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Association of Oncogenic Human Papillomaviruses (HPV 16, 18) with Cervical Intraepithelial Neoplasia and Cervical Cancer

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=Abstract=**The prevalence of oncogenic human papillomavirus (HPV) type 16 and 18 was investigated by polymerase chain reaction (PCR) method in cervical scrapes omitting prior DNA extraction. Samples were obtained from 70 gynecologic inpatients with normal cervix and 160 women with cervical neoplastic lesion (N = 50 in cervical intraepithelial neoplasia (CIN) I, N = 50 in CIN II, N = 30 in CIN III, N = 30 in invasive cervical cancer). Eight members were excluded from the data due to failure of β-globin amplification during the PCR procedure. The HPV 16 prevalence rate was 19.1 % (13/68) in the normal group, 38.8 % (19/49) in CIN I, 57.1 % (28/49) in CIN II, 75.9 % (22/29) in CIN III, 88.9% (24/27) in invasive cancer. For HPV type 18, DNA positivity was 4.4 % (3/68), 8.2 % (4/49), 12.2 % (6/49), 13.8 % (4/29), 18.5 % (5/27), respectively. In the whole series a consistent correlation was found between HPV positivity and severity of cervical lesion. HPV 16 was the more prevalent type and about five times more common than HPV 18. These results suggest that HPV 16 and 18 may be strongly associated with carcinogenesis of cervical cancer. The high risk HPV typing by direct PCR from cervical scrapes can be used as a useful marker for the presence of neoplastic cells and also served as a simple tool in identifying women who are at risk of developing dysplasia and cervical cancer.**

Key Words: *Human papillomavirus, PCR, Cervical intraepithelial neoplasia, Cervix cancer*

INTRODUCTION

Cancer of the uterine cervix is the most common gynecologic malignancy in Korea. Papanicolaou's (PAP) cytologic screening has been used to detect cervical cancer and its precursor lesions and has contributed much in the early diagnosis or cervical neoplastic lesion (Miller 1986). But the difficulty of predicting which cervical lesion will show progression or regression and the high false negative rate (Richard and Barron 1981) require the better or

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additional prognostic markers. Over recent years much interest has focused on human papillomavirus (HPV) as a possible etiologic factor of cervical cancer (Munoz et al. 1988; Van den Brule *et al.* 1989,1990a,1990b; Zur Hausen et al. 1989a, 1989b). Until now over 60 different HPV types are identified by molecular biologic techniques. Among these, HPV type 6 and 11 are mainly detected in benign lesions such as condyloma accuminatum and therefore, they are called the benign low risk types (Gissmann *et al.* 1983; Schneider *et al.* 1987).On the other hand HPV type 16, 18, 31, 33, 35 have been classified as high risk types because they have been much more frequently detected in either CIN or cervical cancer than in normal cervical epithelium (Buckley *et al.* 1981; Maureen *et al.* 1992; Munoz *et al.* 1988; Peter *et al.*1989; Young *et al.* 1989). In vitro studies have also shown that transfection of DNA from HPV 16, 18, 31 and 33(but not HPV 6b and 11) induced immortalization and aneuploidy in normal human genital keratinocyte (Barnes *et al.* 1990; Schlegel *et al.* 1988). This suggests that high risk types are strongly associated with the carcinogenesis of cervical cancer. Among the above high risk HPV types, type 16 and 18 have been found *at a* higher prevalence and HPV type 18 is suspected to be associated with more aggressive and rapidly progressive cancer (De Villiers *et al.*, 1987; Lorincz *et al.* 1987; Mark *et al.* 1991; Walker *et al.* 1989; Xiao *et al.* 1988).Polymerase chain reaction (PCR) can be used for the detection of HPV in crude cervical scrapes and therefore applied to the mass screening of HPV in a rapid, sensitive and reliable manner. But still great variations are present in the HPV prevalence rates among studies probably due to geographical difference, methods applied, sample contamination, HPV subtypes included and so on. To define the level of association of specific HPV types with cervical neoplasia in Korean women, we studied the prevalence rate of HPV type 16 and type 18 by PCR in the crude cervical scrapes from normal cervix, CIN and invasive cancer.

