Lethal acantholytic epidermolysis bullosa due to a novel homozygous deletion in DSP: expanding the phenotype and implications for desmoplakin function in skin and heart

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Abstract

Desmoplakin is the major linker in desmosomes in epithelia and myocardium anchoring intermediate filaments by the C-terminus to plakoglobin and plakophilin in the desmosomal plaque. Mutations in the gene DSP encoding desmoplakin have been associated with various phenotypes affecting skin and/or heart. One of these phenotypes, lethal acantholytic epidermolysis bullosa (LAEB), is characterized by extensive postnatal shedding of epidermis leading to early demise and is caused by recessive mutations in the gene DSP resulting in truncation of the desmoplakin C-terminus. Here we describe two infants born to the same consanguinous parents who suffered extensive epidermal dislodgment and died shortly after birth. In addition, universal alopecia, anonychia, malformed ears, and an enlarged heart were observed. As the clinical diagnosis was LAEB DSP mutation analysis was performed. A homozygous deletion (c.2874del5) abrogating the donor splice site of exon 20 was found. The deletion is predicted to cause read-through in intron 20 with subsequent recognition of a premature termination codon, resulting in desmoplakin lacking its rod domain and C-terminus (Lys959MetfsX5). Electron microscopic analysis of skin biopsies showed absence of the desmosomal inner dense plaque and lack of tonofilament insertion. This is the second report of LAEB. Similar to the previously reported LAEB patient, a recessive DSP mutation was underlying LAEB in our cases. The findings suggest that cardiomyopathy may be part of the LAEB phenotype. Furthermore, they indicate that in addition to the desmoplakin C-terminus, the rod domain is dispensable for intra-uterine development but is essential for the inner dense plaque of desmosomes.
Introduction

Desmoplakin (DP) is an essential constituent of desmosomes, intercellular junctions present in tissues subject to mechanical stress, such as epithelia and myocardium (for review see1-3). DP functions as a link between the cytoplasmic intermediate filament (IF) system and the transmembrane desmosomal cadherins. DP consists of an N-terminal plakin domain followed by a central coiled-coil rod domain involved in homodimerization and a C-terminus containing IF binding sites.4,5 The N-terminus consists of a plakin domain with binding sites for the armadillo proteins plakoglobin, plakophilins, and desmosomal cadherins.6-8 Two DP isoforms, DPI (332 kD, 2871 aa) and DPII (259 kD, 2272 aa), are generated by alternative splicing of the DSP transcript in exon 23 encoding the rod domain responsible for parallel homodimerization.9-11 DPII lacks most of this domain and is thought to form monomers. Both DPI and DPII are present in all epithelia. In myocardium DPI is abundantly present, while only minimal expression of DPII is observed in some parts of the heart.12-14 Studies of DP knockout mice reveal that DP is essential for embryogenesis as reflected by embryonic lethality of DP -/- mice at E6.5 before formation of desmosomes in the embryo.15 Epidermal-specific DP knockout mice show dramatic dislodgment of epidermis.16 Their desmosomes were slightly reduced in number and size but completely lacked the inner dense plaque and IF insertion, and as a whole were torn out off the cell membrane, illustrating the necessity of DP in epithelial sheet formation and the maintenance of epithelial integrity under mechanical stress.

A wide variety of DSP mutations have been implicated in various human hereditary diseases affecting skin, ectodermal tissue and heart, both non-syndromic and in combination, such as striate palmoplantar keratoderma, arrhythmogenic right ventricular cardiomyopathy, Carvajal syndrome, Naxos-like syndrome, and skin fragility-woolly hair syndrome.17 Recently, Jonkman et al. described a new disorder, lethal acantholytic epidermolysis bullosa (LAEB, MIM# 609638), that shares marked similarities with epidermal-specific DP knockout mice with extensive epidermal dislodgment and structurally normal desmosomes, lacking keratin filament insertion, being torn out as a whole.18 The reported infant suffered early demise due to extensive epidermal erosions and massive fluid loss; compound heterozygous nonsense mutations in DSP leading to truncation of almost the complete DP C-terminus in both isoforms were identified.

