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The genetics of human mendelian skin disorders

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The genetics of human mendelian skin disorders

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Editorial: The genetics of human Mendelian skin disorders

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Editorial on the Research Topic
The genetics of human Mendelian skin disorders

Introduction

The skin is comprised of multiple types of cells that serve as a protective barrier. Mutations in the genes that are responsible for protecting the functional integrity of the skin are often found in many inherited skin diseases, more commonly known as the Mendelian human skin disorders. Advances in molecular techniques and sequencing technologies have enabled identification of novel pathogenic variants, which helps to provide insight into genotype–phenotype correlations and to define the genetic basis of these skin disorders. In this Research Topic, a total of ten articles are published, including those describing findings from case studies and original research, as well as a mini review of current genetic diagnosis strategies, novel gene variants, and genotype–phenotype correlations in human Mendelian skin disorders.

Genetic diagnosis of Mendelian skin conditions

Recent developments in genome-wide association studies (GWAS) and next-generation sequencing (NGS) techniques have resulted in an integrative approach to the use of functional genomics and expression data in deciphering the causative genetic variants of inherited skin diseases. Because most of the mutations identified in human Mendelian skin disorders reside in protein-coding genes, whole exome sequencing (WES) has been widely used in the identification of such pathogenic variants, and in the genetic

diagnosis of Mendelian skin conditions with atypical or unique phenotypes. Xu et al. report on the use of WES in identifying a *de novo* pathogenic variant c.2T>C (p.M1T) in *KLHL24* in Chinese twin boys with epidermolysis bullosa simplex. Similarly, Wang et al. demonstrate the detection using WES of a missense mutation, c.1156G > A (p.Ala386Thr) in *DKC1*, which leads to a benign form of dyskeratosis congenita syndrome with the mucocutaneous triad. In another article appearing in the Research Topic, WES analysis also reveals a heterozygous missense mutation c.293G>A in *GJB3*, which is associated with erythrokeratodermia variabilis, ichthyosis, and non-syndromic hearing loss Gao et al.

Although WES is best used to characterize small indels in protein-coding exon regions, the detection of copy number variations, structural variants, and homologous regions remains challenging. Therefore, several studies have combined the use of ultra-high multiplexed PCR and ligation-dependent probe amplification techniques with WES in the form of molecular diagnostics protocols for the identification of exon variants and the development of genetic profiles of patients with human Mendelian skin disorders. This includes the detection of ectodysplasin A exon variants in X-linked hypohidrotic ectodermal dysplasia Wang et al. the genetic profiling of epidermolysis bullosa cases Alharthi et al. and the discovery of a novel CREBBP variant for genetic diagnosis of Rubinstein–Taybi Syndrome Lee et al. Recent evidence from large-scale expression studies (using microarrays and RNA sequencing) has also revealed the roles played by non-coding RNA molecules, such as small non-coding RNAs or miRNAs, in the pathogenesis of several complex skin diseases (Shefler et al., 2022). The discovery and identification of such non-coding RNAs enables them to potentially serve as diagnostic markers.

Novel genetic variants in human Mendelian skin disorders

Molecular genetic studies based on family case reports and large-scale regional profiling analyses often provide significant insight into novel pathogenic variants, thereby helping to extend the spectrum of the genetic profile, improve diagnosis, and establish an improved understanding of human Mendelian skin disorders. In this Research Topic, Alharthi et al. report the discovery of 14 novel mutations in patients with inherited epidermolysis bullosa. This includes novel missense and frameshift mutations in gene *COL7A1* among patients with dystrophic epidermolysis bullosa and frameshift mutations in *COL17A1* and *LAMB3* among patients with junctional epidermolysis bullosa, as well as missense and non-sense mutations in genes *TGM5*, *PLEC*, and *DST* among patients with epidermolysis bullosa simplex. Meanwhile, a novel heterozygous splice-site mutation c.900-1G > C in the *ATP2C1* gene is also identified in a case report on a rare autosomal-dominant blistering disorder known as Hailey–Hailey disease Dai et al. Separately, Lee et al. have established a genetic diagnosis protocol for the detection of Rubinstein–Taybi syndrome, identifying a novel heterozygous non-sense *CREBBP* mutation (NM_004380: c. C2608T: p. Gln870Ter) with a significant pathogenicity score. In addition, a novel homozygous missense mutation (p.L154R) in gene ABHD5 has been detected in a patient with Chanarin–Dorfman syndrome, a rare autosomal recessively inherited genetic disease Liang et al. Finally, Wu et al. also report in this Research Topic on a new case of congenital poikiloderma with a novel missense mutation in the *FAM111B* gene c.1883G>A (rs587777238).

Genotype–phenotype correlations

Certain phenotypic features of inherited skin disorders may be associated with particular gene mutations, but paradigms for clinical genotype–phenotype correlation remain unclear in many instances due to the highly variable phenotypic expressivity of the relevant mutations; these paradigms require further refinement. In this Research Topic, Xu et al. provide an initial description of their discovery of a c.2T>C pathogenic variant in *KLHL24*, affecting twins in China, and its correlation with epidermolysis bullosa simplex. Their research has identified correlations between phenotypes and genotypes in epidermolysis bullosa, in which *KLHL24* pathogenic variants are associated with the mild phenotype. In contrast, the study by Liang et al. on genotype–phenotype analysis in patients with reported Chanarin–Dorfman syndrome reveals no correlation. Meanwhile, as indicated in a review by How et al. there is no significant genotype–phenotype relation in incontinentia pigmenti, a rare type of X-linked dominant genetic disease characterized by ectodermal dysplastic disorder. However, the literature does suggest that variation in a combination of the types of mutations, functional domains affected, X-inactivation, and genomic background may lead to the variability observed in incontinentia pigmenti phenotypes How et al. The authors propose that a detailed understanding of the genotype–phenotype correlation in incontinentia pigmenti will support further investigations concerning prognosis and future reproductive options. Meanwhile, a recent large cohort study involving investigation of genotype–phenotype correlations in patients with autosomal recessive ichthyosis has provided new insights on and definitions of specific phenotypic clues for corresponding genetic mutations (Simpson et al., 2020). In addition to the need for large cohort trials, some researchers have proposed the use of computational approaches to connecting patient phenotypes based on phenotypic co-occurrence, combined with the use of genomic information related to the mutations found in each patient, to correlate genes with phenotypes; this type of approach can be used to investigate the relevant functional systems (Díaz-Santiago et al., 2020).

Conclusion

In summary, this Research Topic enhances our knowledge of recent exciting progress in the field of genodermatosis, including molecular diagnostics protocols, novel pathogenetic variants, and genotype–phenotype correlations. Together, these studies provide value in the form of greater diagnostic precision, a source of information for clinical assessments, and ways to improve clinical care and management.

Author contributions

All authors listed have made a substantial, direct intellectual contribution to the editorial and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Case Report: De novo KLHL24 Gene Pathogenic Variants in Chinese Twin Boys With Epidermolysis Bullosa Simplex

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Objectives: The aim of this study was to determine the molecular etiology and clinical manifestations of a pair of Chinese twins affected with epidermolysis bullosa simplex. Pediatricians should pay attention to the early genetic diagnosis of this disease.

Methods: Histopathological examination of HE-stained skin, electron microscopy of biopsied normal skin, and whole-exome sequencing was performed to assess pathogenicity and conservation of detected mutations. Two years later, the cutaneous and extracutaneous manifestations of the twins were comprehensively evaluated.

Results: *A de novo* pathogenic variant c.2T>C (p.M1T) in *KLHL24* (NM_017,644) was identified in both twins. The characteristics of extensive skin defects on the extremities at birth and the tendency to lesson with increasing age were confirmed. No positive sensitive markers, such as B-type natriuretic peptide, cardiac troponin I, for cardiac dysfunction were detected.

Conclusions: The *de novo* pathogenic variants c.2T>C (p.M1T) in *KLHL24* (NM_017,644) contributes to the development of epidermolysis bullosa. Genetic diagnosis at birth or early infancy can better predict the disease prognosis and guide the treatment.

Keywords: KLHL24, *de novo* pathogenic variants, epidermolysis bullosa, skin defect, follow-up

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INTRODUCTION

Inherited epidermolysis bullosa (EB) is a genetically heterogeneous disorder, characterized by skin fragility annexed with the formation of blisters and skin erosion in response to minor mechanical trauma.1 Currently, over twenty genes that encode structural proteins within keratin intermediate filaments, focal adhesion desmosome cell junctions, and hemidesmosome attachment complexes have been reported in the pathogenesis of EB. (Has and Fischer, 2018; Vahidnezhad et al., 2018). Clinically, EB is classified into four major groups based on the plane of cleavage within the skin, *viz.* epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), dystrophic epidermolysis bullosa (DEB), and Kindler epidermolysis bullosa (KEB) (Bardhan et al., 2020). The diagnosis and classification of EB are based on the morphological analysis of a skin sample using immunohistologic methods and on the analysis of the pathogenic variants of the candidate genes. (Has and He, 2016; Has and Fischer, 2018). As the most common type of EB, EBS is mainly caused by

monoallelic pathogenic variants in *KRT5* (MIM: 148,040) and *KRT14* (MIM: 148,066), which encode keratin 5 and keratin 14, respectively. In addition, some cases of EBS were reported to associate with other pathogenic variants in *PLEC* (plectin), *EXPH5* (exophilin-5), *DST* (dystonin, 230-kDa bullous pemphigoid antigen), and *CD151* (member of the tetraspanin superfamily). (Karamatic Crew et al., 2004; Groves et al., 2010; McGrath et al., 2012; Bolling et al., 2014; Bardhan et al., 2020). Recently, pathogenic variants in *KLHL24* (MIM: 611,295) encoding the Kelch-like protein 24 have been identified in cases with skin fragility and progressive thickening of the nails by whole-exome sequencing. To date, about 40 individuals with monoallelic pathogenic variants of *KLHL24* have been reported. (He et al., 2016; Lin et al., 2016; Lee et al., 2017; Alkhalifah et al., 2018; Yenamandra et al., 2018; Grilletta, 2019; Hachem et al., 2019; Schwieger-Briel et al., 2019). *KLHL24* is part of the family of more than 40 genes with a Kelch-like motif, and it partially forms the ubiquitin-ligase complex. (Dhanoa et al., 2013). These pathogenic variants caused the loss of the first 28 amino acids of the encoded protein. The mutant protein promotes excessive ubiquitination and degradation of KRT14. The above observations have invoked a new mechanism that is germane to inherited skin blistering, namely, dysregulation of autoubiquitination. (He et al., 2016). Most *KLHL24* positive patients carry a heterozygous pathogenic variant in the first codon that affects translation initiation. (He et al., 2016; Lin et al., 2016; Lee et al., 2017; Alkhalifah et al., 2018; Yenamandra et al., 2018).

The clinical diagnosis for EB can be difficult at birth or in the early infancy, even for experienced dermatologists, particularly without an established family history of the disease. An important part of EB research lies in the diagnosis and classification of the disease at the early stage. The optimal treatment regime for disease complications has to be assessed for a long time. Here, we reported a case of twin boys with *de novo KLHL24* pathogenic variants. This is the first study to describe the pathogenic variant in *KLHL24* affecting Chinese twins. The twin brothers were diagnosed, screened, and treated effectively at the early stage by pediatricians. Meanwhile, cutaneous and extracutaneous manifestations were evaluated at the age of two. This case report will help pediatricians, not confined to dermatologists, to pay enough attention to the early diagnosis and long-term management of EB.

CASE REPORT

The dichorionic twin boys in this report were born at the 32nd week of gestation as a result of a large intracranial hemorrhage in their mother, who had a history of multiple spontaneous abortions under diverse complications, including antiphospholipid antibody syndrome and subclinical hypothyroidism during pregnancy, and she took multiple medications during pregnancy, including methylprednisolone, hydroxychloroquine, and aspirin. The older brother's body weight was 1,380 g, less than third percentile of typical boys of the same gestational age. At birth, he presented with extensive areas of denuded skin involving the limbs, knees, wrist joints, and ankle joints (**Figure 1A**). The younger brother's body weight was 1,650 g, which ranges between the 25th and 50th percentiles for boys of the same gestational age. His skin had the same appearance as his brother's. New skin defects occurred on the twins faces after positive-pressure ventilation. At birth, both the white blood cell count and neutrophil count of the two brothers were low. The white blood cell count of the elder brother was $1.94*10^9$/L and the neutrophil count was $0.2*10^9$/L, and that of the younger brother was $1.58*10^9$/L and $0.29*10^9$/L respectively. The course was complicated by *Serratia marcescens* sepsis as a result of preterm labor. The twins homocysteine levels at birth were 4.42 and 4.61 μmol/L (normal ≤15 μmol/L) respectively. The results of echocardiography indicated congenital heart disease and atrial septal defect (secondary foramen type), and the degree of interruption was 6 and 5 mm, respectively. The remaining systemic examination was normal. Histopathological examination of hematoxylin-eosin (HE)–stained skin and electron microscopy (EM) of normal skin biopsy were performed in a reference center for EB. In the older brother, pathology showed no *epidermis* or intradermal vascular hyperplasia (**Figure 1B**). EM revealed cleavage within the epidermal basal layer, some epidermal cells with a large amount of melanin deposition, reduction in the local density of the superficial dermis, and partial basal cell degeneration with vacuolar changes (**Figure 1C**). In his younger brother, histopathological examination showed no *epidermis* and dermis with only a lipid membrane structure. EM also revealed cleavage within the epidermal basal layer and the substrate, incomplete basal cells within the dermis, and reduction in the local density of the superficial dermis (**Figure 1D**). These results collectively suggest EBS. Soon thereafter, a whole-exome sequencing analysis was performed of peripheral blood DNA for this family. We identified the *de novo* variants c.2T>C (p.M1T) in *KLHL24* (NM_017,644) from the two boys, which were previously reported to be pathogenic. (He et al., 2016). We have applied ACMG, PolyPhen-2 and PROVEN criteria to prove the pathogenicity of this mutation c.2T>C. Moreover, variant c.2T>C was absent in cohorts of healthy control subjects in dbSNP, 1,000 Genomes, the Exome Variant Server, and the ExAC Browser in previous report (He et al., 2016). Sanger sequencing confirmed these *de novo* pathogenic variants (**Figure 1E**). Treatment consisted largely of supportive care, including wound care, as well as prevention and treatment of complications. Mupirocin ointment and recombinant bovine basic fibroblast growth factor were mixed at a 1:1 ratio, then above medicine was applied on the oil gauze, and finally the oil gauze was covered the skins wound. The treatment was carried out every other day on the twin boys. After about 1 month, the stability of the skins was enhanced gradually, above skin treatments were carried on when necessary.

At the age of 2 years, we followed up with the two brothers. There were old scars, pigmentation, nail thickening, and yellowing, no joint contracture and functional damage, and few new blisters (**Figure 1F**). Their head circumferences were below the third percentile of boys after adjusted age, and their heights and body weights were between the third and 10th percentiles. Considering the existence of extrauterine growth retardation, it may be related to the lack of functional training, regular follow-up, and the late addition of

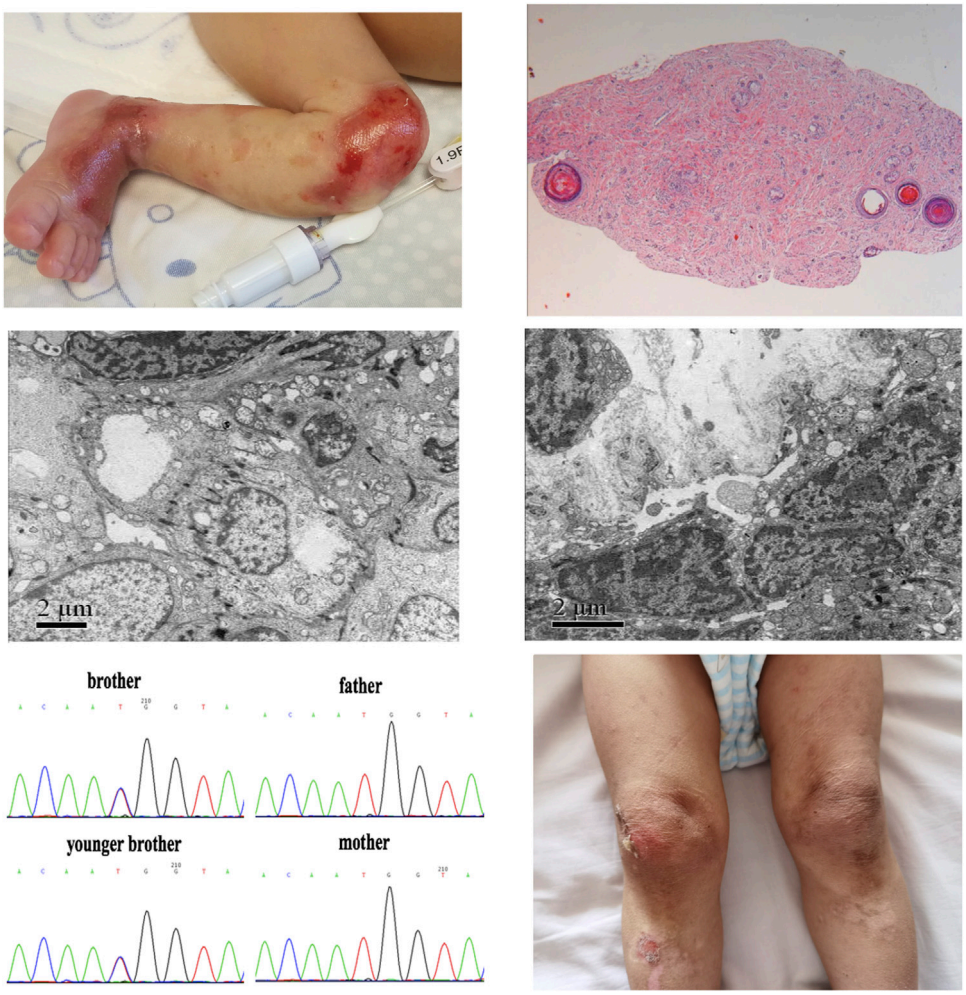

FIGURE 1 | Skin image, pathology, and *de novo* pathogenic variants of *KLHL24* in the twin brothers. **(A)**Picture of the older brother's left leg at birth demonstrated congenital skin defects. **(B)**Histopathological examination of HE-stained skin in the older brother **(C)** Skin performance under EM in the older brother **(D)** Skin performance under EM in the younger brother **(E)** *De novo* pathogenic variants of c.2T>C (p.M1T) in *KLHL24* of this ancestry **(F)** Picture of younger brother's legs at the age of 2 years demonstrated skin damage, pigmentation and scar.

complementary food. Both brothers were assessed for Gesell Developmental Observation, and they showed mild retardation in adaptability, fine movement and personal social interaction, moderate retardation in language, and normal serum myocardial enzymes (except the MB isoenzyme of creatine kinase is higher than normal in elder brother). The elder brother's brain MRI showed myelination delay, and echocardiography showed atrial septal defect (5 mm) and a small amount of tricuspid regurgitation, yet the results of the younger brother's brain MRI and echocardiography were normal.

DISCUSSION

KLHL24 was first reported by He et al., (He et al., 2016), who discovered heterozygous pathogenic variants of this gene in 5 unrelated individuals with EBS. (Lin et al., 2016). *KLHL24* is expressed in multiple tissues, including heart, brain, liver, skeleton muscle, kidney, pancreas, placenta, lung, and peripheral blood, as well as in the main skin cell types: keratinocytes, fibroblasts, and melanocytes. (He et al., 2016; Lin et al., 2016). It was of interest to note that all 26 previously reported patients harbored monoallelic pathogenic variants in the *KLHL24* translation start codon, c.1A > G, c.1A > T, c.2T > C, c.3G > T, c.3G > A, with a high rate of *de novo* and recurrent pathogenic variants. (Has and Fischer, 2018)' (Has, 2017) In our case, it is the first to describe c.2T>C pathogenic variant in *KLHL24* affecting twins in China and it was correlated with EB simplex. He et al. (He et al., 2016) also found truncated *KLHL24* resulting from the start codon mutations and the use of a downstream methionine initiation codon. Abnormal intermediate filaments in keratinocytes and fibroblasts, with evidence for irregular and fragmented KRT14, and data to support an altered balance in the stability and degradation of this keratin (He et al., 2016).

TABLE 1 | Summary of clinical features of individuals with c.2T>C pathogenic variant in *KLHL24*.

Case	Age (Y)	Sex	Areas of birth damage	Scarring at sites of birth damage	Milia	Nail	Oral	Hair	Cardiac symptoms/ Clinical features	CK in U/L (normal value)	CKMB in ng/ml (normal value)	Pro-BNP or BNP in pg/ml (normal value)	Development
1 Lee et al. (2017)	9	M	Legs, wrists	+	+	+	–	–	NA	NA	NA	NA	NA
2 He et al. (2016), Schwieger-Briel et al. (2019)	4	M	Legs, arms, wrists	+	+	+	–	–	Tachycardia, extrasystoles at 6Y	182 (<168)	7.7 (<6)	82 (<390)	Delay of cognitive and motor development
3 Hachem et al. (2019)	7	M	Legs, arms, wrists, buttocks, left mammary region	+	+	+	+	+	Normal	Normal	Normal	Normal	NA
4 Hachem et al. (2019)	5	M	Legs, arms, abdomen, wrists	+	+	+	–	–	Normal	Normal	Normal	Normal	NA
5 (Twin 1, elder brother)	2	M	Legs, arms, wrists	+	+	+	–	–	Atrial septal defect (5 mm) and small amount of tricuspid regurgitation	124 (<200)	4.3 (<4.0)	47 (<100)	Delayed myelination, mild retardation in adaptability moderate retardation in language
6 (Twin 2, younger brother)	2	M	Legs, arms, wrists	+	+	+	–	–	Normal	103 (<200)	2.74 (<4.0)	22.6 (<100)	Mild retardation in adaptability, moderate retardation in language

NA: not applicable

The clinical manifestations of our cases included extensive skin defects on the extremities at birth, leaving hypopigmentation and atrophy with a whirled pattern, in accordance with the characteristic of *KLHL24*. In addition, early blistering occurred often in the trunk and upper limbs, especially after the compression or friction. These lesions were typically healed quickly with subtle atrophic scarring. Based on the results of other studies, blistering persists throughout childhood but tends to lesson with increasing age. (Hachem et al., 2019). Nail defects and oral ulceration are common, whereas transient milia also occur. Dyspigmentation is not a prominent feature. Other reported features include cutaneous findings, such as loss of dermatoglyphics, hypohidrosis, and congenital malrotation of the great toenails, besides mental problems. (Yenamandra et al., 2018). Whether these symptoms are related to *KLHL24* requires further investigations. After 2 years of follow-up in our study, we found that the skin defects became milder, nails became thicken and yellowing, the oral ulceration was not obvious.

Indeed, the wide tissue distributions of *KLHL24* suggest that pathogenic variants could affect organs other than the skin. Schwieger et al. (Schwieger-Briel et al., 2019) found evidence of dilated cardiomyopathy in 8 of 20 EBS-*KLHL24* patients (40%), with the youngest being 25 years. He et al. (He et al., 2016) also noticed dilated cardiomyopathy in a 43 year-old patient, although the age of onset in this patient was unclear. In our report, the cardiac ultrasound examinations of the boys indicated congenital heart disease (atrial septal defect). Other sensitive markers, such as B-type natriuretic peptide, cardiac troponin I, for cardiac dysfunction were proved negative until hospital discharge (except for the MB isoenzyme of creatine kinase is higher than normal in the elder brother). Because of the skin damage and other problems caused by the disease, the children need special care in daily life which produced heavy financial burden for the family. At 2 years of birth, they were comprehensively evaluated by pediatricians, dermatologists, and neurologists. The dichorionic twin boys in this report were born at the 32nd week of gestation. At the 2-year follow-up, except for old scars, we only found the elder brother's brain MRI showed delayed myelination, and echocardiography showed atrial septal defect (5 mm) and a small amount of tricuspid regurgitation. Their phenotypes were compared with previous cases reported with c.2T>C variant in *KLHL24* (**Table 1**). As myelination is a progression phenotype, later examinations will be required to confirm their brain developmental status. Congenital heart disease is very common in premature infants. Clinicians also need further follow-ups to determine the future treatment plan. Future cardiac complications may emerge with age,

and this should be a focus of the treating physician. Considerably different phenotypes of pathogenic variants have been reported within EB subtypes. There were correlations between phenotypes and genotypes in EB. *KLHL24* pathogenic variants were associated with the mild phenotype, such as EB simplex.

Although there is currently no effective treatment for EB, genetic diagnosis at an early age can better predict the prognosis and guide the treatment. We here recommend both genetic and prenatal diagnoses to reduce the incidence of this disease and improve the quality of life.

This study was supported by the Medical and health science and technology innovation project of the Chinese Academy of Medical Sciences (2018-12M-1–003), and approved by the Ethical Committee, China-Japan Friendship Hospital. All study protocols and the report of the clinical research findings are in accordance with federal laws and institutional regulations in China and approved by the institutional review board. Written informed consent was obtained from the father (legal guardian) of the twins to agree to the participation and reporting of the clinical records, as well as the publication of this case report and all information and any accompanying images from the family.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by China-Japan Friendship Hospital. Written informed consent to participate in this study was provided by the participants legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

XX, JZ: Responsible for the diagnosis and treatment of patients, put forward research ideas, collect datas, write papers MR: Responsible for the extraction, examination and interpretation the results of the cutaneous pathological CW, XQ, FY: Responsible for the concrete treatment of patients) MS, KW, QZ: Responsible for guiding and proposing appropriate treatment plans).

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Case Report: A Case of Hailey–Hailey Disease Mimicking Condyloma Acuminatum and a Novel Splice-Site Mutation of ATP2C1 Gene

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Hailey–Hailey disease (HHD) is a rare autosomal-dominant blistering disorder characterized by recurrent vesicular and erosive lesions at intertriginous sites. We described a 24-year-old male who presented with multiple bright red verrucous papules in his mons pubis, bilateral groins, scrotum, perineum, and crissum, clinically resembling condyloma acuminatum. The histopathology showed extensive acantholysis with the characteristic appearance of a dilapidated brick-wall. The mutation analysis revealed a novel splice-site mutation in the *ATP2C1* gene. The patient was definitely diagnosed with HHD. The antibacterial treatments resulted in a dramatic improvement. Our findings help to broaden the understanding of clinical manifestations of HHD and improve the clinical diagnosis and treatment of this disease.

Keywords: hailey–hailey disease, ATP2C1 gene, mutation, acantholytic dyskeratosis, familial benign chronic pemphigus

INTRODUCTION

Hailey–Hailey disease (HHD), also known as familial benign chronic pemphigus, is a rare autosomal-dominant blistering disease with an estimated incidence of approximately 1/50,000 (Ben Lagha et al., 2020). It is characterized by recurrent vesicles, erosions, and macerated plaques involving the intertriginous areas, such as the lateral neck, axillae, groins, and perianal areas. The disease usually gives rise to severe discomfort and chronic relapse, so greatly impacts a patient's quality of life. The affected individuals are usually presented with clinical findings between the third and fourth decades of life. HHD is caused by mutations in the *ATP2C1* gene on chromosome 3q21 encoding the human secretory pathway Ca^{2+}/Mn^{2+} ATPase isoform 1 (hSPCA1) in the Golgi apparatus. hSPCA1, a calcium transporter protein, regulates the concentration of both Ca^{2+} and Mn^{2+} in the Golgi complex (Hu et al., 2000; Sudbrak et al., 2000). The intracellular Ca^{2+} stores play a pivotal role in maintaining epidermal integrity. The loss-of-function mutation in the *ATP2C1* gene leads to defective calcium homeostasis, loss of cell–cell adhesion of keratinocytes, and acantholysis (Fairclough et al., 2003; Vanoevelen et al., 2007). We report a 24-year-old male, who was presented with condyloma acuminatum-like lesions and a novel splice-site mutation in the *ATP2C1* gene from a Chinese family with HHD.

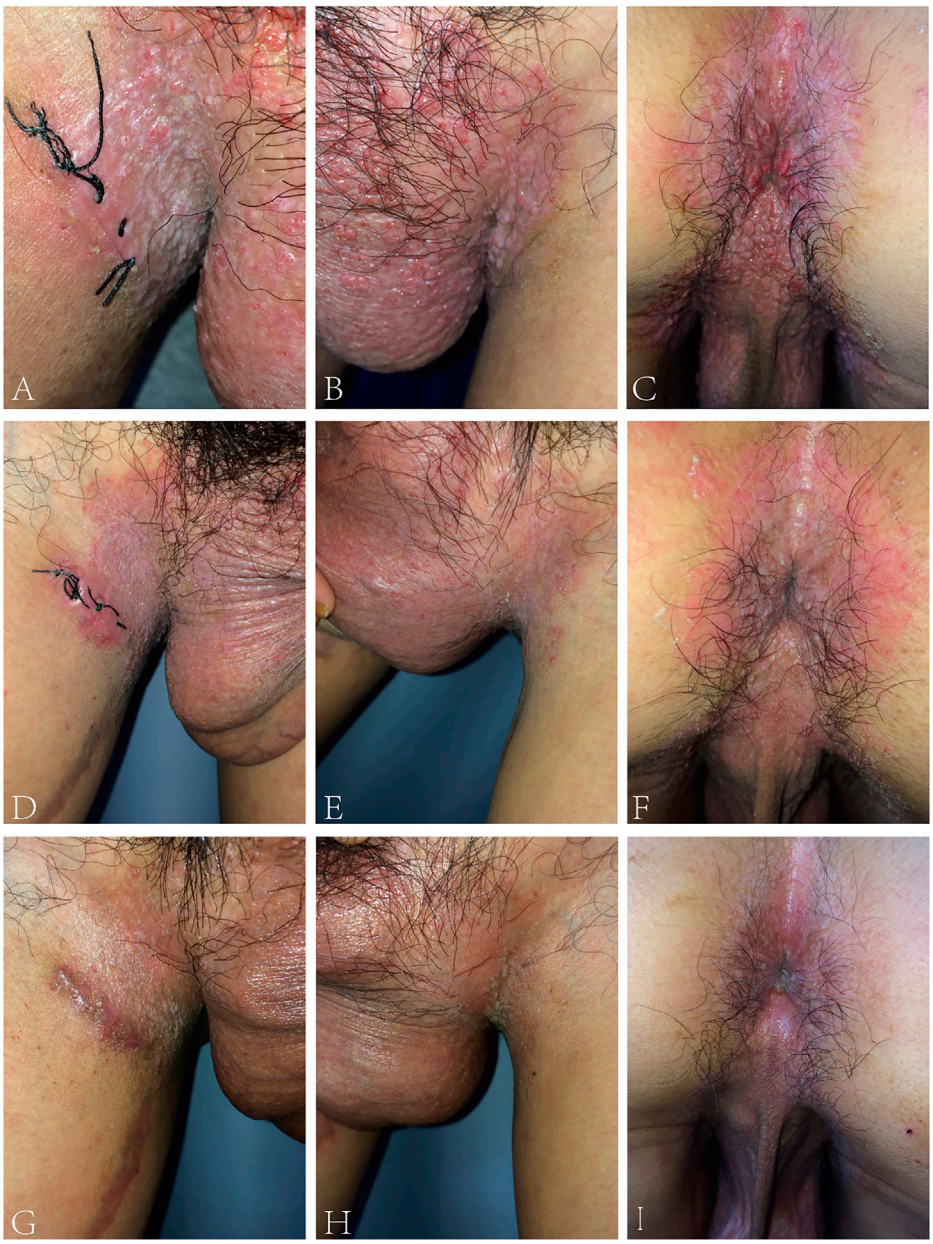

FIGURE 1 | (A–C) Bright red warty papules on bilateral groins, scrotum, perineum, and crissum of the proband. **(D–F)** Almost all of the warty papules subsided after 5 days of treatment. **(G–I)** 4 weeks later, only a few greyish white papules remained.

