ORIGINAL ARTICLE

High β-HPV DNA Loads and Strong Seroreactivity Are Present in Epidermodysplasia Verruciformis

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Epidermodysplasia verruciformis (EV) is a rare disease, characterized by cutaneous warts and associated with a strong predisposition to β-genus human papillomavirus (HPV). Earlier studies reported high copy numbers of HPV-DNA in nearly all skin tumors from EV patients, but neither HPV replication status in non-lesional skin nor anti-HPV seroreactivity in these patients have been reported yet. We therefore performed a comprehensive viral load analysis for the more common β-HPV types on skin samples and plucked eyebrow hairs from four EV patients treated at our dermatology department. The results clearly demonstrate that they carry a multiplicity (up to eighteen types) of β-HPV genotypes in both skin sites. Worthy of note, a high intrapatient concordance for specific types between hair bulbs and skin biopsies was observed and the same β-PV profile was maintained over time. Viral load analysis revealed a load range between less than one HPV-DNA copy per 100 cells to more than 400 HPV-DNA copies per cell in both eyebrow hairs and skin proliferative lesions. Evaluation of seroreactivity to β-HPV types in the four EV patients revealed that antibodies against the 16 β-HPV were significantly more prevalent and showed higher titers than in the controls.

INTRODUCTION

Epidermodysplasia verruciformis (EV) is a rare genodermatosis characterized by development of disseminated flat warts since early childhood (Jablonska and Majewski, 1994). EV patients are highly susceptible to human papillomavirus (HPV) infection, and often develop cutaneous squamous-cell carcinoma (SCC; Orth, 2006).

According to the most updated *Papillomaviridae* classification, the HPV genotypes originally found in EV patients (for example, EV-HPV) and phylogenetically related types are grouped together into the *Betapapillomavirus* genus, whereas the mucosal genotypes (for example, HPV types 16 and 18) belong to the *Alphapapillomavirus* genus (deVilliers *et al.*, 2004).

The HPV types found more frequently in EV skin lesions are 5, 8, 9, 12, 14, 15, 17, and 19–25. HPV types 5 and 8 have been detected in 90% of cutaneous SCC in EV cases, and in two patients, HPV type 5 was detected in the primary tumor and metastasis, suggesting a prominent role for HPV type 5 in SCC (Orth, 1986; Favre *et al.*, 1998; Majewski and Jablonska, 2002; Pfister, 2003). HPV usually persists extra-chromosomally in high copy numbers (100–300 per diploid host genome) in nearly all SCCs from EV patients and is actively transcribed. According to early *in situ* hybridization (ISH) experiments, high copy numbers can be partially traced back to a few carcinoma cells in the tumor, supporting vegetative viral DNA replication (Orth *et al.*, 1971, 1979; Orth, 1980, 1986).

Highly sensitive detection techniques identified genus β-papillomavirus (β-PV) DNA in a substantial proportion (30–50%) of non-melanoma skin cancer in immunocompetent non-EV patients (Boxman *et al.*, 1997; Harwood and Proby, 2002; Purdie *et al.*, 2005; Akgul *et al.*, 2006; Nindl *et al.*, 2007). The viral load in these skin tumors is low, with a single HPV copy being detected in only 10–1,000 dysplastic cells. The viral load may even decrease during skin carcinogenesis (Weissenborn *et al.*, 2005). These results are in clear contrast with those obtained with mucosal HPV, where HPV-induced genital cancers usually carry at least one viral genome per cell, and where high viral load represents an important risk factor for the development of cervical carcinoma (Snijders *et al.*, 2006). Moreover, β-PV DNA is frequently detected in skin swabs and biopsies from normal skin or in plucked hair bulbs from individuals with or without

skin cancer, with a higher prevalence in sun-exposed sites such as the forehead (Antonsson *et al.*, 2000; Struijk *et al.*, 2003; de Koning *et al.*, 2005; Kohler *et al.*, 2007).

The majority of the HPV DNA detection studies performed to localize and estimate the amount of virus present in EV SCC date back to more than thirty years ago (Orth *et al.*, 1971, 1979; Orth, 1980, 1986). Over the last two decades, several PCR-based methods have been developed to detect and quantify a broad range of in part more recently identified cutaneous HPV, and the sensitivity of the ISH technique has been optimized as well. Furthermore, there is increasing evidence that detection of EV-HPV DNA in eyebrow hairs may be an adequate marker for the HPV type present in skin lesions (Boxman *et al.*, 1997, 2000; Struijk *et al.*, 2003; Wolf *et al.*, 2004; Cronin *et al.*, 2008). To further investigate the replication status of these viruses in both lesional and non-lesional skin from EV patients, we performed a comprehensive viral load analysis for the more common β-genus HPV types on skin samples and plucked eyebrow hairs from four EV patients. We used quantitative real-time PCR (Q-PCR) and ISH to determine the quantity and distribution of HPV in skin biopsies and plucked eyebrows. In addition, serologic analysis was performed to detect antibodies against the α, β, γ, μ, and ν genera of HPV in EV patients' sera.

