OXFORD

ORIGINAL ARTICLE

Mutation in exon 1a of *PLEC*, leading to disruption of plectin isoform 1a, causes autosomal-recessive skin-only epidermolysis bullosa simplex

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Abstract

PLEC, the gene encoding the cytolinker protein plectin, has eight tissue-specific isoforms in humans, arising by alternate splicing of the first exon. To date, all *PLEC* mutations that cause epidermolysis bullosa simplex (EBS) were found in exons common to all isoforms. Due to the ubiquitous presence of plectin in mammalian tissues, EBS from recessive plectin mutations is always associated with extracutaneous involvement including muscular dystrophy, pyloric atresia and cardiomyopathy. We studied a consanguineous family with sisters having isolated blistering suggesting EBS. Skin disease started with foot blisters at walking age and became generalized at puberty while sparing mucous membranes. DNA sequencing revealed a homozygous nonsense mutation (c.46C>T; p.Arg16X) in the first exon of the plectin variant encoding plectin isoform 1a (P1a). Immunofluorescence antigen mapping, transmission electron microscopy, western blot analysis and qRT-PCR were performed on patient skin and cultured keratinocytes, control myocardium and striated muscle samples. We found hypoplastic hemidesmosomes and intra-epidermal 'pseudo-junctional' cleavage fitting EBS. Screening for cardiomyopathy and muscle dystrophy showed no abnormalities. We report the first cases of autosomal-recessive EBS from P1a deficiency affecting skin, while mucous membranes, heart and muscle are spared. The dominant expression of the P1a isoform in epidermal basal cell layer and cultured keratinocytes suggests that mutations in the first exon of isoform 1a cause skin-only EBS without extracutaneous involvement. Our study characterizes yet another of the eight isoforms of plectin and adds a tissue-specific phenotype to the spectrum of 'plectinopathies' produced by mutations of unique first exons of this gene.

Introduction

Epidermolysis bullosa (EB [MIM 131900]) is a heterogeneous group of genodermatoses characterized by lifelong skin fragility and extracutaneous complications present from birth (1). Affected individuals suffer from trauma-induced blistering of skin and mucous membranes with severity ranging from mild to lethal. For EB, up until now there have been mutations identified in 18 different genes affecting different proteins in the epidermis and basement membrane zone (BMZ) (1). Depending on the affected protein, the epidermis is rendered fragile by cleavage of

the skin at its biological location. The most commonly occurring subtype is epidermolysis bullosa simplex (EBS) where skin cleavage occurs in the epidermis (2). In basal EBS the split occurs in the basal cell layer as witnessed by immunofluorescence antigen mapping or electron microscopy. Basal EBS is commonly associated with four different genes: KRT5, KRT14, BPAG1 and PLEC. In 8% of EBS cases, PLEC is the mutated gene (3). PLEC encodes the cytolinker protein plectin, which is ubiquitously present in skin, mucous membranes, gut, muscle and heart tissue. The phenotypes associated with PLEC mutations are mucocutaneous blistering, pyloric atresia, muscular dystrophy and cardiomyopathy (4,5). Here, we identify a novel mutation causing cutaneous EBS without extracutaneous involvement. This nonsense mutation found in the distinct first exon 1a encoding the N terminus of plectin isoform 1a (P1a) only disrupts this one isoform whose tissue dominant distribution in the epidermis results in skin-only EBS.

