

Markers of Cutaneous Human Papillomavirus Infection in Individuals with Tumor-Free Skin, Actinic Keratoses, and Squamous Cell Carcinoma

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Abstract

Separately, actinic keratosis (AK) and cutaneous squamous cell carcinoma (SCC) have been associated with cutaneous human papillomavirus (HPV) infections. To further explore the association between HPV infection and SCC development, we determined markers of cutaneous HPV infection within a single population in persons with precursor lesions (AK), cancerous lesions (SCC), and without. Serum and plucked eyebrow hairs were collected from 57 tumor-free controls, 126 AK, and 64 SCC cases. Presence of HPV L1 and E6 seroreactivity and viral DNA were determined for HPV types 5, 8, 15, 16, 20, 24, and 38. Significant positive associations with increasing severity of the lesions (controls, AK, and SCC, respectively) were observed for overall HPV L1 seropositivity (13%, 26%, and 37%) and for HPV8 (4%, 17%, and 30%). In

parallel, the proportion of L1 seropositive individuals against multiple HPV types increased from 14% to 39% and 45%. The overall E6 seroreactivity, however, tended to decline with AK and SCC, especially for HPV8 (21%, 11%, and 2%). HPV DNA positivity was most prevalent in the AK cases (54%) compared with the SCC cases (44%) and the tumor-free controls (40%). Among all participants, there was a positive trend between overall HPV DNA positivity and L1 seropositivity, but not E6 seropositivity. Taken together, our data suggest that cutaneous HPV infections accompanied by detectable HPV DNA in eyebrow hairs and HPV L1 seropositivity, but not E6 seropositivity, are associated with an increased risk of AK and SCC. (Cancer Epidemiol Biomarkers Prev 2006;15(3):529–35)

Introduction

Cutaneous squamous cell carcinoma (SCC), often preceded by a benign lesion called actinic keratosis (AK), is one of the most common cancers among white populations (1). Exposure to sunlight is generally accepted to be the most important environmental risk factor (1, 2). The high prevalence of DNA from β -human papillomaviruses (β -HPV; formerly known as epidermodysplasia verruciformis-associated HPV; ref. 3) in AK and SCC (4-15), as well as the *in vitro* transforming abilities of particular members of this group of viruses (e.g., HPV5 and HPV38; refs. 16-18), suggests a role for HPV infection in the development of these type of skin tumors (4-15).

Recent epidemiologic studies have reported associations between markers of β -HPV infection (presence of viral antibodies in serum and presence of viral DNA in plucked eyebrow hairs) and AK and SCC independently (10, 11, 19, 20). An evaluation of HPV infection markers in the course of SCC development within a single population has not been reported before. In this study, markers of β -HPV infection were monitored in residents of Brisbane, Queensland Australia, who had either tumor-free skin, prevalent AKs, or incident SCCs using serologic and molecular techniques.

Seroreactivity was measured to the HPV major capsid protein L1 and the nonstructural, intracellular protein E6 of β -HPV types 8, 15, 20, 24, and 38 and genital HPV type 16. Simultaneously β -HPV infection was determined in plucked eyebrow hairs by detecting the presence of HPV DNA. This study offered the opportunity to correlate three markers of HPV infection with the presence of AK and SCC within a single population known for its high incidence of SCC.

Materials and Methods

Study Populations. The study was set in Brisbane, the capital city of Queensland, Australia. Three study groups were selected using the following inclusion criteria: (a) SCC patients who had incident, histologically proven SCC within the past 6 months and no history of surgical excision of skin cancers within 2 years before their SCC diagnosis. They may have had prevalent or past AK. (b) People with prevalent AK and no known past history of SCC or any surgical treatment for skin cancer. (c) Controls who had no prevalent skin tumors and no history of any type of treatment for skin tumors. Persons with immunodeficiencies or persons using immunosuppressive or immunomodulatory drugs (i.e., azathioprine, cyclosporine, methotrexate, prednisone, tacrolimus, IFNs, and interleukins) were excluded from this study.

Persons with SCC or AK were recruited from the Royal Brisbane Hospital, a public teaching hospital, and from general practice skin cancer clinics in the same region of Brisbane as the hospital from 2001 to 2002. Tumor-free controls were recruited concurrently from the ophthalmology outpatients' clinic at the Royal Brisbane Hospital.

