



# Next-generation sequencing for typing human papillomaviruses and predicting multi-infections and their clinical symptoms

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## Abstract

Human papillomavirus (HPV) has more than 100 different types, some of which are associated with cancer. The most common example is that of cervical cancer, which is associated with HPV16 and HPV18. Here, we performed next-generation sequencing (NGS) to type 2436 samples obtained from Korean women to elucidate the correlation between multiple infections, virus types, and cytology. NGS revealed that types 58, 56, and 16 were the most common in high-risk (HR) types, whereas types 90, 54, and 81 were the most common in low-risk (LR) types. The incidence of atypical squamous cells of undetermined significance (ASCUS) or high-grade squamous intraepithelial lesion (HSIL) was 11.45% in single-type cases and 27.17% in multiple infections by the two types of HPV. ASCUS or HSIL was 29.79% in only the HR type multiple infections and 29.81% in mixed high- and low-risk types of multiple infections, whereas it was 18.79% in LR type multiple infections ( $P \leq 0.0001$ ). Co-infection by LR-HPV and HR-HPV is therefore more likely to cause cell lesions. Collectively, these results show that the higher the incidence of multiple infections, the greater the frequency of cell lesions. Thus, to predict the clinical symptoms, it would be beneficial to confirm the HPV type and multiple infections using NGS, although this could be relatively expensive.

## KEYWORDS

high-risk type, human papillomavirus, low-risk type, next-generation sequencing, pap smear

## 1 | INTRODUCTION

Human papillomavirus (HPV) has a double-stranded DNA genome with approximately 8000 nucleotides. The HPV genome consists of three regions: an upstream regulatory region, which includes a sequence that regulates translation and replication; an open reading frame (E1, E2, E4, E5, E6, and E7 genes) with various functions, such as trans-activation of translation, translation, and replication; and L1 and L2 capsid proteins, responsible for the viral structure.<sup>1–4</sup> Notably, the prevalence rate of HPV cervical infections varies significantly worldwide and is closely related to the corresponding risk of developing cervical cancer.<sup>5–9</sup>

HPV infection is associated with benign and malignant diseases such as condyloma acuminatum and respiratory papillomatosis (laryngeal papillomatosis), as well as gastrointestinal, cervical, anal, vulval, vaginal, penile, and head and neck cancers.<sup>10–13</sup> A high-risk (HR) HPV type (e.g., type 16 or type 18) is likely to cause cervical cell abnormalities that may eventually lead to cancer.<sup>14,15</sup> HR-HPVs were divided into 14 types presented by the CDC, and the remaining types were identified as low-risk (LR) HPVs.<sup>16,17</sup>

Representative methods for detecting HPV include the pap smear test, DNA microarray, and PCR.<sup>18–20</sup> The pap smear test is the most common screening test for HPV, but it has a high false-positive and false-negative rate and requires the examiner

**Abbreviations:** ASCUS, atypical squamous cells of undetermined significance; HPV, human papillomavirus; HR, high-risk; HSIL, high-grade squamous intraepithelial lesion; LR, low risk; LSIL, low-grade squamous intraepithelial lesion; NGS, next-generation sequencing.

to be highly proficient. The results obtained from these tests are divided into high-grade squamous intraepithelial lesions (HSIL), low-grade squamous intraepithelial lesions (LSIL), and atypical squamous cells of undetermined significance (ASCUS).<sup>21–23</sup> HPV detection using nucleic acids helps to diagnose quickly, easily, and accurately<sup>24</sup> and can be achieved by Southern and northern blots, in situ hybridization, signal amplification molecular technology, and DNA sequencing.<sup>21</sup> PCR can identify genotypes, but such identification is limited to only certain types. Because of the limitations of the commonly used HPV detection methods, platforms to sequence multiple types of HPV must be developed.<sup>21,25</sup> The genotyping of HPV by conventional sequencing is difficult because mixed peaks are observed in the sequencing data of patients with mixed infections.<sup>21,24</sup> Next-generation sequencing (NGS) technology can accurately detect different types simultaneously and overcome these limitations.<sup>26,27</sup>

In this study, the frequency of HPV types in Korean women was evaluated using an NGS panel, and the association between the number of HPV types and cell lesions was identified.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and samples

In this study, 2436 remnant samples were obtained from April to November 2019 for NGS testing after HPV typing was performed. These samples were anonymized and delivered by SML Genetree. Of the total subjects, 1357, 655, 262, 118, and 44 women were < 29, 30–39, 40–49, 50–59, and > 60 years old, respectively.

