Type-Specific Viral Load and Physical State of HPV Type 16, 18, and 58 as Diagnostic Biomarkers for High-Grade Squamous Intraepithelial Lesions or Cervical Cancer

Jongseung Kim, MD, PhD1
Bu Kyung Kim, PhD2
Dongsoo Jeon, MD3
Chae Hyeong Lee, MD, PhD4
Ju-Won Roh, MD, PhD4
Joo-Young Kim, MD, PhD5
Sang-Yoon Park, MD, PhD6

1Department of Family Medicine, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, 2Department of Tumor Biology, Seoul National University, Seoul, 3Department of Obstetrics and Gynecology, Soochunhyang University College of Medicine, Gumi, 4Department of Obstetrics and Gynecology, Dongguk University Ilsan Hospital, Goyang, 5Proton Therapy Center, Research Institute and Hospital, National Cancer Center, Goyang, 6Center for Uterine Cancer, Research Institute and Hospital, National Cancer Center, Goyang, Korea

Correspondence: Ju-Won Roh, MD, PhD
Department of Obstetrics and Gynecology, Dongguk University Ilsan Hospital, 27 Dongguk-ro, Ilsandong-gu, Goyang 10326, Korea
Tel: 82-31-961-7363
Fax: 82-31-961-7155
E-mail: rohjw@hanmail.net

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Purpose
High rate of false-positive tests is a major obstacle to use human papillomavirus (HPV) detection as a diagnostic tool for high-grade squamous intraepithelial lesions or cervical cancer (HSIL+). We investigated whether type-specific viral load or physical state of HPV 16, 18, and 58 are useful biomarkers for HSIL+.

Materials and Methods
Type-specific viral loads of E6 and E2 genes in cervical cells from 240, 83, and 79 HPV 16−, 18−, and 58−infected women, respectively, were determined using real-time polymerase chain reaction. Viral loads were normalized to cellular DNA (copy/cell). Total and integrated viral loads and physical state were compared between HSIL+ and controls, and diagnostic value was determined using receiver operating characteristic analysis.

Results
Viral loads of HPV 16, 18, and 58 were significantly different in lesions in the same pathologic grade. High type-specific total viral loads were significantly associated with HSIL+ (odds ratio [OR], 14.065, 39.472, and 7.103 for HPV 16, 18, and 58, respectively). High integrated viral load was related to HSIL+ in women with HPV 16 (OR, 8.242), and integrated state was associated with HSIL+ in women with HPV 18 (OR, 9.443). Type-specific total viral load was significantly associated with HSIL+ (area under curve, 0.914, 0.937, and 0.971 for HPV 16, 18, and 58, respectively), indicating an excellent performance in detecting HSIL+.

Conclusion
Type-specific total viral load may be a powerful diagnostic marker for HSIL+ in HPV 16−, 18−, and 58−infected HSIL+ lesions. If demonstrated in all other high-risk HPV types, this method can lead to a paradigm shift in the strategy of equivocal cytologic abnormalities.

Key words
Human papillomavirus, Viral load, High-grade squamous intraepithelial lesions, Cervical cancer

Introduction
Uterine cervical cancer is the fourth most commonly diagnosed cancer and the fourth leading cause of cancer death among women, with an estimated 570,000 new cases and 311,000 cervical cancer-related deaths reported worldwide in 2018. In low Human Development Index settings, it ranks second only to breast cancer in incidence and mortality [1]. The causal link between high-risk human papillomavirus (HPV) infection and cervical cancer, including squamous intraepithelial lesion (SIL), has been well established by molecular and epidemiologic studies [2]. However, HPV infection is self-limited and regresses within 1 year in most cases [3].

Cervical cancer screening is mainly based on Papanicolaou (Pap) tests, and Pap test–based screening has played an important role in reducing the incidence of and mortality from this disease. However, their effectiveness is limited by their low sensitivity and reproducibility. Moreover, the rare
incidence of HPV-negative cancers supports the need for
HPV testing for cervical cancer screening. Used either alone
or in combination with cytology, HPV testing is a particu-
larly efficient screening tool [4,5]. However, despite exhibit-
ing better sensitivity and reproducibility than cytology [6],
HPV tests have poor positive predictive value of HPV detec-
tion because only a few women with HPV will progress to a
cervical lesion [7].

