International Journal of Infectious Diseases 78 (2019) 22–30

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Diagnostic performance of the E6/E7 mRNA-based Optimygene HR-HPV RT-qDx assay for cervical cancer screening

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ARTICLE INFO

Article history:
Received 31 August 2018
Received in revised form 18 September 2018
Accepted 18 September 2018
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Cervical cancer
Human papillomavirus
E6/E7 mRNA
DNA
Reverse transcriptase quantitative PCR
Molecular diagnosis

ABSTRACT

Objectives: Pap smear and high-risk human papillomavirus (HR-HPV) DNA testing are the most widely applied methods for cervical cancer screening, but both methods are limited by their low specificity and lack of association with patient prognoses. The aim of this study was to compare the clinical and prognostic significance of HPV E6/E7 mRNA as an early biomarker with cytology and HPV DNA detection in cervical cancer screening.

Methods: This study evaluated the performance of the Optimygene HR-HPV RT-qDx assay, which is an HPV E6/E7 mRNA-based assay, to detect 16 HR-HPV subtypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69. The clinical evaluation was conducted using 563 ThinPrep liquid-based cytology samples and the results were compared to those of cytological and histological diagnoses and HPV DNA testing.

Results: The clinical sensitivity and specificity of the Optimygene HR-HPV RT-qDx assay for the detection of high-grade lesions, according to cervical cytology, were 92.4% (95% confidence interval (CI) 0.9167–0.9972, $p < 0.0001$) and 96.9% (95% CI 0.8632–0.9524, $p < 0.0001$), respectively; they were 85.9% (95% CI 0.7631–0.9211, $p < 0.0001$) and 82.5% (95% CI 0.7491–0.8825, $p < 0.0001$), respectively, for CIN2+. This assay showed a higher specificity and positive predictive value for cytological and histological diagnosis than HPV DNA testing. Overall, the agreement between the Optimygene HR-HPV RT-qDx assay and HPV DNA testing in cytological and histological diagnosis was 87.9% ($\kappa = 0.76$, 95% CI 0.7054–0.8128, $p < 0.0001$) and 90.5% ($k = 0.81$, 95% CI 0.7338–0.8878, $p < 0.0001$), respectively. In this study, the most frequently detected HPV genotypes among HR-HPV-positive women were HPV 16 (37.9%), HPV 33–58 (21.5%), and HPV 18 (11.4%).

Conclusions: These findings suggest that the higher specificity and positive predictive value of the Optimygene HR-HPV RT-qDx assay are valuable for predicting insignificant HPV DNA infections among patients with a borderline cytological diagnosis. This assay could be used to prevent unnecessary biopsy procedures and the over-referral of patients with transient HPV infections, as well as reduce patient anxiety during the follow-up period.

Introduction

Although effective screening programs have reduced the incidence and the mortality of cervical cancer, this disease remains the third most common malignancy after breast cancer or colon cancer in women worldwide. Human papillomavirus (HPV) infection has been found to play a crucial role in causing cervical pre-cancer and invasive cervical carcinoma (ICC) if left untreated. Cervical intraepithelial neoplasia (CIN) and ICC are caused by persistent infection with oncogenic HPV genotypes that belong to a few phylogenetically related high-risk (HR) species (Walboomers et al., 1999; Zeng et al., 2016). Therefore, testing for oncogenic HPV infection in cervical lesions may be an accurate means of identifying women who are at risk of developing cervical cancer. Oncogenic HPV genotypes including HPV 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are detected in over 99.7% of cervical cancer cases. HPV 16 and 18 are the most prevalent types

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https://doi.org/10.1016/j.ijid.2018.09.014

associated with cervical cancer worldwide, causing more than 70% of cases (Zeng et al., 2016).

The most common screening methods for cervical cancer are liquid-based cytology or the Papanicolaou (Pap) test, in which cells are collected using a cervical swab and then analyzed under a microscope to detect premalignant and malignant lesions (Killeen et al., 2014). Pap screening is highly specific but shows low sensitivity for high-grade lesions (CIN2, CIN3, and squamous cell carcinoma (SCC)); therefore, multiple tests must be performed for each case. As HPV cannot be cultured, current tests for HPV infection rely on the detection of viral nucleic acids in the infected region. HPV DNA analysis is conducted as an adjunctive test for all cervical cytology samples in women over 30 years of age according to current recommendations (Cox, 2009). Pap smear and HPV DNA testing are currently the methods most widely applied in screening for cervical lesions, but both methods are limited by a low specificity and lack of association with patient prognoses (Schiffman and Solomon, 2009). Therefore, an effective biomarker and new method of non-invasive screening for the diagnosis of cervical lesions, particularly for the early detection of pro-malignant CIN, is important for improving the diagnosis and treatment of cervical cancer (Fan and Shen, 2018).

A commercial diagnostic kit for cervical cancer screening based on HPV E6/E7 mRNA expression has recently been introduced (Coquillard et al., 2011; Binnicker et al., 2014; Munkhdelger et al., 2014). The E6 and E7 proteins stimulate the cell cycle by binding to and inactivating cellular p53 and pRb, leading to their degradation via the proteosomal pathway and ultimately leading to cervical cancer (McLaughlin-Drubin and Münger, 2009; Brimer and Vande Pol, 2014). These two oncogenes are uniformly retained and highly expressed in cervical cancer cells, and their continuous expression is required to maintain the tumorigenic phenotype.

This pilot study was conducted to assess the analytical and clinical performance of the Optimygene HR-HPV RT-qDx assay (Optipharm, Osong, Republic of Korea) using multiplex qRT-PCR, as a potential screening method to improve the accuracy and predictability of the diagnosis of high-grade lesions and cervical cancer.

Materials and methods

Clinical samples

Liquid-based cytology samples (Table 1) from 563 women were obtained over a 1-year period (from July 2013 to June 2015) from the Department of Pathology, Yonsei University Wonju Severance Christian Hospital, Wonju, Republic of Korea. This study was approved by the institutional review boards of the participating hospitals. All clinical samples were collected using ThinPrep Pap materials (Hologic, Inc., Bedford, MA, USA).

