Novel *TMC8* splice site mutation in epidermodysplasia verruciformis and review of HPV infections in patients with the disease

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Funding sources: none

Conflict of interest: none

Keywords: Epidermodysplasia verruciformis, human papilloma virus, splice site mutation, *TMC6, TMC8*, genetic skin disease, genodermatosis
Abstract

**Background:** Epidermodysplasia verruciformis (EV) is a genodermatosis leading to infections with cutaneous HPV, persistent plane warts and a high rate of non-melanoma skin cancer (NMSC). Biallelic loss-of-function mutations in TMC6 and TMC8 are known to be causative.

**Objective:** The aim of this study was to report EV-causing mutations in four patients with EV and to give an overview of all described EV patients.

**Patients and methods:** We investigated four patients with classical features of EV from two families. All patients were affected by plane warts with typical EV histology since early childhood and β-HPVs were detected on their skin. One patient had recurring cutaneous squamous cell carcinomas (cSCC) and carcinomas in situ (Bowen type). We sequenced both TMC6/8 for disease-causing mutations and quantified levels of gene expression. We also performed a systematic literature review to discuss these patients in the context of previously reported cases, mutations already identified, as well as HPV types.

**Results:** Three patients of one family carried a homozygous splice site mutation in TMC8 resulting in aberrantly spliced transcripts that were not degraded. By contrast, no TMC6/8 mutation was detected in the patient from the other family. A systematic literature review revealed 501 described EV patients. Around 40% of EV patients analysed for genetic alterations carried no mutation in TMC6/8. While β-HPVs were identified in the majority of cases, α-HPVs were detected in several individuals.

**Conclusion:** The relatively high proportion of EV patients without mutation in TMC6/8 indicates the existence of EV-causing mutations in additional, presently unknown gene(s). However, a homozygous TMC8 splice site mutation in our patients resulted in aberrant transcripts which cannot retain the healthy phenotype. The literature review revealed that HPV-5 is the most commonly identified HPV in EV patients, but HPV-3, HPV-14, and HPV-20 were unexpectedly identified more frequently than HPV-8.
Introduction

Epidermodysplasia verruciformis (EV) (OMIM 226400) is a rare, autosomal recessive genodermatosis with unknown prevalence. EV patients develop plane wart-like lesions during childhood and have a high risk for early onset development of non-melanoma skin cancer (NMSC) on UV light exposed skin. Biallelic loss-of-function (LoF) mutations in either TMC6 or TMC8, also named EVER1 or EVER2, both located on chromosome 17q, are causative for EV\(^1,2\). Complete loss of TMC8 protein has been shown in two cases\(^3\). On the other hand, several patients without TMC6/8 mutation are reported. Lesions of EV patients were shown to harbour high copy numbers per cell of cutaneous human papilloma viruses (HPV), mainly β-HPV, which are assumed to be carcinogenic\(^4,5\). Understanding the association between different HPV types and malignant transformation better would contribute to development of improved treatments, but very little is known about the frequency of different HPV types in EV patients or about differences in HPV infection between patients and the general population. In this study, which has been approved by the local ethics committee (EKNZ), we describe the clinical and genetic background of four patients with classical phenotype of EV. By reviewing the literature, we furthermore address the open questions regarding the total number of published EV patients, the proportion of families that carry deleterious mutations in TMC6/8, and the HPV types identified.

Case 1

A Swiss woman aged 49 was examined. Her parents were first cousins. Since early childhood, reddish skin lesions on the back of her hands and the extensor sides of her knees were visible. The lesions extended to the retroauricular area, neck, chest, arms, hands, knees, and lower legs (Fig. 1a-b). They showed the typical histological appearance of plane warts with minimal hyperkeratosis, acanthosis, as well as some cells with perinuclear halos and bluish staining (Fig. 1c). The patient had few persistent palmar lesions similar to palmar pits which were dermatoscopically consistent with plane warts (Fig. 1d). This is a very rare feature of EV which has been described in only three patients so far\(^6,8\). The patient developed multiple cutaneous squamous cell carcinomas (cSCC) and carcinomas in situ (Bowen type),
mainly on her forehead and nose (Fig. 1e). After the patient provided informed consent, blood samples and skin biopsies were taken. The whole coding sequence of TMC6 and TMC8, as well as adjacent intronic regions, was sequenced as described elsewhere. Some common SNPs without effect on the protein sequence were detected, as well as a heterozygous SNP in TMC8 (rs7208422, c.917A>T, p.Asns306Ile, MAF = 0.45), but no LoF mutation was found.

In a skin biopsy, HPV type 5 was detected by using nested PCR with primers specific for the L1 capsid gene and subsequent Sanger sequencing.

