Substantiating atypical phenotypes of epidermolysis bullosa

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Chapter 3

A novel *PLEC* isoform rescues the phenotype in epidermolysis bullosa simplex

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ABSTRACT

Eight percent of all cases of epidermolysis bullosa simplex (EBS) are explained by autosomal recessive and dominant mutations in *PLEC*, encoding plectin. Complete absence of plectin leads to generalized skin fragility and pyloric atresia with death occurring shortly after birth, but other plectinopathies have been reported. Here, we describe how the phenotype in a 17-year-old girl with mild acral blistering since childhood, progressive muscular dystrophy and cardiomyopathy is rescued by unexpected alternative splicing of exon 8. DNA analysis revealed a homozygous deletion of 22 base pairs in intron 8. RT-PCR analysis showed intron 8 retention inducing a premature termination codon. Notably, we also identified an internally truncated, but in-frame RNA transcript resulting from the use of an alternative splice donor site 12 base pairs upstream of the original splice site. This alternatively spliced product, which was also present in control keratinocytes, myocardium and striated muscle, resulted in small amounts of plectin production, as witnessed in our patient's skin. A lethal EBS phenotype in our patient was averted due to the production of this slightly shorter, newly identified plectin isoform.

INTRODUCTION

The most commonly occurring subtype of the group of blistering genodermatoses collectively termed epidermolysis bullosa (EB) is epidermolysis bullosa simplex (EBS).[1] The major subtype of EBS can be divided into suprabasal EBS and basal EBS, from which the latter is caused by mutations in the genes *KRT5, KRT14, DST, EXPH5* and *PLEC*.[2,3] To date, approximately 20% of patients with EBS have no identified genetic cause for their disease.[2]
PLEC encodes the ubiquitously present cytolinker protein plectin, which plays an important role in the hemidesmosome by connecting keratin filaments to the underlying integrin α6β4 subunit.[4,5] Plectin deficiency in skin results in intraepidermal skin cleavage in basal keratinocytes.[6] In muscle, plectin is present at the z-disk, sarcolemma, intercalated discs and neuromuscular junction and is crucial for stability of myocytes, as it links desmin together with the dystrophin–glycoprotein complex.[7,8] In humans, eight distinct plectin isoforms have been identified arising from tissue specific translation. The *PLEC* gene sequences encoding these eight isoforms 1, 1a, 1b, 1c, 1d, 1e, 1f and 1g differ in exon 1 and their own upstream regulatory sequences.[9,10] Additionally, alternative splicing of exon 31 results in a rodless plectin variant, which has been identified in human keratinocytes and skeletal muscle plectin.[11,12] Almost all EBS-causing mutations in *PLEC* lead to absent or reduced protein expression. The resulting phenotype is characterized by skin blistering upon trivial mechanical trauma, and nail anomalies. Depending on the mutation in *PLEC*, EBS can occur with isolated skin disease as seen in EBS-Ogna[13] or EBS-plectin 1a,[14] or associated with pyloric atresia (EBS-PA)[15] or muscular dystrophy (EBS-MD).[16] Complete absence of plectin leads to the more severe phenotype of EBS-PA in which blistering is generalized and death occurs shortly after birth.[17] Here, we present a novel alternative *PLEC* isoform expressed in skin, muscle and heart, which is the result of a yet unknown alternative splicing event in exon 8. Moreover, we show functionality of the slightly shorter plectin protein by studying a patient with EBS-MD in which the lethal EBS-PA phenotype was averted.

