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Detection and Typing of Human Papillomavirus DNA by PCR Using Consensus Primers in Various Cervical Lesions of Korean Women

The association between cervical cancers and human papillomavirus (HPV) is now well established. To estimate the extent of infection with common HPVs among Korean women, we have examined 224 cervical scrapes of various cervical lesions. Detection and typing of HPVs were done by polymerase chain reaction (PCR) using consensus primers followed by restriction enzyme digestion and PCR using type-specific primers. The prevalence of total HPV infection in patients with cervical intraepithelial neoplasia (CIN) and cervical cancer were significantly higher than those in healthy women and patients with atypical squamous cells of undetermined significance (ASCUS). HPV typing in 41 invasive carcinomas of the cervix revealed the prevalence of HPV 16 in 15 cases, followed by HPV 58, 18, 33, 31, 52 and 35. The distribution pattern of HPV types in CIN were not much different from carcinomas. HPV types except HPV 18 had a tendency to show higher prevalence in high-grade squamous intraepithelial lesion (HSIL) than low-grade squamous intraepithelial lesion (LSIL), however, HPV 18 was detected in LSIL but not in HSIL. HPV 18 tended to have the worse clinical stage, although it was not statistically significant. These findings suggest the importance of HPV typing other than HPV 16 and 18 and a different clinicopathologic significance of HPV 18.

Key Words: *Cervical neoplasms; Papillomavirus, human; Polymerase chain reaction; Polymorphism, restriction fragment length*

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INTRODUCTION

Human papillomaviruses are naturally occurring DNA tumor viruses which induce epithelial cell proliferation during the course of a productive infection, and are known to be associated with cervical cancer. Involvement of human papillomavirus (HPV) in the process of carcinoma development of the cervix has been investigated by many workers, and HPV types 16, 18, 31, 33, 35, 52b, and 58 have attracted attention for their close relationship to carcinoma (1, 2). Cervical cancer is the most common cancer in Korean women and the prevalence of this cancer was reported to be 31 out of 100,000 women (3). Since HPV types 16 and 18, which were prevalent in Western countries, were widely known to be associated with cervical cancer, most of the previous Korean studies were mainly concentrated on HPV types 16 and 18 (2, 4-6). However, recent evidence have shown that the prevalence of HPV 18 has profound geographical differences (7-10) and other HPV types such as 31, 33, 35, 52b and 58 have been frequently reported especially in Japan (11-15) although HPV 16 was the most prevalent type associated with cervical malignancy worldwide. Therefore, it is important to identify the presence of other common HPV types known to be associated with cervical malignancy in Korean women than types 16 and 18.

In cervical carcinomas, the genome of integrated HPV has often been found to contain deletions, but the long control region and the E6 and E7 open reading frames (ORFs) are preferentially conserved (16, 17). PCR using consensus primers designed from E6 and E7 ORFs can help us to detect multiple HPV type DNAs and the specific type can be determined by digestion with restriction endonuclease.

This study was performed to determine the 1) prevalence of common genital HPV infection in various cervical lesions in Korean women, 2) prevalent HPV types associated in cervical malignancy and premalignancy in Korean women, and 3) correlation between HPV types and the histological type of invasive carcinoma and clinical stage.

MATERIALS AND METHODS

The samples were collected from 224 Korean women

visiting the Department of Obstetrics and Gynecology, Inha University Hospital, Inchon, Korea. One hundred thirty healthy women, 18 patients whose cytologic smear showed atypical squamous cells of undetermined significance (ASCUS), 35 patients with cervical intraepithelial neoplasia (CIN), and 41 patients with invasive carcinoma were analyzed. All abnormal cases were confirmed histopathologically except for 8 ASCUS which were colposcopically clear.

Cell collection from patients

The samples were collected by scraping the uterine cervical canal with a small cytobrush after PAP smear, and the brush was put into a 15 mL centrifuge tube containing phosphate buffered saline.

DNA extraction from cervical scrapes

DNA was extracted using Wizard genomic DNA purification kit (Promega, U.S.A.). After vortexing the 15 mL centrifuge tube to dissociate cells and centrifugation at 1,200 rpm for 3 min, DNA was isolated from cells by detergent lysis buffer and protease digestion. RNA was removed by digestion with ribonuclease and the DNA was concentrated by ethanol precipitation.

