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Missense Mutation in *LAMA3* Associated with Herlitz Junctional Epidermolysis Bullosa in a Pakistani Family

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Abstract.- Junctional Epidermolysis Bullosa (JEB) affects intra-lamina lucida of skin and is an exclusively autosomal recessive mechanobullous disorder. Its major subtypes include Herlitz (H-JEB; MIM#226700) and non Herlitz-JEB (nH-JEB; MIM#226650), the former being the lethal form. Genetic causes of this disease include mutations in genes encoding for laminin-332 (LM-332) *i.e. LAMA3, LAMB3* and *LAMC2*. The objective of the current study was to elucidate the genetic basis of JEB patients in a consanguineous family of Pakistani origin. Screening of exons and intron-exon boundaries of *LAMA3, LAMB3* and *LAMC2* was carried out. Normal as well as carriers of the affected family, along with 99 healthy control individuals of the same ethnicity, were screened by PCR-RFLP and dye terminator cycle sequencing. Sequence analyses of the patient revealed homozygosity for a missense variant c.4540G>C (p.D1514H) in *LAMA3*, which encodes the alpha chain of LM-332 (a skin adhesion protein) that is of importance for cell surface binding. The majority of cases with H-JEB are caused by truncating mutations with loss of LM-332 whereas missense mutations are extremely rare. These results add to the mutation spectrum associated with JEB and improve our understanding of *LAMA3* in epidermal-dermal integrity.

Key words: Junctional epidermolysis bullosa, Herlitz JEB, mutational analyses, laminin-332.

INTRODUCTION

Junctional Epidermolysis Bullosa (H-JEB) is a subtype of epidermolysis bullosa characterised by distinct site of blister formation localised in lamina lucida within the basement membrane zone. H-JEB is a lethal variant as patients usually do not survive their first year. The phenotype includes generalized external and internal blistering present at birth accompained by a hoarse cry or cough. Non-Herlitz JEB (nH-JEB) is a non lethal variant characterized by generalized blistering involving also the mucosal membranes, scalp, nails and teeth.

Laminin-332 formerly known as laminin-5/Epiligrin/Kalinin/Ladsin is a skin adhesion protein and is encoded by three genes *i.e.*, *LAMA3* (18q11.2), *LAMB3* (1q32) and *LAMC2* (1q25-q31). LM-332 belongs to a family of multifunctional large trimeric glycoproteins, which are important for the Basement membrane (BM) of the skin (Hamill *et al.*, 2010). It has three components: the α-, β- and γ-chains. The alpha chain has five variants, while the beta and gamma chains have three variants each. There are two major transcripts of LM-332, *LAMA3A* and *LAMA3B*. *LAMA3A* is expressed solely in human keratinocytes whereas *LAMA3B1* and *LAMA3B2* are expressed in hKCs and fibroblasts (McLean *et al.*, 2003).

Mutations in *LAMC2* of LM-332 were first identified as a cause of JEB (Fassihi *et al.*, 2005). The most common JEB mutation occurs in *LAMB3* which accounts for 70% (Pfendner and Lucky, 2007). Typically frameshift, splice-site or non-sense mutations cause JEB in which the patient inherits one mutant allele from either parent. The usual mutations associated with H-JEB are premature termination codon (PTC) mutations in LM-332 genes whereas in the case of nH-JEB the mutations are mostly missense or putative splice site mutations in trans (Nakano *et al.*, 2002). There are also some reports of complete paternal uniparental isodisomy

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of chromosome in H-JEB patients (Pulkkinen *et al.,* 1997; Fassihi *et al.,* 2005; Takizawa *et al.,* 1998, 2000).

H-JEB is mainly associated with mutations in *LAMC2* (Posteraro *et al.,* 2004). Different populations show different spectrum of mutations, which is demonstrated in a study of consanguinous JEB families from the Middle East (Nakano *et al.,* 2002). In this study seven novel mutations in *LAMA3, LAMB3, LAMC2* and *COL17A1* were found none of which have been reported in American and European patients. A few mutations in the genes encoding LM-332 are recurrent and indicate mutational hotspots, *e.g.,* p.R650X in exon 16 of *LAMA3*, p.R42X in exon 3, p.Q243X in exon 8, p.957ins77 in exon 10, p.R635X in exon 14 of *LAMB3*, and p.R95X in exon 3 of *LAMC2*. R635X is a recurrent Pakistani mutation, which is also found in other ethnic groups where it accounts for 40% of all H-JEB mutated alleles (Nakano *et al.,* 2000, Kivirikko *et al.,* 1995; McGrath *et al.,* 1995; Pulkkinen *et al.,* 1997).

