Human Papillomavirus Infections in Nonmelanoma Skin Cancers From Renal Transplant Recipients and Nonimmunosuppressed Patients

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Background: **Nonmelanoma carcinomas of the skin represent the most frequent cancers among the Caucasian population worldwide. They occur with high frequency in renal allograft recipient patients after prolonged immunosuppression. *Purpose:* We analyzed tumors obtained from both immunosuppressed and nonimmunosuppressed patients for human papillomavirus (HPV) DNA. *Methods:* Twenty-nine specimens of nonmelanoma carcinomas of the skin were obtained from 19 renal allograft recipient patients; these included 20 specimens of squamous cell carcinoma (SCC) from 11 patients, five specimens of basal cell carcinoma (BCC) from four patients, and four specimens of carcinoma in situ (CIS) from four patients. Forty-one specimens of nonmelanoma carcinomas of the skin were obtained from 32 nonimmunosuppressed patients; these included 26 SCC specimens from 19 patients, 11 BCC specimens from nine patients, and four keratoacanthoma (benign epithelial tumor) specimens from four patients. A polymerase chain reaction method involving use of degenerate oligonucleotide primers, in which the conserved region of the open reading frame of the HPV L1 (major capsid protein) gene is amplified, was used to amplify total cellular DNA purified from individual tumors. The DNA of each specimen was subjected to 16 different amplification reactions; different primer combinations were used in order to increase the sensitivity and specificity of HPV detection. Resulting products were probed with a radioactively labeled, degenerate oligonucleotide. HPV-specific DNA was either sequenced directly after elution from the gel or amplified with semi-nested, degenerate primers, after which the products were cloned and sequenced. Sequences were compared with all known papillomavirus sequences. *Results:* Thirteen (65%) of the 20 SCC specimens and three of the five BCC specimens from immunosuppressed (renal allograft recipient) patients contained identifiable HPV-related sequences, among them 13 putative novel HPV genomes. In addition, all other malignant tumor specimens from this patient group revealed faint signals upon amplification and hybridization; the origin of these signals has not been identified in the present study. In nonimmunosuppressed patients, eight (31%) of 26 SCC specimens and four (36%) of 11 BCC specimens contained sequences of HPV types. Two putative novel HPV sequences could be identified in this group. Faint signals of yet undetermined origin were observed in eight of the SCC specimens and in two of the BCC specimens. Two of four keratoacanthoma specimens contained sequences of known HPV type. (Keratoacanthoma is a nonmalignant lesion for which the natural history has not been defined.) The spectrum of HPV types in both groups of patients differed substantially. *Conclusions:* These data point to the frequent presence of HPV sequences in SCCs and BCCs of the skin. The etiologic relationship of these infections to the respective malignant tumors remains to be evaluated. *Implications:* The presence of HPV DNA in a large percentage of specimens of nonmelanoma carcinomas of the skin from immunosuppressed patients, as well as from nonimmunosuppressed patients, renders a papillomavirus infection as a possible factor in the etiology of this disease. [J Natl Cancer Inst 1996;88:802-11]**

Nonmelanoma carcinomas of the skin represent the most frequent cancers occurring among the Caucasian population worldwide (*1*,*2*). These carcinomas, i.e., basal cell carcinoma (BCC) (71%), squamous cell carcinoma (SCC) (15%), and Bowen's disease (10%), occur much more frequently than malignant

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See "Notes" section following "References."

melanoma (4%) (*3*). Various attempts have been made to determine the factors involved in the etiology of nonmelanoma carcinomas of the skin. It is widely accepted that the UV component of solar radiation is a major environmental cause (*4*). This is reflected in the body site distribution of the lesions, as well as the pattern of the mutations (typical for UV radiation-induced DNA damage) present in the tumor suppressor gene p53 (*2,5*).

Papillomaviruses play a crucial role in the development of certain malignant tumors (*6*). This has been demonstrated clearly in the case of genital tumors such as cervical carcinomas. Specific types of human papillomaviruses (HPVs) ("high-risk" HPVs) have been implicated in neoplastic transformation. The transforming function of the HPV E6/E7 gene products depends on the prior modification and functional elimination of cellular tumor suppressor genes and their products (*7*).

Most studies examining the involvement of papillomaviruses in the development of cutaneous carcinomas have been done on lesions that have developed in patients with the hereditary disease epidermodysplasia verruciformis or in patients subjected to prolonged periods of immunosuppression, e.g., renal allograft recipients. Both types of patients develop extensive cutaneous papillomatous lesions. These lesions initially appear as benign keratoses and frequently progress to dysplasia and then to carcinoma in situ (CIS) or invasive carcinoma on sun-exposed sites (*8-10*). Lesions in epidermodysplasia verruciformis patients contain a specific group of papillomavirus types not commonly found in the general population (*8*).

We have recently been able to demonstrate the presence of known and putative new HPV types in 56% of SCC and BCC specimens from renal allograft recipients (*11*). We did not detect any sequences identical to the epidermodysplasia verruciformis-associated HPV types in these lesions, although the partial sequences of the newly identified HPV types were related to members of this group of HPVs. Using the polymerase chain reaction (PCR) technique, other investigators (*12*) have reported a high prevalence (81%) of epidermodysplasia verruciformis-specific HPV types in cutaneous cancers from renal allograft recipients. An extensive study conducted in Europe (involving Southern blot and PCR analysis) and performed on specimens from the general population led to the detection of known HPV types in only 2.5% of such lesions (*13*).

