INTRODUCTION

Epidermolysis bullosa (EB) is a rare hereditary skin disorder defined by moderate to excessive fragility of epithelial tissues and blister formation. The disease can manifest in many different organ systems, and in severe cases, blisters may occur inside the body, such as the lining of the mouth or the stomach. The human skin is made up of an outer layer...
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rare disorders (Vahidnezhad, Youssefian, Saeidian, & Uitto, 2019).

The advent of high throughput next-generation sequencing (NGS) provides massively parallel or high deep data in a rapid and cost-effective manner. To date, whole-exome sequencing (WES) has become the standard of care for the identification of the functional genomic variants associated with rare disorders (Trujillano et al., 2017). In 2015, the pathogenic mutation identification of nine EB patients, failed by skin biopsy analysis and Sanger sequencing, was performed successfully by WES (Takeichi et al., 2015). The implementation of WES as a diagnostic tool in EB was referred to in several published studies to this point (Gong, Liu, Li, & Xu, 2019; Mahajan et al., 2018; Yenamandra et al., 2017, 2018).

The aim of this study is to identify mutations/genetic variants that might be pathogenic by WES in six EB individuals of five Vietnamese families. In the future, these data could have implications for genetic counseling and prognosis.

2 | MATERIALS AND METHODS

2.1 | Subjects

Six affected individuals of five families with the clinical diagnosis of EB and their parents were recruited from Hanoi Medical University Hospital, Hanoi, Vietnam. Patients present mechano-bullous lesions of the skin with blistering and scarring. The parents of all families were not consanguine and healthy in clinical features. Written informed consent was obtained from all family members before sample collection. This study was approved by the Institute of Genome Research Institutional Review Board, Vietnam Academy of Science and Technology.

For patients and their parents, 2 ml of whole blood was collected, preserved in EDTA-containing tubes, frozen, and stored at −20°C until use. Genomic DNA was extracted from the peripheral blood samples using Exgene™ Blood SV (GeneAll Biotechnology) according to the manufacturer’s protocol. DNA quantification was performed by using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and Eppendorf BioSpectrometer® fluorescence (Eppendorf AG).

2.2 | Methods

2.2.1 | Whole-exome sequencing

For the affected individuals (Figures 2a–6a), the DNA lib-

brane construction was performed by using Sure Select V6-

Post (Agilent Technologies) following the manufacturer’s guidelines. In brief, the sequencing library was prepared by random fragmentation of DNA, following by 5’ and 3’ adapter ligation. Fragments ligated with adapter were sub-

sequently amplified by PCR and gel purified. The Qubit™

dsDNA HS Assay Kit (Thermo Fisher Scientific) was used for enriched library quantification. The library size distribution was checked by bioanalyzer using high sensitivity DNA chip (Agilent Technologies) with an expected size ranging from 200 to 400 bp. The sequencing was performed by using an Illumina NovaSeq 6000 platform (Illumina) with paired reads of 150 bp.

2.2.2 | Sanger Sequencing

The candidate variants were validated by direct Sanger se-

quencing in patients as well as their parents. Primers for PCR and sequencing were provided by PHU SA Biochem Company. For PCR amplification, 10 ng of total genomic DNA was used as a template in 20 µl of reaction mixture containing 1× Neb Master mix (New England Biolabs, Ipswich), 0.8 µl of each primer (10 pmole), and 8.4 µl of deionized water. The thermocycling was 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 68°C for 20 s and a final extension at 68°C for 5 min. The PCR products were purified using Multiscreen PCR 96 Filter Plate (Merck-Millipore), and sequenced by ABI Prism BigDye Terminator Cycle Sequencing Kit, Version 3.1 (Applied BioSystems) on ABI 3500 Genetic Analyzer (Applied BioSystems).

2.2.3 | Variant calling

The reads were mapped to hg19/GRCh37 human reference genome by BWA.v0.7.12 tool (Li & Durbin, 2009), and Picard was used to marking the duplicates. Genome Analysis Tool Kit (GATK) and Samtools were used to detect single nucleotide variants (SNVs) and short insertions/deletions (Indels). To exclude false positive, all variants with depth read lower than 20× were removed. Short Indels in the repeat regions and within the 10 bp range from the start and
end of the read were also excluded. After that, the remaining variants were filtered from the public databases comprising 1000 Genomes and gnomAD. Because the prevalence of EB is about 8.2 per 1,000,000 live births in the global population (Fine, 2010), all variants with a frequency above 0.5% were eliminated.

