

# Identification of a novel *PLS1* heterozygous variant causing autosomal dominant non-syndromic hearing loss

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**Abstract.** Congenital disabling hearing impairment is a prevalent sensory deficit, affecting >30,000 newborns annually in China. Non-syndromic hearing loss (NSHL) accounts for a notable proportion of these cases, ~60% of which are attributed to genetic defects by heterogeneity. Although >120 genes have been linked to NSHL, a definitive molecular diagnosis remains elusive for approximately one-half of the individuals undergoing genetic testing. In the present study, a Chinese family with NSHL was investigated using next-generation sequencing of the affected members, with validation by Sanger sequencing. A novel variant was identified, namely plastin-1 (*PLS1*) c.981+5G>A. Functional analysis by reverse transcription PCR revealed that this variant induces exon skipping, establishing its pathogenic mechanism. *PLS1* encodes the actin-bundling protein plastin-1, which is highly abundant in the stereocilia of hair cells. Recent studies have implicated *PLS1* in hearing loss; therefore, the present study provides direct functional validation of its pathogenicity, expanding the pathogenic variation spectrum of *PLS1* and offering valuable insights into clinical diagnostic advancements and prenatal screening.

## Introduction

Hearing loss is a common congenital sensory impairment, affecting 2-3 out of every 1,000 newborns in China (1). Genetic factors are a primary cause, accounting for ≥50% of cases of severe bilateral neonatal hearing loss. Among these cases, 70% are non-syndromic hearing loss (NSHL), while the remainder are syndromic, presenting with additional abnormalities and clinical features, such as visual impairment and developmental delays. Furthermore, genetic factors are also implicated in delayed childhood hearing loss (2). The majority of NSHL

cases follow monogenic hereditary patterns; however, the high degree of clinical and genetic heterogeneity often complicates the identification of a definitive molecular etiology for numerous individuals (3). The advent of next-generation sequencing has revolutionized the genetic diagnosis of hearing loss; its high-throughput capacity has facilitated the identification of numerous pathogenic variants and novel hearing loss genes. To date, >120 genes have been associated with NSHL (4).

The ability of the auditory system to process sound relies on precise coordination among proteins. Within the inner ear, hair cells function as sensory receptors, converting mechanical sound vibrations into electrical signals (5). The apical surface of hair cells is adorned with stereocilia, which are microvillar projections, including static and motile cilia (6,7). Plastin-1 (*PLS1*) is characterized by four calponin-homology domains and an EF-hand calcium-binding motif for linking filamentous (F)-actin (8). The rigidity and structural integrity of these stereocilia bundles are key in mechanotransduction and are maintained by actin-binding proteins, including *PLS1* (9). The existence of numerous F-actin crosslinkers regulates the rigidity of the stereocilia, thereby modulating the sensitivity of the sound-to-mechanotransduction process (9). Studies on plastin-1-deficient mice have demonstrated that this protein is key for the proper bundling of actin filaments within stereocilia, with its absence leading to stereocilia pathology and hearing impairment (10,11). The key role of *PLS1* in human hearing was first established in a study by Schrauwen *et al* (12), which identified it as a pathogenic gene in an NSHL family. Subsequent reports have further demonstrated that *PLS1* mutations may be a cause of deafness (13-15).

In the present study, the identification of a novel splicing mutation of the *PLS1* gene (c.981+5G>A) in a family with NSHL through whole-exome sequencing is reported. The mutation was investigated to elucidate the molecular mechanism underlying the deafness phenotype in this family. The findings expand the mutation spectrum of *PLS1* and reinforce its key role in hearing, providing valuable insights for improving the clinical and molecular diagnosis, as well as prenatal screening.

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## Materials and methods

*Clinical characteristics.* At the Northwest Women's and Children's Hospital (Xi'an, China), an affected family was

identified in December 2024 when the mother came for advice on having a second child. A 7-year-old girl and her mother, aged 36 years, exhibited congenital hearing loss within this family. Subsequently, clinical information was comprehensively collected from the affected individuals and peripheral blood samples (6 ml each) were obtained from the proband, and her parents and grandparents, in EDTA-anticoagulant tubes. All patients or their legal guardians provided written informed consent for both genetic counseling and molecular genetic testing. The present study was approved by the Research Ethics Committees of Northwest Women's and Children's Hospital and was conducted in accordance with the ethical principles of the Helsinki Declaration for medical research.

