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Seroreactivity to Epidermodysplasia Verruciformis-related Human Papillomavirus Types Is Associated with Nonmelanoma Skin Cancer[1]

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ABSTRACT

DNA from epidermodysplasia verruciformis-related human papillomavirus (EV-HPV) types is frequently found in nonmelanoma skin cancer (squamous and basal cell carcinoma). Epidemiological studies that investigate the relation between EV-HPV infection and nonmelanoma skin cancer are scarce. We designed a case-control study in which we looked for HPV infection in 540 cases with a history of skin cancer and 333 controls. By measuring seroreactivity to L1 virus-like particles of EV-HPV types 5, 8, 15, 20, 24, and 38 and the genital type HPV16 and by estimating the skin cancer relative risk among HPV seropositives, we analyzed whether EV-HPV serorecognition is associated with nonmelanoma skin cancer. Seroreactivity to five of the six EV-HPV types tested (HPV5, 8, 15, 20, and 24) was significantly increased in the squamous cell carcinoma cases. After adjusting for age and sex, the estimated squamous cell carcinoma relative risk was significantly increased in HPV8 and HPV38 seropositives [odds ratio (OR) = 14.7 (95% confidence interval (CI), 1.6–135) and OR = 3.0 (95% CI, 1.1–8.4), respectively]. The estimated relative risk for nodular and superficial multifocal basal cell carcinoma was also significantly increased in the HPV8 seropositives [OR = 9.2 (95% CI, 1.1–78.2) and OR = 17.3 (95% CI, 2.1–143), respectively] and in the HPV20 seropositives [OR = 3.2 (95% CI 1.3–7.9) and OR = 3.4 (95% CI 1.2–9.5), respectively]. The relative risk of developing malignant melanoma was not increased among HPV seropositives, and no associations were found for HPV16. Restricted analyses among the HPV seropositives only, to exclude distortion by interindividual differences in seroresponsiveness, underscored the significance of our findings. Restricted analyses among patients with skin cancer only, however, revealed that EV-HPV seropositivity was not significantly more present in patients with nonmelanoma skin cancer than in those with melanoma skin cancer. Taken together, our results indicate that EV-HPV serorecognition is nonspecifically associated with nonmelanoma skin cancer and suggest that EV-HPV-directed seroresponses are induced upon skin cancer formation, rather than upon infection.

INTRODUCTION

HPVs[3] are involved in the genesis of specific benign and malignant proliferative disorders, such as skin warts (HPV1, 2, and others), genital warts and laryngeal papillomas (HPV6 and 11), cervical cancer (HPV16, 18, and others), and possibly nonmelanoma skin cancer (1). To date, 86 HPV genotypes have been completely sequenced, a quarter of which belong to the so-called EV-HPV types (2). EV-HPV types were originally found in skin lesions from patients with EV (3). EV patients develop multiple macular lesions on sun-exposed sites that progress into SCC in 30% of the cases. HPV5 and 8 have been regularly found in these lesions and are the best-known EV-HPV representatives (4).

Recent studies indicated that EV-HPV types are not restricted to EV patients. SCCs and BCCs in the general population, together referred to as nonmelanoma skin cancer, also frequently contain EV-HPV DNA (5, 6). In renal transplant recipients, who often develop nonmelanoma skin cancer in the years after transplantation, the percentage EV-HPV-positive SCCs increases up to 90% (7–9). EV-HPV is also frequently detected in actinic keratoses (~65%), the presumed SCC precursor (10, 11). Increasing age, male sex, fair skin, sun exposure, and smoking are known risk factors for nonmelanoma skin cancer (12–14). EV-HPV infection may act as an additional risk factor, especially because EV-HPV interacts with UV-induced apoptosis (15).

In most HPV DNA prevalence studies in nonmelanoma skin cancer, no specific EV-HPV type stood out. Usually, a wide spectrum of EV-HPV types is detected, including unassigned new EV-HPV types (5, 11, 16–18), and multiple EV-HPV types are frequently found in single biopsies (7, 11). The complexity of this situation, which hampers the search for oncogenic EV-HPV types, may be explained by the use of different PCR primer sets and the ubiquity of these viruses (18). Alternative strategies to investigate the relation between EV-HPV infection and nonmelanoma skin cancer, such as serology, are therefore needed.

In individuals with a history of different types of skin cancer, we looked for serorecognition of six EV-HPV types (HPV5, 8, 15, 20, 24, and 38) frequently detected in nonmelanoma skin cancer biopsies and plucked hairs (7, 11, 16, 19) and a common genital type associated with cervical cancer (HPV16; Ref. 1). By comparing EV-HPV seroreactivity and seropositivity among skin cancer cases and controls and by estimating skin cancer relative risks, we looked for associations between EV-HPV infection and skin cancer development.