MATERIALS AND METHODS

Study group and sample preparation

Cervical scrapes were obtained from 230 patients who were admitted to the Gynecologic department, Seoul National University Hospital. Normal control cervical samples were taken from 70 gynecologic in-patients who planned to undergo hysterectomy because of non-cervical gynecologic disease, colpos-copically normal cervices and no history of abnormal cervical cytology. Hysterectomy specimens were pathologically reconfirmed to have no neoplastic lesion in the cervix. Pathologic samples were obtained from 130 patients with CIN (N = 50 for CIN I, N = 50 for CIN II, N = 30 for CIN III) and 30 patients with invasive cancer. 8 patients were excluded due to poor amplification of β-globin during the PCR procedure. Cervical cells were collected from the transformation zone and / or endocervical canal with a cytobrush and were suspended in 1 ml cold phosphate buffered saline (PBS). Tubes with exfoliative cervical cells were refrigerated at -20° C if necessary.

The suspension was centrifuged at 4° C for 5 minutes at 6,000 rpm and the pellet was resuspended in 10 μl of 0.1 N NaOH-2 M NaCl solution and vortexed. After boiling at 95° C for 2 minutes, 90μl TE buffer solution (10 mM tris hydrochloride - 1 mM EDTA) was added and the samples were stored at 4 ° C.

In each assay negative(Neuroblastoma tissue DNA) and positive controls (CaSki and HeLa cell DNA for HPV 16 and 18, respectively) were included. Cells were digested at 37° C for 2 hours with 100 μg/ml proteinase K in 10mM Tris-HCl, 1 mM EDTA, 1 % Sodium dodexyl sulfate (SDS). Thereafter DNA was separated by three extractions with phenol and chlorform/isoamyl alcohol (24:1 vol/vol). 50mg/ml RNAse A was added to remove RNA. DNA concentration was determined using a spectrophotometer (O.D.260 / O.D.280 ≒ 1.8).

The genomic region chosen for HPV type 16 and type 18 was within the E6 open reading

frame (ORF) whose DNA remains after viral integration into host genomic DNA. The suitability of the DNA for amplification in each cervical specimen was confirmed by successful amplification of β-globin by using β-globin specific primer (supplied from Clontech Co.) as an internal reaction control. HPV 16 and 18 type-specific primer sequences (by Young) are shown in Table 1. The size of amplified products was 120 base pair (bp) for HPV 16, 100 bp for HPV type 18 and 260 bp for β-globin, respectively.

HPV DNA amplification by PCR

Amplification of HPV DNA was carried out in 100 μl of reaction mixture containing 10 μl of sample (1.0 μl of DNA in CaSki, HeLa and neuroblastoma cells), 25 mM KCl, 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.05 % Tween 20, 100 μl of each dNTP (dATP, dGDP, dCTP and dTTP) (Perkin Elmer Cetus), 0.25 μM of upstream primer, 0.25 μM of down stream primer, 0.15 μM of β-globin primer, 0.01 % gelatin, and 2.5 unit of the thermostable Taq DNA polymerase. The sample was overlaid with mineral oil (100 μl) to prevent evaporation and subjected to 35 cycles of amplification by programmed heat block (Hybaid thermal reactor: Hybaid Ltd., U.K.). Each cycle involved heating to 95° C for 1 minute (Primer annealing), and heating to 72° C for 2 minutes (Chain extension). 10 μl of each PCR mixture was mixed with loading dye and loaded onto an 8 % polyacrylamide gel and electrophoresis was carried out for 50 minutes at 100 volts. The gel was then stained in 1 μg/ml ethidium bromide solution for 30 minutes and visually inspected under ultraviolet light and photographed by black and white polaroid (ASA 3,000).

Statistical analysis

Association between the severity of the lesion and the HPV DNA positive rate was analyzed by score test for trend. A p-value below 0.05 ($P < 0.05$) was considered to indicate a significant difference.

RESULTS

8 out of 230 cases (2 cases in the normal group, 3 cases in the CIN group,3 cases in the invasive cancer group) were excluded because β-globin was not amplified during PCR. There was amplification of CaSki cell DNA (Positive control of HPV 16 DNA) and HeLa cell DNA (Positive control of HPV 18 DNA) by PCR but no amplification in neuroblastoma cell (Negative controls) (Fig. 1). HPV type 16 DNA yielded a band in 120 bp and HPV type 18 DNA in 100 bp, and the bands are seen in Fig. 2. The