CASE PRESENTATION

The proband was a 24-year-old male, who presented with pruritic skin lesions in his genital and perianal regions for more than 7 years. On physical examination, multiple bright red verrucous papules were observed in his mons pubis, bilateral groins, scrotum, perineum, and crissum (**Figures 1A–C**). His general health was normal. Mycological examination of scales showed no hyphae and spores under a light microscope. The acetic acid white test was negative. PCR detection of the HPV DNA showed the absence of HPV. All blood TRUST, TPPA, and anti-HIV antibody tests were also negative. Histopathology of a biopsy from his right groin showed epidermal hyperkeratosis, parakeratosis, downward proliferation with a finger-like protrusion, and acantholysis with the appearance of a dilapidated brick-wall as well as the formation of a blister in the epidermis. In addition, there were vascular dilatation in the dermal papilla and infiltration of lymphocytes and eosinophils in the dermis (**Figure 2**). His mother was a 45-year-old woman, who presented with relapsing flares of mild erythema under her armpits for many years. His father was unaffected. The proband was diagnosed with Hailey–Hailey disease on the

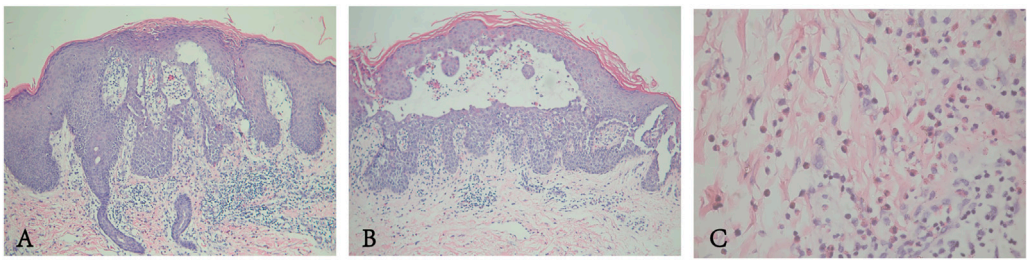

FIGURE 2 | (A,B) Epidermal hyperkeratosis accompanied by parakeratosis, acantholysis with the appearance of a dilapidated brick-wall, and formation of intraepidermal blisters. **(C)** Some lymphocytes and a few eosinophils in the dermis.

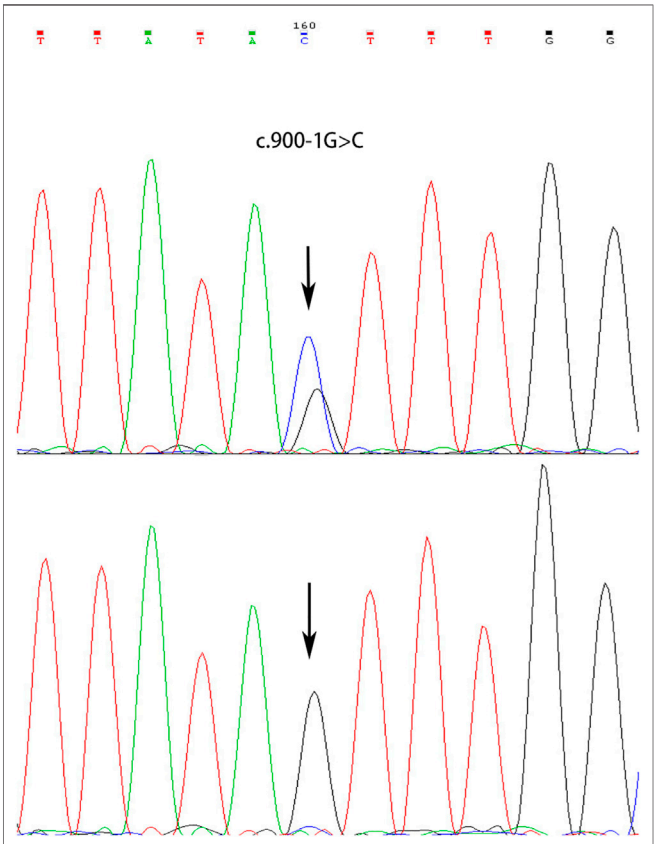

FIGURE 3 | Heterozygous splicing mutation in *ATP2C1* identified in the proband and his mother **(upper)** and sequencing result of his father **(normal, lower)**.

basis of his clinical and laboratory findings. He was administered with the treatment of oral cephradine, cleansing of 1:5,000 potassium permanganate solution, and topical 2% mupirocin ointment. The warty papules were dramatically improved after 5 days of his second visit (**Figures 1D–F**). Oral cetirizine and cyproheptadine were then given to relieve severe itching. Four weeks later, a few greyish white small papules were still present in his bilateral groins (**Figures 1G–I**). Therefore, the combination of tacalcitol ointment and mucopolysaccharide polysulfate cream was then used.

MATERIALS AND METHODS

The peripheral blood of the proband and his parents was collected after obtaining their informed consent and the approval of the Ethics Committee of Anhui Medical University. Genomic DNA was extracted by the DNA extraction kit (Promega, Madison, WI, United States).

Primer Premier 5.0 (Primer Biosystems, Foster City, CA, United States, Resource Identification Portal, RRID: SCR_004098) was used to design primers of all exons of *ATP2C1*. The PCR products of genomic DNA were then sequenced by using an ABI 3730xl DNA analyzer (ABI, Foster City, CA, United States, USEDit, RRID: SCR_018018), and the nucleotide sequences were analyzed by FinchTV (Version 1.4).

The variant was annotated against NCBI RefSeq: NM_001001486.1 and checked for the presence in ClinVar,[1] ExAC, 1000G,[2] and *ATP2C1* LOVD v.3.0 databases[3].

RESULT

A novel heterozygous splice-site mutation c.900-1G > C in the *ATP2C1* gene was identified in both proband and his mother, whereas his father showed a wild-type sequence (**Figure 3**). The mutation was predicted to be "disease-causing" in MutationTaster[4] and "Alteration of the WT acceptor site, most probably affecting splicing" in Human Splicing Finder[5]. The genotype is perfectly co-segregated with the clinical phenotype in this family. The finding of gene mutation analysis provides strong evidence to support the diagnosis of HHD.

[1]http://www.ncbi.nlm.nih.gov/clinvar.
[2]https://www.internationalgenome.org/.
[3]http://lovd.nl/ATP2C1.
[4]http://www.mutationtaster.org/.
[5]http://www.umd.be/HSF3/.

DISCUSSION

Typically, the patients with HHD present with flaccid vesicopustules, crusted erosions, macerations, or fissures in the friction-prone skin folds. However, the vulva, back, or inframammary areas were also affected (Vasudevan et al., 2015; Reyes et al., 2016; Lemieux and Funaro, 2020; Sousa Gomes et al., 2020). Rarely, mucosal involvement was observed including conjunctival, oral, esophageal, and vaginal mucosa (Burge, 1992; Oğuz et al., 1997; Fresco et al., 2020). There are some clinical variants in this disease, such as generalized, segmental, vesiculobullous, condylomatous, circinate or annular, lichenoid, and psoriasiform HHD (Hwang et al., 2003; Vilmer and Dehen, 2004; Ghosh et al., 2017; Plaza et al., 2017; Ni et al., 2018; Leducq et al., 2020; Ting et al., 2021). HHD was concomitant with bullous pemphigoid, eczema herpeticum, and human papillomavirus infection in a few cases (Chan et al., 2007; Shah et al., 2020; Li et al., 2021).

In our study, the proband presented with multiple bright red verrucous papules, clinically resembling condyloma acuminatum. Condyloma acuminatum is a benign proliferative disease of mucocutaneous tissues caused by the infection of human papillomavirus (HPV). Its typical feature is red corolliform or cauliflower-like papules or plaques on the anogenital areas. Usually, histopathological examination demonstrates epidermal hyperkeratosis and koilocytes in the granular and upper spinous layers (Chan, 2019). The diagnosis of condyloma acuminatum can be easily excluded according to his histopathological finding and absence of HPV DNA for the proband.

So far, a total of 250 public pathogenic variants in the *ATP2C1* gene have been described in the *ATP2C1* LOVD v.3.0 database (Accessed on Nov 21, 2021). There are 16.8% variants occurring in the splice region and 29.6% causing frameshift mutations, 25.2% causing missense mutations, 6. 8% causing in-frame deletions, 0.4% causing no protein production, 0.4% causing in-frame indels, 22.8% causing stop changes, and 14.8% are unknown. No significant associations between the genotype and phenotype have been found. The mutation identified in our study is located at the acceptor splice site of intron 11 that probably affects the complete splicing of exon 12. Exon 12 of *ATP2C1* encodes the location of a protein associated with calcium binding (Deng and Xiao, 2017). The mutation c.900-1G > C in the ATP2C1 gene is previously not described.

HHD is one of the acantholytic conditions or papular acantholytic dyskeratosis. The common histopathological findings are the epidermal parakeratosis, dyskeratosis, suprabasal acantholytic cleft or bulla, and the typical appearance of "dilapidated brick-wall." In general, intercellular deposition of IgG and complement 3 (C3) is not detected in the epidermis of HHD patients in contrast to autoimmune pemphigus. However, one HHD patient had linear deposition of C3 along the dermoepidermal junction (Gu et al., 1999). Anti-desmoglein and anti-desmocollin antibodies are found in sera of two cases of HHD patients (Bennani et al., 2012; Ueo et al., 2015). Moreover, fixed and soluble immune complexes are present in the epidermis of patients (Makhneva and Beletskaya, 2007). Regretfully, we did not perform direct immunofluoresence staining and serum autoantibodies detection for the patient. Probably, the formation of anti-desmoglein antibodies, anti-desmocollin antibodies, and immune complexes is associated with the unmasking of desmosomal antigens due to acantholysis. These conditions suggest that immunological factors are also involved in the pathogenesis of HHD in addition to a genetic defect. The speculation could provide a plausible explanation for the use of corticosteroids or immunosuppressants in HHD. In addition, abnormally elevated oxidative stress levels have been found in the keratinocytes of HHD; a small number of patients with refractory symptoms achieved good efficacy with antioxidant drugs (Biolcati et al., 2014).

There are a variety of triggering factors aggravating HHD, such as ultraviolet exposure, skin infection, high temperature, sweating, friction, trauma, menstruation, and pregnancy (Engin et al., 2015). So, these unfavorable factors should be avoided or eliminated. At present, there is no known cure for HHD. Multiple therapeutic options have been reported. Conventional treatments include topical antibacterial or antifungal agents, oral antibiotics, moderate to potent topical corticosteroids, topical tacrolimus ointment, and topical vitamin D3 analogs. Although ultraviolet light may exacerbate HHD, some patients respond well to narrow-band UVB phototherapy (Mizuno et al., 2014; Abaca et al., 2018). Systemic corticosteroids, cyclosporin, methotrexate, acitretin, or alitretinoin may be considered for generalized HHD (Sárdy and Ruzicka, 2014; Ben Lagha et al., 2020); however, long-term use is not recommended because of serious side effects. Multiple new treatments have been demonstrated to be effective in some refractory cases of HHD in recent years, including botulinum toxin, naltrexone, dupilumab, apremilast, photodynamic therapy, common or fractional CO_2 laser, 595-nm pulsed dye laser, and electron beam radiotherapy (Di Altobrando et al., 2020; Michael et al., 2020; Alzahrani et al., 2021; Dulmage et al., 2021; Zhang et al., 2021). Long-term improvement was observed in some patients treated with various laser ablation or electron beam radiotherapy (Leung et al., 2018).

In conclusion, we provided one case of HHD with a rare clinical feature and a novel splice-site mutation in the *ATP2C1* gene. Multiple warty papules dramatically resolved after antibacterial treatment. Our findings help to broaden the understanding of the clinical of HHD and improve the clinical diagnosis and treatment of this disease.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YD conducted Sanger sequencing and wrote the manuscript. LY collected clinical data and blood samples and performed DNA extraction. YW collected the literature and put them in order. MG and PW were responsible for the study design and guiding of the study implementation and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Genetic Profile of Epidermolysis Bullosa Cases in King Abdulaziz Medical City, Riyadh, Saudi Arabia

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Epidermolysis bullosa (EB) is a rare heterogeneous genetic mechanobullous skin disorder that is characterized by increased skin fragility leading to blistering following minor trauma. EB may be inherited as an autosomal dominant or an autosomal recessive disorder and can be classified into dystrophic EB (DEB), junctional EB (JEB), and EB simplex (EBS). A total of 28 Saudi patients with EB were included in this observational, retrospective chart-review study. A consecutive non-probability sampling technique was used to approach all affected patients. Molecular analysis was done to test the patients' genomic DNA using a custom-designed AmpliSeq panel of suspected genes. All disease-causing variants were checked against available public databases. Twelve patients (42.9%) were found to have DEB, 6 patients (21.4%) with JEB, and 10 patients (35.7%) with EBS. The molecular genetic results revealed detections of 24 various homozygous genetic variations in the genes associated with EB, of which 14 were novel mutations. The most frequent variations were detected in *COL7A1* in 12 cases (42.9%), followed by *LAMB3* in 5 cases (17.9%), *TGM5* in 4 cases (14.3%), and other genes. Furthermore, the majority (87.5%) of EB cases were confirmed to have homozygous mutations, and few were documented with positive consanguinity history. Only 3 cases (12.5%) were found to be autosomal dominant displaying heterozygous mutations. This is the first study to establish the EB genetic profile in Saudi Arabia where DEB is the most frequent type. A total of 14 novel mutations were identified that had not been previously reported. Consanguineous marriage is clearly recognized in the Saudi population; therefore, we propose a nationwide EB program that would help extend the spectrum of the genetic profile and help in the diagnosis and better understanding of this disease.

Keywords: epidermolysis bullosa, dystrophic epidermolysis bullosa, junctional epidermolysis bullosa, epidermolysis bullosa simplex, Saudi Arabia

INTRODUCTION

Inherited epidermolysis bullosa (EB) is a heterogeneous group of skin disorders characterized by increased skin fragility leading to blister formation following minor trauma (Fine 2010; Mariath et al., 2020). Worldwide, it is estimated that the EB prevalence is about 19.6 per one million of live-born infants (Fine 2016). EB may be inherited as either autosomal dominant or autosomal recessive. This disorder is caused so far by more than 29 gene mutations encoding structural proteins within the skin with functional absence or loss that leads to instability of the micro-architectural connections between the dermis and epidermis, leading to blister formation (Has et al., 2020; Mariath et al., 2020). To date, there are over 30 subtypes of EB recognized, which are classified into four major groups based on clinical or molecular studies: dystrophic EB (DEB), junctional EB (JEB), EB simplex (EBS), and recently Kindler syndrome (Has et al., 2020). Kindler syndrome is a rare type of EB caused by mutations in the *FERMT1* gene and is inherited in an autosomal recessive pattern. Dystrophic EB is caused by mutations in the gene encoding type VII collagen leading to the separation of the sub-basal lamina. DEB is inherited in an autosomal recessive or autosomal dominant pattern. Junctional EB results from mutations in genes encoding either laminin-332 or collagen type XVII, resulting in blister formation within the lamina lucida of the basement membrane. JEB is inherited in an autosomal recessive pattern. EBS results from intra-epidermal separation with mild systemic involvement ascribed to mutations encoding *KRT5* and *KRT14*, resulting in a disturbance of the stability of the keratin filament network. EBS is usually inherited in an autosomal dominant pattern, but in rare cases, it is inherited as autosomal recessive.

In Saudi Arabia (SA), few EB cases were reported in the Eastern Province among dermatology clinic case reviews without detailing their genetic characteristics (Fine 2016). EB research is scarce in the region unlike in other parts of the world, so this study aims to highlight the genetic perspective in Saudi EB patients at a tertiary healthcare center.

The EB patients' quality of life is highly impacted, as even the mildest form of the disorder leads to blisters and wounds that are quite painful (Abahussein et al., 1993; Tabolli et al., 2009). Potential complications are anemia, vocal cord stenosis, obstructive urethral lesions, and scarring and visual impairment (Abahussein et al., 1993; Fine et al., 2008; Fine and Mellerio 2009a; Fine and Mellerio 2009b). Patients have claimed suffering from physical and psychological restrictions like physical pain, lack of engagement in social activities, and embarrassment owing to their skin appearance (Horn and Tidman 2002a; Fine et al., 2009).

MATERIALS AND METHODS

Subjects

We performed an observational and retrospective chart-review study of 28 Saudi' patients at King Abdulaziz Medical City, a tertiary care hospital in Riyadh, SA. The enrolled patients were diagnosed with EB and skin fragility disorders in the period between 1998 and 2020 and treated at the same center under the divisions of dermatology, general pediatrics, ophthalmology, and dentistry. A consecutive non-probability sampling technique was used to review the files of the patients. All required data were retrieved and gathered from the hospital BestCare system as well as from the database of the molecular pathology and genetics laboratory. Institutional Review Board (IRB) approval was obtained from the ethics committee of King Abdullah International Medical Research Center under RC19/250/R. Data collected from the patients' files include sociodemographic, clinical, laboratory, and genetic data.

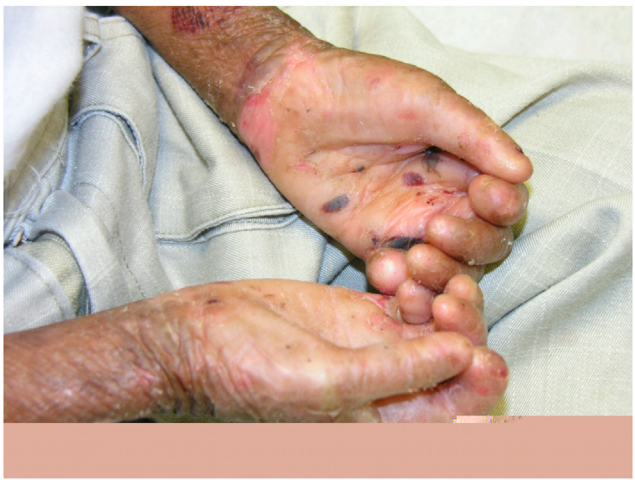

FIGURE 1 | Clinical presentation of dystrophic epidermolysis bullosa.

Genetic analysis

Molecular analysis of these cases was carried out by testing genomic DNA and checking for genetic variations of all exons and exon/intron boundaries using a custom-designed AmpliSeq panel that includes the following genes: *CD151*, *CHST8*, *COL17A1*, *COL7A1*, *CSTA*, *DSG1*, *DSG2*, *DSG3*, *DSG4*, *DSP*, *DST*, *EXPH5*, *FERMT1*, *GRIP1*, *ITGA3*, *ITGA6*, *ITGB4*, *KRT1*, *KRT10*, *KRT14*, *KRT5*, *LAMA3*, *LAMB3*, *LAMC2*, *MMP1*, *NID1*, *PKP1*, *PLEC*, and *TGM5*. All disease-causing variants were checked against the Human Gene Mutation Database (HGMD), ClinVar, the Genome Aggregation Database (gnomAD), and the Exome Aggregation Consortium (ExAC). The in silico tools SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org were used to predict coding variant effects on protein function. The collected data were entered into Microsoft Excel and analyzed using a simple statistical parameter through IBM Statistical Package for Social Sciences (SPSS) version 24. Numerical variables are presented as mean and standard deviation, and categorical variables are presented as frequencies and percentages.

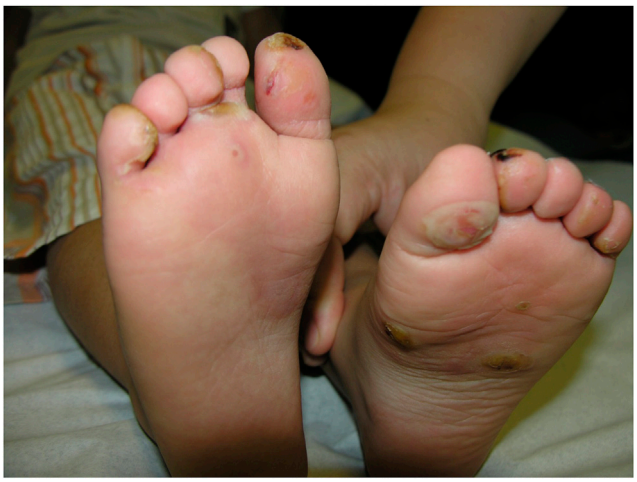

FIGURE 2 | Clinical presentation of epidermolysis bullosa simplex.

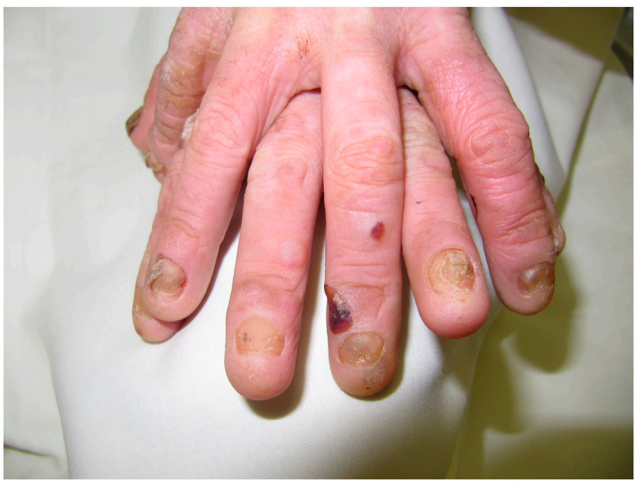

FIGURE 3 | Clinical presentation of junctional epidermolysis bullosa.

RESULTS

The population is represented with a 1.3:1 male-to-female ratio, as male patients were 16 (57%) and female patients were 12 (42.9%). The mean age was 8.9 ± 5.4 years old, the youngest patient was 3 years, and the oldest was 21 years old. Positive consanguinity history was documented in 9 patients, while family history was noted in 6 patients.

Dystrophic EB 12 (42.9%) was the most frequent subtype, followed by EB simplex 10 (35.7%) and junctional EB 6 (21.4%). Phenotypic presentations of each classification are shown in **Figures 1–3**. The mutations were detected in 7 genes: *COL7A1*, *LAMB3*, *TGM5*, *PLEC*, *DST*, *KRT14*, and *COL17A1*. Mutations implicated with *COL7A1* were the most frequent in which they were found in 12 (42.9%) patients, followed by mutations with *LAMB3* in 5 (17.9%) patients, *TGM5* with 4 (14.3%) patients, *PLEC* with 3 (10.7%) patients, *DST* with 2 (7%) patients, *KRT14* with 1 (3.6%) patient, and *COL17A1* with 1 (3.6%) patient (**Table 1**).

TABLE 1 | Frequency of the genes involved in all EB patients.

#	Gene	N=cases	Percentage %
1	COL7A1	12	42.9
2	LAMB3	5	17.9
3	TGM5	4	14.3
4	PLEC	3	10.7
5	DST	2	7.1
6	KRT14	1	3.6
7	COL17A1	1	3.6
Total		28	100

The clinical features of all patients were of the usual phenotype seen in EB patients, namely, mechanobullous fragility and blisters with a wide range of severity according to the genotype. Furthermore, nail deformities, tooth decay, lesions and erosive ulcerations in the oral cavity, and recurrent respiratory and urinary tract infections have been observed. None of patients had any gastrointestinal complications with an exception of one patient who had pyloric atresia. However, we did not detect any unusual other clinical features even in patients with novel mutations.

Genetic analysis of the implicated genes revealed 24 mutations identified among all enrolled cases. Among them, 14 mutations have not been reported to date. Autosomal recessive inheritance prevailed in 25 (89.3%) cases, and only 3 (10.7%) cases were found to be autosomal dominant (**Table 2**). A total of 4 different mutations were found in more than one patient, and those genes were diagnosed within another member of the same family. A total of 3 cases had the same mutation in *COL7A1* (case nos. 6, 7, and 8), and another mutation of *COL7A1* was found in two cases (case nos. 10, 11). Two cases had the same mutation involved in *LAMB3* (case nos. 17 and 18), and two additional patients had the same gene mutations in *TGM5* (case nos. 26 and 27).

DISCUSSION

DEB is a rare inherited EB caused by mutations involving the genes that encode type VII collagen leading to the separation of the sub-basal lamina (Brun et al., 2017). Recessive DEB has a wider array of severity and milder/localized form that has acral and nail involvement, similar to other forms of DEB. In particular, DEB patients have a significant risk of developing aggressive squamous cell carcinoma in chronic lesion sites (Mitsuhashi and Hashimoto 2003). The severe form is characterized by generalized blistering of the hands and feet, usually involving the acral surfaces, leading to pseudosyndactyly and flexural contractures that intensify with age (Bruckner-Tuderman 2010). Clinically, our cases do not differ much from what were described internationally. Although gastrointestinal complications are common in patients with EB, this was not the case in our patients since only one patient had pyloric atresia.

DEB was the most frequent subtype in the study population. Twelve patients with DEB were detected with 10 different homozygous variants in *COL7A1*. A total of 5 mutations are

TABLE 2 | Genes, variants, mutation types, and novelty status per EB classifications.

Case No	Gene	Mutation in cDNA (GenBank ID)	Consequence (protein)	Mutation status	Annotation	*In silico* prediction analysis			Pathogenicity	Reported (dbSNP# or HGMD)
						SIFT	PP	MT		
Dystrophic epidermolysis bullosa										
1	*COL7A1*	c.7768G > C (NM_000094.4)	p.Gly2590Arg	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs2043715843
2	*COL7A1*	c.7411C > T (NM_000094.4)	p.Arg2471*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs121912852/ CM960412
3	*COL7A1*	c.4520G > T (NM_000094.4)	p.Gly1507val	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
4	*COL7A1*	c.4448G > A (NM_000094.4)	p.Gly1483Asp	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs756217590/ CM093143
5	*COL7A1*	c.4864G > C (NM_000094.4)	p.Gly1622Arg	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported CM1618661
6	*COL7A1*	c.4198delG (NM_000094.4)	G1400Vfs*310	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
7	*COL7A1*	c.4198delG (NM_000094.4)	G1400Vfs*310	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
8	*COL7A1*	c.4198delG (NM_000094.4)	G1400Vfs*310	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
9	*COL7A1*	c.611T > G Deletion exons 25–52 (NM_000094.4)	p.Leu204Ser p.?	Heterozygous Heterozygous	Missense Stop gained	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Reported rs745939385 Not reported
10	*COL7A1*	c.1507+1G > C (IVS11+1G > C) (NM_000094.4)	p.?	Homozygous	Splice region	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Reported CS072154
11	*COL7A1*	c.1507+1G > C (IVS11+1G > C) (NM_000094.4)	p.?	Homozygous	Splice region	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Reported CS072154
12	*COL7A1*	c.7442G > A (NM_000094.4)	p.Gly2481Asp	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
Junctional epidermolysis bullosa										
13	*COL17A1*	c.3922delA (NM_000494.4)	p.Ser1308Alafs*4	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
14	*LAMB3*	c.972delA (NM_000228.3)	p.Cys325Serfs*71)	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
15	*LAMB3*	c.972delA (NM_000228.3)	p.Cys325Serfs*71	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
16	*LAMB3*	c.1978C > T (NM_000228.3)	p.Arg660*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs146794392/ CM972912
17	*LAMB3*	c.958_1034dup (NM_000228.3)	p.Asn345Lysfs*77	Homozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs1553277702
18	*LAMB3*	c.1977-1G > A (NM_000228.3)	p.?	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs786205451
Epidermolysis bullosa simplex										
19	*PLEC*	c.4552 C > T (NM_000445.5)	p.Gln1518*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported CM010392

(Continued on following page)

TABLE 2 | (*Continued*) Genes, variants, mutation types, and novelty status per EB classifications.

Case No	Gene	Mutation in cDNA (GenBank ID)	Consequence (protein)	Mutation status	Annotation	*In silico* prediction analysis			Pathogenicity	Reported (dbSNP# or HGMD)
						SIFT	PP	MT		
20	*PLEC*	c.7144C > T (NM_000445.5)	p.Gln2382X*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
21	*PLEC*	c.7144C > T (NM_000445.5)	p.Gln2382X*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
22	*TGM5*	c.1335G > C (NM_004245.4)	p.Lys445Asn	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs606231276/ CM095542
23	*TGM5*	c.1335G > C (NM_004245.4)	p.Lys445Asn	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs606231276/ CM095542
24	*TGM5*	c.1335G > C (NM_004245.4)	p.Lys445Asn	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported rs606231276/ CM095542
25	*TGM5*	c.1138G > C (NM_004245.4)	p.Ala380Pro	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Pathogenic	Not reported
26	*DST*	c.3370C > T	p.Gln1124*	Homozygous	Stop gained	Deleterious	Probably damaging	Disease causing	Pathogenic	Reported CM103946
27	*DST*	c.16496C > G	p.Al5499Gly	Homozygous	Missense	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported
28	*KRT14*	c.1094_1095delGC	p.R365LfsX117	Heterozygous	Frameshift	Deleterious	Probably damaging	Disease causing	Likely pathogenic	Not reported

SIFT, <0.05 as deleterious; PolyPhen-2, the closer to 1 as probably damaging; MutationTaster, disease-causing is pathogenic variation; dbSNP#, The Single Nucleotide Polymorphism database; HGMD, The Human Gene Mutation Database.

novel: 4 missense and 1 frameshift (**Table 2**). The other 5 reported variants are 4 missense mutations and 1 non-sense mutation that were previously reported (Horn and Tidman 2002b; Almaani et al., 2009). These results expand the spectrum of identified mutations implicated with DEB.

