Analysis of Human Papillomavirus Type 16 (HPV16) DNA Load and Physical State for Identification of HPV16-Infected Women with High-Grade Lesions or Cervical Carcinoma\V†

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Integration of human papillomavirus (HPV) DNA into the host cell genome is a frequent event in cervical carcinogenesis, even though this phenomenon does not seem to be mandatory for cervical cancer development. Our objective was to describe the load and physical state of HPV type 16 (HPV16) DNA in a series of cervical samples representative of the natural history of cervical cancer. We used a combination of three real-time PCR assays targeting E6, E2, and albumin genes to calculate HPV16 load (E6 and albumin) and the E2/E6 ratio as a surrogate of integration. This method was applied to 173 HPV16-positive cervical samples. Results show that viral load increases with the lesion grade (from 102 HPV16 DNA copies per 10^3 cells in normal samples up to 56,354 copies per 10^3 cells in cancers), while E2/E6 ratio decreases (from 1 in normal samples down to 0.36 in cancers). We propose that, according to this technique, an HPV16 viral load of higher than 22,000 copies/10^3 cells or an E2/E6 ratio of lower than 0.50 allows the identification of women with prevalent high-grade lesions or worse with a high specificity. In conclusion, both viral load and E2/E6 ratio, used in combination with an appropriate cutoff value, are suitable to screen women with prevalent cervical intraepithelial neoplasia grade 2 or 3 or cancer. Therefore, these assays would be useful in addition to routine HPV testing to more accurately identify women with (pre)cancerous lesions.

Cervical cancer is a worldwide health concern, with approximately 493,000 new cases and 274,000 deaths in 2002 (7, 38). The causality link between human papillomavirus (HPV) infection and this cancer has been well established by molecular and epidemiological studies (5, 52). HPVs belong to a family of circular double-stranded DNA viruses that are classified in terms of genus, species, and type (13). Papillomaviruses associated with cervical diseases are found mainly among the alpha genus. These viruses can be divided into low-risk HPV and high-risk HPV (HR-HPV), according to their oncogenic potentials (35).

In most cases, infection by HR-HPV is transient and cleared within 8 to 13 months (12, 15, 19, 54) and remains asymptomatic. Induction of an efficient and specific immune response that eliminates the virus and/or the infected cells likely plays a pivotal role in the control of HPV infection. Thus, while HPV genital infection is the most common sexually transmitted infection, cervical cancer is a rare consequence of an HR-HPV infection (32). Cervical cancer takes decades on average to arise and follows a progressive histopathological disease pattern. After an infection with an HR-HPV, cervical intraepithelial neoplasia grade 1 (CIN1) can arise. A high proportion of CIN1 regresses spontaneously if left untreated, but if HR-HPV infection persists, especially with HPV16 or HPV18 (23), CIN1 may progress to CIN2/3. Because these lesions are recognized as precancerous lesions, it is recommended to treat them generally through surgical ablation. If not, CIN2/3 may persist or progress toward invasive carcinoma (30, 37). Pap smears, introduced in the 1950s, proved to be an efficient screening tool for cervical cancer prevention (45). It allows the identification of cytological abnormalities evocative of low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and cancer according to the Bethesda system. Some Pap smears are however difficult to categorize and are referred to as atypical squamous cells of undetermined significance (ASC-US) or atypical squamous cells that cannot exclude HSIL (ASC-H).

Several studies have shown that the persistence of the HR-HPV infection is necessary for the development and progression of lesions to CIN2/3 and/or invasive carcinoma (12, 53). Moreover, studies showed that a high viral load was associated with an increased persistence of HPV infection and with an increased risk of developing CIN2/3 or cancer (12, 21, 27). Finally, an increasing viral load in persistently infected women was also shown to be associated with an increased cumulative prevalence of precancerous/cancerous lesions of the cervix (31, 51).
TABLE 1. Cross-tabulation of cytology and histology diagnoses

<table>
<thead>
<tr>
<th>Cytology diagnosis</th>
<th>No. (%) of available samples with histology diagnosis of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N(\text{a})</td>
</tr>
<tr>
<td>WNL (n = 28)</td>
<td>26</td>
</tr>
<tr>
<td>LSIL (n = 45)</td>
<td>33</td>
</tr>
<tr>
<td>HSIL (n = 41)</td>
<td>11</td>
</tr>
<tr>
<td>ASC-US (n = 37)</td>
<td>27</td>
</tr>
</tbody>
</table>

\(\text{a})\) NA, no. of samples with indicated cytology diagnosis that were not available for histology diagnosis.

