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Detection and Typing of Multiple Genital Human Papillomaviruses by DNA Amplification with Consensus Primers

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Many types of human papillomavirus (HPV) are associated with genital lesions. In order to develop simple and sensitive diagnostic procedures for HPV infection, we took advantage of the polymerase chain reaction (PCR). We compared the published nucleotide sequences of the L1 region from six genital HPV types and designed a pair of consensus primers for L1 region. The PCR with the consensus primers for L1 region (L1-PCR) could amplify at least nine genital HPV types, 6, 11, 16, 18, 31, 33, 42, 52 and 58, and the amplified HPV DNA could be typed by subsequent restriction mapping. L1-PCR was compared to Southern blot analysis and also to the consensus primer-mediated PCR for E6 region (E6-PCR) described before. Although both our PCR systems are nonradioactive, the sensitivity in detecting HPV DNA was even better than that obtained by using Southern blot analysis. By means of the PCR systems we detected HPV DNA in 100% of cervical condylomas (10/10), 92% of cervical intraepithelial neoplasias (33/36) and 96% of invasive cervical carcinomas (53/55), while we detected HPV DNA in 12% of normal cervices (12/102).

Key words: Human papillomavirus — Carvical cancer — Polymerase chain reaction

Specific types of human papillomavirus (HPV), especially HPV types 16 and 18, have been studied intensively as a possible etiologic agent of cervical cancers for several years. HPV 16 DNA can transform cultured cells and the open reading frame E7 is responsible for the transformation.[1–5] HPV types 16 and 18 DNA in cancer cells retains E6 and E7 gene regions as well as the transcriptional control region.[6–8] The E7 gene product is the most abundant viral protein in two cell lines (SiHa and CaSKi) derived from the cervical cancer.[9, 10] While these *in vitro* studies support the possibility that HPV plays a causative role in cervical cancers, epidemiologic evidence is still controversial.[11] Positive rates of HPV 16 DNA sequences in cervical cancers have varied from 14% to 92%.[12] In addition to HPV types 16 and 18, HPV types 31, 33, 35, 39, 52 and 58 have been identified, though less frequently, in cervical cancers.[13–19] Although it is important to elucidate the prevalence of various genital HPV types and to define the spectrum of their pathogenicity, the information on these newly cloned HPVs is limited not only because the availability of the appropriate probes for Southern blot analysis is limited, but also because there are too many HPV types to prepare a type-specific probe for each for routine use.

Polymerase chain reaction (PCR)[20, 21] has been applied, with a great success, in the detection of type-specific HPV DNA[22, 23]; however, the type-specific primers can be synthesized only for sequenced HPVs such as types 6, 11, 16, 18, 31 and 33.[24–29] Recently, in an effort to detect a broad spectrum of HPV genotypes easily, consensus or general primers have been elaborated.[30–33] While others chose radioactive hybridization to identify HPV types of the amplified DNA fragments, our PCR system for E6 (E6-PCR) was unique in typing them non-radioactively. In this study we describe a pair of consensus primers in the L1 region which can be used to detect and identify nine types of genital HPVs non-radioactively (L1-PCR). These two PCR systems were applied to specimens from normal controls and from patients with benign condylomas, precancerous lesions or invasive carcinomas. The specificity and the sensitivity of these PCR systems were also compared to each other and to those of Southern blot analysis.[34]

MATERIALS AND METHODS

Materials Eighteen types of cloned HPV DNAs, HPV types la, 2, 3, 5, 6b, 8, 10, 11a, 13, 16, 18, 31, 33, 34, 36, 42, 52b and 58 cloned in pBR322 or pUC9, were used to determine the specificity and sensitivity of two consensus primer-directed PCR systems. The plasmids were digested prior to use with the restriction enzymes used to clone each HPV DNA into the plasmid vectors.

The study group consisted of swab specimens from 102 cytologically normal cervices and biopsied specimens from 10 papillary condylomas, 5 moderate dysplasias, 11 severe dysplasias, 20 carcinomas *in situ* and 55 invasive carcinomas (stages Ib–III) of the uterine cervix. The

range of age and the mean age of these groups were as follows; normal cervices (23–49 and 39.0 yr), papillary condylomas (20–32 and 24.2 yr), moderate dysplasias (29–38 and 33.3 yr), severe dysplasias (32–49 and 35.8 yr), carcinomas *in situ* (30–74 and 42.2 yr) and invasive carcinomas (25–78 and 56.9 yr). Total cellular DNA from these specimens was extracted according to the standard SDS-proteinase K procedure.