Currently, high-grade SIL (HSIL) is the widely accepted
cut-off for treatment, because a large number of low-grade
SIL (LSIL) and lower lesions spontaneously regress [8]. Fol-
lowing this guideline, using diagnostic tests of HPV infection
for cervical cancer screening may produce too many false-
positive results and lead to needless invasive procedures,
prolonged follow-up periods, and psychosocial problems
such as anxiety [9]. Recently, the Cobs HPV test (Roche
Molecular System, Pleasanton, CA), an automated real-time
polymerase chain reaction method using HPV 16 and HPV
18 genotype information, has been introduced with Food and
Drug Administration approval. In the ATHENA study,
although the Cobs HPV test was more sensitive than cytol-
gy in detecting high-grade squamous intraepithelial lesions
or cervical cancer (HSIL+), the positive predictive value for
HSIL+ was from 10.1% (high-risk HPV) to 15.5% (HPV 16,
18, or both), which meant that most women with positive
HPV infection did not need invasive diagnostic procedures
[10]. Therefore, there is a need to identify new, more specific
biomarkers to confirm the subgroup of women with HSIL or
worse (HSIL+) who require treatment from the group of
women with high-risk HPV infections.

Viral load and physical state estimations are very attractive
candidate biomarkers for HSIL+ based on HPV-associated
carcinogenesis. Some previous cross-sectional and longitu-
dinal studies have demonstrated an association between
high viral load and risk of HSIL+ [11-13], whereas other
reports did not find a strong correlation [14]. Integration into
the human genome is also known to be a main event in cer-
vical carcinogenesis [15] and is considered an important viral
marker [12]. Previous studies on the viral load and integra-
tion of HPV 16 alone or in combination have been performed,
yielding variable results [14-17]. Such variability may result
from various factors, such as using semi-quantitative meth-
ods like Hybrid Capture 2 (HC2), heterogeneous specimen
quality, different methods of calculating viral load, non-type-
specific evaluation of viral load, and the presence of multiple
infections as confounding factors. HC2, used in many previ-
ous studies, does not determine viral load considering the
cellular input and cannot distinguish between HPV types or
discriminate monotypic infections from infections with mul-
tiple HPV types [16,17]. Most studies that did focus on HPV
type looked only at HPV 16 or HPV 18 [11,13]. Only a few
small studies have addressed the viral load of other HPV
types, reporting that type-specific viral loads and clinical sig-
nificance were different, type-dependent markers for HSIL+
[12,18]. In our previously published report [19], HPV 58 was
found to be the third most common HPV type related to
HSIL+ and almost the same number of subjects were con-
firmed to have HPV 58 infection. In this study, we investi-
gated whether viral load or integration status could be useful
diagnostic biomarkers for HSIL+ using type-specific evalua-
tion of monotypic HPV 16, 18, and 58 infections.

Materials and Methods

1. Study subjects

We selected 497 patients who were evaluated and treated
for cervical neoplasms at the Center for Uterine Cancer.
Women referred for abnormal cytologic results were consid-
ered tentative cases. Tests using the HC2 (Qiagen, Hilden,
Germany) high-risk HPV kit were performed, and cervical
cells were collected before colposcopy-directed biopsies were
obtained. Ninety-two patients with a previous history of hy-
terectomy or conization, with negative results on a high-risk
HPV test using HC2, or who refused to enroll in the study
were excluded. An additional 1,506 women who received
cervical cytologic examinations and HC2 at the Center for
Cancer Prevention and Early Detection were screened during
the same period. High-risk HPV infections were found in 283
women. After exclusion of 111 women with a previous his-
tory of hysterectomy or conization or who refused to enroll,
172 women were referred to the Uterine Cancer Center.
Among the 577 women with high-risk HPV infections, 453
women were confirmed to be infected with HPV 16 (n=240),
18 (n=83), or 58 (n=79) by polymerase chain reaction (PCR)–
based genotyping (Fig. 1). For clarity, individuals with mul-
tiple high-risk HPV infections were excluded from the
analysis. Pathologic confirmation was performed by col-
poscopy-directed punch biopsy. We defined cases as HSIL+
because these are the targets of cervical cancer screening.
Individuals without pathologic abnormalities, koilocytic
atypia, and LSIL were regarded as controls. All participants
signed an informed consent form.

