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ORIGINAL ARTICLE

Genetic etiology study of four Chinese families with two nonsyndromic deaf children in succession by targeted next-generation sequencing

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Abstract

Background: Genetic components contribute significantly to the cause of hearing loss. Nonsyndromic hearing loss has been shown to have high genetic heterogeneity. For families who had given birth to two nonsyndromic deaf children in succession, it seems that their deafness was highly related to genetics.

Objectives: This study aimed to disclose the genetic causes of the subjects from the four Chinese families with two nonsyndromic deaf children in succession who failed to find the genetic etiology of the hearing loss by common deafness genetic screening (*GJB2*, *GJB3*, *SLC26A4*, and *MT-RNR1*, including 20 hot variants in 4 genes).

Methods: Targeted next-generation sequencing (NGS) of 127 known deafness genes was performed in probands of four families, followed by a series of comprehensive analyses of all family members combined with a literature review of related genes.

Results: We identified pathogenic variants in three families including c.919-2A>G/c.1985G>A in *SLC26A4*; c.109G>A (p.V37I) in *GJB2*; and m.7505T>C in *MT-TS1*. Sanger sequencing confirmed that these variants segregated with the hearing impairment of each family. We also identified c.331C>T/c.625-5C>T/c.5717G>A in *CDH23*; c.138T>C in *POU3F4* in two families, in which the pathogenicity in clinical was likely pathogenic or unknown.

Conclusions: Using the NGS detection technology, we found the genetic etiology of the HL in part of deaf families. Our study provided a useful piece of information for the variant spectrum of hearing loss in Chinese families with two deaf children in succession.

KEYWORDS

deafness, genetic etiology, nonsyndromic, targeted next-generation sequencing

1 | INTRODUCTION

Hearing loss (HL) is the most sensory defect that affects 1–3 in every 1,000 newborns worldwide, and half of these cases are attributed to genetic factors (Morton & Nance, 2006). Newborn hearing concurrent genetic screening for HL has been widely carried out in China. It can not only identify the children with congenital HL but also identify the late-onset

and progressive hearing impairment and aminoglycoside ototoxicity-susceptible children. In this way, we improve the abilities for early diagnosis and intervention for the hearing defects.

Nonsyndromic HL (NSHL) accounts for 70% of inherited hearing deafness (Bayazit & Yilmaz, 2006). To date, more than 120 genes have been identified as responsible for NSHL (Hereditary Hearing Loss Homepage, http://hereditaryhearing loss.org/). NSHL has an autosomal recessive (80%), autosomal dominant (18%), X-linked (1%–3%), or mitochondrial (<1%) pattern of inheritance (Bayazit & Yilmaz, 2006). However, unlike some other well-known genetic disorders caused by a single variant (cystic fibrosis) or variants in a single gene (Duchenne muscular dystrophy), in most cases, there are more than 120 genes and more than 1000 reported deafness-causing variants. This extreme genetic heterogeneity makes genetic diagnosis for NSHL exceedingly difficult (Shearer & Smith, 2015). In recent years, with the wide application of NGS technology in clinical, the genetic diagnosis rate of deafness has been improved, which makes the genetic etiology of many children with HL clear.

China's population control policy, which was implemented nearly 40 years ago, aimed at regulating family size. In recent years, with the implementation of Chinese two-child policy, which allows all families to have two children, more and more families have been found to give birth to two deaf children in succession. For families with two nonsyndromic deaf children in succession, it seems that their deafness was highly related to genetics. In Chinese Hans, variants in three genes, *GJB2*, *SLC26A4*, and *MT-RNR1*, were commonly found in deaf patients, accounting for more than 30% of genetic causes of nonsyndromic deafness (Yang et al., 2013). In this study, targeted NGS (including 127 known deafness genes) technology was used to search for the genetic etiology of the deafness for the families with two deaf children in succession who failed to find the cause of the HL by common deafness genetic screening (*GJB2*, *GJB3*, *SLC26A4*, and *MT-RNR1*, including 20 hot variants in 4 genes). Then, a series of comprehensive analyses were carried out.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

All subjects and their family members gave written, informed consent to participate in this study. This study was approved by the Ethics Committee of Tianjin Women and Children Healthcare Center.

The probands' data used in this study were derived from the healthcare records data from the Tianjin's maternal and child healthcare system. Tianjin's maternal and child healthcare system is a three-tier care system consisting of community-based health centers, district-level Women and Children Healthcare Centers (also including secondary hospitals), and a city-level (Tianjin) Women and Children Healthcare Center (also including tertiary hospitals). Tianjin, which is a municipality in the north of China adjacent to Beijing, has a population of 15 million people with about 100,000 live births annually. All the infants born in Tianjin received both newborn hearing screening (NHS) and deafness genetic screening (20 variants in 4 genes). Written informed consents were obtained from infants' parents or guardians. All newborns' information, including a list of risk factors for deafness, was collected into a newborn hearing screening database in Tianjin's maternal and child healthcare system, with an additional blood spot obtained at the time of newborn metabolic screening. Flowchart of newborn hearing concurrent genetic screening for hearing loss is shown in Figure 1.

2.2 | Newborn hearing screening

A two-step hearing screening was performed in infants. A distortion product otoacoustic emission (DPOAE) test was used as the initial screening at 48–72 h after delivery. If an infant failed to pass the DPOAE test, either bilateral or unilateral, it would be recorded as first-step DPOAE "refer." Our primary NHS rate has reached over 99% in Tianjin. All the first-step DPOAE "refer" or infants that had high-risk factors for deafness were referred to Tianjin Women and Children Healthcare Center (the only diagnosis and treatment center for children with hearing impairment in Tianjin) to receive a repeated test of DPOAE plus AABR (automated auditory brainstem response) at the age of 42 days. Those who failed to pass the two-step test would receive a further comprehensive audiological assessment at the age of 3 months.

