Human Papillomavirus DNA in Non-melanoma Skin Cancers of a Renal Transplant Recipient: Detection of a New Sequence Related to Epidermodysplasia Verruciformis Associated Types

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The detection of human papillomavirus (HPV) types originally isolated from patients with epidermodysplasia verruciformis (EV) in skin tumors of transplant recipients may point to a role of this HPV subgroup in non-melanoma skin cancer in immunosuppressed people. We analyzed 17 formalin-fixed, paraffin-embedded biopsies of benign or malignant skin tumors of a renal transplant patient with unusually widespread cutaneous carcinomas. Using a nested polymerase chain reaction (PCR), HPV-specific DNA was demonstrated in 11 specimens (65%). Analysis of nine PCR amplification products revealed four different sequences related to EV-associated HPVs. Three sequences occurred only in one lesion. In six samples identical sequences were found that differed from all HPV sequences published to date and may therefore represent a novel EV-HPV type, preliminarily labeled RTRX7. RTRX7 was found in benign, premalignant, and malignant skin lesions. Alignments identified HPV12 as the closest relative of RTRX7, both in the DNA (81% homology) and in the amino acid sequence (84% homology). ***Key words: viral carcinogenesis/immunosuppression/genom alignment. J Invest Dermatol 108:53–56, 1997***

Transplantation patients are at increased risk for the development of skin cancer (Hartevelt *et al*, 1990; Espana *et al*, 1995). The detection of human papillomavirus (HPV) types originally isolated from patients with epidermodysplasia verruciformis (EV) in skin tumors of transplant recipients may point to a role of this HPV subgroup in non-melanoma skin cancer in immunosuppressed people (Jablonska, 1990). Mucosal HPV types detected by others (Euvrard *et al*, 1993; Soler *et al*, 1993) were ruled out by De Jong-Tieben, who frequently found EV-associated HPV types in biopsies from malignant and premalignant skin lesions from renal transplant recipients (De Jong-Tieben *et al*, 1995). Recently, seven novel EV-HPV sequences were described (Berkhout *et al*, 1995), suggesting the existence of a large group of unknown types, subtypes, and variants of HPVs. A putatively new sequence related to EV-associated HPVs is described in this study, which was initiated because of unusually widespread cutaneous carcinomas in a renal transplant recipient.

MATERIALS AND METHODS

Clinical Data and Histology A 38-y-old man received a kidney transplant in 1976 after renal failure due to hereditary Alport syndrome (nephropathy combined with perceptual deafness and congenital ocular defects). There was no indication of a preexisting impaired cell-mediated immunity and no known history of non-melanoma skin cancer or unusual warts before the patient underwent transplantation. Immunosuppressive therapy, beginning immediately after transplantation, consisted of azathioprine (100 mg/d) and prednisolone (10 mg/d). Skin tumors started to appear in 1984 and treatment with cryotherapy was initiated. In 1993 four non-melanoma skin cancers were surgically removed in another hospital followed by radiotherapy restricted to several head, neck, and hand areas.

When the patient attended our department in 1994, his examination was remarkable for an unusually large number of keratotic papules and tumors predominantly on the face and the hands. Clinically, some appeared clearly to be basal cell carcinomas, squamous cell carcinomas, solar keratoses, or viral papillomas. Abdominal ultrasound and a chest-x-ray were normal, and an internal malignancy was excluded; 23 skin tumors were surgically resected. The remaining lesions were treated with topical 5-fluorouracil.

Sample Preparation and Polymerase Chain Reaction (PCR) Amplification Formalin-fixed, paraffin-embedded tumor tissues were retrieved from the files of the Department of Dermatology, University of Innsbruck and from the Department of Pathology, Hospital of Feldkirch, Austria. Seventeen samples were processed for PCR as described previously (Greer *et al*, 1994). Briefly, paraffin sections were deparaffinized by two extractions with n-octane and absolute ethanol, followed by a proteinase K digestion (200 μg per ml) (Boehringer Mannheim, Mannheim, Germany) for 3 h at 55°C.