### 2.2.4 | Variant annotation and prediction

The variants were annotated with the ANNOVAR program (Wang, Li, & Hakonarson, 2010). The in silico analysis was performed by SIFT (Hu & Ng, 2013), Polyphen-2 (Adzhubei et al., 2010) and Mutation Taster (Schwarz, Cooper, Schuelke, & Seelow, 2014) to anticipate the functional effect of missense, nonsense variants. For splicing prediction, Human Splicing Finder Professional (HSF Pro) and Alamut Visual (http://www.interactive-biosoftware.com) were used.

### 2.2.5 | Functional analysis of splice site (c.4518+2delT)

Total RNA of patient 4, patient 5, and their parents were isolated from the fresh blood samples using Monarch Total RNA Miniprep Kit (New England Biolabs). Reverse transcription (RT) was performed by ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs) following the manufacturer’s instruction. The cDNA obtained was amplified using the forward primer (5′-GGTGACCGGGGCTTTCCA-3′) and reverse primer (5′-TTCAGGACCCTTGGCTCCAG-3′) located in exons 42 and 44 of COL7A1, respectively. PCR products were separated by electrophoresis in a 3% agarose gel.

### 3 | RESULTS

#### 3.1 | Clinical characteristics of patients

Patient 1 was a 4-year-old male and presented with moderate blisters, erosions, and scarring in the body, such as the shoulder, back, hands, feet, elbows, buttocks, and knees. He had contracted toes and dystrophic nails (Figure 1a). Patient 2 was an 8-years-old girl, who reported severe blistering on the left face and peeling skin on her feet at birth. Pseudosyndactyly, contractures and complete absent nails were observed in hands and feet. In particular, the mitten deformity was developed in feet. The blistering was extended and formed erosions, exudate, crusting, and scarring as she increased in age (Figure 1b). Immunofluorescence antigen mapping showed the reduced stain of type VII collagen (COLVII) at the epidermal side of the blister (data not shown). Patient 3 was about 1-month-old baby girl with blisters occurring in hands, feet, and inside the mouth (Figure 1c). The severe phenotype was also found in two affected brothers, patients 4 (7 years old) and 5 (15 years old). They presented with widespread blistering, extensive erosions, and scarring. The skin was dry and wrinkled in hands and feet, with loss of finger and toenails (Figure 1d). The milder phenotype in patient 6 (Figure 1e) was associated with lesions in the lower back and groin.

#### 3.2 | Molecular findings

Analysis of the WES data revealed eight different COL7A1 (NM_000094.4, MIM:120120) variants, comprising of three novel mutations and five very rare variants (3.99 × 10⁻⁶–1.65 × 10⁻⁵, Table 1). Three novel variants, including one glycine substitution (c.8279G>A, p.G2760E) and two splicing variants (c.4518+2delT and c.5821-2A>G), were not found in any public database (1000 Genomes, dbSNP, gnomAD, HGMD, and COL7A1 database) and in the inhouse database of 500 healthy Vietnamese. Five very rare changes included one missense variant (c.6205C>T, p.R2069C), two stop-gain variants (c.5047C>T, p.R1683* and c.8233C>T, p.R2745*), and two indels (c.2858_2859del and c.6081delC). Analysis by multialignments (www.genome.ucsc.edu) displayed conservation of p.G2760, p.R2745, and p.R2069 among several vertebrates (human, rhesus, mouse, dog, elephant, opossum, and chicken), indicating the critical contribution of these amino acids for normal protein function (Figures 2c, 4c and 6c).

In patient 1, a homozygous change in c.8279G>A in exon 11 was found, leading to the substitution of conserved amino acid glycine by glutamic acid (p.G2760E). This variant was predicted as probably damaging/deleterious by SIFT, Polyphen2, and MutationTaster (Table 1). The heterozygous c.8279G>A variant was observed in both parents by Sanger sequencing (Figure 2b).

A compound heterozygote frameshift deletion in the COL7A1 gene was identified in patient 2. The first variant was c.2858_2859del on exon 22, which has not been found in 1000 Genomes, dbSNP, gnomAD, and HGMD. However, this variant was reported in two patients with generalized severe RDEB (1-year-old girl and 3-year-old boy) and submitted in COL7A1 gene variant database (http://www.col7a1-database.info). The c.2858_2859del resulted in a premature termination codon (PTC) at eighth amino acid downstream of exon 22 (p.E953fs*8) and leading to a deletion of 1984 amino acids downstream. The second variant was c.6081delC in exon 73 and created a PTC at codon 2206 (p.P2029fs*177), which has been reported previously with autosomal RDEB (Christiano, McGrath, & Uitto, 1996; Kern, Kohlhase, Bruckner-Tuderman, & Has, 2006), as well
as in ClinVar with pathogenic classification. Confirmation by Sanger sequencing also showed that the affected child inherited c.6081delC from mother and c.2858_2859del from father (Figure 3b).

