Performance of HPV E6/E7 mRNA Genotyping Test on Paired Cervical Cancer Exfoliated Cells and Formalin Fixed Paraffin Embedded Tissues

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Investigation of human papillomavirus (HPV) in archival formalin-fixed paraffin-embedded (FFPE) material is important for understanding cervical carcinogenesis. The objective of the present study was to identify the high risk HPVs (HR-HPVs) using HPV E6/E7 mRNA testing from archival tissues in cervical cancer and the relation to HR-HPVs genotypes in paired cervical exfoliated cells. HPV E6/E7 mRNA testing and DNA chip testing were performed in 79 paired cervical FFPE tissues and exfoliated cells from women with histologically confirmed squamous cell carcinoma and adenocarcinoma. Overall agreement in HR-HPVs detection from FFPE samples and cytology samples were 98.5% in HPV 16, 100% in HPV 18, HPV 31, HPV 33, HPV 58, HPV 66, and HPV 68. Type-specific agreement between FFPE samples and cytology samples was 89.1% in HPV positive, 93.5% in HPV 16 and more than 70% in the other HR-HPVs. In conclusion, HR-HPVs were reliably detected in paired FFPE and cytology samples with some variation in type-specific detection.

Key Words: Cervical cancer; Ki67, HPV E6/E7, RT-qPCR, High risk HPVs

INTRODUCTION

More than 200 human papillomaviruses (HPV) genotypes have been identified to date. In particular, 14 genotypes (HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66, and HPV 68) have been classified as carcinogenic or high risk HPVs (HR-HPVs), and HPV 16 and HPV 18 are present in over 70% of all cervical cancers worldwide (Munoz et al., 2006; Munoz et al., 2003; de Sanjose et al., 2010; Kim et al., 2012).

The risks of developing cancer as well as the distribution of each of the 15HR-HPVs varies in high-grade cervical lesions and cancer (Carozzi et al., 2000; Quek et al., 2013; Tjalma et al., 2013). Diagnosis of HPV infection has been

performed from cervical cytology specimens and used in natural history studies, implementation of screening programs, and follow-up of vaccination studies (Wheeler, 2010; Tay and Tay, 2011; Lee et al., 2015). However, cytology specimens often contain larger numbers of HPV infections, most of which are thought to be transient infections, whereas it is assumed that a lesion is caused by one genotype (Mehlhorn et al., 2013; Ho et al., 1995; Lee, 2013; Lee et al., 2011). Thus, identification of HPV in tissue specimens is important to ascertain the causal type involved in HPV-related carcinogenesis.

Formalin-fixed paraffin-embedded (FFPE) tissue samples derived from cervical cancer lesion have advantages such as investigating cancer specific characteristics and identifying clinical outcome but have been limitation to PCR-based assays because formalin fixation induces fragmentation of the nucleic acids. Most HPV assays target the L1 gene and some other viral oncogenes E6/E7 (Kim et al., 2015). Amplicon sizes range from 65 base pairs (SPF10 primers) to 450 base pairs (PGMY09/11 primers). Several studies revealed the small amplicon length designed PCR such as SPF-LiPA (65 bp) and Inno-LiPA (65 bp) were successfully amplified from FFPE tissue samples (Castro et al., 2015; Dona et al., 2013; Castle et al., 2006; Tjalma and Depuydt, 2013; Gravitt et al., 2007).

In our previous study, the HPV E6/E7 mRNA test in cervical cytology was developed and evaluated (Wang et al., 2015; Munkhdelger et al., 2014). The amplicon size of HPV E6/E7 mRNA test is 70 bp, so it was assumed to be possible for studying HPV genotypes in FFPE tissue samples. Given the importance of accurately identifying HPV present in FFPE materials and the lack of studies comparing HPV assays in tissue specimens, our aim of this study was to evaluate the performance of HPV E6/E7 mRNA test on FFPE cervical biopsy samples and to compare the DNA chip results from paired cervical exfoliated cells.

