Targeted sequencing identifies 33 novel mutations in 130 ClinGen curated hearing loss genes among 253 pediatric patients: A retrospective case study

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Abstract. Hearing loss (HL) can occur at any age, with hereditary HL being one of the most prevalent congenital disabilities. In the present study, a cohort of pediatric patients with HL was established, comprising 259 individuals at the Children's Hospital of Zhejiang University from 2017-2022. All patients underwent comprehensive diagnostic evaluations, including complete clinical examinations and audiological assessments. Targeted genomic enrichment with massively parallel sequencing was applied to analyze the mutation spectrum of known hearing-loss genes in 253 Chinese children who had positive hearing screening results. Among the 253 patients, 211 (83.40%) exhibited bilateral HL, while 42 (16.60%) had unilateral HL. Targeted sequencing identified 197 variants in 104 genes, yielding a detection rate of 41.1%. A total of 144 genotypes were identified, including 62 heterozygous mutations, 6 hemizygous mutations, 23 homozygous mutations and 48 complex heterozygous mutations. The four most frequently identified genes were *GJB2* (26.5%), *SLC26A4* (13.5%), *MYO15A* (6.5%) and *USH2A* (6.5%). Additionally, 33 novel variants in deafness-associated genes were discovered, comprising 21 novel pathogenic or likely pathogenic variants and 12 variants of uncertain significance. The present results highlight the genetic profile of HL in the Chinese population, with GJB2 being the most prevalent causative gene in early-onset deafness. Furthermore, the current findings provide insight into age- or severity-related gene frequencies for HL. For the genetically unsolved cases, further investigation into digenic inheritance models or other contributing factors is warranted.

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Introduction

Hearing loss (HL) can occur at any age, with hereditary HL being one of the most prevalent congenital disabilities in children, affecting ~2-3 out of 1,000 infants. Genetic causes account for nearly half of these cases, with over 251 genes reported in the hereditary HL database (1). Early diagnosis of HL in newborns, along with genetic counseling, is essential for improving speech and language development (2). In developing countries, newborn hearing screening using transient evoked optoacoustic emissions and automatic auditory brainstem responses is supplemented by genetic screening through heel blood tests for high-risk children, facilitating early diagnosis and intervention.

HL is classified into non-syndromic HL (NSHL), involving only hearing impairment, and syndromic HL, which includes conditions such as Usher syndrome and Waardenburg syndrome. Syndromic HL accounts for ~30% of hereditary HL cases, with over 400 genetic syndromes elucidated to date (3). By contrast, NSHL comprises 70% of hereditary cases. While some children are born with congenital HL, others may have normal hearing at birth or present with delayed-onset symptoms. Therefore, understanding the genetic etiology of HL is essential for clinical evaluation and genetic counseling for families. The HL Gene Curation Expert Panel (HL GCEP) has curated 164 genes associated with hereditary HL, including 105 non-syndromic and 59 syndromic manifestations (4). Among these, mutations in *GJB2*, *SLC26A4* and *GJB3* are the most common, accounting for almost 40% of cases with hereditary HL (5). In previous studies, hotspot mutations were screened using multiplex polymerase chain reaction or microchip technology in deaf children for economic reasons. The most common mutations identified were *GJB2* c.235delC and *SLC26A4* c.919-2A>G, with allele frequencies of 23.8 and 6.8%, respectively, in the Chinese population (6-8). With the application of next-generation sequencing (NGS), more

than 30 new candidate genes have been identified through whole-exome sequencing (9). Moreover, predicted pathogenic variants in known hearing-loss-related genes have frequently been identified (10).

In the present study, targeted genomic enrichment with massively parallel sequencing was applied to investigate the mutation spectrum of known HL genes in 259 Chinese children with positive hearing screening. The enrichment design was coding region of 130 HL genes.

Materials and methods

Ethical approval and consent to participate. The present study was approved (approval no. 2024-IRB-0116-P-01) by the Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine (Zhejiang, China). Peripheral blood samples (2 ml) from patients with hearing loss were collected and extracted genomic DNA by DNA extraction Kits (MyGenostics, Inc.), with informed consent was obtained from all families. Genetics counseling was provided to all patients and their families, and all data analyzed in the present study were anonymized to ensure confidentiality and privacy.