Results

Our index female patient (V:1, EB 315-01) was 27-years old when referred to our clinic for consultation of her unclassified EB subtype. She was one of two affected daughters born to consanguineous Turkish parents (Fig. 1A). Mild blistering began after walking age at 1.5 years and slightly restricted her physical education activities at school. Both hand and toenails were dystrophic from the age of 3 years. Blistering became generalized with pruritus when in puberty at 14 years and lesions healed with scarring and hyperpigmentation. Mucous membranes were not affected and enamel was normal. She displayed excoriated and verrucous papules and intact blisters (Fig. 1B) most pronounced on the acra with residual post-inflammatory hyperpigmentation (Fig. 1C–E). Focal plantar hyperkeratosis and severe nail dystrophy on both hand and toenails was seen (Fig. 1E). Scalp, axillary and pubic hair was normal. Her one-year younger sister (V:2, EB 315-02)
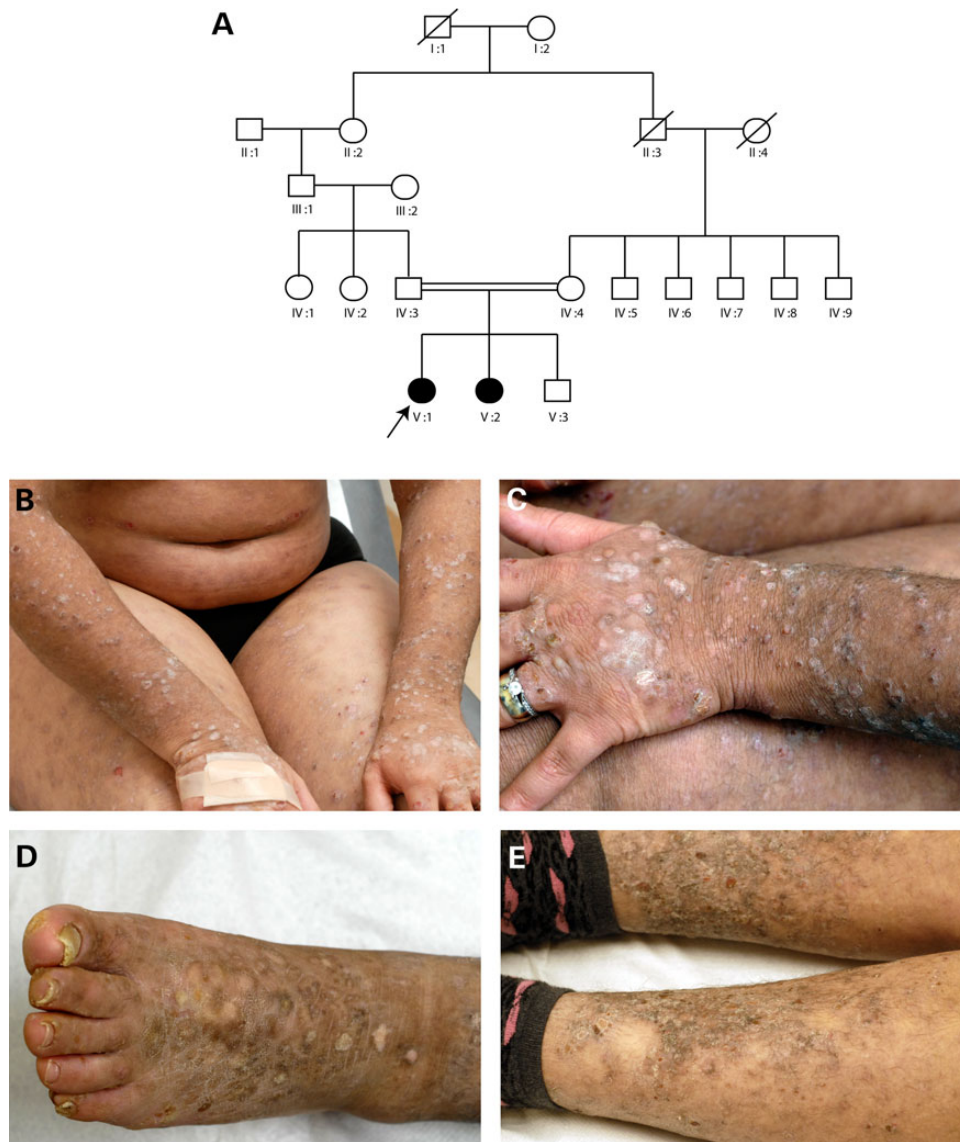
Figure 1. Clinical picture. (**A**) Pedigree of family 1 with EBS-plectin 1a. DNA was obtained from individuals V-1 and V-2. The rest of the family members was unavailable for consultation. (**B–E**) Clinical presentation of patient V:1. Generalized verrucous papules, excoriations and post-inflammatory hyperpigmentation of abdomen, thighs, shins and dorsal surface of arms.