Consensus primers Published DNA sequences were obtained from the GenBank. According to the feature table in each sequence file, L1 regions were selected. The L1 region of HPV types 6b, 11a, 18, 31 and 33 was compared to that of HPV 16 by the computer program, GAP (Sequence analysis software package of the Genetics Computer Group). Based on the comparison and with the help of the program LINEUP (Sequence analysis software package of the Genetics Computer Group), DNA sequences of the L1 region were lined up and areas with high homology were searched. A pair of consensus primers, L1C1 and L1C2, were constructed so that the primers maintain near 90% homology among the above 6 HPV types, although they are not identical to any of them (Fig. 1). The length between L1C1 and L1C2 is 244 bp in HPV types 6 and 11, 253 bp in types 16 and 18 and 256 bp in types 31 and 33. Consensus primers for the E6 region (E6C1; 5′-ACCGAAAACGGTTGAACCGAAAACGGT-3′ and E6C2; 5′-AATAATGTCTATATTCACTAATT-3′) were synthesized as described.[33] The oligonucleotide primers were synthesized by a model 380B DNA synthesizer (Applied Biosystems).

Polymerase chain reaction The PCR protocol employed in this study was 40 cycles of denaturation (95°C, 1.5 min), annealing (48°C, 1.5 min) and extension (70°C, 2 min) on a BioGene PHC-1 (Techne Ltd., Cambridge, England). Each reaction mixture (100 μl) contained 50 mM KCl, 10 mM Tris-Cl pH 8.4, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and 4 units of Taq polymerase (Biotech International Ltd., Bentley, WA). After the reaction, one-tenth (10 μl) of the reaction mixture was electrophoresed through 4% NuSieve™ GTG agarose (Takara Shuzo Co. Ltd.) gel containing 1 μg/ml ethidium bromide and the gel was photographed. About one-fourth (25 μl) to one-tenth (10 μl) was used for digestion with the restriction enzymes according to the intensities of the amplified fragment.

Typing of HPV DNA The amplified HPV fragments could be typed on the basis of the restriction fragment length polymorphisms (RFLPs) among HPVs. Restriction maps of the currently available enzymes were surveyed by MAPSORT (Sequence analysis software package of the Genetics Computer Group) and restriction enzymes useful for typing were chosen (Table I). *Rsa* I and *Sau* 3AI were used to type the HPV fragments amplified by E6-PCR as previously described.[33] In both E6-PCR and L1-PCR, amplification products were almost free from nonspecific bands, and therefore aliquots of the amplification reaction were subjected to restriction digestion without further purification.

RESULTS

Amplification of various HPV types using consensus primers Fig. 1 shows the nucleotide sequences of the consensus primers in the L1 region used in this study as well as the mismatched nucleotides in the corresponding region of each sequenced genital HPV. A panel of 18 cloned HPV DNAs was used to examine the specificity and sensitivity of the PCR using the consensus primers

L1C1	5′-CGTAAACGTTTTCCCTATTTTTTT-3′		Base Position
HPV 6	·····A·····TA·····	87%	5761–5784
HPV11	·····A·····TA·····	87%	5743–5766
HPV16	·····A··A·····	91%	5609–5632
HPV18	·····G·····	95%	5585–5608
HPV31	·····G·AT·A·····	83%	5524–5547
HPV33	·····A·····	95%	5566–5589
L1C2	3′-GTTATGTCTCATAAATCCCAT-5′		
CONS.	5′-CAATACAGAGTATTTAGGGTA-3′		
HPV 6	·····G·····A···G	86%	5984–6003
HPV11	·····T·····G····A····	86%	5966–5986
HPV16	·····G·····AA··	86%	5841–5861
HPV18	·····T·····G	90%	5817–5836
HPV31	·····T··G·····T	86%	5759–5778
HPV33	·····T··G··T·····C	81%	5801–5820

Fig. 1. Sequence comparison of synthesized primers with various HPV types. The 5′ end is on the left side of each sequence except for L1C2, which is the complementary strand of the consensus sequence (CONS.) of this region. For each HPV type, only the nucleotides mismatched with the consensus are shown and the homologous nucleotides are expressed by dots. The % homology of each HPV type with the consensus is shown as well as the corresponding base positions.