\(\text{b})\) N, normal.

Integration of the HPV genome in the host cell DNA represents a key step in the progression of lesions, as it allows the continuous overexpression of E6 and E7. Recently, real-time PCR targeting E6 and E2 was shown to be reliable to study HPV16 DNA physical state (episomal or integrated) (1, 2, 8, 16, 25, 36, 40). As a whole, these studies showed that the proportion of lesions with integrated HPV16 increased with the grade of cervical lesion. However, in most studies, authors have limited their investigation to the description of (i) pure episomes, (ii) mixes of integrated and episomal forms, or (iii) pure integrated forms of HPV16 DNA in clinical specimens. Probably, the difficulties in retrieving a low frequency of integration events due to the high load of episomal forms may have precluded a more detailed description of integration (2).

In this study, after a validation step, we applied E2 and E6 targeting real-time PCR to 173 HPV16-positive cervical samples in order to describe the viral DNA physical state according to the lesion grade. Moreover, we wished to define cutoff values for viral load and integration that would allow the discrimination of prevalent high-grade lesions and/or cancer versus low-grade lesions or normal epithelium.

MATERIALS AND METHODS

Sample collection. The study was retrospectively performed on 289 smears and 22 cervical cancer biopsy samples. One hundred ninety-four smears had been collected at the endo-exocervix junction with a cytobrush and conserved in STM (specimen transport medium; Digene, Gaithersburg, MD). These specimens had been denatured with sodium hydroxide for routine HR-HPV testing with the HCII assay (Digene) and then stored at −20°C until further processing. Ninety-five samples had been similarly collected but stored in PreservCyt (Cytvec, Marlborough, MA) or Cytoscreen (Seroa, Monaco, France) (liquid-based cytology transport medium) at 4°C until further processing for routine HR-HPV screening with HCII.

Among the 289 smears, 151 samples harboring HPV16 were selected for viral load and integration analysis. Twenty-eight were samples within normal limits (WNL), and 45 were diagnosed as LSIL, 41 as HSIL, and 37 as ASC-US (no ASC-H selected). For 54 cases, a histology result obtained from a subsequent biopsy sample or cervical resection was retrieved from medical record: 4 were normal, and 14 were diagnosed as CIN1 and 36 as CIN2/3. However, viral load and E2/E6 ratio for the tissue specimens were not studied. The cross-tabulation of cytology and histology results is shown in Table 1.

As smears evocative of invasive carcinoma were rare, we collected 22 cervical invasive cancers fixed with formaldehyde and paraffin embedded to study viral load and E2/E6 ratio.

DNA extraction. DNA extraction procedures were adapted according to the nature of the specimens as follows. (i) For STM samples, 350 μl of denatured solution was neutralized with 50 μl of 5 M acetic acid and 3 M potassium acetate. Then, 400 μl of AL buffer (Qiagen, Courtabeuf, France) and 40 μl proteinase K (Qiagen) were added and the mix was digested overnight at 56°C. (ii) Four-milliliter portions of samples stored in PreservCyt or Cytoscreen medium were centrifuged for 10 min at 2,500 × g. The pellet was suspended in 400 μl Tris-EDTA buffer (pH 8). Then, 400 μl of AL buffer (Qiagen) and 40 μl proteinase K (Qiagen) were added and the mix was digested overnight at 56°C. All lysates were filtered through the column provided in the kit. After washes, DNA was eluted with 80 μl of molecular biology-grade water.