The combination of nonsense and another variant including missense variant involving an amino acid other than glycine and splicing variant in COL7A1 gene was detected in patients 3, 4, and 5. In patient 3, compound heterozygous
| Patient | Variant change | Amino acid | Zygosity | Type of mutation | Region | Global population frequency | In silico prediction |
|---------|----------------|------------|----------|------------------|--------|-----------------------------|---------------------|
|         |                |            |          |                  |        |                             | Genomes | gnomAD | SIFT | Polyphen2 | MutTaster | HSF | Alamut visual |
| 1       | c.8279G>A      | p.G2760E   | hom      | nonsyn          | exon11 | –                            | –       | –      | D    | D          | D          | –   | –              |
|         |                 | p.E953fs*8 |          |                  |         |                               | –       | –      | –    | –          | –          | –   |                |
| 2       | c.2858_2859del | p.P2029fs*177 | het     | FS del          | exon22 | –                            | –       | –      | –    | –          | –          | –   | –              |
|         | c.6081delC     |            |          |                  |         |                               | –       | –      | –    | –          | –          | –   |                |
| 3       | c.6205C>T      | p.R2069C   | het      | nonsyn          | exon74 | 1.65 × 10⁻⁵                   | –       | –      | D    | B          | A          | –   | –              |
|         | c.8233C>T      | p.R2745*   | het      | stopgain        | exon111 | 3.99 × 10⁻⁶                  | –       | –      | –    | –          | –          | –   |                |
| 4, 5    | c.4518+2delT  | –          | het      | splicing        | exon43 | –                            | –       | –      | –    | –          | –          | –   |                |
| brothers|                 |            |          |                  |         |                               | –       | –      | –    | –          | –          | –   |                |
|         | c.5047C>T      | p.R1683*   | het      | stopgain        | exon54 | 8.48 × 10⁻⁶                  | –       | –      | A    | –          | –          | –   |                |
| 6       | c.5821-2A>G    | –          | het      | splicing        | exon71 | –                            | –       | –      | –    | –          | D          | –   |                |
|         |                 |            |          |                  |         |                               | –       | –      | –    | –          | –          | –   |                |
|         | c.6205C>T      | p.R2069C   | het      | nonsyn          | exon74 | 1.65 × 10⁻⁵                   | –       | –      | D    | B          | A          | –   | –              |

The bold rows indicate novel variants.

Abbreviations: A, disease causing automatic; B, benign; D, damaging/detrerious/disease-causing (in MutTaster); FS del, frameshift deletion. Bold indicates the novel variants; het, heterozygous; hom, homozygous; nonsyn, nonsynonymous; wt, wild type.
of one nonsynonymous SNV (c.6205C>T) and one stop-gain mutation (c.8233C>T) was identified, corresponding to rs121912855 and rs768202310 in dbSNPs, respectively. The c.6205C>T (p.R2069C) was described as pathogenic in ClinVar, involving individuals affected with DEB. The c.8233C>T apparently resulted in the expression of a truncated protein (p.R2745*). Verification by Sanger sequencing indicated that the mother and the father were heterozygous carriers for c.6205C>T and c.8233C>T, respectively. Coexistence of these genetic variants was also not detected in patient’s parents (Figure 4b). In two brothers (patients 4 and 5), two heterozygous variants were identified: a novel deletion point (c.4518+2delT) in intron 43 and a single-base substitution (c.5047C>T) in exon 54 (Figure 5b). In silico splice site prediction by Alamut Visual, the effect of skipping exon 43 very likely for c.4518+2delT was predicted. However, HSF Pro predicted no significant impact on splicing signals (Table 1). In contrast, RT-PCR result showed one amplicon of 125 bp in wild type and two amplicons in mutation with an additional shorter amplicon of 89 bp (Figure 5c), suggesting aberrant splicing, as revealed the same consequence to splice prediction by Alamut Visual. The lack of 36 bp corresponded to the skipping of exon 43, which could be constructed by splice site (c.4518+2delT), leading to the removed 12 amino acids in transcription. The replacement of a single base at the codon 5047 produced a premature stop codon (p.R1683*). The parent was heterozygous for

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**FIGURE 2**  (a) Pedigree chart of family 1 with an RDEB case. Black/white represent patient/not affected individuals. (b) Sanger sequencing showed a homozygous COL7A1 (NM_000094.4) variant in affected individuals and carriers, partial sequence with mutated nucleotides marked with vertical lines and red arrows. (c) The amino acid substitutions at evolutionarily conserved residues were marked with a blue arrow