2.3 | Newborn genetic screening

This genetic screening using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and Sanger sequencing verification technology involve 20 hot spot variants from 4 primary NSHL genes: *GJB2*, *GJB3*, *SLC26A4*, and *MTRNR1* (*12S rRNA*; including *GJB2*–c.235delC, c.299_300delAT, c.167delT, c.176_191dell6, and c.35delG; *GJB3*–c.538C>T and c.547G>A; *SLC26A4*–c.919-2A>G, c.2168A>G, c.1174A>T, c.1226G>A, c.1229C>T, c.1975G>C, c.2027T>A, c.2162C>T, c.281C>T, c.589G>A, and c.IVS15+5G>A; and *MT-RNR1*–m.1555A>G and m.1494C>T). Children who passed the NHS but had positive genetic screening results would receive annual hearing test until 6 years old. Children that had positive genetic screening results and that failed to pass the two-step NHS would receive further comprehensive audiological assessment at the age of 3 months.

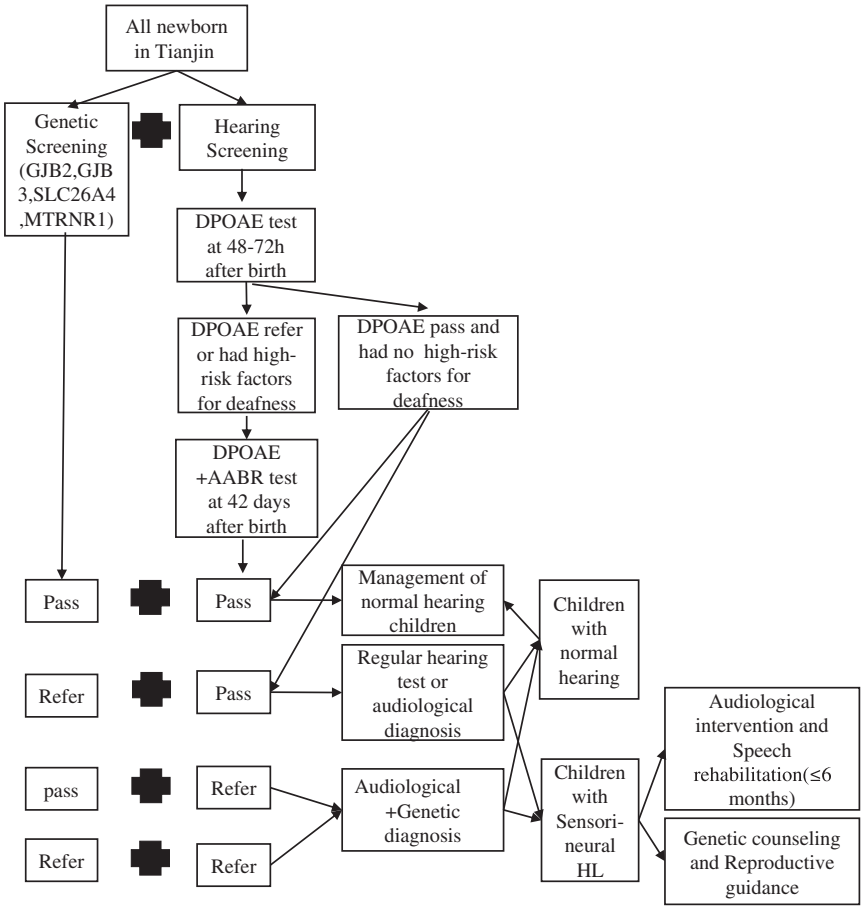

FIGURE 1 Flowchart of newborn hearing concurrent genetic screening for hearing loss (HL). DPOAE, distortion product otoacoustic emission; AABR, automated auditory brainstem response

2.4 | Hearing diagnosis

Children who failed to pass the two-step NHS would receive the first comprehensive audiological assessment (including acoustic immittance [AI], auditory brainstem response [ABR], DPOAE, and cochlear microphonic potential [CM]) at the age of 3 months in Tianjin Women and Children Healthcare Center. Children diagnosed with HL for the first time would undergo a second audiological assessment (including AI, ABR, DPOAE, CM, and auditory steady-state response [ASSR]) at about 6 months after birth. Based on the comprehensive test results, the hearing condition of the children would be evaluated to determine whether there were HL and the degree of the HL. At the same time, combined with imaging (temporal bone computer-assisted tomography [CT] or magnetic resonance imaging [MRI]) examination, appropriate intervention measures were given. Of course, all the information about the children conducting hearing test, such as electronic cases, audiological information of the children (with normal hearing or with HL), and appropriate intervention measure information were entered into the Tianjin's maternal and child healthcare system. For children with HL, audiology test would be carried out every six months.

The V-wave response threshold of Click ABR wave was used as a reference index of assessing 2–4 kHz high-frequency HL in infants. According to the criteria developed by the American Academy of Pediatrics Joint Committee on Infant hearing in 2002 (Academy & of Pediatrics Joint Committee on Infant hearing, 2002), the V-wave response threshold of Click ABR showed that 31–50dBnHL was mild abnormal, 51–70dBnHL was moderate abnormal, 71–90dBnHL was severe abnormal, and ≥91dBnHL was profound abnormal.

2.5 | Family recruited and description and clinical evaluations

The four families who had given birth to two deaf children in succession were recruited from the Department of Otolaryngology, Tianjin Women and Children Healthcare Center, Tianjin, China. The deaf children from the four families failed to find the cause of the HL by common deafness genetic screening. All the subjects were managed through Tianjin Maternal and Child Healthcare System. All the probands had bilateral sensorineural HL and had no obvious syndromic symptoms other than HL. The comprehensive clinical evaluation information, imaging examination results (temporal bone CT or MRI), audiological data (including otoscope examination, pure-tone audiometry (PTA), AI, ABR, DPOAE, CM, and ASSR), and other relevant clinical information were collected for the probands and their family

members. PTA was used to assess the degree of HL in adults and calculated as the average of the hearing level at 0.5, 1.0, 2.0, and 4.0 K Hz for the better ear. The severity of HL was defined as mild (20–40 dB), moderate (41–70 dB), severe (71–95 dB), and profound (>95 dB). The pedigrees of those hearing loss-affected families are shown in Figure 2.