MATERIALS AND METHODS

Study Population. The study described in this paper is embedded in the Leiden Skin Cancer Study that was initiated at the LUMC in 1997 as a case-control study of the causes of skin cancer in the Dutch population (13). The local medical ethical commission approved of the study, and its design was extensively described by De Hertog *et al.* (13). In short, 580 newly diagnosed cases from 1985 to 1997 with histologically confirmed skin cancer were studied: 161 persons with SCC; 301 persons with nBCC; 153 persons with sBCC; and 125 persons with malignant melanoma. The melanoma cases had no known metastases and did not use any systemic treatment for malignant melanoma. Controls ($n = 386$) from the same age range and the same referral area were recruited at the LUMC Ophthalmology outpatient clinic. Eye conditions are not usually associated with skin conditions. Controls were excluded when they had an intraocular melanoma or skin cancer in their history. Both cases and controls were excluded when they were transplant recipients or suffered from hereditary skin disorders with an increased risk of skin cancer. Dark-skinned individuals (skin type ≥ V, Fitzpatrick classification; Ref. 20) were also excluded because they very rarely develop skin cancers. Ninety-three

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[3] The abbreviations used are: HPV, human papillomavirus; EV, epidermodysplasia verruciformis; EV-HPV, epidermodysplasia verruciformis-related HPV; OR, odds ratio; CI, confidence interval; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; nBCC, nodular BCC; sBCC, superficial multifocal BCC; VLP, virus-like particle; C-PBS, 0.2% casein in PBS; LUMC, Leiden University Medical Center; GFP, green fluorescent protein; nt, nucleotide(s).

individuals (40 cases and 53 controls) were excluded from the current study because of lack of antigen to repeat the ELISA for sera that had unacceptable duplicate values (see below) or for sera that were located on plates with unacceptable positive control serum levels (see below).

For the current study, 873 subjects were included: 333 controls without skin cancer; 456 cases with nonmelanoma skin cancer (160 SCC, 291 nBCC, and 141 sBCC); and 84 melanoma cases. Of the nonmelanoma skin cancer cases included, 134 (29.4%) had more than one type of skin cancer, including melanoma. Every nonmelanoma skin cancer case (SCC, nBCC, or sBCC) was analyzed accordingly, regardless of the presence of other skin cancer types, unless stated otherwise. The melanoma cases selected had no other type of skin cancer.

Data and Sample Collection. The visit at the dermatology outpatient clinic was identical for cases and controls and consisted of a standardized interview and a physical examination. During physical examination, a dermatologist recorded skin type and the possible presence of skin cancer. Occasionally, controls appeared to have skin cancer, which was subsequently confirmed by histological examination. These persons were further considered as cases. In the same visit, blood was drawn to collect serum.

Generation of the HPV-L1 Recombinant Baculoviruses. Recombinant baculovirus *Ac*HPV5aL1 (21) was a gift from Drs. G. Orth and M. Favre (Pasteur Institute, Paris, France). *Ac*HPV8L1 (22) and *Ac*HPV15L1 (23) were donated by Drs. H. Pfister and P. Fuchs (Virology Institute, University of Cologne, Cologne, Germany). *Ac*HPV16L1 was provided by Dr. J. T. Schiller (NIH, Bethesda, MD; Ref. 24). HPV20-L1, HPV24-L1, and HPV38-L1 expressing recombinant baculoviruses were made in the Bac-to-Bac expression system (Life Technologies, Inc.) according to the manufacturer's instructions, with slight modifications. The pFastBacDual-GFP expression vector was used (25), which contains the GFP gene cloned in multiple cloning site II. As a result of this insertion, an additional *Hin*dIII site was introduced. This *Hin*dIII site was subsequently removed by the ligation of a mutated *Hin*dIII*-*Kpn*I linker in the vector digested with *Hin*dIII and *Kpn*I. The resulting vector is called pFBD-GFP*.

The L1 genes of HPV20, 24, and 38, respectively, were inserted into multiple cloning site I of pFBD-GFP* by PCR-assisted cloning from pBR322-HPV20 (26), pBR322-HPV24A (27), and pBR322-HPV38 (27), respectively. The primers used are as follows (their restriction site extensions are in italic): 5′-HPV20, nt 5889–5905 (CGA*AGGCCT*CC*ATCGAT*CCGCAGATGGCAGTTTGGC, *Stu*I, *Cla*I); 3′-HPV20, nt 7462–7445 (GG*CTCGAG*CC*ACTAGT*CCGTACCGAAAACGGTCGGG, *Xho*I, *Spe*I); 5′-HPV24, nt 5713–5739 (GCGC*GGATCC*AATATGTCGGTGTGGTTGCCAGCCAGTGGT, *Bam*HI); 3′-HPV24, nt 7251–7231 (CGAC*AAGCTT*ATTTTACGTGCGTTTTCGTTTGGT, *Hin*dIII); 5′-HPV38, nt 5660–5677 (CGT*CTCGAG*CCGATGACACTTTGGCTTCC, *Xho*I); and 3′-HPV38, nt 7209–7193 (GC*AAGCTT*C*ATCGAT*CCGGGCGACCGAAAACGGT, *Hin*dIII, *Cla*I).

The HPV20-L1 PCR product was first ligated into the *Stu*I- and *Xho*I-digested pFastBac1 and subsequently cloned into pFBD-GFP* as a *Stu*I-*Spe*I fragment. The HPV24-L1 PCR product was cloned into pFBD-GFP* as a *Bam*HI-*Hin*dIII fragment. HPV38 was first isolated from pBR322-HPV38 by digestion with *Eco*RI and ligated as a circular fragment. Subsequently, L1 was PCR-amplified from the recirculized HPV38 viral genome and cloned into pFastBac1 after *Xho*I and *Hin*dIII digestion. Subsequently, it was cloned into pFBD-GFP* as a *Sal*I-*Hin*dIII fragment.

pFBD20L1-GFP*, pFBD24L1-GFP*, and pFBD38L1-GFP*, respectively, were used to transform DH10Bac cells. Total DNA was harvested from gentamicin-resistant colonies and used to transfect Sf9 cells that were screened for fluorescence 4 days later. Supernatants from GFP-positive cultures were passaged to new Sf9 cells and checked for the presence of the HPV-L1 gene by PCR. GFP- and L1-positive passage 1 supernatants were plaque-purified and amplified to viral stocks of *Ac*HPV20L1-GFP*, *Ac*HPV24L1-GFP*, and *Ac*HPV38L1-GFP*.

