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A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin

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A pair of degenerate PCR primers (FAP59/64) was designed from two relatively conserved regions of the L1 open reading frame of most human papillomaviruses (HPV). The size of the generated amplicon was about 480 bp. PCR using these primers was found capable of amplifying DNA from 87% (65/75) of the HPV types tested, its sensitivity being 1–10 copies for HPV-5, -20 and -30 clones. HPV was found in 63% (5/8) of tumour samples and in 63% (5/8) of normal skin biopsies from patients with various cutaneous tumours. HPV-5, HPV-8, HPV-12, HPVvs20-4 and six putatively novel HPV types were identified. No correlation was found to exist between specific HPV and tumour types. Skin surface swab samples from one or more sites on three of four healthy volunteers were found to contain HPV, types 12 and 49 being identified, as well as eight novel HPV types, two of which were also found among the patients. In all, HPV was detected in 75% (9/12) of those tested, five HPV types and 12 novel candidate types being identified, and 37% (7/19) of HPV-positive samples were found to manifest more than one HPV type. All the HPV detected manifested high degrees of nucleotide sequence similarity with HPV types associated with skin lesions and epidermodysplasia verruciformis. The overall HPV finding in the skin samples was 50% (20/40) using the FAP primers as compared to 18% (7/40) using another PCR test designed for skin types. The results thus suggest the new method to be sensitive and generally applicable for detecting cutaneous HPV.

Introduction

Of the approximately 80 different types of human papillomavirus (HPV) identified hitherto, more than 25 are known to infect the genital tract, and some have been identified as the causative agent of cervical cancer in at least 90% of cases (zur Hausen, 1996). Over 20 specific HPV types have been identified in patients with epidermodysplasia verruciformis (EV), though only a few of these types, mainly HPV-5 and -8, have been found to be associated with skin cancer (Jablonska & Majewski, 1994). PCR with primers manifesting a high degree of nucleotide identity with conserved sequences in the L1 and E1 open reading frames (ORF) have been successfully used for the detection of a wide range of genital HPV types (Manos *et al.*, 1989; Guerrero & Shah, 1991; Smits *et al.*, 1992; de Roda Husman *et al.*, 1995). PCR with universal primers specially designed for cutaneous HPV types has also been developed (Shamanin *et al.*, 1994 *a*, *b*, 1996; Berkhout *et al.*, 1995; Boxman *et al.*, 1997; de Villiers *et al.*, 1997). However, methods for the detection of cutaneous HPV types have disadvantages such as nested PCR or combinations of several degenerate primers. Moreover, use of the different PCR approaches has resulted in discrepant reports of HPV DNA in skin lesions (Surentheran *et al.*, 1998).

In the present study, we have designed and evaluated a single pair of degenerate PCR primers primarily aimed at the amplification of cutaneous HPV types.

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Methods

■ **Primer design.** Aligned sequences from the L1 ORF of HPV types in the 1996 and 1997 HPV Sequence Database Compendia (Myers, 1996, 1997) were manually reviewed to identify regions characterized by a high