Table 1. Sequences of oligonucleotide primers used in PCR procedure

HPV type Sequence(5'-3')	Genomic location	Size of amplified Products (bp)
Type specific primers#		
HPV 16 A : TCAAAAGCCACTGTGTCCTG	421 - 440	120
B : CGTGTTCTTGATGATCTGCA	521 - 540	
HPV 18 A : ACCTTAATGAAAAACGACGA	463 - 482	100
B : CGTCGTTGGAGTCGTTCCTG	543 - 562	
β-Globin primer†		
HPV 18 A : GAAGAGCCAAGGACAGGTAC		260
B : CAACTTCATCCACGTTCACC		

Data from Young *et al.*(1989)

† Data from Resnick etal. (1990)

A : Upstream Primer, B: Downstream Primer

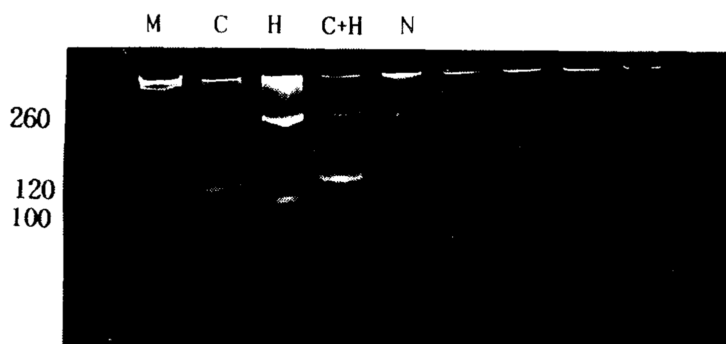

Fig. 1. Polyacrylamide gel electrophoresis of control samples amplified by polymerase chain reaction (PCR). Lane M: Size marker. Lane C: CaSki cell DNA (Positive control for HPV 16). Lane H: HeLa cell DNA (Positive control for HPV 18). Lane C + H: Mixture of CaSki and HeLa cell DNA. Lane N: Neuroblastoma (Negative control).

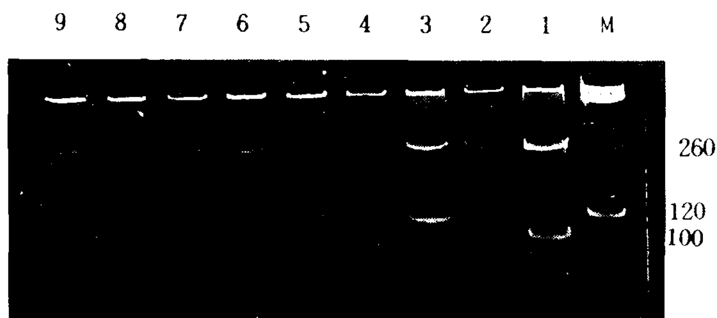

Fig. 2. Polyacrylamide gel electrophoresis of the samples from cervix cancer patients amplified by polymerase chain reaction. Lane 1-9: DNA samples from cervix cancer patients. Lane M: size marker.

prevalence rate of HPV type 16 and HPV 18 DNA is in table 2. HPV 16 DNA prevalence rate was 19.1 % (13/68) in normal cervices, 38.8 % (19/49) in CIN I, 57.1 % (28/49) in CIN II, 75.9 % (22/29) in CIN III, 88.9 % (24/27) in invasive cancer. HPV 18 positive rate was 4.4 % (3/68) in normal cervices, 8.2 % (4/49) in CIN I, 12.2 % (6/49) in CIN II, 13.8 % (4/29) in CIN III and 18.5 % (5/27) in cervical cancer. A consistent correlation was found between the severity of the cervical lesions and the positive rate of the HPV 16 or HPV 18 DNA (χ^2 = 52.7, P < 0.05 for HPV 16. χ^2 = 5.4, P < 0.05 for HPV 18). In the whole series HPV 16 was almost five times more common than HPV 18.

DISCUSSION

Human papillomaviruses (HPV) have been implicated in the development of malignant lesions of the female genital tract. Over 60 different types of HPV types were identified and among these, HPV type 16 and 18 were most frequently detected in dysplastic and malignant lesions of the cervix (De Villiers *et al.* 1987; Mark *et al.*1991; Pater *et al.*1986; Xiao *et al.*1988). It is thought that oncogenic HPV DNA integration into human genome is often accompanied by deletion of parts of the viral genome. For instance, the integration of HPV 16 is often accompanied by a partial deletion of the E1-E2 open reading frame(ORF). It is speculated that the E2 protein has a regulatory function for E6 and E7 gene expression and the deletion of this gene gives rise to consistent E6 - E7 gene transcription with maintenance of oncogenic phenotype (Howley 1988). The E6 and E7 proteins were found to bind to the p53 and the retinoblastoma gene products which are regarded as tumor suppressor genes. Along with this, some cofactors are suspected to play a role in malignant transformation because only a few HPV positive women progress to neoplastic lesion (De Villiers et al. 1992).