2.6 | Targeted next-generation sequencing

To make a precise diagnosis, we then performed targeted NGS of related hearing impairment genes. A capture panel (NimbleGen) of hearing impairment genes was previously designed and assessed by our group. The capture panel comprised 621186 bp that covered all exons together with the flanking exon and intron boundaries (±15 bp) of 127 genes (Additional file Table S1), including 45 genes causing autosomal recessive nonsyndromic hearing impairment, 3 genes causing X-linked hereditary hearing impairment, 29 genes causing autosomal dominant nonsyndromic hearing impairment, 66 genes causing syndromic hearing impairment, and 2 genes causing maternally inherited hearing impairment.

Genomic DNA of all the family members' blood samples was extracted according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen). Then, the genomic DNA of the family was fragmented by Covaris LE220 to generate paired-end library (200–250 bp). The library was enriched by array hybridization according to previously published procedure (Wei et al., 2011), followed by elution and postcapture amplification. The products were then subjected to Agilent 2100 Bioanalyzer and ABI StepOne to estimate the magnitude of enrichment. After quality control, captured library sequencing was carried out on Illumina HiSeq 2500 Analyzers (Illumina), following the manufacturer's standard sequencing protocols, for 90 cycles per read to generate paired-end reads. Image analysis, error estimation, and base calling were performed using Illumina Pipeline software (version 1.3.4) to generate raw data.

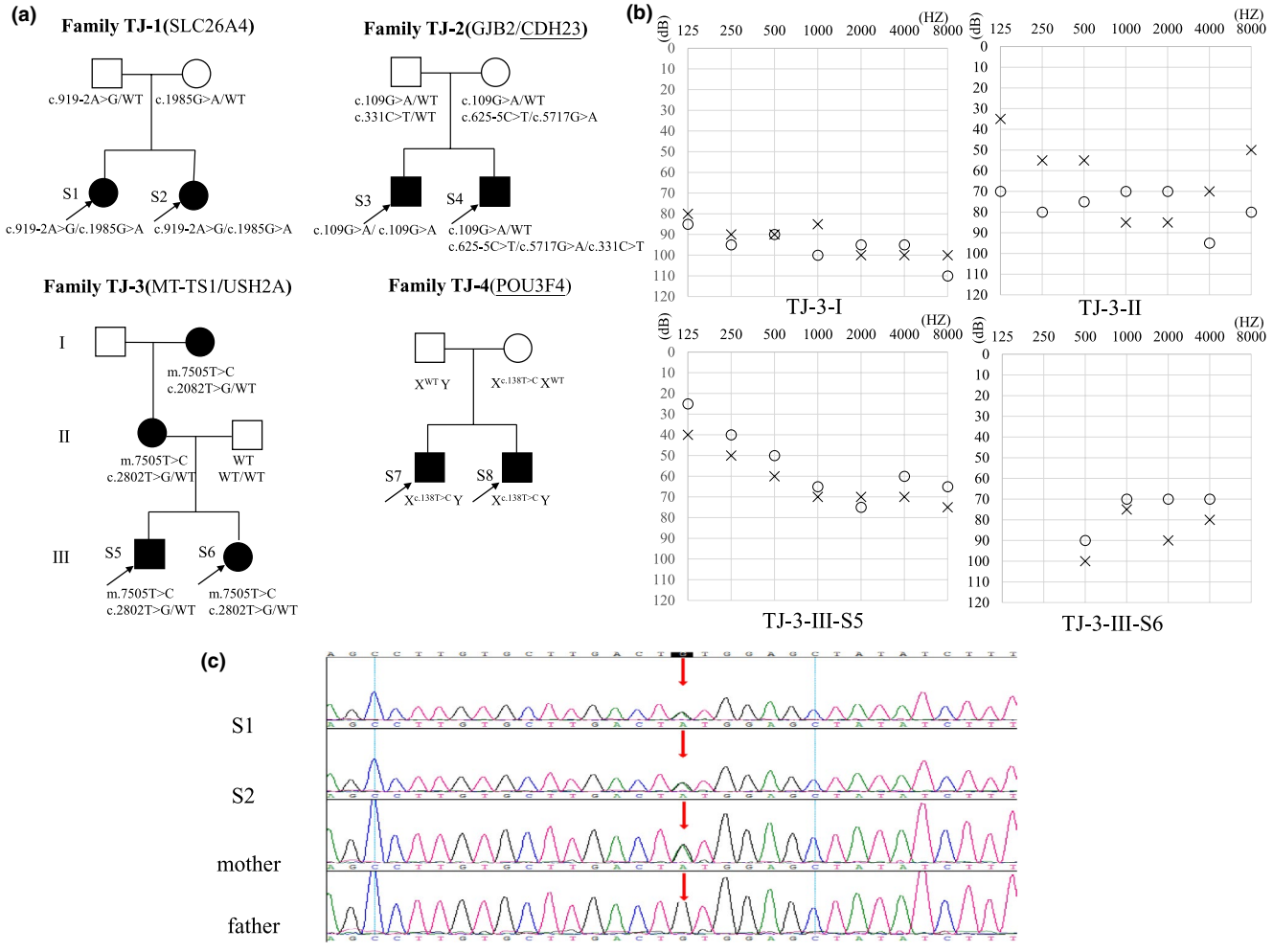

FIGURE 2 (a) Pedigrees and the variants detected in the 4 families (TJ-1–TJ-4), the arrow shows the probands (S1–S8) in each family; (b) air conduction audiogram of the 4 HL members in family TJ-3. Symbols: X—left, O—right ear, due to the young age of Ⅲ-S6, the PTA was replaced by ASSR;(c) the chromatogram of SLC26A4 c.1985G>A of Sanger sequencing in family TJ-1(variant SLC26A4 c.1985G>A was detected in the proband S1 and S2 and the mother)

