Diverse clinical and genetic characteristics of six cases of inherited epidermolysis bullosa

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Received August 3, 2022; Accepted October 3, 2022

DOI: 10.3892/etm.2022.11663

Abstract. Inherited epidermolysis bullosa (IEB) represents a group of rare genetic dermatoses comprising various phenotypes ranging from severe cutaneous and extracutaneous involvement to mild cutaneous fragility. Pathogenic variants have been identified in at least 20 genes responsible for IEB. In the present study, six cases of epidermolysis bullosa were recruited and subjected to a combination of clinical and genetic analysis. The family history of each case was surveyed. Whole exome sequencing was performed to identify the causative variation. The six patients showed typical EB symptoms. In all cases, WES detected the diagnostic variations of the *COL7A1* or *DST* gene. A total of 10 variants were identified and verified. The findings of the present study further expanded the mutation spectrum of IEB, provided evidence for genetic counseling to the affected families, as well as highlighted the complexity of the pathogenesis of IEB.

Introduction

Inherited epidermolysis bullosa (IEB) represents a group of rare heterogeneous genetic dermatoses characterized by mucocutaneous fragility and blister formation, often induced by trivial trauma (1). Patients with IEB can be affected mildly to severely, while in extreme cases, the disease can be debilitating or mortal (1). Patients with severe EB may show the involvement of not only the skin tissue but also any epithelial-lined organ (2).

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Key words: inherited epidermolysis bullosa, whole exome sequencing, *COL7A1* gene, *DST* gene, variations

Recently, due to the emergence of EB-related pathogenic genes and clinical subtypes after a previous classification was revised in 2014 (3), those practitioners studying IEB have to understand it anew. According to the latest consensus report, >30 subtypes of EB comprised four main types: EB simplex (EBS), Junctional EB (JEB), Dystrophic EB (DEB) and the rarely occurring Kindler EB (KEB), generally based on the level of skin cleavage (4). Other disorders with relatively minor skin blisters are also classified into separate categories, including peeling skin disorders, erosive disorders, hyperkeratotic disorders and connective tissue disorders with skin fragility (4). As examples of large cohorts, according to a long-term and large-sample epidemiological survey (the National EB Registry of USA, ~1986-2002), IEB occurred at a rate of ~11.1 cases per million individuals and 19.6 cases per million live births, with no differences among the sexes or ethnic groups (5). EB, in general, or its specific subtypes, has been reported to have a higher prevalence in some ethnic groups, which may simply reflect improved case collection and integrity of molecular diagnostics in these studies (6,7). Among East Asians, the largest ethnic group, the incidence of IEB has not been accurately reported, except for Japan (8). This limits the development of research on the disease in this region.

To date, pathogenetic variations in at least 20 distinct genes encoding proteins influencing cellular integrity and adhesion have been implicated in IEB (1). EBS is the predominant type accounting for ~70% of the EB cases, which could be caused by mutations in *KRT5*, *KRT14*, *PLEC*, *KLHL24*, *DST*, *EXPH5* (syn. *SLAC2B*), *CD151* (syn. *TSPAN24*), *TGM5*, *PKP1*, *DSP* and *JUP* genes. JEB is associated with mutations in *LAMA3*, *LAMB3*, *LAMC2*, *COL17A1*, *ITGA6*, *ITGB4* and *ITGA3,* while DEB and KEB are caused by mutations in *COL7A1* and *FERMT1* (syn. *KIND1*), respectively (4). An accurate diagnosis is established based on multimodal methods consisting of transmission electron microscopy (TEM), immuno-fluorescence antigen mapping of the affected skin and DNA mutational analysis (9). Recently, the advancement in gene sequencing techniques promises faster, cheaper and more comprehensive diagnosis, facilitating the identification of new genes and ultimately personalized treatments (10,11). Precise molecular diagnosis,

although not currently fully functional, is essential to advance the understanding of disease to provide a basis for the potential stratification and prognostication, as well as a platform for tailored or stratified management of disease, including genetic counseling and targeted therapies (12-15).

The present study aimed to provide a definite molecular diagnosis on six enrolled cases with suspected IEB. It conducted a comprehensive survey of clinical and family history and detected mutations using whole exome sequencing. The findings confirmed the complexity of clinical and genetic characteristics for IEB.