had a similar history and distribution of progressive skin lesions, however displayed less pronounced hyperkeratotic papules and plantar hyperkeratosis possibly due to minor pruritus as compared to her sister (Supplementary Material, Fig. S1). Routine histopathology of a papule revealed dyskeratosis with papillar inflammation of the dermis (Supplementary Material, Fig. S2). Immunofluorescence antigen mapping of lesional skin showed a thin granular lining of keratin staining on the blister floor revealing a very low intra-epidermal cleavage in basal cells that led us to the diagnosis of EBS. Double immunofluorescence staining of intact patient's skin with affinity-purified isoform-specific rabbit antibodies to P1a and pan-plectin mouse monoclonal antibody (mAb) 10F6 recognizing all isoforms of plectin was negative for P1a (Fig. 2A), but positive for pan-plectin, producing a linear pattern along the epidermal BMZ, albeit of markedly reduced intensity compared to control skin (Fig. 2B). In the latter, staining for P1a revealed the same pattern and intensity of linear fluorescence along the BMZ as that for pan-plectin (Fig. 2D–F). The expression of integrin α6β4 was normal (data not shown). A 1.5-mm punch biopsy, obtained for fixation and transmission electron microscopy, revealed hypoplastic hemidesmosomes and intra-epidermal pseudo-junctional cleavage with the plasma membrane of basal cells visible on the blister floor (Fig. 2M and N). Together, these findings indicated *PLEC* as a candidate gene. DNA was isolated from peripheral blood and mutation analysis of the coding region of the *PLEC* gene including exon-intron boundaries of all 8 isoforms revealed a homozygous nonsense mutation present in P1a, c.46C>T leading to a premature termination codon p.Arg16X (Fig. 3A and B). The affected sister of the index patient was a carrier of the same homozygous mutation. Further *PLEC* sequence analysis also revealed a nucleotide transition in the promoter sequence of isoform 1, c.-27C>T. Following mutation analysis, immunofluorescence mapping was extended with isoform-specific antibodies to P1a and anti-pan-plectin mAb 10F6 on control striated muscle and myocardium from normal cadavers. P1a was not expressed by myocytes in striated muscle and myocardium, whereas the shared plectin epitope (10F6) was prominently stained at Z-disks of striated muscle (Fig. 2H) and intercalated discs of myocardium (Fig. 2K). We also examined stratified epithelia other than skin of human controls, and found expression of P1a along the basement membrane of the oral cavity mucosa and to a limited extent in the conjunctiva (Supplementary Material, Fig. S3).

When cell lysates of cultured skin keratinocytes from both of the affected sisters were subjected to immunoblotting using mAb 10F6 and antibodies to P1a, reduced 10F6 signals (V:1 16.5% and V:2 15.4%) compared to control cells were observed in both cases (Fig. 3C, first panel), whereas in neither case were P1a-positive bands detectable (Fig. 3C, second panel). Blot staining done with antibodies to desmoplakin as a control, for both patients revealed unchanged signals compared to control cells (Fig. 3C, third panel).

We performed quantitative RT-PCR (qRT-PCR) on cultured cells and found dominance of P1a transcription compared to plectin isoform 1c (P1c) in normal human keratinocytes and fibroblasts (Fig. 3D). The transcription level of P1a, but not of P1c, was reduced in patient keratinocytes and fibroblasts (Fig. 3D).

We also checked the transcription level of plectin isoforms in mouse epidermis and keratinocytes to compare with our human data and to line up with literature (6). Absolute quantification unveiled two important differences in isoform expression between epidermis and primary mouse keratinocytes. While P1c accounts for a substantial fraction (~80%) of the plectin transcript in epidermis, this is no longer the case in primary keratinocytes

(~20%), conversely, P1a transcript levels are higher (more than 50% of total) in primary keratinocytes than in epidermis (~20% of total) (Supplementary Material, Fig. S4).

Considering that plectin mutations leading to EBS can manifest with extracutaneous complications of muscle dystrophy and/or cardiomyopathy (3,4), the index patient was screened by a neurologist and cardiologist respectively. Upon physical examination no symptoms of muscular dystrophy were found and a muscle biopsy was deemed unnecessary. Cardiologic consultation including physical examination, electrocardiogram and an echocardiogram was carried out with no associated findings of cardiomyopathy. Moreover, her affected younger sister had no symptoms of muscular or cardiologic pathology. Currently both sisters, now aged 29 and 30 years, exhibit no signs of muscular dystrophy or cardiomyopathy.