Generation of HPV VLPs. To generate the HPV5, 8, 15, 16, 20, 24, and 38 VLPs, Sf9 cells were infected with *Ac*HPV5aL1, *Ac*HPV8L1, *Ac*HPV15L1, *Ac*HPV16L1, *Ac*HPV20L1-GFP*, *Ac*HPV24L1-GFP*, and *Ac*HPV38L1-GFP*, respectively, at a multiplicity of infection of 5–10. Cells were harvested 3 days after infection, washed with PBS, and disrupted by sonification at 0°C during 2 × 45 s at 18 μm. Protease inhibitors (100 μg/ml pepstatin, 200 μg/ml aprotinin, 50 μg/ml leupeptin, and 10 mM benzamide) were added, and cell debris was spun down. The supernatant was purified on 40% and 70% sucrose cushions at 100,000 × *g* for 2 h at 4°C. The sucrose interface was resuspended in 27% CsCl in PBS (w/w) and centrifuged to equilibrium (200,000 × *g*, 24 h, 4°C). The VLP-containing band with a density of about 1.3 g/ml was isolated from the gradient with a syringe, followed by overnight dialysis against PBS. VLP batches were checked by SDS-PAGE (Fig. 1*A*) and electron microscopy to confirm typical VLP morphology (Fig. 1*B*).

Detection of HPV VLP Serum Antibodies. IgG seroreactivity against the HPV VLPs was measured by ELISA without knowledge of the serum origins. Polysorp microtiter plates (Nunc) obtained from one batch were subdivided into sections and coated overnight at 4°C with a 100 ng/well solution in PBS of each HPV VLP or nothing (henceforth referred to as "uncoated"). Subse-

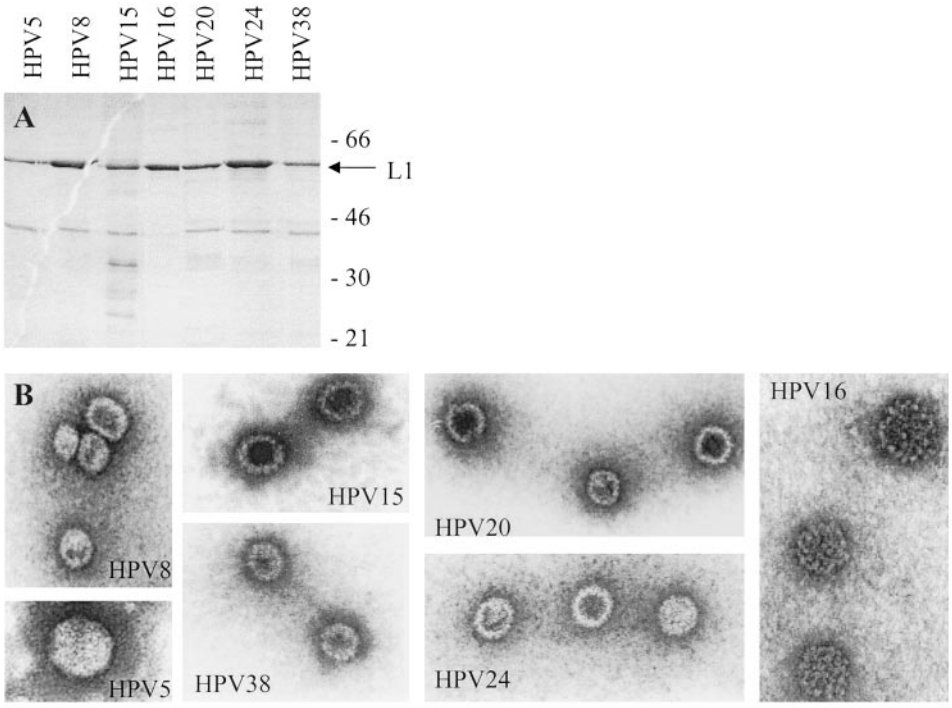

Fig. 1. *A*, Coomassie Blue-stained SDS polyacrylamide gel (12%) of CsCl-purified HPV VLP preparations. *B*, electron micrographs of the HPV VLPs. The VLP preparations were absorbed to carbon-coated grids, stained with 1% uranyl acetate, and examined by transmission electron microscopy.

quently, the plates were stored at −80°C. By including a positive control serum and a negative control (blank) for each antigen on each plate, all sera were tested against the individual HPV antigens under identical conditions. The positive control serum consisted of a pool of four EV-HPV VLP-reactive sera. The blank consisted of only serum dilution buffer, C-PBS. Each antigen-serum combination was tested in duplicate.

At the day of use, plates were thawed, washed three times with PBS, and blocked with a fresh solution of C-PBS for 1 h at 37°C. For 1 h at 37°C, 50 μl serum/well was added, diluted 1:100 in C-PBS. After washing three times in PBS, biotin-labeled antihuman total IgG (1 mg/ml), kindly donated by Dr. L. Aarden (CLB Sanquin, Amsterdam, the Netherlands), was added for 1 h at 37°C diluted 1:2000 in C-PBS, followed by three washes, and then streptavidin-poly-horseradish peroxidase (1 mg/ml, CLB Sanquin) diluted 1:10,000 in 1% casein in PBS was added for 30 min at room temperature. Finally, 50 μl of 0.1 mg/ml tetramethylbenzidine substrate in a 0.03% H_2O_2 0.11 M sodium acetate solution (pH 5.5) were added. The reaction was stopped after 5 min by adding 50 μl of 2 M H_2SO_4. The absorbance of the wells in each plate was measured at 450 nm in an automated plate reader.