Data Collection. Cases and controls were requested to complete a questionnaire with information about their age, skin phenotype, sun exposure, and smoking histories. Sun

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exposure was estimated using a lifetime "jobs" calendar to prompt participants about occupational and recreational exposure during different periods of their life. Six percent of the participants did not return the questionnaire despite persistent follow-up. Participants attended a physical examination, during which a trained graduate researcher recorded their skin type, eye and hair color, and counted actinic keratoses. Venous blood was collected using standard phlebotomy techniques. Six to eight eyebrow hairs were collected from each patient using sterile tweezers and disposable gloves. These hairs were stored at -70°C until laboratory analysis took place. The study adhered to the Helsinki Principles and was approved by the relevant institutional medical ethics committees. All participants gave informed consent.

HPV ELISA. All ELISAs were carried out without knowledge of the subject category of the samples. For the HPV L1 ELISA, L1 viruslike particles of HPV types 8, 15, 16, 20, 24, and 38 were prepared using recombinant baculoviruses as previously described (20). The HPV L1 ELISAs were done as previously described (20). The selected HPV types are frequently detectable in skin tumors, normal skin, and plucked hairs of immunosuppressed as well as immunocompetent individuals (4-15) and are widely distributed in the genus β -papillomavirus (3).

To generate antigen for the HPV E6 ELISAs, GST-E6 fusion proteins were produced. The HPV E6 PCR fragments of HPV types 8, 15, 16, 20, 24, and 38 generated by primers with *Bam*HI and *Sma*I restriction sites in the 5' and 3' primers, respectively, were ligated in the *Bam*HI and *Sma*I restriction sites of the pGEX-5X3 vector (Pharmacia, Uppsala, Sweden). All generated pGEX-HPV E6 DNA constructs were confirmed by DNA sequence analysis. *Epicrician coli* BL21 competent cells (Stratagene, La Jolla, CA) were transformed with the pGEX plasmids and grown at 37°C in Luria Bertani medium. At an A_{600} value between 0.2 and 0.4, recombinant protein expression was induced by adding isopropyl- β -D-thio-galactoside to the medium to an end concentration of 0.25 mmol L^{-1} . After 8 hours of induction at 25°C , bacteria were harvested. The bacterial pellet was resuspended in 1:100 of the starting volume of the bacterial culture in cold NET/N⁺ buffer [100 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA (pH 8.0), 20 mmol L^{-1} Tris-HCl (pH 8.0), 0.5% NP40 plus protease inhibitors: $1\text{ }\mu\text{g mL}^{-1}$ pepstatin, $2\text{ }\mu\text{g mL}^{-1}$ aprotinin, $0.5\text{ }\mu\text{g mL}^{-1}$ 10 mmol L^{-1} benzamide]. Bacteria were lysed using a French press. Lysates were cleared by centrifugation (at 4°C for 30 minutes at $13,000\text{ rpm}$), glycerol was added to an end concentration of 50%, and samples were stored at -20°C . The purity and identity of the GST-fusion proteins were analyzed on a 15% SDS-polyacrylamide gel followed by Western blotting using antibodies directed to GST (Santa Cruz Biotechnology, Santa Cruz CA; data not shown).

Serum samples were tested in a GST capture ELISA, done in a similar way as described by Sehr et al. (21), using GST-E6 fusion proteins from the β -HPV types 8, 15, 20, 24, and 38 and HPV type 16. Briefly, polysorb plates (Nunc, Rochester, NY) were coated with 100 ng glutathione-casein per well overnight at 4°C . Wells were blocked with $100\text{ }\mu\text{L}$ blocking buffer [0.2% (w/v) casein in PBS, 0.05% (v/v) Tween 20] followed by an incubation with $25\text{ }\mu\text{g/well}$ crude extracts from BL21 *E. coli* overexpressing the relevant GST-E6 protein. In the following incubation step, human sera, diluted 1:100 in blocking buffer containing $25\text{ }\mu\text{g mL}^{-1}$ crude extracts from the parental *E. coli* strain BL21, were added to the wells. For the detection, biotin-labeled antihuman total immunoglobulin G antibody (1 mg mL^{-1} , kindly donated by Dr. L.A. Aarden, CLB Sanquin, Amsterdam) was used, diluted 1:5,000 in blocking buffer followed by an incubation with streptavidin-poly-horseradish peroxidase (1 mg mL^{-1} , CLB Sanquin), diluted 1:10,000 in blocking buffer for 30 minutes at room temperature. As

substrate, 0.1 mg mL^{-1} tetramethylbenzidine (in 0.03% H_2O_2 and 0.11 mol L^{-1} NaAc pH 5.5) was used. The reaction was stopped after 5 minutes with 2 mol L^{-1} H_2SO_4 and absorbance was measured at A_{450} . Unless indicated otherwise, all incubation steps were carried out with $50\text{ }\mu\text{L/well}$ for 1 hour at room temperature. Three manual washing steps were done in PBS containing 0.05% (v/v) Tween 20 to remove unbound reagents.