RESULTS

The proband (IV-4, EB 210-01, Figure S1) presented at 17 years of age with complaints of acral skin blistering since the age of 2 years. She was the daughter of consanguineous Moroccan parents. Acral blistering, subtle nail pitting of the hands, focal plantar hyperkeratosis, onychokeratosis and onycholysis were observed (Figure 1). Hair implant and teeth were normal. At the time of consultation she had left-sided ptosis, which would worsen throughout the day. Chewing and swallowing took more time than normal. She complained of muscle weakness in her shoulders and arms, because of which she was not able to brush her hair or lift small objects such as a hairbrush. Walking for short distances quickly resulted in fatigue within 10 minutes. There was no visible muscular atrophy of extremities. Neurological consultation and electromyography revealed signs of myopathy with normal neuromuscular transmission. In her family, only her older brother had suffered from the same skin complaints. Both her father (III-6) and brother (IV-I) had died from sudden cardiac death at ages 43 and 19, respectively. Following her brother's death, the proband and her older sister (IV-3) underwent cardiologic screening, which had disclosed a cardiomyopathy. Immunofluorescence (IF) staining of lesional skin showed intraepidermal cleavage with very thin lining of keratin in the blister floor. Also, staining of laminin 332 and type VII collagen were in the blister floor indicating basal EBS. Staining of intact skin with the monoclonal antibody HD121 (kind gift from Dr. K. Owaribe), recognizing all forms of plectin, revealed reduced staining along the basement membrane zone (BMZ) (Figure 2 a,b). The intensity of the stainings for keratin 5, keratin 14, laminin 332 and type VII collagen were comparable to control skin (data not shown). Transmission electron microscopy (TEM) further visualized pseudojunctional cleavage with the plasma membrane and the lamina densa in the blister floor (Figure 2 c,d). Hemidesmosomes were hypoplastic. Taken together, the data suggested a plectinopathy and *PLEC* as the candidate gene.

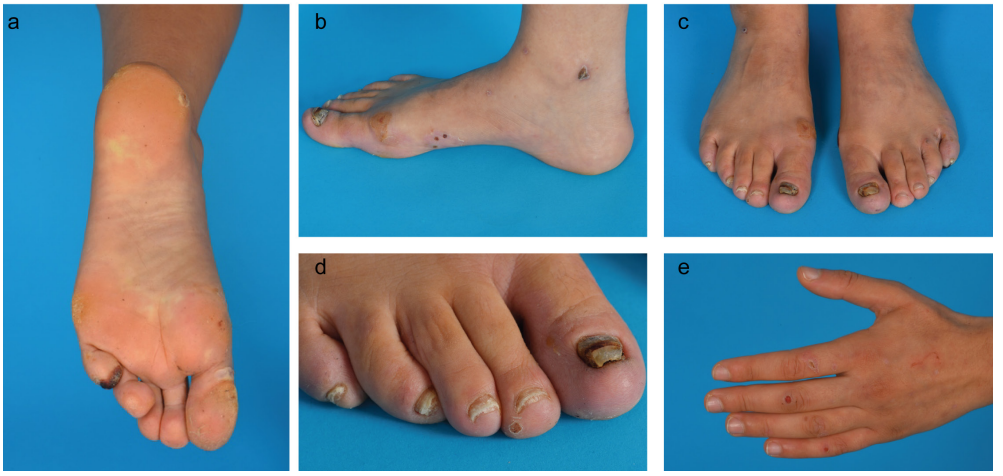


Figure 1: CLINICAL CHARACTERISTICS OF EB 210-01

Focal plantar hyperkeratosis and haemorrhagic blister and erosions (a-c). Onychodystrophy, onycholysis with discoloration seen affecting all toenails (d). On the dorsal side of hands erosions with crusts and hyperpigmentation are seen (e).

