Human Papillomaviruses are Commonly Found in Normal Skin of Immunocompetent Hosts

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We have previously demonstrated, by the combined application of two degenerate polymerase chain reaction primer sets, the presence of human papillomavirus (HPV) DNA in 91% of cutaneous squamous cell cancers from renal allograft recipients, with multiple types being present in one-third of these tumors. Five HPV types – HPV 20, HPV 23, HPV 38, DL40, and DL267 – accounted for 73% of positive results. These HPV types are all related to the epidermodysplasia verruciformis group, and HPV 38 was originally isolated from a melanoma. The aims of this study were to determine: (i) whether HPV DNA could readily be demonstrated in skin tumors, as well as in perilesional skin, of immunocompetent patients using two polymerase chain reaction primer sets; (ii) the prevalence of infections in normal skin; and (iii) the prevalence of HPV 38 or HPV 38 related viruses in melanoma. The HPV types detected in lesions from renal allograft recipient were present not only in the perilesional skin and tumors of immunocompetent patients, but also in 35% of normal skin biopsies. HPV DNA was present in 13% of the melanoma samples, but none harbored HPV 38 DNA. We identified four putatively new HPV types. Infections with different types of human papillomavirus are widespread and often occur in clinically normal skin. *In vitro* studies are required to determine the specific molecular mechanisms by which these HPV types may be involved in the etiology of nonmelanoma skin cancer.

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Nonmelanoma skin carcinoma (NMSC) is the most frequently occurring malignancy worldwide in the Caucasian population (Preston and Stern, 1992). The distribution of these tumors on sun-exposed sites, suggests ultraviolet (UV) radiation as a major environmental factor in their pathogenesis (Frost and Green, 1994). Moreover, the pattern of mutations present in the suppressor gene p53 in these tumors is typical of UV radiation induced DNA damage (Ziegler *et al*, 1993; Rees, 1994). Patients with epidermodysplasia verruciformis are predisposed to develop extensive viral warts that, on sun-exposed sites, frequently progress to NMSC, especially squamous cell carcinomas (SCC). A specific group of closely related human papillomavirus (HPV) types, the epidermodysplasia verruciformis group, especially HPV 5 and HPV 8, have been isolated from over 90% of SCC from these patients (Orth, 1987). Organ allograft recipients also frequently develop multiple cutaneous tumors ranging from viral warts to SCC, and these occur predominantly on sun-exposed sites (Penn, 1991). A possible role for epidermodysplasia verruciformis and other HPV types in the carcinogenic process has been investigated over the past 12 y, but results have shown enormous variation in the frequency and type of HPV DNA detected, depending on the method employed. Moreover, the sensitivity and specificity of the detection methods have improved considerably over the last decade, leading to the identification of many more HPV types; there are now more than 130, of which 82 have so far been fully characterized.

We have recently demonstrated the presence of known, as well as putative new HPV types in 33% of NMSC biopsies taken from immunocompetent patients (Shamanin *et al*, 1996) and in 91% taken from immunosuppressed renal allograft recipients (RAR) (Shamanin *et al*, 1994b, 1996; de Villiers *et al*, 1997). These studies were performed applying a degenerate polymerase chain reaction (PCR) technique including 16 different primer combinations (HD primers) per sample (Shamanin *et al*, 1994a), or, in the latter study, in combination with a modified version of the PCR method (AM primers) described by Berkhout *et al* (1995). Of the 227 samples analyzed, ranging from benign to premalignant and malignant lesions, 47 putative new HPV types were identified, as well as a wide spectrum of fully characterized HPV types. In biopsies obtained from RAR, a combination of the two above-mentioned PCR methods resulted in the detection of more than one HPV type in one-quarter of all specimens tested, and five HPV types (HPV 20, HPV 23, HPV 38, DL40, and DL267) were present in 73% of the cancers (de Villiers *et al*, 1997). HPV 20 and HPV 23 (Kremsdorf *et al*, 1984) were both originally isolated from epidermodysplasia verruciformis lesions, whereas HPV 38 was first isolated from a cutaneous melanoma of the skin (Scheurlen *et al*, 1986). DL 40 and DL 267 are partial sequences of the L1 open reading frame of two putatively new HPV types (de Villiers *et al*, 1997).

