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Exposure Profiles and Human Papillomavirus Infection in Skin Cancer: An Analysis of 25 Genus β-Types in a Population-Based Study

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Abstract

An increasing number of studies report that genus β human papillomaviruses (HPVs) are associated with skin cancer, with suggestions of specificity for squamous cell carcinoma (SCC) of the skin. We have conducted a systematic examination of HPV DNA in tumors from immunocompetent hosts, including SCC and basal cell carcinoma (BCC), using a highly sensitive methodology and population-based samples to test the hypothesis that a differential prevalence of β-HPVs exists between SCC (n = 101) and BCC (n = 101) tumors. When testing for all known β-HPV types, we found no significant difference in HPV prevalence between the two histologies. However, SCC lesions were significantly more likely to be infected with HPV genus β-species 1 (includes types 5 and 8), than BCC samples (P = 0.01); this difference was not observed for any other species. A histologic difference was also observed for those HPV types previously reported to be important in skin cancer (P = 0.003). SCC samples showed a higher rate of infectivity (that is, were positive for multiple types) than BCC tumors (P = 0.02). These data highlight the potential importance of various genus β-HPV types, in particular genus β-species 1 in SCC, and support the hypothesis of a behavioral difference of the virus within the two major histological skin cancers.

INTRODUCTION

Human papillomaviruses (HPVs) are highly prevalent among human populations. Over 100 different types of HPVs have now been identified and can be characterized into phylogenetic genera α, β, γ, ν, and μ (de Villiers *et al*., 2004). Many α-HPVs, most notably HPV 16, can induce papillomatous proliferations with a high risk for malignant progression and are associated with cancer of the cervix uteri, other anogenital cancers, and a subgroup of head-and-neck squamous cell carcinoma (zur Hausen, 2002; Clifford *et al.*, 2003).

The first link between HPV and skin cancers was a rare autosomal-inherited disease called epidermodysplasia verruciformis. The disease is characterized by an abnormal predisposition

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CONFLICT OF INTEREST

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to infection with certain HPV types (now classified as the genus β-HPVs) as well as cutaneous lesions that display a high rate of progression to squamous cell carcinoma (SCC). Although genus β-type HPVs have been frequently detected in non-melanoma skin cancers (NMSC) in immunosuppressed individuals, very little is known about the presence of the virus in immunocompetent individuals. Epidemiologic studies have detected associations between markers of β-HPV infection and SCC, but not basal cell carcinoma (BCC) (Harwood and Proby, 2002; Pfister, 2003; Sterling, 2005; Karagas *et al.*, 2006), suggesting a potential etiological role in SCC. However, the presence of HPV DNA in eyebrow pluckings was found to be nearly ubiquitous (Boxman *et al*., 2000). To better understand whether the virus plays a part in the development of skin cancers, a number of investigations have tested for the presence of the viral DNA in tumors themselves. These, generally small case series studies have demonstrated β-HPV in a higher proportion of SCC, than in BCC tumors from immunocompetent individuals (50%) and immunocompromised individuals (90%) (Orth *et al*., 2001; Harwood and Proby, 2002; Pfister, 2003). Further, 85% of actinic keratoses, precursor lesions of SCCs, showed a high prevalence of β-HPV DNA, indicating a potential carcinogenic role for HPV in the early stages of SCC development (Pfister, 2003). A role in HPV in the development of BCC is even less certain.

Complicating the interpretation of the existing literature is a lack of population-based case sampling, and inconsistent laboratory methods for HPV detection. Specifically, different primer sets have been used for amplification of the β-HPV genome. Furthermore, until recently, direct sequencing was the only viable approach to HPV detection; however, it may have limited sensitivity for HPV detection of highly related genotypes (Gheit *et al*., 2007). Using a newly developed reverse hybridization method for HPV detection (de Koning *et al*., 2006), we tested BCC and SCC tumors from a large epidemiologic case–control study for the presence β-HPVs to determine the differential prevalence of HPV infection in these lesions. Importantly, this new method analyzes the most highly conserved region of the viral genome, allowing us to test for the presence of all 25 known types of β-HPVs without the use of sequencing.

RESULTS

Among all 25 known β-HPV DNA genotypes examined, HPV 15 was most prevalent in SCC samples (25.5%), whereas HPV 76 was most commonly found in BCC samples (25.5%; Table 1). Conversely, no BCC cases were tested positive for HPV 19 and HPV 49; and no cases of either histology contained HPV 25 DNA. The prevalence for commonly known β-types 5 and 8 was low for both types (below 10%), especially BCC (Table 1).