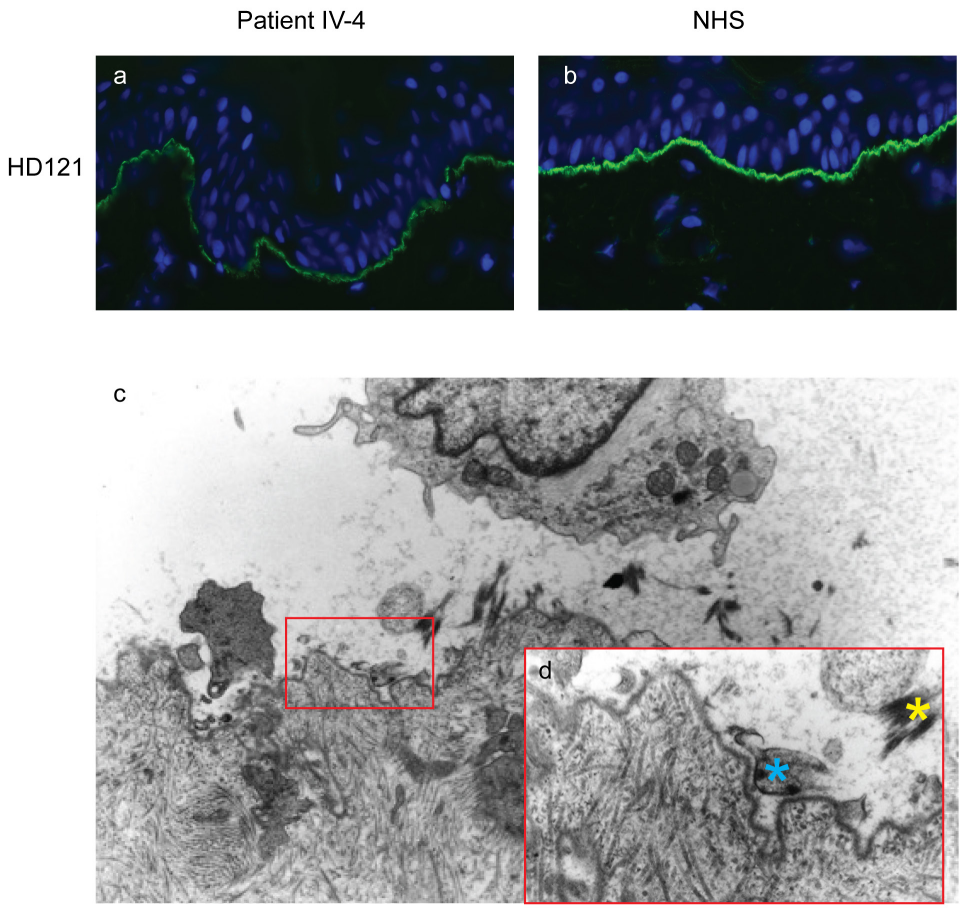


Figure 2: IMMUNOFLUORESCENCE STAINING AND TRANSMISSION ELECTRON MICROSCOPY
Immunofluorescence staining of non-lesional skin of EB 210-01. (a,b) Anti-pan plectin monoclonal antibody HD121 with decreased staining along the BMZ when compared to normal human skin (NHS) in controls. (c,d) Transmission electron microscopy of skin samples from lesional skin of EB 210-01. Blister cavity with a pseudojunctional split with the lamina densa in the blister floor (c). The red square indicates the portion of zoomed in segment depicted by picture (d). The blue asterisk indicates rest segments of the plasma membrane with rests of hemidesmosomes. The yellow asterisk displays broken off keratin filaments with incomplete insertion into the hemidesmosome.

Sanger sequence analysis of the *PLEC* gene (NM_000445.3) encompassing all exons including five to ten nucleotides of intron sequence adjacent to the 5′ and 3′ ends of the exons revealed no mutation. Additional DNA sequences outside the exon-intron regions for standard sequence analysis were analyzed for mutations when protein studies pointed to a mutation in *PLEC*. This revealed a novel homozygous deletion in intron 8, c.906+19_40del. Five different splice site software programs predicted that this deletion does not affect activity of the wildtype donor and acceptor splice sites of intron 8. A novel splice site was not introduced either. Despite these *in silico* results an effect on splicing of this deletion could not be ruled out.

The only other family member available for genetic screening was the patient's mother (III-5), who was heterozygous for the same deletion c.906+19_40del. Parallel to *PLEC* gene analysis, because of the cardiomyopathy, the patient was screened for inherited cardiomyopathy using a targeted next generation sequencing panel of 55 genes (Table S1). [18] No mutations were identified.