To detect the potential variants in the family, we performed bioinformatics processing and data analysis after receiving the primary sequencing data. We used previously published filtering criteria to generate "clean reads" for further analysis (Wei et al., 2011). The "clean reads" (with a length of 90 bp) derived from targeted sequencing and filtering were then aligned to the human genome reference (hg19) using the BWA (Burrows Wheeler Aligner) Multi-Vision software package (Li & Durbin, 2009). After alignment, the output files were used to perform sequencing coverage and depth analysis of the target region, single-nucleotide variants (SNVs), and INDEL calling. We used SOAPsnp software (Li, Li, et al., 2009) and Samtools (Li, Handsaker, et al., 2009) to detect SNVs and indels. All SNVs and indels were filtered and estimated via multiple databases, including NCBI dbSNP, HapMap, 1000 human genome dataset, and database of 100 healthy Chinese adults.

To predict the effect of missense variants, we used dbNSFP (Li, Handsaker, et al., 2009), which contains seven well-established in silico prediction programs (Sorting Intolerant From Tolerant [SIFT], Polyphen2, LRT, Mutation Taster, and PhyloP). Pathogenic variants were assessed under the protocol issued by ACMG (Richards et al., 2015). The Human Gene Mutation Database (HGMD) was used to screen mutation reported in published studies.

All variants and potential pathogenic variants were validated using conventional Sanger sequencing methods. Segregation analysis was performed if DNA from family members was available.

3 | RESULTS

3.1 | Clinical manifestations

The 8 probands (5 males and 3 females) came from 4 Chinese families, and each family had two children with deafness in succession. All the probands, aged 2 to 14 years, were diagnosed with bilateral, sensorineural HL at the age of 3 months to 6 years. The probands had no obvious syndromic symptoms other than the HL. The degree of HL varied from moderate to severe and profound. The probands (S1–S4, S7–S8) did not pass the NHS and were diagnosed as congenital HL. Except for family TJ-3, the other three families had no family history of deafness. Among the probands, 4 cases (S1, S2, S7, and S8) of temporal bone CT results showed enlarged vestibular aqueduct (EVA), while the remaining 4 cases (S3–S6) showed normal CT results. Among them, the probands (S5 and S6) were diagnosed as auditory neuropathy spectrum disorders (ANSD) with severe or profound abnormality of ABR while DPOAE and (or) CM were normal. The pedigrees and the variants detected in the 4 families (TJ-1–TJ-4); air conduction audiogram of the 4 HL members in family TJ-3; and the chromatogram of *SLC26A4* c.1985G>A of Sanger sequencing in family TJ-1 are shown in Figure 2. Clinical and audiology characteristics of HL members in the 4 families are summarized in Table 1.

3.2 | Variant analysis

The probands (S1 and S2) in the first family TJ-1 were detected a heterozygous variant (c.919-2A>G) in *SLC26A4* in intron 7 by deafness genetic screening. Except for the family TJ-1, the results of four common gene screening in the probands of the other 3 families (TJ-1–TJ-3) were all negative. All the probands(S1–S8) from the 4 deaf families were subjected to targeted NGS of 127 known deafness genes. Candidate causative variants are summarized in Table 2.

In the TJ-1 family, the probands(S1–S2) were detected compound heterozygous variant c.919-2A>G in intron 7 and c.1985G>A (p.C662Y) in exon 17 in *SLC26A4*. Both of the two variants were pathogenic. In addition, the heterozygous

TABLE 1 Clinical and audiology characteristics of HL members in the 4 families

ID	Sex	Age	Results of primary NHS(DPOAE)	ABR (L/R)	DPOAE (L/R)	CM (L/R)	AI (L/R)	Degree of HL	Temporal bone CT results
S1	F	14 y	Refer	100/60	–/–	–/–	A/A	moderate	EVA
S2	F	9 y	Refer	95/–	–/–	–/–	A/A	profound	EVA
S3	M	8 y	Refer	60/55	–/–	–/–	A/A	moderate	Normal
S4	M	3 y	Refer	–/–	–/–	–/–	A/A	profound	Normal
S5	M	6 y	Pass	85/85	–/–	+/+	A/A	severe	Normal
S6	F	20 m	Pass	–/–	+/+	+/+	A/A	profound	Normal
S7	M	7 y	Refer	95/95	–/–	––/–	A/A	profound	EVA and MD
S8	M	4 y	Refer	100/–	–/–	–/–	A/A	profound	EVA
TJ-3-Ⅰ	F	63 y	—	–/–	–/–	–/–	A/A	severe	—
TJ-3-Ⅱ	F	37 y	—	–/–	–/–	–/–	A/A	severe	—

Abbreviations: ABR, auditory brainstem response; AI, acoustic immittance; CM, cochlear microphonic potential; DPOAE, distortion product otoacoustic emission; EVA, enlarged vestibular aqueduct; F, female; M, male; MD, mondini dysplasia; NHS, newborn hearing screening.

