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Original Article

Identification of two rare and novel large deletions in *ITGB4* gene causing epidermolysis bullosa with pyloric atresia

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Abstract: Epidermolysis bullosa with pyloric atresia (EB-PA) is a rare autosomal recessive hereditary disease with a variable prognosis from lethal to very mild. EB-PA is classified into Simplex form (EBS-PA: OMIM #612138) and Junctional form (JEB-PA: OMIM #226730), and it is caused by mutations in *ITGA6*, *ITGB4* and *PLEC* genes. We report the analysis of six patients with EB-PA, including two dizygotic twins. Skin immunofluorescence epitope mapping was performed followed by PCR and direct sequencing of the *ITGB4* gene. Two of the patients presented with non-lethal EB-PA associated with missense *ITGB4* gene mutations. For the other four, early postnatal demise was associated with complete lack of β4 integrin due to a variety of *ITGB4* novel mutations (2 large deletions, 1 splice-site mutation and 3 missense mutations). One of the deletions spanned 278 bp, being one of the largest reported to date for this gene. Remarkably, we also found for the first time a founder effect for one novel mutation in the *ITGB4* gene. We have identified 6 novel mutations in the *ITGB4* gene to be added to the mutation database. Our results reveal genotype–phenotype correlations that contribute to the molecular understanding of this heterogeneous disease, a pivotal issue for prognosis and for the development of novel evidence-based therapeutic options for EB management.

Key words: epidermolysis bullosa – genodermatoses – inherited skin diseases – *ITGB4* mutations – pyloric atresia

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Introduction

Epidermolysis bullosa (EB) is a rare genetic autosomal disorder characterized by muco-cutaneous fragility. More than 30 EB subtypes caused by mutations in 18 different genes have been described (1). EB with pyloric atresia (EB-PA) is inherited as an autosomal recessive trait and is classified into Simplex form (EBS-PA: OMIM#612138) or Junctional form (JEB-PA: OMIM#226730) depending on whether the detachment plane is intra-epidermal or within the basal lamina, respectively (1,2). EB-PA is caused by mutations in the *ITGA6* and *ITGB4* genes or in the *PLEC* gene, which encode the structural components of the hemidesmosome-anchoring complex (3,4) $\alpha 6\beta 4$ integrin (5–7) and plectin, respectively (8–10).

EB-PA resulting from defects in $\alpha 6\beta 4$ integrin is clinically heterogeneous with a variable prognosis from lethal in early infancy to very mild. Lethal EB-PA usually results from the presence of a premature termination codon (PTC) in the coding sequence of *ITGA6* and *ITGB4* genes due to direct changes or insertions, deletions or splice-site mutation that cause a frameshift in both alleles, while non-lethal outcome is usually related to the presence of a missense mutation in at least one allele (11,12). However, a few lethal cases resulting from missense/missense or a combination of missense/PTC mutations have also been reported (6,11,13–15), suggesting that not only the nature of the mutation but also its position within the protein and the functional domain affected are key determinants of phenotype severity (6).

Most of the mutation reported in the *ITGB4* gene (16,17) are private mutations and correspond to single base substitution or very small deletions (1–10 bp). In contrast, deletions of more than 10 bp are rare (5,14,18,19), and, to the best of our knowledge, only one large deletion spanning 1.2 kb of *ITGB4* gene has been previously described (18).

In this study, we have characterized six new cases (from five families) and disclosed six novel mutations in the *ITGB4* gene causing pyloric atresia combined with a spectrum of cutaneous and extracutaneous phenotypes, ranging from mild to lethal. Two of these novel mutations are rare deletions, including one that spans 278 bp and encompasses an entire exon and part of the

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downstream intron. In addition, a transition change located at the splice donor site of intron 24, resulting in splicing alterations, has been characterized. Finally, we describe for the first time a founder effect for one novel mutation in the *ITGB4* gene in the Spanish population. Our study contributes to further characterize the genotype–phenotype correlations of this highly heterogeneous disease and provides key information for proper clinical management and genetic counselling.

Materials and methods

Patients and samples

Six patients with PA and clinical features compatible with EB were investigated. When suitable, two skin punch biopsies (4–6 mm) were taken. EDTA blood was obtained from all patients and progenitors. Clinical reports and phenotypic features were collected using a standard clinical quiz. Appropriate informed consent was obtained from all individuals analysed and/or from parents/legal representatives. The study was conducted in accordance with the principles of Declaration of Helsinki and Universal Declaration of UNESCO and approved by the ethics committee of the collaborative hospitals.