HPV detection and typing

PCR using consensus primers

PCR reaction was performed using consensus primers (Table 1). The reaction mixture of 100 μL contained 100 mM-KCl, 20 mM Tris-HCl pH 8.0, 2.0 mM $MgCl_2$, 2.5 mM of each dNTP, 2.5 Units of *Taq* polymerase (TaKaRa biomedicals, Japan), 25 pmol of consensus primers (TaKaRa biomedicals). The mixture was subjected to 30 cycles of amplification using a DNA thermal cycler 9600 (Perkin-Elmer Cetus, U.S.A.). Each cycle included a denaturation step at 94℃ for 30 seconds, an annealing step at 55℃ for 2 min, and a chain elongation step at 72℃ for 2 min. To avoid false positives and false negatives, a reagent control (no template DNA) and known samples containing HPV DNA were included in each amplification. PCR product was electrophoresed on 1.5% 3:1 NuSieve agarose (FMC bioproducts, U.S.A.) gel, stained with ethidium bromide, and photographed under UV light.

Table 1. Sequences of consensus and type specific primers

Consensus primer		
	(F)	5'-TGTCAAAAACCGTTGTGTCC-3'
	(R)	5'-GAGCTGTCGCTTAATTGCTC-3'
Type specific primers		
HPV 16	(F)	5'-TGTCAAAA**G**CC**AC**TGTGTCC-3'
	(R)	5'-GAGCTGTC**AT**TTAATTGCTC-3'
HPV 18	(F)	5'-TG**C**CA**G**AAACCGTTG**AA**TCC-3'
	(R)	5'-**TCTGA**GTCGCTTAATTGCTC-3'
HPV 31	(F)	5'-TGTCAAA**G**ACCGTTGTGTCC-3'
	(R)	5'-GAGCTGTCG**GG**TAATTGCTC-3'
HPV 33	(F)	5'-TGTCAAA**G**ACC**T**TTGTGTCC-3'
	(R)	5'-GAGCTGTC**A**CTTAATTGCTC-3'
HPV 35	(F)	5'-TGTCAAAAACCG**C**TGTGTCC-3'
	(R)	5'-GAGCTGTC**AC**A**C**AATTGCTC-3'
HPV 52b	(F)	5'-TGTCAAA**CG**CC**A**TT**A**TGTCC-3'
	(R)	5'-GAGCTGTC**AC**C**T**AATTGCTC-3'
HPV 58	(F)	5'-TGTCAAA**G**ACC**A**TTGTGTCC-3'
	(R)	5'-GAGCTGTC**AC**A**T**AATTGCTC-3'

Restriction enzyme analysis

Eight μL of PCR product was digested with 8-10 units of *AVa* II, *Afa* I, *Bgl* II, *Acc* I, and *AVa* I in 25 μL reaction mixture at 37℃ for 1 hr. Digestion products were electrophoresed on 2.0% 3:1 Nusieve agarose (FMC bioproducts, U.S.A.) gel, stained with ethidium bromide, and photographed under UV light. Restriction fragment length polymorphism (RFLP) patterns were analyzed (Table 2).

Confirmation of HPV types by using type specific primers

The reaction mixture of 50 μL contained 100 mM-KCl, 20 mM Tris-HCl pH 8.0, 2.0 mM $MgCl_2$, 2.5 mM of dNTP, 1.5 units of *Taq* polymerase (TaKaRa biomedicals, Japan), and 25 pmol of each primer (Table 1). The mixture was subjected to 30 cycles of amplification using a DNA thermal cycler 9600 (Perkin-Elmer Cetus, U.S.A.). Each cycle included a denaturation step at 94℃ for 30 seconds, an annealing step at 55℃ for 2 min, and a chain elongation step at 72℃ for 2 min. To avoid false positives, a reagent control (no template DNA) was included with each amplification. PCR product was electrophoresed on 1.5% 3:1 NuSieve agarose (FMC bioproducts, U.S.A.) gel, stained with ethidium bromide, and photographed under UV light.

