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The Prevalence of Human Papillomavirus Genotypes in Nonmelanoma Skin Cancers of Nonimmunosuppressed Individuals Identifies High-Risk Genital Types as Possible Risk Factors[1]

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ABSTRACT

Nonmelanoma skin cancer is the most commonly diagnosed malignant disease in Caucasians. Known risk factors include fair skin, sun exposure, male gender, advancing age, and the presence of solar keratosis. No viral risk factors have been established thus far. To examine the association between nonmelanoma skin cancer and infection with human papilloma virus (HPV) types, we performed a retrospective study in which skin biopsies were collected from 496 nonimmunosuppressed patients attending dermatologic clinics during a defined period and for whom a biopsy or resection of a tumor was indicated for medical reasons. A total of 390 patients with histologically confirmed diagnosis of warts ($n = 209$), solar keratosis or Bowen's disease ($n = 91$), squamous cell carcinoma ($n = 72$), or basal cell carcinoma ($n = 18$), as well as 106 control patients with normal skin was analyzed for infection with HPV and, if positive, HPV typed by sequencing. Logistic regression was performed to separately investigate association of certain HPV types with the occurrence of warts, precancerous lesions, and skin cancer compared with normal skin. For all three histological groups, both crude risk and risk adjusted for age, sex, and sun exposure were calculated. HPV DNA was detected in only 4.7% of controls, in 90.9% of benign warts, in 60.4% of precancerous lesions, in 59.7% of squamous cell carcinoma, and in 27.8% of basal cell carcinoma, which demonstrates that viral infection is specifically linked to skin disorders. The distribution of viral types found is distinctly different between warts and precancers or cancers, supporting an etiologic role of specific HPV types. This is supported by statistical analysis, where after adjusting for age, gender, and sun exposure, the odds ratio for nonmelanoma skin cancer in patients who were DNA positive for the high-risk mucosal HPV types, 16, 31, 35, and 51 was 59 (95% confidence interval, 5.4–645) with normal skin as controls. These findings suggest that persistent infections of the skin with high risk genital HPV types recently identified as significant risk factors for cervical cancer may also represent a risk factor for nonmelanoma skin cancer in a nonimmunosuppressed population.

INTRODUCTION

NMSC,[3] including both SCC and basal cell carcinoma, is the most commonly diagnosed malignant disease in the Caucasian population. Although the incidence of metastasis is low, treatment can be disfiguring, and diagnosis and management are costly. At current rates, 1 in 5 United States citizens will develop skin cancer during lifetime. In 1997, 800,000 cases of NMSC were diagnosed in the United States, and roughly 2000 deaths cases have been reported (1, 2). Already known risk factors for NMSC include fair skin, sun exposure, male gender, advanced age (3–7), and the presence of solar keratoses as precursor lesion for SCC that progress, however, only in a minor fraction (8).

In contrast to the accepted causal role of high-risk HPVs in the origin of cervical cancer where 15 epidemiologically defined high-risk HPV types (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-68, HPV-73, and HPV-82) have been found to cause a persistent infection of the cervix as a necessary risk factor for the development of cervical cancer (9), thus far there is no such clear epidemiological evidence implicating a role of specific HPV types in NMSC.

One malignant skin disorder known to be associated with HPVs is the rare genetic disorder Ev, where individuals develop early in childhood HPV-induced warts that progress into SCC on sun-exposed skin in 30–60% of the patients (10, 11). These lesions are infected by a subgroup of cutaneous HPVs (Ev-associated HPV types), which are nonpathogenic in the normal population with the exception of lesions of psoriasis patients (12). Cutaneous HPV types with a putative increased malignant potential have been described such as HPV-5 and HPV-8 that were found in >90% of SCCs in a few Ev patients (10, 11). Skin cancers associated with HPV have also been described in immunosuppressed renal transplant recipients having a 65-fold increased risk to develop skin cancer where 65–81% (13, 14) of the tumors were found to be HPV DNA positive (13–19). The majority of these transplant patients develop warts shortly after the onset of immunosuppressive therapy. NMSC arises after 20 years of immunosuppression in 40–70% of patients (20), implying that immunosuppression specifically induces an impaired ability to control tumorigenic viruses. In nonimmunosuppressed skin cancer patients, data on the presence and spectrum of HPV types detected is largely inconsistent. In general, HPV DNA has been found in a lower percentage of SCC and BCC developing in normal individuals as compared with immunosuppressed patients (21–22), and there is no strong epidemiological evidence linking specific HPV types to an increased risk of skin cancer. We aimed to evaluate the role of specific HPV types as a risk factor in the origin of NMSC in nonimmunosuppressed individuals. For this, we performed a study applying a sensitive and broad range PCR method to 181 precancer and cancer of the skin, as well as to 315 benign warts and normal skin samples and defined the HPV type by direct sequencing.