Participant recruitment and study design. A cohort of 259 patients with pediatric-onset HL was established at the Children's Hospital of Zhejiang University from January 2017 to December 2022. After excluding six lost-to-follow-up cases, a total of 253 patients were included in the final study cohort. This cohort was recruited opportunistically, and no formal sample size calculation was performed. All patients underwent comprehensive examinations and audiologic assessments. For patients <5 years, auditory brainstem response (ABR) testing was performed to ensure accuracy. For patients aged ≥5 years, pure-tone audiometry was conducted in addition to ABR testing. In the initial ABR screening, a stimulus intensity of 30 dB nHL was utilized. Replicable wave-V thresholds of 30 dB nHL or less indicated normal hearing, while ABR wave-V thresholds exceeding 30 dB nHL indicated varying degrees of HL, including mild (31 to 50 dB nHL), moderate (51 to 70 dB nHL), severe (71 to 90 dB nHL) and profound (≥91 dB nHL) HL. Conductive HL was excluded through acoustic immittance measurement. The patients were examined and diagnosed by experienced specialists in Otorhinolaryngology and Head and Neck Surgery at the Children's Hospital of Zhejiang University School of Medicine.

Target-region capture and NGS. Genomic DNA from peripheral blood of 253 patients was isolated using a DNA extraction kit (QIAamp Blood Midi Kit; Qiagen, Inc.). The quality of the library was evaluated with Qubit 4.0 (Thermo Fisher Scientific, Inc.). A customized deafness gene panel including 130 genes (OT010 V3) (Table SI) was designed using biotinylated oligo-probes (MyGenosticsGenCap Enrichment technologies). The manufacturer does not provide information on primer sequences, due to commercial protection. These probes covered all exons and 30 bp of flanking intron sequence based on NCBI Build37/UCSC version hg19. After amplification of the enrichment DNA fragment, quantitative QC was passed through Qubit dsDNA HS Assay Kit and length of DNA was detected by Agilent 2100 Bioanalyzer system (Agilent DNA 1000 Kit). The 150 bp reads were paired using an Illumina NextSeq 500 sequencer (Illumina, Inc.) according to the manufacturer's instructions. After sequencing, data processing and variant annotation were performed using standard analyses. The raw data were saved in FASTQ format. The quality of FASTQ files was checked using FastQC. Quality control (QC) filters were applied to remove low-quality reads. The high-quality reads were then assembled and spliced using the second-generation sequencing analysis platform provided by MyGenostics, and the coverage and sequencing quality of the target region were evaluated. High-quality reads were mapped to the human reference genome GRC37/hg19. Small variants were identified using Genome Analysis Toolkit version 3.8 (https://gatk.broadinstitute.org/hc/en-us). Finally, for recessive model analyses, variants with a minor allele frequency of <0.01 in dbSNP138 (https://genome.ucsc.edu/index.html), 1000 Genomes (https://www.coriell.org/), ExAC (https://www.exac.ca/en/) and gnomAD databases (https://www.genomenon.com/) were selected and the possible variation loci were determined. The 130 HL genes were selected from 142 curated HL genes identified by ClinGen expert panel. Four genes (*KITLG, MYO1C, MYO1F* and *TMTC2*) were excluded as they had been refuted or disputed (11). Additionally, 10 loci (*COL2A1, FGFR2, FGFR3, FREM1, MYO1A, POLR1D, PROKR2, PTPN11, SALL1* and *SEMA3E*) were incorporated based on findings in the literature.