Materials and methods

Subjects. The six cases with apparent EB were recruited in the Department of Dermatology, the First Hospital of Hebei Medical University, Shijiazhuang, between January 2018 and December 2021. The clinical evaluation was made by GZ via routine clinical examination, family survey and TEM testing of the skin tissue obtained by biopsy (only for Case 1). Genomic DNA was extracted from the peripheral blood specimens of the patients and their parents using the QIAamp DNA Midi kit (Qiagen GmbH) for further testing.

Whole-exome sequencing (WES). WES was used to detect the sequence variants in the samples of the probands (16). Briefly, the sequences of the target region were enriched using the Agilent Sure Select Human Exon Sequence Capture kit (Agilent Technologies, Inc.). The DNA libraries were tested using quantitative PCR, where the size, distribution and concentration were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). The DNA of ~150 bp paired-end reads was sequenced using the NovaSeq6000 platform (Illumina, Inc.), taking ~300 pM of DNA per sample using the NovaSeq Reagent kit. Sequencing raw reads (quality level of Q30%>90% and the criteria for quality listed at https://www.illumina.com/science/technology/next-generation-sequencing/plan-experiments/quality-scores.html) were aligned to the human reference genome (accession No. hg19/GRCh37) using the Burrows-Wheeler Aligner tool (bwa-0.7.17.tar.bz2) (17), following which, duplicate PCR products were removed using the program Picard v1.57 (https://github.com/broadinstitute/picard). Variant calling was performed using the Verita Trekker® Variants Detection system (v2.0; Berry Genomics Co., Ltd.) and Genome Analysis Tool kit (https://software.broadinstitute.org/gatk/). Then, variants were annotated and interpreted using the ANNOVAR (v2.0) (18) and Enliven® Variants Annotation Interpretation systems (Berry Genomics Co., Ltd.), according to the guidelines by ACMG (American College of Medical Genetics and Genomics) (19). To assist in the interpretation of variant pathogenicity, the present study referred to three frequency databases, namely ExAC_EAS (http://exac.broadinstitute.org), gnomAD_exome_EAS (http://gnomad.broadinstitute.org), 1000G_2015aug_eas (https://www.internationalgenome.org) and Human Gene Mutation Database Pro v2019 (https://www.hgmd.cf.ac.uk/ac/index.php). The Revel score (a combined method of pathogenicity prediction) (20) and pLI score (representing the tolerance for truncating variants) were also employed.

Sanger sequencing. For validation, Sanger sequencing was performed on potentially causative-specific variants using the 3730 DX Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Analysis of conservatism. The evolutionary conservatism of all affected amino acid (AA) residues by the corresponding missense variants was analyzed using the online tool MEGA7 (http://www.megasoftware.net/previousVersions.php) with default parameters.

Results

Clinical manifestations. All six patients included in this study showed EB-like phenotypes shortly after birth, except for Case 4, who began to develop multiple skin breakages and blister formation at ~8 years of age. The parents of this patient denied any family history of genetic disease, except that the elder brother of this patient also had an EB presentation. Specifically, the clinical characteristics and family history of these cases were as follows:

Case 1, a 4-month-old girl, initially exhibited erosion and desquamation at both palms, soles and oral mucosa after birth. Then, her entire body showed recurrent blisters, tatters and scabs, part of which developed infections (Fig. 1A-C). TEM revealed a split epidermis (Fig. 1D). Case 2, a 3.5-year-old boy who displayed mild EB phenotype presenting with localized repeated skin breakages on the fingers, toes, knees and ankles (Fig. 1E-H). In addition, the boy showed autism-like features, such as difficulty in communication and concentration. Case 3, a 34-year-old male who, along with his elder brother, presented with moderate to severe EB that was mainly localized in the back, neck, elbows, lower extremities and fingers (Fig. 2A-F for the patient; G-H for the elder brother). The patient had progressive nail loss. Also, his brother developed subcutaneous pustules and infections at the shins, as well as truncated and fused fingers and toes as the result of prolonged illness and poor care.

Case 4, a 13-year-old boy, who had been exhibiting mild to moderate EB phenotype mainly with erythema blisters on the distal extremities since the age of 8 years (Fig. 3A and B). Case 5, a 4-month-old girl, started showing multiple skin lesions and blistering shortly after birth (Fig. 3C-E). Case 6, a newborn girl, showed multiple skin lesions and strephenopodia (Fig. 3F-H).