MATERIALS AND METHODS

Characteristics of the Study Population. A total of 496 nonimmunosuppressed patients between the ages of 5 and 98 years attending an office-based dermatologist or different university hospitals in northern and southern Germany or a cancer center in southern California was enrolled in the study (Table 1). A large fraction of patients (352), including all diagnosis categories, were attending a dermatology clinic in Germany during a defined enrollment period.

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[3] The abbreviations used are: NMSC, nonmelanoma skin cancer; SCC, squamous cell carcinoma; HPV, human papillomavirus; Ev, Epidermodysplasia verruciformis; OR, odds ratios; CI, confidence interval; BCC, basal cell carcinoma.

Table 1 *Characteristics of the study population*

	Controls[a] (n = 106) (%)	Warts (n = 209) (%)	Solar keratosis and Bowen's disease (n = 91) (%)	SCC (n = 72) (%)	BCC (n = 18) (%)	All subjects (n = 496) (%)
Sex						
Male	53 (50.0)	88 (42.1)	53 (58.2)	45 (62.5)	12 (66.7)	251 (50.6)
Female	48 (45.3)	99 (47.4)	34 (37.4)	21 (29.2)	6 (33.3)	208 (41.9)
Unknown	5 (4.7)	22 (10.5)	4 (4.4)	6 (8.3)	0	37 (7.5)
Age at diagnosis						
≤39 yr	22 (20.8)	110 (52.6)	1 (1.1)	3 (4.2)	1 (5.6)	137 (27.6)
40–69 yr	39 (36.8)	52 (24.9)	30 (33.0)	28 (38.9)	7 (38.8)	156 (31.5)
70–98 yr	33 (31.1)	19 (9.1)	54 (59.3)	34 (47.2)	9 (50.0)	149 (30.0)
Unknown	12 (11.3)	28 (13.4)	6 (6.6)	7 (9.7)	1 (5.6)	54 (10.9)
Region						
Germany	102 (96.2)	208 (99.5)	91 (100)	26 (36.1)	14 (77.8)	441 (88.9)
California	4 (3.8)	1 (0.5)	0	46 (63.9)	4 (22.2)	55 (11.1)
Unknown	0	0	0	0	0	0
Anatomic site						
Extremities	14 (13.2)	157 (75.1)	15 (16.5)	7 (9.7)	0	193 (38.9)
Head, face, neck	51 (48.1)	13 (6.2)	62 (68.1)	38 (52.8)	13 (72.2)	177 (35.7)
Abdomen	35 (33.0)	2 (1.0)	0	4 (5.6)	3 (16.7)	44 (8.9)
Genital, anal	0	0	2 (2.2)	0	0	2 (0.4)
Unknown	6 (5.7)	37 (17.7)	12 (13.2)	23 (31.9)	2 (11.1)	80 (16.1)
HPV						
HPV negative	101 (95.3)	19 (9.1)	36 (39.6)	29 (40.3)	13 (72.2)	198 (39.9)
HPV positive	5 (4.7)	190 (90.9)	55 (60.4)	43 (59.7)	5 (27.8)	298 (60.1)
HPV mucosal[b] positive	1 (20.0)[c]	3 (1.6)[c]	10 (18.2)[c]	9 (20.9)[c]	3 (60.0)[c]	26 (8.7)[c]
HPV Ev[d] positive	1 (20.0)[c]	2 (1.1)[c]	6 (10.9)[c]	4 (9.3)[c]	0	13 (4.4)[c]

[a] Normal skin.
[b] HPV mucosal: 16, 31, 33, 35, and 51.
[c] Percentage of all HPV positive.
[d] HPV Ev: 5, 8, 12, 17, 19, 22, and 36.