TABLE 2 Candidate causative variants detected in 8 probands from 4 Chinese families

ID	Gene	Variant type	Genetic pattern	Nucleotide change (transcript version)	Heterozygosity	Amino acid change	HGMD variation type
S1	SLC26A4	Splicing	AR	c.919-2A>G (NM_000441)	Het	Splicing	Pathogenic
	SLC26A4	Missense	AR	c.1985G>A (NM_000441)	Het	p. C662Y	Pathogenic
S2	SLC26A4	Splicing	AR	c.919-2A>G (NM_0004s41)	Het	Splicing	Pathogenic
	SLC26A4	Missense	AR	c.1985G>A (NM_000441)	Het	p. C662Y	Pathogenic
S3	GJB2	Missense	AR/AD	c.109G>A (NM_004004)	Hom	p. V37I	Pathogenic
S4	GJB2	Missense	AR/AD	c.109G>A (NM_004004)	Het	p. V37I	Pathogenic
	CDH23	Nonsense	AR	c.331C>T (NM_022124)	Het	p. Q111*	Likely pathogenic
	CDH23	Missense	AR	c.5717G>A (NM_022124)	Het	p. G1906E	VUS
	CDH23	—	AR	c.625-5C>T (NM_022124)	Het	—	VUS
S5	MT-TS1	Mitochondrial variant	Mitochondrial	m.7505T>C (NC_012920)	Hom	Mt DNA	Pathogenic
	USH2A	Missense	AR	c.2802T>G (NM_206933.4)	Het	p.C934W	Likely pathogenic
S6	MT-TS1	Mitochondrial variant	Mitochondrial	m.7505T>C (NC_012920)	Hom	Mt DNA	Pathogenic
	USH2A	Missense	AR	c.2802T>G (NM_206933.4)	Het	p.C934W	Likely pathogenic
S7	POU3F4	Synonymous	XR	c.138T>C (NM_000307)	Hem	p. Val46=	VUS
S8	POU3F4	Synonymous	XR	c.138T>C (NM_000307)	Hem	p. Val46=	VUS

Abbreviations: AD, autosomal dominant inheritance; AR, autosomal recessive inheritance; Hem, hemizygous variant; Hom, homozygous variant; mt DNA, mitochondrial DNA, Het, heterozygous variant; XR, X-linked recessive inheritance.

variants of c.919-2A>G and c.1985G>A (p.C662Y) were detected in the father and mother, respectively.

The family TJ-2 was very special, the older brother (S3) had a homozygous variant c.109G>A (p.V37I) in *GJB2*, while the younger brother (S4) had a heterozygous variant. In addition, the younger brother(S4) was also detected three variants in *CDH23*, containing one likely pathogenic variant (c.331C>T (p.Q111*)) and two variants (c.625-5C>T and c.5717G>A (p. G1906E)) of unknown clinical significance.

In family TJ-3(S5, S6), we identified a homoplasmic mitochondrial variant m.7505T>C in *MT-TS1*, which had been identified in one Han Chinese pedigree with maternal transmission of nonsyndromic deafness (Tang et al., 2010). And we found that the mother and grandmother also carried this variant. At the same time, the variant c.2802T>G (p.C934W) in *USH2A* was also detected in family TJ-3. Type 2A of Usher syndrome related to *USH2A* was autosomal recessive.

In addition, in family TJ-4, we identified one hemizygous candidate variant c.138T>C (p. Val46=) in *POU3F4* in probands S5 and S6, which was also detected in their mother. The inheritance mode of the nonsyndromic deafness type 2 associated with *POU3F4* followed X-linked recessive inheritance pattern. The variant c.138T>C (p. Val46=) was a synonymous variant, and the pathogenicity of this site in clinical was unknown.

4 | DISCUSSION

In this study, we identified pathogenic variants in three families, including c.919-2A>G/c.1985G>A in *SLC26A4* in family TJ-1; c.109G>A (p.V37I) in *GJB2* in family TJ-2; and

m.7505T>C in *MT-TS1* in family TJ-3. Sanger sequencing confirmed that these variants segregated with the HL of each family. We also identified c.331C>T/c.625-5C>T/c.5717G>A in *CDH23* in family TJ-2; and c.138T>C in *POU3F4* in family TJ-4, in which the pathogenicity in clinical practice was likely pathogenic or unknown. Now, we will introduce these genes and analyze the causes of deafness in each family.

4.1 | *SLC26A4* and EVA syndrome

Enlarged vestibular aqueduct (EVA), most frequently found by radiological examination (CT or MRI), is the most common form of inner ear abnormality in patients with NSHL (Usami et al., 1999). NSHL with EVA is characterized by pre- or perilingual in onset of sensorineural or mixed HL, which may be fluctuating or progressive (Pryor et al., 2005). In patients with EVA syndrome, sudden HL can be precipitated by minor head trauma or barotrauma. The *SLC26A4* (OMIM #605646), the second most common cause of inherited HL after *GJB2*, is currently recognized as the responsible gene for EVA syndrome. *SLC26A4* is located on chromosome 7q31, and it comprises twenty-one exons and encodes a transmembrane protein pendrin, the latter is mainly expressed in thyroid, inner ear, and kidney. Pendrin is closely related to a number of sulfate transporters, while it mediates mostly the transportation of $I^-$and Cl^- (Scott et al., 1999). Variants in the *SLC26A4* are responsible for Pendred syndrome (PS) and DFNB4 (NSHL with inner ear abnormalities, such as EVA with or without Mondini dysplasia (MD)) (Hone & Smith, 2003; Yuan et al., 2009).

The detection rates of *SLC26A4* variants in patients with EVA are different: 95% in China (Dai, Stew, et al., 2009); 78.1% in Japan (Tsukamoto et al., 2003); 92.31% in South Korea (81% for biallelic alleles and 11% for monoallelic variants; Park et al., 2005); and 40% in Caucasians (24% for biallelic alleles and 16% for monoallelic variants) (Albert et al., 2006). In China, the most common variant of *SLC26A4* is IVS7-2A>G (c.919-2A>G), and the variants c.919-2A>G (73%) and c.2168A-G (p.H723R) (14%) account for 87% of the total variants in the *SLC26A4* variant spectrum (Dai, Stew, et al., 2009). Although the *SLC26A4* is the primary recessive gene that causes EVA/PS, it is not the only genetic factor that causes EVAS/PS. Variants in *FOXI1* and *KCNJ10* genes may also be involved in the pathogenesis of EVAS/PS (Yang et al., ,2007, 2009).

