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

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Abstract

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.

Abbreviations: EV, epidermodysplasia verruciformis; GST, glutathione S-transferase; HPV, human papillomavirus; ISH, in situ hybridization; PV, papillomavirus; Q-PCR, quantitative real-time PCR; SCC, squamous-cell carcinoma

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 extrachromosomally 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.

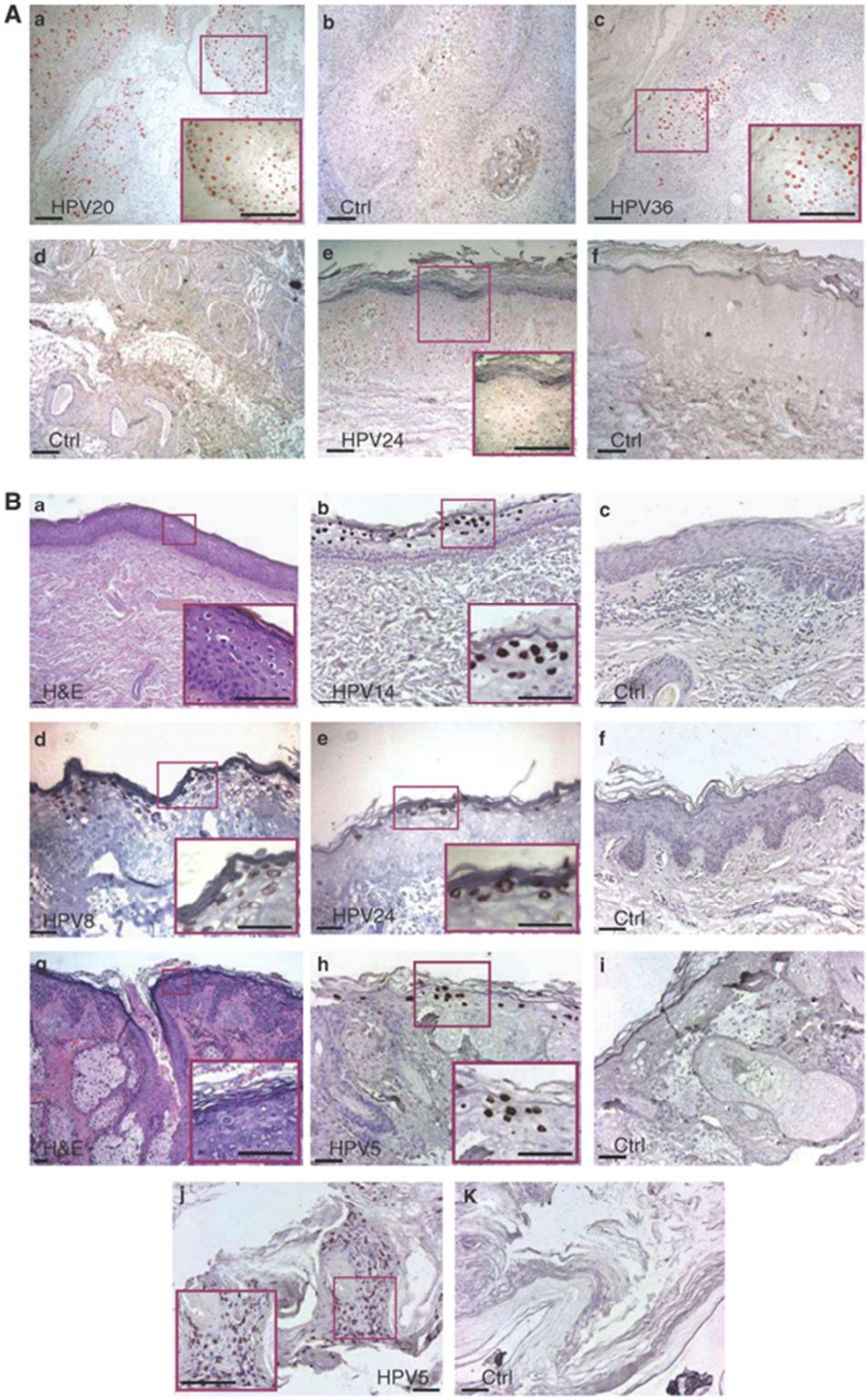

Figure 1. Human papillomavirus (HPV)-DNA detection in formalin-fixed paraffin-embedded skin biopsies by in situ hybridization (ISH). HPV-DNA positive nuclei appear red (AEC staining; scale bar=250 mum). HPV type-specific DNA probes were used. (A) Patient 1. SCC: DNA-probe HPV type 20 (a), and Ctrl* (b); 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.