Cytological and histological diagnosis

The clinical diagnosis of liquid-based cytology slides (Pap smears) was performed primarily by cytopathologists using the 2001 Bethesda System terminology and evaluated according to the 2001 Bethesda System terminology (Solomon et al., 2002) designed by two cytopathologists. Cases with available tissue biopsies were reviewed by two pathologists. Cytological cases of SCC, high-grade squamous intraepithelial lesion (HSIL), atypical squamous cells – cannot exclude HSIL (ASC-H), low-grade squamous intraepithelial lesion (LSIL), atypical squamous cells of undetermined significance (ASC-US), and normal (within normal limits, including reactive change due to inflammation, fungal infection, and atrophy) were included. High-grade lesions in this study (that is, lesions in which the cells appeared very different from normal cells and classified as CIN grade 2 or 3) were those showing major cytological abnormalities especially closely related to cervical cancer and included SCC, HSIL, and ASC-H. After preparing the Pap slide, 1 ml of an exfoliated cervical cell sample was transferred to each of two 1.5-ml microcentrifuge tubes and stored at −70 °C until use.

Table 1
Clinical samples used in this study.

Cytological diagnosis	Age-range (median ± SD)	Number of samples (%)	Histological diagnosis	Number of samples (%)
SCC	32–83 (55 ± 14.1)	51 (9.1)	SCC	19 (8.6)
HSIL	21–84 (47 ± 15.1)	87 (15.5)	SCC	7 (3.2)
			CIN3	18 (8.2)
			CIN2	3 (1.4)
			CIN1	1 (0.5)
ASC-H	57–74 (67 ± 8.7)	20 (3.6)	SCC	3 (1.4)
			CIN3	4 (1.8)
			CIN2	4 (1.8)
			CIN1	5 (2.3)
LSIL	20–60 (45 ± 11.4)	121 (21.5)	SCC	6 (2.7)
			CIN3	8 (3.6)
			CIN2	7 (3.2)
			CIN1	36 (16.4)
ASC-US	25–83 (47 ± 11.5)	157 (27.9)	SCC	3 (1.4)
			CIN3	10 (4.5)
			CIN2	2 (0.9)
			CIN1	33 (15.0)
			Normal	1 (0.5)
Normal	24–78 (41 ± 10.6)	127 (22.6)	Normal	50 (22.7)
Total		563 (100)		220 (100)

SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia.

Total RNA isolation

After cytology slide preparation, the remaining specimen was used for RNA isolation. Total cellular RNA was isolated using Isol-RNA Lysis Reagent (5 Prime, Austin, TX, USA) according to the manufacturer's instructions. Briefly, 1 ml of Isol-RNA Lysis Reagent was added to the cell pellet. Cells were lysed by vortexing or repeated pipetting and left to stand at room temperature for 5 min. Subsequently, 200 μl of chloroform was added and the mixture was shaken vigorously and incubated at room temperature for 3 min before centrifugation at 12 000 × *g* for 15 min. The resulting aqueous layer was transferred to a new tube and an equal volume of isopropanol was added and mixed by inverting the tube. After incubation for 10 min at 25 °C and centrifugation at 12 000 × *g* for 10 min, 1 ml of 75% ethanol was added to the supernatant and mixed by inverting the tube. Finally, the mixture was centrifuged at 7500 × *g* for 5 min and the supernatant was removed. The RNA pellet was dried and eluted in 30 μl of diethylpyrocarbonate-treated water (Intron Biotechnology, Seoul, Republic of Korea). The purity and concentration of total RNA were determined by measuring the absorbance at 260 nm and 280 nm using an Infinite 200 spectrophotometer (Tecan, Vienna, Austria). All steps in the preparation and handling of total RNA were conducted in a laminar flow hood under RNase-free conditions. The isolated total RNA was stored at −70 °C until use.

cDNA synthesis

cDNA was synthesized using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen) according to the manufacturer's recommendations. Briefly, 10 μl of total RNA was added to Master Mix containing 1 μl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP at a neutral pH), 0.25 μg of random hexamers, and 5 μl of diethylpyrocarbonate-treated water in PCR tubes. The reaction mixture was incubated at 65 °C for 5 min and then quickly chilled on ice. A mixture of 4 μl of 5× First-Strand Buffer, 2 μl of 0.1 M dithiothreitol, and 1 μl of M-MLV reverse transcriptase (RT) was added to the reaction mixture, and the cDNA synthesis reaction was performed at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 15 min.

HR-HPV E6/E7 mRNA assay

Detection of HPV E6/E7 mRNA in cervical samples was performed with the type-specific Optimygene HR-HPV E6/E7 mRNA RT-qDx assay (Optipharm), a quantitative reverse transcriptase PCR (RT-qPCR)-based assay, using a CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) for thermocycling and fluorescence detection. The assay consists of three different sets of HR-HPV and detects 16 HR-HPV genotypes in three tubes (group I: HPV 16 (FAM), 33, 58 (HEX), and 31, 35 (Cy5); group II: HPV 18 (FAM), 39, 68 (HEX), and 45–51 (Cy5); group III: HPV 53, 56, 66 (FAM), 59, 69 (HEX), and 52 (Cy5)), by incorporating specific TaqMan probes labeled with different fluorophores (Figure 1). The results can be visualized separately for each group. Real-time PCR amplification of HPV E6/E7 mRNA was performed in a reaction mixture containing 10 μl of 2× Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan), 5 μl of primer and TaqMan probe mixture, 2 μl of template cDNA, and distilled water to a final volume of 20 μl for each sample. To avoid false-negatives because of degradation of mRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and cycle threshold (Ct) values of ≥30 were excluded. Target gene mRNA expression levels relative to GAPDH were calculated automatically using the comparative Ct method with CFX Manager Software v. 3.0 (Bio-Rad) or Genex Software (Bio-Rad).

Positive and negative controls were included throughout the procedure. No-template controls containing sterile distilled water rather than template DNA were incorporated into each run. PCR cycling conditions were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 3 s, and 55 °C for 30 s. The mRNA expression level was quantified by determining Ct, which is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence.