Genetic findings. All six cases showed positive results for WES detection, which was also confirmed by Sanger sequencing. A total of 10 variants distributed in *COL7A1* and *DST* genes were detected. The detailed information of all variants is presented in Table 1, while the pattern shown by the members of each family is presented in Table 1 and Fig. 4.

Specifically, Case 1 harbored a compound heterozygous variation in *COL7A1* that consisted of two variants, c.5404_5405 delGC (p.Ala1802Trpfs*69) and c.4811G>A (p.Gly1604Glu), which were inherited from her parents (Fig. 4: Family 1). Case 2 had a *de novo* heterozygous missense variation, namely *COL7A1*: c.6191G>A (p.Gly2064Glu; Fig. 4: Family 2). Case 3 carried a compound heterozygous *COL7A1* variation consisting of c.5932C>T (p. Arg1978*)

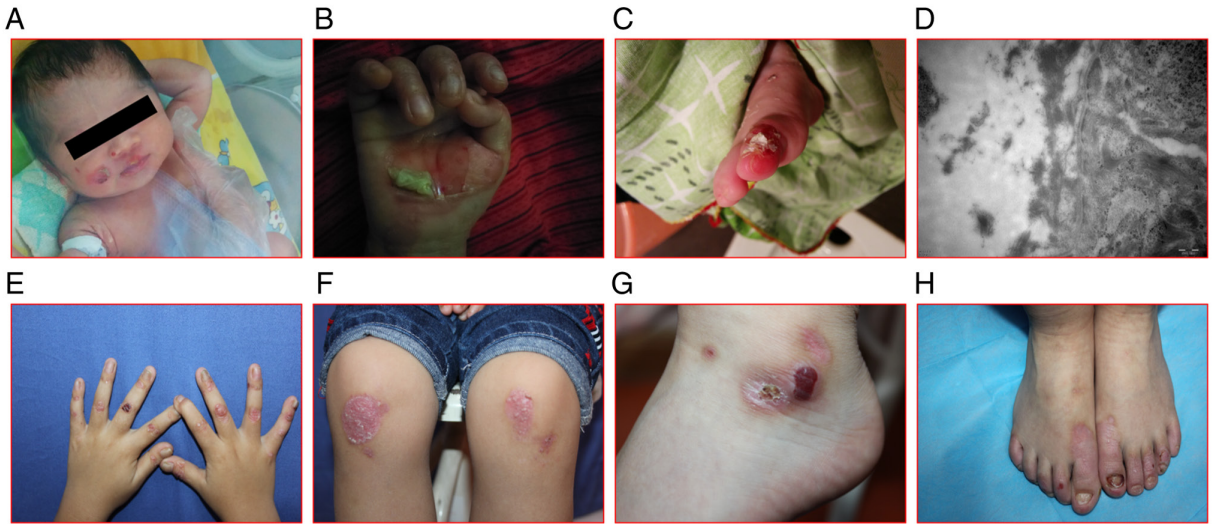

Figure 1. The EB symptoms of (A-D) Case 1 and (E-H) Case 2.