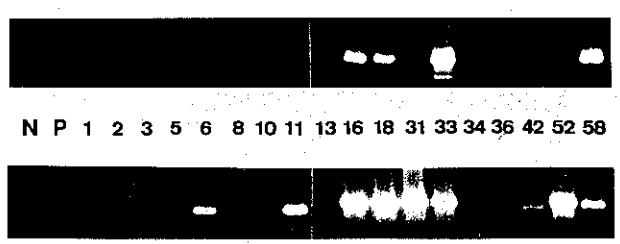

Fig. 2. Amplification of cloned HPV types by the consensus primers for the E6 region (A) and for the L1 region (B). Cloned HPV DNA (0.1 ng) was used for the reaction and 1/10th of each reaction mixture was loaded on the gel. The numbers indicate HPV types. N: PCR reaction without HPV DNA. P: PCR reaction with 0.1 ng of pBR322 DNA.

(Fig. 2). By PCR using the consensus primers in the E6 region (E6-PCR), the fragment of the expected size was easily detected in 4 HPV types as described (Fig. 2A).[33] Nine HPV types (types 6, 11, 16, 18, 31, 33, 42, 52 and 58) were clearly detected and two HPV types (types 34 and 36) were very weakly detected by PCR using the consensus primers in the L1 region (L1-PCR) (Fig. 2B). Although L1-PCR has a wider range of detection than E6-PCR, the level of amplification of HPV 58 was lower than that in E6-PCR and the level of detection of HPV types 34 and 36 did not appear to be high enough for practical use.

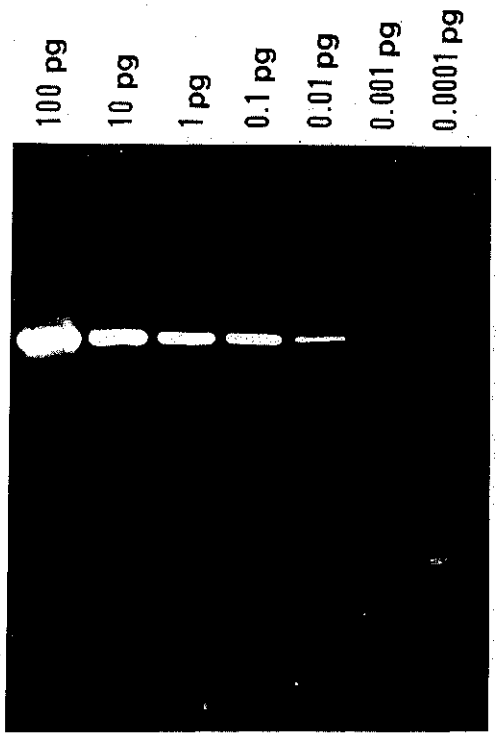

Fig. 3. The sensitivity of L1-PCR to amplify HPV 18 DNA. Cloned HPV DNA was serially diluted in TE and each reaction mixture contained 0.1 μg of human placental DNA.

To define the sensitivity of L1-PCR more precisely, the cloned HPV 18 DNA serially diluted in TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) containing 0.1 μg of human placental DNA was subjected to L1-PCR. One-tenth of each reaction mixture was electrophoresed and the gel was photographed under UV illumination (Fig. 3): 0.01 pg of DNA in the initial material was the minimal amount that could be detected in this way. The genome of HPV is about 10^6 times smaller than that of a human diploid cell. Therefore, detecting 0.01 pg of HPV DNA in the presence of 0.1 μg of human DNA indicates that this system can detect 0.1 copy per cell of HPV 18 DNA in 0.1 μg of the sample DNA. Similar sensitivity tests indicated that L1-PCR could detect 0.01 pg of DNA of HPV types 6, 11, 16, 31, 33 and 52 in addition to type 18, while 0.1 pg of HPV DNA was necessary for HPV types 42 and 58 (data not shown).

Identification of HPV types of the amplified fragments

We have reported that the HPV fragments amplified by E6-PCR could be typed according to RFLPs.[33] The same strategy for typing could be applied to L1-PCR, since the product of L1-PCR was also almost free from nonspecific bands (see below).

