Clinical Validation of Anyplex II HPV HR Detection Test for Cervical Cancer Screening in Korea

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• ***Context.***—**The Anyplex II HPV HR detection kit (Seegene Inc, Seoul, Korea) is a new, multiplex, real-time polymerase chain reaction assay to detect individual 14 high-risk (HR) human papillomavirus (HPV) types in a single tube.**

Objective.—**To evaluate the clinical performance of the HPV HR kit in predicting high-grade squamous intraepithelial lesions and cervical intraepithelial lesions grade 2 or worse in cervical cancer screening.**

Design.—**We analyzed 1137 cervical samples in Huro Path medium (CelltraZone, Seoul, Korea) from Korean women. The clinical performance of the HPV HR kit was compared with Hybrid Capture 2 (Qiagen, Valencia, California) using the noninferiority score test in a routine cervical cancer screening setting. The intralaboratory and interlaboratory agreements of HPV HR were also evaluated.**

Results.—**Overall agreement between the 2 assays was 92.4% (1051 of 1137) with a κ value of 0.787. Clinical sensitivity of HPV HR for high-grade squamous intraepithelial lesions and cervical intraepithelial lesions grade 2 or worse was 94.4% (95% confidence interval [CI], 89.2–99.7) and 92.5% (95% CI, 84.3–100.0), respectively. The respective values for Hybrid Capture 2 were 93.1% (95% CI, 87.2–98.9) and 87.5% (95% CI, 77.3–99.7). Clinical sensitivity and specificity of HPV HR were not inferior to those of Hybrid Capture 2 ($P = .005$ and $P = .04$, respectively). The HPV HR showed good intralaboratory and interlaboratory reproducibility at 98.0% ($\kappa = 0.953$) and 97.4% ($\kappa = 0.940$), respectively.**

Conclusions.—**The HPV HR demonstrates comparable performance to the Hybrid Capture 2 test and can be useful for HPV-based cervical cancer screening testing.**

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Persistent infection with at least one of the high-risk carcinogenic human papillomavirus (HPV) types is the primary risk factor for the development of cervical cancer. Human papillomavirus is found in virtually all cases of cervical cancer and precursor lesions.[1,2] Providing clear information about infected HPV genotypes in the stage of infection, clearance, and persistence is important to manage patient monitoring and treatment. However, conventional cytology tests do not determine HPV infection but detect only cytologic abnormality.

Molecular HPV testing has been considered as an adjunct/follow-up to the Papanicolaou test for cervical cancer in women 30 years and older.[3] Recently, the US Food and Drug Administration approved molecular HPV testing as a first-line, primary screening test for cervical cancer in women 25 years and older.[3]

Several molecular test kits for the detection of high-risk human papillomavirus (hrHPV) are currently available. Evaluation of their clinical performance is critical before they are routinely used in clinical practice. According to international guidelines[4] for validation of hrHPV DNA tests, candidate assays should demonstrate clinical noninferiority to an established and clinically validated reference assay, such as Hybrid Capture 2 (HC2; Qiagen, Valencia, California) or $GP5^+/6^+$ polymerase chain reaction (PCR). The candidate assays should have demonstrable sensitivity for cervical intraepithelial neoplasia (CIN) grade 2 or 3 and treatable cancer of 0.90 or greater and specificity for $CIN2^+$ of 0.98 or greater. In addition, candidate assays should display intralaboratory reproducibility and interlaboratory agreement, with a lower confidence bound of not less than 87% ($\kappa \leq 0.5$).

The recently developed Anyplex II HPV HR detection kit (Seegene Inc, Seoul, Korea) is a new multiplex, real-time PCR assay designed to detect and individually distinguish 14 hrHPV types* using tagging oligonucleotide cleavage and extension (TOCE) technology.[5] The TOCE technology uses artificial, template-based melting temperature instead of the current, probe-based melting temperature and distinguishes multiple targets in a single channel in a real-time PCR reaction.[6] In addition, the cyclic-catcher melting temperature analysis with TOCE enables a semiquantitative

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* *References 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.*

estimation of viral load through repeated melting temperature analysis expression during the TOCE reaction.[7]

Currently, the prevalence of HPV among Korean women with normal cytology results is higher than in more-developed countries.[8,9] Human papillomavirus cytology examination is carried out using a program incorporating a high level of cytopathology control.