Herein, we report the second and third cases of LAEB and add to the phenotype described in the initial LAEB case. A novel, recessive, out-of-frame DSP mutation was found, predicted to result in truncation of the DP rod domain and C-terminus. These findings indicate that in addition to the C-terminus, the DP rod domain is dispensable for embryogenesis and foetal development. However, they also underscore the role of the DP C-terminus in maintaining epithelial and myocardial integrity under mechanical stress and highlight the importance of the DP rod domain for development of the desmosomal inner dense plaque.
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Case report

The proband (EB232-01) is a 2284 g boy born at 36 6/7 weeks gestation to a 23 year-old G3P2 mother of Yemenese descent. The parents are first cousins. Their first child, a male, (EB232-02) died at three days of age of an erosive skin condition consistent with a lethal form of epidermolysis bullosa. A second child is normal. In the third pregnancy (proband) fetal ultrasound at 32 weeks gestation revealed growth parameters consistent with mild in-utero growth restriction. In addition, mild cardiac ventricular hypertrophy with reduced contractility was noted. Amniotic fluid was noted to be debris filled and there was distal tapering of the digits with 5th finger clinodactyly. The fetus was noted to be hairless at both the 32 and 36 week ultrasounds. The findings were consistent with recurrence of the skin condition. Immediately after birth the newborn was vigorous, but extensive cutaneous erosions were noted, and he was transferred to a tertiary level neonatal intensive care unit. Initial evaluation revealed a scalded appearance with extensive epithelial shedding covering at least 50% of the body at the end of the first day. Islands of pale, necrotic epidermis were floating on the erosions (figure 1a), similar as observed in the initial LAEB case (EB114-01) described by Jonkman et al.18 (figure 1b). Blistering was not seen. There was universal alopecia. Nail beds were present without nails and there was mild 2-3, 3-4 and 4-5 finger syndactyly with 5th finger clinodactyly. Extensive milia were seen over the nose. There were no natal teeth. The external ears revealed mild unravelling of the superior helices and retroversion. The ears in the previously affected baby (EB114-01) revealed prominent antihelices and squaring off of the superior helices.18 At the end of the first day the proband needed to be intubated because of respiratory failure. Skin biopsies taken for histology, electron microscopy (EM), and immunofluorescence studies demonstrated dermis without attached epidermis, precluding further analysis. His parents declined further studies, and requested withdrawal of medical intervention. He expired that same day. Autopsy was declined. Histology of the proband’s brother’s archival skin biopsies showed an acantholytic cell-poor intra-epidermal blister with a single row of preserved but partially necrotic basal cells on the blister floor.
Figure 1: Clinical features of the patients with lethal acantholytic epidermolysis bullosa (LAEB). (a) Widespread skin erosions and complete alopecia in the proband, EB232-01. Note the detached epidermis floating on the underlying erosions (arrow). (b) Previously unpublished photo of the original LAEB case, EB114-01. Note the similar findings, including the malformed ear.

Material and Methods

Genomic DNA
After obtaining informed consent of the parents genomic DNA (gDNA) was extracted from peripheral blood leucocytes from the proband (EB232-01). DNA of the affected brother (EB232-02) was extracted from formalin fixed paraffin embedded (FFPE) skin tissue. The complete gDNA coding sequence of DSP was amplified and directly sequenced including exon-intron borders, as previously described. Sequencing of the purified PCR products was performed using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA USA) and the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA USA). In order to confirm the 5 basepair deletion detected on the exon-intron border of exon 20-intron 20 in EB232-01 and EB232-02, and to exclude the mutation in 100 control gDNA samples a restriction enzyme assay (REA) was developed. In this REA the PCR fragments of exon 20 of EB232-01, EB232-02 and healthy controls were restricted with enzyme Tru9I (Fermentas GMBH, St.Leon-Rot, Germany). The restriction reaction was performed according to manufacturer’s protocol. The restriction fragments were visualized on a high quality agarose gel (3%) (RESponse™ Research PCR Agarose, Bioplastics, Landgraaf, The Netherlands). The Generuler™ 50bp DNA ladder (Fermentas GMBH, St.Leon-Rot, Germany) and a 25 bp DNA ladder (Invitrogen, Breda, The Netherlands) were used to asses the size of the resulting bands.