MATERIALS AND METHODS

Study population

A total of 79 paired cervical FFPE tissues and exfoliated cells from women patients between the ages of 30 and 85 years (mean 55 years) were retrospectively obtained at the Department of Pathology, Yonsei University Wonju Severance Christian Hospital, Wonju, Republic of Korea, between January 2010 and December 2014. This study was approved by the Institutional Ethics Committee at Yonsei University Wonju College of Medicine (approval number: YWMR-12-4-010), and all subjects provided written informed consent.

Thin-layer slides were prepared using the Thin Prep 5000 Processor (Product Insight Inc., Acton, MA, USA) according to the manufacturer's instructions. The prepared slides were stained by the Papanicolaou method and evaluated according to the 2001 Bethesda System by certified cytopathologists. After preparing the cytology slide, 1 mL of solution was removed to a 1.5-mL Eppendorf tube for HPV DNA, and stored at -72℃ until used. The tissue biopsy blocks with matched cytology available patients were reviewed by two pathologists. Seventy one squamous cell carcinomas (89.9%) and eight adenocarcinomas (10.1%) were histologically confirmed. Three 10-μm sections from each paraffin block of cervical tissue were used for total RNA extraction (Table 1).

Nucleic acid extraction

DNA preparation was performed using HPV DNA Extraction Solution (M&D, Wonju, Korea), according to the manufacturer's instructions. Briefly, clinical specimens were collected and vortexed for about 1 min. The volume was adjusted to 40 mL with PBS (pH 7.2) and centrifuged at $2,000\times g$ at 4℃ for 30 min. The supernatant was discarded, and 300~500 μL of sterile distilled water was added to the pellet, and the mixture was vortexed and transferred to a 1.5-mL Eppendorf tube. The mixture was then centrifuged at $17,590\times g$ at 4℃ for 5 min, and the supernatant was discarded. DNA extraction solution (100 μL) was added to the pellet. The mixture was vortexed for 1 min, then incubated at 56℃ for 15 min with intermittent tube tapping. After incubation, samples were boiled for 10 min in a heating block or in a boiling water bath (recommended) and centrifuged at $17,590\times g$ for 3 min at 25℃. The supernatant (3~5 μL) was used as a template for PCR.

Three 10-μm sections from each paraffin block of cervical tissue were used for total RNA extraction. Qiagen RNeasy FFPE mini kit (Qiagen, Hilden, Germany) were used

according to the manufacturer's protocol. Purity and concentration of total RNA were determined by measuring absorbance at 260 and 280 nm using a spectrophotometer (Infinite 200, Tecan, Salzburg, Austria). All preparation and handling of total RNA was conducted in a laminar flow hood under RNase-free conditions. Isolated total RNA was stored at -70℃.

DNA chip

HPV genotyping via the Goodgene HPV chip (Goodgene Inc., Seoul, Korea) was carried out according to the manufacturer's recommendations. The genotyping method requires nested PCR to amplify the target region using the MY11 and MY9 primers, followed by the GP5/GP6 primer pair. The nested PCR conditions consist of an initial denaturation step for 5 min at 94℃, followed by 15 cycles consisting of denaturation for 30 s at 94℃ and extension for 30 s at 65℃. These cycles are followed by a subsequent 45 cycles consisting of 30 s at 94℃ and 30 s at 54℃. The final extension step occurs at 72℃ for 7 min. After PCR amplification of the target region, the subsequent steps were performed according to the manufacturer's recommendation. PCR products were loaded onto the probe-labeled glass Goodgene HPV Chip, and the resulting signal was read using a scanner.

HPV E6/E7 mRNA RT-qPCR assays

Complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen) according to the manufacturer's recommendations. Briefly, 10 μL total RNA was added to master mix containing 10 mM dNTP mix at neutral pH, 0.25 μg random hexamers, and 5 μL DEPC-treated water. Reactions were incubated at 65℃ for 5 min and chilled on ice. A mixture of 4 μL 5× First-Strand Buffer, 2 μL 0.1 M dithiothreitol, and 1 μL M-MLV reverse transcriptase (RT) was added, and cDNA synthesis was at 25℃ for 10 min, 37℃ for 50 min, and 70℃ for 15 min.