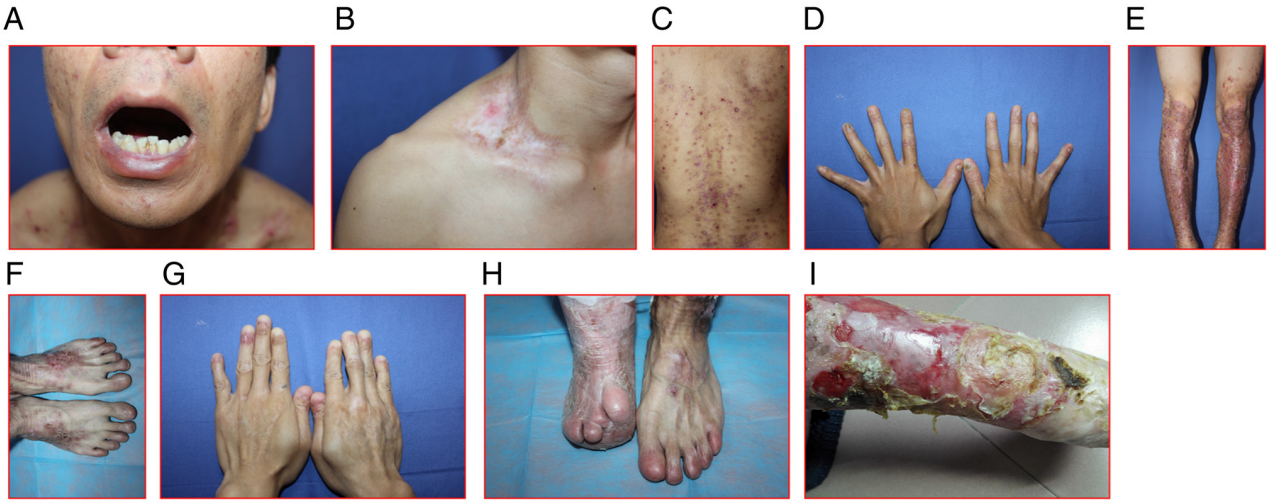

Figure 2. The EB symptoms of Case 3 (A-F) the proband; (G-I) the elder brother of the proband.

and c.8065G>A (p.Gly2689Arg) (Fig. 4: Family 3). Case 4 had a *de novo* heterozygous intergenic deletion, *COL7A1*: c.4783-40_7068+22del (Fig. 4: Family 4). Case 5 carried a compound heterozygous variation in the *DST* gene, consisting of c.7577G>A (p.Ser2526Asn) and c.6905G>A (p.Arg2302His) (Fig. 4: Family 5). Case 6 carried a compound heterozygous *COL7A1* variation, consisting of c.6329delC (p.P2110Lfs*96) and c.3625_3635del (p.S1209Lfs*6) variants (Fig. 4: Family 6). Among these variants, four, namely *COL7A1*: c.4783-40_7068+22del, *DST*: c.7577G>A (p.Ser2526Asn), *DST*: c.6905G>A (p.Arg2302His) and *COL7A1*: c.6329delC (p.P2110Lfs*96), were newly identified.

Regarding Case 2, WES also revealed two suspected variations that might contribute to the autistic symptoms of the patients. One variation was a compound heterozygous variation in the *LFNG* (NM_001166355) gene, consisting of c.139_142del (p.Asp55Serfs*141) and c.142_143insGATG (p.Glu56Glyfs*2), which were inherited from the patient's parents. Another variation was a *de novo* missense variation, namely *SCN9A* (NM_002977): c.554G>A (p.Arg185His). These two variations and their carrying status are presented in Fig. S1.

The conservatism of the amino acid residues affected by missense variations. A total of five missense variants, namely *COL7A1*:c.4811G>A (p.Gly1604Glu), *COL7A1*: c.6191G>A (p.Gly2064Glu), *COL7A1*: c.8065G>A (p.Gly2689Arg), *DST*: c.7577G>A (p.Ser2526Asn) and *DST*: c.6905G>A (p.Arg2302His), were detected in the present study. The homologous sequences of the DST protein have been resolved in only a few species, so the nature of conservatism of the two amino acids (Ser2526 and Arg2302) in it were not analyzed. The MEGA7 analysis demonstrated that the AA residues affected by the three variants in COL7A1 remained highly conserved among multiple species (Fig. 5).

Discussion

IEB comprises various conditions with overlapping skin and epidermal-link phenotypes, each with unique

Table I. Information of the genetic variants identified in this study.

