Human Papillomavirus 16 Load and E2/E6 Ratio in HPV16-Positive Women: Biomarkers for Cervical Intraepithelial Neoplasia ≥2 in a Liquid-Based Cytology Setting?

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Abstract

This retrospective case-control study assessed human papillomavirus 16 (HPV16) viral load and E2/E6 ratio as risk markers for cervical intraepithelial neoplasia (CIN) ≥2 lesions in HPV16-positive women in a routine liquid-based cytology setting. Triplex quantitative PCR for HPV16 E6, E2, and β-globin was done to determine the HPV16 load and the E2/E6 ratio, as a surrogate marker for integration, for women with a negative histologic endpoint (200 controls: 83 normal histology and 117 CIN1) and women with a ≥CIN2 endpoint (180 cases: 41 CIN2, 122 CIN3, and 17 invasive carcinoma). Our analysis showed a significantly higher HPV16 load in the case group, which was completely attributable to the high viral load of samples with invasive carcinoma as histologic endpoint. There was no significant difference in viral load between the other histologic groups. The E2/E6 ratio proved to be lower for the cases. However, the E2/E6 ratio indicated the presence of HPV integration in a considerable amount of control samples (44.3%), which suggests that HPV integration occurs early in the development of cancer and undermines the clinical value of viral integration. Overall, the intrinsic heterogeneous nature of the cervical cytology samples caused a substantial overlap of the HPV16 load and the E2/E6 ratio between controls and cases, which precludes the determination of cutoff values for risk prediction and hampers the clinical applicability in a cervical screening setting. (Cancer Epidemiol Biomarkers Prev 2009;18(11):2992-9)

Introduction

The causal relationship between persistent infection with high-risk human papillomavirus (HR-HPV) and cervical cancer has resulted in the development of HPV detection systems (1, 2). Presence of HR-HPV DNA identifies women who are at particular risk of progression to cervical cancer. The use of HPV testing has been suggested for primary screening (3, 4), the triage of equivocal Papanicolaou smears (5, 6), and the follow-up of patients after treatment for cervical intraepithelial neoplasia (CIN; refs. 7-9). However, because of the high prevalence of transient infections, viral detection has a poor positive predictive value, which makes it an incomplete means of identifying women at risk (3).

HR-HPV load has been suggested as a type-dependent risk marker for high-grade CIN or carcinoma (10-18). Nevertheless, the initial optimism regarding the clinical utility of viral load analysis was tempered by inconsistencies in the association between HPV load and the duration of the infection, HPV clearance, and subsequent risk of acquisition or progression of disease (19).

Measurement of HPV integration has been proposed to further improve the clinical assessment of CIN. HPV16 integration frequency has been reported to increase in parallel with the severity of cervical lesions (20-27). However, several recent studies challenged this view by showing integrated HPV in a significant fraction of low-grade lesions, suggesting that integration is an early event in the cervical carcinogenesis (28-35). Integration of HR-HPV in the human genome interferes with viral control mechanisms through disruption of the viral E2 regulatory gene and its negative feedback control over the oncogene expression (36, 37). Various methodologies have been described to analyze the HPV type, load, and physical state. Our real-time quantitative PCR (qPCR) strategy is based on a test described by Peitsaro et al., which has been extensively used for simultaneous detection of the viral load and physical state of the predominant HR-HPV type 16 (22, 28-34, 38, 39). Recent publications confirmed this test to be sensitive
and specific for the identification of HPV integration (33, 40). For this study, qPCR analysis has been applied on a large number of samples from a routine liquid-based cytology (LBC) setting to assess HPV16 viral load and integration state as risk markers for ≥CIN2 lesions in HPV16-positive women. Based on a method applicable in high-throughput clinical settings, our study unravels the potential role of viral load and integration state in HPV-based screening and triage strategies.

Materials and Methods

Clinical Samples. The Laboratory for Clinical Pathology (Labo Lokeren campus RIATOL) yearly receives 100,000 cervical samples for cytologic evaluation. Cervical cells, collected into an ethanol-based preservative (SurePath; Tripath Imaging) using the Cervex-Brush (Rovers), are processed into thin-layer LBC preparations by the fully robotic Autocyte PREP system (Tripath Imaging). The cytology results are classified according to the Bethesda system 2001. Ancillary to cytologic screening, samples with abnormal cytology are routinely subjected to HPV detection and typing, whereas samples with normal cytology are only tested in function of quality control or at specific request of the clinician (41). Cytologic outcome, HPV state, and available additional information, such as treatment or histologic diagnosis, of screened women have been registered in an internal database since 1998.