DNA from the 22 paraffin-embedded cancer tissues was extracted from 1 to 16 10-μm serial sections (according to the size of the specimen) by use of the EXTRAcell kit (Bioline, Turin, Italy) according to the manufacturer’s instructions. DNA from cultured cell lines was extracted by proteinase K digestion and subsequent phenol-chloroform extraction and served as the control.

Real-time PCR. Quantification of the human albumin gene and the HPV16 E6 and E2 genes was performed with a LightCycler 1.2 (Roche Diagnostics, Meylan, France) using TaqMan technology. Primers and probes used have been described elsewhere and their sequences are provided in Table 2 (26, 40, 41).

Table 2. Sequences of primers and probes used for viral load and E2/E6 ratio determination.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer or probe</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6 HPV16</td>
<td>Forward primer</td>
<td>5'-GAG-AAC-TGC-AAT-GTT-TCA-GGA-CC3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-TGT-ATA-GTT-TTG-AGC-TCT-GTG-C3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>3'BHQ-1-CAG-GAG-CCA-CCC-AGA-AAG-TTA-CCA-CAG-TT-FAM5'</td>
</tr>
<tr>
<td>E2 HPV16</td>
<td>Forward primer</td>
<td>5'-AAC-GAA-GTA-TCC-TCT-CCT-GAA-ATT-ATT-AG3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-CCA-AGG-CCA-CGG-CTT-TG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>3'BHQ-1-CAC-CCC-AGG-GCC-GCC-ACC-CAT-A-FAM5'</td>
</tr>
<tr>
<td>Albumin</td>
<td>Forward primer</td>
<td>5'-GCT-GTC-ATC-TCT-TGT-GGG-CTG-T3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-ACT-CAT-GGG-AGC-TGG-TTG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>3'BHQ-1-GGA-GAG-ATT-TGT-GTG-GCC-ATG-ACA-GG-FAM5'</td>
</tr>
</tbody>
</table>

<sup>a</sup> FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1.

Viral load and physical state determination. The viral load was determined using the HPV16 copy number (real-time PCR targeting HPV16 E6 open reading frame) normalized according to the number of cells (real-time PCR targeting the albumin gene). This normalization is important, as it limits bias linked to the number of cells harvested in smears and to the nature of the cervical samples.
used in this study. Results are expressed as HPV16 copy number per 10^3 cells. The physical state of HPV16 was determined using real-time PCR targeting the E6 and E2 open reading frames of HPV16. Then, the E2/E6 ratio was calculated for each sample. To assess the validity of the method, E2/E6 ratios were determined for mixes containing known E2 and E6 copy numbers. For this purpose, we set up a series made with mixes of pBR322-HPV16 plasmids (representing episomal forms) and SiHa cells DNA (representing integrated forms; two copies/cell) with ratios varying from 0 to 1, with 0.1 increments.

**Statistical analysis.** Data are expressed as medians, ranges, and first and third quartiles. According to data set characteristics (continuous data but small samples; non-Gaussian), differences between groups were assessed using the Kruskal-Wallis test. When differences were significant, post hoc pairwise comparisons between groups were performed using the method described by Siegel and Castelane (47), which includes corrections for significance values to account for multiple comparisons. The level of significance was set to 0.05. Analyses were performed with the SYSTAT package (v 10.0), and StatXact (V5.0) was used to compute Monte Carlo estimates of the exact P values.

**RESULTS**

**HPV16 E2 real-time PCR is sensitive, specific, and reproducible.** First, we validated the E2 real-time PCR. Dilutions of the pBR322-HPV16 plasmid were realized with cellular DNA from HaCat cells to obtain from 1 to 10^6 E2 gene copies per 10^3 cells. The technique was performed six times independently on all diluted samples. The detection limit reached 10^2 copies per 10^3 cells.

The E2 real-time PCR was specific for HPV16, as no cross-reaction was observed for samples previously genotyped and harboring the phylogenetically related HPV31, HPV33, HPV52, HPV18, or HPV56 (data not shown).

The reproducibility of the E2 real-time PCR was assessed using standard curves obtained from 17 independent experiments. The coefficient of variation of crossing point (Cp) obtained at each concentration was less than 4%, showing that the technique was very reproducible (Fig. 1A).