A positive serum control was included on each plate to correct for interplate differences. For each analyzed antigen, the background was defined as the mean of two wells containing antigen without serum. This background and the absorbance values measured against GST alone for each serum sample, defining the background reactivity of a serum sample, were both subtracted from the absorbance value measured against the GST-E6 fusion proteins for each serum sample. All samples were tested in duplicate. Sera that showed background absorbance levels >0.5 measured against GST alone ($n = 1$) were excluded from the study.

Calculation of ELISA Values and ELISA Cutoff Values. Sera for which the difference between duplicate wells exceeded 50% of the duplicate mean were retested. Subsequently, for each antigen, we calculated the mean of the positive control serum values of all test plates. Serum samples from plates with positive control serum value that exceeded this mean value $\pm 2\text{ SD}$ were retested. All serum values were corrected according to the mean positive control serum values that we calculated on each plate.

The ELISA cutoff values to determine the value above which a serum was called seropositive were based on sera from randomly selected Dutch hospital employees. These sera were tested in exact the same manner as the test sera. For each antigen, we calculated the mean of the corrected absorbance values of this population $\pm 3\text{ SD}$, excluding outliers (20). For the L1 viruslike particle ELISA, the calculated cutoff values for HPV8, 15, 16, 20, 24, and 38 were 0.233, 0.391, 0.403, 0.441, 0.289, and 0.325, respectively (based on 50 control serum samples), and for the GST-E6 ELISA these values were 0.123, 0.174, 0.080, 0.116, 0.173, and 0.231, respectively (based on 100 control serum samples). The mean of the absorbance values of the Dutch population selected to determine the cutoff values was similar to the mean absorbance values of the Australian control population for each of the L1 and E6 proteins tested.

HPV DNA Analysis. The DNA extraction from the cells attached to the plucked hairs was described earlier (11) and was done according to the DNA extraction method described (22). For the HPV type-specific PCR, primers were designed for β -HPV types 5, 8, 15, 20, 24, and 38. These β -HPV types are widely distributed in the cutaneous β -subgroup of the phylogenetic tree of papillomaviruses and are regularly found in lesional skin tissues (4, 6, 8, 9, 11, 23, 24). The primers were located in the E7 open reading frame and generated a PCR product between 121 and 147 bp (19). The HPV16-specific PCR was not done on these hair samples because no HPV16 DNA could be detected in a former study analyzing plucked eyebrow hairs from 526 SCC cases and controls (19). The HPV type-specific PCR was done as described earlier (19). Before HPV PCR analysis, the DNA quality of each sample was determined by PCR amplification of a 184-bp fragment of the *A-myb* gene (24). The *A-myb* PCR conditions were the same as for the HPV type-specific PCR using 3.5 mmol L^{-1} MgCl_2 . Four samples showed no *A-myb* PCR product and were subsequently removed from the study.

Statistical Analysis. Analyses were done using SAS Version 8 (SAS Institute Cary, NC). We used logistic regression analyses to estimate odds ratios (OR) for HPV, 95% confidence intervals (95% CI), and *P* values. In each statistical model, tumor-free controls were used as the reference group. All

analyses were adjusted for age and sex. The effect of other potential confounders was assessed using a forward stepwise approach but made no difference to the estimates, and the most parsimonious model was therefore presented.

Positivity to any HPV type was assessed initially. We then categorized people according to the number of HPV types found in the sample (either hair or serum) and assessed associations between skin cancer risk and the presence of multiple HPV types.

Cumulative occupational sun exposure was calculated for each participant by summing the time spent outdoors during the workday for each "job" or activity, weighted by the duration of the job. Cumulative recreational sun exposure was calculated for each participant by summing the time spent outdoors during the leisure time that corresponded to each job weighted by the number of years worked in that job. The cumulative recreational and occupational sun exposure scores were categorized into tertiles for analysis based on the distribution in the tumor-free control group.