MATERIALS AND METHODS

Ethics committee approval and sample collection

This study was approved by the Ethics Committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi and follows the Helsinki declarations. The proband of the family (Fig. 1) was identified from Multan, Pakistan. The patient was initially diagnosed as suffering from JEB by local Dermatologists at Nishtar Hospital, Multan, Pakistan and on the basis of symptoms and photographs; diagnosis was later confirmed by Dermatologists at the Salzburg General Hospital, Austria. Complete medical and family history of the patient was obtained after getting informed written consent. The blood samples were collected in EDTA vacutainer tubes (BD, Germany) from the patient, family members and unaffected controls. Genomic DNA was isolated from whole blood by standard methods.

Sequence analyses

The patient's DNA was amplified by PCR and screened for previously identified recurrent mutations. This was followed by sequencing of the entire coding sequences of *LAMA3, LAMB3* and *LAMC2* using primers described previously (Pulkkinen *et al.,* 1997). The chromatograms were aligned and compared with genomic sequence obtained from the UCSC genome browser using BLAST tool (http://blast.ncbi.nlm.nih.gov) and Chromas.exe (http://technelysium.com.au)**.** The variant was assessed on Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://www.blocks.fhcrc.org/sift/SIFT.html). The multiple sequence alignment of *LAMA3* protein from various species was performed using ClustalW (http://www.clustal.org/).

Restriction digestion

A PCR-RFLP (Restriction fragment length polymorphism) was designed to screen the identified c.4540G>C variant as this change destroys the restriction site for HinfI. DNA from 99 unaffected controls of Pakistani origin and all family members of the patient were also analyzed by this method. A total of 20 µl reaction was prepared for restriction digestion with 10 U of HinfI, incubated at 37°C for 16 hours, followed by inactivation at 80°C. The samples were then electrophoretically separated on 3% agarose gel and visualized under UV-transillumination.

RESULTS AND DISCUSSION

Clinical findings

The female proband was born after an uneventful 9 month gestation and delivery to first-cousin parents. The family comprised several consanguineous loops and both maternal and paternal grandparents were first cousins. The patient was the second affected child in the family and the elder affected sibling died at the age of a few months due to infections. Soon after birth, she presented with fluid filled blisters accompanied by peeling away of the epidermis. At the time of sampling the infant was underweight with blisters all over the body including the scalp, fingers and feet. The groin region was completely affected (Fig. 1B). She had difficulties in feeding and swallowing owing to blistering in the mouth and gastrointestinal tract and she had repeated respiratory tract infections. The inability to feed the child did further

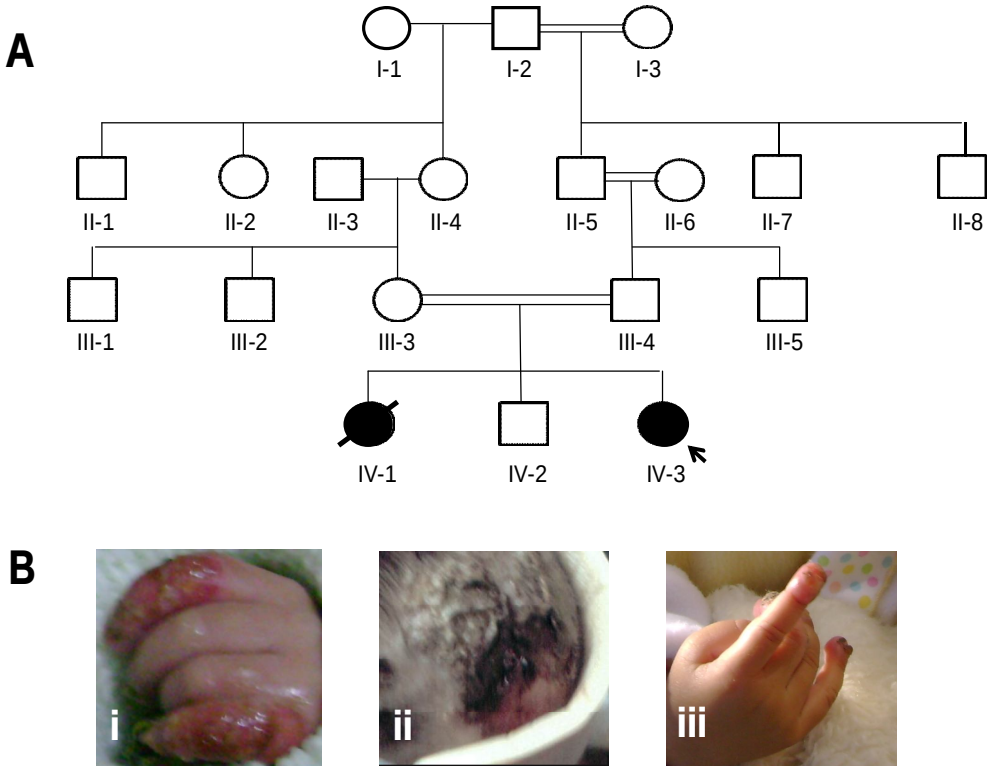

Fig 1. (A) Pedigree of a Pakistani JEB family segregating the c.4540G>C variant resulting in p.D1514H. Affected individuals are indicated by *filled symbols*. The proband IV-3 is labeled by a black arrow, (B) Clinical presentation of the patient studied in this report, showing old blisters on fingers (i), head (ii) and absence of finger nails (iii).

