Association Between Epidermodysplasia Verruciformis-Associated Human Papillomavirus DNA in Plucked Eyebrow Hair and Solar Keratoses

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Epidermodysplasia-verruciformis-associated human papillomavirus DNA has been demonstrated in squamous cell carcinomas and plucked hair from immunocompetent patients and renal transplant recipients. This study investigated the association between infection with epidermodysplasia-verruciformis-associated human papillomavirus, identified by the detection of viral DNA in plucked eyebrow hairs, and solar keratoses. These lesions are strongly predictive of squamous cell carcinoma. In a cross-sectional study 518 individuals were enrolled from a randomly selected sample of a subtropical Australian community. Epidermodysplasia-verruciformis-associated human papillomavirus DNA in eyebrow hair was detected using a nested polymerase chain reaction specific for epidermodysplasia-verruciformis-associated human papillomavirus types. Epidermodysplasia-verruciformis-associated human papillomavirus DNA was present in 121 (49%) of 245 men and 116 (44%) of 262 women. There was a strongly significant increase in epidermodysplasia-verruciformis-associated human papillomavirus infection with age ($p < 0.00001$), with prevalences of 29% in the 25–39 y age group, 42% at 40–59 y and 65% in the 60–79 y age group. Among men there was a strong association between epidermodysplasia-verruciformis-associated human papillomavirus and solar keratoses with an odds ratio, adjusted for age, skin color, and occupational sun exposure, of 3.40 (95% confidence interval, 1.77–6.53). No such association was found among women [odds ratio 1.03 (95% confidence interval 0.59–1.77, after adjustment for the same factors)]. Differences in occupational sun exposure and smoking histories could not explain these apparently different associations between epidermodysplasia-verruciformis-associated human papillomavirus infection and solar keratoses in men and women. In conclusion, epidermodysplasia-verruciformis-associated human papillomavirus infection is associated with solar keratoses in men suggesting that epidermodysplasia-verruciformis-associated human papillomavirus infection, in conjunction with sex specific factors (like androgens), may be involved in neoplastic changes of keratinocytes. ***Key words: epidemiology, human papillomavirus, nested polymerase chain reaction, skin cancer. J Invest Dermatol 117:1108–1112, 2001***

Cutaneous squamous cell carcinomas and basal cell carcinomas, together called nonmelanoma skin cancers, are the most common malignant tumors among white populations (Harvey *et al*, 1989; Goldberg, 1996; Marks, 1996).

Solar keratoses are benign skin tumors predominantly found on the face, backs of hands, and forearms, sites that are chronically exposed to the sun (Frost and Green, 1994). On rare occasions solar keratoses are precursors of squamous cell carcinoma and they share the same risk factors (Frost and Green, 1994; Frost *et al*, 1998). Exposure to sunlight is generally considered to be the most important environmental risk factor for both solar keratoses and cutaneous squamous cell carcinoma (Brash *et al*, 1991; Marks, 1996).

The high prevalence of human papillomavirus (HPV) DNA detected in solar keratoses and squamous cell carcinomas of immunosuppressed patients suggests a potential role for HPV infection in the etiology of these lesions (Berkhout *et al*, 1995; Shamanin *et al*, 1996; Pfister and ter Schegget, 1997). HPV are small double-stranded DNA viruses found in a wide variety of proliferative lesions of epithelial origin. The earliest evidence for the involvement of specific HPV types in human skin cancer originated from studies in patients suffering from the rare hereditary disease epidermodysplasia verruciformis (EV) (Orth *et al*, 1980). These HPV types and phylogenetically related types are commonly

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Abbreviations: EV, epidermodysplasia verruciformis; EV–HPV, epidermodysplasia-verruciformis-associated human papillomavirus.

