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Prevalence of Human Papillomavirus Infection and Genotype Distribution Determined by the Cyclic-Catcher Melting Temperature Analysis in Korean Medical Checkup Population[§]

Yun-Jee Kim[1], Min-Jung Kwon[2], Hee-Yeon Woo[2*], and Soon-Young Paik[1*]

[1]*Department of Microbiology, College of Medicine, The Catholic University, Seoul 137-701, Republic of Korea*
[2]*Department of Laboratory Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul 110-746, Republic of Korea*

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Although cytology screening has reduced the incidence and mortality rate of cervical cancer significantly, its usefulness is limited to samples from the site of the lesion, resulting in its low sensitivity and unsuitability for use in medical checkups. The purpose of the present study was to evaluate the prevalence of HPV infection using genotype distribution and to analyze the correlation of the HPV DNA test results with cytological results. We also evaluated the benefits of quantitative information obtained from cyclic-catcher melting temperature analysis (CMTA) in screening for cervical cancer. We performed cyclic-CMTA using Anyplex™ II HPV28 Detection in combination with cervical cytology for 2,181 subjects. The following HPV positivity types were detected using cyclic-CMTA and HPV positivity was found to increase together with the severity of the cytology results: (1) For 419 HPV positive specimens, HPV DNA was detected in 18.1% of normal specimens, 78.3% of ASCUS, and all of LSIL and HSIL; (2) high-risk HPV DNAs were detected in 63.3% of normal (N=547), 65.9% of ASCUS (N=41), 76.9% of LSIL (N=13), and 88.9% of HSIL (N=9) among total detected HPV DNA regardless multiple detection; (3) multiple HPV genotypes were detected in 4.8% of normal specimens (N=2,146), 52.2% of ASCUS (N=23), 57.1% of LSIL (N=7), and 40.0% of HSIL (N=5). In addition, a high level of viral DNA was observed using cyclic-CMTA in all specimens beyond the LSIL stage according to cytology, while only 6% of specimens with normal cytology showed a correlation with viral quantitation by cyclic-CMTA. The combination of HPV genotyping with a quantitative assay and cytology will allow for a more accurate diagnosis of cervical cancer.

Keywords: *Human papillomavirus*, HPV, cervical cancer, prevalence, CMTA, cyclic-CMTA

*For correspondence. (H.Y. Woo) E-mail: heeyeon.woo@samsung.com; Tel.: +82-2-2001-2387; Fax: +82-2-2001-2364 / (S.Y. Paik) E-mail: paik@catholic.ac.kr; Tel.: +82-2-2258-7342; Fax: +82-2-535-6473
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Introduction

Early diagnosis and treatments based on mass screening have reduced the mortality rate of cervical cancer dramatically (Shin *et al*., 2008). Unlike other cancers, the relatively long precancerous stage of cervical cancer has allowed its early detection through cervical cytology examinations, which may serve as a major factor to reduce the incidence of invasive cervical cancer (Burd, 2003). The introduction of early diagnosis was reportedly responsible for the gradual decrease in the incidence and mortality rate of cervical cancer in Korea (Jung *et al*., 2012). Although cervical cytology is the most common diagnostic method for the detection of cervical cancer, its high false-negative rate due to its low sensitivity is a major issue (Walboomers *et al*., 1995). Moreover, the fact that cervical cytology merely provides information on existing lesions makes such a method inappropriate for determining the potential risks of lesion-free patients.

