Cold cases in epidermolysis bullosa: not the usual suspects
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Heterozygosity for a novel missense mutation in the \textit{ITGB4} gene associated with autosomal dominant epidermolysis bullosa

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List of abbreviations:

DEB: dystrophic epidermolysis bullosa

EB: epidermolysis bullosa

EBS: epidermolysis bullosa simplex

EBS-PA: epidermolysis bullosa simplex with pyloric atresia

HD: hemidesmosome

JEB: junctional epidermolysis bullosa

JEB-loc: junctional epidermolysis bullosa, localized

VWFA: von Willebrand factor type A doma
**Importance** Epidermolysis bullosa (EB) is a group of mechanobullous genodermatoses characterized by the fragility of skin and mucous membranes. Mutations in the *ITGA6* and *ITGB4* genes, encoding the hemidesmosomal protein integrin α6β4, have been involved in the pathogenesis of EB. To date, the inheritance of these particular genes is known to be exclusively autosomal recessive. Here we report a novel heterozygous missense mutation in the *ITGB4* gene exerting a dominant negative effect that co-segregates with the EB phenotype in an extended family.

**Observations** The clinical phenotype of affected individuals is primarily characterized by nail dystrophy, and late onset of mild skin fragility and acral blistering. Some patients developed granulation tissue in the larynx, urethra, lacrimal duct and external auditory canal. Sequencing the complete set of genes associated with EB revealed a heterozygous missense mutation in exon 5 of *ITGB4*: c.433G>T, p.Asp145Tyr. The mutation was found in the affected relatives and was not present in unaffected relatives and control DNA samples.

**Conclusions and relevance** This study highlights, for the first time, the possibility of a dominant mode of inheritance for a missense *ITGB4* mutation in EB, thus expanding the mutational database and genotype-phenotype correlation for this rare disease.

Epidermolysis bullosa (EB) comprises a group of heterogeneous inherited blistering diseases. Mutations in multiple genes encoding proteins responsible for the maintenance of dermal-epidermal adhesion and skin integrity have been implicated in the disease pathophysiology. Based on the level of tissue cleavage, EB is further subdivided into epidermolysis bullosa simplex (EBS) with intra-epidermal cleavage, junctional epidermolysis bullosa (JEB) with intra-lamina lucida cleavage, dystrophic epidermolysis bullosa (DEB) with sublamina densa cleavage and Kindler syndrome (KS) with a mixed cleavage plane. Loss-of-function mutations in the *ITGA6* and *ITGB4* genes encoding the integrin α6 and integrin β4 subunits, respectively, have been implicated in EB. The possible subtypes include: epidermolysis bullosa simplex with pyloric atresia (EBS-PA), junctional epidermolysis bullosa with pyloric atresia (JEB-PA) and junctional epidermolysis bullosa localized (JEB-loc). The latter subtypes are reported solely involving the integrin β4 subunit, whereas the subtypes with pyloric atresia involve either integrin α6 or β4 subunit. The clinical phenotype of the affected individuals presents as a spectrum ranging from neonatal death to mild skin fragility and nail dystrophy. Pyloric atresia and urethral strictures may occur; these symptoms are, however, not obligatory for diagnosis. Integrin α6β4 is a heterodimeric transmembrane polypeptide located at the core of the hemidesmosomes (HDs).
This protein plays a major role in linking the intracellular hemidesmosomal plaque of the basal keratinocytes to the underlying basement membrane, thus providing mechanical resilience to the skin and mucous membranes. Also, integrins are known to execute agile responses to changes in the local environment and mediate transduction of signaling across plasma membranes for important cell functions such as migration, proliferation, and apoptosis.\(^3\) To date, epidermolysis bullosa due to ITGB4 and ITGA6 gene mutations has been reported to be inherited exclusively in an autosomal recessive manner.\(^1\) Here, we report a novel autosomal dominant missense mutation in the von Willebrand factor type A domain (VWFA domain) of integrin \(\beta4\) associated with an EB phenotype.