**Molecular genetics analysis.** Genomic DNA was extracted from peripheral blood samples. Library preparation, sequencing and data analysis were performed as previously described (16). Briefly, the standard procedure included DNA fragmentation, end repair, amplification purification and library quality control. Target regions were captured and enriched to ensure sufficient coverage. Multiplexed sequencing was performed on the AmCareSeq 2000 sequencer (Guangzhou Jiajian Medical Testing Co., Ltd.) using a 2x150 bp paired-end sequencing kit (Guangzhou Jiajian Medical Testing Co., Ltd.). The sequencing covered all coding exons and flanking 20-base pair introns, generating raw FASTQ data with an average depth of ~200x. Raw FASTQ data were processed using a standard bioinformatics pipeline whereby: i) Trimmomatic software (version 0.39) (17) was used to remove low-quality reads (Phred quality score <20; read length <50 bp) and adapter-contaminated reads; ii) clean reads were aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner software package (version 0.7.15) (18) under default parameters; iii) aligned Binary Alignment/Map files were processed with the Genome Analysis Toolkit (version 4.4.0; Broad Institute) for duplicate marking ('MarkDuplicates'), base quality score recalibration, variant calling ('HaplotypeCaller') and outputting variants in Variant Call Format files; and iv) variants were filtered by excluding those with a minor allele frequency (MAF) >1% in public databases [Exome Aggregation Consortium (<https://avillach-lab.hms.harvard.edu/access-data/open-data>); Genome Aggregation Database (<https://gnomad.broadinstitute.org>)] low-quality copy number variation (CNV) and synonymous or deep intronic variants not near splicing sites. Furthermore, pathogenicity and evolutionary conservation analyses were analyzed using the PolyPhen tool (version 2) and VarCards database (version 2.0) (19). The final pathogenicity assessment of variants and CNVs was conducted according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) (20). Primer pairs were then designed to validate these candidate pathogenic variants using Sanger sequencing (Table I).

**In silico analysis of novel splicing mutations and protein structure.** To predict the effect of the novel variant on mRNA transcripts of *PLS1*, the online software AUGUSTUS (<https://bioinf.uni-greifswald.de/webaugustus/>) was used

with scripts and parameters set according to the official manual (21). Prediction results were visualized using the Integrative Genomics Viewer (version 2.19.7) (22). Homology modeling was employed to construct structural models of the *PLS1* and  $\beta$ -actin (*ACTB*) complex using SWISS-MODEL (<https://swissmodel.expasy.org>) and MODELLER (version 10.8) (23). The model was constructed using a multi-template alignment strategy based on the crystal structure of human *PLS1* [Protein Data Bank (PDB): 1AOA], a plant fimbrin structure resolved by cryo-electron microscopy and *ACTB* (PDB: 3BYH). The final structural model was visualized using PyMOL (version 3.1; Schrödinger).

**Identification of splice transcripts by reverse transcription PCR.** Total RNA was extracted from the peripheral blood of all patients and controls using the TRIzol® (Thermo Fisher Scientific, Inc.) method. Furthermore, 1,000 ng of mRNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit according to the manufacturer's instructions (Takara Bio Inc.). Primers were designed (forward, 5'-CTTGGTCTTGGACTTCTCTG-3'; and reverse, 5'-TGTTCTTCCCAA GTTCCACT-3') to amplify the *PLS1* exons of interest in affected family members. Thermocycling conditions were an initial denaturation at 98°C, followed by annealing at 58°C and extension at 72°C for 35 cycles. The amplification products were verified through 1% agarose gel electrophoresis, and visualized by gel imaging system (ChemiDoc™ XRS; Bio-Rad Inc.) and then subjected to Sanger sequencing (conducted by Beijing Tsingke Biotech Co., Ltd.).