Swab samples (2 mL) were collected from each individual in specimen transportation tubes containing PBS and stored at –80°C to maintain DNA quality until further testing could be performed. This study was approved by the Institutional Review Board Committee of Ethics and Research of the Catholic University of Korea (1040395-202003-HBM-901).

### 2.2 | DNA preparation

HPV DNA was extracted from samples using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) according to the manufacturer's instructions, after which the DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Briefly, samples were extracted with lysis buffer and protease K. The DNA was bound to magnetic beads, washed with 99.9% ethanol, followed by two wash buffers, and stored at –80°C until analysis.

### 2.3 | Plasmid DNA synthesis

For analysis performance test and positive control production, plasmid DNA of different HPV types (3, 6, 10, 11, 16,

18, 26, 27, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44/55, 45, 51, 52, 53, 54, 56, 57, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 90, and 102) was prepared using an HPV-positive PCR product. The DNA fragments were purified from the HPV PCR product and inserted into plasmid DNA using the pGEM-T Easy Vector System (Promega) according to the manufacturer's protocol. Plasmid DNAs were extracted using the AccuPrep® plasmid mini extraction kit (Bioneer), and the DNA quantity was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Several plasmid dilutions were tested, varying from  $1 \times 10^2$  to  $1 \times 10^3$  copies/μL. A series of log dilutions of HPV plasmid DNAs was then prepared to establish an external positive control for NGS technology. The positive controls were stored as aliquots at –80°C until use.

### 2.4 | Detection of infected HPV DNA and HPV genotyping

A library was constructed by performing PCR using the Ezplex HPV NGS kit protocol (SML Genetree). This kit was modified by a one-tube nested PCR method using a modified GP5 + /GP6 + primer and a nested PCR primer inside. PCR was performed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific). The PCR conditions were: 95°C for 10 min; 3 cycles of denaturation at 95°C for 20 s, annealing at 45°C for 2 min, and extension at 72°C for 5 min; 32 cycles of denaturation at 97°C for 20 s, annealing at 55°C for 2 min, and extension at 72°C for 1 min, and a final single incubation at 72°C for 5 min. The primer has a barcode and an index, and each of the amplicons is designed to be made into a library and recognized by an NGS instrument. After PCR, 1 μL of each PCR product was added to 4560 μL of sterilized distilled water, and the final concentration was adjusted to 40 pmol. This pooled sample was tested (from the enrichment to the chip loading stage) on an Ion CHEF instrument (Thermo Fisher Scientific) using the manufacturer's protocol. Sequencing was performed on the Ion Torrent S5 XL (Thermo Fisher Scientific) using 520 chips. FastQ file data collection and processing were performed on an Ion Torrent server. Reads of less than 100 bases were filtered out on the S5 XL instrument as per the protocol. HPV genotypes were identified for direct automation. The analysis software (SML Genetree) exported HPV infection and infection type data to Microsoft Excel. The identified HPV types were displayed with their depth coverage and positive levels from 1+ to 3+.

### 2.5 | Detection of cell lesions

The cervical cancer test instrument was inserted into the vagina, sufficiently exposing the cervix. Next, cells were collected from the surface of the cervix using a small brush and fixed in 95% alcohol. The cells or cell suspension from the brush was

smear on a glass slide and observed under a microscope after Papanicolaou staining. The results of these pap smear tests were classified as HSIL, LSIL, and ASCUS, depending on the degree of cell lesions. Cytology results were compiled based on the results of the previous examination of each sample.

## 2.6 | Statistical analyses

The characteristics of subjects at baseline are expressed as numbers and percentages. The frequency of cell abnormalities according to HPV type(s) was examined using the chi-square test. All data were analyzed using SAS software, version 9.4 (SAS Institute). Results with  $P < 0.1$  were considered statistically significant.