HPV DNA genotyping assay

An HPV DNA chip test and PCR-reverse blot assay (REBA) were used for HPV genotyping of cytology and histology samples. Both assays targeted the HPV L1 gene. HPV DNA chip tests were performed at Yonsei University Wonju Severance Christian Hospital as follows: DNA was extracted from residual exfoliative cytology samples after the Pap smear test. HPV genotypes were identified with a Goodgene HPV DNA Genotyping Chip (Goodgene, Inc., Seoul, Republic of Korea) following the manufacturer's recommendations. The Goodgene HPV chip is designed to detect 15 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 69) together with seven low-risk (LR)-HPV genotypes (6, 11, 34, 40, 42, 43, and 44). The genotyping method involves nested PCR to amplify the target region using MY11 and MY9 primers, followed by amplification with the GP5 and GP6 primer pair. Nested PCR conditions consisted of an initial denaturation step for 5 min at 94 °C, followed by 15 cycles of denaturation for 30 s at 94 °C and extension for 30 s at 65 °C. The second amplification involved 45 cycles of 30 s at 94 °C, and 30 s at 54 °C. A final extension step was performed at 72 °C for 7 min. After PCR amplification of the target region, subsequent steps were performed according to the manufacturer's recommendations. For the Goodgene HPV chip, PCR products were loaded onto the probe-labeled glass chip and the resulting signal was interpreted using a GenePix Pro 6.0 scanner (Axon Instruments, Foster City, CA, USA).

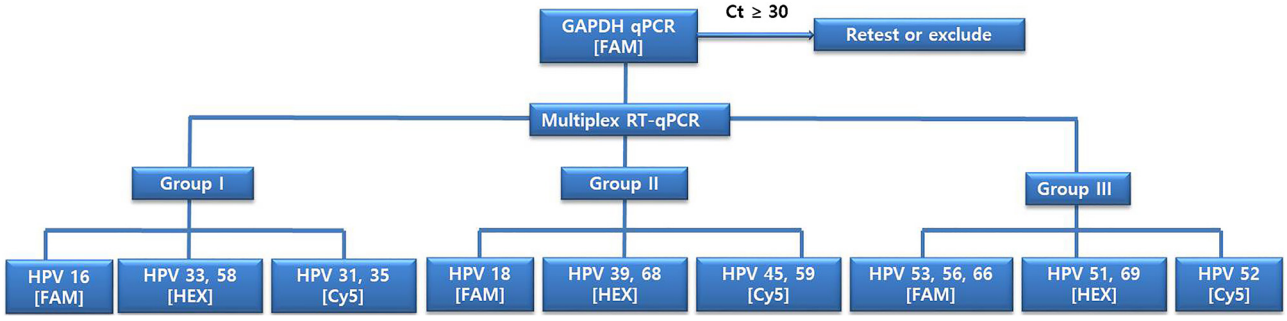

Figure 1. Scheme of the Optimygene HR-HPV RT-qDx assay.

PCR-REBA is based on colorimetric visualized hybridization signals. The fully automated MolecuTech REBA HPV-ID assay (YD Diagnostic, Yongin, Republic of Korea) was used in this study following PCR, and was performed in accordance with the manufacturer's instructions (Munkhdelger et al., 2014). This kit detects 18 HR-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 59, 68, 69, and 73), a medium-risk HPV type (34), and 13 LR-HPV types (6, 11, 32, 40, 42, 43, 44, 54, 70, 72, 81, 84, and 87). PCR was performed in a 20-μl reaction mixture (Genetbio, Daejeon, Republic of Korea) containing 2× Master Mix, 1× primer mixture, 5 μl of sample DNA, and sterile distilled water to a final volume of 20 μl. The first 10 PCR cycles consisted of denaturation at 95 °C for 30 s, followed by annealing and extension at 60 °C for 30 s. These 10 cycles were followed by 40 cycles of denaturation at 95 °C for 30 s and annealing and extension at 54 °C for 30 s. After the final cycle, samples were maintained at 72 °C for 10 min to complete the synthesis of all strands. The amplified target was visualized as a single band corresponding to a length of 150 base pairs using a Chemi Doc system (Vilber Lourmat, Deutschland, Germany).

For REBA HPV-ID, hybridization and washing processes were performed according to the manufacturer's instructions. Briefly, biotinylated PCR products were denatured at 25 °C for 5 min in denaturation solution and diluted in 970 μl of hybridization solution on the REBA membrane strip in the blotting tray provided. Denatured single-stranded PCR products were hybridized to the probes on the strip at 50 °C for 30 min. The strips were then washed twice in 1 ml of washing solution for 10 min at 50 °C with gentle shaking, incubated at 25 °C with 1:2000 diluted streptavidin–alkaline phosphatase (AP) conjugate (Roche Diagnostics, Basel, Switzerland) in conjugate diluents solution for 30 min, and finally washed twice with 1 ml conjugate diluents solution at room temperature for 1 min. Colorimetric hybridization signals were visualized by adding a 1:50 dilution of AP-mediated staining solution, NBT/BCIP (Roche Diagnostics), and incubation until a color change was detected. Finally, the band pattern was read and interpreted.

Sequence analysis

The HPV E6/E7 gene region was sequenced to detect any samples that were inconsistently identified in the two different assays for HPV identification. The primer sets used to amplify the target HPV E6/E7 and L1 gene were 60F-5′-CCGAAAMCGGTKVR-TATAAAAGCA-3′ and 970R-5′-GTACCTKCWGGATCAGCCAT-3′, and GF-5′-TTTGTTACWGTKGTRGATAC-3′ and GR-5′-GAAAAATAAACTG-TAAATCATATTC-3′, respectively. The amplified cDNA was sequenced using the ABI Prism BigDye Terminator and an ABI 3730 automated DNA sequencer (Cosmo Genetech, Seoul, Republic of Korea). The sequences obtained were compared with sequences in the National Center for Biotechnology Information GenBank database for species assignment.

Statistical analysis

The statistical analysis was performed using GraphPad Prism software version 5.02 (GraphPad, Inc., La Jolla, CA, USA) and SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). For the Optimygene HR-HPV RT-qDx assay, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and 95% confidence interval (CI) of predictive ability were estimated. Agreement between the Optimygene HR-HPV RT-qDx assay and HPV DNA testing results was calculated using Cohen's kappa coefficient (κ) and was classified as follows: 0.00–0.19, poor; 0.20–0.39, fair; 0.40–0.59, moderate; 0.60–0.79, strong; 0.80–1.00, excellent (Estrade and Sahli, 2014). Significance between proportions was assessed with McNemar's test. A p-value of <0.05 was considered to indicate a statistically significant difference.