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.

Table 1. Detection of DNA of cutaneous bold beta-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[1]	Viral load	
			HPV type	Copies/cell
1	Wart-like lesion (forearm) 2003	**5**[‡], **14, 15, 20,** 24, 36	5	<0.01
			14	<0.01
M/40[§]			15	<0.01
			20	<0.01
			24	<0.01
			36	<0.01
	SCC (left temporal region) 2003	**20**	na*	na*
	AK (elbow) 2003	5, 15, 20, 24, 36	5	<0.01
			15	<0.01
			20	<0.01
			24	<0.01
			36	38
	Bowenoid lesion	5, 15, 20, 24, **36**	5	<0.01

	(forehead) 2003		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	8, 24, 37	8	472
			24	132

	(forearm) 1999			
	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**[§]	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
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

‡ Normal font numbers: HPV identified by PM-PCR only; Bold numbers: HPV identified previously by CP(62/69)-PCR

§ Consanguinity

* na: not available

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 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 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).

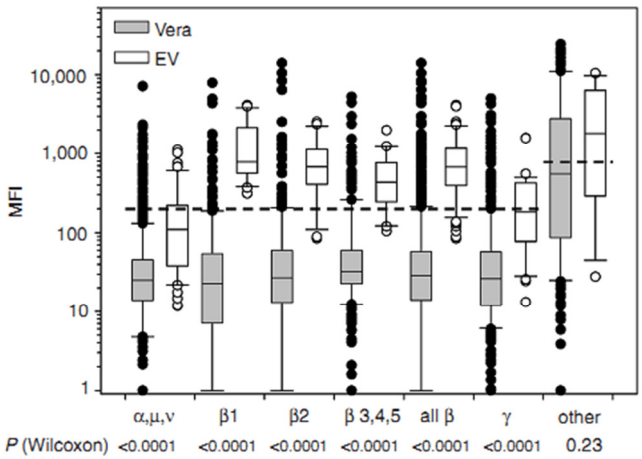

Figure 2: HPV serum antibody reactivity in EV patients (n=4) and controls (n=54). The box plots show the distribution of serum antibody reactivities measured by HPV multiplex serology and expressed as median fluorescence intensity (MFI, logarithmic scale). Sera from 4 EV patients (EV) and 54 age-matched 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.

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 106 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.

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Table S_1. Serum antibody reactivity in EV patients (n=4) and controls (n=54) to 38 HPV types and 3 control antigens.

