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Amplification and Specific Detection of Transforming Gene Region of Human Papillomavirus 16, 18 and 33 in Cervical Carcinoma by Means of the Polymerase Chain Reaction

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We have established a highly sensitive method for specific detection of human papillomavirus (HPV) 16, 18 and 33, by using the polymerase chain reaction (PCR). A HPV-related sequence (140 bp) in the E6 transforming region was specifically amplified and detected by gel electrophoresis and by the use of a specific oligonucleotide probe. The PCR could detect 10^{-5}–10^{-6} copies per cell (maximum sensitivity). Furthermore, HPV 16, 18 and 33 DNAs were synthesized in a common reaction solution and specifically detected by HPV type-specific probes. The PCR detected the HPV sequence from tissues which were negative to Southern hybridization. This detection technique may contribute significantly to the precise analysis of HPV in small proliferative lesions in the cervix.

Key words: Cervical carcinoma — Human papillomavirus — Polymerase chain reaction

An association between human papillomavirus (HPV) and cervical carcinoma has been noted. HPV can be classified into over 60 different types based on DNA hybridization criteria. HPV 16, 18, 31, 33 and/or 35 have been found in cervical dysplasia and carcinoma.[1-4] In previous papers,[5-7] the rate of occurrence of HPV in cervical dysplasia and carcinoma ranges from 17% to 80%. The lower occurrence rates of HPV in some reports may reflect the low sensitivity of the detection systems used. Southern hybridization has hardly been used for the detection of HPV sequences in small dysplastic lesions. Recently, the polymerase chain reaction (PCR) technique has been developed for highly sensitive detection by amplifying a small segment of DNA flanked by oligonucleotide primers of known DNA sequence.[8] In this report, we detected HPV 16, 18, or 33 DNA in cervical carcinoma and cervical intraepithelial neoplasia (CIN) tissues by PCR using common and specific primers for these HPVs and found that the technique was simpler, more sensitive and more specific than the Southern hybridization method.

The HPV 16, 18, or 33 genome is usually integrated into cellular DNA in cervical carcinoma.[9] In some cases, some regions of HPV genome were deleted, but the long-control region (LCR) and the E6 and E7 regions were preferentially conserved.[10-14] We selected 140 bp of the E6 region for amplification. Sequences of primers for amplification and of probes for detection of HPV type are shown in Fig. 1B. The primer p16-1 is located close to the 5′ terminus of the E6 coding sequence of HPV 16, which is 90% (19/21) homologous to the corresponding HPV 18 sequence, and 86% (18/21) homologous to the corresponding HPV 33 sequence. We used p16-1 as the common primer of HPV 16, 18 and 33. The second primer p16-2R corresponds to the E6 antisense sequence of HPV 16, which differs from those of HPV 18 and 33. We also designed specific primers p18-2R and p33-2R for the antisense sequences of HPV 18 and 33, respectively (Fig. 1B). For type-specific detection, specific probes pB16-I for HPV 16, pB18-I for HPV 18 and pB33-I for HPV 33 were synthesized (Fig. 1B). The homology of this region is less than 50% among HPV 16, 18 and 33. DNA from cervical cells was subjected to amplification with the primers p16-1 and p16-2R specific for HPV 16, and analyzed by agarose gel electrophoresis (Fig. 2A). The predominant 140 bp band of HPV 16 DNA was amplified in DNA of SiHa cells which carry HPV 16 DNA, but not amplified in DNA of HeLa cells containing HPV 18. DNAs from several cervical tissues were subjected to amplification. No DNA band was amplified in DNA from normal cervical tissues and condyloma tissues containing HPV 6 or 11 sequences. HPV 16 140 bp band was amplified in DNA from cervical carcinoma tissues containing HPV 16 (Fig. 2A). Reaction products were subjected to hybridization with the probe pB16-I specific for HPV 16 (Fig. 2B). HPV 16 DNA was detected in the reaction product from SiHa cells and cervical carcinoma tissues. The results in Fig. 2 indicate