The efficiency of real-time PCRs was also calculated from plasmid dilutions and from dilutions of clinical samples. Efficiency was equal to 1.9 ± 0.1 and very close to that obtained with the real-time PCR targeting E6 (2 ± 0.1).

**E6 and E2 real-time PCR permit the physical state of HPV16 DNA to be defined.** Mixes of pBR322-HPV16 plasmid and SiHa cell DNA representing E2/E6 ratios from 0 to 1 were subjected to real-time PCR targeting E2 and E6. In Fig. 1B, we plotted experimental E2/E6 ratios (obtained from six independent experiments) against theoretical ratios. Overall, an excellent correlation (r = 0.959; P < 10^-3) was observed. However, we noted that the variability in experimental E2/E6 ratios was more important when ratios were higher than 0.8. One can propose that real-time PCR lacks the accuracy to retrieve high E2/E6 ratios. For this reason, we defined three classes of samples regarding the physical state of HPV16 DNA: (i) E2/E6 values equaling 0 (representing fully integrated genomes), (ii) E2/E6 values between 0 and 0.8 (representing mixed genomes), and (iii) E2/E6 values of more than 0.8 (mostly representing episomes).

After this validation step, we submitted the DNA from the 151 HPV16-positive smears and the 22 HPV16-positive cervical carcinomas to the three real-time PCR assays (E6, E2, and albumin) to investigate the load and physical state of HPV16 DNA.

**HPV16 viral load increases with the grade of the lesion.** Figure 2 represents the medians, ranges, and first and third quartiles of HPV16 viral loads per 10^3 cells in the 151 smears and in the 22 cervical cancer samples.

We observed that the median viral load increases with the lesion grade from 102 HPV16 DNA copies per 10^3 cells in WNL to 270 in LSIL and to 20,974 in HSIL. Statistical analysis revealed differences in viral loads between WNL and HSIL (P < 0.01) and between LSIL and HSIL (P < 0.01). ASC-US samples showed a median viral load of 802 HPV16 DNA copies/10^3 cells. HPV16 loads were significantly higher in ASC-US than in WNL and LSIL samples (P < 0.01). However, they were lower than those noted for HSIL, but no significant difference was observed.

For the smears from women subsequently diagnosed with a CIN2/3 (median load of 7,270 copies/10^3 cells), we observed HPV16 loads higher than those observed for the smears from women with CIN1 (median load of 94 copies/10^3 cells; n = 14) or with normal cervixes (median load of 397 copies/10^3 cells; n = 4). However, no statistical difference was noted.

The highest HPV16 loads (median load of 59,963 copies/10^3 cells; n = 22) were observed for biopsy samples from cervical squamous cell carcinomas.
cancers. These loads were statistically different than those observed for the smears from women with CIN1 only.

Physical state of HPV16 DNA is related to lesion grade. As for cytology results, a majority of WNL (71%) presented E2/E6 values of more than 0.8, indicating that they harbored mostly episomal HPV16 genomes (Fig. 3). The remaining 29% of samples harbored both episomal and integrated HPV16 genomes. None of the samples belonging to this category presented only integrated forms of HPV16 DNA (E2/E6 values equal to 0). In LSIL, the proportion of samples presenting E2/E6 values of >0.8 decreased to 44%, while the proportion of samples with mixed forms increased to 53%. In one smear, E2/E6 reached the value of 0.001, suggesting that most if not all HPV16 genomes were integrated. Unfortunately, the medical record was not available to confirm the final diagnosis. In HSIL, the proportion of specimens with E2/E6 values of more than 0.8 dropped to 15%, while 85% of these samples presented integrated HPV16 DNA associated with episomes. Finally, 76% of ASC-US samples harbored integrated HPV16 DNA (E2/E6 values between 0 and 0.8). Five percent of ASC-US (n = 2) presented E2/E6 values of 0, indicating that HPV16 DNA was totally integrated. The remaining ASC-US specimens (19%) presented an E2/E6 value of >0.8. As a whole, we observed that the proportion of samples with integrated HPV16 genomes increased with the lesion grade (P < 0.01; chi-square test).

