Genetic analysis of seven patients with inherited ichthyosis and Nagashima-type palmoplantar keratoderma

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Abstract. Inherited ichthyosis comprises a series of heterogeneous dermal conditions; it mainly manifests as widespread hyperkeratosis, xerosis and scaling of the skin. At times, overlapping symptoms require differential diagnosis between ichthyosis and several other similar disorders. The present study reports seven patients with confirmed or suspected to be associated with ichthyosis by conducting a thorough clinical and genetic investigation. Genetic testing was conducted using whole-exome sequencing, with Sanger sequencing as the validation method. The MEGA7 program was used to analyze the conservation of amino acid residues affected by the detected missense variants. The enrolled patients exhibited ichthyosis-like but distinct clinical manifestations. Genetic analysis identified diagnostic variations in the *FLG*, *STS*, *KRT10* and *SERPINB7* genes and clarified the carrying status of each variant in the respective family members. The two residues affected by the detected missense variants remained conserved across multiple species. Of note, the two variants, namely *STS*: c.452C>T(p.P151L) and c.647_650del(p.L216fs) are novel. In conclusion, a clear genetic differential diagnosis was made for the enrolled ichthyosis-associated patients; the study findings also extended the mutation spectrum of ichthyosis and provided solid evidence for the counseling of the affected families.

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Introduction

Ichthyosis is an umbrella term for a large heterogeneous group of disorders of cornification, which are usually monogenetic and mainly characterized by widespread hyperkeratosis, xerosis and scaling of the skin; at times, ichthyosis is also associated with syndromic features (1). Hundreds of genes, encoding for their corresponding proteins, have a role in the normal differentiation of keratinocytes and participate in the formation of a functional epidermal barrier (2). Therefore, inherited ichthyosis and related keratinization disorders may have a complex etiology and overlapping manifestations; this poses a huge challenge for clinical interpretation.

In general, four main types of nonsyndromic ichthyosis have been reported, namely ichthyosis vulgaris (I. vulgaris or IV), X-linked ichthyosis, autosomal recessive congenital ichthyosis (ARCI) and keratinopathic ichthyosis (1). Thus far, at least 67 genes associated with various forms of inherited ichthyosis, either syndromic or nonsyndromic, have been identified; these include the filaggrin gene [*FLG*; online mendelian inheritance in man (OMIM) no. 135940], steroid sulfatase (*STS*; OMIM no. 300747), keratin genes (*KRT1*/*KRT10*; OMIM no. 139350/148080) and member 12 of the ATP-binding cassette subfamily A (OMIM no. 607800) (3). Following advances in genetic diagnostic methods based on next-generation sequencing (NGS), ~80-90% of inherited ichthyosis cases can be resolved at present; this is beneficial not only for discovering genetic causes and novel mutations but also for establishing genotype-phenotype associations (4-7).

As the most common type of ichthyosis (prevalence, ~1/300), IV (OMIM no. 146700), with a typical feature of fine, pale-grey scaling, is caused by autosomal, semi-dominant, inherited loss-of-function mutations in the *FLG* gene (8). Patients with biallelic *FLG* mutations, accounting for ~2/3 of all cases, tend to exhibit more severe symptoms than those with a single heterozygous mutation (9,10). To date, >175 *FLG* variants that can cause IV, xerosis cutis or atopic dermatitis have been detected (https://www.hgmd.cf.ac.uk/; pro V2023.2). The second most common type, namely X-linked ichthyosis (XLI), has an approximate prevalence of 1/2,000 in males, and it is caused by STS deficiency (11); this disorder

is frequently associated with other clinical issues, e.g., cryptorchidism or social communication deficits, such as attention deficit or hyperactivity syndrome and autism, which reflects the pleiotropy of the *STS* gene (12,13). Another common group of ichthyosis is ARCI, with a prevalence of ~1/100,000; this disorder is associated with at least 10 genes involved in the biosynthesis of acylceramide, lipid lamellae and cornified lipid envelope (14). Several other rare forms of ichthyosis frequently appear in dermatological clinical settings, which increasingly rely on genetic diagnosis for identification (15).