			genus	μ	μ	ν	α	α	α	α	α	α	α	α	α	α	β	β	β	β	β	β	β	β	β	β	β	β	β	β	γ	γ	γ	γ	γ	γ	γ	γ	γ	control antigens		
			species	1	2	1	2	2	2	4	4	4	8	10	9	7	1	1	1	1	1	2	2	2	2	2	2	3	3	3	4	5	1	1	1	2	3	4	5		*H.pylori*	JCV
			type	**1**	**63**	**41**	**3**	**10**	**77**	**2**	**27**	**57**	**7**	**13**	**16**	**18**	**5**	**8**	**20**	**24**	**36**	**9**	**15**	**17**	**23**	**38**	**107**	**49**	**75**	**76**	**92**	**96**	**4**	**65**	**95**	**48**	**50**	**60**	**88**	**101**	**103**	**CagA**	**OMP**	**VP1**
panel	**serum ID**	**age**	**sex**																																							
VERA	1	34	M	77	26	38	27	38	9	22	11	40	24	37	44	49	293	29	17	9	14	25	62	-8	14	8	33	16	24	33	32	43	13	38	40	7	26	8	6	80	25	399	9	687
VERA	2	34	M	153	15	18	31	30	14	20	36	72	8	48	-2	8	72	36	-53	4	-4	53	17	-32	6	-33	100	11	28	41	26	38	11	68	33	13	8	19	8	71	26	18662	3595	1251
VERA	3	34	M	52	15	28	24	31	43	18	31	153	24	20	25	28	31	16	15	14	15	25	13	22	27	39	13	17	23	28	22	21	210	15	35	24	35	10	18	24	13	44	8	728
VERA	4	35	M	5	6	-3	41	22	14	4	9	62	21	20	-2	35	48	38	-29	44	5	49	23	152	28	8	70	19	34	31	24	47	139	34	46	64	4	12	3	27	10	10796	7632	2472
VERA	5	35	M	6	24	62	17	16	12	16	18	40	15	19	16	38	32	18	1	12	10	20	15	3	15	17	29	19	25	28	20	32	384	43	44	34	25	8	14	54	16	163	563	136
VERA	6	35	M	6	13	4	8	17	9	24	24	27	28	128	10	16	25	15	-1	16	33	28	21	0	18	13	22	28	27	34	40	28	6	36	34	10	12	7	9	47	26	49	9	42
VERA	7	36	M	10	71	9	149	44	19	29	22	75	39	33	59	19	52	24	-22	22	7	32	27	-1	25	-3	63	27	35	41	29	55	8	39	44	13	22	20	21	78	144	138	232	209
VERA	8	36	M	21	-10	-63	29	22	-15	274	-50	149	-16	-26	-38	-71	80	22	-140	-35	-49	9	-23	-102	-47	-100	309	50	2	15	2	-7	-4	2	6	-9	32	333	-27	53	8	20121	3319	1559
VERA	9	37	M	13	23	18	26	41	10	11	16	52	31	28	29	15	54	35	-7	12	10	39	40	9	18	6	32	28	54	41	29	37	11	33	43	19	7	12	15	45	47	17	20	40
VERA	10	37	M	11	52	10	30	31	39	31	43	35	42	75	246	10	62	35	-16	8	13	42	40	26	23	2	35	17	49	27	197	107	13	26	36	55	44	9	22	59	42	76	34	420
VERA	11	37	M	5	38	13	14	38	15	19	28	28	30	24	46	19	26	68	0	12	10	21	14	4	14	8	20	26	37	32	17	45	756	156	37	35	16	6	12	121	118	132	274	20
VERA	12	37	M	423	440	2174	283	284	952	358	593	168	670	1587	96	1427	1377	1742	837	217	285	274	241	688	1333	859	241	896	529	440	1007	474	577	543	541	1935	831	264	1218	404	500	318	218	1190
VERA	13	38	M	23	16	14	23	24	7	14	12	19	27	29	26	25	42	36	37	30	27	21	25	9	18	27	79	25	75	32	26	138	19	48	62	57	11	7	15	66	24	53	151	372
VERA	14	38	M	410	90	108	23	48	19	32	42	50	36	48	29	34	62	346	33	70	16	35	31	26	20	41	30	32	46	61	41	62	11	76	50	30	32	12	17	80	27	57	9	33
VERA	15	38	M	8	10	10	47	34	35	162	169	128	22	51	25	18	28	19	1	12	10	86	119	80	95	137	86	101	214	171	116	32	59	22	40	12	11	5	7	49	18	1245	2631	27