Calculation of ELISA Values and ELISA Cutoff Values. Sera for which the difference between duplicate wells exceeded 50% of the duplicate mean were retested. To correct for antigen-specific background, for each antigen, the blank value was subtracted from the test values. To correct for serum-specific background, for each serum, the uncoated value was subtracted from the test values. Subsequently, for each antigen, we calculated the mean of the positive control serum values of a total of 180 test plates. Serum samples from plates with positive control serum values that exceeded this mean value ± 2× SD were retested. All serum values were corrected according to the mean positive control serum values that we calculated for each antigen.

The ELISA cutoff values to determine the value above which a serum was called seropositive were based on a group of 100 sera from randomly selected anonymous LUMC hospital employees tested at appointment for serum antibodies against hepatitis B virus surface antigen. 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 + 3× SD, excluding outliers. The calculated cutoff values for HPV5, 8, 15, 16, 20, 24, and 38 were 0.620, 0.366, 0.405, 0.456, 0.348, 0.432, and 0.336, respectively. The percentages of seropositivity in this population, 0.0%, 0.0%, 1.0%, 0.0%, 2.9%, 3.7%, and 0.9%, respectively, were comparable with those measured in the study control population (Table 2).

Statistical Analysis. All calculations were performed with the statistical software package JMP version 2 of the SAS Institute Inc. (Cary, NC). The data were analyzed using Student's t test and the χ^2 test. Relative risks were estimated using exposure OR from cross tabulation. Multivariate logistic regression analysis was used to adjust for possible confounding factors.

RESULTS

Population Characteristics. Characteristics of the study population according to skin cancer status were published previously by De Hertog *et al.* (13). Briefly, SCC and nBCC cases were significantly older than controls, whereas the melanoma cases were significantly younger. Males were overrepresented in the nonmelanoma skin cancer cases, and fair skin was significantly more common in all skin cancer cases. Chronic sun exposure was associated with nonmelanoma skin cancer but inversely associated with malignant melanoma. In the SCC cases, significantly more smokers were detected. These data confirmed the known risk factors for nonmelanoma skin cancer (age, male sex, fair skin, and sun exposure) and identified smoking as an additional risk factor for SCC (13).

HPV Seroreactivity and Nonmelanoma Skin Cancer. To investigate the association between EV-HPV infection and nonmelanoma skin cancer, we determined the reactivity in 873 sera against 6 EV-HPV types (HPV5, 8, 15, 20, 24, and 38) and a common genital type (HPV16). The absorbance values measured against each of the antigens are presented separately for cases and controls in Fig. 2. For five of the six EV types tested (HPV5, 8, 15, 20, and 24), we observed a statistically significant increase in seroreactivity in the SCC cases

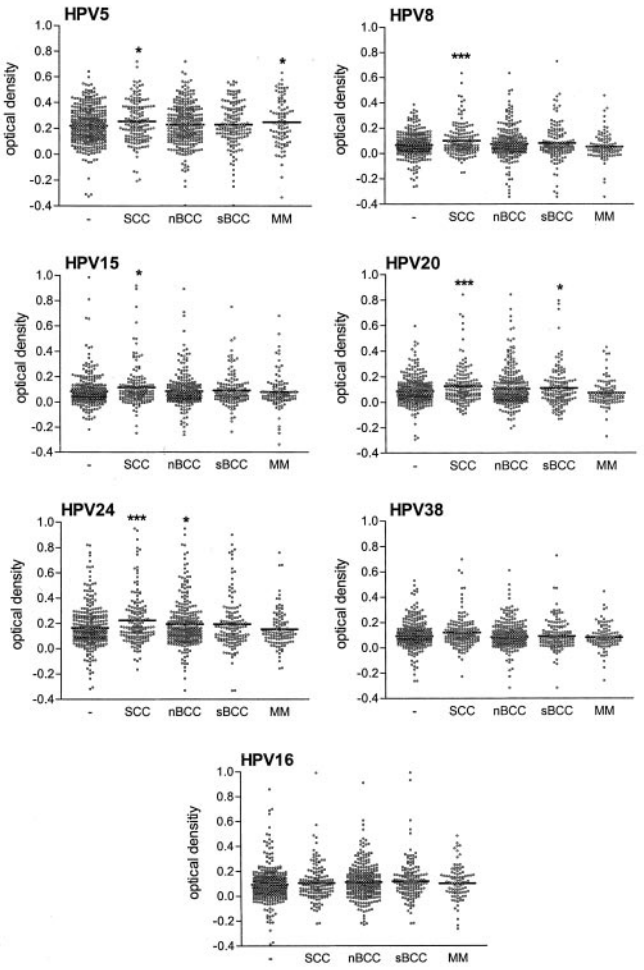

Fig. 2. Seroreactivity against seven HPV types, shown as optical densities measured in ELISA, among skin cancer patients and controls (−). *MM*, malignant melanoma. The *horizontal bars* represent the mean calculated absorbance values for each population. *, $P < 0.05$; ***, $P < 0.001$ compared with controls (Student's t test).

compared with controls. HPV20 and 24 seroreactivity was also increased in the sBCC and nBCC cases, respectively, and HPV5 seroreactivity was increased in the melanoma cases. HPV16 seroreactivity was not increased among the skin cancer cases.