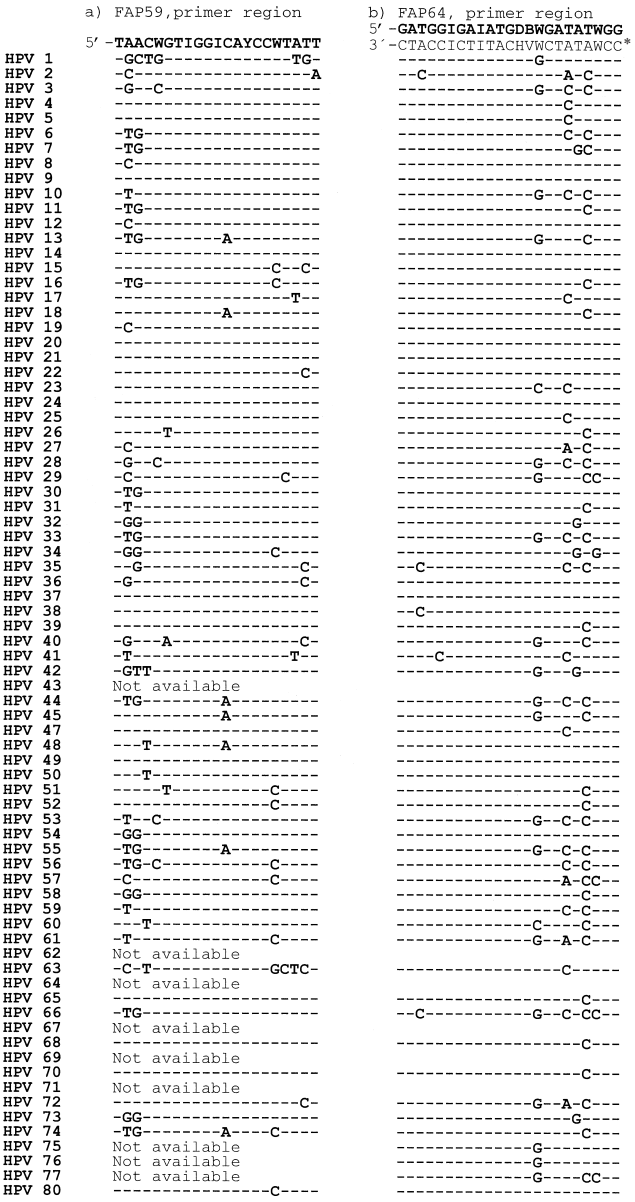

Fig. 1. Alignment of the FAP59 (*a*) and FAP64 primer (*b*) sequences with the corresponding region in the L1 ORF of available HPV types. Lines and characters represent identical and mismatched nucleotides, respectively. The sequence of the FAP64 primer is marked with an asterisk. Degenerate nucleotides of primers: W = T, C; I = inosine; Y = C, T; D = A, G, T; B = G, C, T; H = A, C, T; V = A, C, G.

degree of nucleotide sequence identity. Two regions manifesting a relatively high degree of nucleotide sequence identity were found in the 5′ end of the L1 ORF. From the aligned sequences, primers FAP59 (5′ TAACWGTIGGICAYCCWTATT 3′) and FAP64 (5′ CCWATATCW-VHCATITCICCATC 3′) were designed (Fig. 1). The positions of the primers corresponded to nucleotides 5981–6001 and 6458–6436 of the HPV-8 genome, yielding an amplicon of 478 bp. The FAP59 primer contained two inosine nucleotides and was degenerated at three positions, with a total degeneracy number of 8. The FAP64 primer also contained two inosines and was degenerated at four positions, with a total degeneracy number of 36.

■ **PCR parameters.** The 25 µl PCR mixture contained 2·5 µl of sample, 0·75 µM of each FAP59 and FAP64 primer, 200 µM of each dNTP (Boehringer Mannheim), 0·2% BSA and 0·625 U Taq DNA polymerase (AmpliTaq Gold) and reaction buffer (Perkin Elmer). PCR was carried out in an automated thermocycler (Hybaid Omnigene) programmed for block temperatures, using the following parameters: 10 min at 94 °C and then 45 cycles of 1·5 min at 94 °C, 1·5 min at 50 °C and 1·5 min at 72 °C. Five µl of the amplified material was analysed by electrophoresis in 2% agarose gel (Sea Kem, FMC Bioproducts) in 1 × TBE buffer (Sambrook *et al.*, 1989) containing ethidium bromide (20 µg/ml, Boehringer Mannheim). The HPV-specific amplicon was identified by size determination in UV light.

■ **Generality and sensitivity analysis.** To evaluate the general applicability of the degenerate primers, cloned materials from 73 different HPV types were tested. HPV-19 was not tested since the vector was positioned between the sites of the primers. This was also true of HPV-16 and -51, and therefore the material used for analysis of these two types was obtained from cervical brush samples. HPV types 25, 46, 78 and 79 were not available for analysis. DNA (2·5 pg, corresponding to approximately 2×10^5 copies) of each cloned HPV type was added to each PCR tube. As negative controls, pBR322, proteinase K-treated human embryonal lung (HEL) fibroblasts and H_2O without template were used.