VERA	16	39	M	58	38	28	18	31	12	21	33	26	18	30	856	106	51	62	108	15	33	39	27	39	30	30	25	30	30	24	30	48	100	25	36	17	22	8	14	25	13	1178	12	1031
VERA	17	40	M	9	27	18	121	36	28	31	25	28	30	35	33	23	51	23	8	20	17	28	32	10	18	74	25	24	33	33	29	46	20	39	44	29	30	9	13	94	33	412	55	24
VERA	18	40	M	1191	548	28	29	44	26	23	22	50	42	33	38	41	75	62	29	22	53	129	190	31	164	359	52	355	191	94	133	475	37	216	512	281	26	14	25	198	51	86	24	144
VERA	19	41	M	7	16	8	12	13	6	13	14	23	18	19	10	16	25	12	-1	13	6	52	37	11	17	16	27	12	20	19	32	80	5	19	20	11	8	4	8	45	14	273	6	99
VERA	20	42	M	2	7	14	16	37	6	18	10	12	10	19	15	20	15	18	-7	7	7	42	12	3	20	8	18	9	20	21	12	24	4	17	17	5	5	6	4	28	19	29	40	417
VERA	21	44	M	61	49	0	132	71	6	29	0	122	33	8	14	-5	81	103	-65	2	5	50	13	11	15	-8	143	38	43	35	19	41	26	52	37	18	12	27	2	75	29	16579	3141	89
VERA	22	44	M	917	67	209	29	52	-1	25	-18	118	18	8	-1	-24	87	88	361	20	-5	61	17	-28	23	25	180	-4	20	32	23	47	25	34	43	27	6	19	-2	75	3114	14769	14192	1177
VERA	23	44	M	15	68	-14	38	53	33	28	0	94	14	40	19	18	133	161	66	64	-12	261	364	1	46	196	111	428	81	168	601	32	175	121	127	9	2	12	0	125	30	16472	3272	1861
VERA	24	45	M	16	187	12	23	34	33	11	14	23	14	49	35	13	40	465	43	13	21	19	14	11	24	14	19	23	28	21	43	33	50	17	38	38	29	11	13	28	22	37	2575	857
VERA	25	58	M	29	7197	9	11	31	11	20	14	54	28	51	136	27	94	50	94	59	58	79	71	75	44	72	71	67	63	92	70	105	61	64	95	53	55	6	9	39	22	5727	3343	1331
VERA	26	58	M	7	26	24	15	143	106	16	15	25	35	41	46	10	35	28	30	15	9	28	22	17	21	8	29	17	32	30	32	42	204	74	62	58	27	12	17	57	22	57	29	22
VERA	27	59	M	31	15	-14	42	71	18	30	28	82	87	38	18	10	94	43	-75	1	3	54	36	-30	-4	-45	80	28	48	38	22	55	22	77	43	73	36	12	22	83	37	792	284	1487
VERA	28	59	M	144	13	2	15	17	-1	13	-5	643	14	7	6	-8	25	13	-7	9	-3	13	11	23	5	-4	61	1	6	6	9	13	1	19	14	3	5	7	0	32	15	13568	2174	1624
VERA	29	59	M	100	35	4	22	86	243	11	9	47	15	7	100	5	44	25	-19	11	9	41	15	10	49	33	60	29	40	58	24	65	73	133	151	201	91	13	10	31	29	8092	538	696
VERA	30	60	M	12	36	42	14	19	6	15	11	15	2343	24	25	23	33	24	-1	7	22	23	13	174	11	21	14	24	20	19	21	39	160	18	36	15	20	6	11	40	26	764	121	87
VERA	31	60	M	124	35	-32	22	115	140	5	-38	117	-16	-23	9	-53	73	27	-64	-19	-20	45	-6	26	-26	-48	154	565	78	261	13	12	428	49	32	110	-10	20	-17	51	0	24387	2220	5547
VERA	32	60	M	29	24	14	24	34	5	14	-12	59	13	6	12	-9	94	44	1	-2	52	31	41	49	-3	46	82	28	19	40	23	35	14	18	13	6	2	8	-3	67	13	11583	10209	2650
VERA	33	61	M	20	28	12	21	39	19	20	22	25	38	27	31	28	36	29	2	12	15	28	32	42	31	17	18	26	25	48	23	106	10	38	60	24	29	12	11	58	30	1563	4	231
VERA	34	61	M	42	11	12	36	39	46	9	-5	59	10	14	-1	-9	64	235	-53	7	20	15	5	-23	-5	-14	63	4	38	62	12	22	8	18	23	11	1	7	-3	37	12	6065	2282	500
