

INVESTIGATIVE REPORT

Human Papilloma Virus in Skin, Mouth and Uterine Cervix in Female Renal Transplant Recipients With or Without a History of Cutaneous Squamous Cell Carcinoma

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Some human papilloma viruses are thought to be associated with skin cancer. In this pilot study, 21 female renal transplant carriers, 10 with a history of skin squamous cell carcinoma and 11 without, together with 9 age-matched healthy women were investigated for human papilloma virus DNA in sun-exposed (forehead) and less sun-exposed (buttock) skin, mouth and uterine cervix. Paraffin-embedded tumours from 9 of the patients with a history of squamous cell carcinoma were analysed. Healthy skin from both the healthy and the immunosuppressed individuals harboured a wide variety of papilloma viruses. In the healthy individuals, samples from less sun-exposed skin showed a lower prevalence of human papilloma virus DNA than corresponding samples from the immunosuppressed patients (4/9 and 7/9, respectively). Among the immunosuppressed patients, human papillomavirus DNA was found as frequently in buttock samples (17/21) as in forehead samples (17/20). There was no increased prevalence of human papillomavirus in the cervix or mouth samples from the immunosuppressed patients. **Key words:** human papilloma virus; kidney transplant patients; polymerase chain reaction; squamous cell carcinoma.

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Recipients of organ transplants under long-term immunosuppression are at greatly increased risk of non-melanoma skin cancer (NMSC), and cohort studies have demonstrated a 50–100-fold increased risk of cutaneous squamous cell carcinoma (SCC) and a 5–10-fold increased risk of basal cell carcinoma (BCC) compared with the general population (1–5).

It is established that high-risk human papilloma virus (HPV) types play a causative role in anogenital cancer (6), and there is also evidence for an aetiological role of HPV in oral SCC (7, 8). It has been speculated, although not yet proven, that HPV infections may also

be involved in the pathogenesis of NMSC, probably in conjunction with ultraviolet (UV) light (4, 9–13).

The aim of this study was to investigate whether the prevalence of HPV infection in the uterine cervix, the oral mucosa and the skin is increased in female renal transplant recipients, and whether HPV DNA can be found in specimens from archival cutaneous SCCs. To our knowledge, no search for HPV DNA in all of these locations in the same organ transplant recipients has been described previously.

MATERIALS AND METHODS

Patients and controls

Three groups of female patients were examined. Group 1 comprised 10 women who had been diagnosed with invasive or *in situ* SCC of the skin at least once. Nine patients had had a kidney transplant over 10 years previously, and one patient 6 years previously. The mean age of the patients in this group was 55 years (range 38–80 years). Group 2 comprised 11 women with a mean age of 47 years (range 22–73 years), with renal transplants for more than 10 years, but who never had been diagnosed with SCC. Nine age-matched healthy women with a mean age of 51 years (range 23–78 years) made up the third (control) group.

Samples

Exfoliated cells were collected from the forehead (skin exposed to sunlight), buttock (skin not exposed to sunlight), tongue and tonsils using pre-wetted (0.9% NaCl solution) cotton-tipped swabs that were drawn back and forth over the sampling site. Cervical samples were collected with a cytobrush. All samples were suspended in 0.9% NaCl solution and kept frozen at –20°C until tested.

Tumour tissue (formalin-fixed and embedded in paraffin) was available from 9 of the 10 patients diagnosed with skin cancer (1–3 samples per patient).

Sample preparation

The forehead and buttock samples were tested by polymerase chain reaction (PCR) without previous DNA extraction. The specimens from the tongue, tonsils and uterine cervix were centrifuged at 3000 g for 10 min, and the cell pellets were resuspended in 10 mM Tris-HCl (pH 7.4) to the same volume as before centrifugation. The resuspended samples were then frozen at –20°C overnight and boiled (100°C) for 10 min. before being analysed.

Four 5- μ m sections were cut from the formalin-fixed tumour tissues, and the samples were prepared as described by Wright & Manos (14).

Polymerase chain reaction (PCR)

All specimens were tested for HPV DNA using the primers FAP59 and FAP64 as described by Forslund et al. (15). DNA-free water was used as negative control and a clinical sample containing HPV 20 served as positive control. The specimens from the tongue, tonsils and cervix were also tested with PCR using the primers GP5+ and GP6+ as described by Jacobs et al. (16). A clinical sample containing HPV 11 served as positive control.

The formalin-fixed tumour tissue samples were analysed for HPV DNA by a newly developed nested "hanging droplet" PCR system (17). The amplification cycles and the PCR solutions were prepared as described by Forslund et al. (17), but the primers were replaced with degenerate primers located within the E1/E2 genes (Table I). DNA-free water was used as negative control and HPV 49 (plasmid, 1 pg/ μ l) served as positive control.