The two probands (S1 and S2) in TJ-1 family referred universal newborn hearing screening (UNHS) with two ears and were diagnosed as congenital, bilateral, and sensorineural HL at 3 months old. The temporal bone CT results of the two probands showed no cochlear malformations. However, bilateral EVA was present. The two probands performed with c.919-2A>G single-allele variant detected by deafness genetic screening. After 127 gene sequencing, the sequence analysis of *SLC26A4* indicated that the two probands presented compound heterozygosity of a c.919-2A>G variant in intron 7 and a c.1985G>A (p.C662Y) missense variant in exon 17. Additionally, the parents with normal hearing were verified that each of them carried one pathogenic variant. The father was a heterozygous carrier of the c.919-2A>G variant, while the mother was a heterozygous carrier of the c.1985G>A (p.C662Y) variant. Both the two sisters inherited two pathogenic alleles as a genotype of compound heterozygous variants c.919-2A>G and c.1985G>A, no normal pendrin can be encoded, which explained the HL of the two probands. According to the genetic law, the probability of the parents having children with deafness again is 25%.

The variant of c.1985G>A (p.C662Y) was a missense mutation, although the probability of this site occurring in normal people was extremely low, but there had been relevant reports on the detection of this site in deaf patients. Zhao et al. performed *SLC26A4* sequencing on 1056 Chinese nonsyndromic enlargement of vestibular aqueduct (NSEVA) patients and found that total 925 NSEVA patients carried two-allele *SLC26A4* pathogenic mutations, accounts for 87.59% (925/1056) of the genetic etiology in Chinese NSEVA patients. Among the 1056 Chinese NSEVA patients, only two patients were compound heterozygosity with c.1985G>A(p.C662Y) variant. One carried c.1985G>A(p.C662Y) and c.1687_1692insA variants, while the other carried c.1985G>A(p.C662Y) and IVS7-2A>G variants. The detection rates of variant c.1985G>A (p.C662Y) were about 0.19% (2/1056) in Chinese NSEVA patients (Zhao et al., 2014). Zhang et al. reported that the positive rate of c.1985G>A (p.C662Y) variants in nonsyndromic deafness patients in Linyi with the biggest population and the largest area in Shandong Province located in the east part of China was about 0.15% (Zhang et al., 2016).

4.2 | GJB2(P.V37I) and CDH23

For many populations, the most common cause of autosomal recessive NSHL is variant in connexin 26, a gap junction protein encoded by the *GJB2* (OMIM #220290), which accounts for nearly 20% of all cases of HL, as well as 50% of autosomal recessive NSHL (Estivill, Fortina, et al., 1998; Estivill, Govea, et al., 1998; Gabriel et al., 2001; Minami et al., 2013; Yuan et al., 2009; Zelante et al., 1997). To date, more than 350 variants in the *GJB2* have been identified (http://www.hgmd.cf.ac.uk/ac/index.php). The variant spectrum and prevalence of variants vary widely among different ethnic groups. Certain variants in GJB2 are particularly common in specific populations: 235delC in Asians (Yuan et al., 2009), 35delG in Caucasians (Tekin et al., 2001), and 167delT in Ashkenazi Jewish populations (Morell et al., 1998). In China,

the most common variants of *GJB2* in patients with NSHL are c.235delC (11.90%), c.299delAT (2.22%), c.176del16 (0.65%) and c.35delG (0.27%) (Ji et al., 2011).

The c.109G>A(p.V37I) variation of *GJB2* is significantly frequent in East Asian deaf patients, with a allele frequency of 6.7% in Chinese deafness (Dai, Yu, et al., 2009), 11.1% in Thai deafness (11.1%; Wattanasirichaigoon et al., 2004), 16.5% in Japanese deafness (Tsukada et al., 2010), and 19.4% in Korean deafness (Kim et al., 2013). The pathogenicity of c.109 G>A variant has been controversial in the past, but most experts believe that the variant is still pathogenic (Chan & Chang, 2014; Dai, Yu, et al., 2009; Huang et al., 2015). The study has found that about 65% of the patients with homozygous c.109 G>A (p.V37I) variant have congenital HL and the remaining 35% have a late-onset HL. The HL phenotype ranged from normal to profound, with the severity of HL strongly correlated with age (Bason et al., 2002; Chai et al., 2015).

The proband(S3) in family TJ-2 suffered congenital HL, and the degree of HL was moderate. He had a homozygous pathogenic variation c.109G>A (p.V37I) in *GJB2*, which was the cause of the deafness. However, the younger brother(S4) only had a heterozygous variant c.109G>A in GJB2, while the suspected pathogenic variant c.331C>T (p.Q111*) and clinical significance unknown c.625-5C>T and c.5717G>A (p. G1906E) variants in *CDH23* were also found. The parents with normal hearing were verified that the father was a heterozygous carrier of the c.331C>T variant and the mother was a compound heterozygous carrier of the c.625-5C>T and c.5717G>A variants in *CDH23*. Because the older brother (S3) did not carry any variant in the *CDH23* and their mother had a normal hearing, we could speculate that the heterozygous variant of the c.625-5C>T and c.5717G>A carried by their mother in *CDH23* was from the same chromosome and inherited from the same paternal allele (otherwise, the brother should carry at least one heterozygous variant in *CDH23* inherited from their mother).