RESULTS

The clinical, virologic, genetic, and immunologic characterizations of patients 1 and 2 have already been described (Azzimonti *et al.,* 2005; Zavattaro *et al.*, 2008). The histopathological features of patient 3 and 4—skin biopsies (Figure 1B panels a and g)—together with the clinical history were consistent with a diagnosis of EV. In addition, HPV DNA analysis from both skin biopsies and plucked eyebrows of patients 3 and 4 revealed the presence of multiple HPV genotypes from the β-genus, as expected for EV patients.

Overall, analysis of eyebrow hair bulbs revealed multiple β-genus HPV infections, with between 5 and 18 types of the virus present (Table 1). HPV DNA analysis was performed by using a newly developed broad spectrum PCR (PM-PCR) in combination with a reverse hybridization system; de Koning *et al.*, 2006). To avoid discrepancy due to the PCR technique, specimens from patient 1 and 2 that were previously analyzed by CP(62/69)-based PCR were retested by the PM-PCR. As PM-PCR is more sensitive than the CP(62/69)-PCR, additional β-genus HPV types were identified (Table 1). All previously detected types were confirmed here with PM-PCR. The β-globin gene could no longer be amplified from the DNA of the SCC specimen from patient 1, likely due to DNA degradation. For this sample, ISH analysis was done with HPV type 20, which was previously identified in this lesion (Azzimonti *et al.*, 2005). Interestingly, the highest number of genotypes was found in hair bulbs from both forearm and eyebrows, confirming the hypothesis that this site can function as a reservoir of β-genus HPV types. By contrast, PCR reactions for γ-genotypes gave negative results in all the specimens from the four study patients.

Next, to determine HPV DNA copy numbers and input cell equivalents, type-specific Q-PCR protocols for HPV types 5, 8, 14, 15, 19, 20, 23, 24, 36, 38, 93, and 96 were performed. Overall, HPV DNA loads in EV biopsies ranged between less than 1 HPV DNA copy per less than 100 cells to more than 400 HPV-DNA copies per cell. For patient 1, DNA from three biopsies was available for Q-PCR; HPV type 36 predominated in both the actinic keratosis and the Bowenoid lesion, whereas no HPV-type with a high viral load could be observed for the wart-like lesion. For the papular lesion of patient 2, no predominant type could be identified. For patient 3, high loads could be seen for the wart-like lesion (HPV types 8 and 24), the SCC (HPV types 24, 14, and 19), and the Bowenoid lesion (HPV types 14 and 19). In patient 4, HPV type 5 predominated in terms of viral loads in all four skin biopsies. In the Bowenoid lesion, there were also high load for both HPV types 93 and 96.

Consistent with the results obtained with skin biopsies, HPV-DNA loads in hair bulbs from plucked eyebrows and forearm hairs ranged between less than 1 HPV-DNA copy per 100 cells to more than 400 HPV-DNA copies per cell. In five of the six hair bulb samples multiple HPV-types were seen at high loads. Notably, the viral load of HPV type 5 was the highest among the HPV genotypes quantified in plucked hair bulbs from both eyebrows and forearm hairs from all four patients, suggesting that a predominant replication of this genotype is somehow favored in EV patients.

To localize viral HPV DNA in the context of the surgical lesions, histological slides were prepared to perform ISH. The choice of the probes was made on the basis of the Q-PCR results. As a negative control, samples were hybridized with the empty parental vector probe (data not shown) and an unrelated genotype (HPV type 16). For patient 1, a very strong signal was detected in the SCC lesion with the HPV type 20 probe and in the Bowenoid lesion with the HPV type 36 probe (Figure 1A panels a and c, respectively). Positive nuclei were distributed heterogeneously in the tumor tissues. In the papular lesion from patient 2, a weaker signal was obtained with the HPV type 24 probe (Figure 1A panel e). Serial sections from the skin biopsies of patient 3 showed a strong signal in the Bowenoid lesion with HPV type 14 (Figure 1B panel b) and 19 (data not shown), and in the wart-like lesion with both HPV types 8 and 24 probes (Figure 1B panels d, and e). In these lesions, HPV genomes were mainly detected in the nuclei of the upper superficial layers. In the SCC hybridized with HPV types 24, 14, and 19, the positive nuclei were mostly located in the normal epithelium adjacent to the tumor nests, where only scattered nuclei were positive (data not shown). In both actinic keratosis and Bowenoid lesion from patient 4, a strong signal was obtained with HPV type 5 (Figure 1B panel h and l), 93, and 96 (data not shown), whereas very few positive cells were detectable in both SCC and basal cell carcinoma hybridized with the same DNA probes (data not shown).

