Jpn. J. Cancer Res. 82, 532–538, May 1991

Relatively Low Prevalence of Human Papillomavirus 16, 18 and 33 DNA in the Normal Cervices of Japanese Women Shown by Polymerase Chain Reaction

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Ninety-two cervical scrapes and tissues, obtained from cytologically or histologically normal cervices of Japanese women, were examined for the presence of human papillomavirus (HPV) 16, 18 and 33 DNA by the polymerase chain reaction (PCR) method. Five out of 92 cases were HPV 16 DNA-positive, but neither HPV 18 nor 33 DNA was detected. The HPV (type 16, 18 and 33) prevalence rate in pregnant women, including postpartum, was 10% (3/31), which was higher than that in non-pregnant women. In two HPV 16-positive cases, we detected HPV 16 DNA again 2 months later. HPV (type 16, 18 and 33) prevalence in normal cervices was shown to be relatively low. However, it is very important to follow up the HPV-positive cases in cytologically normal cervices in order to elucidate the relation between HPV infection and the progression of cervical cancer.

Key words: Normal cervix — Human papillomavirus — Polymerase chain reaction

Many experimental data suggest that specific types of human papillomavirus (HPV) infection are involved in the etiology of cervical cancer. Among the over 60 different HPV types, HPV 16, 18, 31, 33, 35, 52b, and/or 58 have been found in cervical dysplasia and carcinoma.[1] For detection of HPV, hybridization techniques such as Southern blot hybridization and *in situ* hybridization have been used[2–8] and we have found that 62% (28/45) of cervical carcinomas and 55% (17/31) of cervical intraepithelial neoplasias (CINs) were HPV DNA-positive by Southern blot hybridization.[9] However, these techniques are not sensitive enough to detect small amounts of HPV DNA from small-sized specimens.

Recently it has become possible to detect even a single copy of a target sequence in a given sample by using polymerase chain reaction (PCR), which is the most sensitive method so far developed.[10] We have previously reported on the sensitive and specific PCR detection of HPV 16, 18, and 33 by amplification of each transforming gene region.[11] By using our PCR method, the HPV detection rate increased to 83.7% (43/36) in cervical carcinomas and 70.4% (19/27) in CINs.[11] The increased HPV detection rate thus appears to reflect the higher sensitivity of the PCR method. Through the use of PCR, an increased HPV detection rate has been reported of up to 91% in cervical carcinomas[12] and 100% in CINs.[13]

To evaluate the role of HPV as a risk factor in cervical carcinoma, it is necessary to know the prevalence rate of HPV DNA in normal cervices and to follow up HPV-positive healthy women. Compared with the high prevalence of HPV in neoplastic lesions, different prevalence rates of HPV in the normal cervix have been reported by various investigators. Here, we investigated HPV 16, 18 and 33 DNA in normal cervices by our PCR method[11] in the Hokkaido area of Japan.

MATERIALS AND METHODS

Patients studied and sample preparation The subjects were 92 women (age 18–73, mean age 38.5) attending the outpatient clinics of the Department of Obstetrics and Gynecology of Sapporo Medical College Hospital and other hospitals in Hokkaido, Japan. We obtained cervical scrapes from 83 women who were diagnosed cytologically as having normal cervices. Out of 83 cervical scrapes, 69 showed Papanicolaou (Pap) class I and 14 cases showed Pap class II, with none of them showing koilocytes in routine cytological examination. Of the 83 women studied, 26 were pregnant (4–36 weeks of pregnancy) and 5 were postpartum (1 month after delivery). We obtained fresh cervical tissues from nine women undergoing hysterectomy for benign non-neoplastic disease who had no previous history of abnormal cytologic studies or treatment of the cervix. The cervical tissues were snap-frozen in liquid nitrogen and tissues close to the samples were diagnosed as normal by histopathologic study.