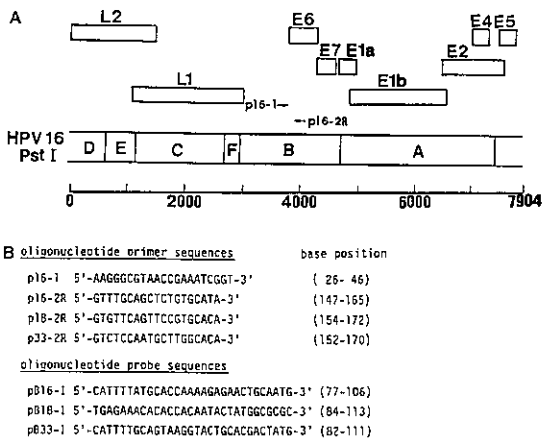

Fig. 1. Sequences of specific oligonucleotide primer and probes used in PCR and their locations in HPV genomes. (A) HPV 16 full-length DNA, *Pst*I cleavage sites, and open reading frames are based on the sequence determined by Seedorf *et al.*[15] Arrows indicate the 5′→3′ orientation of the primer. (B) Primers and probes correspond to sequences homologous to the E6 open reading frame of HPV 16, 18 and 33. Base positions are numbered according to the published sequences.[15–17] The primer p16-1 corresponds to the E6 sense sequence of HPV 16, and is available for amplification of HPV 16, 18 and 33 DNA. The primers p16-2R, p18-2R, and p33-2R correspond to specific E6 antisense sequences of HPV 16, 18, and 33, respectively.

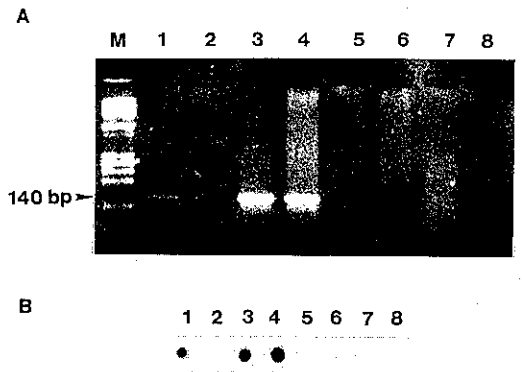

Fig. 2. Amplification and detection of HPV 16 DNA in cervical cells. The PCR was carried out with a Gene-Amp Kit (Perkin-Elmer Cetus) essentially as described.[18] Cellular DNA (1 μg) isolated from SiHa cells (lane 1), HeLa cells (lane 2), cervical carcinoma tissues (lanes 3, 4), condyloma acuminata tissues (lanes 5, 6), and normal cervical tissues (lanes 7, 8) from Myoma uteri 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) gelation), 0.2 m*M* (each) dATP, dGTP, dCTP, dTTP, 0.2 μM (each) primers, 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). (A) 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. (B) A 1 μl aliquot of the reaction mixture was transferred to a nylon filter (Schleicher & Schuell) and hybridized to ^{32}P end-labeled HPV type-specific oligonucleotide probes designated in Fig. 1B. 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 the filters were autoradiographed with Fuji X-ray film RXOH for 3–12 h at −70°C.

that this region of the HPV genome and these primers are useful for specific amplification and detection of HPV 16 E6 region.

In order to test the type specificity of our second primers, PCR was performed by using plasmids which carry the whole HPV16, 18 or 33 genome as a template and the viral-related sequence was detected by the type-specific probe (Fig. 3). HPV 16 sequence and a smaller amount of HPV 18 sequence were synthesized by primers p16-1/p16-2R. From primers p16-1/p18-2R, HPV 18 and HPV 16 sequences were synthesized. The HPV 18 sequence synthesized by primers p16-1/p16-2R hybridized with the probe pB18-I, but not with the probe pB16-I. Also, the HPV 16 sequence synthesized by primers p16-1/p18-2R hybridized with the probe pB16-I, but not with the probe pB18-I. Therefore, these amplified DNAs were discriminated by the type-specific probes pB16-I and pB18-I. HPV 33 DNA was only amplified by primers p16-1/p33-2R and detected with the probe pB33-I. Therefore, combined use of appropriate primers and type-specific probes was effective for HPV type detection.