The protease was inactivated by boiling, and 5 μl of the solution were employed in a nested PCR with degenerate primers CP65/CP70 (0.9 μM) and CP66/CP69 (0.9 μM) designed by Berkhout *et al* (1995) to detect EV HPVs. The amplification was performed exactly as described. The target sequence is located in the 3′-part of the L1 gene [HPV8 nucleotide (nt) 6832–7298 for CP65/CP70 and HPV8 nt 6862–7250 for CP66/CP69]. To avoid PCR product carryover, the suggestions of Kwok (1990) were diligently considered. Four negative controls (PCR reagents with water

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Abbreviations: EV, epidermodysplasia verruciformis.

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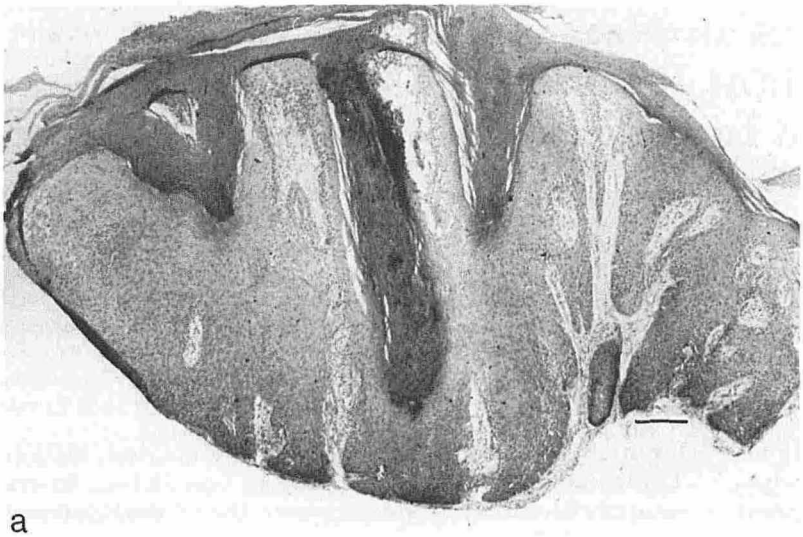

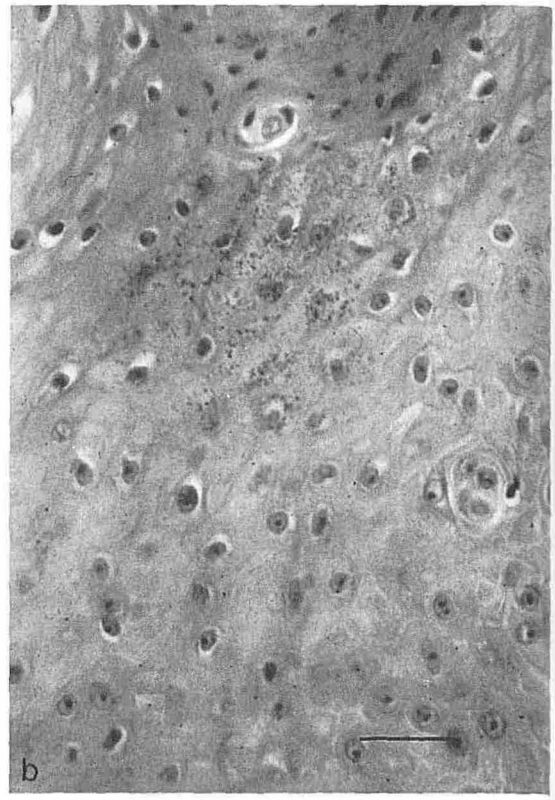

Figure 1. Viral papilloma containing RTRX7. *(a)* Prominent hyper-/parakeratosis, acanthosis, and papillomatosis oriented to a fictive center (hematoxylin-eosin; *scale bar*, 100 μm). High-power view *(b)* with prominent coarse hypergranulosis, hyperparakeratosis, and koilocyte-like features in the stratum spinosum (hematoxylin-eosin; *scale bar*, 10 μm).

instead of patient samples) were included in each PCR, and 0.1 pg of full-length HPV8 plasmid DNA (Steger *et al*, 1990) served as a positive control. Internal PCR products (374–389 bp, depending on the HPV type) were analyzed on ethidium bromide–stained 4% agarose gels (NuSieve 3:1, FMC-BioProducts, Rockland, ME).