**Report of a Pedigree**

The index patient (Figure 1, IV:2, EB 301-03) of this family had progressive nail dystrophy of the hands and feet since birth, and eventually total loss of nails on his feet. After puberty he developed mild acral blistering on palms, soles and wrists (Figure 2 A, B). In addition, this patient suffered from chronic granulation tissue formation in the right external auditory canal, which led to a relapsing obstruction needing repeated surgery. The external auditory canal wound was finally closed by autologous split-skin graft transplantation (Figure 2 C) from a donor site located on the upper left arm. Subsequently, a 3-year long post-operative complication occurred consisting of delayed wound healing at the donor site with hypergranulation of the wound bed. Multiple attempts to facilitate healing with autologous cultured keratinocytes transplants were mostly unsuccessful. He also developed obstructed lacrimal ducts, leading to lacrimation. Another problem was the development of urethral strictures for which an urethroplasty was performed. At physical examination, hair and teeth were unaffected and he had no palmo-plantar keratosis. His younger brother also suffered from external auditory canal and urinary tract involvement. Interestingly, his youngest daughter developed laryngeal stenosis due to exuberant granulation tissue formation at age 5 (Figure 2 D), for which tracheostomy was performed. She underwent several endoscopic procedures, in which the granulation tissue was repeatedly removed by laser therapy, followed by local application of mitomycine. Finally, an endoscopic posterior cricoid split with rib cartilage interposition was performed, which resulted in successful decannulation. The child had one relapse for which she received local application of mitomycine once more. During a two-year follow-up she remains asymptomatic.
The pedigree of this family clearly demonstrates an autosomal dominant mode of inheritance (Figure 1). The clinical phenotype predominantly manifests as pachyonychia dystrophic nails and late-onset, mild, acral blistering. Some family members developed extracutaneous complications including granulation tissue in the larynx and involvement of urethra, lacrimal duct and external auditory canal (Figure 2).

Immunofluorescence microscopy of nonlesional skin of the index patient and his daughters revealed normal staining for both extracellular and intracellular domains of integrin β4 with monoclonal antibodies 58XB4 and clone 7, respectively. Plectin, dystonin-e, type XVII collagen, laminin 332 and type VII collagen were also normally expressed (not shown). The plane of cleavage could not be determined since no lesional skin biopsy was available. Electron microscopy of uninvolved skin showed an adequate number of hemidesmosomes (HDs), however, some were hypoplastic (Figure 3). The lamina densa was structurally abnormal with irregular thickness and some blind off shoots. The other components of the epidermal basement membrane zone were normal.

To identify the underlying genetic mutation for this disease, we applied in the index patient and his affected daughters our diagnostic next generation sequencing gene panel test consisting of a comprehensive set of 33 genes associated with EB, which is based on targeted SureSelect enrichment (Agilent Technologies Inc., Santa Clara, CA USA) and subsequent sequencing on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). We identified a heterozygous c.433G>T substitution in exon 5 of the ITGB4 gene resulting in the p. Asp145Tyr missense mutation (Figure 4 A). Sanger sequencing of genomic DNA confirmed the presence of this mutation in the patient and other affected family members. This mutation was not found in the Genome of the Netherlands,4 1000 genomes, or the ExAc Browser databases, and to our knowledge was not earlier described in the literature. The pathogenicity prediction software tool Alamut, version 2.0 (Interactive Biosoftware, Rouen, France), classifies the missense mutation as probably pathogenic. The p. Asp145Tyr missense mutation changes the acidic side chained aspartate to hydroxyl side chained tyrosine at codon 145 in the extracellular VWFA domain of integrin β4. Alignment of integrin β4 orthologues illustrated that residue p. Asp145 is highly conserved among species, which advocates its functional significance (Figure 4 B). To exclude that this mutation induced alternative splicing, nested PCRs surrounding the mutation were performed with cDNA isolated from skin biopsies from the index patient and his youngest daughter. No alternatively spliced cDNA products were observed (Figure 4 D); this result was in congruence with the assessment of in silico splice-site prediction software.
Discussion

Our findings emphasize the importance of considering heterozygous \textit{ITGB4} gene mutations as a possible cause for EB, where the pachyonychia dystrophic nails are typically the presenting symptom in affected individuals. Also, this study provides information on the natural history of this particular mutation in later life, with prognostic repercussions for younger patients, such as acral blistering after puberty.