Two smears from women with normal biopsy results presented E2/E6 values of more than 0.8, whereas the two others presented both episomal and integrated HPV16 genomes. The proportion of smears with E2/E6 values of more than 0.8 decreased to 36% and to 16% for women with CIN1 and CIN2/3, respectively. The remaining samples presented both episomal and integrated HPV16 genomes.

For cancer biopsy samples, the proportion of specimens with E2/E6 values of more than 0.8 (18%) was close to the one observed for smears from women with a subsequent diagnosis of CIN2/3. In contrast, the proportion of samples with mixed forms decreased to 45% to the benefit of pure integrated HPV16 genomes, which were observed in 37% of cases.

We also noted that the proportion of smears with integrated HPV16 DNA increased with the lesion grade. However, this trend was not statistically significant (P > 0.05; chi-square test).

E2/E6 ratio decreases with lesion grade. The medians, ranges, and first and third quartiles of E2/E6 ratios for each category of samples are presented in Fig. 4. As a whole, the median of E2/E6 ratios decreased with the lesion severity (1 for WNL, 0.64 for LSIL, and 0.46 for HSIL). Whereas no significant difference was found between WNL and LSIL, the E2/E6 ratios were significantly lower for HSIL than for WNL and LSIL (P < 0.01). The E2/E6 ratios for ASC-US (median of 0.55) were also significantly lower than for WNL (P < 0.01). A
similar trend was observed when the 54 smears were classified according the subsequent histological data. Indeed, the median of E2/E6 decreased from 0.75 for smears from women with normal histologies to 0.58 for smears from women with CIN1 and 0.51 for smears from women with CIN2/3. No difference between E2/E6 ratios was observed. The lowest E2/E6 ratio median (0.36) was noted for cancer samples. However, no statistical difference was observed in comparison to ratios calculated from smears.

**Appropriate cutoffs permit identification of women with high-grade lesions.** Figure 5 shows the receiver operating characteristic (ROC) analysis results of viral load and E2/E6 ratios in discriminating WNL and LSIL from HSIL samples.

For viral load, the cutoff with the highest positive likelihood ratio (LR⁺) (LR⁺ of 5.1), was 21,907 copies/10⁶ cells. At this threshold, HPV16 load allowed the identification of women with HSIL with a specificity of 90% and a sensitivity of 50%. We then used this cutoff to discriminate smears from women with normal cervixes or with CIN1 from smears from women with CIN2/3. The specificity and the sensitivity decrease to 83% and 40%, respectively, with the LR⁺dropping to 2.3 (area under the curve, 0.68; 95% confidence interval [95% CI], 0.52 to 0.85).

For the E2/E6 ratio, the cutoff with the highest LR⁺ (LR⁺ of 3) was 0.52, with a specificity of 78% and a sensitivity of 66%. This cutoff was not used to discriminate smears from women with normal cervixes or with CIN1 from smears of women with CIN2/3, as the ROC analysis was not satisfactory (area under the curve, 0.66; 95% CI, 0.50 to 0.81).

**DISCUSSION**

In this study, we quantified HPV16 E6 and E2 DNA copy numbers by real-time PCR to analyze the load and physical state of the HPV16 genome in a series of 173 cervical samples. Several studies have reported the use of this methodology to characterize integration (1, 2, 8, 16, 25, 36, 40), even though some authors have highlighted limitations concerning the use of real-time PCR targeting E2 (2, 3, 29, 44). In order to preclude the erroneous interpretation of real-time PCR results, we validated our method with mixes of pBR322-HPV16 plasmids (mimicking episomal forms) and SiHa cell DNA (two integrated HPV16 genomes per cell) representing E2/E6 ratios from 0 to 1 with increments of 0.1. Moreover, we confirmed that E6 and E2 real-time PCR assays have almost equal efficiencies and that the calculation of E2/E6 ratios was accurate.