Table I shows a list of the restriction enzymes and the restriction fragment lengths useful for typing the HPV DNA fragments amplified by L1-PCR. Fig. 4 shows ex-

Table I. Restriction Enzymes Useful for Typing L1-PCR Product and the Lengths of Restriction Fragments[a]

Enzyme	HPV6	HPV11	HPV16	HPV18	HPV31	HPV33	HPV42	HPV52	HPV58
Rsa I	–[b]	204, 40	–	–	216, 40	141, 62 40, 13	–	190, 70	195, 65
*Dde*I	–	–	169, 84	216, 37	–	–	–	180, 80	170, 90
*Hae*III	207, 37	207, 37	200, 53	210, 43	122, 91 43	112, 91 43, 10	140, 60[c]	190, 50[c]	200, 40[c]
*Hin*fI	–	147, 97	–	141, 112	–	–	–	–	–
*Xba*I	143, 101	143, 101	–	146, 107	–	–	–	–	–
*Acc*I	–	–	193, 60	–	196, 60	–	–	–	–
*Pst*I	–	–	–	190, 63	–	–	–	–	–
*Kpn*I	–	–	–	–	–	218, 38	–	–	–

a) The lengths of restriction frements of the sequenced HPV types were obtained by MAPSORT as described in "Materials and Methods." The lengths of restriction fragments of the unsequenced HPV types were obtained by experiment.
b) There is no restriction site in the sequenced HPV types. For the unsequenced HPV types, the migration of the amplified fragment did not change after digestion.
c) Apparently there should be a smaller fragment which could not be seen on the gel.

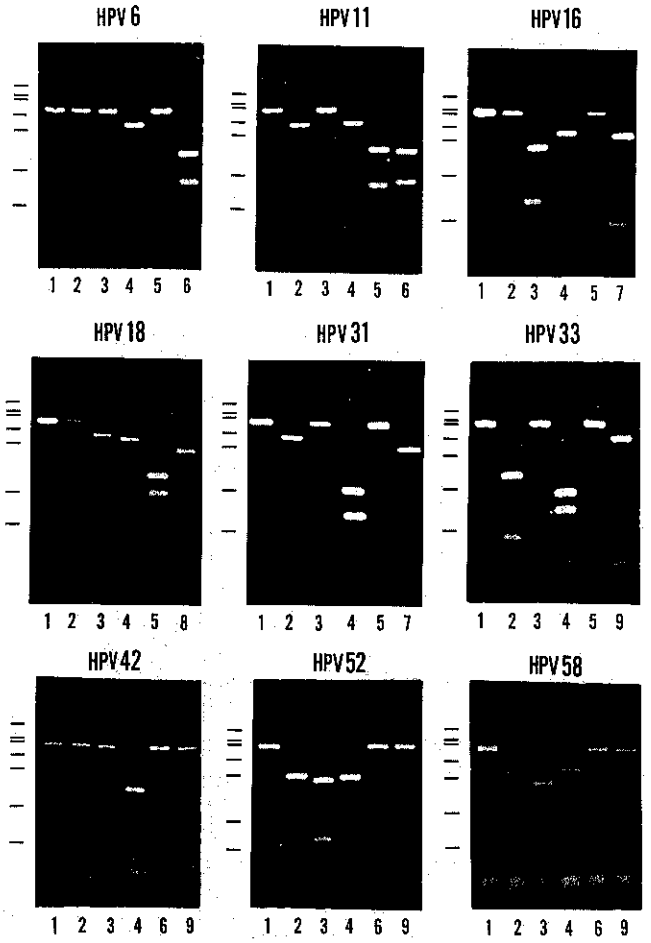

Fig. 4. Restriction mapping of the L1-PCR product. Nine cloned HPV types were subjected to L1-PCR. About 1/10th of the product was digested with the following restriction enzymes. 1, uncut; 2, *Rsa* I; 3, *Dde* I; 4, *Hae* III; 5, *Hinf* I; 6, *Xba* I; 7, *Acc* I; 8, *Pst* I; 9, *Kpn* I. After electrophoresis, the gels were stained and photographed. Bars on the left side of each panel are molecular markers (ϕx174 digested with *Hae* III): From the top; 310, 281, 271, 234, 194, 118 and 72 bp.