We present the results of the first comparative evaluation of HPV HR relative to HC2 according to international guidelines[4] in the setting of Korean cervical cancer screening.

MATERIALS AND METHODS

Cervical Sample Collection

Specimens (n = 1137) from Korean women were randomly selected from the liquid-based cervical specimens submitted to the Seegene Medical Foundation (Seoul, Korea) between January and June 2014. All specimens were collected with a cytobrush, and were placed in a Huro Path liquid-based cytology sampling device (CelltraZone, Seoul, Korea). They were stored at –70°C until HPV testing. Each specimen was divided into 2 aliquots and then used for HPV detection with HC2 (4 mL) and HPV HR (1 mL) tests. The cervical cytologic examination was performed by experienced cytopathologists. To examine the clinical performance of HPV assays for histology of CIN2$^+$, the medical records were reviewed to gather information about colposcopic examination and histologic diagnosis. The study protocol was approved by the institutional review board of the Seegene Medical Foundation (Seoul, Korea; No. ND-IRB-2013-06).

HC2 Test

The HC2 testing was performed according to the manufacturer's instructions. The RNA cocktail probes in the HC2 assay detect 13 hrHPV genotypes (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Briefly, the specimens were denatured at 65°C for 45 minutes and hybridized with a mixture of RNA probes. The DNA-RNA hybrid was captured by an anti–DNA-RNA antibody on the surface of the microtiter plate wells and reacted with an alkaline phosphatase-conjugated antihybrid monoclonal antibody. Chemiluminescence substrate was added and the light emitted by the bound enzyme-antibody conjugate was measured with a luminometer. All specimens with a relative light unit:cutoff ratio of 1.0 or greater were considered positive. An HC2 sample conversion kit (Qiagen) was used with cervical specimens collected in Huro Path solution. The validity of placing specimens in Huro Path solution for the HC2 test, instead of PreservCyt solution (ThinPrep, Cytyc, Boxborough, Massachusetts), was confirmed by comparison of results from duplicate serial 10-fold dilutions from 10^5 to 10^{-1} copies of HPV DNA of HeLa (HPV-18) and SiHa (HPV-16) cell lines spiked into Huro Path and PreservCyt.

HPV HR Test

Liquid-based cytology samples (1 mL) were centrifuged at 15 000*g* for 15 minutes. Each cell pellet was resuspended in 450 μL of phosphate-buffered saline and added to the Microlab STARlet automated purification system (Hamilton, Reno, Nevada). For each sample, HPV detection and genotyping were performed according to the manufacturer's instructions for the HPV HR test using a CFX96 real-time thermocycler (Bio-Rad, Hercules, California). The TOCE technology of the HPV HR is initiated with hybridization of the dual priming oligonucleotide primers and the "Pitcher" to the target sequence. *Taq* polymerase, with 5′-nuclease activity, encounters the target-bound Pitcher and releases the tagging portion of the Pitcher. The sequence of the released tagging portion is complementary to the capturing portion of the "Catcher" as an artificial template. As the tagging portion is fully extended on the Catcher to create the "Duplex Catcher," quenching is diminished, and the fluorescent signal can be detected. Briefly, each PCR reaction was performed in a 20-μL reaction consisting of 5 μL extracted DNA, 4× HPV HR TOCE oligo mix, and Anyplex PCR mix containing uracil-DNA glycosylase. The cycling conditions were initial incubation at 50°C for 4 minutes for activation of the uracil-DNA glycosylase system to prevent contamination, denaturation at 95°C for 15 minutes, followed by 50 cycles of denaturation (30 seconds at 95°C), annealing (1 minute at 60°C), and elongation (30 seconds at 72°C). Cyclic-catcher melting temperature analysis was performed after PCR cycles 30, 40, and 50. Catcher melting temperature analysis conditions were as follows: cooling the reaction mixture to 55°C, holding at 55°C for 30 seconds, and heating from 55°C to 85°C (5 s/0.5°C). The L1 gene of HPV DNA and the human housekeeping gene (human β-globin) were coamplified simultaneously, and the human housekeeping gene was used as an internal control to monitor DNA purification efficiency, PCR inhibition, and cell adequacy. Data interpretation was automated with the Anyplex software (Seegene) according to the manufacturer's instructions.