At present the only reliable way to detect and type HPV is nucleic acid detection method, and there are numerous methods of detecting HPV DNAs such as Southern blot hybridization, dot spot method, filter in situ hybridization (FISH), DNA/RNA in situ hybridization and polymerase chain reaction. The Southern blot method is considered sensitive (detect a level up to 1 pg HPV DNA) and specific but laborious and requires a large amount of DNA (10 μg). Moreover there have been questions about the reproducibility and specificity in HPV typing by this method. Dot spot technique requires relatively small quantities of DNA (0.3 -1 μg) and can detect a level as low as 0.5 - 1 pg HPV DNA. But this procedure can only be performed

Table 2. HPV 16 and HPV 18 positive rate by cervical histology

	Total entries	Cases included	Positive rate		
			HPV 16	HPV 18	HPV 16 or 18
Normal	70	68	13 (19.1%)	3 (4.4%)	15 (22.1%)
CIN I	50	49	19 (38.8%)	4 (8.2%)	22 (44.9%)
CIN II	50	49	28 (57.1%)	6 (12.2%)	31 (63.3%)
CIN III	30	29	22 (75.9%)	4 (13.8%)	24 (82.8%)
Cancer	30	27	24 (88.9%)	5 (18.5%)	25 (92.6%)
			$P < 0.05$ †	$P < 0.05$ †	$P < 0.05$

CIN : Cervical Intraepithelial Neoplasia
† : Score test for trend

in highly stringent conditions and it is difficult to differenciate related viral types (Roman and Fife 1989). FISH does not require extraction of DNA but it lacks sensitivity. Besides this, FISH is hampered by high background signals (Wagner *et al.* 1984). The advantage of the in situ hybridization technique is the preservation of morphology which permits exact location of HPV within tissue, but the necessity of biopsy makes this method unsuitable for screening purposes. The development of polymerase chain reaction (PCR) (Saiki *et al.* 1985) can be considered as one of the major advances in molecular virological diagnostics and is known to be more sensitive than in situ hybridization or FISH in the detection of HPV DNA. PCR has been used to identify as few as 1–2 copies of an HPV genome in a sample of only 10 cells and it should allow an absolute prevalence of HPV infection to be estimated. It also takes less time than FISH. Our study was based on direct PCR technique which has allowed us to apply this directly on cervical scrapes, omitting the laborious DNA purification procedure. In agreement with recent available data (Van den Brule *et al.* 1990; Pasetto *et al.* 1992; Peter *et al.* 1989) the prevalence of HPV type 16 and 18 increased with the severity of pathologic lesion and nearly all cervical cancer tissue carries HPV 16 or HPV 18, which suggests an important role for these HPV types in the development of CIN and cervical cancer. But studies performed by different investigators show large variations in the prevalence of HPV 16 and 18 normal cervix and cervical neoplastic lesion. The positive rate ranges from 0 % to 80 % in normal cervices (Melchers *et al.* 1989), and from 40% to 100 % in women with cervical cancer (Munoz *et al.* 1988). While this may reflect true geographical prevalence difference, it is far more likely that bias is attributed to different definitions of the normal group, contamination during experiment, methodological factors in PCR, the HPV subtypes included, and interlaboratory variations. Because PCR is a very sensitive method, special care must be taken to avoid contamination of clinical samples during all experimental procedures. We used positive and negative controls to improve the specificity of the PCR. Besides progressive increase in HPV positive rate with severity of lesion, HPV 16 is about five times more prevalent agent as compared with HPV 18 and this is consistent with other report (Mark *et al.* 1991).

Even though additional modifications of the host cell genes controlling HPV expression are required for the development of a malignant lesion, HPV infection is suspected as an essential etiologic factor. Even though cytologic screening is still the most widely used method for the early detection of premalignant and malignant cervical lesions it has some limitations. High risk HPV typing in the cervical scrapes by direct PCR can be used as a fast

and sensitive marker for the presence of neoplastic cells and may be able to identify women who might carry an increased risk of developing cervical cancer.

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