Human Papillomavirus E6 and E7 mRNA Testing in Detection of Uterine Cervical Lesions

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Directed by Professor Kwang Hwa Park

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ABSTRACT

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Background: HPV DNA testing, commonly used for cervical cancer screening along with cytology test has shown high sensitivity but low specificity. Recently, it was suggested that detection of E6/E7 oncogene transcripts of high-risk HPV types shows higher specificity for detection of high grade cervical squamous lesions but less sensitivity due to few HPV types targeted. There is still no reliable method with equal high sensitivity and specificity for the detection of HPV in cervical intraepithelial neoplasia (CIN) and cancers. **Methods:** Primers for real-time PCR to detect HPV E7 mRNA of HPV types 16,

18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69 were developed and the clinical application was evaluated and compared to commercial HPV DNA and mRNA assays. In total of 180 Thin Prep® samples, the remaining fluid after the cytology slide preparation were tested with Goodgene HPV DNA chip (DNA assay), NucliSENS EasyQ® HPV E6/E7 mRNA assay (EasyQ assay) and real-time PCR with new HPV E7 primers, which we developed (mRNA qRT-PCR). The sensitivity and specificity of each test were calculated with histologically CIN2+ (CIN, grade 2 or higher) lesions as the disease endpoint. **Results:** Among them, 54 (30%) were positive for mRNA qRT-PCR while 127 (71%) and 54 (30%) were positive for DNA assay and EasyQ assay, respectively. DNA assay was positive in all abnormal cytology cases. In cytology groups of squamous cell carcinoma (SCC), high grade squamous intraepithelial lesion (HSIL), atypical squamous cells – cannot exclude HSIL (ASC-H), low grade squamous intraepithelial lesion (LSIL) and atypical squamous cells of undetermined significance (ASC-US), the mRNA qRT-PCR test showed 100%, 100%, 100%, 25%, and 18% positivity while EasyQ assay showed 73%, 74%, 60%, 56%, and 32% positivity, respectively.

In normal cytology cases, the positivity rates were 3%, 7% and 51% by mRNA qRT-PCR, EasyQ assay and HPV DNA assay, respectively. For the detection of CIN2+ lesions, the sensitivity of mRNA qRT-PCR and DNA assay was 100% each, while EasyQ assay showed sensitivity of 72%. HPV E7 mRNA qRT-PCR showed the highest specificity (92%) among the three tests (DNA assay: 36% and EasyQ assay: 83%). **Conclusion:** It is suggested that HPV E7 mRNA qRT-PCR can overcome the shortcoming of low specificity in DNA assay as well as the low sensitivity of EasyQ assay for clinical detection of high grade cervical lesions and malignancies.

Key words: Human papillomavirus, genotyping, E6/E7 oncogene, cervical cancer, real-time PCR.

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INTRODUCTION

Cervical cancer is one of the most common cancers in women, ranked third by mortality rate in women cancer death by 2008.[1] In United States, it was the 13th most common cancer in women by 2008, showing more than 75% decline of annual rate over the past half century through cervical cytology screening program (Papanicolaou test).[2] Nevertheless, the incidence of cervical cancer is disproportionately high (>80%) in the developing countries, where cytology screening programs are either not in place or are not efficient.[3] Cytology screening programs, which require several

frequent visits to health services and expensive quality assurance are costly and difficult to set or maintain in undeveloped countries. As high-risk Human Papillomavirus (HPV) is defined as one of important pathogens of cervical cancer, HPV detection assays and HPV vaccines have been developed and HPV testing seems to be more sensitive than cytology for detection of precancerous lesions.[4]

Squamous cell carcinoma (SCC) is the most common histological subtype of cervical cancer, accounting for approximately 80% of cervical cancer cases. Lately, in regions with effective cervical cancer screening programs, the percentage of adenocarcinoma has increased (15-20%), because they arise from the poorly sampled glands of the cervical canal or from poorly recognized precursor lesions.[3] SCC develops through its precursor lesion, i.e. cervical intraepithelial neoplasia (CIN). It is now understood that persistent infection of high-risk HPV (HR-HPV) types plays an etiologic role in both SCC and its immediate precursor lesion, cervical intraepithelial neoplasia grade 3 (CIN 3).

Human Papillomavirus

In mid nineteen seventies, for the first time, researchers started to postulate and analyse a possible role of HPV in cervical cancer development. HPV type 16 and 18 were first isolated from the cervical cancer tissues in 1983 and 1984, respectively.[5-7] Later, numbers of studies were performed and provided more detailed information about HPV types, its structure and oncogene functions.[7]

More than 150 types of HPVs have been identified to date. About 40 of them can infect cervix and are divided into high and low risk group based on their frequent detection in carcinoma (including high grade lesions) and low grade lesions, respectively.[1] A pooled analysis of 11 case-control studies, performed in nine countries has proposed that HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 should all be considered as potentially carcinogenic. They classified HPV types 26, 53, and 66 as probable high-risk types and HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 as low-risk types, respectively.[8] From HR-HPV types, HPV type 16 and 18 are the most prevalent types associated with cervical cancer, accounting

together for 73% and individually accounting for 57% (HPV type 16) and 16% (HPV type 18), respectively.[9]

HPVs are non-enveloped, 8000-base pair, double-stranded, and circular DNA viruses. HPV genome has two coding regions (early "E" and late "L") and a non-coding regulatory region (NCR) (also referred to as "Long Control Region", "LCR" or "Upstream Regulatory Region", "URR") (Figure 1).

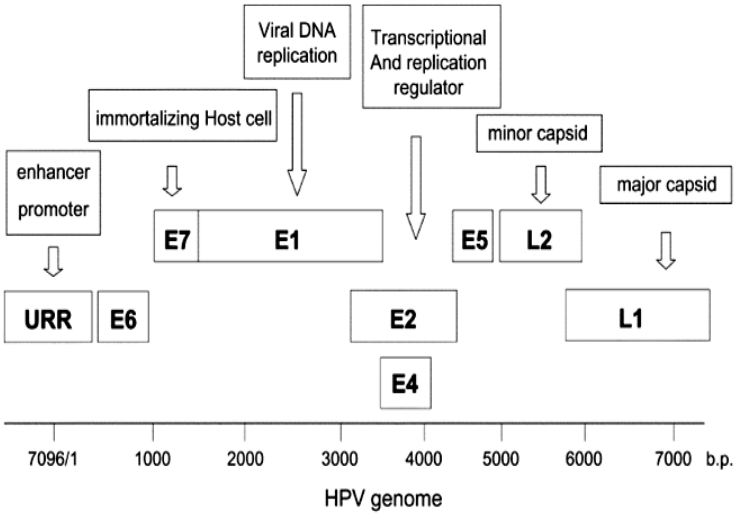


Figure 1. HPV genomic structure. HPV genome consists of six early (E) and two late (L) regions and an upstream regulatory region (URR).

Adopted from J Microbiol. 2004 Dec;42(4):255-66.

Six early genes E1, E2&E4-6, and E7 are involved in viral replication and two late genes L1&L2 are involved in capsid protein formation. Among three genes with proliferation-stimulating activity (E5, E6 and E7), E5 is deleted together with some part of the genome during viral DNA integration into host-cell DNA. Among several functions described for E6 and E7 oncoproteins, the most important one is attributed to tumor suppressor proteins p53 and retinoblastoma gene product (pRb), respectively. The oncoprotein E6 initiates degradation of the cellular tumor suppressor protein p53 via ubiquitin-mediated pathway, while E7 inactivates the pRB.[3, 10, 11] The synergistic effect of E6-E7 proteins would result in disturbance of cell cycle regulation, prevention of apoptosis, and further transformation and maintenance of neoplastic and dysplastic cells.

HPV infection and cancer development

Mucosal HPV infections are associated with a variety of diseases, ranging from benign genital warts to carcinomas of the cervix and anogenital region. HPV infection is the most common sexually transmitted disease and the great majority of sexually active women and men are infected with HPV at least once in their lifetime.[1] In most cases, HPV infection is resolved by itself within 6-12 months, occasionally causing mild morphologic changes (Figure 2). On the contrary, some HPV infections persist, showing increase of cancer risk.[2]

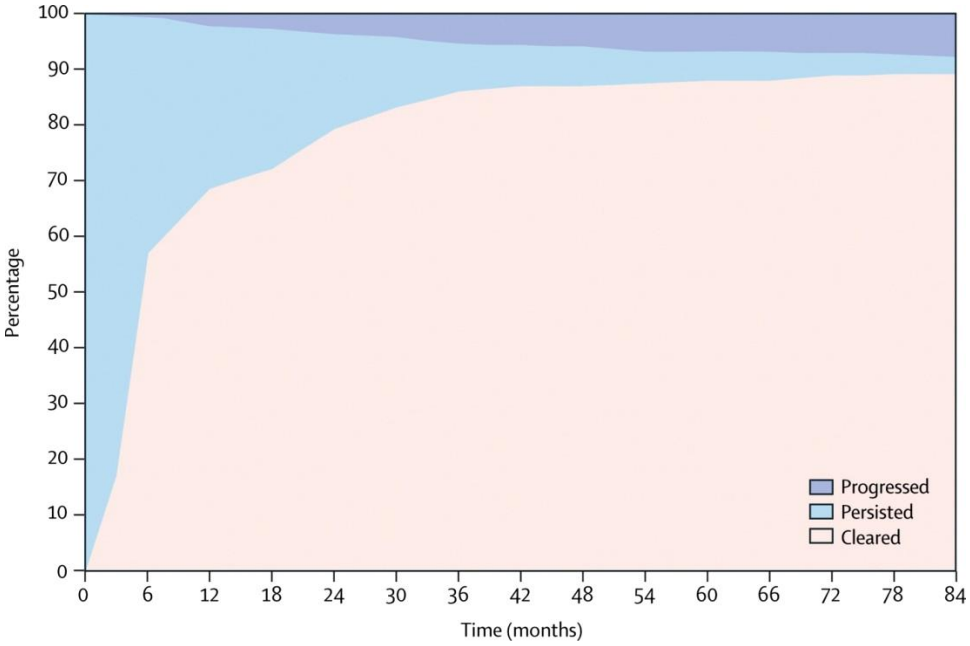


Figure 2. Clearance, persistence, and progression of HPV infections. While most of HPV infections clear by themselves, small percentage remains persistent and further progresses to cancer.