Results

Cytological and histological diagnosis

The characteristics of the population examined in this study are shown in Table 1. The age range of the women included in the study was 20–87 years, with a mean age of 47.8 years (standard deviation ±12.5 years, 95% CI 46.3–49.2 years). Of the 563 liquid-based cytology samples, 51 were classified as SCC, 87 as HSIL, 20 as ASC-H, 121 as LSIL, 157 as ASC-US, and 127 as normal. The histological diagnosis was confirmed by excisional biopsy in 220 of these 563 cases: 38 cases were identified as SCC, 40 as CIN grade 3 (CIN3), 16 as CIN grade 2 (CIN2), 75 as CIN grade 1 (CIN1), and 51 as normal.

Positivity of HR-HPV RT-qDx and DNA assays in the different cytological diagnoses

To determine the positivity of the HR-HPV RT-qDx assay, multiplex RT-qPCR was performed and the positive rates of the HR-HPV RT-qDx assay according to cytological grade were compared to those of HPV DNA testing (Goodgene HPV DNA Genotyping Chip or MolecuTech REBA HPV-ID). All 563 ThinPrep Pap samples were positive for GAPDH mRNA expression. The comparison of positive results of the HPV E6/E7 mRNA and DNA assays for the different cytological diagnoses is shown in Figure 2A. For SCC, 90.2% of cases ($n = 16$) were positive for HPV mRNA and 90.2% of cases ($n = 16$) for HPV DNA; for HSIL, results were 93.1% ($n = 81$) for HPV mRNA and 91.9% ($n = 80$) for HPV DNA; for ASC-H, results were 80% ($n = 16$) for both HPV mRNA and HPV DNA; for LSIL, results were 33.1% ($n = 40$) for HPV mRNA and 52.1% ($n = 63$) for HPV DNA; for ASC-US, results were 20.4% ($n = 32$) for HPV mRNA and 37.6% ($n = 59$) for HPV DNA; finally, for normal cases, 3.1% ($n = 4$) were positive for HPV mRNA and 16.5% ($n = 21$) for HPV DNA. Of the 285 HPV DNA-positive samples, 218 (76.5%) were positive and 67 (23.5%) were negative in the HR-HPV RT-qDx assay. Of the 67 HR-HPV RT-qDx assay-negative samples, 50 showed low-grade lesions (LSILs and ASC-US) and 17 showed normal results (Table 2).

Positivity of HR-HPV RT-qDx and DNA assays in the different histological diagnoses

Among the 220 histologically diagnosed cases, the positive rates in SCC cases were 86.8% ($n = 33$) for HPV mRNA and 92.1% ($n = 35$) for HPV DNA; for CIN3, results were 85% ($n = 34$) for HPV mRNA and 95% ($n = 38$) for HPV DNA; for CIN2, results were 75% ($n = 12$) for HPV mRNA and 87.5% ($n = 14$) for HPV DNA; for CIN1, results were 29.3% ($n = 22$) for HPV mRNA and 46.7% ($n = 35$) for HPV DNA; finally, for normal cases, 0% ($n = 0$) were positive for HPV mRNA and 3.9% ($n = 2$) were positive for HPV DNA (Figure 2B).

Sensitivity and specificity of HR-HPV RT-qDx and DNA assays

The clinical sensitivities of the HR-HPV RT-qDx and HPV DNA assays for detecting high-grade lesions according to cervical cytology were 92.4% (95% CI 0.9167–0.9972, $p < 0.0001$) and 91.7% (95% CI 0.8632–0.9524, $p < 0.0001$), respectively, and the clinical specificities were 96.9% (95% CI 0.8282–0.9772, $p < 0.0001$) and 83.5% (95% CI 0.7597–0.8900, $p < 0.0001$), respectively. Also, the clinical sensitivities of the HR-HPV RT-qDx assay and HPV DNA testing for detecting CIN2+ were 84% (95% CI 0.7521–0.9020, $p < 0.0001$) and 92.6% (95% CI 0.8518–0.9659, $p < 0.0001$), respectively, and the clinical specificities were 82.5% (95% CI 0.7491–