Considering the dominant mode of inheritance of this disorder and the predominant feature of pachyonychia nail dystrophy, we considered pachyonychia congenita (PC) as a differential diagnosis. Other typical PC symptoms, such as oral leukokeratosis, cysts, and follicular keratosis were not part of the phenotype. In addition, involvement of the external auditory canal, lacrimal duct and urinary tract, delayed wound healing, and granulation tissue formation in the larynx have been reported in JEB, but not in PC.\textsuperscript{5,6} PC was excluded ultimately as diagnosis through our extended EB gene panel test, as no mutations in the genes $KRT6A$, $KRT6B$, $KRT6C$, $KRT16$ and $KRT17$, known to underlie PC,\textsuperscript{6} were identified.

Notably, granulation tissue formation in the larynx of individual V:2 is a clinical feature reminiscent of junctional epidermolysis bullosa- laryngo-onycho-cutaneous syndrome (JEB-LOC). This diagnosis was, however, excluded since no mutations have been found in \textit{LAMA3} and its isoform \textit{LAMA3A}.

The immunofluorescence studies with monoclonal antibodies against integrin $\beta 4$ in the index patient and his daughters were normal, which may not be surprising considering that the heterozygous missense mutation is not likely to result in diminished protein production. Notably, unaltered immunofluorescence staining for integrin $\alpha 6\beta 4$ has also been reported in a patient with homozygous missense mutations in \textit{ITGB4}.\textsuperscript{2} The EM findings in the skin sample of the index patient revealed a combination a normal and hypoplastic HDs. Integrin $\alpha 6\beta 4$ is essential for the assembly of HDs.\textsuperscript{7} In fact, this protein is one of the first to emerge at the basement membrane zone and is the nucleating factor for HD formation. The occurrence of hypoplastic HDs in the presence of this mutation confirms the important role for integrin $\beta 4$ in the maturation of these structures. In non-lethal cases of JEB due to mutations in the \textit{ITGB4} gene, HDs are present, although they are often incomplete.\textsuperscript{8} Abnormal assembly of mutated integrin $\beta 4$ and normal integrin $\alpha 6$ polypeptides could explain the occurrence of hypoplastic HDs. EM also revealed abnormal architecture of the basement membrane, which may be caused by the central role which integrin beta4 plays in its formation\textsuperscript{9} or due to
EM findings in the skin sample of the index patient revealed a combination a normal wound healing, and granulation tissue formation in the larynx have been reported involvement of the external auditory canal, lacrimal duct and urinary tract, delayed cysts, and follicular keratosis were not part of the phenotype. In addition, a differential diagnosis. Other typical PC symptoms, such as oral leukokeratosis, feature of pachyonychia nail dystrophy, we considered pachyonychia congenita (PC) as Considering the dominant mode of inheritance of this disorder and the predominant prognostic repercussions for younger patients, such as acral blistering after puberty. typically the presenting symptom in affected individuals. Also, this study provides mutations as a possible cause for EB, where the pachyonychia dystrophic nails are

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β4 and normal integrin α6 polypeptides could explain the occurrence of hypoplastic nucleating factor for HD formation. The occurrence of hypoplastic HDs in the presence of this mutation confirms the important role for integrin β4 in the maturation of these proteins and extracellular domains of transmembrane proteins. In fact, Yalak et al. reported six experimentally verified phosphorylation sites within the extracellular domain of integrin β4, including one on a tyrosine residue. In regard to our study, the p.Asp145Tyr substitution is intriguing because such event may create a novel phosphorylation site in the extracellular domain of integrin β4 and modify its function.