Case No.	Carrier ID	Gene[a]	DNA variation	Protein variation	Variation frequencies in 3 databases[b]	HGMD rating[c]	CADD_ PHREAD score[d]	Revel score[e]	Pathogenicity rating (evidences)[f]
1	1.1;1.2	*COL7A1*	c.5404_5405delGC	p.Ala1802Trpfs*69	-; -; -	DM	/	/	LP (pvs1+pm2)
	1.1;1.3	*COL7A1*	c.4811G>A	p.Gly1604Glu	-; -; -	DM	27.6	0.936	VUS (pm2+pm5+pp3)
2	2.4	*COL7A1*	c.6191G>A	p.Gly2064Glu	-; -; -	DM	28.4	0.982	LP (pm2+pm5_strong+pp3)
3	3.1;3;3;3.4	*COL7A1*	c.5932C>T	p. Arg1978*	-; -; -	DM	35	/	LP (pvs1+pm2)
	3.2;3.3;3.4	*COL7A1*	c.8065G>A	p.Gly2689Arg	-; -; 0.000054	DM	31	0.982	LP (pm2+ps1+pp3)
4	4.3	*COL7A1*	c.4783-40_7068+22del	/	-; -; -	/	/	/	P (one copy loss of 2 exons)
5	5.1;5.3	*DST*	c.7577G>A	p.Ser2526Asn	-; 0.000356; 0.000390	/	19.47	0.039	VUS (pm2+bp4)
	5.2;5.3	*DST*	c.6905G>A	p.Arg2302His	-; 0.000204; 0.000250	/	23.8	0.267	VUS (pm2)
6	6.1;6.3	*COL7A1*	c.6329delC	p.P2110Lfs*96	-; -; -	/	/	/	LP (pvs1+pm2)
	6.2;6.3	*COL7A1*	c.3625_3635del	p.S1209Lfs*6	-; -; -	DM	32	/	P (pvs1+pm2+ps4_supporting)

[a]Gene transcript version: COL7A1, NM_000094.3; DST, NM_001144769.3. [b]Three databases: 1000g2015aug_eas (https://www.internationalgenome.org/); ExAC_EAS (http://exac.broadinstitute.org); gnomAD_exome_EAS (http://gnomad.broadinstitute.org/). [c]HGMD, Human Gene Mutation Database (Professional Version 2021.10). [d]CADD score: A comprehensive scoring algorithm for the harmful degree of mutation (http://cadd.gs.washington.edu/score). [e]Revel: An ensemble method for predicting the pathogenicity of missense variants on the basis of individual tools: MutPred, FATHMM, VEST, PolyPhen, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP, SiPhy, phyloP andphastCons (http://dx.doi.org/10.1016/j.ajhg.2016.08.016). [f]Pathogenicity level rating by ACMG (The American College of Medical Genetics and Genomics); P, pathogenic; LP, likely pathogenic; VUS, variants of unknown significance.

