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ARTICLE

Carriers with functional null mutations in *LAMA3* have localized enamel abnormalities due to haploinsufficiency

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The hereditary blistering disease junctional epidermolysis bullosa (JEB) is always accompanied by structural enamel abnormalities of primary and secondary dentition, characterized as amelogenesis imperfecta. Autosomal recessive mutations in *LAMA3*, *LAMB3* and *LAMC2* encoding the heterotrimer laminin 332 (LM-332) are among the genes causing JEB. While examining pedigrees of JEB patients with *LAMA3* mutations, we observed that heterozygous carriers of functional null mutations displayed subtle enamel pitting in the absence of skin fragility or other JEB symptoms. Here, we report two new *LAMA3* functional null mutations: nonsense c.2377C>T p.(Arg793Ter) and splice-site c.4684+1G>A mutation in heterozygous carriers exhibiting enamel pitting. Both parents had offspring affected with JEB and displayed subtle enamel pitting of secondary dentition without any sign of skin blistering. The reported enamel abnormality in *LAMA3* mutation carriers could be attributed to a half dose effect of the laminin α3 chain (haploinsufficiency).
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INTRODUCTION

Epidermolysis bullosa (EB) is an inherited blistering disease affecting the adhesion of epithelial cells.[1] Autosomal recessive (and very seldom dominant) mutations in the genes *COL17A1*, *ITGA3, ITGA6*, *ITGB4, LAMA3*, *LAMB3* and *LAMC2* cause the junctional subtype (JEB).[1] The latter three genes encode the individual chains of the heterotrimer laminin 332 (LM-332).[1] In our Dutch EB cohort, 60% of JEB cases are caused by mutations in the LM-332 genes, of which 28% are in *LAMA3* (unpublished data). Clinically, patients with JEB exhibit skin and mucous membrane blistering, nail dystrophy, and structural enamel abnormalities termed amelogenesis imperfecta.[2] Primary and secondary dentition are always affected in JEB.[2] Enamel in JEB is thin, hypoplastic and contains a 20% lower mineral content than that of healthy individuals.[3,4] Recently, we reported that heterozygous carriers of loss of function (null) mutations in *LAMA3* exhibit dental abnormalities without skin symptoms.[5] Carriers exhibited localized enamel pitting and hypoplasia of secondary dentition. Here, we report two new heterozygous carriers affected with dental abnormalities from loss of function mutations in *LAMA3*.

SUBJECTS AND METHODS

We studied two kindreds of Dutch origin, of which the index patients were referred to our clinic with suspected EB. Assessment of skin fragility was based on review of patient history and specific anamnestic questions (ie, tolerating Band-Aids, wound healing) and dermatological physical examination. We harvested and processed skin specimens for immunofluorescence antigen mapping (IF) and transmission electron microscopy (TEM) of the JEB index patients as described before.[6,7] IF and TEM were not performed in carriers, as no biopsies were available. Genomic DNA was isolated from peripheral blood from the index patients and parents, and sequenced using Sanger's sequencing by a candidate gene approach. Phenotype and variant information were submitted to Leiden Open Variant database http://dna2.leeds.ac.uk/LOVD/genes/LAMA3 (patient IDs 00000159 and 00000160). Immunoblotting of cell lysates from cultured patient keratinocytes (not carriers) was performed as described before.[8] Dental examination of the index patients and parents was performed by an experienced maxillofacial dentist (CS), with more than 15 years of experience treating EB patients. Appropriate informed consent was obtained and patients were treated in accordance with the principles of the Declaration of Helsinki.

