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CLINICAL REPORT

Structural Defects of Laminin β3 N-terminus Underlie Junctional Epidermolysis Bullosa with Altered Granulation Tissue Response

May EL HACHEM[1], Paola FORTUGNO[2], Antonio PALMERI[3], Manuela HELMER CITTERICH[3], Andrea DIOCIAIUTI[1], Vittoria PROTO[1], Renata BOLDRINI[4], Giovanna ZAMBRUNO[1] and Daniele CASTIGLIA[2]

[1]Dermatology Division and [4]Department of Pathology, Bambino Gesù Children's Hospital, IRCCS, [2]Laboratory of Molecular and Cell Biology, Istituto Dermopatico dell'Immacolata-IRCCS, [3]Centre for Molecular Bioinformatics, Department of Biology, University of Rome Tor Vergata, Rome, Italy

Mutations in the laminin-332 (α3Aβ3γ2) genes cause junctional epidermolysis bullosa (JEB), a recessively inherited disease characterized by blistering and altered wound repair. In addition, specific mutations that affect the N-terminus of the α3A chain cause a JEB-related non-blistering condition characterized by chronic production of granulation tissue, suggesting a critical role of this region in epithelial-mesenchymal communication. We report here a 9-year-old patient with JEB with a few long-standing skin ulcers with prominent granulation tissue in the absence of active blistering. He bears a homozygous missense mutation, p.Gly254Asp, within the first laminin epidermal growth factor-like (LE) repeat of the β3 short arm. We show that p.Gly254Asp causes misfolding of the LE motif, leading to reduced secretion of laminin-332 and structural alterations of the cutaneous basement membrane zone. These findings demonstrate, in a patient *in vivo*, that the β3 short arm is also involved in the outcome of the granulation tissue response. *Key words: genodermatosis;* LAMB3*; chronic skin ulcer; laminin EGF-like domain; protein folding.*

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Daniele Castiglia, Laboratory of Molecular and Cell Biology, Istituto Dermopatico dell'Immacolata-IRCCS, via dei Monti di Creta 104, IT-00167 Rome, Italy. E-mail: d.castiglia@idi.it

Junctional epidermolysis bullosa (JEB) is a genetic disease characterized by skin fragility resulting in blistering of the skin and mucous membranes. Some patients also present exuberant granulation tissue formation, leading to chronic wounds (1, 2). Morphological hallmarks of the disease are tissue cleavage within the lamina lucida of the cutaneous basement membrane (BM) and dysfunctional/altered hemidesmosome-anchoring filament complexes (3). Clinically, the most common JEB subtypes are JEB generalized severe, which usually results in early lethality, and JEB generalized intermediate (JEB-gen intermed), which comprises phenotypes of mild to moderate severity and normal life expectancy (3). Recessive mutations in either of 3 genes (*LAMA3A, LAMB3* and *LAMC2*) encoding for laminin-332 (LM332) chains (α3Aβ3γ2) can cause both lethal and milder forms according to the mutation effects on gene expression (2, 4, 5).

LM332 is a multi-domain protein of the BM crucial for epidermal–dermal cohesion. It is assembled in the endoplasmic reticulum of basal keratinocytes and then secreted and incorporated in the extracellular matrix (ECM) (6, 7). The trimeric molecule has the form of a cross with 1 long and 3 short arms. Opposite ends of the molecule have distinct functional roles: C-terminal LG globules harbour binding sites for cell surface receptors, such as integrins and syndecans, while domains of N-terminal short arms, specifically the unique LN and multiple LE motifs, connect to the dermal components collagen VII and other BM laminins (8). Of note, a few mutations that affect the α3A N-terminus cause laryngo-onycho-cutaneous syndrome (LOCS), a JEB-related non-blistering condition characterized by markedly increased production of vascularized granulation tissue, mainly affecting the eye, larynx and nail bed. Ocular and laryngeal involvement can lead to blindness and respiratory tract obstruction (9, 10). In addition to underscoring the role of LM332 in wound repair (11), LOCS molecular findings also show that the phenotypic spectrum associated with defects in this protein is wider than expected and ranges from severe blistering to negligible skin fragility. We report here a 9-year-old patient with JEB characterized by the persistence over the years of a few non-healing skin ulcers with prominent granulation tissue but minimal skin fragility. The identified mutation and its consequence on the structure of a LE module of β3 short arm highlight the role of this region in controlling granulation tissue formation and BM structure.