According to previous reports, 47% of patients with invasive cervical cancer in the U.K. had been screened for cervical cancer within 5 years of their diagnosis (Sasieni *et al*., 1996). In order to overcome this weakness of cytological screening, molecular diagnostic methods that directly detect human papillomavirus (HPV), a major cause of cervical cancer, have been emphasized. Although 80–90% of HPV infections resolve naturally through self-clearance, a persistent HPV infection over 2 years reportedly leads to a 200 times higher chance of developing a high-grade squamous intraepithelial lesion (HSIL) compared to HPV-negative subjects (Ho *et al*., 1998; Nobbenhuis *et al*., 1999; Chen *et al*., 2011). It has also been shown that HPV viral load is highly correlated with cytomorphological changes (Snijders *et al*., 2003). In addition, co-infection or sequential infections of multiple HPV types, e.g., HPV types 18 and 51, reportedly increase the risk of cervical cancer synergistically (Trottier *et al*., 2006). Patients infected with multiple HPV types require intensive care to prevent cancer progression and death (Bachtiary *et al*., 2002), which indicates that monitoring the genotype and quantity of HPV may provide critical information in the diagnosis of infections for future patient management and prognosis.

Anyplex™ II HPV28 Detection is designed for HPV genotyping and quantitation to meet these current demands for HPV detection. It simultaneously detects 19 high-risk HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53,