HPV Seropositivity and Population Characteristics. A total of 136 individuals (15.6%) were HPV seropositive: 6 for HPV5 (0.7%); 19 for HPV8 (2.2%); 24 for HPV15 (2.7%); 18 for HPV16 (2.1%); 37 for HPV20 (4.2%); 75 for HPV24 (8.6%); and 24 for HPV38 (2.7%). With respect to the population characteristics, we found statistically significant associations between HPV24 seropositivity and age, as well as male sex, and a weak association between HPV38 seropositivity and fair skin (skin types I and II; Table 1). Neither cumulative sun exposure nor smoking was associated with HPV seropositivity (data not shown).

HPV Seropositivity and Nonmelanoma Skin Cancer Risk. To estimate the skin cancer relative risks, we calculated the ORs to have had skin cancer among the HPV-seropositive individuals, adjusted for age and sex. Especially among HPV8 seropositives, the relative risks to have had one of the nonmelanoma skin cancer types was high (between 9 and 17; Table 2). HPV20-seropositive individuals had a 3-fold increased risk to have had a nBCC or sBCC, respectively. In the calculation adjusted for age and sex, HPV38-seropositive individuals also had a 3-fold increased chance to have had SCC (Table 2).

To exclude confounding by the occurrence of different types of skin cancer within one individual, we repeated the relative risk calculations

Table 1 *HPV seropositivity in relation to population characteristics*

		Seropositive (n = 136)[a]						
	Seronegative (n = 737)	HPV5 (n = 6)	HPV8 (n = 19)	HPV15 (n = 24)	HPV20 (n = 37)	HPV24 (n = 75)	HPV38 (n = 24)	HPV16 (n = 24)
Age (yrs)								
Mean ± SD	59.6 ± 11.4	63.5 ± 10.6	62.4 ± 10.5	62.7 ± 9.8	62.6 ± 10.6	63.2 ± 10.2[b]	59.1 ± 12.5	60.0 ± 9.3
Range	24.1–79.9	45.8–74.1	45–76.6	41.3–76.6	41.0–77.4	38.3–78.1	28.6–78.1	45.7–74.9
Sex, n (%)								
Male	349 (47.4)	4 (66.7)	10 (52.6)	12 (50.0)	18 (48.7)	48 (64.0)[b]	12 (50.0)	10 (55.6)
Female	388 (52.6)	2 (33.3)	9 (47.4)	12 (50.0)	19 (51.3)	27 (36.0)	12 (50.0)	8 (44.4)
Skin type, n (%)								
I or II	398 (54.0)	3 (50.0)	13 (68.4)	11 (45.8)	16 (43.2)	47 (62.7)	18 (75.0)[c]	12 (66.7)
III or IV	339 (46.0)	3 (50.0)	6 (31.6)	13 (54.2)	21 (56.8)	28 (37.3)	6 (25.0)	6 (33.3)

[a] The added number of HPV type-specific seropositive cases exceeds the total number of seropositive cases because 45 individuals were seropositive to more than one HPV type.
[b] $P < 0.01$, compared with HPV seronegatives.
[c] $P < 0.05$, compared with HPV seronegatives.

Table 2 *Estimated relative risks of different types of skin cancer according to HPV seropositivity*

	Seropositivity					ORs[a]			
	Controls (n = 333)	SCC (n = 160)	nBCC (n = 291)	sBCC (n = 141)	MM[b] (n = 84)	SCC	nBCC	sBCC	MM
	n (%)	n (%)	n (%)	n (%)	n (%)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
No HPV	289 (86.8)	128 (80.0)	239 (82.1)	112 (79.4)	75 (89.3)	1	1	1	1
Any EV-HPV	41 (12.3)	31 (19.4)	49 (16.8)	26 (18.4)	8 (9.5)	1.4 (0.83–2.5)	1.3 (0.83–2.1)	1.6 (0.90–2.7)	0.74 (0.31–1.8)
EV-HPV5	1 (0.3)	2 (1.3)	3 (1.0)	0 (0.0)	1 (1.2)	2.6 (0.21–31.9)	3.7 (0.35–38.4)	Not possible	8.2 (0.48–141)
EV-HPV8	1 (0.3)	7 (4.4)	7 (2.4)	7 (5.0)	1 (1.2)	**14.7 (1.6–135)**[c]	**9.2 (1.1–78.2)**	**17.3 (2.1–143)**	2.1 (0.13–34.0)
EV-HPV15	7 (2.1)	7 (4.4)	7 (2.4)	4 (2.8)	2 (2.4)	1.8 (0.57–5.6)	1.2 (0.41–3.6)	1.5 (0.43–5.3)	1.1 (0.21–6.1)
EV-HPV20	7 (2.1)	8 (5.0)	20 (6.9)	9 (6.4)	2 (2.4)	2.2 (0.75–6.7)	**3.2 (1.3–7.9)**	**3.4 (1.2–9.5)**	1.0 (0.18–5.7)
EV-HPV24	22 (6.6)	21 (13.1)	31 (10.7)	17 (12.1)	3 (3.6)	1.5 (0.75–2.9)	1.5 (0.83–2.7)	1.9 (0.94–3.6)	0.59 (0.16–2.3)
EV-HPV38	9 (2.7)	9 (5.6)	6 (2.1)	4 (2.8)	2 (2.4)	**3.0 (1.1–8.4)**	0.88 (0.30–2.6)	1.2 (0.35–3.9)	0.57 (0.11–3.1)
HPV16	7 (2.1)	4 (2.5)	5 (1.7)	5 (3.5)	1 (1.2)	1.5 (0.38–5.6)	0.90 (0.27–3.0)	1.8 (0.56–5.9)	0.54 (0.06–4.7)

[a] The ORs are adjusted for age and sex.
[b] MM, malignant melanoma.
[c] Statistically significant associations are shown in bold.

after exclusion of nonmelanoma skin cancer cases with more than one type of skin cancer. In addition, we confined these calculations to the seropositive persons to exclude a possible bias introduced by unknown differences in social behavior or immune responsiveness between HPV-seropositive and -negative individuals. Roughly, the same associations were observed (Table 3) compared with those calculated in the complete dataset, although statistical significance was sometimes lost. This was mainly caused by the limited power of the smaller subgroups.