RESULTS

HPV DNA was detected by PCR with consensus primers to generate a fragment of about 250 bp. The 250 bp fragment was further digested by different restriction enzymes (*AVa* II, *Afa* I, *Ava* I, *Bgl* II, and *Acc* I) for HPV typing. Restriction fragment length polymorphism patterns of HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 52b, and HPV 58 DNAs contained in cervical scrapes are shown (Fig. 1). In panel HPV 16,

Table 2. Restriction enzyme fragment sizes of PCR products using consensus primers

Restriction enzyme	HPV type						
	HPV 16	HPV 18	HPV 31	HPV 33	HPV 35	HPV 52b	HPV 58
Total length (bp)	238	268	232	244	232	231	244
Ava II	157/81	172/96	NC	136/108	NC	NC	NC
Afa I	NC	NC	117/115	NC	NC	NC	NC
Ava I	NC	NC	NC	NC	186/46	NC	NC
Bgl II	NC	NC	NC	NC	NC	176/55	NC
Acc I	NC	NC	NC	NC	NC	NC	126/118

NC, not cut

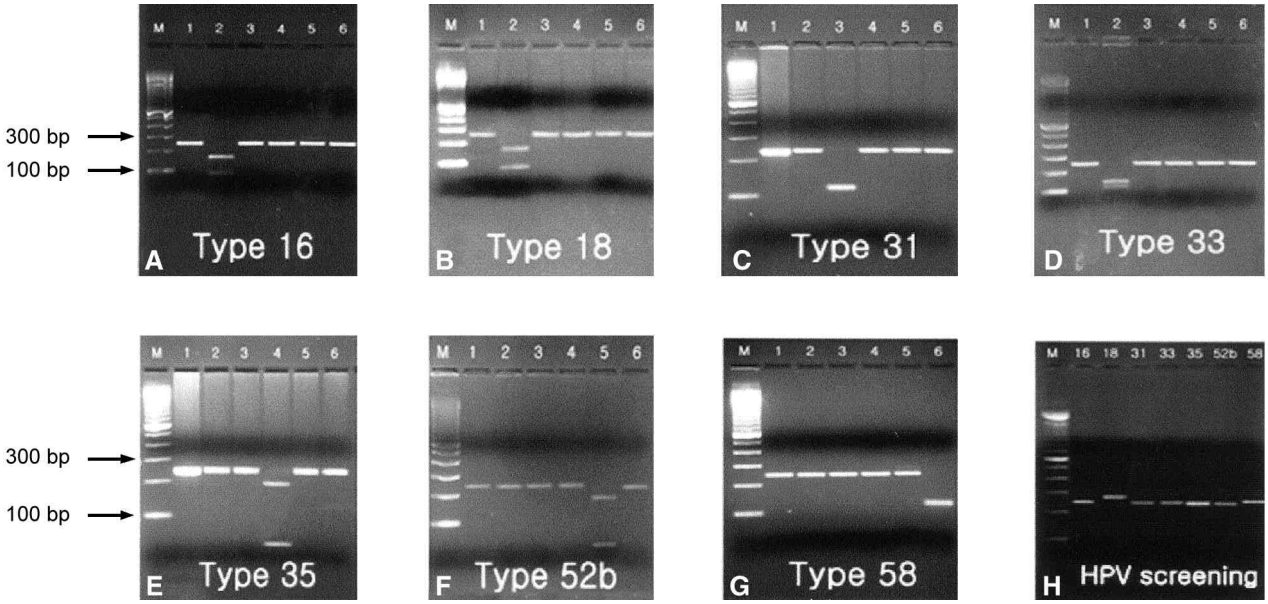

Fig. 1. Determination and confirmation of HPV type by RFLP pattern (A-G) and PCR using type specific primers (H).

lane 1 contained PCR products using consensus primers; lane 2 contained 157 and 81 bp *AVa* II digested fragments; lanes 3, 4, 5, and 6 did not contain cutting sites for *Afa* I, *AVa* I, *Bgl* II, and *Acc* I. In panel HPV 18, lane 1 contained PCR products using consensus primers; lane 2 contained 172 and 96 bp *AVa* II fragments; lanes 3, 4, 5, and 6 did not contain cutting sites for *Afa* I, *AVa* I, *Bgl* II, and *Acc* I. In panel HPV 31, lane 1 contained PCR products using consensus primers; lane 3 contained 117 and 115 bp *Afa* I fragments; lanes 2, 4, 5, and 6 did not contain cutting sites for *AVa* II, *AVa* I, *Bgl* II, and *Acc* I. In panel HPV 33, lane 1 contained PCR products using consensus primers; lane 2 contained 136 and 108 bp *AVa* II fragments; lanes 3, 4, 5, and 6 did not contain cutting sites for *Afa* I, *AVa* I, *Bgl* II, and *Acc* I. In panel HPV 35, lane 1 contained PCR products using consensus primers; lane 4 contained 186 and 46 bp *AVa* I fragments; lanes 2, 3, 5, and 6 did not contain cutting sites for *AVa* II, *Afa* I, *Bgl* II, and *Acc* I. In panel HPV 52b, lane 1 contained PCR products using consensus primers; lane 5 contained 176 and 55 bp *Bgl* II fragments; lanes 2, 3, 4, and 6 did not contain cutting sites for *AVa* II, *Afa* I, *AVa* I, and *Acc* I. In panel HPV 58, lane 1 contained PCR products using consensus primers; lane 6 contained 118 and 126 bp *Acc* I fragments; lanes 2, 3, 4, 5 did not contain cutting sites for *AVa* II, *Afa* I, *Ava* I and *Bgl* II.