Six patients with JEB were among the study population. A total of 3 patients had novel mutations, and the other 3 patients had previously reported mutations. Two novel homozygous mutations were detected in 2 genes, *COL17A1* and *LAMB3*; 1 mutation with frameshift in *COL17A1*; and 1 frameshift in *LAMB3* gene (**Table 2**). In those with consanguineous history, homozygous mutations were identified. The reported mutations are 1 non-sense, 1 frameshift, and 1 missense in *LAMB3* genes, which were previously reported by Christiano et al. and Pulkkinen *et al.* (Christiano et al., 1996; Pulkinnen and Uitto, 1999).

EBS was diagnosed in 10 patients with 8 different mutations in 4 genes: *TGM5*, *PLEC*, *DST*, and *KRT14*. Four are pathogenic mutations that are not reported to date. The 4 novel mutations of EBS in 3 different genes are 1 missense in *TGM5*, 2 non-sense in *PLEC*, and 1 missense in *DST* (**Table 2**). The other 5 reported mutations are 2 missense in *TGM5*, one non-sense mutation involving *PLEC*, and one frameshift in *KRT14* gene that were previously reported (Fine and Mellerio 2009b; Fine et al., 2008; Mitsuhashi and Hashimoto 2003). *TGM5* mutations may belong to other disorders with skin fragility and not to classical EB (Has et al., 2020). Almost all cases (**Table 2**) were inherited in an autosomal recessive pattern harboring either homozygous or compound heterozygous allele variants, although the loss-of-function mutations might have resulted from missense or frameshift mutations through the mechanism of non-sense-mediated mRNA decay. Indeed, it seems likely that the presence of homozygosity in our Saudi patients conformed to a high fertility rate and consanguinity rate, which reached more than 50% in some areas (Scott et al., 2016).

It is worth mentioning that all newly reported variants in this study were evaluated for their impact on protein function and structure using in silico prediction tools such as Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2), and MutationTaster. However, the final protein function pathogenicity effect of these mutant variants and the association with the EB disease may require further functional verification.

Study limitation

The study was conducted at a single tertiary care center at central SA, yet it is the first pillar to establish the Saudi EB genetic profile. Secondly, the retrospective design has made the study more subjective to missing some relevant data. However, the effects do not have any major impact on the findings. We propose therefore further collaboration between various centers from different parts of the region to have substantial effects on the sample size, including diversifying the patients' backgrounds.

CONCLUSION

The study elaborates and reports on the genetic profile and prevalence of EB in Saudi patients at a single tertiary

healthcare center in central SA. Dystrophic EB was the highest reported subcategory among all the other EB classifications. *COL7A1* was the most common gene identified among the patients. Positive family history of consanguinity was evident as expected; further highlighting its role through education is needed among Saudi EB patients.

This study entails an impact on the future of identifying the genetic characteristics of Saudi EB patients, along with emphasizing on the need to launch an entity that would be responsible for directing the efforts of initiating a national center for EB.

AUTHOR'S NOTE

This manuscript is dedicated to Ahad Alharthi one of our interns who participated in this research and passed away on 27th July 2021. Despite her short professional life as a medical student and intern, she participated in many researches and was a leader in many skin awareness campaigns.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by King Abdullah International Medical Research Center (RC19/250/R). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHORS CONTRIBUTIONS

RA, MuAA, AA, and SA were involved in collection of the patients' clinical information, data analysis, and drafting of the manuscript. SA-K reviewed the patients' clinical presentation, genetic data, and reviewed and revised the manuscript. MoAA designed and supervised the entire research and reviewed and revised the manuscript. All authors have read and approved the final version of manuscript.

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frontiers in Genetics

CASE REPORT
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Case Report: Chanarin-Dorfman Syndrome: A Novel Homozygous Mutation in ABHD5 Gene in a Chinese Case and Genotype-Phenotype Correlation Analysis

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The Chanarin–Dorfman syndrome (CDS) is a rare, autosomal recessively inherited genetic disease, whch is associated with a decrease in the lipolysis activity in multiple tissue cells. The clinical phenotype involves multiple organs and systems, including liver, eyes, ears, skeletal muscle and central nervous system. Mutations in ABHD5/CGI58 gene have been confirmed to be associated with CDS. We performed whole exome sequencing on a Chinese CDS patient with skin ichthyosis features mimicking lamellar ichthyosis, ectropion, sensorineural hearing loss, and lipid storage in peripheral blood neutrophils. A novel homozygous missense mutation (p.L154R) in ABHD5 gene was detected in this patient. Genotype-phenotype analysis in reported CDS patients revealed no particular correlation. Our findings further enrich the reservoir of ABHD5 mutations in CDS.

Keywords: ichthyosis, ABHD5/CGI-58 gene, Chinese, Jordan's anomaly, Chanarin-Dorfman syndrome

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INTRODUCTION

Chanarin-Dorfman syndrome (CDS; OMIM 275630) is an extremely rare, multisystemic, autosomal recessive neutral lipid storage disorder (NLSD) arising from impaired lipid metabolism (Demerjian et al., 2006). CDS is associated with a multitude of clinical symptoms, the most prominent of which is icthyosis, especially non-bullous congenital ichthyosiform erythroderma. Patients can be born as collodion babies, occasionally accompanied by bilateral ectropion and eclabion. Other manifestations include liver steatosis, myopathy, sensoryneural hearing loss, and cataract (Yamaguchi and Osumi, 2009). To date, approximately 120 cases of CDS have been reported around the world, but mainly in Mediterranean and Middle Eastern countries, especially in Turkey (Incecik et al., 2018; Eskiocak et al., 2019; Louhichi et al., 2019; Niculescu et al., 2019; Al-Hage et al., 2020; Dabas et al., 2020; Cakmak and Bagci, 2021; Jiang et al., 2021; Tavian et al., 2021). So far, only three patients of CDS have been reported from China (Takeichi et al., 2016; Jiang et al., 2021).

CDS is caused by mutations of the abhydrolase domain containing 5 gene (*ABHD5*)/comparative gene identification-58 (*CGI-58*) on chromosome 3p21, leading to insufficient fatty acids (FAs)

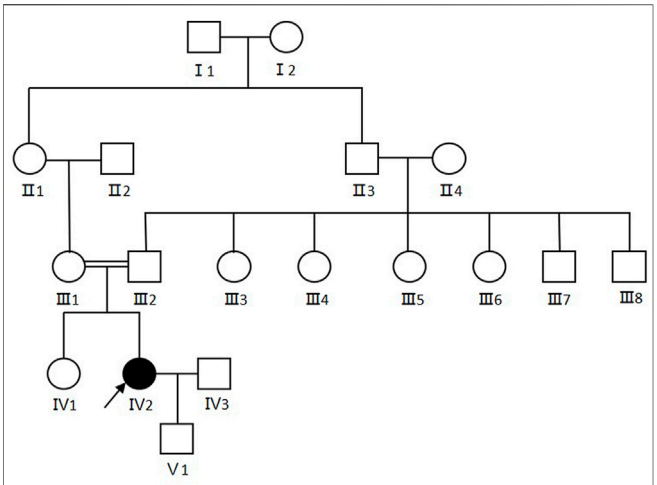

FIGURE 1 | Pedigree of the CDS family. Arrows show the proband. Females were indicated by circles while males were indicated by squares. Blackened symbols represented patients who were carried the mutation through mutation sequencing.

mobilization within the cell and systemic triglyceride accumulation in cytosolic droplets in multiple tissues. These lipid droplets have been observed in hepatocytes, intestinal mucosa, blood, bone marrow, skin fibroblasts, myocytes, central nervous system cells and many other types of cells.

The diagnosis is based on the presence of ichthyosis and identification of lipid droplets in granulocytes (Jordan's anomaly) in peripheral blood smear. For patients with CDS, dietary modification has been reported to be an effective treatment, with no deleterious effects on liver function (Kakourou et al., 1997). Herein, we present a Chinese patient with CDS caused by a novel homozygous missense mutation, p. L154R, in *ABHD5* gene, and the genotype-phenotype correlation analysis was also conducted.

MATERIALS AND METHODS

Case Report

The proband (IV2) in this study was a 30-year-old female displaying diffuse erythema, fine scaling on the body, and sensorineural hearing loss since her birth as a collodion baby. The severity of the ichthyosiform erythroderma had lessened as she aged. The condition was severe in winter and mild in summer. Her nails, teeth and hair appeared normal. No additional involvement of muscular system and central nervous system was found. Her parents were consanguineous (first cousins), and there were no other affected family members. Her son was 1 year old and was normal (**Figure 1**).

Physical examinations of the proband revealed coarse facial features, including ptosis, bilateral extropion of the eyelids, broad forehead, depressed nasal bridge, and extensive erythematous patch and plaques accompanied by fine scaling covering the body (**Figures 2A,B**). Dermatoscopy showed dilation of twisted capillaries and diffused white scales (**Figure 2C**). Laboratory findings revealed high levels of alanine aminotransferase (ALT, 108 U/L; normal 7–40 U/L), aspartate aminotransferase (AST, 87 U/L; normal 13–35 U/L), creatine kinase (CK, 400 U/L; normal 100–250 U/L), and low levels of urea (2.42 mmol/L;

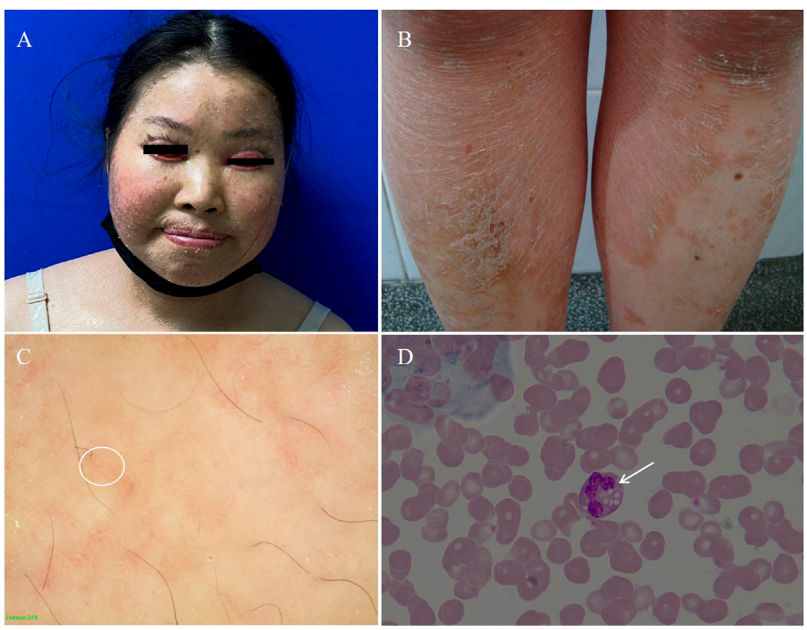

FIGURE 2 | (A) Coarse facies of the proband with ptosis, bilateral extropion of the eyelids, broad forehead, depressed nasal bridge. **(B)** Nonbullous ichthyosiform erythroderma fine scales on the trunk. **(C)** The dermatoscopic appearance of the lesion on the trunk white scales and the diffuse, punctate haemorrhage of apparent blood capillaries (white circle as shown, 20×). **(D)** Peripheral blood smear. The arrow shows lipid vacuolization in leukocytes observed in blood smear (Jordan's Anomaly) (Wright's stain, 100×).

normal 2.60–7.50 mmol/L), and vitamins D (28 ng/ml; normal 30–100 ng/ml). Triglycerides and total cholesterol levels were normal. Abdominal ultrasound showed moderate fatty infiltration of the liver without splenomegaly. Test of the peripheral blood revealed distinct lipid accumulation in polymorphonuclear cells (Jordan's anomaly, **Figure 2D**).

Peripheral Blood Collection and DNA Extraction

After obtaining informed consent from all participants and approval from Clinical Research Ethics Committee of Anhui Medical University, EDTA anticoagulated venous blood samples were collected from the family. Genomic DNA was extracted using a Flexi Gene DNA Kit (250) in a standard procedure and stored at −80°C. The procedures were in accordance with the Helsinki Declaration of 1975, as amended in 1983.

Whole Exome Sequencing

WES was performed in the proband (IV2). Genomic DNA fragments corresponding to all exons in genome were amplified by PCR and subjected to automatic DNA sequencing after purification. Agilent SureSelect XT Library Prep Kit and SureSelect Human All Exon V6 kit were used for the library preparation and capture. Illumina Hiseq XTen platform was used for the sequencing. Screening for disease-associated deleterious mutations was made with emphases on all the possible pathogenic variations in reported *ABHD5* gene.

Sanger Sequencing

The possible pathogenic variations identified by WES were confirmed by Sanger sequencing in the proband's father (III2) and sister (IV1) to detect genotype-phenotype co-segregation. Primers flanking all coding regions of the possible variation were designed using software Primer Premier 5.0 (Primer Biosystems, Foster City, CA, United States). PCR products from genomic DNA were sequenced using an ABI 3730XL DNA Analyzer (ABI, Foster City, CA, United States). The sequencing results were analyzed using Finch TV (Version 1.5), and the newly discovered mutation was named referring to the principle of the Human Genome Variation Society (HGVs).

Review of the Literature

Articles published between 1974 and 2021 were searched on PubMed by using the following keywords singly or in various combinations: "Chanarin–Dorfman Syndrome", "Dorfman–Chanarin syndrome", "congenital ichthyosiform erythroderma", "neutral lipid storage disorder", "*ABHD5*/*CGI-58* mutation" and "Jordan's anomaly". The patients' race, age, gender, clinical symptoms, genetic mutations were all evaluated.

RESULTS

WES Results and Co-Segregation Analysis

WES revealed a novel homozygous missense mutation c.461T > G (NM_016,006) in *ABHD5* gene, resulting in the substitution of amino acid arginine for leucine at position 154 (p.L154R), which is a highly conserved amino acid leucine across multiple species (**Figures 3A,B**). The mutation was predicted by REVEL to be pathogenic and by SIFT to be damaging, with scores of 0.959 and 0, respectively. Sanger sequencing revealed the mutation was homozygous in the proband and heterozygous in her father and sister. (**Figure 3A**). The mutation was not found in 100 control individuals from the same ethnicity, and was not recorded in the database of genomAD.

Genotype-Phenotype Correlation Analysis

We found 106 CDS patients (58 male) reported in literature in whom the molecular analysis of *ABHD5* gene were performed (**Supplementary Table S1**). The age of the patients varied from 4 months to 67 years. A total of 45 mutations in *ABHD5* have been identified, including 37 homozygous mutations and 8 compound heterozygous mutations (**Supplementary Table S1**). The mutations identified in the patients included missense, nonsense, insertions, deletions, and frameshift mutations. Irrespective the nature of the mutation, all CDS patients showed the typical skin features of non-bullous congenital ichthyosiform erytroderma and Jordan's anomaly, followed by hepatomegaly and hepatosteatosis. The most common mutation in patients is p. N209X (26/45, 57.8%). Within the group of N209X mutation patients, the CDS phenotype was homogeneous. This mutation was identified in 26 cases (23 from Turkey), and is rare in other populations. The other mutations mostly appear to be familial or local. No particular genotype-phenotype correlation were found in the literature.

DISCUSSION

In the present study, the clinical features, laboratory findings and genetic results of the proband were consistent with the diagnosis of CDS. And mutation analysis of *ABHD5* using WES and Sanger sequencing revealed a new homozygous mutation. For this mutation, p. L154R, leucine is a hydrophobic amino acid, while arginine is an alkaline amino acid. The transition of the amino acid polarity may affect the structure and function of ABHD5 protein.

To date, 45 different mutations in the *ABHD5* gene have been reported (Incecik et al., 2018; Eskiocak et al., 2019; Louhichi et al., 2019; Niculescu et al., 2019; Al-Hage et al., 2020; Dabas et al., 2020; Cakmak and Bagci, 2021; Jiang et al., 2021; Tavian et al., 2021), among which the homozygous mutation of p. N209X is the most common. A comparison of findings in patients with the common N209X mutation and other mutations did not show major differences, and does not point to a particular genotype-phenotype correlation, which is consistent with previous researches (Aggarwal et al., 2012; Nur et al., 2015). The variability of clinical symptoms in patients with CDS depends on a large number of mutations involved, and the severity of the phenotype can be quite variable. Ichthyosis from birth was a universal presentation, followed by liver disease. It is reported that there was an intrafamilial

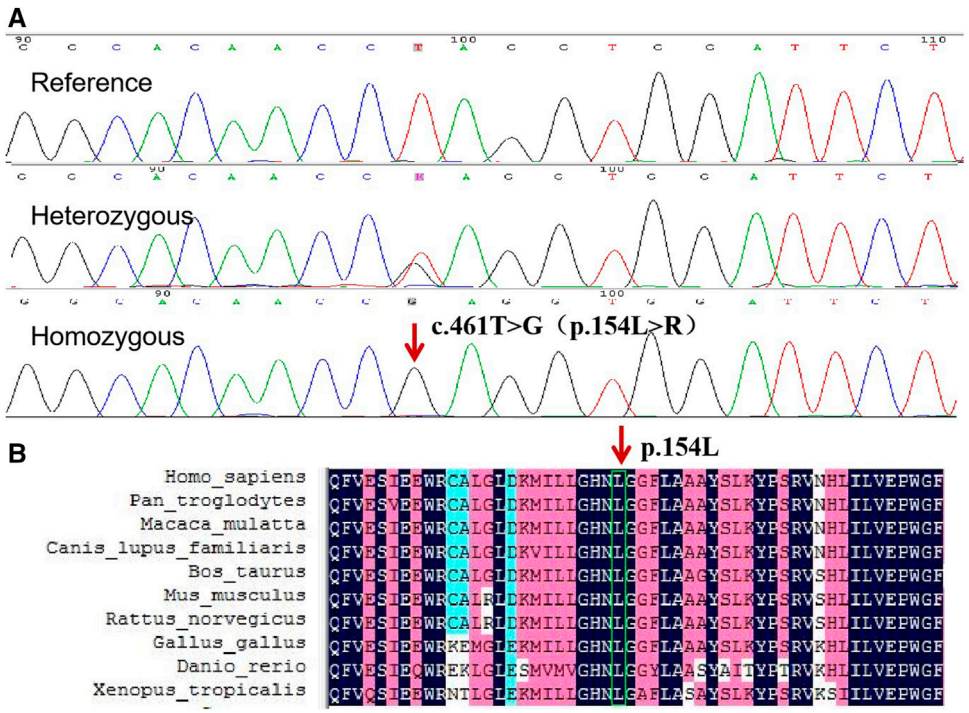

FIGURE 3 | (A) Sequence analysis of *ABHD5* gene in the proband and her sister. The arrow indicates the homozygous mutation c.461T > G, which results in the protein change, p. L154R. **(B)** Conservation analysis using DNAMAN revealed that ABHD5 amino acid sequence at position 154 are highly conserved across multiple species.

phenotypic heterogeneity in the alive affected individuals, which led to the hypothesized that mutations in other genes might have affected the phenotypes through modifier effects (Takeichi et al., 2016). Furthermore, the lack of correlation between the genotype and the severity of the disease may be explained by the role of epigenetic and environmental factors. Liver involvement is an important cause of mortality and morbidity in CDS patients. Most of the patients with cirrhosis identified in the literature had advanced age (Cakmak and Bagci, 2021). However, it is reported that the cirrhosis may develop at an early age depending on the nature of the mutations (Aggarwal et al., 2012; Tamhankar et al., 2014). So, it is possible that there is some genotype-phenotype correlation. More CDS cases with mutation data are needed to confirm the genotype-phenotype correlations in *ABHD5* mutations.

Mutations in *ABHD5* may lead to the accumulation of long-chain fatty acids, energy deficiency in cells and affect the skin barrier (Tamhankar et al., 2014). The ABHD5 protein has been studied as a cofactor for adipose triglyceride lipase (ATGL). Ujihara er al. revealed that the triglycerides levels in the scales from the patient were positively correlated with the severity of ichthyosis (Ujihara et al., 2010). The level of triglyceride in this patient was normal, which may be a reason for the mild clinical manifestation of this patient. It has been shown that ABHD5 participates in the assembly and release of hepatitis C virus particles by mobilizing the lipid pool of cytoplasmic lipid droplets, therefore, CDS patients may show certain resistance to hepatitis C virus infection (Vieyres et al., 2016).

The diagnosis of CDS can be confirmed by performing a peripheral blood smear to show lipid vacuoles in granulocytes, myocytes, hepatocytes, fibroblasts and keratinocytes, a feature called Jordan's anomaly. Ichthyosiform erythroderma, as a usual symptom of CDS, is a typical manifestation in congenital ichthyosis syndromes. Studies found that the co-existence of Jordan's anomaly and ichthyosis provided the definitive diagnosis in CDS, regardless of the *ABHD5* gene mutation (Cakmak and Bagci, 2021). Therefore, PBS is necessary to examine for the presence of Jordan's anomaly. In peripheral blood smears taken from our patients, lipid vacuolization was seen in the cytoplasm of leukocytes. This finding supports the presence of a natural lipid storage disorder.

There has been no curative treatment of CDS so far. Various topical therapies, including emollients and keratolytic agents, have been proposed to improve ichthyosis, with mostly unsatisfactory results. While systemic therapy with retinoids combined with dietary modification has been used successfully in patients with skin and muscle manifestations (Gandhi et al., 2007; Israeli et al., 2012), co-morbidities limit its use in CDS. Niculescu et al. proposed tazarotene 0.015% cream as a potential topical agent for patients with ichthyosis, including patients with systemic involvement (Niculescu et al., 2019). In the present study, a diet low in fat and rich in short/medium-chain fatty acids and emollients were administered. After 1 year

of treatment, the skin lesions improved, and the patient was satisfied with the treatment effect and attended the scheduled follow-up visits.

In conclusion, the patient we presented showed ichthyosiform dermatosis, and mutation analysis eventually confirmed the diagnosis of CDS. CDS presents suffer from damage of many systems, and the severity of the phenotype can be quite variable. The diagnosis can be established by the clinical features and a blood smear, which can be confirmed by the molecular analysis. The mutation found in this patient enriched our understanding of pathogenic mutations for CDS. As it is not easy to obtain an accurate diagnosis only based on the dermal features, a blood smear and mutational analysis are required for patients suspected with congenital ichthyosis (Cheng et al., 2020).

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BL conducted Sanger sequencing and wrote the manuscript. HH and JZ performed whole exome sequencing and wrote the manuscript. GC, XK, and MZ collected clinical data and blood samples, and performed DNA extraction. LT performed data analysis of the whole exome sequencing. PW and LT were responsible for the study design and guiding of the study implementation, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.847321/full#supplementary-material

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CASE REPORT
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Case Report: A Missense Mutation in Dyskeratosis Congenita 1 Leads to a Benign Form of Dyskeratosis Congenita Syndrome With the Mucocutaneous Triad

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Background: Dyskeratosis congenita (DC) is a rare inheritable disorder characterized by bone marrow failure and mucocutaneous triad (reticular skin pigmentation, nail dystrophy, and oral leukoplakia). Dyskeratosis congenita 1 (*DKC1*) is responsible for 4.6% of the DC with an X-linked inheritance pattern. Almost 70 *DKC1* variations causing DC have been reported in the Human Gene Mutation Database.

Results: Here we described a 14-year-old boy in a Chinese family with a phenotype of abnormal skin pigmentation on the neck, oral leukoplakia, and nail dysplasia in his hands and feet. Genetic analysis and sequencing revealed hemizygosity for a recurrent missense mutation c.1156G > A (p.Ala386Thr) in *DKC1* gene. The heterozygous mutation (c.1156G > A) from his mother and wild-type sequence from his father were obtained in the same site of *DKC1*. This mutation was determined as disease causing based on *silico* software, but the pathological phenotypes of the proband were milder than previously reported at this position (HGMDCM060959). Homology modeling revealed that the altered amino acid was located near the PUA domain, which might affect the affinity for RNA binding.

Conclusion: This *DKC1* mutation (c.1156G > A, p.Ala386Thr) was first reported in a Chinese family with mucocutaneous triad phenotype. Our study reveals the pathogenesis of *DKC1* c.1156G > A mutation to DC with a benign phenotype, which expands the disease variation database, the understanding of genotype–phenotype correlations, and facilitates the clinical diagnosis of DC in China.

Keywords: dyskeratosis congenita syndrome, *DKC1*, missense mutation, c.1156G > A, p.Ala386Thr

INTRODUCTION

Dyskeratosis congenita (DC) is a rare inheritable disorder characterized by bone marrow failure and mucocutaneous triad (skin pigmentation, dystrophy nails, oral leukoplakia) (1). So far, several genes have been identified to be associated with DC, including dyskeratosis congenita 1 (*DKC1*), CTS telomere maintenance complex component 1 (*CTC1*), regulator of telomere elongation

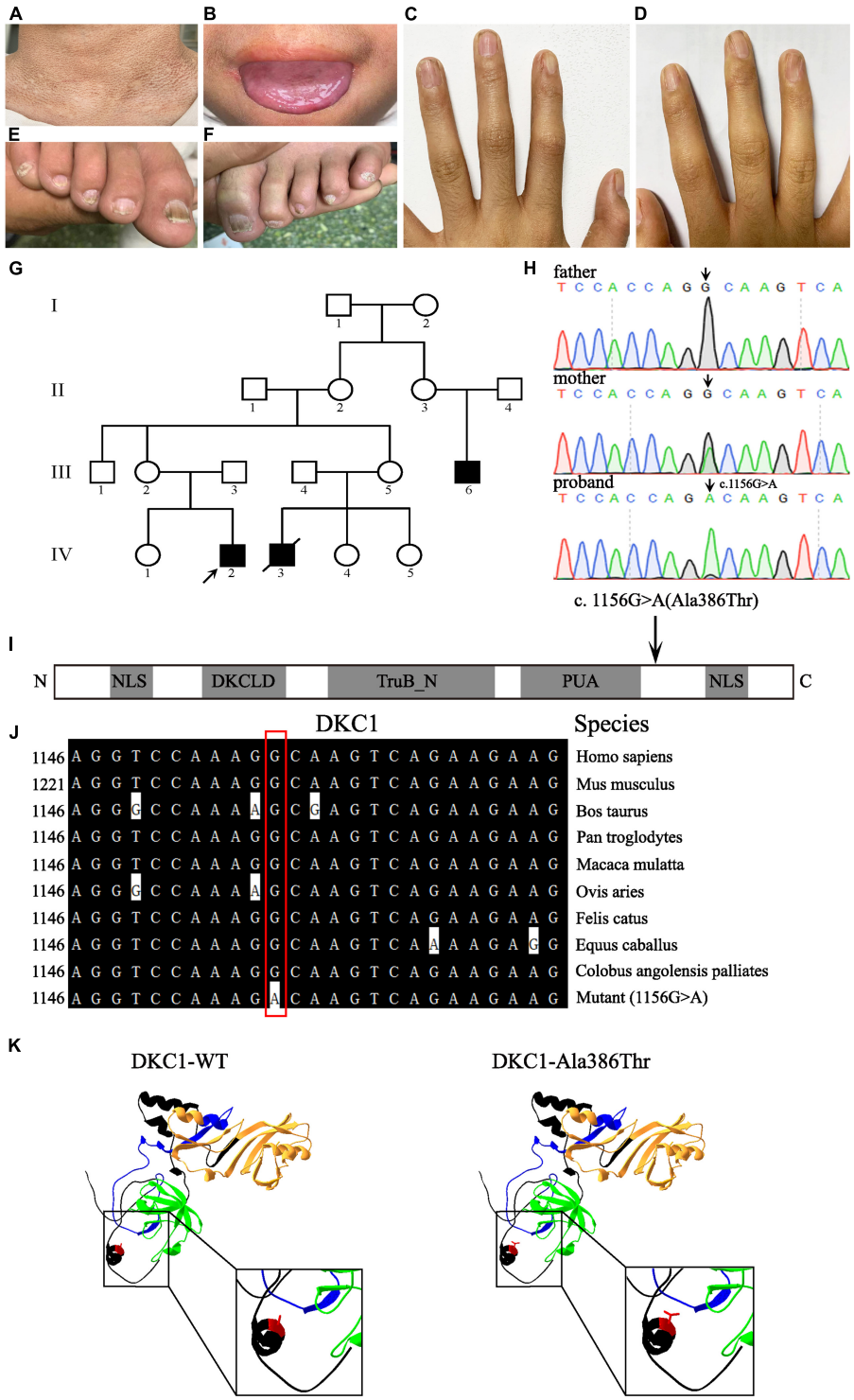

FIGURE 1 | Clinical features of the proband and pedigree, sequencing analysis, and DKC1 mutation investigations. Pigmentation on the neck **(A)**, mucosal leukoplakia on the tongue **(B)**, finger nail ridging, toenail ridging, and longitudinal splitting **(C–F)** in the proband. **(G)** The pedigree of the family. The arrow indicates the proband. **(H)** Sequencing chromatograms show the proband with a hemizygous mutation DKC1 c.1156G > A, the proband's mother with the same heterozygous mutation; the black arrow indicates the position of the nucleotide mutation. **(I)** A linear representation of the DKC1 protein shows the location of the N-terminal nuclear localization signals (NLS), DKCLD, TruB_N, and PUA domains. The black arrow shows the positions of the amino acid substitutions. **(J)** The mutant site (c.1156G > A) of DKC1 is highly conserved phylogenetically among the indicated species. **(K)** The mutant proteins were structured by the Swiss-Model online software and compared with the wild type. Ribbon representation of the human DKC1 and map of the studied variant localization obtained by homology modeling analysis. The wild-type and mutant monomers are shown in black; DKCLD, TruB_N, and PUA domains are shown in blue, orange, and green, respectively. Amino acid Ala386 is shown as red.

TABLE 1 | Bioinformatics prediction of a pathogenic variant.

Mutation prediction	Prediction score values			
Tool	**Mutation Taster**	**SIFT**	**PolyPhen-2**	**PROVEAN**
c.1156G > A	D (0.99)	D (0.02)	B (0.122)	D (−3.121)

Mutation Taster: D, disease causing; P, polymorphism.
SIFT: D, damaging; T, tolerated.
PolyPhen-2: D, probably damaging; P, possibly damaging; B, benign.
PROVEAN: D, deleterious; N, neutral.

helicase 1 (*RTEL1*), TERF 1-interacting nuclear factor 2 (*TINF2*), telomerase RNA component (*TERC*), telomerase reverse transcriptase (*TERT*), adrenocortical dysplasia homolog (*ACD*), NHP2 ribonucleoprotein (*NHP2*), NOP 10 ribonucleoprotein (*NOP10*), poly(A)-specific ribonuclease (*PARN*), nuclear assembly factor 1 *(NAF1)*, and WD repeat containing antisense to TP53 (*TCAB1*), and DKC1 is responsible for 4.6% of the DC (2, 3). Almost 70 dyskeratosis congenita 1 (DKC1) variations causing DC have been reported in the Human Gene Mutation Database (HGMD[1]); the gene encoding a nucleolar protein is called dyskerin, which is involved in both ribosome biogenesis (4) and telomere maintenance (5). Here, we found a DC patient in a Chinese family. The clinical data of the patient and literature review of DC are described.