2. HPV genotyping

Cervical cells were centrifuged immediately after collec-
tion at 3,000 xg and the resulting pellet was diluted in 1 mL
of 1× phosphate-buffered saline and stored at –70°C until
DNA extraction. DNA was isolated using the AccuPrep
genomic DNA extraction kit according to the manufacturer’s
Fig. 1. Flowchart of the study subjects. Pap, Papanicolaou; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; HSIL+, high-grade squamous intraepithelial lesions or cervical cancer.

instructions (Bioneer, Daejeon, Korea) within 1 week of sample collection and stored at −70°C until use. Analysis of HPV genotypes was carried out using PCR-based dot blot hybridization with HPV type-specific oligonucleotide probes [20]. HPV DNA was amplified using MY09/11 and GP5+/6+ L1 consensus primers with AmpliTaq Gold polymerase as previously described [21]. Thermocycling conditions were as follows: initial denaturation at 95°C for 9 minutes, 40 cycles at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds, and a final 5-minute extension at 72°C. The MY09/11 and GP5+/6+ systems amplify approximately 450- and 150-base pair segments in the L1 region, respectively. During each PCR run, samples were tested together with one negative control (water) and one positive control (cells harboring HPV 16). β-Globin gene was amplified from each sample for the confirmation of presence and amount of DNA. PCR products were analyzed using gel electrophoresis and then dot blot hybridization was conducted as described previously [20]. Briefly, the PCR products were denatured in 0.4 mol/L sodium hydroxide and 10 mmol/L EDTA and applied to a nylon membrane that was premoistened with 6× saline sodium citrate (SSC) using a 48-well dot blot apparatus (Life Technologies, Grand Island, NY). DNA was then UV cross-linked to the membrane and dried. The nylon membrane was placed in prehybridization buffer (6× SSC, 0.5% sodium dodecyl sulfate [SDS], 1 mmol/L EDTA, and 100 μg/mL denatured salmon sperm DNA) for 2 hours at 68°C, followed by overnight incubation at 42°C in hybridization buffer mixed with biotin-labeled type-specific oligonucleotide probes for 16 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69). After washing with 2× SSC and 0.5% SDS, bound probes were detected with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). Specimens that were positive for HPV DNA but did not match any of the 16 probes were labeled high-risk HPV-negative.

3. Viral load determination using real-time PCR

Quantification of β-actin and type-specific HPV E6 and E2 genes was performed with an ABI Prism 7900HT System and TaqMan Universal PCR Master Mix (PE Applied Biosystems,
Perkin-Elmer, Foster City, CA). Sequence information of each primer used is available in S1 Table. For determination of integrated viral load, the primers and probes were designed for targeting the E2 hinge regions, which are known to be deleted most frequently during viral integration [22]. β-Actin DNA loads were used for normalization. Fifty nanograms of DNA from each sample was used in each reaction and standard curves were produced by amplification of a dilution series of 10^4–10^7 copies of a clone of type-specific HPV or β-actin DNA in the pGEM-T vector. Control reactions without template were included in each test and all experiments were performed in triplicate. The mean value was used if the three values were similar, whereas the mean of two values was used if the third was an outlier. If the test was negative, direct sequencing of the PCR product with the L1 consensus primer was performed to confirm the HPV genotype. If the sample was confirmed as HPV 16, 18, or 58, quantification was repeated. We used the following formula to convert plasmid DNA into copy number: (number of base pairs)×(average weight of double-stranded DNA)/Avogadro’s number=μg/plasmid molecule, (μg/μL)/(μg/plasmid molecule)=plasmid molecules/μL or copies/μL. The viral load was normalized to cellular DNA input as indicated below: HPV DNA load (HPV copies/cell)=number of HPV copies/ (number of β-actin copies/2) [23,24]. A normalized HPV load over the 80th percentile of controls was considered a high viral load.

