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Posted Date: 30 October 2024

doi: 10.20944/preprints202410.2339.v1

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## Article

# Next Generation Sequencing of Chinese Children with Congenital Hearing Loss Reveals Rare and Novel Variants in Known and Candidate Genes

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**Abstract:** Hearing loss(HL) is the most common disorder in newborns with a highly heterogeneous genetic background. Despite significant progress in screening and identifying genes related with congenital hearing loss, there are still candidate genes implicated in HL that remain undiscovered. We investigated HL in 43 China families that segregate bilateral sensorineural via whole-exome sequencing(WES) and Sanger sequencing. Variants were found in 10 known non-syndromic hearing loss (NSHL) genes, 5 known syndromic hearing loss(SHL) genes and a candidate HL genes *ATP7B*. RNA sequencing reveals *ATP7B* mRNA expression of in developing and adult cochlea. Immunohistochemistry of the adult mouse cochlear tissue revealed prominent expression of *ATP7B* in the organ of Corti and the spiral ganglion neuron. Overall, we propose a new candidate genes *ATP7B* for congenital hearing loss and novel variants in known HL genes, which expand our understanding of the etiology of HL.

**Keywords:** congenital hearing loss; congenital deafness; etiology; gene diagnosis

## 1. Introduction

Hearing loss(HL) is the most common disorder that has an incidence of 2–3 out of 1000 live births [1]. Genetics accounted for at least 50% of congenital hearing loss [2]. Regional and ethnic differences of genetics between populations exist [3]. To date, more than 120 non-syndromic hearing loss(NSHL) genes have been identified [4,5]. *GJB2* is the most common gene associated with autosomal recessive (AR) NSHL among East Asian population [6,7].

Identifying the genetic etiology of HL is essential for customizing personalized treatment plans, evaluating hearing recovery after cochlear implant, and genetic counseling [8,9]. Patients carrying different pathogenic variants exhibit a wide spectrum of phenotypes and vastly differing outcomes after cochlear implantation, thus a precise genetic diagnosis for HL patients may predict the potential benefits of cochlear implants [10]. Variants limited to presynaptic areas such as cochlear hair cells, supporting cells, or stria vascularis regions may result in better hearing recovery effects after cochlear implantation, while variants limited to the postsynaptic to auditory central region may result in uneven or poor hearing recovery after cochlear implantation [10–12]. Genetic testing combined with genetic counseling for congenital hearing loss may also reduced the incidence of congenital hearing loss in newborns.

Despite significant progress in screening and identifying genes related with congenital hearing loss, there are still candidate genes implicated in HL that remain undiscovered [13–16]. Whole-exome sequencing (WES) have recently enhanced the discovery of rare or novel variants in known and candidate genes related hearing loss. In this study, we investigated HL in 43 China families that

segregate bilateral sensorineural HL via WES and Sanger sequencing, and identified rare and novel variants in known and candidate genes, which are valuable for a deeper understanding of the relationship between genotype and phenotype of HL.

## 2. Materials and Methods

### *Sample Collection and Clinical Evaluation*

Participants were enrolled from our outpatient department. Approval from the Institutional Review Boards of Tongji Medical College, Huazhong University of Science and Technology (2022 S041). Before participation, the study was clearly explained to each participant or their parents. All participant or their parents and they have signed written initial informed consent per the Declaration of Helsinki. All of the probands underwent evaluation include family and clinical history, available medical records, physical examination, tympanometry and audiological examinations, such as pure tone audiometry (PTA), audiometry Distortion Product Otoacoustic Emission (DPOAE), and auditory steady-state-evoked responses (ASSR) or auditory brainstem response (ABR). 3-5ml of peripheral blood samples from the probands and related family members was collected and subjected to whole-exome sequencing. Further clinical evaluation is used to determine whether the patient and family members have the syndrome. Temporal bone magnetic resonance imaging(MRI) or computed tomography(CT) is used to identify any possible developmental malformation.

### *Whole-Exome Sequencing and Data Analyses*

Genomic DNA (gDNA) samples from 43 probands from 43 families underwent WES. The method has been described in detail in our previous articles. gDNA was extracted from the samples using the QIAamp DNA Blood Maxi Kit. Fragmentation of the genomic DNA was performed to generate the exome library. The libraries were sequenced on the BGISEQ-500 platform. Sequencing data were compared with the human genome reference (GRCh37/hg19) to detect target regions, single-nucleotide variants (SNVs), and INDEL calling. Identified SNVs and indels were compared with the information available in multiple databases (National Center for Biotechnology Information GenBank database, the Database of Single Nucleotide Polymorphisms, the 1000 Genomes Database). Pedigree mapping of the suspected variants was amplified via Sanger sequencing or WES.

### *RNA Expression Profiling of ATP7B in Mice Cochlear Tissues*

Publicly available datasets in Gene Expression Omnibus (GEO) database and SHIELD (Shared Harvard Inner-Ear Laboratory Database) were utilized for statistical analysis and evaluation of *ATP7B* RNA expression levels in the mice cochlea. RNA sequencing data for hair cells and surrounding cells from the cochlea and utricle at different developmental stages (E16, P0, P4, and P7) originated from the dataset (GSE60019). We also analyzed the expression of the *ATP7B* genes in adult CBA/J mice cochlear RNA sequencing data from dataset (GSE111347) that includes the transcriptome of cochlear pillar and Deiters' cells of adult CBA/J mice (28–35 days after birth), and dataset (GSE56866) that also includes the transcriptome of cochlear inner hair cells (IHC) and outer hair cells (OHC) of either sex adult CBA/J mice (25–35 days after birth). Cell-specific expression of the *ATP7B* gene in two datasets containing adult mouse cochlear RNA sequencing was comprehensively analyzed using Transcripts Per Kilobase Million (TPM).