For this study, the database was searched for women with a known histologic outcome and a HPV16 infection in their clinical history. The women were classified in two groups, controls and cases, based on their histopathologic diagnosis. For each woman, all archival DNA samples or LBC leftovers, dated before the histologic endpoint, were retrieved.

The study was done in accordance with the guidelines of the local ethical committee. Study-specific patient identification codes were assigned and transmitted in accordance with patient confidentiality standards.

DNA Extraction from Cervical Cells. DNA was obtained by automatic nucleic acid preparation (MultiPROBE II; Perkin-Elmer). The absorbance of each LBC leftover was measured at 405 nm to assess the cellularity (VICTOR3; Perkin-Elmer). This measurement determined the presence of HPV in clinical samples. All standard curves were produced in quadruplicate and crossing points were plotted versus the log of copy number or DNA concentration.

Quantification of HPV16 Load and Integration State. qPCR analysis was done according to the original report by Peitsaro et al., with some modifications (30). The strategy involved the measurement of the total viral load by quantification of the HPV16 E6 gene. To determine the physical state of the virus, the E2 gene was quantified and the E2/E6 ratio was calculated. This approach is based on the fact that episomes present an identical amount of E2 and E6, whereas integration induces loss of E2.

Because addition of a known amount of DNA to each PCR is not practical for a large series of clinical samples, our approach included normalization for sample cellularity by means of β-globin quantification. qPCRs for E6, E2, and β-globin were done in a triplex format to minimize inaccuracies in the quantification of the DNA concentration (Table 1).

Standard curves for HPV16 E6 and E2 were constructed based on pH16 plasmids containing the entire genome of HPV16 (pHPV16; Clonit). A 10-fold dilution series was made ranging between 5 × 10−3 and 0.5 copies/μL. Each dilution was completed with 32.14 ng/μL female human DNA (Promega) to mimic the complex nucleic acid environment present when amplifying genomic DNA.

A β-globin standard curve was obtained by amplification of a 2-fold dilution series of female human DNA between 112.5 and 1.75 ng/μL (Promega). To each dilution, 71,428 copies of HPV16 were added to take into account the presence of HPV in clinical samples. All standard curves were produced in quadruplicate and crossing points were plotted versus the log of copy number or DNA concentration.

qPCRs were based on Taqmam technology and done in a 5 μL volume containing LightCycler 480 Probes Master (Roche Applied Science), 900 nmol/L of each primer, 300 nmol/L probe, and 1 μL template DNA. The thermocycling profile consisted of 45 two-step cycles: 15 s at 95°C and 50 s at 60°C.

The amount of genomic DNA (ng) present in each sample was divided by the weight of 1 genome equivalent (6.6 pg/cell) to obtain the number of cells in the sample. Viral load was expressed as the number of E6 per cell and the ratio E2/E6 was calculated to assess the viral integration state. An experimental threshold for 100% episomal DNA was established. Plasmids containing the entire HPV16 physical state of the virus, the E2 gene was quantified and the E2/E6 ratio was calculated. This approach is based on the fact that episomes present an identical amount of E2 and E6, whereas integration induces loss of E2.

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genomic load (pH16) or only HPV16 E6 (pE6; Addgene plasmid 8642) provided standards for episomal DNA (100% pH16) and mixed DNA (pHPV16 spiked with 10%, 20%, 30%, or 50% pE6; refs. 32, 42). The E2/E6 ratio was determined in 12-fold for the standards and for two clinical samples with a mean E2/E6 ratio equal to 1 to calculate 99% confidence intervals and coefficients of variation (CV).

To determine the intrarun variability, four repeated measurements were done on six clinical samples with crossing point values across the entire PCR dynamic range. To determine the intrarun variability, 12 clinical samples with crossing point values across the entire PCR dynamic range were analyzed in three independent qPCR runs.

Several precautions were taken to prevent false-positive results. Different steps such as DNA extraction, sample preparation, amplification, and post-PCR were done in strictly separated rooms. The negative PCR control included all PCR components without template DNA.

**Statistical Analysis.** Statistical analysis was done using the R package (R version 2.6.2, The R Foundation for Statistical Computing). For continuous variables, nonparametric tests (Mann-Whitney and Kruskal-Wallis) were used to compare the distributions of two or more populations. To show these distributions graphically and to interpret inferential conclusions, logarithmic transformations were applied. Such monotone transformations symmetrize the distributions, leading to improved visualizations, but they do not affect the results of the nonparametric tests. χ² tests were done to assess the independence of two categorical variables. When the tables were too sparse, the Fisher’s exact test was used. In all tests, P values < 0.05 were considered statistically significant.