Out of a total of 224 cervical scrapes from women with various cervical lesions, HPV DNA was identified in 84 cases (37.5%). The HPV types in various cervical lesions were summarized (Table 3). Genotyping by RFLP and PCR using type specific primers (Fig. 1) revealed that HPV type 16 was the most frequent type of infection comprising 26 cases (11.6%), followed by HVP type 58 in 12 cases (5.4%), HPV 18 in 8 cases (3.6%), HPV 33 in 8 cases (3.6%), HPV 52b in 8 cases (3.6%) and HPV 31 in 5 cases (2.2%), and HPV 35 in 1 case (0.4%).

HPV DNA was detected in 13 cervical scrapes (10.0 %) out of 130 healthy women. HPV typing in normal

Table 3. Prevalence of HPV types in various cervical lesions

Diagnosis	PCR positive, No (%) for												Total
	Consensus	16	18	31	33	35	52b	58	16+18	52+58	Unidentified	Negative	
Total	84 (37.5)	26 (11.6)	8 (3.6)	5 (2.2)	8 (3.6)	1 (0.4)	8 (3.6)	12 (5.4)	1 (0.4)	1 (0.4)	14 (6.3)	140 (62.5)	224
Normal	13 (10)	4 (3.1)	1 (0.8)	0	0	0	2 (1.5)	3 (2.3)	1 (0.8)	0	2 (1.5)	117 (90)	130
ASCUS	7 (38.9)	2 (11.1)	0	0	1 (5.6)	0	0	1 (5.6)	0	0	3 (16.7)	11 (61.1)	18
CIN	26 (74.3)	5 (14.3)	3 (8.6)	2 (5.7)	3 (8.6)	0	4 (11.4)	2 (5.7)	0	0	7 (20)	9 (25.7)	35
LSIL	6 (75.0)	0	3 (37.5)	0	0	0	2 (25.0)	0	0	0	1 (12.5)	2 (25.0)	8
HSIL	20 (74.1)	5 (18.6)	0	2 (7.4)	3 (11.1)	0	2 (7.4)	2 (7.4)	0	0	6 (22.2)	7 (25.9)	27
Carcinoma	38 (92.7)	15 (36.6)	4 (9.8)	3 (7.3)	4 (9.8)	1 (2.4)	2 (4.9)	6 (14.6)	0	1 (2.4)	2 (4.9)	3 (7.3)	41
Sq. cell ca.	36 (94.7)	15 (39.5)	2 (5.3)	3 (7.9)	4 (10.5)	1 (2.6)	2 (5.3)	6 (15.8)	0	1 (2.6)	2 (5.3)	2 (5.3)	38
Aden. ca.	0	0	0	0	0	0	0	0	0	0	0	0	0
Adenosq. ca.	2 (100)	0	2 (100)	0	0	0	0	0	0	0	0	0	2
Glassy cell ca.	0	0	0	0	0	0	0	0	0	0	0	1 (100)	1

cytologic specimen revealed the prevalence of HPV 16 in 4 cases (3.1%), followed by HPV 58 in 3 cases (2.3%), HPV 52b in 2 cases (1.5%) and HPV 18 in 1 case (0.8 %). One case showed double infection of HPV 16 and 18. Two cases showed amplified product with consensus primers which were not digested by any restriction enzyme.

HPV DNA was detected in 7 cervical scrapes (38.9%) out of 18 patients with cytologic features showing ASCUS. HPV typing in 18 ASCUS revealed the prevalence of HPV 16 in 2 cases (11.1%), followed by HPV 33 in 1 case (5.6%) and HPV 58 in 1 case (5.6%). Three cases showed amplified product with consensus primers which were not digested by any restriction enzyme.