In this study, we have extended the results obtained in our previous study (*11*). We analyzed the DNA from the same malignant tissue specimens, but we applied a less degenerate PCR method and amplified the DNA from each sample with 16 different primer combinations. By this method, we could increase the sensitivity and specificity of this PCR test to such an extent that we could identify HPV DNA sequences in samples that had previously resulted in a positive signal, which, however, was too faint to be further characterized. In addition, we included a series of nonmelanoma carcinomas of the skin from nonimmunosuppressed patients and amplified the DNA obtained from these lesions in the same manner as that used for the samples from the renal allograft recipients.

Materials and Methods

Clinical Samples

Specimens from the 19 renal allograft recipients were obtained from a group of patients attending the Royal London Hospital during 1991-1992 inclusive (*11*). They included 20 SCC specimens from 11 patients, five BCC specimens from four patients, and four CIS specimens from four patients. Samples of nonmelanoma carcinomas of the skin (26 SCC specimens from 19 patients, 11 BCC specimens from nine patients, and four keratoacanthoma [benign epithelial tumor] specimens from four patients) from nonimmunosuppressed patients were collected over a period of 11 years (1984-1995) from various hospitals throughout the Federal Republic of Germany. All specimens were immediately frozen after collection and stored at –70 °C until use.

The approval of the Ethics Committee for obtaining and using patient material was obtained where necessary.

PCR Analysis

All specimens were processed at the Deutsches Krebsforschungszentrum in Heidelberg, Federal Republic of Germany. Total cellular DNA was extracted from all the specimens as previously described (*14*).

The PCR method was performed as follows: DNA (100 ng) was amplified in the DNA Thermal Cycle (The Perkin-Elmer Corp., Foster City, CA) with 1 U *Taq* polymerase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) by use of the buffer provided by the supplier; the buffer was in a total volume of 50 μL containing 50 pmol of each primer. An initial denaturation at 94 °C for 4 minutes was followed by 40 cycles of amplification (94 °C for 1 minute, 52 °C for 2 minutes, 72 °C for 1 minute, and an extension at 72 °C for 2 seconds per cycle); the final extension was at 72 °C for 6 minutes in the last cycle, followed by cooling to 4 °C. In the previous study (*11*), each sample was amplified with the primers HVP2 (forward) and C (backward), or F14 and F15, respectively. These primers covered a highly conserved region of approximately 650-700 base pairs (bp) in the open reading frame (ORF) of the HPV L1 (major capsid protein) gene. In the present study, the products of the amplified DNA were detected either by ethidium bromide staining or, in the case of weaker signals, only after hybridization with the degenerate probe P1 [MTXSARGAYGSX-GAYATG, where M represents A or C; R is A or G; S is C or G; Y is C or T; and X is A, G, C, or T (*15*)]. The amplified products of these samples were eluted from the gel and reamplified by use of the same primers as used in the first round. In a number of cases, an amplified band (in some cases, even two bands) of the expected size (approximately 650-700 bp) became visible after ethidium bromide staining (Fig. 1). In other cases, this second amplification remained negative, and the specific DNA could not be isolated and characterized. In these cases, a DNA sample from each specimen was amplified by use of the 16 primer combinations of the degenerate oligonucleotide primers described previously by Shamanin et al. (*15*). These primers were each less degenerate, thus increasing the sensitivity and specificity to detect HPV sequences when compared with those of the primer combinations HVP2/C and F14/F15, which can be regarded as a pool of these less degenerate primers. These less degenerate primers (i.e., the forward primers A1, F10, F11, and F12 and the backward primers B5 and B6) amplified sequences corresponding to those found in the majority of the known HPV genomes. In addition, use of the forward primers F21, F22, F23, and F24 with the backward primers B11 and B12 led to amplification of sequences corresponding to sequence patterns characteristic of those of the following group of HPV types: HPV4, HPV48, HPV50, HPV60, and HPV65. This latter group of primers amplifies a broader spectrum of HPV types when compared with that of primers F14 and B15, which we previously described for use in the amplication of HPV4, HPV60, and HPV65 DNA sequences (*11*). To facilitate the subsequent cloning of the PCR products, the sequence of each primer was modified to include the nucleotide T at the 5′ end. The DNA from each specimen analyzed in this study was amplified in 16 different PCR assays by use of the following primer combinations (forward/backward): A1/B5, F10/B5, F11/B5, F12/B5, A1/B6, F10/B6, F11/B6, F12/B6, F21/B11, F22/B11, F23/B11, F24/B11, F21/B12, F22/B12, F23/B12, and F24/B12 (*15*). In each experiment, a sample of placenta DNA and a sample containing water instead of cellular DNA were included as controls. In addition, an HPV type that would give a marginally amplified signal with the respective primer combination was included as a positive control.