### *Immunofluorescence of ATP7B Expression in Cochlear Tissue*

To observe the regions where *ATP7B* is expressed in the inner ears of auditory mature mice, we prepared cochlear samples and conducted immunofluorescence staining. Cochlear tissues were dissected from C57BL/6 mice at postnatal day 18(P18). After the mice were euthanized, the cochlear tissue were carefully dissected from the temporal bones and rapidly moved into 4% paraformaldehyde at 4°C overnight and then treat the cochlear tissues with 10% EDTA-Na<sub>2</sub> at 4°C overnight to soften the tissues. The cochlear tissues were embedded in optimal cutting temperature compound (Servicebio, G6059) and sectioned on a microtome (Leica,CM1950) to 10 μm sections at -

25°C. The prepared tissue sections are stored at -80°C. The cochlear tissue specimens were incubated in 10% donkey serum for 1 h at room temperature, and then placed in ATP7B primary antibody(ABclonal, A5676, 1:200 diluted) and incubated overnight at 4°C. Then the cochlear tissue specimens were washed with 0.1% Tween20 in PBS for four times and incubated with Alexa Fluor®488 Donkey anti Rabbit secondary antibodies (AntGene, ANT024s) for 2 hour at room temperature. After incubation with a secondary antibodies, specimens werewashed with 0.1% Tween20 in PBS for three times. DAPI (AntGene, ANT165) and phalloidin (Yeasen, 40736ES75) were used for nuclear and F-actin staining, respectively. Images were obtained with a Nikon laser scanning confocal microscope.

3. Results

*The Pedigree Analysis of the Families in this Cohort*

This cohort consisted of 43 unrelated Chinese families. The participants in this study were recruited across the country with the majority from Hubei province. 79.1% (34/43) families could be explained by carrying genomic variants leaving 20.9% (9/43) families without genetic causes identified through WES (Figure 1. A). The number of NIHL family and SHL family was 36 (84.8%) and 7 (15.2%) , respectively (Figure 1. B). 76.5% (26/34 genetic causes) families exhibited a likely AR mode of inheritance. 20.6% (7/34 genetic causes) families showed a pattern of an autosomal dominant (AD) mode of inheritance. 2.9% family (1/34 genetic causes) had a possible X-linked dominant mode of inheritance(Figure 1. C). 79.1% (34/43) families have members with prelingual HL (Figure 1. D).

*Bioinformatic and Molecular Analysis*

Identified variants in this study include missense variants (27), insertion/deletion (23) , nonsense (6), synonymous (1), and non-coding variants (6) (Figure 1. E). Ten known NSHL genes were identified in 27 of the 43 families (Table 1). These genes included *GJB2*, *SLC26A4*, *MYO7A*, *DIAPH3*, *PTPRQ*, *LOXHD1*, *CDH23*, *MPZL2*, *EYA4*, and *OTOA*. Five known SHL genes were identified in 6 of the 43 families (Table 2). These genes included *TCOF1*, *EYA1*, *FDXR*, *AIFM1*, and *SOX10*. *ATP7B* was segregate in SHL family as a candidate SHL gene (Table 3). Variants in *GJB2* (14 families, 32.6%) and *SLC26A4* (5 families, 11.6%) accounted for the majority (44.2%). *EYA1* variants were found in two families, and variants in 13 other genes were only observed in one family. Twenty of the variants have been reported to be associated with the occurrence of hearing loss, while thirteen of the novel variants were found to segregate in 11/43 HL families, include *DIAPH3* c.2256\_2257insT, *PTPRQ* c.6293T>C, *CDH23* c.4859T>A, *MPZL2* c.393\_436+21del, *TCOF1* c.3997\_4007del, *LOXHD1* c.2438T>A and c.1759C>T, *EYA1* c.1350\_1353delTAATinsCAGACA, *FDXR* c.1069G>T and c.364C>T, *ATP7B* c.4014T>A, *SOX10* c.133del, *AIFM1* c.1771-14T>A.

**Table 1.** Variants identified in the non-syndromic hearing loss families.

Family	Gene	Nucleotide change	Protein change	Inh	GT	Mutation type
Fam1	<i>GJB2</i>	c.235del	p.Leu79CysfsTer3	AR	Hom	Deletion
Fam2	<i>GJB2</i>	c.99del	p.Met34Ter	AR	Het	Deletion
		c.299_300del	p.His100ArgfsTer14	AR	Het	Deletion
Fam3	<i>GJB2</i>	c.235del	p.Leu79CysfsTer3	AR	Hom	Deletion Deletion
Fam4	<i>GJB2</i>	c.109G>A	p.Val37Ile	AR	Hom	Missense
Fam5	<i>GJB2</i>	c.139G>T	p.Glu47Ter	AR	Het	Nonsense
		c.176_191del	p.Gly59AlafsTer18	AR	Het	Deletion

Fam6	GJB2	c.109G>A	p.Val37Ile	AR	Hom	Missense
Fam7	GJB2	c.235del	p.Leu79CysfsTer3	AR	Hom	Deletion
Fam8	GJB2	c.235del	p.Leu79CysfsTer3	AR	Het	Deletion
		c.176_191del	p.Gly59AlafsTer18	AR	Het	Deletion
Fam9	GJB2	c.109G>A	p.Val37Ile	AR	Hom	Missense
Fam10	GJB2	c.109G>A	p.Val37Ile	AR	Hom	Missense
Fam11	GJB2	c.235del	p.Leu79CysfsTer3	AR	Het	Deletion
		c.299_300del	p.His100ArgfsTer14	AR	Het	Deletion
Fam12	GJB2	c.235del	p.Leu79CysfsTer3	AR	Hom	Deletion
						Deletion
Fam13	GJB2	c.257C>G	p.Thr86Arg	AR	Het	Missense
		c.109G>A	p.Val37Ile	AR	Het	Missense
Fam14	GJB2	c.299_300delAT	p.His100ArgfsTer14	AR	Het	Deletion
		c.176_191del	p.Gly59AlafsTer18	AR	Het	Deletion
Fam15	SLC26A4	c.919-2A>G	-	AR	Het	Noncoding
		c.281C>T	p.Thr94Ile	AR	Het	Missense
Fam16	SLC26A4	c.1594A>C	p.Ser532Arg	AR	Het	Missense
		c.2168A>G	p.His723Arg	AR	Het	Missense
Fam17	SLC26A4	c.919-2A>G	-	AR	Hom	Noncoding
Fam18	SLC26A4	c.2009T>C	p.Val670Ala	AR	Hom	Missense
Fam19	SLC26A4	c.919-2A>G	-	AR	Het	Noncoding
		c.2168A>G	p.His723Arg	AR	Het	Missense
Fam20	MYO7A	c.1183C>T	p.Arg395Cys	AR	Het	Missense
		c.3696_3706del	p.Arg1232SerTer72	AR	Het	Deletion
Fam21	DIAPH3	c.2256_2257insT	p.Ser752SerfsTer12	AD	Het	Deletion Insertion
Fam22	PTPRQ	c.6293T>C	p.Leu2098Ser	AD	Het	Missense
Fam23	LOXHD1	c.2438T>A	p.Leu813Ter	AR	Het	Nonsense
		c.1759C>T	p.Arg587Trp	AR	Het	Missense
Fam24	CDH23	c.4859T>A	p.Val1620Glu	AR	Hom	Missense
Fam25	MPZL2	c.220C>T	p.Gln74Ter	AR	Het	Nonsense
		c.393_436+21del	-	AR	Het	Noncoding
Fam26	EYA4	c.1759C>T	p.Arg587Ter	AD	Het	Nonsense
Fam27	OTOA	c.2359G>T	p.Glu787Ter	AR	Het	Nonsense

c.2353A>C	p.Thr785Pro	AR	Het	Missense
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<sup>1</sup> Inh mode of inheritance, hom homozygote, het heterozygote, GT patient genotype.