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Table I. Characteristics of persons with and without solar keratoses

	Persons without solar keratoses[a] n (%)	Persons with solar keratoses[a] n (%)	Odds ratio (95% confidence interval)
Number[b]	231	276	
Age categorized			
25–39	67 (29.0)	22 (8.0)	1
40–59	128 (55.4)	135 (48.9)	3.21 (1.82; 5.71)
60–79	36 (15.6)	119 (43.1)	10.07 (5.25; 19.45)
Sex			
Female	140 (60.6)	122 (44.2)	1
Male	91 (39.4)	154 (55.8)	1.94 (1.34; 2.81)
Tanning ability			
Tan only	29 (12.6)	24 (8.7)	1
Burn then tan	169 (73.2)	197 (71.6)	1.41 (0.76; 2.61)
Always burn		33 (14.3)	54 (19.6) 1.98 (0.93; 4.28)
Skin color			
Olive/brown	31 (13.4)	14 (5.1)	1
Medium	102 (44.1)	102 (37.1)	2.21 (1.06; 4.67)
Fair	98 (42.4)	159 (57.8)	3.59 (1.74; 7.50)
Occupational sun exposure			
Mainly indoors	119 (51.5)	94 (34.2)	1
In and outdoors	76 (32.9)	109 (39.6)	1.82 (1.20; 2.76)
Mainly outdoors	36 (15.6)	72 (26.2)	2.53 (1.52; 4.23)
Smoking			
Nonsmokers	136 (59.4)	151 (55.3)	1
Ex-smokers	66 (28.8)	94 (34.4)	1.28 (0.85; 1.93)
Current smokers	27 (11.8)	28 (10.3)	0.93 (0.50; 1.73)
Skin cancer[c]			
No skin cancer	187 (80.9)	137 (49.6)	*d*
SCC only	0	15 (5.4)	
SCC + BCC	0	9 (3.3)	
BCC only	15 (6.5)	54 (19.6)	
Not characterized	29 (12.6)	61 (22.1)	
EV–HPV in eyebrow hair			
Negative	148 (64.1)	122 (44.2)	1
Positive	83 (35.9)	154 (55.8)	2.25 (1.55; 3.28)

[a]Mean (± SD) age in years of (i) persons without solar keratoses: 47.4 (± 11.4), and (ii) persons with solar keratoses: 57.8 (± 11.8). Difference: 10.4 y (8.37; 12.43).
[b]Numbers do not always add to the total due to missing information.
[c]SCC, squamous cell carcinoma; BCC, basal cell carcinoma.
[d]Odds ratios were not calculated because of blank values in some of the groups.

referred to as EV–HPV types and include HPV 5, 8, 9, 12, 14, 15, 17, 19–25, 36–38, and 47.

As well as being detected in solar keratoses and squamous cell carcinoma, EV–HPV DNA has also been found in plucked hair from both immunosuppressed and immunocompetent persons (Boxman *et al*, 1997), in skin scrapings and biopsies from psoriatic patients (Favre *et al*, 1998; Weissenborn *et al*, 1999); and from patients with bullous disease (Favre *et al*, 2000). These findings indicate that EV–HPV are seen not only in certain groups of skin cancer patients, but are widely distributed among the general population. To date, the role of EV–HPV in the pathogenesis of solar keratoses and squamous cell carcinoma and also in psoriasis is unclear.

Recently, we successfully described a noninvasive technique, plucking hairs, to obtain biologic samples from all individuals (cases and controls) in a nested case–control study in order to determine their EV–HPV status. Recently, we detected the same EV–HPV types in multiple lesions from the same individual collected over time (Berkhout *et al*, 2000) and in hair samples from different body sites collected at the same time point (Boxman *et al*, 1997). This suggests that viral DNA isolated from hairs might be a marker of HPV infections elsewhere on the body. As squamous cell carcinoma and solar keratoses commonly occur on the face, eyebrow hairs were chosen to identify those individuals infected with EV–HPV. Participants were drawn from a much larger community study of skin cancer in Nambour, Queensland (Boxman *et al*, 2000). A nonsignificant negative association between the presence of EV–HPV DNA in eyebrow hair and basal cell carcinoma was found and a nonsignificant positive association with squamous cell carcinoma, the estimated relative risk of developing squamous cell carcinoma among HPV-infected individuals being 2.00 (95% confidence interval 0.50–8.00). Given the shortcomings of a previous study due to limited numbers of cases with squamous cell carcinomas (n = 25), we decided to investigate the association between the presence of EV–HPV DNA in eyebrow hair and the more prevalent solar keratoses, in a larger cross-sectional study. The purpose of this study was to establish the prevalence of EV–HPV DNA in eyebrow hair, to examine factors that may be associated with EV–HPV infection, and to address the question of whether EV–HPV infection is associated with the presence of solar keratoses.