Immunomapping

Immunofluorescence analysis (IF) for dermo-epidermal junction (DEJ) antigens was performed on frozen skin sections (20). Monoclonal antibodies used are listed in Supplementary Information S1. IF and electron microscopy reports were available for patients 1 and 3, respectively.

Mutation detection

Genomic DNA (gDNA) was extracted from peripheral blood (Qiagen, Vento, The Netherlands). Specific primers for PCR amplification were designed to amplify all 41 exons and intron boundaries of *ITGB4* gene (21). Fragments were sequenced using Big Dye Terminator V.1.1 Cycle Sequencing kit and examined on a 3730 DNA Analyser (Life Technologies, Carlsbad, CA, USA).

Genotyping of patients 3, 5 and 6 by allele separation was performed as at least one mutation was not clearly identified by direct sequencing of PCR products. Briefly, PCR fragments were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and cloned using pGEM®-T Vector System I (Promega Co., Madison, WI, USA). At least ten clones were sequenced in each case to detect all allelic variants.

As patients 2 and 4 were carrying a common mutation, gDNA of their relatives was examined for six polymorphic microsatellite markers that scan the neighbouring regions of the *ITGB4* gene (D17S1864, D17S1535, D17S1301, D17S1839, D17S1603 and D17S785). Genetic distance and microsatellite information were taken from the Genethon human linkage map and from the Marshfield chromosome 17 map (22). Fluorescently labelled alleles were analysed on an ABI PRISM 3100 automated DNA genetic analyser (Applied Biosystems, Foster City, CA, USA).

The frequency of the three novel mutations found in Spanish patients was tested by screening 100 unrelated ethnically matched healthy individuals, and was less than 1% in all cases. Sequence nomenclature was based on the NCBI RefSeq for *ITGB4*: NM_001005731.1 and NM_000213.3.

Keratinocyte culture and RNA isolation

Keratinocytes were isolated from non-lesional skin biopsies and cultured as previously described (23). The novel mutation found in patient 3 was characterized in cDNA by RT-PCR. RNA was extracted from keratinocyte culture with TRIzol® Reagent (Life Technologies) and transcribed to first-strand cDNA using SuperScript_III First Strand (Life Technologies). Sequences of specific primers designed to amplify regions of interest spanning exons 4–8 or exon 21–27 are listed in Supplementary Information S2.

Results

Clinical and genetic findings in patients carrying *ITGB4* mutations

A summary of the genotypic and phenotypic patient features is presented in Table 1.

Patient 1

A girl born by Caesarean section at 31 weeks of a pregnancy complicated with polyhydramnios (Fig. 1a). She is the first daughter of healthy consanguineous parents from Pakistan. There was no family history of skin or gastrointestinal disorders. At birth, she presented limited blistering on limbs and ears. On the first day of life, distended stomach and PA were surgically repaired. At the age of 3 years, she exhibited onychodystrophy and subungual hyperkeratosis in toenails and severe ocular lesions. The patient was diagnosed with EBS-PA based on the intra-epidermic level of cleavage. IF report depicted a deficiency of β4 integrin at the dermo-epidermal junction (DEJ). A previously reported (15) homozygous missense mutation c.3674G>A (p.R1225H) in *ITGB4* gene was disclosed. Both progenitors were healthy heterozygous carriers of this mutation.

Patient 2

A girl of Spanish origin, born at 32 weeks of a gestation, complicated with polyhydramnios. Her parents were non-consanguineous and had an older child who had developed anal stenosis but no other remarkable clinical findings. There was no family history of skin blistering disorders. Patient 2 developed cutaneous lesions, mucosal involvement and onychodystrophy. JEB diagnosis was made by electron microscopy. At 3 weeks of age, PA was surgically corrected. At 19 months of age, the patient also had developed oral, ocular and urinary involvement. Additionally, she presented signs of malnutrition and suffered frequent infections. At the age of 3 years, Patient 2 had an acceptable quality of life, was of normal height and suffered two small active erosions, nail dystrophy, alterations of enamel and hoarseness. At present, the patient, who is 8 years old, is almost free from skin lesions, but presents onychodystrophy and toenail loss, enamel hypoplasia and caries (Fig. 1b). The ultrastructural report indicated the presence of areas of dermo-epidermal detachment at the lamina lucida level. IF analysis revealed a linear labelling of the basal lamina (comparable to controls) for all antigens studied, including β4 and α6 integrins, in the areas without detachment (Fig. 2; P2).