Sequence Analysis and Alignments HPV typing was performed by comparison of the newly determined sequences with already known HPV L1 sequences. For sequencing of PCR products the internal primers were used at a concentration of 0.4 μM, and one of them was biotinylated. A polymerase with proofreading activity was used for all amplifications (Ultma DNA Polymerase, Perkin-Elmer, Branchburg, NJ). Direct solid phase sequencing of PCR products was performed as described recently (Wieland *et al*, 1994). Briefly, biotinylated PCR products were immobilized on magnetic streptavidin-coated beads (Dynabeads M-280 Streptavidin, Dynal, Hamburg, Germany) and denatured with 0.1 M NaOH. The immobilized single-stranded templates were sequenced by the dideoxy chain termination method using α-(35S)dATP (Amersham, Braunschweig, Germany), T7 DNA polymerase, and reagents and conditions supplied by Pharmacia Biochemicals, Inc. (T7 sequencing kit, Pharmacia, Freiburg, Germany). CP66 (0.8 mM) was used for plus-strand sequencing and CP69 was used for minus-strand sequencing. Samples were electrophoresed on 8% polyacrylamid/7 M urea gels, washed (10% acetic acid), dried, and exposed to Hyperfilm-βmax films (Amersham) overnight.

Sequence analysis and alignments were performed with the sequence analysis softwares MacVector 4.1.1. (Kodak, New Haven, CT) and AssemblyLign 1.0.5. (Kodak). Published HPV L1 sequences of 85 HPV types served as reference sequences for the alignments [Entrez Document Retrieval System, Release 17.0 (1995), National Center for Biotechnology Information, Bethesda, MD].

RESULTS

Histology With lesions retrieved from the files of another hospital, a total number of 27 were available for routine histopathology. Two of the skin tumors appeared to be viral papillomas with prominent warty epidermal hyperplasia, columns of parakeratosis, and characteristic koilocytes, shown in **Fig 1*a*,*b***. Bowenoid features were noted in one of the warts. The other 25 lesions were clinically and histopathologically classified as non-melanoma skin cancers or precursors as follows: eight squamous cell carcinomas, four basal cell carcinomas, nine Bowen's diseases **(Fig 2*a*,*b*)**, and four pre-malignant solar keratoses. Some of the malignant lesions revealed minimal focal changes reminiscent of a viral infection.

PCR Data DNA extracts of the kidney transplant recipient's paraffin-embedded skin biopsies were employed in nested EV-specific HPV PCR. The PCR protocol used here works with degenerate broad-spectrum primers and has been shown to be suitable for the amplification of the L1 gene fragments of all known EV-associated HPV types (Berkhout *et al*, 1995). In six of the 17 samples processed for PCR, no HPV-specific signal could be detected: two basal cell carcinomas, two solar keratoses, one Bowen's disease, and one virus papilloma. HPV-specific DNA was demonstrated in 11 lesions (65%) listed in **Table I**. Only one of these tumors histologically appeared to be a viral papilloma, whereas the remaining 10 lesions comprised three invasive carcinomas (two squamous cell carcinomas, and one basal cell carcinoma), four carcinomas *in situ* (Bowen's disease), and three precancerous lesions (solar keratoses).

Sequence Analysis and Alignments HPV L1 gene DNA sequences of nine of the 11 PCR-positive samples were determined by direct solid phase sequencing of the internal (CP66/CP69) PCR products. HPV typing was performed by comparison with known HPV L1 sequences. The HPV sequences obtained from the renal transplant patient were aligned to each other and to 85 published EV and non-EV HPV L1 gene sequences. The same was done for the deduced amino acid sequences. Results are listed in **Table I**. Three of the nine L1 sequences were identical to previously published ones corresponding to HPV38 and to two new isolates, tentatively labeled RTRX1 and ICPX1 (Berkhout *et al*, 1995). In the remaining six samples, identical sequences were found that differed from all hitherto published L1 sequences and may therefore represent a novel EV-HPV type. With reference to these authors (Berkhout *et al*, 1995), who have recently named novel EV-HPV sequences found in renal transplant recipients RTRX1-RTRX6, our sequence has preliminarily been labeled RTRX7. RTRX7-DNA was found both in benign and in malignant skin lesions. Representative histopathologies are shown in **Figs 1 and 2**.