## Results

**Clinical characteristics of patients.** Within the present study, the two affected individuals exhibiting hearing loss, were members of a three-generation Chinese family from Shanxi (Fig. 1A). The proband (III-1), a 7-year-old girl, was diagnosed with congenital hearing loss, failing newborn hearing screening, which included otoacoustic emissions and automatic auditory brainstem response (Fig. 1B). The patient also exhibited an abnormal V wave response in the right ear. At age 6, pure-tone audiometry revealed bilateral symmetric severe hearing loss at medium-to-high frequencies (250-8000 Hz), with abnormal air and bone conduction thresholds (Fig. 1C). Tympanometry showed a 'type As' tympanogram in the right ear and a 'type B' in the left. Typical inflammatory symptoms were absent, ruling out conditions such as otitis media. The proband's mother, aged 36 years, also exhibited congenital hearing loss, with pure-tone audiometry demonstrating levels markedly above the normal threshold ( $\leq 25$  dB HL) (Fig. 1D). At present, both mother and daughter require hearing aids for daily life. Furthermore, the father and maternal grandparents of the proband currently exhibit no hearing abnormalities.

**Identification of a novel *PLS1* mutation through whole-exome sequencing.** To determine the genetic etiology of the family's congenital hearing loss, whole-exome sequencing was performed on the proband. After filtering out variants with an MAF >0.01 in public databases, the hearing loss-related

Table I. Primers of the Sanger sequencing performed in the present study.

Primer	Sequence
<i>PLS1</i> -E9F	5'-GAATCCAGGGAAGGCATACG-3'
<i>PLS1</i> -E9R	5'-AGGGCTGAGGGCTCTACTGG-3'
<i>ELMOD3</i> -12F	5'-GGAAAGTTACTAAAGGTCACTGA-3'
<i>ELMOD3</i> -12R	5'-AGCCAGCAGGTGGAGTAGAG-3'
<i>TNC</i> -3F	5'-GTCTGCGAACCTGGCTGGAA-3'
<i>TNC</i> -3R	5'-CATGTGCCGTGCTCCTCACT-3'

*PLS1*, plastin-1; *ELMOD3*, ELMO domain containing 3; *TNC*, tenascin-C; F, forward; R, reverse.