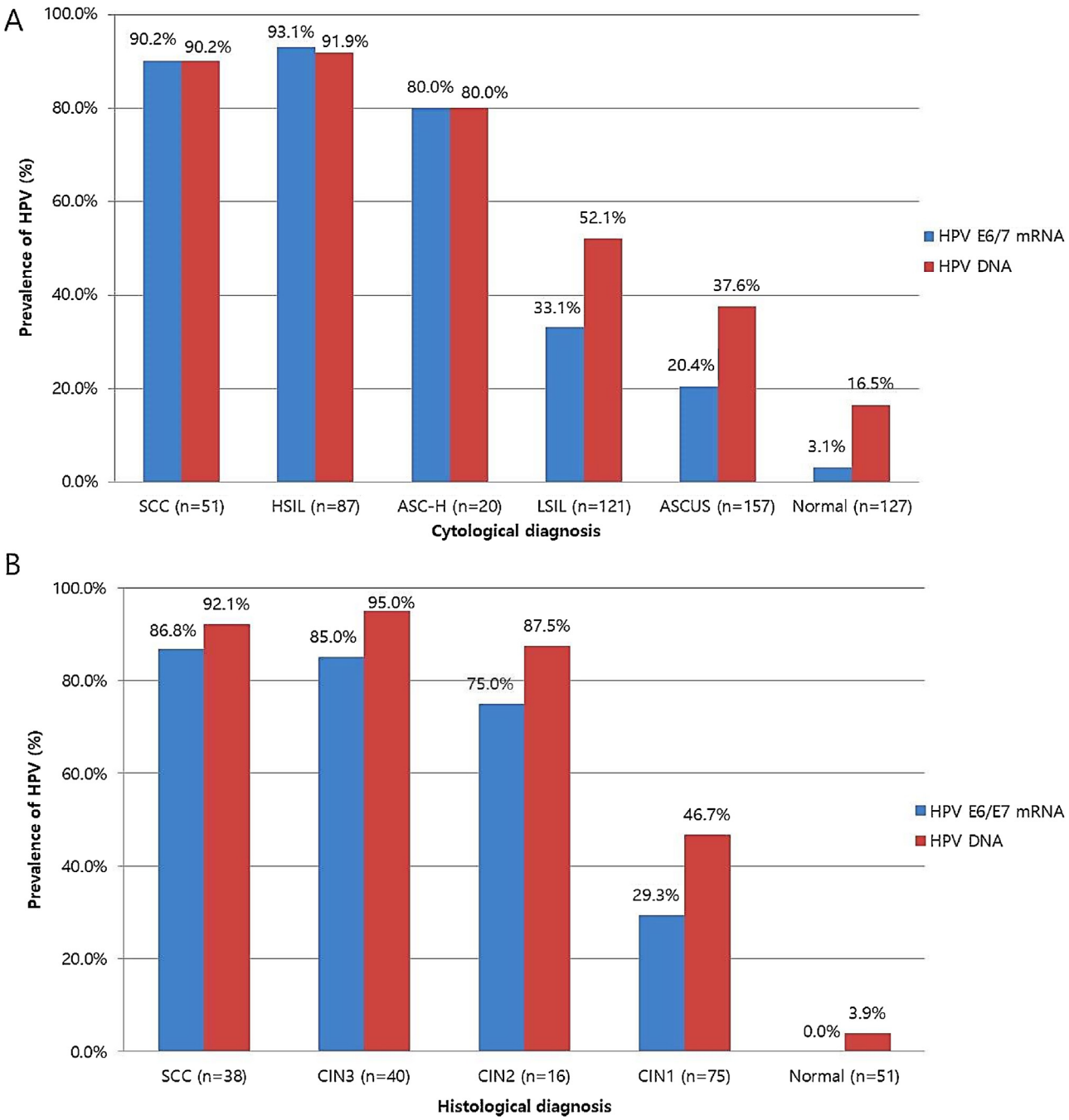

Figure 2. Positive rates of the Optimygene HR-HPV RT-qDx assay as determined by multiplex quantitative reverse transcriptase polymerase chain reaction in cytologically (A) and histologically (B) diagnosed lesions. HR-HPV, high-risk human papillomavirus; SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; CIN3, cervical intraepithelial neoplasia grade 3; CIN2, cervical intraepithelial neoplasia grade 2; CIN1, cervical intraepithelial neoplasia grade 1.

0.8825, $p < 0.0001$) and 70.6% (95% CI 0.6214–0.7790, $p < 0.0001$), respectively (Table 3).

Agreement between HR-HPV RT-qDx and HPV DNA results

Overall, agreement between the HR-HPV RT-qDx and HPV DNA assays by cytological and histological diagnosis was 87.9% ($\kappa = 0.76$, 95% CI 0.7054–0.8128, $p < 0.0001$) and 90.5% ($k = 0.81$, 95% CI 0.7338–0.8878, $p < 0.0001$), respectively, showing good agreement (Table 4).

Comparison of HR-HPV genotype results based on HR-HPV RT-qDx and HPV DNA assays in the different cytological grades

The proportions of HR-HPV genotypes detected using the two molecular assays according to the cytological grade are shown in Table 5. The distribution of HPV genotypes differed between high-grade and low-grade lesions in the two molecular assays. Although the detection rates of HR-HPV genotypes for the two methods were similar in high-grade lesions (90.5% for HPV E6/E7 mRNA assay vs. 89.9% for HPV DNA testing), in the 12 cases showing discordant

Table 2
Positivity by HR-HPV RT-qDx assay and HPV DNA testing in cytologically diagnosed samples.

Cytology	Number of positive results by the assays (%)				κ coefficient (95% CI)	*p*-Value
	HR-HPV RT-qDx	95% CI	HPV DNA	95% CI		
SCC ($n=51$)	46 (90.2)	0.86–0.99	46 (90.2)	0.86–0.99	1 (0.90–1.0)	<0.0001
HSIL ($n=87$)	81 (93.1)	0.85–0.97	80 (91.9)	0.84–0.96	0.9 (0.75–1.0)	
ASC-H ($n=20$)	16 (80)	0.57–0.92	16 (80)	0.57–0.92	1 (0.79–1.0)	
LSIL ($n=121$)	40 (33.1)	0.25–0.41	63 (52.1)	0.43–0.61	0.6 (0.49–0.76)	
ASC-US ($n=157$)	32 (20.4)	0.14–0.27	59 (37.6)	0.30–0.45	0.6 (0.46–0.73)	
Normal ($n=127$)	4 (3.1)	0.01–0.08	21 (16.5)	0.11–0.24	0.2 (0–0.59)	
Total ($n=563$)	219 (38.9)		285 (50.6)			

SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance.

genotypes, the results showed 100% concordance with the results of the HR-HPV RT-qDx assay when sequencing analysis was used as a confirmative tool (Table 6). Additionally, one sample classified as HPV 16 in the HR-HPV RT-qDx assay was not detected on HPV DNA testing. For low-grade lesions, HPV DNA testing detected more HPV genotypes (37 HR-HPV and 13 LR-HPV) than the HR-HPV RT-qDx assay in 50 samples. The difference in detection rates between the HR-HPV RT-qDx assay and DNA tests of HR-HPV types in the overall frequency of HPV infections was significant in women with low-grade lesions (McNemar's test, $p < 0.0001$). The most common HPV genotypes in descending order of prevalence were HPV 16 (39.9%, $n=63$), 33–58 (21.5%, $n=34$), 31–35 (7%, $n=11$), and 18 (6.3%, $n=10$) in high-grade lesions. In low-grade lesions, prevalent HPV genotypes detected were HPV 39–68 (6.1%, $n=17$), 16 (5%, $n=14$), 53–56–66 (4.7%, $n=13$), and 18 (4.3%, $n=12$) (Figure 3).

Discussion

An optimal strategy for cervical cancer screening should efficiently and accurately identify precancerous lesions that are likely to progress to invasive cancer and avoid the detection and unnecessary treatment of transient HPV infections and associated benign lesions (Saslow et al., 2012). Although current HPV DNA testing shows adequate analytical sensitivity, its poor specificity can lead to unnecessary colposcopy and biopsy examinations in women with abnormal Pap smear results who are positive for HR-HPV DNA (Naucler et al., 2009). In Korea, an HPV DNA test was approved as a primary screening method by the Food and Drug Administration in 2014; however, the committee stated that it is unclear whether the HPV DNA test reduces the incidence of cervical cancer compared to cervical cytology and whether negative effects after false-positivity are increased (Min et al., 2015). Therefore, more specific methods are needed to overcome these limitations in cervical cancer screening. Previous studies have evaluated the clinical performance of the equivalent E6/E7 mRNA assay as a triage test for cytology, and HPV DNA testing showed that triage of HPV DNA-positive women with the E6/E7 mRNA test reduced referral for colposcopy more drastically than cytology, but at the cost of lower sensitivity (Benevolo et al., 2011).

