The Seoul Journal of Medicine
Vol. 36, No. 2 : 53-60, June 1995

Prognostic Significance of Human Papillomavirus Type 16 and 18 in Cervical Cancer

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= Abstract = **To assess the prognostic significance of Human papillomavirus (HPV) type 16 and 18 which have been supposed as high risk oncogenic viruses in cervical cancer, polymerase chain reaction (PCR) was used to determine HPV type in formalin fixed paraffin embedded tissue blocks. Samples were obtained from patients with stage I and II cervical cancer who underwent surgery from 1985 to 1986 at Seoul National University Hospital. In total 65 patients were enlisted, but 9 patients were excluded in data analysis because of poor amplification of β-globin during the PCR procedure. Cilnicopathologic factors (age, stage, histologic type, tumor size, lymph node metastasis) and 5 year survival rate were compared between HPV positive and HPV negative groups. The survival difference between HPV 16 positive and HPV 18 positive cases was also analyzed. HPV 16 / 18 positive rate in cervical cancer is 80.4% (45 / 56), and there is no significant difference in age, stage, histologic type, tumor size, nodal metastasis by HPV infection status. No difference was found in 5 year survival rate between HPV positive and negative groups. When compared with the HPV 16 positive group, the HPV 18 positive group did not show any difference in prognosis. These data suggested that the presence of HPV 16 or HPV 18 in cervical cancer had no relation with known prognostic factors. Either HPV positivity or HPV type (HPV 16 vs. HPV 18) did not have any prognostic significance in cervical cancer.**

Key words: *Cervix Cancer, Human Papillomavirus, PCR, Prognosis*

INTRODUCTION

Invasive cancer of the uterine cervix is the most common gynecologic malignancy in Korea. Epidemiologic studies suggest that cervical cancer is a sexually transmitted disease (Reeves

*이 연구는 1993년도 서울대학교 병원 지정진료비 (02-93-098) 보조로 이루어진 것임.
서울대학교 의과대학 산부인과학교실 : 송용상, 박노현, 강순범, 이효표
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et al., 1989; Slattery *et al.*, 1989; Syrjanen K, 1989) and Human papillomavirus (HPV) may play a major role in the development of this disease (Lancaster WD *et al.*, 1986; Ostrow RS *et al.*, 1987; Xiao X *et al.*, 1988; Syrjanen K, 1989; Zur Hausen H, 1989). Until now over 65 HPV types have been identified and type 16 and 18 are suspected to have high oncogenic potential and be the most prevalent types in cervical cancer (Class ECJ, 1989; Young LS *et al.*, 1989; Ji HX, 1990). Shibata *et al.* (1988), described polymerase chain reaction in paraffin embedded tissues which is a highly sensitive method for HPV typing. This technology made it possible to detect HPV in old tissue samples and to compare tumor histology and HPV viral DNA in the same specimen. The association of these viruses with known high risk prognostic factors and survival rate has been under study but remains to be established (Gordon AN *et al.*, 1989; King LA *et al.*, 1989; Liou G *et al.*, 1990; Walker J *et al.*, 1989; Ji HX *et al.*, 1991; Maureen AJ *et al.*, 1992; Ulla Hording *et al.*, 1992). Some reports suggested that HPV type 18 related cervical cancer showed poorer prognosis than HPV 16 associated cancer, further studies are needed to come to a conclusion. If the presence of these oncogenic HPV in cervix cancer was associated with invasiveness and patient's survival, it would be possible to use these biologic data in the management of cervical cancer. To test the above hypothesis in this retrospective study, we obtained HPV 16 and 18 DNA prevalence rates in cervical cancer tissues. The relationship between the presence of HPV DNA and patient's prognostic factors was analyzed, and survival rate according to HPV positivity and HPV type were compared.