This combination of methods has not been used previously to analyze SCC and basal cell carcinoma (BCC) from immunocompetent

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Abbreviations: BCC, basal cell carcinoma; IEC, intraepidermal carcinoma; NMSC, nonmelanoma skin cancer; RAR, renal allograft recipient.

patients for the presence of HPV DNA. Using these methods our aims in this study (which was not intended to be an epidemiologic study) were to determine whether the same range of HPV types as detected in RAR could be demonstrated in tumors from immunocompetent patients. In addition, we wished to determine whether HPV DNA was present in perilesional skin adjacent to tumors, as well as in normal skin samples obtained from patients undergoing cosmetic surgery. Finally, we analyzed several biopsies from melanoma for the presence of HPV DNA, especially HPV 38 DNA.

MATERIALS AND METHODS

Patients Twenty-one skin biopsies from immunocompetent patients were examined, including one biopsy from a self-healing epithelioma, one sebaceous epithelioma, one verrucous keratosis, two actinic keratoses, six intraepidermal carcinoma (IEC), three BCC, one SCC, and six samples of uninvolved perilesional skin (which had histologically been confirmed as normal skin) adjacent to the sebaceous epithelioma, verrucous keratosis, one actinic keratosis, two IEC, and one BCC. These biopsies were taken from seven male and 16 female patients, with a mean age of 78 y (range 44–99). The diagnoses were confirmed by histology in all cases. Fifteen biopsies from melanomas were obtained from 13 patients, nine male and four female, mean age 66 y (range 47–85). Twenty samples of normal eyelid skin were obtained from 15 patients, 10 male and five female, mean age 51 y (range 41–69), undergoing cosmetic surgery. All biopsies were snap frozen and stored at −70°C until use. Patient consent was obtained for removal of all material and use where necessary.

DNA extraction Samples were digested with Proteinase K (100 μg per ml) in the presence of 0.5% sodium dodecyl sulfate for 2 h at 65°C. This was followed by phenol-chloroform-isoamyl alcohol extraction and precipitation with ethanol. At every stage of the analysis, great care was taken to avoid contamination of samples.

PCR analysis Samples of total cellular DNA (100 ng) were amplified by PCR using either one or both sets of degenerate primers (HD and AM). These two sets of primers cover adjacent sequence stretches within a highly conserved region of the L1 open reading frame of papillomaviruses. The first set (HD) consisted of 16 different primer combinations described by Shamanin *et al* (1994a), as well as two additional combinations (de Villiers *et al*, 1997). Eighteen rounds of amplification, each time using a different primer combination, were performed in 40 cycles after an initial denaturation at 94°C for 4 min. Each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 52°C, and 1 min at 72°C with an auto-extension of 2 s per cycle at 72°C. The final extension was for 6 min at 72°C in the last cycle followed by cooling at 4°C. All amplified products were subjected to electrophoresis and the subsequent Southern blots hybridized to a radiolabeled degenerate probe (Shamanin *et al*, 1996). Virus-specific PCR products were gel-purified and subjected to a semi-nested PCR amplification as previously described (Shamanin *et al*, 1996). The amplified DNA was gel-purified and cloned by use of the pGEM-T Vector Cloning Kit (Promega, Madison, WI). The second set of primers (AM) consisted of the forward primer CP65 and the backward primer CP70 for the initial amplification, followed by a nested amplification with the primers CP66 (forward) and CP69 (backward), as described previously (Berkhout *et al*, 1995). This method was modified by adding a BamH1-linker to each primer to facilitate subsequent cloning of the products, and by lowering the annealing temperature in each cycle from 55°C to 50°C. All amplified products obtained after the nested amplification were purified and the products cloned using the Original TA Cloning Kit with the vector pCR™ 2.1 (Invitrogen, Carlsbad, CA). HPV 38 specific primers were selected in the region of the L1 open reading frame in which the CP66/CP69 primers are located. The forward primer used was (5′-3′)ttggatccTGGTATTTTATGGGGCAATC (nt 990–1010 of HPV 38) and the backward primer used was ttggatccCAATTTTTCAGT-CATGTCCACA (5′-3′) (nt 1375–1396 of HPV 38; Hajo Delius, Deutsches Krebsforschungszentrum, Germany, unpublished results. Southern blots containing the amplified products were hybridized to a radiolabeled probe specific for HPV 38 (5′-ATGCTGAAGTGCTGACACAG-3′, nt 1182–1201). Amplified bands were purified and cloned as described above.