When comparing prevalence of any β-HPV type between histologies, no statistical difference was observed in the overall prevalence of HPV DNA (Tables 1 and 2). In additional to overall HPV prevalence, we explored the modifying effects of factors such as sex, age, and skin factors, including skin sensitivity to sunlight, number of painful lifetime sunburns, occupational sun exposure, as well as sun exposure at the tumor site. The results are presented in Table 2. Among strata, no significant differences in the prevalence of any β-HPV between histologies were observed. It may be noteworthy that 93.6% of the non-smoking SCC cases were HPV DNA-positive versus 79.1% of former or current smoking SCC cases, a trend that was recently reported in a study of head and neck cancer (Applebaum *et al*., 2007).

However, when the 25 β-HPV types are limited by phylogenic species or by those types previously implicated in NMSC, a significant difference in prevalence is observed among histological subgroups. Using unconditional multivariate logistic regression, odds ratios (ORs) and 95% confidence intervals (CIs) for odds of infection according to histological subgroup were calculated, adjusted for age, sex, education, smoking status, skin sensitivity to sunlight, as well as number of lifetime sunburns (Table 1).

When it was restricted to β-types previously associated with NMSCs, including types 5, 8, 15, 20, 24, 36, and 38 (Bouwes Bavinck *et al*., 2000; Harwood *et al*., 2000; Feltkamp *et al*., 2003; Masini *et al*., 2003; Karagas *et al*., 2006; Struijk *et al*., 2006), we detected a higher prevalence among SCC tumors than in BCC tumors (OR = 2.6, 95% CI = 1.4–5.1). Also, we previously reported an association between positive serology for more than one β-HPV type and SCC but not BCC case status (Karagas *et al*., 2006). When comparing the two histologies on the presence of multiple HPV types, we found that SCC cases were more often infected with two or more HPV types than BCC cases (OR = 2.0, 95% CI = 1.0–3.9). When 25 β-types were limited to those types classified as species 1 (types 5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47, and 93), SCC tumors were significantly more likely to be infected with these HPV genotypes (OR = 2.0, 95% CI = 1.1–3.6). This was not observed for the species 2 types (OR = 1.4, 95% CI = 0.7–2.5) or species 3, 4, and/or 5 (OR = 1.0, 95% CI = 0.5–2.0) (Table 1).

DISCUSSION

We investigated the role of β-HPVs in NMSC, using innovative methods to test population-based samples for the presence of HPV DNA belonging to all known sequenced types of the genus β. Although all 196 tumors tested for the current analysis were from unique patients, we do not have a confirmed record of prior NMSC as part of this study.

We tested the hypothesis that prevalence of HPV DNA differed between SCC and BCC samples and observed no significant difference between the two histologies overall. We did, however, find that SCC samples were more often infected with those types belonging to species 1 of the β genus, which includes HPV 5 and HPV 8, than in BCC samples. Although HPV 36 was detected most often within this species among SCC cases, the presence of HPV 24 differed most between the two histological groups. Interestingly, antibodies to HPV 24 (Feltkamp *et al*., 2003) and HPV 24 DNA (Struijk *et al*., 2003) have been positively associated with SCC in addition to HPV 24 DNA detected in the malignant lesions of epidermodysplasia verruciformis patients (Orth, 1987; Gubinelli *et al*., 2003). Furthermore, SCC tumors had a higher level of infectivity (positive for multiple types) by β-HPVs compared with BCC samples.

Nearly, all previous studies suggest an association between SCC and β-HPV DNA, yet comparison with these studies is difficult, in part, due to small sample sizes, and hence variable prevalence rates. Furthermore, studies used inconsistent methods that differ in both sensitivity and specificity (Table 3). For example Surentheran *et al*. (1998) tested SCC tumors from six immunocompetent patients using PCR primer pairs lying within the open reading frame of the viral gene encoding the structural protein L1 and analyzing the sequence of the amplified gene fragment. But, recent investigations have shown that the L1 gene is less conserved than originally assumed (Iftner *et al*., 2003). Consequently, studies (for example, Pfister *et al*., 2003 as well as Forslund *et al*., 2007) using this methodology may have had limited ability to detect several HPV types (including, HPV 2–6, 8, 10, 11, 16, 31, 41, and 57).