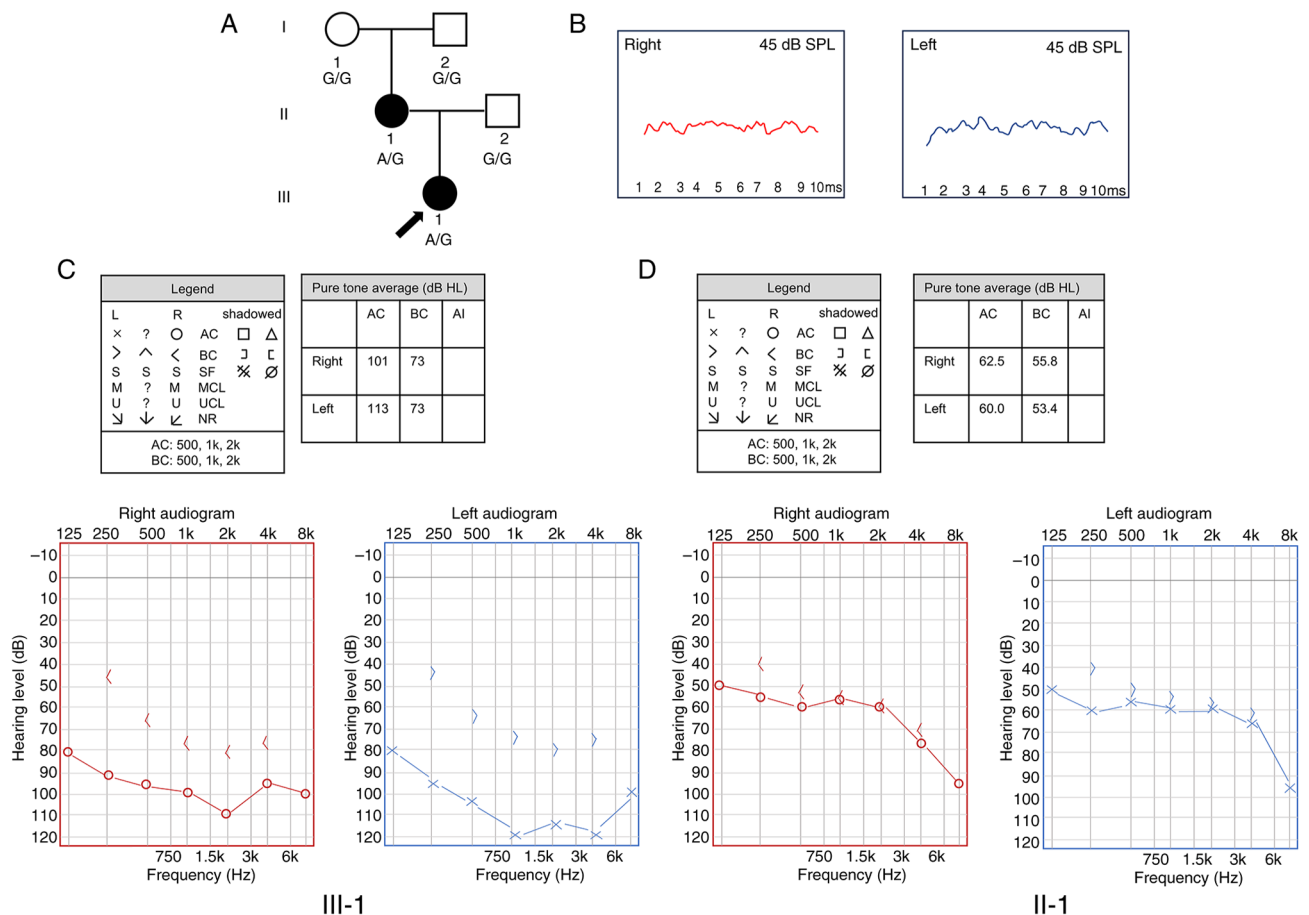


Figure 1. Identification of a family with non-syndromic hearing loss. (A) Pedigree of the family. The arrow indicates the proband. Affected family members are shown in black. (B) Results of auditory brainstem response testing at the proband's newborn hearing screening, showing an abnormal wave in the right ear. (C) Pure-tone hearing testing results of the proband at 250 Hz-8k Hz, showing flat or declining curves in the bilateral ears. Pure tone averages indicate severe-to-profound hearing loss. (D) Pure-tone hearing testing results of the proband's mother at 250 Hz-8k Hz, showing flat or declining curves in the bilateral ears. Pure tone averages indicate severe-to-profound hearing loss. AC, air conduction; BC, bone conduction; SF, sound field; MCL, most comfortable level; UCL, uncomfortable level; NR, no response; AI, asymmetry index; HL, hearing level; SPL, sound pressure level.

genes matching the patient phenotype were focused on. Subsequent bioinformatics analysis identified a novel mutation, *PLS1* c.981+5G>A (reference sequence: NM\_001145319.2), as the most likely candidate variant. Sanger sequencing was applied to further demonstrate segregation of this mutation within the disease presented by the family (Fig. 2A). The mutation was found to be present in the affected mother but absent in the healthy grandparents (data not shown), indicating the *de novo* nature of the mutation within the mother.

According to the ACMG guidelines and specialized criteria for hereditary hearing loss (24), the *PLS1* c.981+5G>A variant is classified as a Variant of Uncertain Significance (VUS) based on the following evidence codes: i) PM2 (absent or very low frequency in normal population databases); ii) PM6-P (assumed *de novo* without confirmed maternity and paternity. A phenotype consistent with the genotype but not highly specific and with a high degree of genetic heterogeneity); and iii) PPI (co-segregation with disease). Supporting