Sera from the four EV patients and 54 age- and sex-matched control individuals were analyzed in parallel for antibodies to 38 HPV types. The sera were also analyzed for three other control antigens that have a high seroprevalence (VP1 from human polyoma virus JC; CagA and OMP from *Helicobacter pylori*). In EV patients, antibodies against the 16
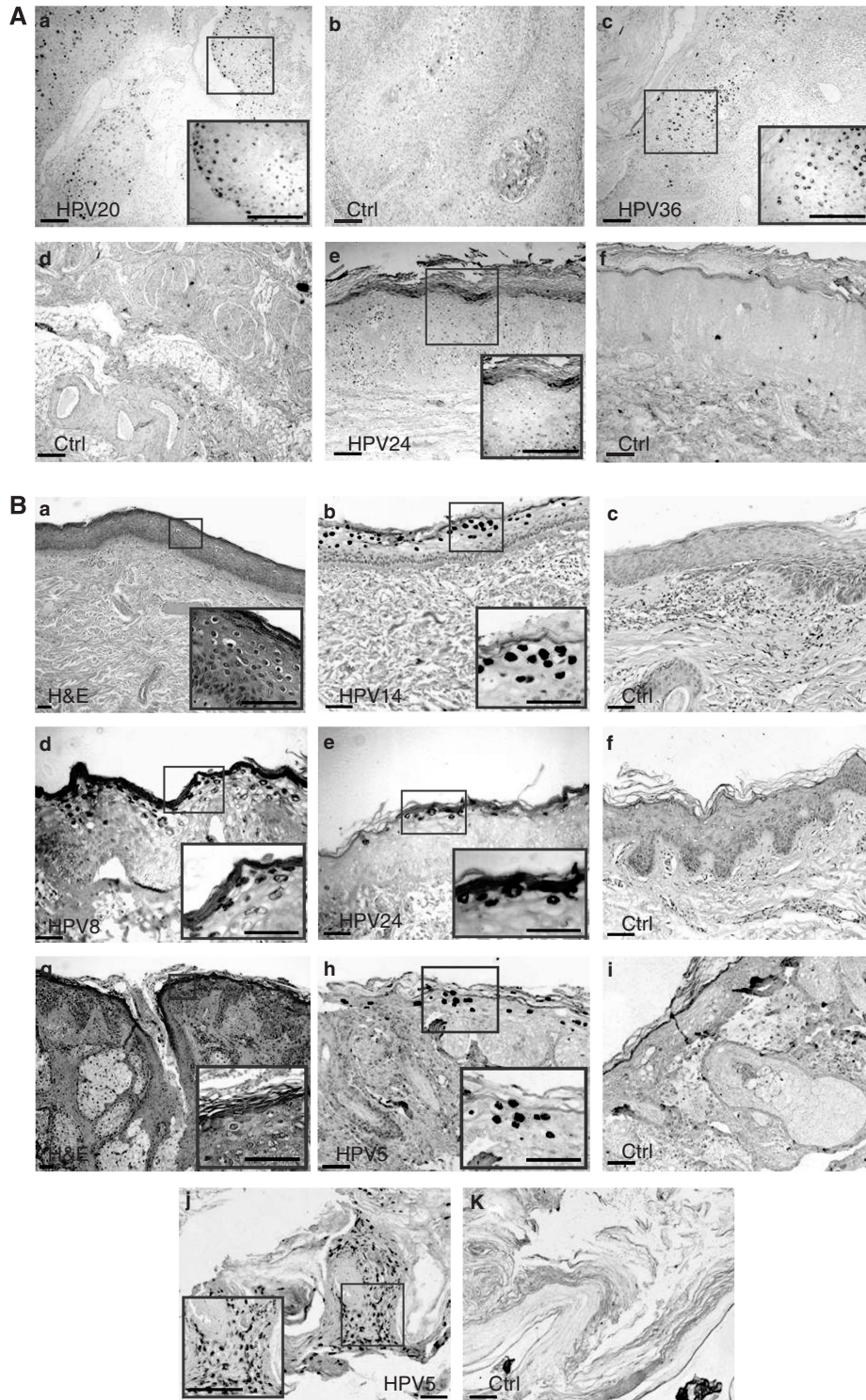
Figure 1. **Human papillomavirus (HPV)-DNA detection in formalin-fixed paraffin-embedded skin biopsies by *in situ* hybridization (ISH).** HPV-DNA positive nuclei appear red (AEC staining; scale bar = 250 μm). HPV type-specific DNA probes were used. (**A**) Patient 1. SCC: DNA-probe HPV type 20 (a), and Ctrl* (b); Bowenoid lesion: DNA-probe HPV type 36 (c), and Ctrl (d). Patient 2. Papular lesion: DNA-probe HPV type 24 (e), Ctrl (f). (**B**) Patient 3. Bowenoid lesion: H&E**(a), DNA-probe HPV type 14 (b), and Ctrl (c); Wart-like lesion: DNA-probe HPV type 8 (d), HPV type 24 (e), and Ctrl (f). Patient 4. AK: H&E (g), DNA-probe HPV type 5 (h), and Ctrl (i); Bowenoid lesion: DNA-probe HPV type 5 (l), and Ctrl (m). *Ctrl: tissue specimens hybridized with the unrelated genotype HPV type 16; **H&E: Hematoxylin-eosin staining.