Two novel point mutations were disclosed in the *ITGB4* gene, c.997T>G in exon 8 and c.1370G>A in exon 11, resulting in the substitution of two highly conserved amino acids in the extracellular domain of β4 integrin, p.Y333D and p.C457Y, respectively. Cys 457 residue is part of the cysteine-rich tandem repeats. The p.Y333D mutation was also found in Patient 4. Segregation of both mutations was confirmed in family members.

Patient 3

A male of Romanian origin born by Caesarean section at 36 weeks of gestation due to polyhydramnios. His parents were non-consanguineous. At birth, Patient 3 presented widespread aplasia cutis

Table 1. Summary of clinical and molecular findings in EB-PA patients

Patient	Sex	Birth	EB type	Paternal mut	Maternal mut	Immunomapping[1]	PA	AC	Muco-cutaneous blistering, nails and teeth	Renal manifestations	Ocular findings
1	Female	2010 Alive	Simplex	c.3674G>A(15) p.R1225H	c.3674G>A p.R1225H	Intra-epidermic α6 ↓ β4 -[2] Plectin -	Yes	No	Localized blistering Onychodystrophy		Painful conjunctival ulcerations, corneal scars and leucoma
2	Female	2005 Alive	**Junctional**	**c.1370G>A p.C457Y**	**c.997T>G p.Y333D**	Lamina basal α6+ β4 +	Yes	No	Localized blistering, mostly oral (tongue) Onychodystrophy and nail loss enamel hypoplasia and caries	Urinary retention	Conjunctivitis
3	Male	2011 Demise	**Junctional**	**c.470_566+182del p.A157Gfs*2**	**c.2783-2A>G p.D928Gfs*20**	Lamina basal α6 - β4 -	Yes	Yes		Ureterohydronephrosis and deficient cortico-medullary differentiation (right and left kidney, respectively)	Conjunctivitis corneal ulcers corneal opacity since birth.
4	Male	2010 Demise	**Junctional**	c.3321_3331del p.S2008Lfs*25	**c.997T>G p.Y333D**	Lamina basal α6 - β4 -	Yes	Yes	Oral mucosa blistering Onychodystrophy		
5	Male	2012 Demise	**Junctional**	**c.701G>T p.G234V**	**c.3707_3725del19 p.T1236Sfs*28**	Lamina basal α6↓ β4 -	Yes	Yes	Oral mucosa blistering Onychodystrophy	Bladder urothelial detachment, bilateral ureterohydronephrosis and severe electrolytic imbalance	
6	Male	2012 Demise	**Junctional**	**c.701G>T p.G234V**	**c.3707_3725del19 p.T1236Sfs*28**	Lamina basal α6↓ β4 -	Yes	Yes	Oral mucosa blistering Onychodystrophy	No	

Novel mutations found in this cohort are in bold, and references for previously reported ones are provided.
Expression of plectin in patients 2, 3, 4, 5 and 6 was similar to control skin.
[1]↓ reduced; − absent; + present.
[2]Performed with clone 7 antibody recognizing integrin beta 4 endodomain.

including left leg and left parieto-occipital area (Fig. 1c) with severe ocular findings. Abdominal echography showed severe renal manifestations. There was no family history of skin or gastrointestinal disorders. PA required a termino-terminal anastomosis. The patient had two septic episodes and died at postnatal day 20 due to metabolic imbalance and renal failure.

Immunomapping showed cleavage at the level of the basal lamina with collagen XVII at the blister roof whereas laminin 332 and collagen VII mapped to the floor (data not shown). In areas without dermo-epidermal detachment, the signal for these markers was comparable to the control. The expression of both, α6 and β4 integrins, was almost absent (Fig. 2; P3).

We found that Patient 3 is a compound heterozygous carrier of two novel mutations in *ITGB4* gene leading to a PTC in both alleles. A 278-bp deletion comprising the whole exon 6 and 182 pb of the following intron (c.470_566+182del) was found (Fig. 3a). In the other allele, the mutation c.2783-2A>G alters the splice acceptor site consensus sequence of intron 24 (Fig. 3c). To analyse the effect of these mutations on gene transcription, we performed RT-PCR. Using primers spanning exons 4–8, two different transcripts were obtained (Fig. 3b; line P3), one corresponding to the wild-type sequence (black arrowhead) and another lacking exon 6 (red arrowhead). Using primers spanning exons 21–27, four different products were obtained (Fig. 3c; line P3). One of them corresponded to the wild-type cDNA sequence (black arrowhead), and the other three were aberrant transcripts (red arrowheads). Sequencing of these transcripts (Fig. 3c; line P3: central red arrow) revealed that in one of them intron 24 was retained, showing a G to A single base substitution at intronic position (−2) (c.2783-2A>G). The other two aberrant transcripts lacked exon 26, and one of them also retained intron 24 (Fig. 3c; line P3: upper and lower red arrows, respectively). All three aberrant transcripts were absent in mRNA from healthy controls.