Subsequent RT-PCR analysis was performed in order to investigate the effect of the intronic deletion in *PLEC* on the mRNA. RT-PCR analysis on RNA isolated from a patient's skin biopsy using primers surrounding intron 8 (Supplementary table 2) revealed intron 8 retention leading to a frameshift and a premature termination codon (PTC), p.Val303_Pro313ins11* (Figure 3). Consequently, this homozygous mutation is predicted to result in a complete loss of plectin expression. More importantly, an additional novel transcript was also identified that was 12 nucleotides shorter than wildtype. This product was the result of the use of an alternative splice site 12 base pairs upstream from the wildtype exon 8 splice donor site. The resulting plectin protein was predicted to be four amino acids shorter in length, missing amino acids 299-302 when compared to wildtype plectin. This shorter mRNA transcript was also found in skin of healthy control individuals at the same level (Figure 3).

Additionally, western blot analysis with HD121 antibody on patient keratinocytes and fibroblasts revealed 30% and 10% plectin protein expression, when compared to healthy control keratinocytes and fibroblasts, respectively (Figure 4 b,c). These findings led us to further investigate whether the alternatively spliced transcript was also present in muscle and myocardium. RT-PCR analysis on RNA isolated from healthy human striated muscle and myocardium samples showed indeed the presence of the alternatively spliced transcript (Figure 4a).

DISCUSSION

Here, we describe physiological alternative splicing of exon 8 resulting in a novel four amino acid shorter, yet in-frame plectin isoform that is present in skin, myocardium and striated muscle of healthy human controls. Moreover, we show that this isoform is functional in an EBS-MD patient.

Earlier reports have shown that homozygous nonsense mutations predominantly found in the exons preceding or following exon 31 result in the development of EBS-PA. [17,19,20] Our patient carries a homozygous intronic deletion in intron 8 of the *PLEC* gene, affecting all known plectin isoforms. This deletion leads to the retention of intron 8, resulting in a premature termination codon, which was predicted to cause complete loss of plectin expression and in turn, the development of a lethal EBS-PA phenotype. [11,17,21] However, none of the characteristic EBS-PA features were observed in the proband: i.e. early demise within months, cutis aplasia congenita and widespread, severe blistering. [16,19,20] Rather than developing EBS-PA, the patient exhibited a less severe 'forme fruste' of EBS-MD. The onset of all complaints complies with that in reported EBS-MD cases. [15,16,22] Additionaly, the EBS-MD phenotype was reflected with the following phenotype characteristics: acral skin blistering, nail dystrophy of the hands, progressive skeletal muscle dystrophy lacking signs of atrophy, and cardiomyopathy.However,characteristics that were missing from classic EBS-MD were: muscular atrophy, major respiratory infections, and mucosal involvement. Her skin involvement was mild in comparison to published cases. [15,16,22] Also, severe oesophageal and urethral complications requiring dilatations were not seen. [20,23] Moreover, otologic and

ocular problems have been described in the long-term follow up of a large EBS-MD family, but were absent in the proband. [22] Accordingly, our results imply that the patient is protected from a severe phenotype by expression of a novel plectin isoform.

The alternative splice variant explains the retained low expression levels of plectin observed in patient skin and the EBS-MD phenotype. The shorter plectin protein lacks the amino acids 299-302, which are part of the highly conserved actin-binding domain of plectin. [6] As can be seen in Figure 4d, the four amino acids are strongly conserved with exception of the amino acid on position 301. It is yet unknown whether the absence of these amino acids affects functionality. The protein is at least partially functional, as the phenotype is much milder than EBS-PA and not classic EBS-MD. It could even be that the shorter plectin protein has the same functionality as full-length plectin, as the amount of plectin protein production is lower, i.e. 30% in keratinocytes and 10% in fibroblasts, compared to normal control skin, which alone might explain the occurrence of a phenotype. Recent studies by the group of Sonnenberg have shown that the rodless plectin resulting from alternative splicing of exon 31, which is maintained in EBS-MD, can functionally compensate for the full-length plectin in mice. [12] In contrast to the rodless mouse model and despite the expression of rodless plectin, EBS-MD patients still develop the disease. This may be due to the lower expression level of rodless plectin in EBS-MD patients than in rodless plectin mice. [12]

