Detection and Typing of Genital High-Risk HPV DNAs in Cervical Scrapes Using the E6E7-Specific Consensus PCR

Yuko INOUE[1,2], Toshiharu YAMASHITA[1], Setsuko ISHIDA[1], Akira NISHIKAWA[2], Yukako FUJINAGA[1], Ryuichi KUDO[2] and Kei FUJINAGA[1]

[1]*Department of Molecular Biology, Cancer Research Institute and*
[2]*Department of Obstetrics and Gynecology, Sapporo Medical University School of Medicine, South1, West17, Chuo-ku, Sapporo 060, Japan*

ABSTRACT

Specific types of human papillomaviruses (HPVs) are closely associated with the development of genital carcinomas. We previousy reported a PCR method which amplifies the E6E7 sequence from 6 different high-risk genital HPVs (HPV16, 18, 31, 33, 52 and 58) (J Gen Virol 1991, 72: 1039-1044.).

To amplify broader types of genital high-risk HPVs, we have modified our consensus primers by extending 9 nucleotides at the 3' end of the sense primer and changing 5 nucleotides at the 5' end of the anitisense primer. Genotype diagnosis was carried out by *Ava*II plus *Rsa*I digestion. This modified PCR method enabled the detection of trace levels of at least 11 types of genital high-risk HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58), at subpicogram to subnanogram amounts of cloned DNA, amplified after the consensus PCR.

We applied this method to analyze 155 cervical scrapes from patients who had been diagnosed with premalignant or malignant cervical lesions. HPVs were detected in 63.0% of mild dysplasia (17/27), 100% of moderate dysplasia (all 12 cases), 91.7% of severe dysplasia (11/12), 95.8% of carcinoma *in situ* (23/24), and 80.0% of invasive cervical cancer (52/65). HPV16 was present predominantly (60.9%), followed by HPV58 (15.7%), HPV52 (13.9%), HPV31 (13.0%), HPV18 (6.1%), HPV35 (2.6%), HPV51 (2.6%), HPV56 (2.6%), HPV33 (1.7%) and HPV39 (1.7%). Five cases contained unclassified types (4.3%). The results indicate that this modified E6E7 consensus PCR method provides a quick and easy way to detect and diagnose genotypes of the high-risk genital HPVs from scraped cells.

Key words: Human papillomavirus, E6, E7, Polymerase chain reaction, Consensus primer, Cervical cancer, Cervical intraepithelial neoplasia

INTRODUCTION

At present, more than 70 types of human papillomaviruses (HPVs) have been characterized(1, 2). About 30 types of those HPVs were isolated from anogenital and oral mucosae(1, 2), and have been divided into high-risk and low-risk HPVs on the basis of their nucleotide sequences and oncogenic potentials (3-5). The high-risk types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 68 are associated with cervical intraepithelial neoplasia (CIN) and cancer of the uterine cervix(1-3, 6-9). HPV6, 11, 42, 43 and 44 are classed as the low-risk(3), since they are rarely associated with genital cancers and usually found in benign pathological changes such as condyloma acuminatum(6, 10-13). Thus, close relationships have been established between the pathogenesis of cervical proliferative lesions and HPV genotypes. In order to evaluate the prognosis of a cervical lesion, it is necessary to investigate for the presence or absence of the high-risk HPVs. For this purpose a nontedious, rapid and accurate method is required for the detection and type diagnosis of genital HPVs.

Recently, the polymerase chain reaction (PCR)(14, 15) had been broadly applied to the identification of HPVs, since it enables to detect low copies of HPVs in infected cells. PCR methods have been reported which amplify the viral sequences specifically from high-risk HPVs(16, 17). To detect a broader spectrum of genital HPVs, the consensus PCR methods have been developed which amplify E1(18), L1(19), L1 and E1(20), L1 and E6(21-23) open reading frames (ORFs). We previously reported a consensus PCR which amplifies E6E7 sequences from HPV6, 11, 16, 18, 31, 33, 52 and 58(24). The E6 and E7 ORFs are preferentially retained in cervical cancer cell lines which contain HPV16 or HPV18 sequences(25-28). E6 and E7 collaborate to immortalize human keratinocytes(29-32), via conceivably complex formations with p53 and Rb tumor suppressor proteins(33, 34). Thus, the E6E7 region is considered to be the best candidate for the target of consensus PCR.

Recently, at least 26 HPV types have been reported to be detectable in cervical malignant and premalignant lesions(1-5). In this study, we modified the E6E7 consensus PCR method to detect broader spectrum of high-risk HPVs. We applied this HPV detection system to cervical scrapes from the patients with CIN and cervical carcinoma. We show the detection rate of high-risk HPVs from cervical dysplasias, carcinomas *in situ* (CIS) and invasive carcinomas.

MATERIALS AND METHODS

HPV plasmid

Genital HPV DNAs of 23 genotypes, HPV6(10), 11(35), 16(36), 18(37), 30

(38), 31(39), 33(40), 34(41), 35, 39(12), 40(42), 42(12), 43(13), 45(7), 51 (43), 52(44), 53(45), 54(46), 55(46), 56(8), 58(47), 59 and 61 cloned in the plasmid were used to evaluate the specificity and sensitivity of the consensus PCR. The cloned HPV59 and 61 were kindly provided by Dr. T. Matuskura. HPV35 was isolated from a cervical cancer tissue and verified by sequence comparison(48) by Dr. K. Okazawa in our laboratory.

DNA preparation

Human placental DNA was contained in the PCR of the sensitivity test. We obtained human placenta soon after delivery and stored fragments in liquid nitrogen. Two cervical scrapes were taken from each subject using sterilized cotton tips, placed in 3 m*l* of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.5% sodium dodecyl sulfate (SDS), and stored at −20℃ until use.

DNA extraction was carried out according to the standard technique(49). Total cellular DNA from cervical scrapes and human placenta was purified by lysing cells in 0.5% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, containing 1 mg of pronase per m*l*, and incubated overnight at 37℃. DNA samples were extracted twice with phenol, and twice by chloroform-isoamyl alcohol (24 : 1, vol./vol.). Nucleic acid was precipitated with ethanol and dissolved in H_2O.

PCR

Primers were synthesized on a DNA synthesizer (MilliGen 7500) and purified on an Oligonucleotide Purification Cartridge (Applied Biosystem). The β-globin primers (sense primer, GH20: GAAGAGCCAAGGACAGGTAC in noncording region of exon 1 and antisense primer, KM38: TGGTCTCCTTAAACCTGT CTTG in intron 1) were obtained from Takara Shuzo Co., Ltd.. The β-globin primers amplify a 325 base pair (bp) band from human genomic DNA.