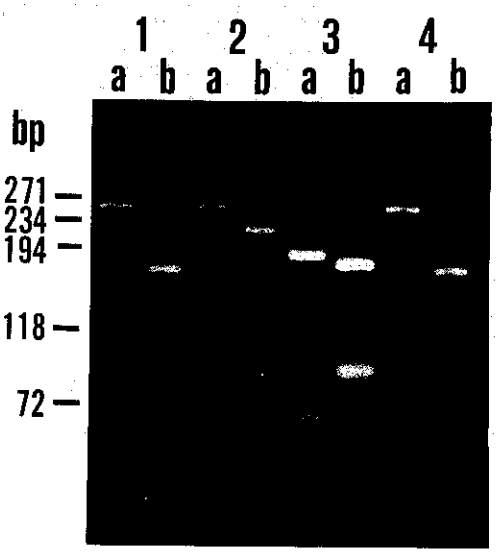

Fig. 5. Initial typing of the L1-PCR products from biopsied specimens (invasive carcinomas). Each sample was digested with *Rsa* I (a) or *Dde* I (b). The size markers are shown on the left.

amples of the actual electrophoretic pattern. There was no discrepancy between the actual digest and the expected fragment length in the sequenced HPVs (types 6, 11, 16, 18, 31 and 33). The fragment lengths of the unsequenced HPVs were deduced from the experiments. Because it is not practical to use all the enzymes listed in Table I for routine typing, we chose two enzymes, *Rsa* I and *Dde* I, as primary screening enzymes. The fragments of HPV types 11, 31 and 33 could be digested with *Rsa* I but not with *Dde* I (HPV 33 has a unique cleavage pattern with *Rsa* I). The fragments of HPV types 16 and 18 could be cleaved with *Dde* I but not with *Rsa* I. Moreover, *Dde* I cut HPV types 16 and 18 into different sizes. The fragments of HPV types 52 and 58 could be cleaved with both of the enzymes. Although the difference in the restriction pattern between HPV types 52 and 58 was small, the two types could be distinguished by running the samples side by side. The fragments of HPV types 6 and 42 could not be cleaved with either of the enzymes. The HPV types in the samples identified or grouped as above were further confirmed by using at least two additional restriction enzymes listed in Table I. Fig. 5 shows examples of the amplification of HPV DNA from the biopsied specimens. Samples 1, 2 and 4 could be cleaved with *Dde* I but not with *Rsa* I, while sample 3 could be cleaved with both enzymes. Based on the digestion patterns, samples 1 and 3 were judged as type 16, while sample 2 and sample 4 were judged as type 18 and type 52, respectively. Subsequent analysis with additional enzymes confirmed the accuracy of the above typing (data not shown).

Fig. 6 shows representative confirmatory analyses of samples typed as HPV 18 in the initial examination. The initial examination with *Rsa* I and *Dde* I could thus be applied successfully to clinical specimens.

Consensus primer-mediated PCR versus Southern blot analysis We have described PCR systems using the consensus primers for E6[33] and L1 regions (this report). Although the sensitivity and the specificity of each PCR system to detect the cloned HPV DNAs has been described, we wished to compare the efficacies of the two PCR systems with that of Southern blot analysis in clinical specimens.

We previously studied the presence of HPV 16 and HPV 18 in DNA samples from invasive cervical carcinomas by using Southern blot analysis under a stringent condition (Tm-18°C).[34] Most of the samples were still available and we had collected some more cases after publication. We studied those DNA samples with E6-PCR and L1-PCR. Table II shows the numbers of positive samples by each method. The same 31 samples out of 55 were positive and typed as HPV 16 by both PCR systems. All the 20 samples in which the presence of HPV 16 DNA was proven by Southern blot analysis were positive and typed as HPV 16 by both PCR systems. Therefore, both PCR systems are very specific and more sensitive than Southern blot analysis. The sensitivity to detect HPV 16 DNA is almost the same in E6-PCR and L1-PCR. In terms of HPV 18, both PCR systems detected and correctly typed all three samples that were HPV 18-positive by Southern blot. L1-PCR appeared slightly more sensitive to detect HPV 18 than E6-PCR.