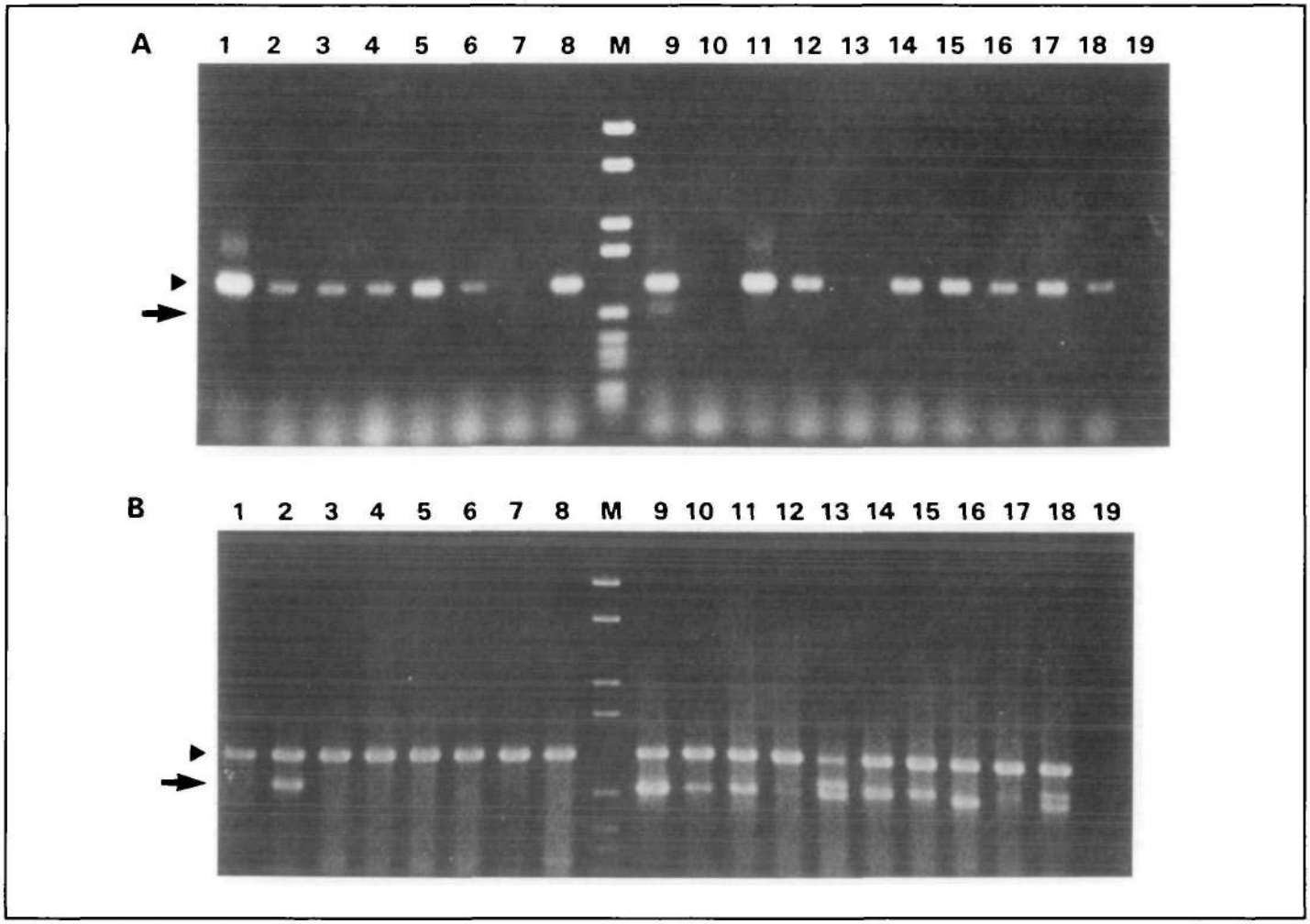

Fig. 1. Example of reamplification of the polymerase chain reaction product after agarose gel purification. Ethidium bromide-stained gel after the first round of DNA amplification using primers HVP2/C (**A**) and the corresponding samples after the second round of amplification with the same primers (**B**). Triangle indicates amplified cellular sequences, and arrow indicates the expected size (650-700 base pairs [bp]) of the HPV-specific sequences. Double bands represent two different HPV types. The individual samples are shown in the various lanes. Lane 1: human placenta DNA; lane 2: WV-8537 (wart with atypia); lane 3: WV-8533 (squamous cell carcinoma [SCC]); lane 4: WV-8496 (SCC); lane 5: WV-8467 (SCC); lane 6: WV-8461 (SCC); lane 7: WV-8367 (wart with atypia); lane 8: WV-8336 (SCC); lane 9: WV-8550 (wart with atypia); lane 10: WV-8499b (wart); lane 11: WV-8484 (SCC); lane 12: WV-8470 (wart); lane 13: WV-8466 (wart); lane 14: WV-8465 (wart); lane 15: WV-8464 (wart); lane 16: WV-8362 (dysplastic wart); lane 17: WV-8348 (wart with atypia); lane 18: WV-8326 (wart); and lane 19: WV-8238a (dysplastic wart). The following samples became positive only after a third round of amplification with the same primers: WV-8496, WV-8461, WV-8336, and WV-8238a. M = size marker (bp, top to bottom): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, and 154.

After PCR amplification, products were subjected to agarose gel electrophoresis and Southern blot analyses. The radiolabeled degenerate oligonucleotide probe P2 used for hybridization is a modification of the previously described P1 probe (*15*). Extra variation (deduced from the amino acid sequence) was included in the nucleotide sequence in order to cover the HPV60 sequence as well: MTXSARGAYGSXGAXATG (the position differing from P1 is shown in bold).

Semi-nested PCR

Virus-specific PCR products were purified from the agarose gel and reamplified as described previously (*11*), except that a nested forward primer NPR2 was used in combination with the backward primer used in the primary PCR. The sequence of this NPR2 consists of the P2 sequence tailed at the 5′ end with the *Bam*H1 linker: 5′ ttggatccMTXSARGAYGSXGAXATG. The expected size of an HPV-specific product resulting from this reaction would be approximately 400 bp.