*Non-syndromic hearing loss gene variants.* Fourteen families segregated variants in *GJB2* (OMIM: 121011), while five families segregated variants in *SLC26A4* (OMIM: 605646), which are the most common HL genes in East Asia [17,18]. All the variants (N=12) identified in these families were classified as pathogenic and likely pathogenic based on the ACMG-AMP classification guidelines for HL. *MYO7A* (OMIM: 276903) variants c.1183C>T (p.Arg395Cys) and c.3696\_3706del (p.Arg1232SerTer72) were segregated from NSHL phenotype of proband in Fam 20. It has been Fam 21 was found to have a *DIAPH3* (OMIM: 603550) c.2256\_2257insT (p.Ser752SerfsTer12) variant segregating with the HL phenotype. In Fam 21, the mother of the proband was confirmed by Sanger sequencing to carry *DIAPH3* c.2256\_2257insT (Figure 2.A). The mother's three brothers also affected HL, although they did not undergo genetic testing. *PTPRQ* (OMIM: 603317) is associated with ADHL (OMIM: 617663) and ARNSHL (OMIM: 613391). Novel heterozygous variants *PTPRQ* c.6293T>C (p.Leu2098Ser) were segregating in Fam 22 (Figure 2.B). Proband showed normal vestibular function until now. *LOXHD1* (OMIM: 613072) is associated with ARNSHL (OMIM: 613079). Novel compound heterozygous variants *LOXHD1* c.2438T>A (p.Leu813Ter) and c.1759C>T (p.Arg587Trp) were segregating in Fam 23 (Figure 2.C). *CDH23* (OMIM: 605516) is involved in Usher syndrome 1D (OMIM: 601067) and ARNSHL (OMIM: 601386). Fam 24 was found to have Homozygous *CDH23* c.4859T>A (p.Val1620Glu) segregating with the profound HL phenotype (Figure 2.D). The proband was 13 years old at the time of testing and had not yet exhibited vestibular dysfunction or pigmentary retinopathy. Fam 25 segregates compound heterozygous variants *MPZL2* (OMIM: 604873) c.393\_436+21del and c.220C>T (p.Gln74Ter) (Figure 2.E). *MPZL2* has been associated with ARNSHL(OMIM: 618145). The feature of Fam26 are an HL father and two HL boys, which both carrying *EYA4* (OMIM: 603550) c.1759C>T ( p.Arg587Ter) variant (Figure 3.F). *EYA4* has been associated with ARNSHL(OMIM: 601316). No clinical signs were observed after the genetics tests that would indicate that affected family members have disorder affecting other organs relate to syndromic HL. We also identified a novel compound heterozygous variants c.2359G>T(p.Glu787Ter) and c.2353A>C(p.Thr785Pro) in *OTOA* (Figure 2.G). *OTOA* (OMIM: 607038) has been associated with ARNSHL(607039).

**Table 2.** Variants identified in the syndromic hearing loss families.

Famil y	Gene	Nucleotide change	Protein change	Inh	G T	Mutation type
Fam2 8	<i>TCOF1</i>	c.3997_4007del	p.Ser1333GlnfsTer16	AD	He t	Deletion
Fam2 9	<i>EYA1</i>	c.1372C>G	p.Pro458Ala	AD	He t	Missense
Fam3 0	<i>EYA1</i>	c.1350_1353delTAT insCAGACA	p.Asn451ArgfsTer18	AD	He t	Deletion/ Insertion
Fam3 1	<i>FDXR</i>	c.1069G>T	p.Val357Leu	AR	He t	Missense
	<i>FDXR</i>	c.364C>T	p.Arg122Cys	AR	He t	Missense
Fam3 3	<i>SOX10</i>	C.133del	p.Gly38AlafsTer71	AD	He t	Deletion

	SLC26A4	c.1975G>C	p.Val659Leu	AR	Het	Missense
	SLC26A4	c.1919G>A	p.Trp640Ter	AR	Het	Nonsense
Fam34	AIFM1	c.1771-14T>A	-	X-link	Het	Noncoding

<sup>2</sup> Inh mode of inheritance, hom homozygote, het heterozygote, GT patient genotype.