MATERIALS AND METHODS

Study population In 1986, 2095 residents of the township of Nambour in Queensland, Australia, who were randomly selected from the electoral roll (enrollment is compulsory) attended a skin cancer survey (Green *et al*, 1988). In 1992, 1626 of these participants agreed to be part of a skin cancer prevention trial involving randomization to sunscreen and/or β-carotene (Green *et al*, 1994, 1999). Half of the 1250 participants attending the 1996 skin examinations were invited to participate in the present cross-sectional study and 518 persons took part with their informed consent. All received whole-body skin examinations by experienced dermatologists in 1992, 1994, and 1996.

Table II. Characteristics of persons with and without HPV infection

	Persons without HPV infection[a] n (%)	Persons with HPV infection[a] n (%)	Odds ratio (95% confidence interval)
Number[b]	270	237	
Age categorized			
25–39	63 (23.3)	26 (11.0)	1
40–59	153 (56.7)	110 (46.4)	1.74 (1.01; 3.03)
60–79	54 (20.0)	101 (42.6)	4.53 (2.49; 8.30)
Sex			
Female	146 (54.1)	116 (48.9)	1
Male	124 (45.9)	121 (51.1)	1.23 (0.85; 1.77)
Tanning ability			
Tan only	26 (9.7)	27 (11.4)	1
Burn then tan	197 (73.2)	169 (71.3)	0.83 (0.45; 1.53)
Always burn	46 (17.1)	41 (17.3)	0.86 (0.41; 1.80)
Skin color			
Olive/brown	23 (8.6)	22 (9.3)	1
Medium	104 (38.7)	100 (42.2)	1.01 (0.50; 2.01)
Fair	142 (52.8)	115 (48.5)	0.85 (0.43; 1.67)
Occupational sun exposure			
Mainly indoors	132 (49.1)	81 (34.2)	1
In and outdoors	88 (32.7)	97 (40.9)	1.80 (1.18; 2.73)
Mainly outdoors	49 (18.2)	59 (24.9)	1.96 (1.20; 3.22)
Smoking			
Nonsmokers	157 (58.6)	130 (66.5)	1
Ex-smokers	80 (29.8)	80 (34.2)	1.21 (0.80; 1.81)
Current smokers	31 (11.8)	24 (10.3)	0.93 (0.50; 1.74)
Skin cancer[c]			
No skin cancer	183 (67.8)	141 (59.5)	1
SCC only	3 (1.1)	12 (5.1)	5.19 (1.33; 23.64)
SCC + BCC	5 (1.9)	4 (1.7)	1.04 (0.23; 4.55)
BCC only	28 (10.4)	41 (17.3)	1.90 (1.09; 3.33)
Not characterized	51 (18.9)	39 (16.5)	0.99 (0.60; 1.63)
Solar keratoses			
Absent	148 (54.8)	83 (35.0)	1
Present	122 (45.2)	154 (65.0)	2.25 (1.55; 3.28)

[a]Mean age (± SD) in years of (i) persons without HPV infection: 49.2 (± 11.4) and (ii) persons wth HPV infection: 57.5 (± 12.7). Difference: 8.3 (6.20; 10.40) years.
[b]Numbers do not always add to the total due to missing information.
[c]SCC, squamous cell carcinoma; BCC, basal cell carcinoma.

Data collection At the 1996 skin examination, dermatologists counted and mapped the positions of all solar keratoses present on 13 body sites. Those with one or more solar keratoses at any site were defined as having prevalent solar keratoses. As well, about eight to 10 hairs were plucked from eyebrows by one of the investigators or a trained assistant using a disposable pair of tweezers and gloves for each individual. Only hairs that contained hair follicles were collected and samples were stored at –70°C until analysis.

Standard questionnaires were used to obtain data on known or suspected risk factors for solar keratoses, including skin color, hair color, eye color, tanning ability, occupational sun exposure at baseline, lifetime and recent sunburn histories and smoking.