PCR was done by the method previously described(14, 15) with a slight modification. One μg of template DNA was incubated for 10 min. at 94℃ and chilled quickly for denaturation. The reaction mixture of 100 μl contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 200 mM of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.01% (vol./vol.) gelatin, 2.5 units of Taq polymerase (Takara Shuzo Co., Ltd.) and 100 pmol of each primers. The mixture was overlaid with several drops of mineral oil and subjected to 30 cycles of amplification for β-globin PCR and 50 cycles for the consensus PCR using a DNA Thermal Cycler (Perkin-Elmer Cetus). Each cycle included denaturation at 94℃ for 1 min., annealing at 55℃ for 2 min., and elongation at 72℃ for 2 min. A reaction without template DNA was included in each amplification(50, 51). PCR product (10 μl) was electrophoresed on a composite gel consisting of 3%

NuSieve Agarose (FMC Bioproducts) and 1% Seakem Agarose (FMC Bioproducts), and photographed under U. V. light after staining with ethdium bromide.

To assess the sensitivity of the consensus PCR method, each of the different concentrations of HPV plasmid was mixed with 1 μg of human placental DNA and subjected to PCR.

Restriction enzyme analysis

PCR product (90 μl) was purified with phenol-chloroform-isoamyl alcohol (25 : 24 : 1) extraction followed by ethanol precipitation, and suspended in 34 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). A quarter of the product was mixed with 4 units each of *Ava*II and *Rsa*I in one reaction tube using a buffer containing 33 mM Tris-HCl, pH 7.9, 10 mM magnesium-acetate, 0.5 mM dithiothreitol, 66 mM potassium-acetate and 0.01% bovine serum albumin in 10 μl reaction mixture. The mixture was electrophoresed after incubation for 1.5 hours at 37℃. Amplified products which were not cleaved *Ava*II nor *Rsa*I were then digested with *Ava*I, *Bgl*II and *Acc*I, independently. Digested products were analyzed on composite agarose gels described above. All restriction enzymes were obtained from Takara Shuzo Co. Ltd..

Preparation of clinical samples

Cervical scrapes were obtained from 155 women who were treated at Sapporo Medical University Hospital, Sapporo, Japan, and other associated hospitals from April 1991 through November 1994. This selected group had cytological abnormalities. Forty-four cases had previously been histologically diagnosed by punch biopsy and were being followed up at an outpatient clinic. One-hundred and eleven cases were surgically treated for cervical cancer or severe dysplasia. Pathological examination was performed by several pathologists in each case. The study group is consisted of 31 mild dysplasias, 13 moderate dysplasias, 13 severe dysplasias, 28 CIS and 70 invasive carcinomas (stage Ia-IIIb) of the uterine cervix. Two scrapes each were collected with cotton tips by several consulting gynecologists and used for the HPV tests. The first smear, with a spatula, was made for the routine cytological examination.

RESULTS

design of consensus primers

We have previously reported two pairs of E6E7 consensus primer which were able to amplify 6 types of high-risk genital HPV (HPV16, 18, 31, 33, 52 and 58) and 2 types of low-risk HPV (HPV6 and 11)(24). The pU-1M and pU-2R

primers are designed in the E6E7 regions conserved among the genital high-risk HPVs (Fig. 1). This primer also detected HPV35, 39, 45, 51 and 56 DNA with lower amplification efficiencies than other high-risk types (data not shown). We modified the primer sequence to reduce mismatches between the primer and HPV DNA sequences. The pU-1M and pU-2R and their modified versions were aligned with the corresponding sequences of 11 genotypes (Fig. 2). The modified sense primer, pU-1M-L, contains 9 extended bases at the 3' end of pU-1M. Five nucleotides of the 3' end of the original pU-1M had 2 or 3 mismatches to the sequences of HPV18, 39, 45, 51 and 56, while that of pU-1M-L had none or only one to these HPVs (Fig. 2, A). We also changed 5 nucleotides (GAGCT) at the 5' region of pU-2R to TCTGA (pU-2R-N), which is identical to the sequence of HPV18, 39, 45, 51 and 56 (Fig. 2, B).

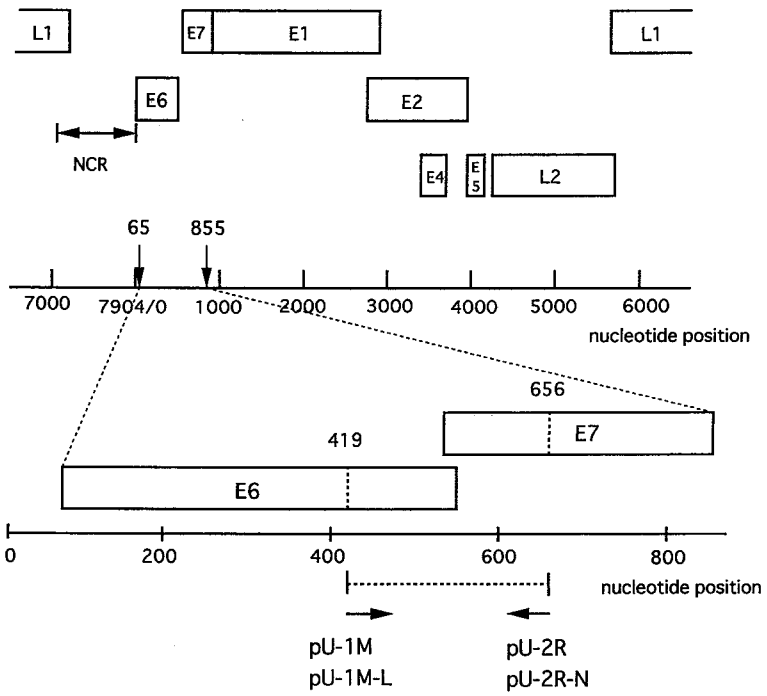

Fig. 1 Locations of the consensus PCR primers on the HPV16 genome. Open boxes indicate ORFs. NCR is the non-coding region between the L1 and E6 ORFs. Diagram of structure of HPV16 ORFs are shown based on the published DNA sequence(52). The pU-1M, pU-1M-L, pU-2R and pU-2R-N primers were designed in the E6E7 region.