The results of the present study also showed that compared to HPV DNA testing, the HR-HPV RT-qDx assay yielded a significantly lower rate of positive results in patients with a normal cytology

Table 3
Clinical sensitivity and specificity of the HR-HPV RT-qDx assay and HPV DNA testing stratified by high-grade cytological and histological diagnosis of cervical samples.[a]

Molecular assays	Number of cytological diagnoses		Sensitivity	Specificity	PPV	NPV	Agreement	κ coefficient (95% CI)	*p*-Value[b]
	Positive	Negative	% (n) (95% CI)						
HPV E6/E7 mRNA									
Positive	146	4	92.4 (146/158) (0.9167–0.9972)	96.9 (123/127) (0.8282–0.9772)	97.3 (146/150) (0.9311–0.9919)	91.1 (123/135) (0.8498–0.9497)	94.4 (269/285) (0.9101–0.9658)	0.89 (0.8333–0.9409)	<0.0001
Negative	12	123							
HPV DNA testing									
Positive	145	21	91.7 (145/158) (0.8632–0.9524)	83.4 (106/127) (0.7597–0.8900)	87.3 (145/167) (0.8079–0.9120)	89.1 (106/119) (0.8208–0.9363)	88.1 (251/285) (0.8376–0.9137)	0.76 (0.6804–0.8336)	<0.0001
Negative	13	106							
Molecular assays	Number of histological diagnoses		Sensitivity	Specificity	PPV	NPV	Agreement	κ coefficient (95% CI)	*p*-Value
	Positive	Negative	% (n) (95% CI)						
HPV E6/E7 mRNA									
Positive	79	22	84 (79/94) (0.7521–0.9020)	82.5 (104/126) (0.7491–0.8825)	78.2 (79/101) (0.6915–0.8522)	87.4 (104/119) (0.8013–0.9232)	83.2 (183/220) (0.7765–0.8758)	0.66 (0.5596–0.7596)	<0.0001
Negative	15	104							
HPV DNA testing									
Positive	87	37	92.6 (87/94) (0.8518–0.9659)	70.6 (89/126) (0.6214–0.7790)	70.2 (87/124) (0.6157–0.7753)	92.7 (89/96) (0.8547–0.9666)	80 (176/220) (0.7518–0.8558)	0.62 (0.5219–0.7263)	<0.0001
Negative	7	89							

PPV, positive predictive value; NPV; negative predictive value; 95% CI, 95% confidence interval.
[a] HPV DNA testing was performed by Goodgene HPV detection chip kit or MolecuTech REBA HPV-ID. High-grade lesions in cytological diagnosis included SCC, HSIL, and ASC-H groups.
[b] The *p*-value was calculated using McNemar's test, where $p < 0.05$ is significant.

Table 4
Overall agreement between the HR-HPV RT-qDx assay and HPV DNA testing stratified by cytological and histological diagnosis.[a]

HR-HPV RT-qDx	Number of HPV DNA testing			
	Agreement, % (*n*)	95% CI	κ coefficient (95% CI)	*p*-Value[b]
Cytology	87.9 (495/563)	0.8496–0.9037	0.7591 (0.7054–0.8128)	<0.0001
Histology	90.5 (199/220)	0.8496–0.9037	0.8108 (0.7338–0.8878)	

95% CI, 95% confidence interval.
[a] HPV DNA testing was performed by Goodgene HPV detection chip kit or MolecuTech REBA HPV-ID.
[b] The *p*-value was calculated using McNemar's test, where $p < 0.05$ is significant.

Table 5
Comparison of HPV genotype results by HR-HPV RT-qDx assay and HPV DNA testing in cytologically diagnosed samples.

Molecular assay	Genotypes[a]	Number of positive results (%) in the respective cytological lesions						
		SCC (*n* = 51)	HSIL (*n* = 87)	ASC-H (*n* = 20)	LSIL (*n* = 121)	ASC-US (*n* = 157)	Normal (*n* = 127)	Total (*n* = 563)
HPV E6/E7 mRNA	HR-HPV	46 (90.2)	81 (93.1)	16 (80)	40 (33.1)	32 (20.4)	4 (3.1)	219 (38.9)
	HPV 16	28 (60.9)	32 (39.5)	9 (56.3)	6 (15)	8 (25)	–	83 (37.9)
	HPV 33–58	12 (26.1)	26 (32.1)	1 (6.3)	2 (5)	5 (15.6)	1 (25)	47 (21.5)
	HPV 31–35	1 (2.2)	8 (9.9)	3 (18.8)	2 (5)	1 (3.1)	–	15 (6.8)
	HPV 18	4 (8.7)	5 (6.2)	1 (6.3)	5 (12.5)	7 (21.9)	3 (75)	25 (11.4)
	HPV 39–68	–	1 (1.2)	–	8 (20)	9 (28.1)	–	18 (8.2)
	HPV 45–59	–	–	–	2 (5)		–	2 (0.9)
	HPV 53–56–66	–	3 (3.7)	1 (6.3)	12 (30)	1 (3.1)	–	17 (7.8)
	HPV 51–69	–	1 (1.2)	–	3 (7.5)	1 (3.1)	–	5 (2.3)
	HPV 52	1 (2.2)	5 (6.2)	1 (6.3)	–	–	–	7 (3.2)
HPV DNA	Total HPV	46 (90.2)	81 (93.1)	16 (80)	62 (51.2)	59 (37.6)	21 (16.5)	285 (50.6)
	HR-HPV	46 (90.2)	80 (91.9)	16 (80)	60 (49.6)	49 (31.2)	21 (16.5)	272 (48.3)
	HPV 16	29 (63)	30 (37)	7 (43.8)	12 (19.4)	17 (28.8)	11 (52.4)	106 (37.2)
	HPV 33–58	10 (21.7)	25 (30.9)	3 (18.8)	8 (12.9)	7 (11.9)	3 (14.3)	56 (19.6)
	HPV 31–35	2 (4.3)	10 (12.3)	2 (12.5)	4 (6.5)	2 (3.4)	–	20 (7)
	HPV 18	4 (8.7)	5 (6.2)	1 (6.3)	6 (9.7)	8 (13.6)	5 (23.8)	29 (10.2)
	HPV 39–68	–	1 (1.2)	–	11 (17.7)	9 (15.3)	–	21 (7.4)
	HPV 45–59	–	–	–	2 (3.2)	–	1 (4.8)	3 (1.1)
	HPV 53–56–66	–	3 (3.7)	2 (12.5)	12 (19.4)	1 (1.7)	1 (4.8)	19 (6.7)
	HPV 51–69	–	1 (1.2)	–	3 (4.8)	1 (1.7)	–	5 (1.8)
	HPV 52	1 (2.2)	5 (6.2)	1 (6.3)	2 (3.2)	4 (6.8)	–	13 (4.6)
	LR-HPV	–	1 (1.2)	–	2 (3.2)	10 (16.9)	–	13 (4.6)

SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; HR-HPV, high-risk human papillomavirus; LR-HPV, low-risk human papillomavirus.
[a] HPV genotypes were separated by type-specific probes of HR-HPV E6/E7 mRNA assay.