Adopted from Lancet 2007, 370:890-907.

Cervical cancer develops through four stages – HPV transmission, viral persistence, progression to precancerous lesion, and invasion. Backward steps also can occur, mainly clearance of HPV infection and less frequently, precancerous lesion regress to normal.[3]

HPV transmission: Anogenital HPV infections usually transfer via skin-to-skin or mucosa-to-mucosa contact. Transmission routes of all HPV types are same, therefore the rate of concurrent infections with several different types are high (20-30%).[3]

HPV persistence and clearance: More than 90% of HPV infections clear by themselves within a few years. The clearance rate is high within the first months after infection and decreases over time. Although the HPV clearance is defined when the HPV infection is no longer detectable by sensitive tests, the latent state of HPV is not well understood. The small proportion (less than 10%) of HPV infection persists for several years or more, remaining as persistent infection.[1, 3]

Progression to precancerous lesion and invasion: CIN 3 and carcinoma in situ are regarded as precancerous lesions while CIN 1 is not precancerous lesion but only a sign of HPV infection. However,

CIN 2 is equivocal in cancer potential. Persistent infections of HPV carry an increased risk of cancer precursors and cancer development. The time between HPV infection and development of CIN 3 is within 5 years, much shorter than development of invasive cancer from CIN 3.[1, 3] Therefore, the main target of cervical cancer screening programs is to diagnose CIN 3 as early as possible and to treat it before progression to invasive cancer.

CINs, histology and cytology

Uterine cervical cancer and CINs are screened and diagnosed on cytological and histological examination by the pathologists. Normally, uterine ectocervix and endocervix are lined by stratified squamous epithelium and glandular epithelium, respectively. The zone where these two epitheliums co-exist is called “transformation zone” and cervical cancers arise primarily from this zone. Several cytopathologic classifications were developed and used for cervical precancerous lesions (Table 1). The oldest one had four categories depending on the degree of dysplasia of epithelial cells. Next classification system is CIN system, which is commonly used in histological diagnosis. This system has three categories depending on the extent of dysplastic change within epithelium (Figure 3). The recent classification system has changed three-tiered system to two-tiered system, with CIN 1 renamed as low-grade squamous intraepithelial lesion (LSIL) and both CIN 2 and CIN 3 combined into one category, high-grade squamous intraepithelial lesion (HSIL).[12]

Table 1. Classification systems for squamous cervical lesions

Dysplasia/Carcinoma in Situ, Old Classification	Cervical Intraepithelial Neoplasia (CIN)	Squamous Intraepithelial Lesion (SIL), Current Classification
Mild dysplasia	CIN 1	Low-grade SIL (LSIL)
Moderate dysplasia	CIN 2	High-grade SIL (HSIL)
Severe dysplasia	CIN 3	High-grade SIL (HSIL)
Carcinoma in situ	CIN 3	High-grade SIL (HSIL)

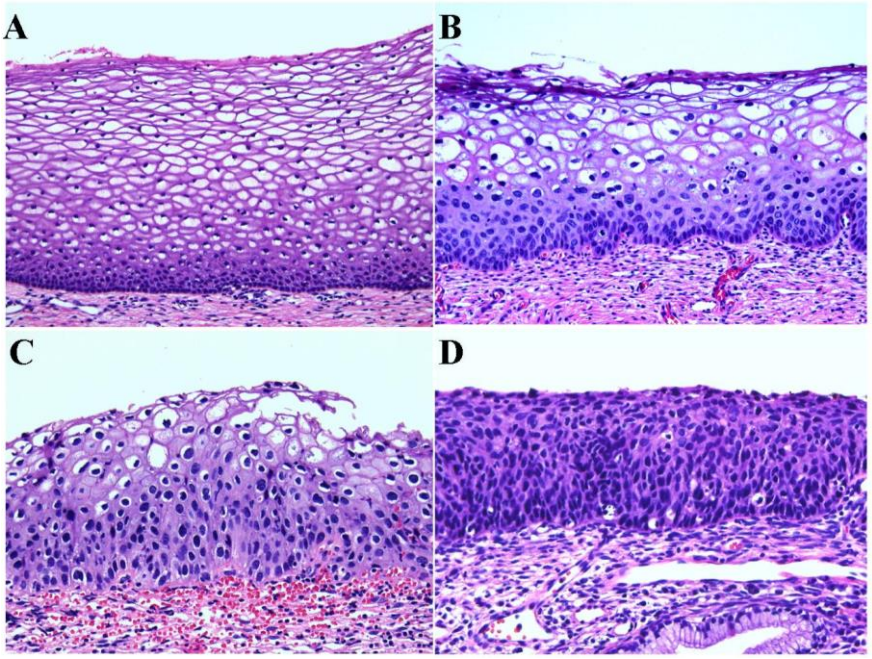


Figure 3. Cervical intraepithelial neoplasias (CINs).

A) Normal squamous epithelium; B) CIN grade 1 with koilocytic atypia and expansion of the immature basal cells within lower third of the epithelial thickness; C) CIN grade 2 with progressive atypia and expansion of the immature basal cells above the lower third of the epithelial thickness; D) CIN grade 3 with diffuse atypia with expansion of the immature basal cells to the epithelial surface.

The Bethesda System is used for reporting cervical cytology diagnosis. In this system, there are additional categories; atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H), each covers atypical cells that cannot fulfill the criteria of LSIL and HSIL, respectively (Table 2).[13]

Table 2. Bethesda System for cervical cytology report

The Bethesda System for Pap Smear Report (Squamous Cell Abnormalities)	Abbreviation
Squamous Cell Abnormalities	
- Atypical squamous cells of undetermined significance	ASC-US
- Atypical squamous cells cannot exclude HSIL	ASC-H
Squamous Intraepithelial Lesion	
- Low-grade squamous intraepithelial lesion	LSIL
- High-grade squamous intraepithelial lesion	HSIL
Squamous cell carcinoma	SCC

Uterine cervical screening

Cytology is the main tool for cervical screening program. However, a single cytological screening shows low sensitivity (about 50-60%) in detection of CIN 3.[14] Because a single-negative cytology test cannot provide stronger reassurance of not developing precancerous lesions, cervical screening requires repeated rounds of cytology tests. In addition to cytology screening, many studies have shown the benefit of HPV DNA detection for screening of high-grade CINs and SCCs.[15-17] HPV DNA testing is more sensitive than cytological screening and gives stronger reassurance (5-10 years) of not developing precancerous lesion and cancer for HPV DNA-negative women. High negative predictive value provides lengthening of cervical screening intervals, making cervical screening more cost-effective.[1, 18, 19] Therefore, co-testing for HPV DNA and cervical cytology is recommended and started being used for women 30 and older who have passed the peak age of acute, typically transient infections.[19, 20] Although HPV DNA testing is the main method used in cervical screening, it has some limitations.

HPV DNA testing

A variety of HPV DNA tests are currently available. Among them, Hybrid Capture® 2 (Qiagen Corporation, Gaithersburg, MD, USA) was the first to be approved by Food and Drug Administration of United States. This method is based on DNA/RNA hybridization with signal amplification and detects 13 high risk and 5 low risk HPV types. This assay is widely used in many clinical diagnostic laboratories, but it has a disadvantage that it cannot provide HPV genotyping within the low-risk or high-risk groups.[21, 22] Lately, HPV genotyping tests based on PCR assays, especially chip-based assays have been developed.[23] In South Korea, several DNA chip-based tests are commercially available and are used widely for cervical screening in clinical field.[22, 24]

Since the transient and asymptomatic HPV infection is highly prevalent in contrast to the small population with persistent infection and risk for precancerous lesion, the HPV DNA testing has high sensitivity (0.70-1.00) but low specificity (0.28-0.56) for detection of precancerous lesion and cancer.[25] Several studies have suggested that detection of E6/E7 oncogene transcripts of HR-HPV types would offer

higher specificity for screening the risk of development of high-grade CINs and SCCs.[26-28]

HPV E6/E7 mRNA testing

There are three commercially available HPV E6/E7 mRNA tests, the NucliSENS EasyQ® HPV (bioMérieux, Rome, Italy) and the PreTect™ HPV-Proofer (NorChip, Klokkarstua, Norway), which are based on the same technique, but manufactured by different companies, and the APTIMA® HPV Assay (Gen-Probe Incorporated, San Diego, CA, USA).[25] The NucliSENS EasyQ® HPV uses nucleic acid sequence-based amplification (NASBA) technique. This assay utilizes molecular beacon probes for the real-time detection and typing of E6/E7 mRNA from HPV type 16, 18, 31, 33, and 45.[29] NASBA is a sensitive isothermal (41°C), transcription-based amplification method which amplifies single stranded nucleic acids or RNA equivalents even in a background of double-stranded DNA.[30] Several studies using this assay for the detection of HPV E6/E7 mRNA have shown its higher specificity but lower sensitivity compared to DNA based assay tests.[28, 31-35] The average sensitivity and specificity of NucliSENS EasyQ® HPV assay in detection of CIN2+ lesions were 0.41-0.86 and 0.63-0.85, respectively.[25]

The APTIMA® HPV Assay detects HPV E6/E7 mRNA from 14 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) but does not provide genotyping. The APTIMA® HPV Assay has three main steps: (1) capture of the target mRNA, (2) target mRNA amplification using transcription-mediated amplification (TMA), and (3) detection of the amplification products using the Hybridization Protection Assay (HPA).[36] The APTIMA® HPV Assay reveals higher sensitivity (0.91-0.95) but lower specificity (0.42-0.56) in detection of CIN2+ lesions compared to NucliSENS EasyQ® HPV assay. However, both mRNA assays have not been tested in South Korean population until now.

Purpose of the study

Although HPV E6/E7 mRNA tests suggest higher specificity than HPV DNA tests, its clinical applicability is still controversial and additional clinical studies are needed.[25] From three existing commercial HPV E6/E7 mRNA tests, none has been tested in South Korean population yet. The purpose of this study is 1) to test the commercial HPV E6/E7 mRNA assay in South Korean population, 2) to develop new HPV E7 mRNA qRT-PCR primers and test them using clinical samples, and 3) to compare the two HPV mRNA tests with HPV DNA test.