Cycle Sequencing

The PCR-amplified putative HPV DNA that appeared as a visible band after ethidium bromide staining was eluted from the agarose gels and purified with glass milk by use of a Geneclean Kit (BIO 101). This product was subsequently sequenced by cycle sequencing by use of the ^{33}P-labeled backward primer as a sequencing primer.

Cloning of PCR Products

The purified, amplified DNA was cloned by use of the pGEM-T Vector Cloning Kit (Promega Corp. Madison, WI). Three to five recombinant plasmids harboring inserts of the expected size were randomly picked and sequenced (Sequenase 2.0 DNA Sequencing Kit; U.S. Biochemical, Cleveland, OH). Only one DNA strand was initially sequenced (about 250 bp). If this sequence shared 90% or more homology to any known HPV sequence (definition of a papillomavirus type), it was regarded as that HPV type; if this homology was below 90%, both strands of this cloned insert were sequenced in full.

Computer-Assisted Sequence Analysis

The sequences obtained were compared with the available HPV sequences in the European Molecular Biology Laboratory data bank (European Bioinformatics Institute, Cambridge, U.K.) and GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). They were also compared with unpublished HPV sequences provided by Dr. Hajo Delius

(Deutsches Krebsforschungszentrum), using the HUSAR software package. A phylogenetic tree of the sequences was constructed using the PILEUP program available in the HUSAR software package.

Results

PCR Analysis

No papillomavirus DNA could be detected in several samples in our previous study (*11*). To determine whether HPV DNA was present in these samples, we modified the PCR by applying different combinations of less degenerate primers on each of these specimens; to do so, we subjected each specimen obtained from the malignant lesions to 16 distinct PCR amplifications and analyses. We subjected 41 specimens obtained from nonimmunosuppressed patients to a similar analysis. The results varied from positive signals with several primer combinations (suggestive of mixed infections) to negative signals in all primer combinations. Of the 16 primer combinations tested, seven (A1/B5, F24/B11, F23/B12, F24/B12, F10/B5, F11/B5, and F12/B5) proved to be the most informative. Visible ethidium bromide-stained bands of the expected size (approximately 650-700 bp in the first PCR or about 400 bp in the semi-nested PCR) were purified from the agarose gels and sequenced. In several cases, a mixture of sequences was observed, after which the purified DNA was first cloned and then sequenced. Between 30% and 90% of the clones contained inserts of the expected size. We determined the sequences of three to five randomly picked recombinants per DNA sample.

HPV DNA in Nonmelanoma Carcinomas of the Skin and CIS From Renal Allograft Recipients

The nonmelanoma carcinomas of the skin (20 SCC specimens and five BCC specimens obtained from a total of 15 patients) as well as CIS (four specimens from four patients) obtained from renal allograft recipients were analyzed in two steps. The preliminary analyses were done with the use of the more degenerate HVP2 and C primers (*11*). Of 20 SCC samples, HPV41 was detected in one sample, the newly identified 29-related HPV type (vs93-1) was detected in three samples, and HPV69 was detected in four (total positive = 8; 40%) (Table 1, A). Faint, unidentifiable signals were obtained in an additional seven (35%) of these samples. HPV20 was detected in one

Table 1. Detection of human papillomavirus (HPV) sequences in nonmelanoma carcinomas of the skin from renal allograft recipients

Carcinoma	Patient No	Specimen No.	Primer combination*										HPV type
A)†			HVP2/C										
Squamous cell	1	WV-8324											HPV69
	2	WV-8336											HPV69
		WV-8337											HPV69
		WV-8338‡											HPV69
		WV-8461											vs93-1
	3	WV-8456											HPV41
	4	WV-8484											vs93-1
	5	WV-8496											vs93-1
In situ	6	WV-8539											HPV20
B)			F22/B11	F23/B11	F24/B11	F23/B12	F24/B12	A1/B5	F10/B5	F11/B5	F12/B5	F10/B6	F12/B6	
Squamous cell	2	WV-8239	++		+								+	vs19-6
		WV-8240‡									++		++	HPV54
														HPV61
		WV-8467‡												
		WV-8543‡												
	7	WV-8331					+							vs201-1
	8	WV-8359												
	9	WV-8476												
	4	WV-8480												
		WV-8481												
	10	WV-8490a		++	++	++	++	+	++	++	++			vs205-1
														vs206-2
														vs207-22
														vs20-4
	11	WV-8533							++		+		+	HPV16
	12	WV-8541												
Basal cell	13	WV-8327		++	+	+			++		++	+	+	HPV56
														HPV60
	14	WV-8329						+		+			+	HPV51
	14	WV-8330												
	10	WV-8489			+			+		++				HPV51
														vs204-4
														vs206-2
	15	WV-8638												
In situ	16	WV-8471				+								vs203-2
														vs206-2
	2	WV-8335	+		+		+							vs19-6
														vs202-8
	10	WV-8642			+									VS204-4

*Only the primer combinations that resulted in positive signals are listed. + = positive signal visible only after hybridization; ++ = positive signal visible after ethidium bromide staining.

†Data obtained with HVP2/C primers (*11*).

‡WV-8240 was a primary tumor and WV-8338, WV-8467, and WV-8543 were the first, second, and third tumor recurrences, respectively, at that tumor site.

sample of CIS, and in another sample of CIS an uncharacterized signal was obtained. The analyses of the BCC samples revealed that three (60%) of the five samples tested exhibited previously uncharacterized signals. All the samples resulting in such uncharacterized signals, as well as those that had tested negative, were analyzed in the present study. HPV DNA sequences could be isolated from five additional SCC samples (total positive = 13/20; 65%), whereas faint signals were detected in the remaining seven samples.