Detection of HPV E6/E7 mRNA in cervical specimens was performed using OPTIMYGENE HPV E6/E7 mRNA RT-qDx assay kits (Optipharm, Osong, Republic of Korea) based on RT-qPCR TaqMan assays using a CFX-96 real-time PCR system (Bio-Rad). Real-time PCR amplification

Table 1. Clinical characteristics on 79 paired cytology and FFPE samples

Characteristics	n (%)
Ages	
30~39 years	14 (17.7)
40~49 years	21 (26.6)
50~59 years	20 (25.3)
60~69 years	8 (10.1)
70~79 years	11 (13.9)
> 80 years	5 (6.3)
Cytology	
ASCUS	2 (2.5)
ASC-H	2 (2.5)
HSIL	13 (16.5)
SCC	51 (64.6)
Adenoma	4 (5.1)
Adeno-Squamous	1 (1.3)
Not done	6 (7.6)
Histology	
SCC	71 (89.9)
ADC	8 (10.1)
Total	79 (100)

Abbreviation: atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells - cannot exclude HSIL (ASC-H), high-grade squamous intraepithelial lesion (HSIL), Squamous cell carcinoma (SCC), adenocarcinoma (ADC), and adeno-squamous cell carcinoma (Adeno-Squamous)

of HPV E6/E7 mRNA was in 10 μL 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 μL primer and TaqMan probe mixture, 2 μL template cDNA, and DW to final volume 20 μL. Multiplex RT-qPCR assays detected HPV E6/E7 genes simultaneously in a single tube by incorporating three target-specific TaqMan probes labeled with different fluorophores (FAM, HEX, and Cy5).

Sequence analysis

The HPV E6/E7 gene region of HPV E6/E7 mRNA positive samples in FFPE cervical cancer tissue was sequenced for differentiating HPV genotypes. Primer sets used to amplify the target HPV E6/E7 gene were 60F-5'-CCGA-AAMCGGTKVRTATAAAAGCA-3' and 970R-5'-GT-ACCTKCWGGATCAGCCAT-3'. Amplified cDNA was

sequenced using an ABI Prism BigDye Terminator and ABI 3730 automated DNA sequencer (Cosmo Genetech, Seoul, Republic of Korea). Sequences were compared with the National Center for Biotechnology Information GenBank database for species assignment.

Statistical analyses

HPV genotyping concordance was tested by calculating overall and type-specific percentages of agreement. HPV genotyping results from FFPE specimens was compared to results from cytology specimens as described above, and by creating three categories of agreement (1) Concordant: the number of HPV positive and their specific types are same in both tests. (2) Compatible: the number of HPV positive is same but their specific types are different in both tests. (3) Discrepant: the number of HPV positive and their specific types are different in both tests. The agreement between concordant samples in FFPE and cytology samples was evaluated using the Cohen kappa statistic. Observed *P*-values of less than 0.05 were considered significant.

RESULTS

Cytologic and histologic diagnosis

The characteristics of seventy nine samples in this study

are shown in Table 1. A total of 79 cytology samples were diagnosed as 2 atypical squamous cells of undetermined significance (ASC-US) (2.5%), 2 atypical squamous cells - cannot exclude HSIL (ASC-H) (2.5%), 13 high-grade squamous intraepithelial lesion (HSIL) (16.5%), 51 squamous cell carcinoma (SCC) (64.6%), 4 adenocarcinoma (ADC) (5.1%), and 1 adenosquamous cell carcinoma (Adeno-Squamous) (1.3%) and 6 were not tested (7.6%). Among the 79 FFPE cervical tissue samples were 71 squamous cell carcinomas (89.9%) and 8 adenocarcinomas (10.1%) were histologically confirmed (Table 1).