Histopathological and immunohistochemical analysis
Unfortunately, no skin samples containing epidermis from the proband (EB232-01) were available. An FFPE skin sample containing epidermis was available from his brother (EB232-02). In order to study the effect of the mutation at the protein level, immunofluorescence antigen mapping with monoclonal antibodies against different parts of the DP protein was performed.
as previously described\textsuperscript{21}, using archived fresh frozen skin of the previously reported LAEB\textsuperscript{18} case (EB114-01) for comparison. DP C-terminal directed antibodies (20B6, 2A5 and 5A3) were kindly provided by Dr K. Green (Philadelphia, USA) and Dr J.K. Wahl III (Omaha, USA). In addition, antibody Dp2.15 (Boehringer, Mannheim, Germany) against the DP N-terminus was used.

**Electron microscopy**

Electron microscopic (EM) analysis of archival glutaraldehyde fixed and epon-embedded skin blocks obtained from the proband and the proband’s affected brother was performed as previously described.\textsuperscript{21}

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**Figure 2.** DSP mutation analysis. (a) A homozygous 5 bp deletion (TAAGG, c.2874del5) in DSP was found in gDNA of EB232-01 and EB232-02. (b) The deletion comprised the four most 3’ nucleotides of exon 20 and the first nucleotide of intron 20 (red underline). Read-through in intron 20 would lead to a premature termination codon (PTC, red box) after 16 bp from the frameshift. (c) Schematic view of the desmoplakin (DP) protein with the location of the mutation just before the rod domain and affecting both DP isoforms (red double arrow). The compound heterozygous mutations of the previous LAEB case EB114-01 are also depicted (orange lines).
LAEB due to novel homozygous DSP mutation

Results

Genomic DNA

Sequencing results of the complete DSP gene of the proband (EB232-01) revealed a homozygous 5 bp deletion in the exon-intron boundary of intron 20 (figure 2a). The location according to the genomic NCBI sequence of DSP in chromosome 6 (RefSeq NC_000006) is 35403del5. The mRNA location is 3153del5 (2874del5 when starting from A of the first ATG in RefSeq NM_004415.2).

This 5 bp deletion comprises the four most 3’ nucleotides of exon 20 and the first nucleotide of intron 20 hereby completely disrupting the donor splice site of intron 20 (figure 2b). Splice site prediction programs (www.cbs.dtu.dk/services/NetGene2 and www.fruitfly.org) indicated that the donor splice site is not recognized as such anymore.22, 23 In the protein the mutation is located at the end of the DP N-terminus (figure 2c). The third nucleotide of ATT encoding isoleucine (I) at aminoacid position 958, and the complete AAG encoding lysine (K) at aminoacid position 959 are deleted. Read-through in intron 20 would lead to a premature termination codon (PTC) after 16 bp (p.Lys959MetfsX5).

![In silico REA with Tru1I on the PCR product of DSP exon 20 of control and EB232 gDNA.](image)

**Figure 3.** Restriction enzyme analysis (REA). (a) *In silico* REA with Tru1I on the PCR product of DSP exon 20 of control and EB232 gDNA. (b) Gel analysis of the products of REA shows a band of 158bp (163 minus the 5bp deletion) in EB232-01 material instead of two bands of 51bp and 112bp as in control.

Immunohistochemical analysis

Immunofluorescence microscopy on fresh frozen skin samples of the previously reported LAEB case (EB114-01,18) with the DP C-terminal truncating mutations showed negative staining for the antibodies against the DP C-terminus while antibodies against the DP rod and the N-terminus (Dp2.17 against DPI (Boehringer Mannheim GmbH, Mannheim, Germany) and Dp2.15 against DPI/DPII (Progen Biotechnik GmbH, Heidelberg, Germany)) were positive (supplemental figure S1). The anti-DP C-terminal antibodies thus provide a sensitive and quick screening method for
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DP C-terminal truncating mutations. Unfortunately the antibodies did not work on control FFPE tissue. In general the FFPE tissue of EB232-02 was of such poor quality that no conclusions could be derived at all. RNA extraction of patient FFPE tissue failed as well.