For HPV detection, cervical scrapes were collected from the cervical transformation zone using sterilized cotton swabs, and they were then put into 2 ml of phosphate-buffered saline (PBS). Samples of scrapes were vigorously vortexed and centrifuged for 10 min at

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Table I. Sequences of Oligonucleotide Primers and Probes[11]

	Sequences (5′-3′)	Genomic location
Primer		
p16-1	AAGGGCGTAACCGAAATCGGT	26–46
p16-2R	GTTTGCAGCTCTGTGCATA	147–165
p18-2R	GTGTTCAGTTCCGTGCACA	154–172
p33-2R	GTCTCCAATGCTTGGCACA	152–170
GH20[a]	GAAGAGCCAAGGACAGGTAC	
GH21[a]	GGAAAATAGACCAATAGGCAG	
Probe		
pB16-I	CATTTTATGCACCAAAAGAGAACTGCAATG	77–106
pB18-I	TGAGAAACACACCACAATACTATGGCGCGC	84–113
pB33-I	CATTTTGCAGTAAGGTACTGCACGACTATG	82–111

a) GH20 and GH21 (Takara Shuzo Co., Ltd.) are β-globin primers as described by Saiki *et al.*[15]

3000 rpm. Cell pellets were resuspended in 0.5 ml of 0.01 *M* Tris, 0.005 *M* EDTA, 0.5% SDS and 50 μg/ml proteinase K and kept at 37°C overnight. Genomic DNAs were prepared by phenol/chloroform extraction, chloroform extraction and ethanol precipitation.[14] Cervical tissue DNA was isolated according to the standard procedure, essentially as described.[14]

Polymerase chain reaction (PCR) Amplification of target HPV DNA from normal cervical scrapes or tissues was performed by using the PCR method as previously described by Shimada *et al.*[11] We used p16-1 as a common sense primer of HPV 16, 18 and 33, and p16-2R, p18-2R and p33-2R as type-specific antisense primers of HPV 16, 18 and 33, respectively (Table I). For type-specific detection, specific oligonucleotide probes, pB16-I, pB18-I and pB33-I, were used for identification of HPV 16, 18 and 33, respectively (Table I). These primers and probes correspond to sequences homologous to the E6 open reading frame of HPV 16, 18 and 33.[16–18] As an internal reaction control, β-globin-specific primers were used to confirm that our templates were appropriate for amplification.[15] All primers and probes were synthesized on a Milligen 7500 DNA synthesizer and purified by HPLC. The PCR was carried out with a Gene-Amp kit (Perkin-Elmer Cetus) essentially as described.[15] Cellular DNA (1 μg) was denatured at 94°C for 10 min, and placed in 100 μl of 1×PCR buffer (10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin), 0.2 m*M* of each dNTP (i.e., dATP, dGTP, dCTP and dTTP), 0.4 μM p16-1, 0.2 μM each of p16-2R, p18-2R and p33-2R, and 2.5 units of Taq-polymerase. To amplify HPV DNA, 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 2 min) were done on a DNA Thermal Cycler (Perkin-Elmer Cetus). After amplification, a 10 μl aliquot of the reaction mixture was subjected to electrophoretic analysis on 3% NuSieve and 1% Seakem agarose (FMC) and stained with ethidium bromide.

Dot blot analysis of PCR products To identify HPV type-specific sequences, we analyzed PCR products by dot blot hybridization. A 1 μl aliquot of the reaction mixture was transferred to a nylon filter (Schleicher & Schuell). Hybridization was performed at 37°C in 5× SSC, 0.1% SDS, 5×Denhart's solution and 100 μg/ml herring testis DNA with each of the ^{32}P-end-labeled HPV-specific oligonucleotide probes (pB16-I, pB18-I and pB33-I). Filters were washed twice in 2×SSC, 0.1% SDS for 10 min at room temperature and washed twice in 0.2 ×SSC, 0.1% SDS for 20 min at 55°C. Finally filters were autoradiographed with Fuji X-ray film RXOH for 1–3 h at −70°C.