Pathogenic intron mutations in the *PLEC* gene are rare and have been presented in two earlier reports as compound heterozygous mutations together with exon mutations resulting in a PTC. The intron mutations resulted in aberrant splicing in all affected patients, who suffered from EBS-PA and EBS-MD with myopathy respectively. The first patient c.[3342-2A>G];[3902_3903del] born prematurely died in the first day of life from extensive cutis aplasia congenita, pyloric atresia and extensive blistering of the oesophagus, trachea and megacolon. [20] The second patient c.[7804C>T];[4126-4A>G] showed congenital myopathy as predominant feature, while skin involvement was limited to a single blister. [24, 20] No reports of cardiomyopathy were disclosed in the reported patients.

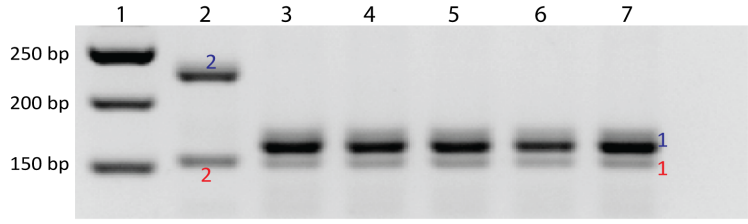
Standard sequencing techniques such as Sanger sequencing and whole exome sequencing target coding regions of genes and generally cover intron/exon borders spanning about 50-100 nucleotides. [25] Our data show that pathogenic intronic mutations may be missed by standard sequencing techniques, and that in the approximately 20% of patients with EBS without an identified mutation, intronic mutations in candidate EBS genes might explain the disease. We expect that RNA analysis by RT-PCR or RNA sequencing of known EBS genes would resolve more EBS cases. This has recently been exemplified by Chmel et al. who reported a patient with another subtype of EB, Kindler syndrome, in whom a FERMT1 mutation was later found deep in intron 9 (c. IVS9+740G>A) leading to aberrant splicing and absence of kindlin-1. [26] The same mutation was later confirmed in other unsolved cases of Kindler syndrome. This mutation would have been missed with Sanger or exome sequencing.

The role of the lower plectin expression in the occurrence of the cardiomyopathy in our patient is yet unclear. It certainly cannot be excluded that it has played a role, as no mutations were found in 55 known cardiomyopathy genes. However, as the cause of the cardiomyopathy in the other affected family members, the father and two siblings (III-6, IV-1 and IV-3), is also unknown, and a skin phenotype was only reported in the proband's brother, an other,

autosomal dominant mutation in a cardiomyopathy-associated gene should still be considered. Ideally, screening of the entire family for *PLEC* and the inherited cardiomyopathies would give more insight into phenotypic differences in this family.

In conclusion, our results reveal the presence of a novel *PLEC* isoform due to alternative splicing of exon 8. The functionality of the produced shorter length plectin protein is witnessed by salvage of the otherwise lethal EBS phenotype into mild EBS-MD in the presented patient. This alternative splicing mechanism may mitigate the effect of other mutations disrupting the constitutive splice donor or nonsense mutations in one of the four skipped amino acids. Consequently, this may preclude the development of the most severe plectinopathy phenotypes in patients carrying such mutations.