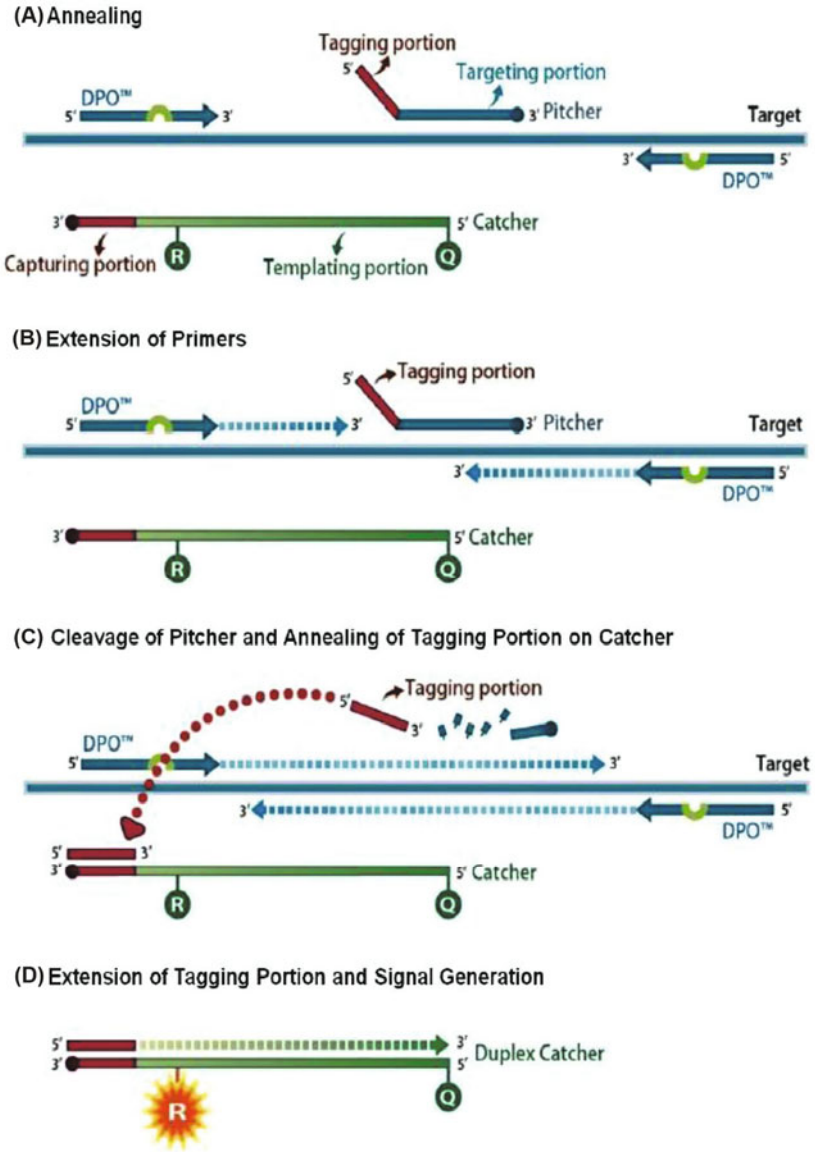

Fig. 1. Schematic diagram of TOCE assay. (A) Two outer DPO™ primers and a Pitcher molecule hybridize to their specific sequences. (B) As DNA polymerase extends the DPO™ primers, its exonuclease activity cleaves the tagging portion of the Pitcher. (C) The released tagging portion is now able to hybridize to capturing portion, which contains sequences complementary to the tagging portion. (D) As the tagging portion is extend, fluorescent reporter molecule is separated from its quencher molecule and signal is generated. Reprint with permission of Seegene Co.

56, 58, 59, 66, 68, 69, 73, and 82) and 9 low-risk HPV types (types 6, 11, 40, 42, 43, 44, 54, 61, and 70) from a single specimen using tagging oligonucleotide cleavage and extension™ (TOCE) technology. Melting temperature analysis (MTA) using "Catcher" as an artificial template facilitates the analysis of multiple analytes in a single fluorescent channel to overcome the multiplexing limitations of conventional real-time PCR (Lee, 2012) (Fig. 1). Furthermore, the use of cyclic-catcher melting temperature analysis (cyclic-CMTA) with TOCE enables a quantitative estimation of viral load through repeated MTAs during the TOCE reaction, thereby exceeding the constraint of endpoint MTA methods, which only provide qualitative analysis (Hwang, 2012).

In this study, we assessed the prevalence of HPV infections using genotype distribution and the correlation of the HPV DNA test with cytology. We also evaluated the benefits of quantitative information obtained from cyclic-CMTA as a screening test for cervical cancer.

Materials and Methods

Specimens

A total of 2,181 cervical swabs were obtained at the Total Health Care Center, Kangbuk Samsung Hospital (Seoul, Korea), from female healthcare examinees aged 21–76 years. The medical records of each subject were reviewed using an electronic medical records system to collect information on their age and cytology results. The specimens were collected using a DNA PAP™ Cervical Sampler™ (QIAGEN, USA).

Nucleic acid extraction and HPV testing

Nucleic acids were extracted from a 350-μl sample using a MICROLAB® STARlet automated purification system (USA).

For each sample, HPV detection and genotyping were performed according to the manufacturer's instruction using Anyplex™ II HPV28 Detection (Seegene, Korea) and a

Table 1. Cytology results according to age

Age group	Total N (%)	Normal N (%)	ASCUS N (%)	LSIL N (%)	HSIL N (%)
20–29 yrs	42 (100)	42 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
30–39 yrs	929 (100)	912 (98.2)	9 (1.0)	4 (0.4)	4 (0.4)
40–49 yrs	1,021 (100)	1,008 (98.7)	10 (1.0)	2 (0.2)	1 (0.1)
50–59 yrs	157 (100)	153 (97.5)	3 (1.9)	1 (0.6)	0 (0.0)
≥ 60 yrs	32 (100)	31 (96.9)	1 (3.1)	0 (0.0)	0 (0.0)
Total	2,181 (100)	2,146 (98.4)	23 (1.1)	7 (0.3)	5 (0.2)

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions

CFX96 real-time thermocycler (Bio-Rad, USA). Briefly, each PCR reaction was performed in a 20-μl reaction consisting of 5 μl extracted DNA, 1× HPV28 TOM, and Anyplex™ PCR Mix. The thermal cycler conditions consisted of an initial incubation at 50°C for 4 min for the activation of the UDG system to prevent contamination, denaturation at 95°C for 15 min, followed by 50 cycles of denaturation (30 sec at 95°C), annealing (1 min at 60°C), and elongation (30 sec at 72°C). Cyclic-CMTA was performed after PCR cycles 30, 40, and 50. CMTA was performed by cooling the reaction mixture to 55°C, holding at 55°C for 30 sec, and heating from 55°C to 85°C (5 sec/0.5°C) with continuous fluorescent monitoring. L1 gene of HPV DNA was amplified and simultaneously human housekeeping gene (Human beta-globin) was co-amplified as an internal control to monitor DNA purification efficiency, PCR inhibition, and cell adequacy.