EV-HPV Seropositivity and Skin Cancer Type Specificity. The seroreactivity and seropositivity data shown in Fig. 2 and Tables 2 and 3 suggest that HPV type-specific serorecognition is often associated with more than one type of nonmelanoma skin cancer. We questioned the skin cancer type specificity of EV-HPV serorecognition by recalculating the relative risks of SCC, nBCC, and sBCC exclusively among the skin cancer cases. In these calculations, the melanoma cases were considered as the reference population. Most calculated ORs decreased, and statistical significance was lost in all cases (Table 4), partly due to considerable broadening of the CIs.

DISCUSSION

In this case-control study of four types of skin cancer, significant associations were found between serorecognition of EV-HPV types 8, 20, and 38 and a history of nonmelanoma skin cancer. These associations were irrespective of the presence of more than one nonmelanoma skin cancer type in the affected individuals and seemed independent of host factors, such as immune responsiveness to HPV antigens. As anticipated, malignant melanoma was not associated with EV-HPV serorecognition. No associations were found for HPV16

Table 3 *Estimated relative risks of different types of skin cancer, restricted to HPV-seropositive individuals with only one type of skin cancer*

	Seropositivity					ORs[a]			
	Controls[b] (n = 44)	SCC (n = 17)	nBCC (n = 27)	sBCC (n = 9)	MM[c] (n = 9)	SCC	nBCC	sBCC	MM
	Pos./Neg.	Pos./Neg.	Pos./Neg.	Pos./Neg.	Pos./Neg.	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
HPV5[d]	1/42	1/16	2/25	0/9	1/7	2.1 (0.09–47.6)	4.4 (0.34–57.1)	Not possible	24.0 (0.85–676)
HPV8	1/43	4/13	5/22	4/5	1/8	**36.6 (1.7–772)**[e]	**14.7 (1.4–154)**	**69.1 (4.2–1127)**	2.0 (0.10–38.8)
HPV15	7/37	5/12	4/23	2/7	2/7	2.7 (0.59–12.2)	1.0 (0.25–4.2)	1.5 (0.25–8.8)	1.3 (0.18–9.2)
HPV20	7/37	5/12	10/27	3/6	2/7	3.1 (0.64–15.0)	**3.5 (1.1–11.6)**	2.7 (0.52–13.7)	1.1 (0.14–7.9)
HPV24	22/22	12/5	17/10	4/5	3/6	1.2 (0.29–4.8)	1.4 (0.84–2.3)	0.81 (0.17–3.8)	0.62 (0.12–3.3)
HPV38	9/35	3/14	2/25	2/7	2/7	4.1 (0.58–28.9)	0.39 (0.07–2.0)	1.0 (0.17–6.3)	1.1 (0.19–6.6)
HPV16[d]	7/36	1/16	1/26	2/7	1/7	0.60 (0.05–6.6)	0.23 (0.03–2.1)	1.5 (0.25–8.7)	0.58 (0.06–6.2)

[a] The ORs are adjusted for age and sex.
[b] Referent population. Pos., positive; Neg., negative.
[c] MM, malignant melanoma.
[d] For HPV5 and HPV16, 21 sera were excluded because of reproducibly poor duplicate values.
[e] Statistically significant associations are shown in bold.

Table 4 *Estimated relative risks of nonmelanoma skin cancer types compared with individuals with malignant melanoma according to HPV seropositivity*

	Seropositivity				ORs[a]		
	MM[b] (n = 84)	SCC (n = 160)	nBCC (n = 291)	sBCC (n = 141)	SCC	nBCC	sBCC
	Pos./Neg.	Pos./Neg.	Pos./Neg.	Pos./Neg.	OR (95% CI)	OR (95% CI)	OR (95% CI)
HPV5[c]	1/74	2/153	3/282	0/137	0.17 (0.01–2.8)	0.59 (0.03–11.4)	Not possible
HPV8	1/83	7/153	7/284	7/134	1.7 (0.14–22)	1.7 (0.15–19)	3.3 (0.35–30)
HPV15	2/82	7/153	7/284	4/137	1.4 (0.12–16.5)	0.98 (0.12–7.8)	1.3 (0.18–10)
HPV20	2/82	8/152	20/271	9/132	1.8 (0.19–17)	2.9 (0.53–16)	3.2 (0.57–18)
HPV24	3/81	21/139	31/260	17/124	3.2 (0.55–19)	3.3 (0.80–14)	3.4 (0.81–14)
HPV38	2/82	9/151	6/285	4/137	2.4 (0.42–14)	0.42 (0.06–3.1)	0.73 (0.11–5.0)
HPV16[c]	1/74	4/151	5/280	5/132	1.2 (0.09–15)	0.76 (0.07–7.9)	1.3 (0.13–13)

[a] The ORs are adjusted for age and sex.
[b] Referent population. MM, malignant melanoma.
[c] For HPV5 and HPV16, 21 sera were excluded because of reproducibly poor duplicate values.

serorecognition, and therefore we doubt whether this oncogenic genital HPV type is related to skin cancer.