Overall HPV prevalence in paired cervical cancer FFPE tissues and exfoliated cells

In order to identify the HPV genotypes presented in cervical cancer lesion, HPV E6/E7 mRNA expression and its genotypes in 79 cervical cancer FFPE tissues were performed by multiplex RT-qPCR and sequencing. Among the 79 cervical cancer tissues, 65 cases (82.3%) were HPV E6 E7 mRNA positive and 14 cases (17.7%) were HPV mRNA E6/E7 negative. Specifically, HPV 16 and HPV 18 accounted for 68.4% (54/79) and 10 HR-HPVs accounted for 13.9% (11/79), respectively. In paired cytology samples, the prevalence of HPV 16 and HPV 18 were identical (68.4%). The prevalence of 10 HR-HPVs and HPV negative in cytology

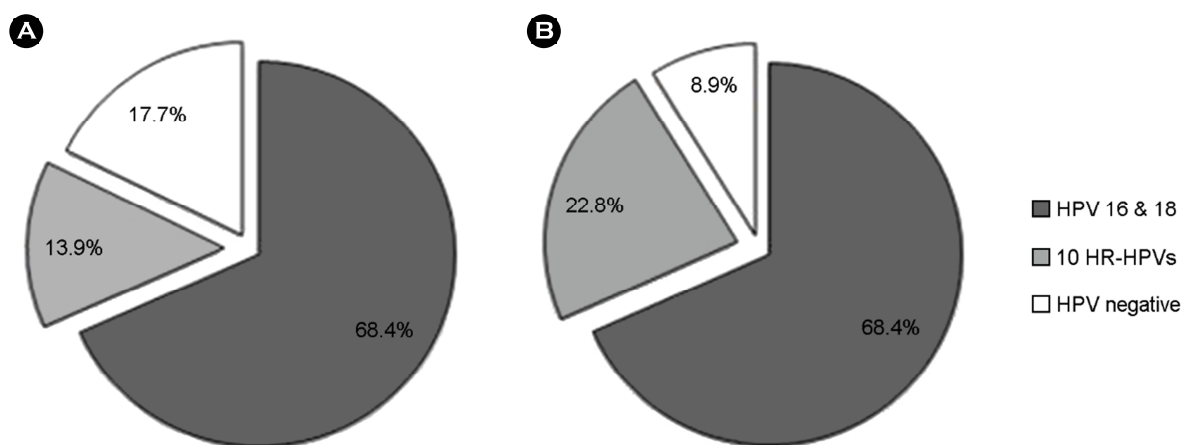**Fig. 1. Overall HPV prevalence in paired FFPE samples (A) and cytology samples (B).** In 79 cervical cancer FFPE tissues, HPV 16 and HPV 18 accounted for 68.4% (54/79) and 10 HR-HPVs and HPV negative accounted for 13.9% (11/79) and 17.7% (14/79), respectively. In paired cytology samples, the prevalence of HPV 16 and HPV 18 were 68.4% (54/79) and 10 HR-HPVs and HPV negative accounted for 22.8% (18/79) and 8.9% (7/79).

samples accounts for 22.8% and 8.9%, respectively (Fig. 1).

Prevalence of type-specific HR-HPVs in paired cervical cancer FFPE tissues and exfoliated cells

The most prevalent genotypes in cervical FFPE cancer tissues were HPV 16 (57.0%), HPV 18 (7.6%), HPV 33 (5.1%), HPV 58 (3.8%), HPV 31 (2.5%), HPV 66 (1.3%), and HPV 68 (1.3%), respectively. Three cases (3.8%) were multi-infection including HPV 16 with 2 cases (2.5%) and HPV 18 with 1 case (1.3%). The prevalence of genotypes in paired cytology samples was similar to those in FFPE cancer tissues. The most prevalent type was HPV 16 (57.0%) and followed by HPV 18 (8.9%), HPV 33 (3.8%), HPV 58 (2.5%), HPV 31 (2.5%), HPV 66 (2.5%), and HPV 68 (1.3%). Among the HR-HPVs, one of HPV 39, HPV 45, and HPV 56 each (1.3%, respectively) were detected. Four cases of other HPVs (other than 16 HR-HPVs, 5.1%) were detected (Table 2).