MATERIALS AND METHODS

Study group

Invasive cervical cancer patients who were surgically treated at the Department of Obstetrics and Gynecology, Seoul National University Hospital from Feburary 1985 to December 1986 were identified from the tumor registry. Their pathologic files were reviewed and 65 cervical cancer specimens were selected among them. The Hematoxyline-eosin (H-E) stained sections of the paraffin embedded tissues (PETs) were histologically reassessed by a pathologist and tissue blocks which showed the largest percentage of tumor with the least amount of necrotic debris were chosen. 50 μm sections from each block for PCR and 5 μm sections for H-E staining were made to confirm histology. 9 cases were excluded due to failure of β-globin expression during the PCR procedure, therefore the remaining 56 cases were included in data analysis. Data were compiled regarding HPV status, clinico-pathologic findings, and survival for these patients.

Sample preparation

DNA extraction from the paraffin embedded tissue sections was done according to the method as described by Wright and Manos (Wright DK, 1990). The 50 μm tissue section was placed in a 0.5 ml polypropylene tube and deparaffinized in xylene at 37℃ for 30 minutes, centrifuged twice at 6,000 rpm for 3 minutes, washed twice in 1 ml absolute ethanol for 30 minutes and air dried. The sample was digested in 100 μl of buffer containing 10 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, and Proteinase-K 5 μl (2 mg/ml). After incubation at 50℃ for 2 hours, and 95℃ for 5 minutes to inactivate the protease, the sample was centrifuged briefly and the supernatant was frozen at −20℃ for later PCR amplification.

PCR amplification

10 μl of sample supernatant was added to the 90 μl PCR reaction buffer solution to a total volume of 100 μl containing 100 μM of each of the four nucleotide triphosphates, 20 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, 1 IU of Taq polymerase. The reaction mixture included 0.25 μM each of a pair of synthetic primers specific either for a 260 bp sequence of the human β-globin genome (Internal control), for a 120 bp region of the HPV 16 genome, or for a 100 bp region of HPV 18. Primers were supplied from Clontech Co. (Palo Alto, California, USA) (Table 1). The samples were overlaid with drops of mineral oil to prevent

Table 1. Sequences of oligonucleotide primers

HPV[a] type Sequence(5'-3')	Genomic location	Size of amplified Products (bp)
Type specific primers[b]		
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[c]		
A[d] : GAAGAGCCAAGGACAGGTAC		260
B[d] : CAACTTCATCCACGTTCACC		

[a] : Human Papillomavirus
[b] : Data from Young LS et al.
[c] : Data from Resnick et al.
[d] : A : Upstream primer, B : Downstream primer

evaporation and subjected to 35 cycles of amplification in a DNA thermal cycler (Hybaid thermal reactor : Hybaid Ltd., U.K.). Each cycle consists of a 96℃ denaturation step of 60 seconds, a 56℃ primer annealing step of 60 seconds, and a 72℃ primer extension step of 120 seconds. In the first cycle the denaturation step extended to 5 minutes at 96℃, and in the final cycle the primer extension step was prolonged to 5 minutes at 72℃.

Electrophoresis and DNA staining

10 μl of each PCR mixture was loaded onto an 8% polyacrylamide gel and electrophoresis carried out. The gel was stained in 1 μl/ml ethidium bromide solution for 30 minutes and visually inspected under ultraviolet light and photographed by black and white polaroid (ASA 3000).

Statistical analysis

Student's t-test was used to examine the difference in mean age and average tumor size between HPV type 16 or type 18 positive group and HPV negative group. The relationship between HPV status and patient chracteristics such as age (<50 vs. ≥ 50), histologic type (squamous cell carcinoma vs. adeno or adenosquamous cell carcinoma), tumor size (≤ 3cm vs. >3cm), L/N metastasis (positive vs. negative) was examined using Chi-square or Fisher's exact test. Cochran-Mantel-Haenszel test was used to see linear association between HPV status and stage. 5 year survival estimates were based on Kaplan-Meier method and the comparison of survival by HPV status was made by Log-rank test. The above analyses were performed using SAS statistical software and considered to be significant if P-value <0.05.