Skin Samples. For all patients, a skin biopsy or resection of a nonmelanoma skin tumor was indicated for medical reasons. All diagnosis of SCC, BCC, solar keratoses, Bowen's disease, and normal skin were histologically confirmed. Normal skin biopsies (dog ears) were obtained from surgical procedures because of different medical reasons. Diagnoses of plantar warts and warts at other sites were clinical. Biopsy specimens were immediately snap frozen in liquid nitrogen after excision and stored at −70°C.

Laboratory Methods. DNA extraction process and all pre- and post-PCR procedures were carried out in separate rooms and cabinets. Buffer and blank controls were always included in different positions of the extraction protocol to obtain sufficient numbers of negative controls to monitor contamination events. All samples were tested for integrity of the DNA by PCR using primers for the human *β-globin* gene. The following primer combinations were used for HPV DNA amplification: CP4 (5′-nt1942-ATG-GTA-CAR-TGG-GCA-TWT-GA-nt1961–3′) taken together with CP5 (5′-nt2400-GAG-GYT-GCA-ACC-AAA-AMT-GRC-T-nt2378-3′), as well as PPF1 (5′-nt2082-AAC-AAT-GTG-TAG-ACA-TTA-TAA-ACG-AGC-nt 2108-3′; according to the sequence of HPV16W12E GenBank accession no. AF125673) together with CP5. All PCR reactions were performed in quadruple with three dilutions of input DNA (undiluted, 1:2, 1:5, and 1:20). The sensitivity of our PCR system for different HPV types was determined by testing serial-fold dilutions of HPV DNA plasmids for the following types: HPV1-HPV8, HPV10-HPV19, HPV21-HPV26, HPV30-HPV38, HPV40, HPV45–47, and HPV60 in the absence and presence of human placenta DNA (1 μg). The sensitivity observed was in the range of 0.1 fg (10 plasmid copies) to 100 fg (10,000 plasmid copies) of HPV plasmid DNA. The highest sensitivity was found for the detection of HPV-7, HPV-10, HPV-13, HPV-21, HPV-32, HPV-33, HPV-36, and HPV-45 and the lowest for HPV-3, HPV-14, HPV-15, HPV-26, HPV-31, HPV-40, and HPV-46. These different levels of sensitivity were, however, not reflected in the distribution of HPV types finally detected. The detection range of this PCR method includes at least 64 different HPV types. Direct sequencing of the amplimers was performed in 47-cm capillaries with the use of an ABI 310 sequencer (PE Biosystems), and the obtained sequence was compared with the GenBank database (National Center for Biotechnology Information, Bethesda, MD) by using the Blast program. A nucleotide sequence was regarded as a distinct HPV type if it shared ≥90% homology with a known type. Sequence homologies < 90% were regarded as related types. The term mixed infection was attributed to 32 samples where PCR was repeatedly positive and sequence reactions resulted in electropherograms with clearly superimposed sequences. Eleven samples were designated as HPV-X because PCR was repeatedly positive but sequencing gave no reproducible results.

Statistical Analysis. ORs, corresponding 95% CIs, and *P*s were calculated using the software package SAS (version V8e). Association of HPV infection and certain HPV types with benign tumors (warts), preinvasive cancer (solar keratosis and Bowen's disease), or invasive cancer (SCC or BCC) were investigated. Reference category used as controls were people with normal skin biopsies. Crude ORs and ORs adjusted for age, gender, and sun exposure were calculated by unconditional logistic regression. Sun exposed body parts (head, face, neck, forearm, hands and lower leg) *versus* nonexposed (abdomen, upper arm, thigh, feet, genital, and anal region) were used as a surrogate to control for sun exposure.

RESULTS

A two-step protocol was used to identify and type HPV in skin samples. The initial screening was done by PCR using degenerate consensus primers followed by direct sequencing of the amplimer to identify the underlying HPV type. This method permits the identification of at least 64 HPV types from both the mucosal and the cutaneous/Ev group with a sufficiently high sensitivity. No regional differences in the incidence and types of HPV were detected when lesions from patients originating from southern and northern Germany and California were analyzed. Only 4.7% (5 of 106) of histologically proven normal skin samples contained HPV DNA (Fig. 1; Table 1), including one biopsy with an HPV type found exclusively in normal skin (HPV-12). Of 102 palmoplantar warts and 107 warts from other body sites, we found 91% to be positive for HPV DNA (Fig. 1). The most prevalent HPV types found in these benign tumors were HPV-1 (27.3%), HPV-27 (12.9%), HPV-57 (11.5%), HPV-2 (9.6%), HPV-10 (9.0%) HPV-4 (7.6%), and HPV-65 (3.8%; Fig. 2). Only 3 of 209 warts (1.4%) contained DNA of the high-risk HPV types 16, 31 and 33.