HPV DNA was detected in 26 cervical scrapes (74.3 %) out of 35 CIN patients. Typing in 35 CIN revealed the prevalence of HPV 16 in 5 cases (14.3%), 52b in 4 cases (11.4%), HPV 18 in 3 cases (8.6%), HPV 33 in 3 cases (8.6%), HPV 31 in 2 cases (5.7%), HPV 58 in 2 cases (5.7%). Seven cases (20.0%) showed amplified product, but 3 cases were not digested by any restriction enzyme and 4 cases were not digested by *AVa* II, but the procedure could not be completed due to a lack of DNA. Among 8 low-grade squamous intraepithelial lesion (LSIL), 6 cases (75.0%) revealed HPV DNA and among 27 high-grade squamous intraepithelial lesion (HSIL), 20 cases (74.1%) revealed HPV DNA.

HPV DNA was detected in 38 cervical scrapes (92.7 %) out of 41 invasive carcinoma patients. HPV typing in 41 invasive carcinomas of the cervix revealed the prevalence of HPV 16 in 15 cases (36.6%), followed by HPV 58 in 6 cases (14.6%), HPV 18 in 4 cases (9.8%), HPV 33 in 4 cases (9.8%), HPV 31 in 3 cases (7.3%), HPV 52b in 2 cases (4.9%), and HPV 35 in 1 case (2.4 %). One case (2.4%) showed double infection of HPV 52b and 58. Two cases (4.9%) showed amplified product with consensus primers which were not digested by any restriction enzyme. Among squamous cell carcinomas, HPV 16 was the most prevalent type, followed by types 58, 33, and 31, 52b, 18 and 35. Two adenosquamous cell carcinoma cases revealed HPV 18 DNA.

Table 4. Correlation between HPV types and clinical stage of invasive squamous carcinoma

HPV type	Clinical stage				Total
	I	II	III	IV	
16	10	2	1	1	14
18	0	2	0	0	2
31	3	0	0	0	3
33	2	2	0	0	4
35	1	0	0	0	1
52b	1*	0	0	0	1
58	4*	1	0	0	5
Unidentified	1	1	0	0	2
Negative	2	1	0	0	3
Total	24	9	1	1	35

*These results include a double infection of HPV 52b and 58

The correlation between HPV subtypes and the clinical stage in 35 squamous cell carcinoma of the cervix was analyzed (Table 4). There was no significant correlation between HPV genotype and the clinical stage of invasive squamous cell carcinoma although there was a tendency for HPV 18 to have the worse clinical stage.

DISCUSSION

Our findings show that HPV infection was quite rare in normal cervical tissues, contrasting some previous

studies in other countries, which also employed the PCR technique and showed quite high prevalence rates for HPV infection in normal cervical tissues (6, 18, 19). Such differences may be partly due to some geographical and/or racial factors. One must also be cautious about PCR results, since the technique is notorious for false-positivity due to laboratory contamination. Furthermore, PCR techniques with higher detection sensitivities, such as two-step PCR, might increase the rate of HPV detection. Indeed, some of the papers were later retracted (19). A recent study employing primers flanking HPV cloning sites (anti-contamination primers) showed much lower HPV prevalence rates in cervical scrapes and biopsies cytologically classified as normal (20).

We found HPV infection in 38 (92.7%) out of 41 cancer patients. This result was somewhat higher than other reports showing 68 to 84% positivity (8, 9, 21, 22). This is most likely due to the consensus primers we used which were able to detect 7 different HPV types. Fifteen (36.6%) of the 41 cervical cancer tissues screened were shown to be infected with HPV 16 followed by HPV 58 in 6 cases (14.8%) and HPV 18 in 4 cases (9.8%). Twenty six (74.3%) out of 35 CIN were shown to be infected with HPVs, and HPV 16 was the most commonly detected type and was present in 5 cases (14.3%) followed by HPV 52b in 4 cases (11.4%) and HPV 18 in 3 cases (8.6%).

