High HPV viral load has been shown to be associated with the microscopic diagnosis of a concurrent lesion (9-14). In addition, high HPV viral load, especially HPV16 viral load, may be an indicator that an HPV infection is likely to progress to precancer and cancer. In a case-control study, Josefsson et al. (15) reported that women with increasingly higher HPV16 viral load, measured using quantitative PCR on cells scraped from archival Pap smears, were at an increasingly greater risk for development of carcinoma in situ (roughly equivalent to CIN3) up to 13 years before diagnosis. In a related study, repeat detection of high HPV16 viral load by quantitative PCR has been associated with increased risk of carcinoma in situ compared with women with low HPV16 viral load (16). Another study using quantitative PCR found that high HPV16 viral load predicted progression to CIN2/CIN3 and conferred a decreased likelihood of viral clearance compared with low viral load (17). Finally, a study using a PCR-based method for a semiquantitative assessment of viral load in cervical scrapes found that among women with no evidence of cytologic squamous intraepithelial lesions (SIL), high viral load was associated with a 3- to 5-fold increase risk for the development of cytologic high-grade SIL during a follow-up period of up to 8 years compared with low viral load (18).

In contrast, we reported no link between semiquantitative baseline measurement of oncogenic HPV viral load for a pool of oncogenic types and subsequent histologic CIN3 or cancer (≥CIN3) in a 10-year cohort study of women attending a health maintenance organization in Portland, Oregon (19). In that study, we used a hybridization and signal amplification–based probe cocktail to test for 13 oncogenic HPV types (Hybrid Capture 2, Digene Corporation, Gaithersburg, MD); values progressively greater than the positive cut point were considered as a surrogate for oncogenic HPV viral load in toto, which has been shown to correlate with quantitative PCR–measured viral load (20, 21). In an attempt to synthesize the apparently discrepant results from our group and others, we recently retested the Hybrid Capture 2 test positives from our earlier study specifically for HPV16 using a semiquantitative test method (22). Specifically, we wished to determine whether increased viral load of HPV16 specifically might convey an appreciably greater risk of ≥CIN3.

Materials and Methods

Study Subjects. Between April 1, 1989, and November 2, 1990, 23,702 women were enrolled in a natural history study of HPV infection at the Kaiser Permanente, Northwest Region prepaid health plan in Portland, Oregon, as previously described (23, 24). Both NIH and Kaiser Permanente Institutional Review Boards approved the study. Subjects were ages ≥16 years with a mean age of 35.9 years (range 16-94 years). A main analysis cohort of 20,810 women was established and followed passively as part of standard cytologic screening for cervical neoplasia (24). This analysis cohort excluded women...
who (a) refused to participate \((n = 1,107)\), (b) had undergone hysterec-
yomy \((n = 1,406)\), (c) had an inadequate specimen for HPV

testing \((n = 195)\), (d) had unsatisfactory or missing

enrollment cervical smears \((n = 85)\), or (e) underwent colpo-

scopy rather than Pap smear screening at enrollment \((n = 99)\).

**Enrollment Examination.** According to Kaiser Institutional

Review Board guidelines, subjects underwent a routine pelvic

examination \((23)\). Exfoliated cervical cells were typically

collected with an Ayre spatula and a cytobrush for Pap test

screening. Next, each subject underwent a cervicovaginal

lavage to collect specimens for HPV testing. Lavages were
done by rinsing the cervical os with 10 mL sterile physiologic saline using a syringe fitted with an intracatheter extender and
then collecting the pooled fluid in the vaginal fornix using the same device \((23)\). Lavages were refrigerated within 1 hour of collection and shipped to a central laboratory for processing \((22, 23)\).