**Electron microscopy**
While the FFPE samples contained a few keratinocytes, the epidermis in the EM blocks was better preserved. The interfollicular epidermis showed acantholysis in the entire epidermis (figure 4a). The keratinocytes were incompletely separated and showed swollen mitochondria implicating cell distress. Desmosomes were reduced in number and very hypoplastic. Most desmosomes were torn out the cell membrane as a whole with the outer dense plaques still glued together. Half-desmosomes were seen as well and seemed to involve the most hypoplastic desmosomes, as if the separation through the densal midline was the last phase of pin-point hypoplasia (figure 4b). The inner dense plaque was completely absent, as was IF insertion in all desmosomes (figure 4b-inset). Outer dense plaques showed reduced diameter: 200 ± 50 nm (control 366.3 ± 6.9 nm)	extsuperscript{24}. The tonofilament skeleton showed midcytoplasmatic retraction (figure 4a). The basal keratinocytes were well-connected to the basement membrane although the hemidesmosomes appeared slightly smaller and with reduced tonofilament insertion as well (figure 4a).
Supplemental Figure S1: Immunofluorescence antigen mapping of skin of the previously reported LAEB case (EB114-01) with anti-DP antibodies shows positive staining with antibody Dp2.15 against the DP N-terminus, and negative staining with all anti-C-terminal antibodies. Original magnification 20x.
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Figure 4: Electron microscopy of LAEB patient EB232-02. (a) Acantholysis depicted in the stratum basale and lower stratum spinosum. Note the retraction of tonofilaments (white arrows). (b) Magnification of widened intercellular space shows small desmosomes lacking inner dense plaques (arrow) being torn out the cell membranes as a whole, and hypoplastic desmosomes separated into half-desmosomes (arrowhead). (b-inset) Desmosome lacking its inner dense plaque and tonofilament insertion.

Discussion

In 2005, Jonkman et al. published the first case of LAEB and delineated the role of truncating DSP mutations as the aetiology of the disorder.18 We present two additional cases of LAEB with confirmed mutations in the DSP gene encoding DP. These new cases, and additional studies performed on the previously reported LAEB case, expand the phenotype of LAEB and our understanding of the role of DP in epithelial and cardiac function.

A clinical diagnosis of LAEB was suspected in the proband (EB232-01) because of the striking phenotypic findings: extensive non-bullous epidermal dislodgment, universal alopecia, anonychia, and rapid post-natal demise. Comparison of this case with the first LAEB case also
revealed similarly malformed ears. In the first LAEB case (EB114-01), post-mortem examination revealed a dilated myocardium\textsuperscript{16}; this could not be attributed to DP alterations with certainty as secondary heart failure due to fluid loads and post-mortem tissue changes were plausible explanations. However, an enlarged heart with poor myocardial contractility was documented in the proband \textit{in utero}. Cardiac pathology was not directly confirmed as the family refused autopsy, but the findings of poor contractility in both cases strongly supports an association of LAEB with cardiomyopathy. From a mechanistic standpoint, cardiac involvement can be expected in LAEB as C-terminal truncating mutations cause loss of DP binding sites for desmin IFs in myocardium.\textsuperscript{25} All reported DSP mutations affecting the DP C-terminus have been associated with cardiomyopathy. However, the cardiomyopathy in these cases was never present at birth. A possible explanation for the early cardiac abnormalities in LAEB might be the severity of the mutations, causing complete truncation of the C-terminus and thus IF anchorage, while in the other mutations still a part of the C-terminus remained allowing some IF-binding (for review see also chapter 6 of this thesis).\textsuperscript{26}