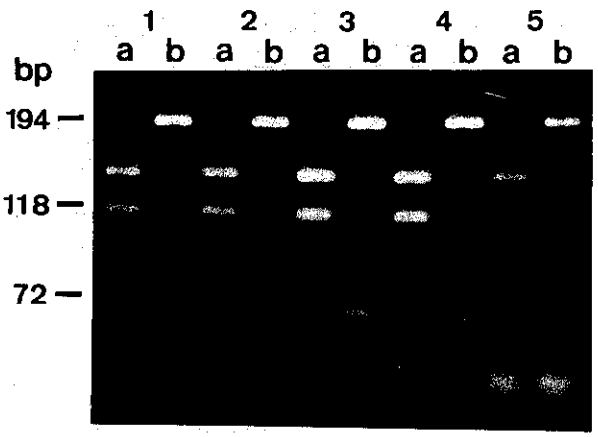

Fig. 6. Confirmatory typing of 5 samples from invasive carcinoma typed initially as HPV 18. Each sample was digested with *Hinf* I (a) or *Pst* I (b). The size markers are shown on the left.

Table II. Comparison of Methods for Detecting HPV in Invasive Cervical Carcinomas (n=55)

Methods	HPV 16-positive	HPV 18-positive
Southern blot	20	3
E6-PCR	31	6
L1-PCR	31	7

The prevalence of HPV in DNA samples from cervical neoplasias and normal controls E6- and L1-PCR were applied to DNA samples obtained from biopsied specimens of cervical neoplasia. DNA samples from exfoliated cells of asymptomatic women with normal cytology were also examined. HPV DNA was detected in 10 out of 10 (100%) papillary condylomas, in all 5 (100%) moderate dysplasias, in all 11 (100%) severe dysplasias, in 17 out of 20 (85.0%) carcinomas *in situ* and in 53 out of 55 (96.4%) invasive carcinomas of the cervix, while it was detected in 12 out of 102 (11.8%) normal controls (Table III).

The results of the two PCR systems coincided for HPV types 16 and 33. HPV 18 and HPV 58 were more frequently detected by L1- and E6-PCR, respectively, due to the difference in the sensitivities. Samples positive for HPV types 31 and 52 were detected only by L1-PCR. The type of HPV was identified in 87.0% (94/108) of HPV-positive samples. Fourteen samples grouped as X in Table III gave rise to a sharp, strong band of the expected size (12 in L1-PCR only and 2 in both E6-PCR and L1-PCR), but the restriction map did not conform to any of the above nine HPV types. We categorized those tentatively as type X.

DISCUSSION

We designed a pair of consensus primers in the L1 region that can amplify and determine at least nine types

Table III. Summary of Cervical Lesions Examined and Detection of HPV DNA

Lesions	6	11	16	18[a]	31	33	42	52	58[b]	X[c]	HPV+
Papillary condyloma	8	1	0	1	0	0	0	0	0	0	10/10
Moderate dysplasia	0	0	1	1	0	0	0	1	0	2	5/5
Severe dysplasia	0	0	3	2	2	1	0	3	0	0	11/11
Carcinoma *in situ*	0	0	9	1	0	3	0	1	1	2	17/20
Invasive carcinoma	0	0	31	7	2	1	0	3	4	5	53/55
Normal controls	0	0	2	0	0	0	0	5	0	5	12/102

a) Three of 12 samples were amplified only by L1-PCR.
b) Two of 5 samples were amplified only by E6-PCR.
c) Twelve of 14 samples were amplified only by L1-PCR and two were amplified by both E6-PCR and L1-PCR.

of genital HPV DNA. L1-PCR was superior in range of detection to E6-PCR, because the L1 region is more conserved than the E6 region among HPVs.[35] We checked that there is no interruption within the target sequences for both E6- and L1-PCR in cloned HPV DNA of types 6b, 11a, 16, 18, 31, 33 (from the literature), 52b and 58 (Dr. Yajima and Dr. Matsukura, respectively, personal communications), but there might be HPV types that were not amplified due to the interruption created by cloning. The sensitivity of L1-PCR was comparable to that of E6-PCR in detecting HPV types 16, 18 and 33 (0.1 copy/cell), but lower in detecting HPV 58 (1 copy/ cell versus 0.1 copy/cell). The sensitivity of these nonradioactive detection systems appears sufficient for clinical use.