A maximum of 10 recombinant clones per sample per primer pair was sequenced (Sequenase 2.0 DNA Sequencing Kit, U.S. Biochemical, Cleveland, OH).

Sequence analysis The sequences obtained were compared with the available HPV sequences in the EMBL and GenBank Databanks using the HUSAR software package (Deutsches Krebsforschungszentrum). Only one strand was initially sequenced. If a sequence shared 94% or more homology to any known HPV sequence, it was regarded as that type. If the homology was below 94%, both strands of this cloned insert were sequenced in full. Both DNA strands of the cloned PCR fragment were sequenced in full. The respective nucleotide lengths and accession numbers in the EMBL Nucleotide Sequence Database are as follows: GA1–3 437 bp (AJ000151), GA3–1 437 bp (AJ001058), GA6–2 389 bp (AJ001059), and GA9–4 380 bp (AJ001060).

GA1-3:
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1   AATCAACTGT TTATTACAGT GGTGGACAAC ACAAGAAACA CAAACTTCAG
51  TATTAGTGTG TATAGTGAAG CAGGTAAAGT AAAGGATATT TCAGATTATG
101 ATGCAAACAA ATTTAGGGAA TATCAAAAAC ATGTAGAAGA ATATGAAATT
151 TCTTTAATAT TACAACTATG TAAGATACCT TTAAAAGCCG ACGTGTTGGC
201 ACAAATTAAT GCAATGAATC CATCGTTATT AGAAGAGTGG CAACTGGGGT
251 TTGTACCTGC ACCAGACAAT CCATTGCAAA GTACCTATAG GTATATCGAT
301 AGCTTGGCCA CACCATGTCC TGATAAAGTG CCTACCAAAG AAAAGGAAGA
351 TCCATATGCT CCGTTTACAT TTTGGAACGT TGATTTGACA GAAAGACTTT
401 CCTTGGAACT GGATCAATAT TCTCTGGGAC GAAAGTT
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GA3-1:
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1   AATCAGATGT TTATTACTGT TGTAGACAAC ACACGCAGCA CAAATTTTAG
51  TATATCAGTT CACACAGAAA ATCAAGATAT ATCTAAAATT GACAGTTTTG
101 ATGCAACTCA GTTTAGGGAA TACTTAAGAC ATGTAGAGGA ATATGAGATT
151 TCTATAATAT TACAGTTATG TAAGATTCCT CTGAAAGCAG AAGTCTTAGC
201 ACAAATTAAT GCAATGAATT CTTCATTACT TGAAGACTGG CAACTTGGCT
251 TTGTGCCGAC GCCTGATAAT CCAATTCATG ATACGTACAG ATATATTGAT
301 TCTTTGGCAA CACGGTGCCC TGATAAGACG CCTCCAAAGG AAAAACCTGA
351 TCCATATGAA AAGTTACATT TTTGGAATGT GGACCTTACC GAACGTCTGT
401 CTTTAGATTT AGATCAATAT CCTCTGGGAC GAAAGTT
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GA6-2:
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1   GTTAGATCTA CATTCCAGAA TGTTAATTTT TCATAAGGAT CAGGTTTTTC
51  CTTTGGTGGA TTTTTATCGG GACACCTGGT AGCTAAGGAA TCAATAAATC
101 TGTATGTGTC CTGGATAGGA TTATCGGGAG TTGGCACAAA TCCTAACTGC
151 CAATCCTCTA GTAAGGAGGG GTTCATTGCA TTTATTTGTG CTAAAACTTC
201 TGCTTGTAGT GGAATCTTAC ACAACTGTAA AATAATAGAA ATTTCATATT
251 CTTCTACGTG CCTTAAATAT TCCCTAAACT TTTGTGAGTC ATATGATTGT
301 ATTTGCTGTA TATCTTGATT TTCACTAGAT ATAGATATAC TGAAATTGGT
351 GTTTCGCGTG TTGTCTACAA CTGTAATAAA CATTTGATT
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GA9-4:
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1   AATCAACTGT TTGTTACAGT TGCAGATAAT ACAAGGAATA CCAATTTTAC
51  TATAAGTGTA ACATCTAATG GTACCCCCAT AGCAGAATAT GATTCCAAAA
101 CTATTAGAGA ATTTTTAAGG CACGTAGAAG AATATCAGTT GTCCATGATA
151 TTGCAATTAT GTAAAGTACC TTTAAAAGCA GAAGTTTTAT CCCAGATTAA
201 TGCTATGAAT TCAGGTATTT TGGAGGAGTG GCAGTTAGGT TTTGTGCCTA
251 CACCAGACAA CTCTGTACAT GATATTTATA GATATATTGA CTCAAAAGCA
301 ACAAAATGTC CCGATGCAGT GCCTGCAAAA GAAAAAGAAG ATCCATTTGA
351 CAAATATACA TTTTGGAATG TAGATCTAAC
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Figure 1. Sequences of newly identified HPV types.

