Human Papillomavirus-DNA Loads in Actinic Keratoses Exceed those in Non-Melanoma Skin Cancers

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Recent studies suggest a role of cutaneous human papillomaviruses (HPV) in non-melanoma skin cancer (NMSC) development. In this study viral DNA loads of six frequent HPV types were determined by quantitative, type-specific real-time-PCR (Q-PCR) in actinic keratoses (AK, n = 26), NMSC (n = 31), perilesional tissue (n = 22), and metastases of squamous cell carcinomas (SCC) (n = 8) which were previously shown to be positive for HPV5, 8, 15, 20, 24, or 36. HPV-DNA loads in AK, (partially microdissected) NMSC, and perilesional skin ranged between one HPV-DNA copy per 0.02 and 14,200 cell equivalents (median: 1 HPV-DNA copy per 344 cell equivalents; n = 48). In 32 of the 79 HPV-positive skin biopsies and in seven of the eight metastases viral loads were even below the detection limit of Q-PCR. Low viral loads in NMSC were confirmed by *in situ*-hybridization showing only a few HPV-DNA-positive nuclei per section. Viral loads in SCC, basal cell carcinomas, and perilesional tissue were similar. But, viral loads found in AK were significantly higher than in SCC ($p = 0.035$). Our data suggest that persistence of HPV is not necessary for the maintenance of the malignant phenotype of individual NMSC cells. Although a passenger state cannot be excluded, the data are compatible with a carcinogenic role of HPV in early steps of tumor development.

Key words: actinic keratoses/human papillomavirus (HPV)/*in situ*-hybridization (ISH)/non-melanoma skin cancer (NMSC)/viral load
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Non-melanoma skin cancers (NMSC), which comprise basal cell carcinomas (BCC), squamous cell carcinomas (SCC), and Bowen's disease (BD), represent the most common human cancers in fair-skinned populations (Alam and Ratner, 2001). NMSC mainly occur on sun-exposed sites and UV radiation has been recognized as the main risk factor for NMSC development. Immunosuppressed organ transplant recipients have a greatly increased risk of multiple and aggressive NMSC, especially SCC, indicating a crucial role for the immune system in SCC prevention (Berg and Otley, 2002; Euvrard *et al*, 2003).

Recent studies suggest an association of NMSC development and human papillomaviruses (HPV) (for a review, see Harwood and Proby, 2002; Pfister, 2003), whose oncogenic potential is fully accepted for genital cancer (Bosch *et al*, 2002; zur Hausen, 2002). The involvement of cutaneous HPV in human skin cancer has first been demonstrated in patients with the rare hereditary disease epidermodysplasia verruciformis (EV) (reviewed in Pfister and ter Schegget, 1997). Seroepidemiology has further established a linkage between NMSC and HPV (Feltkamp *et al*, 2003; Masini *et al*, 2003). HPV-DNA was detected in about 20%–50% of BCC and 30%–60% of SCC from immunocompetent patients (Pfister and ter Schegget, 1997; Harwood and Proby, 2002; Pfister, 2003). The highest prevalence was shown with up to 93% in actinic keratoses (AK), the precursor lesions of SCC (Forslund *et al*, 2003b; Pfister *et al*, 2003). In immunosuppressed patients HPV-DNA prevalence is even higher, sometimes reaching 100% (Pfister, 2003). Some light was shed on the high prevalence of HPV in skin tumors by Forslund *et al* (2004), who showed that removal of the outer stratum corneum layers by tape stripping led to a significant reduction of HPV positivity.

HPV-DNA detection usually requires nested PCR protocols implying low HPV-DNA copy numbers in NMSC samples. To verify this, we developed quantitative real-time PCR (Q-PCR) protocols for six frequent HPV types. The goal of this study was to measure viral loads in NMSC, AK, and perilesional tissue with special attention to different HPV-DNA loads in premalignant (AK) and malignant (BCC, SCC) tumors. To our knowledge, we are the first to show viral load differences between AK and SCC. Furthermore, we were able to localize viral DNA in NMSC by *in situ*-hybridization (ISH).