Both E6-PCR and L1-PCR permitted typing by restriction analysis and staining. Both PCR systems had target sequences long enough to be analyzed by restriction mapping and produced almost no co-amplification of cellular DNA. Recently, Snijders *et al.*[36] described a PCR system with two pairs of consensus primers, one for HPV types la, 5 and 8 and the other for HPV types 6b, 11, 16, 18, 31 and 33. They used different target sequences from the present study and reported the detection of 11 HPV types (types la, 6, 8, 11, 13, 16, 18, 30, 31, 32 and 33). However, in their trials non-radioactive typing of HPV was impossible due to the co-amplified cellular DNA sequences.

We compared the detectability of HPV types 16 and 18 in cervical carcinomas between PCR systems and Southern blot analysis. Both PCR systems had higher sensitivity than Southern blot analysis. In our laboratory, the limit of sensitivity of Southern blot analysis for typing from *PstI* -cleavage patterns is at the level of 0.5 copy per diploid cell (data not shown), while the sensitivity of both PCRs exceeds the level of 0.1 copy per diploid cell. The HPV 16-positive rate increased from 36.4% to 56.4% and the HPV 18-positive rate from 5.5% to 12.7%.

We detected HPV DNA in all cervical condylomas (10/10) and in over 90% of cervical intraepithelial neoplasias (dysplasias plus carcinomas *in situ*) (33/36 [91.7%]) and invasive cervical carcinomas (53/55 [96.4%]). It should be emphasized that the very high positive rate was a consequence of not only the high sensitivity but also the wide range of detection. The high incidence of detection of HPV in cervical intraepithelial neoplasias and invasive carcinomas supports the hypothesis that HPV infection is a necessary condition for the development of cervical carcinomas, although it may not be sufficient.

The incidence of each HPV type varied with the severity of cervical lesions. Benign cervical condylomas had preferential association with HPV 6 and 11, although one case had HPV 18, which is only rarely detected in cervical condylomas.[37,38] The incidences of HPV 16 and 18 gradually increased with the progression of the lesion from moderate dysplasia to invasive carcinoma, suggesting that patients with HPV 16 or HPV 18 infection are at high risk. Multiple other types such as HPV 31 (2/55 [3.6%]), HPV 33 (1/55 [1.8%]), HPV 52 (3/55 [5.5%]), HPV 58 (4/55 [7.3%]) and HPV X (5/55 [9.1%]) were also associated with invasive cervical carcinoma.

The incidence of HPV infection in normal controls was 11.8%(12/102). The types of infecting HPV were as follows; HPV 16 (2/102 [2.0%]), HPV 52 (5/102 [5.0%]) and HPV X (5/102 [5.0%]). The HPV-positive incidence in normal controls was apparently lower than that in cervical neoplasias. The results were consistent with our previous data obtained by filter *in situ* hybridization to HPV 11, HPV 16 and HPV 18, which showed HPV 11, HPV 16 and HPV 18 infections in 0.9%, 1.8% and 0.6% out of 666 normal control cases.[38]

In the present study there was no specimen in which we detected two different types of HPV by Southern blot or PCR. Koutsky *et al.*[39] stated in a review that the reported frequency of dual infection is significantly (two- to 20-fold) different according to the method used and that the frequency of dual infection was usually less than 10% when Southern blot analysis was performed. Further analysis is necessary to determine the efficiency of our PCR systems to detect dual infection. We did not encounter any case whose typing conflicted between different methods (Southern blot versus PCR or E6-PCR versus L1-PCR). However, point mutants of known HPV types may yield amplified fragments with unexpected restriction maps.

We established consensus primer-mediated PCR systems in the E6 and L1 regions and confirmed the usefulness of the two PCR systems for detection of multiple genital HPV types in clinical samples. Comparison between the data obtained by E6-PCR and L1-PCR showed that HPV-positive specimens conserved the L1 region as well as E6 region of HPV DNA sequences. L1-PCR would become more convenient if the sensitivity for detection of HPV 58 can be increased. Very recently we obtained the unpublished sequence of HPV 58 from Dr. T. Matsukura, and we have obtained evidence that the changes from G to A of the 6th base and from T to C of the 9th base from the 3′ end of L1C2 (new L1C2; 5′-TACCCTAAATACCCTATATTG-3′) increase the sensitivity of L1-PCR to HPV 58 without decreasing the sensitivity to other HPV types. Further confirmation using clinical samples is in progress.

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