To determine the smallest amount of template which would synthesize viral sequences, cloned plasmid pHPV 16 was serially diluted and subjected to PCR. One microgram of genomic DNA from cells which carry the HPV sequence at one copy per cell contains 10^6 copies of HPV

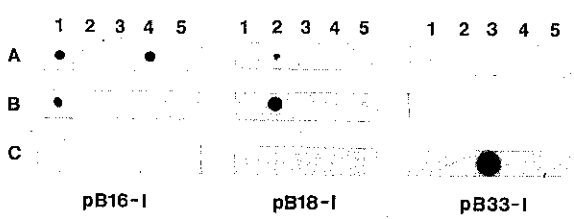

Fig. 3. Type-specific detection of HPV DNA. Aliquots (1 ng) of plasmid pHPV16 (lane 1), pHPV18 (lane 2) and pHPV33 (lane 3) cloning HPV 16,[1] 18[2] and 33[4] full genomes, respectively, and 1 μg of SiHa (lane 4) or HeLa (lane 5) DNA were subjected to amplification with specific primers (A) p16-1 and p16-2R (B) p16-1 and p18-2R (C) p16-1 and p33-2R. A 1 μl aliquot of reaction mixture was hybridized to the specific probe pB16-I, pB18-I, or pB33-I, respectively, as described in Fig. 2.

genome. Therefore, the result in Fig. 4 demonstrates that our method makes it possible to detect 10^{-5} to 10^{-6} copies of HPV DNA per cell, which corresponds to almost a single HPV 16 gene.

Several investigators have reported that HPV incidence among Japanese patients was 40–50%.[19-21] We attempted to detect HPV 16, 18 or 33 DNA in cervical carcinoma tissues in patients from Hokkaido. A genomic DNA was prepared by proteinase K digestion followed by phenol extraction and ethanol precipitation. For Southern hybridization, 10 μg of DNA was digested with *Bam*HI or *Bam*HI and *Pst*I (HPV 16), *Eco*RI (HPV 18), or *Bgl*II (HPV 33), electrophoresed on a 1% agarose gel, and blotted onto a nylon membrane. Hybridization was performed at 68°C in 6×SSC, 0.1% SDS, 5×Denhart's solution, and 1 mg/ml salmon testis DNA with each of ^{32}P-labeled HPV 16, 18 and 33 probes. The nylon membrane was washed twice in 2×SSC, 0.1% SDS at room temperature and washed twice in 0.1×SSC, 0.1% SDS at 55°C. Finally, the membrane was autoradiographed with Fuji X-ray film. Amplification by PCR was performed with mixed primers (0.2 μM each of p16-1, p16-2R, p18-2R, and p33-2R) and the amplified DNA was detected with type-specific probes (pB16-I, pB18-I, pB33-I). The detection rate in the same population group was compared to that found by Southern hybridization (Table I). HPV 16, 18 or 33 DNA was found in 60.4% of cases (26/43) by Southern hybridization, but in 83.7% (36/43) by PCR. HPV 16 was detected in 76.7% (33/43), HPV 18 in 27.9% (12/43), and HPV 33 in 14.0% (6/43). In some cervical carcinomas, two or three types of HPV sequences were detected in a single tissue. HPV 16 and 18 were detected from 7 cases (16.3%), HPV 16 and 33 from 4 cases (9.3%) and HPV 16, 18 and 33 from 2 cases (4.7%) out of 43 independent tissues (Table I). The PCR could detect the HPV sequence in carcinoma tissue which was negative in Southern hybridization. The limit of the ability of the Southern hybridization to detect HPV DNA was at the level of about 0.2 copies per cell. The PCR could detect 10^{-5}–10^{-6} copies per cell (Fig. 4). The increased HPV detection rate thus appears to reflect the higher sensitivity of the PCR. It is clear that there are some carcinoma tissues in which only a small population of cells contains HPV DNA sequence. HPV might play a key role in initiation of carcinogenesis but not in its further progression as suggested by *in situ* hybridization analysis.[22] Further investigation is necessary on the role of the PCR-detected population in the oncogenic process.

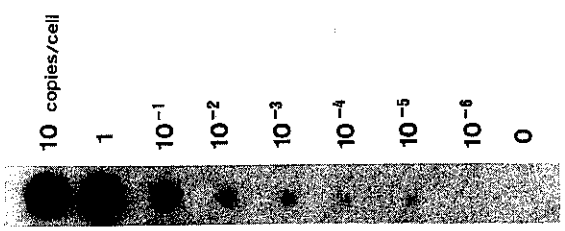

Fig. 4. The sensitivity of detection of HPV DNA. The plasmid pHPV 16 was diluted to 10 copies HPV DNA/cell with normal cervical genome DNA (10 copies/cell = 40.6 pg pHPV16/1 μg genome DNA). The 10 copies/cell DNA was diluted serially with normal cervical genome DNA. The samples were subjected to 30 cycles of amplification and hybridized to the probe pB16-I as described in Fig. 2. Numbers indicate copy number/cell.