A)

Sense primer	5'	3'	5' Nucleotide
pU-1M	TGTCAAAAACCGTTGTGTCC		
pU-1M-L	TGTCAAAAACCGTTGTGTCCAGAAGAAAA		
HPV 16	--------G--AC-------T---C----		419
HPV 18	--C--G---------AA----G-A-----		426
HPV 31	-------G--------------------		423
HPV 33	-------G---T--------TC-------		424
HPV 35	----------C---------TT-----		425
HPV 39	----TG------C---------C------		432
HPV 45	--C--G-----A---AAC----C------		419
HPV 51	-------G---AC-TG-G--T--------		412
HPV 52	-------CG--A--A-----T--------		418
HPV 56	-------GT-----AAC---G--G-----		425
HPV 58	-------G---A---------C-------		425

B)

Anti-sense primer		
pU-2R	GAGCTGTCGCTTAATTGCTC	
HPV 16	--------AT----------	656
HPV 31	---------GG---------	654
HPV 33	--------A----------	667
HPV 35	--------A-AC--------	652
HPV 52	--------A----------	648
HPV 58	--------A-A---------	668
pU-2R-N	TCTGAGTCGCTTAATTGCTC	
HPV 18	--------------------	693
HPV 39	-----C--T-C---------	712
HPV 45	-----C--T-C---------	687
HPV 51	------CT-TCA--------	654
HPV 56	------CT-TCC--------	665

Fig. 2 Alignments of 11 genital HPV sequences corresponding to consensus primers. Primer sequences are shown with HPV sequences aligned below. Dashes represent nucleotides that match the primer sequences, and mismatches are shown at their respective positions. The 5' nucleotide position on the viral genome is given at right. (A) Alignments of sense primer pU-1M, pU-1M-L and corresponding sequences of 11 genital HPV DNA. (B) Alignments of antisense primer pU-2R and corresponding sequences of HPV16, 31, 33, 35, 52 and 58, and alignments of antisense primer pU-2R-N and corresponding sequences of HPV18, 39, 45, 51 and 56.

Specificity and sensitivity of consensus PCR

To learn the genotypes detected by and the sensitivity of the modified E6E7 consensus PCR, DNA of each cloned HPV was serially diluted in TE containing 1 μg of human placental DNA and subjected to the consensus PCR. As shown in Fig. 3, it produced clear bands without a human genomic nonspecific background. Out of 23 types of genital HPVs, 11 (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58) were amplified, and the predicted 231 to 271 bp bands we obtained varied in accordance with HPV types (Fig. 3). The detectable level was 4.06×10^{-2} pg (approximately 10^{-2} copies per cell) for HPV16 to 4.06×10^{2} pg (approximately 10^{2} copies per cell) for HPV51 (Table 1). Fig. 4 shows the result of the PCR to define the sensitivity using the HPV16 plasmid (pHPV16) as a substrate. The 231-bp band specific for HPV16 was clearly observed in the PCR product from the 4.06×10^{-2} pg pHPV16DNA (Fig. 4, lane 6). None of the cloned DNA of 12 genotypes (HPV6, 11, 30, 34, 40, 42, 43, 53, 54, 55, 59 and 61) was amplified from the 4.06×10^{4} pg amount by the pU-1M, pU-1M-L/pU-2R, pU-2R-N primers (data not shown).

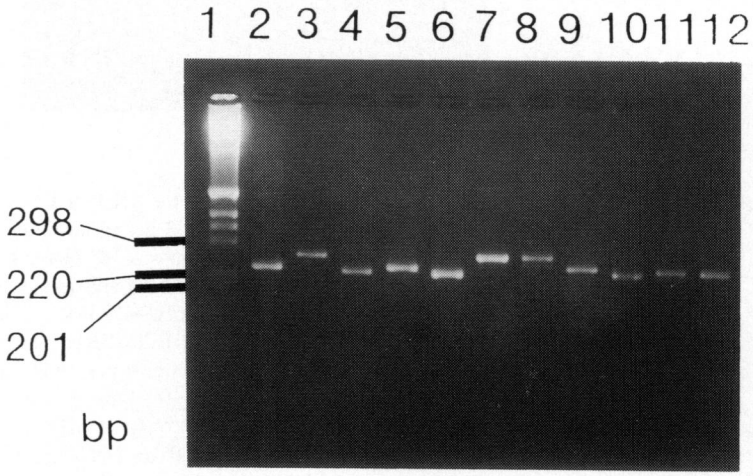

Fig. 3 Amplification of HPV DNA from HPV plasmids by the modified E6E7 consensus PCR. Each type of HPV was amplified from 4.06×10^{3} pg of pHPV DNA containing 1 μg of human placental DNA. Fifty cycles of PCR were performed as described in "Materials and Methods. Lane 1, DNA molecular weight marker, 1 kb Ladder (GIBCO BRL); lane 2, HPV16 DNA; lane 3, HPV18 DNA; lane 4, HPV31 DNA; lane 5, HPV33 DNA; lane 6, HPV35 DNA; lane 7, HPV39 DNA; lane 8, HPV45 DNA; lane 9, HPV51 DNA; lane 10, HPV52 DNA; lane 11, HPV56 DNA; lane 12, HPV58 DNA. The DNA bands were produced clearly without a human genomic nonspecific background.

Table 1 *Sensitivity of the consensus PCR in amplification of each type HPV*

HPV type	16	18	31	33	35	39	45	51	52	56	58
4.0.×	10^{-2}	10	1	10^{-1}	1	10	1	10^{2}	1	10	1

(pg pHPV DNA/1 μg cellular DNA/100 μl reaction mixture)

1 copy/cell=4.06 pg pHPV DNA/1 μg cellular DNA

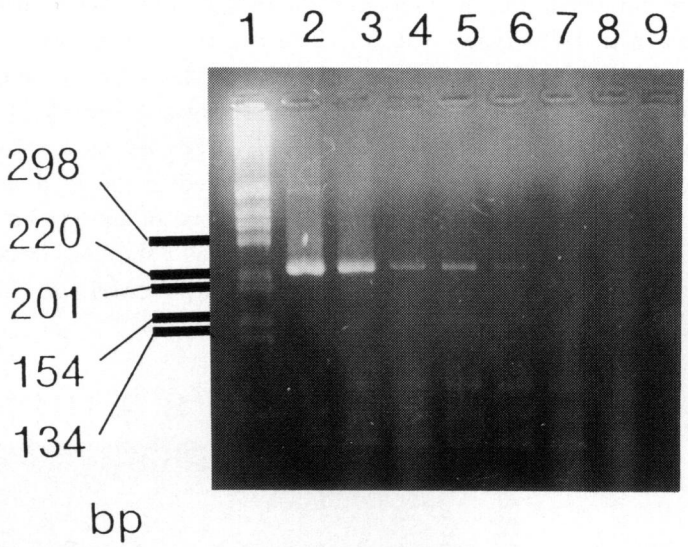

Fig. 4 The sensitivity of modified E6E7 consensus PCR for HPV16 DNA. For PCR, cloned pHPV16 DNA was serially diluted in TE and an aliquot was mixed with 1 μg of human placental DNA. The size of DNA band is agreeable with the estimated size. The HPV16 E6E7 band is clearly observed after the amplification from 4.06×10^{-2} pg pHPV16 DNA. Lane 1, DNA molecular weight marker, 1 kb Ladder (GIBCO BRL); lane 2, 4.06×10^{2} pg of pHPV16 DNA (approximately 10^{2} copies per cell); lane 3, 4.06×10^{1}pg of pHPV16 DNA (10 copies per cell); lane 4, 4.06 pg of pHPV16 DNA (1 copy per cell); lane 5, 4.06×10^{-1} pg of pHPV16 DNA (10^{-1} copies per cell); lane 6, 4.06×10^{-2} pg of pHPV16 DNA (10^{-2} copies per cell); lane 7, 4.06×10^{-3} pg of pHPV DNA16 (10^{-3} copies per cell); lane 8, negative control 1 (no pHPV DNA, placental DNA only); lane 9, negative control 2 (no DNA).