*Syndromic hearing loss gene variants.* Novel heterozygous variants *TCOF1* (OMIM: 606847) c.3997\_4007del (p.Ser1333GlnfsTer16) were identified to be likely cause of SHL in Fam 28 (Figure 3.A). The proband in Fam28 has been clinically diagnosed with Treacher Collins syndrome (OMIM:154500). It was reported that clinical features of include downward-slanting palpebral fissures, malar hypoplasia, conductive deafness, mandibular hypoplasia, atresia of external ear canal, microtia, coloboma of the lower eyelid, asymmetry, projection of scalp hair onto the lateral cheek, cleft palate, choanal stenosis/atresia, cardiac malformation. Two families (Fam29 and Fam30) segregate variants *EYA1* (OMIM: 601653) c.1225C>T (p.Asn451ArgfsTer18) and c.1350\_1353delTAATinsCAGACA (p.Pro458Ala) (Figure 3.B,C). *EYA1* is associated with Branchiootic syndrome 1 (OMIM: 602588). Both probands were observed to have clinical features of branchial cervical fistulae, preauricular pits and cup shaped Pinnae. Novel compound heterozygous variants *FDXR* c.1069G>T (p.Val357Leu) and c.364C>T (p.Arg122Cys) were identified to be likely cause of SHL in Fam 31 (Figure 3.D). *FDXR* (OMIM: 103270) variants *FDXR* c.364C>T (p.Arg122Cys) and c.1069G>T (p.Val357Leu) could cause Auditory neuropathy and optic atrophy (OMIM: 617717) or Multiple mitochondrial dysfunctions syndrome 9B (OMIM: 620887). Proband with severe HL in Fam 31 have bilateral optic nerve atrophy and retinitis pigmentosa, while the brother of the probands in Fam 31 have Down syndrome(OMIM: 190685). Sanger sequencing confirms that the mother of the proband carries a heterozygous variant c.364C>T (p.Arg122Cys) in *FDXR*, and the father of the proband does not carry *FDXR* variant. In Fam 33, novel heterozygous variants *SOX10* (OMIM: 602229) C.133del (p.Gly38AlafsTer71) and heterozygous variants *SLC26A4* (OMIM: 605646) c.1975G>C were both identified in proband (Figure 3.E). *SOX10* variants has been associated with Waardenburg syndrome 2E (OMIM:611584) and Waardenburg syndrome 4C (OMIM:613266). The proband have blue eyes and profound HL. The mother of the proband in Fam 33, also have blue eyes and HL, and was identified carrying heterozygous variant C.133del in *SOX10* by WES sequencing. The father of the proband in Fam 33, with the HL phenotype, and was identified carrying compound heterozygous variants c.1975G>C and c.1919G>A (p.Trp640Ter) in *SLC26A4*. In addition, the younger brother of the proband's father in Fam 33 also suffers from HL and was identified to carry a compound heterozygous variant c.1975G>C and c.1919G>A in *SLC26A4*. Additionally, the proband in Fam 34 was identified to carry novel variants *AIFM1* c.1771-14T>A (Figure 3.F). *AIFM1* (OMIM: 300169) have been associated with X-linked deafness-5 with peripheral neuropathy (DFNX5, OMIM: 300614), Cowchock syndrome (OMIM: 300169), and Combined oxidative phosphorylation deficiency 6 (OMIM: 300816). The proband in Fam 34 showed delayed development at 6 months after birth. Then the proband underwent a series of clinical examinations and was diagnosed with profound sensorineural hearing loss. MRI shows bilateral widening ventricular. The proband has multiple epileptic seizures. By age 5 years, the proband could not stand or walk alone, has poor eye contact, and is almost unresponsive to external sound stimuli. At present, the proband underwent cochlear implantation at the age of 6 and received continuous speech and motor rehabilitation training. However, the parents of the proband do not believe that cochlear implantation and speech rehabilitation training increase the proband's response to external sound stimuli. The parents of the proband do not carry the variant in *AIFM1*. The *AIFM1* variant in the proband may be spontaneous mutation.

**Table 3.** Hearing loss candidate variants segregated in Fam32.

Famil y	Gene	Nucleotide change	Protein change	In h	GT	Mutation type
Fam32	ATP7 B	c.4014T>A	p.Ile1338Ile	AR	He t	Synonymou s
	ATP7 B	c.3446G>A	p.Gly1149Glu	AR	He t	Missense

<sup>3</sup> Inh mode of inheritance, hom homozygote, het heterozygote, GT patient genotype.

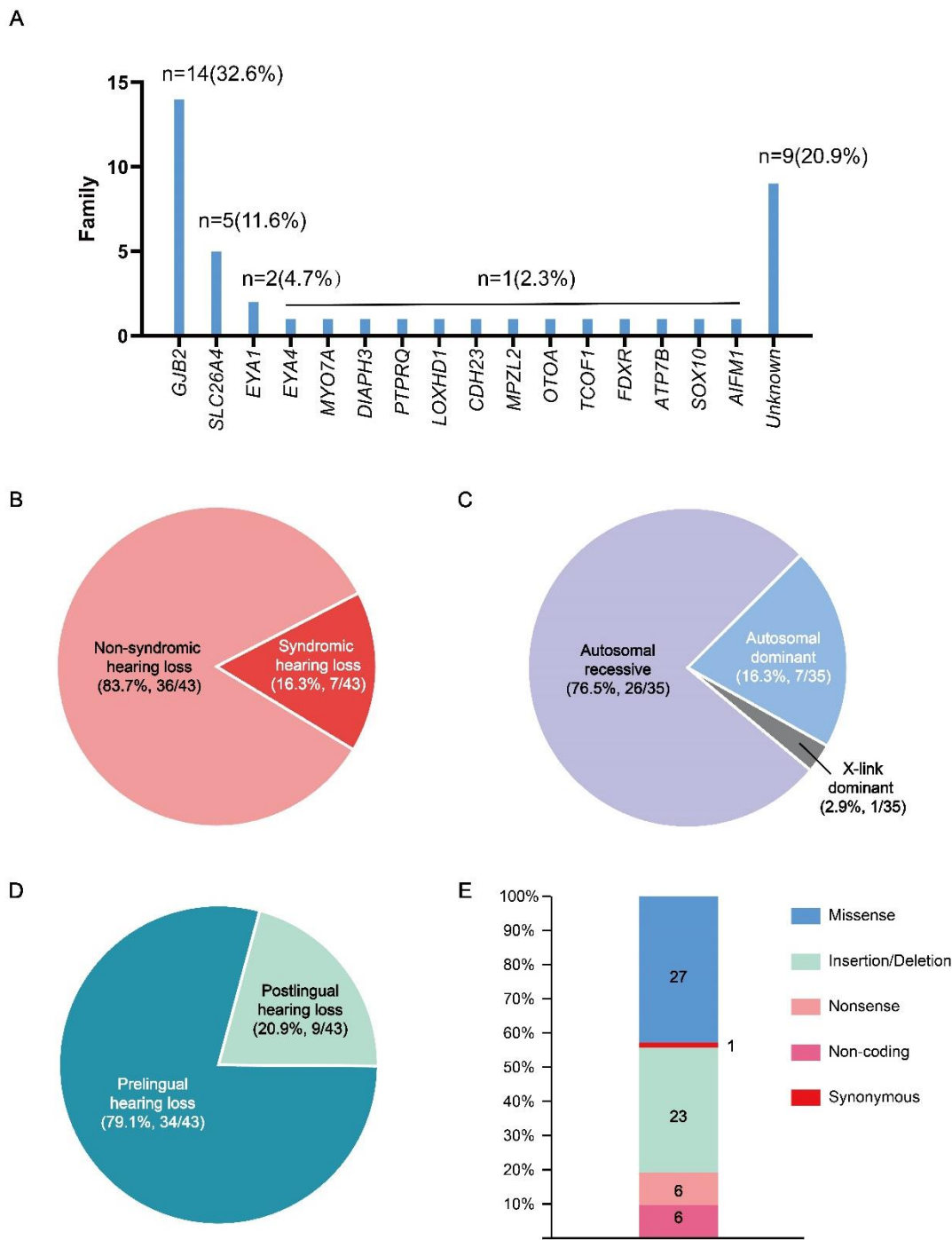
*Hearing loss candidate genes variants.* ATP7B (OMIM: 606882) is with autosomal recessive disorder Wilson disease (OMIM: 277900). In Fam 32, the proband was identified to carry compound heterozygous variant of ATP7B c.4014T>A (p.Ile1338Ile) and c.3446G>A (p.Gly1149Glu) (Figure 4. A). Variant ATP7B c.3446G>A was predicted to be likely pathogenic [19], while the clinical features of ATP7B c.4014T>A carrier could be conflicting interpretat according to gnomAD. It has been reported that synonymous mutation c.4014T>A(p.Ile1338Ile) could cause exon skipping in the ATP7B mRNA transcript [20].

