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ORIGINAL ARTICLE

A Novel Mutation p.L461P in *KRT5* Causing Localized Epidermolysis Bullosa Simplex

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Background: Epidermolysis bullosa (EB) is a rare genetic disease with widely different clinical manifestations, but the relationship between genotype and phenotype is not fully understood. In the present study, we recruited a Chinese family in which two members had been diagnosed with localized EB simplex (EBS), with clinical manifestation, including blisters and erosions on the soles of the feet since infancy. **Objective:** To identify and confirm the genetic variation in a Chinese family diagnosed as localized EBS. **Methods:** Our study included two patients, other healthy members of the family, and 100 normal controls. Genomic DNA samples were isolated from each participant, and then polymerase chain reaction (PCR) direct sequencing was performed. **Results:** The results of PCR direct sequencing revealed a novel heterozygous missense mutation in codon 461 of exon 7 of *KRT5* (c.1382T>C), which led to an amino acid change (p.L461P) in the patients with EBS but was absent in unaffected family members and 100 unrelated control samples. **Conclusion:** The present study broadens the mutational spectrum of EBS, and this knowledge could be harnessed for prenatal screening, gene diagnosis, and gene therapy for localized EBS. **(Ann Dermatol 33(1) 11~17, 2021)**

-Keywords-
Epidermolysis bullosa simplex, Keratin 5, Mutation

INTRODUCTION

Epidermolysis bullosa (EB) comprises a group of hereditary disorders, in which blisters occur spontaneously or after minor injury or friction. The phenotypic spectrum of EB is highly variable, and there are differences in severity and associated extraneous manifestations between different types of EB. However, all types of EB present with traumatic blistering and fragility[1,2]. Based on the level of the dermal-epidermal separation relative to the basement membrane, EB can be divided into four subtypes—simplex, junctional, dystrophic, and Kindler syndrome[3]. In the most common subtype, EB simplex (EBS), separation occurs at or above the basal layer of keratinocytes in the epidermis. Statistics from 1986 to 2002 showed that the prevalence of EB was about 11 cases per 1 million residents and the incidence rate was about 19 cases per 1 million live births[4]. EBS shows significant genetic and clinical heterogeneity. EBS can be subdivided into the generalized severe (Dowling-Meara), generalized intermediate (Koebner), and localized subtypes[5]. Localized EBS (OMIM 131800) is the mildest subtype, in which bullous lesions usually occur during the birth or infancy period, although they may also appear during adolescence or early adulthood. Clinically, the vesicles and bullae are primarily confined to the palmar and plantar regions without nail and mucosal damage[6].

Almost all EBS mutations follow the pattern of autosomal dominant inheritance[7]. EBS is mainly associated with mutations in two genes, *KRT5* and *KRT14*. *KRT5* and *KRT14*

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genes encode basal epidermal keratin 5 and 14, respectively[8]. Both keratin 5 and keratin 14 have a central rod-like alpha helix structure and a non-helix structure (head and tail) on both sides. At the beginning and end of the central rod-like region, there are two highly conserved amino acid sequences, called helix initiation peptide (HIP) and helix termination peptide (HTP)[9]. In most cases, the location of the mutation determines the severity of the clinical phenotype. For example, mutations in HIP and HTP that affect the assembly of keratin filaments lead to the most severe Dowling-Meara EBS (EBS-DM; OMIM 131760); Mutations often lead to localized EBS in nonhelical linker regions, while Koebner (EBS-K; OMIM 131900) mutations are more widely distributed along both keratin 5 and keratin 14 polypeptides[10]. To date, more than 150 different pathogenic mutations associated with these genes have been described[10], most of them are associated with the more severe EBS-DM[5].

In the present study, two patients from a Chinese family who had been diagnosed with localized EBS were evaluated, and a novel missense mutation c.1382T>C (p.L461P), which was located at the 2B segment of the KRT5, confirmed a diagnosis of localized EBS. Identification of new mutations and genotype-phenotype associations leads to a better understanding of the underlying pathophysiologic basis of localized EBS and is important for disease course prediction, gene diagnosis, genetic counseling, and gene therapy.