Precautions for avoiding false-positive results In order to avoid false-positive results owing to contamination, we carried out PCR with special precautions as follows. We prepared sample DNAs and PCR mixtures in a special room which was separated from the room used for treating cloned HPV DNAs and PCR products. A sterile hood was used when the PCR mixture was prepared. All buffers were sterilized by UV irradiation[19] after filtration through a 0.20 μm filter and provided in a small amount for each single experiment. Pipettes, tips and tubes were UV-irradiated, and all other equipment and experimental areas were wiped with sodium hypochlorite solution after each experiment. We chose cervical carcinoma DNA containing 100 copies of HPV DNAs per cell as the positive control. To check for contamination, we included "no DNA" reagent controls with each set of DNA preparation and amplifications.[20–22] HPV-negative human DNA was used as a negative control to check for

non-specific random polymerization. We employed meticulous care in laboratory techniques as described by Kwok and Higuchi.[20,21]

RESULTS

Sensitivity and specificity of PCR for HPV detection The sensitivity of PCR was assessed with various concentrations of plasmid DNA containing HPV 16 DNA.[11] As little as 10^{-5}–10^{-6} copies of HPV 16 DNA per cell could be detected by dot blot analysis. This implies that PCR can detect almost a single HPV 16 gene. In order to test the type specificity of our methods, PCR was performed by using plasmids which carry the whole HPV6,[23] 11,[24] 16,[8] 18,[25] 31,[26] 33,[27] 52b[28] or 58[29] genome as a template. HPV16, 18 or 33 related sequence was detected by dot blot hybridization with the type-specific probe as described before.[11] None of HPV6, 11, 31 and 52b DNAs was amplified by this PCR system. HPV58 DNA was amplified by HPV33 primers (p16-1/p33-2R). However, this amplified DNA was not hybridized with any of the HPV16, 18 and 33 specific probes (pB16-I, pB18-I and pB33-I) (data not shown). As a result, specific detection of HPV16, 18 and 33 DNA was possible by using our PCR system.

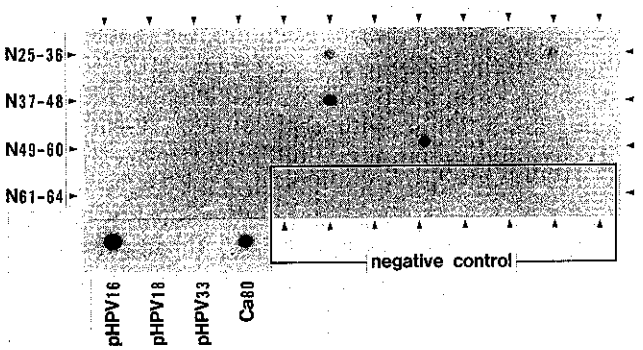

Fig. 1. Dot blot hybridization for HPV 16-specific detection. A one μl aliquot of the reaction mixture was transferred to a nylon filter (Schleicher & Schuell) and hybridized to a ^{32}P-end-labeled HPV 16-specific oligonucleotide probe (pB16-I). Filters were washed twice in 2×SSC containing 0.1% SDS for 10 min at room temperature and washed twice in 0.2×SSC containing 0.1% SDS for 20 min at 55°C. Finally the filters were autoradiographed with Fuji X-ray film RXOH for 1–3 h at −70°C. The four upper lanes (N25–N64) are normal cervical samples and N30, N35, N42 and N56 are HPV 16-positive. At the bottom starting from the left, an aliquot (1 ng) of plasmid pHPV 16,[8] pHPV 18[25] and pHPV 33[27] containing HPV 16, 18 and 33 full genomes, respectively, and 1 μg of cervical carcinoma DNA (Ca80) were subjected to amplification with mixed specific primers, p16-1, p16-2R, p18-2R and p33-2R. Negative controls consisted of "no DNA" reagent controls at each step of DNA preparation and PCR amplification and HPV-negative human DNA sample controls to check for non-specific random polymerization.

HPV prevalence in normal cervices Genomic DNAs of 83 cytologically normal cervical scrapes and 9 histologically normal cervical tissues were subjected to HPV detection by PCR. After 30 cycles of amplification using mixed HPV-specific primers (p16-1, p16-2R, p18-2R and p33-2R), 1 μl of reaction mixture was analyzed by dot blot hybridization. Dot blot hybridizations for HPV 16, 18 and 33 specific detection were carried out using HPV 16, 18 and 33 specific oligonucleotides as probes (pB16-I, pB18-I and pB33-I). DNAs extracted from normal cervical scrapes, except for three samples (N35, N42 and N56), were not hybridized to pB16-I as shown in Fig. 1. One ng of plasmid pHPV 16[8] containing the HPV 16 full genome to test the PCR specificity and 1 μg of cervical carcinoma DNA sample (Ca80) containing HPV 16 DNA as a positive control were used with each set of amplifications. Neither "no DNA" reagent controls nor HPV-negative sample controls were hybridized to pB16-I with any set of amplifications.