**Cases 2-4**

Three Turkish siblings aged 12, 17, and 18 years, as well as their healthy parents, who were second-degree cousins, were included in our study. The siblings developed plane warts during early childhood (Fig. 2a-b). The lesions had an appearance typical for EV and histopathological examination confirmed the diagnosis based on the presence of so-called blue cells (Fig. 2c). These three patients have not yet developed any cancerous lesions, probably because of their young age. Two aunts and one uncle of the patients were affected by EV as well. Blood samples were obtained from the siblings and their parents after they provided informed consent. Sanger sequencing of all coding exons, as well as adjacent intronic regions of TMC6 and TMC8, revealed a homozygous splice site mutation in the donor splice site of IVS9 of TMC8 (c.1127+1G>C) in all patients (Fig. 2d). Both parents were heterozygous carriers of the identified mutation. Exon-overlapping RT-PCR on mRNA from blood including exon 9 and 10 yielded no product in EV-affected patients, while we obtained the expected PCR-product in parents and all control individuals. Quantitative RT-PCR (qRT-PCR) was performed on TMC6 exons 4-5 and 10-11, as well as on TMC8 exons 1-2, 7-8, 9-10, and 14-15. Results were normalized by using GAPDH, TBP, and GUSB with qbase+ (Biogazelle, Belgium). All examined exons of TMC6, as well as exons 1-2, 7-8, and 14-15 of TMC8, showed stable expression in the patients, their parents and the controls. However, exons 9-10 of TMC8 could not be amplified in any affected child, and expression was reduced in heterozygous parents compared to controls (Fig. 2e). To identify the main aberrant splice products, RT-PCR products of TMC8 exons 6-11 were investigated by Sanger sequencing. All
splice products identified in the patients lacked complete exon 9, and a subset additionally lacked exon 10 (Fig. 2f). The former transcript is predicted to result in a frameshift and premature stop codon after 30 amino acids, while the latter transcript would result in an in-frame deletion of 88 amino acids. With the methods described above, infections with β-HPV (type 5 and 9) were detected in all three patients’ plane warts.

Discussion

All cases presented in this study showed the typical clinical, histopathological, and viral phenotype of EV. In addition to these characteristic EV features, one individual presented palmar warts, which constitutes a rarely described feature of EV. This patient was affected by diverse NMSCs, including a cSCC on the side of her nose. Since she had never developed plane warts in this region before, a pre-existing lesion does not seem to be a prerequisite for development of NMSC. Reports about the frequency of precancerses and NMSCs in EV range from none\textsuperscript{10} to numerous\textsuperscript{11}. Our systematic literature review summarizes cases with congenital EV reported to date, including the novel patients described in this study (table 1).

Patients were excluded if they had acquired EV (onset after HIV infection or immunosuppression) or had a very atypical phenotype (excessive wart growth). In total, 501 cases belonging to 347 families have been described with slightly more male patients. The actual number of diagnosed individuals is probably even higher since not every case is presumably published. For example, a clinic in Iraq observed 4-6 affected families per year, mostly from two areas, without reporting details\textsuperscript{12}, and Japanese clinics reported 66 patients in a questionnaire survey\textsuperscript{13}. There are no conclusive studies about the life expectancy of EV patients. However, under regular treatment of precancerses and NMSCs, patients can reach old age\textsuperscript{11}.

Despite the EV-typical appearance of patient 1, we were unable to detect any LoF mutation in \textit{TMC6} or \textit{TMC8}. This was in contrast to the second family (cases 2-4), in whom we detected a splice site mutation in \textit{TMC8}. Nevertheless, we were able to measure regular amounts of aberrantly spliced \textit{TMC8} transcripts, which is consistent with a recent report of regular level of
transcripts with in-frame deletions and protein expression in one patient with splice site mutations in TMC6\textsuperscript{10}. These findings exclude the mechanism of nonsense mediated decay (NMD), which has been reported in patients with TMC8 premature stop mutation\textsuperscript{3}. In contrast to these cases, our patients have not developed any NMSC so far. Moreover, the other EV patient with splice site mutations and stable aberrant transcripts did not develop NMSC either\textsuperscript{10}. These findings suggest that the stable transcript and evading NMSC are potentially correlated, which needs to be proven in further studies and a follow-up of our young patients.

Since 2002, the TMC6 and TMC8 genes have been analysed in 32 families, but in approximately 40\% of them (13 families), no LoF mutation could be detected. These findings suggest mutations of at least one more gene connected to EV. Nearly all families with either TMC6 or TMC8 deficiency carry a homozygous private mutation indicating consanguinity, which is reported in about half of all patients.

HPV analyses in samples from 208 patients showed a positive result in all except one case\textsuperscript{14}. HPV type was determined in 180 of these cases (table S1). Beta-HPV was detected in 159 patients, α-HPV in 57 patients, and γ-HPV in one patient (Fig. 3). Most α-HPVs were detected along with β-HPVs. However, some α-HPV infections were reported without co-infection with β-HPV. These reports should be interpreted with caution. In some studies, the methods used had been developed to find α-HPVs, but were unable to detect β-HPVs. Other studies analysed only perianal samples. In several older studies, HPV-3 was detected by using methods relying on DNA hybridisation or restriction enzyme digestion, without amplification or sequencing. These methods had limited sensitivity and did not differentiate between all types known today. Nevertheless, a recent report has described one individual with a cutaneous HPV-10 (α-HPV) infection without concurrent β-HPV infection, despite a methodical search\textsuperscript{16}. HPV-5 and HPV-8 are often mentioned as the most frequent EV-related HPV types. In contrast to that current opinion, our literature review shows that HPV-14 and HPV-20 have been reported more frequently than HPV-8, which shows the need to pay attention to HPV types other than 5 and 8 in future studies. In addition, EV lesions were shown to be coinfected with β-HPVs and polyomaviruses\textsuperscript{17}. We conclude that further investigations on the effect of β-
HPV types and polyomaviruses on malignant transformation are necessary to better understand the relation of EV and HPV.