Receiver operating characteristic curve analysis was done for total viral load, episomal viral load, and E2/E6 ratio.

**Results**

**Optimization of the qPCR Method.** Optimization of the qPCR method was based on numerous experiments to evaluate primers, probes, fluorochromes, cycling parameters, and the effect of background DNA. Comparison of uniplex and triplex reactions showed no evidence to suggest competition between the PCR targets in the multiplex format. The triplex qPCR proved to be highly sensitive, detecting 50 copies of HPV E6 and E2.

Final external calibration curves for E6, E2, and β-globin were constructed by averaging four replicate standard curves to minimize the effect of interrun variation. Tight correlations were observed between the values for each sample in the quadruplicate runs. For each qPCR, a highly significant linear regression was seen between the log of HPV copy number or log of DNA concentration and crossing points. The slopes of the calibration curves indicated that the amplification efficiencies of E6 and E2 PCR were equivalent.

The type of archival material, LBC leftovers or DNA, did not influence the amplification ability nor did the age of the samples. Assessment of the intrarun and inter-run variability based on clinical samples proved that the repeatability (CV HPV16 E6 qPCR: 0.39%, CV HPV16 E2 qPCR: 0.48%, and CV β-globin qPCR: 0.81%) and reproducibility (CV HPV16 E6 qPCR: 0.38%, CV HPV16 E2 qPCR: 0.64%, and CV β-globin qPCR: 0.93%) of the qPCR method are excellent.

**Definition of the Threshold for 100% Episomal Infections.** The E2/E6 ratio of the episomal DNA solution (100% pH16) showed a 99% confidence interval of 0.96 to 1.03 (CV, 3.68%) and was significantly different from that of the mixed solution containing 10% pE6 (99% confidence interval, 0.78-0.88; CV, 4.58%). The DNA solutions containing 20%, 30%, or 50% pE6 showed 99% confidence interval of 0.71 to 0.81 (CV, 5.24%), 0.58 to 0.71 (CV, 10.39%), and 0.48 to 0.51 (CV, 2.19%) respectively. As expected, the clinical samples showed a wider 99% confidence interval (0.94-1.05 and 0.92-1.04) and a higher CV (6.10% and 6.55%). Based on the lowest 99% confidence limit of 0.92, all samples with a ratio ≥0.92 were considered as 100% episomal infections.

**Study Population.** Women with a defined histologic endpoint and a HPV16-positive cytology sample preceding the biopsy were included and assigned to the control and the case group (Table 2). For each woman, all DNA samples or LBC leftovers were retrieved in a retrospective manner.

The total population of 380 women yielded 639 samples with a mean number of 2.16 ± 0.043 samples per patient. The median age of the controls (31.0 years) was significantly lower than that of the cases (53.0 years).

qPCR analysis resulted in 46 (7.2%) samples with a negative β-globin PCR result or with an extremely low DNA concentration (number of cells <1) because of the small residual sample volume. Of the remaining 593 (92.8%) samples, 71 (11.1%) were both negative for HPV16 E6 and E2 PCR and 12 (1.9%) were only positive for E2 PCR. A remaining 510 (79.8%) samples of 342 patients showed reliable results for all three qPCRs and were included in the analysis. The histopathologic distribution of the final sample population is shown in Table 2. The mean time interval between the histologic endpoints and the corresponding cytology samples was 210 ± 274 days (range, 0-1,396 days). This time interval was significantly shorter for the cases (mean, 175 ± 15 days; median, 53 days) than for the controls (mean, 250 ± 19 days; median, 113 days).

**HPV16 State.** The distributions of DNA concentration and sample cellularity were not significantly different between controls (median, 9.46 ng/μL; 1433.34 cells/μL) and cases (median, 10.10 ng/μL; 1530.30 cells/μL).