Interestingly, the distribution of HPV types that were detected in 41 of 71 solar keratoses, in 14 of 20 Bowen's disease (equivalent to SCC *in situ*), in 43 of 72 squamous carcinomas, and in 5 of 18 basal cell carcinomas was distinctly different from the one found in benign warts (Table 1; Fig. 2). Solar keratoses and Bowen's disease contained cutaneous types (HPV-1, HPV-2, HPV-3, HPV-4, HPV-7, HPV-27, and HPV-57) usually present in benign warts, low-risk

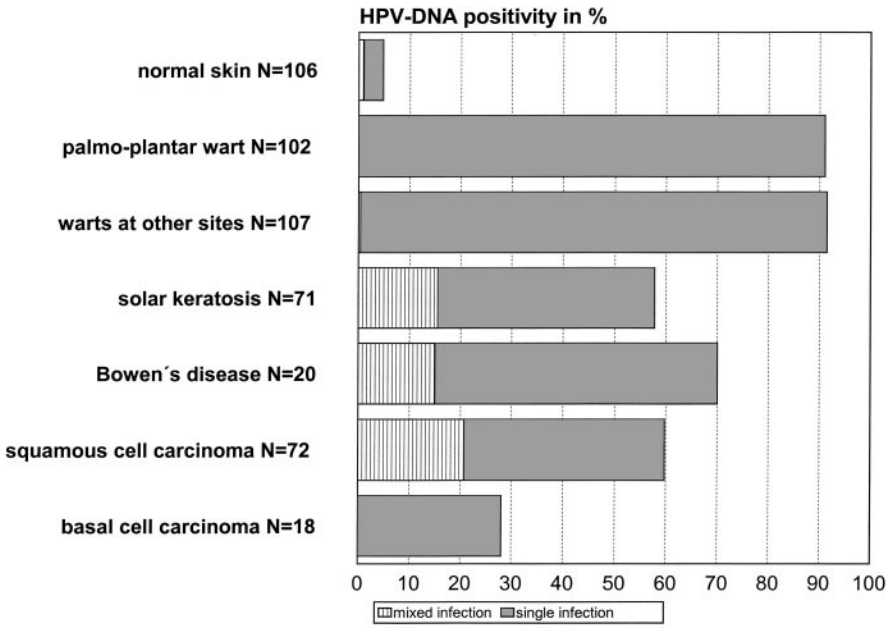

Fig. 1. Frequency of HPV DNA detection in normal skin and different skin diseases. *Horizontal bars* indicate the percentage of HPV DNA positivity for each category.

(HPV-6) and high-risk (HPV-16, HPV-33, and HPV-35) mucosal types, as well as Ev-related (HPV-5, HPV-8, HPV-19, and HPV-36) types. We found 48 of 90 (53.3%) of NMSC to contain HPV DNA, with HPV-33 (9.7%) and HPV-4 (12.5%) being the most prevalent HPV types detected. Only 5 of 18 (27.8%) of basal cell carcinomas were HPV positive as described earlier (12, 13) with DNA for HPV-16, HPV-27, and HPV-33. We can, however, not exclude the possibility that in samples defined negative for HPV DNA, other HPV types are present that were not detectable by our method. Crude ORs and ORs adjusted for age, sex, and sun exposure were defined for all different groups of lesions with respect to HPV positivity, mucosal high risk types, and Ev-associated HPV types (Table 2). When warts were compared with normal skin samples as a reference group a highly significant risk association with HPV infection was observed (Table 2; adjusted OR, 210; 95% CI, 61–729). Similar associations were detected for the precancerous conditions solar keratosis and