In the present study, seven patients with ichthyosis or similar conditions were recruited and subjected to a genetic analysis by whole-exome sequencing (WES). The detected variants were distributed among several genes, thus reflecting the heterogeneity and complexity of this disease. These variants enriched the mutation spectrum of ichthyosis and provide strong evidence for genetic counseling provided to the affected families.

Patients and methods

Subjects. This study was approved by the Ethics Committee of the First Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 20210095). Informed consent for genetic testing and the results to be used in the study was obtained from all participants. Furthermore, written informed consent was obtained from the parent/legal guardian of the patients for the publication of the details of their medical case and any accompanying images. All procedures performed in the present study were in accordance with the Declaration of Helsinki (1964) and its later amendments or comparable ethical standards.

Between January 2018 and December 2021, patients with hyperkeratosis, xerosis and scaling of the skin, particularly those with a family history of the symptoms, were collected. The principle of subject inclusion was based on the 'receivables' policy. The patients were clinically evaluated by physicians on the basis of routine clinical examination and family surveys. Genomic DNA was extracted from the peripheral blood specimens of the patients and their parents by using the QIAamp DNA Midi Kit (Qiagen GmbH) for further analysis.

WES. WES was used to detect the sequence variants in the probands' samples, as described in a previous study by our group (16). In brief, target-region sequence enrichment was performed using the Agilent Sure Select Human Exon Sequence Capture Kit (Agilent Technologies, Inc.). DNA libraries were tested by quantitative PCR (17), wherein the size, distribution and concentration were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). By utilizing ~150 bp pair-end reads, the NovaSeq6000 platform (Illumina, Inc.) was used for DNA sequencing with ~300 pM per sample with the NovaSeq Reagent kit (Illumina, Inc.). Sequencing raw reads (quality level Q30>90%) (17) were aligned to the human reference genome (accession no. hg19/GRCh37) by using the Burrows Wheeler Aligner tool (https://www.geneticsmr.com/keywords/burrow-wheeler-aligner-tool). PCR duplicates were removed using Picardv1.57 (https://github.com/broadinstitute/picard). Variant calling was conducted with the Verita Trekker® Variants Detection system (v2.0; Berry Genomics) and the Genome Analysis Tool Kit (https://software.broadinstitute.org/gatk/). The variants were annotated and interpreted using ANNOVAR (v2.0) (18) and the Enliven® Variants Annotation Interpretation system(Berry Genomics)according to the common guidelines issued by the American College of Medical Genetics and Genomics (ACMG) (19). To accurately interpret variant pathogenicity, we referred to three frequency databases (ExAC_EAS; http://exac.broadinstitute.org;gnomAD_exome_EAS,http://gnomad.broadinstitute.org;and 1000G_2015aug_eas, https://www.internationalgenome.org) and the Human Gene Mutation Database (HGMD) pro V2021.10 (https://www.hgmd.cf.ac.uk/ac/index.php). Revel score (a combined method of pathogenicity prediction, with a threshold for damage of ≥0.700) (20) and pLI score (which represents the tolerance for truncating variants) were also used. Sanger sequencing (21) was performed on the suspected variants as the validation method, with the 3730 DX Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Conservation and structural analysis of missense variants. The evolutionary conservation of all affected amino acid residues by the corresponding missense variants was analyzed using the online tool MEGA7 (http://www.megasoftware.net/.php; accessed on September 25, 2022), with default parameters. Furthermore, the SWISS-MODEL online program (https://swissmodel.expasy.org/) with default parameters was used to generate models and compare the structures of wild-type (WT) and mutant (MT) proteins with the missense variants.

Results

Clinical manifestations. A total of seven patients with possible ichthyosis were recruited at the First Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 20210095) between February 2018 and December 2021. All seven patients showed a dermatological phenotype that was suspected to be associated with ichthyosis; however, there were certain differences and each patient had their own characteristics (Table I). Case 1 was a 15-year-old female patient with ichthyosis vulgaris. Case 2 was a 4-year-old male patient whose father and grandfather were also affected by ichthyosis. Case 3 was a 17-year-old female patient whose main clinical presentation was ichthyosis and atopic dermatitis. Case 4 was a 2-month-old male with ichthyosis and cryptorchidism as the main clinical symptoms. Case 5 was an 18-year-old female with the main clinical symptoms of ichthyosis and attention deficit; this patient's father also had ichthyosis. Case 6 was a 1-month-old male with the main clinical symptoms of hyperkeratosis and scaly skin. Case 7 was a 16-year-old female with the main clinical symptoms of palmoplantar hyperkeratosis and acanthosis. Fig. S1 shows the representative clinical features of several patients (based on the discretion of patients or their guardians to disclose privacy, clinical images of only part of the patients were included).