**HPV16 Testing.** We tested frozen aliquots (\(-70^\circ\)C) of lavages from a stratified sample \(^3\) of 4,321 women for HPV16 DNA by a type-specific adaptation of the hybrid capture method using HPV16-specific capture oligonucleotides and a HPV16 RNA probe as previously reported \((22)\). Signal strengths in relative light units (RLU) were compared with 1 pg/mL HPV16 DNA–positive controls (RLU/PC). Based on a receiver operating characteristic analysis, a 0.6 RLU/PC (pg/mL) positive cut point was selected \((22)\). The progression of numerical RLU/PC values above the positive cut point was taken to represent a surrogate for HPV viral load as shown in previous studies \((20, 23)\).

**Follow-up.** Subjects were followed up to 122 months, undergoing a median of three repeat smears, with 83% of

women having at least one repeat smear. Women with negative enrollment cervical smears had a median follow-up of 6 years. Those with abnormal cytology were managed according to standard practice guidelines; HPV data were not unmasked or used for clinical management.

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\(^3\)All 171 cases of ≥CIN3 diagnosed over the 10-year study; 867 women who were not diagnosed with ≥CIN3 but who were previously reported to be HPV DNA positive by MY09/11 consensus primer PCR \((n = 855\) results, 98.6%) all 2,260 women who were either not tested or negative by PCR but positive by Hybrid Capture 2 \((n = 2,253\) results, 99.7%); 23 women with an enrollment Pap smear interpreted as LSIL or more severe and not captured by the above strata \((n = 23\) results, 100%); a 6% random sample of the remaining 17,489 women not included in the above strata was tested \((n = 1,1043, 100%)\).

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### Table 1. Relationships between HPV viral load and development of ≥CIN3 during a 10-year follow-up

<table>
<thead>
<tr>
<th>Time after baseline (mo)</th>
<th>Viral load (RLU/PC)</th>
<th>Women seen</th>
<th>Women with CIN3+</th>
<th>Absolute risk</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Absolute risk</td>
<td>(95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>All</td>
<td>Cancer or negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(ASCUS or LSIL)</td>
<td>(ASCUS or LSIL)</td>
</tr>
<tr>
<td>0–9</td>
<td>0.60–1.33</td>
<td>241</td>
<td>4</td>
<td>1.7</td>
<td>1.0 (reference group)</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>212</td>
<td>9</td>
<td>7.6</td>
<td>0.83 (0.33–2.1)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>161</td>
<td>10</td>
<td>5.6</td>
<td>3.4 (1.1–11)</td>
</tr>
<tr>
<td></td>
<td>≥21.12</td>
<td>135</td>
<td>12</td>
<td>5.6</td>
<td>3.4 (1.1–11)</td>
</tr>
<tr>
<td>9–69</td>
<td>0.60–1.33</td>
<td>198</td>
<td>7</td>
<td>3.5</td>
<td>1.0 (reference group)</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>120</td>
<td>8</td>
<td>6.7</td>
<td>1.9 (0.71–50)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>120</td>
<td>7</td>
<td>6.5</td>
<td>1.8 (0.7–53)</td>
</tr>
<tr>
<td>69–122</td>
<td>0.60–1.33</td>
<td>129</td>
<td>1</td>
<td>0.8</td>
<td>1.0 (reference group)</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>73</td>
<td>1</td>
<td>1.4</td>
<td>1.8 (0.11–28)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>72</td>
<td>1</td>
<td>1.4</td>
<td>1.8 (0.11–28)</td>
</tr>
<tr>
<td>≥21.12</td>
<td>0.60–1.33</td>
<td>129</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>93</td>
<td>6</td>
<td>5.7</td>
<td>1.4 (0.52–3.7)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>123</td>
<td>7</td>
<td>7.4</td>
<td>1.8 (0.65–51)</td>
</tr>
<tr>
<td>9–122</td>
<td>0.60–1.33</td>
<td>197</td>
<td>8</td>
<td>4.1</td>
<td>1.0 (reference group)</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>124</td>
<td>9</td>
<td>7.3</td>
<td>1.8 (0.71–4.5)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>123</td>
<td>7</td>
<td>5.7</td>
<td>1.4 (0.52–3.7)</td>
</tr>
<tr>
<td>≥21.12</td>
<td>0.60–1.33</td>
<td>95</td>
<td>7</td>
<td>7.4</td>
<td>1.8 (0.65–51)</td>
</tr>
<tr>
<td></td>
<td>1.34–4.32</td>
<td>163</td>
<td>18</td>
<td>11.0</td>
<td>2.2 (1.1–4.5)</td>
</tr>
<tr>
<td></td>
<td>4.33–21.11</td>
<td>165</td>
<td>17</td>
<td>10.3</td>
<td>2.1 (1.0–4.3)</td>
</tr>
<tr>
<td>≥21.12</td>
<td>0.60–1.33</td>
<td>124</td>
<td>18</td>
<td>13.4</td>
<td>2.7 (1.4–5.5)</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not applicable because no cases observed in the reference group.