MATERIALS AND METHODS

Patient samples, immunofluorescence, ultrastructural and molecular analyses

Perilesional skin biopsies and a blood sample were obtained with the patient's informed consent, and with the approval of the Bambino Gesù Children's Hospital and IDI Ethics Committees, in conformity with the guidelines of the Declaration of Helsinki. Immunofluorescence analysis and electron microscopy of a patient's skin biopsy were performed as described previously (12). Genomic DNA, purified from the blood of the patient and his parents, was used for mutation detection and confirmation, as described pre-

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viously (13). The mutation was numbered according to the translation initiation codon of the GenBank reference sequence L25541 for the *LAMB3* cDNA. Total RNA, purified from the remaining skin biopsy was used for reverse transcriptase PCR (RT-PCR) analysis with primers (F) 5'-gcctaatgcacgcctaaatg (exon 6) and (R) 5'-acatgtctctgagtgcccat (exon 10) spanning the mutation site.

Modelling of mutant β3 LE tertiary structure

The 3-dimensional model of human LE motif 1 of the β3 short arm (β3-LE1) was generated using Modeller v9.10 software (14) and the murine laminin β1 structure (Protein Data Bank (PDB) code: 4AQS) as template (sequence identity 46%). To validate structure prediction for the p.Gly254Asp substitution, the probability density for φ and ψ angles of aspartate residues in high-quality protein structures selected from the PDB using the PISCES server (http://dunbrack.fccc.edu/PISCES.php) (15) was compared with that of mutant β3 structure and visualized using a Ramachandran map ([φ,ψ]-plot).

RESULTS

A patient with chronic wounds and prominent granulation tissue

A 9-year-old Tunisian child with epidermolysis bullosa was referred to the Dermatology Unit of our hospital. The patient was the fifth child of healthy consanguineous parents and had been born at term by vaginal delivery. During the first months of life, he developed a few cutaneous blisters and erosions, initially localized on the right cheek and then on the trunk and extremities. Over time skin fragility markedly improved, with only occasional blisters elicited by mechanical traumas, while skin wounds were characterized by a chronic course and slowly enlarged over the years. At approximately 7 years of age, the patient started to experience constipation and recurrent abdominal pain. Family history revealed 2 healthy sisters and 2 brothers affected with a similar blistering disease. The affected brothers were deceased at the age of 14 and 21 years, respectively. The first died of unspecified complications after cystostomy for a urethral stenosis; the second died of cachexia. Both of them presented stenosis of the lacrimal duct and chronic skin ulcerations resistant to treatment. Physical examination of our proband revealed only a few wounds and no blisters. The skin lesions were localized to the face, back and perianal region. A superficial ulceration of the right cheek (Fig. 1), present since the first months of life, showed prominent granulation tissue and sharp borders. Similarly, a long-standing annular wound with raised margins encircled the anus (Fig. 1). The patient also showed enamel hypoplasia with multiple caries and periodontitis with gingival hypertrophy, but no ocular, respiratory or genitourinary involvement. In view of the limited extension of the skin wounds, topical treatment with silver-containing creams and contact layer dressings was started, resulting in a reduction in exuberant granulation tissue, but not in wound size. However, the treatment was discontinued one month after hospital discharge due to product unavailability in the patient's country.