By running PCR on plasmids with inserts of 76 different HPV types, HPV 5, 9, 12, 14, 15, 20, 21, 22, 23, 36, 37, 38, 47, 49, 54, 55, 60, 70, 74, 76 and 92 could be amplified by the method.

As a control for PCR inhibition, all samples were subjected to a PCR test for the human β -globin gene (18).

DNA cloning and sequence analysis

All positive PCR products were cloned, and three clones per sample were subjected to DNA sequencing and comparison with available sequences in the GeneBank database using the BLAST server (<http://www.ncbi.nlm.nih.gov/blast/>).

RESULTS

The skin samples collected from the foreheads of both transplanted patients and controls had equally high prevalence of HPV DNA (17/20 and 7/9, mean 83%), while the total number of HPV types or putative types was almost double in the transplant recipients compared with the healthy controls. The buttock samples from the transplanted patients had the same high HPV DNA prevalence (17/21, mean 81%), while only 4 out of the 9 samples from the healthy controls were HPV DNA positive. There were also fewer HPV types or putative types in the buttock samples from the control individuals (Table II).

Taking all 59 skin samples into account, 45 (76%) were found to be HPV DNA positive, and from these 45 positive samples 60 different HPV types or putative types were identified. Epidermodyplasia verruci-

Table I. Primer sequences of degenerate primers used for analysis of formalin-fixed paraffin-embedded tumour tissue. R=A or G; M=A or C; V=A, C or G; S=G or C; W=A or T; Y=C or T; H=A, C or T; D=A, G or T; K=G or T

Outer primers:	E1 2482F: 5' CTT ACT GAC CAA AGC TGG RMR TC 3'
	E2 2601R: 5' CTS WYT CAT AHA DKK YCA TTA 3'
Inner primers:	E1 2503F: 5' TCT TTT TTT VAA AGG CTT TGG A 3'
	E1 2582R: 5' TTS TCT TGY AGT RCA TTG AAA 3'

Table II. Number of human papillomavirus (HPV)-positive samples in two groups of female renal transplant recipients and in matched healthy controls by sampling site. (Numbers in parentheses refer to the number of HPV types or putative types found in each group and sampling site)

	Forehead	Buttock	Cervix	Mouth	Tumour
Diagnosed with SCC	6/9 (15)	8/10 (16)	1/10 (2)	0/10	2/9 (3)
Not diagnosed with SCC	11/11 (20)	9/11 (16)	0/11	0/11	–
Healthy controls	7/9 (9)	4/9 (5)	1/9 (2)	0/9	–

SCC: squamous cell carcinoma.

formis (EV) types were detected equally frequent in all three groups of patients. Two of the types detected on skin were of mucosal type (HPV 6 and HPV 42) and were found in the buttock sample of one of the transplanted women with a history of skin cancer. The cervical sample from this patient contained HPV 42 in addition to a previously described HPV candidate, CP6108. Identical HPV types or putative types were found in both the forehead and buttock samples of 3 patients with skin SSC, in 2 patients without skin SSC, and in one of the healthy controls. Thirty-eight of the 60 HPV types or putative types were found in one skin sample only. The most common type was HPV 5, which was seen in 6 samples, followed by HPV 20 and FA 38, each found in 4 samples. In total, 14 previously un-described putative HPV type candidates were identified (Table III).

Fifteen samples of formalin-fixed, paraffin-embedded tumour tissue from nine patients were analysed. Two samples were excluded because of negative β -globin PCR. In total, three samples were positive for HPV DNA, containing one genotype each. Samples from two

Table III. Fourteen new putative human papillomavirus (HPV) type candidates and their most closely related HPV types or putative type candidates

Putative HPV type	Fragment size (base pairs)	Most closely related HPV type	% DNA sequence homology	Accession number (GeneBank)
FA 98 ^a	440	HPV65	68	AY204681
FA 99 ^a	443	HPV95	69	AY204682
FA 100 ^a	440	HPV60	68	AY204683
FA 101 ^a	431	HPV4	69	AY204684
FA 102 ^a	215	HPV60	69	AY204685
FA 104 ^a	221	HPV8	66	AY204687
FA 105 ^a	431	HPV95	71	AY204688
FA 106	435	HPV48	69	AY204689
FA 107.1 ^a	443	HPV60	68	AY204690
FA 107.2 ^a	443	HPV60	68	AY204691
FA 108	437	HPV80	76	AY204692
FA 109 ^a	434	HPV65	74	AY204693
FA 110 ^a	440	HPV95	66	AY204694
FA 111 ^a	437	HPV50	68	AY204695
FA 113 ^a	437	HPV 38	87	AY546000

^aPutative types found in renal transplant recipients.

different tumours from one patient contained HPV 22 and HPV 38, and a tumour sample from another patient was positive for HPV 93.