Comparison of HPV genotypes in paired FFPE cervical tissues and cervical exfoliated cells

The paired cervical exfoliated cells among 65 HPV positive cervical cancer FFPE tissues were shown 64 HPV positive cases (98.5%), except one HPV negative exfoliated cells in HPV 16 positive cervical cancer tissues. The result of the HPV positive between FFPE samples and cytology samples was shown good agreement (Cohen kappa test =

0.95; concordant rate = 98%). To be specific, type-specific agreement between FFPE samples and cytology samples was 89.1% (57/64) in HR-HPVs positive, 93.5% (43/46) in HPV 16, 71.4% (5/7) in HPV 18, 75% (3/4) in HPV 33,

Table 2. HPV genotyping results of paired FFPE samples and cytology samples

	FFPE samples		Cytology samples	
	Cases, n	%	Cases, n	%
Single infection	62	78.5%	69	87.3%
HPV 16	45	57.0%	45	57.0%
HPV 18	6	7.6%	7	8.9%
HPV 31	2	2.5%	2	2.5%
HPV 33	4	5.1%	3	3.8%
HPV 58	3	3.8%	2	2.5%
HPV 66	1	1.3%	2	2.5%
HPV 68	1	1.3%	1	1.3%
HPV 39	0	0.0%	1	1.3%
HPV 45	0	0.0%	1	1.3%
HPV 56	0	0.0%	1	1.3%
Other	0	0.0%	4	5.1%
Multi infection	3	3.8%	3	3.8%
HPV 16 included	2	2.5%	1	1.3%
HPV 18 included	1	1.3%	1	1.3%
HPV negative	14	17.7%	7	8.9%
Total	79	100.0%	79	100.0%

Table 3. Comparison of HPV genotypes in paired FFPE cervical tissues and cervical exfoliated cells

	HPV positive cases in paired cervical exfoliated cells (%)	Concordant cases with cervical exfoliated cells	Compatible cases with cervical exfoliated cells
HPV positive	64/65 (98.5)	57/64 (89.1)	7/64 (10.9)
HPV 16[*]	46/47 (97.9)	43/46 (93.5)	3/46 (6.5)
HPV 18[*]	7/7 (100)	5/7 (71.4)	2/7 (28.6)
HPV 31	2/2 (100)	2/2 (100)	0/2 (0)
HPV 33	4/4 (100)	3/4 (75.0)	1/4 (25.0)
HPV 58	3/3 (100)	2/3 (66.7)	1/3 (33.3)
HPV 66	1/1 (100)	1/1 (100)	0/1 (0)
HPV 68	1/1 (100)	1/1 (100)	0/1 (0)
HPV negative	6/14 (42.9)	6/6 (100)	0/14 (0)

*HPV multi-infection were included in HPV16 and HPV18 each

66.7% (2/3) in HPV 58, and 100% in HPV 31, HPV 66, and HPV 68, respectively. The compatible cases in paired cervical exfoliated cells and cancer FFPE cells were 7 cases (10.9%) but the detected HPV genotypes were involved in 12 HR-HPVs (Table 3).

HPV negative in cervical cancer FFPE tissues

Among the 14 HPV negatives cases in cervical cancer FFPE tissues, cytological diagnosis and its HPV genotypes were compared. Seven cases were shown HPV negative in cervical exfoliated cells. HPVs in seven cases cervical exfoliated cells were detected; two HPV16, HPV 18, HPV 39, HPV52, 66, 68, and three other HPVs were detected. There were no tendency according to ages and cytological diagnosis (Supplementary Data, Table 1). The HPV negative rates in high grade cervical lesions among women ranged from 6.2% to 18.2%. Regardless of geographic area and sample sizes, approximately 10% HPV negative were reported (Supplementary Data, Table 2).

DISCUSSION

Several studies have evaluated the performance of HPV detection from FFPE specimens, but few studies have been compared HPV detection on FFPE samples and paired cytological samples. In the present study, we observed good overall performance for two methods in the detection of HPV from paired FFPE and cytological samples. Our findings are very much in agreement with other reports indicating a good performance of HPV E6/E7 mRNA testing (Kim et al., 2007). Castro et al. showed the 81.7% to 91.7% agreement using four different HPV genotyping methods in same FFPE samples (Castro et al., 2015). Our data also showed similar results (82.3% HPV positive in FFPE samples) to Castro et al., and importantly, oncogenic E6/E7 types of HPV in this study were detected compared to L1 gene based studies. There were some variations of HPV genotypes in three samples for HPV 16 and two samples for HPV 18. The short amplicon size and different collected lesions from cervix such as FFPE samples collected from cancer tissue and cytology samples collected from cervical exfoliated cells may lead to misclassifications of genotypes.