Table 1. Comparison of Anyplex II HPV HR Detection Kit (HPV HR)[a] and Hybrid Capture 2 (HC2)[b] Results by Cytology and Histology[c]

Cytology/ Histology	HPV HR Result	HC2 Result, No.		
		Positive	Negative	Total
Less than HSIL[d]	Positive	154	41	195
	Negative	40	830	870
	Total	194	871	1065
HSIL[e]	Positive	65	3	68
	Negative	2	2	4
	Total	67	5	72
CIN2^{+}[f]	Positive	35	2	37
	Negative	0	3	3
	Total	35	5	40

Abbreviations: CIN2$^+$, cervical intraepitherial neoplasia grade 2 or 3 and treatable cancer; 95% CI, 95% confidence interval; HSIL, high-grade squamous intraepithelial lesion.

[a] Seegene Inc, Seoul, Korea.
[b] Qiagen, Valencia, California.
[c] Overall agreement, 92.4%; $\kappa = 0.787$; 95% CI, 0.744–0.830.
[d] Agreement on less than HSIL, 92.4%; $\kappa = 0.745$; 95% CI, 0.693–0.798.
[e] Agreement on HSIL, 93.1%; $\kappa = 0.408$; 95% CI, 0.000–0.829.
[f] Agreement on CIN2$^+$, 95.0%; $\kappa = 0.724$; 95% CI, 0.366–1.000.

HPV HR Reproducibility Test

To assess the reproducibility of the HPV HR, 504 samples, including 152 HC2$^+$ samples, were used. Intrareproducibility tests were performed by retesting HPV HR 4 to 6 weeks after the initial test. Interlaboratory reproducibility studies were conducted at 2 additional laboratories of Seegene Medical Foundation and Seegene Institute of Life Science.

Statistical analyses

All statistical analyses were carried out using GraphPad Prism (GraphPad Software, La Jolla, California) and Excel 2007 (Microsoft, Seattle, Washington). Agreement between the HC2 test and HPV HR was assessed with the Cohen's κ statistic, with values of 0.00 to 0.20 indicating poor agreement, 0.21 to 0.40 fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement, and 0.81 to 1.00 almost perfect agreement. Clinical sensitivity, clinical specificity, positive predictive value, and negative predictive value were calculated with the conventional contingency tables, and 95% confidence intervals (95% CI) were computed using the exact binomial method. The clinical sensitivity and specificity of the HPV HR for high-grade squamous intraepithelial lesion (HSIL) were compared with those of HC2 with a noninferiority score test as previously described.[4] In addition, differences in the clinical sensitivity and specificity

Table 2. Comparison of Anyplex II HPV HR Detection Kit (HPV HR)[a] and Hybrid Capture 2 (HC2)[b] for 280 Abnormal Cytologic Grades

Agreement	No.	Cytology Grades (No.)	Genotyping Results by the HPV HR (No.)
Concordant	251		
HPV HR/HC2 ($^+/^+$)	163		
Single infection	116	HSIL (47), LSIL (54), ASCUS (15)	HPV-16 (37), HPV-18 (2), other types[c] (77)
Multiple infections	47	HSIL (18), LSIL (24), ASCUS (5)	HPV-16 and other types[c] (23); HPV-18 and other types[c] (4); HPV-16, HPV-18 and other types[c] (3); other types[c] (17)
HPV HR/HC2 ($^-/^-$)	88		
Discordant	29		
HPV HR/HC2 ($^+/^-$)	15		
Single infection	14	HSIL (3), LSIL (6), ASCUS (5)	HPV-16 (2), HPV-18 (1), other types[c] (11)
Multiple infections	1	HSIL (0), LSIL (1), ASCUS (0)	HPV-18 and other types[c] (1)
HPV HR/HC2 ($^-/^+$)	14		

Abbreviations: ASCUS, atypical squamous cell of undetermined significance; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

[a] Seegene Inc, Seoul, Korea.
[b] Qiagen, Valencia, California.
[c] Non-HPV-16 and non-HPV-18.

between the two methods were assessed using the McNemar exact χ^2 test. A P value less than .05 was considered significant.