Since 73 different HPV clones were tested in the generality analysis, there could be a theoretical risk that cross-contamination between the clones had taken place. Therefore, amplicons of two plasmids (HPV-5 and HPV-30) were selected for direct DNA sequencing. They were found to contain the expected and correct HPV type-specific sequences, ruling out the possibility of contamination.

For analysing the sensitivity of the PCR, 10-fold dilution series starting from 1×10^3 copies of cloned HPV-5, -20 and -30 were used. Each PCR contained a background of 100 pg human placental DNA (Sigma) in TE buffer (10 mM Tris–HCl, 0·1 mM EDTA, pH 8·0).

■ **Clinical samples.** From eight patients with different skin tumours, both tumour biopsies and biopsies from normal skin of the volar aspect of the forearm were collected and immersed in 0·9% NaCl for transport to the laboratory. The patients' characteristics are presented in Table 1. The samples were stored at −20 °C (without 0·9% NaCl) until analysed. Then DNA was extracted using a simple phenol-free method. The sample was incubated overnight at 37 °C, with 400 ml 'lysis buffer' (10 mM Tris–HCl, 10 mM NaCl, 10 mM EDTA, pH 7·8) with 4% SDS and proteinase K (200 µg/ml) (Boehringer Mannheim). Then, 120 µl of saturated NH_4Ac was added and the tube was vortexed for 20 s and centrifuged at 17 600 ***g*** for 15 min. The supernatant was transferred to a new tube and the DNA precipitated with 900 µl ethanol for 30 min at −20 °C. After centrifugation at 17 600 ***g*** for 7·5 min, the resulting pellet was washed once with 500 µl 95% ethanol. The pellet was dried briefly and then dissolved in 100 µl TE buffer.

From three healthy volunteers, swab samples were collected from each of six sites: the forehead, the volar aspect of the left and right forearms, the upper abdomen, and the anterior aspect of the left and right thighs. Furthermore, a forehead sample was collected from a fourth healthy individual. All samples were taken with a prewetted (0·9% NaCl) cotton-tipped swab (Bio Hospital) which was drawn to and fro five times within an area of about 5 × 15 cm, and suspended in 1 ml 0·9% NaCl. The samples were stored at 4 °C until analysed.

Aliquots (2·5 µl) of each sample were added to 22·5 µl PCR mixture and 45 cycles of amplification were performed as described above. The positive controls used in the PCR were two clinical samples, one

Table 1. HPV DNA in biopsies of skin tumours and normal skin from eight patients

All samples from normal skin were taken as 3 mm punch biopsies from the volar aspect of the right forearm.

Patient no.	Male/ Female	Age	Diagnosis	Tumour localization	Amplicon yield	HPV	No. of clones
1	M	74	Basal cell carcinoma	Left external ear	–	–	
			Normal		+	FA1.2	3
2	M	75	Basal cell carcinoma	Nose	++	FA1.3	1
						FA1.2	1
						FA4	1
			Normal		+	Failure	*
3	F	73	Actinic keratosis	Upper lip	+	FA5	1†
			Normal		+	FA5	3‡
4	M	74	Melanoma	Abdomen	+	HPV-5 NA1	3
			Normal		(+)	HPVvs20-4	2
5	M	87	Squamous cell carcinoma	Left hand, dorsal	–		
			Normal		–		
6	M	74	Actinic keratosis	Left hand, dorsal	++	FA1.1	3
			Normal		–		
7	M	65	Seborrhoeic keratosis	Left scapula	–		
			Normal		–		
8	M	79	Benign adnexal tumour	Right cheek	+	HPV-8	1
						FA12	2
			Normal		++	HPV-12	2
						FA9	1
						FA13	1

* Sequencing failed in seven out of eight attempts. The eighth attempt yielded a human mRNA sequence.
† Sequenced eight times, FA5 found once, human mRNA five times and at two attempts no sequence was obtained.
‡ A fourth sequence analysis yielded a human mRNA sequence.

containing HPV-20, the other HPV-6. As negative control, H_2O without template was used. Five µl of the amplified material was analysed by electrophoresis as described above.