EV–HPV analysis Hair samples were lyzed in L6 buffer for 1 h. One portion of the buffer was supplemented with diatoms (Boom *et al*, 1990) and incubated for a further hour. DNA was isolated according to a method described in Boom *et al* (1990). The remaining portion of L6 buffer was stored at −70°C. A nested polymerase chain reaction (PCR) method was used to detect EV–HPV DNA. The degenerate primers of the first PCR (CP62/CP70a) (Berkhout *et al*, 1995; Boxman *et al*, 1997) and nested PCR (CP65/CP69a) (Berkhout *et al*, 1995; Boxman *et al*, 1997) were located in the L1 open reading frame and designed to detect the subgroup of known EV–HPV types. Eight to 10% of the extractable DNA was used as input to the first step PCR. All PCR reactions were performed as described by Boxman *et al* (1997). A negative control (water) was included between each set of two DNA samples and processed in the same way as the hair samples throughout DNA preparation and both PCR steps. No negative controls were positive for EV–HPV. To determine the efficacy of the nested PCR, 10-fold dilutions of DNA of a plasmid containing HPV15 were used as positive controls. The detection limit for this plasmid was 0.1 fg, representing about 10 molecules of HPV DNA.

HPV typing of 89 positive PCR samples confirmed that only EV–HPV DNA was amplified by the PCR used (Boxman *et al*, 2000). In addition, because none of the negative controls were positive in the PCR, no further sequence analyses were performed in our study.

Statistical analysis Calculations were performed with the statistical software package of the SAS Institute Inc. (Cary, NC). The data were analyzed using Student's t test and the chi-square test. Odds ratios were estimated from logistic regression. Multivariate logistic regression analysis was used to adjust for confounders. Factors that were considered were age, sex, tanning ability, skin color, occupational sun exposure, smoking, and trial design factors [daily sunscreen application (*vs* discretionary sunscreen application) and of β-carotene (*vs* placebo)] (Green *et al*, 1999).

In addition, Mantel–Haenszel weighted odds ratios were calculated to adjust for age groups by using Epi-Info (version 6.04b, Centers for Disease Control & Prevention, U.S.A. and World Health Organization, Geneva, Switzerland).

RESULTS

Among the 518 individuals who agreed to participate there were five whose samples were lost during transport or storage, and of the remaining 513 individuals, the presence of solar keratoses was scored in 507 individuals. Analyses were restricted to these 507, of whom 245 (48%) were male. The mean age of men taking part was 53.0 y, and of women, 53.1 y.

Characteristics of persons with and without solar keratoses are presented in **Table I**. Persons with solar keratoses were significantly older, were more often male, had fairer skin, less often had an outdoor occupation, and had more frequently developed skin

Table III. Risk of solar keratoses dependent on the presence of HPV DNA in plucked eyebrow hair stratified according to age groups

		Persons without solar keratoses n (%)	Persons with solar keratoses n (%)	Odds ratio	Pooled odds ratio[a]
Men					
All ages	HPV neg.	65 (71.4)	59 (38.3)	1	1
	HPV pos.	26 (28.6)	95 (61.7)	4.03 (2.22; 7.33)	2.65 (1.40; 5.11)
25–39 y	HPV neg.	29 (85.3)	8 (53.3)	1	
	HPV pos.	5 (14.7)	7 (46.7)	5.07 (1.05; 25.97)	
40–59 y	HPV neg.	32 (68.1)	35 (47.3)	1	
	HPV pos.	15 (31.9)	39 (52.7)	2.38 (1.04; 5.50)	
60–79 y	HPV neg.	4 (40.0)	16 (25.0)	1	
	HPV pos.	6 (60.0)	49 (75.0)	2.04 (0.42; 9.74)	
Women					
All ages	HPV neg.	83 (59.3)	63 (51.6)	1	1
	HPV pos.	57 (40.7)	59 (48.4)	1.36 (0.81; 2.29)	1.11 (0.63; 1.94)
25–39 y	HPV neg.	22 (67.7)	4 (57.1)	1	
	HPV pos.	11 (33.4)	3 (42.9)	1.50 (0.21; 10.22)	
40–59 y	HPV neg.	49 (60.5)	37 (60.7)	1	
	HPV pos.	32 (39.5)	24 (39.3)	0.99 (0.48; 2.07)	
60–79 y	HPV neg.	12 (46.2)	22 (40.7)	1	
	HPV pos.	14 (53.8)	32 (59.3)	1.25 (0.44; 3.55)	

[a]Adjustment for age groups, using Mantel–Haenszel weighted odds ratio.

cancer. There was a strong association between the presence of solar keratoses and the presence of EV–HPV in plucked eyebrow hair ($p < 0.00001$).