VERA	35	62	M	27	45	14	53	60	60	61	26	55	26	56	62	33	54	31	33	40	23	41	45	62	34	74	48	40	54	41	84	106	48	105	87	61	59	14	27	61	44	1095	2800	120
VERA	36	62	M	2	10	0	14	18	9	11	38	29	12	92	25	8	21	8	-9	-1	1	17	15	-6	8	-15	23	8	26	21	11	62	1	12	26	9	8	4	11	30	34	32	1	181
VERA	37	56	F	257	323	9	30	49	20	29	28	57	23	75	36	30	189	330	480	278	109	217	193	143	170	384	88	555	209	121	1549	1194	1008	1143	904	539	40	10	16	50	43	128	5433	164
VERA	38	57	F	7	8	207	43	61	192	43	94	42	16	23	93	0	181	805	-37	8	-3	34	24	-33	27	180	62	7	23	36	113	40	6	32	19	6	23	5	-2	43	21	9288	6239	1889
VERA	39	57	F	26	8	-22	25	28	-8	15	-15	85	4	36	-7	-18	45	32	-63	-9	-8	26	7	-31	-3	-20	96	-9	7	19	8	25	8	17	20	-5	-1	12	3	38	16	19211	4392	50
VERA	40	57	F	40	23	2	235	41	-1	21	-2	89	13	7	-6	-14	101	672	12	225	41	1142	885	1646	75	1514	382	812	754	260	513	219	69	245	39	246	11	16	5	83	37	19419	4178	246
VERA	41	57	F	288	331	1764	244	262	704	244	520	151	542	1465	45	1156	1107	1797	583	134	214	213	270	562	930	697	273	589	374	350	768	416	477	368	469	1695	533	186	888	371	464	215	855	16
VERA	42	58	F	360	19	19	31	41	19	32	60	83	15	64	16	16	77	37	-27	4	5	13	17	1	15	303	66	21	27	26	34	28	27	28	34	1538	19	11	10	120	16	8743	13589	368
VERA	43	59	F	14	93	10	36	24	17	16	13	25	22	46	20	11	55	25	1	8	18	37	19	5	57	80	34	15	65	139	46	43	6	52	41	10	10	8	18	37	23	1038	13403	2443
VERA	44	59	F	14	16	18	14	17	12	16	15	25	22	31	26	21	201	22	7	75	20	15	21	18	15	17	13	15	20	16	31	31	20	24	26	14	15	6	7	32	69	56	646	35
VERA	45	59	F	224	24	30	31	53	3	66	-11	123	50	466	16	-39	120	70	0	-8	36	53	32	-55	5	-51	233	-1	22	59	4	104	26	80	47	24	17	17	7	150	86	11171	9052	1262
VERA	46	60	F	60	566	121	83	32	20	58	43	74	2325	273	70	178	1235	4981	7939	4379	1250	8399	6451	14034	2544	10523	1272	5303	2388	1006	4471	2993	2847	4333	4301	1387	5035	214	16	2542	77	99	7161	446
VERA	47	61	F	12	36	22	23	42	25	75	28	28	29	36	28	40	38	23	29	41	23	36	65	20	44	30	25	49	65	55	55	53	31	78	82	33	39	11	19	99	63	285	37	93
VERA	48	61	F	24	40	63	15	36	29	37	29	1224	47	57	69	21	81	33	-2	17	40	36	34	5	32	173	30	22	66	104	59	82	84	216	166	107	59	12	31	97	103	183	1666	115
VERA	49	63	F	19	4	-24	24	25	10	16	-7	91	11	11	3	7	54	20	-63	-4	-3	29	19	-8	-9	2	119	11	41	40	22	40	6	49	27	12	19	18	19	62	42	8841	1505	86
VERA	50	63	F	241	47	53	12	23	9	17	29	28	18	20	21	25	31	19	17	15	32	45	26	52	20	33	17	43	26	27	28	28	200	218	36	17	25	7	12	285	54	70	13	399
VERA	51	63	F	27	143	30	32	45	35	66	25	45	39	58	31	35	60	45	-13	18	13	58	44	11	35	17	41	43	41	38	52	51	25	40	60	27	16	16	26	51	25	3343	796	1622
VERA	52	64	F	20	10	7	93	322	457	36	23	37	18	60	83	14	22	23	-2	19	7	15	13	0	41	6	35	14	23	37	13	29	552	335	160	26	15	9	11	35	18	2797	985	28
VERA	53	64	F	15	33	0	66	120	52	42	152	110	25	135	723	149	55	29	-42	18	6	29	10	-1	54	8	102	20	52	49	17	29	30	61	54	22	11	24	18	62	36	13385	9160	1783