## 3 | RESULTS

### 3.1 | Sample characteristics

In total, 2436 samples were collected from subjects of different age groups as follows: below the age of 29 years ( $n = 1357$ ), in their 30s ( $n = 655$ ), in their 40s ( $n = 262$ ), in their 50s ( $n = 118$ ), and in their 60s ( $n = 44$ ). Cytological data showed that 300 samples were positive for cytological lesions containing HSIL, LSIL, and ASCUS, while the remaining 2136 samples were negative. The NGS typing data revealed 289 cases of only HR type, 501 cases of only LR type, 426 cases where both HR and LR types were detected together, and 1220 negative cases. NGS typing results revealed 594 and 622 cases of mono-infections and mixed infections, respectively (Table 1). Of the 2436 samples, 262 were detected positive by both NGS and cytology, 38 were detected positive by cytology alone, and 954 were detected positive by NGS alone, whereas 1182 cases tested negative for both cytological lesions and by NGS (Figure 1).

### 3.2 | HPV type frequency detected by NGS typing

An NGS typing test was performed to identify HPV genotypes. In HR cases, types 58 ( $n = 149$ ; 15.27%), 56 ( $n = 107$ ; 10.96%), 16 ( $n = 103$ ; 10.55%), and 52 ( $n = 85$ ; 8.71%) were the most abundant. In LR cases, types 90 ( $n = 165$ ; 11.27%), 54 ( $n = 134$ ; 9.15%), 81 ( $n = 106$ ; 7.24%), 53 ( $n = 105$ ; 7.17%), and 62 ( $n = 94$ ; 6.42%) were the most abundant. Because the number of LR types was quite large, types 4, 55, 91, 30, 11, 34, 72, 86, 32, 74, 74, 74, 82, 26, 102, 71, 3, and 5 were collectively indicated as other types (Table 2).

### 3.3 | Cell abnormalities according to HPV type

Cytology data were organized in accordance with the HPV NGS typing data. There were 61 positive cases and 228 negative cases

TABLE 1 Sample characteristics ( $n = 2436$ )

Category		<i>n</i>	% <sup>a</sup>
Age	≤29	1357	55.71
	30–39	655	26.89
	40–49	262	10.76
	50–59	118	4.84
	≥60	44	1.81
Cytology	Positive	300	12.32
	Negative	2136	87.68
NGS	High-risk type	289	11.86
	Low-risk type	501	20.57
	High-risk + Low-risk types	426	17.49
	Negative	1220	50.08
	Negative for NGS	1220	50.08
Infection type	Single <sup>b</sup>	594	24.38
	Multi <sup>c</sup>	622	25.53
	Negative for NGS	1220	50.08
Total		2436	100

NGS, next-generation sequencing.

<sup>a</sup>Values are expressed as numbers and percentages (%).

<sup>b</sup>Single = Case where NGS typing results detected only one type of HPV infection.

<sup>c</sup>Multi = Case where NGS typing results detected more than one type of HPV infection.

among the samples infected only by HR-HPV (including single and multiple infections;  $n = 289$ ). Among the LR-HPV samples (including single and multiple infections;  $n = 501$ ), 64 cases were positive, and 437 cases were negative. Additionally, among the samples infected by both HR- and LR-HPV ( $n = 426$ ), 137 cases were positive, and 289 cases were negative ( $P \leq 0.0001$ ; Table 3).

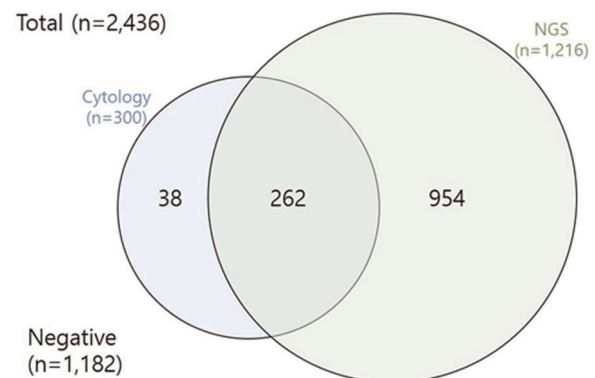


FIGURE 1 Venn diagram of human papillomavirus (HPV) test results using next-generation sequencing (NGS) and cytology [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/1348-0421.12927)]