We aimed to develop new HPV E7 mRNA qRT-PCR primers with high sensitivity and specificity. Commercial HPV E6/E7 mRNA assays have shown higher specificity than DNA tests, but optimizing its clinical sensitivity is required. To get optimal sensitivity as well as specificity, careful selection of HPV types which are targeted is required. Data collected from women of different world regions showed heterogeneity in HPV type distribution among women from different populations, especially in Asian countries and recommended that it should be taken into

account when developing screening tests for the HPV detection.[37] In South Korea, the most common HPV types associated with cervical cancer are type 16, 18, 31, 33, 35, 52, and 58,[38-40] while in European countries those are type 16, 18, 45, 33, and 31. Multi-target HPV E7 mRNA quantitative real-time RT-PCR primers were developed and tested on clinical samples, evaluated its sensitivity and specificity and compared to those of commercially available HPV DNA and mRNA tests.

MATERIALS AND METHODS

Patients and samples

Liquid-based cytology samples collected with ThinPrep® PAP test (Hologic, Marlborough, MA, USA), from 180 women between the age of 20 to 83 years (mean ± SD, 46.97 ± 11.40 and median age 46 years) were retrieved from the Department of Pathology, Wonju Severance Christian Hospital, between January 2010 and December 2011. Ethical approval was obtained from the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (YWMR-12-4-010). Cytology slides (Pap smear) were evaluated by pathologist according to the 2001 Bethesda System. Cytology cases of benign (within normal limit, reactive change due to inflammation, fungal infection, and atrophy), ASC-US, ASC-H, LSIL, HSIL, and SCC were included. Cases with available tissue biopsies were reviewed by pathologists. The remaining specimen after the cytology slide preparation was used as the sample for HPV DNA and RNA detection.

DNA extraction

DNA preparation was performed using the HPV DNA Extraction Solution (M&D, Wonju, South Korea), according to the manufacturer's instructions. Briefly, clinical specimens were collected in 50 ml tubes 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*, 4°C for 5 min. The supernatant was discarded. DNA extraction solution (100 μl) was added to the pellet. Then we vortexed the mixture for 1 min, and incubated at 56°C for 15 min with intermittent tube tapping. After incubation, samples were boiled for 10 min on a heating block or in a boiling water bath and centrifuged at 17,590 × *g* at 25°C for 3 min, and then supernatant (3-5 μl) was used as a template for PCR.

HPV DNA detection and genotyping

HPV genotyping using the Goodgene HPV chip (Goodgene Inc., Seoul, South Korea) was carried out according to the manufacturer's recommendations. Goodgene HPV chip is designed to detect 15 HR-HPVs together with 7 low-risk HPVs. The genotyping method required nested PCR to amplify the target region using MY11 and MY9 primers, followed by the P5/GP6 primer pair. Nested PCR conditions consisted of an initial denaturation step for 5 min at 94ºC; 15 cycles comprising of denaturation for 30 sec at 94ºC, and extension for 30 sec at 65ºC. The subsequent 45 cycles were 30 sec at 94ºC, and 30 sec at 54ºC. The final extension step was performed at 72ºC for 7 min. After PCR amplification of the target region, the following 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 read using GenePix Pro6.0 (Axon Instruments, Foster City, CA, USA) scanner.

RNA extraction

We examined and verified the MagNA Pure LC RNA Isolation Kit III on the MagNA Pure LC 1.0 Instrument (Roche Diagnostics, Mannheim and Penzberg, Germany). Tissue from specimens was extracted thirty-times in one series via the High Performance Protocol. In this automated process, the samples were lysed in a buffer containing a chaotropic salt and an RNAase inactivator. Nucleic acids were bound to the surface of glass magnetic particles. After a DNAase digestion step, unbound substances were removed by several washing steps, and purified RNA was eluted (elution volume, 50 $\mu\ell$) with a low-salt buffer followed by spectrophotometric quantification of RNA. After RNA isolation, RNA yield and purity were determined by spectrophotometric (OD260) measurement and calculation of OD260/280 ratio, respectively.

Real-time multiplex NASBA (NucliSENS EasyQ® HPV v1.1)

HPV mRNA extracts were tested with the NucliSENS EasyQ® HPV v1.1 test according to the manufacturer's instructions. NASBA is based on a repeated process of primer annealing, formation of double stranded DNA containing a T7 promoter site and T7 RNA polymerase-mediated transcription of multiple anti-sense copies of the RNA target sequences (amplicons).

Six different molecular beacons are used in the NucliSENS EasyQ® HPV v1.1 assay to detect the amplicons of the five HPV types (16, 18, 31, 33, and 45) and the U1 small nuclear ribonucleoprotein specific protein A (U1A protein). The use of two fluorescent dyes 6-carboxy fluorescein for the HPV types 16, 31 and 33 and 6-carboxy-X-rhodamine for the U1A protein and HPV types 18 and 45 allows simultaneous duplex amplification. Kinetic analysis of the fluorescent signals reveals the transcription rates of U1A protein and all five HPV RNA targets.

Briefly, three premixes were made by the reconstitution of reagent spheres in the reagent sphere diluents followed by the addition of either U1A/HPV type 16, HPV type 18/31, or HPV type 33/45

primer/molecular beacon mixes and KCl stock solution. Ten microliters of premix was distributed to each well, followed by 5 μℓ of each RNA, and incubated for 5 min at 65°C and 2 min at 41°C. The reaction was started by the addition of enzymes and measured in real time using the NucliSENS EasyQ® analyzer at 41°C. Data analysis was performed using the NucliSENS EasyQ® HPV assay software. Result analysis is preferably determined automatically using NucliSENtral™ HPV software (bioMérieux, Rome, Italy). The NucliSENS EasyQ® HPV test includes primer pairs targeting U1A mRNA as intrinsic control to determine the sample validity. If U1A is positive or one or more HPV targets are positive, the sample is valid. If U1A is negative and no HPV targets are positive, the sample is invalid. To evaluate the run validity, positive controls for U1A/HPV type 16, HPV type 18/31, and HPV type 33/45 are included.

Reverse transcription PCR

Complementary DNA (cDNA) was synthesized by Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. Shortly, the first mixture for reverse transcription was incubated at 65℃ for 5 min and quickly chilled on ice. Then the second mixture was added to first master mix in PCR tubes and the cDNA synthesis reaction consisted of 10 min at 25℃, 50 min at 37℃, and 15 min at 70℃.

HPV mRNA real-time RT-PCR

The TaqMan real-time PCR assay targets 16 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69) using E7 region primers and probes.

PCR primers and corresponding TaqMan probes were designed for three different sets of HPVs, targeting their common sequence (Set 1: Type 16, 31, 33, 35, 52,& 58; Set 2: Type 18, 39, 45, 51, 59,& 68; Set 3: Type 53, 56, 66,& 69). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for sample adequacy. HPV E7 mRNA and GAPDH gene primers were designed by Beacon Designer 3.0 software (Bio-Rad, Hercules, CA, USA).

Primers and probes were verified by analysing serial dilutions of plasmids containing DNAs of each HPV type. In each HPV type, plasmids were prepared using TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) with pCR2.1 plasmid (Invitrogen, Carlsbad, CA, USA).

HPV E7 mRNA qRT-PCR was applied to clinical samples. Briefly, 3 µl of cDNA was added to 10 µl universal PCR master mix

(Roche Diagnostics, Branchburg, NJ, USA) with 1 μl primers, 1 μl probes, and 4 μl nuclease-free water. After uracil DNA glycosylase activation at 50°C for 2 min and initial denaturation at 95°C for 10 min, the PCR for DNA detection was run for 45 cycles (15s at 95°C, 60s at 55°C) on an ABI 7300 instrument (Applied Biosystems, Carlsbad, CA, USA). The threshold cycle (*CT*) value for each reaction was recorded (a low *CT* value indicates a large amount of target). Only samples yielding a *CT* value for GAPDH below 38 were included in analysis.

Statistical analysis

All analyses were performed with SAS 9.2 Ver. (SAS Inc., Cary, NC, USA) statistical software. Sensitivity, specificity, accuracy and predictability of each DNA and RNA tests were estimated with histologically CIN2+ cases serving as the disease end point. Sensitivity and specificity values were calculated using the conventional contingency tables and, 95% confidence intervals (CIs) were computed using exact binomial methods. Accuracy of HPV detection test was calculated as the percentage of the correct results by each HPV test compared to the cytology diagnosis. The predictability was measured by area under the receiver operating characteristic curve (ROC) (AUC).

RESULTS

Cytological and histological diagnosis

Cytological and histological diagnostic findings are shown in Table 3. From 180 cytology samples, 45 (25%) cases were confirmed histologically, using biopsy or excision specimens at the same time; all ASC-H, HSIL and cancer cases had histological confirmation. Ninety nine (55%) cases were benign, 22 (12%) were ASC-US, 16 (9%) were LSIL, 5 (3%) were ASC-H, 23 (13%) were HSIL and 15 (8%) were cancer on cytology. Among the cancer cases, there was one case of microinvasive SCC and remaining cases were all SCCs. Among HSIL cases, 22 cases were diagnosed as CIN 3/SCC in situ, and 1 case was microinvasive SCC on histology. Three cases of ASC-H were CIN 2 on histology and the other two cases were CIN 3. Among the LSIL cases, two cases had histological confirmation and both cases were CIN 1 on histology.

Table 3. Cytological and histological diagnoses

Diagnosis		No. of samples (%)	
		Cytology (n = 180)	Histology (n = 45)
Benign		99 (55)	-
ASC-US		22 (12)	-
LSIL	CIN 1	16 (9)	2 (5)
ASC-H	CIN 2	5 (3)	3 (7)
	CIN 3		2 (5)
HSIL	CIN3/SCC in situ	23 (13)	22 (48)
	Microinvasive SCC		1 (2)
Malignancy	Microinvasive SCC	15 (8)	1 (2)
	SCC		14 (31)

ASC-US, atypical squamous cells of undetermined significance; LSIL, low grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; HSIL, high grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; CIN 1, cervical intraepithelial neoplasia, grade 1; CIN 2, cervical intraepithelial neoplasia, grade 2; CIN 3, cervical intraepithelial neoplasia, grade 3.