Overall HPV prevalence in the FFPE specimens was lower than in cytology specimens, although the sample size was limited for a formal test. In order to overcome the limitation of the fragmented FFPE samples, the amplicon size of HPVs was designed to be 90 bp and endogenous control GAPDH was designed to be 70 bp. GAPDH CT of the 14 HPV negative cases in FFPE samples ranged from 19.4 to 31.2 (Mean 24.6 ± SD 3.2) was shown valid samples for PCR In addition to amplicon size factor, Donà et al. demonstrated that the sample less detection compared to cytology test, RNA fragmentation by xylene treatment as well as the presence of paraffin, can impact PCR amplification efficiency.

Moreover, in terms of epidemiology, 6.2% to 18.2% (approximately 10%) HPV negative cases in high grade cervical lesions were reported in several studies. Our results also indicated that 8.9% and 17.7% of HPV negative cases in cytology and histology samples, respectively. In this study, the 7 cases (8.9%) were both HPV negative in paired cytology and histology samples. Moreover, Rodriguez-Carunchio et al recently reported that HPV negative patients were associated with poor prognosis compared to HPV positive patients (Rodriguez-Carunchio et al., 2015). Therefore, detection of HPV in cervical cancer is highly important, but other molecular markers complementing diagnosis of cervical cancer will be needed.

Conflict of interest
None.

REFERENCES

Carozzi F, Ronco G, Confortini M, Noferini D, Maddau C, Ciatto S, Segnan N. Prediction of high-grade cervical intraepithelial neoplasia in cytologically normal women by human papillomavirus testing. British Journal of Cancer. 2000. 83: 1462-1467.

Carozzi M, Tornesello L, Burroni E, Loquercio G, Carillo G, Angeloni C, Scalisi A, Macis R, Chini F, Buonaguro M, Rossi G, Grp W. Prevalence of human papillomavirus types in high-grade cervical intraepithelial neoplasia and cancer in italy. Cancer Epidemiology Biomarkers & Prevention. 2010. 19: 2389-2400.

Castle E, Sadorra M, Garcia F, Holladay B, Kornegay J. Pilot study

of a commercialized human papillomavirus (HPV) genotyping assay: comparison of HPV risk group to cytology and histology. J Clin Microbiol. 2006. 44: 3915-3917.

Castro A, Koshiol J, Quint W, Wheeler M, Gillison L, Vaughan M, Kleter B, Van J, Chaturvedi K, Hildesheim A, Schiffman M, Wang S, Zuna E, Walker L, Dunn T, Wentzensen N. Detection of HPV DNA in paraffin-embedded cervical samples: a comparison of four genotyping methods. BMC Infect Dis. 2015. 15: 544.

De sanjose S, Quint G, Alemany L, Geraets T, Klaustermeier E, Lloveras B, Tous S, Felix A, Bravo E, Shin R, Vallejos S, De ruiz A, Lima A, Guimera N, Clavero O, Alejo M, Llombart-Bosch A, Cheng C, Tatti A, Kasamatsu E, Iljazovic E, Odida M, Prado R, Seoud M, Grce M, Usubutun A, Jain A, Suarez A, Lombardi E, Banjo A, Menendez C, Domingo J, Velasco J, Nessa A, Chichareon C, Qiao L, Lerma E, Garland M, Sasagawa T, Ferrera A, Hammouda D, Mariani L, Pelayo A, Steiner I, Oliva E, Meijer J, Al-jassar F, Cruz E, Wright C, Puras A, Llave L, Tzardi M, Agorastos T, Garcia V, Clavel C, Ordi J, Andujar M, Castellsague X, Sanchez I, Nowakowski M, Bornstein J, Munoz N, Bosch X. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010. 11: 1048-1056.

Dona G, Ronchetti L, Giuliani M, Carosi M, Rollo F, Congiu M, Mazza D, Pescarmona E, Vocaturo A, Benevolo M. Performance of the linear array HPV genotyping test on paired cytological and formalin-fixed, paraffin-embedded cervical samples. J Mol Diagn. 2013. 15: 373-379.