It was not possible to characterize these faint signals with the presently applied method. These faint signals could indicate the presence of yet unidentified HPV sequences that share a very distant DNA homology to known types, or they could result from nonspecific amplification and hybridization of cellular sequences. Similar results were obtained in the analysis of the specimens from BCC lesions. HPV DNA sequences were identified in three (60%) of five samples; the remaining two samples yielded faint signals (Table 1, B). The analysis of the CIS lesions revealed three of four samples containing HPV sequences, all putatively new HPV types. Two of these HPV types, vs206-2 and vs204-4, were previously identified in one SCC sample and in one BCC sample, respectively.

Several specimens gave positive signals with more than one primer combination (Table 1, B). Only a few recombinant clones from individual tumors were subsequently sequenced. The specimens WV-8240, WV-8490a, WV-8327, and WV-8489 each contained more than one HPV DNA sequence. WV-8240 harbored HPV54 as well as HPV61. WV-8490a harbored the previously described putatively new HPV sequence vs20-4 (*11*) as well as sequences of three additional putatively new HPV types, vs205-1, vs206-2, and vs207-22. WV-8327 contained HPV56 and HPV60 DNA. WV-8489 contained HPV51 DNA, as well as the newly identified vs204-4 and vs206-2 sequences. The results are summarized in Table 2.

Table 2. Summary of the human papillomavirus (HPV) sequences identified in skin tumors from renal allograft recipients

Sequence	No. of HPV-positive tumors		
	Carcinoma in situ	Squamous cell carcinoma	Basal cell carcinoma
Known HPV			
HPV16		1	
HPV20	1		
HPV41		1	
HPV51			2
HPV54		1	
HPV56			1
HPV60			1
HPV61		1	
HPV69		4	
Putative new HPV			
vs19-6	1	1	
vs20-4		1	
vs93-1		3	
vs201-1		1	
vs202-8	1		
vs203-2	1		
vs204-4	1		1
vs205-1		1	
vs206-2	1	1	1
vs207-22		1	
No. of HPV DNA positive/ total No. (%)			
Specimens	4/4 (100)	13/20 (65)	3/5 (60)
Patients	4/4 (100)	8/11 (73)	3/4 (75)

In a few cases, several specimens originating from a single patient were analyzed. An example of the HPV DNA sequences detected in such a group of specimens is shown in Table 3. This table includes results obtained in the previous study (*11*) and in

Table 3. Multiple lesions obtained from an individual renal allograft recipient

Type of lesion	Specimen No.	Location	HPV*
Common wart	WV-8464	Left arm	vs73-1
	WV-8466	Upper left arm	HPV6b + vs75-3
	WV-8465	Left shoulder	vs73-1 + vs93-1
Dysplastic wart	WV-8238	Scalp	HPV54
	WV-8460	Left forehead	vs93-1
	WV-8334	Scalp	HPV69
Carcinoma in situ	WV-8335	Left hand	vs19-6 + vs202-8
Squamous cell carcinoma	WV-8239	Right hand	vs19-6
	WV-8461	Right forehead	vs93-1
	WV-8240†,‡	Left hand	HPV54 + HPV61
	WV-8336	Right forearm	HPV69
	WV-8337	Right cubital fossa	HPV69
	WV-8338†,§	Left hand	HPV69
	WV-8467†,‖	Left hand	Uncharacterized
	WV-8543†,¶	Left hand	Negative
No. HPV DNA positive/ total No. of samples (%): 13/21 (62)			

*HPV = human papillomavirus.
†Recurrent lesion.
‡Primary tumor.
§First recurrence.
‖Second recurrence.
¶Third recurrence.

the present study. A total of 21 specimens, ranging from benign to malignant and taken from different locations on the body, were analyzed. Of these, 13 contained detectable HPV DNA, including known as well as putatively new HPV types (HPV6b, HPV54, HPV61, HPV69, vs73-1, vs75-3, vs93-1, vs19-6, and vs202-8). Four biopsy specimens of one of the recurrent lesions on the left hand of the patient (as indicated in Table 3) were analyzed. The HPV types detected in each recurrent specimen differed from the HPV types in the lesion removed before.

HPV DNA in Nonmelanoma Carcinomas of the Skin and in Keratoacanthoma From Nonimmunosuppressed Patients

This group of samples (26 SCC, 11 BCC, and four keratoacanthoma samples) was analyzed by use of only the presently described primer combinations. The results are summarized in Table 4. The positive signals obtained were generally much weaker than those observed for the renal allograft recipient group, and fewer primer combinations gave positive signals within one sample tested (Table 4). Positive hybridization signals were obtained in 16 (62%) of 26 SCC specimens. HPV-specific DNA sequences were identified in eight (31%) of 26 SCC specimens, whereas the signals (i.e., the hybridization-positive amplification products) from the additional eight specimens could not be sequenced. Four (36%) of the 11 BCC samples contained HPV DNA sequences, and faint signals were seen within two additional samples (18%), which resulted in six (55%) of 11 with a positive hybridization signal. Most positive samples contained sequences of known HPV types, and only

Table 4. Detection of human papillomavirus (HPV) sequences in nonmelanoma carcinomas of the skin and keratoacanthoma* from nonimmunosuppressed patients