Acknowledgements

The authors would like to thank all patients for participating in this study, W. Bayard and C. Forno for referring patients, C. Reinbold for helpful comments to this manuscript, and G. Orth for providing primer sequences for *TMC6* and *TMC8*.

Conflict of interest

None declared.
References


**Figure 1:** EV lesions on (a) knees and (b) neck of patient 1. (c) Haematoxylin-eosin (HE) stained Bowenoid lesion. Typical pale blue cytoplasm and slightly polymorphic keratinocytes are visible. (d) As a rare feature, the patient showed persistent palmar lesions (marked with blue circles). (e) cSCC developing on the nose of the patient where no previous EV lesions had been present.
Figure 2: EV lesions on (a) chest and (b) hands of patient 2. (c) HE stained EV lesion of patient 4 with typical blue cytoplasm of keratinocytes and slightly enlarged and hyperchromatic cells mainly in stratum granulosum. (d) Sanger sequencing of TMC8 revealed a mutation at the donor splice site of intron 9 (TMC8 c.1127+1G>C), which was found to be homozygous in all three affected siblings and heterozygous in the parents. A wildtype control is shown. (e) Relative expression of exons 1-2 and 9-10 of TMC8 measured by qRT-PCR. Each symbol represents the average of one individual measured in three replicates. The grey horizontal bars indicate the group average. Whereas no expression could be detected for exon 9-10, a regular expression level is observed for exon 1-2. (f) TMC8 splice products identified in controls and patients. The region covered by sequencing is indicated in black. The splice site mutation is indicated by an arrow. In healthy controls as well as in patients, a splice variant with a shortened exon 10 can be found additionally to products with full length exon 10, corresponding to ENST00000590184.1. All identified splice products in the patients are lacking at least exon 9, a subset additionally lacks exon 10.
Figure 3: Diagram showing the number of patients by detected HPV infections. Most patients were solely infected by cutaneous β-HPV but some were co-infected by cutaneous or mucosal α-HPV. Only once γ-HPV were identified and in three samples only mucosal α-HPV were detectable.
Table 1:
Summary of congenital EV cases reported in the literature, including the presented patients.
All percentages were calculated respective to all patients or families for whom the information is available.

<table>
<thead>
<tr>
<th>Number of published cases</th>
<th>501 patients from 347 families</th>
</tr>
</thead>
<tbody>
<tr>
<td>male / female</td>
<td>229 (60.7%) / 148 (39.3%) patients</td>
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<tr>
<td>consanguineous parents</td>
<td>65 families</td>
</tr>
<tr>
<td>non consanguineous parents</td>
<td>68 families</td>
</tr>
<tr>
<td>HPV detection positive / negative</td>
<td>208 / 1 patients</td>
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<tr>
<td>HPV type determined</td>
<td>180 patients</td>
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<tr>
<td>α-HPV detected</td>
<td>57 (31.7%)</td>
</tr>
<tr>
<td>β-HPV detected</td>
<td>159 (88.3%)</td>
</tr>
<tr>
<td>γ-HPV detected</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td>TMC6 and TMC8 sequenced</td>
<td>32 families</td>
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<tr>
<td>LoF mutations in TMC6</td>
<td>8 (25.0%)</td>
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<tr>
<td>LoF mutations in TMC8</td>
<td>11 (34.4%)</td>
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<tr>
<td>no mutation in TMC6/8</td>
<td>13 (40.6%)</td>
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</table>
Supporting information

Table S1:
Number of EV patients with different HPV-types and species\textsuperscript{15} without consideration of combinations in single patients. In total, HPV detection had been performed in 209 patients and many patients were positive for more than one HPV type. The various studies used different methods for HPV detection with different sensitivities and detection ranges.

<table>
<thead>
<tr>
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<td>5</td>
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<tr>
<td>41</td>
<td>3</td>
<td>α2</td>
</tr>
<tr>
<td>36</td>
<td>20</td>
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<td>31</td>
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<tr>
<td>28</td>
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<td>-</td>
</tr>
<tr>
<td>26</td>
<td>17</td>
<td>β2</td>
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<td>4, 6, 11, 18, 27, 32, 34, 37, 53, 75, 76, 92, 96, 111</td>
<td>γ1, α6, α6, α7, α4, α1, α11, β2, α10, β3, β3, β4, β5, β2</td>
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<tr>
<td>1</td>
<td>no HPV detected</td>
<td>-</td>
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Dermatol Surg 11:01-11 2005


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