RESULTS

In Family 289, the index patient (EB-289) was a 2-year old boy and the third child of healthy, unrelated parents (Figure 1a). Beginning at 6 months of age, he began to develop blistering of hands, legs and ears together with nail dystrophy. He had generalized enamel pitting throughout his entire primary dentition, termed hypoplastic amelogenesis imperfecta (AI; Figure 1b). IF of intact skin showed normal expression of LM-332 (3+) when stained with the monoclonal antibody GB3 directed against the conformation epitope in the γ2 chain. Staining of the individual laminin α3 subunit with monoclonal antibody BM165 (gift from Dr Marinkovich, Stanford University) was decreased (2+) when compared with control (3+, Figure 1c). TEM of lesional skin on the left elbow confirmed cleavage through the lamina lucida fitting with JEB. Sequencing of DNA isolated from peripheral blood revealed that the patient was compound heterozygous for mutations in *LAMA3* (NM_000227.4) c.(2377C>T);(4484C>T), concluding the diagnosis of JEB-generalized intermediate (previously JEB non-Herlitz).[1] Additional screening of *LAMB3* and *COL17A1*

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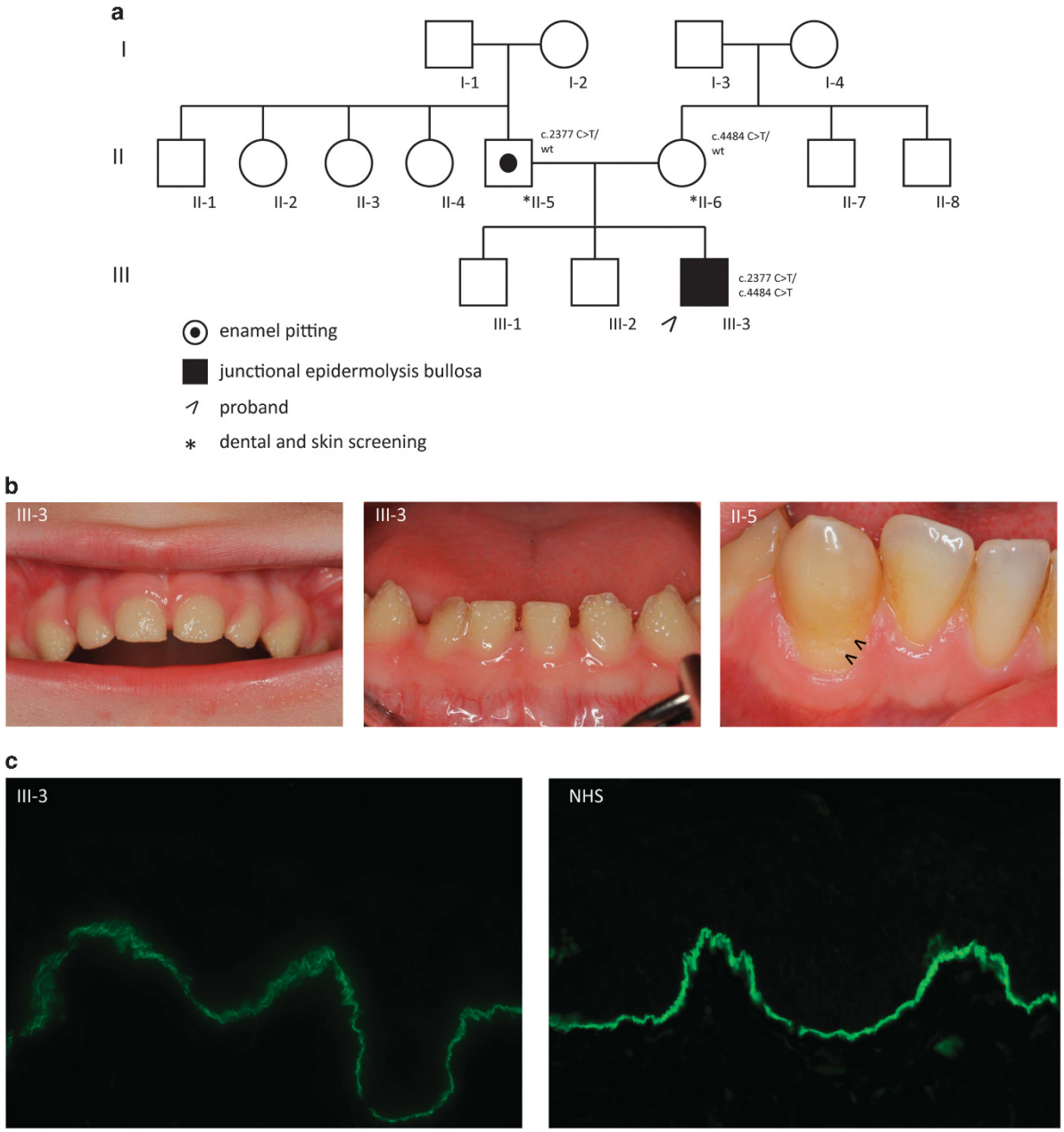