CASE PRESENTATION

Clinical Manifestations and Family History

Three affected males (III-6, IV-2, and IV-3) and 14 unaffected individuals are involved in this family and are recruited from Shanxi Province, China (**Figure 1G**). The proband IV-2 is a 14-year-old boy with abnormal skin pigmentation on the neck (**Figure 1A**), oral leukoplakia (**Figure 1B**), and nail dysplasia on his hands and feet (**Figures 1C–F**). III-6 presents with similar phenotypes. II-2, II-3, III-2, and III-5 are mutation carriers without any mild signs of congenital dyskeratosis.

Sequencing Analysis of the Patient and His Family

Whole-exome sequencing (WES) data were functionally annotated and filtered using cloud-based rare disease NGS analysis platform,[2] based on the Ensembl (GRCh37/hg19), dbSNP, EVS, 1000 genome, ExAC, and GnomAD databases. Exonic sequence alterations and intronic variants at exon–intron boundaries, with unknown frequency or minor allele frequency (MAF) < 1% and not present in the homozygous state in those databases, were retained. Filtering was performed for variants in genes associated with DC. Then the only DC-related gene mutation *DKC1* mutation (c.1156G > A, p.Ala386Thr) was identified.

[1]http://www.hgmd.org/; 2021.2.

[2]http://www.gene.ac/

TABLE 2 | Main clinical features of dyskeratosis congenita (DC) patients in the Human Gene Mutation Database (HGMD)/literature.

Mutation on cDNA/protein	Ethnic origin	Gender	Age (years)	Mucocutaneous triad	Bone marrow failure	Anemia	Thrombocytopenia	Telomere shortening	Pulmonary fibrosis	References
5C > T/A2V	Egyptian	Male	40	+	NT	NT	NT	NT	+	(8)
29C > T/P10L	-	-	-	+	+	NT	NT	NT	NT	(4)
91C > A/Q31K	Japan	Male	11	+	NT	NT	+	NT	NT	(9)
91C > G/Q31E	United States	Male	33	+	+	NT	NT	+	NT	(10)
106T > G/F36Y	Belgian	Male	30	+	NT	NT	+	NT	+	(11)
113T > C/I38T	Italy	Male	0.75	+	+	+	NT	NT	NT	(11)
114C > G/I38M	United Kingdom	-	-	+	+	NT	NT	NT	NT	(12)
115A > G/K39E	United Kingdom	Male	-	-	-	-	-	-	-	(13)
119C > G/P40R	United Kingdom	Male	14	+	NT	NT	NT	-	-	(14)
121G > A/E41K	Turkey	Male	-	-	-	-	-	-	-	(13)
127A > G/K43E	Germany	-	-	-	-	-	-	-	-	(15)
146C > T/T49M	United Kingdom	Male	3	NT	NT	NT	NT	NT	NT	(16)
			2.6	NT	NT	NT	NT	NT	NT	(16)
			5	+	NT	NT	NT	NT	NT	(16)
145A > T/T49S	United States	Male	49	NT	+	+	+	+	+	(1)
		Female	25	NT	NT	NT	NT	NT	NT	(1)

(Continued)

TABLE 2 | (Continued)

Mutation on cDNA/protein	Ethnic origin	Gender	Age (years)	Mucocutaneous triad	Bone marrow failure	Anemia	Thrombocytopenia	Telomere shortening	Pulmonary fibrosis	References
160C > G/L54V	United States	Female	65	+	NT	+	NT	NT	NT	(17)
		Female	65	+	NT	NT	NT	NT	NT	(17)
		Male	45	+	+	NT	NT	+	NT	(17)
166_167invCT/L56S	Russian	Male	14	+	NT	+	+	NT	NT	(18)
189T > G/N63K	Canada	Male	24	+	+	+	+	+	+	(19)
194G > A/R65K	Japan	Male	46	+	+	NT	NT	+	+	(20)
194G > C/R65T	Germany	-	-	-	-	-	-	-	-	(13)
198A > G/T66A	United States	-	-	-	-	-	-	-	-	(13)
200C > T/T67I	-	-	-	+	+	NT	NT	NT	NT	(4)
204C > A/H68Q	-	-	-	+	+	NT	NT	NT	NT	(4)
203A > G/H68R	Spain	Male	36	+	NT	+	+	NT	NT	(21)
202C > T/H68Y	United States	Male	-	NT	+	NT	NT	NT	NT	(12)
209C > T/T70I	United States	-	-	-	-	-	-	-	-	(22)
214C > T/L72F	China	Male	7	+	+	+	+	NT	NT	(23)
227C > T/S76L	United States	-	-	NT	+	NT	NT	NT	NT	(12)
361A > G/S121G	United Kingdom	Male	1.5	NT	NT	NT	NT	NT	NT	(16)
247C > T/R158W	United States	-	-	-	-	-	-	-	-	(24)
838A > C/S280R	United States	-	-	-	-	-	-	-	-	(24)
911G > A/S304N	United States	Male	-	-	-	-	-	-	-	(25)
941A > G/K314R	-	-	-	+	+	NT	NT	NT	NT	(4)
942G > A/K314K	United States	Male	65	NT	+	NT	+	+	+	(26)
949C > G/L317V	United States	Male	-	-	-	-	-	-	-	(25)
949C > T/L317F	Germany	-	-	-	-	-	-	-	-	(27)
961C > A/L321I	China	Male	4.3	+	NT	+	NT	NT	NT	(28)
961C > G/L321V	Italy	Male	-	-	-	-	-	-	-	(13)
965G > A/R322Q	Germany	-	-	-	-	-	-	-	-	(27)
1049T > C/M350T	United Kingdom	-	-	-	-	-	-	-	-	(13)
1050G > A/M350I	Austria	-	-	-	-	-	-	-	-	(13)
1050G > C/M350I	Germany	Male	40	+	NT	+	+	NT	NT	(29)
1051A > G/T351A	-	Male	7	+	NT	+	NT	NT	NT	(30)
1054A > G/T352A	-	Male	31	+	NT	NT	NT	NT	NT	(3)
1058C > T/A353V	Brazil	Male	3	+	+	+	NT	NT	+	(31)
	India	Male	12	+	NT	NT	NT	NT	NT	(32)
1066T > C/S356P	Portugal	Male	15	+	NT	NT	+	NT	NT	(33)
			10	+	+	NT	+	NT	NT	(33)
1069A > G/T357A	Japan	Male	10	+	+	+	+	NT	NT	(9)
1072T > G/C358G	Germany	Male	0.6	+	NT	NT	+	NT	NT	(34)
1075G > A/D359N	-	-	-	+	NT	NT	NT	NT	NT	(4)
1133G > A/R378Q	United States	-	-	NT	+	NT	NT	+	NT	(12)

(Continued)

TABLE 2 | (Continued)

Mutation on cDNA/protein	Ethnic origin	Gender	Age (years)	Mucocutaneous triad	Bone marrow failure	Anemia	Thrombocytopenia	Telomere shortening	Pulmonary fibrosis	References
1151C > T/P384L	United States	-	-	-	-	-	-	-	-	(24)
1156G > A/A386T	-	-	-	+	NT	NT	NT	NT	NT	(4)
1186G > A/K390Q	United States	-	-	-	-	-	-	-	-	(22)
1177A > T/I393F	India	Male	21	+	NT	+	NT	+	NT	(35)
1193T > C/L398P	Japan	Male	-	-	-	-	-	-	-	(36)
1204G > A/G402R	India	-	-	-	-	-	-	-	-	(13)
1205G > A/G402E	United States	Male	-	-	-	-	-	-	-	(37)
1213A > G/T405A	United States	Male	65	+	NT	NT	NT	NT	+	(38)
			69	NT	NT	NT	NT	NT	+	(38)
1223C > T/T408I	-	-	-	+	NT	NT	NT	NT	NT	(4)
1226C > G/P409R	United States	Male	46	+	NT	NT	NT	NT	NT	(1)
		Male	40	+	NT	NT	NT	NT	NT	(1)
		Female	16	+	NT	+	NT	NT	NT	(1)
		Female	16	+	NT	NT	NT	NT	NT	(1)
		Female	8	+	NT	NT	NT	NT	NT	(1)
	China	Male	24	+	NT	NT	NT	NT	NT	(7)
			20	+	NT	NT	+	NT	NT	(7)
1226C > TP409L	China	Male	20	+	NT	NT	NT	NT	NT	(39)
IVS1 ds592C_G	Belgium	Male	30	+	NT	NT	+	NT	+	(24)
			10	NT	NT	NT	+	NT	NT	(24)
			56	+	NT	NT	NT	NT	+	(24)
IVS2 as-15 T-C	China	Male	8	+	+	NT	NT	NT	NT	(40)
IVS2 as-5 C-G	Spain	-	-	-	-	-	-	-	-	(13)
IVS12 ds + 1 G-A/A386fsX1	Italy	Male	0.3	NT	NT	NT	NT	NT	NT	(41)
IVS14 as-2 A-G	-	-	-	+	+	NT	NT	NT	NT	(4)
-141C > G	Spanish	Male	13	NT	NT	NT	NT	+	NT	(24, 42)
-141C > G	United States	-	2	NT	NT	NT	NT	NT	+	(22)
103_105delGAA/E35del	United States	Female	10	+	NT	NT	NT	NT	NT	(17)
106_108delCTT/L36del	Caucasian	-	-	-	-	-	-	-	-	(37)
1168_1170delAAG/K390del	Spanish	Male	32	+	NT	NT	NT	NT	NT	(21)
1495_1497delAAG/K499del	United Kingdom	-	-	-	-	-	-	-	-	(43)
112_116delATCAAinsTCAAC/ T38SfsX31	Canada	Male	-	-	-	-	-	-	-	(44)
14_215CT > TA/L72Y	United Kingdom	Male	-	-	-	-	-	-	-	(37)
1258,1259AG > TA/S420Y	-	-	-	+	+	NT	NT	NT	NT	(4)
Duplication of ∼14 kb (described at genomic DNA level)	United States	Male	-	NT	NT	NT	NT	NT	NT	(45)
1493A > G/S485G	Germany	-	-	NT	NT	NT	NT	NT	NT	(46)

+, presents positive expression; NT, presents negative expression; -, presents not in detail.

TABLE 3 | The Asian and outside Asian variations and the main clinical phenotypes.

Mutation percent	Age (year)	Male	Mucocutaneous triad	Bone marrow failure	Anemia	Thrombocytopenia	Telomere shortening	Pulmonary fibrosis
Asian (19.07%)	4.3–46	100%	78.57%	35.7%	35.7%	28.57%	14.28%	7.17%
Outside Asian (81.11%)	0.3–69	84.09%	70.59%	37.5%	19.6%	27.45%	13.72%	23.53%

Peripheral blood samples were collected from this family, which includes three individuals (III-2, III-3, and IV-2); a recurrent *DKC1* hemizygous mutation (c.1156G > A) in exon 12 was confirmed in the proband (IV-2) by using Sanger sequencing (**Figure 1H**). Furthermore, a heterozygous mutation (c.1156G > A) in his mother (III-2) and a wild-type sequence in his father (III-3) were obtained on the same site of *DKC1* (**Figure 1H**). The original contributions presented in the study are publicly available. These data can be found here: ClinVar Wizard Submission ID: SUB11097305; Accession: SCV002097631.

Pathogenicity Prediction of Variant

The effect of the missense variant was computationally analyzed by four prediction programs: Mutation Taster, SIFT, PolyPhen-2, and PROVEAN. The outcomes are summarized in **Table 1**.

Molecular Analysis

Evolutionary conservation of amino acid residue showed that the impaired amino acid residues Ala386 were highly conserved in different species (**Figure 1J**). The eukaryotic DKC1 protein presents three well-characterized domains: DKCLD (amino acids 49–106), TruB_N (amino acids 107–247), and PUA (amino acids 297–371) besides nuclear and nucleolar localization signals (amino acids 11–20; 446–458) (6, 7). Bioinformatic and biochemical assessment on the effect of the altered amino acid on the functions of DKC1 shows that the missense mutation was concentrated near the PUA domain (**Figures 1I,K**), which is crucial for the RNA binding of telomerase (7). *DKC1* mutations concentrated in or near the PUA domain decrease the affinity for RNA binding (6). In conclusion, the recurrent *DKC1* pathogenic variant was identified by WES and Sanger sequencing in a Chinese DC family.

DISCUSSION

Here, we report a case of DC in a Chinese pedigree with a mutation c.1156G > A (p.Ala386Thr) in *DKC1*. The affected amino acids are located near the PUA kinase domain from the linear structure, indicating that the mutation might result in defect on the affinity for RNA binding (6). Evolutionary conservation analysis of amino acid residue showed that the amino acid residue Ala386 is highly conserved among DKC1 protein from different species, indicating that the mutation is likely pathological.

We have reviewed articles describing cases of DC using the Human Gene Mutation Database and NCBI—PubMed, with the search term "dyskeratosis congenita" from January 1998 to November 2021 (**Table 2**). Among the studies, we identified 74 variations in *DKC1* with 85 individuals for analysis. Most publications were case reports so that the clinical data were not comprehensive. There were 87.5% male patients, 12.5% female patients, and 29 patients without gender description in the patients, indicating that males were the dominant patients of DC.

We find that the clinical symptoms of these DC patients are varied, but skin pigmentation, nail dystrophy, mucosal leukoplakia, and bone marrow failure are the most classic symptoms in patients. In this analysis, the incidence of skin pigmentation, nail dystrophy, and mucosal leucoplakia are nearly 86.58, 78.048, and 64.63%, respectively. Moreover, apart from the mucocutaneous triad, anemia can be another routine clinical sign of DC. Missense mutation is the most common mutation type among all the variations and shows higher incidence of the typical clinical symptoms of DC, but only one patient with c.194G > C (p.R65K) had mild symptoms such as pulmonary symptoms (20). The patient with mutation of small indel (c.166_167invCT) only suffer from thrombocytopenia and anemia (18). The patients with mutations of regulatory (c.-142C > G or c.-141C > G) only suffer from short telomere or pulmonary fibrosis (22, 24).

We also found 13 variants of *DKC1* in Asia with 100% male (7, 9, 13, 20, 23, 28, 32, 35, 36, 40), 52 variants in non-Asia with 84.8% male (1, 8, 10–14, 16–19, 21, 24–26, 29, 31, 33, 34, 37, 38, 41, 42, 44, 45), and 10 variants with unknown nationality (3, 4, 30). Asians develop DC at a younger age than non-Asians, between 4.3 and 46 years old (1, 7–12, 14, 16–24, 26, 28, 29, 31–35, 38–42). The incidence of the mucocutaneous triad (skin pigmentation, nail dystrophy, and mucosal leukoplakia), bone marrow failure, thrombocytopenia, and telomere shortening in Asia are similar to that of non-Asia (**Table 3**; 1, 3, 4, 7–12, 14, 16–24, 26, 28–35, 38–42, 45, 46). However, the DC-Asians are more likely to develop anemia instead of pulmonary fibrosis than non-Asians apart from the mucocutaneous triad (**Table 3**; 1, 3, 4, 7–12, 14, 16–24, 26, 28–35, 38–42, 45, 46). Unfortunately, the patient involved in our study did not present with anemia; the reason could be due to the lower incidence (35.7%) of anemia in Asian DC population.

The *DKC1* variation of c.1156G > A (p.Ala386Thr) was also reported from a DCR216-family in 2006 (4). The patient presents both the features of classic DC and Hoyeraal Hreidarsson (HH) syndrome, including intrauterine growth retardation, developmental delay, microcephaly, cerebellar hypoplasia, immunodeficiency, or bone marrow failure (4). However, the patient involved in our study only presents with benign phenotype of the mucocutaneous triad without any other abnormality, which provides more information on the mutation phenotype spectrum of DC. A similar case occurs for the *DKC1* c.1226C > G (p.P409R) mutation. This mutation was first identified in the patient with the features of liver cirrhosis, frequent caries, low platelets, gray hair, and tongue cancer in 2013 (1). However, the patient with the same mutation

was reported from China in 2020 presenting fewer symptoms of reticulate interspersed pigmentation with hypopigmented macules on the neck, fingernail ridging and longitudinal splitting, and mucosal leukoplakia on the tongue (7). Those results demonstrate that there is no specific relationship between the genotype and phenotype.

Our findings indicate *DKC1* missense mutation c.1156G > A leads to a benign phenotype, which expands the disease variation database, the understanding of genotype–phenotype correlations, and facilitates the clinical diagnosis of DC in China. However, the mechanism of *DKC1* mutation resulting in DC should be investigated further.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Clinvar [accession: SCV002097631].

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Shanxi University (SXULL2021080). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LW wrote the manuscript and performed the practical work. JL collected patients' data. PL and QX analyzed the patients' data. PL designed the study. Y-AZ, PL, and CW conceived the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Genetic Diagnosis of Rubinstein–Taybi Syndrome With Multiplex Ligation-Dependent Probe Amplification (MLPA) and Whole-Exome Sequencing (WES): Case Series With a Novel *CREBBP* Variant

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Rubinstein–Taybi Syndrome (RSTS) is a rare congenital disease with distinctive facial features, broadening of the thumbs and halluces, and developmental delay. RSTS is caused by *de novo* genetic alterations in *CREBBP* and the homologous *EP300* genes. In this study, we established a genetic diagnostic protocol by integrating multiplex ligation-dependent probe amplification (MLPA) and whole-exome sequencing (WES). Five patients clinically diagnosed with RSTS were enrolled for genetic testing. Germline DNA was extracted from the peripheral blood of the patients and their families. One patient (case 1) was identified as harboring a large heterozygous deletion in the 16p13.3 region, spanning the *CREBBP* gene. Three patients (Cases 2–4) harbored different *CREBBP* variants (c.2608C>T:p.Gln870Ter,c.4404_4405del: p.Thr1468fs,c.3649C>T:p.Gln1217Ter). No causative variants were identified for the fifth RSTS patient (case 5). Here, we propose a molecular diagnostic protocol that identified causative genetic alterations in 4/5 of the patients, yielding a molecular diagnostic rate of 80%. Given the rarity of RSTS, more research is needed to explore its pathogenesis and mechanism.

Keywords: rubinstein-taybi syndrome, multiplex ligation-dependent probe amplification, whole-exome sequencing, next-generation sequencing, genetic diagnosis, novel variant, CREBBP (Crebb binding protein)

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INTRODUCTION

Rubinstein–Taybi Syndrome (RSTS, Broad thumb–hallux syndrome, MIM 180849) is a rare congenital malformation syndrome that affects approximately 1 in 100,000–125,000 newborns. RSTS is characterized by the broadening of the thumbs and halluces, developmental delay, moderate to severe intellectual disability, proneness to keloids, and distinctive facial features such as a large beaked nose and a low-hanging columella (Roelfsema and Peters, 2007).

Genetic research on RSTS has mainly focused on mutations in the genes encoding CREB binding protein (*CREBBP*, MIM 600140) and its homolog, E1A-binding protein P300 (*EP300*, MIM 602700). *CREBBP* is located at 16p13.3, while *EP300* is located at 22q13.2. Both CBP and p300 are highly homologous transcriptional coactivators sharing conserved protein–protein interaction domains including the enzymatic histone acetyltransferase (HAT) domain. Acetylation disrupts the DNA–histone interaction by neutralizing the positively charged lysine residues in histones and enabling increased accessibility for transcription factors to activate gene expression (Kalkhoven,

TABLE 1 | Summary of causative genetic alterations found in this study.

Case	Gender	Age at diagnosis (months)	Phenotype Description at Diagnosis	Molecular Genetic Diagnosis	
				MPLA	WES
Case 1	Female	1	Short stature[a], microcephaly[b], cyanotic congenital heart disease, pulmonary hypertension, prominent nose, broad thumbs and/or halluces, keloids	16p13.3 (3,728,096-3,962,938)del	Low read coverage across the mutation span
Case 2	Male	36	Prominent nose, mild intellectual disability, mild mental retardation, broad thumbs and/or halluces, keloids	Normal[c]	**CREBBP*:exon14:c.2608C>T: p.Gln870Ter (CADD:21.4, DANN: 0.9986)
Case 3	Female	27	Short stature[a], microcephaly[b], broad thumbs and/or halluces	Normal[c]	*CREBBP*:exon27:c.4404_4405del: p.Thr1468fs (CADD:NA, DANN:NA)
Case 4	Male	8	Short stature[a], ventricular septal defect, patent ductus arteriosus, hearing loss, bilateral hearing impairment, cryptorchidism, broad thumbs and/or halluces	Normal[c]	*CREBBP*:exon19:c.3649C>T: p.Gln1217Ter (CADD:44, DANN: 0.9987)
Case 5	Male	32	Short stature[a], microcephaly[b], congenital glaucoma, broad thumbs and/or halluces	Normal[c]	Normal[d]

[a]≤3rd percentile for height.
[b]≤3rd percentile for occipital frontal circumference.
[c]No large intragenic deletions detected in CREBBP or EP300.
[d]No pathogenic SNP or indels detected.
*novel variant.

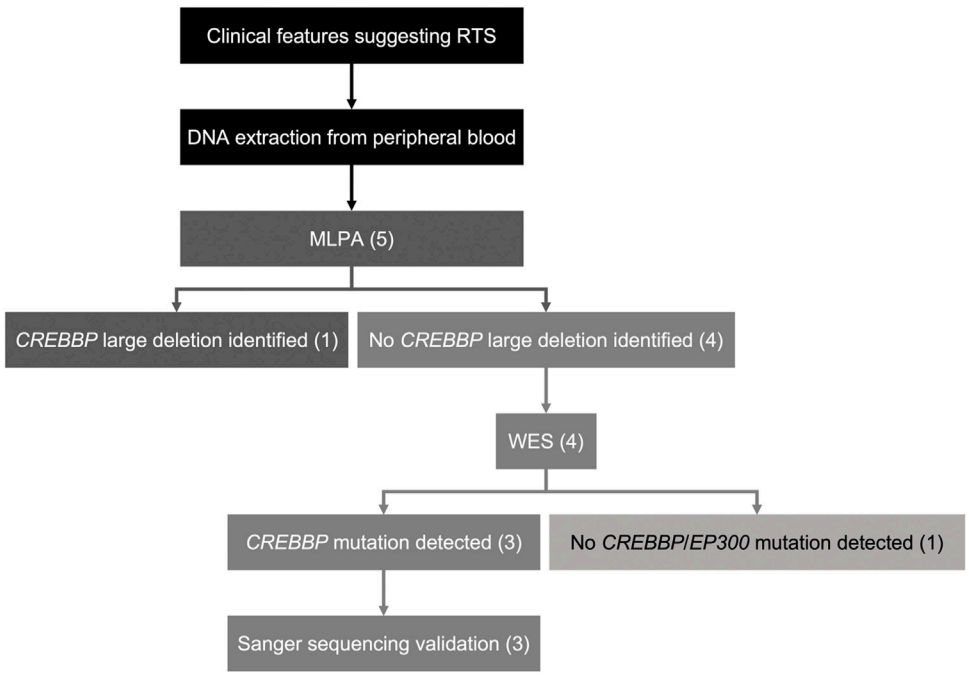

FIGURE 1 | Schematic of the diagnostic workflow in this study. Five patients clinically diagnosed with RSTS were enrolled. MLPA was done on DNA samples of all the RSTS patients. 1/5 patient (20%) was found to harbor a large *de novo* deletion spanning *CREBBP*. WES was performed on DNA samples of the other four patients showing negative MLPA results. 3/5 patients (60%) were found to have novel *CREBBP* mutations with high pathogenicity scores. 1/5 patient (20%) showed negative results for MLPA and WES.

2004; Oliveira et al., 2006). Among patients with RSTS, roughly 50–60% harbor pathogenic variants in *CREBBP* (RSTS type I), while only 3–8% of the patients harbor mutations in *EP300* (RSTS type II) (López et al., 2018). The vast majority (about 99%) of RSTS cases occur sporadically from *de novo* heterozygous *CREBBP* mutations, although vertical transmission has also been documented in a handful of cases (Bartsch et al., 2010). The spectrum of reported genetic alterations regarding RSTS includes point mutations (i.e., frameshift, nonsense, missense, and splice-site mutations), intragenic or large deletions, translocations and inversions (Negri et al., 2019).

Whole-exome sequencing (WES), which utilizes the "shotgun-based" approach of next-generation sequencing, is known for its efficiency and effectiveness in detecting single-nucleotide polymorphisms (SNPs) and small insertions/deletions (indels). Structural variants and large deletions, on the other hand, remain challenging for WES due to its reliance on the short-read approach (Chong et al., 2015; Chiu et al., 2021). Multiplex ligation-dependent probe amplification (MLPA), on the other hand, although unable to recognize SNPs and indels, shows strengths in detecting genomic variations, such as copy number variations (CNVs) and large-spanning deletions by comparing the differences in PCR-amplified fluorescently labeled primers binding to the probes. Although molecular diagnosis can reveal the genetic alterations in most RSTS cases, a sizeable subset of patients (30%) remain undiagnosed when this method is used (Tajir et al., 2013). In this study, we leveraged the distinct advantages of WES and MLPA by combining the two methods in our genetic diagnostic protocol for RSTS. Here, we report three patients with pathogenic *CREBBP* mutations and one with a large deletion spanning *CREBBP* in a five-patient cohort of clinically diagnosed RSTS individuals.

MATERIALS AND METHODS

Patients

The inclusion criteria included concurrent presentation of common RSTS clinical characteristics (distinctive facial features, broadening of the thumbs and halluces, short stature) unexplained by other systemic syndromes (**Figure 2**). Five patients (3 males and 2 females) fulfilled the criteria and were enrolled for further molecular genetic diagnosis. Detailed clinical data of the cases were documented. 3/5 (60%) of the cases also had microcephaly, another common presentation of RSTS. Additional clinical findings relevant to RSTS, including cyanotic heart disease, pulmonary hypertension, intellectual disability, and other neurological impairments, were found in some cases. The clinical manifestations of each case enrolled in this study are summarized in **Table 1**. Ethics approval was granted by the Ethics Committee of National Cheng Kung University Hospital (A-BR-104-052). Informed consent was obtained from the patients' parents. Peripheral blood specimens were collected from the patients. DNA was extracted using the Qiagen FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed on DNA from each patients via the Affymetrix 750K array platform (Thermo Fisher Scientific, Waltham, United States) using a human microdeletion syndrome probe set (SALSA P096; MRC-Holland, Amsterdam, Netherlands). On the whole, microdeletions in the 4p, 5p15, 8p, 8q24, 11p13-14, 16p13.3, and 21q22.2 regions were detected with the probe set and were thus analyzed. MLPA reveals the relative quantification of the number of copies of targeted DNA sequences. Data were analyzed using GeneMarker software (SoftGenetics, State College, PA, United States). For the purpose of this study, heterozygous or homozygous deletions in the 16p13.3 region, which spans the *CREBBP* gene, were considered abnormal positives.

In one patient showing an abnormal large deletion spanning the *CREBBP* gene (case 1), MLPA was also done on the germline DNA from the parents of the proband to examine whether the mutation occurs *de novo*. As for the patients showing negative results for *CREBBP* large deletions, WES was performed on the DNA specimen to detect pathogenic SNPs and indels in the genes *CREBBP* and *EP300*.

Whole-Exome Sequencing and Sanger Sequencing

Germline DNA extracted from the probands was used for paired-end library preparation using the SureSelect All Exon 50 Mb Version 4.0 kit (Agilent, Santa Clara, CA, United States) according to the manufacturer's recommendations. Sequencing was carried out by massively parallel sequencing with 100-bp paired-end reads using the HiSeq-2000 platform (Illumina, CA, United States). The Novoalign software package (Novocraft Technologies Sdn Bhd) was used to align reads generated to the reference human genome. Reads mapping to multiple locations on the reference human genome were excluded from downstream analysis. The BedTools package was used to calculate the depth and breadth of sequence coverage (Quinlan and Hall, 2010). Single-nucleotide substitutions and small indels were detected with the SamTools package (Li et al., 2009). Sequence variants were annotated with the Annovar tool (Wang et al., 2010). To assess the pathogenicity of the candidate variants, an in-house variant-filtering pipeline was used. Nonsense variants or indels resulting in frame shifts in *CREBBP* or *EP300* with minor allele frequencies (MAF) of less than 0.5% in the 1,000 Genomes Project (Siva, 2008) and Exome Aggregation Consortium (ExAC) were included. The damage prediction criteria for filtering the candidate variants included a Combined Annotation Dependent Depletion (CADD) score of above 15, a Deleterious Annotation of Genetic variants using

Neural Networks (DANN) score of above 0.95, and a Polymorphism Phenotyping v2 (PolyPhen-2) score of above 0.95. Variants with MAF exceeding 0.5% or with damage prediction scores not fulfilling our criteria were excluded as non-pathogenic.

BAM files of WES were visualized via Integrative Genomics Viewer (IGV) (Robinson et al., 2011). Confirmative polymerase chain reaction (PCR) and Sanger sequencing tests were performed on the DNA from the probands and their parents to validate the filtered variants detected by WES and for segregation analysis. Primers were designed using the Ensembl database (Howe et al., 2021) and Primer3 (Untergasser et al., 2012) online software. The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA806385.

RESULTS

Genetic alterations relevant to RSTS identified in this study by both MLPA and WES are summarized in **Figure 1**. The characteristic clinical manifestations of RSTS for all five cases are summarized in **Figure 2**. As a whole, among the patients enrolled, MLPA showed a large 16p13.3 DNA deletion spanning the *CREBBP* gene in 1/5 (20%) patient, while causative *CREBBP* variants were detected in 3/5 (60%) patients by WES and were confirmed by Sanger sequencing. Through MLPA and WES, causative variants were identified in 4/5 (80%) of the RSTS patients and are summarized in **Table 1**.