■ **Cloning and DNA sequence analysis.** For improving the quality of direct DNA sequencing, 5 µl of the amplicon was reamplified in 50 µl PCR mixture, which was subsequently separated in 1·5 % agarose gel (Sea Kem) in 1 × TAE buffer (Sambrook *et al*., 1989). The amplicon was then cut out from the gel and purified with a spin column (QIAquick Gel Extraction kit, Qiagen) and eluted in 30 µl H_2O. Eight µl was used for the cycle sequencing reaction (ABI Prism, Dye Terminator Cycle Sequencing ready reaction kit FS, Perkin Elmer). The products were separated and analysed with an automated DNA sequencer (model 373A, Perkin Elmer). In cases where low quality DNA sequences were obtained, the amplicons were cloned using the pCR-Script cloning kit (Stratagene).

At least three recombinant clones were sequenced from each sample. Amplicons from samples collected with cotton swabs were cloned without reamplification and the cloned materials were sequenced with forward and reverse primers.

Obtained sequences were compared to available HPV sequences in the GenBank database, by using the Blast server.

■ **Nucleotide sequence accession numbers.** The obtained DNA sequences have been submitted to GenBank under the following numbers: FA1.1, AF121419; FA1.2, AF121420; FA1.3, AF121421; FA2.1, AF121422; FA2.2, AF121423; FA2.3, AF121424; FA3, AF121425; FA4, AF121426; FA5, AF121427; FA6, AF121428; FA7, AF121429; FA8, AF121430; FA9, AF121431; FA11, AF121432; FA12, AF121433; and FA13, AF121434.

■ **Alternative PCR.** The ability of the FAP59/64 PCR to detect HPV from the 40 skin samples was compared with that of an HPV skin type PCR described by Berkhout *et al*. (1995). Briefly, 5 µl of sample was used in the first-step PCR with the CP65/CP70 primer set. In the nested PCR with the CP66/CP69 primers, 3 µl from the first step was used as input. As positive controls HPV-5 and -20 were used, and as negative controls H_2O without template. Five µl of the amplified material was analysed by electrophoresis as described above.

■ **Statistical analysis.** Statistical analysis of the HPV DNA results obtained by the FAP PCR and the alternative PCR test was done by using McNemar's test (corrected for continuity).

Results

Generality and sensitivity of the PCR

Amplicons could be detected by gel electrophoresis from 65 of the 75 different HPV types tested. Fifty-nine HPV types yielded distinct bands, and weak bands were yielded by HPV-15, -33, -38, -56, -67 and -72. No band was detected for HPV types 1, 2, 35, 41, 44, 55, 63, 66, 71 or 74. HPV-40 and HPV-58 gave rise to bands of about 700 bp and 260 bp, respectively