MATERIALS AND METHODS

Clinical evaluation and DNA sampling

In total, five members of the localized EBS pedigree (Fig. 1) were enrolled in the study. Members II:1 (42-years-old female) and III:1 (15-years-old female; proband) presented to our hospital. Our study also included proband's father, grandparents, and 100 normal control subjects. Written informed consent was obtained from all participating individuals (or their guardians), and 5 ml blood samples were drawn from each participant for DNA extraction. Clinical examinations of all participants were carried out by a dermatologist. The current research, which was performed in compliance with the principles of the Declaration of Helsinki, was authorized by the Institutional Review Board of Tongling People's Hospital. We received the patient's consent form about publishing all photographic materials. Ethical approval was gained from the Ethics Committee of Tongling People's Hospital (2020001).

Mutational analysis and multiple sequence alignment

We used the polymerase chain reaction (PCR) to amplify genomic DNA samples (Humphries et al. 1996)[11], after which PCR products were sequenced with Sanger sequencing. Consulting the National Center for Biotechnology Information (NCBI) cDNA reference sequences NM_000424.3 for *KRT5* and NM_000526.4 for *KRT14*, we identified a specific mutation present only in clinically affected family members. Multiple online bioinformatics software programs, including follows, PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.bii.a-star.edu.sg), and Mutation Taster (http://www.mutationtaster.org/), were used to predict whether the resulting amino acid substitution could have an effect on the structure and function of the KRT5 and KRT14. We aligned the proteins of different species, including *Bos taurus*, *Gallus gallus*, *Gorilla gorilla*, *Mus musculus*, and *Oryctolagus cuniculus*. The change of structure between wild-type and mutation (p.L461P) was predicted by SWISS-MODEL (https://swissmodel.expasy.org, PBD: 3TNU). A structural comparison of the wild-type and mutation revealed notable differences between them.

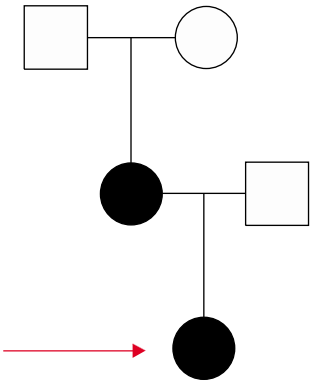

Fig. 1. Family pedigree. The family tree shows two affected members across three generations. The unfilled symbols represent the unaffected members of the pedigree; the dark symbols indicate the affected members. Circles and squares represent females and males, respectively. The proband of pedigree has been marked with a red arrow.

RESULTS

Phenotypic features of the affected patients

There were two affected people in this family. The proband, a 15-year-old Chinese girl, was the only daughter of unrelated Chinese parents. She and her mother have similar clinical symptoms, and there is no other family history. Since birth, the proband and her mother have been prone to blisters after mechanical injury to the foot, but the blisters heal without scarring. The symptoms were worse in the summer and improved with age. Physical examinations were normal except the blisters (Fig. 2). Pathologic examination of the right plantar blister of the proband re-