Only two of the 59 women in the study had HPV-positive cervical samples, both of whom had dual infections (Table II). HPV 22 and HPV CP6108 were both detected in one of the transplanted women with skin SSC, and HPV 16 and HPV 67 were detected in one of the healthy controls.

All of the tongue and tonsil samples, from all three groups, were negative for HPV DNA when tested with both the FAP59/64 and the GP5+/6+ primer combinations.

The test for human β -globin DNA was positive for all samples from tongue, tonsil and cervix, while 3 forehead and 11 buttock samples were negative. All but two of the β -globin DNA negative samples were, however, positive for HPV DNA.

DISCUSSION

Organ transplant recipients undergoing long-term immunosuppression are prone to develop NMSCs, mainly SCC and to a lesser extent BCC, as well as benign HPV-induced warts (3, 19). The risk of cutaneous SCC is related to the length and degree of immunosuppression, i.e. the stronger the immunosuppressive therapy, the higher the risk (20).

This small study confirms and strengthens previous observations that: (i) there is an astonishing polymorphism of HPV genotypes in normal human skin. In the limited number of 59 samples from normal skin, 45 were positive for HPV DNA. From these 45 specimens, 15 HPV types and 45 putative HPV types, 14 of which have not been described previously, were identified; (ii) in healthy individuals, samples from non-sun-exposed skin (buttock) show lower prevalence of HPV DNA than do samples from sun-exposed skin (forehead). Prolonged sun exposure may promote increased HPV prevalence through local immunosuppressive effects from ultraviolet (UV) radiation (21); (iii) in immunosuppressed patients, skin not exposed to sunshine has a higher prevalence of HPV DNA than corresponding samples from healthy individuals; (iv) HPV DNA can be detected in skin SCC tissue but only in low copy numbers.

Our study did not show an increased prevalence of HPV infection in either mouth samples or cervical samples of the transplanted patients compared with the healthy controls, and the low prevalence found is in agreement with that expected from healthy individuals without head and neck or cervical malignancies (22, 23).

Of the 14 skin samples that were negative when tested for human β -globin DNA, 12 were positive for HPV DNA. This might indicate that even if the human cellular DNA is degraded in the desquamated cells, the viral DNA contained in the virions is not.

Other epithelial malignancies, such as SCC of the uterine cervix, anus, and oral cavity (especially the oropharynx), aetiologically strongly correlated to HPV infections, are only slightly more common in transplant recipients (24). The relationship between skin cancer in transplant recipients and the underlying biological and epidemiologic factors is complex (1), and the aetiology of NMSC is far from elucidated (25–30). UV light is, however, a documented risk factor and most of the tumours appear in sun-exposed skin (3). It is also intriguing that increased incidence of skin warts in patients such as those suffering from epidermodysplasia verruciformis (EV) or those undergoing immunosuppression, goes hand in hand with an increased incidence of skin cancer. Recently, it has been demonstrated clearly that a great number of HPV types are ubiquitous in normal skin (31). If the superficial epidermal layer, rich in HPV DNA, is stripped off using tape before taking biopsies, there is now evidence that skin tumours harbour more HPV than healthy skin from the same individuals. However, the number of HPV DNA copies is only in the range of one in a hundred to one in several thousand cells (32). It has also been shown that actinic keratoses have higher viral load than SCC (33).

One could speculate that HPV plays an essential role in oncogenesis through a hit-and-run mechanism or, alternatively, that HPV is not associated with the genesis of skin cancer. The pathogenesis of NMSC is not understood, but there is a growing consensus that HPV may be contributing, at least in immunosuppressed transplant recipients, in co-operation with the mutagenic effects of UV light (34). This hypothesis would not require high viral load because it proposes that the anti-apoptotic mechanism contributed by HPV would allow for persistent accumulation of UV-induced mutations within a HPV infected keratinocyte that would subsequently lead to a transformed and genetically unstable clone. Once mutations or genetic instability is established, there would be no further requirement for HPV (9, 34).

Even though it has been concluded that immunosuppression does promote HPV infections of the skin, the role of HPV in the pathogenesis of NMSC still needs to be elucidated.

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REFERENCES

1. Sheil AGR. Skin cancer in renal transplant recipients. *Transplant Sci* 1994; 4: 42–45.
2. Blohmé I, Larkö O. Skin lesions in renal transplant patients after 10 to 23 years of immunosuppressive therapy. *Acta Derm Venereol* 1990; 70: 491–494.