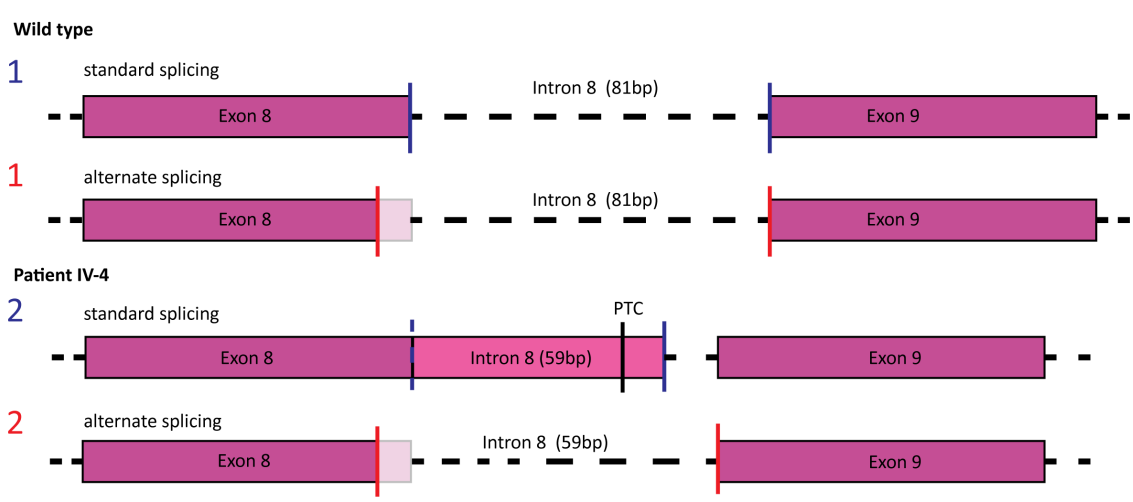
a


1
2
3
4
5
6
7
250 bp
200 bp
150 bp
2
2
1
1

b


AGGATGGG
GTGAGGGCCAAC
gtgagtgggggcccggagggcaggggcttgggcctggacgccccgacgaccctgacagccgcccgtgcctgcccgcag
GAGCTGCAGCT
Exon 8
Intron 8
Exon 9

c


Wild type
1
standard splicing
Intron 8 (81bp)
Exon 8
Exon 9
1
alternate splicing
Intron 8 (81bp)
Exon 8
Exon 9
Patient IV-4
2
standard splicing
PTC
Exon 8
Intron 8 (59bp)
Exon 9
2
alternate splicing
Intron 8 (59bp)
Exon 8
Exon 9

d

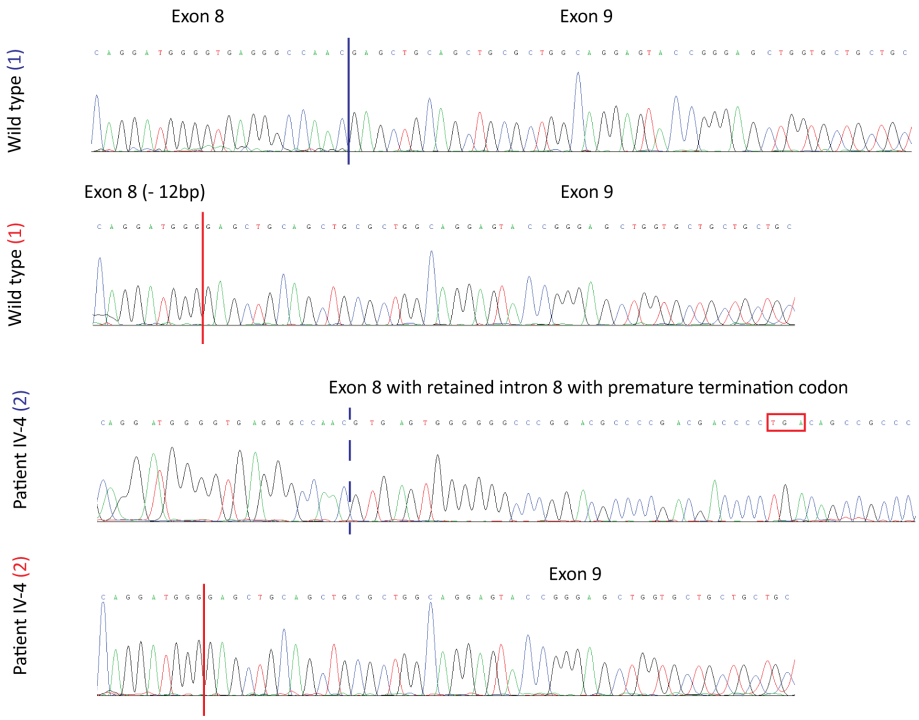
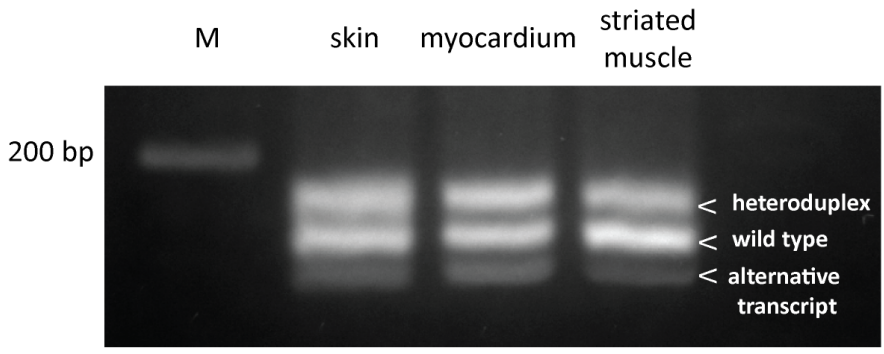