RESULTS

Our study included 1137 cervical cell specimens collected in Huro Path medium from patients whose mean age was 41.8 years (range, 18–96 years; median age, 41 years). The cytologic grades of specimens were classified as normal (n = 857; 75.4%), atypical squamous cell of undetermined significance (n = 85; 7.5%), low-grade squamous intraepithelial lesion (n = 123, 10.8%), and HSIL (n = 72, 6.3%). Cervical biopsies were performed for 75 women, and 40 samples from women with a biopsy of CIN2$^+$ (CIN2, 12; CIN3, 24; and 4 squamous cell carcinomas) within 12 months were included to evaluate clinical sensitivity for CIN2$^+$.

Of the 1137 specimens, HPV HR detected 263 (23.1%) cases of hrHPV and HC2 detected 261 (23.0%) cases, with no statistically different detection rate evident between them ($P = .91$) (Table 1). According to the cytologic grades and histology, the hrHPV$^+$ rates by the HPV HR and the HC2 were 9.9% (85 of 857) and 9.8% (84 of 857) in normal cases, 29.4% (25 of 85) (identical) in cases of atypical squamous cell of undetermined significance, 69.1% (85 of 123) (identical) in cases of low-grade squamous intraepithelial lesion, 94.4% (68 of 72) and 93.1% (67 of 72) in cases of HSIL, and 92.5% (37 of 40) and 87.5% (35 of 40) in CIN2$^+$ cases, respectively. Overall agreement between the HPV HR and HC2 was 92.4% (1051 of 1137), with a κ value of 0.787 (95% CI, 0.744–0.830). Agreement for cases with less than an HSIL diagnosis was 92.4% (984 of 1065), with a κ value of 0.745 (95% CI, 0.693–0.798), whereas the agreement for cases with an HSIL diagnosis was 93.1% (67 of 72), with a κ value of 0.408 (95% CI, 0.000–0.829). Agreement for the CIN2$^+$ was 95.0%, with a κ value of 0.724 (95% CI, 0.366–1.000). The HC2 and HPV HR test cytologic findings and histology are shown in Table 1.

Of the 280 specimens with abnormal cytologic findings, 251 (89.6%) specimens had concordant results in both assays. Of these, 46.2% (116 of 251) had a single genotype (including HPV-16, n = 37; HPV-18, n = 2), whereas 18.7% (47 of 251) had multiple genotypes (including HPV-16, n = 23; HPV-18, n = 4; HPV-16 and HPV-18, n = 3) ranging between 2 and 3 different genotypes by the HPV HR. Also, 35.1% (88 of 251) of specimens were not detected by both methods. Of the 29 specimens showing discordant results and having abnormal cytologic findings, 15 specimens (51.7%) were positive in only the HPV HR and 14 specimens (48.3%) were positive in only the HC2 (Table 2).

The clinical sensitivity, clinical specificity, positive predictive value, and negative predictive value of the HPV HR and HC2 are shown in Tables 3 and 4. The clinical sensitivity of the HPV HR and HC2 for the detection of HSIL was 94.4% (95% CI, 89.2–99.7) and 93.1% (95% CI, 87.2–98.9), respectively, and clinical specificity was 81.7% (95% CI, 79.4–84.0) and 81.8% (95% CI, 79.5–84.1), respectively. Using a noninferiority score test, the clinical sensitivity and specificity of the HPV HR were not inferior to those of the HC2 assay ($P = .005$ and $P = .04$, respectively) (Table 3).