**FIGURE 3**  (a) Pedigree chart of family 2 with an RDEB case. Black/white represent patient/not affected individuals. (b) Electropherogram of the patient with COL7A1 (NM_000094.4) compound heterozygote, which was inherited from the parents. Mutated nucleotides were marked with vertical lines and red arrows. (c) The amino acid substitutions at evolutionarily conserved residues were marked with a blue arrow.
FIGURE 4  (a) Pedigree chart of family 3 with an RDEB case. Black/white represent patient/not affected individuals. (b) Compound heterozygous of COL7A1 (NM_000094.4) was detected in patient 3 and father/mother carried once. Mutated nucleotides were marked with vertical lines and red arrows. (c) The amino acid substitutions at evolutionarily conserved residues were marked with a blue arrow.

FIGURE 5  (a) Pedigree chart of family 4 with two RDEB cases. Black/white represent patient/not affected individuals. (b) Sequencing analysis indicated that two siblings carried a compound heterozygous COL7A1 (NM_000094.4) variant and inherited from mother and father. Mutated nucleotides were marked with vertical lines and red arrows. (c) Splice defect of c.4518+2delT. Agarose gel electrophoresis of RT-PCR products, the longer band with 125 bp in length presented the wild-type (WT) transcript, the shorter band with 89 bp in length presented the mutated transcript (MT), the bottom bands are primer dimer.
c.4518+2delT and c.5047C>T, corresponding to the maternal and paternal variants, respectively (Figure 5b).

The conjunction of a splicing variant (c.5821-2A>G) and a missense variant (c.6205C>T) was found in patient 6. The c.5821-2A>G was a novel substitution variant in intron 71. This variant was predicted in “the skipping exon 71 very likely” and “alteration of the WT acceptor site, most probably affecting splicing as a consequence of substitution in intron 71” by Alamut Visual and HSF Pro, respectively (Table 1). The c.6205C>T was a pathogenic variant and was also found in patient 3. Sequencing analysis indicated that both mother and father were heterozygous carriers for c.C6025T and c.5821-2A>G, respectively (Figure 6b).

4 | DISCUSSION

The mutation in the COL7A1 gene causes the dystrophic epidermolysis bullosa (DEB), which is inherited in an autosomal dominant (DDEB) or an autosomal recessive (RDEB) manner. The diagnosis of DEB base on the clinical manifestations and the variant causing in COL7A1. Up to now, more than 800 genetic variants of COL7A1 are related to DEB (http://www.col7a1-database.info) (Wertheim-Tysarowska et al., 2012). Among these, almost all mutations are glycine substitutions located in triple helix domain (THD; Leverkus et al., 2011). The clinical phenotypes of DDEB are comparatively milder than that of RDEB. In DDEB, the blistering is often limited to hands, feet, knees, and blows. It is relatively benign but nonetheless heals with scarring. RDEB is a severe inherited skin disorder characterized by extensive blisters, atrophic scarring, milia, minor dystrophic, or absent nails (Laimer, Prodinger, & Bauer, 2015). Although almost all mutations in DDEB were found in exons 73–75, mutations including insertions, deletions, single-base substitution, and splice junction alteration in RDEB occur in both alleles that result in either null allele or out-of-frame mutations (Christiano, Amano, Eichenfield, Burgeson, & Uitto, 1997; Fine et al., 2014; Van den Akker et al., 2011). In our study, a homozygous and four compound heterozygous variants in COL7A1 were found in six EB cases, and the heterozygous carriers of each variant did not show any symptoms, leading to the prediction of recessive in inheritance trait.

Compound heterozygous PTC variant (c.2858_2859del and c.6081delC) was identified in patient 2 with severe phenotype (Figures 1b and 3). Widespread blistering, extensive scarring, flexion contractures of limbs, and pseudosyndactyly with mitten deformity were observed in clinical findings. Genotype to phenotype correlation indicates that PTC mutations in both alleles of COL7A1 known as the main mutation of RDEB generalize severe (RDEB-GS) cases, causing in nonsense-mediated decay or truncated polypeptides, resulting in significant reduction or complete absence of type VII collagen (COLVII) or anchoring fibrils (AFs) (Christiano et al., 1997; Fine et al., 2014; Van den Akker et al., 2011). In few cases, RDEB-GS could be caused by compound heterozygosity of two missense mutations or the conjunction of a PTC and a missense mutation (Christiano, McGrath, Tan, & Uitto, 1996). In cases of two brothers (patients 4 and 5), the severe phenotype consists of extensive blisters, erosions, scarring in the body, pseudosyndactyly, and contractures.