Gravitt E, Van doorn J, Quint W, Schiffman M, Hildesheim A, Glass G, Rush B, Hellman J, Sherman E, Burk D, Wang S. Human papillomavirus (HPV) genotyping using paired exfoliated cervicovaginal cells and paraffin-embedded tissues to highlight difficulties in attributing HPV types to specific lesions. J Clin Microbiol. 2007. 45: 3245-3250.

Ho Y, Burk D, Klein S, Kadish S, Chang J, Palan P, Basu J, Tachezy R, Lewis R, Romney S. Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. J Natl Cancer Inst. 1995. 87: 1365-1371.

Kim GH, Kim SH, Park SJ, Park SJ, Han L, Yubo R, Li Y, Lee IS, Lee JD, Lee DS. Human papillomavirus prevalence and distribution according to age among korean and chinese women. Korean J Clin Lab Sci. 2015. 47: 259-266.

Kim SH, Lee DS, Kim Y, Kim GH, Park SJ, Choi YI, Kim TU, Park KW, Lee HY. Clinical evaluation of human papilloma-

virus DNA genotyping assay to diagnose women cervical cancer. Biomedical Science Letters. 2012. 18: 123-130.

Kim YG, Seo CW, Kim SH, Park YP. Analysis of telomerase activity by HPV E6/E7 expression in SW13. Korean J Clin Lab Sci. 2007. 39: 1-6.

Lee DS, Kim GH, Kim SH, Park SY, Wang HY, Park SJ, Lin H, Ren Y, Yingxue L, Park KW, Lee HY. Human papillomavirus distribution among women in western shandong province, east china using reverse blot hybridization assay. Biomedical Science Letters. 2015. 21: 32-39.

Lee KE. Down-regulation of miR-34a expression in cervical intraepithelial neoplasia with human papillomavirus infection and its relationship with p53 expression. Biomedical Science Letters. 2013. 19: 348-352.

Lee YJ, Jung JH, Park SJ, Jung DY. The relationship between papanicolaou smear test and human papilloma virus DNA chip test in the uterine cervix. Korean J Clin Lab Sci. 2011. 43: 26-32.

Mehlhorn G, Obermann E, Negri G, Bubendorf L, Mian C, Koch M, Sander H, Simm B, Lutge M, Banrevi Z, Weiss A, Cathomas G, Hilfrich R, Wilhelm M, Griesser H. HPV L1 detection discriminates cervical precancer from transient HPV infection: a prospective international multicenter study. Mod Pathol. 2013. 26: 967-974.

Munkhdelger J, Choi Y, Lee D, Kim S, Kim G, Park S, Choi E, Jin H, Jeon Y, Lee H, Park H. Comparison of the performance of the NucliSENS EasyQ HPV E6/E7 mRNA assay and HPV DNA chip for testing squamous cell lesions of the uterine cervix. Diagn Microbiol Infect Dis. 2014. 79: 422-427.

Munoz N, Bosch X, De S, Herrero R, Castellsague X, Shah V, Snijders J, Meijer J. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003. 348: 518-527.

Munoz N, Castellsague X, De B, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine. 2006. 24 Suppl 3: S3/1-10.

Oh K, Alemany L, Suh I, Rha H, Munoz N, Bosch X, Quint W, Lloveras B, Klaustermeier E, De sanjose S, Shin R. Type-specific human papillomavirus distribution in invasive cervical cancer in Korea, 1958-2004. Asian Pac J Cancer Prev. 2010. 11: 993-1000.

Quek C, Lim K, Domingo E, Soon R, Park S, Vu V, Tay H, Le T, Kim T, Vu Q, Cao T, Limson G, Pham T, Molijn A, Ramakrishnan G, Chen J. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical

intraepithelial neoplasia across 5 countries in asia. International Journal of Gynecological Cancer. 2013. 23: 148-156.

Rodriguez L, Soveral I, Steenbergen D, Torne A, Martinez S, Fuste P, Pahisa J, Marimon L, Ordi J, Del M. HPV-negative carcinoma of the uterine cervix: a distinct type of cervical cancer with poor prognosis. BJOG. 2015. 122: 119-127.