Genetic findings. Table I summarizes the detailed information of each genetic variation, including the frequency in the three population-based databases, the index status in the HGMD database, the Revel score and the pathogenicity level

Table I. Clinical indications and genetic variations of the seven cases in this study.

Case no.	Age	Sex	Main clinical indication	Gene	Genomic variation	Protein variation	Frequency in 3 databases[a]	HGMD pathogenicity level	Revel[b] score	ACMG level (evidence)	Allele frequencies in gnomAD	CADD score
1	15 years	F	Ichthyosis vulgaris	*FLG*	c.7945delA	p.S2649Vfs*94	0.004; 0.0035; 0.0020	DM	/	P (PVS1+PP5+PM2)	$9.98x10^{-5}$	/
2	4 years	M	Ichthyosis vulgaris	*FLG*	c.6950_6957del	p.S2317fs	0.005; 0.0032; 0.0027	DM	/	P (PVS1+PP5+PM2)	$1.31x10^{-4}$	/
3	17 years	F	Ichthyosis vulgaris; atopic dermatitis	*FLG*	c.3321delA	p.G1109Efs*13	0.0069; 0.0090; 0.0098	DM	/	P (PVS1+PP5)	$2.32x10^{-4}$	/
4	2 months	M	Ichthyosis; cryptorchidism	*STS*	chrX:6968331_7894165del (0.93Mb)	Whole protein absence	0; 0; 0	DM	/	P (PVS1+PS4_Supporting +PM2+PP4)	0	/
5	18 years	F	Ichthyosis; attention deficit	*STS*	c.452C>T	p.P151L	0; 0; 0	/	0.897	LP (PM1+PM2+PM5+PP4)	0	0.453
6	1 month	M	Hyperkeratosis; scaly skin; keratin clumping?	*KRT10*	c.449T>C	p.M150T	0; 0; 0	DM	0.954	P (PS3+PP3+PP5+PM1 +PM5+PM2)	0	4.387
7	16 years	F	Palmoplantar hyperkeratosis; acanthosis	*SERPINB7*	c.796C>T	p.R266*	0.0072; 0.0070;0.0119	/	/	LP (PP5+PM2)	$2.50x10^{-4}$	/
					c.647_650del	p.L216fs	0.0007; 0.0009; 0.003	/	/	LP (PVS1+PP5+PM2)	$6.16x10^{-6}$	/

[a]Three databases: 1000g2015aug_eas (https://www.internationalgenome.org/); ExAC_EAS (http://exac.broadinstitute.org); gnomAD_exome_EAS (http://gnomad.broadinstitute.org/). [b]Revel is an ensemble method for predicting the pathogenicity of missense variants on the basis of individual tools: MutPred, FATHMM, VEST, PolyPhen, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP, SiPhy, phyloP and phast Cons (http://dx.doi.org/10.1016/j.ajhg.2016.08.016). ACMG, American College of Medical Genetics and Genomics; HGMD, Human Gene Mutation Database (Professional Version 2021.10); DM, disease-causing mutation; P, pathogenic; LP, likely pathogenic; PVS1, variation criterion 'pathogenetic very strong'; PS1-4, variation criterion 'pathogenetic strong'; PM1-6, variation criterion 'pathogenetic moderate'; PP1-5, variation criterion 'pathogenetic supporting'; M, male; F, female; STS, steroid sulfatase; FLG, filaggrin; KRT, keratin; ABCA, ATP-binding cassette subfamily A.