Figure 1 Family 289 (**a**) Family pedigree with indicated genotypes of studied individuals (wt: wild type). (**b**) Generalized hypoplastic amelogenesis imperfecta in primary teeth of the index patient (III-3) His father (II-5) showed clinically silent localized enamel pitting on the cervical area of the lower canine as indicated by arrows. (**c**) IF antigen mapping of the α3 chain with BM165 showed 2+ expression when compared with 3+ in normal human skin (NHS) in the index patient.

revealed no mutations in the proband. We performed *LAMA3* allele carrier analysis, and skin and dental examination in both parents. Exons are numbered like in NG_007853.2. The father (II-5) carried the novel c.(2377C>T); p.Arg793Ter mutation located in exon 19. *In silico* analysis of the mutation using AlamutVisual software (version 2.6.1, alamut.interactive-biosoftware.com) with protein and splice-site prediction programs for the paternal c.(2377C>T) mutation showed no alternate splicing, thus predicting a premature termination codon (PTC) p.(Arg793Ter). The father displayed subtle enamel pitting of secondary dentition (Figure 1b) and had no history of skin blistering. The mother (II-6) carried the missense mutation c.(4484C>T); p.(Ala1495Val) in exon 33, a mutation earlier reported in Yuen *et al.*[5] Dental screening for pathology was negative in the mother, and dermatological examination was unremarkable. The parents reported that the index patient's two older brothers (III-1 and III-2) had no skin disease or dental problems, but were unavailable for objective examination and mutation analysis.

The female child (EB-351, III-1) of Family 351 was seen in our clinic shortly after birth. The age of gestation was 35 weeks, and the parents were unrelated (Figure 2a). The child presented with skin fragility and absent nails of digits 1-4 of both hands. IF of lesional skin indicated strongly reduced staining of GB3 (1+) compared with