Serorecognition of HPV8 in particular posed a significant risk to have had nonmelanoma skin cancer. Because of the high ORs observed, it is tempting to believe that HPV8 is the cutaneous oncogenic EV-HPV type. However, the broad CI observed, due to the low HPV8 seroprevalence in our population, does not allow us to draw any definite conclusions yet regarding the oncogenic potential of this EV-HPV type. Because HPV8, 20, and 38 do not belong to the same EV-HPV subgroup (11), we cannot relate nonmelanoma skin cancer to a specific EV-HPV subgroup.

The possible relationship between EV-HPV infection and skin cancer was also the subject of earlier, smaller serological studies. Favre *et al.* (28) could not detect any seropositivity in BCC and SCC patients against the HPV5a VLPs. This is in agreement with our finding that HPV5 seropositivity was extremely low in our study populations. Stark *et al.* (22) used similar HPV8 VLPs and also reported increased seroprevalence of HPV8 antibodies in patients with nonmelanoma skin cancer. The differences in HPV8 seroprevalence measured in our study compared with that measured by Stark *et al.* (22) are probably based on differences in ELISA methodology. We reported previously (29) that HPV8 VLP seropositivity was significantly associated with development of multiple actinic keratoses on the tropical island of Saba. In this population, SCC development was also associated with HPV8 seropositivity, although it did not reach significance, perhaps because of the low number of SCC cases. HPV15 VLP seropositivity measured by Wieland *et al.* (23) was comparable with ours and also showed no significant differences among BCC cases and controls.

As a measure of EV-HPV infection, we chose detection of serum antibodies. These are expected against HPVs that have infected the host, provided that this infection leads to immune response induction and not to immune neglect or tolerance. In the case of natural cutaneous EV-HPV infection, which may be persistent (11), very little is known about the immune response induced. In particular, the onset and the longevity of these responses are unknown. In view of the relatively low number of seropositives, the ubiquity of the virus (18), and the associations found between serorecognition and nonmelanoma skin cancer, the majority of EV-HPV-directed antibodies are probably generated later in infection, probably when tumor formation occurs. This belief is supported by our very recent findings that EV-HPV prevalence measured as the presence of type-specific EV-HPV DNA in plucked eyebrow hairs in the same population exceeds the EV-HPV seroprevalence about five times.[4] Induction of EV-HPV-specific antibodies along with tumor formation may be a reflection of increased viral load or local inflammation. Because, in our population, the period between skin cancer diagnosis and serum collection was not associated with seropositivity (data not shown), we have no indication that the measured IgG responses are short-lived.

Throughout the study, particularly for HPV8, EV-HPV type-specific serorecognition was often associated with more than one skin cancer type. This prompted us to look into a possible association with skin cancer as such. In the calculations shown in Table 4, where the melanoma cases served as a control for the individual SCC, nBCC, and sBCC cases, all associations found for the individual nonmelanoma skin cancer types were lost. This suggested to us that EV-HPV serorecognition is rather associated with skin cancer as such and not so much with a particular skin cancer type. If seroresponses to EV-HPV are induced upon skin cancer formation, they may be considered as a skin cancer epiphenomenon. As a consequence, HPV8 and 20, in particular, are expected to be relatively abundant in these cancers. Whether these types are involved in carcinogenesis remains to be solved. The concurrent appearance of EV-HPV seroresponses is probably not unique to skin cancer, because HPV5 antibodies were described as accompanying the epidermal repair process after extensive burns (28).

To monitor whether EV-HPV infection precedes nonmelanoma skin cancer development, the EV-HPV serological approach that we used is probably not the most appropriate. To this purpose, accurate studies that monitor EV-HPV type-specific infection in and outside skin tumors, *e.g.*, by detection of EV-HPV type-specific DNA and RNA, remain indispensable. Ideally, these studies should be performed in a follow-up setting, because only then we will know if EV-HPV infection precedes nonmelanoma skin cancer development and in potential might be causally involved.

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[4] L. Struijk, J. N. Bouwes Bavinck, P. Wanningen, E. van der Meijden, R. G. J. Westendorp, J. ter Schegget, and M. C. W. Feltkamp. Presence of human papillomavirus DNA in plucked eyebrow hairs is associated with a history of cutaneous squamous cell carcinoma, manuscript submitted for publication.