An alternative approach is to use various PCR primer pairs lying within the most highly conserved, virus-specific E1 gene, which encodes an ATP-dependent DNA helicase. However, it has been recently reported that the primer pair CP4/CP5, used previously (for example, by Iftner and colleagues with 72 SCC and 18 BCC tumors), is not suitable for amplification due to interference in typing by sequencing (Tieben *et al*., 1993). To avert this problem, we used an approach that involves PCR amplification of a small 114 bp nucleotide sequence from the HPV E1 open reading frame followed by a reverse hybridization step. The high degree of conservation of the viral E1 gene, as compared with that of the viral L1 gene, ensures that all 25 β-HPV types can be detected using a single amplicon with a sensitivity as high as 10 viral copies per cell. In comparison with PCR-based methods that have detection limits as high as

5×10^{15}, the analytical sensitivity of the primer set for each of the 25 β-HPV types in the reverse hybridization assay method is between 10 and 100 copies per PCR. Using this method, we found a substantial number of lesions (77.6% of BCCs and 83.7% of SCCs) from immunocompetent individuals tested positive for β-HPV. Natural HPV contamination (detection of passenger HPV types), although unlikely, cannot be fully excluded. Arguing against this is a study that examined this issue in eyebrow pluckings and observed unique HPV profiles among five individuals who shared a student household (de Koning *et al*., 2007).

Using the reverse hybridization assay method, we detected nearly all β-HPV types in our study. In total, 24 of 25 known β-HPV types were detected; HPV 25 was not observed. Although HPV 25 has been previously found in the normal skin and flat wart-like lesions, the same studies also found no evidence of HPV 25 in any malignant skin tumors. Although no significant difference was found between SCC and BCC samples with regard to β-HPVs overall, there was a statistical difference when the types were limited to those belonging to species 1, emphasizing the potential importance of this highly related (70% nucleotide identity) subgroup of viruses.

In agreement with previous studies (Berkhout *et al*., 2000; Karagas *et al*., 2006), multiple β-HPV infections were detected in 67.3% of SCC samples, as compared with 50.0% of BCC lesions. Competition among different genotypes and the preferential use of a subset of PCR primers in the mix of broad-spectrum primers could have affected our results. On the other hand, if one HPV genotype is present in high molar excess over the other, the minor genotype could be out-competed and remain unidentified. The phenomenon is a common problem in broad-spectrum PCRs and can lead to an underestimation of the number of HPV genotypes within the same sample; however, we would expect any possible misclassification of the minor genotype to be non-differential by histology. Furthermore, the direct comparison of HPV profiles from matched normal skin to tumor profiles from each patient may strengthen our findings and help clarify which HPV types may be more important in tumor formation and/or progression.

HPVs encompass the most common “carcinogenic” viruses. Although a connection was discovered nearly a century ago, an etiological relationship between cutaneous HPV infection and skin cancers remains speculative. Our findings suggest that a large fraction of SCC and BCC tumors contain HPV DNA of the genus β-types, and specifically indicate a potential role of β1 and possibly other β-types in SCC occurrence.

MATERIALS AND METHODS

Study population and specimens

Cases included individuals with histologically confirmed squamous cell and basal cell skin cancers newly diagnosed between July 1997 and March 2000 (sampled at a ratio of 1:1), identified through a collaborative network of dermatologists, dermatopathologists, and pathologists throughout the state of New Hampshire and border regions, with over 90% participation (Karagas *et al*., 1999). Participants gave their written informed consent. All 196 tumors tested for the current analysis were from unique patients. Tumor specimens (that is, paraffin blocks) were retrieved from the pathology laboratories and the percentage of tumorous tissue, along with the histopathologic diagnosis of each specimen was documented on a standardized form. In addition to tumor morphology, the study dermatopathologist documented tumor grade, perineural invasion (yes/no), associated actinic keratoses (yes/no), and extent of solar elastoses (absent, mild, moderate, and severe). The slide review ensured that specimens were appropriate for DNA analysis. All elements of the research design were approved by the Dartmouth Medical School and Harvard School of Public Health Institutional Review Boards

and informed consent was provided by all study participants. This study adhered to the Declaration of Helsinki Principles.

DNA isolation from tumors

DNA was isolated from 60 μm of paraffin-embedded biopsy material by adding 500 μl of histochoice clearing agent (Sigma, St Louis, MO) to each tube, followed by a 5-minute incubation, allowing the paraffin to dissolve. Subsequently, tubes were centrifuged at high speed for 1 minute and the histochoice liquid layer removed. The pellet was suspended in 250 μl of 100% ethanol. Following a second high-speed centrifugation, the liquid layer was again removed. If necessary, a second ethanol wash was performed. Next, the pellet was suspended in 250 μl of PBS, centrifuged, and supernatant removed. The pellet was digested overnight at 55 °C using an SDS-lysis solution with proteinase K. A volume of 50 μl of 5 M NaCl was added to each sample to allow de-crosslinking (65 °C for 4–12 hours). Samples were cooled down to room temperature and DNA purified using the Omega Wizard DNA kit as per manufacturer's directions.