β-PV were significantly more prevalent (89% of all reactions positive versus 10% in the controls, $P < 0.0001$; Figure 2), and EV patient sera showed higher titers than the controls ($P < 0.0001$; Figure 2). Also, antibodies to γ-PV were more prevalent in EV patients (42 versus 10%, $P < 0.0001$) and elevated in titer ($P < 0.0001$; Figure 2). This was also true for

Table 1. Detection of DNA of cutaneous β-PV genotypes and assessment of viral load in biopsy specimens and plucked hair bulbs from EV study patients

Patient Sex/Age	Histology (location)	HPV type	Viral load	
			HPV type	Copies/cell
1 M/40[1]	Wart-like lesion (forearm) 2003	5[2], 14, 15, 20, 24, 36	5	<0.01
			14	<0.01
			15	<0.01
			20	<0.01
			24	<0.01
			36	<0.01
	SCC (left temporal region) 2003	20	NA[3]	NA[3]
	AK (elbow) 2003	5, 15, 20, 24, 36	5	<0.01
			15	<0.01
			20	<0.01
			24	<0.01
			36	38
	Bowenoid lesion (forehead) 2003	5, 15, 20, 24, 36	5	<0.01
			15	<0.01
			20	<0.01
			24	<0.01
			36	16
	Plucked hair bulbs (eyebrows) 2007	5, 9, 12, 15, 20, 21, 22, 24, 36	5	416.7
			15	147
			20	270
			24	<0.01
			36	100
2 F/60	Papular lesion (forearm) 2005	5, 14, 15, 24	5	<0.01
			14	<0.01
			15	<0.01
			24	<0.01
	Plucked hair bulbs (eyebrows) 2007	5, 9, 14, 15, 24	5	312
			14	<0.01
			15	<0.01
			24	<0.01
3 M/39	SCC (right temporal region) 1999	14, 19, 24	14	148
			19	143
			24	88
	Bowenoid lesion (right temporal region) 1999	14, 19, 24	14	33.3
			19	46.1
			24	0.1
	Wart-like lesion (forearm) 1999	8, 24, 37	8	472
			24	132
	Plucked hair bulbs (eyebrows) 2007	5,8, 9, 12, 14, 19, 21, 23, 24, 25, 36, 37, 38, 47, 49, 75	5	14
			8	0.25
			14	<0.01
			19	<0.01
			23	<0.01
			24	0.09
			36	0.02
			38	<0.01
	Plucked hair bulbs (forearm) 2007	5, 8, 9, 12, 14, 17, 19, 21, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76	5	125
			8	3.3
			14	<0.01
			19	0.25
			23	0.02
			24	1
			36	0.23
			38	0.02
4 M/60[1]	BCC (medial left eyelid) 2007	5, 8, 15, 20, 21, 23, 25, 93, 96	5	4.6
			8	<0.01
			15	0.09
			20	0.02
			23	<0.01
			93	0.02
			96	0.02
	AK (right cheek bone) 2007	5, 19, 20, 21, 24, 25, 47	5	445
			19	<0.01
			20	<0.01
			24	<0.01
	SCC (medial left eyelid) 1993	5, 20, 21, 25, 93, 96	5	3.4
			20	<0.01
			93	<0.01
			96	<0.01

Table 1. Continued

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Patient Sex/Age	Histology (location)	HPV type	Viral load	
			HPV type	Copies/cell
	Bowenoid lesion (back) 1990	5, 93, 96	5	618
			93	53
			96	73
	Plucked hair bulbs (eyebrows) 2008	5, 8, 14, 20, 21, 25, 36, 93, 96	5	111
			8	<0.01
			14	4.4
			20	0.54
			36	<0.01
			93	1.1
			96	0.65
	Plucked hair bulbs (forearm) 2008	5, 8, 14, 20, 21, 24, 25, 36, 47, 93, 96	5	71.8
			8	<0.01
			14	4.6
			20	5.3
			24	<0.01
			36	<0.01
			93	<0.01
			96	0.9