Case 1. was a 4-year-old female showing typical clinical features, including a high-arched palate, broadened thumbs and halluces, and a tendency to keloid development (**Figure 2A**). Congenital

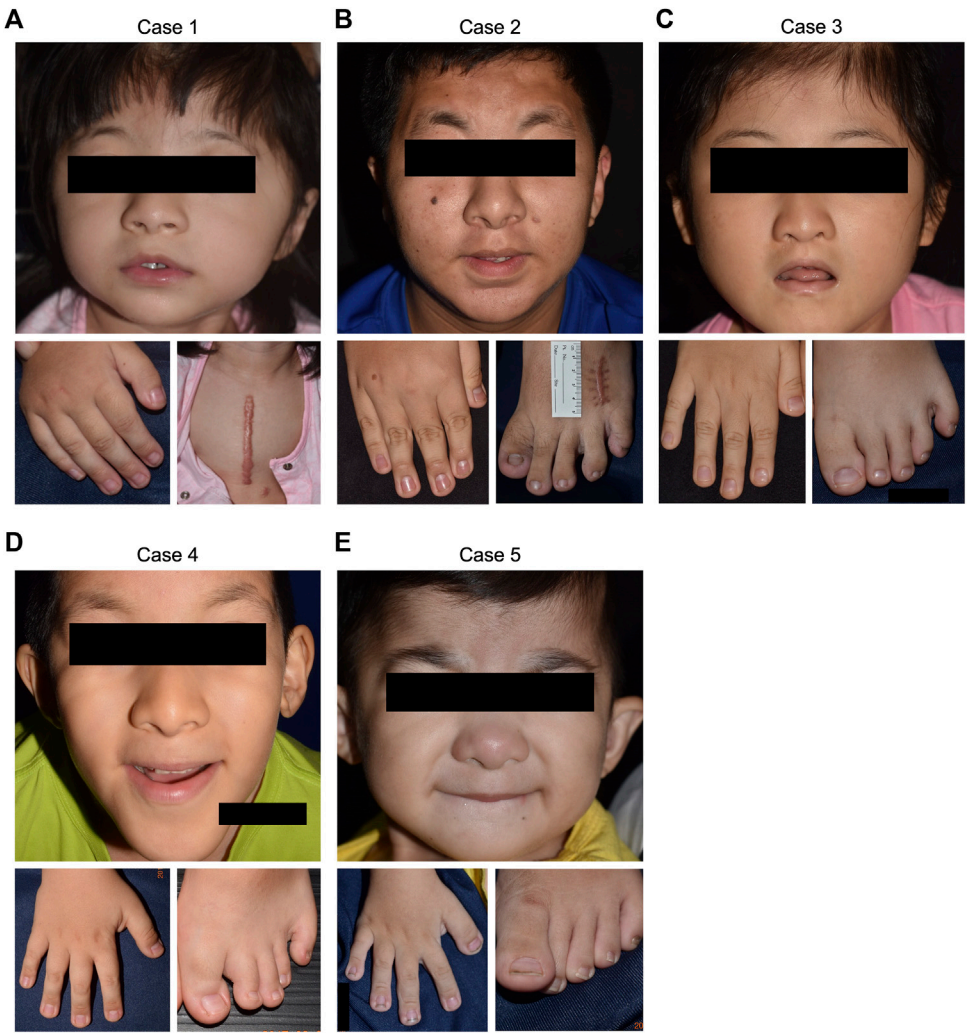

FIGURE 2 | Clinical manifestations of the five RSTS cases. Positive findings of common RSTS characteristics, including distinctive facial features, and broadened thumbs and halluces, were noted in all of the enrolled cases. **(A)** case 1. **(B)** case 2. **(C)** case 3. **(D)** case 4. **(E)** case 5.

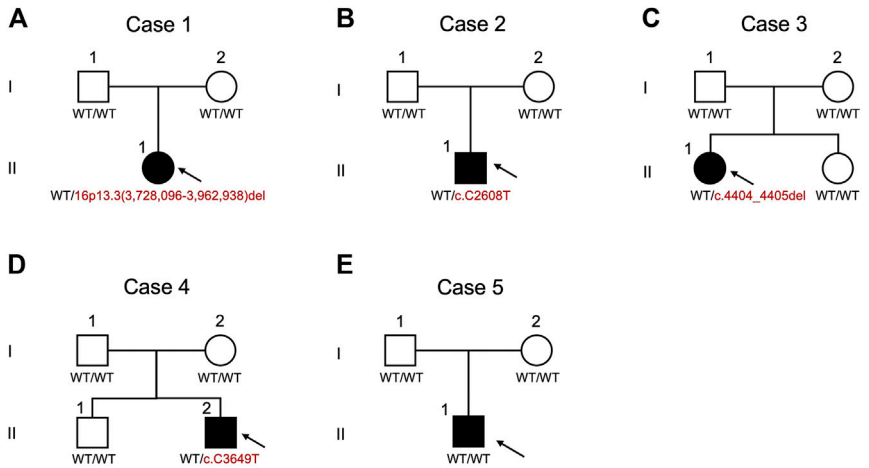

FIGURE 3 | Pedigrees and schematic summary of genetic alteration discoveries in this study. **(A)** case 1. **(B)** case 2. **(C)** case 3. **(D)** case 4. **(E)** case 5. WT, wild type.

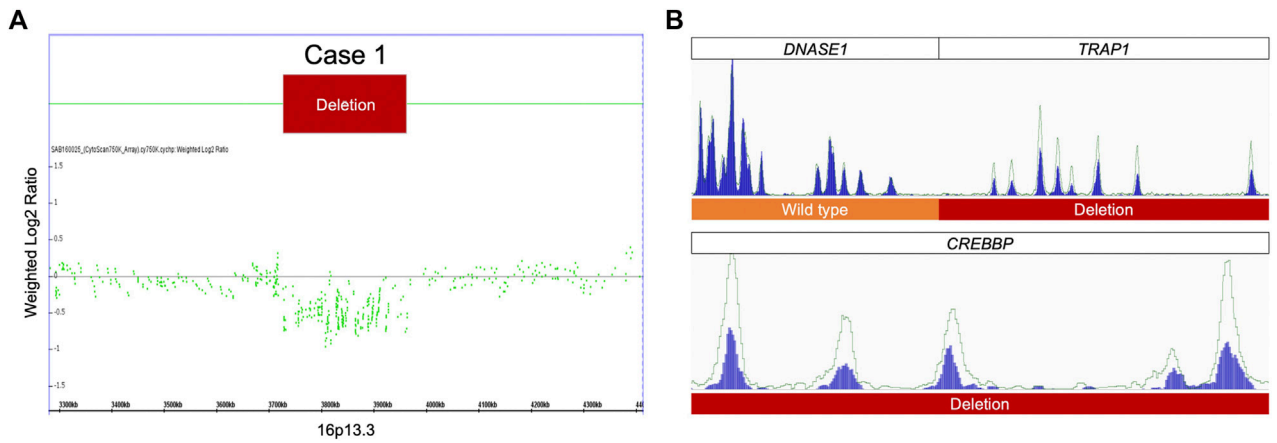

FIGURE 4 | MLPA and WES results for case 1. **(A)** MLPA of the proband showing heterozygous deletion at 16p13.3. **(B)** A visualization of the WES results for case 1 show fewer reads in the proband than in the healthy control, confirming the heterozygous deletion detected by MLPA.

cyanotic heart disease were also found, including hypoplastic aortic isthmus with mild coarctation of the aorta, perimembranous ventricular septal defect, large right patent ductus arteriosus (PDA), and anomalous left pulmonary artery arising from left side PDA. MLPA of DNA from the proband and the parents revealed a *de novo* heterozygous 16p13.3:3,728,096-3,962,938 deletion that spanned the loci of *TRAP1* and *CREBBP* (**Figures 3A**, **4A**). To validate the results, we also performed WES on the proband's DNA, and a visualization of the WES results in IGV revealed low read coverage across the mutation span (**Figure 4B**). Surgical repair of the patient's congenital cyanotic heart disease was performed to alleviate the cardiac symptoms, and diflucortolone ointment was prescribed for the treatment of the developed keloid. The developmental delay was managed by follow-up treatments in the outpatient department and diet education.

Case 2. was a 15-year-old male with typical clinical features suggesting RSTS (**Figure 2B**). MLPA of case 2 showed no detectable deletions in *CREBBP*. WES of the proband's germline DNA revealed a novel *CREBBP* heterozygous nonsense mutation (NM_004380:c.C2608T: p.Gln870Ter) with a significant pathogenicity score (CADD:21.4, DANN:0.9986). Further segregation analysis by Sanger sequencing confirmed the variant detected through WES and revealed both parents to harbor homozygous wild-type alleles at the mutation site, confirming that the proband's mutation had occurred *de novo* (**Figures 3B**, **5A**). Since that the patient also presented with intellectual disability and mental retardation, the patient was referred to neurologists and psychiatrists at our hospital for diagnostic interviews and further psychological assessments.

Case 3. was a 9-year-old female with typical clinical features suggesting RSTS (**Figure 2C**). MLPA did not reveal any large

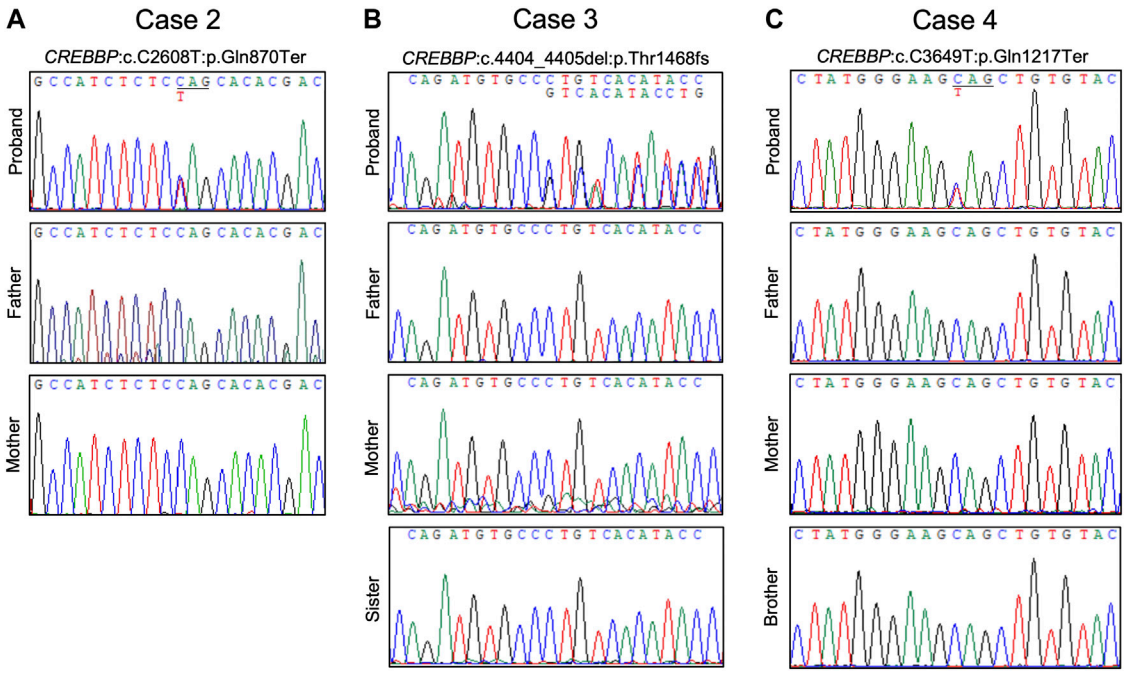

FIGURE 5 | Chromatograms of confirmative PCR-based Sanger sequencing and segregation analysis results. **(A)** A *de novo* nonsense *CREBBP* mutation in case 2. **(B)** A *de novo CREBBP* 2-bp deletion in case 3. **(C)** The *CREBBP* nonsense mutation was detected in both case 4 and the proband's mother.

deletions spanning *CREBBP*. WES showed a 2-bp deletion leading to a frame-shift in the *CREBBP* locus (NM_004380: c.4404_4405del:p.Gly1469AlafsTer9), which had been reported in another RSTS patient (Murata, 2001). Segregation analysis showed a wild-type genotype for both parents at the mutation site (**Figures 3C**, **5B**), indicating that the mutation had occurred *de novo*.

Case 4. a 10-year-old male, also showed features of RSTS (**Figure 2D**). No large deletions were found in the patient's DNA by MLPA, but WES revealed a *CREBBP* nonsense mutation (NM_004380:c.C3649T:p.Gln1217Ter) with a significant pathogenicity score (CADD:44, DANN:0.9987) that had been reported in a previous RSTS patient (Saettini et al., 2020). Further segregation analysis showed that the mutation had occurred *de novo* (**Figure 5C**). The patient also showed microcephaly and white matter hyperintensities under magnetic resonance imaging (MRI) examination. Rehabilitation programs and psychological assessments were arranged on a regular time course for the neurological symptoms.

Case 5. was a 4-year-old boy also with typical features of RSTS (**Figure 2E**). MLPA showed no large deletions in *CREBBP*. Unexpectedly, WES also failed to identify pathogenic variants within the *CREBBP* and *EP300* loci (**Figure 3E**). Therefore, the causative genetic or genomic variant for case 5 remains unknown. Since the patient also manifested congenital glaucoma, trabeculectomy including peripheral iridectomy under the microscope was performed.

DISCUSSION

Of the five patients whose clinical presentation suggested RSTS, pathogenic variants in *CREBBP* were identified in four patients (80%) using an MLPA–WES genetic diagnostic workflow. However, the causative variant for 1/5 (20%) patient, remains uncertain. Among the sequencing methods used in this study, although WES efficiently detects small indels in protein-coding (exonic) DNA regions, the detection of copy number variations (CNVs), structural variants, and homologous regions of the genome remains challenging for WES. Given that pathogenic variants in RSTS comprise single-nucleotide or oligonucleotide variations as well as large deletions, genomic assays that can screen for large genomic variants (e.g., MLPA) are highly desirable. In this study, we integrated MLPA and WES in our molecular diagnostic protocol. PCR-based Sanger sequencing was also utilized to confirm the variants identified by WES. Indeed, a similar workflow has been applied to a Korean cohort (Choi et al., 2021), leading to the detection of causative variants in 80% of their patients, which is similar to our diagnostic yield.

Given that RSTS is a rare disorder arising from *de novo* mutations, the number of reported cases remain limited. In the Taiwanese population, one study reported the clinical and molecular data of 10 RSTS patients (Hou, 2005). In that study, chromosomal deletions over the 16p13 region (responsible for the *CREBBP* gene)

were detected by fluorescence *in situ* hybridization (FISH). Only 30% of the RSTS patients were detected as having interstitial submicroscopic deletions in the 16p13 region. The low diagnostic rate from the use of FISH alone might lie in its ability to resolve only large DNA deletions. Thus, pathogenic single-nucleotide variants would be neglected. Similarly, among the five RSTS patients collected in our study, only 1/5 (20%) patient showed a large DNA deletion spanning *CREBBP* (case 1). Our study highlights the potential gains from harnessing various genetic assays.

Mutation hotspots in the *CREBBP* and *EP300* loci have yet to be documented. In a previous report, mutations in the highly conserved HAT domain were regarded as leading to high pathogenicity (López et al., 2018). However, no mutations resided within the HAT domain among our patients. Previous studies have further reported that frameshift mutations are the most prevalent type of mutation in RSTS patients (López et al., 2018; Choi et al., 2021). Although limited in number, more nonsense mutations (cases 2, 4) than frameshift mutations (case 3) were observed in the current study.

In our diagnostic workflow, the causative genetic alteration in 1/5 (20%) RSTS patient was not identified. Given that the pathogenesis of RSTS is not yet fully understood, the possibility remains that mutations occurring outside the *CREBBP* and *EP300* loci may contribute to the RSTS phenotype. *CREBBP* and *EP300* are both epigenetics-associated factors that alter acetyltransferase activity. In a previous study, experiments on a developmental animal model for RSTS were conducted using mice deficient in CREB-binding protein (CBP), and these $CBP^{+/-}$ mice were found to exhibit an abnormal RSTS skeletal pattern (Tanaka et al., 1997). Furthermore, transgenic mice generated by Korzus et al. expressing reversible CBP that lacked HAT activity showed deficits in long-term memory, indicating that the HAT activity of CBP is essential for brain information processing and cognitive function (Korzus et al., 2004; Korzus, 2017). Although the genetic and epigenetic functions of *CREBBP* genes in rodents have been thoroughly investigated, the complete pathogenicity of RSTS remains to be explored.

CONCLUSION

Here, we summarize the molecular genetic diagnostic progress of five patients clinically diagnosed with RSTS. Diagnostic workflow combining MLPA and WES was carried out to identify causative genetic alterations in *CREBBP* and *EP300*. Four of the five patients were found to harbor causative mutations in *CREBBP*. As the exact pathogenesis of RSTS has yet to be completely elucidated and genetic alterations in roughly 20% of the patients have yet to be found, more research is needed on the roles of genetic and epigenetic alterations in RSTS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA806385.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Y-RL: experiment performing, case collection, project managing. Y-CL: original manuscript drafting, figures arrangement, manuscript revision. Y-HC: figures arrangement, project design. H-YH: bioinformatic processing and interpretation. Y-KH: experiment performing, project design. WA: manuscript revision. W-TT: project design, case collection. M-CT: project design, case and clinical data collection. Y-YC: project design, case and clinical data collection. CH: project design, case and clinical data collection, supervision.

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A Connexin Gene (*GJB3*) Mutation in a Chinese Family With Erythrokeratodermia Variabilis, Ichthyosis and Nonsyndromic Hearing Loss: Case Report and Mutations Update

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Background: Gap junctions formed by connexins are channels on cytoplasm functioning in ion recycling and homeostasis. Some members of connexin family including connexin 31 are significant components in human skin and cochlea. In clinic, mutations of connexin 31 have been revealed as the cause of a rare hereditary skin disease called erythrokeratodermia variabilis (EKV) and non-syndromic hearing loss (NSHL).

Objective: To determine the underlying genetic cause of EKV, ichthyosis and NSHL in three members of a Chinese pedigree and skin histologic characteristics of the EKV patient.

Methods: By performing whole exome sequencing (WES), Sanger sequencing and skin biopsy, we demonstrate a Chinese pedigree carrying a mutation of *GJB3* with three patients separately diagnosed with EKV, ichthyosis and NSHL.

Results: The proband, a 6-year-old Chinese girl, presented with demarcated annular red-brown plaques and hyperkeratotic scaly patches on her trunk and limbs. Her mother has ichthyosis with hyperkeratosis and geographic tongue while her younger brother had NSHL since birth. Mutation analysis revealed all of them carried a heterozygous missense mutation c.293G>A of *GJB3*. Skin biopsy showed many grain cells with dyskeratosis in the granular layer. Acanthosis, papillomatosis, and a mild superficial perivascular lymphocytic infiltrate were observed.

Conclusion: A mutation of *GJB3* associated with EKV, ichthyosis and NSHL is reported in this case. The daughter with EKV and the son with NSHL in this Chinese family inherited the mutation from their mother with ichthyosis. The variation of clinical features may involve with genetic, epigenetic and environmental factors.

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INTRODUCTION

Gap junctions are channels or hemichannels assembled by connexins mediating cell-cell or cell-environment communication. Ions and small molecules can pass through gap junctions and guide embryonic development or pathogenic processes. Connexin 31(Cx31) coded by *GJB3* (NM_024009.3), is one important member of connexin family. Highly expressed in upper differentiating epidermis (Di et al., 2001) and cochlear (Richard et al., 2000), mutations of *GJB3* can result in different diseases including erythrokeratodermia variabilis (EKV) and non-syndromic hearing loss (NSHL) ranging from profound congenital deafness to mild, progressive hearing loss in late childhood.

EKV is a rare autosomal dominant skin disease featuring transient red patches that change over hours and days, along with fixed localized or generalized keratotic plaques. The disease is mainly caused by mutations in the *GJB3*, *GJB4*, and *GJA1* genes, all coding members of connexin (Cx) family (Ishida-Yamamoto, 2016). Clinical presentation of EKV associated with *GJB3* mutation can be variable ranging from typical keratotic lesions (Ishida-Yamamoto, 2016) to grey-brown and verrucous hyperkeratosis up to 2 cm thick (Glatz et al., 2011).

NSHL is a type of hereditary hearing loss without defects in other body parts and can be categorized as autosomal dominant, autosomal recessive, X-linked or mitochondrial mutation-related disease. Mutation of some important genes have been identified as the cause of NSHL, including *GJB2*, *GJB3*, and *GJB6*, which are all members of connexin family and generally involve with autosomal recessive or dominant hearing loss (Meena and Ayub, 2017). The Cx31 mutations lead to both recessive and dominant NSHL and severity can vary widely, from late-onset moderate deafness affecting high frequencies to congenital deafness (Liu et al., 2000).

Herein, we report a Chinese family with a missense mutation of *GJB3* associated with different clinical symptoms covering EKV, ichthyosis and NSHL.

MATERIALS AND METHODS

Participants

The study cohort includes a pair of parents, their daughter and son in a Chinese pedigree. The proband was a 6-year-old girl with demarcated annular red-brown plaques of variable sizes and colors spreading over the extensor side of right lower limb (**Figure 1A**), the right side of her chest (**Figure 1B**) and lumbar region (**Figure 1C**). Hyperkeratotic scaly patches were present mainly on the right thigh and knee. These manifestations had presented 6 months earlier, initially appearing on the right lower leg. No involvement of hair or nails was observed and no hearing impairment was found. Her mother has ichthyosis with hyperkeratosis on her limbs and geographic tongue while the patient's younger brother was diagnosed with NSHL during hearing screening since birth. The father is an unaffected individual. The pedigree is shown in **Figure 2A**. The study was approved by institutional review board of Chinese Academy of Medical Sciences. Written informed consent was obtained from all participants, or from legal guardians in the case of minors.

Genomic DNA Extraction

The genomic DNA was extracted from peripheral blood samples of all four participants using the QIAamp DNA Blood Mini Kit

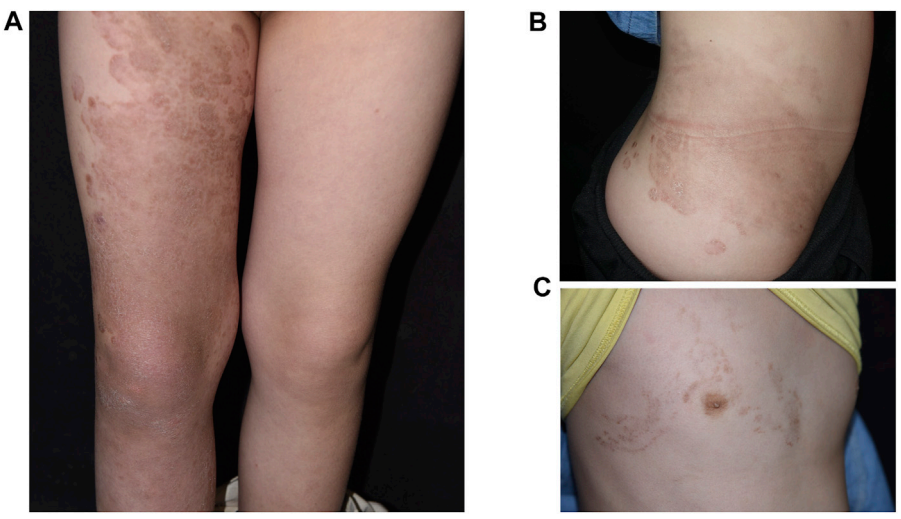

FIGURE 1 | Clinical images showing demarcated, annular, red-brown plaques over the extensor side of right lower limb **(A)**, lumbar region **(B)**, and right side of the patient's chest **(C)**.

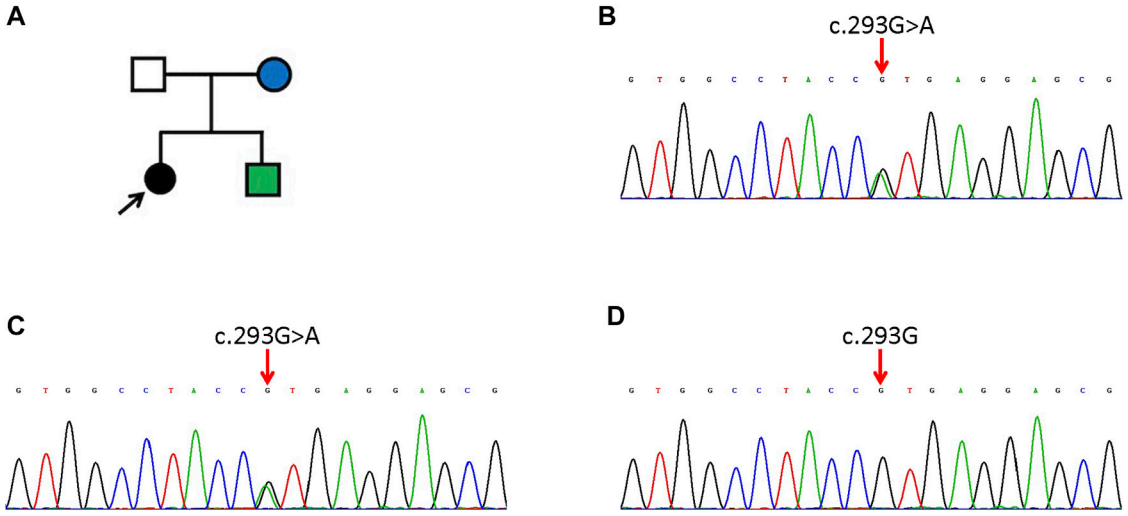

FIGURE 2 | The pedigree of the family **(A)** and WES results of the proband **(B)**, her mother **(C)** and her father **(D)**. **(A)** The arrow indicates the proband. Black means EKV. The green one has NSHL and the blue one has ichthyosis. **(B,C)** The proband and her mother have a heterogenous missense mutation of c. 293G>A in *GJB3*. **(D)** The proband's father has no mutation (c. 293G) in *GJB3*.

(QIAGEN, Hilden, Germany), according to the standard protocol and quantified by NanoDROP 2000 Spectrophotometer (Thermo Scientific; Waltham, MA, United States).

Whole Exome Sequencing, Sanger Sequencing and Mutation Analysis

Whole exome sequence (WES) was conducted in the proband and her mother in Novogene company (Beijing, China) by using Illumina Novaseq plat, and the average sequencing depth is 100X. Sanger sequencing was performed in the proband's brother for hot spot variants in NSHL-related genes (*GJB2*, *GJB3*, *SLC26A4*, and *MT-RNR1*). Raw sequence results were aligned to the human reference genome (GRCh37/hg38) annotated to get the candidate variants. Then the candidate variants were validated by Sanger sequencing to confirm the results of WES. And the primers were designed using primer3 Input for the suspected disease-causing genes.

The strategies of WES data filtering are as follows: 1) Variants with minor allele frequency (MAF)>0.01 were excluded, which were screened in normal population variant databases, including 1000G, ESP6500siv2 and gnomAD. 2) Variants in exons or alternative splicing regions were retained. 3) Synonymous mutations variants were removed, which were not located in highly conserved regions and would not affect splicing according to the same prediction software; and small non-frameshift insertion or deletion variants in the repeat regions were eliminated. 4) Variants that matched one of the following conditions were included: a) Variants were predicted to be pathogenic by at least one of the following programs including SIFT, Polyphen, MutationTaster, CADD. b) Variants were predicted to affect splicing by dbscSNV. 5) The remaining data were filtered by inheritance patterns and cutaneous phenotypes.

Skin Biopsy

A skin biopsy was taken from the proband's right thigh and viewed under the microscope for histopathological examination after hematoxylin-eosin staining.

RESULTS

WES Result

Genetic tests revealed a highly pathogenic heterozygous missense mutation of *GJB3* in the daughter and mother (**Figure 2B, C**). Sanger sequencing confirmed the existence of the same mutation in the younger brother. This mutation (NM_024009.3; c.293G>A; p.R98H) resulted in a change from a highly alkaline arginine residue at codon 98 to a slightly alkaline histidine residue, between the second transmembrane helix and intracellular domain of Cx31. The mutation was not detected in the father or healthy controls (**Figure 2D**). A diagnosis of EKV was made for the proband.

Histopathological Result

Histopathological examination showed many grain cells with dyskeratosis in the granular layer. Acanthosis, papillomatosis, and a mild superficial perivascular lymphocytic infiltrate were observed (**Figure 3**).

DISCUSSION AND LITERATURE REVIEW

Gap junctions are important for exchange of metabolites, ions and secondary messengers, especially in skin and cochlea. There

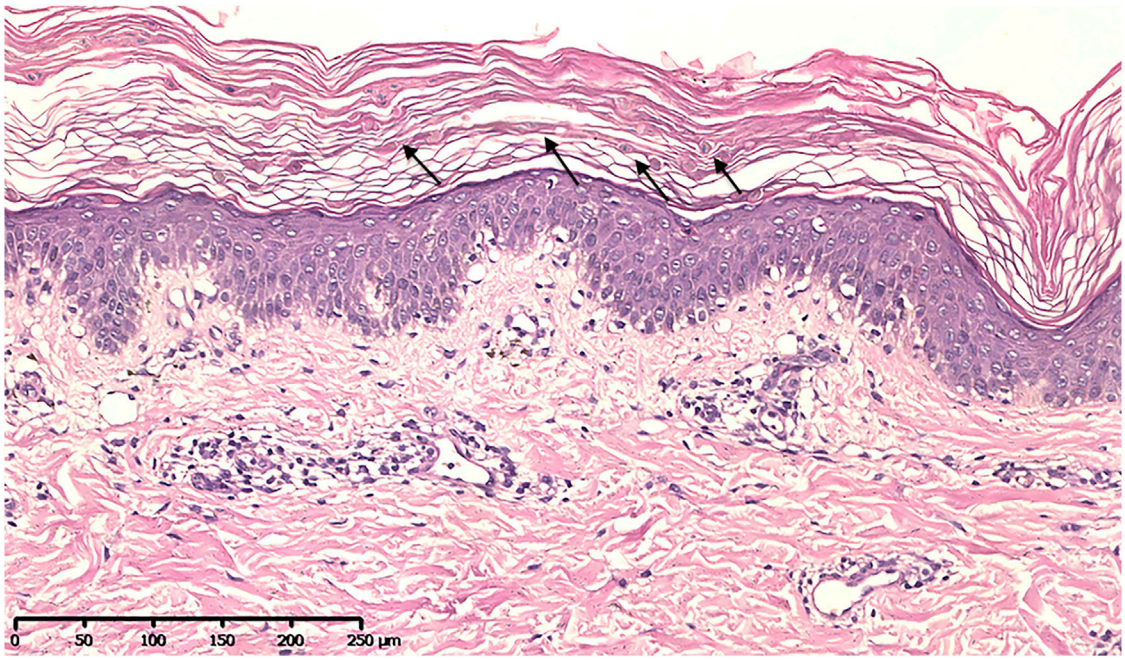

FIGURE 3 | Histopathological image showing many grain cells with dyskeratosis in the granular layer, acanthosis, papillomatosis, and a mild superficial perivascular lymphocytic infiltrate (H&E).

are more than eight kinds of connexins expressed in skin epidermis, which contribute to its differentiation (Richard et al., 2000). Exchange of ions and small molecules helps maintain unique electrochemical environments which is important for cochlea normal function (Cohen-Salmon et al., 2002). *GJB3* encodes Cx31 and is highly expressed in epidermis and cochlea, forming gap junctions (Scott and Kelsell, 2011), which is important in differentiation of keratinocytes and transfer of nerve pulses (Martinez et al., 2009). Gap junctions can be homomeric (consisting of one connexin type) or heteromeric (consisting of more than one connexin type) within the same cell (Kelly et al., 2015). Therefore, the connexons formed in epidermis and cochlea are intricate and delicate to guide the differentiation and maintain normal function.