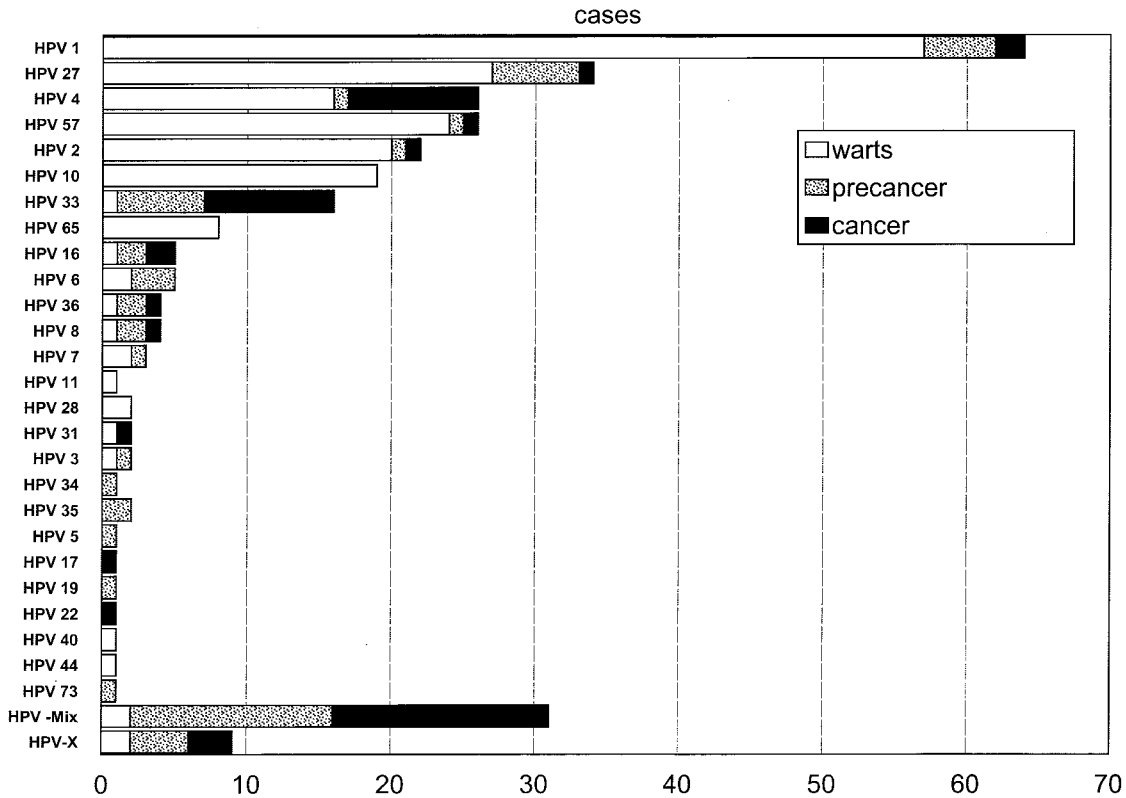

Fig. 2. HPV type prevalence in different groups of skin diseases such as benign warts, precancerous conditions, and NMSC. *Horizontal bars* indicate the frequency (number of cases) of individual HPV types found in 496 patients with warts, precancers, and nonmenlanoma skin cancers. HPV-mix denotes samples with multiple HPV infections and HPV-X samples with HPV types that could not be identified by direct sequencing.

Table 2 *OR for different skin diseases and nonmelanoma skin cancer associated with HPV and adjusted for other risk factors with normal skin as controls*