Patient 4

A male of Spanish origin born by Caesarean delivery at 35 weeks of gestation due to presumptive duodenal atresia. The parents were non-consanguineous, and they had no other children. There was no family history of skin or intestinal disorders. At birth, Patient 4 presented widespread aplasia cutis involving legs, feet, hands and face. Erosions in the nose and penis glans, blisters in the oral cavity (Fig. 1d) and onychodystrophy were also present. The duodenal atresia was surgically treated at postnatal days 3 and 9, leaving an ileostomy in the second intervention. The patient died at postnatal day 33 as a consequence of multiple organ failure.

A complete loss of β4 integrin expression was found by IF staining. Expression of α6 integrin was located at both blister sides (roof and floor) denoting the level of split at the basal lamina (Fig. 2; P4). In areas without detachment, immunoreactivity against other basement membrane components was comparable to the control (data not shown).

Patient 4 presented an 11-bp deletion in exon 28 of the *ITGB4* gene, (c.3321del11) causing a PTC 84-bp downstream. This mutation had been previously described in homozygous state in another Spanish patient with lethal outcome (5). The second mutation, c.997T>G in exon 8 of *ITGB4* gene, was novel and also found in Patient 2. It results in the substitution of an amino acid, p.Y333D, located at the extracellular domain of β4 integrin. Microsatellite study of the region around the *ITGB4* gene revealed that the c.997T>G mutation segregates with a common haplotype shared by the two families carrying it (Fig. S1).

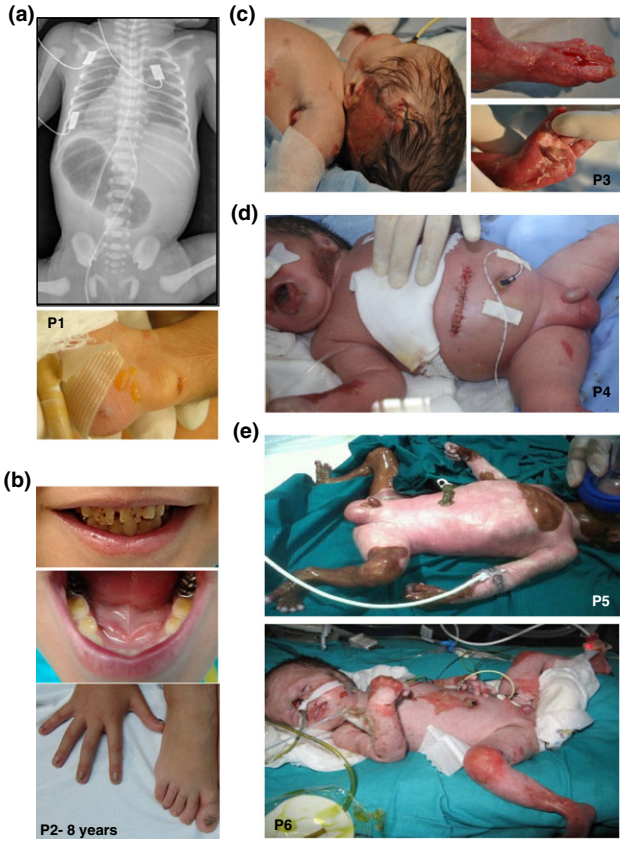

Figure 1. Clinical presentation of EB-PA patients. (a) X-ray image of patient 1 in which distension of stomach is evident (upper panel) and a picture showing blisters on baby's heel; (b) Patient 2 exhibited enamel hypoplasia, caries and nail loss and onychodystrophy as the main clinical features; (c) Clinical pictures of patient 3 showing toenail loss and thumb articular retraction besides aplasia cutis in the left side of the head and left limbs; (d) Patient 4 presented aplasia cutis in neck and extensive erosion in his right forearm. Surgical scar resulted from gastrointestinal atresia correction; (e) Clinical pictures of Patients 5 and 6, dizygote twins who exhibited extensive areas of aplasia cutis and body widespread erosion.