RESULTS

9 cases were excluded because β-globin was not amplified and the remaining 56 cases were included in data analysis. HPV 16 and 18 DNA positive rate was 42/56 (75.0%) and 9/56 (16.1%) respectively. Total HPV positive rate 80.4% (45/56) and both types were positive in 6 cases. There is no difference in the clinicopathologic characteristics between the HPV positive group and the HPV negative group except for average umor size (Table 2). There is no significant difference in 5 year survival rate according to the absence or presence of HPV (80% vs 75.7%) and this fact also applied to HPV type between HPV 16 and 18 (75.9% vs 71.4%) (Table 3) ($P>0.05$).

Table 2. Clinicopathologic characteristics of patients according to HPV status

Variable	Number of cases	HPVa (+) cases		HPV (−) cases		P-Value
		Number	%	Numbe	%	
Age						
Mean(Range)		48.5(25-70)		52.8(30-75)		P=0.26b
<50	28	22	78.6	6	21.4	NS[c]
≤50	28	23	82.1	5	17.9	
Stage						
Ia	5	4	80.0	1	20.0	NS[d]
Ib	20	18	90.0	2	10.0	
IIa	28	20	71.4	8	28.6	
IIb	3	3	100.0	0	0.0	
Histology						
squamous	50	41	82.0	9	18.0	NS[c]
non-squamous	6	4	66.7	2	33.3	
Tumor size(No. =51)						
mean		3.0		4.35		P=0.007[b]
S.D.		1.3		1.49		
≤3cm	28	25	89.3	3	10.7	NS[c]
>3cm	23	16	69.6	7	30.4	
L/N metastasis						
Positive	13	11	84.6	2	15.4	NS[b]
Negative	43	34	79.1	9	20.1	

[a]: Human papillomavirus
[b]: Student t-test
[c]: x^2 test or Fisher's exact test
[d]: Cochrane Mantel Haenszel test

DISCUSSION

While the definite etiologic role of human papillomavirus in the carcinogenesis of cervical cancer is still unknown, a strong association with HPV type 16 and 18 has been established. Polymerase chain reaction (PCR) has been widely used as a sensitive HPV detection method in various specimens such as cervical scrapes, paraffin embedded tissues and so on. The availability of HPV detection in paraffin-embedded tissues by PCR method provides the opportunity to study previously treated patients retrospectively. There has been concern about the prognostic value of HPV in cervical carcinoma and arguments about this issue. Some reports said that HPV infection is not associated with age, stage, tumor grade, lymph node metastasis survival (King LA *et al.*, 1989; Ji HX *et al.*, 1991), but others found that HPV positive patients had a better prognosis than HPV negative counterparts (Riou G *et al.*, 1990). It has also been suggested that certain HPV types, especially type 18, were related to poor clinical outcome and poor prognosis (Walker J *et al.*, 1989, Arends MJ

Table 3. 5 year survival estimates by stage and HPV[a] status

Variable	number of cases	estimated 5 year survival rate(%)	univariate log-rank test
Stage			
Ia	5	100.0 %	P = 0.007
Ib	20	87.2 %	
IIa	28	73.0 %	
IIb	3		
HPV status			
Positive	45	75.7 %	NS[b]
Negative	11	80.0 %	
HPV type	Type 16(+)	42	76.0 %
Type 18(+)	9	71.4 %	NS