EKV is a rare autosomal dominant skin disease associated with mutation of connexin genes, including *GJB3*, *GJB4*, and *GJA1* (Ishida-Yamamoto, 2016). Several cases of autosomal recessive mutations of *GJB3* causing EKV have also been reported (Gottfried et al., 2002; Terrinoni et al., 2004; Fuchs-Telem et al., 2011; Deng et al., 2019). Transient red patches and keratotic plaques are two prominent features of EKV. In this case, the patient with EKV and her mother both carry R98H mutation in Cx31 but the mother only shows the symptom of keratotic plaques and were diagnosed with ichthyosis. A severe case of EKV with grey-brown and verrucous hyperkeratosis up to 2 cm thick was reported caused by mutation of *GJB3* (Glatz et al., 2011). Therefore, clinical symptoms of EKV may be diverse. Other genetic, epigenetic, and environmental factors are probably the explanation for variation of symptoms (Renner et al., 2008). Deep investigation is still needed. For the younger brother, no manifestation of skin is probably due to late onset characteristic of EKV or other factors related to genetics and environment.

Many kinds of connexins have been identified in cochlea and among them, Cx26 and Cx30 are predominant components while other types are limited (Wingard and Zhao, 2015). The mutations of Cx26 account for at least half of NSHL cases, while mutation of Cx31 is also a cause (Rabionet et al., 2000). Clinical symptoms of hearing loss resulted by *GJB3* mutations range from congenital hearing loss since birth to late-onset hearing loss during childhood (Wingard and Zhao, 2015). Most NSHL cases related to Cx31 mutation are autosomal recessive while a few autosomal dominant cases were also reported (Liu et al., 2012; Oh et al., 2013). However, no case carrying the Cx31 mutation with both EKV and NSHL was reported but a pedigree with both Cx26 and Cx31 mutation presented hearing loss and palmoplantar keratoderma (Kelsell et al., 2000). Therefore, one possible explanation is that other connexin protein may make up the function loss of Cx31 in skin or cochlea while more studies are still required. In this family, three people harbor the same mutation but only the son has NSHL, which is probably due to partial penetrance. In earlier reports, female carriers with *GJB3* dominant mutations in two deafness families have subclinical deafness or normal hearing while male carriers have NSHL (Xia et al., 1998), which indicates partial penetrance involving sex may be the reason of different symptoms of carriers.

How the mutation in Cx31 affects cell function is believed to be related to where the mutation site lies (Sugiura et al., 2015). The structure of Cx31 mainly contains four transmembrane domains (M1-4) linked by one intracellular loop (CL) and two

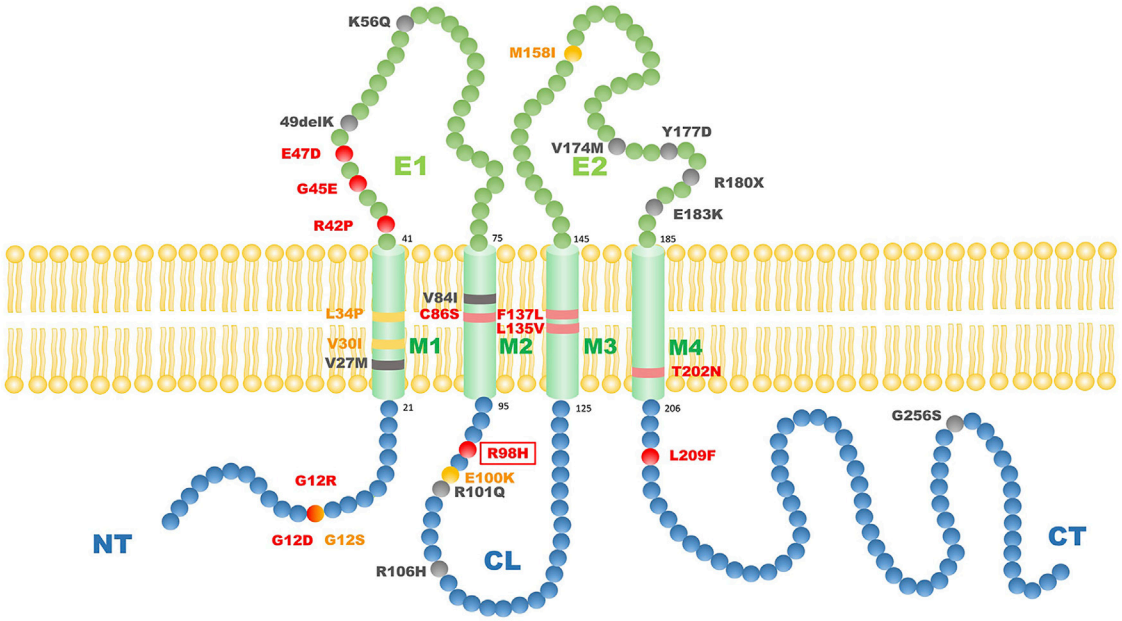

FIGURE 4 | The scheme of reported GJB3 mutation related to EKV and autosomal dominant GJB3 point mutations related to NSHL. Red balls indicate autosomal dominant mutations with EKV phenotypes; yellow balls indicate autosomal recessive mutations with EKV phenotypes; black balls indicate common autosomal dominant GKB3 mutation related to NSHL. The red frame indicates the mutation we report in this case. M1–M4 refers to transmembrane domains. E1 and E2 refer to extracellular domains. CL refers to cytoplasmic loop. NT refers to cytoplasmic amino terminus. CT refers to cytoplasmic carboxy terminus.

extracellular loops (E1 and E2) with conserved cysteine residues while N- and C-termini (NT and CT) are lying inside the cell (Kelly et al., 2015; **Figure 4**). The E1 domain plays an important role in formation of the gap junction channel (Richard et al., 2000). The M2 domain is known for function in voltage gating (Rabionet et al., 2000). The extracellular domain E2 probably functions in interaction between different types of connexin and formation of heterotypic connexons (Sugiura et al., 2015). Mutations of *GJB3* resulting in NSHL mainly locate in E2 domain, which may interfere the interaction between Cx31 and Cx26 and damage the function of heterotypic connexons on the membrane of cochlear cells (Sugiura et al., 2015). However, there is seemingly no relationship between the mutation locus and phenotypes of EKV patients. Most mutations related to EKV are autosomal dominant while a few recessive mutations were also found (shown in **Figure 4**). Interestingly, a compound heterozygous case with two recessive mutations in *GJB3* presented a mutation lying in E2, which was the first pathologic mutation involved with EKV identified in this domain (Deng et al., 2019). This patient had no symptoms of hearing loss probably because this mutation in E2 domain is recessive. By systematically searching the PubMed, Embase and Web of Science, we summarized all the *GJB3* mutations reported leading to EKV and phenotypes in each case (**Table 1**) and autosomal dominant *GJB3* mutation related to NSHL (**Figure 4**; Richard et al., 1998; Xia et al., 1998; Wilgoss et al., 1999; Lopez-Bigas et al., 2000; Richard et al., 2000; Gottfried et al., 2002; Alexandrino et al., 2004; Terrinoni et al., 2004; Common et al., 2005; Feldmeyer et al., 2005; Morley et al., 2005; Yang et al., 2007; Renner et al., 2008; Li et al., 2010; Fuchs-Telem et al., 2011; Glatz et al., 2011; Scott and Kelsell, 2011; Wang et al., 2011; Liu et al., 2012; Torres et al., 2012; Wang et al., 2012; Ikeya et al., 2013; Oh et al., 2013; Otaguchi et al., 2014; Beck et al., 2015; Sugiura et al., 2015; Takeichi et al., 2016; Deng et al., 2018; Imura et al., 2020). In this case, the substitution of R98H lying in the border of M2 and CL, which are important in voltage and pH gating (Richard et al., 2000), is the first mutation found involving both EKV and NSHL. The exact mechanism behind needs more investigation.

Although the phenotypes of different pathologic mutations may be the same, the mechanisms behind them are likely different. In many *vitro*-studies, overexpression of Cx31 with the same mutation in cells may obtain different conclusions about pathogenic mechanisms possibly due to different experimental conditions. But overall, the viability of cells with EKV-related mutated Cx31 was decreased, while that of cells with NSHL-related Cx31 mutation was not (He et al., 2005; Tattersall et al., 2009; Easton et al., 2019). The mechanisms behind can be concluded into mainly two ways: 1) The mutated Cx31 protein accumulates in endoplasmic reticulum (ER) due to misfold, leading to ER stress response and finally cell death (Di et al., 2002; Tattersall et al., 2009; Chi et al., 2012). 2) Mutated Cx31 can be transferred to the cell membrane but only form dysfunctional gap junctions which may even interfere the normal function of plasma membrane (Rouan et al., 2003). However, a kind of rare mutation of Cx31 with G45E exhibits a new way to damage cells by inducing necrosis (Easton et al., 2019). Overexpression of Cx31G45E-GFP within HeLa cells and HaCaT cells led to expansion of the ER due to accumulation of mutated protein and finally cell necrosis rather than ER stress responses (Easton et al., 2019). Also, the

TABLE 1 | Reported pathogenic mutations in *GJB3* related to EKV and phenotypes.

No	Hereditary mode	Erythematous plaques distribution	Palmoplantar keratoderma	Nucleotide change	Amino acid change	Protein domain	Mutation type	Novel or reference
1	AD	/	/	c.34G>C	P. G12R	NT	Missense	(Richard et al.,1998)
2	AD	/	/	c.35G>A	p. G12D	NT	Missense	(Richard et al.,1998)
3	AR	Face, limbs, buttocks, and chest	Y	c. 34G>A	p. G12S	NT	Missense	(Deng et al., 2018)
4	AR	Back	Y	c.88G>A	p. V30I	M1	Missense	(Fuchs-Telem et al, 2011)
5	AR	Abdomen, trunk, earlobes and extensor aspects of the upper and lower limbs	N	c. 101T>C	p. L34P	M1	Missense	(Gottfried et al., 2002)
6	AD	① / ② Buttocks, lower back, neck and four limbs	① Y ② Y	c.125G>C	p. R42P	E1	Missense	① (Richard et al., 2000) ② (Wilgoss et al., 1999)
7	AD	① Whole body ② The extensor sides of the extremities and the face	Y	c.134G>A	p. G45E	E1	Missense	① (Wang et al., 2012) ② (Renner et al., 2008)
8	AD	Body and limbs	Y	c. 141G>C	p. E47D	E1	Missense	(Wang et al., 2011)
9	AD	① /	/	c.256T>A	p. C86S	M2	Missense	(Richard et al.,1998)
10	AD	right side of chest, waist, and extensor side of right leg	N	c.293G>A	p. R98H	CL	Missense	Novel
11	AR	Whole body	Y	c. 829G>A	p. E100K	CL	Missense	(Terrinoni et al., 2004)
12	AD	Trunk and limbs	/	c. 403C>G	p. L135V	M3	Missense	(Scott et al., 2011)
13	AD	① Four extremities ② Back and four limbs ③ Face, upper trunk, arms, and buttocks	① Y ② / ③ Y	c. 409 T>C	p. F137L	M3	Missense	① (Richard et al.,2000) ② (Glatz et al., 2011) ③ (Imura et al., 2020)
14	AR	Face, limbs, buttocks, and chest	Y	c. 474G>A	p. M158I	E2	Missense	(Deng et al., 2018)
15	AD	Trunk and the extremities	Y	c. 605C>A	p. T202N	M4	Missense	(Sugiura et al., 2015)
16	AD	① Forehead, cheeks, extremities and buttocks ② Back and limbs ③ Extensor surfaces and buttocks; buttocks, trunk, face and extremities and extensor surfaces; limbs and buttocks; buttocks and right arm.	① Y ② Y ③ Y in 2 women and 1 man, N in 1 man	c. 625C>T	p. L209F	CT	Missense	① (Morley et al., 2005) ② (Otaguchi et al., 2014) ③ (Feldmeyer et al., 2005)

AD, autosomal dominant; AR, autosomal recessive; Y, yes; N, no.

interaction between mutated Cx31 and other wild-type connexins enables the accumulation of normal connexin in ER, which decreases the gap junctions on the cell membrane and interferes with normal function (Easton et al., 2019). The pathogenic mechanism of R98H in Cx31 needs experiments *in vitro* to identified.

In this case, we report a Chinese family with a mutation associated with EKV, ichthyosis and NSHL. The daughter with EKV and the son with NSHL in this Chinese family inherited the mutation from their mother with ichthyosis. The variation in clinical features may involve with genetic, epigenetic and environmental factors. One shortage of our research is that further experiments *in vitro* are needed to identify the possible pathogenic mechanism of this mutation. Our results indicate an important mutation site of Cx31 leading to EKV and NSHL with partial penetrance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: GenBank database, accession number OL471368.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Chinese Academy of Medical Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

TW participated in data receipt, clinical data collection and paper revision; YG participated in paper writing and literature review; QZ conducted gene mutation detection and verification and literature review. SZ and LY participated in case data collection; YPL and YHL were in charge of the research and revision of the paper.

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Three Variants Affecting Exon 1 of *Ectodysplasin A* Cause X-Linked Hypohidrotic Ectodermal Dysplasia: Clinical and Molecular Characteristics

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Background: Ectodysplasin A (EDA) variations are major pathogenic factors for hypohidrotic ectodermal dysplasia (HED), the most common form of ectodermal dysplasia (ED), characterized by hypotrichosis, hypohidrosis, hypodontia, and other oral features.

Methods: Molecular genetic defects in three HED families were detected by whole-exome sequencing and confirmed by Sanger sequencing or multiplex ligation-dependent probe amplification. The effect of splicing variant was further verified by EDA minigene *in vitro* analysis. De novo deletion was confirmed by chromosomal microarray analysis.

Results: Three variants (c.396 + 1 G > C, c.171-173 del GTT, and exon 1 deletion) were identified, all affecting exon 1 of the *EDA* gene. Variants c.396 + 1 G > C and c.171-173 del GTT were first identified. Minigene analysis of the splicing variant (c.396 + 1 G > C) displayed a prolonged EDA-A1 transcript containing extra 699 bp at the start of intron 1, representing a functional cryptic splice site formation *in vitro*. Combining the results of chromosomal microarray analysis and whole-exome sequencing, the deletion variant was over 87 kb. Three variants were predicted to affect protein function to differing degrees, and were responsible for X-linked HED with varying phenotype.

Conclusion: Investigating the clinical and molecular characteristics of these variations broadens our understanding of *EDA* gene variants, supporting clinical diagnosis, genetic counseling, and prenatal diagnosis of HED.

Keywords: hypohidrotic ectodermal dysplasia, EDA, whole-exome sequencing, splicing variant, cryptic splice site, HED, ectodysplasin A

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INTRODUCTION

Ectodermal dysplasia (ED) is a set of genetic diseases with two or more abnormally developed ectoderm-derived structures, including hair, teeth, nails, skin, and sweat glands (Pigno et al., 1996; Vaidya et al., 2013; Wright et al., 2019). Of the 200 different types of ED reported, ~30 causative genes have been identified at the molecular level (Itin and Fistarol, 2004). Genetic variations in ectodysplasin A (EDA) pathway genes, such as *EDA*, ectodysplasin A receptor (*EDAR*), and EDAR-associated adapter protein (*EDARADD*) are known to be associated with hypohidrotic

ED (HED); the prevalence of which is ~1/100,000 (Khabour et al., 2010; Deshmukh and Prashanth, 2012; Okita et al., 2019). *EDA* is a unique gene involved in the pathogenesis of X-linked hypohidrotic ectodermal dysplasia (XLHED; OMIM 305100), which accounts for 95% of patients with HED, and the remaining 5% are mainly due to autosomal dominant or recessive inheritance (Clarke et al., 1987; Clauss et al., 2008; Deshmukh and Prashanth, 2012; Wang et al., 2020). XLHED is characterized by a clinical triad of hypodontia (congenital absence of teeth), hypoplasia of sweat glands, and hypotrichosis (sparse hair) (Moura et al., 2019).

The *EDA* gene is located on chromosome Xq12-q13.1, which has nine exons, and consists of four domains: a transmembrane domain, furin recognition sequences, a collagen domain, and β-sheets A–H of the tumor necrosis factor (TNF) homology domain, encoded by exon 1, 3, 5–6 and 7–9, respectively (Schneider et al., 2001). It was identified as a membrane-bound signaling molecule of the TNF superfamily (Trzeciak and Koczorowski, 2016). Variations in the *EDA* gene lead to loss or dysfunction of EDA1, associated with the signaling of the epithelial–mesenchymal transition during embryogenesis, as well as the initiation and development of ectodermal derivatives (Korber et al., 2020). At least 100 variants of the *EDA* gene have been identified as pathogenic mutations in the NCBI ClinVar database (http://www.ncbi.nlm.nih.gov) based on published papers and submissions (Liu et al., 2012; Huang et al., 2015).

In the present study, we identified three variants of the *EDA* gene in three Chinese families with XLHED, and demonstrated that variants led to varying clinical phenotypes through different molecular mechanisms affected by exon 1 of the *EDA* gene.

MATERIALS AND METHODS

Case Information

Patients and their parents attended the Medical Genetics Centre, Gansu Provincial Maternity, and Childcare Hospital (Lanzhou, China), seeking genetic diagnosis of congenital symptoms (no sweating, pyrexia, and dysplasia of hair and teeth). Proband 1 was a 30-year-oldman; proband 2 was a 10-month-old boy; and proband 3 was a 20-day-old boy. Their parents were healthy and unrelated. All participants gave their signed informed consent for genetic studies before collecting blood samples or performing clinical evaluations.

DNA Extraction

Peripheral blood (3–5 ml) was collected from proband family members for DNA extraction using a Genomic DNA Extraction kit (Tiangen Biotech, Beijing, China), and extracted genomic DNA was subsequently used for targeted whole-exome sequencing (WES) and Sanger sequencing.

Whole-Exome Sequencing

Trio WES was carried out by MyGenostics Co., Ltd (Beijing, China). Briefly, qualified genomic DNA was fragmented randomly to an average size of 180 bp with a Bioruptor sonicator (Diagenode, Liege, NJ, United States). The fragmented DNA was then repaired and A-tails were ligated to the 3' end. Next, Illumina adapters (Illumina Inc., United States) were ligated to the fragments, and adapted DNA templates were amplified by PCR. DNA was then captured using a GenCap Custom Enrichment kit (MyGenosticsGenCap Enrichment Technologies, Beijing, China) and sequenced on an Illumina HiSeq 2500 platform (Illumina Inc.) as paired-end 90 bp reads. The mean sequencing depth was >100. N20 reads covered targeted bases by >95%.

Bioinformatics Analysis

Using the Trim Galore program, reads of low quality and adapters were filtered out after sequencing. SOAP aligner (SOAP v2.21) was used to align clean reads to the h19 human reference genome (UCSC Genome Browser hg19). Insertions, deletions, and single-nucleotide polymorphisms (SNPs) were identified by the Burrows–Wheeler alignment program (0.7.12-r1044) and tested using a GATK tool kit. The exome assistant program was used to annotate the locations of exonic, intronic, and intergenic regions, as well as protein-coding effects such as synonymous, missense, nonsense, and frameshift (Xu et al., 2015). Frequency and function were the main factors used to obtain candidate variants for further analysis. For the frequency filter, a 0.01 cut-off was applied according to allele frequency estimates from NCBI dbSNP (v152; http://www.ncbi.nlm.nih.gov/projects/SNP/), 1,000Gome (http://www.ncbi.nlm.nih.gov/Ftp/), and Exome Aggregation Consortium (http://gnomad.broadinstitute.org/) databases. Synonymous and missense variants, which were predicted to be benign or tolerated in Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, Mutation Taster, and GERP++, were removed by the functional filter. Splicing variants were evaluated by MaxEntScan and dbscSNV11.

Sanger Sequencing Validation

In order to identify the target variants associated with HED in family members, Sanger sequencing was performed. Direct PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, CA, United States) and analyzed on an ABI3500DX Genetic Analyzer (Applied Biosystems, Warrington, United Kingdom) using *EDA* primers (pedigree 1, Forward, 5′-actccactctgactcccaggac-3'; Reverse, 5′-ctggtcctgccctctaaattg-3'; pedigree 2, Forward, 5′-gcctcaagagagtgggtgtc-3'; Reverse, 5′-gtcctgggagtcagagtgga-3′).

Minigene Construct Generation, Transfection, and RT-PCR

In order to further investigate the pathogenic mechanism of the splicing variant at the 5' (donor) splice site (5'ss; c.396 + 1 G > C), a minigene containing exon 1, partial intron 1, and exon 2 of the *EDA* gene were designed using exon-trapping pEGFP-C1 plasmids. Specifically, exon 1, the first and last 1,000 bp of intron 1 (the intermediate sequence of intron 1 was deleted), and exon 2 encoding wild-type (WT) or mutant type (MT)

sequences were incorporated into the pEGFP-C1 vector within the 5′ *Xho*l and 3′ *Bam*HI restriction enzyme sites using specific primers. WT and MT expression vector construction was performed by Hitrobio Tech, Beijing, China. All recombinant plasmids were validated by direct sequencing. Human embryonic kidney 293T (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, MA, United States), penicillin (100 U/L), and streptomycin (100 mg/L) at 37°C with 5% CO_2. Transfection was performed using HEK 293T cells grown to 70–80% confluence in the serum-free medium by Lipofectamine 2000 Reagent (Thermo Fisher Scientific) following the manufacturer's instructions, and cells were collected at 48 h after transfection. For RT-PCR analysis, a MiniBEST Universal RNA Extraction kit (Takara, Dalian, China) was used to extract total RNA and a PrimerScript RT Reagent kit (Takara) was used for reverse transcription. PCR amplification of minigene transcripts was conducted using vector-specific forward primer pEGFP-C-5 F (5′-CATGGTCCTGCTGGAGTTCGTG-3′) and reverse primer pEGFP-C-3 R (5′-ATCTCAGTGGTA TTTGTGAGC-3′). PCR products were identified by 1% agarose gel electrophoresis, and Sanger sequencing was performed to analyze mutant patterns.

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed on the pedigree 3 using a SALSA MLPA Probemix P183 kit (MRC-Holland, Amsterdam, the Netherlands; http://www.mlpa.com). This kit included probes from all exons of *EDARR*, *EDAR*, *EDA*, and *WINT10A*, all associated with HED. The assay was performed according to the manufacturer's protocol. Briefly, 100 ng genomic DNA was denatured at 98°C for 5 min then allowed to hybridize to the MLPA probe overnight. Ligation reactions were then performed using Ligase-65 enzyme and PCR was carried out with SALSA PCR primers. PCR products were separated by capillary electrophoresis on an ABI 3500 Genetic Analyzer (Applied Biosystems). Original data were analyzed by Gene mapper 4.0 and Coffalyser.Net software, and copy number was calculated according to the MLPA kit instructions.

Chromosomal Microarray Analysis

CMA was performed with a CytoScan 750K array (Affymetrix, Santa Clara, CA, United States) according to the manufacturer's recommendations. Genomic DNA of 40 ng/μL was digested, ligated with adaptors, amplified, purified, labeled, and then hybridized into the array. After the completion of hybridization, the array was washed with buffer, stained, and scanned with a laser scanner. Data were analyzed with Chromosome Analysis Suite (ChAS) (version 4.2.0.80) software. The hg19/GRCh37 genome was used for genomic assembly. All identified variants were further analyzed with reference to public databases including Database of Genomic Variants (DGV, http://projects.tcag.ca/variation), the 1,000 Genome Project (http://browser.1000genomes.org), DECIPHER (http://decipher.sanger.ac.uk/), gnomAD (http://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and OMIM (https://www.omim.org).

RESULTS

Clinical Examination

Proband 1 presented a distinctive facial appearance with sparse hair and eyelashes, and no eyebrows, along with a flat nose, thick lips, and albino around the lips (**Figure 1A**). His uncle (III3) and uncle's grandpa (II7) were both HED patients presenting the same symptoms. Probands 2 and 3 showed a similar phenotype with sparse hair, dry skin, and frequent fever. As early as the fetus stage, alveolar bone dysplasia was identified in proband 3 by sonographic examination, indicating thinner upper alveolar bones and fewer tooth germs compared to normal fetus, as we reported previously (Li et al., 2021). After birth, his skin appeared abnormally dry and wrinkled (**Figure 3B**) and his upper alveolar bones were thinner compared with normal. Proband 2 also appeared to have thinner alveolar bone at 10 months. A preliminary diagnosis of HED was made by a dermatologist based on the clinical manifestations presented by all probands (Julia et al., 2018). The varying symptoms of the three patients are shown in **Table 1**.

Identification of *EDA* Gene Variants

A hemizygous splicing variant of the *EDA* gene (c.396 + 1 G > C) was identified in pedigree 1 (**Figure 1C**). The variant resulted in the destruction of the splicing donor. There is no information in the 1,000 Genomes, MyGenosticsInhouse, ESP6500, EXAC, or ExAC_EAS population databases about this variant. It was predicted to be deleterious by MaxEntScan following analysis of variants near the 5′ and 3′ splice sites. Additionally, dbscSNV11 predicted that the variant was deleterious. The hemizygous variant c.396 + 1 G > C of the *EDA* gene was verified by Sanger sequencing, and it was judged to be pathogenic based on American College of Medical Genetics and Genomics (ACMG) Guidelines. Furthermore, II2, III4, and IV2 carried the same variant, while III3 was a patient and died (**Figure 1B**). Employing amniotic fluid puncture for prenatal diagnosis, fetus V2 was detected as a carrier with *EDA* gene heterozygous variant c.396 + 1 G > C. Similarly, proband 2 and proband 3 were identified as c.171-173 del GTT and exon 1 deletion hemizygous variants of the EDA gene respectively. Sanger sequencing was then performed on available lineage members (**Figure 2B**). The results showed that the variant in proband 2 (c.171-173 del GTT) was inherited from his mother (**Figure 2A**). However, MLPA showed the mother of proband 3 did not carry the same variant, indicating a *de novo* deletion variant (**Figure 3D**). Based on raw data of WES and probe coverage of MLPA, boundaries of this deletion established at the upstream and downstream regions of exon 1. The length of the deletion can be roughly estimated by CytoScan750k gene chip for its composition of 550,000 non-polymorphic CNV probes and more than 200,000 SNP probes. According to the position of the missing SNP, there is a deletion of 82,724 bp in the X chromosome (chrX: 68,748,640–68,831,364), which belong to the upstream region of EDA gene (**Figure 3E**). At downstream

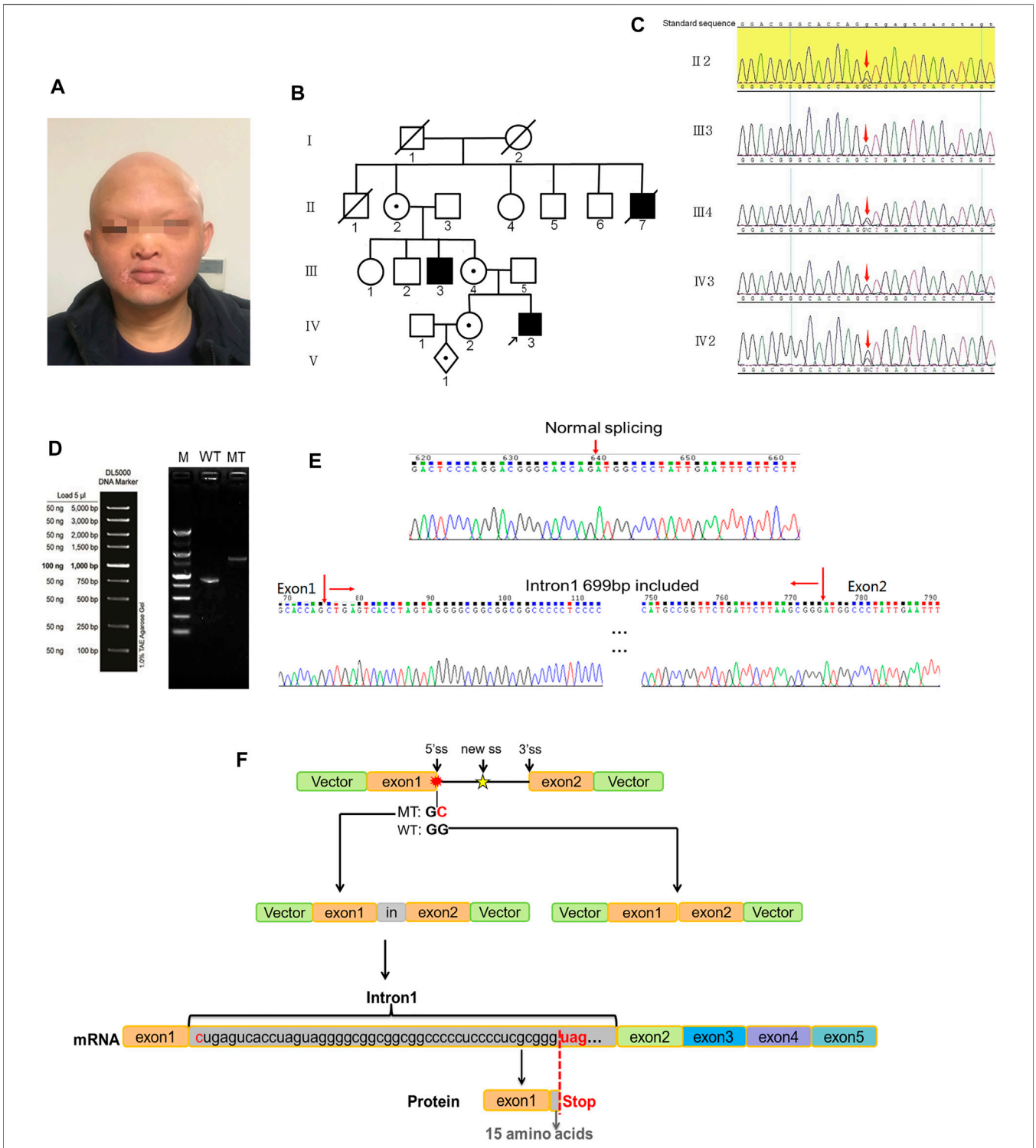

FIGURE 1 | Variants in case 1. **(A)** Frontal photograph of proband 1 showing various developmental defects of ectodermal appendages including protuberant lips, short chin, and flat nose. **(B)** Family tree showing that the proband's uncle (III3) and uncle's grandpa (II7) were HED positive. **(C)** Validation of the mutation site by Sanger sequencing. The red arrow indicates the mutated base. **(D)** Electrophoretogram results of transcripts generated from the transfected WT and MT EDA minigenes. Lane M, 5,000 bp markers; Lane WT, WT EDA minigene transcripts showing a single band of 821 bp; Lane MT, MT minigenes containing mutant alleles showing a longer band (the empty lane has been cropped). **(E)** Inclusion of an extra 699 nucleotides in intron 1 validated by Sanger sequencing. **(F)** The splicing and transcription pattern of MT EDA pre-mRNA.