As detailed in the results, this report focuses on a group of 516 (2.5%) of the 20,810 baseline cohort members who had satisfactory Pap smears and tested positive for HPV16 DNA using a single type-specific probe for HPV16 (see below). Of these 516 HPV16-positive women, 64 (12.4%) were diagnosed with ≥CIN3 histology during follow-up using a rigorous pathologic definition involving independent review \((25)\).

**Analysis.** Follow-up time was crudely divided into an initial period of 9 months (Pap smears that were rapidly repeated, presumably prompted by a previous cytologic abnormality or suspicious symptoms) followed by yearly intervals for a total time of 122 months. These 12-month intervals roughly paralleled the intervals at which women returned for annual smears. As a consequence of a small number of newly diagnosed cases during follow-up, we then combined time intervals into periods of 0 to 9, 9 to 69, and 69 to 122 months, which would categorize outcomes as prevalent, early-incident, and late-incident disease.

We stratified semiquantitative viral loads among HPV16-positive women into tertiles \((0.60–1.33, 1.34–4.33, 4.33–21.11, \text{and} ≥21.12 \text{RLU/PC})\) to examine the association of viral load and risk of ≥CIN3. We also used strata defined by log units of viral load \((0.60–5.99, 6.00–59.99, 60.00–599.99, \text{and} ≥600 \text{RLU/PC})\). Extrapolating from testing results for the sampling fractions to the entire population \((an\ estimated 699 HPV16 positives; ref. 11)\), we calculated absolute risk and relative risk \((RR)\) for ≥CIN3 with 95% confidence intervals \((95\% CI)\) for three time periods \((0–9, 9–69, \text{and} 69–122 \text{months})\, for 9 to 122 months, and for the entire 122-month follow-up. Confidence intervals were based on the actual number of women tested. Results were also stratified on baseline cytologic interpretations \([cytologic negative (including reactive changes) or atypical squamous cells of unknown significance (ASCUS)/low-grade SIL (LSIL)]\).

To evaluate whether there was any bias in the frequency of screening or any censoring related to viral load, we examined the relationships of HPV16 viral load to number of screening visits, to having a mild cytologic abnormality, and to undergoing ablative surgery during follow-up for the 452 HPV16-positive women who did not become cases during follow-up \((<CIN3)\). Pearson \(\chi^2\) test and the Mantel extension test for trend were used to evaluate the relationships of viral load and these follow-up screening and treatment characteristics.

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\(^3\)CIN3 histology during follow-up using a rigorous pathologic definition involving independent review \((25)\).
Results

For the entire 122-month follow-up, women with the highest three quartiles of HPV16 viral load had similarly elevated RRs for ≥CIN3 of 2.2 (95% CI, 1.1-4.5; 2nd quartile), 2.1 (95% CI, 1.0-4.3; 3rd quartile), and 2.7 (95% CI, 1.4-5.5; 4th quartile) compared with the lowest quartile of viral load (Table 1). The greatest period of elevation of risk for ≥CIN3 was the first 9 months with RRs for ≥CIN3 of 3.4 (95% CI, 1.1-11; 2nd quartile), 3.4 (95% CI, 1.1-11; 3rd quartile), and 5.4 (95% CI, 1.8-16; 4th quartile). Stratified on cytologic interpretation at baseline, cytologic-negative women with higher HPV16 viral loads were at elevated risk of ≥CIN3 during intervals of 0 to <9 and 9 to <69 months, but there was again no difference in risk between the upper three quartiles of viral load. No elevated risk for higher viral loads was observed for women with ASCUS/LSIL cytology at quartiles of viral load. No elevated risk for higher viral loads was again no difference in risk between the upper three quartiles of viral load (data not shown).