Restriction enzyme analysis of PCR product

Table 2 shows the restriction enzymes used and sized of restriction fragments of PCR products predicted from published sequences(8, 43, 44, 47, 52-57). We previously reported that PCR products amplified by the pU-1M/pU-2R primer pair were successfully differentiated according to the size and number of restriction fragments(24). In our original method, the amplified product was indepen-

dently cleaved by *Ava*II and *Rsa*I. We simplified this process by digesting the PCR product with the *Ava*II plus *Rsa*I in one reaction tube. The PCR products from HPV16, 18, 31, 33, 39, 45, 51 and 56 have either or both of *Ava*II and *Rsa*I recognition sites; thus, we can differentiate these 8 types of HPVs in one test run (Table 2 and Fig. 5, A). PCR products of HPV35, 52 and 58 were cleaved neither by *Ava*II nor *Rsa*I, but have single *Ava*I, *Bgl*II and *Acc*I sites (Table 2 and Fig. 5, B). Sites for these 3 restriction enzymes are not present in the other 8 HPVs (Table 2).

HPV detection in cervical scrapes

A modified E6E7 consensus PCR using mixed primers (pU-1M, pU-1M-L/pU-2R, pU-2R-N) was applied to cervical scrapes. Out of 155 samples, DNA band for β-globin PCR was not produced from 15 samples (9.6%). We analyzed the remaining 140 samples by consensus PCR. Ten types of genital high-risk HPV were found in 115 cervical scrapes (82.1%) including 27 of multiple infections (19.3%) (Table 3): 2 types in 25 cases and 3 types in 2 cases. A part of the result is shown in Fig. 6. DNA bands of about 230-250 bp were amplified from 4 materials (Fig. 6, A; lane 2-5), while no band was produced from a case of mild dysplasia (Fig. 6, A; lane 6). The sample from invasive carcinoma (IC) case No.1 (Fig. 6, A; lane 2) was revealed to contain HPV16 from the *Ava*II plus *Rsa*I-cleavage patterns (Fig. 6, B, lane 2) and IC case No.2 (Fig. 6, A; lane

Table 2 *Restriction fragment sizes of consensus PCR products*[a]

HKV type / Dnzyme	16	18	31	33	35	39	45	51	52	56	58
Total Length (bp)	238	268	233	244	232	271	271	246	231	250	244
Ava II	157	172	119	136	—[b]	104	100	110	—	86	—
plus	81	96	114	108		100	93	85		84	
Rsa I						51	78	51		80	
						16					
Ava I	—	—	—	—	177	—	—	—	—	—	—
					55						
Bgl II	—	—	—	—	—	—	—	—	176		
									55		
Acc I	—	—	—	—	—	—	—	—	—	—	126
											118

a) The length of the restriction fragments was estimated from the published sequence.
b) Minus (−) means that there is no restriction site in the PCR product.

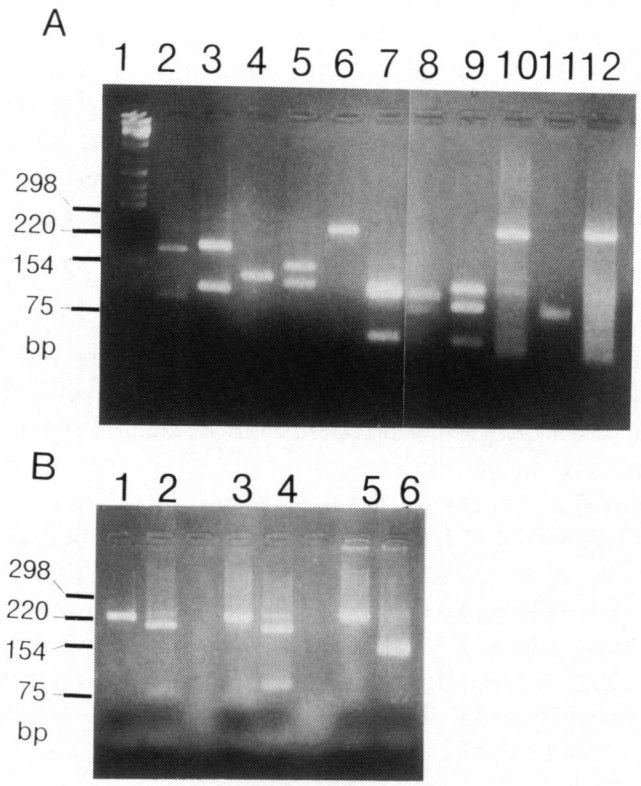

Fig. 5 Restriction enzyme cleavage of PCR products. (A) *Ava*II plus *Rsa*I digestion; lane 1, DNA molecular weight marker, 1 kb Ladder (GIBCO BRL); lane 2, HPV16 DNA; lane 3, HPV18 DNA; lane 4, HPV31 DNA; lane 5, HPV33 DNA; lane 6, HPV35 DNA (not cleaved); lane 7, HPV39 DNA; lane 8 HPV45 DNA; lane 9, HPV51 DNA; lane 10, HPV52 DNA (not cleaved); lane 11 HPV56 DNA; lane 12, HPV58 DNA (not cleaved). (B) *Ava*I, *Bgl*II and *Acc*I digestion; lane 1, intact HPV35 DNA; lane 2, HPV35 DNA, *Ava*I degestion; lane 3, intact HPV52 DNA; lane 4, HPV52 DNA, *Bgl*II digestion; lane 5, intact HPV58 DNA; lane 6, HPV58 DNA, *Acc*I digestion.