Exon 8
Exon 9
Wild type (1)
Exon 8 (- 12bp)
Exon 9
Wild type (1)
Exon 8 with retained intron 8 with premature termination codon
Patient IV-4 (2)
Exon 9
Patient IV-4 (2)

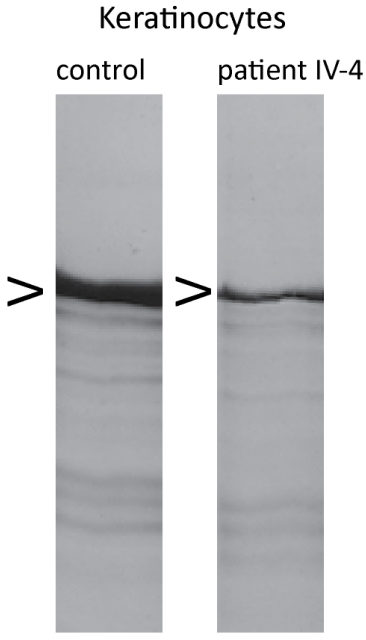
Figure 3: ALTERNATE SPLICING IN EXON 8 OF *PLEC*

(a) RT-PCR analysis of patient RNA isolated from a frozen skin biopsy showed two products (lane 2). The larger product of 223 kb contains the retention of shortened intron 8 of 59 bp. This intron retention results in a PTC, and absence of protein (p.Val303_Pro313ins11*). The smaller product of 152 kb had a deletion of 12 nucleotides, and results in a protein that is four amino acids shorter (p.Val299_Asn302del4). The smaller, alternatively spliced product was observed in all healthy controls as depicted in lanes 3-7. (b) Schematic representation of the exon-intron junction of exons 8 and 9 of *PLEC* while depicting the described splice sites and intronic deletion seen in the proband. The intronic deletion is depicted in green lower case letters, whereas the wildtype splice site is marked with a blue line, and the alternative splice site with a red line. (c) The numbers in blue and red correspond with the RNA transcripts that are observed in the agarose gel in (a). (d) The numbers in blue and red correspond to (c).

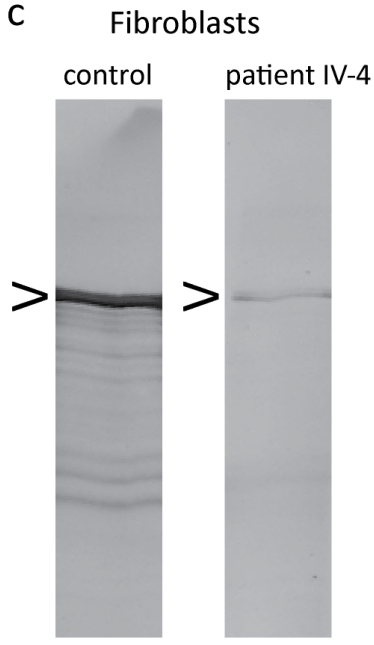
a


M
skin
myocardium
striated muscle
200 bp
heteroduplex
wild type
alternative transcript