With CIN2$^+$ as reference standard, clinical sensitivity and specificity was analyzed in 40 samples with CIN2$^+$ and 892 samples with normal cytology and without evidence of a CIN2$^+$ diagnosis within a 12-month period. The clinical sensitivity of the HPV HR and HC2 for the detection of CIN2$^+$ was 92.5% (95% CI, 84.3–100.0) and 87.5% (95% CI, 77.3–99.7), and clinical specificity was 88.0% (95% CI, 85.9–

Table 3. Clinical Sensitivity and Clinical Specificity of Anyplex II HPV HR Detection Kit (HPV HR)[a] and Hybrid Capture 2 (HC2)[b] for the Detection of High-Grade Squamous Intraepithelial Lesion

Test	Clinical Sensitivity, % (95% CI)	Clinical Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
HPV HR	94.4 (89.2–99.7)	81.7 (79.4–84.0)	25.9 (20.6–31.1)	99.5 (99.1–100.0)
HC2	93.1 (87.2–98.9)	81.8 (79.5–84.1)	25.7 (20.4–31.0)	99.4 (98.9–99.9)

Abbreviations: 95% CI, 95% confidence interval; NPV, negative predictive value; PPV, positive predictive value.

[a] Seegene Inc, Seoul, Korea.
[b] Qiagen, Valencia, California.

Table 4. Clinical Sensitivity and Clinical Specificity of Anyplex II HPV HR Detection Kit (HPV HR)[a] and Hybrid Capture 2 (HC2)[b] for the Prediction of Cervical Intraepithelial Neoplasia Grade 2 or 3 and Treatable Cancer				
Test	Clinical Sensitivity, % (95% CI)	Clinical Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
HPV HR	92.5 (84.3–100.0)	88.0 (85.9–90.1)	25.7 (18.6–32.8)	99.6 (99.2–100.0)
HC2	87.5 (77.3–99.7)	87.9 (85.8–90.0)	24.5 (17.4–31.5)	99.4 (98.8–99.9)

Abbreviations: 95% CI, 95% confidence interval; NPV, negative predictive value; PPV, positive predictive value.

[a] Seegene Inc, Seoul, Korea.
[b] Qiagen, Valencia, California.

90.1) and 87.9% (95% CI, 85.8–90.0), respectively. Using a noninferiority score test, the clinical sensitivity and specificity of the HPV HR were not inferior to those of the HC2 assay ($P = .007$ and $P = .02$, respectively) (Table 4).

The HPV HR exhibited excellent intralaboratory and interlaboratory reproducibility, showing 98.0% (494 of 504; $\kappa = 0.953$; 95% CI, 0.925–0.982) and 97.4% (491 of 504; $\kappa = 0.940$; 95% CI, 0.908–0.972), respectively (Tables 5 and 6). For both the intralaboratory and interlaboratory reproducibility, the lower confidence bounds were less than 87%, with κ values less than 0.5. The results complied with the international guidelines.[4] For each genotyping agreement in the intralaboratory and interlaboratory reproducibility test of HPV HR, the lower confidence bounds were greater than 87%, with κ values greater than 0.5. The results complied with the international guidelines (data not shown).[4]

COMMENT

The purpose of this study was to evaluate the clinical performance of the novel, real-time, multiplex HPV HR using TOCE technology for the detection of 14 hrHPV genotypes. We compared HPV HR to HC2 as an established reference method in the setting of Korean cervical cancer screening based on international guidelines. To use routinely requested liquid-based cervical cytology samples in Huro Path medium, we validated the HC2 test using Huro Path medium versus PreservCyt medium. There was an excellent agreement between HC2 results using the 2 different media, demonstrating Huro Path medium to be an appropriate alternative to PreservCyt medium.

In comparative analysis of HPV HR, high agreement between the results of HPV HR and HC2 was observed. For samples diagnosed as less than HSIL, HSIL, and CIN2$^+$, the agreement rates between the 2 tests was similar at 92.4% ($\kappa = 0.408$), 93.1% ($\kappa = 0.745$), and 95.0% ($\kappa = 0.724$), respectively. A recent study[10] compared the HPV 28 test (Seegene) with the HC2 test for the detection of 13 hrHPV genotypes (HPV HR provides 14 hrHPV genotypes, whereas the HPV 28 test provides 19 hrHPV genotypes, including 14 hrHPV genotypes of the HPV HR and 9 low-risk genotypes). The overall agreement between 2 assays was 91.4% with $\kappa = 0.50$, which was similar to our results.