We also tried to detect HPV 16, 18 or 33 DNA in CIN tissues (Table II). The detection rate was also increased from 48.1% (13/27) by Southern hybridization to 70.4% (19/27) by PCR. HPV genotype detection rates in CIN tissues were similar to those in carcinoma tissues; 70.4% (19/27) for HPV 16, 22.2% (6/27) for HPV 18 and 14.8% (4/27) for HPV 33. HPV DNA was detected in tissues of all the progressive stages of CIN except for moderate dysplasia (0/1). We carried out PCR with special precautions to prevent DNA contamination, as Kwok and Higuchi proposed.[23] In our experimental procedure, no HPV-related sequence was found in DNA from 9 samples of normal tissues after at least 30 cycles of amplification. On the other hand, as shown in Fig. 3,

Table I. Detection of Human Papillomavirus DNA in Cervical Carcinomas

	HPV										
	16	18	33	16/18	16/33	18/33	16/18/33	16 (total)[a]	18 (total)	33 (total)	Total
Southern hybridization	19	4	0[b]	2	0[b]	0[b]	1[b]	22/43 (51.2%)	7/43 (16.3%)	1/14[b] (7.1%)	26/43 (60.4%)
PCR	20	3	0	7	4	0	2	33/43 (76.7%)	12/43 (27.9%)	6/43 (14.0%)	36/43 (83.7%)

a) Total number of cases which contained HPV16, 18, or 33 sequences detected alone or with other types.
b) Fourteen cases out of forty-three were tested with the HPV33-specific probe.

Table II. Detection of Human Papillomavirus DNA in Cervical Intraepithelial Neoplasia (CIN)

	HPV										
	16	18	33	16/18	16/33	18/33	16/18/33	16 (total)[a]	18 (total)	33 (total)	Total
Southern hybridization	11	1	nd[b]	1	nd	nd	nd	12/27 (44.4%)	2/27 (7.4%)	nd	13/27 (48.1%)
PCR	9	0	0	6	4	0	0	19/27 (70.4%)	6/27 (22.2%)	4/27 (14.8%)	19/27 (70.4%)
mild dysplasia	3	0	0	0	2	0	0	5/8	0/8	2/8	5/8
moderate dysplasia	0	0	0	0	0	0	0	0/1	0/1	0/1	0/1
severe dysplasia	4	0	0	2	1	0	0	7/8	2/8	1/8	7/8
CIS[c]	2	0	0	4	1	0	0	7/10	4/10	1/10	7/10

a) Total number of cases which contained HPV16, 18, or 33 sequences detected alone or with other types.
b) nd: not determined.
c) CIS: carcinoma *in situ*.

HPV16, 18 and 33 DNA were synthesized in a common reaction solution and specifically detected with each specific probe DNA. Thus, we consider that our results reflect the true occurrence rates of HPV in displasia and carcinoma. Melchers *et al.*[24] have amplified the L1 region of HPV by PCR and detected HPV DNA. But the L1 region of the integrated HPV genome might be deleted in some cases, as Shirasawa *et al.*[11] pointed out. Also Shibata *et al.*[25] have amplified the E6 region (bases 320–429 of HPV 16) by PCR. They used DNA polymerase I for amplification and their primers had A-T-rich sequences. So the primer had to be annealed at lower temperature (at 37°C for example), and HPV-unrelated DNA in cervical tissues might possibly be amplified as the result of nonspecific annealing. Our region is more specific for amplification because of the higher annealing temperature of the primer (at 55°C). We also set up 89 bp of the E7 region (bases 661–749 of HPV 16) for effective amplification and obtained similar results to those reported in this paper (data not shown). We could detect mRNA of the E6 and E7 regions in cervical dysplasia tissues by PCR using these E6 and E7 regions for amplification (data not shown). Our technique may contribute to evaluation of the role of HPV as a risk factor in cervical carcinoma.

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