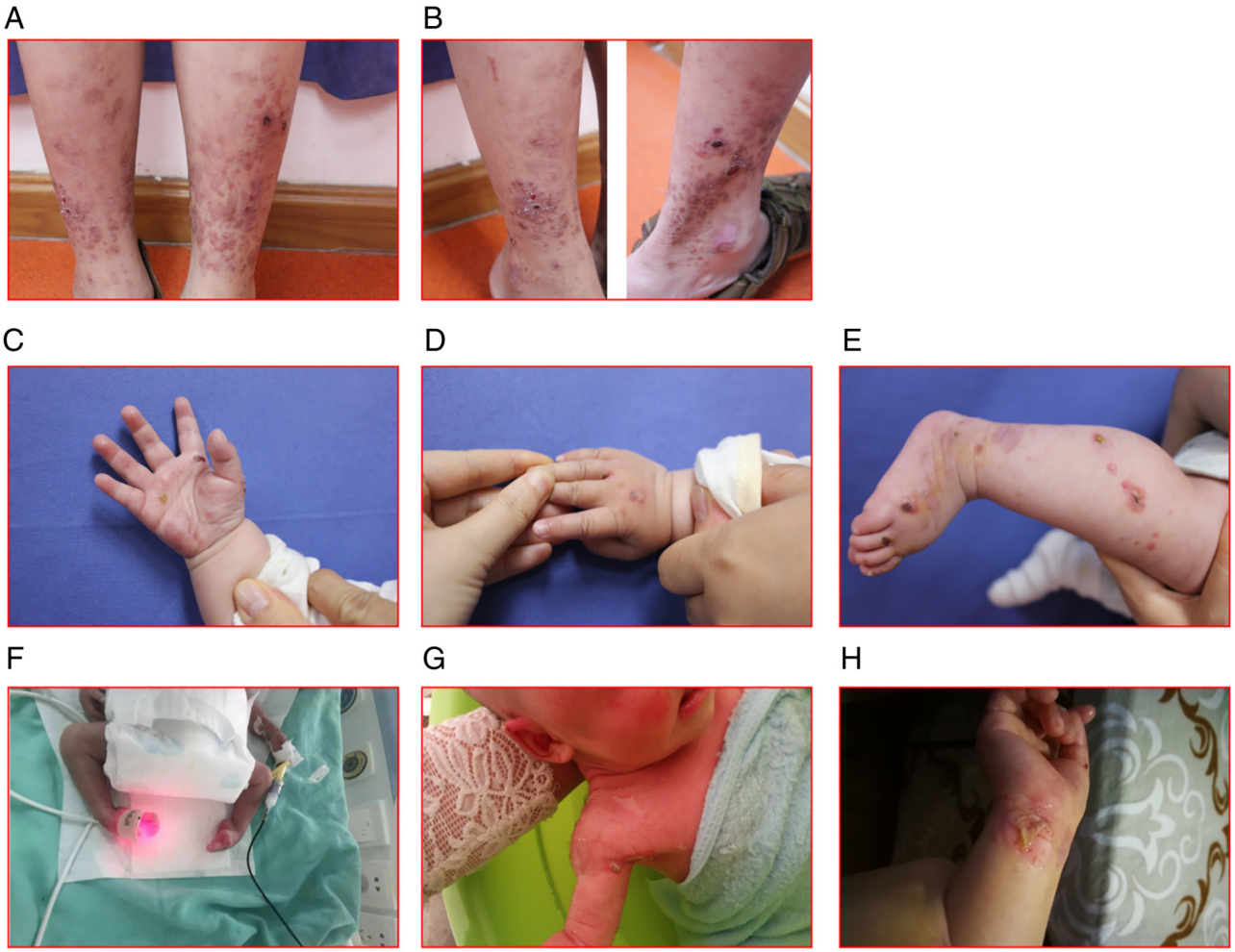

Figure 3. The EB symptoms of (A and B) Case 4, (C-E) Case 5 and (F-H) Case 6.

characteristics (1,21-23). Clinically, it is difficult to accurately diagnose the subtype of IEB, especially in newborns. However, an accurate diagnosis is vital for prognostics, genetic counseling and patient management (12,24). The present study used WES to directly detect the causative gene of EB in six Chinese families and found disease-associated variants in the known EB genes of *COL7A1* and *DST*. The distribution of the subtypes of EB is different in different countries. Worldwide variations in the population and level of immigration (ethnic background, consanguineous marriages and spectrum of mutations) may affect the epidemiology and distribution of the subtypes of EB per region (1). Research indicates that the recurrent mutations R578X, 7786delG and R2814X in *COL7A1* seem to be exclusive to a specific ethnic group, the British population; in addition, the mutations 5818delC, 6573+1G->C and E2857X are present only in individuals of Japanese ethnic origin (8). However, due to the limited sample size, a larger screening effort is necessary for us to further clarify whether there are ethnic difference between Asian patients and non-Asian patients (1,25).

Dystrophic EB (DEB, MIM #131750 and #226600) is characterized by the cleavage of the upper dermis (22). DEB arose from the *COL7A1* (MIM *120120) mutations that resulted in mutant type VII collagen and disrupted anchoring fibrils (1,22). In the present study, DEB accounted for the majority (five) of the six cases, which was not consistent with the situation in other studies, in which EBS had the highest incidence. This may be attributed to the small sample size of the present study or differences in our ability and standard of clinical identification (5,15,26). Among the five subjects with DEB, two (Case 2 and 4) had *de novo COL7A1* variations and conformed to the autosomal dominant pattern, while three (Case 1, 3 and 6) carried compound heterozygous variations in *COL7A1* and conformed to the autosomal recessive pattern. Mutations of *COL7A1* were linked to ADDEB in 1991 by Ryynänen *et al* (27) and ARDEB in 1993 by Christiano *et al* (28). Until now, ~1,000 variants of *COL7A1* have been related to DEB (http://www.col7a 1-datab ase.info; http://www.hgmd.cf.ac.uk/ac/index.php) (29). Generally, the symptoms of ARDEB are more severe, including skin fragility that is manifested by blistering with minimal trauma that heals with milia and scarring, while in ADDEB, blistering is often mild and limited to the hands, feet, knees and elbows, although it heals with scarring (22). The clinical phenotypes of our five DEB cases were consistent with this pattern; thus, Cases 1, 3 and 6 showed more severe and widespread symptoms. However, it was also observed that the brothers in Case 3 showed some difference in phenotypic severity, which suggests that there may be other factors regulating DEB phenotypes, which need further clarification (30).