4. Determination of the physical state of HPV

The physical state of HPV was determined using normalized viral loads of type-specific HPV E2 and E6. The integrated viral load was determined by subtracting normalized E2 load from normalized E6 load. Then, the physical state was calculated by the ratio of integrated load (E6-E2)/total load (E6), which varied from 0 to 1. The definition of integrated physical state was an (E6-E2)/E6 ratio greater than 0.8. A ratio of 0.8 or less was considered episomal.

5. Statistical analyses

Continuous variables were assessed for normality distribution (Kolmogorov–Smirnov test) and are expressed as median with interquartile ranges. Analyses using the Kruskal-Wallis or the Mann-Whitney U tests were performed to determine statistical significance as appropriate. Categorical variables were evaluated via chi-square analysis and logistic regression analysis to obtain odds ratios (ORs). Receiver operating characteristic (ROC) analyses and determination of the area under the curve (AUC) were performed to evaluate whether a parameter could differentiate between HSIL+ and controls. Three replicates were used to monitor the performance of each experiment. p < 0.05 was considered statistically significant. IBM SPSS Statistics ver. 21.0 (IBM Corp., Armonk, NY) was used for all statistical analyses.

6. Ethical statement

This study was conducted between March 2003 and June 2008 after receiving approval by the Institutional Review Board of the National Cancer Center of Korea (NCCNHS-03022). All study participants provided informed consent.

![Figure 2](image_url)

**Fig. 2.** Type-specific normalized total viral load of human papillomavirus (HPV) 16, 18, and 58 in patients with cervical lesions at the same pathologic grade. Box plots show median and interquartile range. Controls include patients without pathologic abnormalities or with low-grade squamous intraepithelial lesions. p-values were evaluated using Kruskal-Wallis tests. HSIL, high-grade squamous intraepithelial lesions; ICC, invasive cervical cancer.
Table 1. Type-specific total viral load and pathologic grade of cervical lesion

<table>
<thead>
<tr>
<th>HPV type</th>
<th>HPV 16</th>
<th>HPV 18</th>
<th>HPV 58</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Viral load (copy/cell)</td>
<td>No.</td>
<td>Viral load (copy/cell)</td>
</tr>
<tr>
<td>Control</td>
<td>77</td>
<td>0.000 (0.000-0.001)</td>
<td>24</td>
<td>0.000 (0.000-0.002)</td>
</tr>
<tr>
<td>HSIL</td>
<td>36</td>
<td>0.208 (0.034-31.177)</td>
<td>6</td>
<td>0.062 (0.032-0.272)</td>
</tr>
<tr>
<td>ICC</td>
<td>127</td>
<td>1.505 (0.202-11.724)</td>
<td>53</td>
<td>0.175 (0.032-0.769)</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; ICC, invasive cervical cancer. aP-value of the difference according to pathologic severity in the same HPV type.

Table 2. Differences in type-specific viral loads in the HSIL+ group

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Control</th>
<th>HSIL+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Viral load (copy/cell)</td>
<td>No.</td>
</tr>
<tr>
<td>HPV 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>0.001 (0.000-0.001)</td>
<td>163</td>
</tr>
<tr>
<td>Integrated</td>
<td>0.000 (0.000-0.000)</td>
<td>0.267 (0.008-2.795)</td>
<td></td>
</tr>
<tr>
<td>HPV 18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>0.000 (0.000-0.002)</td>
<td>59</td>
</tr>
<tr>
<td>Integrated</td>
<td>0.000 (0.000-0.000)</td>
<td>0.031 (0.000-0.239)</td>
<td></td>
</tr>
<tr>
<td>HPV 58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>0.002 (0.000-0.020)</td>
<td>51</td>
</tr>
<tr>
<td>Integrated</td>
<td>0.000 (0.000-0.000)</td>
<td>0.006 (0.000-0.542)</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as median (interquartile range). HSIL+, high-grade squamous intraepithelial lesions or cervical cancer; HPV, human papillomavirus.

