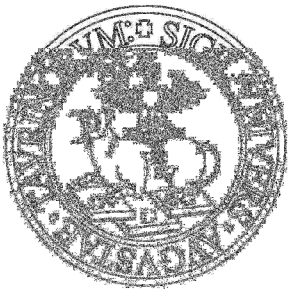
**Lack of EVER2 Protein in Two Epidermodysplasia Verruciformis Patients with Skin Cancer Presenting Previously Unreported Homozygous Genetic Deletions in the EVER2 Gene**



UNIVERSITÀ DEGLI STUDI DI TORINO

**Lack of EVER2 Protein in two Epidermodysplasia Verruciformis patients with Skin Cancer Presenting Novel Homozygous Genetic Deletions in the EVER2 Gene.**

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**Short Title:** Genetic and viral profile of two EV patients

**Abbreviations:** EV, Epidermodysplasia Verruciformis; HPV, human papillomavirus; NMSC, non-melanoma skin cancer.

**TO THE EDITOR**

Epidermodysplasia Verruciformis (EV) is a rare, lifelong, autosomal recessive skin disease (OMIM number 226400) associated with an unusual susceptibility to infections with ubiquitous beta human papillomaviruses (β-HPV), but not to infections with other pathogens, including cutaneous and genital HPVs of the alpha, gamma, mu, or nu genera (Jablonska and Majewski, 1994; Orth, 2006).

Beta-HPVs are evolutionarily distinct from the other HPV genera and are associated with widespread in-apparent or asymptomatic infections in the general population (Bernard *et al.*, 2010; Bravo *et al.*, 2010). In immunosuppressed individuals, however, and in individuals suffering from the rare inherited disease EV, these viruses can replicate unchecked and have been implicated in the development of non-melanoma skin cancer (NMSC) (Akgul et al., 2006; Bouwes Bavinck *et al.*, 2011; Proby *et al.*, 2011).

The sensitivity of EV patients to β-HPVs has been linked to homozygous mutations in two genes (EVER1 and EVER2) ( Ramoz *et al.*, 2002). The mutation of either of the EVER genes holds the potential to alleviate EVER-mediated host restriction and favor β-HPV replication and skin carcinogenesis, as is the case in EV patients (Lazarczyk *et al.*, 2009).

In this report, we describe the presence of two novel homozygous mutations in the EVER2 gene in two Italian EV patients, each of whom have already developed more than ten NMSC.

EV patient 4 (EVpt4) has been previously described by our group (Dell'Oste *et al.*, 2009) and his clinical picture is presented in Figure 1a. EV patient 5 (EVpt5) is a 58-year-old male who was referred to our hospital with a diagnosis of EV in 2009. Since the age of 42, he has had 12 tumours resectioned from his face (forehead) (Figure 1b) and back. HPV14 was consistently found in all lesions (CB and MG, manuscript in preparation). HPV DNA analysis of plucked eyebrow hairs revealed the presence of multiple HPV genotypes of the β-genus, with HPV5 and HPV14 viral loads being the highest (Figure 1a and b, below the forehead images). Both patients are HIV-negative. As shown in the representative Bowen's disease tumor sections in Figure 1c and d, the unequivocal

histological features of EV (defined by acanthosis containing a majority of cells that show perinuclear halos and blue-gray pallor) are present in both patients' tumors.

Genomic DNA extracted from blood samples of both patients was used to perform genetic analysis of the EVER1 and EVER2 genes (see Supplementary information). In these patients, no mutations were detected in the EVER1 gene, while they harbor novel invalidating deletions in the EVER2 gene which create shifts in the reading frame and introduce a premature termination codon (PTC) as shown in Figure 1e and f. Neither of these frameshift mutations had previously been reported in EV. We confirmed the lack of the EVER2 protein by Western blot analysis. As shown in Figure 1h, lysates obtained from keratinocytes isolated from the patients lacked a protein of about 85 kDa, the molecular weight (mw) expected for the EVER2 protein, which was present in keratinocytes obtained from healthy donors. No extra bands with lower mw were detected in EV patients' extracts indicating that production of the predicted truncated proteins (Figure 1g) did not occur as a consequence of PTC introduction but rather transcripts are eliminated by nonsense-mediated RNA degradation (Bhuvanagiri et al, 2010).