**Table 2. Histologic outcome of all women in the control and case groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Histologic outcome, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>Normal: 83 (21.8)</td>
</tr>
<tr>
<td></td>
<td>CIN1: 117 (30.8)</td>
</tr>
<tr>
<td></td>
<td>All controls: 200 (52.6)</td>
</tr>
<tr>
<td>Case group</td>
<td>CIN2: 41 (10.8)</td>
</tr>
<tr>
<td></td>
<td>CIN3: 122 (32.1)</td>
</tr>
<tr>
<td></td>
<td>Invasive carcinoma: 17 (4.5)</td>
</tr>
<tr>
<td></td>
<td>All cases: 180 (47.4)</td>
</tr>
<tr>
<td></td>
<td>All histology: 380 (100)</td>
</tr>
</tbody>
</table>
Figure 1. Box-plots (left) and density plots (right) for log(E6/cell+1; top), log(E2/cell+1; middle), and the ratio E2/E6 (bottom). Histologic groups: normal histology (0), CIN1 (1), CIN2 (2), CIN3 (3), and invasive carcinoma (4). Density plots: overall, controls with normal histology and CIN1; cases with CIN2, CIN 3, and invasive carcinoma.
The distribution of the total viral load of the controls (median, 43.21 E6/cell) was significantly different from that of the cases (median, 79.12 E6/cell). A closer look revealed that this was due to the different distribution of the total viral load in the invasive carcinoma histologic group. Figure 1 (top) shows box-plots (left) and density plots (right) of the log-transformed total viral load log(E6/cell+1) for all histologic groups (median, 55.28 for normal samples, 40.32 for CIN1, 48.47 for CIN2, 78.22 for CIN3, and 576.55 for invasive carcinoma). Whereas the viral load on the original scale was highly skewed for all groups, logarithmic transformation made the distributions much more symmetric, improving graphical comparison. The distribution of log (E6/cell+1) in the invasive carcinoma group appeared to be remarkably different: without the left peak, which is based on only three observations, the distribution with the right peak is clearly more concentrated, indicating a smaller variance, and shifted to the right, when compared with the distribution of the other histologic groups. A highly significant difference in E6/cell was found between the invasive carcinoma group and all others together (P = 0.0024). When excluding the invasive carcinoma group, the significant difference in the distribution of E6/cell across the histologic groups disappeared (P = 0.1867).

The distribution of episomal viral load was not significantly different between controls (median, 39.33 E2/cell) and cases (median, 75.55 E2/cell). However, the distribution of the episomal viral load in the invasive carcinoma group (median, 444.50 E2/cell) significantly differed from that in the other four groups together (median, 50.97 E2/cell; P = 0.0055). Figure 1 [middle; episomal viral load on the logarithmic scale, log(E2/cell+1)] depicts very similar qualitative characteristics for the distribution of log(E2/ cell+1) across the different histologic groups as for log (E6/cell+1).

The distribution of the E2/E6 ratio proved to be significantly different between controls and cases (P = 0.0002). Figure 1 (bottom), which shows the distributions of the E2/E6 ratio on the original scale, illustrates that these are not only shifted, which is characterized by the median of 0.94 for controls and the median of 0.86 for the cases, but also strongly differ in variance. When taking all histologic groups into account, similar conclusions can be drawn (median, 0.94 for normal samples, 0.95 for CIN1, 0.87 for CIN2, 0.85 for CIN3, and 0.865 for invasive carcinoma; P = 0.0067). Figure 1 (bottom) shows that the distribution of the invasive carcinoma group is now less distinctive.

Receiver operating characteristic curve analysis confirmed the poor diagnostic accuracy of the viral parameters. Neither the total viral load, the episomal viral load, nor the E2/E6 ratio enabled discrimination between cases and controls (Fig. 2).

Based on the E2/E6 ratio, the HPV16 physical state was determined in terms of episomal (E2/E6 ≥ 0.92), mixed (0 < E2/E6 < 0.92), and integrated (E2/E6 = 0). Physical state statistically differed between controls and cases (χ² test) and between the different histologic groups (Fisher’s exact test). Table 3 shows the observed frequencies.

Discussion

Cervical carcinoma is the second most common cancer in women worldwide. As a result of organized screening programs, it is only the 10th most common cause of cancer death in women in Europe (43). Because cervical cancer is the only cancer that is almost completely preventable through regular screening, further implementation of effectively organized screening programs and improvement of existing screening strategies and technologies would certainly decrease the burden of this disease (4).

Establishment of the critical role of HPV in the carcinogenesis of cervical carcinoma has led to the development of new applications to identify cancer precursors. The European Genital Tract Infection and Tumour Research Organisation and the American Society of Colposcopy and Cervical Pathology have suggested HR-HPV DNA detection as a routine screening tool in combination with cytology or even instead of it. This study investigates whether...
HPV16 viral load and/or integration state could be useful as additional predictive markers for risk assessment of CIN progression in HPV16-positive women in a LBC setting.