Results

1. Difference in viral loads according to HPV type and SIL pathologic severity

The distribution of HPV 16, 18, and 58 viral loads in a cell with the same pathologic grade of cervical lesion is shown in Fig. 2. We observed that total viral loads were different from type to type in lesions with the same pathologic grade (p < 0.001). The viral load of HPV 58 was the highest, whereas the viral load of HPV 18 was the lowest (Table 1). The normalized total viral loads of HPV 16, 18, and 58 (copy/cell) increased with the pathologic severity of SIL (p < 0.001) (Table 1).

2. Type-specific total and integrated viral load and physical state of HSIL+

The median total viral loads of HPV 16, 18, and 58 were significantly higher in the HSIL+ group than in controls (p < 0.001 for all) (Table 2). In addition, the median integrated viral loads of HPV 16, 18, and 58 were significantly higher in HSIL+ cases than in controls (p < 0.001 for HPV 16 and 18; p=0.007 for HPV 58). Logistic regression analysis revealed that high total viral load was an independent risk factor for all HPV types. The ORs of high viral load of HPV 16, 18, and 58 were 14.065 (95% CI, 5.126 to 38.634), 39.472 (95% CI, 2.814 to 554.821), and 7.103 (95% CI, 2.523 to 20.005), respectively (Table 3). However, high integrated viral load was an independent risk factor only for HPV 16 (OR, 8.242; 95% CI, 2.633 to 25.825), and integration into the host genome was an independent risk factor only for HPV 18 (OR, 9.443; 95% CI, 1.454 to 61.605).

3. ROC analysis of viral load as a diagnostic biomarker of HSIL+

We next evaluated whether type-specific viral load could differentiate between the control and HSIL+ groups. ROC curves for controls versus HSIL+ are shown in Fig. 3. Type-specific viral load was associated with HSIL+ (AUC, 0.914,
Table 3. Logistic regression analysis of type-specific viral loads and physical state for detection of HSIL+

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HSIL+</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPV 16</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>62</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>15</td>
<td>145</td>
<td>14.065 (5.126-38.634)</td>
</tr>
<tr>
<td>Integrated viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>62</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>15</td>
<td>139</td>
<td>8.242 (2.633-25.825)</td>
</tr>
<tr>
<td>Physical status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>70</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>Integrated</td>
<td>7</td>
<td>75</td>
<td>2.421 (0.684-8.645)</td>
</tr>
<tr>
<td><strong>HPV 18</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>19</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>57</td>
<td>39.472 (2.814-554.821)</td>
</tr>
<tr>
<td>Integrated viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>19</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>50</td>
<td>0.071 (0.041-1.386)</td>
</tr>
<tr>
<td>Physical status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>21</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Integrated</td>
<td>3</td>
<td>47</td>
<td>9.443 (1.454-61.605)</td>
</tr>
<tr>
<td><strong>HPV 58</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>44</td>
<td>7.103 (2.523-20.005)</td>
</tr>
<tr>
<td>Integrated viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>27</td>
<td>1.654 (0.474-5.794)</td>
</tr>
<tr>
<td>Physical status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>27</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>Integrated</td>
<td>1</td>
<td>3</td>
<td>0.872 (0.063-12.991)</td>
</tr>
</tbody>
</table>

HSIL+, high-grade squamous intraepithelial lesions or cervical cancer; OR, odds ratio; CI, confidence interval; HPV, human papillomavirus; high viral load= viral load higher than the 80th percentile of controls; integration=integrated viral load / total viral load > 0.8.

0.937, and 0.971 for HPV 16, 18, and 58, respectively), indicating that it has excellent diagnostic value in differentiating HSIL+ cases from controls. For integrated viral load, the AUCs of HPV 16, 18, and 58 were 0.871 (95% CI, 0.818 to 0.924), 0.840 (95% CI, 0.757 to 0.922), and 0.676 (95% CI, 0.560 to 0.793), respectively, and thus inferior to total viral load as a diagnostic biomarker of HSIL+.

**Discussion**

We have shown here that type-specific normalized total viral load is a powerful diagnostic biomarker of HSIL+. This observation may help overcome the limitations of using HPV testing for cervical cancer screening.