[a]: Human papillomavirus

[b]: not significant

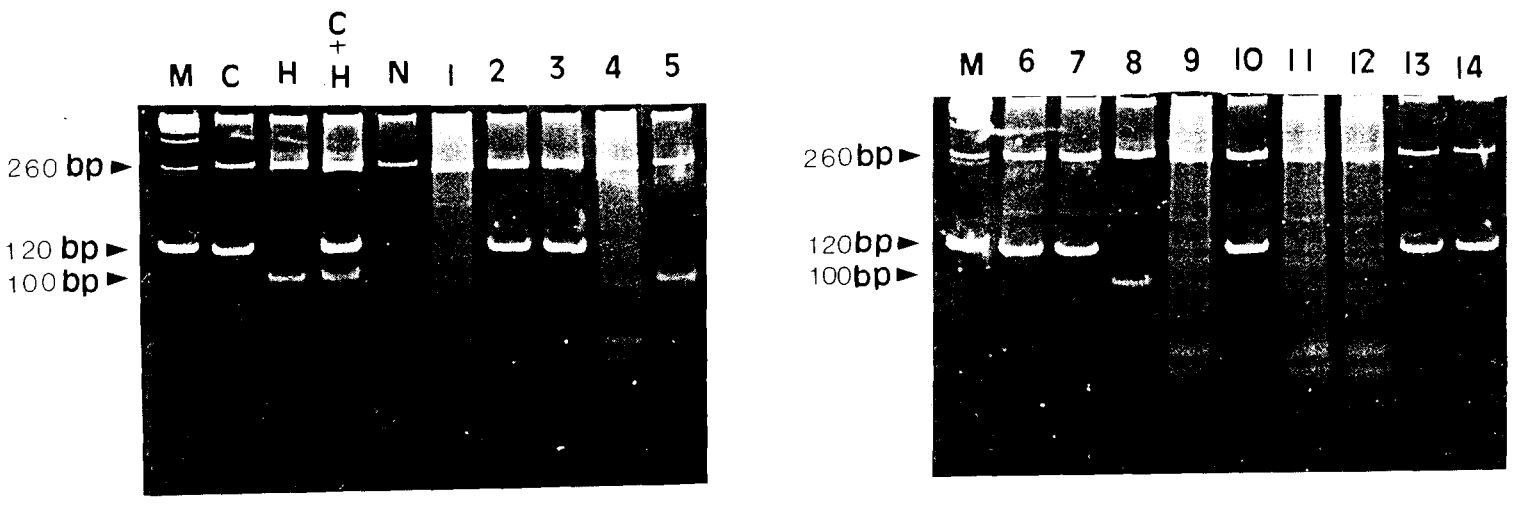

Fig. 1. Polyacrylamide gel electrophoresis following polymerase chain reaction(PCR) with control samples.
Lane M : Size marker,
Lane C : CaSki cell DNA (positive control for HPV 16),
Lane H : HeLa cell DNA (positive control for HPV 18),
Lane C + H : Mixture of CasKi and HeLa cell DNA,
Lane N : Neuroblastoma (Negative controls),
Lane 1-14 : DNA samples from cervix cancer patients

et al., 1993). In the pre. nt study, HPV 16/18 DNA was found in 80.4 % (45/56) and both types were positive in 6 cases. There was no relationship between HPV positivity and clinicopathologic and prognostic factors such as age, stage, histologic type, tumor size, L/N metastasis. No survival difference was noted between HPV positive and negative groups, both had approximately a 75 % 5 year survival rate. Considering all the facts above, HPV 16 or 18 DNA positivity could not be regarded as a prognostic factor, and cervix cancer with HPV DNA did not show a poorer prognosis than HPV negative cancer. Kurman RJ *et al.* (1988), suggested that HPV 18 might have higher oncogenic potential because this type is more frequently found in

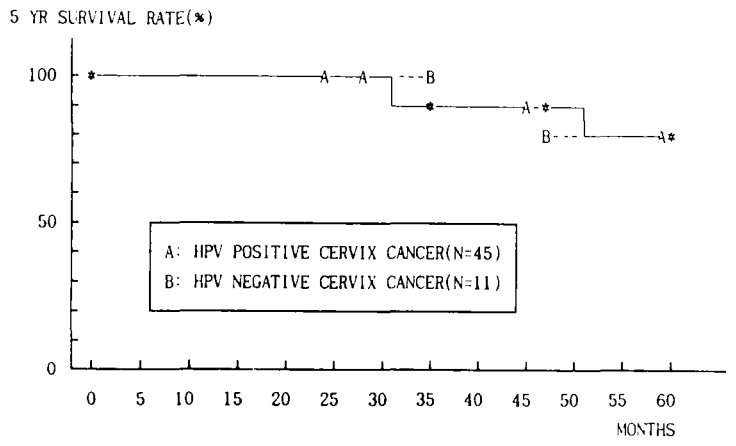

Fig. 2. 5 year survival rate by presence of human papillomavirus(HPV)