HPV DNA genotyping

From total 180 cases, 127 (71%) were positive for 161 HPV DNAs of 15 types (Table 4). HPV DNA assay showed 100% and 51% positivity in abnormal and normal cytology cases, respectively (Figure 4).

Type-specific HPV DNA prevalence in benign, ASC-US, LSIL, ASC-H, HSIL, and cancer groups are shown in Table 4. By overall distribution, the most prevalent HPV type was HPV16 (n = 60, 33%) followed by HPV18 (n = 24, 13%), HPV33 (n = 19, 11%), HPV58 (n = 11, 6%), HPV35, HPV56 and HPV66 (n = 8, 4%, respectively).

In 15 cases of cancer, 4 types of HPVs, 16 (n = 9, 60%), 33 (n = 4, 27%), and 31 and 58 (n = 1, 7%, each) were detected. Nine HPV types found in 23 HSIL cases were HPV type 16 (n = 11, 48%), 33 (n = 6, 26%), 18 and 35 (n = 2, 9%, each), and types 31, 52, 56, 58 and 66 (n = 1, 4%, each). In ASC-H group, HPV type 16 (n = 2, 40%), 31, 35, and 52 (n = 1, 20%, each), were detected.

HPV type 18 was the most prevalent type (n = 7, 44%) in the 16 LSIL cases, followed by HPV type 16 (n = 6, 38%), 33 (n = 3, 19%), 56 and 66 (n = 2, 13%, each), and 31, 35, 39, 51, 58, 59, and 69 (n = 1, 6%, each). In ASC-US group, HPV type 16 (n = 13, 59%), 18 (n = 4, 18%), 35, 58 and 66 (n = 2, 9%, each), and 33, 39, 45, and 69 (n = 1, 5%, each) were detected.

Out of 99 normal cases, 50 (51%) were HPV DNA positive and 49 (49%) cases were HPV DNA negative. The five most prevalent types in normal cases were HPV type 16 (n = 19, 19%), 18 (n = 11, 11%), 58 (n = 6, 6 %), 33 and 56 (n = 5, 5%, each).

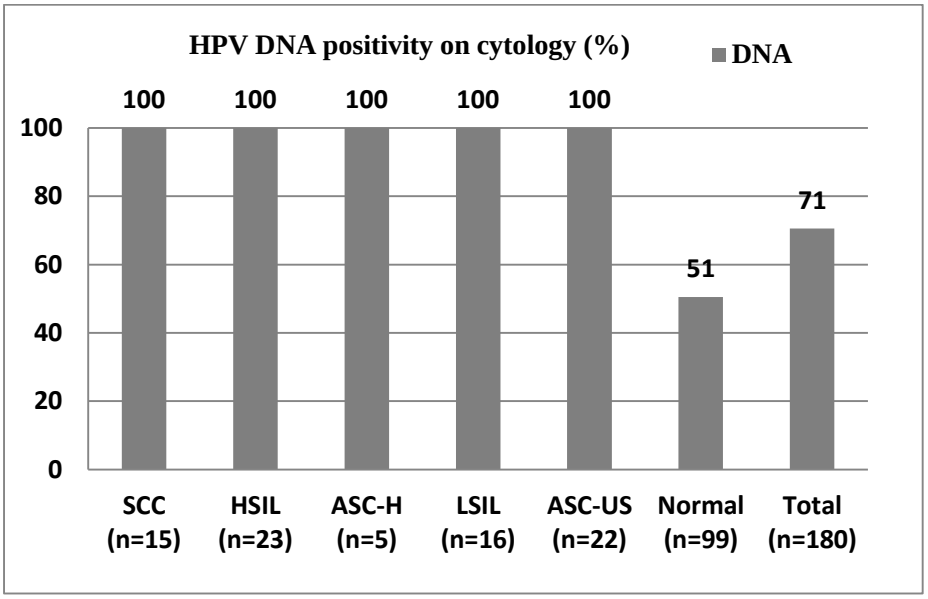


Figure 4. The HPV positivity on DNA assay. The HPV DNA showed similar high positivity in each abnormal cytology group.

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.

Table 4. Type-specific distribution of HPV DNA on cytology

HPV types	Cytological diagnosis (%)						Total* (n = 180)
	SCC (n = 15)	HSIL (n = 23)	ASC-H (n = 5)	LSIL (n = 16)	ASC-US (n = 22)	Normal (n = 99)	
16	9 (60)	11 (48)	2 (40)	6 (38)	13 (59)	19 (19)	60 (33)
18	0 (0)	2 (9)	0 (0)	7 (44)	4 (18)	11 (11)	24 (13)
31	1 (7)	1 (4)	1 (20)	1 (6)	0 (0)	1 (1)	5 (3)
33	4 (27)	6 (26)	0 (0)	3 (19)	1 (5)	5 (5)	19 (11)
35	0 (0)	2 (9)	1 (20)	1 (6)	2 (9)	2 (2)	8 (4)
39	0 (0)	0 (0)	0 (0)	1 (6)	1 (5)	1 (1)	3 (2)
45	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	2 (2)	3 (2)
51	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	0 (0)	1 (1)
52	0 (0)	1 (4)	1 (20)	0 (0)	0 (0)	3 (3)	5 (3)
56	0 (0)	1 (4)	0 (0)	2 (13)	0 (0)	5 (5)	8 (4)
58	1 (7)	1 (4)	0 (0)	1 (6)	2 (9)	6 (6)	11 (6)
59	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	1 (1)	2 (1)
66	0 (0)	1 (4)	0 (0)	2 (13)	2 (9)	3 (3)	8 (4)
68	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
69	0 (0)	0 (0)	0 (0)	1 (6)	1 (5)	1 (1)	3 (2)
Total*	15 (100)	26 (113)	5 (100)	27 (169)	27 (123)	61 (62)	161 (89)

*Each HPV type in a multiple infection is counted, which describes the cumulative percentage of more than 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.

Multiple HPV infection was detected in 21 (12%) cases (Table 5). From those, 14 cases showed double HPV infection and 7 cases showed triple HPV infection. On cytology, multiple infection was found in 9 (9%), 5 (22%), and 5 (31%) cases of normal, ASC-US and LSIL cases, respectively. From high grade lesions, HSIL group showed 2 (9%) cases of multiple HPV infection while cancer and ASC-H groups did not show any multiple infection.

Table 5. Multiple HPV infection patterns on cytology.

HPV types	Cytological diagnosis (%)						Total
	SCC (n = 15)	HSIL (n = 23)	ASC-H (n = 5)	LSIL (n = 16)	ASCUS (n = 22)	Normal (n = 99)	(n = 180)
16, 18	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	1 (1)	2 (1)
16, 45	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (0.6)
16, 58	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	1 (1)	2 (1)
18, 35	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.6)
18, 56	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.6)
18, 58	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (0.6)
18, 66	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	1 (0.6)
33, 56	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.6)
33, 58	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	1 (1)	2 (1)
35, 66	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.6)
56, 69	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.6)
16, 18, 33	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	1 (1)	2 (1)
16, 18, 35	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	0 (0)	1 (0.6)
16, 18, 39	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	0 (0)	1 (0.6)
16, 18, 56	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.6)
16, 33, 66	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.6)
18, 56, 59	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	0 (0)	1 (0.6)
Total	0 (0)	2 (9)	0 (0)	5 (31)	5 (22)	9 (9)	21 (12)

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.

The most prevalent HPV types in high grade lesions and low grade lesions showed significant difference (Figure 5). While HPV type 33 was the second most common type (22%) in high grade lesions, it formed 8% of HPV infections in low grade lesions. In contrast, HPV type 18, the second most prevalent type (19%) in low grade lesions, was detected in only 4% of HPV infections in high grade lesions.

The 4 most common HPV types in the 43 cases of histologically CIN2+ were HPV type 16, 33, 31, and 35 in descending order (Figure 6).

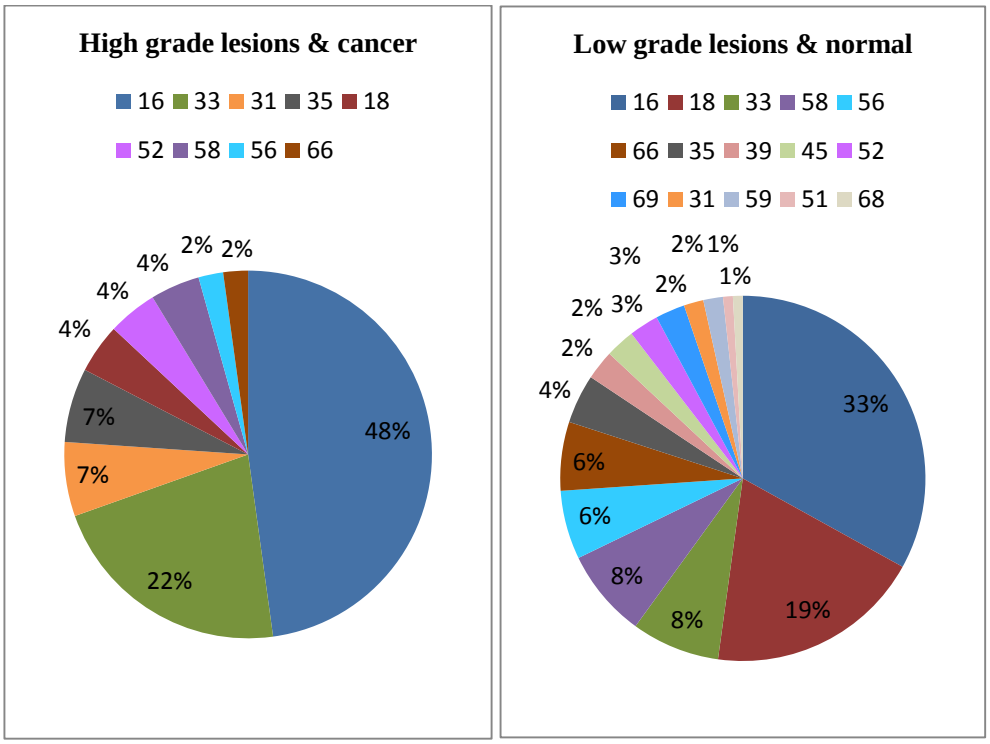


Figure 5. HPV type differences in high and low grade lesions. Although HPV16 is the most common HPV type in both high and low grade lesions, the remaining HPV infections show significant difference in type distribution.