Discussion

The spectrum of diseases in humans caused by *PLEC* mutations, 'the plectinopathies', continues to expand and be classified. The cytolinker scaffold protein plectin is present in essentially all mammalian tissue. This cardinal supporting structure is expressed in tissues such as heart, muscle, striated epithelia as well as the blood–brain barrier. At the cellular level, plectin strengthens tissues in regions of mechanical stress, such as linking desmin and the dystrophin glycoprotein complex in myocytes of the heart. In skin, plectin is a fundamental building block of the hemidesmosome, anchoring basal keratinocytes of the epidermis to the underlying dermis (7). *PLEC* is a large gene consisting of 32 exons with 8 tissue-specific isoforms which arise by alternative 5′splicing of transcripts (8–10). Molecularly, the plectin protein is built of a central α-helical coiled-coil rod domain flanked by N- and C-terminal globular domains (11). All 8 isoforms share exons 2-32 and differ only by distinct first exons (4) (Supplementary Material, Table S1). The various isoforms of plectin continue to be classified according to occurrence and tissue specificity arising from the alternate promoter regions and *cis* splicing from several different first coding exons (4,7). The most prominently expressed isoform of plectin in mouse and human cultured keratinocytes is P1a, followed by P1c and other isoforms among them P1, plectin isoform 1f (P1f) and P1b (Supplementary Material, Fig. S4) (see also Ref. 12).

That P1a is also dominantly expressed at the dermal–epidermal junction of human epidermis is suggested by (i) the identical pattern and intensity of P1a and pan-plectin staining using double immunofluorescence labeling of normal human skin (Fig. 2F), (ii) the severe reduction of the pan-plectin signal in patient skin (Fig. 2B), (iii) the ~84% reduction of pan-plectin in patient keratinocyte extracts (Fig. 3C, first panel) and (iv) qRT-PCR showing a higher level of P1a than P1c transcripts in cultured human keratinocytes in normal control cell populations (Fig. 3D). The dominance of P1c transcripts in mouse epidermis (Supplementary Material, Fig. S4A) is simply explained by the fact that primary keratinocytes lack the suprabasal layers where P1c is predominantly expressed while they are rich in hemidesmosome-like structures where P1a is found.

P1a was expressed also in oral mucosa, and to a limited extent in conjunctiva (Supplementary Material, Fig. S3). Since P1a is expressed in these epithelia in normal human controls one may have expected mucosal lesions in the patients. The fact that they were absent suggests that in these tissues P1a's loss was compensated by other proteins or isoforms of plectin.