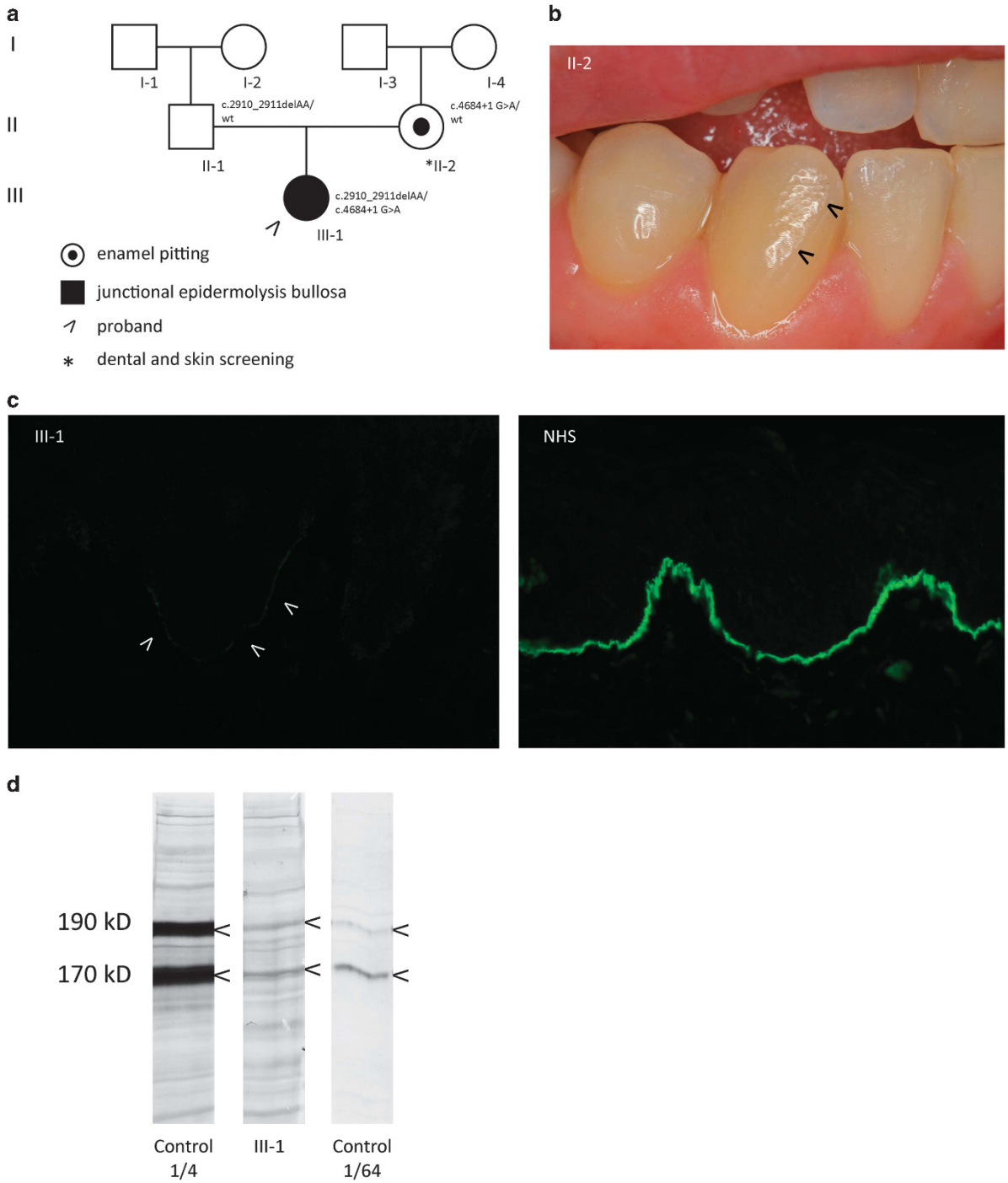

Figure 2 Family 351 (**a**) Family pedigree. (**b**) Dental examination showing with arrows subtle enamel pitting on the labial surface of the lower canine by the mother (II-2) who carried the c.4684+1G>A mutation. Her upper and lower central and lateral incisors displayed the same focal pitting and grooves. (**c**) IF antigen mapping with BM165 showing severely diminished expression as compared with normal human skin (NHS). (**d**) Immunoblotting of cell lysates from cultured keratinocytes derived from healthy human skin (control) and from the index patient III-1. The α chain of LM-332 stained with monoclonal antibody BM165 exists in a processed (190 kDa) and non–processed (170 kDa) form. Patient extract (middle lane) compared with 1/4 diluted (left) and 1/64 diluted (right) extracts of control healthy keratinocytes, indicated that the amount of α chain expressed by the patient was <2% of normal. The blots were cropped for presentation clarity and alignment.

control (3+). Antigen mapping showed GB3 exclusively in the blister roof and type VII collagen exclusively in the blister floor fitting a diagnosis of JEB. Additional staining with BM165 (Figure 2c) for the laminin α3 chain was strongly reduced (1+) when compared with healthy controls (3+). Genetic analysis revealed compound heterozygous mutations c.2910_2911delAA, in exon 22 and c.4684+1G>A in intron 34 of the *LAMA3* gene. She was diagnosed with JEB-generalized severe (former JEB-Herlitz).[1] DNA analysis of the

Table 1 Reports of Isolated Enamel Abnormalities in Heterozygous Carriers of EB Gene Mutations