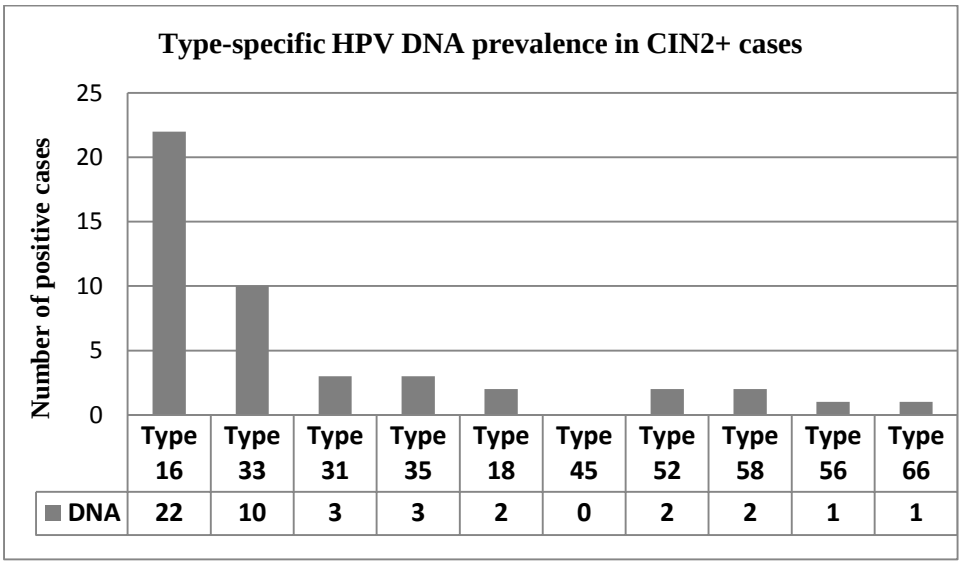


Figure 6. Type-specific distribution of HPV DNA in CIN2+ cases. DNAs from nine HPV types were detected in CIN2+ cases.

HPV E6/E7 mRNA NucliSENS EasyQ® assay

The NucliSENS EasyQ® was positive for 54 (30%) cases out of total 180 cases. The positivity rate in each cytology group is shown in Figure 7. The mRNA test showed higher expression of E6/E7 proteins in high grade lesions compared to those in low grade lesion and normal cases. The positivity rates were 73%, 74%, 60%, 56%, 32% and 7% for SCC, HSIL, ASC-H, LSIL, ASC-US, and normal groups, respectively.

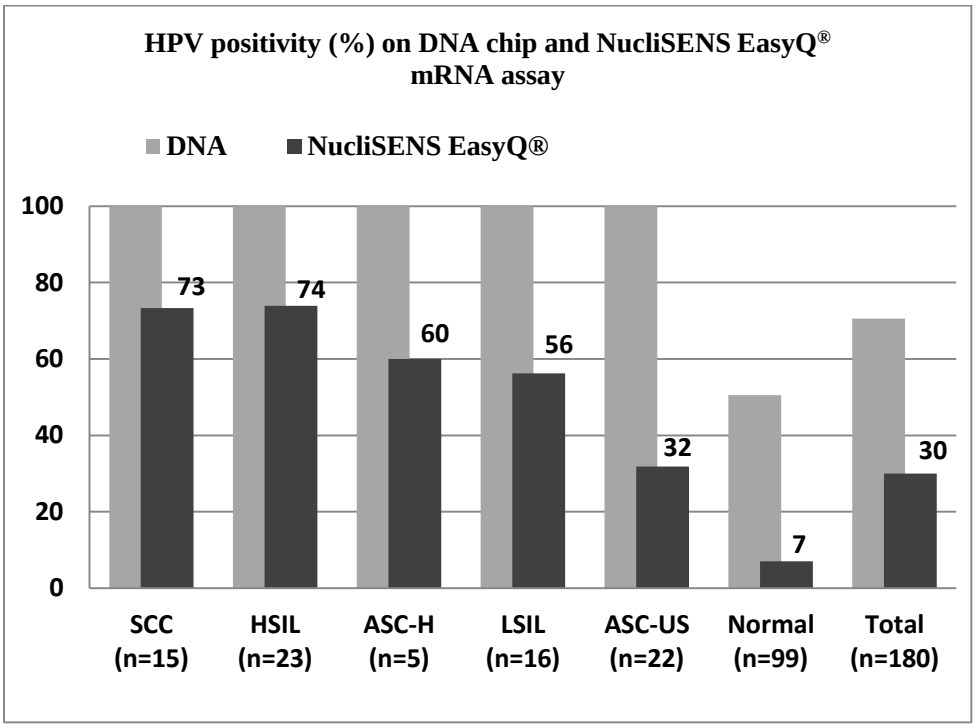


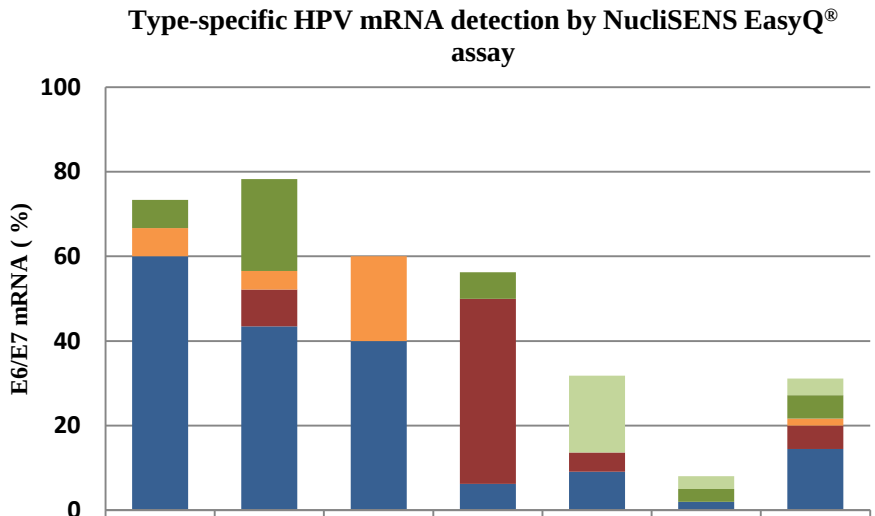
Figure 7. The differences of HPV positivity on DNA assay and mRNA NucliSENS EasyQ® assay. The positivity of HPV by mRNA test is enhanced along with the increase in the degree of dysplasia while the DNA assay shows same high HPV positivity in all abnormal cytology groups.

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.

Type-specific detection rates of HPV mRNA according to cytology are shown in Figure 8. Among total 180 cases, the most common HPV types expressing E6/E7 mRNA was HPV type 16 (n = 26, 14%), followed by 33 (n = 10, 6%), 18 (n = 10, 6%), 45 (n = 7, 4%), and 31 (n = 3, 2%). In 15 cancer cases, mRNA from HPV types 16 (n = 9, 60%) and 31, and 33 (n = 1, 7%, each) were detected. From the four negative cases, three were positive for HPV type 33 and one was positive for 58 by DNA genotyping. In HSIL group, HPV types that expressed HPV E6/E7mRNA were HPV type 16 (n = 10, 43%), 33 (n = 5, 22%), 18 (n = 2, 9%) and 31 (n = 1, 4%). In the six mRNA negative cases, DNAs from HPV types 18, 33, 35, 52, and 58 were detected on DNA genotyping. In both SCC and HSIL groups, HPV E6/E7 mRNA from HPV type 45 was not found. Out of five ASC-H cases, two cases were positive for HPV type 16 mRNA (n = 2, 40%), and one case was positive for HPV type 31 mRNA (n = 1, 20%).

In LSIL group, mRNAs from HPV type 18 (n = 7, 44%), 16 (n = 1, 6%) and 33 (n = 1, 6%) were detected. In ASC-US group, mRNAs from HPV type 45 (n = 4, 18%), 16 (n = 2, 9%) and 18 (1,

5%) were detected. In normal 99 cases, 7 cases (7%) showed positivity of mRNAs from HPV type 33 (3, 3%), 45 (3, 3%), and 16 (2, 2%).



	SCC (n=15)	HSIL (n=23)	ASC-H (n=5)	LSIL (n=16)	ASCUS (n=22)	Normal (n=99)	Total (n=180)
Type 45	0	0	0	0	18	3	4
Type 33	7	22	0	6	0	3	6
Type 31	7	4	20	0	0	0	2
Type 18	0	9	0	44	5	0	6
Type 16	60	43	40	6	9	2	14

Figure 8. Type-specific distribution of HPV E6/E7 mRNA on cytology. From five targeted HPV types, mRNA from HPV45 was not detected in high grade lesions or cancer group. In contrast, HPV31 mRNA was not detected in low grade lesions or normal group.

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.

Total 3 (2%) cases showed mRNA expression of two HPV types. From HSIL group, one cases showed E6/E7 mRNA from HPV type 16 and 18. One case of ASC-US showed mRNA from HPV type 16 and 45. HPV mRNA of HPV types 33 and 45 were detected in one case of normal cytology group.

The HPV types that showed mRNA expression in the 43 cases of histologically CIN2+ cases were HPV type 16, 33, 18, and 31 in descending order (Figure 9).

The comparison of NucliSENS EasyQ® HPV mRNA assay and HPV DNA assay in the CIN2+ cases is shown in Table 6. While all 43 cases were positive for HPV DNA assay, 12 cases showed negativity for HPV mRNA by NucliSENS EasyQ® HPV mRNA test.