REFERENCES

1. zur Hausen, H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst. (Bethesda), *92*: 690–698, 2000.
2. de Villiers, E. M. Taxonomic classification of papillomaviruses. Papillomavirus Report, *12:* 57–63, 2001.
3. Jablonska, S., and Orth, G. Epidermodysplasia verruciformis. Clin. Dermatol., *3:* 83–96, 1985.
4. Orth, G. Epidermodysplasia verruciformis. *In:* N. P. Salzman and P. M. Howley (eds.), The Papovaviridae, Vol. 2, 1st ed., pp. 199–243. New York: Plenum Press, 1987.
5. Pfister, H., and ter Schegget, J. Role of HPV in cutaneous premalignant and malignant tumors. Clin. Dermatol., *15*: 335–347, 1997.
6. Harwood, C. A., and Proby, C. M. Human papillomaviruses and non-melanoma skin cancer. Curr. Opin. Infect. Dis., *15:* 101–114, 2002.
7. Berkhout, R. J., Tieben, L. M., Smits, H. L., Bouwes Bavinck, J. N., Vermeer, B. J., and 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., *33:* 690–695, 1995.
8. Shamanin, V., Glover, M., Rausch, C., Proby, C., Leigh, I. M., zur Hausen, H., and de Villiers, E. M. Specific types of human papillomavirus found in benign proliferations and carcinomas of the skin in immunosuppressed patients. Cancer Res., *54:* 4610–4613, 1994.
9. Harwood, C. A., McGregor, J. M., Proby, C. M., and Breuer, J. Human papillomavirus and the development of non-melanoma skin cancer. J. Clin. Pathol., *52:* 249–253, 1999.
10. de Jong-Tieben, L. M., Berkhout, R. J., Smits, H. L., Bouwes Bavinck, J. N., Vermeer, B. J., Van der Woude, F. J., and ter Schegget, 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. Investig. Dermatol., *105* : 367–371, 1995.
11. Berkhout, R. J., Bouwes Bavinck, J. N., and ter Schegget, J. Persistence of human papillomavirus DNA in benign and (pre)malignant skin lesions from renal transplant recipients. J. Clin. Microbiol., *38:* 2087–2096, 2000.
12. Bouwes Bavinck, J. N., Vermeer, B. J., Van der Woude, F. J., Vandenbroucke, J. P., Schreuder, G. M., Thorogood, J., Persijn, G. G., and Claas, F. H. Relation between skin cancer and HLA antigens in renal-transplant recipients. N. Engl. J. Med., *325:* 843–848, 1991.
13. De Hertog, S. A., Wensveen, C. A., Bastiaens, M. T., Kielich, C. J., Berkhout, M. J., Westendorp, R. G., Vermeer, B. J., and Bouwes Bavinck, J. N. Relation between smoking and skin cancer. J. Clin. Oncol., *19:* 231–238, 2001.
14. Marks, R. Squamous cell carcinoma. Lancet, *347:* 735–738, 1996.
15. Jackson, S., and Storey, A. E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. Oncogene, *19:* 592–598, 2000.
16. de Villiers, E. M., Lavergne, D., McLaren, K., and Benton, E. C. Prevailing papillomavirus types in non-melanoma carcinomas of the skin in renal allograft recipients. Int. J. Cancer, *73:* 356–361, 1997.
17. Harwood, C. A., Spink, P. J., Surentheran, T., Leigh, I. M., de Villiers, E. M., McGregor, J. M., Proby, C. M., and Breuer, J. Degenerate and nested PCR: a highly sensitive and specific method for detection of human papillomavirus infection in cutaneous warts. J. Clin. Microbiol., *37:* 3545–3555, 1999.
18. Antonsson, A., Forslund, O., Ekberg, H., Sterner, G., and Hansson, B. G. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J. Virol., *74:* 11636–11641, 2000.
19. Boxman, I. L., Berkhout, R. J., Mulder, L. H., Wolkers, M. C., Bouwes Bavinck, J. N., Vermeer, B. J., and ter Schegget, J. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. J. Investig. Dermatol., *108:* 712–715, 1997.
20. Fitzpatrick, T. B. The validity and practicality of sun-reactive skin types I through VI. Arch. Dermatol., *124:* 869–871, 1988.
21. Favre, M., Orth, G., Majewski, S., Baloul, S., Pura, A., and Jablonska, S. Psoriasis: a possible reservoir for human papillomavirus type 5, the virus associated with skin carcinomas of epidermodysplasia verruciformis. J. Investig. Dermatol., *110:* 311–317, 1998.
22. Stark, S., Petridis, A. K., Ghim, S. J., Jenson, A. B., Bouwes Bavinck, J. N., Gross, G., Stockfleth, E., Fuchs, P. G., and Pfister, H. Prevalence of antibodies against virus-like particles of epidermodysplasia verruciformis-associated HPV8 in patients at risk of skin cancer. J. Investig. Dermatol., *111:* 696–701, 1998.
23. Wieland, U., Ritzkowsky, A., Stoltidis, M., Weissenborn, S., Stark, S., Ploner, M., Majewski, S., Jablonska, S., Pfister, H. J., and Fuchs, P. G. Papillomavirus DNA in basal cell carcinomas of immunocompetent patients: an accidental association? J. Investig. Dermatol., *115:* 124–128, 2000.
24. Kirnbauer, R., Hubbert, N. L., Wheeler, C. M., Becker, T. M., Lowy, D. R., and Schiller, J. T. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J. Natl. Cancer Inst. (Bethesda), *86:* 494–499, 1994.
25. Van Hulten, M. C., Westenberg, M., Goodall, S. D., and Vlak, J. M. Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology, *266:* 227–236, 2000.
26. Kremsdorf, D., Favre, M., Jablonska, S., Obalek, S., Rueda, L. A., Lutzner, M. A., Blanchet-Bardon, C., Voorst Vader, P. C., and Orth, G. Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. J. Virol., *52:* 1013–1018, 1984.
27. Scheurlen, W., Gissmann, L., Gross, G., and zur, H. H. Molecular cloning of two new HPV types (HPV 37 and HPV 38) from a keratoacanthoma and a malignant melanoma. Int. J. Cancer, *37:* 505–510, 1986.
28. Favre, M., Majewski, S., Noszczyk, B., Maienfisch, F., Pura, A., Orth, G., and Jablonska, S. Antibodies to human papillomavirus type 5 are generated in epidermal repair processes. J. Investig. Dermatol., *114:* 403–407, 2000.
29. Bouwes Bavinck, J. N., Stark, S., Petridis, A. K., Marugg, M. E., ter Schegget, J., Westendorp, R. G., Fuchs, P. G., Vermeer, B. J., and Pfister, H. The presence of antibodies against virus-like particles of epidermodysplasia verruciformis-associated humanpapillomavirus type 8 in patients with actinic keratoses. Br. J. Dermatol., *142:* 103–109, 2000.