GENE-PROTEIN	*Mutation*	*Effect on protein*	*Protein expression*	*Clinical phenotype*	*Remarks*	*Report*
LAMA3 -3333 amino acids	c.488delG	p.Gly163AspfsTer30	Absent	Mild enamel pitting and susceptibility to caries		Yuen *et al.*[5]
	c.2377C>T	p.(Arg793Ter)	Absent	Focal, asymptomatic enamel pitting		This article
	c.4684+1G>A	Splicing defect, 2% of wild-type protein production	Severely reduced	Focal, asymptomatic enamel pitting		This article
LAMB3 -1172 amino acids	c.124C>T	p.Arg24Ter	Absent	Hypoplastic	No dental pictures were present, limited description and it was only found in one individual carrying the c.124C>T mutation	Prasad *et al.*[16]
	c.1903C>T	p.Arg635Ter	Absent	AI hypoplastic	No dental pictures were present, and limited description. Further, the mother who was carrier of the same mutation did not have a phenotype.	Prasad *et al.*[16]
	c.3357_3358insC	p.Met1120fsTer40	Truncated protein with 39 novel amino acids instead of a C-terminal with 53 amino acids in the wild-type protein	Generalized irregular hypoplastic enamel in all primary teeth		Lee *et al.*[12]
	c.3392_3393insG	p.Glu1133GlyfsTer27	Truncated protein with 26 novel amino acids instead of a C-terminal with 40 amino acids in the wild-type protein	Irregular hypoplastic AI, frequent pain and aesthetic problems, multiple dental extractions and restorative dental care		Poulter *et al.*
	c.3431C>A	p.Ser1144Ter	Truncated, 1143 amino acids	Generalized enamel hypoplasia, thermal hypersensitivity, deep grooves and pits, vertical linear grooves in incisors		Kim *et al.*
	c.3446_3453delGACTGGAG	p.Gly1149GlufsTer8	Truncated protein with 8 novel amino acids instead of a C-terminal with 24 amino acids in the wild-type protein	Enamel hypoplasia in all primary and permanent dentition, deep grooves and pitting defects, generalized enamel hypoplasia on radiographic examination. Taurodontism, vertical linear grooves in incisors, thermal hypersensitivity		Kim *et al.*
	c.3463_3475delGAGCAGATCCGTG	p.Glu1155fsTer51	Truncated protein of 1204 amino acids with 50 novel amino acids replacing the C-terminal 18 amino acids	Enamel hypoplasia in all primary and permanent teeth, hypoplastic grooves and pits		Lee *et al.*"T4"
	c.3466C>T	p.Gln1156Ter	Truncated, 1155 amino acids	Enamel hypoplasia in all primary and permanent teeth, hypoplastic grooves and pits		Wang *et al.*[13]
COL17A1-1497 amino acids	c.823delA	p.Thr239fsTer52	Absent	Extensive enamel pitting horizontal ridging of incisors and loss of enamel in molars		Murrell *et al.*[14]
	c.1141+1G>A	p.(0?)	Most likely either: 1) skipping of the 54 amino acids of exon 14, or 2) intron retention leading to a premature termination codon, and absence of protein.	Hypoplastic enamel		Prasad *et al.*[16]
	c.1646G>A	p.(Trp549Ter)	Absent	Hypoplastic enamel		Prasad *et al.*[16]
	c.1873C>T	p.(Arg625Ter)	Absent	Hypoplastic, hypomature		Prasad *et al.*[16]
	C.1880G>T	p.Gly627Val	Present with one different amino acid	Extensive enamel hypoplasia and pitting		McGrath *et al.*[2]; Almaani *et al.*[17]
	c.2407G>T	p.(Gly803Ter)	Absent	Isolated AI. Hypoplastic enamel.		Prasad *et al.*[16]

AI: amelogenesis imperfect. Note: To date there have been no isolated enamel abnormalities in carriers of LAMC2, ITGA3, ITGA6, and ITGB4 mutations reported.[18,19]

father (II-1) revealed a heterozygous mutation in *LAMA3*: c.2910_2911delAA. The c.2910_2911delAA mutation caused a frameshift leading to a PTC, p.(Lys970AsnfsTer8). He was not available for clinical assessment. DNA analysis of the mother (II-2) revealed a heterozygous c.4684+1G>A mutation affecting the consensus donor splice site sequence of intron 34. Dental examination showed focal enamel pitting and grooves in some of her secondary dentition that had gone earlier unnoticed (Figure 2b). Skin fragility was absent. To further investigate the consequence of the maternal splice-site mutation on mRNA, we performed RT-PCR and subsequent sequencing of RNA isolated from the index's keratinocytes. Three cDNA products of different length were identified. The shortest product resulted from skipping of exon 34 and 35 sequences (291 base pairs length) encoding an in-frame transcript. The second product showed skipping of exon 34 (160 base pairs) resulting in an out-of-frame transcript with introduction of a PTC. The third was a full-length wild-type transcript. Subsequently, we performed immunoblot in order to assess LM-332 protein expression using cell lysates of skin keratinocytes as described earlier.[9] Staining with BM165 indicated a severely reduced amount of polypeptide of less than 2%, when compared with normal human control keratinocytes (Figure 2d). No smaller protein products were observed, suggesting that only the wild-type transcript from the maternal allele attributed to laminin-α3 expression.