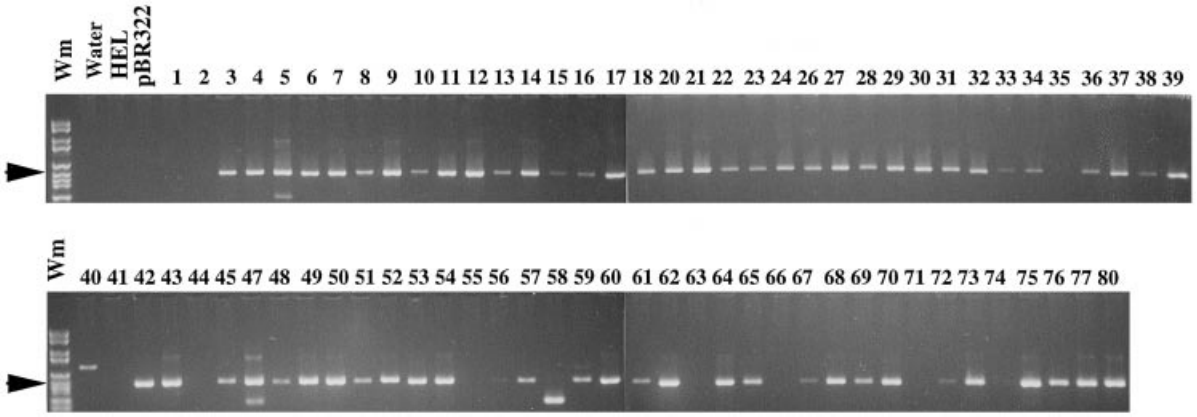

Fig. 2. Amplicon yield from 2·5 pg of 73 cloned HPV types by PCR with FAP59 and FAP64 primers. For HPV-16 and -51, material from cervical brush samples was used. HPV types 19, 25, 46, 78 and 79 were not analysed. Water, proteinase K-treated human embryonal lung (HEL) fibroblasts and 2·5 pg plasmid pBR322 were used as negative controls. Wm, DNA molecular mass marker VI (Boehringer Mannheim). Arrowheads indicate the position of amplicon of 480 bp.

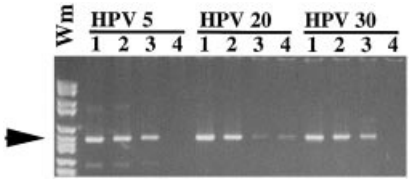

Fig. 3. Sensitivity analysis of PCR with FAP59 and FAP64 primers. Cloned HPV-5, -20 and -30 were amplified in a background of 100 pg placental DNA. Lanes 1–4 show 1000, 100, 10 and 1 input copies of HPV DNA in the PCR, respectively. Wm, DNA molecular mass marker VI (Boehringer Mannheim). The arrowhead indicates the position of amplicon of 480 bp.

(Fig. 2). PCR of clinical samples containing HPV-40 or -58 manifested the same amplicon size as the cloned materials (data not shown).

The sensitivity limit of the PCR was between 1 and 10 copies for HPV-5, -20 and -30 (Fig. 3).

Clinical samples

In the eight patients with various skin tumours, HPV was found in 63% (5/8) of both the normal skin samples and tumours. No correlation between certain HPV types and tumours was seen. One patient manifested the same HPV DNA (FA5) both in normal skin and in an actinic keratosis. In these eight patients, HPV-5, HPV-8, HPV-12, HPVvs20-4 and six different isolates of previously uncharacterized HPV types were identified (Table 1).

Of the four healthy volunteers, three were found to harbour HPV on their skin. Of the samples from one of the male volunteers (no. 4), those from four of the five sampling sites were HPV-positive, HPV-12, -49 and an HPV isolate provisionally designated FA9 being found in the left thigh sample, and five different HPV isolates of previously undefined

Table 2. HPV DNA findings of skin from healthy volunteers

Volunteer no.	Male/ Female	Age	Left forearm	Right forearm	Upper abdomen	Left thigh	Right thigh	Forehead
1	M	55	–	–	–	–	–	–*
2	F	26	–	–	–	–	–	FA6*(3)†
3	F	27	NS	NS	NS	NS	NS	FA2.1 (1) FA7 (2)
4	M	37	FA3 (3)	FA2.1 (3)	FA2.1 (1) FA2.3 (1) FA8 (1)	FA9 (1) HPV-12 (1) HPV-49 (1)	–	NS
4‡			FA2.2 (3)	FA1.1 (1) FA11 (2)	–	FA1.1 (3)	–	(+)§

* Forehead sample collected 6 months after the other samples.
† Number of sequenced clones from each sample.
‡ Samples collected 16 days later.
§ Sample collected 6 months after the other samples. Low yield was found but was not sequence analysed.
NS, No sample.