Table 2. Relationships of HPV16 viral load to the number of screening visits (A), to any mild cytologic abnormality (B), and to undergoing an ablative treatment (C) during follow-up of noncases

<table>
<thead>
<tr>
<th>Viral load (RLU/PC)</th>
<th>1-2 Visits</th>
<th>3-5 Visits</th>
<th>5-7 Visits</th>
<th>8 Visits</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60-1.33</td>
<td>39</td>
<td>23</td>
<td>13</td>
<td>26</td>
<td>117</td>
</tr>
<tr>
<td>1.34-4.32</td>
<td>42</td>
<td>23</td>
<td>15</td>
<td>12</td>
<td>110</td>
</tr>
<tr>
<td>2.03-6.48</td>
<td>50</td>
<td>24</td>
<td>17</td>
<td>23</td>
<td>114</td>
</tr>
<tr>
<td>6.12-21.11</td>
<td>35</td>
<td>27</td>
<td>13</td>
<td>20</td>
<td>111</td>
</tr>
<tr>
<td>21.12</td>
<td>31.5%</td>
<td>22.5%</td>
<td>27.9%</td>
<td>18.0%</td>
<td>452</td>
</tr>
<tr>
<td>Total</td>
<td>166</td>
<td>102</td>
<td>96</td>
<td>88</td>
<td>452</td>
</tr>
</tbody>
</table>

P = 0.5, Pearson χ²; P_trend = 0.9

A. Number of screening visits during follow-up

B. Any mild cytologic abnormality during follow-up

C. Underwent surgery during follow-up

<table>
<thead>
<tr>
<th>Viral load (RLU/PC)</th>
<th>No</th>
<th>Yes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60-1.33</td>
<td>95</td>
<td>22</td>
<td>117</td>
</tr>
<tr>
<td>1.34-4.32</td>
<td>89</td>
<td>61</td>
<td>142</td>
</tr>
<tr>
<td>2.03-6.48</td>
<td>89</td>
<td>61</td>
<td>142</td>
</tr>
<tr>
<td>21.12</td>
<td>68</td>
<td>31</td>
<td>111</td>
</tr>
<tr>
<td>Total</td>
<td>303</td>
<td>149</td>
<td>452</td>
</tr>
</tbody>
</table>

P = 0.001, Pearson χ²; P_trend = 0.001

P = 0.02, Pearson χ²; P_trend = 0.008

Among the noncases, we examined the effects of HPV16 viral load on follow-up (Table 2). We found no relationship between viral load and the number of follow-up screening visits (P_trend = 0.9), suggesting that there was no significant difference in women’s screening behavior according to their viral load. However, women with higher HPV16 viral loads were both more likely to have a cytologic abnormality (P_trend = 0.001) and have ablative surgery (P_trend = 0.008) during follow-up. Inclusion of cases did not alter these findings (data not shown).

Discussion

We found that HPV16-infected women with higher RLU/PC signal strength over a broad range were at a slightly higher risk of ≥CIN3 than women with the lowest signal strength. This finding differs from our previous finding (19) in which we did not observe an elevated risk for women with higher viral load for a pooled probe test (Hybrid Capture 2) for 13 oncogenic types that included HPV16. Although this finding is consistent with the idea that higher viral load for HPV16 confers elevated risk for ≥CIN3, we observed the greatest risk elevation within the first 9 months of follow-up, suggesting that most of these cases were missed prevalent at baseline but detected with a delay at follow-up. This is consistent with earlier findings in which high viral load, especially for HPV16, was elevated among those of concurrent abnormal pathology (9-14, 26); the relationship of degree of severity and viral load is less certain (11) perhaps because of the influence of low-grade lesions on the viral load measurement for high-grade lesions (27).