	No. (%) patients	No. (%) controls	Crude OR (95% CI)	*P*	Adjusted OR[a] (95% CI)	*P*
Wart	209 (66.3)	106 (33.7)				
HPV negative	19 (9.1)	101 (95.3)	Reference		Reference	
HPV positive	190 (90.9)	5 (4.7)	202 (73–557)	<0.0001	210 (61–729)	<0.0001
HPV high-risk mucosal[b] positive	3 (1.6)	1 (20.0)	16 (1.6–162)	0.019	13 (1.2–149)	0.036
HPV Ev[c] positive	2 (1.1)	1 (20.0)	10.6 (0.9–123)	0.059	37 (2.5–550)	0.008
Solar keratosis and Bowen's disease	91 (46.2)	106 (53.8)				
HPV negative	36 (39.6)	101 (95.3)	Reference		Reference	
HPV positive	55 (60.4)	5 (4.7)	31 (11.5–83)	<0.0001	26 (7.3–91)	<0.0001
HPV high-risk mucosal[b] positive	10 (18.2)	1 (20.0)	28 (3.5–227)	0.002	[d]	
HPV Ev[c] positive	6 (10.9)	1 (20.0)	16.8 (2.0–145)	0.010	9.2 (1.0–80)	0.046
SCC and BCC	90 (45.9)	106 (54.1)				
HPV negative	42 (46.7)	101 (95.3)	Reference		Reference	
HPV positive	48 (53.3)	5 (4.7)	23 (8.6–62)	<0.0001	21 (7.2–63)	<0.0001
HPV high-risk mucosal[b] positive	12 (25.0)	1 (20.0)	29 (3.6–229)	0.002	59 (5.4–645)	0.001
HPV Ev[c] positive	4 (8.3)	1 (20.0)	9.6 (1.0–89)	0.046	6.4 (0.6–65)	0.118
SCC	72 (40.4)	106 (59.6)				
HPV negative	29 (40.3)	101 (95.3)	Reference		Reference	
HPV positive	43 (59.7)	5 (4.7)	30 (10.9–83)	<0.0001	32 (10–100)	<0.0001
HPV high-risk mucosal[b] positive	9 (20.9)	1 (20.0)	31 (3.8–258)	0.001	[f]	
HPV Ev[c] positive	4 (9.3)	1 (20.0)	13.9 (1.5–130)	0.020	9.6 (0.9–100)	0.058
BCC	18 (14.5)	106 (85.5)				
HPV negative	13 (72.2)	101 (95.3)	Reference		Reference	
HPV positive	5 (27.8)	5 (4.7)	7.8 (2.0–30)	0.003	7.3 (1.7–30)	0.006
HPV high-risk mucosal[b] positive	3 (23.1)	1 (20.0)	23.3 (2.3–241)	0.008	26 (2.1–318)	0.011
HPV Ev[c] positive	0	1 (20.0)	[e]		[e]	

[a] OR adjusted for age, sex, and sun exposure (sun exposed: head, face, neck, forearm, hands, and lower limb).
[b] HPV high-risk mucosal types: 16, 31, 33, 35, and 51.
[c] HPV Ev types: 5, 8, 12, 17, 19, 22, and 36.
[d] All cases are 50 years ≥, no control age ≥50 years is HPV mucosal positive.
[e] No BCC case is HPV Ev positive.
[f] No control age ≥50 years is HPV mucosal positive.

Bowen's disease, as well as for skin cancers, with adjusted ORs of 26 (95% CI, 7.3–91) and 21 (95% CI, 7.2–63), respectively. This association remains unchanged when 26 cancers (24 SCCs and 2 BCCs) of unknown anatomical site were excluded from the analysis (data not shown). When analysis was performed separately for SCC and BCC adjusted ORs were 32 (95% CI, 10–100) and 7.3 (95% CI, 1.7–30), respectively. When all HPV types associated with Ev (HPV-5, HPV-8, HPV-12, HPV-17, HPV-19, HPV-22, and HPV-36) were grouped together, a significant association was only observed for warts with an adjusted OR of 41 (95% CI, 2.7–608), whereas the adjusted OR for SCC and BCC was 6.4 (95% CI, 0.6–65). In contrast, the presence of DNA from other cutaneous HPV types usually found in benign warts was not associated with increased risk for squamous dysplasia or invasive cancer. Multiple infections were significantly elevated in preinvasive and cancer lesions (15–20%) in comparison to benign warts (Fig. 1) but could not be additionally genotyped because of overlapping electropherograms. Because only very few samples in the normal skin control group were HPV positive, an additional type-specific statistical discrimination analysis was not possible.