*Expression of HL Candidate Genes ATP7B in the Mouse Inner Ear*

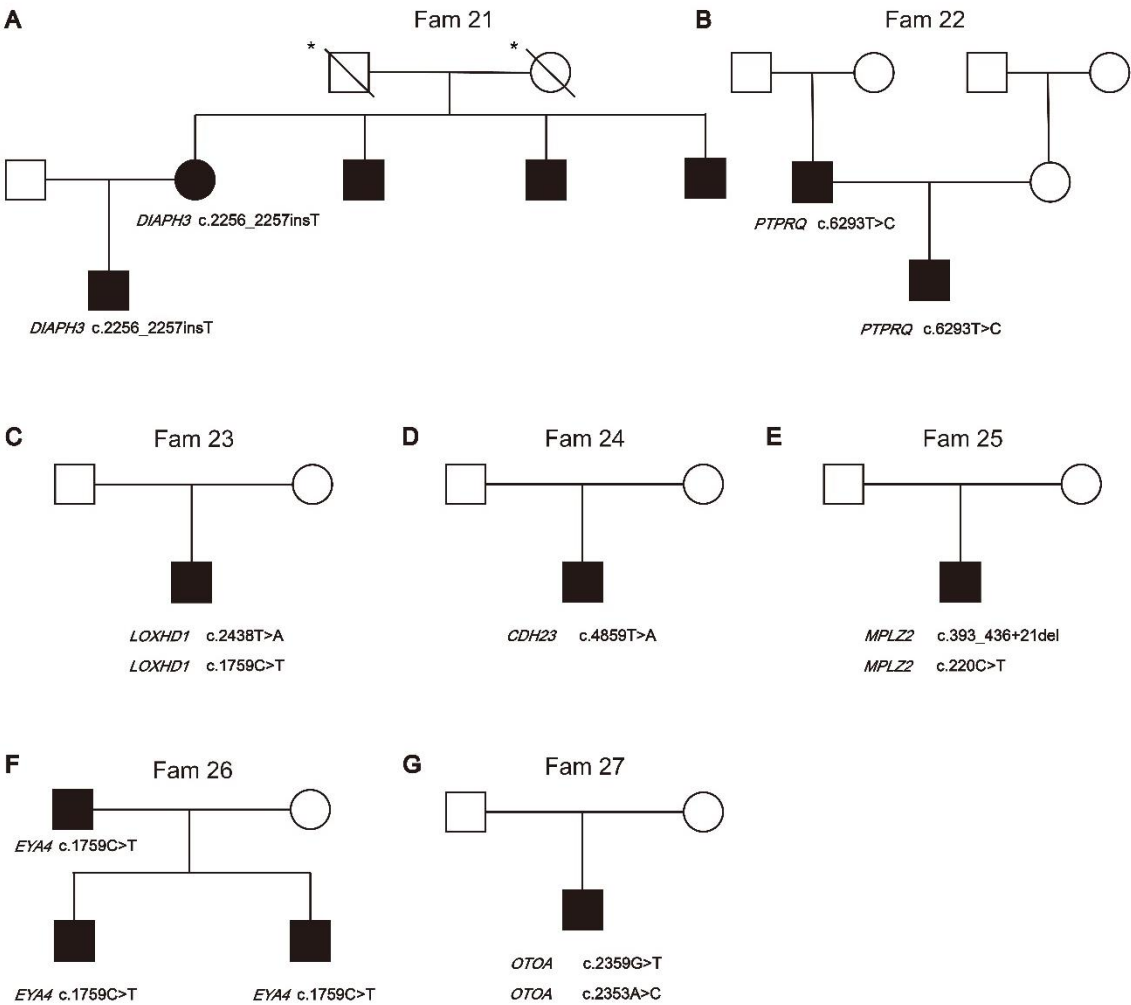
We used various publicly available RNA sequencing and microarray data sets to investigated expression of HL candidate gene ATP7B in the developing and adult mouse cochlear. ATP7B shows a low mRNA expression in the developing cochlear epithelium include cochlear hair cells(HC) and cochlear surrounding cells(SC) throughout E14, P0, P4 and P7 (Figure 4. B). The expressions of ATP7B mRNA were detected in pillar cells (PC) at adult CBA/J mice cochlear, while less ATP7B mRNA were detected in inner hair cells (IHC), outter hair cells (OHC), and Deiters' cells (DC) (Figure 4. C).

The onset of mice hearing arround P12 and the hearing threshold of mice remains stable around P18. To detect the expression of ATP7B protein in the adult mouse cochlea, we performed immunofluorescence staining on cochlear tissues of C57BL/6 mice at P25. ATP7B were detect to mainly expressed in the spiral ganglions (SGN) and organ of Corti (OC), include IHC, OHC, PC, DC, and Claudius' cells (CC) (Figure 5. A-L). Immunofluorescence results also demonstrates that ATP7B were expressed in the cytoplasmic region of the cochlear outer hair cells (Figure 5. M-O) and spiral ganglions cell (Figure 6. A-H).