Type of lesion	Specimen No.	Primer combination†					HPV type
		A1/B5	F10/B5	F11/B5	F12/B5	F22/B11	
Keratoacanthoma	WV-7153						
	WV-7235				+		HPV6b
	WV-7263						
	WV-8396		+				HPV34
Squamous cell carcinoma	WV-6878						
	WV-6880		+		+		HPV9
	WV-6884c						
	WV-7135ii						
	WV-7394						
	WV-7457						
	WV-7669i						
	WV-7669ii						
	WV-7675						
	WV-7680ii		+				HPV23
	WV-7770i						
	WV-7770ii						
	WV-7802i						
	WV-7802ii						
	WV-7804i						
	WV-7804ii						
	WV-7804iii		+				HPV42
	WV-7804iv		+				HPV42
	WV-7805						
	WV-8067						
	WV-8251i		++		+		HPV32
	WV-8251ii						
	WV-8417	+		++			HPV51
	WV-8632						
	WV-8680i					++	HPV4
	WV-8705		++		+		HPV8
							vs208-1
Basal cell carcinoma	WV-6462						
	WV-6463	++	+	+			HPV7
	WV-6464						
	WV-6465	+					vs200-1
	WV-6495	+				++	HPV25
							HPV4
	WV-7641i		+				HPV6b
							HPV8
	WV-7663i						
	WV-7663ii						
	WV-7663iii						
	WV-7678						
	WV-8797ii						

*Keratoacanthoma is a nonmalignant lesion of the skin for which the natural history has not been defined.

†Only the primer combinations that resulted in positive signals are listed. + = positive signal only after hybridization; ++ = positive signal after ethidium bromide staining.

two specimens harbored putatively new HPV sequences (vs208-1 and vs200-1). The HPV DNA sequences detected in the non-immunosuppressed patients are summarized in Table 5.

Putative New HPV Types

We reported the identification of nine putative novel HPV types in the previous study (*11*). Most of these sequences shared a nucleotide homology to known epidermodysplasia verruciformis–HPV types. A total of 10 newly identified HPV DNA sequences resulted from this study. Eight of the 10 shared a sequence homology to viruses in the HPV4, HPV48, HPV50, HPV60, and HPV65 groups. The remaining two, vs200-1 and vs208-1, shared homology to HPV24 and HPV68, respectively. Each of these sequences was cloned into the pGEM-T vector, and both strands of the inserted DNA were sequenced. By sequence comparison with all the known papillomavirus genomes, each one of these sequences shared less than 90% homology to any known type (Table 6), which thus identified them as fragments of yet to be isolated HPV genomes. The sequences of the cloned fragment of the putative novel types will appear in the nucleotide sequence databases of the European Molecular Biology Laboratory, GenBank, and the DNA Databank of Japan (National Institute of Genetics, Mishima, Shizuoka, Japan) under the following accession numbers: vs19-6—X89876, vs200-1—X89877, vs201-1—X89878, vs202-8—X89879, vs203-2—X89880, vs204-4—X89881, vs205-1—X89882, vs206-2—X89883, vs207-22—X89884, and vs208-1—X89885.

Discussion

Different groups of investigators have screened various numbers of samples of nonmelanoma carcinomas of the skin from immunosuppressed and nonimmunosuppressed populations for the presence of HPV DNA sequences; this screening resulted in a diverse spectrum, not only of the percentage of positive samples, but also in the types of HPV detected (*10-13,16-19*). This result can be attributed largely to the different tests applied in the analyses. The sensitivity of the methods (ranging from in situ hybridization assays and PCR analyses) varied dramatically. The number of probes used differed in each study; as a result, the number of known HPV types that could be detected was limited. Several groups investigating lesions from renal allograft recipients reported the presence of epidermodysplasia verruciformis-specific HPV types. The methodology utilized involved PCR with use of either specific (*10*) or degenerate (*12,19*) primers designed to detect primarily epidermodysplasia verruciformis-associated HPV types.

As we recently reported (*11*), we detected a broad spectrum of HPV types, including known as well as newly identified ones, in benign and malignant lesions obtained from a group of renal allograft recipient patients. HPV41 and the newly identified vs93-1 (HPV29-related) sequences were detected in 20% of the malignant lesions. A large number of the SCC and BCC samples contained faint signals that could not be characterized by the PCR method used at the time. We extended this study by splitting the primers into a larger series of primer combinations, each with decreased degeneracy (*15*). Additional DNA samples were included, i.e., specimens obtained from nonimmunosuppressed patients. These two groups of samples differed markedly in the HPV DNA detectability as well as in the HPV types present. HPV DNA could be amplified in samples from the renal allograft recipient group with at least 11 of the 16 primer combinations used. As many as eight primer combinations yielded positive signals (several of equal intensity) in a single sample (Table 1). Different HPV sequences could be detected in the different products (e.g., WV-8327). In contrast, only a limited number (n = 5) of primer combinations yielded positive results in the nonimmunosuppressed group, possibly indicating the presence of a limited number of HPV sequences. No more than two primer combinations gave positive results within one specimen. These signals varied in intensity; however, upon sequencing of both products, the presence of only one HPV type was revealed.