b


Keratinocytes
control
patient IV-4

c


Fibroblasts
control
patient IV-4

d

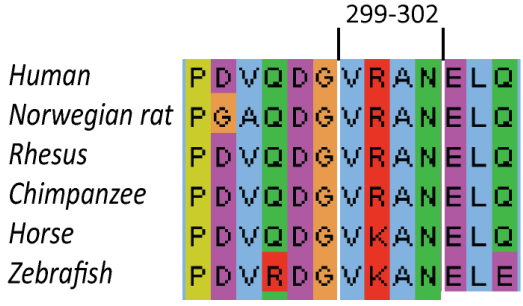

299-302
Human PDVQDGVRANELQ
Norwegian rat PGAQDGVRANELQ
Rhesus PDVQDGVRANELQ
Chimpanzee PDVQDGVRANELQ
Horse PDVQDGVKANELQ
Zebrafish PDVRDGVKANELE

Figure 4: ALTERNATIVE SPLICING PRESENT IN MYOCARDIUM AND STRIATED MUSCLE, IMMUNOBLOTTING OF PATIENT CELLS AND CONSERVED AMINO ACIDS

(a) RT-PCR analysis on healthy human skin, healthy human myocardium and healthy human striated muscle samples. The alternatively spliced product is present in skin and muscle samples of healthy controls. The heteroduplex product is a combination of wildtype product and alternatively splice product.

(b) & (c) Immunoblotting with monoclonal antibody HD121 of cell lysates from cultured keratinocytes (b) and fibroblasts (c) derived from normal humans (control) and the proband (IV-4). Arrows indicate plectin (molecular mass ~500 kDa). The plectin production in the patient's keratinocytes is 30% of the production in control keratinocytes (b), whereas the production in patient's fibroblasts is even less, i.e. 10% of control keratinocytes (c). (d) Conservation of plectin residues 299-302 (p.Val303_Pro313ins11*), which are omitted when the alternate splice site is used.

MATERIALS & METHODS

Patient

The patient (EB 210-01) was referred to our clinic for further analysis and subtype classification of EB. All experiments performed were done with material obtained for diagnostic purposes, which did not require extra approval from the institutional ethical committee. The patient gave informed consent for publication of photographs and the use of tissue samples. She was seen with her mother who consented for molecular carrier analysis. All non-patient tissue samples were contributed and analyzed anonymously. All experiments were conducted according to the principles of the Declaration of Helsinki.

Immunofluorescence antigen mapping

Four-mm skin biopsies of fresh blisters and healthy skin under the left arm were taken for immunofluorescence antigen mapping of the proband, and processed as described before. [27] Mouse monoclonal antibodies HD121 (gift of Dr. K. Owaribe) and 10F6 (Santa Cruz Biotechnology, Santa Cruz, CA) were directed against the plectin rod domain.

Electron microscopy

Two-mm punch biopsies of perilesional and non-lesional skin were taken from the upper arm and prepared as described previously. [27]

Molecular analysis

Genomic DNA was extracted from peripheral blood lymphocytes using standard laboratory methods. The *PLEC* gene including all 8 isoforms (1, 1a, 1b, 1c, 1d, 1e, 1f and 1g) was screened for mutations by sequencing analysis of all exons including exon-intron boundaries. Primer sequences can be found in Supplementary table 2. *In silico* analysis of the mutation using Alamut®Visual software (version 2.6.1, alamut.interactive-biosoftware.com) with protein and splice site prediction programs was used.

Western blotting

Western blotting was performed as described previously. [14] Extracts from cultured keratinocytes and fibroblasts were used from the proband and healthy controls as a substrate. To calculate the relative protein contents of the extracts, serial dilutions were run by SDS-PAGE. The gel was then stained by Blue Silver staining and the intensities of the lanes calculated using Quantiscan® software. Serial dilutions of all extracts were immunoblotted and plectin was visualized with antibody HD121. The relative plectin expression was calculated from the intensity of staining using Quantiscan® software.

RNA analysis

RT-PCR was performed on RNA isolated from primary cultured keratinocytes, fibroblasts, and cryosections. The RNeasy Micro Kit (Qiagen) was used for the isolation of RNA. The primers used for the PCR after reverse transcription are listed in Table S2.

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