3) contained HPV16 and 58 from the *Ava*II plus *Rsa*I-and *Acc*I-cleavage patterns (Fig. 6, C, lane 2 and 5). A case of moderate dysplasia (Fig. 6, A; lane 4) contained HPV35 from the *Ava*I-cleavage pattern (Fig. 6, D, lane 3) and mild dysplasia case No.1 (Fig. 6, A, lane 5) contained HPV58 from the *Acc*I-cleavage pattern (Fig. 6, E, lane 5). The HPV positive rates are summarized in Table 3. The mild, moderate and severe dysplasias contained high-risk HPV in 63.0% (17/27), 100% (all 12 cases) and 91.7% (11/12) of the competent cases, respec-

Table 3 *The prenence of HPV and pathological diagnosis*

Pathological Diagnosis / presence or absense of HPV	Mild Dysplasia	Moderate Dysplasia	Severe Dyspasia	Carcinoma *in situ*	Invasive Carcinoma	Total
HPV DNA detected	17 (63.0%)	12 (100%)	11 (91.7%)	23 (95.8%)	52 (80.0%)	115 (82.1%)
[Multiple infection	6 (22.2%)	4 (33.3%)	1 (8.3%)	5 (20.8%)	11 (16.9%)	27 (19.3%)
HPV not detected	10 (37.0%)	0	1 (8.3%)	1 (4.2%)	13 (20.0%)	25 (17.9%)
Total	27	12	12	24	65	140

Multiple infection=2 or 3 genotype HPVs were detected

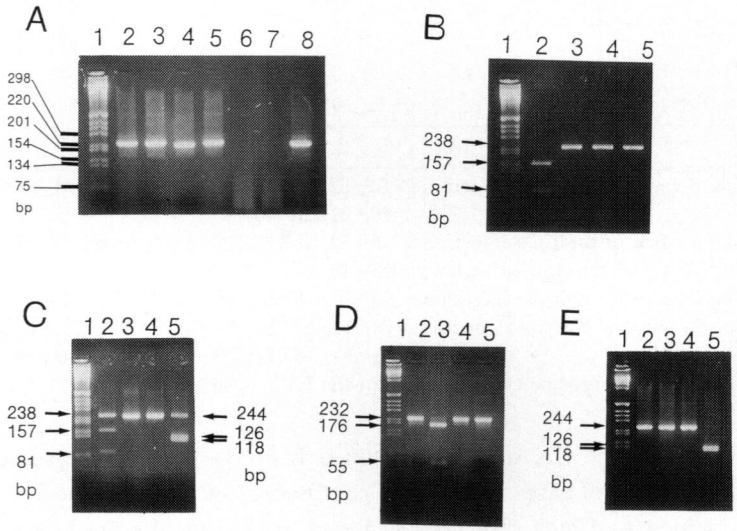

Fig. 6 Amplification and typing of HPV DNA band from clinical samples. Cervical scrapes from patients with cervical lesions were analyzed by the modified E6E7 consensus PCR as described in Materials and Methods. (A) Lane 1, DNA molecular weight marker, 1 kb Ladder (GIBCO BRL); lane 2, PCR band amplified from cervical scrapes of a patient with invasive carcinoma (IC), case No. 1; lane 3, IC No. 2; lane 4, moderate dysplasia; lane 5, mild dysplasia case No. 1; lane 6, mild dysplasia case No. 2; lane 7, negative control, 1 μg of human placental DNA; lane 8, positive control about 1 ng pHPV16 DNA plus 1 μg human placental DNA were subjected to the modified E6E7 consensus PCR. (B, C, D and E) Genotype analysis of HPV positive samples. Lane 1, DNA molecular weight marker, 1 kb Ladder (GIBCO BRL); lane 2, *Ava*II+*Rsa*I digestion; lane 3, *Ava*I degestion; lane 4, *Bgl*II digestion; lane 5, *Acc*I digestion. (B) IC case No. 1, HPV16. (C) IC case No. 2, HPV16+58. (D) moderate dysplasia, HPV35. (E) mild dysplasia case No. 1, HPV58.

Table 4 *Specific disease association of 11 HPVs types at varying grades in the clinicopatholgical spectrum*

pathology / HPV type	Mild Dysplasia	Moderate Dysplasia	Severe Dysplasia	Carcinoma *in situ*	Invasive Carcinoma	Total (%)[f]
16	8	6	5	16	35	70 (60.9)
18	1	1	2	0	3	7 (6.1)
31	4	0	1	4	6	15 (13.0)
33	0	0	0	0	2	2 (1.7)
35	1	1	0	0	1	3 (2.6)
39	1	0	0	0	1	2 (1.7)
45	0	0	0	0	0	0 (0)
51	2	0	0	0	1	3 (2.6)
52	4	3	1	2	6	16 (13.9)
56	1	1	0	1	0	3 (2.6)
58	3	2	3	5	5	18 (15.7)
Unclassified	0	2	0	0	3	5 (4.3)
Total	25[a]	16[b]	12[c]	28[d]	63[e]	144/115

a) including 6 cases of multiple infection: 16+52+51, 39+58, 56+31, 16+58, 16+51, 16+31+52
b) including 4 cases of multiple infection: 16+52, 18+52, 16+unclassified, 56+52
c) including 1 case of multiple infection: 16+18
d) including 5 cases of multiple infection: 16+52, 16+31, 16+31, 16+52, 31+56
e) including 11 cases of multiple infection: 18+51, 16+52, 16+31, 16+52, 16+31, 16+52, 16+31, 16+58+52+33, 18+52, 16+58
f) the incidence of the specific types of HPV in the HPV positive 115 case

tively. HPV specific DNAs were amplified from 23 out of 24 cases of CIS (95.8%) and 52 out of 65 cases of invasive carcinoma (80.0%) (Table 3).

The incidence of high-risk HPVs in each pathological state is summarized in Table 4. HPV16 was found predominantly (60.9%), followed by HPV58 (15.7%), HPV52 (13.9%), HPV31 (13.0%), HPV18 (6.1%), HPV35 (2.6%), HPV51 (2.6%), HPV56 (2.6%), HPV33 (1.7%) and HPV39 (1.7%). HPV45 was not detected in our study group. In 5 cases (4.3%), a single discrete band of 230-250 bp was amplified by the modified E6E7 consensus PCR (data not shown). However, we failed to determine their genotypes since they were not cleaved by *Ava*II, *Rsa*I, *Bgl*II, *Acc*I or *Ava*I.

DISCUSSION

By dint of much effort, it has been revealed that specific types of genital HPVs closely associate with CIN and cervical cancer(1-5). The presence of high-risk HPVs in cervical lesions is believed to be the most significant risk fac-

tor for them to progress into carcinoma(58). Thus, an easy and accurate method which specifically detects high-risk HPVs will be quite useful for evaluation of cervecal proliferative lesions.

Southern blot analysis has frequently been used to search for HPVs from cervical tissue; however, this method is not always adequate to examine clinical samples such as scraped cells, because it requires appreciable amounts (5-10 μg) of cellular DNA. On the other hand, PCR is capable of detecting subpicogram to subnanogram amounts of viral DNA from 1 μg of infected cell DNA.