EVpt4 has consanguineous parents (they were first cousins). He is married to a healthy, unrelated woman, has one son and one daughter who are both unaffected, and a brother who is unaffected. To verify the presence of the 13 nucleotide deletion detected in EVpt4, the genomic DNA extracted from blood samples of all of his family members was amplified by exon 4 specific primers and analyzed by agarose gel electrophoresis with high resolution. As shown in Figure 2, both parents carried the 13 nucleotide deletion in the heterozygous state, but not inherited by the other sibling. In contrast, both the proband's son and daughter inherited the mutated allele in the heterozygous state.
HPV-DNA genotyping and determination of DNA copy numbers by type-specific Q-PCR protocols were performed with DNA extracted from the plucked eyebrows of all family members (see Supplementary information). Some of the genotypes present in the proband were consistently found in all family members, including HPV5 and 20. Of note, the HPV5 viral load in his mother was 15 DNA copies/cell. This finding is consistent with the predominant high viral load for HPV5 reported

in all 5 EV patients enrolled by our group (Azzimonti *et al.*, 2005; Zavattaro *et al.*, 2008, Dell'Oste et al., 2009) and suggests that even in the heterozygous genetic background HPV5 replication may be favored by a reduction in EVER2 protein availability. HPV5 and 20 are highly persistent in this family, despite the fact that the family members are of very different ages and live in different houses. The presence of HPV5 with a viral load of 4 DNA copies/cell in his wife suggests that close and frequent skin contacts with an individual carrying high viral loads for a certain genotype can facilitate virus transmission and sharing of such specific genotype between family members.

This report describes, for the first time, a correlation between genetic status and β-HPV carriage in EV family members. Collectively, our findings strengthen the hypothesis that the EVER2 gene plays a crucial role in the development of EV and in susceptibility to specific β-HPV genotypes. The detection of HPV5 and 20, both of which belong to the β1-species, in all family members confirms that infection by these subsets of β-genotypes is favored by the EV genetic background and passed between individuals via skin contact (Weissenborn *et al.*, 2009).

Following the pioneering work by Ramoz and colleagues in 2002, which showed frameshift mutations in the EVER2 gene in an Algerian/Colombian consanguineous EV family, this is the second report to demonstrate an EVER2 autosomal recessive trait in EV. Another three studies have described novel nonsense mutations in the EVER2 gene: a Chinese EV patient born from consanguineous parents (Sun *et al.*, 2005), three siblings from a Brazilian family (Rady *et al.*, 2007), and a Hispanic man (Berthelot *et al.*, 2007). Here, we demonstrate two novel deletions, in exons 4 and 6 respectively, in two Italian EV patients. In one patient, we also show that the EVER2 deletion was carried in the heterozygous state by his parents and inherited in an heterozygous manner by his children.

This study provides additional evidence indicating that the lack of the EVER2 protein favors the infection and persistence of specific β1-genotypes, in particular, HPV5, which is often the type presenting the highest viral loads in EV hair bulbs. In addition, the findings of this study strengthen

the notion that EVER2 is a key host factor in controlling β-HPV infection and virus-induced skin carcinogenesis in EV patients.

## Conflict of Interest

The authors state no conflict of interest.


## Acknowledgments

We thank the EV patients, their family members for their co-operation, and Mandar Bawadekar for his comments on the manuscript. E. Z. is funded by Regione Piemonte (LR 4/2006- DR 225/2009). This study was supported by MIUR (PRIN 2008: S. L. and M. G.; FIRB - Futuro in Ricerca 2008: M. DA), and Fondazione Banca Popolare di Novara BPN (M. G). C. B. is on sabbatical at the NIMR in London on a fellowship from FEMS.