Immunopathological and ultrastructural findings

Immunofluorescence studies with monoclonal antibody GB3 directed to trimeric LM332 showed a reduced staining along the BM compared with control skin (Fig. 2a). Similar results were obtained using antibodies that recognize individual chains of LM332 (BM165 to the α3 chain, K140 to β3, and a polyclonal antibody to the γ2) (not shown) (13). In addition, a granular labelling was focally present within the cytoplasm of basal and some suprabasal keratinocytes. Cytoplasmic deposits were irregularly distributed along the epidermis. Interestingly, collagen VII and collagen IV immunoreactivity along the BM zone appeared thickened and focally extended into the papillary dermis (Fig. 2a). Finally, linear staining for integrin α6β4 and collagen XVII appeared focally interrupted (not shown). Ultrastructural analysis showed cleavage at the level of the lamina lucida of the cutaneous BM (not shown). In surrounding areas, hemidesmosomes appeared hypoplastic with attenuated cytoplasmic plaques (Fig. 2b). Of note, the lamina densa was focally thickened and presented duplications extending in the papillary dermis. Numerous hypoplastic anchoring fibrils were observed in association with lamina densa duplications (Fig. 2b).

Mutation identification and modelling of laminin β3 domains

Based on clinical, immunopathological and ultrastructural findings JEB due to laminin-332 defects was

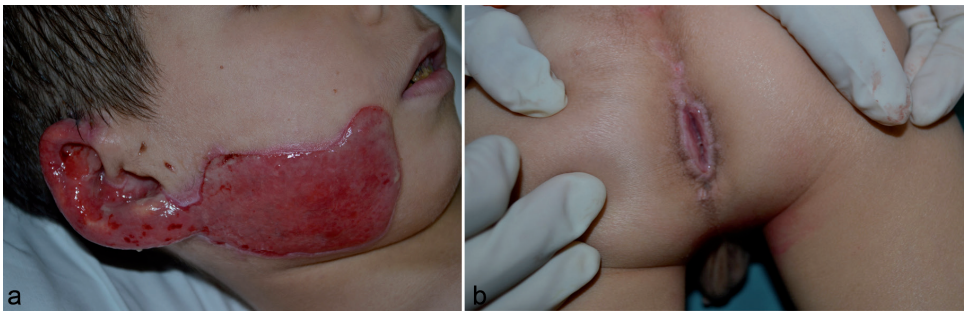

Fig. 1. Clinical findings in a patient with junctional epidermolysis bullosa (JEB) with altered wound repair. Large area of ulceration on the face with sharp margins and prominent granulation tissue present since infancy. The lesion involves the ear pinna and external part of the ear canal and extends to most of the cheek (*left panel*). Long-standing annular wound with raised margins encircling the anus (*right panel*). Note the absence of blistering lesions on the buttocks and thighs.