Abbreviations: AK, actinic keratoses; BCC, basal cell carcinoma; EV, epidermodysplasia verruciformis; HPV, human papillomavirus; ISH, *in situ*-hybridization; NMSC, non-melanoma skin cancer; Q-PCR, quantitative real-time PCR; RT, room temperature; SCC, squamous cell carcinoma; TSA, tyramide signal amplification

Results

HPV-DNA load in biopsies For the determination of viral DNA loads of six cutaneous EV-HPV-types (HPV5, 8, 15, 20,

24, 36) 57 lesional skin biopsies, 22 samples of perilesional skin, and eight SCC metastases were available. In 39 of these samples HPV-DNA load was below the detection limit of quantitative PCR (six AK, seven BCC, one BD, 10 SCC, eight perilesional samples, seven metastases). Nine of these (one AK, one BCC, four SCC, three perilesional tissue) were excluded from statistical analyses because of low cellular DNA input (<2000 β-globin gene copies per assay) (Table I). In the 48 samples with quantifiable HPV-DNA load viral loads ranged from 1 HPV-DNA copy per 14,200 cell equivalents to 1 HPV-copy per 0.02 cell equivalents (50 HPV-DNA copies per 1 cell equivalent). Mean and median HPV-DNA loads were 1 HPV-DNA copy per 1430 cell equivalents and 1 HPV-DNA copy per 344 cell equivalents, respectively. Viral load distribution was similar in samples that carried only quantifiable types and in samples that carried additional types. In almost all metastases (seven of eight) and in about half of the BCC and SCC/BD samples viral load was below detection limit of the quantitative PCR (Table I). In contrast, only 20% of the AK samples were within this category and viral loads of 1 HPV-DNA copy per less than five cell equivalents were only observed with AK. The HPV-DNA load difference between AK and SCC/BD was shown to be significant ($p = 0.035$; Mann–Whitney *U* test, two-sided).

From eight SCC microdissected malignant epithelial cells could be analyzed (Fig 1). In seven of these HPV5-, 8-, 15-, or 20 positive samples HPV-DNA load was below the detection limit of the quantitative PCR. In one sample 1 HPV5-DNA copy per 551 cell equivalents was observed (Fig 1).

To compare HPV-DNA loads in NMSC and perilesional skin we analyzed 16 homologous pairs that carried the same HPV type (six BCC; 10 SCC of which eight were microdissected). Three pairs of microdissected SCC and perilesional biopsies had to be excluded from this analysis because of low β-globin DNA input. Load differences within a half-log scale (i.e. 3-fold difference or lower) were defined as being similar, larger differences were regarded as significant. Trends for load differences between tumor and perilesional skin could neither be observed for BCC nor for SCC pairs. Of the six BCC/perilesional pairs two had higher loads in the tumor, two had higher loads in the perilesional tissue and two had similar loads in the tumor and the perilesional tissue. Of the five microdissected SCC/perilesional pairs included in the analysis, three fell into the category "similar viral load in tumor and perilesional tissue" and two into the category "higher viral load in the tumor". Of the two non-microdissected SCC/perilesional pairs one had a higher viral load in the tumor than in the perilesional tissue, and the other one vice versa. In six perilesional biopsies that had been positive for HPV15, 20, or 24 by screening PCR and for which the corresponding NMSC had been HPV negative or carried HPV types for which no Q-PCR was available, HPV-DNA loads ranged between below detection limit of Q-PCR and 1 HPV-DNA copy per 159 cell equivalents. Viral loads in the small number of samples from immunosuppressed patients were comparable with the loads found in samples of immunocompetent individuals.

Detection of HPV-DNA in skin biopsies by ISH Tissue for ISH was available from three of the NMSC analyzed by Q-PCR: one SCC (1 HPV15 copy per 708 cells) (Fig 2), two BCC (1 HPV8 copy per 244 and per 455 cells), and the respective perilesional tissues from three immunocompetent patients. From each biopsy 3–12 sections were analyzed. A maximum of 20 HPV-positive nuclei per section could be observed in the SCC. In most sections only one to four positive nuclei were detected and some sections did not show any signals (Fig 2, perilesional tissue with 1 HPV copy per 2332 cells in Q-PCR). HPV-positive nuclei were found both in the superficial more differentiated part of the SCC and in the invasive part (Fig 2). The low number of HPV-positive nuclei in NMSC of immunocompetent patients was confirmed by the analysis of three further tumor/perilesional tissue pairs that had been positive for the HPV-types 37 (SCC), RTRX5 (SCC) or RTRX9 (BD). In summary, some HPV-DNA-positive nuclei could be detected in all biopsies with the exception of one perilesional sample. Taking

Table I. Highest HPV-DNA loads are found in actinic keratoses

Load categories	Metastases (n = 8 (%))	Perilesional[a] skin (n = 19 (%))	BCC[a] (n = 13 (%))	SCC/BD[a] (n = 13 (%))	AK[a] (n = 25 (%))
Below detection limit [b]	87	32[c]	46	54[c]	20
One HPV copy per					
>500 cells	13	37	15	23[d]	32
51–500 cells	0	21[e]	31	8	8
5–50 cells	0	10	8	15	28
<5 cells	0	0	0	0	12

[a]The observed HPV-DNA load difference between AK and SCC/BD was shown to be significant ($p = 0.035$; Mann–Whitney *U* test, two-sided). p-values for HPV-DNA load differences between the other sample groups were: AK *versus* BCC 0.084; AK *versus* perilesional skin 0.104; SCC/BD *versus* perilesional 0.379; BCC *versus* perilesional 0.739; SCC/BD *versus* BCC 0.679.