Table 3. New HPV candidate types and their nucleotide sequence similarity to known HPV types

HPV candidate	Closest related HPV type	Nucleotide sequence similarity (%)	Size of fragment (bp)*
FA1.1	HPV-48	68	437
FA2.1	HPV-50	67	437
FA3	HPV-24	69	446
FA4	HPV-50	67	440
FA5	HPV-80	78	440
FA6	HPV-60	68	446
FA7	HPV-12	73	440
FA8	HPV-50	69	437
FA9	HPV-4	71	440
FA11	HPV-49	70	434
FA12	HPV-4	68	443
FA13	HPV-48	70	440

* Primer sequences were not included in the comparison analysis.

types in samples from the other sites (Table 2). At repeat testing of this HPV-positive healthy volunteer 16 days later, samples from three of five sites were again found to be HPV-positive, manifesting three putatively novel HPV types, two of which had not been found in the earlier samples. Thus, in all, four different HPV types, including two which were previously unknown, were isolated from the left thigh of this healthy volunteer. Three of the four volunteers yielded HPV-positive forehead samples, one putatively novel type in one case, and two putatively novel HPV types in another, no typing having been performed in the third case (Table 2).

HPV-12 and the novel HPV candidate types FA1.1 and FA9 were found both in the healthy male volunteer and in patients with skin tumours. FA1.1 was detected on the right forearm and left thigh of a healthy male volunteer (no. 4), as well as in an actinic keratosis in one of the patients (no. 6) (Tables 1 and 2). Among the patients, the novel HPV variant FA1.2 was found in a normal skin biopsy, and FA1.3 in a basal cell carcinoma. Three variants each of FA1 and FA2 were found. FA1.2 differed by one nucleotide from FA1.1, and FA1.3 differed by two nucleotides, all differences occurring in the third nucleotide position within a codon. FA2.2 differed by one nucleotide from FA2.1, and FA2.3 differed by three nucleotides, all but one of the differences occurring in the third nucleotide position within a codon. The putative novel HPV types manifested the closest nucleotide sequence similarity with skin and EV types (Table 3). FA1.1 and FA13 manifested only 50% nucleotide sequence similarity, although both were related most closely to HPV-48. FA4 and FA8 manifested 65% sequence similarity with each other, and 67% and 69%, respectively, with HPV-50. FA9 and FA12 manifested 62% nucleotide similarity, and both were related most closely to HPV-4. Totally, HPV DNA was detected on skin samples from nine of 12 individuals; and of the 19 HPV-positive samples, seven manifested more than one HPV sequence simultaneously. From the nine HPV-positive individuals, HPV-5, -8, -12, -49, HPVvs20-4 and 12 previously unreported HPV types were identified.

Table 4. Comparison between HPV findings from skin samples using PCR with FAP59/64 primers and a nested skin type-aimed PCR test (Berkhout *et al.*, 1995)

		FAP59/64 PCR		
		+	−	Total
Nested PCR	+	6	1	7
	−	14	19	33
Total		20	20	40

The alternative skin type HPV PCR detected seven HPV DNA-positive samples out of 40 tested, as compared to 20 by the FAP PCR ($P < 0{\cdot}001$) (Table 4).

Discussion

Here we describe a PCR technique with a pair of degenerate HPV primers (FAP59 and FAP64) that enabled detection of a broad range of HPV types. The method showed high sensitivity, optimized to allow detection of less than 10 copies of certain cloned HPV genomes. The technique was also found to detect significantly higher numbers of HPV-positive skin samples as compared to the nested PCR test described by Berkhout *et al.* (1995). Furthermore, sampling from skin with cotton swabs proved to be a feasible means of obtaining

sufficient material for HPV DNA analysis, and no DNA extraction was needed from the cotton swab samples prior to PCR.

We also found AmpliTaq Gold to be the only DNA polymerase from *Thermus aquaticus* that gave the desired amplicon without non-specific background on the gels.