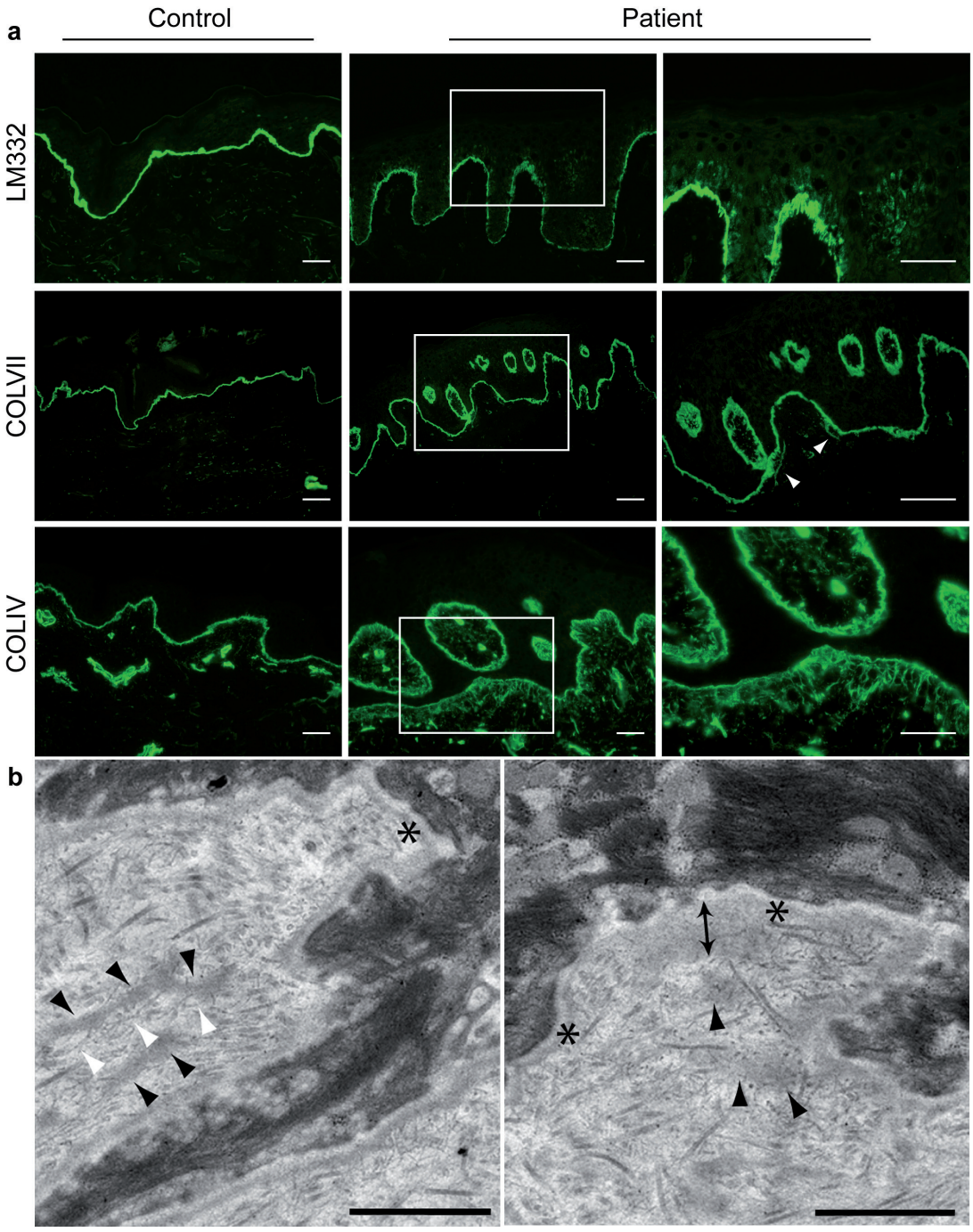

Fig. 2. (a) Immunofluorescence analysis of patient (*middle and right panels*) and control skin (*left panels*). The laminin-332 (LM332) staining (GB3 antibody) along the basement membrane zone (BMZ) is reduced in patient skin (*middle panel*) compared with control skin (*left panel*). In addition, intracytoplasmic laminin-332 granular deposits are focally visible within basal and some suprabasal keratinocytes, as better shown in the overexposed right panel image (*magnification of the middle panel inset*). Collagen VII (COLVII) and collagen IV (COLIV) immunoreactivity along the BMZ appears thickened and more intense than in control skin. Collagen VII labelling shows short traits of duplication beneath the BMZ (*right panel, white arrowheads*). The reticular collagen IV staining extending within the papillary dermis in patient skin is better visible in the right panel. *Scale bars*: 20 µm. (b) Ultrastructural analysis shows hypoplastic hemidesmosomes with attenuated cytoplasmic plaques (*asterisks*), irregular thickness of the lamina densa, which is often enlarged (*up and down arrow*) and presents focal duplications extending in the papillary dermis (*black arrowheads*). Numerous hypoplastic anchoring fibrils insert on lamina densa duplications (*white arrowheads*). *Scale bars*: 2.0 µm.

diagnosed, and molecular analysis of laminin-332 genes performed. We detected the homozygous c.761G>A transition in *LAMB3* resulting in the p.Gly254Asp substitution within the β3-LE1 (Fig. S1a[1]). The muta-

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tion was excluded in 51 unrelated ethnically matched healthy controls, in 1,000 Genomes and the Exome variant server and confirmed at the heterozygous state in both proband parents. Sequence alignment of the amino acid sequence of LE modules revealed that the Gly254 residue is strictly conserved across orthologous and paralogous β1 and β2 proteins (not shown). Mutation consequences on *LAMB3* pre-mRNA splicing were excluded by RT-PCR analysis.