DISCUSSION

Most studies of HPV in NMSC are small case series often without a control group. HPV types found covered a broad range of mucosal, cutaneous, and especially Ev types, and frequently multiple infections have been described previously (13–19). However, the demonstration of a group of specific HPV types has not been consistent (15, 22), and no epidemiological evidence could be obtained linking the HPV types detected to an increased skin cancer risk. Some of the earlier case studies had additional limitations in the laboratory methods using type-specific PCR primers, *in situ* hybridization, or Southern blot analysis and were therefore biased in the detectability of individual types. However, for a thorough assessment, it is necessary to apply a sufficiently sensitive method that is able to detect many mucosal, cutaneous, and Ev types with similar sensitivity in cases and controls. Because of the genomic diversity consensus primers for PCR are necessary to detect the 90 fully described HPV genomes known to date. We used primers derived from the highly conserved helicase region of the E1 gene, which is involved in the replication of the viral DNA, in a single-step PCR that has a detection range of at least 64 individual HPV types with a similar sensitivity for different types. In histologically proven normal skin, we found, in contrast to previous studies (23), only a very low prevalence (4.7%) of HPV infection. This difference could be either because all normal skin biopsies were carefully cleaned with disinfectant before they were taken, which may have reduced the observed high skin surface contamination with virus particles (24) or to the circumstance that earlier studies used skin biopsies from unusual anatomical sites such as eyelids as control samples. The known causal relationship between papillomavirus infection and wart development could be fully proven by the high prevalence of 90.9% HPV DNA we observed in these benign tumors. When compared with precancerous conditions and SCC, we found a lower prevalence of 60.4 and 59.7%, respectively, that was still significantly higher than in normal skin. Importantly, the distribution of viral types found in warts is distinctly different from that in precancers or cancers, which points to an etiologic role of specific HPV types. These observations are supported by the statistical analysis performed. Because it is known that advanced age and male gender increase the risk for the development of NMSC, we calculated ORs adjusted for these risk factors. Additionally, we used sun exposed body parts (head, face, neck, hands, forearm, and lower leg) *versus* nonexposed (abdomen, genital and anal region, upper arm, thigh, and feet) as a surrogate to control for UV exposure. As a result, we obtained an OR of 59 (95% CI, 5.4–645) for NMSC in patients who were DNA positive for the high-risk mucosal HPV types, 16, 31, 35, and 51 with normal skin as controls. For the group of HPV types associated with Ev (HPV-5, HPV-8, HPV-12, HPV-17, HPV-19, HPV-22, and HPV-36), the adjusted OR for SCC and BCC was only 6.4 (95% CI, 0.6–65).

There are some limitations to our study. Controls were not matched for age to cases at inclusion into the study. However, age is controlled for in the adjusted logistic regression models. There are a number of missing values for anatomical site and age. However, the exclusion of SCC of unknown site from the analysis does not change the results. Because two-thirds of patients with SCC were recruited at a Californian clinic, whereas most of the other patients and controls were enrolled in German clinics, a potential regional effect cannot be totally disregarded. In addition, our method of direct sequencing of the PCR product leads to an underestimation of the number of mixed infections.

Our finding that high-risk genital HPV types, which are already linked to the development of cervical cancer, implies an excess risk to NMSC in nonimmunosuppressed individuals is in line with other studies describing the presence of mucosal HPV types such as 6, 32, 34, 42, and 51 in skin tumors of nonimmunosuppressed patients (13) or HPV-16 and HPV-33 in extragenital Bowen's disease (25, 26). In addition, a recent nested case-control study combining serological and PCR analysis described high-risk HPV-type 16 as a risk factor for SCC of the head and neck (27). The fact that all other HPV types that are similarly transmitted do not represent a significant risk factor for preinvasive cancer or invasive cancer of the skin suggests that the HPV-associated risk is not confounded by differences in lifestyle. Although, we controlled for the main risk factors of NMSC, age, gender, and sun exposure, the possibility of bias or confounding because of other factors cannot be totally disregarded.

In summary, these data provide some evidence that persistent infections of the skin with high-risk genital HPV types recently identified as significant risk factors for cervical cancer may also represent a risk factor for NMSC in a nonimmunosuppressed population. These results differ from previous investigations attempting to identify HPV types as possible high-risk candidates for skin cancer, which were simply based on frequency analysis in malignant lesions. The epidemiological association of high-risk HPV types with NMSC demonstrated here is no proof of a causal relationship. Additional experiments have to be performed to determine the viral load and transcriptional activity of these viruses in cancer cells of NMSC. However, our observation makes sense in terms of biology because HPV-5 and HPV-8 or HPV-16, HPV-31, HPV-33, and HPV-35 have already been linked to skin cancer in Ev patients or to cervical cancer, respectively, and were shown to posses transforming activity in tissue culture (28–31).

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