TABLE 1 | The genetic characteristics of *EDA* gene in patients.

No.	Age	Gene	Gender	Phenotype					Chromosome Position	Transcript/Exon	Mutation Analysis	Mutation type	MAF	SIFT	PolyPhen_2	REVEL	ACMG Classification (Evidence of pathogenicity)
				Hypodontia	Hypotrichosis	Fever	Dry Skin	Special Face									
1	30 Years	EDA	Male	+	+++	+	+	+	chrX-68836549	NM_001005609 exon1	c.396 + 1 G > C	Splicing mutation	-	unknown	benign	unknown	PVS + PM2 (likely pathogenic)
2	10 Months	EDA	Male	+	+	+	+	-	chrX-68836322–68836325	NM_001399 exon1	c.171_173 delGTT (p.57_58days elTLinsT)	Indels mutation	-	unknown	unknown	unknown	PM1+PM2+PM4+PP4 (likely pathogenic)
3	20 Days	EDA	Male	+	+++	+	+++	+	exon1	NM_001399 exon 1	del exon 1	Indels mutation	-	-	-	-	PVS1+PS1+PS2+PP4 (pathogenic)

Note: means without corresponding phenotype; + means with corresponding phenotype; +++ means with severe phenotype; MAF: minor allele frequency; ACMG: American College of Medical Genetics and Genomics; PS: pathogenic strong; PP: pathogenic supposing; PM, pathogenic moderate; PVS, pathogenic very strong.

of missing SNP region, there are 6314 bp length without SNP coverage which contain upstream of exon 1, exon 1 and partial intron 1 of EDA gene. Combining the results of CMA, MLPA, and WES, the absolute deletion length extended to downstream of exon 1, which is 87,938 bp (chrX: 68,748,640–68,836,578) including upstream of exon 1 (87,513 bp) and exon 1 (396 bp), and downstream of exon 1 (29 bp). In fact, the deletion length might be longer. Detailed information for *EDA* variations is shown in **Table 1**. Variants c.396 + 1 G > C and c.171-173 del GTT are reported herein for the first time.

RT-PCR Analysis of *EDA* Minigenes

RT-PCR assays were performed to investigate transcripts generated from transfected WT and MT *EDA* minigenes. The WT *EDA* minigenes produced a single band of 821 bp comprising the expected pEGFP-C-5′, exon 1, exon 2, and pEGFP-C-3′ regions. However, the MT RT-PCR products showed a longer band (**Figure 1D**). Sequence analysis of the abnormal products revealed the inclusion of the first 699 nucleotides from intron 1, which may be attributed to the presence of a cryptic 5′ splice site in intron 1 because the AG sequence was observed at positions 700 and 701 of intron 1 (**Figure 1E**).

Protein Function Prediction

For proband 1, inclusion of the first 699 nucleotides of intron 1 could potentially result in a redundant reading frame in exon 1 of the *EDA* gene, and ultimately generated a longer peptide. However, there was a stop codon at positions c.396 + 46–48 that could lead to abnormal truncated EDA-A1 proteins or nonsense-mediated mRNA decay (NMD; **Figure 2F**). Therefore, c.396 + 1 G > C was judged to be a loss-of-function variant.

For proband 2, variants of c.171-173 del GTT resulted in a single amino acid deletion of the leucine codon (**Figure 2C**). The mutation taster tool predicted this to be a disease-causing variation. Comparative sequence alignment was performed across most mammals using the T-Coffee Multiple Sequence Alignment Program (http://www.ebi.ac.uk/Tools/msa/tcoffee). The results showed that this amino acid has been conserved in mammals during evolution, and is therefore important for protein structure and/or function (**Figure 2D**). Furthermore, this variation might cause changes in the α-helical and β-sheet secondary structure components of the EDA protein predicted by Psipred 4.0 software (**Figure 2E**).

For proband 3, MLPA was applied to confirm the absence of exon 1 of the *EDA* gene (**Figure 3D**). The results showed that two peaks representing exon 1 were absent in proband 3 but not in his parents. The detection of CMA indicated that there was a large fragment deletion, including the upstream region (87 kb) of the EDA gene, which completely destroyed the initiation of transcription of *EDA* and caused the failure of EDA protein synthesis (**Figure 3C**).

DISCUSSION

According to the HGMD Professional database (2017.2), there are 355 registered variations of the *EDA1* gene, of which 31 occur in the intron region. In the three HED pedigrees, an unreported

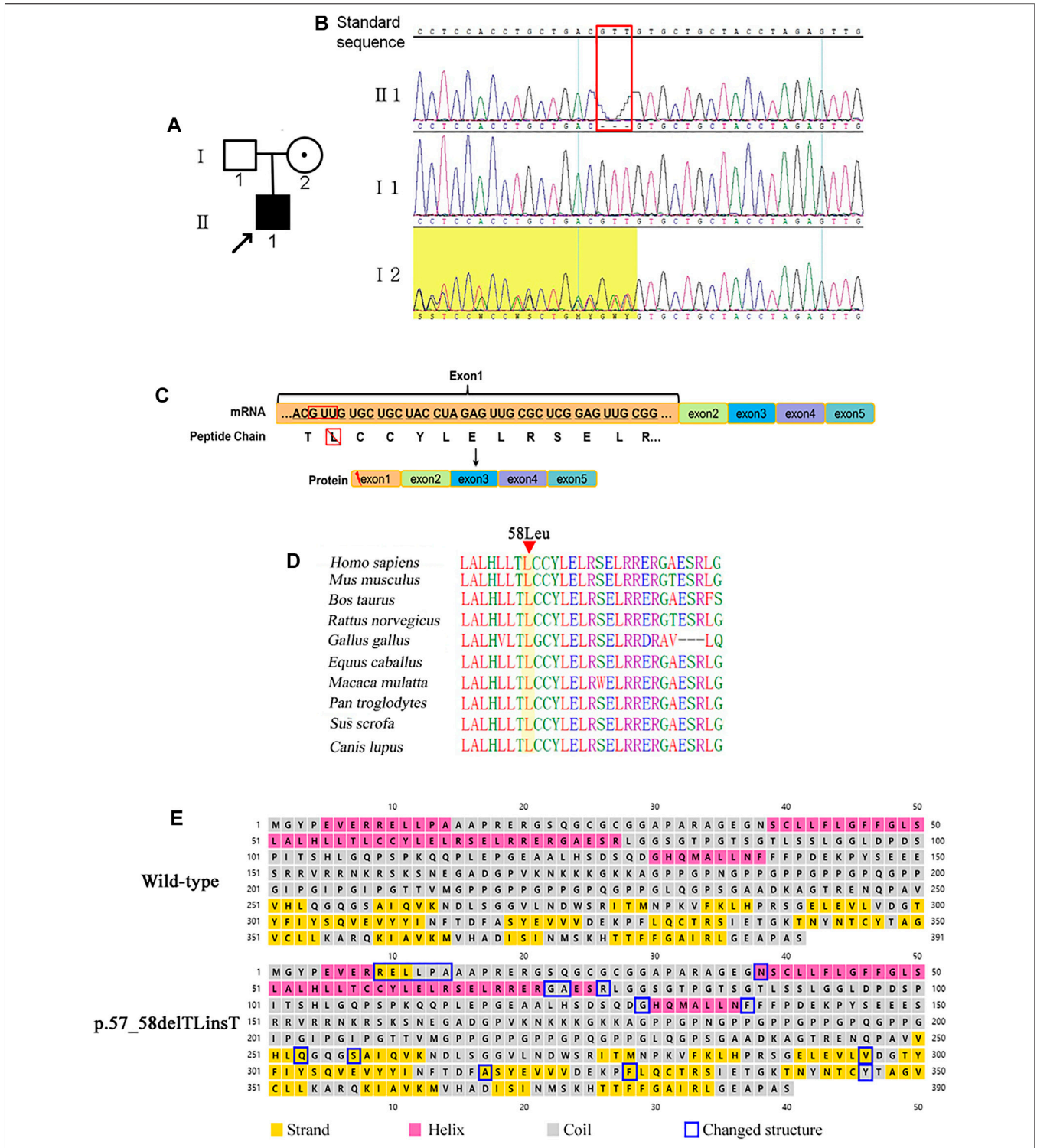

FIGURE 2 | Variants in case 2. **(A)** Family tree showing the mutations inherited from I2. **(B)** Validation of the mutation site (c.171_173delGTT) by Sanger sequencing. **(C)** Transcription pattern. **(D)** Conservation analysis of affected amino acids in the EDA protein among 10 different mammalian species. **(E)** Secondary structure prediction of the WT EDA protein and the p.57_58delTLinsT mutant.

5'ss variant (c.396 + 1 G > C) of the *EDA* gene was found, and the splicing alteration mechanism was confirmed by minigene *in vitro*. The variant led to aberrant pre-mRNA splicing in exon 1 of the *EDA* gene, which generates a longer transcript with an extra 699 nucleotides in intron 1. In most cases (98.7%), GT and AG sequences are canonical splice sequences at the 5′ and

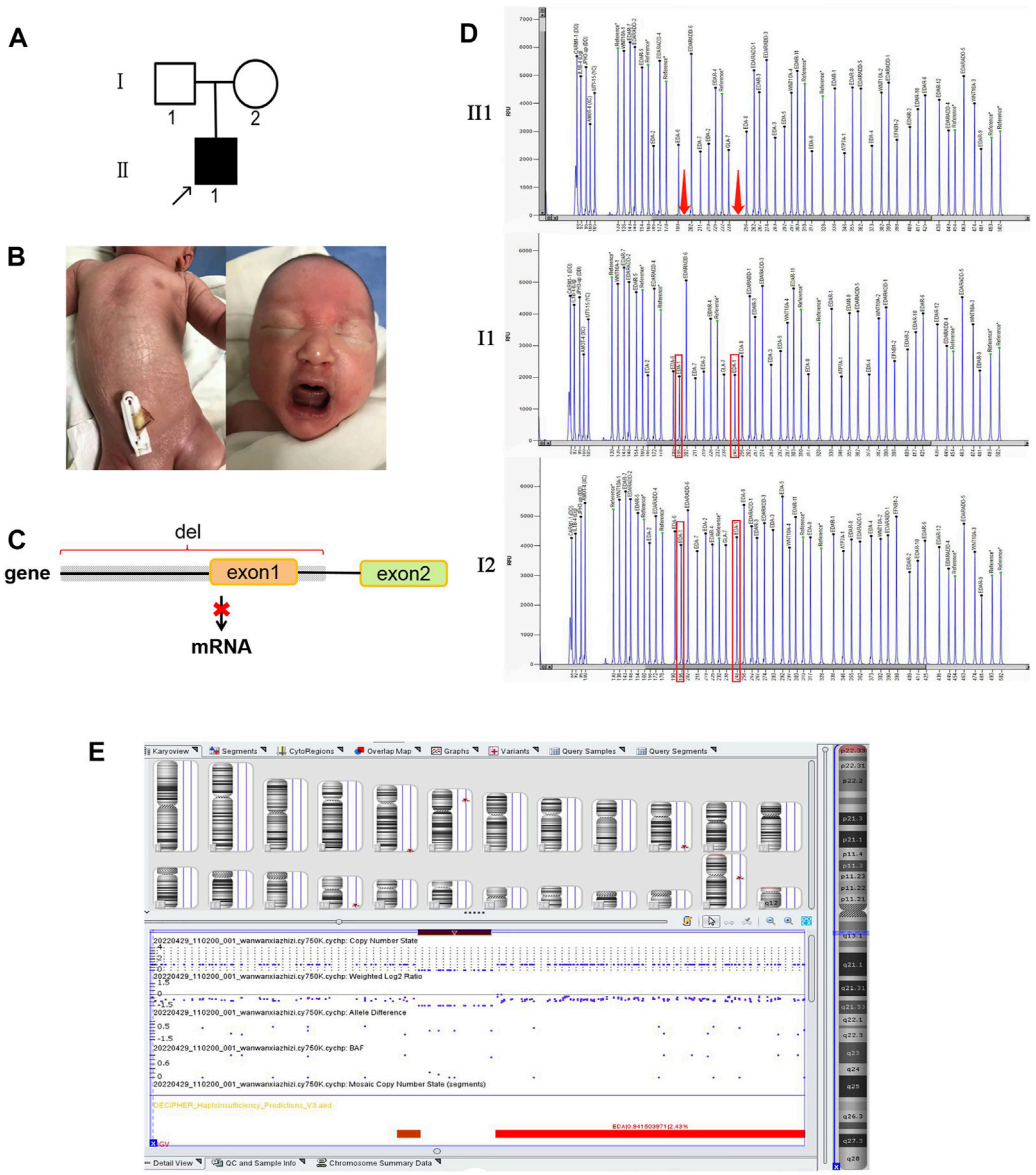

FIGURE 3 | Variants in case 3. **(A)** Family tree showing a *de novo* mutation. **(B)** Dry skin of proband 3. **(C)** Transcription pattern. **(D)** MLPA results show deletion of exon 1. **(E)** CMA profile showing a loss of 82 kb of Xq (chrX: 68,831,364–68,748,640; hg19) (dark red box) which is upstream of the *EDA* gene. Images were generated using the Chromosome Analysis Suite (ChAS) software.

3′ ends of the intron, respectively, that define exon–intron boundaries for spliceosome recognition. The 5′ donor splice site variants at + 1 and +2 positions, as well as the 3′ acceptor splice site variants at−1 and −2 positions, are considered pathogenic (Caridi et al., 2016; Anna and Monika, 2018; Ma et al., 2019). Furthermore, variant c.526+1G > A in the *EDA* splice donor site caused the complete omission of exon 3, which resulted in abnormal truncated EDA-A1 proteins (Liu et al., 2018). In addition, c.396 + 5 G > A and c.396 + 2 T > C variants were also reported in HED cases without further research.

Splicing variants can be summarized into four types according to the impact on the final composition of mRNA: 1) Exon skipping, in which an authentic splice site variant and a variant within an exon usually result in whole-exon skipping; 2) cryptic exon inclusion, in which inclusion of a subsequent intron (pseudo exon) is caused by a deeply intronic nucleotide variant; 3) exon sequence removal, in which activation of cryptic splice sites caused by a single-nucleotide variant in an exon results in exclusion of exonic sequences; and 4) intron inclusion, in which intronic sequence inclusion generated by a variant in the intron or authentic site leads to the generation of a cryptic intronic splice site (Wimmer et al., 2007; Anna and Monika, 2018). Variants at the canonical splice sequence either result in skipping of one or more adjacent exons, or activation of a cryptic

splice site of the same type in a neighboring exon site. In case 1, the positions of c.369 + 700–701 present alternative GT nucleotides as a stronger cryptic intronic splice site described in type 4, resulting in inclusion of an intron fragment instead of exon skipping. For 5'ss, a highly significant correlation was observed between the mutational consequences at the RNA level and the number of potentially utilizable cryptic donor splice sites in the region around the affected splice site (Buratti et al., 2007). More potential splice sites in the region around the affected splice site mean an increased probability of inclusion of introns (Kovacova et al., 2020). In case 1, 32 potentially utilizable GT nucleotides upstream of the chosen splice site were ignored, and only one aberrant transcript was generated in the minigene assay. Studies show that recognition of the 5'ss in a pre-mRNA is initiated by the formation of a base pairing interaction between the splice site sequence and particular sequences in the U1 snRNA of the spliceosome (Liu et al., 2017). Hence, the chosen cryptic donor splice site may be associated with a strong splicing motif for greater sequence complementarity, leading to the 5′ end of U1 snRNP binding with higher affinity.

Variant c.171-173 del GTT leads to p.57-58 del TL ins T in the *EDA* gene, predicted as unknown by PolyPhen_2, SIFT, and REVEL. In addition, the HGMD database has no relevant reports for this locus, hence it was evaluated as uncertain by ACMG. However, the symptoms of hypohidrosis, hypotrichosis, and thickness of the alveolar arch strongly support the clinical diagnosis of XLHED. This represents new pathogenic evidence for this variant. Variant with full deletion of exon 1 was first reported in Finland (Pääkkönen et al., 2001). In China, we previously reported the ultrasonic phenotype of this XLHED with thinner alveolar bone than a normal fetus. After birth, the infant's upper alveolar bones were also thinner compared with normal. Hence, it is important to take the thickness of the alveolar arch into consideration for investigating intrauterine or infancy dental development.

Although all three variants affected exon 1, the results were different between variants, as we predicted. For proband 1, there was an extra stop codon in intron 1 that could lead to NMD and decreased expression of the abnormal truncated EDA-A1 protein. For proband 2, the variant led to loss of leucine in exon 1, which could affect the transmembrane transportation domain of the EDA-A1 protein. For proband 3, the variant led to loss of exon 1 and upstream region, which completely destroyed the initiation of transcription and translation. The wild-type EDA protein could not be produced normally. Correspondingly, the symptoms in proband 3 were the most severe in terms of skin and hypotrichosis, whereas proband 2 did not exhibit severe dry skin.

In conclusion, we investigated the genetic and clinical features of patients with XLHED. Three variants located in or affecting exon 1 of the *EDA* gene were identified, including two novel variants of the splicing donor site (c.396 + 1 G > C) and c.171-173 del GTT. We further demonstrated the role of c.396 + 1 G > C in altering gene transcription (creating a cryptic 5′ splice site in exon 1) *in vitro*, which facilitates accurate prenatal diagnosis and genetic counseling for other family members of pedigree 1. As there is still no effective treatment for XLHED, our findings also broaden our knowledge of the *EDA* gene in HED patients, and can be used as a reference for clinical disease screening, diagnosis, and genetic counseling.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Review Committee of Gansu Provincial Maternity and Child-Care Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW: conceptualization, methodology, writing (original draft preparation); CZ, BZ, LH, LZ: software, data mining, revising and approval of the version; XC, SW, LY, SH: experiment conduction, data mining, investigation; QZ: supervision, writing (reviewing), editing.

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Case Report: Diverse phenotypes of congenital poikiloderma associated with *FAM111B* mutations in codon 628: A case report and literature review

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Congenital poikiloderma is an extremely rare autosomal dominant genetic syndrome, characterized by a combination of early onset poikiloderma, telangiectasia, and epidermal atrophy. *FAM111B* gene with multiple mutations has been identified as a potential causative gene for congenital poikiloderma. In this report, we described a boy with congenital poikiloderma confirmed by clinical manifestations. Next-generation sequencing based on a gene probe panel consisting of 541 genetic loci of genodermatoses, was used to screen mutations of the proband and his parents. Results showed that a missense mutation in the *FAM111B* gene c.1883G>A (rs587777238) was identified in the proband, but absent in his parents, indicating the mutation is *de novo*. In conclusion, a new case of congenital poikiloderma in China was reported, and the hotspot mutations in codon 628 of *FAM111B* gene was reviewed, as well as authenticating the uncertain association between genotypes and phenotypes in this rare disease.

KEYWORDS

congenital poikiloderma, FAM111B, mutation, case report, literature review

Introduction

Congenital poikiloderma (hereditary fibrosing poikiloderma) is an extremely rare syndromic form of the autosomal dominant disease. It is characterized by a combination of early onset poikiloderma, telangiectasia, epidermal atrophy, tendon contractures, myopathy, and pulmonary fibrosis (POIKTMP), accompanied with the deficiency of eccrine sweat glands (also called hypohidrosis), sparse scalp hair and absent body hair, including eyebrows and eyelashes (Rayinda et al., 2021). In 2013, family with sequence similarity 111 member B (*FAM111B*) mutations were reported to be responsible for congenital poikiloderma (Mercier et al., 2013). Its mode of inheritance and primary clinical features were first described in two generations of a multiplex South African family. *FAM111B* has also been reported to be associated with inherited exocrine

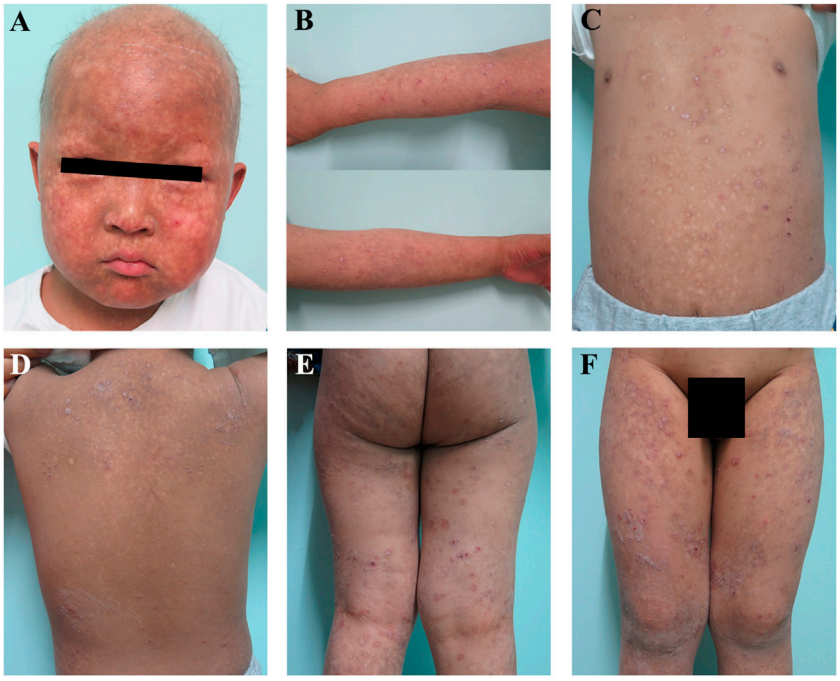

FIGURE 1
Clinical features of the patient **(A,B)** Congenital poikiloderma, including mottled pigmentation, telangiectasia, epidermal atrophy, sparse scalp hair, as well as absent eyelashes and eyebrows **(C–F)** The patient also had eczematous lesions on the trunk and legs.

pancreatic dysfunction and prostate cancer (Akamatsu et al., 2012; Seo et al., 2016). In addition, *FAM111B* has been confirmed as a direct target of p53 and identified as an oncogene for lung adenocarcinoma (Sun et al., 2019). However, the underlying pathogenic mechanism concerning *FAM111B* mutations is still unclear.

Herein, we reported a 5-year-old boy with mottled pigmentation, telangiectasia, epidermal atrophy, and a missense mutation (c.1883G>A) of *FAM111B* gene was identified. Furthermore, the mutations in codon 628 of *FAM111B* gene were reviewed and the uncertain association between genotypes and phenotypes in this rare disease was also authenticated.

Case report

Ethical approval

The current study conformed to the tenets of the Helsinki declaration and was approved by Ethical Committee of Shanghai skin disease hospital. The proband, his parents and 120 ethnically matched control individuals were informed regarding the purpose of the study and written consent was provided prior to recruitment and sampling.

Case description

A 5-year-old boy was admitted to the Shanghai Skin Disease Hospital outpatient department for developed blisters on the scalp, that were present 1 month after his birth, which gradually spread to the whole body and turned into poikiloderma after 3 months (Figure 1). The lesion was predominantly located on the face and in the other sun-exposed areas, which were typical manifestations for the diagnosis of congenital poikiloderma (Figures 1A,B). He had hypohidrosis and also eczematous lesions on the trunk and legs (Figures 1C–F). No lymphoedema of the upper or lower extremities was observed. Since the onset of the disease, the rash has occurred repeatedly and aggravated in winter. His scalp hair was sparse, with eyelashes and eyebrows absent. His nails and teeth were normal. In addition, from the first year of life, elevated liver transaminase levels were observed on repeat blood samples, including aspartate aminotransferase (316 U/L; normal range, 15–40 U/L), alanine transferase (354 U/L; normal range, 9–50 U/L), γ-glutamyl transferase (334 U/L; normal range, 10–60 U/L), alkaline phosphatase (532 U/L; normal range, 0–500 U/L) and lactate dehydrogenase (362 U/L; normal range, 120–230 U/L). Vasodilation and hyperemia were also observed. The results were consistent with the manifestation in congenital poikiloderma.

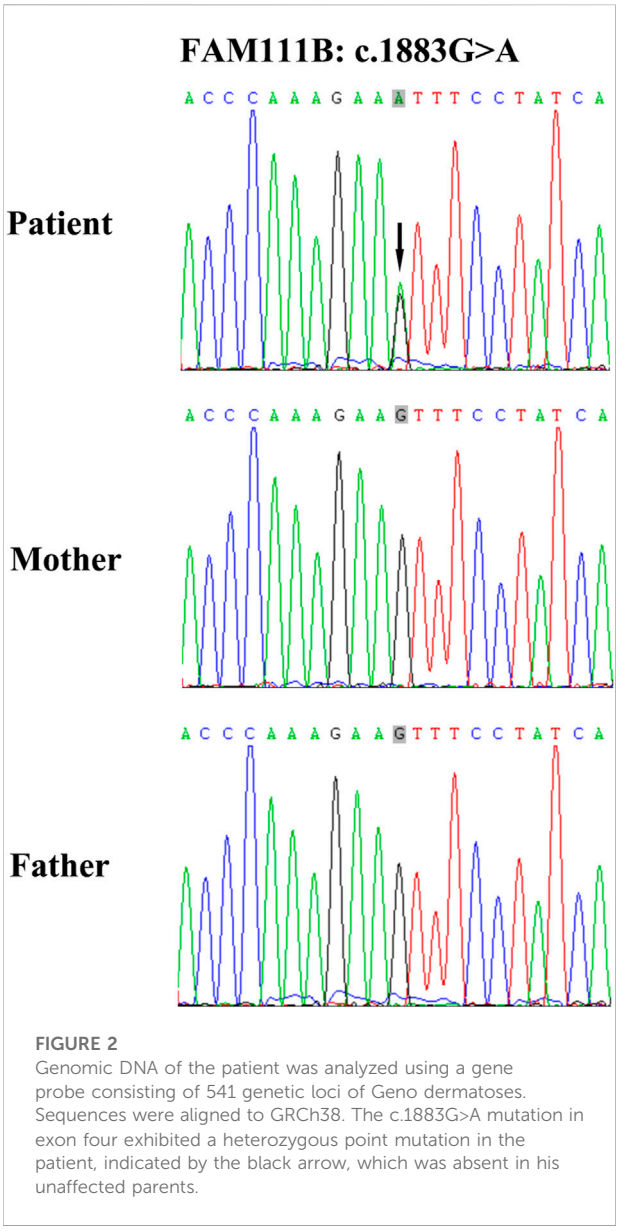

FIGURE 2
Genomic DNA of the patient was analyzed using a gene probe consisting of 541 genetic loci of Geno dermatoses. Sequences were aligned to GRCh38. The c.1883G>A mutation in exon four exhibited a heterozygous point mutation in the patient, indicated by the black arrow, which was absent in his unaffected parents.

Multi-gene panel sequencing

To investigate the underlying mutation of congenital poikiloderma, next-generation sequencing based on a multi-gene probe panel consisting of 541 genes of monogenic hereditary diseases was used to screen mutations of the proband and his parents. In detail, genomic DNA was extracted from the peripheral blood using the Wizard Genomic DNA purification kit (Promega Corporation). A total of 120 unrelated population-matched control samples were also used to exclude the possibility that these were polymorphisms. Total DNA was isolated from peripheral blood using QIAamp DNA Mini kit (Qiagen, Inc.) according to the manufacturer's instructions. DNA was concentrated and quality controled using a Qubit 3.0 Fluorometer instrument (Invitrogen; Thermo Fisher Scientific, Inc.) to ensure the concentration was higher than 40 ng/μL. The Illumina Hiseq X Ten sequencing platform (Illumina, Inc.) was used, with an average sequencing depth >200× and Q30 > 90%. To verify the accuracy of the identified mutation, direct Sanger sequencing was performed to confirm whether the variants co-segregated with the disease phenotype in the proband and his parents using an ABI PRISM 3730XL automated sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequencing reactions were all performed in forward and reverse directions. The American College of Medical Genetics (ACMG) classification of the variant was performed using the online tool Varsome (https://varsome.com/) (Kopanos et al., 2019).

Genetic analysis

A heterozygous point mutation, c.1883G>A (rs587777238) in *FAM111B* was detected, leading to an amino acid alternation from serine to asparagine (*p*.628S > N) (Figure 2). This mutation was absent from his unaffected parents, which indicates that it is a *de novo* event. According to the ACMG variant classification guideline, this variant was categorized as a pathogenic variant. Moreover, it was not found in any of the healthy controls also showing that it is a novel pathogenic mutation, not a common polymorphism. This mutation causes protein structural and functional changes, which induces the occurrence of this disease.

Literature review

The following terms were combined in the search strategy [FAM111B (Title/Abstract)] AND [poikiloderma (Title/Abstract)] from PubMed database. Then, the retrieved literatures were analyzed in full text. Mutations in condon 628 of *FAM111B* identified in congenital poikiloderma were summarized.

Phenotypic heterogeneity for codon 628 in FAM111B

Previous studies suggested that codon 628 of *FAM111B* could be a mutation hotspot. A total of 8 cases with mutations in codon 628 were retrieved from PubMed and results from the present case report were also illustrated for comparison (Supplementary Table S1). Poikiloderma and hypohidrosis were found in every patient carrying *FAM111B* mutations in codon 628. In contrast to the other patients, the proband in our report showed more severe liver damage, while muscle weakness was not found. Patients mostly present with hypotrichosis, but patients in one pedigree reported by Goussot et al. showed no signs of the symptom (Goussot et al., 2017).

TABLE 1 A comparison of clinical features of different mutation spots of FAM111B.