PCR using consensus primers for E6 and E7(24), E1(18), L1(19), L1 and E1 (20), L1 and E6(21-23) can detect multiple types of HPV. The amplification of E6E7 has implications different from E1- or L1-consensus PCR. The E6 and E7 ORFs are preferentially retained in cervical carcinomas(25-28), probably because of their roles as the viral oncogenes(33, 34). Our original E6E7 consensus primers amplified the high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 45, 52 and 58) effectively, and HPV39, 51 and 56 with lower efficiency. Modification of the 3' end by extending 9 nucleotides in the sense primer and of the 5' end by changing 5 nucleotides in the antisense primer has made it possible to amplify 11 genital HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58). Similar to pU-1M/pU-2R set(24), mixed primers (pU-1M, pU-1M-L/pU-2R, pU-2R-N) did not amplify the low-risk HPVs (HPV6, 11, 42 and 43) from 4.06×10^4 pg pHPV DNA (approximately, 10^4 copies per cell) (data not shown). Other consensus primers designed for E6, E1 and L1 ORFs have amplified not only high-risk but also low-risk HPVs simultaneously(30-35); therefore, our consensus PCR appears to be the most powerful method presently available to amplify and detect high-risk HPVs separately and specifically.

Out of 155 cervical scrapes, sufficient cellular DNAs for PCR were obtained from 140 cases (90.3%). β-globin was not amplified from cellular DNA in 15 cases, including 5 of invasive carcinoma. The reason the cellular DNA was not sufficient for the PCR source was probably that the tissues had been contaminated by necrosis, since several of those patients had received radiation therapy and/or chemotherapy before the operation. Cases like these must be examined by other approaches such as DNA *in situ* hybridization.

The HPV detection rates (63.0%, 100%, 91.7% and 95.8% in mild, moderate, severe dysplasia and CIS, respectively) are comparable to the previous reports (3, 22, 59), while the incidence in invasive carcinomas (80.0%) seems to be relatively low (Table 3).

The sensitivity of the modified E6E7 PCR was comparable to that of other consensus PCR(18, 19, 22) in detecting HPV16, 31, 33, 35, 45, 52 and 58 (10^{-2} to 1 copy per cell), but lower in detecting HPV18, 39 and 56 (10 copies per cell)

and HPV51 (10^2 copies per cell), as shown in Table 1. The sensitivity of this nonradioactive method appears sufficient for clinical use, though for detecting HPV51 a more sensitive method may be required. The effectivity of our modified consensus PCR could be improved with hybridization, which detects 10^{-5} to 10^{-6} HPV copies per cell(16), or double (two-step) consensus PCR, which detects 10^{-2} HPV copies per cell(60).

We detected 10 types of genital high-risk HPVs in the cervical scrapes. Similar to the previous reports(3, 22, 59), HPV16 was most frequently detected in our study group (70/115, 60.9%), followed by HPV31, 52 and 58 (13-15%), HPV18, 33, 35, 39, 51 and 56 (1-5%) (Table 4). HPV45 was not detected in our study group, probably because of its low prevalence (about 1%) (3). In conclusion, our modified E6E7 consensus PCR has a broad detection spectrum for high-risk HPVs with adequate sensitivity.

We could detect 10 types of high-risk HPV genotypes in 115 samples including 5 unclassified cases (4.3%). Point mutations in the restriction site for the PCR product can mislead typing. We have previously reported that HPV16 E7 sequences in human cervical carcinoma did not contain point mutations in the restriction sites within our E6E7-consensus-PCR target region(61, 62). It is possible that the unclassified cases contain uncharacterized, novel HPV type (s) which have not yet been reported.

From the result that 2 or 3 genotypes of HPV were detected at the same time in 27 samples (19.2%), the incidence of mixed or multiple infections appeared to be more frequent than estimated in previous studies by Southern blot analysis (2.4 to 14%)(63-67). However, the frequency of mixed infection estimated by PCR was 8 to 48%(67-69). Further study is required to conclude the incidence of mixed infection.

HPV16, 18, 45 and 56 have been discriminated from other high-risk HPVs, since these HPVs were more frequent in cervical cancer than in high grade CIN (4). The present results confirm that hypothesis, since HPV16 was found in CIS and invasive carcinoma (55-57%) more frequently than in cervical dysplasia (32-42%). It is difficult to discuss other high-risk HPVs because of the small number of samples. We would like to continue measurements of high-risk HPVs in CIN and cervical cancer.

We previously reported relatively low prevalence of HPV16, 18 and 33 DNA in the normal cervix using the type-specific PCR(70). Further study is in progress to detect and type the high-risk HPVs in cervical scrapes from women with cytologically normal or low grade of CIN such as condyloma using the modified E6E7 consensus PCR.

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REFERENCES

1. de VILLIERS E-M. Heterogeneity of the human papillomavirus group. J Virol 1989, 63: 4898-4903.
2. de VILLIERS E-M. Human pathogenic papillomavirus types: an update. In: zur Hausen H ed. Human pathogenic papillomaviruses. Curr Top Microbiol Immunol 1994, 186: 1-12.
3. LORINCZ AT, REID R, JENSON AB, GREENBERG MD, LANCASTER W, KURMAN RJ. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol 1992, 79: 328-337.
4. RANST MV, KAPLAN JB, BURK RD. Phylogenetic classification of human papillomaviruses: correlation with clinical manifestations. J Gen Virol 1992, 73: 2653-2660.
5. CHAN SY, BERNARD HU, ONG CK, CHAN SP, HOFMANN B, DELIUS H. Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. J Virol 1992, 66: 5714-5725.
6. LORINCZ AT, TEMPLE GF, KURMAN RJ, JENSON AB, LANCASTER WD. Oncogenic association of specific human papillomavirus types with cervical neoplasia. J Natl Cancer Inst 1987, 79; 671-677.
7. NAGHASHFAR ZS, ROSENSHIEN NB, LORINCZ AT, BUSCEMA J, SHAH KV. Characterization of human papillomavirus type 45, a new type 18-related virus of the genital tract. J Gen Virol 1987, 68: 3073-3079.
8. LORINCZ AT, QUINN AP, GOLDSBOROUGH MD, McALLISTER P, TEMPLE GF. Human papillomavirus type 56: a new virus detected in cervical cancers. J Gen Virol, 1989, 70: 3099-3104.
9. FUKUSHIMA M, YAMAKAWA Y, SHIMANO S, HASHIMOTO M, SAWADA Y, FUJINAGA K. The physical state of human papillomavirus 16 DNA in cervical carcinoma and cervical intraepithelial neoplasia. Cancer 1990, 66: 2155-2161.
10. de VILLIERS E-M, GISSMANN L, zur HAUSEN H. Molecular cloning of viral DNA from human genital warts. J Virol 1981, 40: 932-935.
11. GISSMANN L, WOLNIK L, IKENBERG H, KOLDOVSKY U, SCHNURCH HG, zur HAUSEN H. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acal Sci USA 1983, 80: 560-563.
12. BAUDENON S, KREMSDORF D, OBALEK S, JABLONSKA S, PEHAU-ARNAUDET G, CROISSANT O, ORTH G. Plurality of genital human papillomaviruses: characterization of two new types with distinct biological properties. Virology 1987, 161: 374-384.