Mutations in the exons common to all 8 isoforms (2-32) of this one gene continue to be reported and classified within the main
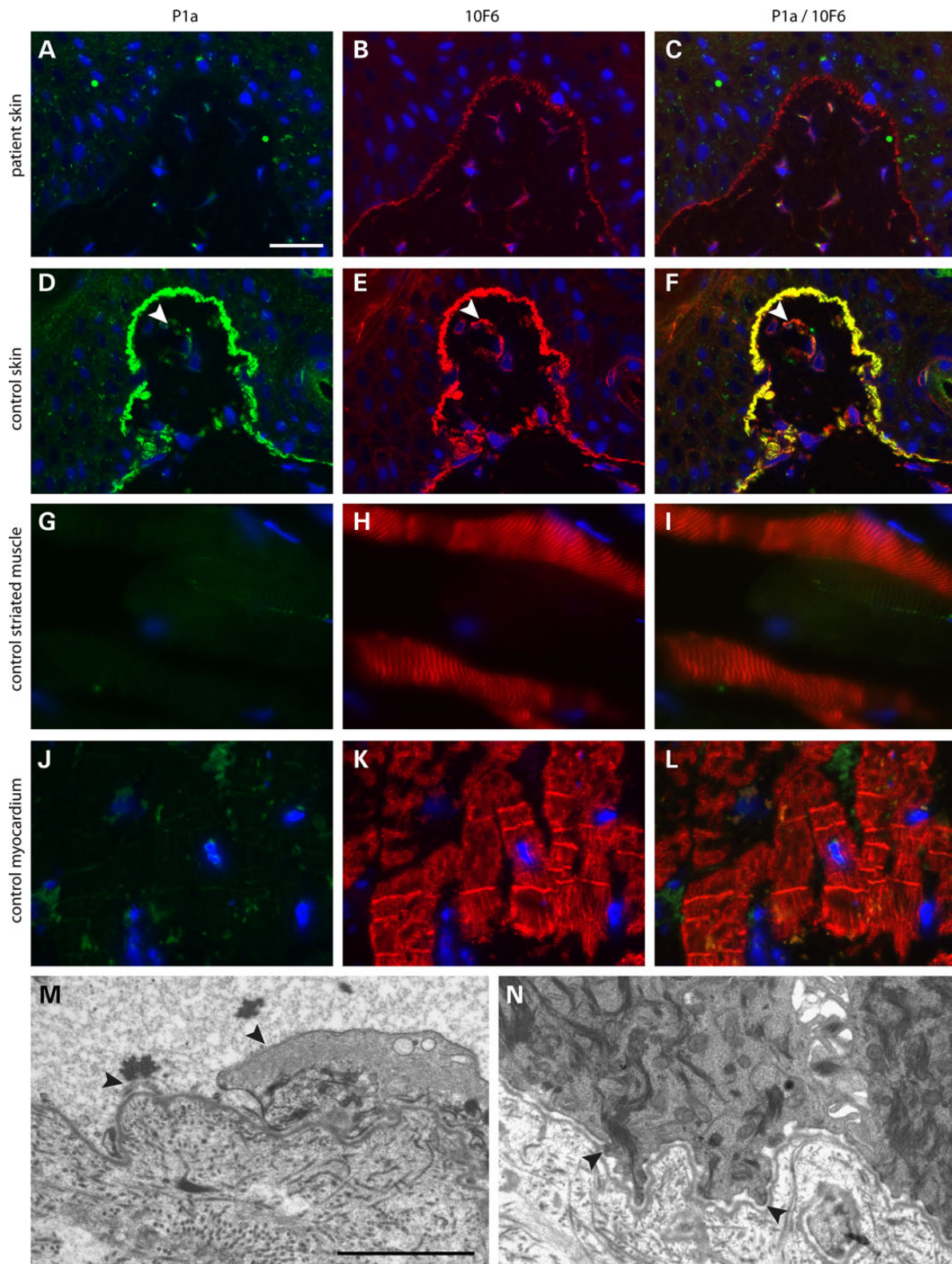
Figure 2. Double immunofluorescence microscopy of human skin, muscle and heart tissue using antibodies to P1a (green) and an epitope shared between plectin isoforms (red) and transmission electron microscopy of skin. In skin of patient V:1, P1a (**A**) is absent, whereas 10F6 staining at the BMZ is reduced (**B and C**). In control skin, P1a (**D**) and the shared epitope of all plectin isoforms (10F6) (**E**) display a strong linear staining that co-localizes (**F**) at the BMZ. Note that the staining of dermal blood vessels (arrowhead) with antibodies 10F6 is more dominant than that for P1a. Striated muscle of healthy controls shows no expression of P1a (**G**), whereas Z-disks clearly stain for the shared epitope of plectin (**H and I**). Myocardium of healthy controls shows no expression of P1a (**J**), whereas Z-disks and intercalated disks stain strongly for the shared plectin epitope (**K and L**). Bar in A corresponds to 25 μm for all immunofluorescence images. Transmission electron microscopy of ultrathin sections of patient skin (**M and N**). Lesional skin (**M**) reveals remnants of keratinocyte membranes (arrowheads) lining the blister floor fitting with a 'pseudo-junctional' intra-epidermal split level. The intact skin (**N**) shows hypoplastic hemidesmosomes (arrowheads). Bar in M corresponds to 2 μm for both EM images.
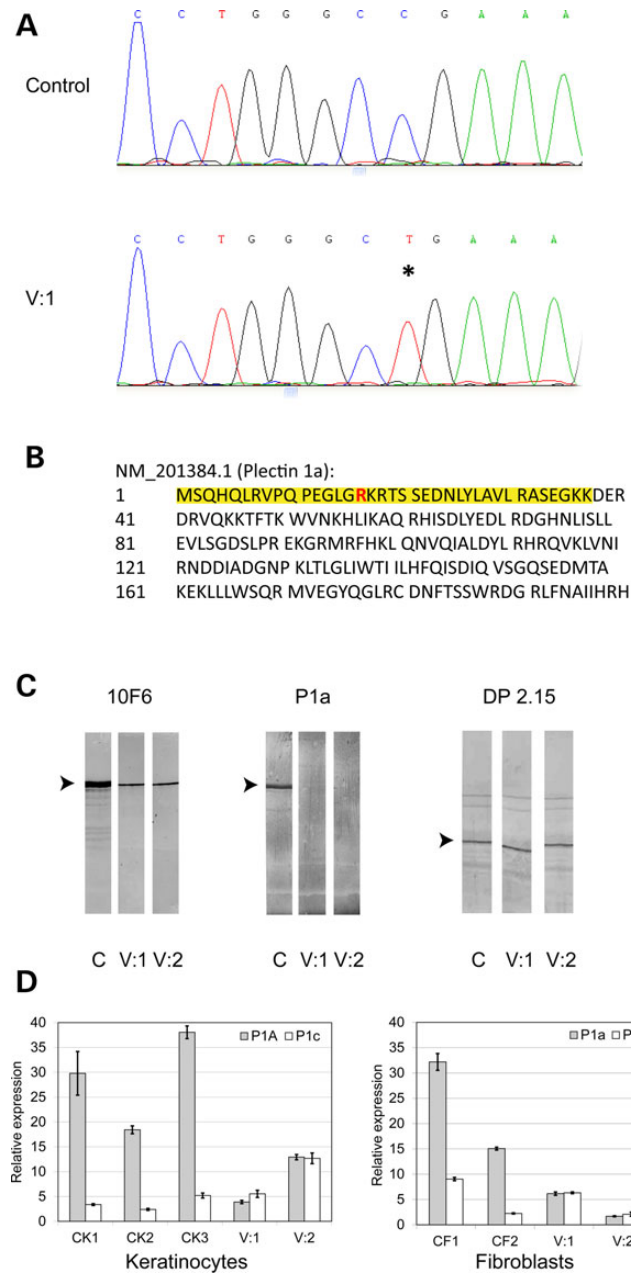