In the present study, as with other Asian and African studies (6, 9, 10, 14), the detection rate for HPV 16 (36.6%) in cervical carcinomas was lower than that reported in Europe and the U.S.A. (2, 18, 23), where 60-84% of cervical carcinomas had detectable HPV 16. It might be attributed in part to geographical and/or racial differences. However, PCR using consensus primers enhanced the detection rate of HPV subtypes other than HPV 16 as well as the overall detection rate, resulting in a relative decrease in the HPV 16 infection rate. Although HPV 16 infection rate in the present study was lower than other reports, HPV 16 was still the most common HPV type. Comparing the prevalence of different HPV type in different countries, Japanese studies showed a relatively lower prevalence of HPV 16 and HPV 18, but a higher prevalence of HPV 52 and HPV 58 (13, 24, 25). The studies showed HPV 16 to be 33% and HPV 18 to be 5% of cervical cancer in one study (24) and HPV 16 to be 20-22% (13, 25), HPV 58 to be 8% (25), HPV 52 to be 20% of cervical cancer in other studies (13). In the United States and Germany, studies showed a higher prevalence of HPV 16 and HPV 18, but a lower prevalence of HPV 52 (2, 18, 23, 26). They showed HPV 16 to be 40-60%, HPV 18 to be 15-25% and HPV 52 to be 2% of the cervical cancer. Our study revealed an intermediate prevalence between Japanese and Western reports. The prevalence of different HPV types in different geographical locations may indicate different etiologies of cervical cancer. Different genetic and environmental factors may contribute differently to the mechanism of cervical cancer induction by different types of HPV.

In the present study, the incidence of HPV 16, 31, 33, and 58 was higher in cervical carcinoma tissues than in HSIL biopsies (18.5%) and none were found in LSIL tissues. Labeit et al. also observed increased detection of HPV 16 in invasive cervical cancers compared with early cervical lesions (27). These results may suggest that premalignant lesions infected with HPV 16, 31, 33, and 58 are more likely to progress to malignancy than those infected with other HPV types.

Two of 38 squamous cell carcinomas and both of 2 adenosquamous cell carcinomas showed HPV 18 which comprised in 9.8% of 41 invasive cervical carcinomas. The prevalence of HPV 18 infection in cervical cancer in other countries varied from 1.5 to 25% (2, 8, 9, 14, 21). When we focused only on squamous cell carcinoma, the prevalence of HPV 18 in the present study decreased by about 5%. According to Matsukura's study, HPV 18 was identified in CIN I or II but not in CIN III, while HPV 16, 31, 33, 35, 52b, and 58 were identified in CIN III as well as in CIN I and II. They could not identify HPV 18 in any invasive cervical carcinomas. MaLachin et al. also reported that HPV 18 was frequently identified in low grade intraepithelial lesions than high grade intraepithelial lesions (28). In the present study, HPV 18 was identified in 3 of 8 LSIL, but not in any of the 7 HSIL, whereas HPV 16, 31, 33, 52b, and 58 were more frequently identified in HSIL or at least equally identified in HSIL and LSIL. Referring to the above findings, HPV 18 appears to have a different clinicopathologic significance from other HPV types studied, and the mechanisms leading to these differences remains to be determined.

Only two cases had detectable dual HPV infections. This is probably an underestimation of the dual infections, as the PCR using consensus primers favors amplification of the more prevalent HPV type, and the type having the least mismatches with the primers. There are also at least 22 different HPV types occurring in the genital tract (29), and in the present study specific primers were available for only seven different HPV types. Therefore, double infections, with an HPV type for which specific oligonucleotide probe were not available, could not be detected. Cases which revealed the amplified product with consensus primers but was not digested by any of the restriction enzymes used in this study may have the HPV types other than HPV 16, 18, 31, 33, 35, 52b, and 58.

It is difficult to evaluate the correlation between HPV types and histological type of cervical carcinomas since most of the cases were squamous cell type. However, we suggest that HPV 18 is more prevalent in adenosquamous cell carcinomas as in other reports.

The result of the present study show that HPV 18 has a tendency to be associated with the advanced stage, supporting other researchers' opinion that HPV 18 containing tumors have a more aggressive clinical course than do similar cervical cancers with other HPV types.

In conclusion, the overall prevalence of HPV infection by PCR using consensus primer for HPV types 16, 18, 31, 33, 35, 52b, and 58 in 224 Korean women was 37.5% and the prevalences paralleled with the degree of cervical dysplasia. HPV 16 was the most frequent type of infection in all groups studied, however, the detection rate of this type in cervical carcinoma (36.6%) was lower than those reported in Europe and the U.S.A., and other types (especially HPV 58, 18, 33) comprised rest of the cancer patients. Therefore, it is important to identify the presence of HPV types other than HPV 16 and 18. HPV 18 appeared to have different clinicopathologic significances and the mechanisms leading to these differences needed to be evaluated.

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