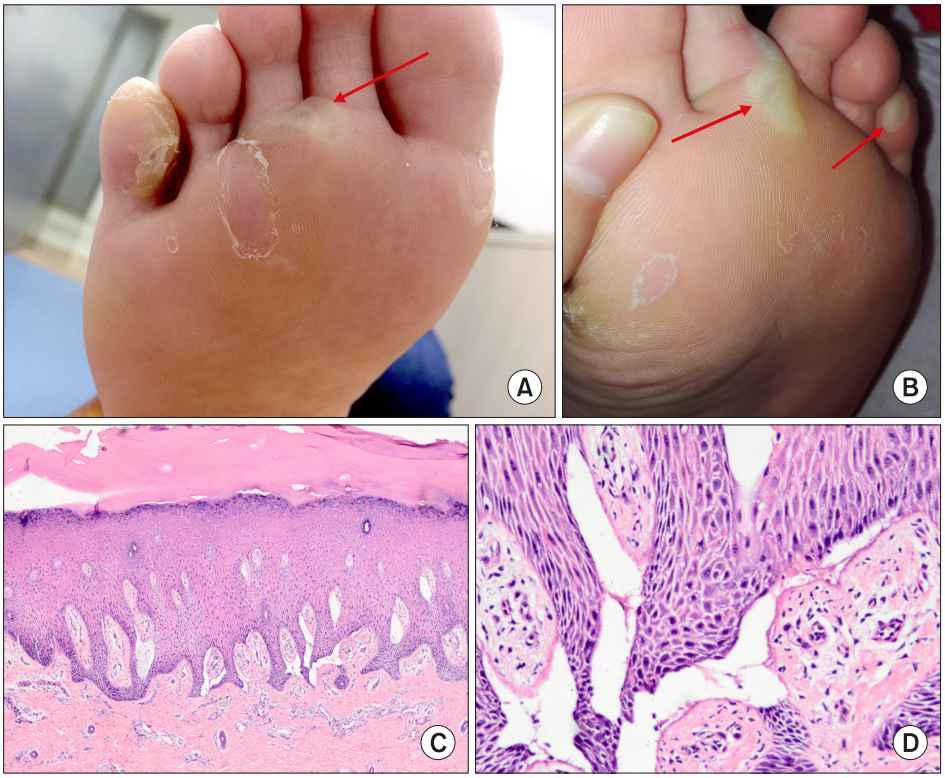

Fig. 2. Clinical manifestations of the patients with localized epidermolysis bullosa simplex. The blisters in the figures has been marked with a red arrow. (A) Blisters and erosions are observed on the proband's right foot. (B) Similar blisters are found in the mother's left sole. (C) The epidermal epithelium is hyperkeratinized, the spinous layer is thickened, and the vesicles in the epidermis (H&E, ×40). (D) The fissure is located inside the epidermis (H&E, ×200).

vealed hyperkeratosis of the epidermis, thickening of the granulosa and spinous layer, and vesicles form in the epidermis. The vessels in the dermal papillary layer were slightly dilated, with little inflammatory cell infiltration, and the perivascular stroma was slightly mucinous (Fig. 2).The diagnosis of this disease was based on the combination of typical clinical manifestations, family history and pathological examination, so it was diagnosed as localized EBS. The molecular tests we performed further confirmed the diagnosis.

Mutation analysis and protein structure modeling

Localized EBS is one of the most common clinical variant diseases of EBS, as well as the mildest type. According to published data, mutations in many genes are associated with EBS phenotype, including *TGM5*, *PLEC*, *PKP1*, *KRT5*, *KRT14*, *DSP*, *JUP*, *DST*, and *EXPH5*, but more than 75% of EBS cases are attributed to the mutation of *KRT5* or *KRT14* genes[12]. Thus, here we selected these two genes as the candidate causative genes to screen for mutation.

Molecular analysis of the proband's DNA indicated a heterozygous T to C transition at nucleotide position 1382 (c.1382T>C) in exon 7 of the *KRT5* gene. The mutation leads to the substitution of proline residue for leucine at codon 461 (p. Leu461Pro). The identical mutation in *KRT5* was detected in the proband's mother. The mutation is located at the 2B segment of the *KRT5*, confirming a diagnosis of localized EBS. No additional mutation was identified in *KRT5* or *KRT14*. No mutation was found in unaffected family members or the 100 population-matched healthy controls (Fig. 3). PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.bii.a-star.edu.sg), and Mutation Taster (http://www.mutationtaster.org/) all predicted that the mutation c.1382T>C (p.L461P) was likely to disrupt protein function, although proline and leucine have similar polarity.