\textit{DSP} mutation analysis in the proband and his brother revealed a homozygous 5 bp deletion in \textit{DSP}, confirming the diagnosis. The 5 bp deletion in \textit{DSP} involved the 3' site of exon 20 and the donor splice site of intron 20. Unfortunately no representative samples were available for mRNA extraction or to perform immunohistochemical staining to investigate the effect of the mutation on mRNA or protein expression. This 5 bp deletion, however, has several possible consequences for the DP protein. First, inclusion of intron 20 due to absence of the donor splice site would lead to a premature termination codon (PTC) after 16 bp in the new reading frame due to the deletion. This is predicted to elicit nonsense mediated RNA decay.\textsuperscript{27} However, nonsense mediated RNA decay would result in loss of DPI and II expression as the mutation is homozygous. We consider this highly unlikely as DP is essential for intra-uterine development indicated by \textit{DSP} knockout mice which die early in embryogenesis.\textsuperscript{15} Moreover, no homozygous DP null mutations in humans have been described. Therefore a more likely result of the PTC is truncation of both DPI and DPII after 963 residues, lacking the rod domain and C-terminus (figure 2c) but still containing the ability to bind PG and plakophilins and target to desmosomes, hereby rescuing embryonic and extra-embryonic tissue development during embryogenesis.\textsuperscript{6, 7} Another consequence of the intron 20 donor splice site deletion could be recognition of alternative splice sites with consequent exclusion of one or more exons, either leading to a frameshift and a subsequent PTC, or being in frame and leading to DP lacking part of its N-terminal plakin domain. As the previously described LAEB patient (EB114-01) lacked the DP C-terminus in both isoforms with loss of keratin filament insertion, and the phenotype was strikingly similar to cases in this present study, we favour the first hypothesis of a truncated DP. The previously reported LAEB case (EB114-01) with DP truncation after 1933 and 2058 aminoacids (figure 2c) showed that presence of the DP N-terminus and rod domain is sufficient for fetal development and tissue integrity \textit{in utero}.\textsuperscript{18} In this light it is also interesting to mention two other recessive C-terminal DP mutations that were both associated with the desmosomal
cardiocutaneous syndromes Carvajal syndrome [OMIM #605676] and Naxos-like syndrome. These syndromes are clinically characterized by the quartet of clinical features woolly hair, palmoplantar keratoderma, skin fragility and cardiomyopathy. The Carvajal syndrome mutation, p.7622delG (when starting from A of the first ATG in RefSeq NM_004415.2) was homozygous and caused truncation of the C-terminal part of the DP C-terminus (figure S2). The other mutation is a homozygous missense mutation, p.Gly2375Arg. These mutations show skin and cardiac abnormalities, but not as severe and early lethal as in the patients with LAEB, probably because some IF-binding takes place by means of the remaining parts of the C-terminus, while the mutations associated with LAEB in this study and in Jonkman et al. lead to absence of the complete C-terminus and therefore loss of DP-IF interaction (see also figure S2).

Supplemental Figure S2. The C-terminal affecting DSP mutations (above), with the resulting (predicted) DP proteins below.
**LAEB due to novel homozygous DSP mutation**

In utero amniotic fluid provides a protection against mechanical stress and allows development of epidermal tissue, even in the absence of appropriate keratin filament anchorage to desmosomes. Peri- and postnatal, proper keratin anchorage becomes essential because of the mechanical forces exerted on the skin. The present LAEB cases indicate that in addition to the C-terminus, the rod domain is dispensable for intra-uterine development as well. Interestingly, the ultrastructural data suggest that the DP rod represents the desmosomal inner dense plaque as this was lacking in skin of the proband (EB232-02) but present in skin of the previous reported LAEB case (EB114-01). Desmosomes in epidermal DP-knockout mice rescued by a transgene encoding the DPΔC-terminus ultrastructurally resemble the desmosomes observed in skin of the first LAEB case (EB114-01) with presence of normally structured desmosomes but lacking IF insertion.\(^\text{16, 18}\) Mice lacking both the DP rod domain and C-terminus showed loss of the inner dense plaque as well.\(^\text{30}\)

Archival fresh frozen samples from the previously reported LAEB case elegantly showed complete absence of staining with the DP C-term directed antibodies while Dp2.15 and Dp2.17 showed normal expression levels hereby providing a valuable immunofluorescence diagnostic for LAEB due to DP C-terminal truncating mutations. Immunofluorescence antigen mapping with a panel of antibodies against different epitopes of DP can thus provide a valuable diagnostic parameter for early diagnosis and guiding mutation analysis in newborns exhibiting extensive skin blistering with the suspicion for LAEB. Important is an appropriate fresh frozen skin sample containing at least some epidermis. DP C-terminal directed antibodies will show absence of staining in LAEB.

In summary, we have presented two new cases of LAEB associated with a novel homozygous deletion mutation in **DSP** predicted to lead to the most severe DP truncation reported yet, involving both the DP C-terminus and the complete rod domain. Contrary to the normal desmosomes in the previously reported LAEB case lacking its DP C-terminus, EM analysis showed lack of the desmosomal inner dense plaque, similar to mice lacking their DP rod and C-terminus, thus indicating that the DP rod comprises the inner dense plaque. This report furthermore suggests that cardiomyopathy and malformed ears may be part of the syndrome. Immunofluorescence analysis of fresh skin biopsies with anti C-terminal DP antibodies can provide in a rapid diagnosis of LAEB.

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References