To rationalize the effect of the p.Gly254Asp substitution on the laminin-332 β3 structure, we built a homology model (14) using the murine laminin β1 structure of the region encompassing N-terminal domains LN-LE1-4 as template (sequence identity 46%, Fig. S1b[1]). From a visual inspection of the wild-type model structure, we noticed that Gly254 is at the edge of a turn connecting 2 cysteine residues, Cys250 and Cys259, involved in a disulphide bridge (Fig. S1b[1]) (8). We hypothesized that the mutation of Gly254 to other residues might interfere with the disulphide bridge formation. Indeed, other residues, such as aspartate, which are less flexible than glycine, might impose higher constraints on the local structure, preventing or hindering the formation of the disulphide bridge. In order to check this hypothesis we plotted a Ramachandran map of aspartate, highlighting in red the Asp254 of the mutant model structure (Fig. S1c[1]). The contours in the graph show the probability density for φ and ψ angles of aspartate residues from high-quality structures selected from the PDB using PISCES (15) (percentage identity ≤ 50, resolution 0.0–2.5, R-factor 1, sequence length> 40). In the mutated structure, Asp254 has a combination of φ and ψ angles that occurs with lower probability with respect to the background distribution, suggesting that this residue at position 254 might cause deformations in the structure, thus interfering with the formation of the Cys250–Cys259 disulfide bridge. Moreover, when mutation p.Gly254Asp was introduced in the model, we observed rearrangements of residues in the neighbourhood of the mutation, which is located in the loop connecting LN with LE1 domains. Therefore p.Gly254Asp is likely to have a significant impact on the structural relationships between LN and LE1 domains.

DISCUSSION

Defective dermal–epidermal adhesion and altered wound repair characterize JEB caused by complete or partial deficiency of LM332. However, the main phenotype in JEB is trauma-induced blistering, while overt wound healing defects are clinically evident in a subset of patients (1, 16, 17). LM332 supervises these processes using different regions of its structure (8). In particular, it is believed that N-terminal domains normally act to signal to the underlying mesenchyme that the BM is intact. When a hit breaches the BM, LM332 synthesis is rapidly up-regulated and its deposition at the leading edge of the newly-forming epithelial tongue promotes keratinocyte migration during wound re-epithelialization. Following BM reconstitution, LM332 N-terminal domains may signal to fibroblasts and/or endothelial cells to terminate the mesenchymal wound healing response (11). Although the mechanisms of this regulation have yet to be determined, the model fits well with the physiopathology of LOCS where the lack of α3A N-terminus consequent to peculiar mutations in *LAMA3A* gene alters the mesenchymal tissue formation response (9, 18). The phenotypic features of our patient recall, in part, manifestations of LOCS, in that he showed almost no new blisters in the presence of a limited number of long-standing, slowly enlarging skin wounds characterized by excessive granulation tissue. However, his nails were not affected and mucosal involvement was limited to periodontitis and gingival hypertrophy, thus lacking major clinical features for LOCS. Most likely, the minimal skin fragility coupled with abnormal granulation tissue formation and wound repair block in our patient is due to both a reduced and/or delayed secretion of mutant LM332 consequent to the local mis-folding of the LE1 structure, as documented by the intracytoplasmic protein retention observed in immunofluorescence, and an altered functionality of the mutant p.Gly254Asp β3 polypeptide in the deposited LM332.