HPV DNA detection in tumors

β-HPV detection and genotyping were carried out as described previously (de Koning *et al*., 2006, 2007) using the Diassay BV Skin (β) HPV kits (Diassay, Rijswijk, The Netherlands). Briefly, detection is achieved using a reverse hybridization assay. Part of the E1 region of the β-papillomavirus genome is amplified by PCR. The 117 bp biotin-labeled amplicon is hybridized with specific oligonucleotide probes, which are immobilized as parallel lines on membrane strips. After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Subsequent incubation with the BCIP/NBT (bromochloroindolylphosphate/nitroblue tetrazolium) substrate allows visual identification of HPV types on the genotype strip. This method allows identification of 25 HPV types within a single sample using a single amplicon, with a detection limit of 10 copies per sample. To ensure that there was no HPV contamination during amplification, a known negative tumor sample was included with each PCR run. The quality of the isolated DNA samples was checked by amplifying a similarly sized fragment from the β-globin gene (Resnick *et al*., 1990).

Statistical analysis

We first examined the association between HPV DNA positivity (any type positive) and known skin cancer risk factors. These risk factors included age, sex, level of education (elementary/high school, college, or graduate/professional), smoking status (never or former/current), skin sensitivity as measured by skin reaction after 1 hour of sun exposure the first time in summer (burner is defined as severe sunburn with blistering or painful sunburn with peeling, tanner is defined as mild sunburn with tanning or tanning), number of lifetime painful sunburns (0, 1–2, and 3 +), lifetime occupational sun exposure (hours), and anatomical tumor site (sun exposed is defined as head or neck, non-sun exposed is defined as all other sites). Unconditional logistic regression was used to estimate the ORs and 95% CIs between any β-HPV DNA positivity (yes or no), previously reported types (5, 8, 15, 20, 24, 36, and/or 38), number of types positive (none/one or multiple), and species-specific positivity (β1–5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47, and 93; β2–9, 15, 17, 22, 23, 37, 38, and 80) in SCC versus BCC tumors, adjusting for age, sex, education, smoking, skin sensitivity to sunlight, and number of lifetime sunburns.

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Abbreviations

BCC basal cell carcinoma

CI confidence interval

HPV human papillomavirus

NMSC non-melanoma skin cancer

OR odds ratio

SCC squamous cell carcinoma

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Table 1

ORs and 95% CIs for HPV infection according to tumor histology

HPV DNA positivity	BCC (*n*=98)	SCC (*n*=98)	OR (95% CI)[1]
Overall			
No β-types positive	22 (22.4)	16 (16.3)	Referent
Any β-HPV	76 (77.6)	82 (83.7)	1.5 (0.7–3.5)
Previously reported types			
No or other β-types	58 (59.2)	37 (37.8)	Referent
HPV 5, 8, 15, 20, 24, 36, and/or 38	40 (40.8)	61 (62.2)	2.6 (1.4–5.1)
Multiple infection			
No or 1 β-type	49 (50.0)	32 (32.7)	Referent
More than 1 β-type	49 (50.0)	66 (67.3)	2.0 (1.0–3.9)
Phylogenic species			
No β1 HPV	52 (53.1)	35 (35.7)	Referent
Any β1 HPV	46 (46.9)	63 (64.3)	2.0 (1.1–3.6)
HPV 5	5 (5.1)	6 (6.1)	
HPV 8	4 (4.1)	6 (6.1)	
HPV 12	1 (1.0)	5 (5.1)	
HPV 14	1 (1.0)	4 (4.1)	
HPV 19	0 (0.0)	3 (3.1)	
HPV 20	7 (7.1)	9 (9.2)	
HPV 21	3 (3.1)	6 (6.1)	
HPV 24	9 (9.2)	15 (15.3)	
HPV 25	0 (0.0)	0 (0.0)	
HPV 36	18 (18.4)	20 (20.4)	
HPV 47	5 (5.1)	5 (5.1)	
HPV 93	18 (18.4)	15 (15.3)	
No β2 HPV	42 (42.9)	32 (32.7)	Referent
Any β2 HPV	56 (57.1)	66 (67.3)	1.4 (0.7–2.5)
HPV 9	18 (18.4)	23 (23.5)	
HPV 15	14 (14.3)	25 (25.5)	
HPV 17	16 (16.3)	24 (24.5)	
HPV 22	2 (2.0)	5 (5.1)	
HPV 23	19 (19.4)	20 (20.4)	
HPV 37	12 (12.2)	12 (12.2)	
HPV 38	10 (10.2)	15 (15.3)	
HPV 80	9 (9.2)	15 (15.3)	
No β3, β4, and/or β5 HPV	69 (70.4)	66 (67.3)	Referent
Any β3, β4, and/or β5 HPV	29 (29.6)	32 (32.7)	1.0 (0.5–2.0)
HPV 49	0 (0.0)	6 (6.1)	
HPV 75	1 (1.0)	3 (3.1)	
HPV 76	25 (25.5)	21 (21.4)	
HPV 92	2 (2.0)	7 (7.1)	
HPV 96	2 (2.0)	3 (3.1)	