In eukaryotic cells phosphorylation usually occurs on serine, threonine and tyrosine residues. Latest advances in extracellular signaling research have provided mounting evidence of extracellular phosphorylation for a large number of extracellular matrix proteins and extracellular domains of transmembrane proteins. In fact, Yalak et al. reported six experimentally verified phosphorylation sites within the extracellular domain of integrin β4, including one on a tyrosine residue. In regard to our study, the p.Asp145Tyr substitution is intriguing because such event may create a novel phosphorylation site in the extracellular domain of integrin β4 and modify its function.

The lack of literature reports regarding dominant integrin β4 subtypes of EB may be better comprehended if only particular mutations, such as p. Asp145Tyr, in very specific regions, lead to clinical manifestations. For instance, in the case of EBS-Ogna, a plectinopathy, the dominant p. Arg2000Trp substitution results in a diseased phenotype. Another example is EBS with mottled pigmentation, a rare entity due to the p.Pro24Leu substitution in keratin 5 or the p.Met119Thr in keratin 14. Evidently, more investigation is necessary to address the exact mechanism by which the p.Asp145Tyr mutation changes dynamics of ligand interaction and signal transduction
in this particular mutated protein domain of integrin β4. Nevertheless, the extensive segregation in a dominant manner, exclusion of mutations in other EB related genes, and the typical clinical features suggest that this mutation is responsible for the EB phenotype in the affected individuals.

Given that not all patients developed exuberant granulation tissue in the mucous membranes, it is possible that other unidentified modifying genetic or epigenetic factors determine whether the patient will develop this particular clinical feature.

Conclusions

In contrast to previous concept, our data indicate that heterozygous missense mutations can cause an autosomal dominant subtype of EB. This expands our current knowledge on the genotype-phenotype correlation of EB. The phenotype of the affected individuals primarily includes pachyonychia nail dystrophy and mild acral skin fragility developing after puberty. Some patients developed extracutaneous complications in the external auditory canal, lacrimal duct, larynx and urethra. The molecular mechanism by which this particular mutation modifies integrin β4 function and leads to EB is yet to be established.

Acknowledgements

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Figure 1. Clinical pedigree of family with autosomal dominant EB due to ITGB4: p. Asp145Tyr mutation. DNA was obtained from individuals III:2, IV:2, IV:4, V:1, V:2, V:3; m/wt, mutation/wildtype, or wt/wt, wildtype/wildtype, respectively, underlines the genotype of the particular individual.
Figure 2. Clinical features of affected family member. A, Pachyonychia nail dystrophy consisting of nail thickening, transverse overcurvature, discoloration and brittleness in the index patient (IV-2 in Figure 1); B, Blistering on the sole of the index patient. C, Postoperative circular external auditory canal stenosis, due to granulation tissue formation in the index patient. D, Granulation tissue (arrow) in the larynx of his youngest daughter (V-2 in Figure 1).
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Figure 3. Transmission electron microscopy of nonlesional skin in the index patient. Both normal (solid arrow) and hypoplastic (dotted arrow) hemidesmosomes in adequate numbers along the epidermal basement membrane zone. The lamina densa displays irregular thickness and some blind off-shoots (asterisk). The intermediate tonofilaments are well inserted and the anchoring fibrils are present. Bar: 0.5 μm
**Figure 4.** Schematic representation of the integrin β4 subunit, conservation of residues and RT-PCR analysis. A, All known ITGB4 missense mutations involved in epidermolysis bullosa (EB) are indicated above the schematic polypeptide with evident clustering within the Von Willebrand factor type A (VWFA) domain. The heterozygous p. Asp145Tyr substitution reported in this study is shown in red below the schematic structure. B, Conservation of integrin β4 residue Asp^{145} (D letter code) between species. C, Alignment of integrin β subunits sequences shows evident conservation (except integrin 88) of DDL peptide sequence (boxed area). D, Electrophoresis gel analysis of mRNA amplified by nested PCR’s in the index patient and his youngest daughter identified no alternatively spliced products in the index patient (IV:2, lane 3) and his youngest daughter (V:2, lane 2), compared to control (lane 1).
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