RESULTS AND DISCUSSION

Papillomavirus infections have been confirmed as the most important factor in the etiology of squamous carcinomas of the genital tract. The two transforming genes E6 and E7 of the "high-risk" genital HPV types HPV 16 and HPV 18, have been shown to interact with cellular factors in deregulating the normal growth of the cell (reviewed in zur Hausen, 1994, 1996). In addition, HPV 16 DNA by itself is capable of inducing modifications in the host cell and thereby immortalizing human keratinocytes. The "low-risk" genital HPV types HPV 6 and HPV 11, in contrast, probably depend on the influence of external factors to cause such mutations and do not readily immortalize human keratinocytes *in vitro*.

The anatomical distribution of the NMSC suggests that UV radiation is a major exogenous factor by causing point mutations in genes controlling the cell cycle (Ziegler *et al*, 1993), thereby leading to deregulation of normal cell growth. The presence of HPV DNA (including three putative new HPV types identified in this study) in 35% of normal skin samples tested, suggests that HPV DNA may be widely distributed in normal skin of the immunocompetent population in whom an intact immune system probably inhibits the development of clinical disease. A similar detection rate has been reported for HPV infections in the normal tissue of the genital tract (reviewed in IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 1995).

Previous studies on HPV DNA detection by PCR demonstrated a wide spectrum of HPV types in benign and malignant cutaneous lesions occurring in immunosuppressed populations (Shamanin *et al*, 1994b, 1996; Berkhout *et al*, 1995; de Jong-Tieben *et al*, 1995). In our previous study, in which we combined available PCR methods, HPV DNA was demonstrated in 91% of the SCC obtained from RAR, with HPV 20, HPV23, HPV 38, DL40, and DL267 present in

Table I. HPV DNA in tumors and perilesional skin from immunocompetent patients

Patient	Ref. No.	Site	Diagnosis[a]	HPV	
				HD[b]	AM[c]
KL	WV-9086	back	self-healing epithelioma	neg	neg
HC	WV-9095	ear	sebaceous epithelioma	neg	neg
	WV-9094	ear	perilesional skin	neg	20, DL267
HA	WV-9080	wrist	verrucous keratosis	75	DL231, GA6–2
	WV-9079	wrist	perilesional skin	neg	20, 24
MM	WV-9085	forehead	actinic keratosis	neg	neg
	WV-9084	forehead	perilesional skin	neg	9, 38
MK	WV-9093	upper lip	actinic keratosis	vs73–1	DL267
CH	WV-9075	leg	IEC	neg	neg
MJ	WV-9076	nose	IEC	neg	DL287, GA1–3
ED	WV-9078	hand	IEC	neg	20
JD	WV-9082	leg	IEC	neg	neg
	WV-9081	leg	perilesional skin	neg	neg
	WV-9087	scalp	IEC	neg	neg
CG	WV-9092	cheek	IEC	neg	20
	WV-9091	cheek	perilesional skin	neg	neg
JG	WV-9077	nose	BCC	neg	neg
IM	WV-9089	temple	BCC	neg	neg
	WV-9088	temple	perilesional skin	neg	neg
HJ	WV-9090	back	BCC	neg	neg
VH	WV-9083	leg	SCC	neg	neg

[a]SCC, squamous cell cancer; BCC, basal cell cancer; IEC, intraepidermal cancer.
[b]Primer set described by Shamanin *et al* (1996).
[c]Primer set described by Berkhout *et al* (1995).