The present study has several advantages over previous studies. First, we normalized viral load to cellular input DNA, which is a very objective and reproducible method. Many studies reported that high HPV viral load is associated with persistent infection and may be useful in predicting HSIL+, but the proposed cut-off values of viral load differed greatly [12-14,24,25]. These different cut-off values result from various factors including differences in experimental methods and different methods of estimating viral load. Because the ratio between the number of HPV DNA copies and the number of cells in the tested specimen seems the most credible and reproducible means of measuring viral load, we used the number of copies of HPV DNA per cell as
an expression of viral load. We suggest that normalizing viral load to cellular DNA input can produce reliable and reproducible findings for clinical applications. Specimen homogeneity, including absolute amount of input DNA, may be an important issue in this kind of study, because the number of cells in the specimen may affect viral load quantification [26]. The range of viral load overlaps with the pathologic group if the degree of specimen heterogeneity is significant.

The viral loads determined in our results were relatively well quantified by normalization to cellular input DNA. Second, we analyzed the diagnostic roles of viral load and physical state stratified by type. The few, small studies that have been performed on viral loads of other HPV types reported that viral loads were type-dependent markers for HSIL+ [12,18]. However, most studies on viral load and HPV integration focused only on type 16 or pooled data irrespective of type [11,27], a few looked at type 18 [13,28,29], and very few examined other types [18,30]. This is because sample size is a major obstacle to accurate evaluation of the role of type-specific viral load except in the case of HPV 16. The number of study subjects infected only with HPV 18 or 58 in the current study is the largest to our knowledge, and thus our findings are more reliable in assessing the effect of viral load and its diagnostic value in cervical cancer screening. HPV 58 is a high-risk type with low prevalence in Eastern countries but considerably more prevalent in Asian countries [19,30]. There have been very few studies on the correlation between HPV 58–specific viral load and integration and disease severity [18]. Although previous studies related to HPV 58 produced unclear results, our study supports the usefulness of viral load as a diagnostic marker of HSIL+, but not integration or physical status. Compared with previous studies, our study featured a larger sample size, fewer confounders (as subjects had only a single infection), and colposcopy-directed biopsy confirmation. From our findings, we can conclude that the mechanism of carcinogenesis differs depending on HPV type and the effect of viral load and physical state may differ from type to type. Thus, type-specific analysis is desirable for accurate evaluation. Third, viral load and integration, the most important viral markers of HSIL+, were considered at the same time. Although not only total viral load but also integrated viral load seem to be valuable for the diagnosis of HSIL+ in subjects with HPV 16, only total viral load, not integrated viral load, was useful in cases of HPV 18 or 58 in our results.

A few recent studies observed higher viral loads of HPV 18 in subjects with higher pathologic grade cancers [28,29], whereas many other studies failed to observe a significant effect of HPV 18 viral load on disease severity [13]. In our results, the viral load of HPV 18 was significantly lower than that of HPV 16 and 58, and was distributed more narrowly, than that of HPV 16 or 58. These results are consistent with a recently published report using the Cobas HPV test with normalization [29]. However, the authors could not find a linear relationship of HPV 18 viral load, but only increased viral loads in invasive cervical cancer (ICC) compared to HSIL [29]. This discrepancy may result from the small sample size in both studies. In the report by Wu et al. [29], the number of HPV 18–positive HSIL and ICC samples was only 10 and 27, respectively. Similarly, in our study, the number

**Fig. 3.** Receiver operating characteristic curve analysis of human papillomavirus (HPV) 16, 18, and 58 for the detection of high-grade squamous intraepithelial lesions or cervical cancer by type-specific total and integrated viral load. AUC, area under the curve.
of HPV 18–positive HSIL and ICC samples was only 6 and
53, respectively. Although we found a statistical difference
in viral load according to pathologic severity, it seems insuf-
icient for a final decision on linear trends considering these
controversial results. Assuming that specific adjuvant factors
or carcinogenic mechanisms could result in a powerful onco-
genic potential despite a low viral load, early and high-fre-
cuency integration may have an important role in HPV
18–related cervical malignancies. We found that integration
state was an independent risk factor in cases of HPV 18.