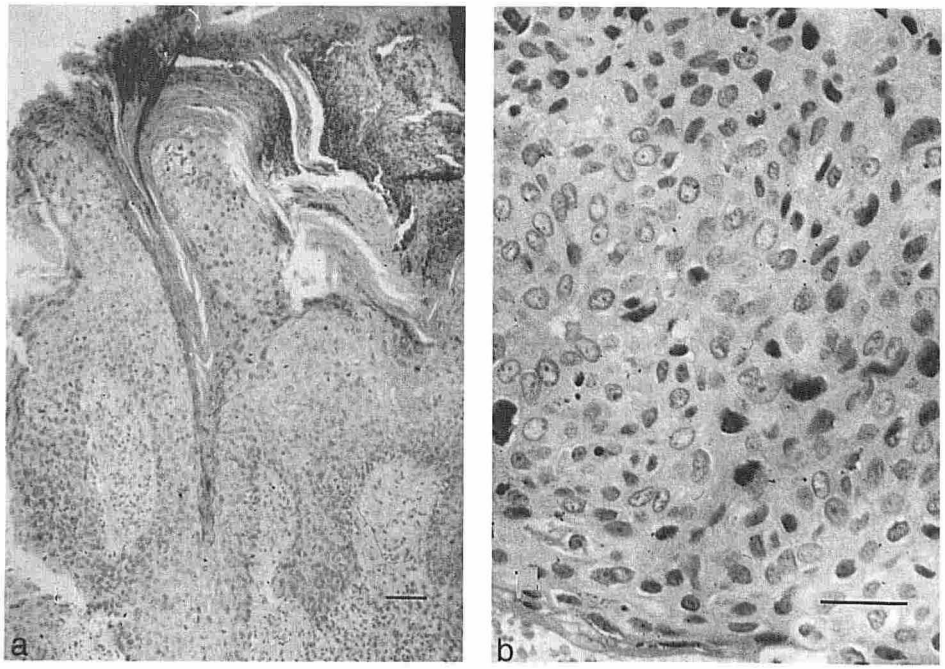

Figure 2. Bowen's disease containing RTRX7. Scanning magnification *(a)* shows prominent hyper-/parakeratosis, irregular hyperplasia, and cellular atypia (hematoxylin-eosin; *scale bar*, 20 μm). High-power view *(b)* depicting loss of normal stratification as well as prominent cellular atypia (hematoxylin-eosin; *scale bar*, 10 μm).

Sequence alignments identified HPV12 as the closest relative of RTRX7 both in the DNA and the amino acid sequences **(Fig 3)**. The analyzed RTRX7 L1 fragment (220 nt, 73 amino acids) differed from the corresponding HPV12 L1 fragment (HPV12 nt 6897–7116, Delius and Hofmann 1993, NCBI sequence ID 396910) in 19% of the nucleotides and in 16% of the deduced amino acids. Due to its relationship to HPV12 and to other HPV types related to HPV12, such as HPV5b, HPV8 and HPV47, RTRX7 was assigned to the EV-HPV subgroup A, as defined by Berkhout *et al* (1995).