Table 6
Comparison of the results of the discrepant HR-HPV genotypes detected using the HR-HPV RT-qDx assay and HPV DNA testing with regard to high-grade lesions.

Cytology	Discrepant results between three molecular assays					
	HR-HPV RT-qDx	*n*	HPV DNA	*n*	Sequencing	*n*
SCC	HPV 16	1	HPV 33–58	1	HPV 16	1
	HPV 33–58	3	HPV 16	2	HPV 33	3
			HPV 31–35	1		
HSIL	HPV 16	3	HPV 31–35	2	HPV 16	3
			HPV 33–58	1		
	HPV 33–58	2	HPV 16	2	HPV 33	2
ASC-H	HPV 16	2	HPV 33–58	1	HPV 16	2
			HPV 53–56–66	1		
	HPV 31–35	1	HPV 33–58	1	HPV 31	1
	Total	12		12		12

SCC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; HR-HPV, high-risk human papillomavirus.

(3.1% for HR-HPV RT-qDx vs. 16.5% for HPV DNA testing; $p < 0.05$). Similarly, for patients with low-grade lesions, HPV DNA testing gave a higher rate of positive results compared to the HR-HPV RT-qDx assay (39.2% vs. 25.9%). These results are similar to those of a previous study that compared the performance of the NucliSENS Easy Q HPV test (bioMérieux) and HPV-HR genotyping multiplex real-time PCR test (Sacace) in clinical specimens (Duvlis et al., 2015). These results suggest that the selective targeting used in the HR-HPV E6/E7 mRNA assay can reduce the positive detection rate of HPV infections in women with normal or low-grade lesions. Therefore, the HR-HPV RT-qDx assay may be a more accurate predictor of cervical cell dysplasia. However, women who are HPV DNA-positive but HPV E6/E7 mRNA-negative for low-grade lesions may have cervical neoplasia and require further follow-up.

In this study, the HR-HPV RT-qDx assay had a similar sensitivity (92.4% vs. 91.7%) but a significantly higher specificity (96.9% vs. 83.5%) compared to HPV DNA testing. Additionally, the sensitivity and specificity of the HR-HPV RT-qDx assay for detecting high-grade CIN3+ by histological diagnosis were 89.3% (95% CI 0.8010–0.9474, $p < 0.0001$) and 82.5% (95% CI 0.7491–0.8825, $p < 0.0001$), respectively. The results showed that the HR-HPV RT-qDx assay was less sensitive than HPV DNA testing (89.3% vs. 92.5%), but had higher specificity for CIN3+ compared to HPV DNA testing (82.5% vs. 72.2%). The reason for the low sensitivity was that eight cases (six of CIN3 and two of CIN2) with low-grade lesions in the cytological results were not detected in the HR-HPV RT-qDx assay but were detected by HPV DNA testing.

Although there were no significant differences in the total positive rates ($\kappa = 0.29$, 95% CI 0.2154–0.3596, $p < 0.0001$), the agreement between the HR-HPV RT-qDx assay and high-grade lesions was 94.4%, implying good agreement ($\kappa = 0.89$, 95% CI 0.8333–0.9409, $p < 0.0001$), which was higher than that of HPV