Table 5. Summary of the human papillomavirus (HPV) sequences identified in skin tumors from nonimmunosuppressed patients

Sequence	No. of HPV-positive tumors		
	Keratoacanthoma	Squamous cell carcinoma	Basal cell carcinoma
Known HPV			
HPV4		1	1
HPV6b	1		1
HPV7			1
HPV8		1	1
HPV9		1	
HPV23		1	
HPV25			1
HPV32		1	
HPV34	1		
HPV42		2	
HPV51		1	
Putative new HPV			
vs200-1			1
vs208-1		1	
No. HPV DNA/ total No. positive (%)			
Specimens	2/4 (50)	8/26 (31)	4/11 (36)
Patients	2/4 (50)	7/19 (37)	4/9 (44)

Table 6. Putative new human papillomavirus (HPV) sequences identified in skin tumors

Sequence	Closest prototype*	% similarity
vs200-1	HPV24	80.7
vs19-6	HPV65	69.1
vs201-1	HPV48	69.4
vs202-8	HPV48	66.3
vs203-2	HPV65	66.9
vs204-4	HPV65	66.4
vs205-1	HPV4	69.1
vs206-2	HPV48	68.7
vs207-22	HPV48	76.6
vs208-1	HPV68	81.8

*Homology corresponding to the following nucleotides in the respective L1 open reading frames (nucleotide [nt] numbers in the context of the complete genomic sequence, except for HPV68, for which the complete genomic sequence is not available): HPV4 = nt 5924-6345; HPV24 = nt 6295-6728; HPV48 = nt 5769-6190; HPV65 = nt 5905-6326; HPV68 = nt 620-1004.

The HPV types detected in the malignant lesions of the renal allograft recipient group included eight known HPV types and 10 putatively new HPV types (including vs93-1 [HPV29-related], of which the complete genome has recently been cloned). Two of the known types, HPV41 and HPV60, had been previously isolated and associated with cutaneous lesions (*20-22*). The remaining six HPV types (HPV16, HPV51, HPV54, HPV56, HPV61, and HPV69) all have been associated with mucosal lesions, mainly malignant genital lesions (*23-26*). This finding is very unusual, because even though HPV16 DNA has been demonstrated regularly in premalignant cutaneous changes of the hand [(*27*); reviewed in (*28*)], these other mucosal HPV types have not yet been detected in lesions of cutaneous origin.

The putative new HPV types detected in the renal allograft recipient group are all related to the HPV4 group of viruses, thus enlarging this group of phylogenetically related HPV types from four to 11 (Fig. 2).

The HPV types identified in the specimens from the nonimmunosuppressed group fall into a completely different range. We could identify sequences of 11 known HPV types (Table 5). HPV8, HPV9, HPV23, and HPV25 belong to the epidermodysplasia verruciformis-specific HPV types; HPV8 is associated with malignant lesions, and HPV9, HPV23, and HPV25 are associated with benign lesions (*8,29*). HPV7, detected in these lesions, was originally associated with the so-called "butchers' wart" (*30*) and subsequently was detected frequently in oral mucosal and facial skin lesions in individuals infected with the human immunodeficiency virus (*31*). HPV7 has not been detected previously in malignant lesions. In addition, the presence of a number of mucosa-associated HPV types has again been demonstrated, although most of them are usually grouped with the low-risk genital types (*32*). HPV6b and HPV42 are associated with condylomata acuminata (*26,33*). HPV34 was isolated from Bowen's disease of the skin (*34*), but subsequently it was associated more frequently with genital intraepithelial neoplasias. HPV32 has been isolated from an oral lesion of focal epithelial hyperplasia (*35*) and has also been detected in large numbers of oral papillomas [(*36*); our unpublished results], but to date, it has not been detected in cutaneous lesions. HPV51 has been isolated from a benign genital lesion (*37*), but subsequently, it was grouped with the high-risk genital HPV types (*26*). Thus, with the exception of HPV51, the mucosal HPV types detected in these skin lesions have not been reported to cause malignant transformation in tissues. Only two putatively new HPV sequences could be isolated. The sequence of the first one, vs200-1, is related to HPV24 (an epidermodysplasia verruciformis-specific HPV type), whereas the sequence of the other, vs208-1, is related to HPV68 (another high-risk genital HPV type) (*25*).

Allograft recipients are at increased risk of developing nonmelanoma carcinomas of the skin; constant immunosuppressive therapy, solar radiation, and viral warts are the main risk factors (*38,39*). A high percentage of these lesions develop within the first 10 years after transplantation. Nonmelanoma carcinomas of the skin in the general population usually present in patients older than 60 years of age. A changing pattern in incidence rates for this latter group has been reported (*3,4,40*); UV radiation is the most important cofactor, causing mutations in the host DNA as detected in the high rate of mutations in the p53 gene (*2,5*).