AK, actinic keratosis; BCC, basal cell carcinoma; HPV, human papillomavirus; SCC, squamous-cell carcinoma.
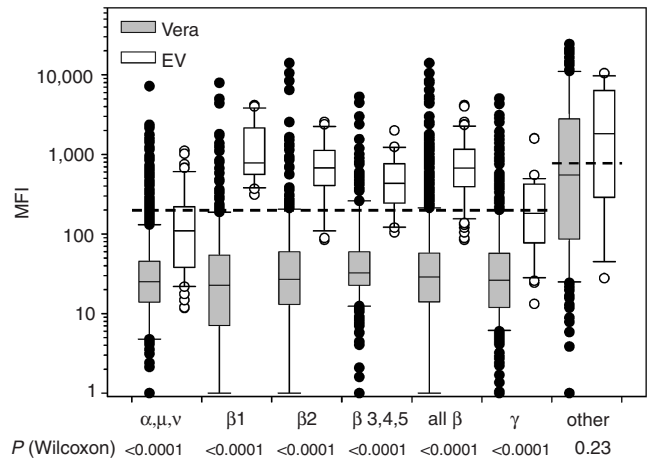[1]Consanguinity.
[2]Normal font numbers: HPV identified by PM-PCR only; Bold numbers: HPV identified previously by CP(62/69)-PCR.
[3]NA: not available.

Figure 2. **HPV serum antibody reactivity in EV patients ($n=4$) and controls ($n=54$).** The box plots show the distribution of serum antibody reactivities measured by HPV multiplex serology and expressed as median fluorescence intensity (MFI, logarithmic scale). Sera from 4 EV patients (EV) and 54 age-matched control individuals of a population-based nutrition survey (VERA) were analyzed with major capsid proteins L1 from 38 HPV types grouped by phylogeny, and three other control antigens (VP1 from human polyoma virus JC; CagA and OMP from *Helicobacter pylori*) with high seroprevalence. The group α, μ, ν includes HPV types 2, 3, 7, 10, 13, 16, 18, 27, 57, 77, 1, 41 and 63 group β1 types 5, 8, 20, 24, and 36, group β2 types 9, 15, 17, 23, 38, and 107, group β3, 4, 5 types 49, 75, 76, 92, and 96, and group γ types 4, 48, 50, 60, 65, 88, 95, 101, and 103. Boxes include the 25th to 75th percentile of measurements, and the line within shows the median. Whiskers below and above describe the 10th and 90th percentile. Values outside these boundaries are shown by circles. The dotted lines represent the arbitrarily chosen uniform cut-off of 200 MFI to define seropositivity for all HPV antigens and 800 for the control antigens.

the cutaneous and mucosal α- and cutaneous μ-, and ν-PV. However, median β-PV titers in EV patients were 13 (β-species 3, 4, 5) to 34 (β-species 1) times higher than controls, whereas median titers for γ-PV and α-/μ-/ν-PV were only increased seven- and fourfold, respectively. The three control antigens did not differ significantly between the two groups (Figure 2). In the individual patients, the antibody responses against the β-PV types with high viral load (>one copy per cell) did not appear to differ from the response against the other β-PV types (Table S1).

DISCUSSION

For the first time to our knowledge, this study characterizes HPV infection in EV patients with precise typing, quantitative tests, *in situ* localization, and antibody analysis. Despite the small number of patients due to the extremely low incidence of the disease, this study offered the unique possibility of thoroughly evaluating different virologic parameters in EV patients in both lesional and non-lesional skin. The results obtained clearly demonstrate that EV patients carry a multiplicity (up to 18 types) of β-PV genotypes in both eyebrow

and hair bulbs from different skin sites, confirming and expanding previous findings done on skin scrapes from EV lesions (Orth, 1986). The most likely explanation for this broader spectrum of genotypes is that the techniques used in the past were less sensitive or the number of HPV probes available was certainly much smaller. The rate of infection in EV patients is much higher than that reported for either the immunocompetent or immunosuppressed non-EV population (Boxman *et al.*, 1997, 1999, 2000). De Koning *et al.*, (2007), by investigating 23 healthy individuals over time for the presence of β-PV DNA in plucked hairs with the same test applied here, found a multiplicity of HPV types restricted in the range of one to 10 β-PV types present simultaneously, with 10 types found in one individual only.