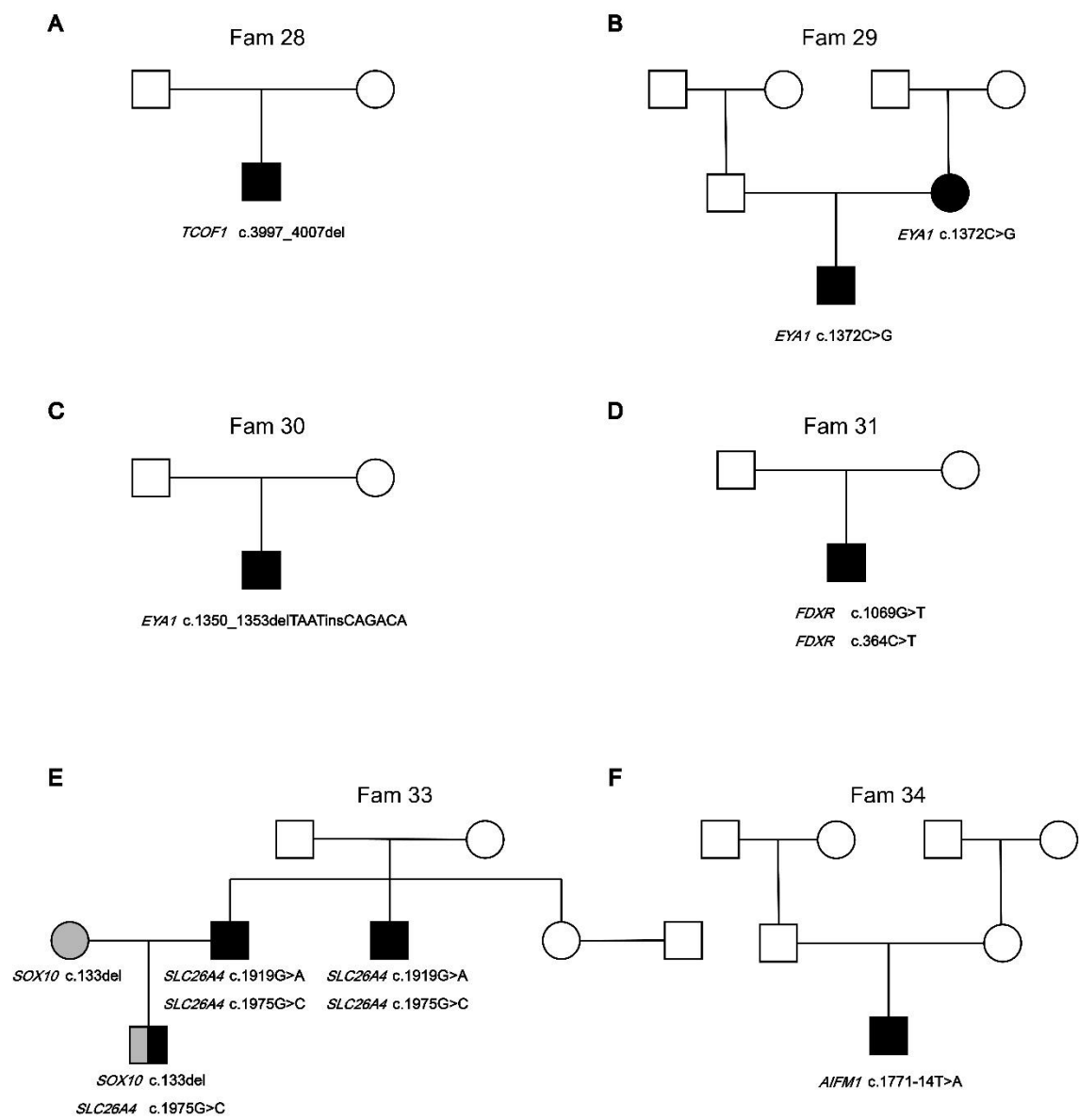
3.2. Figures, Tables and Schemes



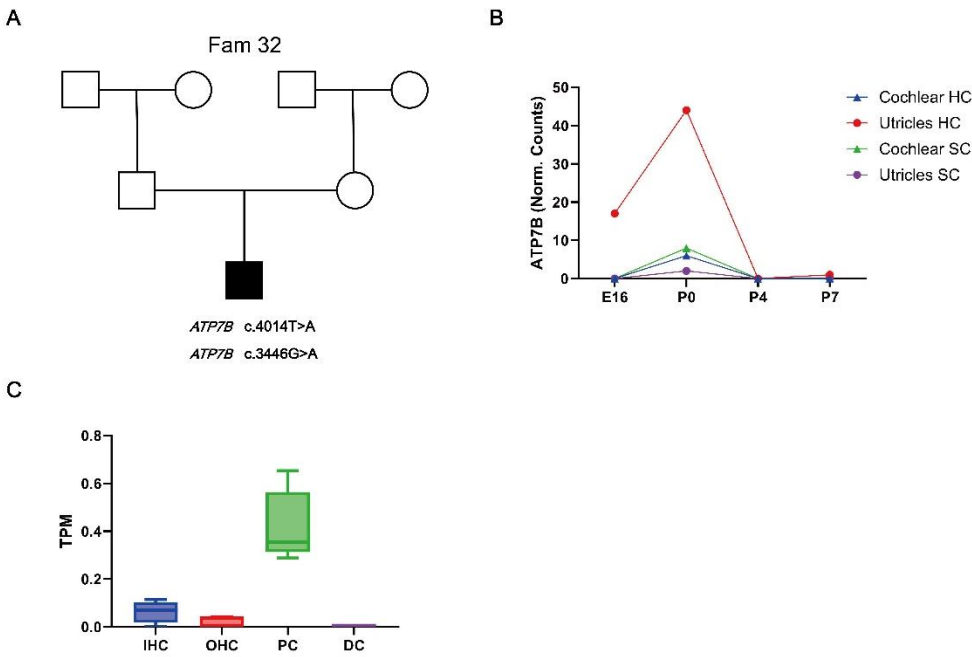
**Figure 1.** Principal component analysis and overview of gene variants in this cohort. **A:** Frequency of the known HI and candidate genes in Chinese families included in this cohort. **B:** Proportions of non-syndromic hearing loss and syndromic hearing loss families in this cohort. **C:** Proportions of hereditary mode of variants in known and novel candidate HL genes were identified. **D:** Proportions of pre-lingual and post-lingual hearing loss of the probands in this cohort. **E:** Proportions of variant types in known and novel candidate HL genes were identified.