Results

Characteristics of the Study Population. We recruited 58 tumor-free controls, 129 prevalent AK cases, and 64 incident

SCC cases. The response rate was between 75% and 85% in all groups. Eyebrow hair was collected from 57 controls, 126 AK, and 63 SCC cases, and blood from 53 controls, 118 AK, and 55 SCC cases. The median age of the AK and SCC cases was slightly higher than the controls, being 65 years in controls and 69 and 68 years in AK and SCC cases, respectively. Males were increasingly overrepresented in the AK and SCC cases. Statistically significant associations were observed between the propensity to sunburn, the inability to tan and fair skin, and AK and SCC (Table 1). Neither high recreational nor occupational sun exposure was associated with AK or SCC, consistent with previous findings in a similar population (25). Smoking was not associated with AK or SCC.

HPV Seroreactivity. The absorbance values measured against each of the HPV capsid proteins L1 are represented separately for AK and SCC cases and controls in Fig. 1A. In the AK and SCC cases, the mean seroreactivity for HPV8 and 20, respectively, was significantly and increasingly higher than in controls. The mean seroreactivity for HPV15 and HPV38 was significantly higher in the SCC cases only. The mean seroreactivity against HPV16 and HPV24 was also higher in the AK and SCC cases but this was not statistically significant.

The absorbance values measured against the intracellular, putative oncoprotein E6 showed no increase of the mean

Table 1. Characteristics of the study population drawn from residents of Brisbane, Australia (n = 251)

	Controls	Actinic keratoses		Squamous cell carcinoma	
	n (%)	n (%)	OR (95% CI)*	n (%)	OR (95% CI)*
Age (y)					
35-54	13 (22)	10 (8)	1.0	10 (16)	1.0
55-74	36 (62)	90 (70)	3.25 (1.3-8.1)	39 (61)	1.41 (0.6-3.6)
>75	9 (16)	29 (22)	4.2 (1.4-12.7)	15 (23)	2.17 (0.7-7.0)
Subtotal	58	129		64	
Sex					
Female	25 (43)	45 (35)	1.0	15 (23)	1.0
Male	33 (57)	84 (65)	1.4 (0.8-2.7)	49 (77)	2.5 (1.1-5.4)
Subtotal	58	129		64	
Propensity to sunburn					
Rarely/never burn	20 (40)	10 (9)	1.0	4 (6)	1.0
Sometimes burn	15 (30)	34 (29)	4.0 (1.5-11.1)	15 (24)	4.8 (1.3-18.4)
Mostly burn	8 (16)	29 (25)	9.0 (2.8-28.9)	18 (29)	12.7 (3.0-53.8)
Always burn	7 (14)	44 (38)	13.6 (4.3-42.8)	26 (42)	22.4 (5.3-95.3)
Subtotal	50†	117		63	
Tanning ability					
Very brown/deeply tanned	18 (36)	15 (13)	1.0	3 (5)	1.0
Moderately tanned	26 (52)	42 (36)	1.8 (0.7-4.4)	17 (27)	3.2 (0.8-13.1)
Slightly tanned	4 (8)	33 (28)	12.6 (1.2-3.9)	28 (44)	33.5 (6.5-171)
Not tanned at all	2 (4)	28 (24)	22.0 (4.1-119)	15 (24)	37.2 (5.3-261)
Subtotal	50	118		63	
Skin color‡					
Olive/medium	45 (79)	30 (23)	1.0	11 (17)	1.0
Fair	12 (21)	99 (77)	13.0 (5.9-28.6)	53 (83)	18.9 (7.3-49.0)
Subtotal	57	129		64	
Occupational sun exposure§					
Low	16 (34)	28 (24)	1.0	10 (17)	1.0
Medium	15 (32)	32 (27)	0.9 (0.4-2.4)	26 (44)	2.4 (0.8-6.7)
High	16 (34)	59 (50)	1.5 (0.6-3.7)	23 (39)	2.0 (0.7-5.6)
Subtotal	47	119		59	
Recreational sun exposure					
Low	15 (32)	40 (34)	1.0	14 (24)	1.0
Medium	16 (34)	33 (28)	0.9 (0.3-2.5)	11 (19)	0.5 (0.1-1.8)
High	16 (34)	46 (39)	0.9 (0.3-2.3)	34 (58)	1.4 (0.5-4.1)
Subtotal	47	119		59	
Smoking status					
Never smoker	25 (51)	63 (54)	1.0	31 (50)	1.0
Ex-smoker	18 (37)	41 (35)	0.8 (0.4-1.8)	24 (39)	0.8 (0.3-1.9)
Current smoker	6 (12)	12 (10)	0.9 (0.3-2.8)	7 (11)	0.8 (0.2-3.1)
Subtotal	49	116		62	

*Adjusted for age and sex.