Figure 3. DNA sequence analysis, immunoblotting and RT-PCR of P1a in patients. DNA sequencing of *PLEC* (**A**) revealed a homozygous mutation in exon 1a encoding the isoform-specific sequence of P1a in patient V:1. A cytosine at position 46 is exchanged for a thymine (**c.46C>T**). This homozygous nucleotide change results in an arginine at position 16 rendering a stop codon (**p.Arg16X**) in isoform 1a but not in any other plectin isoform (**B**). The isoform-specific exon 1a is highlighted in yellow. (**C**) Immunoblotting of cell lysates from cultured keratinocytes derived from normal humans (lanes C) and patients (lanes V:1 and V:2). First panel (10F6): A marked reduction (80–84%) of band intensity is seen for both patients using mAbs 10F6 which bind to the rod domain common to all isoforms of plectin, including P1a. Because of the absence of P1a, the 10F6 signal (representing total plectin in skin) is reduced as expected. Arrow, position of plectin (molecular mass ~ 500 kDa) deduced from the position of molecular size markers (not shown). Second panel (P1a): P1a-specific antibodies show no detectable expression of the protein in keratinocytes from patients, whereas P1a was markedly expressed in normal human keratinocytes. Arrow, as in first panel. Third panel (DP2.15): Antibodies to human desmoplakin I (250 kDa) and II (215 kDa) show normal expression of both proteins in both patients, similar to cultured keratinocytes from unaffected humans. Arrow, position of desmoplakin I. (**D**) Quantitative RT-PCR of P1a (gray) and P1c (white) mRNA expression in cultured keratinocytes and fibroblasts from human control (CK1-3 and CF1,2, respectively) and corresponding patient (V:1,V:2) specimens. Bars represent expression relative to household gene B2M. Error bars, standard deviations of experiments run in triplicates. Note the predominance of P1a above P1c in control keratinocytes (CK1-3), and the diminished expression in patient cells.