Viral load analysis for the more common β-genus HPV types in skin samples and plucked hair bulbs revealed a viral load range between one DNA copy per 100 cells to more than 400 DNA copies per cell. These finding are comparable to those reported earlier by Orth *et al.* (1971, 1979) and Orth, (1980, 1986), but much higher than those observed in our previous study of a series of both premalignant and malignant lesions from immunocompetent individuals (Weissenborn *et al.*, 2005). Importantly, in eyebrow hair bulbs from all four patients, very high HPV loads were observed for some (for example, HPV types 5) but not all types. These high loads exceeded median loads observed in hair bulbs of

immunocompetent and immunosuppressed patients from a multicenter study by four orders of magnitude. In these groups, less than 5% of the patients had loads comparable to those of EV-patients (SJ Weissenborn *et al.*, unpublished). Despite the broad spectrum of β-genotypes found in the four patients, a high intrapatient concordance for specific types between hair bulbs and skin biopsies was observed. Worthy of note, the same β-PV signature was maintained over time, consistent with previous findings indicating that an individual becomes colonized with a particular profile of β-PV, probably from early infancy, which tends to persist (de Koning *et al.*, 2007; Cronin *et al.*, 2008). In patients 2, 3, and 4 the HPV type 5 load was dominant, and in patient 1, although other genotypes with high viral load were also present, HPV type 5 again displayed the highest load (416 copies per cell versus 270 for HPV type 20, 147 for HPV type 15, and 100 for HPV type 36).

It has been speculated that HPV replication, and presumably enhanced, gene expression may stimulate keratinocyte proliferation and contribute to carcinogenesis in the early stages of non-melanoma skin cancer development by inhibition of apoptosis in response to UV damage and binding of a protein required for repair of single-strand DNA breaks (Jackson and Storey, 2000; Iftner *et al.*, 2002; Weissenborn *et al.*, 2005). In line with this hypothesis, the increased risk for SCC development in EV patients might be due to a larger number of HPV-positive cells or higher viral copy numbers per cell associated with an increased concentration of viral oncoproteins, thus facilitating the accumulation of UV-induced mutations and oncogenic transformation in a larger fraction of cells.

There is increasing evidence that the clinical course of EV can be radically different. The benign form only presents with flat, wart-like lesions over the body, whereas the malignant form shows a higher rate of polymorphic skin lesions and development of multiple cutaneous tumors (Cortes-Franco *et al.*, 1997; de Oliveira *et al.*, 2003; Gul *et al.*, 2007). Accordingly, the clinical course of patient 2 versus patients 1, 3, and 4 was radically different because the latter had already developed several malignant and premalignant lesions early in life, whereas patient 2 had not even though she was almost 60. A possible explanation for this difference may be found in the lower multiplicity of genotypes reported in patient 2.

To localize viral HPV DNA, ISH was performed. Nests of cells with strong signals were scattered in the Bowenoid lesions from all patients and the SCC from patient 1, but not in patients 3 and 4. However, the absence of signals in other cells does not exclude the presence of a few viral copies escaping detection by this technique, which is estimated to be able to detect less than 10 copies per cell as deduced from ISH experiments in transgenic mouse tissues (Weissenborn *et al.*, 2005). On the other hand, these results indicate viral loads higher than those given in Table 1, and point to viral replication in ISH-positive cells. The presence of cell nests with active replication in the normal epithelium surrounding the tumor area might also explain the weak signal in the SCC lesion from patient 3 with HPV type 24, 14, and 19. As suggested by the high viral loads, more distant serial sections might have resulted in a different staining pattern. For both papular and wart-like lesions, HPV genomes were mainly detected in the nuclei of the superficial layers of the lesions, as expected for productive lesions.

Epidemiological studies in immunocompetent individuals revealed that the presence of β-genus HPV DNA, evaluated as the number of specific types in eyebrow hairs, was significantly associated with a history of SCC, but none of the HPV types found was predominantly associated with skin cancer (Boxman *et al.*, 1997; Struijk *et al.*, 2003; Bouwes Bavinck *et al.*, 2008). By contrast, in EV patients a predominant association of skin cancer with HPV type 5 was reported (Orth *et al.*, 1971, 1979; Orth, 1980, 1986). In our study, we found several β-genotypes in the skin cancers of EV patients (for example, 14, 19, 20, 24, and the genotypes HPV types 93 and 96) never or rarely reported before. Despite the presence of high viral loads in hair bulbs from all EV study patients, HPV type 5 was only detected in the skin cancers from patient 4. We can only speculate about possible reasons for not finding a predominant oncogenic role for HPV type 5. It may be related to different degree of UVB exposure, genetic background or simply the small cohort of patients. Worthy of note, only patient 4 carried a nonsense mutation in the EVER2 gene, together with a nonsynonymous single nucleotide polymorphism in the same gene (rs7208422) that was present in all four patients (Zavattaro *et al.*, 2008; E.Z. and M.G. unpublished observations).