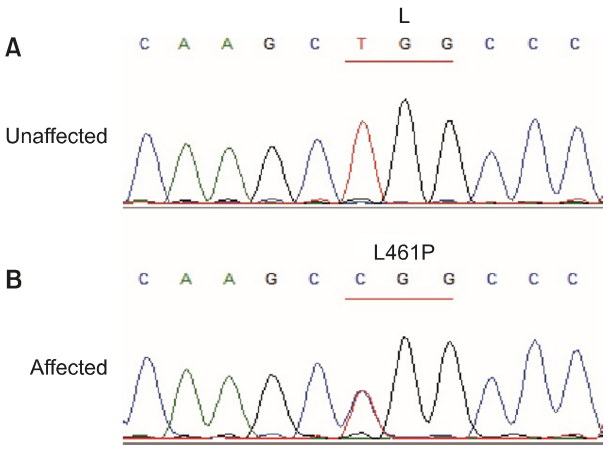

Fig. 3. Mutation analysis of *KRT5*. The molecular analysis demonstrates a *de novo* heterozygous missense mutation c.1382T>C (p.Leu461Pro) in the *KRT5* gene. (A) Unaffected members, (B) affected members.

We compared proteins from different species and obtained structural-based multi-sequence alignment of KRT5 from different species, showing that the mutation (p. l 461pro) is located in a highly conserved region (Fig. 4).
In wild-type KRT5, Leu461 forms two hydrogen bonds, with Met457 and Val465, while the mutant Pro461 only forms one hydrogen bond, with Val465. The reduction in hydrogen bonds may impact the stability of 2B, alpha-helical domains whose spatial structure is maintained by hydrogen bonds (Fig. 5). The c.1382T>C (p.L461P) mutation occurred in the non-helical junction region, and the proline of the same polarity replaced the leucine, which resulted in the existence of lightest EBS. Collectively, these results indicate that p.L461P in *KRT5* is a causative mutation for localized EBS in this family.

Retrieved reported KRT5 mutation and analysis

To investigate whether there are any mutation-rich exons for KRT5 mutations, we retrieved all the reported mutation in KRT5. As shown in Table 1[8,10,13-25], about 28 novel KRT5 mutations responsible for EBS have been found since January 1, 2008. Mutations in the tail domain of KRT5 are often associated with EBS-migratory circinate erythema (EBS-Migr) and pigment forms of the disease, suggesting that this region may play a role in the regulation of pigmentation and inflammation. Also, as to the genetic diagnose, more attention should be paid to these exons coding for tail domains when screening for mutations causing EBS. We noticed that only a few mutations are associated with localized EBS, which are distributed in all regions of KRT5 but mainly gathered in the head and the non-helical linker regions. Examples include p.Val133Met, p.Asn146Lys, p.Met327Thr, p.Asp328Gly and the novel mutation (p.Leu 461Pro) in our study[5,11,13]. It revealed that if the patients are diagnosis as localized EBS, sequences coding for head and the non-helical linker regions of KRT5 should have propriety for the mutation screening. Because the process of gene expression is complicated and can be influenced by other genes and external factors, it may be cautious in predicting the relationship between genotype and phenotype[14].

DISCUSSION

KRT5 and *KRT14* genes encode the intermediate filament (IF) proteins in the basal layer of the epidermis and related complex epithelia[26]. The keratin intermediate filament (KIF) network is attached to desmosomes and hemi-desmosomes, connecting keratinocytes to adjacent cells and basement membranes, forming intercellular adhesions that protect epithelial cells from mechanical and other stresses[27]. EBS is closely related to mutations in *KRT5* and *KRT14*, but the mechanism by which mutations cause the formation and collapse of keratin networks remains unclear[28].
It is reported that disruption of KRT5/KRT14 filament network architecture can cause the fragility of basal keratinocytes, making the cells unable to bear mechanical pressure, then further leading to intracellular vacuoles, which eventually result in blistering of the skin. Sawant et al.[29] found that threonine 150 (T150) KRT5 phosphorylation plays an important role in the formation of the KIF network, relating to the pathogenesis of EBS. In addition, other