3. Lindelöf B, Sigurgeirsson B, Gäbel H, Stern RS. Incidence of skin cancer in 5356 patients following organ transplantation. *Br J Dermatol* 2000; 143: 513–519.
4. Harwood CA, Suretheran T, Sasieni P, Proby CM, Bordea C, Leigh IM, et al. Increased risk of skin cancer associated with the presence of epiderodysplasia verruciformis human papillomavirus types in normal skin. *Br J Dermatol* 2004; 150: 949–957.
5. Pfister H. Human papillomaviruses and skin cancer. *J Natl Cancer Inst Monogr* 2003; 31: 52–56.
6. Longworth MS, Laimins LA. Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev* 2004; 68: 362–372.
7. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000; 92: 709–720.
8. Mork J, Lie AK, Glattre E, Clark S, Hallmans G, Jellum E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *N Engl J Med* 2001; 344: 1125–1131.
9. Akgül B, Cook JC, Storey A. HPV-associated skin disease. *J Pathol* 2006; 208: 165–175.
10. de Villiers EM, Lavergne D, McLaren K, Benton EC. Prevaling papillomavirus types in non-melanoma carcinomas of the skin in renal allograft recipients. *Int J Cancer* 1997; 73: 356–361.
11. Meyer T, Arndt R, Nindl I, Ulrich C, Christophers E, Stockfleth E. Association of human papillomavirus infections with cutaneous tumors in immunosuppressed patients. *Transpl Int* 2003; 16: 146–153.
12. Orth G. Human papillomaviruses associate with epidermodysplasia verruciformis in non-melanoma skin cancers: guilty or innocent? *J Invest Dermatol* 2005; 125: xii–xiii.
13. Harwood CA, Suretheran T, McGregor JM, Spink PJ, Leigh IM, Breuer J, et al. Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. *J Med Virol* 2000; 61: 289–297.
14. Wright DK, Manos MM. Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR protocols: a guide to methods and applications*. San Diego, CA: Academic Press, Inc., 1990: p. 153–158.
15. Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. *J Gen Virol* 1999; 80: 2437–2443.
16. Jacobs MV, Snijders PJ, van der Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6 (+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997; 35: 791–795.
17. Forslund O, Hoang L, Higgins G. Improved detection of cutaneous human papillomavirus DNA by single tube nested “hanging droplet” PCR. *J Virol Meth* 2003; 110: 129–136.
18. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; 230: 1350–1354.
19. Adami J, Gäbel H, Lindelöf B, Ekstrom K, Rydh B, Glimelius B, et al. Cancer risk following organ transplantation: a nation wide cohort study in Sweden. *Br J Cancer* 2003; 89: 1221–1227.
20. Jensen P, Hansen S, Moller B, Leivestad T, Pfeffer P, Geiran O, et al. Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. *J Am Acad Dermatol* 1999; 40: 177–186.
21. Kelly DA, Young AR, McGregor JM, Seed PT, Potten CS, Walker SL. Sensitivity to sunburn is associated with susceptibility to ultraviolet radiation-induced suppression of cutaneous cell-mediated immunity. *J Exp Med* 2000; 191: 561–566.
22. Morrison EA, Dole P, Sun XW, Stern L, Wright TC Jr. Low prevalence of human papillomavirus infection of the cervix in renal transplant recipients. *Nephrol Dial Transpl* 1996; 11: 1603–1606.
23. Hansson BG, Rosenquist K, Antonsson A, Wennerberg J, Schildt EB, Bladstrom A, et al. Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. *Acta Otolaryngol* 2005; 125: 1337–1344.
24. Zur Hausen H. Papillomavirus infections – a major cause of human cancers. *Biochim Biophys Acta* 1996; 1288: 55–78.
25. Grossman D, Lifell DJ. The molecular basis of non-melanoma skin cancer: new understanding. *Arch Dermatol* 1997; 133: 1263–1270.
26. McGregor JM, Proby CM. Skin cancer in transplant recipients. *Lancet* 1995; 346: 964–965.
27. McGregor JM, Proby CM. The role of papillomaviruses in human non-melanoma skin cancer. *Cancer Surv* 1996; 26: 219–236.
28. Berg D, Otley CC. Skin cancer in organ transplant recipients: Epidemiology, pathogenesis, and management. *J Am Acad Dermatol* 2002; 47: 1–17.
29. Harwood CA, Proby CM. Human papillomaviruses and non-melanoma skin cancer. *Curr Opin Infect Dis* 2002; 15: 101–114.
30. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *N Engl J Med* 2003; 348: 1681–1691.
31. Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG. The ubiquity and impressive genomic diversity of human skin papillomavirus suggest a commensalic nature of these viruses. *J Virol* 2000; 74: 11636–11641.
32. Hazard K, Eliasson L, Dillner J, Forslund O. Subtype HPV38b[FA125] demonstrates heterogeneity of human papillomavirus type 38. *Int J Cancer* 2006; 119: 1073–1077.
33. Weissenborn SJ, Nindl I, Purdie K, Harwood C, Proby C, Breuer J, et al. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J Invest Dermatol* 2005; 125: 93–97.
34. Storey A. Papillomaviruses: death-defying acts in skin cancer. *Trends Mol Med* 2002; 8: 417–421.