VERA	54	65	F	20	25	-22	41	65	-1	19	-35	151	-8	-21	-1	-37	125	-41	-105	-8	-23	35	-13	-86	-37	-36	257	-14	12	30	12	26	17	7	27	-4	-10	29	1	216	12	21304	2185	172
			mean	106	205	95	47	57	67	43	41	98	130	103	62	65	135	240	183	111	46	228	181	327	114	292	104	196	117	86	198	149	169	179	169	168	138	29	49	128	108	5470	2728	752
			median	24	27	14	28	38	18	21	20	54	23	34	26	18	55	32	0	13	13	36	25	10	21	17	61	25	34	37	29	43	26	41	40	24	19	11	11	58	27	915	920	370
			pos (n)	9	6	4	3	3	4	3	2	2	4	4	3	2	5	9	5	4	3	6	5	4	3	7	7	8	6	5	6	6	11	9	5	8	3	3	2	5	3	27	28	18
			pos (%)	17	11	7	6	6	7	6	4	4	7	7	6	4	9	17	9	7	6	11	9	7	6	13	13	15	11	9	11	11	20	17	9	15	6	6	4	9	6	50	52	33
EV	1	40	M	36	187	115	71	173	42	97	206	69	205	387	189	179	794	531	2028	689	370	901	461	563	457	801	89	105	317	211	137	442	179	319	464	184	100	24	29	189	50	7888	6621	403
EV	2	60	F	37	35	92	680	129	78	105	165	58	99	107	407	124	2171	1164	473	1209	660	1157	1016	2109	1245	748	130	965	120	343	1242	1124	181	425	229	1576	431	70	46	361	100	540	85	5565
EV	3	39	M	453	1113	22	12	18	23	22	15	12	30	27	43	45	558	4163	759	2059	313	390	477	370	2383	613	84	774	703	398	444	220	101	125	125	325	142	26	13	70	56	252	28	3113
EV	4	60	M	182	225	1018	125	112	407	225	307	90	312	754	29	675	2370	2351	3994	574	772	859	926	2557	593	1542	172	647	428	355	724	1996	443	552	476	1591	408	119	476	188	424	1780	10499	1863
			mean	177	390	312	222	108	137	172	173	57	162	319	167	256	1473	2052	1813	1133	529	827	720	1400	1170	926	119	623	392	327	637	945	226	355	323	919	270	60	141	202	158	2615	4308	2736
			median	110	206	104	98	120	60	101	185	63	152	247	116	151	1483	1758	1393	949	515	880	702	1336	919	774	110	710	372	349	584	783	180	372	347	951	275	48	38	188	78	1160	3353	2488
			pos (n)	1	2	1	1	0	1	1	2	0	2	2	1	1	4	4	4	4	4	4	4	4	4	4	0	3	3	4	3	4	1	3	3	3	2	0	1	1	1	2	2	3
			pos (%)	25	50	25	25	0	25	25	50	0	50	50	25	25	100	100	100	100	100	100	100	100	100	100	0	75	75	100	75	100	25	75	75	75	50	0	25	25	25	50	50	75
			means	1,7	1,9	3,3	4,7	1,9	2,1	2,6	4,3	0,6	1,2	3,1	2,7	3,9	10,9	8,6	9,9	10,2	11,5	3,6	4,0	4,3	10,3	3,2	1,1	3,2	3,3	3,8	3,2	6,3	1,3	2,0	1,9	5,5	2,0	2,1	2,9	1,6	1,5	0,5	1,6	3,6
ratio (EV/VERA)			medians	4,6	7,7	7,6	3,5	3,2	3,4	4,8	9,4	1,2	6,7	7,2	4,5	8,4	26,8	54,4	np	73,5	40,2	24,4	28,2	131,4	44,8	44,3	1,8	28,7	10,9	9,3	20,1	18,4	6,9	9,0	8,6	38,9	14,6	4,3	3,4	3,2	2,9	1,3	3,6	6,7
			pos (%)	1,5	4,5	3,4	4,5	0,0	3,4	4,5	13,5	0,0	6,8	6,8	4,5	6,8	10,8	6,0	10,8	13,5	18,0	9,0	10,8	13,5	18,0	7,7	0,0	5,1	6,8	10,8	6,8	9,0	1,2	4,5	8,1	5,1	9,0	0,0	6,8	2,7	4,5	1,0	1,0	2,3

color code for MFI, means, medians: 200-400, 401-800, >800
color code for ratios of means, medians, pos (%): 2.0-3.0, 3.1-4.0, >4.0
np, not possible