**HPV16 DNA load analysis.** First, we observed that HPV16 load increased with the lesion grade. An appropriate statistical analysis permitted us to retrieve significant differences between WNL and HSIL samples as well as between LSIL and HSIL samples. This trend was further confirmed when histology results were taken into account. Thus, our data confirm previous observations suggesting that the higher the grade of the lesion, the higher is the viral load, even though statistical analyses were not always available (1, 9, 11, 18, 24, 25, 39, 50, 51, 55, 56). We would like to point out that a direct comparison between our results and those obtained in previous reports may be difficult because no standardized procedures were reported. (i) Original material differed widely. (ii) Different methods for HPV detection, more or less quantitative, were used: MY09/ MY11 PCR followed by type-specific dot blot hybridization (24), PCR followed by an enzyme-linked immunosorbent assay (56), conventional nested PCR (50), real-time PCR using SYBR green (11, 39), real-time PCR using the TaqMan technology (9, 18, 25, 55), and consensus real-time PCR (1). (iii) Results were expressed by different means: copies per cell (1, 56), copies per ng of DNA (11, 25), copies per sample (39, 50), and qualitatively (18, 24). Thus, the value of viral load calculated is likely to be dependent on the procedures used to quantify HPV DNA. This may explain the large range of values obtained for a given lesion grade in the different studies.

We would like to stress the fact that the viral load values observed for invasive cancers were very high. This observation is in contrast with the results described by Cricca and coworkers, who noticed the lowest viral load for samples of invasive cancers (11). This can be attributed to the starting material and the number of samples analyzed (n = 11). Moreover, smears evocative of invasive cancer may include not only cancer cells but also normal cells or cells from associated low-grade lesions, leading to an overall underestimation of the viral load (46). Here, we noticed that among cancer samples, those with the highest viral loads also presented the highest E2/E6 ratios. In contrast, cancer samples with most if not all HPV genomes integrated (E2/E6 values equal to 0) presented the lowest viral load values (data not shown). These observations are likely to...
be consistent with the fact that integration prevents HPV replication (14).

Physical state of HPV16 DNA according to lesion grade. The 3’-terminal part of E2, including the hinge region (nucleotides 3352 to 3535) and the DNA binding domain, is the most often deleted or disrupted part of E2 following HPV16 DNA integration in the cellular genome (2, 22). For that reason, authors used real-time PCR targeting E2 and E6 to study the physical state of HPV16. Here, we defined three classes of samples according to the value of the E2/E6 ratios: class I, with E2/E6 values equal to 0, representing fully integrated genomes; class II, with E2/E6 values between 0 and 0.8, representing mixes of episomal and integrated genomes; and class III, with E2/E6 values of more than 0.8, mostly representing episomes.

In our series, we noticed that the most frequently observed E2/E6 ratios were between 0 and 0.8. This means that the majority of samples harbored mixes of episomes and integrated forms of HPV16 genomes independently of the lesion grade. Our results also confirm that integration can be an early event, because 29% of the normal smears harbored a fraction of integrated HPV16 genomes. This is in accordance with previously published data (8, 11, 17, 25, 28, 40). Some authors do not agree with this observation (20, 49). But their methods (two-dimensional gel electrophoresis [20] and classic PCR [49]) were probably not sensitive enough to detect low-frequency integration events. We also observed that the proportions of samples with integrated forms increased with the lesion grade. This is consistent with the literature, with some variations within each category of smears, however (1, 8, 11, 16, 25, 40, 49). For example, we found that 44% of LSIL (corresponding to 36% of smears from women with CIN1) harbored only episomal HPV16 genomes. This is close to the value (33.3%) we previously published on a series of CIN1 samples (8) but higher than the values from Cricca et al. (15.4%) (25) and lower than the values from Cricca et al. (72.2%) (11), Tonon et al. (92%) (49), and Hudelist et al. (100%) (20). As for viral load, these discrepancies could be explained by the lack of standardized protocols to investigate integration and by definitions of mixed forms of HPV16 genomes. As reported in a previous study, pure integrated forms (E2 values equal to 0) were rarely found for precancerous lesions (8). In the present study, 100% integration was observed for several cancer samples, as already described (3, 10, 24, 40). This is consistent with the HPV-associated mechanism of carcinogenesis. Indeed, tumors grow from cellular clones selected for their proliferative advantages, which are driven by integration and continuous overexpression of E6 and E7 (33).