In previous studies, a high HPV16 load has been reported to be associated with persistent infection and the development of ≥CIN2 lesions (10-18). However, conflicting data have been published, suggesting that viral load may not be an adequate predictor of cervical carcinoma (21, 44-46).

Our analysis showed a cross-sectional association between HPV load and histopathologic outcome. The median viral load was significantly higher in the case group (79.12 E6/cell) than in the control group (43.21 E6/cell), but this difference could be completely assigned to the remarkable high load in the invasive carcinoma group (576.55 E6/cell). In contrast, the mean viral load was higher for the controls (797.93 ± 248.57 E6/cell) than for the cases (432.77 ± 66.78 E6/cell) due to several extreme values in the CIN1 group compared with the other histologic groups (Fig. 1, box-plots in top left). These high measurements reflect the occurrence of productive HPV infections, which do not pose a carcinogenic threat and appear cytologically as low-grade squamous intraepithelial lesions and histologically as CIN1. Such specimens contain an overload of maturing dysplastic squamous cells among which koilocytes, showing lowering amounts of viral DNA per cell (46). If low-grade lesions surround high-grade lesions in ≥CIN2 patients, cervical scraping results in heterogeneous cytology samples with a variable fraction of abnormal cells. The share of mild dysplastic cells with a high viral load could explain the wide viral load range in the CIN2 and CIN3 groups (Fig. 1, box-plots in top left). Therefore, the strong influence of cells from the surrounding tissue on the absolute viral load limits its use as risk marker to develop cervical cancer (21, 44, 46). The substantial overlap of the viral load between controls and cases precludes the determination of cutoff values for risk prediction, as such limiting the clinical applicability of viral load analysis. Receiver operating characteristic curve analysis established the poor diagnostic accuracy of viral load to distinguish cases and control (Fig. 2).

As mentioned above, the distribution of viral load in the invasive carcinoma group strongly differs from that in the other histologic groups and shows a distinct peak profile (Fig. 1, density plot in top right). The high viral load peak could be explained by the uniform sampling of HPV-positive tumor cells. The peak in the low viral load range represents only three specimens, which could have been inappropriately sampled or rich in inflammatory cells. Another possible explanation is a low intrinsic viral load of the carcinoma cells according to a molecular mechanism described by Pett et al., which involves the loss of E2-expressing episomes and subsequent selection of keratinocytes with integrated virus (47, 48). Nevertheless, the divergence within the group of invasive carcinomas further underlines the impracticability of viral load as a prognostic marker for cervical disease in a LBC setting.

In our study, great care was taken to technically validate the qPCR methodology based on HPV16 plasmids and clinical samples. Our methodology proved to be sensitive, reproducible, and suitable for high-throughput purposes. To efficiently test large series of exfoliated cervical cells, cellular DNA was determined based on β-globin quantification instead of adding known amounts of DNA to each PCR.

Previous reconstitution experiments have shown that the ability of the method to detect integration might deteriorate when integrated forms are showered by an excess of episomal forms (38). In this regard, Ruutu et al. state that an E2/E6 ratio ≥1 cannot entirely exclude the possibility of integration (40). However, the authors believe that this would be a rare phenomenon and emphasize the great sensitivity of the method.

Despite the elaborate technical evaluation, a recent study by De Marco et al. was the first to perform a systematic investigation of the reliability of the E2/E6 method as an integration assay by DIPS-PCR and sequencing. The sensitivity and specificity for the identification of HPV integration in cervical samples were confirmed, but further biological validation through comparison with alternative integration detection methods seems desirable.

Our study established the presence of HPV integration in a significant amount of patients with normal cervical epithelium and CIN1 lesions (107 of 238; Table 3), which supports the theory that HPV integration occurs early in the development of dysplasia and cancer (28-35). This concept has been proposed by numerous studies applying the E2/E6 methodology (28-34) but also by others based on Southern blot hybridization (49) or in situ hybridization signal patterns (50). A recent longitudinal cohort study proved that disruption of the E2 gene is a common and early event in the natural history of HPV infection (51). All these studies sustain the model that viral oncogene expression at the early stages of dysplasia induces genomic instability and therefore facilitates HPV integration. The relationship between HR-HPV integration and genomic instability was confirmed by the correlation between the frequency of DNA copy number imbalances and E2 gene loss (52) and the finding that aneuploidization precedes integration of HR-HPV genomes in the progression of cervical dysplasia (53).