†Some of the participants did not return the questionnaire despite persistent follow-up resulting in missing information.

‡Skin color recorded by the examiner.

§Categorized into tertiles according to the distribution in the control group.

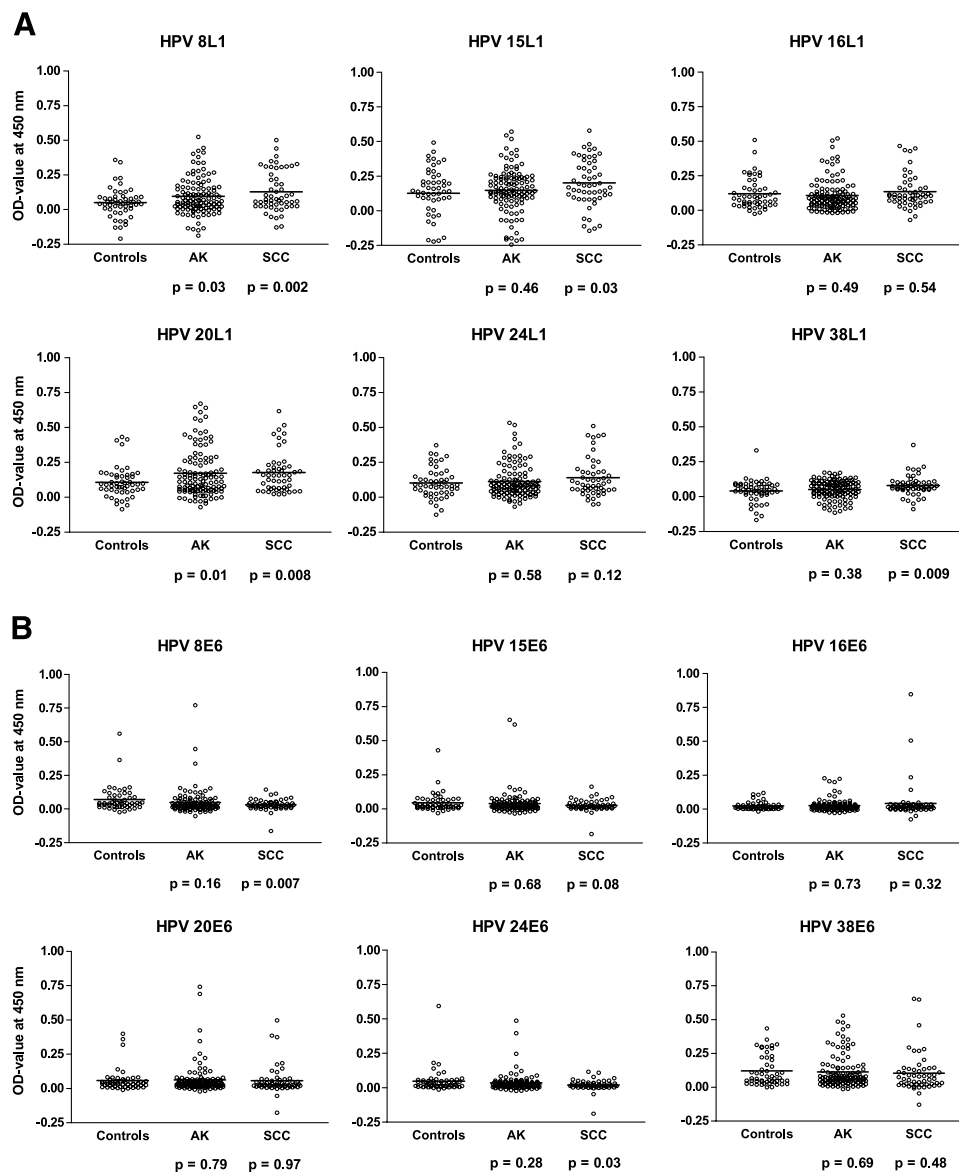


Figure 1. Seroreactivity against L1 viruslike particles (VLP; **A**) and GST-E6 proteins (**B**) of six HPV types, shown as absorbances measured in ELISA among controls, AK, and SCC patients. Horizontal bars, mean calculated absorbance values for each population. $P < 0.05$ was considered to be statistically significant (Student's t test).

seroreactivity in the AK and SCC cases compared with the controls for all tested β -HPV types (Fig. 1B). On the contrary, against most E6 proteins, the mean seroreactivity was lower in the AK and SCC cases compared with the controls. The mean seroreactivity to the E6 protein of HPV8 and HPV24 was significantly lower in the SCC cases compared with controls (Fig. 1B).