CDH23-related nonsyndromic deafness type 12 (DFNB12: OMIM #601386) is autosomal recessive inheritance, and related Usher syndrome type 1D (USH1D: OMIM #601067) is autosomal recessive inheritance (https://www.omim.org/). According to the laws of genetics, the proband S4 may had DNFB12 caused by compound heterozygous variants c.331C>T (inherited from the father) compound c.625-5C>T and c.5717G>A (from the same chromosome inherited from the mother) in *CDH23*. Otherwise, Usher syndrome (USH) is an autosomal recessive disease characterized by the association of sensorineural HL, retinitis pigmentosa (RP), and, in some cases, vestibular dysfunction (Aparisi et al., 2014). According to the severity and progression of the disease, three clinical types are distinguished. Type I (USH1) is defined by profound congenital HL, onset of RP usually within the first decade of life, and an absence of vestibular function (Aparisi et al., 2014). The proband (S4) had no other systemic symptoms and was diagnosed as NSHL, but he was at the age of 3 years when diagnosed with severe HL without ocular phenotype. It remains to be seen if he will develop late-onset RP as other USH1D patients do. Whether compound heterozygous variants c.331C>T (inherited from the father) compound c.625-5C>T and c.5717G>A (from the same chromosome inherited from the mother) in *CDH23* caused USH1D in probands S4 remains to be verified by subsequent clinical manifestations. Therefore, based on the current targeted NGS of 127 known deafness gene results at present, we cannot determine whether the proband S4 has DNFB12 or USH1D. However, the detection of a mild visual phenotype requires patients to be followed with ophthalmoscopy and at least one electroretinography. This will provide more precise genotype–phenotype correlations.

4.3 | MT-TS1

Mitochondrial HL constitutes less than 1% of all hereditary HL cases. A mitochondrion has a 16,569-bp genome containing 22 tRNA and 2 rRNA, which codes for 13 proteins (Bayazit & Yilmaz, 2006). Only maternal DNA is inherited to the child, while paternal DNA is not. Therefore, all children are at risk of having hearing impairment when the mitochondrion of the mother possesses a disease-causing variant. Mitochondrial HL may be nonsyndromic or syndromic as some other associated disorders can occur in addition to HL. These disorders are neuropathy, myopathy, cardiomyopathy, retinal degeneration, diabetes mellitus, Parkinson's disease, encephalopathy, lactic acidosis, epilepsy, and stroke (Bayazit & Yilmaz, 2006).

Among these, the 1555A>G and 1494C>T variants in the mitochondrial *12S rRNA* are two of the most common variants associated with aminoglycoside-induced and non-aminoglycoside-induced nonsyndromic SNHL (Estivill, Fortina, et al., 1998; Estivill, Govea, et al., 1998; Guan, 2004; Fischel-Ghodsian, 1999; Prezant et al., 1993). *MT-TS1*(OMIM #590080), the mitochondrial *tRNA Ser (UCN)*, appears to be another hot spot for variants associated with nonsyndromic SNHL. Nine variants, 7472insC, 7444G>A, 7445A>C, 7445A>G, 7497G>A, 7505T>C, 7510T>C, 7511T>C, and 7512T>C, in *MT-TS1* have been reported in multiple deaf families from various ethnic groups (https://www.omim.org/). Different variants in *MT-TS1* may show different clinical phenotypes. For example, the variant 7512T>C may cause MERRF (myoclonic epilepsy and ragged red fibers) and MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) overlap syndrome or mitochondrial cytochrome c oxidase deficiency (Jaksch et al., 1998; Nakamura et al., 1995). The variant 7445A>G may also cause palmoplantar keratoderma in addition to HL (Reid

et al., 1994; Sevior et al., 1998), while the variant 7497G>A may cause exercise intolerance, muscle pain, and lactic acidemia (Grafakou et al., 2003). Besides, 7472insC variant that can cause either isolated HL or HL may coexist with neurologic disorders such as ataxia, dysarthria, and myoclonus (Tiranti et al., 1995). Some variants such as 7445A>C, 7510T>C, 7511T>C, and 7505T>C may just cause nonsyndromic forms of SNHL (Hutchin et al., 2000; Jin et al., 2007; Sue et al., 1999; Tang et al., 2010).

The two probands (S5, S6) in family TJ-3 passed primary NHS (DPOAE) with two ears at birth but did not have a hearing rescreen (DPOAE+AABR) at 42 days after birth. Because the brother and sister could not speak clearly, they came to otology clinic of Tianjin Women and Children Healthcare Center and were diagnosed as ANSD at the age of 6 years, and 20 months, respectively. Their mother and grandmother also experienced the HL at early age onset of 16 years and 20–30 years. After audiological examination, both the mother and grandmother were diagnosed as SNHL, but different from probands (S5, S6), their DPOAE and CM did not lead out. After 127 gene sequencing, we identified a homoplasmic mitochondrial variant m.7505T>C in *MT-TS1* in the two probands, which was also detected in the mother and grandmother. The variant m.7505T>C in *MT-TS1* had been reported to be associated with NSHL in previous study (Tang et al., 2010). Tang et al. (2010) found that the m.7505T>C variant disrupted a conservative base pairing on the DHU stem of tRNA-ser. The variant was not found in 449 Chinese controls. The amount of tRNA-ser in patient cells was significantly decreased (about 35% of control values). Tang (Tang et al., 2010) suggested that the shortage of this protein may lead to a reduced rate of mitochondrial protein synthesis and cellular respiration defects. So far, only one report about mitochondrial variant m.7505T>C in *MT-TS1*, which had been identified in one Han Chinese pedigree with maternal transmission of nonsyndromic deafness, was found (Tang et al., 2010). But different from Tang's report (Tang et al., 2010), our probands (S5, S6) were characteristic of ANSD, while their probands showed SNHL. Many scholars had found that the patients in clinical with HL were caused by coexisting variants in some related genes and the mitochondrial gene was not uncommon (Chai et al., 2014; Huang et al., 2013). On the other hand, due to the marked genetic heterogeneity of NSHL, variants in more than one causative gene may indeed coexist within the same proband. So, if whole-exome sequencing (WES) were applied, we may find additional or alternative pathogenic causes that cause the phenotype different from Tang's report (Tang et al., 2010). Maybe nuclear-modified genes, epigenetic or environmental factors also contribute to the phenotypic variability of this variant in *MT-TS1* (Chen et al., 2016; Guan, 2004). However, different from the probands (S5, S6), the mother and grandmother audiological performance showed SNHL. We speculate that with the increase in age, the function of cochlear hair cells gradually lost, leading to the failure of CM and DPOAE. This needs to be verified by regular audiological follow-up of the probands (S5, S6). So, further research is needed.