Statistical analysis

Prevalence and 95% confidence intervals (CIs) were calculated for the overall genotypes and each individual HPV genotype. The prevalence of HPV within the categories was analyzed by 1) age group (20–29, 30–39, 40–49, and ≥50 years); 2) cytology results based on the Bethesda system (normal, atypical squamous cells of undetermined significance [ASCUS], low-grade squamous intraepithelial lesion [LSIL], and HSIL); and 3) semi-quantitative levels (+, ++, and +++) obtained by Anyplex™ II HPV28 Detection. Differences of the prevalence between bivariate conditions were tested for statistical significance ($P<0.05$) using the χ^2 test.

Results

Cytology results

A total of 2,181 specimens were collected for three weeks in September 2012. The cytology results according to age distribution are described in Table 1. The average (±SD) age of the examinees was 40.1 years (±6.8). No carcinoma *in situ* or invasive cervical cancer was detected.

We analyzed the cytology results to determine the relationship between age and potential risk for cervical dysplasia. Normal cytology was observed in 98.4% of all samples. Approximately 1–3% of samples from women aged older than 30 years showed abnormal cytology, while no samples from women in their 20s showed abnormal cytology. However, age distribution was not significantly associated with the cytology results (Table 1).

Prevalence of HPV infections and HPV genotype distribution

Among the 2,181 specimens, HPV DNA was detected in 419 (19.2%) specimens. The overall prevalence of high- and low-risk HPV infections was 18.0% and 12.2%, respectively, regardless of multiple infections. The most common HPV genotype was type 52 (2.4%; Fig. 2). The frequency of types 16 and 18, which are recognized as the major causes of cervical cancer, reached 1.6% and 0.7%, respectively. Although types 6 and 11 are known as the most common low-risk HPV genotypes found in genital warts, both were detected rarely (type 6, 0.5%; type 11, 0.2%).

We analyzed the HPV positivity of each age group (data not shown). Women aged 20–29 years displayed approxi-

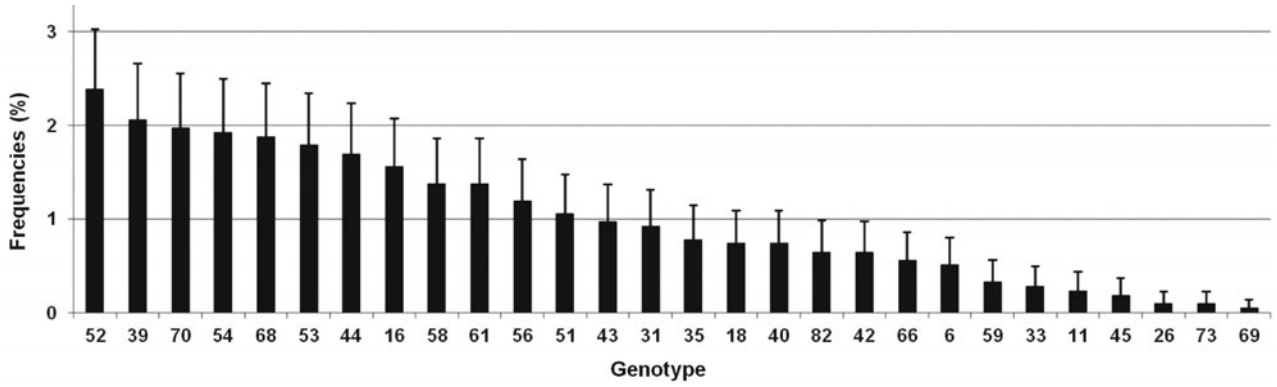

Fig. 2. Distribution of HPV genotypes. Distribution of HPV genotypes in 2,181 medical checkup specimens. Genotypes were determined by the Anyplex™ II HPV28 Detection. Error bars indicate 95% confidence intervals (CI).