HPV Seropositivity. A significant positive trend in overall β -HPV L1 seropositivity was observed with increasing severity of skin tumors, with 13% positive serum samples in the tumor-free controls, 26% in AK cases, and 37% in the SCC cases (P for trend = 0.03), resulting in adjusted ORs of 2.3 (95% CI, 0.85-4.9) and 3.9 (95% CI, 1.4-10.7), respectively (Table 2A). For HPV8, L1 seropositivity was increasingly associated with AK and SCC. For HPV20, a similar trend was observed but associations between L1 seropositivity and AK and SCC could not be calculated because the L1 seropositivity in the controls was negative. For the other β -HPV types and HPV16, the number of seropositives was also increased with increasing severity of the lesions (none, AK, and SCC) but these differences were not statistically significant (Table 2A).

In contrast to L1, the overall E6 seroprevalence declined with increasing severity of the skin lesion, with 35% in the

controls, 25% in the AK, and 24% with SCC cases. This was particularly clear for HPV8 E6 where 21% of the controls, 11% of the AK, and only 2% of the SCC cases were seropositive (Table 2B).

Overall, HPV L1 seropositivity did not predict E6 seropositivity ($P = 0.69$). Only 19 of the 223 individuals (9%) tested were seropositive for both L1 and E6. Seven of these 19 double-seropositives (37%) were positive for the same HPV type. There was no association between the likelihood of being both L1 and E6 seropositive and AK and SCC.

HPV DNA Positivity. A higher proportion of AK patients were overall β -HPV DNA positive (54%) than those with SCC (44%) or tumor-free controls (40%; Table 2C). For most tested HPV types, the highest prevalence of viral DNA was found in the AK cases, and for HPV20 significance was reached (Table 2C).

Multiple HPV Infection. In 38% of the HPV L1 seropositive individuals, HPV L1 antibodies were detected against multiple HPV types. The proportion of HPV L1 seropositives with multiple L1 antibodies increased with increasing severity of the skin lesion, with 14% (1 of 7) in the controls, 39% (12 of 31) in the AK cases, and 45% (9 of 20) in the SCC cases (P for trend = 0.35; Table 3). Including all study participants, rather than

just those seropositive, we observed a similar trend in the proportion of those with multiple HPV infections, ranging from 2% in the controls to 10% in the AK cases and 17% in the SCC cases ($P = 0.06$). Among those who were HPV E6 seropositive, fewer individuals had evidence of multiple infection (26%). No inverse association was observed between multiple HPV E6 antibodies and the development of AK and SCC (Table 3).

In 47% of the β -HPV DNA positive individuals, multiple HPV types were detected in the eyebrow hairs. Multiple HPV DNA positivity was not associated with prevalence of AK or incidence of SCCs (Table 3).

HPV Seropositivity and HPV DNA Positivity. Among all participants, there was a positive trend between HPV DNA positivity and L1 seropositivity ($P = 0.07$). Of those whose hair samples were negative for HPV, 22% were positive for L1 antibodies compared with 33% of those who were HPV DNA positive. This association was present in the controls ($P = 0.03$) and in the AK cases ($P = 0.04$) but not in the SCC cases ($P = 0.34$). Of the 223 participants tested, 37 (17%) were both HPV DNA positive and HPV L1 seropositive. Of these, 12 (32%) were positive for the same HPV type in their hair and serum samples. There was no association between HPV DNA positivity and E6 seropositivity among all participants ($P = 0.78$) or when stratified by tumor group (data not shown).

Discussion

This study offered for the first time the opportunity to correlate markers of β -HPV infection across a spectrum of UV light-associated benign and malignant skin tumors varying

from tumor-free skin to actinic keratoses and SCCs. Three markers of β -HPV infection were used; seroreactivity to the viral capsid protein L1 and the intracellular protein E6 and the presence of β -HPV DNA in plucked eyebrow hairs. In addition to investigating the association between these markers and varying severity of the skin lesions, this study also enabled us to correlate the markers of β -HPV infection with each other.