TABLE 2 HPV type frequency in NGS typing test

High-risk types				Low-risk types			
HPV Type (14 types)	N	% <sup>a</sup> (N/Total sample)	% (N/Total HR)	HPV Type (35 types)	N	% <sup>a</sup> (N/Total sample)	% (N/Total LR)
58	149	6.12	15.27	90	165	6.77	11.27
56	107	4.39	10.96	54	134	5.50	9.15
16	103	4.23	10.55	81	106	4.35	7.24
52	85	3.49	8.71	53	105	4.31	7.17
66	80	3.28	8.20	62	94	3.86	6.42
51	76	3.12	7.79	84	77	3.16	5.26
39	68	2.79	6.97	70	72	2.96	4.92
68	60	2.46	6.15	43	70	2.87	4.78
18	53	2.18	5.43	6	70	2.87	4.78
35	49	2.01	5.02	67	69	2.83	4.71
59	46	1.89	4.71	87	59	2.42	4.03
45	36	1.48	3.69	42	57	2.34	3.89
31	33	1.35	3.38	40	55	2.26	3.76
33	31	1.27	3.18	Other <sup>b</sup>	331	13.59	22.61
Total	976	40.07	100.00	Total	1464	60.10	100.00

HPV, human papillomavirus; NGS, next-generation sequencing.

<sup>a</sup>The sum of % (N/Total sample) may be greater than 100 owing to multi-infections.

<sup>b</sup>Other low-risk types include 44, 55, 91, 30, 11, 34, 72, 89, 86, 32, 73, 114, 74, 61, 82, 83, 26, 69, 102, 71, 3, and 5.

### 3.4 | Association between HPV multi-infections and cell abnormalities

Cytology data in accordance with the infection types determined through HPV NGS typing are as follows: among the negative specimens determined by NGS ( $n = 1220$ ), 38 cases were detected to be positive by cytology, and 1182 cases were negative (Figure 1). Single-type infection samples ( $n = 594$ ) included 78 positive cases and 516 negative cases. Samples infected by two HPV types ( $n = 303$ ) included 77 positive cases and 226 negative cases, whilst those infected

by three abnormality types ( $n = 319$ ) included 107 positive cases and 212 negative cases ( $P \leq 0.0001$ ; Table 4).

### 3.5 | Cell abnormality according to HPV type in multiple infections

Among the samples multi-infected only by HR-HPV ( $n = 47$ ), 16 were positive, and 31 were negative. Among the samples multi-infected only by LR-HPV ( $n = 149$ ), 31 were positive, and 118 were negative. Moreover, among the samples multi-infected by both HR-HPV and LR-HPV ( $n = 426$ ), 137 were positive, and 289 were negative ( $P = 0.0258$ ; Table 5).

## 4 | DISCUSSION

This study confirmed the relationship and consistency between the results obtained from cytology and HPV genotyping tests. The HPV types in each sample were identified, and the ratio of each type was checked using the Ezplex HPV NGS kit, which allows us to identify 100 different types simultaneously. This helped us to overcome the limitation of the number of types that existing kits can detect. HPV genotype data were compared with the cytology test results when infected by HR, LR, or both HR and LR types. The rates of infection with LR-HPV and HR-HPV types were similar, but the rate of multiple infections with both HR-HPV and LR-HPV types was the highest.