[b]Mean β-globin gene input copy numbers per reaction were 10280, 5914, 8712, 3242, 37724 in metastases, perilesional skin, BCC, SCC/BD, and AK, respectively. Nine samples (one BCC, four SCC (three of which were microdissected), one AK, three perilesional (microdissected) skin biopsies) were excluded from analysis because of low cellular DNA input (< 2000 β-globin gene copies/assay).

[c]Ten samples were microdissected (five SCC and five perilesional biopsies): including three microdissected perilesional samples and four microdissected SCC, respectively.

[d]Including one microdissected SCC.

[e]Including two microdissected perilesional samples.

BCC, basal cell carcinoma; SCC, squamous cell carcinoma; BD, Bowen's disease; AK, actinic keratosis; HPV, human papillomavirus.

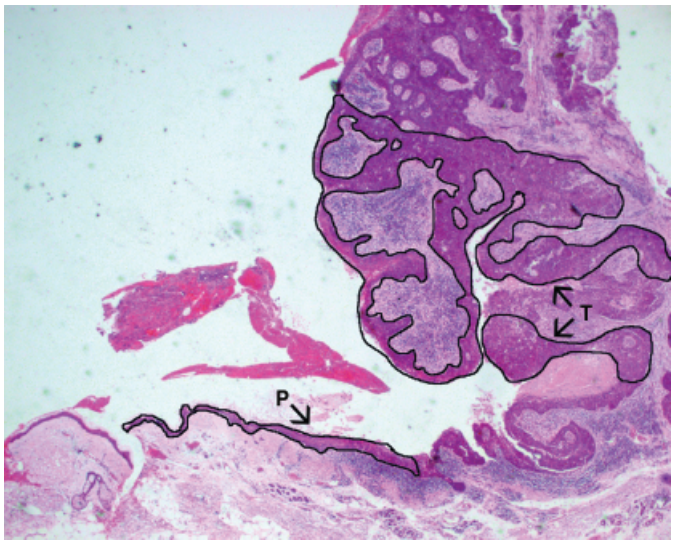

Figure 1
Microdissection of tumorous and perilesional areas of a squamous cell carcinoma (SCC). The black lines demarcate the microdissected epithelial areas. T, tumorous/invasive area; p, perilesional area. The SCC had 1 HPV5 copy per 551 cells in the microdissected area as determined by quantitative real-time PCR. The viral load of the corresponding microdissected perilesional tissue (normal epidermis) was 1 HPV5 copy per 71 cells.

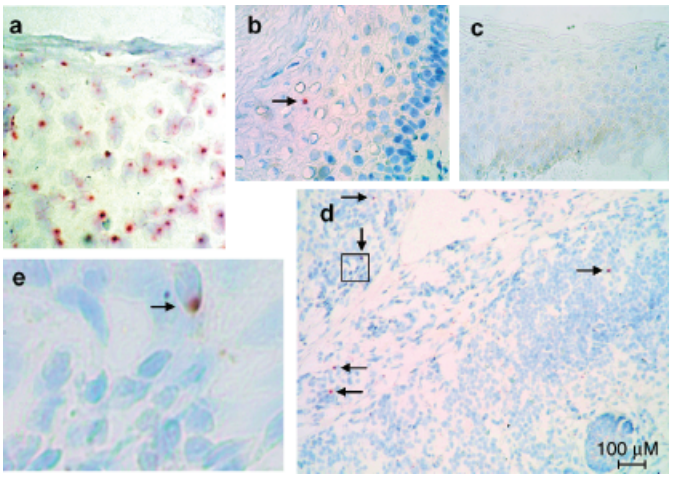

Figure 2
Human papillomavirus (HPV)-DNA detection in cryosections from skin biopsies by *in situ* hybridization. HPV-DNA positive nuclei (*arrows*) appear red (AEC staining; original magnification 100 and 400-fold). HPV8 (*a*) or HPV15 (*b–e*) specific DNA probes were used. (*a*) Skin tumor of an HPV8-transgenic mouse with 27 copies of a 5111 bp HPV8 genome fragment per cell (positive control). (*b*) Detail of an HPV15-positive squamous cell carcinoma (SCC) (viral load as measured by quantitative real-time PCR (Q-PCR) was 1 HPV15 copy per 708 cells). (*c*) Homologous perilesional tissue without detectable HPV-signals (viral load as measured by Q-PCR was 1 HPV15 copy per 2332 cells). (*d*) Invasive part of the same HPV15-positive SCC shown in *b*. The framed rectangle is magnified in (*e*).

into account the proportion of epithelial cells per section, detection rates by ISH were similar for NMSC and perilesional skin in all samples analyzed.