DISCUSSION

We found EV-HPV–specific sequences in 65% of 17 benign, pre-malignant, or malignant skin tumors of a renal transplant recipient. We did not observe the characteristic cytopathic effects described by others in lesions of EV patients, such as clarification of cells with small pyknotic nuclei and unevenly distributed granules of various sizes in the upper layers (Gross *et al*, 1988; Majewski and Jablonska, 1995). This was also noted by Obalek *et al* and may indicate that these viruses replicate poorly even in benign tumors (Obalek *et al*, 1992). Only HPV types of the EV group were detected, although the primers used are also suitable for the amplification of mucosal HPVs (Berkhout *et al*, 1995). This finding is in accordance with recently published results (Tieben *et al*, 1994; Berkhout *et al*, 1995; De Jong-Tieben *et al* 1995). These authors only found EV or EV-related HPVs in malignant skin lesions of immunocompromised patients. Our result is in sharp contrast, however, to the observations of others (Euvrard *et al*, 1993; Soler *et al*, 1993) who chiefly detected HPV6, 11, 16, and 18 in a high percentage of squamous cell carcinomas in immunosuppressed individuals. Shamanin mainly found non-EV HPVs, such as HPV41 or an HPV29-related type, in malignant skin tumors of renal transplant recipients (Shamanin *et al*, 1994). Whereas Berkhout

Table I. Histology and HPV Types Found in 11 EV-HPV PCR-Positive Cutaneous Lesions

Diagnosis	HVP Type	EV Subgroup[a]
Squamous cell carcinoma	RTRX7	A
Squamous cell carcinoma	RTRX7	A
Bowen's disease **(Fig 2)**	RTRX7	A
Bowen's disease	RTRX1	F
Bowen's disease	HPV38	E
Bowen's disease	n.d.	
Basal cell carcinoma	ICPX1	A
Solar keratosis	RTRX7	A
Virus papilloma **(Fig 1)**	RTRX7	A
Solar keratosis	RTRX7	A
Solar keratosis	n.d.[b]	

[a] EV subgroups as described by Berkhout *et al* (1995).
[b] n.d., not determined.

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HPV12  . . . . . Y . D . . N V H
RTRX7  T N F S I S I S S E N Q D I Q
RTRX7  ACCAATTTCAGTATATCTATATCTAGTGAAAATCAAGATATACAG
HPV12  .....T.AC.....T......A..G....C

HPV12  D . P N . . . . . .
RTRX7  Q I Q S Y D S Q K F R E Y L R
RTRX7  CAAATACAATCATATGACTCACAAAAGTTTAGGGAATATTTAAGG
HPV12  G.C..T.C.AAT.....T..T.....A.....A..........A

HPV12  . . . . . L . . . . . .
RTRX7  H V E E Y E I S I I L Q L C K
RTRX7  CACGTAGAAGAATATGAAATTTCTATTATTTTACAGTTGTGTAAG
HPV12  ..T.....G.............C..........A..A.....A

HPV12  V . . K . . . . . .
RTRX7  I P L Q A E V L A Q I N A M N
RTRX7  ATTCCACTGCAAGCAGAAGTTTTAGCACAAATAAATGCAATGAAC
HPV12  G....TT.AA.G........G........G.............

HPV12  S . . . . . .
RTRX7  P S L L E D W Q L G F V P
RTRX7  CCCTCCTTACTAGAGGATTGGCAGTTAGGATTTGTGCCAA
HPV12  T.T..T..G..G.....C.....A.....C..C.......
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Figure 3. RTRX7 Sequence alignment. The analyzed DNA sequence of RTRX7 (220 nt of the L1 gene, obtained by direct sequencing of PCR products) and the deduced amino acid sequence found in six different biopsies of a renal transplant recipient. The corresponding DNA and amino acid sequences of HPV12, the HPV type that is most closely related to RTRX7, are shown for comparison (HPV12 nt 6897–7116/Delius and Hofmann 1993, NCBI sequence ID 396910). The points indicate positions that are conserved between HPV12 and RTRX7.

found more than one HPV type per lesion in over 30% of squamous cell carcinomas of renal transplant patients (Berkhout *et al*, 1995), our direct sequence analyses mostly gave unambiguous results, indicating that only one HPV type predominated in a single EV-HPV PCR-positive sample.

Bernard *et al* (1994) have previously shown that HPV sequences derived from sequence analysis of PCR products of the L1 open reading frame are suitable for HPV typing. Comparison of the nine HPV L1 sequences obtained by us with 85 known L1 sequences revealed that the renal transplant patient harbored at least four different EV-HPV isolates, namely HPV38, the recently identified isolates RTRX1 and ICPX1 (Berkhout *et al*, 1995), and a possibly novel EV HPV type, tentatively named RTRX7. RTRX7 was found in six different benign, pre-malignant, and malignant skin lesions, whereas the other three HPVs occurred only in one lesion each. ICPX1 was previously found in an immunocompetent patient (Berkhout *et al*, 1995), whereas it was detected in an immunocompromised individual in this study.