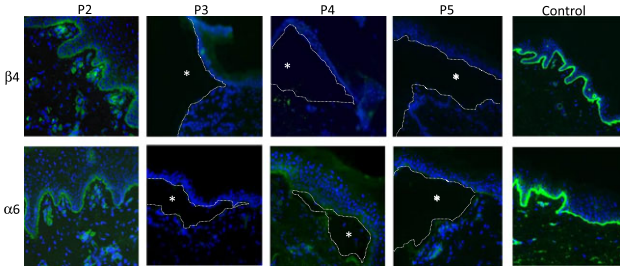

Figure 2. Immunofluorescent staining pattern of α6 and β4 integrins in the skin of EB-PA patients. Specific antibodies against α6 integrin subunit (CD49f) or β4 integrin subunit (3E1) were used. In patient 2, a continuous staining along the intact DEJ is indistinguishable from control. Junctional detachment and absence of integrin β4 are depicted in blisters (*) in patients 3, 4 and 5. Expression of α6 integrin is absent in patient 3 and reduced in patients 4 and 5. White dashed lines delimited blister floor and ceiling.

Patient 5 and Patient 6

Patient 5 and Patient 6 were Spanish male newborn dizygotic twins derived from *in vitro* fertilization of oocyte donor. Delivery was induced by Caesarean section at 34 weeks of gestation due to polyhydramnios and presumption of intestinal atresia. At birth, Patient 5 presented extensive aplasia cutis involving neck, thorax and limbs and widespread cutaneous blistering (Fig. 1e; P5). PA was confirmed and surgically treated. Bladder urothelial detachment, bilateral ureterohydronephrosis, severe electrolytic imbalance and sepsis caused by *Candida albicans* were also diagnosed. The baby died at postnatal day 17. The second twin also presented aplasia cutis, cutaneous blistering (Fig. 1e; P6) and pyloric and oesophageal atresia but no urinary involvement and died at 1 month of age due to respiratory complications and a severe infection.

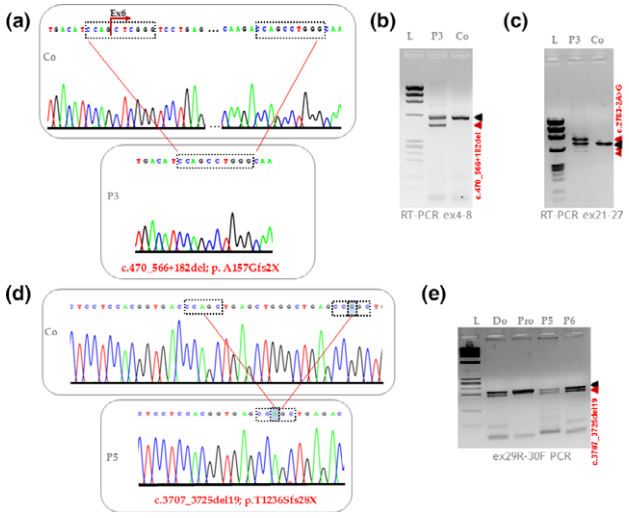

Figure 3. Analysis of *ITGB4* gene deletions in patients 3 and 5 (a) Electropherogram corresponding to the sequence of PCR clones containing exon 6. Mutant sequence from patient 3 (P3, bottom panel) and control wild-type sequence (Con, upper panel). Homologue sequences found at the deletion break point are boxed. (b) Electrophoresis analysis of RT-PCR products with cDNA of keratinocytes from patient 3 (P3 line) and a control (Con line) using primers for *ITGB4* exons 4–8. Black arrowhead points to the wild-type allele present in both, P3 and unaffected Con. Red arrowhead points to the band corresponding to mutant allele shorter in length due to c.470_566+182del. (c) Electrophoresis analysis of RT-PCR products with cDNA of keratinocytes from patient 3 (P3 line) and a control (Con line) using primers to *ITGB4* exons 21–27. Black arrowhead point wild-type allele present in both, P3 and unaffected Con. Three additional bands pointed by red arrowheads corresponding to aberrant transcripts are present in P3. A major band, longer than wild-type allele, corresponds to the mutant allele due to retention of intron 24. Lower size, attenuated bands correspond to alleles lacking exon 26. (d) Electropherogram corresponding to the sequence of PCR clones containing exon 29. Mutant sequence from patient 5 (P5, bottom panel) and control wild-type sequence (Con, upper panel). Homologue sequences found at the deletion break point are boxed. (e) Electrophoresis analysis of PCR products with gDNA from patient 5 (P5 line), patient 6 (P6 line), their progenitor (Pro line) and the oocyte donor (Do line) using primers for *ITGB4* exons 29-30. The black arrowhead indicates the band corresponding to the wild-type allele that is also present in unaffected individual. The red arrowhead indicates the band corresponding to mutant allele, shorter in length than wild type, which is present in P5, P6 and Do who is the carrier of deletion.