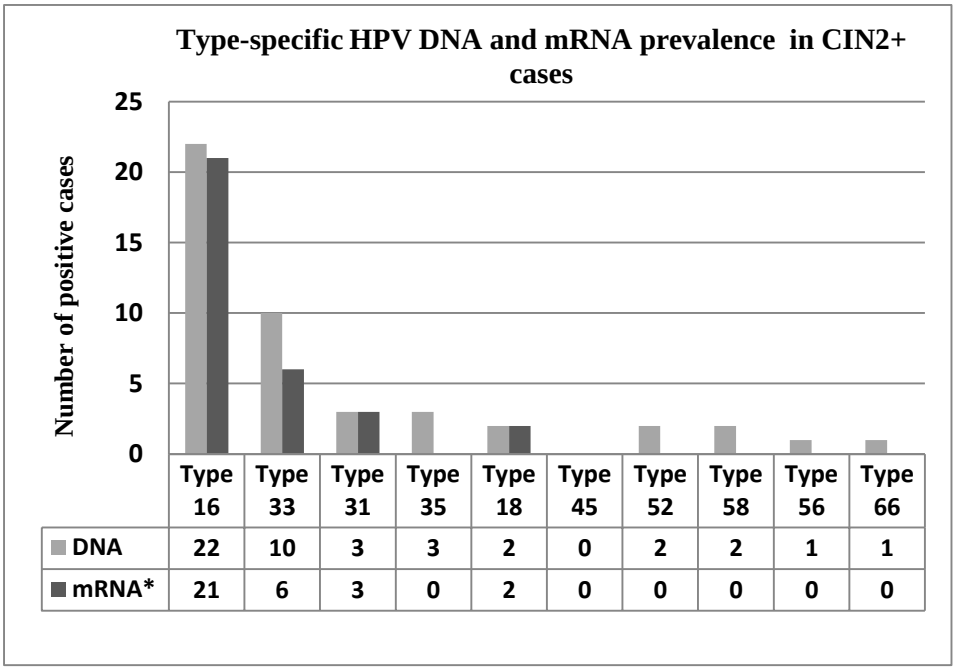


Figure 9. Type-specific distribution of HPV DNA and mRNA in CIN2+ cases. HPV45 was not detected in both HPV DNA and mRNA tests.

*mRNA detection was available for only 5 HPV types, 16, 18, 31, 33, and 45.

Table 6. Comparison between the NucliSENS EasyQ® mRNA assay and Goodgene DNA assay for detection of CIN2+*

NucliSENS EasyQ®	Goodgene, number of samples		
	Positive	Negative	Total
Positive	31	0	31
Negative	12	0	12
Total	43	0	43

* CIN2+: Cervical intraepithelial neoplasia grade 2 or higher

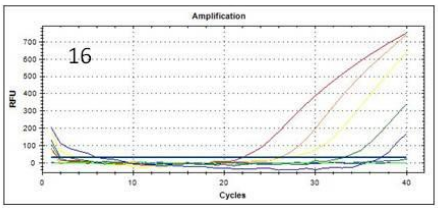
HPV E7 mRNA real-time RT-PCR

HPV mRNA qPCR for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69 showed high sensitivity in testing with serial dilutions of each HPV type plasmids (Figure 10-13).

In clinical samples, the test showed GAPDH positivity as control in all 180 cases. The HPV E7 mRNA qPCR was positive for 54 (30%) cases out of total 180 cases. The positivity rate in each cytology group is shown in Figure 14. The mRNA qPCR showed 100% expression of E7 proteins in high grade lesions and reveals significant low positivity in low grade lesion and normal cases. The positivity rates in each cytology groups were 100%, 100%, 100%, 25%, 18% and 3% for SCC, HSIL, ASC-H, LSIL, AS-CUS, and normal, respectively.

Although its overall positivity rate is same with the NucliSENS EasyQ® assay (30%), HPV E7 mRNA qPCR test showed 100% positivity in high grade lesions (SCC, HSIL, ASC-H) similar to the DNA assay. In contrast, in the low grade lesions and normal cases, HPV E7 mRNA qPCR showed the lowest positivity rate among these

three tests (25%, 18% and 3% in LSIL, ASC-US, and normal groups, respectively).



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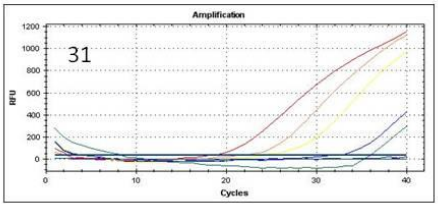
2	Unkn	1pg	1.2×10^5	25.99
3	Unkn	100fg	1.2×10^4	26.82
4	**Unkn**	**10fg**	**1.2×10^3**	**33.06**
5	Unkn	1fg	1.2×10^2	37.17
6	Unkn	N	0 copy	N/A



No.	Plasmid	Conc.	Copy	Ct
1	Unkn-31	10pg	1.2×10^6	18.46
2	Unkn	1pg	1.2×10^5	22.17
3	Unkn	100fg	1.2×10^4	24.27
4	**Unkn**	**10fg**	**1.2×10^3**	**31.55**
5	Unkn	1fg	1.2×10^2	36
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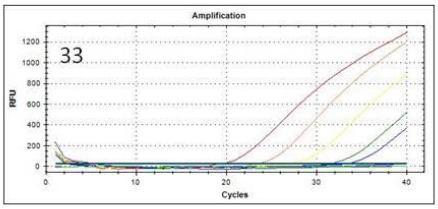

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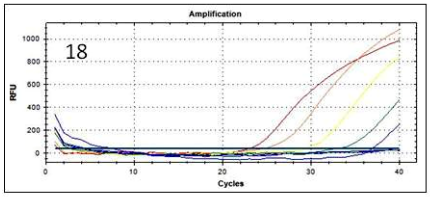
3	Unkn	100fg	1.2×10^4	27.23
4	**Unkn**	**10fg**	**1.2×10^3**	**31.66**
5	Unkn	1fg	1.2×10^2	33.56
6	Unkn	N	0 copy	N/A

Figure 10. The sensitivity of HPV E7 mRNA qRT-PCR in HPV 16, 31, and 33 plasmids. Using serially diluted HPV type-specific plasmids, the sensitivity of HPV E7 mRNA real-time RT-PCR was analyzed. The signal was detected from reactions with $1.2x10^2$ to $1.2x10^6$ copies of HPV type 16, 31, and 33 plasmids and blank control reaction without plasmid.



No.	Plasmid	Conc.	Copy	Ct
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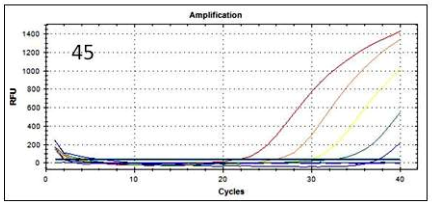
3	Unkn	100fg	1.2×10^4	29.42
4	**Unkn**	**10fg**	**1.2×10^3**	**32.88**
5	Unkn	1fg	1.2×10^2	36.92
6	Unkn	N	0 copy	N/A



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2	Unkn	1pg	1.2×10^5	26.02
3	Unkn	100fg	1.2×10^4	29.84
4	**Unkn**	**10fg**	**1.2×10^3**	**32.51**
5	Unkn	1fg	1.2×10^2	37.5
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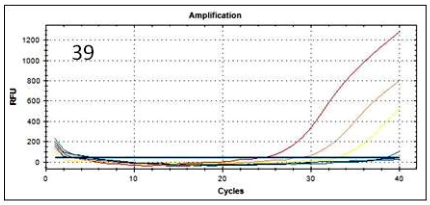

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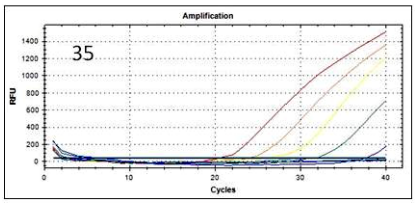
2	Unkn	1pg	1.2×10^5	28.06
3	**Unkn**	**100fg**	**1.2×10^4**	**32.88**
4	Unkn	10fg	1.2×10^3	38.38
5	Unkn	1fg	1.2×10^2	N/A
6	Unkn	N	0 copy	N/A

Figure 11. The sensitivity of HPV E7 mRNA qRT-PCR in HPV 18, 45, and 39 plasmids. Using serially diluted HPV type-specific plasmids, the sensitivity of HPV E7 mRNA real-time RT-PCR was analyzed. The signal was detected from reactions with 1.2×10^2 to 1.2×10^6 copies of HPV type 18, 45, and 39 plasmids and blank control reaction without plasmid.



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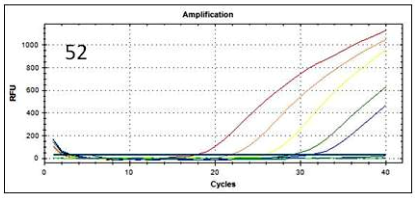
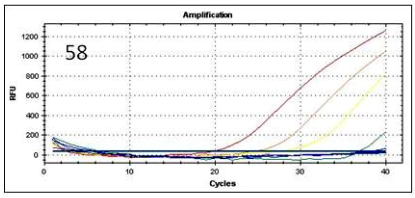
2	Unkn	1pg	1.2×10^5	23.74
3	Unkn	100fg	1.2×10^4	26.06
4	**Unkn**	**10fg**	**1.2×10^3**	**30.94**
5	Unkn	1fg	1.2×10^2	37.5
6	Unkn	N	0 copy	N/A



No.	Plasmid	Conc.	Copy	Ct
1	Unkn-52	10pg	1.2×10^6	17.7
2	Unkn	1pg	1.2×10^5	21.71
3	Unkn	100fg	1.2×10^4	25.69
4	Unkn	10fg	1.2×10^3	29.01
5	**Unkn**	**1fg**	**1.2×10^2**	**31.16**
6	Unkn	N	0 copy	N/A



No.	Plasmid	Conc.	Copy	Ct
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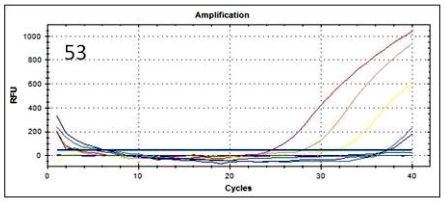
2	Unkn	1pg	1.2×10^5	24.46
3	Unkn	100fg	1.2×10^4	27.41
4	**Unkn**	**10fg**	**1.2×10^3**	**36.87**
5	Unkn	1fg	1.2×10^2	38.3
6	Unkn	N	0 copy	N/A

Figure 12. The sensitivity of HPV E7 mRNA qRT-PCR in HPV 35, 52, and 58 plasmids. Using serially diluted HPV type-specific plasmids, the sensitivity of HPV E7 mRNA real-time RT-PCR was analyzed. The signal was detected from reactions with 1.2×10^2 to 1.2×10^6 copies of HPV type 35, 52, and 58 plasmids and blank control reaction without plasmid.