phenotypic groups (Table 1). New *PLEC* mutations resulting in EBS are frequently reported and account for 8% of all cases of EBS (3). However, except for one (13), all of them reside within exons that are common to all isoforms (4). Epidermolysis bullosa simplex with pyloric atresia (EBS-PA, [MIM 612138]) is characterized by neonatal blistering, pyloric atresia and rapid clinical decline (14). Epidermolysis bullosa simplex with muscular dystrophy (EBS-MD,[MIM 226670]) displays congenital blistering and myopathy onset in later life (15). Additional myasthenic symptoms have been reported in several patients having EBS-MD characterizing yet another subtype, EBS-MD-Mys (16). An autosomal dominant (AD) disease is EBS Ogna (EBS-O [MIM 131950]) with mild

Table 1. The plectinopathies

Subtype	Exons affected	Inheritance	Organs affected	Severity	First reported case
EBS-MD	9–32	AR	Skin, striated muscle, myocardium	Severe and progressive	Smith *et al.* (15)
EBS-MD-Mys	13,31,32	AR	Skin, striated muscle	Severe and progressive	Selcen *et al.* (16)
EBS-PA	Distal exons	AR	Skin, pylorus	Lethal[a]	Charlesworth *et al.* (29)
EBS-Ogna	31	AD	Skin	Mild	Koss-Harnes *et al.* (17)
EBS-plectin 1a	1a	AR	Skin	Moderate	This article
LGMD2Q	1f	AR	Striated muscle	Severe and progressive	Gundesli *et al.* (13)

Subtypes listed: epidermolysis bullosa simplex-muscular dystrophy (EBS-MD), epidermolysis bullosa simplex-muscular dystrophy with myasthenic syndrome (EBS-MD-Mys), epidermolysis bullosa simplex with pyloric atresia (EBS-PA), epidermolysis bullosa simplex type Ogna (EBS-Ogna), epidermolysis bullosa simplex with pathogenic mutation in the first coding exon (exon 1a) of isoform 1a (EBS-plectin 1a), limb-girdle muscular dystrophy (LGMD2Q). Inheritance: Autosomal recessive (AR), Autosomal dominant (AD).
[a]One nonlethal case has been reported (5).

skin fragility (17) that renders the plectin polypeptide vulnerable to proteolysis (6). Of particular interest are the consequences of mutations in the various first exons because it is their tissue specificity which dictates disease manifestation. Comprehensive understanding of isoform expression in different tissues is a work in progress shared by different medical specialties. Recently, Gundesli *et al.* reported and characterized that mutations in the first coding exon of plectin isoform P1f are responsible for limb-girdle muscular dystrophy (LGMD2Q) without any skin involvement (13). Andrä *et al.* first reported and characterized the targeted cellular localization of P1a as the essential plectin isoform present in the hemidesmosome co-localizing with the integrin α6β4 subunit in mice (12). They suggested that this isoform was the culprit responsible for hereditary EBS in humans. This was later confirmed by Walko *et al.* who showed that hemidesmosome failure was caused by decreased levels of isoform P1a in knock-in mice for dominantly inherited epidermolysis bullosa simplex type Ogna (EBS-Ogna) (6). In mice, P1a is the predominant isoform that localizes to the epidermal BMZ (12). The persistent expression of common plectin epitopes in the epidermal BMZ of our patients suggests that in humans, like in mice, other plectin isoforms are co-expressed in the epidermal BMZ. Compensation of the additional non-mutated isoforms known to be expressed in the epidermis (i.e. P1c) did not preserve the hemidesmosome architecture at adult age and led to skin fragility. However, both studied patients did not develop generalized lesions until puberty implicating that perhaps compensation occurred from their non-mutated isoforms during childhood.

The expression of P1a was absent in both normal human striated and cardiac muscle tissues as shown in this study. Due to the fact that the pathogenic mutation did not affect coding exons shared by other isoforms whose role has previously implicated Z-disks of striated muscle and intercalated disks of myocardium, we do not expect development of myopathy or cardiomyopathy. Moreover, late-onset plectin-mediated (cardio) myopathy develops before the age of 30 years (18). The consequence at the protein expression level of the homozygous c. −27C > T variant of plectin isoform P1 is not known. In most genes at this position TATA box promoter sequence elements are surrounded by GC-rich sequences for transcription regulation (19). In the case of c.−27C > T no such sequences can be distinguished. It is possible that c. −27C > T might be a rare polymorphism, commonly present in the Turkish population.