In this study, we were able to identify HPV DNA sequences in 64% of the nonmelanoma carcinomas of the skin (i.e., squamous cell and basal cell carcinomas) obtained from renal allograft recipients and in 32% of those obtained from nonimmunosuppressed patients. Our results suggest that another factor, HPV infection, may be involved in the etiology of these carcinomas. Our results also indicate a probable difference in the HPV types involved in the etiology of the nonmelanoma carcinomas of the skin developing in renal allograft recipients or in nonimmunosuppressed patients. The high frequency of HPV types usually associated with mucosal but not cutaneous lesions indicates that the presence of papillomavirus DNA is much more widespread than previously thought and that these infections probably remain subclinical or latent under normal circumstances. It is likely that the mutagenic effects of cofactors such as UV radiation (over a prolonged period of time) result in changes mainly affecting the cellular genome that lead directly or indirectly to the activation of viral growth-promoting genes. The suppression of the immune system by immunosuppressive drugs appears to be responsible for the failure to eliminate these cells. The HPV types detected in the nonmelanoma carcinomas of the skin developing in renal allograft recipients after a relatively short latency after onset of the immunosuppressive therapy belong to the group of genital HPV types. The spectrum of HPV types detected in genital lesions of immunosuppressed patients does not, however, seem to vary from those types detected in the immunocompetent population, although the risk for such infections is increased in the immunosuppressed patients. Immunosuppression also seems to lead to an increased HPV replication (*41*). A number of genital HPV types detected in the cutaneous lesions described in the present study readily immortalize primary keratinocytes (*6*). Through its binding to p53, the E6 gene of these HPVs plays an active role in the degradation of this protein (*42*), hindering DNA repair. As a result, chromosomal instability occurs, which probably leads to the inactivation of cellular genes whose normal function is to suppress the functional activity of the HPV genome (*7*). Additional studies are required to demonstrate whether the E6 gene of the mucosal HPV types detected in the nonmelanoma carcinomas of the skin from nonimmunosuppressed patients does not cause this chromosomal instability and may therefore be dependent on cofactors that inactivate p53 and possibly other cellular regulatory genes.

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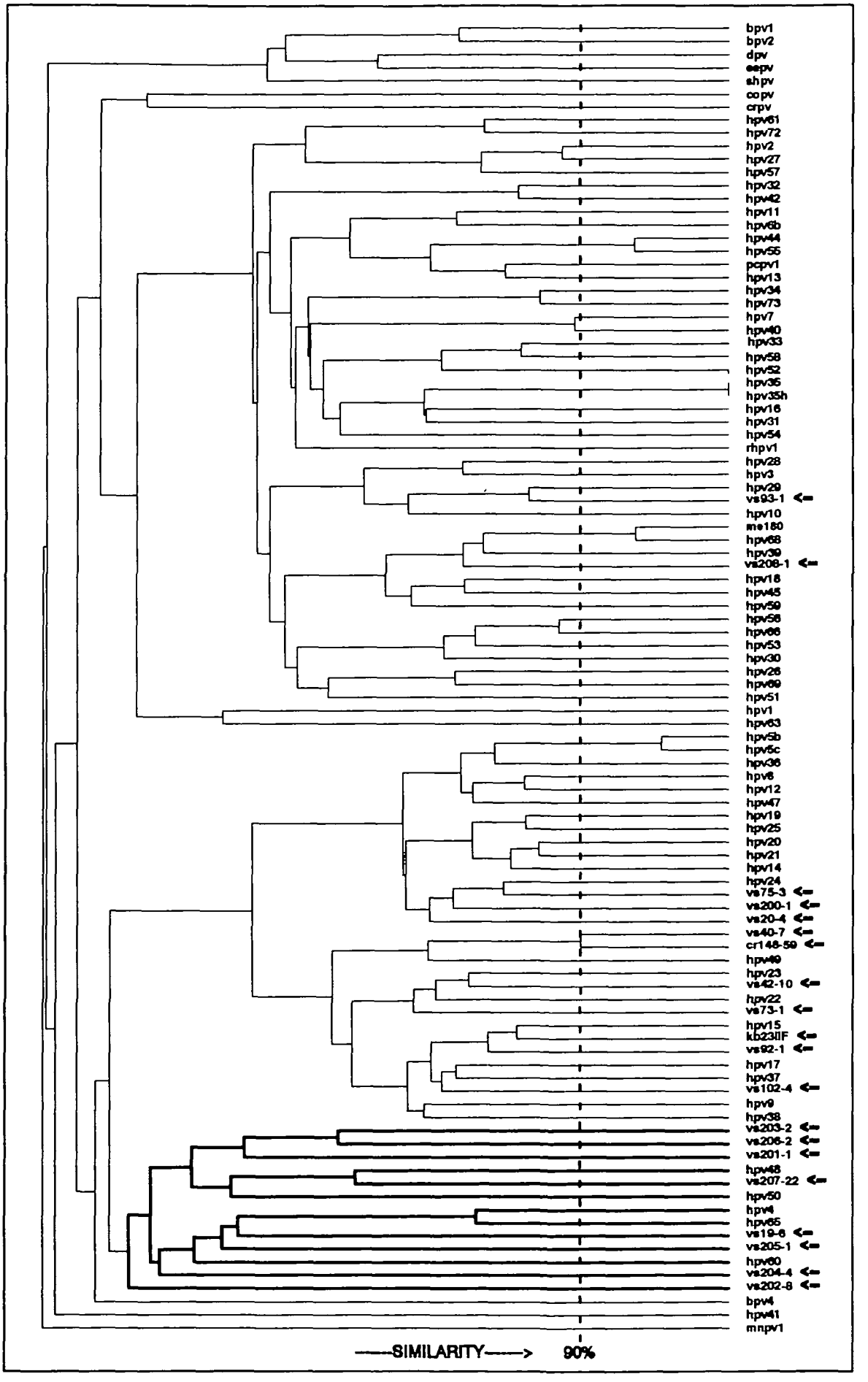

Fig. 2. Phylogenetic tree based on the partial sequence of the L1 open reading frame. Distance along the horizontal axis is proportional to the similarity between sequences. Putative new sequences are marked by an arrow.

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Notes

Supported by the Bundesministerium für Gesundheit, Bonn.

We thank Marion Frick, Helene Rahn, and Armin Keller for excellent technical assistance.

Manuscript received October 27, 1995; revised March 8, 1996; accepted March 21, 1996.