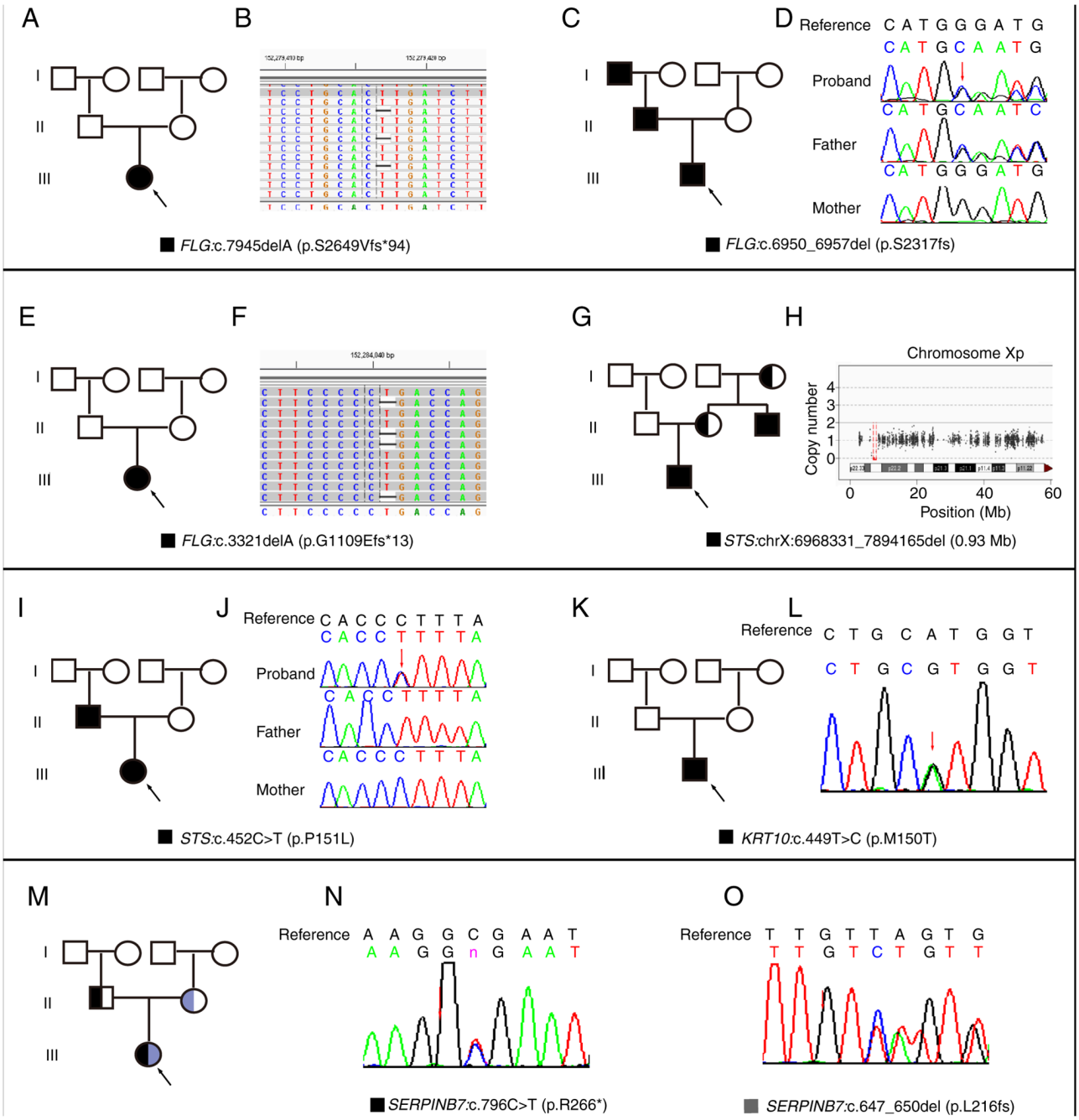

Figure 1. Pedigree diagrams and genetic variations of the seven cases. (A) Pedigree of Case 1. (B) Screenshot of the Integrative Genomics Viewer of the BAM files of the heterozygous *FLG*:c.7945delA variant in the proband of Case 1. (C) Pedigree of Case 2. (D) Sanger sequencing results of the *FLG*:c.6950_6957del variant in each family member of Case 2. (E) Pedigree of Case 3. (F) BAM image of the heterozygous *FLG*:c.3321delA variant in the proband of Case 3. (G) Pedigree of Case 4. (H) Gross copy number calling indicating the one-copy loss of the *STS* gene in Case 4. (I) Pedigree of Case 5. (J) Sanger sequencing results of the *STS*:c.452C>T variant in each family member of Case 5. (K) Pedigree of Case 6. (L) Sanger sequencing result of the *KRT10*:c.449T>C variant in the proband of Case 5. (M) Pedigree of Case 7. (N) Sanger sequencing result of the *SERPINB7*:c.796C>T variant. (O) Sanger sequencing result of the *SERPINB7*:c.647_650del variant. STS, steroid sulfatase; FLG, filaggrin; KRT, keratin; ABCA, ATP-binding cassette subfamily A.

according to the ACMG criteria. Fig. 1 shows the results of NGS or Sanger sequencing verification, together with the pedigree diagram and the variant carrying status of each family.

In all seven patients, the diagnostic variations were identified in ichthyosis-associated causative genes. Cases 1-3 were associated with IV by known *FLG* variants reported by previous studies (21-23); Case 1 (Fig. 1A and B) and Case 3 (Fig. 1E and F) were *de novo*, and Case 2 was familial (Fig. 1C and D). Cases 4 and 5 were XLI due to *STS* variations; Case 4 had a previously reported variant with deletion of the entire *STS* gene (24,25), inherited from his heterozygous carrier mother (Fig. 1G and H), while Case 5 carried a novel variant, namely *STS*:c.452C>T(p.P151L), inherited from her symptomatic father (Fig. 1I and J). Case 6, with a *de novo* known heterozygous *KRT10*: c.449T>C(p.M150T) variant (26), was determined to have epidermolytic hyperkeratosis (OMIM no. 113800; Fig. 1K and L). Case 7 had a compound heterozygous variation in *SERPINB7*, which comprised c.796C>T(p.R266*) and c.647_650del(p.