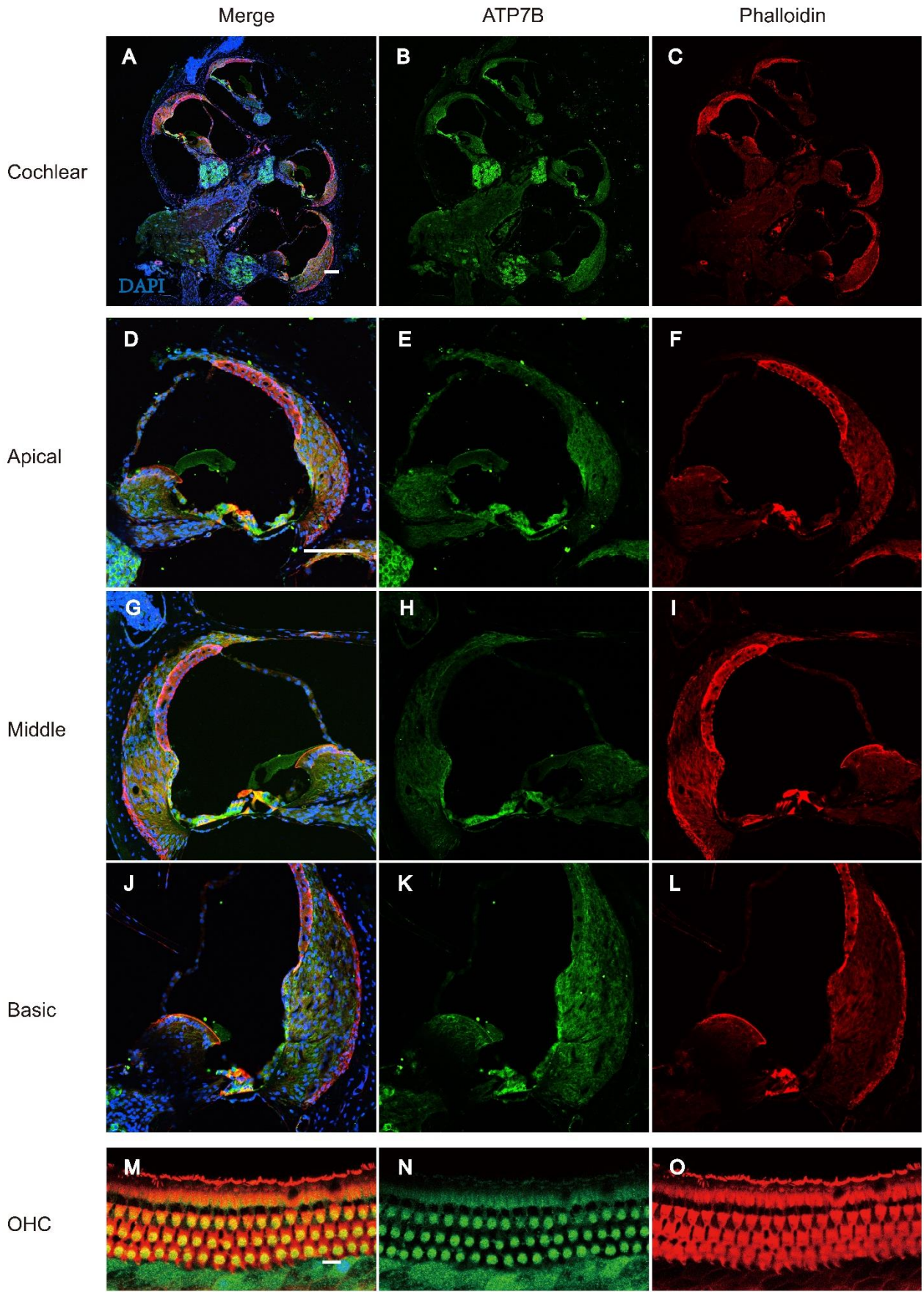
**Figure 2.** Pedigrees of families segregation novel non-syndromic hearing loss genes variants. The segregation of non-syndromic hearing loss genes was shown in the respective families. **A:** Fam21, **B:** Fam22, **C:** Fam23, **D:** Fam24, **E:** Fam25, **F:** Fam26, **G:** Fam27. \* Individuals whose audiological examination results can not be obtained.



**Figure 3.** Pedigrees of families segregation syndromic hearing loss genes variants. The segregation of syndromic hearing loss genes was shown in the respective families. **A:** Fam28, **B:** Fam29, **C:** Fam30, **D:** Fam31, **E:** Fam33, **F:** Fam34.