DISCUSSION

Various degrees of defects of amelogenesis have been observed in mutation carriers of several JEB genes (*COL17A1*, *LAMA3* and *LAMB3*, Table 1).[2,5,10–14] In the examined heterozygous null carriers in both our families, dental pathology was localized focally, clinically asymptomatic and therefore not brought to attention until we examined JEB in their offspring. Since the first report of enamel abnormalities in *LAMA3* null mutation carriers by Yuen *et al.*,[5] we began to screen carriers of *LAMA3* mutations for dental pitting. The low incidence of *LAMA3* mutations, subtle nature of changes and lack of direct screening may explain the scarcity of reports of enamel defects in this population. In teeth, LM-332 is known to be a principal anchoring protein actively involved in the secretory stage of ameloblasts, an early stage of differentiation.[10] Amelogenesis is a tightly regulated process in which ameloblasts express LM-332.[3] Replacement of the lamina basale during amelogenesis is impaired if LM-332 is altered.[3] Enamel and skin apparently respond differently to heterozygous mutations affecting the α chain, because skin fragility was absent in carriers. In teeth, a single allele defect in the α chain in null mutation carriers is enough to disrupt ameloblast-coordinated replacement of basement membrane macromolecules causing enamel pitting.[4] The mechanism by which the heterozygous functional null mutations in *LAMA3* result in disease appears to be haploinsufficiency, as we suggested before.[5] In skin, keratinocyte adhesion is redundantly regulated, and if LM-332 is half dose, it is most likely sufficiently compensated for by other epidermal proteins, such as integrins.[5,15] The splice-site mutation c.4684+1G>A behaves like a *LAMA3* null mutation, as <2% expression of intact laminin α3 protein was detected in the immunoblot (Figure 2d). The low amount of polypeptide can be explained by the detrimental effect on protein stability of the deleted amino acids in the G5 subdomain (codons 1509–1606) encoded by exons 34 and 35. Another possibility could be that the BM165 antibody recognizes an epitope for binding, which is missing from the internally truncated protein that is formed by this mRNA transcript. Although this cannot be completely excluded, it is highly unlikely, since the affected child in Family 351 had the most severe form of JEB due to almost complete loss of LM-332 and deceased at 6 months of age.

LAMA3 null mutation carriers show minor enamel changes, whereas carriers with *LAMB3* mutations have deeper pits and visible grooves requiring medical attention.[10–12] Extensive restorative dental care and multiple extractions such as described by Poulter *et al.* for *LAMB3* (NM_000228.2) carriers were not present in our *LAMA3* carriers. A difference in the reported cases is that in *LAMB3* carriers, dental abnormalities are associated with mutations that are predicted to escape nonsense mediated RNA decay (NMD, Table 1). All mutations, except for one, were located in the last exon which predicted formation of a shortened laminin-β3 peptide truncated at the carboxy-terminus.[12] The formation of abnormal truncated laminin β3 is more detrimental on enamel development than haploinsufficiency of laminin α3. Of note is that in a very recent publication two *LAMB3* null mutations were described resulting in hypoplastic enamel.[16] However, in this publication there was only a minor description of the phenotype, for the c.1903C>T (p.R635X) mutation there was no clear segregation with the dental phenotype, and for the c.124C>T mutation only one individual was affected with dental abnormalities. It yet remains to be determined whether the dental abnormalities were indeed due to the *LAMB3* null mutation, or whether other mutations or factors were the underlying cause. For the *COL17A1* gene both null mutations as well as one missense mutation have been described.

In conclusion, our observations confirm the hypothesis that carriers of functional null mutations in *LAMA3* can exhibit subtle, localized enamel pitting due to haploinsufficiency. We advise dental screening of parents of JEB patients and siblings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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