In our case, the replacement of Asp for Gly at codon 254 of the β3-LE1 motif may affect the formation of the Cys250–Cys259 disulphide bridge, as evidenced by visual analysis of the model generated by homology modelling and supported by the structural distortion introduced by substitution of glycine 254 with aspartate. Connectivity of the 8 cysteine residues in position C1–C3, C2–C4, C5–C6 and C7–C8 is a common feature of LE motifs, thus perturbation of Cys250–Cys259, which correspond to C1–C3 in the 8-Cys pattern, is expected to alter the proper folding of β3-LE1 (residues 249–314). Moreover, contiguity and integration of the LE1 into LN globular domain (residues 18–248) indicate that the mutation could also affect LN (8). Indeed, the substitution of glycine 254 with aspartate introduces significant modifications distributed throughout the local network of residue-residue interactions, potentially impairing physiological connections between the LN and LE1 domains, given the location of the mutation at the hinge between the 2 domains. A survey of the *LAMB3* mutation database (http://www.hgmd.cf.ac.uk/ac/all.php) shows that LE motifs of the short arm are targeted by several missense mutations, which, however, were found in combination with null mutations on the other allele and resulted in JEB forms with generalized blistering as major clinical feature (4). Of note, skin grafts derived from keratinocytes selectively engineered to express a laminin β3 chain lacking the N-terminal tip

showed increased granulation tissue production (19). These findings also directly implicate the β3 N-terminus in the regulation of mesenchymal tissue formation during wound repair. Our study attests at this role for the first time in a patient *in vivo*.

Furthermore, the BM structural abnormalities in our patient's skin suggest that mutation p.Gly254Asp can affect LM332 incorporation into the BM. Two mechanisms could be involved: (*i*) the mutation may perturb the interactions with type VII collagen, specifically with its N-terminal globular domain NC1, and hence keratinocyte adhesion (20–22); and (*ii*) it may affect binding to other laminins, in particular laminin-311 (LM311). Indeed, complexes formed by LM332 and LM311 self-associate and are incorporated into the BM with the contribution of additional ECM proteins, such as nidogen and collagen IV (23, 24). LM332/311 complexes are most likely formed through binding between the unique LN domain of LM332 β3 short arm and a LE domain of LM311 α3 short arm. In our patient laminin polymerization could be perturbed due to the structural defect of the N-terminal tip of the β3 short arm, and the ultrastructural changes observed at the level of the lamina densa in the patient's skin could result from defective laminin polymerization.

In conclusion, our data further supports the crucial role of LM332 N-terminus in orchestrating a correct wound healing response, and point to a specific role of the tip of the β3 short arm.