Table 2. Multi-infection pattern analysis of HPV types

Number of HPV genotypes	Number of specimens (%)			
	HR+LR	HR+HR	LR+LR	Total
Double	44 (56.4)	24 (30.8)	10 (12.8)	78 (100)
Triple	19 (73.1)	5 (19.2)	2 (7.7)	26 (100)
Quadruple	10 (76.9)	2 (15.4)	1 (7.7)	13 (100)
Quintuple	4 (100)	0	0	4 (100)
Septuple	1 (100)	0	0	1 (100)
Total	78 (63.9)	31 (25.4)	13 (10.7)	122 (100)

HR, high-risk; LR, low-risk

mately 2 times higher positivity (40.5%) compared to the other age groups over 30 years. Both the single genotype (26.2%) and multiple (14.3%) infection rates were the highest in women in their 20s and similar multiple infection rates were observed in the age groups over 30 years. The results for multiple infections are summarized in Table 2. Out of the 419 HPV-positive specimens, 122 (29.1%) showed multiple infections. Out of the 122 multiply infected samples, 78 samples were infected with 2 HPV types, 26 were infected with 3 HPV types, 13 were infected with 4 HPV types, 4 were infected with 5 HPV types, and 1 was infected with 7 HPV types. Multiple infections with both high- and low-risk HPV types were the most common (63.9%), while multiple infections with different high-risk types were observed in 25.4%, and multiple infections with more than 2 low-risk types were observed in 10.7%.

Correlation of the HPV DNA test with cytology

We analyzed the distribution of single and multiple genotype HPV infections according to the cytology results. Among the 2,146 normal cytology specimens, 389 samples (18.1%) were positive for HPV DNA (single infection: 13.3%; multiple infection: 4.8%). Multiple infections increased rapidly with the severity of cervical dysplasia. In the ASCUS stage, 18 out of 23 total samples (78.3%) were positive for HPV DNA (single infection: 26.1%; multiple infection: 52.2%) and HPV DNA was found in all LSIL (single infection: 42.9%; multiple infection: 57.1%) and HSIL (single infection: 60.0%; multiple infection: 40.0%) specimens (Fig. 3A, Supplementary data Table S1). While the single infection rate was approximately 2 times higher in the ASCUS stage than in normal cytology, the multiple infection rate was more than 10 times higher in the ASCUS stage.

Next, we compared the proportion of high-risk HPV types according to the cytology results. In normal specimens, the proportion of high- and low-risk HPV genotypes was 63.3% and 36.7%, respectively (Fig. 3B). With the increased severity of the cytology results, the proportion of high-risk HPV gradually increased to 88.9% in HSIL specimens. The frequency of HPV types 16 and 51 was higher in the cervical dysplasia specimens than in the normal specimens (data not shown).

Quantitative analysis of HPV DNA using cyclic-CMTA

The quantity of HPV DNA was categorized automatically into +, ++, or +++, which was determined by the copy number of target HPV DNA (+, $<10^2$ copies/reaction; ++, $\geq 10^2$ and $<10^5$ copies/reaction; +++, $\geq 10^5$ copies/reaction), allowing the determination of the major and minor HPV genotypes in multiple infections. Among the 2,146 speci-