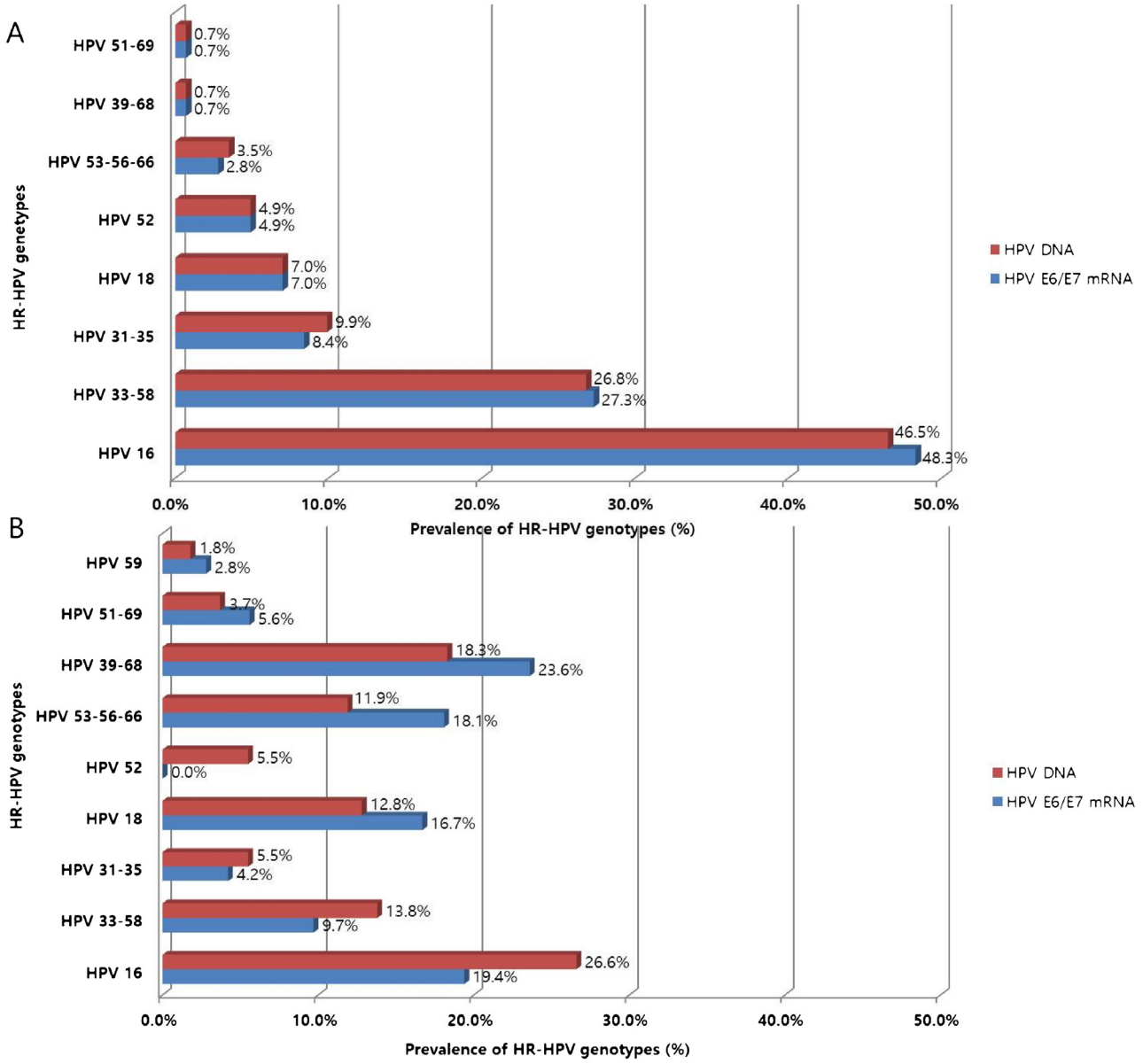

Figure 3. Comparison of the prevalence of oncogenic high-risk (HR) specific HPV genotypes between the Optimygene HR-HPV RT-qDx assay and HPV DNA testing in high-grade (A) and low-grade (B) lesions of liquid-based cytology samples.

DNA testing at 88.1% ($\kappa = 0.76$, 95% CI 0.6804–0.8336, $p < 0.0001$). Furthermore, the agreement between the HR-HPV RT-qDx assay and HPV DNA testing for both high-grade cytological and histological diagnosis were 87.9% ($\kappa = 0.76$, 95% CI 0.7054–0.8128, $p < 0.0001$) and 90.5% ($\kappa = 0.81$, 95% CI 0.7338–0.8878, $p < 0.0001$), respectively, revealing good agreement. Importantly, this high agreement was observed for both the HPV status and genotyping results.

Additionally, this assay may be helpful for identifying HPV genotypes, as the results were well-matched with the sequencing results for inconsistent HPV DNA testing, as well as with the results for HPV 16 identified in the other assay. The oncogenic process of cervical cancer is initiated by HR-HPV infection and mediated by upregulation of HPV E6/E7 oncoproteins. This overexpression, which can be measured as increased E6/E7 mRNA transcripts, is associated with a significantly increased risk of CIN and cervical cancer (Persson et al., 2012). The oncogenic potential of these HR genotypes resides in their ability to express the HPV-encoded oncogenes E6 and E7. Therefore, detection of oncogene E6/E7 expression may be more specific and a better predictor of cervical cancer risk than detecting HPV DNA (Varnai et al., 2008). A previous study suggested that a clinical 'test and treat' approach to HPV E6/E7 mRNA testing can contribute to better clinical safety for women over 40 years of age and for women with a high-grade lesion (Sørbye et al., 2010).

In a worldwide study of the HPV type distribution in cervical cancer, HPV 16 and 18 were the most common types, accounting for 71% of all HPV types detected in cervical cancer (de Sanjose et al., 2010). The present study showed that HPV 16 (43.7%) was the most common type in patients with high-grade lesions (especially, 60.9% of SCC). These results are consistent with those of previous studies showing HPV 16 to be the most common type in patients with precancerous neoplasia lesions in Korea (So et al., 2016) and worldwide (Kjaer et al., 2014; Krashias et al., 2017). In this study, the most frequently detected HPV genotypes among HR-HPV-positive women were HPV 16 (37.9%), HPV 33–58 (21.5%), HPV 18 (11.4%), HPV 39–68 (8.2%), and HPV 53–56–66 (7.8%), which does not agree with results reported previously by Kim et al.: 14.5% of

HPV 53, 11.8% of HPV 58, 11.7% of HPV 52, and 8.8% of HPV 16 (Kim et al., 2013). However, because the patients in the study by Kim et al. were recruited from a geographically limited area, the results for the distribution of HPV types are unlikely to reflect national averages. Additionally, the prevalence of HPV infection was not significant at different ages ($p = 0.2$), but was significantly higher in patients aged 30–50 years ($p < 0.01$). Our results demonstrate that the HPV test is more useful in women aged 30–50 years for early cervical cancer screening.

Current HPV vaccines (Cervarix, Gardasil, and Gardasil 9, by L1 protein) are effective for preventing HPV infections caused by the targeted types and neoplastic disease. However, these vaccines are not effective for eliminating pre-existing infections, as the L1 major capsid proteins are not expressed in infected basal epithelial cells (Hildesheim et al., 2016). Thus, therapeutic vaccines targeting the HPV E6 and E7 oncoproteins, which are associated with cell-mediated immune responses, have recently been developed to treat chronic HPV infections and cervical lesions (Kim et al., 2014; Trimble et al., 2015). As monitoring vaccine efficacy is important, a complementary diagnostic method for detecting changes in the epidemiology of viral genotypes and a screening test for the HR-HPV E6/E7 assay is needed.

There were some limitations to this study. First, information for the prediction of clinical outcomes for patients with low-grade lesions and negative results for HR-HPV E6/E7 mRNA or CIN2+ was not provided. To conclusively demonstrate the utility of these assays as a predictive marker for screening, it will be necessary to conduct additional tests for low-grade lesions. Second, HPV DNA testing was performed using two methods, the Goodgene DNA chip or REBA HPV-ID. Although both detection methods are based on reverse hybridization, the use of two different methods may be less accurate than the use of only one HPV genotyping method.

In conclusion, the results of this study suggest that the HR-HPV RT-qDx assay could serve as a complementary approach for cervical cancer screening because it reduces false-positive results by improving the specificity and PPV.

Ethical approval

This study was approved by the Institutional Ethics Committee at Yonsei University Wonju College of Medicine and all subjects provided written informed consent.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

YHW performed evaluation of the experiments, analyzed the data, and drafted the manuscript. KWP provided clinical samples and clinical information. HK revised the manuscript. All authors read and approved the final version of the manuscript.

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