Eighty-seven out of the 92 cases were HPV 16, 18 and 33 DNA-negative by dot blot analysis, but the β-globin gene in all of these samples could be amplified by PCR using β-globin-specific primers. Five out of the 92 cases were positive for only HPV 16 DNA by dot blot analysis as shown in Table II. HPV prevalence in normal cervices was about 5% (5/92).

In two of the five HPV DNA-positive samples, electrophoretic analysis revealed the expected amplified band of DNA (data not shown). However, in three of the five HPV DNA-positive cases, no HPV-specific band could be found by electrophoretic analysis, but HPV 16 DNA could be detected by dot blot hybridization. We considered that the copy numbers of HPV DNA in these samples were so small that they could be detected only by the more sensitive dot blot hybridization. In each of these samples, some non-specific bands were found by electrophoretic analysis, but they did not hybridize to HPV-specific probes.

HPV prevalence in pregnant women and reevaluation of HPV-positive cases Among the HPV 16-positive

Table II. Detection of HPV DNA in Normal Cervices by PCR

	HPV 16	HPV 18	HPV 33	Total
Cervical tissues	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)
Cervical scrapes	5/83 (6%)	0/83 (0%)	0/83 (0%)	5/83 (6%)

women, two were in their 1st trimester of pregnancy (age 32, 31), one was postpartum (age 32), one had senile colpitis (age 59) and one woman was attending the clinic for cancer screening (age 52). The postpartum woman was Pap class II and the others were Pap class I by cytological diagnosis. We compared the HPV detection rate of pregnant and postpartum women with that of non-pregnant women and found it was 10% (3/31) in pregnant and postpartum women, and 4% (2/52) in non-pregnant women (Table III). Cervical scrapes were re-collected from 3 of the 5 HPV 16 DNA-positive women 2 months after the first sampling, and HPV 16 DNA could still be detected in 2 cases, but not in the third (Table IV).

Table III. Detection of HPV DNA in Pregnant and Non-pregnant Women

	No.	HPV 16 positive	%
Pregnant and postpartum women	31	3	10
Non-pregnant women[a)]	52	2	4

a) Non-pregnant women attending the gynecological clinic.

DISCUSSION

The frequency of the presence of HPV DNA in normal cervices as determined by various hybridization tech-

Table IV. Follow-up Study of HPV-positive Cases

Name	Age	Clinical diagnosis	Detection of HPV 16	
			Cytology	1st exam. → 2nd exam. (2 mo. later)
K.K.	32	Pregnancy (1st trimester)	I	+ → +
N.K.	31	Pregnancy (1st trimester)	I	+ → −
S.I.	59	Senile colpitis	I	+ → +

Table V. Detection of Human Papillomavirus in Normal Cervices

Material	Method	HPV type	Total HPV-positive rate	Country	Reference
Cervical scrapes	PCR	16	11% (9/85)	USA	30
			0% (0/32)		
			22% (6/27)		
		L1 consensus	41% (35/85)		
		system	31% (10/32)		
			44% (12/27)		
Cervical scrapes	PCR	16, 18, 33	5% (5/92)	Japan	Author
Cervical scrapes	PCR	6, 11, 16, 18, 33	6% (13/220)	Holland	31
			12% (14/120)		
Cervical scrapes	PCR	General primer	25% (21/83)	Holland	12
Cervical scrapes	PCR	16	26% (13/53)	Germany	32
Cervical scrapes	PCR	6, 11, 16, 18, 33	42% (43/102)	Taiwan	33
Cervical scrapes	PCR	16	51% (23/45)	England	34
Cervical scrapes	PCR	11, 16	70% (7/10)	England	13
Paraffin-embedded tissue	PCR	16, 18, 31	30% (3/10)	USA	35
Cervical biopsy	Southern[a)]	16, 18	11% (1/9)	England	2
Cervical biopsy	Southern[a)]	16	30% (6/20)	England	3
Cervical biopsy	Southern[a)]	16	35% (9/26)	England	4
Cervical tissue	*in situ*[b)]	6, 11, 16, 18	57% (24/42)	Hungary	36
Cervical scrapes	FISH[c)]	6, 11, 16, 18	3% (22/666)	Japan	5
Cervical scrapes	FISH[c)]	6, 11, 16, 18	9% (806/8755)	Germany	6
Cervical scrapes	FISH[c)]	6, 11, 16, 18	11% (4/36)	Germany	7