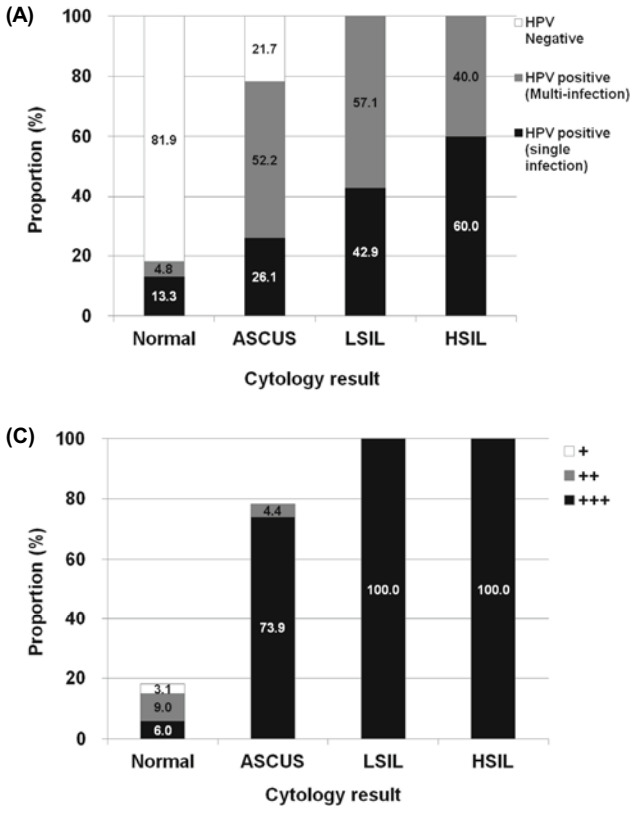

Fig. 3. Prevalence analysis by cytology test results. (A) Proportions of single and multi-infection of HPV according to cytology severity. Significant difference of the proportion were observed ($P<0.05$). (B) Proportions of high risk HPV infection compared to low-risk HPV infection according to cytology test results. High risk HPV can lead to cancers of the cervix, vulva, vagina, and anus in women. In men, it can lead to cancers of the anus and penis. Low risk HPV can cause genital warts. (C) Results of quantitation of HPV according to cytology test results. *P*-value of χ^2 tests for prevalence analysis between cytology groups was <0.001

mens with a normal cytology result, only 129 (6.0%) were identified as +++. However, the proportion of high level of HPV DNA (+++) increased with the severity of the cytology results (73.9% in 17 ASCUS and 100% in 7 LSIL and 5 HSIL) (Fig. 3C). These results indicate that cervical dysplasia is associated with a high amount of HPV DNA.

Discussion

A recent study emphasized that the risk level of cervical cancer depends on the HPV genotypes as well as the manner in which the disease progresses (Bulkmans *et al*., 2007). Patients diagnosed with abnormal cytology reportedly have a 1.5 times lower 18-month clearance rate for most HPV types when compared to those with normal cytology, and HPV16 infections showed particularly the lowest 18-month clearance rate followed by HPV31, HPV33, and HPV18 infections (Bulkmans *et al*., 2007). Other studies also indicated that a persistent HPV infection is critical for the development of cervical cancer (Ho *et al*., 1995, 1998; Wallin *et al*., 1999; Dalstein *et al*., 2003).

In the present study, in which we examined 2,181 women who requested a screening test for cervical cancer during a medical checkup, approximately 20% were positive for HPV DNA and the most frequent types were 52, 39, 70, 54, 68, and 53 in order of frequency. These results also indicated the higher frequency of HPV types in the 50s (types 51, 52, 53, 54, and 58) and a particularly high frequency of HPV type 70. This frequency pattern was similar to those in several studies (Shin *et al*., 2003, 2004; Bae *et al*., 2008; Oh *et al*., 2009). When dividing the subjects into 2 groups according to their cytology results, i.e., normal and abnormal groups, the frequency of the high-risk HPV types 16, 51, and 35 was higher in the abnormal group than in the normal group. However, as the abnormal group comprised only 1.6% of all samples, additional analysis in a larger abnormal group may be needed to determine differences in HPV type distribution between the normal and abnormal groups.

The proportion of high-risk HPV types clearly increased with the severity of cytology in our study: 63.3% in normal, 65.9% in ASCUS, 76.9% in LSIL, and 88.9% in HSIL. The low-risk HPV types displayed an opposite trend, as reported previously (Kim, 2009).