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**Figure legends**

**Figure 1. Clinical, histological, virologic, and genetic findings from the EV study patients.** Forehead regions of EVpt4 (**a**) and EVpt5 (**b**) showing erythematous and hyperkeratotic plaques. Biopsy specimens from Bowen's disease lesions (**c** EVpt4 and **d** EVpt5) demonstrate typical histological features of EV. Scale bars, 100 μm. HPV genotyping in eyebrow hair bulbs is reported for each patient below the forehead images. The viral load values, expressed as DNA copy number/cell, are shown in brackets. na: not available. (**e**) The automated sequencing of the PCR fragments derived from the EVER2 gene revealed a 13 nucleotide deletion g.6609_6621delACTTCACCTTCCT in exon 4 in EV-pt4. The deletion is predicted to change the reading frame and result in a premature stop codon (PTC) after 9 amino acids (p.Y109fsX118). (**f**) In patient 5, a single nucleotide deletion was detected in exon 6 of EVER2 (g.7668delG) which is predicted to change the reading frame and result in a PTC in exon 7 (p.V191fsX226). **(g)** Schematic representation of the WT EVER2 gene with indication of the mutations detected in the two study patients. The encoded WT protein along with the variant proteins as predicted by computational analysis are also shown. Green boxes represent the altered protein encoded after deletion, which was interrupted downstream by the predicted PTC. **(h)** Lysates (30 μg) harvested from EV-derived keratinocytes and healthy donors (Ctrl) were analyzed by Western blot for levels of the EVER2 protein. Antibodies were generated against the first 80 amino acids of EVER2 as described in the Supplementary information. β-actin served as a loading control.

**Figure 2. Genetic status and detection of β-HPV DNA in EVpt4's family members.** The pedigree of EV pt4's family is shown together with β-HPV carriage in eyebrows hair bulbs of each individual. The viral load values expressed, as DNA copy number/cell, are shown in curved brackets or not reported when they were less than 1 copy/cell. Squares and circles indicate males and females respectively, underneath the year of birth. Double lines are indicative of

consanguineous unions. Filled symbols indicate the homozygous genotype and half-filled symbols indicate the heterozygous genotype of the EVER2 gene deletion. The arrow indicates the proband.