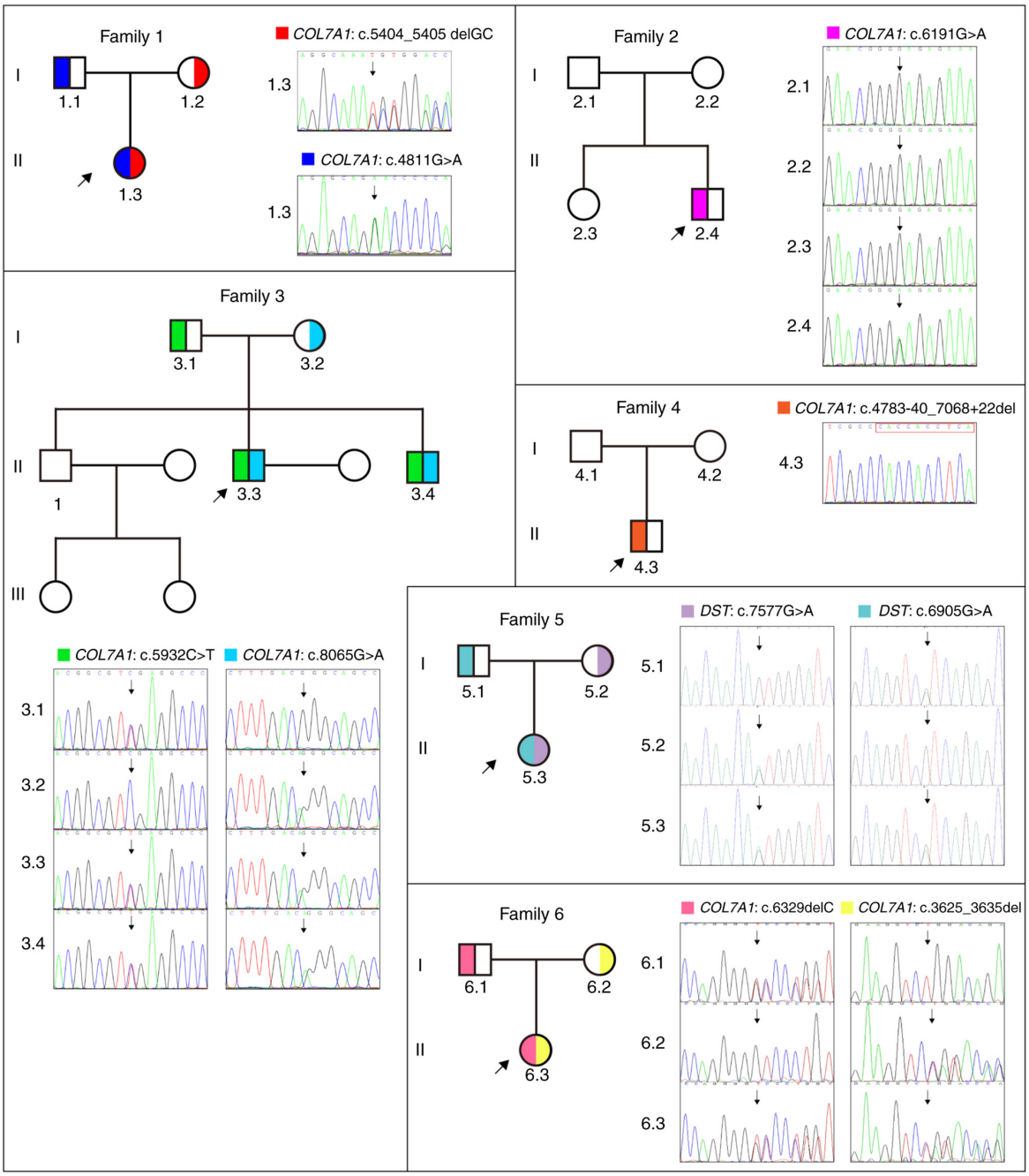

Figure 4. The genetic variants of the six cases and their carrying status in each family.