impair wound healing. The disease appeared for the first time in the current generation and had a recessive mode of inheritance. The proband passed away at the age of 11 months. Subtyping of the disease on the basis of Immunofluorescence antigen mapping (IMF) was not possible as punch biopsy was not taken at the time of sampling for several reasons, later the patient died and was not accessible for sampling. It was clinically sub-classified as H-JEB on the basis of progressive worsening of skin and mucosal involvement in the neonatal period with death within infant period despite getting proper medical attention.

Genetic analyses

Sequencing of *LAMA3*, *LAMB3* and *LAMC2* revealed several variations in the patient's genome. A single potential pathogenic variant was identified in a homozygous state in *LAMA3* exon34, where a G was replaced with C at nucleotide position c.4540 (NM_000227), which resulted in the replacement of aspartate (D) for histidine (H) (Fig.2). Three variants were identified in *LAMA3* of which c.1287C/G in exon 11 and c.3871C/T in exon 30 have been reported previously (Nakano *et al.*, 2002), while a c.371-7G/C (silent variation) in exon 28 identified in the current study has not been reported earlier. Analysis of *LAMB3* revealed 6 SNPs all of which have been reported previously and included: c.291A/C (Fassihi *et al.* 2005) in exon 4, c.384T/C (Fassihi *et al.*, 2005) in exon 6, c.1716C/T (Takizawa *et al.*, 1998) in exon 14, c.2554A/T (Fassihi *et al.*, 2005) in exon 17, c.2673A/G (Fassihi *et al.*, 2005) in exon 18, c.3432A/G (Fassihi *et al.*, 2005) in exon 23. Six silent SNPs were also present in *LAMC2*, all of

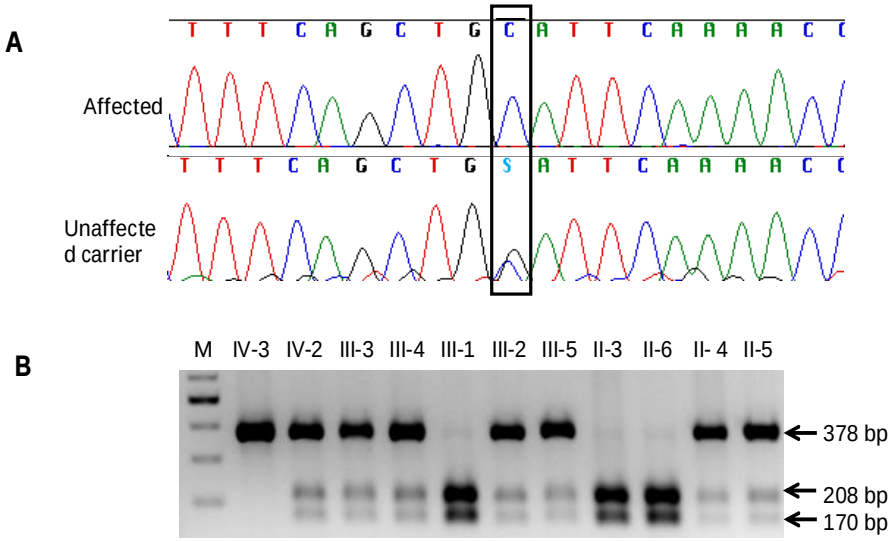

Fig. 2. (A) Genomic DNA sequence of *LAMA3* exon 34 from two individuals (affected and unaffected carrier) demonstrating the missense mutation (p.D1514H) highlighted by black rectangular box (B) PCR-RFLP using HinfI: loss of restriction site due to c.4540G>C in homozygous condition (C/C) resulted in undigested fragment of 378bp. Homozygous normal (G/G) showed complete digestion of PCR product resulting in two bands of 208bp and 170bp where as carriers of the mutation (G/C) has 3 bands of 378, 208 and 170 bp, M: Marker in first lane (GeneRuler DNA Ladder Mix (Fermentas)

which have not been reported previously, these included c.297T/C in exon 3, c.483T/C in exon 4, c.798G/T in exon 7, c.1539T>C in exon 11, c.2688A>G in exon 18 and 3671+13T/G after exon 23.