Quantitative analysis of E2/E6 ratios. We observed that the median of E2/E6 ratios decreased with the increasing grade of the lesions. This indicates that the proportion of episomes decreases to the benefit of integrated forms of HPV16 genomes in the higher-grade lesions. The methods used in this and other studies do not allow a determination of whether some cells harbor episomal forms and others integrated forms or if the same cells harbor both episomes and integrated forms. One possibility to address this question is to analyze the physical state of the HPV16 genome at the single-cell level, for example after the isolation of single abnormal cells by laser microdissection followed by whole-genome amplification and HPV16 DNA analysis. Such experiments would give interesting clues regarding the impact of HPV DNA integration and E2 gene alterations for the progression of HPV16-infected cells.

Optimal cutoffs to identify women with prevalent high-grade lesions. From a practical point of view, we propose that a high viral load or a low E2/E6 ratio is clinically associated with a prevalent precancerous lesion. Thus, we propose that relevant cutoffs of viral load and of E2/E6 ratio might permit the identification of women with high-grade lesions. We chose to discriminate normal plus LSIL versus HSIL or normal and CIN1 versus CIN2/3, since low-grade lesions spontaneously regress in most women within 3 years (32). Even though these lesions need to be monitored, they do not necessarily need aggressive management as high-grade lesions do.

Using the ROC analysis, we have set optimal cutoffs at 21,907 copies/10^3 cells and at an E2/E6 ratio of 0.52 to discriminate WNL plus LSIL from HSIL. These cutoffs allow the identification of women with prevalent high-grade lesion with a good specificity (90% for viral load and 78% for E2/E6). Moreover, we attempted to apply the viral load cutoff using a histology result as the end point. Interestingly, we were able to identify women with prevalent CIN2/3 with a specificity of 83%. E2/E6 ratios were not informative enough to discriminate smear from women with normal cervixes or CIN1 from smears of women with CIN2/3. As expected, the sensitivities remained modest with these cutoffs, which were not designed for primary screening purposes.

We deliberately decided to exclude ASC-US samples from ROC analyses. Indeed, ASC-US can be suggestive of a wide range of histological diagnoses from a totally normal cervix to infiltrating carcinoma, as recently reported by Boulanger and Sevestre (CIN1 or condyloma in 20 to 54% of cases, CIN2 or CIN3 in 5 to 18% of cases, and invasive cancer in 0 to 4% of cases) (6). Thus, we noticed that HPV16 parameters (load, E2/E6 ratio) presented intermediate values, reflecting the diversity of this category of samples.

In summary, we observed that the viral load and E2/E6 ratios were related to the lesion grade. Moreover, we confirmed that integration is an early event in the course of natural HPV infection. Based on our results, we propose that among HR-HPV infected women, those with an HPV16 load of above 22,000 copies/10^3 cells or with an E2/E6 ratio below 0.5 are likely to present a high-grade lesion. These cutoff values, however, are relevant for HPV16 only, which is detected for 34 to 52% of patients with CIN2/3 and for 52 to 58% of cancers (4, 10, 34, 48) (62% of CIN2/3 and in 73% of cancers in France [42, 43]). Because other HR-HPVs (e.g., HPV18, HPV31, and HPV45) are also responsible for cervical lesions, relevant cutoffs should be determined for these genotypes. Now, prospective cohort studies are necessary to evaluate the clinical relevance of these viral parameters. Moreover, there is an obvious need for standardized procedures, as our results may be dependent on the protocol we have used. Standardization would allow the comparison of viral load and integration results from different studies and laboratories. In this respect, we recently worked on the homogenization of techniques between different labs. Preliminary results indicate that it is possible to achieve very good inter-/intra-laboratory and inter-/intraobserver reproducibility.
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