13. LORINCZ AT, QUINN AP, GOLDSBOROUGH MD, SCHMIDT BJ, TEMPLE GF. Cloning and partial DNA sequencing of two new human papillomavirus types associated with condylomas and low-grade cervical neoplasia. J Virol 1989, 63: 2829-2834.
14. SAIKI RK, SCHARF S, FALOONA F, MULLIS KB, HORN GT, ERLICH HA, ARNHEIM N. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985, 230: 1350-1354.
15. SAIKI RK, GELFAND DH, STOFFEL S, SCHARF SJ, HIGUCHI R, HORN GT, MULLIS KB, ERLICH HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988, 239: 487-491.
16. SHIMADA M, FUKUSHIMA M, MUKAI H, KATO I, NISHIKAWA A, FUJINAGA K. 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. Jpn J Cancer Res 1990, 81: 1-5.
17. van den BRULE AJC, CLAAS EG, du MAINE M, MELCHERS WJ, HELMERHORST T, QUINT WG, LINDEMAN J, MEIJER CJLM, WALBOOMERS JMM. Use of anticontamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. J Med Virol 1989, 29: 20-27.
18. GREGOIRE L, ARELLA M, CAMPIONE-PICCARDO J, LANCASTER WD. Amplification of human papillomavirus DNA sequences by using conserved primers. J Clin Microbiol 1989, 27: 2660-2665.
19. SNIJDERS PJF, van den BRULE AJC, SCHRIJNEMAKERS HFJ, SNOW G, MEIJER CJLM, WALBOOMERS JMM. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol 1990, 71: 173-181.
20. van den BRULE AJC, SNIJDERS PJF, GORDIJN RLJ, BLEKER OP, MEIJER CJLM, WALBOOMERS JMM. General primer-mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. Int J Cancer 1990, 45: 644-649.
21. MANOS MM, TING Y, WRIGHT DK, LEWIS AJ, BROKER TR, WOLINSKY SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cell 1989, 7: 209-214.
22. YOSHIKAWA H, KAWANA T, KITAGAWA K, MIZUNO M, YOSHIKURA H, IWAMOTO A. Amplification and typing of multiple cervical cancer-associated human papillomavirus DNAs using a single pair of primers. Int J Cancer 1990, 45: 990-992.
23. RESNICK RM, CORNELISSEN MTE, WRIGHT DK, EICHINGER GH, FOX HS, SCHEGGET J, MANOS MM. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. J Natl Cancer Inst 1990, 82: 1477-1484.
24. FUJINAGA Y, SHIMADA M, OKAZAWA K, FUKUSHIMA M, KATO I, FUJINAGA K. Simultaneous detection and typing of genital human papillomaverus DNA using the polymerase chain reaction. J Gen Virol 1991, 72: 1039-1044.
25. SCHWARZ E, FREESE UK, GISSMANN L, MAYER W, ROGGENBUCK B, STREMLAU A, zur HAUSEN H. Structure and transcription of human papillomavirus sequences in cervical

carcinoma cells. Nature 1985, 314: 111-114.
26. CHOO KB, PAN CC, HAN SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 1987, 161: 259-261.
27. SPENCE RP, MURRAY A, BANKS L, KELLAND LR, CRAWFORD L. Analysis of human papillomavirus sequences in cell lines recently derived from cervical cancers. Cancer Res 1988, 48: 324-328.
28. MATSUKURA T, KANDA T, FURUNO A, YOSHIKAWA H, KAWANA T, YOSHIIKE K. Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J Virol 1986, 58: 979-982.
29. BARBOSA MS, SCHLEGEL R. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage *in vitro* transformation of human keratinocytes. Oncogene 1989, 4: 1529-1532.
30. HAWLEY-NELSON P, VOUSDEN KH, HUBBERT NL, LOWY DR, SCHILLER JT. HPV 16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J 1989, 8: 3905-3910.
31. MUNGER K, PHELPS WC, BUBB V, HOWLEY PM, SCHLEGEL R. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989, 63: 4417-4421.
32. HUDSON JB, BEDELL MA, McCANCE DJ, LAIMINS LA. Immortalization and altered differentiation of human keratinocytes *in vitro* by the E6 and E7 open reading frames of human papillomavirus type 18. J Virol 1990, 64: 519-526.
33. DYSON N, HOWLEY PM, MUNGER K, HARLOW E. The human papillomavirus-16 E7 oncoprotein is able to bind the retinoblastoma gene product. Science 1989, 243: 934-937.
34. SCHEFFNER M, WERNESS BA, HUIBREGTSE JM, LEVINE AJ, HOWLEY PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990, 63: 1129-1136.
35. GISSMANN L, DIEHL V, SCHULTZ-COULON HJ, zur HAUSEN H. Molecular cloning and characterization of human papilloma virus DNA derived from a Laryngeal papilloma. J Virol 1982, 44: 393-400.
36. DURST M, GISSMANN L, IKENBERG H, zur HAUSEN H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci USA 1983, 80: 3812-3815.
37. BOSHART M, GISSMANN L, IKENBERG H, KLEINHEINZ A, SCHEURLEN W, zur HAUSEN H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J 1984, 3: 1151-1157.
38. KAHN T, SCHWARZ E, zur HAUSEN H. Molecular cloning and characterization of the DNA of a new human papillomavirus (HPV30) from a laryngeal carcinoma. Int J Cancer 1986, 37: 61-65.
39. LORINCZ AT, LANCASTER WD, TEMPLE GF. Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J Virol 1986, 58: 225-229.
40. BEAUDENON S, KREMSDORF D, CROISSANT O, JABLONSKA S, WAIN-HOBSON S, ORTH G.

A novel type of human papillomavirus associated with genital neoplasias. Nature 1986, 321: 246-249.