## Figures

**Figure 1**

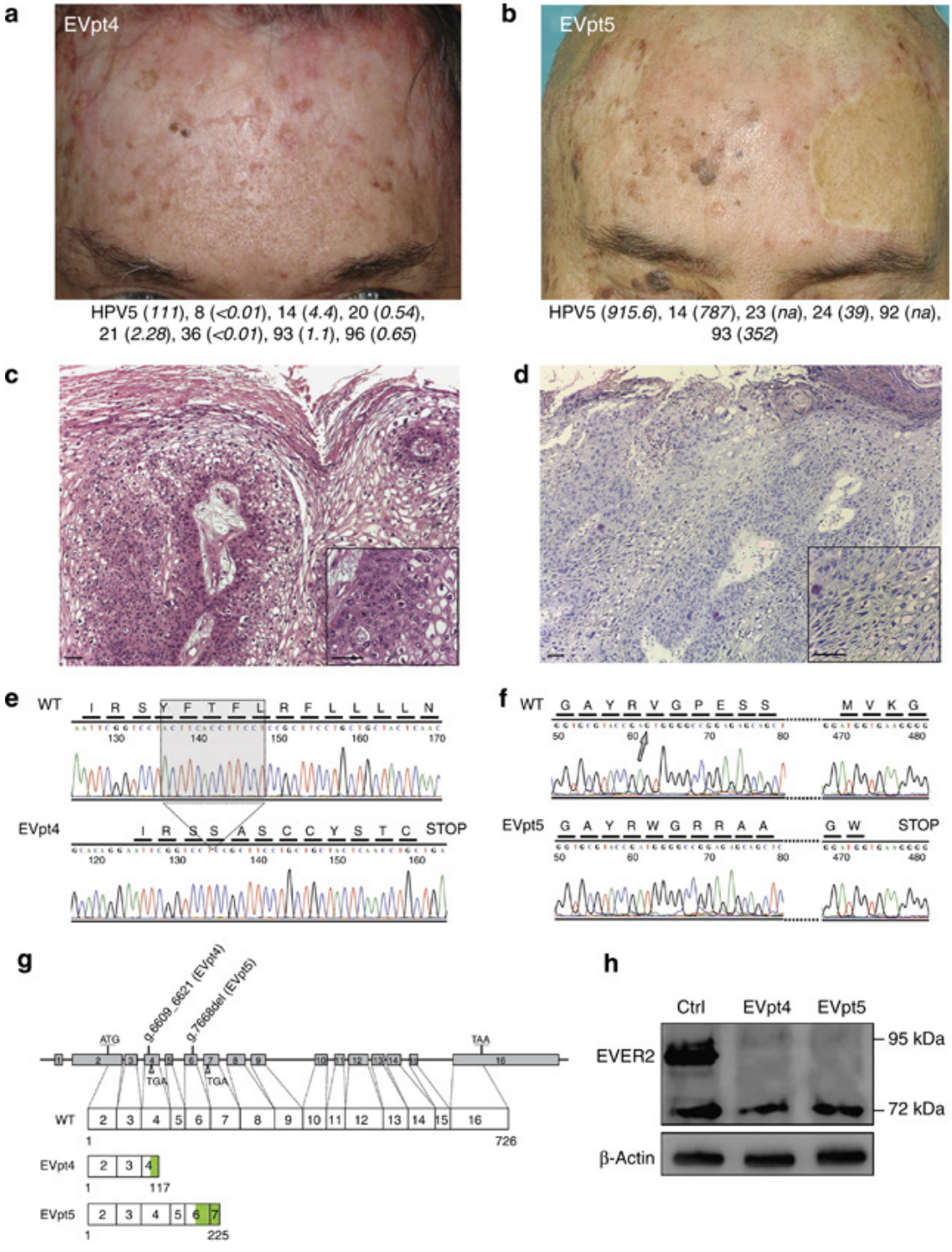
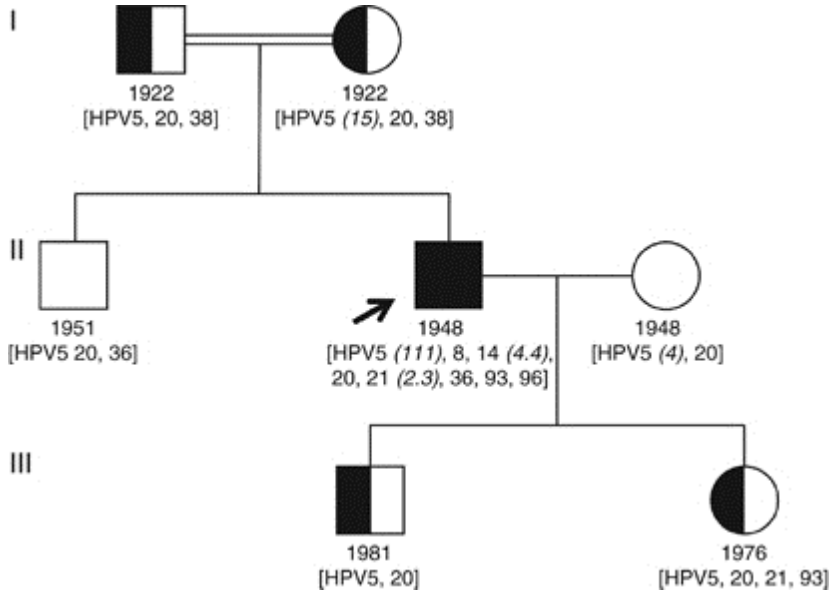


**Figure 2**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1922
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1922
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II
1951
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1948
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III
1981
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1976
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