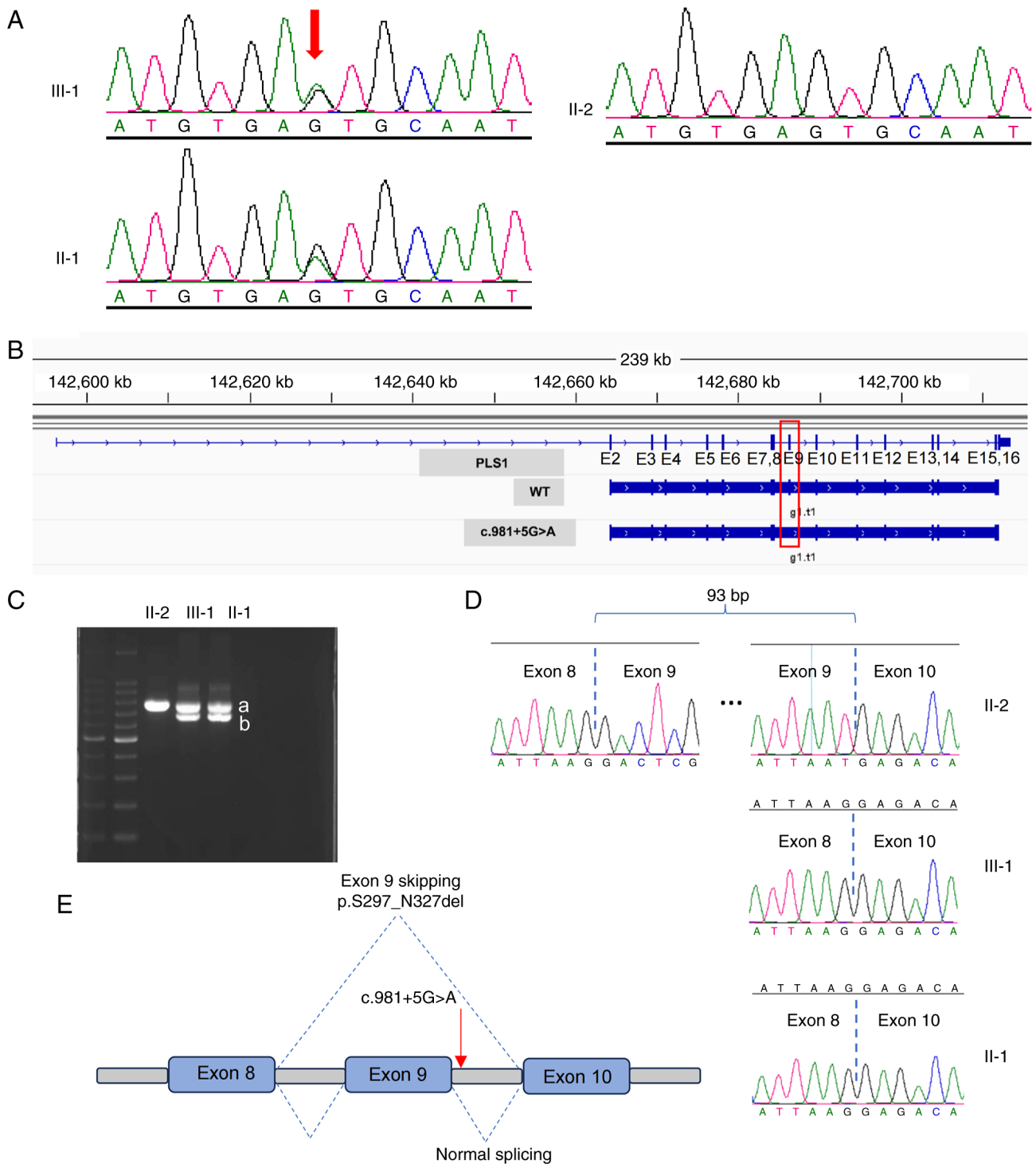


Figure 2. Effects of the novel splicing mutation c.981+5G>A in *PLS1*. (A) Sanger sequencing results demonstrating the presence of heterozygous *PLS1* c.981+5G>A in the proband (III-1) and her affected mother (II-1), but the absence of the variant in the healthy father (II-2). The arrows indicate mutated bases. (B) AUGUSTUS software results indicating that the *PLS1* c.981+5G>A variant leads to exon 9 skipping. (C) Reverse transcription PCR results showing that the proband and her mother both present with an extra band. (D) Sanger sequencing showing that band b is an aberrant transcript, which presents with 93 bp less compared with the normal band a. (E) Schematic of exon 9 skipping due to mutation c.981+5G>A in *PLS1*. *PLS1*, plastin-1; E, exon; WT, wildtype.

its potential pathogenicity, the mutation was predicted to alter *PLS1* splicing by SpliceAI (Illumina, Inc.).