Analysis of the variant using the online mutation prediction tool Polyphen-2 was done, the reference range of this prediction tool is 0-1, with the threshold of probably damaging at 0.85. A score of 0.987 was given for this variant prediciting it to be probably damaging. The SIFT software gives scores in range of 0 to 1. The amino acid substitution is predicted damaging if the score is <= 0.05, and tolerated if the score is > 0.05. The SIFT score for this variation was 0.01 which further proves it to be a damaging mutation. The p.D1514H variant has been reported as an extremely rare SNP *i.e.* rs139393524 with minor allele frequency (MAF), C=0.001/1 in the world population where it is listed in a heterozygous state, so it has never been described as homozygous (C/C) variant. Also it has never been associated with EB. Thus, the finding is potentially unique in the sense that missense mutations has never been reported previously to cause lethal forms of H-JEB.

Analysis of the parents showed that they were heterozygotes (C/G) for this variant consistent with a recessive mode of inheritance (Fig. 2A). Other ten family members and 99 ethnically matched healthy controls were analysed for the variant mutation using restriction digestion with HinfI. The results clearly indicated that the mother and father as well as two grandparents were heterozygous for c.4540G>C (Fig. 2B), and the mutation was excluded on 198 chromosomes from 99 healthy individuals of the same ethnicity. The heterozygous family members (parents and grandparents) had skin with normal apperance. This strengthens the association between the *LAMA3* variant c.4540G>C and disease.

N-terminal of LAMA3A subunit consists of coiled coil domain (LCC) of importance for assembly with the beta and gamma subunits of LM-332. The LCC is followed by five globular domains, *i.e.* LG1-5 (Hamill *et al.*, 2010) and the C-terminal LG4–LG5 pair carries binding sites for heparin,

sulfatides and the cell surface receptor dystroglycan (Ott *et al.*, 1982; Ervasti and Campbell, 1993; Gee *et al.*, 1993; Smalheiser, 1993; Sung *et al.*, 1993; Talts *et al.*, 1999). Detailed investigation of the LAMA3A structure in this study revealed that D1514 residue lies in the C-terminal globular portion of the protein (LG4) that is conserved in mammals including human (ENST00000269217), chimpanzee (ENSPTRT00000018220) and mouse (ENSMUST00000092070) (Fig. 3). The LG domains play role in chain assembly and secretion of LM-332 (Hirosaki *et al.*, 2000) as well as for binding with the cell surface receptor and assembly of hemidesmosomes (Hamill *et al.*, 2010). Absence or reduction along with abberant structure of hemidesmosomes is considered as a marker of JEB. Deletion at N-terminal of the laminin α3a isoform leads to the laryngo-onycho-cutaneous syndrome (LOCS) (McLean *et al.*, 2000). Thus, the position of the p.D1514H mutation in LG4 of LM-332 supports a pathogenic effect.

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human          PTNSFVGCLKNFQLDSKPLYTPSSSFGVS
chimpanzee     PTNSFVGCLKNFQLDSKPLYTPSSSFGVS
mouse          PQHSFVGCLRNFQLDSKPLDSPSARSGVS
chicken        PMKSFKGCLRNFKMNGKVMNTPQQKKDVL
zebrafish      PQKSIIGCIRELRVSKLLLMNPAVNQGAT
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Fig. 3. Multiple sequence alignment of a fragment from LG4 domain of alpha residue of laminin-332 from diverse organisms showing conservation of the D1514 residue in human, chimpanzee and mouse labeled in red.

The C-terminal LG4–LG5 pair, can be released by limited proteolysis (E3 fragment) (Tisi *et al.*, 2000). There are two calcium binding sites on LG4-LG5 pair, these include Asp2982, Asp3055 of laminin α2 chain (Schneider *et al.*, 2007). It also suggested that D1514H might have caused loss of ubiquitinition at K1509 of LAMA3 protein, which could possibly affected the proteolysis of LG4-LG5 from the rest of protein. This separation is critical for the maintenance of BM zone.

Missense mutations may lead to misfolded proteins which causes either malfunctioning or intracellular degradation. Here we reported a change from acidic Aspartic acid to basic Histidine. Our results suggest that this substitution might cause a misfolded LG4 domain which in turn may cause intracellular degradation of LAMA3A protein. If the the alpha chain is altered in structure or missing, assembly with the beta and gamma subunits would result in a non-functional Laminin-332 as suggested from the severe phenotype of the patient with peeled away epidermis.

In conclusion, we suggest this case of lethal Herlitz Junctional Epidermolysis Bullosa to be caused by missense mutation p.D1514H in *LAMA3*. This molecular finding can be used for prenatal diagnosis for subsequent pregnancy in this family.

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Conflict of interest

No conflict of interest.

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