The FAM111B mutations	Location	Clinical features
Codon 416, 430	Outside the putative protease domain	Poikiloderma, Atopecia, Sclerosis, lymphoedema, bullous lesions, and pancreatic cancer
Codons 621, 625, 627, and 628	Within the putative protease domain	Poikiloderma, Atopecia, telangiectasia, epidermal atrophy, tendon contractures, myopathy, liver damage and pulmonary fibrosis

Discussion

Congenital poikiloderma is primarily characterized by early onset poikiloderma, combining with several symptoms, such as telangiectasia and epidermal atrophy, which occurs in neonates and infants. The susceptible gene, *FAM111B*, was identified by Mercier *et al.* (Chen et al., 2019) in 2013, which is the second member of the two-gene "family with sequence similarity 111" gene family. *FAM111B* contains four exons and encodes 734 amino acids, which is likely to contain a trypsin-like cysteine/serine peptidase domain. The identification of mutations in *FAM111B* provided definitive evidence for POIKTMP and distinguishes it from other types of hereditary poikiloderma, such as Rothmund-Thomson syndrome (RTS), hereditary sclerosing poikiloderma of Weary, Kindler syndrome and Clericuzio-type poikiloderma with neutropaenia (Arnold et al., 2010; Küry et al., 2016; Gatinois et al., 2020). In approximately 50% of affected individuals, *FAM111B* pathogenic variant is *de novo* (Mercier et al., 1993), which is the same as the present study.

In the present study, a rare case of congenital poikiloderma with a missense mutation (c.1883G>A) in *FAM111B* was reported. This mutation was within the putative protease domain and predicted to be pathogenic by Varsome database (https://varsome.com/), revealing that the mutation would promote the development of this disease. Recent studies found that disease-associated *FAM111B* mutants forms a complex with Family with sequence similarity 111 member A (*FAM111A*), hyperactivating the intrinsic protease activity of *FAM111A via* a common gain-of-function mechanism, which may become the cause of the hereditary fibrosing poikiloderma syndrome (Hoffmann et al., 2020).

Inter-familial phenotypic variability has been observed in congenital poikiloderma, indicating that it may be a multisystem disorder. The same mutation could lead to different phenotypes and an association between genotypes and phenotypes was not established (Mercier et al., 2013; Mercier et al., 2015; Goussot et al., 2017), suggesting that other factors, such as racial factor and environmental variables, might influence the clinical characteristics of this disease. To date, including our case in the present study, a total of 37 patients with this rare disorders have been reported globally (Arowolo et al., 2022a). For patients with congenital poikiloderma, the predominant manifestation is early onset poikiloderma, telangiectasia, and epidermal atrophy. However, patients display a wide spectrum of disease phenotypes. With respect to the genotype-phenotype association, the mutations in the *FAM111B* gene can be classified into two categories according to their positions (Table 1). The codons 621, 625, 627, and 628 are located within the putative protease domain of *FAM111B*, which may be associate with more severe clinical symptoms in skin, muscle and internal organs, and worse prognosis (Arowolo et al., 2022b). The clinical manifestations in affected individuals with mutations located outside the domain, such as codons 416 and 430, may be characterized by sclerosis, lymphoedema, bullous lesions, and pancreatic cancer (Takeichi et al., 2017; Arowolo et al., 2022b). In our reported case, the patient showed no symptoms or had mild symptoms such as tendon contractures and myopathy, which might be a result of the young age. Longer-term clinical follow-up is required.

In conclusion, we reported a new case of congenital poikiloderma with *FAM111B* mutation c.1883G>A in China. Diverse phenotypes of congenital poikiloderma associated with FAM111B mutations in codon 628 were observed. Our results will expand the current knowledge and also verify the incomplete association between genotypes and phenotypes of this extremely rare disorder.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Author contributions

YW performed the gene analyses and data interpretation. YW and LW wrote the manuscript. PW contributed to the acquisition of clinical data. XW and GZ contributed to the conception and design of the study, critically revised the

manuscript and provided final approval of the version to be published.

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Conflict of interest

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Supplementary material

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Uncovering incontinentia pigmenti: From DNA sequence to pathophysiology

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Incontinentia pigmenti (IP) is an X-linked dominant genodermatosis. The disease is known to be caused by recurrent deletion of exons 4–10 of the Inhibitor Of Nuclear Factor Kappa B Kinase Regulatory Subunit Gamma (*IKBKG)* gene located at the Xq28 chromosomal region, which encodes for NEMO/IKKgamma, a regulatory protein involved in the nuclear factor kappa B (NF-κB) signaling pathway. NF-κB plays a prominent role in the modulation of cellular proliferation, apoptosis, and inflammation. *IKBKG* mutation that results in a loss-of-function or dysregulated NF-κB pathway contributes to the pathophysiology of IP. Aside from typical skin characteristics such as blistering rash and wart-like skin growth presented in IP patients, other clinical manifestations like central nervous system (CNS) and ocular anomalies have also been detected. To date, the clinical genotype-phenotype correlation remains unclear due to its highly variable phenotypic expressivity. Thus, genetic findings remain an essential tool in diagnosing IP, and understanding its genetic profile allows a greater possibility for personalized treatment. IP is slowly and gradually gaining attention in research, but there is much that remains to be understood. This review highlights the progress that has been made in IP including the different types of mutations detected in various populations, current diagnostic strategies, *IKBKG* pathophysiology, genotype-phenotype correlation, and treatment strategies, which provide insights into understanding this rare mendelian disorder.

KEYWORDS

incontinentia pigmenti, *IKBKG/NEMO*, NFκB pathway, pathophysiology, molecular diagnosis, genotype-phenotype

Introduction

Incontinentia Pigmenti (IP; OMIM 308300), an ectodermal dysplastic disorder, is a rare type of X-linked dominant genetic disease. It is caused by mutation of the *IKBKG* gene, which is located at Xq28. It encodes a vital component of the transcription of the nuclear factor kappa B (NF-κB) signaling pathway (1, 2). IP occurs

in approximately 1:40,000 to 1:50,000 births (3, 4). The disease has a prevalence of approximately 0.7/100,000 with female patients being the affected population (5). Two major allelic mutations have been described, namely amorphic allele and hypomorphic allele. In amorphic allele, female survival is attributed to selective skewed X chromosome inactivation. This type of mutation is generally lethal in males, except for cases with XXY chromosome disorder or individuals with somatic mosaicism. On the other hand, the hypomorphic allele leads to mild IP in females, and affected males suffer from ectodermal dysplasia with immune deficiency (EDA-ID) (6). According to the biobank for IP (IPGB, https://www.igb.cnr.it/ipgb/) more than 75% of female and all male IP cases are sporadic (7).

IP can be clinically diagnosed based on the updated Landy and Donnai diagnostic criteria. This involved characteristic cutaneous manifestation, and abnormalities found on hair, nail, central nervous system, eye, orodentofacial, nipple, and breast. These criteria also take into consideration previous male miscarriages and family history of IP (8). Cutaneous manifestation occurs within the first few weeks of life. The skin lesions evolve through four stages, which begin with vesiculobullous eruption (Stage I), followed by verrucous stage (Stage II), hyperpigmented stage (Stage III), and atrophic, hypopigmented stage (Stage IV) (5, 9, 10). Not all stages occur and overlapping clinical manifestation is not uncommon. Eye anomalies are found in 35% of the patients. Retinal anomalies are by far the most common and include retinal vascular anomalies, retinal detachment, and retinal pigment epithelium anomalies. Areas of ischemia may induce neovascularization, which lead to gross intraocular scarring with severe visual loss (11). CNS anomalies can occur in up to 30% of IP patients. They commonly start in the early infantile period and adult-onset neurological symptoms are unlikely related to IP. Convulsive disorders are found to be the most common, followed by paralytic disorders, motor impairment, and intellectual disability. Other manifestations include odentofacial, breast, hair, and nail presentations. On rare occasions, skeletal, cardiac, and other organs may be also affected (12–14).

This review outlines current understanding of molecular diagnosis, pathophysiology, and the genotype-phenotype correlation.

Current technologies for the diagnosis of incontinentia pigmenti

Analytical approaches for the molecular diagnosis of IP should be approached by considering the index case's gender. This is because in an IP female the variant is in a constitutively heterozygous state. This indicates that it can be found in all cells in the body. However, if the postzygotic mutation occurs in a male population, embryonic mosaicism allows two groups of genetically distinct populations to coexist in the same individual (6). In addition, the cell expressing the *IKBKG*/NEMO variant may gradually be eliminated and finally cleared, making the diagnosis in male patients extremely difficult. Simple PCR to detect the genomic deletion of exon 4–10 remains the recommended technique for *IKBKG* variant screening as the recurrent deletion account for 79% of female IP case (15) or 70% of total IP cases (7). If the exon 4–10 deletion is not detected, Sanger sequencing can be used to screen for point mutation and indel along the *IKBKG* coding region and intron-exon junctions will improve diagnostic sensitivity by 9%. On top of those above, qPCR can be employed to detect larger arrangements other than the classical exon 4–10 deletion that account for about 4% of IP cases (7). Despite next generation sequencing being more widely available and cheaper, it has been deemed unusable for IP diagnosis due to the presence of the pseudogene, *IKBKGP1*. Both *IKBKG* and *IKBKGP1* are located in the Xq28 region within and share 99% of their identity (16). The presence of this pseudogene makes the traditional capture probe data analysis difficult, as it reduces the read depth, decreases the mapping quality, and contributes to a poor alignment read, resulting in false-positive results (16, 17). However, a bioinformatics tweak masking the *IKBKGP* in the Next Generation Sequencing (NGS)/Whole Exome Sequencing (WES) pipeline analysis harnesses the technology, acting as a powerful tool in detecting mutations in *IKBKG* (18). With such innovations, NGS/WES undoubtfully accelerates the *IKBKG* mutational screening as an alternative to or in addition to the traditional Sanger (19). Low level mosaicism that happens in male patients, may escape molecular investigation if methodology in relation to female patients is used. Rather than having the genomic DNA extracted from peripheral blood, testing should be done using the tissue of choice from the suspected phenotype (i.e., skin) and analysis of multiple tissues, namely blood, fresh skin, saliva, and sperm samples to detect low-level mosaicism (7, 8, 20). Thus, the latter is more expensive and requires more specific competencies and infrastructure (19, 21).

Incontinentia pigmenti: Genetics and pathophysiology

Genetic variants of incontinentia pigmenti in various populations

The most common genetic mutation in IP is an approximately 11.7-kb deletion in the *IKBKG* gene that removes exons 4 through 10. This mutation accounts for 70–80% of patients with IP worldwide (22–24). This is found in European (25–27), Chinese (24, 28), Japanese (29–31), Korean (32, 33), and Indian (34) populations (Supplementary Table 1). Apart from the 11.7-kb deletion, IP can also arise due to other types of mutations along the *IKBKG* genes that include

single nucleotide substitution, point mutation, and small insertion/deletion (indel). A point mutation can be a non-sense mutation that leads to premature protein translation termination or a missense mutation that leads to amino acid change. Small indel may lead to frame-shift or in-frame amino acid deletion. Both point mutation, as well as indel, may also cause aberrant splicing of the *IKBKG* mRNA. These mutations can result in the absence of or defective *IKBKG* protein, which yields a phenotype of IP (24, 28–31). Other than mutations involving exons, a single nucleotide polymorphism involving intron 8 was also reported by Chinese populations (28). Though less commonly reported, this polymorphism was also reported among Caucasian populations (35). While most reports on IP cases came from western population cohorts and certain East Asian regions, IP cases have also been observed in other populations such as African (36, 37), Indian (34, 38, 39), Malaysian (40), and Brazilian (41).

IKBKG pathophysiology in incontinentia pigmenti

The IκB kinase (IKK) protein complex comprises the catalytic subunits IKKα and IKKβ, and IKKγ (NEMO) (42). The *IKBKG* gene is responsible for encoding for IKKγ (NEMO), which is responsible as the regulatory subunit of the inhibitor kappaB (IκB) kinase (IKK) complex essential for NF-κB pathway activation required in many elementary physiological functions (43). IκB protein phosphorylation, ubiquitination, and degradation upon the activation of the IKK complex results in the removal of the inhibitor that activates the NF-κB complex (44). The absence of IκB allows NF-κB to translocate into the nucleus, where the transcription of targeted genes can occur. Activated NF-κB has been reported to execute immune and inflammatory responses and is involved in the protection against apoptosis induced by signaling proteins (30, 42, 45–47). Thus, a lost-of-function or absence of the *IKBKG* gene contributes to the dysfunction of IKK and consequent termination of NF-κB activity. Without NF-κB, IP cells are highly sensitive to pro-apoptotic signal (43, 48–51).

In the cases of mosaicism in males and lyonization of the X chromosome in females, the neighboring keratinocytes without *IKBKG* gene mutation expressing IKKγ (NEMO) protein can undergo NF-κB activation upon receiving activating signals from *IKBKG*-deficient keratinocytes that are undergoing apoptosis or necrosis (15). Activating signals produced from apoptotic or necrotic cells include danger-associated molecular patterns (DAMPs) as well as "find me" signals such as lysophoshatidylcholine (LCP), sphingosine 1-phosphate (S1P), nucleotide ATP/AUP and Tumor Growth Factor (TGFβ) and others (52). Activation of NF-κB in nearby *IKBKG*-expressing keratinocytes will lead to the production of chemokines such as regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP-1) and eotaxin which recruits eosinophils cells. Besides, pro-inflammatory cytokines such as IL-1, TNF-α, IFN-γ, Lymphotactin will be produced (50, 53–55). Studies found that IL-1 and TNF-α can upregulate eotaxin production which attracts eosinophils migration. Eosinophils recruited will undergo degranulation and the release of proteases (42, 50, 56), leading to inflammation in the epidermis and other areas of the body (Figure 1).

In the epidermis, proteases degrade tonofilaments and desmosomes which result in intracellular oedema (spongiosis) and ultimately blistering, which is observed in the first stage of IP (56, 57). Gradual clearance of skin lesions occurs upon the reduction of *IKBKG*-deficient keratinocytes due to increased apoptosis and progressive replacement by *IKBKG*-expressing keratinocytes as well as subsiding of inflammation (50, 56, 58). Moreover, TNF and other cytokines that may be produced in the epidermis during the early inflammatory phase and could play a role in the process of directly eliminating the *IKBKG*-deficient keratinocytes (58, 59). However, residual *IKBKG*-deficient keratinocytes that managed to escape and survive the elimination process can undergo second episodes of the first stage in IP due to the reoccurrence of keratinocyte hyperproliferation and subsequent inflammation reactions (50, 56).

In the event where NF-κB-deficient endothelial cells and other cells throughout the body have overexpression of chemotactic factors such as eotaxin, specific to eosinophils, this may result in systemic eosinophilia (42, 60, 61). The presence of eosinophils in combination with other inflammatory factors would lead to extensive inflammation. Endothelial inflammation will result in vaso-occlusion and ischemia, contributing to the retinal and neurologic manifestation. The occlusion of retinal arteries leads to areas of avascularity and under-perfusion, precipitating ischemia. Neovascularization occurs as sequelae to this (62). In CNS, brain atrophy and other neurological sequelae are thought to have shared similar vaso-occlusive ischemia pathophysiology in retinal ischemia events (63).

NF-κB plays a role in protecting the integrity of brain endothelial cells and the blood-brain barrier. A defect of such makes endothelial cells susceptible to a variety of potential stimuli, including infections. These stimuli upregulate proinflammatory cytokines, such as IL-6,−8, and−10, leading to endothelium inflammation and subsequent arteriopathy (64). This explains the role of systemic anti-inflammation in the treatment of neurological manifestation in IP patients. However, the exact pathogenesis in CNS lesions is still controversial.

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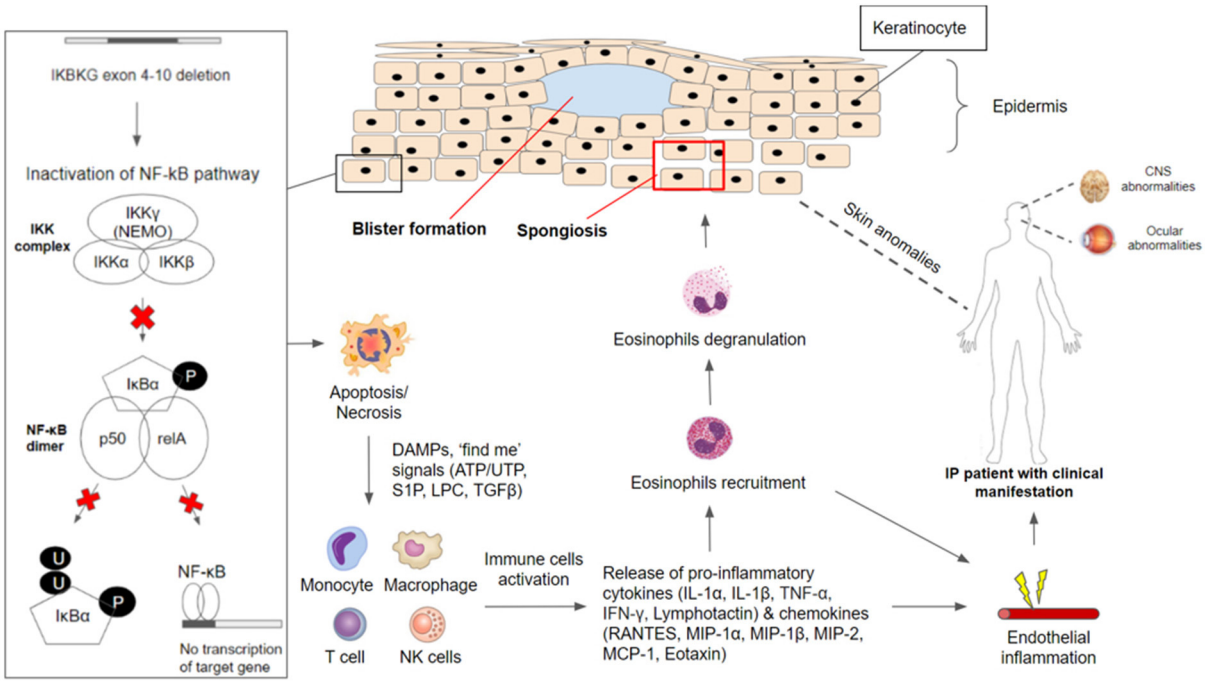

FIGURE 1
IKBKG/NF-κB pathophysiology in incontinentia pigmenti. The *IKBKG* gene is required for activation of the nuclear factor-kappa B (NF-κB) signaling pathway. Under non-stimulated conditions, NF-κB remained inactive in the cytoplasm through association with NEMO/IKKgamma (encoded by *IKBKG*). Phosphorylation of inhibitor NF-κB (IκB) proteins by the IKK complex results in their proteosomal degradation and subsequent release of NF-κB dimer (composed of p50 and relA subunits). Most affected individuals with IP carry a common pathogenic variant on the *IKBKG* gene with exon 4–10 deletion which caused inactivation of the NF-κB signaling pathway. *IKBKG*-deficient keratinocytes are susceptible to apoptosis/necrosis due to the loss of protection against cell death. DAMPS and 'find me' signals (ATP/UTP, S1P, LPC, TGFβ) are released and serve as activating signals which stimulate immune-inflammatory responses. Monocytes, macrophages, T cells, and NK cells have been shown to release cytokines (IL-1α, IL-1β, TNF-α, IFN-γ, Lymphotactin) and chemokines (RANTES, MIP-1α, MIP-1β, MIP-2, MCP-1, Eotaxin), leading to the recruitment of eosinophils. Recruited eosinophils undergo degranulation to release proteases that aid in degrading adhesions between keratinocytes. This results in spongiosis and blister formation which can be observed frequently in the first stage of clinical manifestation in IP patients. Besides the major presentation of skin conditions, IP patients have often reported manifesting CNS and ocular abnormalities. NF-κB-deficient endothelial cells and other cells throughout the body have overexpression of chemotactic factors, leading to eosinophilia, which triggers extensive inflammation. Endothelial inflammation will result in vaso-occlusion and ischemia, contributing to the retinal and neurologic manifestation.

Genotype-phenotype correlation in incontinentia pigmenti

Studies on genotype-phenotype correlation are rare. A study on 10 Japanese patients and three of their mothers revealed no definite difference in extracutaneous manifestation between those with or without *IKBKG* gene rearrangement (30). On a separate note, a study conducted by Wang et al. (65) on 42 IP patients, identified that those with positive *IKBKG* pathogenic variants appeared to have different clinical variations in comparison to those without. It was observed that patients with positive *IKBKG* mutation had a higher frequency of hair (50 vs. 14%), dental (70 vs. 21%), ocular anomalies (45 vs. 29%), and lower frequency of CNS anomalies (20 vs. 35%) (65). This difference suggests that there is a need for in depth evaluation of the key phenotype and genotyping differences between these groups. Past studies found that the clinical phenotype of IP is widely variable as it can range from mild skin alterations (mild IP) to stroke and functional CNS abnormalities (severe IP) (25). Dangouloff and colleagues (66) reported that severe CNS abnormalities have random X-inactivation whereas no or mild CNS abnormalities have skewed inactivation. On the other hand, mutation type (common deletion vs. point mutation) was found to not correlate with disease severity (33). This may be true as the NEMO/IKKgamma protein play a role in a complex signaling pathway that regulates the expression of various genes, its mutation produces different phenotypic outcomes which may explain the entire spectrum of anomalies observed in IP (43).

A phenotype scoring system used by Fusco et al. (25) to examine the correlation between the mutation type and clinical presentation of IP patients showed a high variability of phenotype scores in patients with exon 4–10 *IKBKG* deletion and hypomorphic mutations may have broader phenotypic consequence due to it being still partially active early after

TABLE 1 Extracutaneous difference between common exon 4-10 deletion vs. others.

(*n*), population, gender	Results (positive result/total, %)	Extracutaneous phenotype, *n* (%)		Conclusion	References
		Positive genetic test (delExon 4–10)	Negative genetic test (Exon 4–10)		
n = 10 Japanese, F	5/10, 50%	Alopecia, 3/5 (60) Opthalmic manifestation 3/5 (60) Neurological manifestation 1/5 (20) CVS manifestation—none No dental and nail abnormalities described	Alopecia—none Ophthalmic manifestation 1/5 Neurological manifestation 1/5 CVS manifestation—none	No definite difference in extracutaneous manifestation in their studies and others	(30)
n = 42, M & F White, 27/42 (64%) Hispanic, 1/42 (2%) Asian, 9/42 (21%) Black, 4/42 (10%)	20/34, 58.8%	Hair—10/20 (50) Nail—2/20 (10) Dental-—14/20 (70) Palate—0/20 (0) Ocular—9/20 (45) CNS—4/20 (20)	Hair—2/14 (14) Nail—2/14 (14) Dental—3/14 (21) Palate—1/14 (7) Ocular—4/14 (29) CNS—5/14 (36)	There is a clinical difference between *IKBKG* pathogenic variant positive and negative IP cohort	(65)
n = 25, Korean, F	20/25, 80% (common Exon 4–10) 5/25, 20% (intragenic sequence variants)	Hair—5/20 Nail—2/20 Dental—3/20 Ocular—4/20 CNS—5/20	Hair—0/5 Nail—0/5 Dental—0/5 Ocular—3/5 CNS—2/5	No statistically significant differences in frequencies of extracutaneous manifestations or phenotype scores	(33)
n = 122, France, Detailed phenotype described only in 60 patients	73/122, 59.8%	Hair—8/50 (16) Nail—7/50 (14) Dental—22/50 (44) Ocular—8/50 (16) CNS—4/50 (8)	Only described those with novel mutation identified (*n* = 10/49, 40.2%)—small nucleotide mutation, K90 (266-269delAGA) and H360MfsX449 (1077–1078delC)—high phenotype score compared to those with missense mutation (169G → A, 367 → T)) or non-sense mutation (715C → T, 1150 C → T) Nervous system defects were observed in 44% of patients carrying point-mutations and ocular defects in 55% of patients, whereas only 8% of IP patients with genomic deletion suffered nervous system defects and 16% had ocular defects.	Patients with in frame deletion mutation K90 (266-269delAGA) and frameshift mutations such as H360MfsX449 (1077-1078delC) & P372PfsX450 (1115-1116delT) suffer more severe disease compared with patients with missense or non-sense mutations	(25)
n = 18, France, F	All 15/18, 83% (common Exon 4–10) 3/18, 17% (missense or non-sense mutation)	Only included neurological symptoms 3 main neuroimaging identified Normal (5/18) Mild anomalies of periventricular white matter with T2 weighted hyper signal (7/18), mild cortical atrophy (5/7), and atrophy of corpus callosum (5/7) Severe cortical anomalies suggestive of vascular diseases (7/18)		Mutation type (delete vs. missense/non-sense) had no correlation with MRI Random X inactivation had more severe MRI anomalies	(66)

M, Male; F, Female; CNS, Central Nervous system.

the X-inactivation process. Thus, the mutations preserving some activity show an atypical phenotype characterized by the involvement of much more tissues compared to the classical IP phenotype. This can be observed in IP patients having more severe CNS and ocular defects as skewed X-inactivation is likely to modulate the severity of the disease (25). Besides, the variability in disease expression for patients carrying the same *IKBKG* mutation in different genomic backgrounds may be explained by the additional genetic factors such as modifier genes observed in many mendelian diseases (53). To date, there is no significant genotype-phenotype relation in IP. However, studies have proposed that a combination of the mutation type, the function domain affected, X-inactivation and genomic background may lead to the variability observed in IP phenotypes (25, 33, 53). Comparisons are detailed in Table 1.

Current treatment strategies

The current treatment strategies require multidisciplinary experts (including but not limited to dermatology, neurology, pediatric, geneticist, and ophthalmologist). The treatment approach involves symptom control, rehabilitation, and preventing complications (1).

Among those with skin presentation severely inflamed verrucous lesions can be treated with topical or systemic steroids and/or topical calcineurin inhibitors. Retinoids have been reported to regress painful, verrucous tumors. Physicians shall not be tempted to treat pigmentation with lasers, it may potentially flare skin inflammation. Photoprotection should be emphasized as ultraviolet exposure was found to aggravate cutaneous lesions (1).

An eye examination should be done as soon as IP diagnosis is concluded as this may be visual protective. A protocol used to screen for retinopathy of prematurity should be utilized. Evidence of peripheral vasculopathy warrants an examination under general anesthesia with fundus photography and fluorescein angiography. Argon laser can be used to treat the non-perfusion zone and repeated laser photocoagulation may be required. Ranibizumab had been described to treat refractory proliferative retinopathy (67) adjunctive to failed laser photocoagulations. Strabismus and retinal detachment can be repaired through surgery (68, 69). Propranolol was mentioned as a potential treatment for retinopathy of prematurity (70).

Early neonatal neurological manifestation determines long term patient prognosis and occurrence of disabilities. Most that without neonatal CNS abnormalities usually have normal physical and cognitive development. Thus, it is crucial for a detailed early neurological examination to be done after an accurate dermatological examination. Seizures should be investigated with an electroencephalogram (EEG) and a brain MRI. The two main treatment objectives during the neonatal period include antiepileptic treatment and anti-inflammatory drugs. Antiepileptic of choice will depend on the seizure semiology and the age of the patient (71–73), while steroid is the anti-inflammatory drug of choice. Recently, anti-TNF had been used with success. Gene therapy is under investigation for its potential in correcting severe cerebrovascular pathology (74, 75). Those who suffer from neurological sequelae should be managed by a rehabilitation team including a physician, physiotherapist, speech therapist, and occupational therapist as early as possible to alleviate neurocognitive and orthopedic complications. Those without neurological manifestation should still be routinely followed up in order to detect new neurological, neurocognitive, and/or epileptological manifestations.

Children should be under have a regular dental follow-up to pick up dental manifestation and maintain teeth functioning. Issues that may arise include multiple agenesis, coronary morphological abnormalities, dentofacial orthopedics anomalies, and delayed or absent tooth eruption. Interim dentures and prosthodontic treatment could be used to replace lost dentition and for tooth relocation and alignment. Definitive implant-prothetic and orthodontic rehabilitation can be initiated when growth has halted. Multidisciplinary assessment involving an implantologist periodontologist, and specialist in dentofacial orthopedics and prosthesis may be required (1).

Future directions and conclusion

Aside from having PCR and Sanger sequencing as the gold standard method for genetic testing in IP, further innovation and advancement of NGS with established strategies are needed to increase the sensitivity and specificity of IP molecular diagnosis. Clinical variations between positive and negative *IKBKG* pathogenic variant cohorts indicate the need for in-depth analysis of the key genotypic and phenotypic differences between these groups. A greater extent of understanding of the genotype-phenotype correlation of IP will support clinicians to direct investigations and counseling for affected individuals and their families regarding prognosis and future reproductive choices. Lastly, clinical treatment which involves identifying possible early immunosuppressants to reduce inflammatory markers could be a potential treatment strategy to reduce disability leading, such as retinal and cerebral ischemia. This may aid in the prevention and optimal management of serious complications of IP.

Author contributions

WY and KH conceptualized the project. KH, HL, ZP, and WY wrote the manuscript. HL designed the figure and prepared the table. KH, WY, HL, ZP, KL, and ZL provided vital guidance and insight to the work. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.900606/full#supplementary-material

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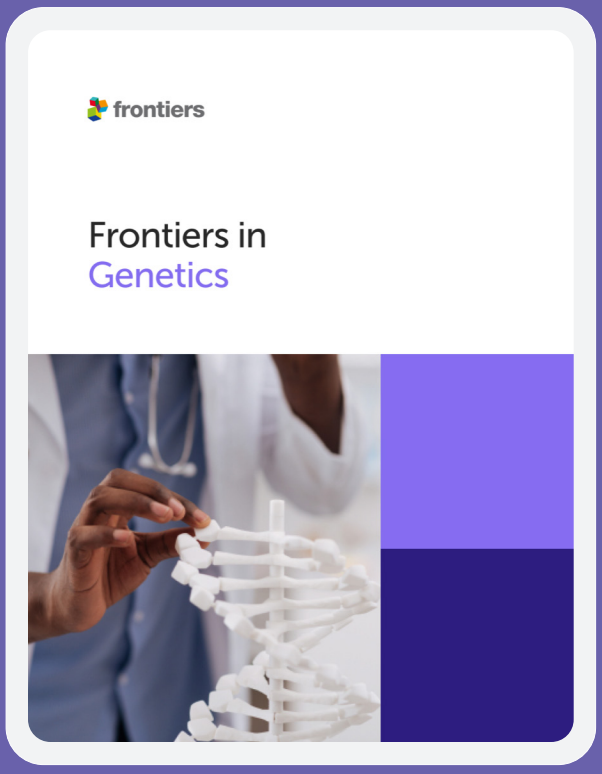

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