FIGURE 6  (a) Pedigree chart of family 5 with an RDEB case. Black/white represent patient/not affected individuals. (b) Sanger sequencing confirmation indicated a compound heterozygous of COL7A1 (NM_000094.4) in patient 6, and each variant was carried from the parents. Mutated nucleotides were marked with vertical lines and red arrows. (c) The amino acid substitutions at evolutionarily conserved residues were marked with a blue arrow.
of the hands and feet, with absent nails on fingers and toes (Figure 1d). The combination of a PTC (p.R1683*) and a splice site variant (c.4518+2delT) was shown in Figure 5. The milder RDEB was often caused by the conjunction of one PTC mutation and one missense mutation (Shimizu, McGrath, Christiano, Nishikawa, & Uitto, 1996). Patient 3 presented with a mild phenotype characterized by the limited blistering on hands, feet, and tongue (Figure 1c), who carried compound heterozygosity, including a PTC variant (p.R2745*) and a missense variant (p.R2069C; Figure 4). Other COL7A1 mutations that did not cause PTCs codon usually produce less severe disease. This could be explained in patients 1 and 6, who were characterized with moderately blistering, erosions, scarring in body (patient 1, Figure 1a), hypopigmentation in the lower back and groin area, limited oral opening, ankyloglossia, and tongue lesion (patient 6, Figure 1e). In detail, the homozygous glycine substitution (p.G2760E, Figure 2) and compound heterozygous variant (c.5821-2A>G, c.6205C>T, Figure 6) were detected in patients 1 and 6, respectively. Due to the genotypes to phenotypes observed from the studied patients, PTC mutations resulted in the most serious cases, it was the same aspect of previously published studies.

Three novel COL7A1 variants were revealed from WES data of RDEB patients. The novel homozygous glycine substitution (p.G2760E) in collagenous encoding domain would lead to the instability and affect critical amino acids in the triple helix structure. The recessive glycine substitution lead to impairment of COLVII secretion as well as AFs assemblage or a conjunction thereof (Uitto, 2011). The other single base substitution (c.8278G>A), that resulted in the glycine substitution at the same codon 2760 and produced a missense mutation (p.G2760R) was previously reported (Yan et al., 2018). The two novel splicing variants residing in a donor splice site (c.4518+2delT) and an acceptor splice site (c.5281-2A>G) of COL7A1. These exon/intron boundary sequences are highly conserved, which consist of GT and AG motifs at 5′ and 3′ end of the intron, respectively. Splice site affecting +1 and +2 residues at 5′ donor site and −1 and −2 residues at the 3′ acceptor had been known as the most classical variants. The mutation in canonical sequence can alter the interaction between pre-mRNA and protein, which responsible for the intron removal (Abramowicz & Gos, 2018). Functional splicing prediction by Alamut Visual indicated that the consequence of exon skipping in both variants. Nevertheless, the variant c.5281-2A>G in intron 70 was predicted by HSF to probably affect splice site signal. Despite of HSF prediction resulted in none splicing defect of c.4518+2delT, this variant was demonstrated in RNA level of two siblings (patients 4 and 5) with complete in-frame skipping of exon 43 (12 amino acids) within THC domain of COLVII.

Our research finding suggested the complete inactivation of COL7A1 and loss of COLVII function. Since the RNA of patient 6 was not available, functional analysis by another assay should be considered to further investigate the c.5281-2A>G impact.

5 | CONCLUSION

In conclusion, this is the first report on mutational screening of DEB patients in Vietnam, providing an important genetic variant spectrum for Vietnamese EB. To sum up, our study had identified 3 novel mutations (p.G2760E, c.4518+2delT, and c.5281-2A>G) and 5 very rare variants (p.E953*8, p.R1683*, p.P2029*177, p.R2069C, and p.R2745*). In addition, pre-mRNA splicing confirmed that the c.4518+2delT led to an in-frame deletion of exon 43. The results of the molecular tests would be valuable for appropriate genetic counseling and providing a theoretical prognosis for the progression of the disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: NDT, NVH; Funding acquisition: NDT; Data curation, Formal analysis, and Investigation: MTHT, NHH, NTTH, NDB, LTLA, VTH, TVK, TTS; Methodology; Roles/Writing—original draft: MTHT, NHH, NDT, NVH; Writing—review and editing: MTHT, NHH, NDT, NVH.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study may be requested from the corresponding author.

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