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

Sunyoung Park1, Hyeyoung Wang2, Sunghyun Kim3, Geeyuk Kim1, Sungyoung Bong1, Hyoungsoo Jang1, Sangjung Park4, Kooyeon Hwang5 and Dongsup Lee6,†

1Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju, Gangwon 26493, Korea
2M&D, Inc., Wonju Eco Environmental Technology Center, Wonju, Gangwon 26493, Korea
3Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 46252, Korea
4Department of Biomedical Science, College of Life and Health Sciences, Hoseo University, Asan, Chungcheong 31066, Korea
5Department of Clinical Laboratory Science, Seoyeong University, Gwangju 61268, Korea
6Department of Clinical Laboratory Science, Hyejeon College, Hongseong, Chungnam 32244, Korea

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°C 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×g at 4°C 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×g at 4°C 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°C 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×g for 3 min at 25°C. 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°C.

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°C, followed by 15 cycles consisting of denaturation for 30 s at 94°C and extension for 30 s at 65°C. These cycles are followed by a subsequent 45 cycles consisting of 30 s at 94°C and 30 s at 54°C. The final extension step occurs at 72°C 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°C 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°C for 10 min, 37°C for 50 min, and 70°C 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 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-AAMCGTKVRTATAAAAGCA-3′ and 970R-5′-GT-ACCTKCWGGATCAGCCAT-3′. Amplified cDNA was

| 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**            |                  |
| ASC-US                  | 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)
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) and HPV negative accounted for 17.7% (14/79), 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).](image-url)

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,

| HPV genotype | FFPE samples | Cytology samples |
|--------------|--------------|------------------|
| Single infection | 62 | 69 |
| HPV 16 | 45 | 45 |
| HPV 18 | 6 | 7 |
| HPV 31 | 2 | 2 |
| HPV 33 | 4 | 3 |
| HPV 58 | 3 | 2 |
| HPV 66 | 1 | 1 |
| HPV 68 | 1 | 1 |
| HPV 39 | 0 | 1 |
| HPV 45 | 0 | 1 |
| HPV 56 | 0 | 1 |
| Other | 0 | 4 |
| Multi infection | 3 | 3 |
| HPV 16 included | 2 | 1 |
| HPV 18 included | 1 | 1 |
| HPV negative | 14 | 7 |
| Total | 79 | 79 |

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

| 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.

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