*PLS1* c.981+5G>A mutation generates a novel transcript through exon 9 skipping. The *PLS1* c.981+5G>A variant is located near a splicing site. Therefore, using the AUGUSTUS program, this mutation was predicted to cause the skipping

of exon 9 (Fig. 2B). To validate this, total RNA was extracted from the lymphocytes of all patients, including the control (the father) and fragments were analyzed through reverse transcription PCR and agarose gel electrophoresis. In addition to the normal band, the proband and her affected mother exhibited an unexpected, lower band that was absent in the control (Fig. 2C). Sanger sequencing demonstrated that this extra band

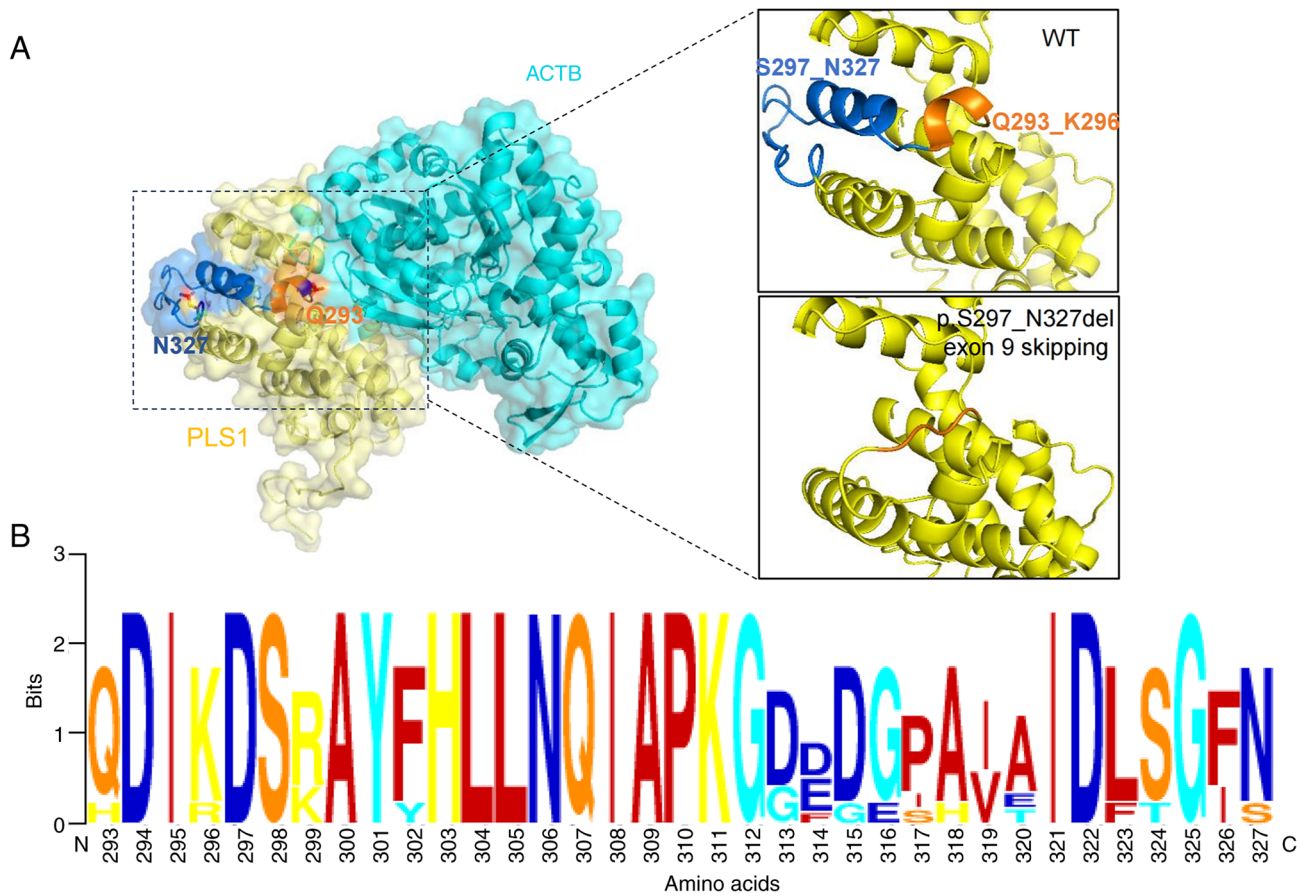


Figure 3. Mutation c.981+5G>A in *PLS1* leads to the dysfunction of plastin-1. (A) Structural model of WT and mutant (p.S297\_N327del) PLS1 (yellow) interacting with ACTB (light blue). The region at amino acid positions 297-327 is shown in dark blue. The  $\alpha$ -helix at amino acid positions 293-296 is shown in orange. (B) Conservation analysis of amino acids encoded by exon 9. PLS1, plastin-1; WT, wild-type; ACTB,  $\beta$ -actin.

corresponded to a transcript lacking the entire exon 9 (Fig. 2D). These findings demonstrate that the *PLS1* c.981+5G>A mutation causes exon 9 skipping, generating an aberrant splicing product.