IF staining for α6 integrin was reduced and was localized to the floor and roof of the blister, whereas staining intensity for laminin 332 was normal and restricted to the floor, showing that the cleavage occurred at the basal lamina level. Signal for β4 integrin was completely absent (Fig. 2; P5).

Both siblings inherited the same two novel mutations. A point mutation in exon 7, c.701G>T, of *ITGB4* gene was disclosed, leading to the substitution of a strictly conserved amino acid, p.G234V, placed in the von Willebrand factor type A domain of the β4 integrin extracellular domain. In addition, both patients had a deletion of 19 bp in exon 30 (c.3707-3725del19) resulting in a frameshift from codon 1236, which predicts a protein with 27

new amino acids and a premature stop codon, p.T1236Sfs*28 (Fig. 3d,e).

Discussion

We have described six patients with EB-PA caused by mutations in the *ITGB4* gene. Six of the mutations disclosed here are novel, and two have been previously described (5,15). In 5 patients, immunomapping revealed cleavage within the DEJ; therefore, these patients were diagnosed with JEB-PA, the most frequent clinical presentation associated with mutations in the *ITGB4* gene.

Patient 1 was the only patient diagnosed with EB simplex as the level of split occurs mainly within the basal layer. EB simplex is relatively rare in patients carrying *ITGB4* mutations. Patient 1 carried a homozygous mutation c.3674G>A resulting in an amino acid substitution, p.R1225H, in the intra-cytoplasmic moiety of β4 integrin (24). This missense mutation disrupts the interaction of β4 integrin with plectin, preventing the localization of plectin to the hemidesmosome (25), which might explain the intra-epidermal cleavage (H & E staining; data not shown). Crystallographic analysis suggests that these plectin-binding sites, which are part of one of the second fibronectin III repeats, are involved in the allosteric control of integrin β4 adhesion properties (26). The p.R1225H mutation had been previously described in heterozygosity in a patient carrying two other mutations in the other allele of the *ITGB4* gene (c.3112-3C>T, p.L336P) (15) that are likely responsible for the manifestation of a more severe phenotype. Remarkably, Patient 1 exhibited corneal scarring, a symptom mainly observed in severe and generalized phenotypes associated with Dystrophic EB and Herlitz-JEB subtypes (27). A previous report by Dang *et al.* described that of a total of 49 patients with *ITGB4* mutations, three of them also presented corneal scarring. Two of them exhibited a non-lethal phenotype (6,15). Accordingly, Patient 1 showed a complete lack of plectin expression similar to that found in these non-lethal cases. Interestingly, the absence of β4 integrin staining in one of the patients was related to a homozygous mutation in the third fibronectin type III repeat of the intra-cytoplasmic domain of β4 integrin, which interacts with type XVII collagen that in turn, is also a plectin partner (28).

In our study, all six patients exhibited pyloric atresia during pregnancy and two of them were alive at the age of 4 and 9 years old. This finding supports that PA is not an appropriate criterion to predict a lethal outcome (19). On the other hand, in our cohort, aplasia cutis was only present in lethal cases although it has been clearly associated with all clinical forms of EB, including those with milder phenotypes (29,30).

The two surviving patients, Patient 1 and Patient 2, carried missense point mutations in *ITGB4* in both alleles (p.R1225H/p.R1225H and p.C457Y/p.Y333D, respectively). Our result confirms previous observations that non-lethal cases are usually associated with missense (or non-PTC splice-site mutations) in at least one allele resulting in integrin β4 proteins that might retain some functionality (2,19,20). Multiple sequence alignment of β4 integrin subunits between different species reveals strict conservation of both cysteine at positions 457 and tyrosine at position 333. However, alignment of different human integrin β-chains (β1-β6) shows that only Y333 residue is conserved among different subunits. The relative pathogenic potential of the two novel point mutations, which might correlate with the different degree of conservation of these two residues, might explain the differences observed between the two patients carrying the Y333D mutation. Both mutations were discarded as allelic variants in an ethnically matched healthy control population.

Lethal cases (Patient 3, Patient 4, Patient 5 and Patient 6) showed null expression of β4 integrin and significant reduction or absence of α6 integrin, in agreement with previous reports (6,15). In Patient 3, null expression of β4 integrin resulted from *ITGB4* mutations (c.470_566+182del/c.2783-2A>G) leading to PTC in both alleles (p.A157Gfs*2/p.D928Gfs*20). Although most PTC-containing transcripts are degraded by non-sense-mediated mRNA decay, these transcripts are readily detectable. The c.2783-2A>G changes the consensus splice acceptor sequence of intron 24, and results in 3 aberrant transcripts, one enriched product retaining intron 24 and two less abundant that lack exon 26. One of these two minor transcripts also keeps intron 24, suggesting that the elimination of exon 26 in both alleles might be mediated by additional changes in introns 25 or 26.