The HPV type most closely related to RTRX7 was HPV12, with differences between the corresponding sequences being 19% (DNA) and 16% (amino acids). These numbers are similar to those previously found for the potentially novel EV-HPV types, RTRX1-RTRX6, and their closest relatives, respectively (Berkhout *et al*, 1995). Almost half of the sequence variations found in RTRX7 led to amino acid exchanges compared to HPV12.

The complete genome of RTRX7 must be cloned before it can definitely be considered a novel EV-HPV type. Due to its relationship to HPV12 and to related EV-HPV types, RTRX7 can be grouped into the EV subgroup A according to Berkhout *et al* (1995) comprising the EV-associated HPVs 5, 8, 12, 36, and 47, some of which are most prevalent in EV skin cancers. The other HPV types found in the investigated patient are members of the EV subgroups A (ICPX1), E (HPV38), and F (RTRX1).

Concerning the HPV status of the PCR-negative samples, several possibilities exist. These samples could have carried no or only very few HPV sequences, HPV DNA could have been damaged during the fixation process, or HPV sequences might have been present that were not amplified by the primers used.

The isolation of novel nonmucosal HPV types by the PCR technique fuels the debate on HPV and its role in skin cancer in general (Tieben *et al*, 1994; De Jong-Tieben *et al*, 1995, Berkhout *et al*, 1995; this paper). The relevance of these HPV types is not yet determined. They may persist only in low copy number because they were missed in previous screening studies of pre-malignant and malignant skin tumors of immunosuppressed patients by Southern blot hybridization. Using this technique, HPV5, 8, and related HPVs were identified in warts together with HPV2 or 3 (van der Leest *et al*, 1987; Obalek *et al*, 1992), occasionally in clinically and histopathologically EV-like lesions (Rüdlinger *et al*, 1986), and very rarely in basal cell and squamous cell skin carcinomas (Lutzner *et al*, 1983; Kawashima *et al*, 1990). In view of extensive sequence homology between the novel types and the probes applied for Southern blot hybridization, the detection rate should have been higher in earlier analyses if there was more than one viral genome per tumor cell. In the case of smaller copy numbers, a role of the virus in the maintenance of the malignant phenotype would be unlikely. So far, it cannot be excluded that latent infections by novel types are unrelated to the carcinogenesis process. The extraordinary widespread non-melanoma skin cancers in the patient examined in this study, however, may increase speculation that RTRX7 has a high oncogenic potential. In addition, the well known co-carcinogenic effect of ultraviolet light may have been augmented by radiotherapy in this case (Kopelson *et al*, 1994). In the future, wider use of novel HPV isolates in probe panels will improve our knowledge about the association of HPV with non-melanoma skin cancer in immunosuppressed and immunocompetent individuals.

REFERENCES

Berkhout RJM, Tieben LM, Smits HL, Bouwes Bavinck JN, Vermeer BJ, ter Schegget J: Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. *J Clin Microbiol* 133:690–695, 1995

Bernard HU, Chan S, Manos M, Ong C, Villa L, Delius H, Peyton C, Bauer H, Wheeler C: Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphism, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* 170:1077–1085, 1994

De Jong-Tieben LM, Berkhout RJM, Smits HL, Bouwes Bavinck JN, Vermeer BJ, van der Woude FJ, ter Scheget J: High frequency of detection of epidermodysplasia verruciformis-associated human papillomavirus DNA in biopsies from malignant and premalignant skin lesions from renal transplant recipients. *J Invest Dermatol* 105:367–371, 1995

Espana A, Redondo P, Fernandez AL, Zabala M, Herreros J, Llorens R, Quintanilla E: Skin cancer in heart transplant recipients. *J Am Acad Dermatol* 32:458–465, 1995