In previous studies, the ratio of multiple infections appeared to be very low at about 10%,<sup>28,29</sup> or relatively high in certain groups, such as those with cell lesions with HSIL or more, or those infected with HIV.<sup>30,31</sup> However, out of the 2436 women in this study, 49.9% ( $n = 1216$ ) were HPV-positive, and half of these positive patients ( $n = 622$ ) were multiple infection cases. These results are in contrast to the results from a previous study, in which only approximately 10% of women who were HPV-positive had multiple infection types.<sup>28</sup> The difference may result from the detection of far more HPV types than in previous studies.<sup>28–35</sup>

Previous studies have shown that the HPV types 16, 52, and 56 among the HR types, and types 6, 53, 62, and 70 among the LR types were the most common. In addition, only HR types and approximately 20 LR types have been studied

TABLE 3 Cell abnormalities according to human papillomavirus (HPV) type

NGS typing results for HPV									
High-risk type ( <i>n</i> = 289)			Low-risk type ( <i>n</i> = 501)		High-risk + Low-risk type ( <i>n</i> = 426)		Total ( <i>n</i> = 1216)		
<i>N</i> %			<i>N</i> %		<i>N</i> %		<i>N</i> %		
Cytology result	Positive	61	21.11	64	12.77	137	32.16	262	21.55
	Negative	228	78.89	437	87.23	289	67.84	954	78.45
	Total	289	100.00	501	100.00	426	100.00	1216	100.00

$P \leq 0.0001$ .



**TABLE 4** Association between multiple infections and cell abnormalities

NGS typing results for HPV												
		Positive for NGS (n = 1216)										
		Negative for NGS (n = 1220)		Multi (≥2; n = 622)								
				Single <sup>a</sup> (n = 594)	Multi (=2; n = 303) <sup>b</sup>				Multi (≥3; n = 319) <sup>c</sup>		Total (n = 2436)	
					N		%		N	%		
Cytology result	Positive	38	3.11	78	13.13	77	25.41	107	33.54	300	12.32	
	Negative	1182	96.89	516	86.87	226	74.59	212	66.46	2136	87.68	
	Total	1220	100	594	100	303	100	319	100	2436	100	

HPV, human papillomavirus; NGS, next-generation sequencing.

<sup>a</sup>Single = Case where NGS detected only high-risk HPV.<sup>b</sup>Multi (=2) = Two types of infections in the NGS typing test.<sup>c</sup>Multi (≥3) = More than three types of infections in the NGS typing test.*P* ≤ 0.0001.**TABLE 5** Cell abnormalities according to HPV type in multiple infections

NGS typing results for HPV									
	High-risk type ( <i>n</i> = 47)		Low-risk type ( <i>n</i> = 149)		High-risk + low-risk type ( <i>n</i> = 426)		Total ( <i>n</i> = 622)		
	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%	
<b>Cytology result</b>	Positive	16	34.04	31	20.81	137	32.16	184	29.58
	Negative	31	65.96	118	79.19	289	67.84	438	70.42
	Total	47	100.00	149	100.00	426	100.00	622	100.00

HPV, human papillomavirus; NGS, next-generation sequencing.

*P* = 0.0591.

previously.<sup>28–36</sup> According to our results, the frequency of the HR types was similar to that described in previous studies,<sup>28–35</sup> however, the frequency of the LR types was very different, owing to the detection of 14 HR types and 35 LR types. Interestingly, type 90, which was rarely reported in previous studies, had the highest frequency.

Moreover, the association between HPV genotyping frequency and cytology testing results was confirmed. There were more cases of cell lesions, such as ASCUS or LSIL, and HSIL in cases of multiple infections (*n* = 426) of LR and HR types than in cases where only LR types (*n* = 149) were present. The frequencies of ASCUS, LSIL, and HSIL were higher when multiple genotypes of HPV were observed.

Taken together, previous studies identified only a small number of HPV types (10 to 46) at a time<sup>28–34</sup> and mostly focused on HR types, such as 16 and 18, and on some LR types, such as 53, 62, 66, and 70.<sup>28,32,35,36</sup> In contrast, this study identified 100 types simultaneously and detected LR types such as 54, 81, and 90, which had not been reported previously. However, further investigation is required to elucidate how this relates to actual disease status or progression. It has also been shown in this study that multiple infection cases in ASCUS, LSIL, and HSIL can be a marker of disease severity.

In conclusion, we investigated the frequencies of 100 HPV types simultaneously. Studying the association between various infection cases and actual diseases can help to identify not only cervical cancer but also to promote the early detection of risk factors for cancers.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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