Patient 3 also carried a deletion that eliminates the entire exon 6 and part of the downstream intron. Remarkably, the deletion break point contains the CCAGC motif, also present in the so called recombination hotspot of the Alu core sequence, as a part of the *chi* element (CCACCAGC) of *Escherichia coli*, and, partially, in the non-homologous recombination breakpoint of the immunoglobulin loci (31). Interestingly, this motif has been frequently identified in or nearby the breakpoint of previously reported *ITGB4* deletions (see Table S1). Moreover, and independently of the presence of this motif in the breakpoint vicinity, a clear homology between the downstream and upstream sequences flanking the deletions is observed. It is known that deletions can be produced by the repair mechanisms acting when DNA damage, that is double-strand break (DSB), occurs. The repair machinery occasionally uses short (5–25 bp) homologous sequences near the DSB through a process called micro-homology-mediated end joining (MMEJ) that always results in deletions (32,33). The presence of small-size repeats such as the ones found in the region flanking deletions 2 and 3 (14,18) (Table S1), predisposes to regional genomic instability (34). Moreover, small-size repeats are implicated in deletions affecting genes such as CTRF (35) and NRXN1 (34) that are associated to other pathologies.

The combination of missense/PTC mutations rarely results in lethal outcomes (5,36). However, patients Patient 4, Patient 5 and Patient 6 in our study died shortly after birth. Patient 4, a carrier of compound heterozygous mutations, a deletion of exon 28 (c.3321del11) and a single amino acid substitution (c.997T>G; p.Y333D), completely lacked β4 integrin expression, as assessed by immunostaining and, resulted in a lethal outcome, while p.Y333D substitution resulted in only a mild phenotype when combined with C457Y amino acid substitution in Patient 2. Expression from the C457Y allele might therefore account for β4 protein detection in Patient 2. Similarly, in Patient 5 and Patient 6 the substitution of a highly conserved glycine for valine at codon 234 combined with a deletion of 19 bp in the other allele resulted in early demise (Patient 5 and 6). These findings, together with protein sequence conservation at these positions across species, suggest that Y333 and G234 play a crucial role in *ITGB4* function or stability. Both residues are located in the integrin von Willebrand A domain. G234 is the second glycine residue contained in the PEGG motif, which

is implicated in metal coordination (37). The first G in this motif is mutated in *ITGB3* gene (p.G247D) in patients diagnosed with Glanzmann thrombasthenia and prevents expression of the integrin complex on the platelet membrane (38).

The Y344 residue in the integrin $\beta 3$ subunit, corresponding to Y333 in the integrin $\beta 4$ subunit, is also mutated in Glanzmann thrombasthenia patients (p.Y344C, p.Y344S). Residues 324–366 constitute a cysteine-rich region important for heterodimer intersubunit surface interactions (39).

Interestingly, the c.3321del11 mutation, causing a PTC, was also present in homozygous state in another lethal case of Spanish ancestry (15). The presence of this mutation in two separate patients could indicate the existence of a hotspot. However, as both patients are of Spanish origin, a founder effect could not be excluded. In the case of the p.Y333D mutation, also found in Patient 2, a common ancestor was confirmed by microsatellite analysis (Fig. S1). This is the first founder effect described in the Spanish EB-PA population and second in the world population (40).

In conclusion, we have identified and described novel mutations in *ITGB4* in the Spanish EB population and found that the genotype–phenotype correlations in our patients can be explained by functional inferences based on the location of these residues in the $\beta 4$ integrin protein. In addition, we have designed two PCR assays that could be used as a prenatal or pre-implantational test for the deletions described in this paper in future pregnancies and for the study of carriers in these families. The mechanism that might have originated these deletions was also discussed. The knowledge gained in this study contributes to further understand this disease, and to provide more accurate diagnosis and genetic counselling as well as new ideas and opportunities for both basic and translational research (41).

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Conflict of interest

The authors have declared no conflicting interests.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. Pedigrees of families from patients 2 and 4.

Table S1. Common motif flanking large deletions (represented *in italics)* in *ITGB4* gene.