Euvrard S, Chardonnet Y, Pouteil-Noble C, Kanitakis J, Chignol MC, Thivolet J, Touraine JL: Association of skin malignancies with various and multiple carcinogenic and noncarcinogenic human papillomaviruses in renal transplant recipients. *Cancer* 72:198–206, 1993

Greer CE, Wheeler CM, Manos MM: Sample preparation and PCR amplification from paraffin-embedded tissues. *PCR Methods Appl* 3:113–122, 1994

Gross G, Ellinger K, Roussaki A, Fuchs PG, H-H Peter, Pfister H: Epidermodysplasia verruciformis *in a patient with Hodgkin's disease:* characterization of a new papillomavirus type and interferon treatment. *J Invest Dermatol* 91:43–48, 1988

Hartevelt MM, Bouwes Bavnick JN, Kootte AMM, Vermeer BJ, Vandenbroucke JP: Incidence of skin cancer after renal transplantation in the Netherlands. *Transplantation* 49:506–509, 1990

Jablonska S: Human papillomaviruses in skin carcinomas. In: Pfister H (ed.). *Papillomaviruses and Human Cancer.* CRC Press, Boca Raton, FL, 1990, pp 46–71

Kawashima M, Pavre M, Obalek S, Jablonska S, Orth G: Premalignant lesions and cancers of the skin in the general population: evaluation of the role of human papillomvirus. *J Invest Dermatol* 95:537–542, 1990

Kopelson PL, Nguyen QH, Moy RL: Verruca vulgaris and radiation exposure are associated with squamous cell carcinoma of the finger. *J Dermatol Surg Oncol* 20:38–41, 1994

Kwok S: Procedure to minimize PCR-product carry-over. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.). *PCR Protocols.* Academic Press, San Diego, CA, 1990, pp 142–145

Lutzner MA, Orth G, Dutronquay V, Ducasse MF, Kreis H, Crosnier J: Detection of human papillomavirus type 5 DNA in skin cancers of an immunosuppressed renal allograft recipient. *Lancet* 2:422–424, 1983

Majewski S, Jablonska S: Epidermodysplasia verruciformis as a model of human papillomavirus-induced genetic cancer of the skin. *Arch Dermatol* 131:1312–1318, 1995

Obalek S, Favre M, Szymanczyk J, Misiewicz J, Jablonska S, Orth G: Human papillomavirus (HPV) types specific of epidermodysplasia verruciformis detected in warts Induced by HPV3 or HPV3-related types in immunosuppressed patients. *J Invest Dermatol* 98:936–941,1992

Rüdlinger R, Smith IW, Bunney MH, Hunter JAA: Human papillomavirus infection in a group of renal transplant recipients. *Br J Dermatol* 115:681–692, 1986

Shamanin V, Glover M, Rausch C, Proby C, Leigh IM, zur Hausen H, de Villiers EM: Specific types of human papillomavirus found in benign proliferations and carcinomas of the skin in immunosuppressed patients. *Cancer Res* 54:4610–4613, 1994

Soler C, Chardonnet Y, Allibert P, Euvrad S, Schmitt D, Mandrand B: Detection of mucosal human papillomavirus types 6/11 in cutaneous lesions from transplant recipients. *J Invest Dermatol* 101:286–291, 1993

Steger G, Olszewsky M, Stockfleth E, Pfister H: Prevalence of antibodies to human papillomavirus type 8 in human sera. *J Virol* 64:4399–4406, 1990

Tieben LM, Berkhout RJM, Smits HL, Bouwes Bavinck JN, Vermeer BJ, Bruijn JA, van der Woude FJ, ter Schegget J: Detection of epidermodysplasia verruciformis-like human papillomavirus types in malignant and premalignant skin lesions of renal transplant recipients. *Br J Dermatol* 131:226–230, 1994

van der Leest RJ, Zachow KR, Ostrow RS, Bender M, Pass F, Faras AJ: Human papillomavirus. Heterogeneity in 36 renal transplant recipients. *Arch Dermatol* 123:354–357, 1987

Wieland U, Hartmann J, Suhr H, Salzberger B, Eggers HJ, Kühn JE: *In vivo* genetic variability of the HIV-1 vif gene. *Virology* 203:43–51, 1994