Altogether, our results demonstrate that (1) in all four patients HPV type 5 is the predominant genotype in eyebrows; (2) β-PV carriage in both hair bulbs and skin biopsies was highly concordant with a broad spectrum of genotypes; and (3) patients infected with HPV types 5 or 8 may develop cancer not attributable to either of these HPV types.

In addition, this is the first report describing antibody response to a broad variety of HPV types in EV patients. Favre *et al.* (1998) had already noted that in EV patients, antibodies to HPV type 5, but not to HPV type 1 are elevated. We show here that the four EV patients, in comparison to age- and sex-matched control individuals, have significantly elevated antibody reaction to almost all of the 16 β-PV and to a lesser extent to the 9 γ-PV. The enhanced, genus-restricted antibody response is thought to reflect higher viral load of β-PV in the patients and is in line with the multiple β-PV detected by DNA analysis. For γ-PV, the slightly elevated antibody response is not matched by positive DNA findings, which might be due to lower sensitivity of the γ-PV PCR used here, or to only localized γ-PV infections in contrast to the ubiquity of β-PV. It cannot be excluded that the strong reactivity to β-PV might be accompanied by some cross-reactivity to other HPV, however, among the control sera significant single reactions with one HPV type only are present whereas closely as well as distantly related HPV types showed no reaction (see Table S1). The serological results thus reiterate the restriction of the HPV replication control defect to a specific genus in EV patients. The high antibody response also suggests that the EV patients do not have a

defect in mounting an effective antibody response even though the virus is not cleared.

Although interpretation of the study results is limited by the small number of EV patients, several aspects of the virological data obtained in this study deserve to be stressed: (1) eyebrows from EV patients contain more HPV types than those from the normal population, and they may define the β-PV profile of these patients; (2) a predominantly high viral load of HPV type 5 in the eyebrows can be detected as a representative marker of the disease; (3) high viral loads are maintained in skin proliferative lesions; and (4) HPV seroreactivity is strongly elevated to almost all β-PV.

MATERIALS AND METHODS

Patients

Four unrelated Caucasian patients with EV were included in this study. Patients 1 and 2 were previously reported (Azzimonti *et al.*, 2005; Zavattaro *et al.,* 2008). The third EV patient was a 39-year-old man. Diagnosis of EV was made when he was 31 based on the histological examination of two crusty and erythematous lesions resected from the forehead that were diagnosed as microinvasive SCC and an intraepithelial Bowenoid-type neoplasia, respectively. He was the only person with EV in his family, and he was not the result of a consanguineous marriage. The fourth EV patient was a 60-year-old man attending the ''San Gallicano'' Dermatologic Institute, IRCCS-Rome. He was the only person with EV in his family, and he was the result of a consanguineous marriage. Since the age of 42, he underwent resection of several lesions from the forehead and back that were diagnosed as either premalignant or malignant (see Table 1). Medical history of both patients 3 and 4 was significant because of the presence since childhood of numerous flat and papular lesions resembling verruca plana on his forearms, chest, neck, upper limbs, and forehead, and whitish pityriasis versicolor-like lesions on his back. All four patients were HIV negative.

Written informed consent was obtained from the patients, and ethical approval for this study was granted by the ''Maggiore Hospital'' Research Ethics Committee, Novara. The present study was conducted according to the Declaration of Helsinki principles.

HPV DNA detection

For hair samples 8–10 hairs were plucked from eyebrows, 4–5 from each side. Only hairs that contained hair follicles were collected, snap frozen, and stored at −70 °C until analysis. DNA was isolated from hairs using a QIAamp DNA Mini Kit and eluted in 70 μl AE buffer (Qiagen, Hilden, Germany). DNA extraction from formalin-fixed paraffin-embedded skin biopsies was performed by using the QIAamp Tissue Kit (Qiagen), according to the manufacturer's instruction. All specimens were examined by β-globin PCR to estimate the quantity of DNA and to control its quality. HPV DNA analysis for the β-genus was performed by using a PM-PCR in combination with a reverse hybridization system (Skin (β) HPV assay; Diassay BV, Rijswijk, The Netherlands; de Koning *et al.*, 2006) following the manufacturer's instructions. The test comprises the PM-PCR, generating a biotinylated amplimer of 117 bp from the E1 region, and a reverse hybridization assay, able to simultaneously identify 25 β-PV types. This test has an analytical sensitivity of 10–100 viral genomes. Negative controls (water or human placental DNA) and positive controls (1000 copies HPV type 8 plasmid) were included in each analysis.