References

1 Fine J D, Eady R A, Bauer E A *et al.* J Am Acad Dermatol 2008: **58**: 931–950.
2 Chung H J, Uitto J. Dermatol Clin 2010: **28**: 43–54.
3 Christiano A M, Uitto J. Exp Dermatol 1996: **5**: 1–11.
4 Pulkkinen L, Uitto J. Exp Dermatol 1998: **7**: 46–64.
5 Ashton G H, Sorelli P, Mellerio J E *et al.* Br J Dermatol 2001: **144**: 408–414.
6 Dang N, Klingberg S, Rubin A I *et al.* Acta Derm Venereol 2008: **88**: 438–448.
7 Vidal F, Aberdam D, Miquel C *et al.* Nat Genet 1995: **10**: 229–234.
8 Charlesworth A, Chiaverini C, Chevrant-Breton J *et al.* Br J Dermatol 2013: **168**: 808–814.
9 Nakamura H, Sawamura D, Goto M *et al.* J Mol Diagn 2005: **7**: 28–35.
10 Pfendner E, Uitto J. J Invest Dermatol 2005: **124**: 111–115.
11 Pulkkinen L, Kim D U, Uitto J. Am J Pathol 1998: **152**: 157–166.
12 Pulkkinen L, Rouan F, Bruckner-Tuderman L *et al.* Am J Hum Genet 1998: **63**: 1376–1387.
13 Masunaga T, Ishiko A, Takizawa Y *et al.* Exp Dermatol 2004: **13**: 61–64.
14 Micheloni A, De Luca N, Tadini G *et al.* Br J Dermatol 2004: **151**: 796–802.
15 Nakano A, Pulkkinen L, Murrell D *et al.* Pediatr Res 2001: **49**: 618–626.
16 Stenson P D, Mort M, Ball E V *et al.* Hum Genet 2014: **133**: 1–9.
17 Yuen W Y, Sinke R J, Jonkman M F. Br J Dermatol 2013: **168**: 432–434.
18 Birnbaum R Y, Landau D, Elbedour K *et al.* Am J Med Genet A 2008: **146A**: 1063–1066.
19 Schumann H, Kiritsi D, Pigors M *et al.* Br J Dermatol 2013: **169**: 115–124.
20 Ruzzi L, Gagnoux-Palacios L, Pinola M *et al.* J Clin Invest 1997: **99**: 2826–2831.
21 del Castillo I, Villamar M, Moreno-Pelayo M A *et al.* N Engl J Med 2002: **346**: 243–249.
22 Dib C, Faure S, Fizames C *et al.* Nature 1996: **380**: 152–154.
23 Rheinwald J G, Green H. Cell 1975: **6**: 331–343.
24 Jonkman M F, Pas H H, Nijenhuis M *et al.* J Invest Dermatol 2002: **119**: 1275–1281.
25 Koster J, Kuikman I, Kreft M *et al.* J Invest Dermatol 2001: **117**: 1405–1411.
26 de Pereda J M, Lillo M P, Sonnenberg A. EMBO J 2009: **28**: 1180–1190.
27 Fine J D, Johnson L B, Weiner M *et al.* Am J Ophthalmol 2004: **138**: 254–262.
28 Borradori L, Koch P J, Niessen C M *et al.* J Cell Biol 1997: **136**: 1333–1347.
29 Chiaverini C, Charlesworth A, Fernandez A *et al.* Br J Dermatol 2014: **170**: 901–906.
30 Smith S Z, Cram D L. Arch Dermatol 1978: **114**: 81–84.
31 Rudiger N S, Gregersen N, Kielland-Brandt M C. Nucleic Acids Res 1995: **23**: 256–260.
32 McVey M, Lee S E. Trends Genet 2008: **24**: 529–538.
33 Verdin H, D'Haene B, Beysen D *et al.* PLoS Genet 2013: **9**: e1003358.
34 Chen X, Shen Y, Zhang F *et al.* Am J Hum Genet 2013: **92**: 375–386.
35 Tang S, Moonnumakal S P, Stevens B *et al.* J Cyst Fibros 2013: **12**: 290–294.
36 Iacovacci S, Cicuzza S, Odorisio T *et al.* Exp Dermatol 2003: **12**: 716–720.
37 Bhattacharya A A, Lupher M L Jr, Staunton D E *et al.* Structure 2004: **12**: 371–378.
38 Jallu V, Dusseaux M, Panzer S *et al.* Hum Mutat 2000: **31**: 237–246.
39 Kannan M, Ahmad F, Yadav B K *et al.* J Thromb Haemost 2009: **7**: 1878–1885.
40 Natsuga K, Nishie W, Shinkuma S *et al.* Exp Dermatol 2011: **20**: 74–76.
41 Has C, Kiritsi D. Exp Dermatol 2015: **24**: 325–331.