Another important finding of this study is that the fre-
cuency of integration of HPV 58 in patients with HSIL+ was
very low compared to that of HPV 16 or 18. Previously, inte-
gration was considered a critical event in carcinogenesis of
cervical cancer as it results in loss of the episomal type. It has
been reported that intact E2 expression derived from epis-
omal HPV DNA has an inhibitory effect on viral oncogene
expression [15], and integrated HPV survive during carci-
genesis because of a relative priority in cell proliferation and
avoidance of the host immune response [31]. In contrast,
some studies demonstrated the presence of integrated HPV
16 only in 28% to 67% of subjects [3], and the frequency of
integration was markedly different in cases with different
HPV types [32]. For HPV 58, the integration frequency in cervices
cancer was only 8.3% in our results, which is in agree-
ment with data previously reported by Ho et al. [18], who
found the integrated from of HPV 58 in only 12.5% of cervical
cancers. Our findings are also similar to those of a study that
reported that the integration frequency of HPV 18 is the high-
est of all the types [18]. The high prevalence of the episomal
form in HPV 58–related cervical cancer supports the conclu-
sion that E2 disruption is not mandatory for enhanced onco-
genic expression, and the incidence of integration probably
is different from type to type. There is a need to investigate
alternative mechanisms of going beyond a limit during the
carcinogenesis of HPV 58. There have been possible expla-
nations for the episomal form of HPV 58, including the sig-
nificant proportion of HPV 16 in cervical cancers. These include
epigenetic controls such as methylation of viral and
host DNA and mutations in the long control region, which
may lead to the acquisition of invasiveness [27]. Differences
in the frequency of integration of HPV types suggest that
integration is not a precondition for cervical carcinogenesis,
nor is it adequate as a diagnostic tool irrespective of HPV
type.

The present study has some limitations. First, the sample
size of subjects infected with HPV 18 or 58 was small. Espe-
cially for HPV 18, the HSIL group had only six samples, and
the differences in viral load between HSIL and ICC was not
large compared to that of HPV 16 (2.5 times and 7.2 times,
respectively). Considering the controversial results relative
to linear relationship of viral load according to disease sever-
ity [29], a larger number of subjects will be needed to resolve
this issue. Second, we performed a cross-sectional study.
Although our results show that type-specific viral load is cor-
related to the pathologic grade of cervical neoplasia, these
results cannot be used to predict the risk of disease progress-
ion. Clinically, predicting the risk of lesion progression is
quite important, considering that a large proportion of cer-
vical intraepithelial neoplasia cases spontaneously regress.
Longitudinal studies that investigate both the load and phys-
ical state of HPV are needed to evaluate their usefulness in
predicting the risk of cervical cancer in the future. Third, it
is both a strong and a weak point that our subjects included
only those with a single type of infection, because significant
proportions of HSIL+ individuals have multiple infections.
Which factor, specific HPV type or viral load, is more reliable
in predicting HSIL+ needs to be examined in the future.
Lastly, we cannot draw any conclusions regarding the diag-
nostic value of HPV types other than 16, 18, and 58, as we
did not look into other types. Large-scale trials are necessary,
because the incidence of other types is low enough to present
a statistical challenge. We are now performing a multicenter
trial with the help of the Korean Gynecologic Oncology
Group to reach this goal. The technical development of
molecular testing of HPV using real-time PCR have resulted
in an increasingly advantageous method. Similarly to the
Cobas HPV test, recent HPV tests using real-time PCR can
provide multiple results in one test run with multiple fluo-
rescent dyes. Once the clinical relevance of viral load is con-
firmed, commercially useful HPV tests using viral load
information will be developed.

In conclusion, type-specific total viral load can be a useful
diagnostic biomarker of HSIL+, at least in HPV 16–, 18–, and
58–infected women. Collectively, our findings can help
increase the diagnostic accuracy of HPV tests, leading to a
paradigm shift in the management of equivocal cytologic
abnormalities in cervical cancer screening.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and
Treatment website (https://www.e-crt.org).

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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