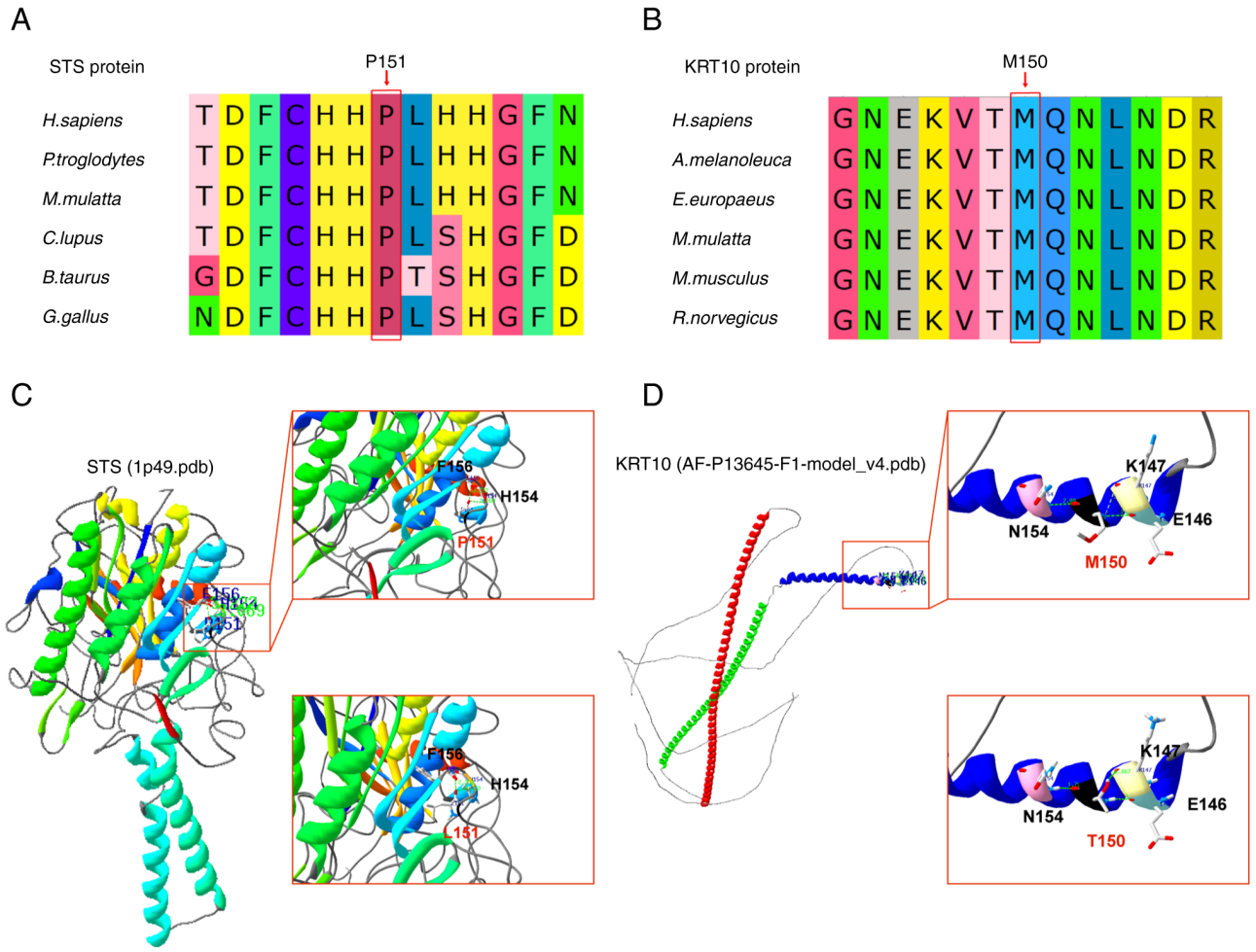

Figure 2. Conservation and structural analysis of the missense variants. (A) Conservation of the residue STS:P151 among the species. (B) Conservation of the residue KRT10:M150 among the species. (C) Structural comparison of the WT and P151L mutant of STS protein segments. The length of the hydrogen bonds was altered. (D) Structural comparison of the WT and M150T mutant of KRT10 protein segments. The length of the hydrogen bonds was altered. STS, steroid sulfatase; KRT, keratin; WT, wild-type.

L216fs); thus, this patient was determined to have palmoplantar keratoderma, Nagashima-type (OMIM no. 615598). These two variants were inherited from her parents (Fig. 1M-O).

Conservation and structural analysis of the associated missense variants. Among the variants, there were two missense variants, namely *STS*: c.452C>T(p.P151L) and *KRT10*: c.449T>C(p.M150T). Conservation analysis for the amino acid residues affected by each of them indicated that these residues were highly conserved across species (Fig. 2A and B). The protein databank (PDB; (https://www.rcsb.org/structure/1P49 for STS:p.P151L) and alpha fold (AF; AF-P13645-F1; https://alphafold.ebi.ac.uk/entry/P13645 for KRT10:p.M150T) were used for prediction and structural analysis of these two missense variants. Of note, the results showed that neither mutant significantly affected the protein structure but altered only the length of the local hydrogen bonds, which may affect the stability of the protein structure (Fig. 2C and D).

Discussion

Inherited ichthyosis and keratodermas include a phenotypically heterogeneous group of disorders; these conditions are associated with several genes involved with the structural components of the epidermis, epidermal lipid metabolism, cell-cell adhesion or keratinocyte differentiation (3). Patients with these disorders generally manifest with dry and scaly skin, with considerable phenotypic variation. Among the traditional diagnostic methods, electron microscopy and tissue immunofluorescence can enable the identification of specific types; however, the cost, invasiveness and tediousness of these methods limit their application (27,28). NGS, with its advantages of high-throughput, efficiency and accuracy, is gradually becoming the first-line technology for diagnosing inherited dermal disorders (5,6,9,16).

The Xp22.31 microdeletion containing the *STS* gene accounts for >80% of the pathogenesis of XLI (13). In Case 5 in the present study, the proband carried a paternally inherited heterozygous *STS* missense variant and the proband's father also showed typical ichthyosis symptoms. This situation is rare, because XLI is usually considered as an X-linked recessive disease, and female carriers with Xp22.31 duplication tend to manifest this disease with dermal symptoms such as blistering/desquamation (29). Hence, it is interesting to further investigate how this missense variant affects the function of STS and causes indications in this female carrier in Case 5.