biopsies from cervical carcinoma than from intraepithelial lesions, while HPV 16 was seen at equal frequencies. Walker *et al* (1989) demonstrated that patients with HPV 18 containing tumor tended to have recurrences more frequently. We could not find any evidence that HPV 18 harboring tumor had a more invasive character or higher risk of metastasis or poorer prognosis than HPV 16 or non-HPV associated cervical cancer. The Association of HPV 18 with adenocarcinoma has also been reported (Wilczynski SP *et al.*, 1988; Tase T *et al.*, 1989; Walker J *et al.*, 1989; Riou G *et al.*, 1990), but our data did not show this.

Even though there is general agreement about the concept of an intimate relationship between HPV and cervical cancer carcinogenesis, the presence of their DNA in cancer tissue does not seem to have prognostic significance. HPV 18 harboring tumor does not have a more invasive character nor does it show a poorer prognosis than its HPV 16 positive counterpart. The extension and improvement of the polymerase chain reaction method, such as quantitative

Table 4. 5 year survival estimates by clinical stage and HPV status

Number Variable	censored of cases	estimated 5 year cases (%)	Univariate survival rate (%)	log-rank test
Age				
<50	28	10 (35.7%)	90.0 %	P = 0.3
≥ 50	28	8 (28.6%)	63.3 %	0.27
Stage				
Ia	5	3 (60.0%)	100.0 %	P = 0.007
Ib	20	6 (30.0%)	87.2 %	0.0001
IIa	28	8 (28.6%)	73.0 %	
IIb	3	1 (33.3%)		
Tumor size(N=51)				
≤ 3 cm	28	9 (32.1%)	75.1 %	P = 0.4
> 3 cm	23	6 (26.1%)	78.6 %	0.79
L/N metastasis				
Positive	13	2 (15.4%)	49.5 %	P = 0.048
Negative	43	16 (37.2%)	87.3 %	0.12
HPV status				
Positive	45	16 (35.6%)	75.7 %	P = 0.54
Negative	11	2 (32.1%)	80.0 %	0.59
HPV type				
Type 16(+)	42	16 (38.1%)	76.0 %	P =
Type 18(+)	9	3 (33.3%)	71.4 %	

[c] χ^2 test or Fisher's exact test

[@] Cochrane Mantel Haenszel test

Table 5. Survival rate by HPV DNA positivity related to age, stage, histologic type, tumor size, depth of invasion, lymph node metastasis.

Variable	Number of cases	HPV (+) cases (%)		HPV types		P-Value
		Number	percentage	HPV 16	HPV 18	
Age (n=61)[a]						
< 50	31	25	80.6	25	3	NS
≥50	30	24	80.0	21	7	
Stage(n=61)[b]						
0	5	4	80.0	4	1	NS
Ia	5	4	80.0	4	0	
Ib	20	18	90.0	18	5	
IIa	28	20	71.4	17	3	
IIb	2	2	100.0	2	1	
III	1	1	100.0	1	0	
Histology						
squamous	55	44	80.0	41	9	NS
adeno	5	4	80.0	4	1	
adenosquamous	1	1	100.0	1	0	
Tumor size(N=51)[a]						
≤3 cm	28	25	89.3	24	6	NS
>3cm	23	16	69.6	14	3	
Depth of invasion (N=55)[b]						
inner 1/3	20	19	95.0	19	2	P<0.05
middle 1/3	10	9	90.0	6	3	
outer 1/3	13	8	66.7	7	2	
full thickness	12	8	66.7	7	2	
L/N metastasis						
Positive	13	11	84.6	9	3	NS
Negative	43	30	78.9	29	6	

[a] χ^2 test

[b] Kendall's tau test

PCR, will make it possible to define the prognostic significance of HPV more clearly.

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