Discussion

Early analyses of skin tumors by Southern blot hybridization, sensitive enough to detect one HPV genome per cell, very rarely demonstrated viral sequences (Kawashima *et al*, 1990), indicating low viral loads in tumors only positive in highly sensitive PCR assays. We developed sensitive, type-specific real-time PCR protocols for six frequent EV-associated HPV-types and found very low viral loads in 25 AK, 13 SCC/BD, and 13 BCC. HPV-DNA loads of individual HPV types were similar (data not shown). Forslund *et al* (2003a) have analyzed HPV-DNA loads of six HPV92-positive tumors, two AK, two SCC, and two BCC. The reported viral loads for AK and SCC are in line with our results. In contrast, one and 94 HPV-copies per cell were found in two BCC. We never observed loads higher than 1 HPV-DNA copy per 21 cell equivalents in 13 BCC. It remains to be established if the higher loads in the HPV92-positive BCC reflect characteristics of HPV92 or the individual tumors.

In almost half of our tumor samples, which were all shown to be positive for EV-HPV-DNA by nested PCR, the amount of viral DNA was below the detection limit of real-time PCR. Viral loads of 1 HPV-DNA copy per less than 500 cells were measured in 39% of BCC and 23% of SCC and viral loads of 1 HPV-DNA copy per less than 50 cells were determined in only 8% of BCC and 15% of SCC. Forslund *et al* (2004) compared HPV-DNA positivity of swab samples from the top of skin-tumors and biopsies which were tape stripped to remove superficial layers. They found only a small fraction of stripped BCC and SCC positive, which was interpreted as an indication that HPV positivity frequently merely reflects contamination of the lesion surface. Interestingly, Forslund *et al*'s proportion of positive stripped biopsies is similar to the proportion of NMSC with higher viral loads in our study. We cannot exclude that the tumors with very low viral loads of our study include cases of surface contamination. In the majority of microdissected SCC the amount of viral DNA was indeed below the detection limit of real-time PCR. But, in one HPV-positive SCC and in two HPV-positive BCC with only 1 HPV genome per 708–244 cells, a few positive nuclei, scattered both in the *in situ* and the invasive part of the tumor, could be detected by the highly sensitive TSA method. This ISH picture is fully compatible with the overall viral load, which furthermore excludes the possibility of viral DNA persistence in the majority of tumor cells below the detection limit of ISH. In clear contrast to cervical cancer (zur Hausen, 1996), the presence of HPV therefore does not seem to be necessary for the maintenance of the malignant phenotype of the individual tumor cells in NMSC. This view is also supported by our finding of one HPV copy in more than 500 cells in only one of eight metastases. We could not observe significant viral load differences between SCC, BCC, and perilesional skin. It is tempting to conclude from these data that HPV is more a passenger than a driver in NMSC. The demonstration of HPV-DNA, however, in a varying fraction of NMSC tumor cells may have an impact on growth and invasion properties of the tumor as a whole, if the virus would trigger, for example, the secretion of cytokines or metalloproteinases. Our study revealed significantly higher viral loads in AK, which are the precursor lesions of SCC, than in SCC. Viral loads of 1 HPV-DNA copy per less than 50 cells were measured in 40% of AK. The higher viral loads in AK

are likely to reflect enhanced HPV-DNA replication. This may be because of intense keratinocyte proliferation and differentiation in AK favoring amplification of commensalic HPV. Active HPV replication and presumably enhanced gene expression may in turn stimulate keratinocyte proliferation and contribute to carcinogenesis in these early stages of NMSC development. HPV-E6 proteins were recently shown to inhibit UV-induced apoptosis by abrogation of Bak in response to UV damage (Jackson and Storey, 2000) and to bind a protein required for repair of single strand DNA breaks (Iftner *et al*, 2002). Thereby, accumulation of UV-induced mutations and oncogenic transformation might be facilitated in cases of active HPV infection.