No.	Plasmid	Conc.	Copy	Ct
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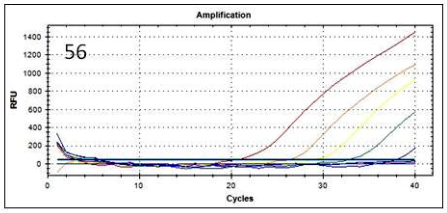
2	Unkn	1pg	1.2×10^5	27.63
3	**Unkn**	**100fg**	**1.2×10^4**	**31.26**
4	Unkn	10fg	1.2×10^3	37
5	Unkn	1fg	1.2×10^2	37.06
6	Unkn	N	0 copy	N/A



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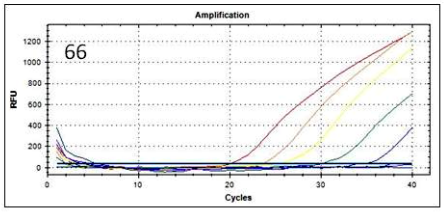
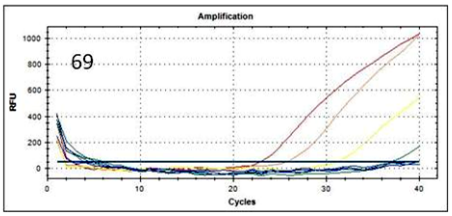
3	Unkn	100fg	1.2×10^4	28.78
4	**Unkn**	**10fg**	**1.2×10^3**	**32.75**
5	Unkn	1fg	1.2×10^2	38.01
6	Unkn	N	0 copy	N/A



No.	Plasmid	Conc.	Copy	Ct
1	Unkn-66	10pg	1.2×10^6	20.14
2	Unkn	1pg	1.2×10^5	23.58
3	Unkn	100fg	1.2×10^4	25.27
4	**Unkn**	**10fg**	**1.2×10^3**	**30.19**
5	Unkn	1fg	1.2×10^2	34.94
6	Unkn	N	0 copy	N/A



No.	Plasmid	Conc.	Copy	Ct
1	Unkn-69	10pg	1.2×10^6	22.85
2	Unkn	1pg	1.2×10^5	25.61
3	**Unkn**	**100fg**	**1.2×10^4**	**31.1**
4	**Unkn**	**10fg**	**1.2×10^3**	**36.93**
5	Unkn	1fg	1.2×10^2	39.68
6	Unkn	N	0 copy	N/A

Figure 13. The sensitivity of HPV E7 mRNA real-time RT-PCR in HPV 53, 56, 66 and 69 plasmids. Using serially diluted HPV type-specific plasmids, the sensitivity of HPV E7 mRNA real-time RT-PCR was analyzed. The signal was detected from reactions with 1.2×10^2 to 1.2×10^6 copies of HPV type 53, 56, 66, and 69 plasmids and blank control reaction without plasmid.

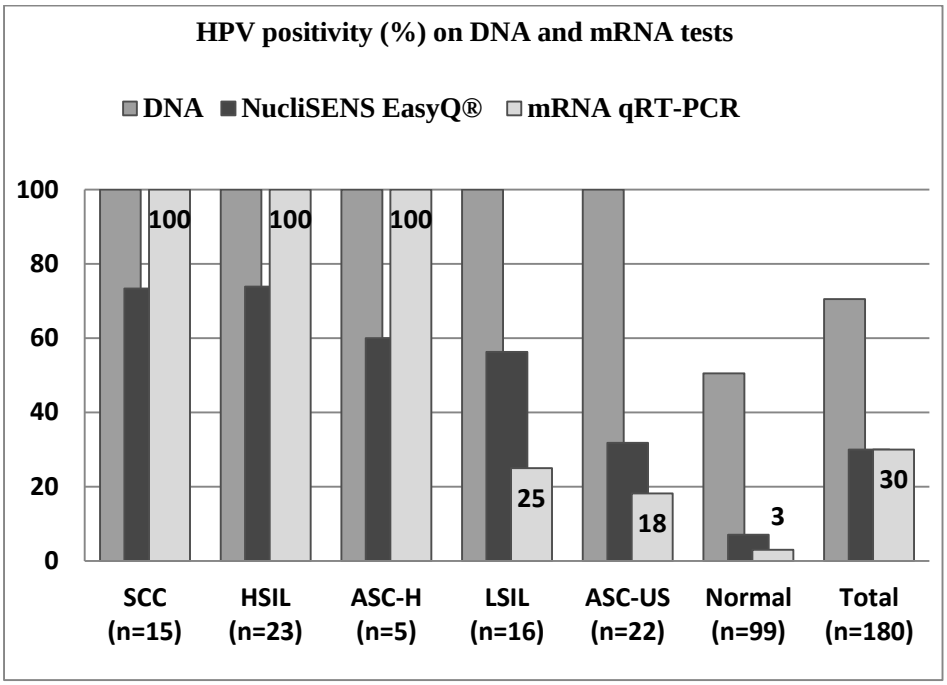


Figure 14. The differences of HPV positivity on DNA assay, mRNA NucliSENS EasyQ® assay and mRNA qRT-PCR. HPV E7 mRNA qRT-PCR showed same high positivity with DNA assay in high grade lesions and the lowest positivity among the three tests in low grade lesions.

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.

The comparison of HPV E7 mRNA qPCR to NucliSENS EasyQ® HPV mRNA assay in the CIN2+ cases is shown in Table 7. While all 43 cases were positive for HPV E7 mRNA qPCR, 12 cases showed negativity for HPV mRNA by NucliSENS EasyQ® HPV mRNA test.

Further NucliSENS EasyQ® HPV mRNA assay negative but HPV E7 mRNA qPCR positive cancer and HSIL cases were tested with type-specific HPV E7 mRNA qPCR. All ten cases were positive for HPV E7 mRNA from HPV types 18, 33, 35, 52, and 58. While five cases were positive for HPV types 35, 52, and 58, which are not covered in NucliSENS EasyQ® HPV mRNA, other five cases showed HPV mRNA positivity of HPV18 and HPV33, which are targeted with NucliSENS EasyQ® HPV mRNA.

Table 7. Comparison between the HPV mRNA qRT-PCR and NucliSENS EasyQ® mRNA assay for detection of CIN2+*

HPV mRNA qRT-PCR	NucliSENS EasyQ® mRNA assay		
	Positive	Negative	Total
Positive	31	12	43
Negative	0	0	0
Total	31	12	43

* CIN2+: Cervical intraepithelial neoplasia grade 2 or higher

Sensitivity and specificity of HPV genotyping and HPV E6/E7 mRNA tests

Diagnostic indices of the three tests are shown in Table 8. Although the sensitivity was the highest (100%) among the three methods, the specificity (36%), accuracy (51%) and predictability (0.67) were the lowest in HPV DNA assay. The NucliSENS EasyQ® HPV mRNA assay showed a moderate sensitivity of 72% as well as specificity (83%), accuracy (80%) and predictability (0.77). The HPV E7 mRNA qPCR showed the highest sensitivity of 100% as well as specificity (92%), accuracy (94%) and predictability (0.96) among the three tests compared.

Table 8. Diagnostic indices of the three tests for detection of CIN2*+ lesions.

Diagnostic index	HPV mRNA qRT-PCR % (95% CI)	NucliSENS EasyQ® % (95% CI)	Goodgene DNA chip % (95% CI)
Sensitivity	100% (100% - 100%)	72% (59% - 85%)	100% (100% - 100%)
Specificity	92% (87% - 96%)	83% (77% - 89%)	36% (28% - 44%)
Accuracy	94% (90% - 97%)	80% (75% - 86%)	51% (44% - 58%)
AUC** predictability	0.96	0.77	0.67

* CIN2+: Cervical intraepithelial neoplasia grade 2 or higher

**AUC: Area under the receiver operating characteristic (ROC) curve.

DISCUSSION

Indeed, HPV mRNA tests have proven to be more effective than HPV DNA tests in cervical cancer screening. In total 180 cervical cytology samples of normal and abnormal cases, the detection rate was significantly higher by HPV DNA assay (71%) compared to HPV mRNA tests (30% each). While DNA assay showed non-specific high positivity in all abnormal (100%) and half of normal (51%) cases, the two HPV mRNA tests showed significant low positivity (7% and 3%, respectively) in normal cases and ascending rate together with dysplasia and malignancy cases. It shows that every HPV infection doesn't express E6/E7 oncogenes, but ones with high risk for dysplasia and malignant change do.

The high detection rate of HPV DNA in low grade lesions and normal cases shows that acute non-carcinogenic transient HPV infections are popular in women, thus it makes the specificity of HPV DNA assay very low (36%). Although its clinical specificity is low, HPV DNA tests have still remained as a test used for cervical screening together with cytology screening. It is partly due to the fact

that HPV E6/E7 mRNA tests have been recently developed and more studies are still required for their application in cervical screening.

Goodgene DNA Chip, which was used in the current study, has an advantage of simultaneous HPV DNA genotyping in addition to detection and further to observe the positive HPV type difference in DNA and mRNA tests. The most prevalent HPV types in CIN2+ lesions and low grade lesion and normal groups showed several significant differences. While HPV types found in cancer and high grade lesions were HPV type 16, 33, 31, 35, 18, 52, and 58, the most common types in low grade lesions and normal cytology were HPV type 16, 18, 33, 58, 56, and 66. HPV type 33, the second most common type found in CIN2+ lesions was 22% among the HPV infection but in low grade lesions and normal cases it formed 8% of all infection. In contrast, HPV type 18 which is the second most common type (19%) in low grade lesion and normal was found only in 4% of HPV infections in CIN2+ cases.