41. KAWASHIMA M, JABLONSKA S, FAVRE M, OBALEK S, CROISSANT O, ORTH G. Characterization of a new type of human papillomavirus found in a lesion of Bowen's disease of the skin. J Virol 1986, 57: 688-692.
42. de VILLIERS E-M, HIRSCH BA, von KNEBEL DC, NEUMANN C, zur HAUSEN H. Two newly identified human papillomavirus types (HPV40 and 57) isolated from mucosal lesions. Virology 1989, 171: 248-253.
43. LUNGU O, CRUM CP, SILVERSTEIN S. Biologic properties and nucleotide sequence analysis of human papillomavirus type 51. J Virol 1991, 65: 4216-4225.
44. YAJIMA H, NODA T, de VILLIERS E-M, YAJIMA A, YAMAMOTO K, NODA K, ITO Y. Isolation of a new type of human papillomavirus (HPV52b) with a transfroming activity from cervical cancer tissue. Cancer Res 1988, 48: 7164-7172.
45. GALLAHAN D, MULLER M, SCHNEIDER A, DELIUS H, KAHN T, de VILLIERS E-M, GISSMANN L. Human papillomavirus type 53. J Virol 1989, 63: 4911-4912.
46. FAVRE M, KREMSDORF D, JABLONSKA S, OBALEK S, PEHAU-ARNAUDET G, CROISSANT O, ORTH G. Two new human papillomavirus types (HPV54 and 55) chatacterized from genital tumours illustrate the plurality of genital HPVs. Int J Cancer 1990, 45: 40-46.
47. KIRII Y, IWAMOTO S, MATSUKURA T. Human papillomavirus type 58 DNA sequence. Virology 1991. 185: 424-427.
48. LORINCZ AT, QUINN AP, LANCASTER WD, TEMPLE GF. A new type of papillomavirus associated with cancer of the uterine cervix. Virology 1987, 159: 187-190.
49. SAMBROOK J, FRITSCH EF, MANIATIS T. Molecular cloning, a laboratory manual. New York, Cold Spring Harbor Laboratory Press, 1989.
50. LO YMD, MEGAL WZ, FLEMING KA. False positive results and the polymerase chain reaction. Lancet 1988, 2: 679.
51. KWOK S, HIGUCHI R. Avoiding false positives with PCR. Nature 1989, 339: 237-238.
52. SEEDORF K, KRAMMER G., DURST M., SUHAI S., ROWEKAMP WG. Human papillomavirus type 16 DNA sequence. Virology 1985, 145: 181-185.
53. COLE ST, DANOS O. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. J Mol Biol 1987, 193: 599-608.
54. GOLDSBOROUGH MD, DISILVESTRE D, TEMPLE GF, LORINCZ AT. Nucleotide sequence of human papillomavirus type 31: a cervical neoplasia-associated virus. Virology 1989, 171: 306-311.
55. COLE ST, STREECK RE. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J Virol 1986, 58: 991-995.
56. MARICH JE, PONTSLER AV, RICE SM, McGRAW KA, DUBENSKY TW. The phylogenetic relationship and complete nucleotide sequence of human papillomavirus type 35. Virology 1992, 186: 770-776.
57. VOLPERS C, STREECK RE. Genome organization and nucleotide sequence of human papillomavirus type 39. Virology 1991, 181: 419-423.
58. MUNOZ N, BOSCH FX, de SANJOSE S, TAFUR L, IZARZUGAZA I, GILI M, VILADIU P, NAVARRO C, MARTOS C, ASCUNCE N, GONZALEZ LC, KALDOR JM, GEURRERO E, LORICZ

AT, SANTAMARIA M, ALONSO de RUIZ P, ARISTIZABAL N, SHAH K. The causal link between human papillomavirus and invasive cervical cancer: a population-based case-control study in Colombia and Spain. Int J Cancer 1992, 52: 743-749.

59. HUSMAN AMR, WALBOOMERS JMM, MEIJER CJLM, RISSE EKJ, SCHIPPER MEI, HELMERHORST TM, BLEKER OP, DELIUS H, van den BRULE AJC, SNIJDERS PJF. Analysis of cytomorphologically abnormal cervical scrapes for the presence of 27 mucosotropic human papillomavirus genotypes, using polymerase chain reaction. Int J Cancer 1994, 56: 802-806.
60. FUJINAGA Y, INOUE Y, ISHIDA S, SHIMADA M, OKAZAWA K, YAMAKAWA Y, FUKUSHIMA M, KATO I, FUJINAGA K. The double polymerase chain reaction with consensus primers permits rapid and sensitive detection of genital human papillomavirus oncogenes. Tumor Res 1993, 28: 101-110.
61. FUJINAGA Y, OKAZAWA K, OHASHI Y, YAMAKAWA Y, FUKUSHIMA M, KATO I, FUJINAGA K. Human papillomavirus type 16 E7 gene sequence in human cervical carcinoma analysed by polymerase chain reaction and direct sequencing. Tumor Res 1990, 25: 85-91.
62. FUJINAGA Y, OKAZAWA K, NISHIKAWA A, YAMAKAWA Y, FUKUSHIMA M, KATO I, FUJINAGA K. Sequence variation of human papillomavirus type 16 E7 in preinvasive and invasive cervical neoplasias. Virus Genes 1994, 9: 85-92.
63. KOUTSKY LA, GALLOWAY DA, HOLMES KK. Epidemiology of genital human papillomavirus infection. Epidemiol Rev 1988, 10: 122-163.
64. LANGENBERG A, CONE RW, McDOUGALL J, KIVIAT N, COREY L. Dual infection with human papillomavirus in a population with overt genital condylomas. J Am Acad Dermatol. 1993, 28: 434-442.
65. HINRICHS S, SMITH L, GHAZVINI S, KIRVEN K, PALLADINO H, SPAULDING D. Multiple HPV infection: microanatomy by *in situ* hybridization and immunohistochemistry. Mod Pathol 1992, 5: 257-261.
66. LAURICELLA LM, PIETTE J, LIFRANGE E, LAMBOTTE R, GERARD P, RENTIER B. High rate of multiple genital HPV infections detected by DNA hybridization. J Med Virol 1992, 36: 265-270.
67. NUOVO GJ, DARFLER MM, IMPRAIM CC, BROMLEY SE. Occurrence of multiple types of human papillomavirus in genital tract lesions. Analysis by *in situ* hybridization and the polymerase chain reaction. Am J Pathol 1991, 138: 53-58.
68. WILLIAMSON AL, BRINK NS, DEHAECK CM, OVENS S, SOETERS R, RYBICKI EP. Typing of human papillomaviruses in cervical carcinoma biopsies from Cape Town. J Med Virol 1994, 43: 231-237.
69. HILLMAN RJ, RYAIT BK, BOTCHERBY M, TAYLOR RD. Changes in HPV infection in patients with anogenital warts and their partners. Genitourin Med. 1993, 69: 450-456.
70. NISHIKAWA A, FUKUSHIMA M, SHIMADA M, YAMAKAWA Y, SHIMANO S, KATO I, FUJINAGA K. Relatively low prevalence of human papillomavirus 16, 18 and 33 DNA in the normal cervices of Japanese women shown by polymerase chain reaction. Jpn J Cancer Res 1991, 82: 532-538.