HPV DNA positivity	BCC (*n*=98)	SCC (*n*=98)	OR (95% CI)[1]

CI, confidence interval; BCC, basal cell carcinoma; HPV, human papillomavirus; OR, odds ratio; SCC, squamous cell carcinoma.

[1] ORs are adjusted for age, sex, education, smoking, skin sensitivity to sunlight, and lifetime sunburns.

Table 2

Percent positivity of tumor samples according to histology for variables such as age, sex, and sun-related factors

Variable	Number of samples analyzed		% β-HPV-positive	
	BCC	SCC	BCC	SCC
Overall	98 (100)	98 (100)	77.6	83.7
Sex				
Male	43 (43.9)	58 (59.2)	76.7	82.8
Female	55 (56.1)	40 (40.8)	78.2	85.0
Age (*years*)				
64 and younger	62 (63.3)	52 (53.1)	70.0	77.6
65 or older	36 (36.7)	46 (46.9)	89.5	89.8
Education				
Elementary/high school	36 (36.7)	42 (42.9)	80.6	83.3
College	36 (36.7)	35 (35.7)	66.7	82.9
Graduates/professional	26 (26.5)	21 (21.4)	88.5	85.7
Smoking				
Never	45 (45.9)	31 (31.6)	75.6	93.6
Former/current	53 (54.1)	67 (68.4)	79.3	79.1
Skin sensitivity[1]				
Tanner	49 (50.0)	59 (60.2)	79.6	83.1
Burner	48 (49.0)	38 (38.8)	75.0	84.2
No. of painful sunburns[1]				
0	24 (24.5)	20 (20.4)	79.2	90.0
1–2	13 (13.3)	18 (18.4)	76.9	88.9
3 or more	50 (51.0)	47 (48.0)	78.0	80.9
Occupational sun exposure				
Less than 3,350 hours	57 (58.2)	54 (55.1)	82.5	85.2
3,350 hours or more	41 (41.8)	44 (44.9)	70.7	81.8
Anatomical site				
Sun exposed	59 (60.2)	53 (54.1)	76.3	84.9
Non-sun exposed	39 (39.8)	45 (45.9)	79.5	82.2

BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

[1] Missing data: skin sensitivity (n=1) and no. of painful sunburns (n=11).

Table 3

Previous Studies of β-HPV DNA detection in NMSC tumor samples of immunocompetent individuals

Author (year)	Sample size	Methods[1]	β-HPV types detected	Main findings
Forslund *et al.* (2007)	82 SCCs 126 BCCs	Single and nested PCR of L1 region using CP65/CP70 and FAP primers[2]	8, 9, 12, 14, 15, 19, 20, 21, 23, 24, 37, 38, 80, 93, 96	Association between β-species 2 and SCC
Pfister *et al.* (2003)	20 SCCs	Nested PCR of L1 region using CP62/CP70a and CP65/CP69a primers	5, 8, 12, 14, 15, 19, 21, 24, 25, 37, 38	80% of AK and 40% of SCC lesions were positive for βHPV DNA
Iftner *et al.* (2003)	72 SCCs 18 BCCs	PCR of E1 region using CP4/CP5 and PPF1/CP5 primers	5, 8, 12, 17, 19, 22, 36	Association between β-HPV DNA and NMSC (OR 6.4)
Boxman *et al.* (2000)	14 BCCs	Nested PCR of the L1 region using CP62/CP70a and CP65/CP69a primers	5, 8, 12, 14, 15, 17, 25,37, 38	43% of BCC tumors were β-HPV DNA-positive
Surentheran *et al.* (1998)	6 SCCs	Degenerate and nested PCR of the L1 region using HPV2/B5, F14/B15, MY09/11, CP62/69	5, 8, 19, 20, 21, 23, 36	All SCC samples were negative most likely due to low viral copy number

AK, actinic keratoses; BCC, basal cell carcinoma; CI, confidence interval; HPV, human papillomavirus; NMSC, non-melanoma skin cancer; OR, odds ratio; SCC, squamous cell carcinoma

[1] All PCR-based methods were followed by direct sequencing.

[2] Pooled results from three laboratories using different PCR methods.