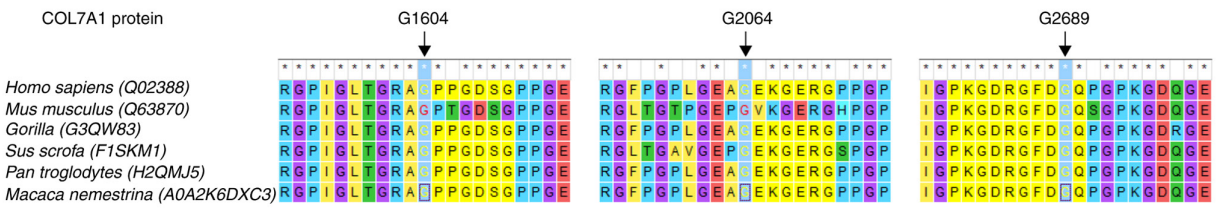

Figure 5. The evolutionary conservatism of the three amino acid residues that were affected by the *COL7A1* missense variants.

To the best of the authors' knowledge, the patient in Case 2 was the first known case in which both DEB and autism were involved. Intriguingly, mutations in two genes possibly contributed to the patient's autism-like phenotype. The *SCN9A* gene (MIM *603415), which encodes a voltage-gated sodium channel that is enriched in the nociceptive and sympathetic neurons of the peripheral nervous system, is involved in a group of nociception-related neuropathies (31,32). Another

gene *LFNG* (MIM *602576), is the causative gene for the autosomal recessive spondylocostal dysostosis 3, a rare skeletal dysplasia (33). Generally, the existing evidence is insufficient to support our diagnosis of these two gene variations and further functional studies are required.

The affected child in Case 5 was a patient of EBS, the pathogenic variation of which possesses a compound heterozygous variation of the *DST* gene (MIM *113810), a rare non-keratin cause for EBS. So far, only 10 variants of *DST*, almost all of which are truncating variants, have demonstrated clear associations with EBS (http://www.hgmd.cf.ac.uk/ac/index.php) and lack universal distribution across ethnic groups (34-36). Moreover, the function of the *DST* gene in human diseases has been under-studied. Based on the available evidence, the present study could only identify two variants as variant with unknown significance at the genetic level. The findings of the present study may contribute to the expansion of the mutation spectrum for this disease in the Chinese population, although further studies, including *in situ* electron microscopy, immunofluorescence assays and possibly functional experiments, are needed to clarify the pathogenicity of the novel variations. In addition, *in silico* structural analysis would contribute to elucidating the pathogenicity of these missense variants. The cross-species conservatism nature of the amino acid residues affected by the three missense variants in *COL7A1* supports their pathogenicity. It also demonstrates that *in silico* methods play an increasingly important role in the analysis of rare disease mutations (37).

The findings of the present study may also have some implications for the recent insights into the pathogenesis of IEB and the emerging potential for new therapies. For example, a recent study on applied Adenine Base Editors to correct the pathogenic mutation of *COL7A1* or to bypass a premature stop codon in fibroblasts of patients produced encouraging results (38). Another study identified several molecules that effectively increased the expression of type 7 collagen in keratinocytes, showing some therapeutic promise (39). The enzymatic modification of structural proteins by non-structural proteins such as *PLOD3, USB1, EXPH5 and KLHL24* may be an important supplement to the pathogenesis of IEB (40).

In conclusion, the findings of the present study established the genetic diagnosis of six IEB cases, expanded the mutation spectrum of the related genes and diseases and provided a solid basis for further analysis of the disease prognosis, treatment design and reproductive guidance for the affected families.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Project Funded by China Postdoctoral Science Foundation (grant no. 2022T150445).

Availability of data and materials

The datasets generated or analyzed during the current study are available in the figshare repository, https://doi.org/10.6084/m9.figshare.20979547.v1. Whole-exome sequencing data are not publicly available due to patient privacy, but are available from the corresponding author on reasonable request.

Authors' contributions

GQZ designed this study. YY and LXZ recruited the case and did the clinical examination. KY, YY and KQ performed the genetic and *in silico* studies. YY, KQ and LXZ analyzed the experimental data and composed all figures and tables. GQZ wrote this manuscript. YY and GQZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of the First Hospital of Hebei Medical University (approval no. HMU-FH-2020-02). Informed consent was signed by all the participants or their guardians for participation in this study. All procedures performed in the present study were following the Declaration of Helsinki 1964 and its later amendments or comparable ethical standards.

Patient consent for publication

Informed consent was signed by all the participants or their guardians for the images of this manuscript to be published.

Competing interests

The authors declare that they have no competing interests.

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