**Mutant PLS1 protein impairs the PLS1-ACTB interaction.** To understand the physiological impact of the exon 9 skipping, a structural model of the actin-binding domain 1 (ABD1) of PLS1 complexed with ACTB was constructed based on homology modeling (Fig. 3A) using the SWISS-MODEL program. Structural analysis revealed that the 31 amino acid residues (p.S297\_N327) encoded by exon 9 were part of the calponin-homology (CH)-2 domain within ABD1. The deletion of this region results in the loss of an  $\alpha$ -helix (residues 293-296), which directly faces the ACTB (Fig. 3A). Therefore, the present study proposes that this structural alteration disrupts the PLS1-ACTB interaction. Furthermore, residues 293-327 are evolutionarily conserved across species, underscoring their functional importance (Fig. 3B). Collectively, these data suggest that the *PLS1* c.981+5G>A mutation impairs the binding between PLS1 and ACTB.

## Discussion

Within the present study, two additional VUSs in the probands mother were also identified by deafness gene panel, namely

ELMO domain containing 3 (*ELMOD3*) c.815+1G>C and tenascin-C (*TNC*) c.625G>A. While both genes are associated with hearing loss [Online Mendelian Inheritance in Man (OMIM)\_619500 and OMIM\_615629, respectively], they are linked to postlingual, progressive, high-frequency hearing impairment with an onset of 8-30 years of age (25,26). By contrast, both affected individuals in the present family exhibited prelingual deafness, consistent with other reported NSHL cases caused by *PLS1* mutations (12-15). Furthermore, neither of these mutations were inherited by the proband (Fig. S1) and two primer pairs were designed to rule them out (Table I). Consequently, *ELMOD3* c.815+1G>C and *TNC* c.625G>A were excluded as candidates. This underscores that the clinical features, particularly the age of hearing loss onset, are key to an accurate molecular diagnosis, especially given the large number of genes associated with hearing loss. Auditory brainstem response is the most commonly employed test to identify post-cochlear lesions and central nervous system disorders. In the present study, since the auditory brainstem response data originate from the newborn hearing screening of the proband when the patient was born, only waveforms less than a sound pressure level of 45 dB could be obtained. Although the lack of additional waveform analysis under multiple stimulation is a limitation of the present study, we consider that integrating all hearing test findings provides an adequate basis for diagnosing the congenital hearing loss of the proband.