Tay K, Tay K. The impact of cytology screening and HPV vaccination on the burden of cervical cancer. Asia Pac J Clin Oncol. 2011. 7: 154-159.

Tjalma A, Depuydt E. Cervical cancer screening: which HPV test should be used--L1 or E6/E7? Eur J Obstet Gynecol Reprod Biol. 2013. 170: 45-46.

Tjalma A, Fiander A, Reich O, Powell N, Nowakowski M, Kirschner B, Koiss R, O'leary J, Joura A, Rosenlund M, Colau B, Schledermann D, Kukk K, Damaskou V, Repanti M, Vladareanu R, Kolomiets L, Savicheva A, Shipitsyna E, Ordi J, Molijn A, Quint W, Raillard A, Rosillon D, De souza C, Jenkins D, Holl K. Differences in human papillomavirus type distribution in high-grade cervical intraepithelial neoplasia and invasive cervical cancer in Europe. Int J Cancer. 2013. 132: 854-867.

Wang Y, Park S, Kim S, Lee D, Kim G, Kim Y, Park H, Lee H. Use of hTERT and HPV E6/E7 mRNA RT-qPCR TaqMan assays in combination for diagnosing high-grade cervical lesions and malignant tumors. Am J Clin Pathol. 2015. 143: 344-351.

Wheeler M. HPV genotypes: implications for worldwide cervical cancer screening and vaccination. Lancet Oncol. 2010. 11: 1013-1014.

Supplementary

Table 1. Characteristics of HPV negative cervical cancers in histological and cytological diagnosis

HPV negative cervical tissues	Histologic diagnosis	Ages	Cytological diagnosis	HPV genotypes from cervical exfoliated samples
Patient 001	Invasive squamous cell carcinoma	48	SCC	Negative
Patient 002	Adenocarcinoma, poorly differentiated	49	ADC	Negative
Patient 003	Invasive squamous cell carcinoma	56	SCC	Negative
Patient 004	Invasive adenocarcinoma	57	–	Negative
Patient 005	Endocervical adenocarcinoma	62	ADC	Negative
Patient 006	Invasive squamous cell carcinoma	69	SCC	Negative
Patient 007	Invasive squamous cell carcinoma	34	SCC	Other
Patient 008	Squamous cell carcinoma	84	H-SIL	Other
Patient 009	Invasive squamous cell carcinoma	85	H-SIL	Other
Patient 010	Invasive squamous cell carcinoma	52	SCC	16
Patient 011	Invasive squamous cell carcinoma	56	SCC	16
Patient 012	Invasive squamous cell carcinoma	71	SCC	18
Patient 013	Invasive squamous cell carcinoma	35	SCC	39
Patient 014	Microinvasive squamous cell carcinoma	40	SCC	52, 66, 68

Abbreviation: high-grade squamous intraepithelial lesion (HSIL), Squamous cell carcinoma (SCC), and adenocarcinoma (ADC)
*Other: universal HPVs is detected, but not included 12 HR-HPVs

Table 2. Prevalence of HPV negative cervical cancers in reference study

Authors	Types of samples	Geographic area	Sample size, N	HPV negative rates, %	Reference
This study	Cervical cancer cells (cytology/histology)	South Korea	79	8.9 (7/79)/ 17.7 (14/79)/	
Sanjose et al.	Invasive cervical cancers	38 countries	10,575	15 (1,598/10,575)	(de Sanjose et al., 2010)
Tjalma et al.	High grade cervical intraepithelial neoplasia Invasive cervical cancer	Europe	3,162	9.2 (259/3,162)	(Tjalma et al., 2013)
Quek et al.	CIN 2/3/AIS, invasive cervical cancer	5 countries (South Korea)	500	6.2 (12/145)	(Quek et al., 2013)
Carozzi et al.	CIN2, CIN3, invasive cervical cancer	Italy	1,162	6.9 (80/1,162)	(Carozzi et al., 2010)
Oh et al.	Invasive cervical cancer	South Korea	742	9.2 (68/742)	(Oh et al., 2010)

Abbreviation: Cervical intraepithelial neoplasia (CIN), adenocarcinoma in situ (AIS)