The generality of the designed primers was satisfactory for most of the EV and other cutaneous types, since only HPV-1, -2, -41 and -63 could not be detected. The reason for the negative result with these HPV types appears to have been inefficient base pairing in the 3′ region between one or both the primers and the templates. The negative result for HPV-35 was also probably due to inefficient base pairing in the 3′ region of both primers. The detection failures for HPV-44 and -55 could be traced to three mismatches in each of the primers, and the negative result of HPV-66 was probably due to five mismatches of the primer FAP64. The reason for the failure to detect HPV-71 could not be determined, since no sequence data were available. The negative result of HPV-74 was probably due to four mismatches of the primer FAP59.

The reduced amplification yield of HPV-15 might have been due to the presence of two mismatches, one and four nucleotides from the 3′ terminal nucleotide of the primer FAP59. The poor amplification of HPV-33 was unexpected, since the mismatches were manifested close to the 5′ terminus of both primers. Surprisingly, the low HPV-38 amplification efficiency could be explained by only one A mismatch (A·C, primer/template) two nucleotides from the 3′ terminal nucleotide of the primer FAP64. The low efficiency of HPV-56 amplification was probably attributable to four mismatches of the primer FAP59. The weak amplification of HPV-72 might have been due to a mismatched T (T·G, primer/template) one nucleotide from the 3′ terminal nucleotide of the primer FAP59, in combination with three mismatches in the 5′ region of the primer FAP64.

The shorter amplicon of HPV-58 was calculated to be 264 bp, since 10 matching nucleotides (positions 6003–6012) at the 3′ terminus of the FAP59 were found downstream of the predicted primer site. The longer amplicon of HPV-40 was calculated to be 740 bp, if seven nucleotides (positions 5658–5664) upstream of the ordinary primer site matching the 3′ terminus of the FAP59 were considered. The ordinary FAP59 primer site was probably affected by a mismatched T (T·G, primer/template) one base from the 3′ terminal nucleotide.

Although no correlation was found to exist between certain HPV types and skin tumours in our small series, all HPV findings were associated with the skin and EV types. By means of different PCR approaches, the EV types have also been found to predominate in skin tumours from renal transplant patients (Shamanin *et al.*, 1994*b*; Berkhout *et al.*, 1995; de Jong-Tieben *et al.*, 1995; de Villiers *et al.*, 1997; Hopfl *et al.*, 1997; Bens *et al.*, 1998). The EV HPV types have been found to occur at high frequency in hairs plucked from normal skin, suggesting HPV to be subclinically present in normal skin (Boxman *et al.*, 1997). Possibly, the stem cells of the hair follicles represent a reservoir for HPV of the skin. However, in our material the HPV DNA was frequently found on the skin by sampling with cotton swabs.

In recent years, a substantial number of putatively novel HPV types has been presented in studies of renal transplant patients (Shamanin *et al.*, 1994*b*; Berkhout *et al.*, 1995; de Jong-Tieben *et al.*, 1995; de Villiers *et al.*, 1997; Hopfl *et al.*, 1997; Bens *et al.*, 1998). In our series, 12 novel HPV candidate types were found. However, it cannot be ruled out that some of these have been reported earlier by others, since the region for the amplicon was positioned upstream of the amplicons presented in other studies.

Direct sequencing of the amplicons from clinical material was unsuccessful, yielding overlapping peak patterns, indicating the presence of more than one type of HPV template in each sample. This was confirmed in 37% of the samples through sequence analysis of a number of clones from each sample. Findings of multiple HPV types from skin lesions have also been reported by others (Shamanin *et al.*, 1994*b*; Berkhout *et al.*, 1995; de Jong-Tieben *et al.*, 1995; Boxman *et al.*, 1997).

Finally, by using the described PCR method we suggest that it will be possible to perform reliable studies of HPV prevalence in larger series of skin carcinomas, benign lesions and normal skin of the healthy population. Such studies might not only contribute to our understanding of the involvement of HPV in the pathogenesis of skin cancer, but also prove to be useful in investigating the general epidemiology of cutaneous HPV types.

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