In conclusion, we present and characterize the first human phenotype of the genodermatosis EBS caused by a homozygous nonsense mutation in exon 1a of the *PLEC* gene. Our findings uphold those of previous ones that have implicated P1a as the dominant isoform upon which hemidesmosome stability is dependent (20).

Materials and Methods

Patients

The patients (EB 315-01, V:1 and EB 315-02, V:2), were two sisters diagnosed with EBS based on clinical findings, immunofluorescence microscopy, electron microscopy and molecular analysis. All experiments performed were done with material obtained earlier for diagnostic purposes which did not require extra approval from the institutional ethical committee. Patients gave informed consent for the use of photographs and tissue samples. 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 immunofluorence antigen mapping from both affected individuals. Immunofluorescence antigen mapping of skin biopsies and cultured human keratinocytes were done as described before (21). The monoclonal antibodies and their origin have been described previously (6,22,23). Mouse monoclonal antibodies 10F6 were directed against an epitope in plectin's rod domain (24). Isoform-specific antibodies to P1a, raised in rabbit against a synthetic peptide corresponding to a 12-amino acid residue-long sequence (KRTSSEDNLYLA) encoded by human exon 1a, were used after affinity-purification (6,12,25); the sequence of the peptide used as immunogen is conserved between man, mouse and rat. Antibodies were diluted in PBS/OVA 1% and sections were incubated at room temperature for 45 min. The secondary antibodies used for double staining were green FITC-conjugated F(ab')2 goat anti-rabbit IgG (1:50; Zymed Laboratories, Carlsbad, CA) and red LRSC-conjugated donkey anti-mouse IgG (1:50; Jackson Immuno Research, Newmarket, UK). Validity of species-specific binding was ascertained by cross experiments. Hoechst 33342 was used to stain nuclei. Pictures of sectors were merged with Adobe Photoshop CS3 software.

Electron microscopy

Two-mm punch biopsies of perilesional and non-lesional skin taken from the upper arm were obtained and prepared as described previously (21).

Molecular analysis

Genomic DNA was extracted from peripheral blood lymphocytes using standard laboratory methods. The *PLEC* gene encompassing the isoforms 1, 1a, 1b, 1c, 1d, 1e, 1f and 1g were screened for mutations by sequencing analysis of all exons including exon–intron boundaries. Primer sequences are available upon request.

Western blotting

SDS–PAGE and western blotting were performed as described previously (26,27). As substrate we used extracts from cultured keratinocytes from our patients and healthy controls. After SDS–PAGE and transfer onto nitrocellulose, the proteins were identified with the same antibodies as used for immunofluorescence microscopy; in addition antibodies DP 2.15 directed against desmoplakin I/II (Abcam®) were used. To calculate plectin expression total protein content of patient and control extracts were compared by running serial dilutions of all extracts on SDS–PAGE gels. After visualization of the proteins with 'Blue Silver' staining (28) the gels were scanned and the intensity of staining of the lanes was used to calculate the relative protein content of the different extracts. Next, serial dilutions were run on one SDS–PAGE gel, and after western blotting plectin was visualized with antibody 10F6. The blot was scanned and the relative plectin expression was calculated from the intensity of staining using Quantiscan software.

Quantitative RT-PCR (qRT-PCR)

For human samples, transcriptor universal cDNA master (Roche, Basel, Switzerland) was used for reverse transcription of a single RNA isolation of each cell population. P1a, P1c and the housekeeping gene beta-2-microglobulin (*B2M*) were verified by qPCR using FastStart Universal SYBR Master (Rox) reagent and an Applied Biosystems 7900ht (Foster City, CA, USA). Primers used are in Supplementary Material, Table S2.

Quantitative RT-PCR of mouse samples was performed as previously described (Fig. S8 in ref 6). Estimated absolute copy numbers of plectin isoforms were calculated from standard curves obtained by serial dilutions of plasmids harboring the different exons. Values were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) transcript levels.

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

Funding

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