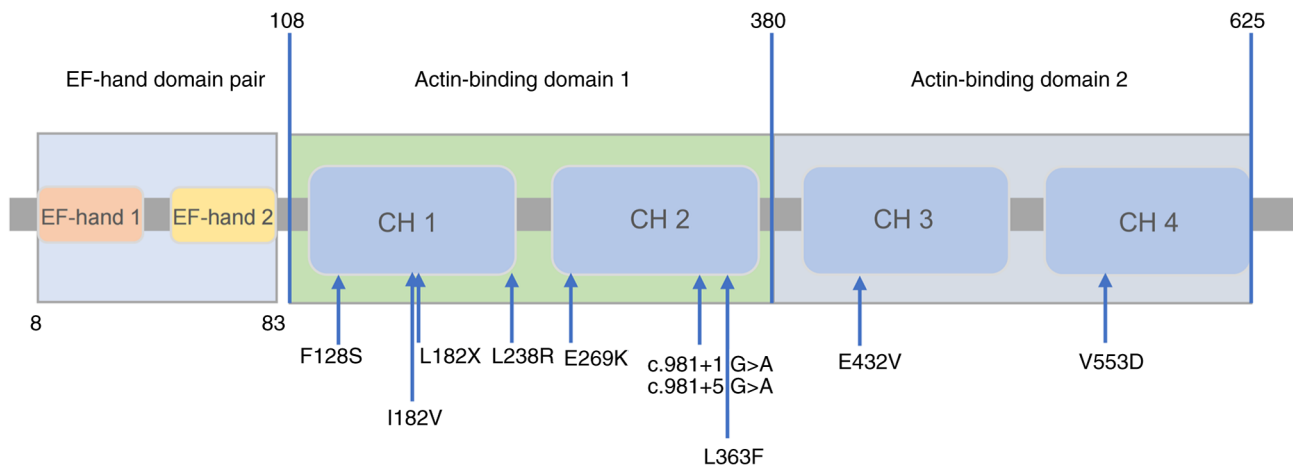


Figure 4. Schematic of plastin-1 protein. Plastin-1 includes an N-terminal regulatory region with two EF-hand domains. Following this, actin-binding domains 1 and 2 are present. Each of these domains consists of two CH domains. The majority of the currently reported mutations map in the first actin-binding domain, except for the mutations p.E432V and p.V553D. CH, calponin-homology.

*PLS1* encodes plastin-1 (also known as fimbrin), a 629-amino acid protein containing two EF-hand domains and two actin-binding domains (ABD1 and ABD2), each composed of CH domains (27). Reported cases of hearing loss due to *PLS1* mutations are scarce. To the best of our knowledge, including the present study, only 12 families with NSHL caused by *PLS1* mutations have been described (12-15), eight of which are from Europe. The remaining three originate from Asia, two of which belong to the Han Chinese population. While this may suggest a higher incidence in Europe, more data is needed to validate this. To date, 10 *PLS1* mutations causing autosomal dominant deafness have been identified, including seven missenses, two splice sites and one frameshift. Notably, both reported splicing mutations were identified in Chinese families. As summarized in Fig. 4, the majority of variants are located within the CH domains of ABD1. *PLS1* crosslinks actin filaments by using its two ABDs to recruit and bind ACTB molecules on opposite faces (28). Therefore, structural defects in ABD1 or ABD2, whether caused by missense or splicing variants, likely impair actin binding, ultimately disrupting stereocilia formation and causing hearing loss.

In the present study, results suggested that the novel c.981+5G>A variant caused the skipping of exon 9, which aligns with the effect of a previously reported mutation at another position, namely c.981+1G>A (15). It has been demonstrated that the deletion of 31 amino acids (p.S297\_N327) causes abnormal inner ear phenotypes in zebrafish through mRNA microinjection experiments (15), supporting the deleterious nature of the variant identified in the present study. This functional evidence meets the ‘PS3\_Moderate’ criterion under ACMG guidelines. Therefore, the c.981+5G>A variant was reclassified from a VUS to ‘likely pathogenic’. Moreover, RNA-sequencing analysis in HEI-OC1 cells suggests that *PLS1* contributes to hearing loss by modulating the PI3K-AKT signaling pathway and focal adhesion (15). However, the precise molecular mechanisms require further investigation.

In conclusion, the present study reports on a family with autosomal dominant NSHL caused by a novel *PLS1* splicing variant, demonstrating that the variant causes exon 9 skipping. The resulting truncation of the protein causes the loss of key

amino acids, which is predicted to disrupt the actin-binding function of *PLS1* and impair stereocilia formation. The present study therefore provides a conclusive genetic diagnosis for this family.

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#### Availability of data and materials

The data generated in the present study may be found in the China National GeneBank database under accession number CNP0008613 or at the following URL: [http://db.cngb.org/cnsa/project/CNP0008613\\_283e261b/reviewlink/](http://db.cngb.org/cnsa/project/CNP0008613_283e261b/reviewlink/).

#### Authors' contributions

CY, YX and DW performed the bioinformatics analysis and experiments. CY and JS designed the present study. CY wrote the manuscript. JS supervised the present study. CY and YX confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Research Ethics Committees of Northwest Women's and Children's Hospital (Xi'an, China; approval no. 202500802). All patients provided written informed consent for both genetic counseling and molecular genetic testing. The written informed consent of minors/children in the present study was provided by their legal guardian.

## Patient consent for publication

The proband, her father (II-2), mother (II-1), grandmother (I-1) and grandfather (I-2) provided written informed consent for the publication of all associated data.

## Competing interests

The authors declare that they have no competing interests.

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