Although interpretation of the study results is limited by the relatively small number of study participants, our results clearly indicated an increase of overall L1 seroreactivity with increasing severity of the skin lesion. In parallel, among the L1 seropositives, the proportion of patients who were seropositive against multiple β -HPV types increased from 14% in controls to 39% in AK and 45% in SCC cases, and among the total study population from 2% to 10% and 17%. Similar trends were observed when only single HPV type L1 seropositives were taken into account, but these seldom reached significance, probably because of the reduction in sample size (data not shown). Together, these results may indicate that β -HPV infections increase the risk of AK and SCC but, alternatively, tumors of increasing severity may promote β -HPV L1 seroresponses more efficiently, possibly as a result of increased local inflammation.

In contrast to the L1 results, seroreactivity to the HPV E6 proteins seemed to diminish with the severity of the lesions. Remarkably, E6 and L1 antibodies were hardly ever found concomitantly (data not shown). Perhaps antibody responses to the early (nonstructural, intracellular) and late (structural, also extracellular) β -HPV proteins take place at different times and phases during HPV infection or HPV-associated tumor development.

Table 2. Calculated OR of actinic keratoses and squamous cell carcinomas according to HPV seroreactivity (A and B) and HPV DNA positivity (C)

	Controls, N/total* (%)	AK, N/total* (%)	AK, adj OR [†] (95% CI)	P	SCC, N/total* (%)	SCC, adj OR [†] (95% CI)	P
(A) Seropositivity to L1							
No β -HPV	45/52 (87)	86/117 (74)	1		34/54 (63)	1	
Any β -HPV	7/52 (13)	31/117 (26)	2.3 (0.85-4.9)	0.07	20/54 (37)	3.9 (1.4-10.7)	0.01
β -HPV8	2/46 (4)	17/102 (17)	4.5 (0.98-20.9)	0.05	14/47 (30)	9.3 (1.9-45.6)	0.01
β -HPV15	3/47 (6)	8/93 (9)	1.8 (0.42-7.5)	0.43	10/43 (23)	3.8 (0.91-15.8)	0.07
β -HPV20	0/44 (0)	11/96 (11)	— [‡]	—	5/38 (13)	—	—
β -HPV24	4/48 (8)	11/96 (11)	1.5 (0.42-5.0)	0.54	8/41 (20)	2.6 (0.70-9.7)	0.15
β -HPV38	0/44 (0)	0/85 (0)	—	—	1/34 (2)	—	—
HPV16	1/45 (2)	3/88 (3)	1.5 (0.15-14.9)	0.74	4/37 (11)	7.9 (0.76-81.7)	0.08
(B) Seropositivity to E6							
No β -HPV	34/52 (65)	88/117 (75)	1		41/54 (76)	1	
Any β -HPV	18/52 (35)	29/117 (25)	0.60 (0.29-1.3)	0.17	13/54 (24)	0.45 (0.19-1.1)	0.08
β -HPV8	8/39 (21)	10/88 (11)	0.44 (0.15-1.3)	0.13	1/36 (2)	0.08 (0.01-0.66)	0.02
β -HPV15	2/33 (6)	2/80 (3)	0.31 (0.04-2.5)	0.27	0/35 (0)	—	—
β -HPV20	5/36 (14)	12/90 (13)	0.96 (0.35-3.1)	0.95	8/43 (15)	1.1 (0.32-4.0)	0.85
β -HPV24	2/33 (6)	3/81 (4)	0.53 (0.08-3.5)	0.51	0/35 (0)	—	—
β -HPV38	10/41 (24)	17/95 (18)	0.69 (0.27-1.7)	0.42	7/42 (13)	0.49 (0.16-1.5)	0.22
HPV16	4/35 (11)	8/86 (9)	0.79 (0.21-3.0)	0.73	4/39 (7)	0.87 (0.20-3.9)	0.87
(C) DNA positivity							
No β -HPV	34/57 (60)	58/126 (46)	1		35/63 (56)	1	
Any β -HPV	23/57 (40)	68/126 (54)	1.6 (0.81-3.0)	0.19	28/63 (44)	0.93 (0.43-2.0)	0.86
β -HPV5	11/45 (24)	32/90 (36)	2.4 (0.86-6.6)	0.36	8/43 (19)	0.59 (0.16-2.2)	0.35
β -HPV8	2/36 (6)	3/61 (5)	1.3 (0.18-9.2)	0.49	2/37 (5)	0.44 (0.05-4.1)	0.70
β -HPV15	1/35 (3)	2/60 (3)	0.71 (0.06-8.8)	0.79	1/36 (3)	0.64 (0.04-11.0)	0.76
β -HPV20	6/40 (15)	31/89 (35)	4.7 (1.3-16.6)	0.03	11/46 (24)	0.86 (0.20-3.7)	0.72
β -HPV24	7/41 (17)	30/88 (34)	2.8 (0.82-9.7)	0.36	9/44 (20)	0.78 (0.19-3.1)	0.95
β -HPV38	15/49 (31)	27/85 (32)	1.7 (0.66-1.6)	0.88	8/43 (19)	0.32 (0.09-1.2)	0.10

*Comparisons are with those who are HPV negative in all analyses.