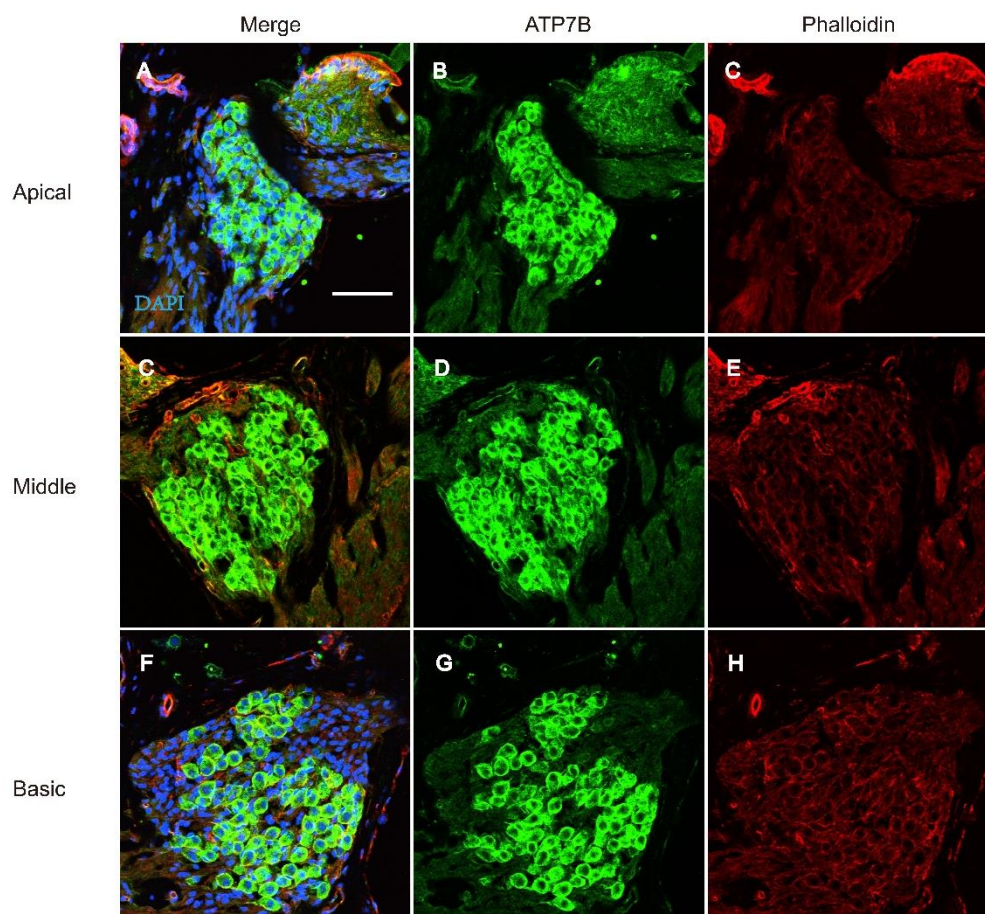


**Figure 4.** Pedigrees of Fam32 segregation *ATP7B* variants and expression of novel candidate gene *ATP7B* in the development and adult cochlear. **A:** Pedigrees of Fam32 segregation *ATP7B* variants. **B:** *ATP7B* mRNA expression in the hair cells(HC) and surrounding cells(SC) of mouse cochlea and utricle during four different developmental stages: E16, P0, P4, and P7. RNA-sequencing data of hair cells (GFP+) and surrounding cells (GFP-) from the cochlear and utricles of mouse expressing EGFP under the Pou4f3 promoter during developmental stages. **C:** *ATP7B* mRNA expression in the inner ear cells of adult CBA/J mice. *ATP7B* mRNA expression in Deiters' cells (DC), Pillar cells (PC), Inner Hair Cells (IHC), and Outer Hair Cells (OHC) were evaluated. The y-axis represents the gene expression normalized to transcripts per million (TPM). Data for (A,B) were obtained from Gene Expression Omnibus (GEO) database and SHIELD (Shared Harvard Inner-Ear Laboratory Database).



**Figure 5.** Immunostaining of ATP7B in the cochlear of C57BL/6 mice at P18. **A-C:** Whole mount immunostaining of the cochlear of P18 C57BL/6 mice. Immunostaining of ATP7B (Green) is observed in both spiral ganglions cell and organ of Corti. Scale bar: 100µm. **D-L:** Immunostaining of ATP7B

(Green) in regions of the OC at the apical, medial and basal turns of the cochlea specimens of C57BL/6 mice at P18. Immunostaining of ATP7B (Green) is observed in both IHCs, OHCs, DCs, PCs, and claudius cell. Scale bar: 100 $\mu$ m. **M-O**: Immunostaining of ATP7B (Green) in cytoplasmic region of the cochlear outer hair cells of C57BL/6 mice at P18. Nucleus was stained with DAPI (Blue). F-actin was stained with phalloidin (red). Scale bar:10 $\mu$ m.



**Figure 6.** Immunostaining of ATP7B in the spiral ganglion cell of C57BL/6 mice at P18. **A-H**: Whole mount immunostaining of ATP7B in the spiral ganglion cell of P18 C57BL/6 mice. Nucleus was stained with DAPI (Blue). F-actin was stained with phalloidin (red). Immunostaining of ATP7B (Green) is observed in spiral ganglions cell at apical, middle and basic turn. Scale bar: 50 $\mu$ m.

#### 4. Discussion

In this study, we investigated HL in 44 China families that segregate bilateral sensorineural HL. Totally, we identified variants in 15 known HL genes. Genetic causes account for at least 79.1% of the studied families, which is higher than the earlier result [21]. Via the present data, we re-evaluated the contribution of GJB2 to 32.6% (14/43) among all families investigated, while the contribution of SLC26A4 to 11.6% (5/43), which suggests the prioritization of GJB2 and SLC26A4 in clinical practice of NIHL. This is consistent with the results in the previous Chinese HL population cohort [22–24].

83.7% family (36 /43) family was diagnosed NSHL. It should be noted that patients with hereditary hearing loss may only have isolated hearing loss phenotypes in childhood, which are often misdiagnosed as non syndromic hearing loss in clinical practice, ignoring other systemic diseases that may occur after puberty or adulthood [25–27]. Genetic diagnosis helps detect additional phenotypes earlier or alert patients to potential risks. MYO7A variants may causes either NSHI or Usher syndrome (USH). Liu et al reported a NSHL proband carried MYO7A heterozygous variants of c.1183C>T and c.1496T>C [28]. Xia et al reported the co-segregation of the homozygous MYO7A c.3696\_3706del variant with the phenotype of deafness and progressive visual loss in the USH family

[29]. Until now, the proband in Fam 20 carried MYO7A heterozygous variants of c.1183C>T and c.3696\_3706del has not shown any ophthalmic symptoms. Similarly, PTPRQ variants could cause autosomal recessive or autosomal dominant congenital sensorineural hearing loss, with or without vestibular dysfunction in infancy or early childhood [30]. It cannot be ruled out that patients currently diagnosed with NSHL may develop late-onset syndrome symptoms in the future. We will closely follow up with the proband in order to intervene in time.

Accurate genetic diagnosis could help us develop targeted and efficient strategies for SHL patients [31–34]. In this study, SHL patients found in seven families (16.2%). It has been reported that prolonged treatment with riboflavin may result in some mild improvement in the ataxia caused by AIFM1 mutation. Ghezzi et al. reported that riboflavin supplementation could partially correct increased cell death and severe respiratory-chain deficiency [35,36]. After the proband was identified as carrying the AIFM1 variant, riboflavin therapy has been recommended. So far, no significant improvement in the patient's symptoms has been observed.

ATP7B encode a copper transporter. Variant in the ATP7B result in a rare autosomal recessive disorder of copper metabolism, Wilson disease (WD), also called progressive lenticular degeneration [37–39]. Copper excessive accumulates in the WD patient's body and its toxic effects causes significant damage on various organs and systems (most characteristically in brain and liver), leading to variability of symptoms such as liver dysfunction, neuropsychiatric disorders, Kayser–Fleischer rings on the cornea, and hemolysis caused by acute liver failure [40–43]. The age range of onset of WD is wide, and symptoms of WD can appear at any age [44,45]. In adult, ATP7B protein express in OC and SGN cells. We reported a patient with sensorineural hearing loss carrying a compound heterozygous variant c.4014T>A and c.3446G>A of ATP7B. Variant ATP7B c.4014T>A could cause exon skipping in the ATP7B mRNA transcript, and the ATP7B mutant may cause gradually affects the function and survival of HC and SGN cells in a dose and time-dependent manner.

**Author Contributions:** Y.S. and W.C. designed the project provided clinical examinations for the participants. Q.Z., Y.X., Y.H., and X.H.H collected and analyzed the data. Y.J. and Z.X.L., wrote the article. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Key Program of the National Natural Science Foundation of China (No. 82430035), the Foundation for Innovative Research Groups of Hubei Province (No. 2023AFA038), the National Key Research and Development Program of China (Nos. 2021YFF0702303, 2023YFE0203200), the Fundamental Research Funds for the Central Universities (No.2024BRA019), and the National Natural Science Foundation of China (No. 82071058).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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