a) Southern blot hybridization. *b*) *in situ* hybridization. *c*) Filter *in situ* hybridization.

niques ranged from 3.5% to 57% (Table V). Using PCR, the HPV occurrence rate in normal cervices ranged from 0% to 70% (Table V). HPV detection rates in normal cervices vary considerably and these differences may occur due to geographic differences, variety of sample status (e.g. sexual activity, age and/or pregnancy, etc.), or technical problems (specificity, sensitivity and contamination). We could detect HPV 16, 18 and 33 DNA in only 5% of normal cervix samples by using sensitive PCR with special precautions. The sensitivity of our method using PCR for HPV detection was approximately 10^{-5}–10^{-6} copies per cell, as mentioned in a previous paper.[11]

One of the most pernicious problems plaguing PCR techniques is contamination with previously amplified materials or plasmids containing the HPV insert. Previously, a high rate of HPV 16 subtype infection was reported to have been found in normal cervices using PCR,[37] but that finding was a consequence of accidental contamination of DNA samples in the laboratory by products of an earlier PCR reaction.[38] Furthermore, Beyer-Finkler *et al.* demonstrated by the use of anti-contamination primers that contamination with HPV-containing plasmids frequently occurred.[32] In addition to the above-mentioned reports, Kwok and Higuchi described many precautions for avoiding false-positive results.[20, 21] It is probable that various forms of contamination give rise to false-positive results. We also think that the reported very high prevalence of HPV 16 in normal cervices should be reevaluated, and standard procedures for avoiding false-positive results are essential.[19–21, 39]

Several investigators have reported increased prevalence of HPVs in the uterine cervices of pregnant women.[40, 41] Rando *et al.*[40] reported that the HPV detection rate was 20.9% during the first trimester of pregnancy, and increased during the third trimester (53.7%), and then decreased at the time of postpartum examination. Schneider *et al.* detected HPV DNA in 28.3% of pregnant women and 12.5% of non-pregnant women.[41] We detected HPV 16 DNA in 10% of pregnant and postpartum women, which was a higher incidence than that in non-pregnant women (4%). Thus, pregnancy seems to represent a risk factor for infection with HPV. Although the mechanism of increased prevalence of HPV in pregnant women is unknown, it appears to correlate with the natural immunologic and hormonal environment of the pregnant women.

Previously, a prospective study showed that of 26 women who had cytological, colposcopic and histological evidence of progression from CIN I to CIN III, 22 (85%) were positive for HPV 16 DNA by filter hybridization of a cervical smear 8 weeks after recruitment.[42] These results suggest that the finding of HPV 16 DNA associated with mild cervical atypia may define women at high risk of rapid progression to CIN III. Follow-up examination of HPV-positive women revealed persistence of HPV in cervices,[43] and we also found the persistence of HPV DNA in normal cervices for 2 months. But it is not clear whether or not HPV-positive normal cervical epithelium tends to progress to neoplastic lesions. We think that follow-up studies of HPV-positive women with undetectable cytological abnormality have practical implications with respect to controlling disease. As it seems that the HPV copy number in normal cervices is small, our detection method using PCR is most suitable for this study.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture. We thank Drs. H. zur Hausen and L. Gissmann (types 6, 11, 16 and 18 through the Japanese Cancer Research Resources Bank), A. T. Lorincz (type 31), G. Orth (type 33), Y. Ito (type 52b) and T. Matsukura (type 58) for providing cloned HPV DNAs.

(Received December 25, 1990/Accepted February 28, 1991)

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