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REFERENCES

1. Yuen WY, Duipmans JC, Molenbuur B, Herpertz I, Mandema JM, Jonkman MF. Long-term follow-up of patients with Herlitz-type junctional epidermolysis bullosa. Br J Dermatol 2012; 167: 374–382.
2. Kiritsi D, Has C, Bruckner-Tuderman L. Laminin 332 in junctional epidermolysis bullosa. Cell Adh Migr 2013; 7: 135–141.
3. Fine JD, Bruckner-Tuderman L, Eady RA, Bauer EA, Bauer JW, Has C, et al. Inherited epidermolysis bullosa: updated recommendations on diagnosis and classification. J Am Acad Dermatol 2014; 70: 1103–1126.
4. Nakano A, Chao SC, Pulkkinen L, Murrell D, Bruckner-Tuderman L, Pfendner E, et al. Laminin 5 mutations in junctional epidermolysis bullosa: molecular basis of Herlitz vs. non-Herlitz phenotypes. Hum Genet 2002; 110: 41–51.
5. Posteraro P, De Luca N, Meneguzzi G, El Hachem M, Angelo C, Gobello T, et al. Laminin-5 mutational analysis in an Italian cohort of patients with junctional epidermolysis bullosa. J Invest Dermatol 2004; 123: 639–648.
6. Matsui C, Wang CK, Nelson CF, Bauer EA, Hoeffler WK. The assembly of laminin-5 subunits. J Biol Chem 1995; 270: 23496–23503.
7. Sugawara K, Tsuruta D, Ishii M, Jones JC, Kobayashi H. Laminin-332 and -511 in skin. Exp Dermatol 2008; 17: 473–480.
8. Rousselle P, Beck K. Laminin 332 processing impacts cellular behavior. Cell Adh Migr 2013; 7: 122–134.
9. McLean WH, Irvine AD, Hamill KJ, Whittock NV, Coleman-Campbell CM, Mellerio JE, et al. An unusual N-terminal deletion of the laminin alpha3a isoform leads to the chronic granulation tissue disorder laryngo-onycho-cutaneous syndrome. Hum Mol Genet 2003; 12: 2395–2409.
10. Barzegar M, Mozafari N, Kariminejad A, Asadikani Z, Ozoemena L, McGrath JA. A new homozygous nonsense mutation in LAMA3A underlying laryngo-onycho-cutaneous syndrome. Br J Dermatol 2013; 169: 1353–1356.
11. Iorio V, Troughton LD, Hamill KJ. Laminins: roles and utility in wound repair. Adv Wound Care (New Rochelle) 2015; 4: 250–263.
12. Castiglia D, Posteraro P, Spirito F, Pinola M, Angelo C, Puddu P, et al. Novel mutations in the LAMC2 gene in non-Herlitz junctional epidermolysis bullosa: effects on laminin-5 assembly, secretion, and deposition. J Invest Dermatol 2001; 117: 731–739.
13. Castori M, Floriddia G, De Luca N, Pascucci M, Ghirri P, Boccaletti V, et al. Herlitz junctional epidermolysis bullosa: laminin-5 mutational profile and carrier frequency in the Italian population. Br J Dermatol 2008; 158: 38–44.
14. Webb B, Sali A. Protein structure modeling with MODELLER. Methods Mol Biol 2014; 1137: 1–15.
15. Wang G, Dunbrack RL Jr. PISCES: a protein sequence culling server. Bioinformatics 2003; 19: 1589–1591.
16. Laimer M, Lanschuetzer CM, Diem A, Bauer JW. Herlitz junctional epidermolysis bullosa. Dermatol Clin 2010; 28: 55–60.
17. Yancey KB, Hintner H. Non-Herlitz junctional epidermolysis bullosa. Dermatol Clin 2010; 28: 67–77.
18. Cohn HI, Murrell DF. Laryngo-onycho-cutaneous syndrome. Dermatol Clin 2010; 28: 89–92.
19. Sakai N, Waterman EA, Nguyen NT, Keene DR, Marinkovich MP. Observations of skin grafts derived from keratinocytes expressing selectively engineered mutant laminin-332 molecules. J Invest Dermatol 2010; 130: 2147–2150.
20. Rousselle P, Keene DR, Ruggiero F, Champliaud MF, van der Rest M, Burgeson RE. Laminin 5 binds the NC-1 domain of type VII collagen. J Cell Biol 1997; 138: 719–728.
21. Chen M, Marinkovich MP, Veis A, Cai X, Rao CN, O'Toole EA, et al. Interactions of the amino-terminal noncollagenous (NC1) domain of type VII collagen with extracellular matrix components. A potential role in epidermal-dermal adherence in human skin. J Biol Chem 1997; 272: 14516–14522.
22. Chen M, Marinkovich MP, Jones JC, O'Toole EA, Li YY, Woodley DT. NC1 domain of type VII collagen binds to the beta3 chain of laminin 5 via a unique subdomain within the fibronectin-like repeats. J Invest Dermatol 1999; 112: 177–183.
23. Champliaud MF, Lunstrum GP, Rousselle P, Nishiyama T, Keene DR, Burgeson RE. Human amnion contains a novel laminin variant, laminin 7, which like laminin 6, covalently associates with laminin 5 to promote stable epithelial-stromal attachment. J Cell Biol 1996; 132: 1189–1198.
24. Cheng YS, Champliaud MF, Burgeson RE, Marinkovich MP, Yurchenco PD. Self-assembly of laminin isoforms. J Biol Chem 1997; 272: 31525–31532.