The relationship between viral load in HPV infection and the risk of cervical dysplasia was demonstrated in quantitative analysis using cyclic-CMTA and multiple infection rates in our study. Regardless of the presence of single or multiple infections, at least 1 HPV type was detected as +++ in 100% of the LSIL and HSIL groups, while only 6% of the normal cytology group showed +++. These results indicate that the severity of cytology increases with viral load and suggests that HPV DNA should be quantified together with HPV genotyping. Dalstein *et al*. (2003) also reported that a high viral load tends to result in persistent infection and disease progression, while a lower viral load is associated with viral clearance, supporting the importance of viral load in HPV diagnosis (Snijders *et al*., 2003).

Sasagawa *et al*. (2001) reported that multiple HPV infections may weaken the natural immune barrier, leading to a persistent infection as well as susceptibility to additional multiple infections. In the present study, 4.8% of 2,146 samples with normal cytology were multiply infected, while more than 50% of samples with abnormal cytology (ASCUS, LSIL, and HSIL) were infected with multiple HPV types, exhibiting an approximately 10 times higher rate of multiple infections in the abnormal group compared to the normal group. This also supports the hypothesis that multiple infections might be more likely to be associated with cervical cancer. Out of the 122 multiply infected samples, the rate of multiple infections by high- and low-risk HPV types was 2.5 times higher than just by high-risk HPV types. This high frequency of high- and low-risk HPV co-infection drew out attention to infections by low-risk HPV types. A small number of studies on low-risk HPV types all suggested that there is only a weak causative relationship between infections by the low-risk types alone and cervical cancer. Our data suggest that additional studies are required in order to clarify the role of low-risk HPV infections in the presence of an existing high-risk HPV infection. Meanwhile, it was difficult to associate severity with the number of HPV types in multiply infected subjects in this study. Due to the small sample size of subjects with multiple HPV infections (only 44 samples were infected with 3 or more HPV types), further research into the relationship between severity and the number of HPV types may also be required.

As awareness of health issues has increased with improvement of the quality of life, the diagnostic process for cervical cancer, which until now has consisted solely of a cytology test and detection of HPV DNA, is in need of improvement. It is now critical to gain quantitative information on various HPV genotypes and identify the presence of multiple infections to initiate early treatment and predict accurate prognosis so that further changes in disease progression can be monitored. The U.S. Preventive Services Task Force with the American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology published updated screening guidelines for cervical cancer in 2012, advising HPV screening and genotyping to be carried out in addition to cytological and histopathological assessments to improve diagnosis, surveillance, monitoring, and treatment of cervical cancer in women aged over 30 years. They also strongly recommended the use of HPV genotyping tests to predict the potential severity of a precancerous state with abnormal cytology, even if the patient is a young woman (Saslow *et al*., 2012).

In fact, taking into account many factors, e.g., the high incidence of HPV infection and its mortality rate, age distribution, the cost of screening and treatment among different countries, and the differences in the frequency of various HPV genotypes, it is necessary that large-scale cohort studies of HPV infections in the general population are pursued more actively to improve the diagnostic and treatment modalities for HPV infections within a single population. A more profound understanding of the epidemiologic distribution of HPV infections will most definitely provide a foundation for the development of vaccines customized to specific populations. The correlation of cervical dysplasia with HPV type and viral load in the present study strongly suggests the need for diagnostic methods capable of quantitative HPV

DNA screening for cervical cancer. The addition of a simultaneous HPV genotyping and quantitative assay to the current cytology-based screening approach will ultimately contribute to a great leap in the prevention, treatment, and long-term management of cervical cancer in the near future.

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Ethical Aspects : This study has been approved by the Ethics Committee of Kangbuk Samsung Hospital (Kangbuk Samsung Hospital Institutional Review Board, IRB No. KBC12139). Participants were informed of HPV test and asked to sign a consent form.

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