HPV16 integration induces deregulation of the E6 and E7 oncogene expression through the disruption of the E2 gene and loss of negative feedback control. Subsequently, the increased E6 and E7 expression in cells with integrated virus results in a selective growth advantage (37). In that context, we believe that HPV integration without

### Table 3. Histologic distribution of all samples in the control and case groups and the physical state of HPV16 (integrated, mixed, or episomal) in different histologic groups

<table>
<thead>
<tr>
<th>Histologic groups</th>
<th>Total (n)</th>
<th>Integrated</th>
<th>Mixed</th>
<th>Episomal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>83 (16.3)</td>
<td>0</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>CIN1</td>
<td>155 (30.4)</td>
<td>1</td>
<td>68</td>
<td>86</td>
</tr>
<tr>
<td>All controls</td>
<td>238 (46.7)</td>
<td>1</td>
<td>106</td>
<td>131</td>
</tr>
<tr>
<td>Case group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN2</td>
<td>77 (15.1)</td>
<td>2</td>
<td>44</td>
<td>31</td>
</tr>
<tr>
<td>CIN3</td>
<td>179 (35.1)</td>
<td>7</td>
<td>112</td>
<td>60</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>16 (3.3)</td>
<td>1</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>All cases</td>
<td>272 (53.3)</td>
<td>10</td>
<td>167</td>
<td>95</td>
</tr>
<tr>
<td>All groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All histology</td>
<td>510 (100)</td>
<td>11 (2.2)</td>
<td>273 (53.5)</td>
<td>226 (44.3)</td>
</tr>
</tbody>
</table>


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disruption of the E2 gene is possible but that mainly the E2-disrupted integration contributes to cancer progression (28). The E2 amplicon detected by our qPCR method is representative for E2 disruption, but its size is constrained. Therefore, it is plausible that integration was detected less frequently than when full-length E2 would be amplified. Integration in other genomic regions than the E2 gene would also be missed and the way in which the threshold for episomal infections was defined might imply an underestimation of the fraction of mixed infections.

These qPCR deficiencies could be accountable for not detecting integrated virus in 95 of 272 cases, among which 4 invasive cancers. However, the establishment of invasive cancers without integrated virus could also imply that a substantial part of cervical tumors reaches this stage via integration-independent pathways. In these cases, some cellular transcripts, such as Yin Yang1 and C/EBPβ, may be involved in the control of viral oncoprotein expression independent of viral integration (54, 55). Additionally, methylation of the E2 binding site can reduce loss of E2 repressor activity without a change in E2 sequence (56).

To gain further insights in the correlation between HPV16 integration state and disease progression, this study will be extended with the follow-up of the control group. We anticipate that a substantial fraction of women with pure episomal HPV16 will show spontaneous viral clearance, whereas persistent infections in women with integrated HPV16 may lead to disease progression.

The statistical analyses as presented here have to be interpreted with some caution. Repeated measurements on the same individuals have been included, leading to correlated data. Moreover, the number of samples per woman varies as well as the time interval between the histologic endpoint and the different cytologic samples. These population characteristics compromise our analyses, as models for correlated data, such as (generalized) linear mixed models (subject-specific type of model) or Generalized Estimating Equations models (marginal type of models), have to be applied. This is beyond the scope of the present article but fits into the application of more complex multivariate statistical models, which will be a topic of further research. Nevertheless, we believe that the statistical analyses presented in this article are indicative and that the main conclusions will not be fundamentally affected.

In conclusion, we believe that the use of viral load and integration state as surrogate markers for ≥CIN2 in a LBC setting is limited. The intrinsic nature of cervical samples hampers the clinical applicability of analysis of viral load and integration state for the prediction of ≥CIN2 lesions. Our qPCR method proved to be sensitive, reliable, and practical for the screening of large sample volumes. It seems unlikely that methodologic improvements would benefit the prognostic value of viral load and integration state in a LBC setting. Although all biological evidence puts these parameters forward to improve HPV-based screening and triage strategies, this study undermines the benefit of quantification of the total HPV state in LBC samples. All HPV16-positive women should be screened regularly and monitored closely.

Conclusions concerning the clinical value of viral load and integration state may not be extrapolated between HR-HPV types, as differences have been reported for the association between viral load and disease for different HR-HPV types (57); the frequency of integration was shown to be type-dependent (20, 28, 58). In that regard, our findings are limited to HPV16, which can be considered as the predominant HR-HPV type, responsible for 33.1% of all ≥CIN2 (59). However, our technical concerns about the quantification of viral load and integration state in LBC samples seem extendable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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