The results of the conservation analysis were supportive of the pathogenicity of this variant.

Keratinopathic ichthyosis (KI) is caused by mutations in the keratin genes, namely *KRT1*, *KRT10* and *KRT2*, with mutations in *KRT10* accounting for >50% of cases (15). The *de novo* missense variant carried by Case 6 of the present study was originally detected by Paller *et al* (26) as early as 1994 and was found to be associated with epidermolytic hyperkeratosis, a severe subtype of KI; this finding indicates that this variant is valuable as a screening target for patients with KI. The amino acid affected by this variant remains highly conserved across species, further supporting its pathogenicity. The results of the structural analysis of this case and the missense variant in Case 5 were not unequivocally strongly supportive; however, more detailed analyses, such as molecular dynamics simulations and *in vitro* functional experiments, are required to clarify the mechanism of specific variations (30).

Case 7, which was initially a confusing case, was finally determined to not be associated with typical ichthyosis by genetic diagnosis. The patient had Nagashima-type palmoplantar keratosis (NPPK; OMIM no. 615598). NPPK was initially thought to be caused by biallelic putative loss-of-function mutations in *SERPINB7* (OMIM no. 603357), and the c.796C>T(p.R266*) mutation was considered a major founder mutation in the East Asian population (31). To date, >20 causative variants have been identified by several studies [STS: c.452C>T(p.P151L) (https://www.hgmd.cf.ac.uk/) (32-36)]. Our finding of Case 7 not only confirmed the widespread prevalence of this c.796C>T variant but also contributed one novel variant, c.647_650del(p.L216fs), to extend the mutation spectrum of the *SERPINB7* gene. A noteworthy finding is that the proband's other siblings exhibited a 25% likelihood of developing this disease; hence, appropriate genetic testing is recommended.

Patients with hyperkeratosis, xerosis and scaling of the skin were recruited into the present study. A total of seven patients with suspected ichthyosis were recruited in our centers. Based on the genetic findings, appropriate counseling was offered to their families and it was recommended that the patients' relatives should also undergo targeted testing. For the families with a familial inheritance pattern, such as Cases 2, 4, 5 and 7, it was recommended that they consider prenatal diagnosis of subsequent pregnancies to determine the course of pregnancy or to plan for adequate caretaking of the newborns. For the families with the *de novo* pattern (Cases 1, 3 and 6), they were reminded that the risk of future recurrence is not extremely low because of the possibility of gonad mosaicism (37). As different variants lead to different phenotypes in these seven patients, the genotype-phenotype correlation of ichthyosis requires further elucidation with more genetic data.

In conclusion, in the present study, a comprehensive clinical and genetic analysis of seven patients with ichthyosis and NPPK was conducted and their respective diagnostic mutations were detected. WES detected the diagnostic variants of the *FLG*, *STS* and *KRT10* genes in these seven patients. A total of eight variants were detected and confirmed. Among all the variants, two were identified for the first time, namely *STS*: c.452C>T(p.P151L) and c.647_650del(p.L216fs). The current findings further enrich the mutant spectrum of ichthyosis and a new variant in the *SERPINB7* gene was identified in a patient with NPPK; this finding provides a basis for further investigation of its pathogenesis.

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Availability of data and materials

The data that support the findings of this study are available in the figshare repository at https://doi.org/10.6084/m9.figshare.22680832. Due to patient self-determination, only 4/7 cases of data were publicly released and the remaining data maybe requested from the corresponding author.

Authors' contributions

GQZ initiated the project and was involved in the conception and design of the study. JZ and YY collected cases and performed clinical evaluation. YT, HYH and JZ performed experimental validation. LXZ analyzed the data. JZ wrote the manuscript. GQZ revised the manuscript. All authors have read and approved the manuscript. YT and YY checked and confirmed the authenticity of the raw data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 20210095). All research participants or their legal representatives signed informed consent forms for participation in clinical and genetic testing. All methods were carried out in accordance with relevant guidelines and regulations.

Patient consent for publication

All study participants or their legal representatives provided written consent to publish their clinical details and images.

Competing interests

The authors declare that they have no competing interests.

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