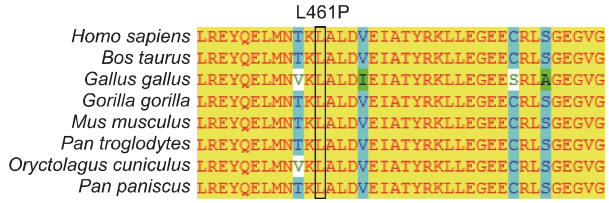

Fig. 4. Multiple-sequence alignment of KRT5 from different species. Structure-based multiple-sequence alignment of KRT5 from different species revealed that the mutation (p.Leu461Pro) was located within a highly conserved region.

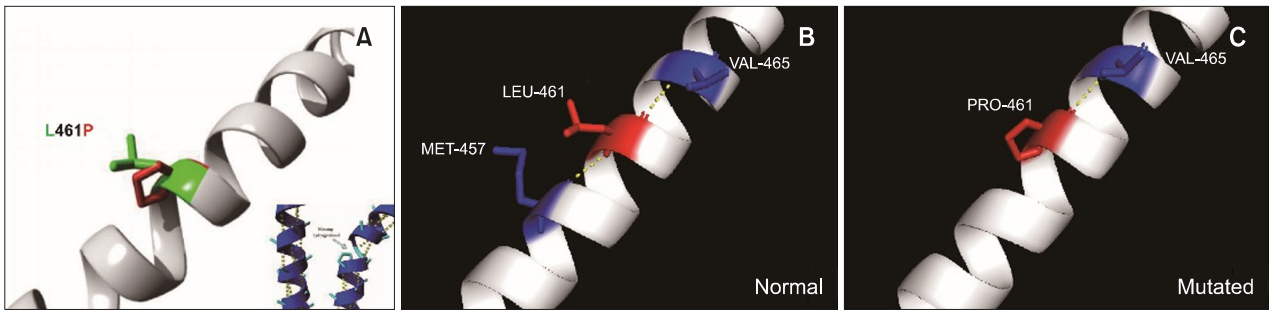

Fig. 5. Structural model of the L461P mutation. The yellow dashed lines indicate the hydrogen bonds between two amino acids and demonstrate the difference between the structures. (A) LEU-461 and PRO-461 are in green and red, respectively. (B) In wild-type KRT5, LEU-461 forms two hydrogen bonds (with MET-457 and VAL-465). (C) The mutant PRO-461 only form sone hydrogen bond (with VAL-465).