In addition, the single round primer pair C4F-C4R was used to detect genus γ-PV (HPV 4, 48, 50, 60, and 65) as previously reported (Harwood *et al.*, 1999).

Quantitative Real-Time PCR

Type-specific Q-PCR protocols for HPV types 5, 8, 15, 20, 23, 24, 36, 38, 14, 19, 93, and 96 were performed on the LightCycler system (Roche Diagnostics, Mannheim, Germany), as previously described (Wieland *et al.*, 2000; Weissenborn *et al.*, 2003; primer and probe sequences for HPV types 14, 19, 23, and 38 are available on request from SW). Primer- and probe-sequences for the quantification of HPV types 93 and 96 were taken from Vasiljević *et al.* (2007) and protocols were adapted to the LightCycler system. HPV DNA copy numbers were determined by using standard curves, generated in the same PCR run with HPV plasmid dilutions ranging from 5 to 10^6 copies per sample in a human placental DNA solution (4 ng μl^{-1}). Analytical sensitivity was 5–50 HPV DNA copies per reaction when duplicate testing was performed. 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 Diagnostics; Weissenborn *et al.*, 2003). In each PCR run, 5 μl of EV patient DNA and 2 μl of plasmid DNA were employed, respectively.

In situ hybridization

ISH was performed on formalin-fixed and paraffin-embedded skin biopsies, applying the mild ISH protocol described in Hopman *et al.*, 2005. Briefly, tissue sections were dewaxed and digested with 8 mg ml^{-1} pepsin (Sigma Chemical Co., St Louis, MO) in 0.2 M HCl for 10 minutes at 37 °C. Complete digoxigenin-labeled (DIG-Nick Translation Mix; Roche Diagnostics GmbH, Mannheim, Germany) genomic HPV plasmid DNA was used as the probe (100 ng ml^{-1}). Probe and target DNA were denatured simultaneously for 6 minutes at 90 °C prior to hybridization. HPV probes were detected using the tyramide signal amplification procedure, according to manufacturer's instructions (PerkinElmer Life and Analytical Sciences Inc., Shelton, CT).

Measurement of HPV antibodies

Frozen serum samples were shipped on dry ice to Dr Pawlita's laboratory at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. Sera from 36 male and 18 female participants (all aged within 5 years of the EV patients) of a German nutrition study (Verzehr, Ernährung, and Risikoanalyse VERA) were used as controls (Anders *et al.*, 1990). Cutaneous HPV antibodies were measured from all samples in one batch. Sera were tested for antibodies to the major capsid protein L1 of genus α-PV (HPV types 2, 3, 7, 10, 13, 16, 18, 27, 57, and 77), genus β-PV (HPV types 5, 8, 9, 15, 17, 20, 23, 24, 36, 38, 49, 75, 76, 92, 96, and 107), genus γ-PV (HPV types 4, 48, 50, 60, 65, 88, 95, 101 and 103), the genus μ-PV types 1 and 63 and the genus ν-PV type 41.

The antibody detection method was based on glutathione S-transferase (GST) capture as described in Sehr *et al.* (2001, 2002) in combination with fluorescent bead technology (Luminex) as recently described (Waterboer *et al.*, 2005, 2006). Detailed

monoclonal antibody analysis with mucosal HPV has shown that GST-L1 proteins like HPV L1 virus-like particles display conformational type-specific as well as cross-reactive epitopes (Rizk *et al.*, 2008). Generation and performance of the cutaneous GST-L1 fusion proteins in serological assays has been described in detail (Michael *et al.*, 2008). Briefly, full-length viral proteins that were fused with a N-terminal GST domain were expressed in bacteria. Glutathione cross-linked to casein was coupled to fluorescently labeled polystyrene beads, and GST fusion proteins were directly affinity purified on the beads in a one-step procedure. Bead sets of different color and carrying different antigens were mixed and incubated with human sera. Antibody bound to the beads via the viral antigens was stained by biotinylated anti-human-Ig and streptavidin-R-phycoerythrin. Beads were analyzed in a Luminex analyzer that identifies the bead color—and thus the antigen carried by the bead—and quantifies the antibody bound to viral antigen via the median R-phycoerythrin fluorescence intensity (MFI) of at least 100 beads of the same internal color.

Statistical analysis

Statistical analysis of differences in seroprevalence were performed by χ^2-tests. The Fisher's exact test was used when frequencies were smaller than or equal to five. Comparisons of seroreactivity (MFI values) were performed by the Wilcoxon rank sum test. All tests were two-sided, and *P*-values below 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Table S1. Serum antibody reactivity in EV patients ($n = 4$) and controls ($n = 54$) to 38 HPV types and 3 control antigens.

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