Table II. Putative new HPV types and their homology to known HPV types

Putative new HPV type	Closest related HPV type	% Homology	Fragment size
GA1–3	HPV 24	80	437 bp
GA3–1	HPV 8	79	437 bp
GA6–2	HPV 12	81	389 bp
GA9–4	HPV 15	84	380 bp

73% of the NMSC tested. These HPV types were often found in combination with another HPV type within the same lesion (de Villiers *et al*, 1997). In this study of immunocompetent patients, "double" infections with these HPV types could also be demonstrated in biopsies from lesional and normal perilesional skin.

The 21 samples from 14 immunocompetent patients were examined using both primer sets (**Table I**). Lesions containing HPV DNA included an actinic keratosis, a verrucous keratosis, and three of six (50%) IEC (**Table I**). Three of six (50%) of the perilesional skin samples contained HPV DNA. The corresponding lesional skin was negative in two cases, and the third harbored HPV types different from those detected in the adjacent lesion. The HPV types detected in these samples included three of the HPV types previously occurring most frequently in nonmelanoma skin cancer of RAR, i.e., HPV 20, HPV 38, and DL267 (de Villiers *et al*, 1997). Two putative new HPV types were also identified in this group. GA6–2, in addition to DL231, was detected in a verrucous keratosis and GA1–3 with DL287 in an IEC. A summary of the four putative new HPV types described in this study, with their homologies to the closest known HPV types, is given in **Table II**.

HPV 38 DNA was originally isolated from a melanoma (Scheurlen *et al*, 1986). We therefore tested 15 melanoma biopsies using PCR with the CP65/CP69 primers and subsequent nested amplification. Only two of 15 biopsies (13%) contained HPV DNA sequences, i.e., DL297 (HPV 5 related) and DL284 (HPV 20 related). All these samples were subsequently amplified with the HPV 38 specific primers, but all were negative for HPV 38 or related DNA sequences. HPV 38 and a newly identified HPV type, DL267 (closest related to HPV 38), were demonstrated in nine of 33 (30%) of the malignant lesions tested in our previous study (de Villiers *et al*, 1997); however, the detection of other HPV types in only two of 15 (13%) samples from melanomas does not support a specific role for HPV 38 in the pathogenesis of this tumor.

We examined normal skin from randomly selected patients, i.e., undergoing cosmetic surgery, in addition to the samples of perilesional skin. PCR was performed using the second set of primers (AM) only. HPV DNA was detected in seven of 20 samples (35%) from six of 15 patients. Two biopsies each were taken from three of the patients with positive samples. Both samples in the one patient contained the putative new HPV type GA1–3, and another putative new HPV type, GA9–4, was isolated from one of the two samples available from the second patient. A third putative new HPV type, GA3–1, was present in another sample of normal skin. HPV DNA has also recently been detected in plucked hairs from renal transplant recipients (92% positive), as well as from healthy volunteers (53% positive) (Boxman *et al*, 1997). These hair samples were taken from different areas of the body. In the results reported in this study, HPV DNA was not only detected in the normal skin from the eyelids, but also in samples from other body sites (wrist, ear, forehead, temple, cheek, and leg).

The presence of HPV, and specifically some of these HPV types, in normal skin supports our previous observation (de Villiers *et al*, 1997) that infection with a broad spectrum of HPV types occurs frequently, but that the immune system may inhibit the development of clinical disease. Further studies are clearly required to determine the specific molecular mechanisms by which these HPV types may contribute to the carcinogenic process. *In vitro* studies may show whether a virus–virus interaction is required in a complementation of otherwise defective HPV types, or whether an indirect mechanism is involved, e.g., through the activation of cellular factors that in turn activate the second virus. UV radiation may participate either through the induction of the HPV genes or cellular factors, or by mutational inactivation of existing cellular genes normally suppressing the HPV gene activity.

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