Table 1. Novel *KRT5* mutations for localized epidermolysis bullosa simplex

Author (year)	Exon	Nucleotide change	Protein change	Keratin domain
Jerábková et al. (2010)[15]	1	c.428T>C	p.V143A	Head
Wertheim-Tysarowska et al. (2016)[13]	1	c.436A>T	p.N146Y	Head
Kim et al. (2017)[16]	1	c.464T>C	p.L155P	Head
Kim et al. (2017)[14]	1	c.502G>C	p.E168Q	Head
Minakawa et al. (2013)[17]	1	c.505C>G	p.R169G	1A
Arin et al. (2010)[10]	1	c.514A>G	p.I172V	1A, HIP
Kim et al. (2017)[14]	1	c.535T>C	p.F179L	1A
Glász-Bóna et al. (2009)[18]	1	c.547A>G	p.I183V	1A
García et al. (2011)[19]	1	c.557T>A	p.V186E	1A
Glász-Bóna et al. (2009)[18]	1	c.570G>C	p.E190D	1A
Bowden et al. (2009)[20]	1	c.593C>G	p.T198S	1A
Flohil et al. (2010)[8]	1	c.596A>T	p.K199T	1A
Gao et al. (2015)[21]	1	c.605T>C	p.L202Q	1A
Cho et al. (2014)[22]	1	c.608T>C	p.L203P	L1
García et al. (2011)[19]	3	c.961A>C	p.T321P	L12
Chiang et al. (2008)[23]	3	c.971T>C	p.V324A	L12
Wertheim-Tysarowska et al. (2016)[13]	3	c.974T>C	p.L325P	L12
Arin et al. (2010)[10]	3	c.991C>A	p.R331S	L12
Glász-Bóna et al. (2009)[18]	3	c.991C>G	p.R331G	L12
Lev-Tov et al. (2012)[24]	6	c.1270G>C	p.A424P	2B
García et al. (2011)[19]	6	c.1283G>A	p.A428T	2B
Minakawa et al. (2013)[17]	6	c.1327A>G	p.K443E	2B
Arin et al. (2010)[10]	6	c.1362del4insAGCTGGTA	p.E455AfsX117	2B
Wertheim-Tysarowska et al. (2016)[13]	7	c.1412G>A	p.R471H	2B
Arin et al. (2010)[10]	7	c.1438A>G	p.R480G	2B, HTP
Arin et al. (2010)[10]	8	c.1636C>A	p.L546I	Tail
Minakawa et al. (2013)[17]	9	c.1644del4	p.G550AfsX82	Tail
Bchetnia et al. (2012)[25]	9	c.1675C>T	p.R559X	Tail

HIP: helix initiation peptide, HTP: helix termination peptide.

pathological mechanisms play important roles in the pathophysiology of EBS, including preinflammatory cytokines, the tumor necrosis factor, interleukin-1 β (IL-1 β), and signaling pathways which associated with them. Russell et al.[30] found that the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB) signal transduction channels of ERK and PKB due to mechanical stresses were involved in EBS, leading to the resistance of keratin mutated cells to apoptosis after channel activation[9]. Beyond that, there are other mechanisms involved in the occurrence of disease, including impaired mechanical stress recovery, downregulation of cellular junction elements, and destruction of epithelial cell adhesion[31]. So far, we do not know how the mutation (p.L461P) causes clinical manifestation in this family; further studies are needed to provide insights into the detailed molecular pathogenesis of the mutations identified in the present study.

At present, there are no effective treatment methods for EBS; the main treatment is symptomatic management. For example, plantar injection of botulinum toxin, as a safe and long-lasting method, can effectively block the occurrence of blisters[32]. In addition, it has been reported that long-term oral erythromycin may be effective on EBS-DM[33]. A 1% diacerein ointment is considered to be a well-tolerated and safe targeted therapy that significantly reduces blisters in most EBS-DM patients[34]. Because of the overexpression of Th17 cytokines found in the blister roof and fluid in the skin of EBS-DM patients[35], with anti-IL-17 agents treatment, a sharp reduction in the number of blisters in all patients has been observed[36]. The natural chemical sulforaphane produced by broccoli has been shown to treat blisters in KRT14 deficient mice by inducing the expression of KRT16 and KRT17 in the epidermal basal layer and improving the EBS phenotype[37]. Recently, Peking et al.[27] successfully developed an *ex vitro* gene therapy using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to correct a recurrent *COL7A1* mutation of dystrophic EB in a xenograft mouse model. Clearly, considerable progress has been made in the treatment of EB with RNA, but there are limitations. Since it is a genetic disease, genome editing as state-of-the-art gene therapy approach may be harnessed for clinical treatment. Specifically, because the mutation is c.1382T>C in *KRT5* gene, it could be corrected with cytidine base editor to convert C-to-T conversion[38].

Taken together, this is the first study reporting the KRT5: p.L461P missense mutation at the 2B helix, or any mutation in codon 461 of KRT5. Identification of this novel mutation (p.L461P) in KRT5 would lead to a better understanding of the underlying pathophysiologic basis of localized EBS, which may be harnessed for gene diagnosis, genetic counseling, and gene therapy. Also, it provides more insight into the phenotype-genotype correlation in EBS.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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