Recently, a large meta-analysis data described HPV types 16, 18, 58, 33, and 52 as the most prevalent HPV types associated with cancer in South Korean women,[38] which differ from the most prevalent

HPV types in other countries.[8, 41, 42] In this study, it was found that the most common HPV types in cancer and high grade lesions were HPV type 16, 33, 31, 35, 18, 52, and 58. The discrepancy between our results and theirs can be explained; it is because the subjects of this study group were residents of a single province. In the cancer group, the second most prevalent HPV type 33 which accounts for 22% was significantly higher compared to their result (4%). In HSIL group, similar difference was also observed. The detected HPV types were 16 (48%), 33 (26%), 18 (9%), 35 (9%), and types 31, 52, 56, 58, and 66 (4% each) in this study while the most common types according to Bae *et al.*[38] were HPV type 16 (40%), 58 (14%), 18 (7%), 52 (5%) and 33 (5%). These findings could be due to the difference between the subject numbers of the two study population. Taken together, we can suggest that the geographic difference, in the prevalence of HPV types of the target population, must be considered while developing screening tests for the HPV detection.

This was the first study to evaluate NucliSENS EasyQ® HPV E6/E7 mRNA assay performance in South Korean population. Although HPV E6/E7 mRNA positivity in each cytology groups

showed differences according to the degree of dysplastic change, the positivity rate in cancer and HSIL groups (73% and 74%), were low compared to those of HPV DNA test. Thus, the sensitivity of NucliSENS EasyQ® HPV E6/E7 mRNA in detecting CIN2+ lesions were low (72%). Several studies have been done in Europe to evaluate the clinical performance of HPV E6/E7 mRNA assays, NucliSENS EasyQ®, and PreTect™ HPV-Proofer in comparison with DNA testing.[25, 28, 43, 44] Most of these studies used Hybrid Capture® 2 (HC2) test for HPV DNA detection. HC2 uses a DNA cocktail of 13 HR-HPV types, thus does not provide genotyping. Cattani *et al*.[35] suggested that mRNA assay has approximately the same sensitivity as DNA assay (81% and 86%, respectively) and a higher specificity (73% and 56%, respectively) in setting of total 180 cases.[35] Compared to their result (sensitivity of 81% and specificity of 73%), this study showed lower sensitivity (72%) but a higher specificity (83%) of NucliSENS EasyQ® assay. It can be explained that the different prevalence of five HPV types targeted by NucliSENS EasyQ® in Europe and Asia has a possibility to make discrepancy. The most common HPV types detected in cervical cancer in Asia were HPV types 16, 18, 58, 33, and

52 while HPV types 16, 18, 33, 31, and 45 were the most prevalent in Europe.[9] Type-specific E6/E7 prevalence showed that E6/E7 mRNA from HPV type 45 was not found in high grade lesions or cancer group. Another study performed in Canada showed sensitivity of 78% and specificity of 75% for HPV E6/E7 mRNA assay.[33] My result shows a slightly higher specificity compared to the above-mentioned studies. Detection of E6/E7 mRNA from only five HPV types is one of the reasons of low sensitivity of NucliSENS EasyQ® HPV E6/E7 mRNA assay. In CIN2+ cases, the most common HPV types were 16, 33, 31, 35, 18, 52, and 58, three of which were not included in the mRNA assay. Increasing the number of HPV types targeted by NucliSENS EasyQ® can be helpful in solving the low sensitivity issue of this test.

In order to increase the sensitivity of HPV E6/E7 mRNA tests, we developed the HPV E7 mRNA qRT-PCR primer which covered 16 HR-HPV types, tested in clinical samples and compared to the two tests described above. Although the overall positivity was the same in both mRNA tests, the positivity in each cytology group showed difference. HPV E7 mRNA qRT-PCR showed higher positivity in high

grade lesions and lower positivity in low grade lesions compared to those of NucliSENS EasyQ® mRNA assay. Partly, the difference in the detection rate can be affected by the difference in number of HPV types covered by each test. NucliSENS EasyQ® only detects five high risk HPV types, one of which (HPV type 45) was not detected in high grade lesions but was found in low grade lesions. On the contrary, the new HPV E7 mRNA primer covers 16 high risk types of HPV. Another reason for the difference of mRNA tests results is the target sequence of the tests. NucliSENS EasyQ® HPV mRNA test uses HPV E6 an E7 genes as a target sequences, but HPV mRNA qRT-PCR targets only E7 regions of each HPV types.

In terms of clinical performance, HPV E7 mRNA qRT-PCR showed the highest specificity together with high sensitivity, accuracy and predictability among the three tested methods. NucliSENS EasyQ® HPV E6/E7 mRNA assay showed a moderate specificity (83%) but the lowest sensitivity (72%) among the three tests. Accuracy and predictability were both higher than DNA assay but lower than new HPV E7 mRNA PCR.

High sensitivity (100%) and high specificity (92%) of the new HPV E7 mRNA qRT-PCR can overcome the disadvantages of low specificity of DNA assay and low sensitivity of NucliSENS EasyQ® HPV E6/E7 mRNA assay. These findings show that HPV E7 mRNA qRT-PCR is effective in detecting the risk of development of high-grade CINs and SCCs despite the high prevalence of HPV infection. Moreover, because HPV E7 mRNA qRT-PCR showed low rate of HPV E7 mRNA in low grade lesions, it will therefore give strong reassurance of not developing cancer or precancerous lesion in HPV E7 mRNA negative women. The disadvantage of the new HPV E7 mRNA qRT-PCR is that it cannot provide HPV genotyping.

CONCLUSION

This study was conducted to develop a new HPV E7 mRNA qRT-PCR primer set and to verify its clinical application compared to NucliSENS EasyQ®, a commercial HPV E6/E7 mRNA assay and Goodgene DNA Chip. The results of the this study suggests that HPV E7 mRNA qRT-PCR has higher sensitivity and specificity and can overcome the shortcoming of low specificity and sensitivity of HPV DNA and NucliSENS EasyQ® HPV E6/E7 mRNA assay for clinical detection of high grade cervical lesions and the risk of their development in HPV infected women. Expanding the types of HPV targeted by mRNA assay increased the sensitivity as well as specificity of detection of high grade cervical lesions. Furthermore, since there is geographic difference in the prevalence of HPV types especially in Asia, it is crucial to consider including the most prevalent HPV types in the panel according to the target population while developing screening tests for the HPV detection.

It is suggested that new HPV E7 mRNA qRT-PCR may be superior to the available HPV DNA and E6/E7 mRNA tests in

detection of high grade cervical lesions and cancer. Further clinical application of the new test in cervical screening will not only improve the screening effectiveness, but will reduce the unnecessary doctor visits and laboratory tests in general population by lengthening the screening intervals.

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국문요약

자궁 경부 병터의 검사로 사람 유두종 바이러스 E6, E7 mRNA 의 적용

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연구배경: 사람 유두종 바이러스 (HPV) DNA 검사법은 자궁경부암 검진용으로 세포검사와 함께 널리 사용되고 있으나 민감도가 높지만 특이도가 낮다. 최근에 고위험군 HPV 유형 E6/E7 암유전자의 전사물을 검출함으로 고등급 편평상피병터의

발견에 높은 특이도가 보인다고 알려지고 있다. 하지만 소수의 HPV 유형만을 대상한 이유로 낮은 민감도가 낮다. 자궁경부암과 상피내병터의 HPV 발견으로 높은 민감도와 특이도를 동시에 만족시키는 신뢰성있는 검사방법은 아직까지 없다. **연구방법:** HPV 유형 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69 E7 mRNA 의 실시간 연쇄중합효소법 (real-time PCR)용 길잡이 (primer)와 다듬자 (probe)를 개발하여 임상적으로 평가하였고 상업용 HPV DNA, mRNA 검사법과 비교하였다. ThinPrep® 세포검사 후에 남은 180 예의 액체를 이용하여 굿젠 HPV DNA 검사와 NucliSENS EasyQ HPV E6/E7 mRNA 검사 (EasyQ), 그리고 새로 개발한 mRNA qPCR 검사를 각각 시행하여 비교하였다. 각 검사의 민감도와 특이도를 조직학적으로 확인된 고등급 상피내병터 (상피내병터 2 등급 이상)으로 계산하였다. **연구결과:**

검사의 양성률은 mRNA qPCR 은 54 (30%), DNA 검사는 127 (71%), EasyQ 검사는 54 (30%) 였다. DNA 검사는 모든 비정상 세포검사 예에서 양성이었다. 편평상피암, 고등급 편평상피내 병터 (HSIL), 고등급 편평상피내 병터 배제될 수 없는 비정형 편평상피세포 (ASC-H), 저등급 편평상피병터 (LSIL), 의미불확실 비정형 편평 상피세포 (ASC-US)에서 EasyQ 는 각각 73%, 74%, 60%, 56%, 32% 양성률을 보인 반면 mRNA qPCR 검사는 100%, 100%, 100%, 25%, 18% 의 양성률을 보였다. 정상 세포검사의 양성률은 HPV DNA 검사 50%, Easy Q 7%, mRNA qPCR 각각 3% 였다. 고등급 편평상피내 병터 이상 병변에서 DNA 검사와 mRNA qPCR 의 민감도는 모두 100% 였으나 EasyQ 는 71%의 민감도를 보였다. mRNA qPCR 은 세가지 검사 중에서, DNA 검사 36%, EasyQ 83% 인데 비하여 가장 높은 특이도 92% 를 보였다. **결론:**

임상적으로 자궁경부암, 고등급 편평상피내 병터의 발견을 위하여 HPV mRNA qPCR 검사는 EasyQ 의 낮은 민감도뿐만 아니라 DNA 검사의 낮은 특이도를 극복할 수 있을 것으로 생각된다.

핵심되는 말: 사람 유두종 바이러스, 유전자형 분석, E6/E7 종양 유전자, 자궁 경부암, 실시간 연쇄 중합효소법.