Characteristics of persons with and without EV–HPV infection are presented in **Table II**. The overall prevalence of EV–HPV infection was 49.4% (121 of 245) for men and 44.3% (116 of 262) for women. There was a strongly significant increase in EV–HPV infection with age ($p < 0.00001$), with prevalences of 29% in the 25–39 y age group, 42% at 40–59 y, and 65% in the 60–79 y age group. Occupational sun exposure was significantly associated and sex, tanning ability, skin color, and smoking were not statistically significantly associated with EV–HPV infection (see **Table II**).

From the outset our analyses showed that there was a strong association between EV–HPV infection and solar keratoses among men but no such association among women, and thus the association between HPV infection and solar keratoses have been presented separately for men and women. To calculate the association between HPV infection and solar keratoses, we adjusted for age, skin color, and occupational sun exposure. No adjustments were made for past or contemporary skin cancer history, because solar keratoses are potential precursors of squamous cell carcinoma.

Among men there was a strong association between presence of EV–HPV in plucked eyebrow hair and presence of solar keratoses with a crude odds ratio of 4.03 (95% confidence interval 2.22–7.33). The pooled odds ratio adjusted for age groups by use of the Mantel–Haenszel test was 2.65 (95% confidence interval 1.40–5.11) (**Table III**). After adjustment for age, skin color, and occupational sun exposure by use of a multivariate model, the odds ratio was 3.40 (95% confidence interval, 1.77–6.53). No such association was found among women, who had a crude odds ratio related to the presence of solar keratoses of 1.36 (95% confidence interval 0.81–2.29) (**Table III**), which was reduced to 1.03 (95% confidence interval 0.59–1.77) after adjustment for age, skin color, and occupational sun exposure by use of a multivariate analysis. Although individuals participating in this study were randomly selected from a much larger skin cancer prevention trial involving randomization to sunscreen and/or β-carotene (Green *et al*, 1994, 1999), adjustment for these interventions had no effect on the association between solar keratoses and HPV infection (data not shown).

DISCUSSION

In the Nambour population (Queensland, Australia), the presence of EV–HPV DNA in plucked eyebrow hair was clearly associated with the presence of solar keratoses. Age was strongly associated with both the presence of EV–HPV infection in eyebrow hair and solar keratoses. This study data confirm these associations. We speculate that individuals are gradually persistently infected with a large range of cutaneous HPV types during their lifetime. Nevertheless, increasing age only partly explains the association between HPV infection and solar keratoses (see **Table III**). Surprisingly, the association between HPV infection and solar keratoses was observed among men but not women. An intriguing question is why. As the overall EV–HPV prevalence in men and women is similar, other factors than HPV infection *per se* may be important for this difference between the genders. The higher level of occupational sun exposure and smoking among men did not explain this apparent difference. Thus, if this sex-specific association is confirmed in this study or by others, new sex-specific factors (such as androgens) will need to be identified that can explain the difference between men and women regarding the association between EV–HPV infection and solar keratoses.

The pathogenesis of solar keratoses and the subsequent squamous cell carcinomas is a complex interplay between immunologic, genetic, environmental, and possibly hormonal factors. The observed association between solar keratoses, EV–HPV infection and age might indicate that elderly patients not only more frequently develop solar keratoses but also have a less effective immunologic response against EV–HPV infections. On the other hand in addition to sun exposure, EV–HPV infection may play a direct part in the pathogenesis of solar keratoses. Sun exposure might directly activate functions of the viral genes (Purdie *et al*, 1999) or sun-exposure may enhance HPV replication in the host by inducing (local) immunosuppression and/or inactivation of keratinocyte growth regulating host genes (e.g., p53) (Brash *et al*, 1991). Recently, it was found that the E6 early protein from a range of cutaneous HPV types (including the EV–HPV type 5) effectively inhibits apoptosis by abrogation of Bak in response to ultraviolet damage, which might be important for malignant progression of HPV-containing lesions (Jackson and Storey, 2000; Jackson *et al*, 2000). As EV–HPV is widely distributed, as shown in the present

and other studies (Boxman *et al*, 1997, 1999), we hypothesize that individuals who are infected by EV–HPV are at an increased risk of developing solar keratoses and squamous cell carcinomas, possibly by preventing ultraviolet-induced apoptosis.

Molecular biologic studies are required to clarify the mechanisms by which the interplay between EV–HPV genes, ultraviolet exposure, and keratinocyte cell growth and differentiation takes place.

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