Regarding the genotype detection results, of 163 abnormal cytologic cases that were positive in both assays (Table 2), 5 cases (3.1%) showed only a single HPV-66 genotype in HPV HR (data not shown). This genotype is not covered by HC2, but the HC2 yielded a positive result. Even though we did not evaluate this result with an additional reference test, such as a sequencing analysis, because of inadequate sample volume, this result supports a previous report[11] of cross-reactivity of high-risk probes of HC2 with low-risk HPV types in the HC2 assay. The HPV HR using the type-specific primer amplification technique might report the more-accurate results without cross-reactivity. Further studies are needed to confirm the current outcome.

The clinical sensitivity and specificity of the HPV HR were noninferior to those of HC2 using the predetermined thresholds of 90% and 98%, respectively.[4] The HPV HR showed good intralaboratory and interlaboratory reproducibility because the lower confidence bound of the percentage of agreement was higher than 87% and the corresponding κ value was higher than 0.5, as suggested by guidelines.[4] Poljak et al[12] have reported that the multiplex, real-time PCR showed discordant results on mixed HPV infections, probably because of competition in amplification. However, the level of intralaboratory reproducibility of the HPV HR in this study was comparable with that of previous assays.[13–15]

The HPV HR has some advantages for hrHPV detection and extended genotyping. It can detect both single infections and multiple infections for 14 hrHPV genotypes using the TOCE technology. Other commercial kits[13–17] can report 3 kinds of results, such as HPV-16 and HPV-18, and a pool of 12 carcinogenic HPV genotypes, whereas the HPV HR can detect and distinguish all 14 hrHPV types individually. Presently, of the 251 specimens with positive results by both HPV HR and HC2 tests, 47 specimens (18.7%) had multiple infections for the hrHPV genotypes. Considering the strong relationship between multiple infections and cervical cancer,[18] the hrHPV genotype information provided by the HPV HR can serve as an

Table 5. Intralaboratory Reproducibility[a] Over Time for the Anyplex II HPV HR Detection Kit[b]			
	Second Results		
First Results	Positive	Negative	Total
Positive	150	8	158
Negative	2	344	346
Total	**152**	**352**	**504**

[a] Agreement, 98.0%; $\kappa = 0.953$; 95% confidence interval, 0.925–0.982.
[b] Seegene Inc, Seoul, Korea.

Table 6. Interlaboratory Agreement[a] Over Time for the Anyplex II HPV HR Detection Kit[b]			
	Second Results		
First Results	Positive	Negative	Total
Positive	150	12	162
Negative	1	341	342
Total	**151**	**353**	**504**

[a] Agreement, 97.4%; $\kappa = 0.940$; 95% confidence interval, 0.908–0.972.
[b] Seegene Inc, Seoul, Korea.

additional tool in treating patients infected with HPV. The exact identification of the HPV genotypes could help assess whether an HPV infection is transient or persistent by comparing the results of the primary cervical screening and follow-up. In addition, because a high viral load is closely associated with persistent infection, the results of HPV genotyping, including semiquantitative information (ie, viral load) using the cyclic-catcher melting temperature analysis, by the HPV HR could provide more accurate diagnosis of cervical cancer.[19,20]

This is the first study, to our knowledge, comparing the HPV HR and the HC2, based on the Meijer guidelines,[4] in the Korean population. There are several potential limitations. For the samples showing discordant results between HPV HR and HC2, further genotypes were not confirmed. Histologic diagnosis was possible only in women who were referred to colposcopy and was not performed in women with HPV^{+} but cytology-negative results. Nevertheless, considering that the overall concordance rate between the cytology and histologic diagnoses was 93.0% with a κ value of 0.77 in a Korean population study[21] and a high cytology-histology correlation in a Croatian population study,[22] the comparative analysis data for HSIL detection might also be useful for evaluation of the clinical performance of the HPV HR.

CONCLUSIONS

In conclusion, our data indicate that the HPV HR is highly comparable to HC2 for clinical equivalence and reproducibility based on international guidelines. The individual typing information for 14 hrHPV genotypes, including HPV-16 and HPV-18, provided by the HPV HR can be an important tool in patient management.

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