The elevated risk was primarily observed in women who were cytologically negative or had benign reactive changes. In our large cohort, there was no added risk attributed to higher HPV16 viral load for women with equivocal (ASCUS) or mildly abnormal (LSIL) cytology. Given the well-recognized relationship of abnormal cytology and histologically diagnosed CIN with higher HPV viral loads (11), it seems likely that women with abnormal cytology overall had higher viral loads as we found to be true in this study (Kruskal-Wallis, P = 0.0001). Thus, cytologic abnormality and higher viral load are “correlates” of each other, and knowing either one reduces the predictive value (for ≥CIN3) of knowing the other.

We note that observed increases in risk were quite modest, with no stepwise trend, compared with those previously reported (15, 17, 18). We offer several explanations. First, by using a signal amplification–based HPV test as a surrogate for viral load, we did not detect the extreme low end of viral load that can be detected by quantitative PCR. The cut point for these single probes and pooled probe assays were selected based on a receiver operating characteristic analysis versus high-grade cervical neoplasia and cancer and, thus, represent the optimal clinical cut point for detection of prevalent high-grade cervical neoplasia rather than mere detection of HPV DNA. Therefore, infections detected by quantitative PCR with a very high analytic sensitivity that would have been called negative by hybridization methods likely represent extremely low viral load, possibly clinically irrelevant infections. Thus, using extremely low viral load infections (an equivalent of using a lower cut point) as the reference group would have inflated estimates of risk for all HPV viral load levels detected.

We also found significant correlation between increased viral load and ablative treatments. Women in the Portland Kaiser plan commonly underwent ablative treatments for cytologic abnormalities in accordance with an aggressive clinical management. Among controls, women who had higher viral loads, whether for HPV16 or another type (data not shown), were more likely to have abnormal cytology follow-up that resulted in ablative treatment. If ablative treatment tends to censor women at highest risk, as suggested by the data and

NOTE: The bottom set of numbers for each viral load range are row percentages.
clinical judgment, estimates of absolute risk and RR of progression to ≥CIN3 reflect local screening and clinical practice (19); particularly, we expect the effects of viral load on the risk of ≥CIN3 in an aggressive and effective cytology screening program, like Portland Kaiser’s, to be muted compared with the effect in a setting with a less effective program. The relatively poor sensitivity of cytology, coupled with long screening intervals in some countries with less aggressive screening and clinical management than the United States, may produce a stronger association of HPV viral load with missed occult CIN3 that is later diagnosed and interpreted as incident disease. To further examine these issues, we are now evaluating the impact of HPV16 and HPV18 viral load in our population study in Guanacaste, Costa Rica, where management of cytologic abnormalities is less conservative.  

Finally, some prior studies may have considered some LSIL and atypical squamous cell cytology as negative. It is now apparent that there are significant regional/national differences in threshold of cytologic abnormality (28). HPV-positive cytology is more likely to be reclassified as SIL upon review than HPV-negative cytology, with an attendant higher risk of subsequent CIN3 and cancer.  

Despite the large size of our 20,000-woman HPV-screened cohort, one of the largest research cohorts reported, only 516 HPV16 infections were identified at enrollment in the sampling fraction and a small number of cases. Consequently, we had unstable estimates of risk with wide confidence intervals.  

Despite this limitation, we have shown that the association between high HPV16 viral load and risk of ≥CIN3 was too weak to form the basis for clinical management in this population. Differences in the definition of HPV16 positivity (and, therefore, the definition of the reference viral load category) and in the threshold (sensitivity) for cytologic abnormalities make the meaning of viral load too uninformative, given the tendency to treat women before the advent of CIN3 in a well-screened population.

References

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