Material and Methods

Patient materials By screening 363 skin biopsies from recent investigations, 87 biopsies could be identified as positive for the HPV types 5, 8, 15, 20, 24, or 36 (Wieland *et al*, 2000a; Pfister *et al*, 2003; Nindl *et al*, unpublished; Purdy *et al*, unpublished). Additional HPV types were found in 28 biopsies. Samples comprised 26 AK, three BD biopsies, 14 SCC, eight metastases of SCC, 14 BCC, and 22 biopsies from perilesional skin. Perilesional skin was collected from areas directly flanking the tumors. Eight pairs of SCC biopsies and perilesional skin consisted of microdissected invasive tumor area and adjacent epidermal cells, respectively (Fig 1). With the exception of the lesional–perilesional sample pairs and one patient of whom two BCC were obtained, only one sample per patient was analyzed. Patients' (58% male, 42% female) mean age was 69.9 y, ranging between 44 and 86 y. Patients had attended the Dermatology Departments of the Universities of either Warsaw, Berlin, Cologne, or London. Of the 87 samples, 43 were from German, 28 from Polish and 16 (the eight pairs of microdissected SCC/perilesional tissue) from British patients. The majority of samples ($n = 68$) had been collected from immunocompetent patients. Only one BCC, five SCC, 10 perilesional skin biopsies and three SCC metastases were collected from immunosuppressed transplant recipients. The study was conducted according to Declaration of Helsinki Principles.

Real-time PCR Type-specific Q-PCR protocols for the cutaneous EV-associated HPV-types 5, 8, 15, 20, 24, and 36 were developed on the LightCycler system (Roche, Mannheim, Germany) and performed and evaluated as previously described (Wieland *et al*, 2000b; Weissenborn *et al*, 2003). Analytical sensitivity was two HPV-DNA copies per reaction when duplicate testing was performed. Binding sites of type specific primers and probes were located within the L1 genes. Type-specificity was shown for all protocols as described before (Weissenborn *et al*, 2003). The supplemental material provides primer and probe sequences, annealing temperatures, magnesium chloride concentrations, and cycling conditions (Table S1). To correct for PCR efficiency and DNA integrity and to determine the number of input cell equivalents, the single-copy gene β-globin was quantified using the "LightCycler-Control Kit DNA" (Roche) (Weissenborn *et al*, 2003). Because of the small HPV-DNA copy numbers found, HPV-DNA loads were defined as 1 HPV-DNA copy per x cell equivalents. Two β-globin-gene copies were taken as a cell equivalent. For statistical analysis (SPSS 10.0.7, SPSS, Illinois) HPV-DNA loads were categorized as specified in Table I and the number of samples falling into the five viral load categories were used as input. Each sample was quantified in duplicate (mean of the relative coefficient of variation 25.7%) and mean values were used as results. Two negative controls (human placental DNA) were included in each run and never yielded fluorescence signals above background.

ISH with tyramide signal amplification (TSA) Cryosections (5 μm) were prepared from 12 fresh frozen HPV-positive skin biopsies (six NMSC and six homologous perilesional tissues) from immunocompetent patients (Wieland *et al*, 2000a and unpublished). According to previous typing results, complete biotin labelled genomic HPV plasmid DNA (HPV8, 15, or 37) or PCR-generated 420 bp L1-gene DNA fragments (RTRX5, RTRX9; Berkhout *et al*, 1995; Bens *et al*, 1998) were used as probes. ISH was performed under high stringency conditions ($T_m - 17.5^\circ C$). Experimental details are published online as supplemental material. As positive control (Fig 2*a*) skin tumor sections of an HPV8-transgenic mouse were used which carried 27 copies of a 5111 bp HPV8 genome fragment per cell (Schaper *et al*, 2005). This corresponds to 18 complete HPV8 genome (7654 nt) copies per cell. As we detected strong signals in almost all nuclei, the sensitivity can be regarded as high. Type specificity of the hybridization was shown by a lack of signals using an HPV15 probe instead of an HPV8 probe.

Microdissection Formalin-fixed paraffin-embedded skin biopsies that had been histologically confirmed as SCC were manually dissected into perilesional and invasive tumor components under microscopic control. Sections (5 μm) were cut onto glass slides, one of which was deparaffinized and stained with hematoxylin and eosin. Using this section as a reference, separate sterile disposable scalpels were used to scrape perilesional and tumor epidermal tissue into sterile tubes. Dissections were performed in a class 2 cabinet which was UV irradiated after every sample as a precaution against contamination.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23733/JID23733.htm

Table S1. Primers, probes and PCR-conditions for the quantification of DNA of HPV-types 5, 8, 15, 20, 24 and 36 by real time PCR.

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