4.4 | POU3F4

1%–3% of human hereditary HL is caused by X-linked variants (Bayazit & Yilmaz, 2006). So far, six deafness loci (DFNX1-6) and five genes (*PRPS1* for DFNX1, *POU3F4* (OMIM #6300039) for DFNX2, *SMPX* for DFNX4, *AIFM1* for DFNX5, and *COL4A6* for DFNX6) have been found for X-linked HL (http://hereditaryhearingloss. org/). In fact, *POU3F4* located on Xq21.1 is found in nearly 50% of all families carrying X-linked NSHL (Corvino et al., 2018). *POU3F4* encodes a member of a transcription factor family that contains a POU domain. POU superfamily genes are involved in organ formation and cell differentiation. Inner ear development is closely associated with *POU3F4* (Corvino et al., 2018). Patients with *POU3F4* variants are mainly male and can display conductive, mix, or sensorineural deafness. Clinical characteristics of *POU3F4* include partial hypoplasia of cochlea, enlarged internal acoustic canal, and a characteristic stapes gusher upon surgery and stapes fixation (Cremers et al., 2002; de KoK et al., 1995). Anatomical anomalies of the temporal bone revealed by CT include dilatation of the lateral end of the internal acoustic canal, abnormally wide communication between the internal acoustic canal and inner ear compartment, and, sometimes, partial hypoplasia of the cochlea (Phelps et al., 1991). As a result of the widening of the internal acoustic canal, cerebrospinal fluid can enter the vestibule, which leads to the reported "gusher" phenomenon, described as fluid gushing out upon removal of the stapes footplate during corrective surgery (Cremers et al., 2008).

In the family TJ-4, the temporal bone CT result of the proband (S7) showed EVA with MD, while the CT result of the proband (S8) showed EVA with a reduction and widening of the spiral structure of bilateral cochlea. After sequencing of 127 genes, no variant was found in *SLC26A4*, the most common gene for EVA, but a hemizygous variant c.138T>C (p. Val46=) was found in *POU3F4*, which was also detected in the mother. The inheritance mode of *POU3F4* is X-linked recessive inheritance. Incomplete partition type III (IP-III) is a typical inner ear anomaly in DFNX2 persons (Altay et al., 2008). Patients with X-linked deafness, carrying variants in *POU3F4*, showed characteristic inner ear radiological features compatible with IP-III, including absent modiolus and lamina spiralis but preserved interscalar septum in a normal-sized cochlea and abnormal dilatation of the lateral end of the internal auditory canal (IAC) (Wu et al., 2020). However, some experts had found that in addition to the above findings (de KoK et al., 1995),

there were also absence of cochlear axis and abnormality of vestibular aqueduct (Talbot & Wilson, 1994). And in 2006, Sennaroglu et al. proposed a new classification for this type of inner ear malformation, namely IP-III, in which the cochlea lacks the entire modiolus and cribriform plate, interscalar septa are present, the vestibule is normal, and the bilateral vestibular aqueduct (VA) is enlarged (Sennaroglu et al., 2006). Kanno et al. (2017) also demonstrated patients with *POU3F4* variants may have an enlargement of VA at the end near the vestibule and lack of enlargement at the operculum is the distinctive feature of *POU3F4* variants, which may relate to the lack of fluctuation of hearing level in contrast to the *SLC26A4* variants (Kanno et al., 2017).

Although the probability of variant c.138T>C (p. Val46=) in *POU3F4* occurring in normal people was extremely low, no relevant reports on the pathogenicity of this site had been found, and the pathogenicity of this site in clinical was unknown. In family TJ-4, both of temporal bone CT results of the proband (S7) and the proband (S8) showed EVA without other inner ear deformities such as the absent modiolus and lamina spiralis and abnormal dilatation of the lateral end of the IAC. Furthermore, the variant c.138T>C (p. Val46=) in *POU3F4* was a synonymous variant. So, the variant is unlikely pathogenic in light of the phenotype of family TJ-4.

In this study, we successfully found the genetic etiology of the HL in 5 probands (S1–S3, S5, and S6) from 3 Chinese families (TJ-1, TJ-2, and TJ-3) by targeted NGS. We identified likely pathogenic and unknown variants in probands S4 from family TJ-2, in which the genetic diagnosis depends on the progression of the disease and the subsequent follow-up. We were not able to obtain a genetic diagnosis for the 2 probands (S7 and S8) from family TJ-4 using the current NGS panel. Further studies including the WES may be needed.

5 | CONCLUSIONS

In conclusion, we successfully identified pathogenic and likely pathogenic variants in 3 Chinese families with two nonsyndromic deaf children in succession by targeted NGS. Families who have had a deaf child or families with a family history of deafness should do genetic counseling before giving birth again. Our study also found that the audiological performance of patients with the variant m.7505T>C in *MT-TS1* was different from that previously reported.

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CONFLICTS OF INTEREST

The authors declare there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Chen Y was responsible for the design and revision of a manuscript; Xiao C, Liu S, Wang H, and Liu H were responsible for genetic test, data collection, and analysis; Xiao C wrote the manuscript; and Ding Y revised the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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