[†]Adjusted for age and sex.

[‡]Impossible to calculate ORs because of zero value in the control column.

Table 3. Calculated ORs of actinic keratoses and squamous cell carcinomas according to a multiple HPV infection

	Controls		Actinic keratosis		Squamous cell carcinoma	
	n (%)	n (%)	OR*	n (%)	OR*	
No. L1 antibodies detected						
0	45 (87)	86 (74)	1.00	34 (63)	1.00	
1	6 (12)	19 (16)	1.72 (0.63-4.74)	11 (20)	2.53 (0.80-7.94)	
2+	1 (2)	12 (10)	6.11 (0.74-50.12) <i>P</i> = 0.06	9 (17)	11.58 (1.36-98.72) <i>P</i> = 0.006	
No. E6 antibodies detected						
0	34 (65)	88 (75)	1.00	41 (76)	1.00	
1	14 (27)	20 (17)	0.52 (0.23-1.17)	11 (20)	0.50 (0.19-1.31)	
2+	4 (8)	9 (7)	0.91 (0.25-3.31) <i>P</i> = 0.34	2 (4)	0.28 (0.05-1.76) <i>P</i> = 0.44	
No. positive HPV types in hair sample						
0	34 (60)	58 (46)	1.00	35 (56)	1.00	
1	11 (19)	33 (26)	1.59 (0.70-3.6)	19 (30)	1.27 (0.50-3.2)	
2+	8 (14)	19 (15)	1.51 (0.67-3.4) <i>P</i> = 0.42	8 (13)	0.61 (0.22-1.7) <i>P</i> = 0.45	

*Adjusted for age and sex.

The inverse relationship between overall E6 seropositivity and AK and SCC tempts to speculate that SCC patients may not be able to induce immune responses to cutaneous HPV E6 proteins or that E6-reactive antibodies to some extent protect against AK and SCC. To investigate these matters properly, follow-up studies are needed that include also measurements of cellular immunity because tumor protection exclusively through humoral immune responses is unlikely. Overall, it seems that HPV seroresponses in conjunction with skin (pre)malignancies behave differently than those in conjunction with genital HPV-related (pre)malignancies, where E6 seroresponses prevail over L1 seroresponses in the cancerous stages (26-28).

In line with previous studies in Europe, the strongest associations were observed for HPV8 (20, 29, 30). Despite the small study population, statistical significance was reached for both the positive and the negative SCC association with HPV8 L1 and E6 seropositivity, respectively. Taken together, this suggests that HPV8 is a cutaneous high-risk HPV type, not only in Europe but also probably worldwide.

Present β -HPV infection measured in the plucked eyebrow hairs was highest in the AK cases. This result contrasted with our recent study in the Netherlands (19) in which we found a statistically significant association between detectable β -HPV DNA in plucked eyebrow hairs and SCC. Differences may be explained by the larger sample size and higher proportion of HPV DNA-positive hair samples in the Dutch study (60% versus 48% in this current study). The latter may be explained by the fact that the present study analyzed fewer hairs per person, 6 to 8 hairs instead of 8 to 10 hairs in the Dutch study population, and included only incident SCC cases rather than people with a past history of SCC. In addition, differences in study outcome may be related to geographic differences between the two study populations, the Australian population being highly sun-exposed such that UV-induced skin carcinogenesis may be less dependent on additional effects of β -HPV infection. AK was not analyzed as a subject category in the Dutch study.

The highest prevalence of β -HPV infection in the plucked hairs from AK cases fits with recent histologic studies in AK and SCC biopsies that showed a substantially higher prevalence of β -HPV DNA and a higher HPV DNA load in AK opposed to SCC tissue (5, 31). Together with experimental observations indicating that cutaneous HPV types seem to impair the process of DNA repair and apoptosis after exposure to UV light (16, 17, 32, 33), our HPV DNA data support the idea that, if so, β -HPVs are involved in early carcinogenesis rather than in cancer maintenance. Overall, this study has generated some interesting new insights in HPV prevalence

and immunity that may help to understand the role that β -HPV infections play in skin cancer development.

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