Variant identification and validation. 'Loss-of-function' (LOF), 'pathogenic' and 'likely pathogenic' variants are identified as follows: The LOF variants (including nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single, or multi-exon deletion) can often be assumed to disrupt gene function by leading to a complete absence of the gene product by lack of transcription or nonsense-mediated decay of an altered transcript. To identify 'pathogenic' variants, the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/validate.php) and the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) were searched. 'Pathogenic' variant should be found and Sanger sequencing validated in cases. To determine 'likely pathogenic' variant, it should be found and Sanger sequencing validated in cases. It should not be found in the Exome Sequencing Project or the Exome Aggregation Consortium database. It should be an exon coding non-synonymous variant. If it is a missense variant, it should be predicted conservatively by Philip's score. It should be predicted as damaging by both SIFT (<0.05) and Polyphen-2 (>0.85) scores. The pathogenicity of variation loci was also analyzed according to ACMG (American College of Medical Genetics and Genomics) genetic variation classification criteria and guidelines. The Sanger sequencing method was applied using an ABI3730xl sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the results were compared with capture sequencing results to identify candidate mutations and assess their genetic patterns through family segregation studies (Fig. S1).

Statistical analysis. Analyses were performed using SPSS software 20.0 (IBM Corp.). The difference between the two groups was analyzed by the χ^2 test. A two-sided P-value of less

than 0.05 (P<0.05) was considered to indicate a statistically significant difference.

Results

Patient and clinical information. Among the 253 patients with pediatric-onset HL, 211(83.40%) had bilateral HL, including 96 with severe-to-profound HL and 48 with moderate-to-mild HL. The remaining 42 (16.6%) patients exhibited unilateral HL, of whom 15 had profound HL and 10 had moderate-to-mild HL. Age groups were classified as follows: 0-1 month (2.3%), 2-12 months (42.5%), 1-3 years (25.1%), 3-6 years (18.9%), 6-10 years (7.3%) and >10 years (3.9%). The study cohort consisted of 253 patients with a male-to-female ratio of 1.47:1 (151 males and 102 females). The age range of the participants was from 1 month to 27 years, with a median age of 1.16 years. A total of 16 patients had a clear family history, while the family history for the others was unknown. Growth retardation occurred in 7 patients and facial abnormalities were observed in 2 patients.

Genetic finding. Targeted sequencing identified 197 variants in 104 out of 253 HL patients, resulting in a diagnostic rate of 41.11%. Among these variants, there were 190 single nucleotide variants, which included 103 missense mutations, 11 nonsense mutations, 5 in-frame mutations, 9 splice mutations, 61 frameshift mutations and 1 synonymous mutation. Additionally, microdeletions in this cohort were detected through copy number variation (CNV) analysis. Whole-gene deletions of *STRC* and *USH2A* were identified in two patients, respectively. Two previously reported mitochondrial DNA mutations (chrM-1555 and chrM-11778) were also identified in three patients with bifocal deafness (7). A total of 144 mutation types were identified, comprising 62 heterozygous mutations, 6 hemizygous mutations, 23 homozygous mutations, and 48 complex heterozygous mutations. Among these, there were 13 *de novo* variants (8.50%), 25 maternal variants (16.34%), 21 paternal variants (13.73%), 55 variants inherited from both parents (35.95%), and 39 variants of unknown origin (25.49%), respectively. A total of 197 variants associated with deafness were detected in the present study, and the summary information of the 197 variants, including the position, reference allele, mutated allele and allele frequencies of all variants is included in Table SII. According to the regulations of the Human Genetic Resources Administration of China (HGRAC), the sequencing data and information of study participants are not public to prevent the disclosure of personal genetic identities. Collaborating researchers may apply for access to the sequencing data for further analysis upon approval by HGRAC.

Recurrent variants. As previously reported in HL studies, the four most frequently identified genes in our cohort were *GJB2* (26.5%),*SLC26A4* (13.5%),*MYO15A* (6.5%) and *USH2A* (6.5%). Other commonly observed genes included *LOXHD1*, *MITF*, *MYH9*, *KCNQ4*, *MYO7A* and *TVC*, each with at least three variants detected in our population study. The top four genes accounted for 54.5% of HL cases, while the other common genes contributed 17.5%, and the remaining genes contributed 27%. The most common variants were p.V37I (13/200), p.L79Cfs*3 (26/200), and p.H100Rfs*14(6/200) (6/200) in *GJB2*, as well as the splicing variants *c.919-2A>G*(8/200) in *SLC26A4*. The 33 novel variants in the HL gene panel are listed in Table I.

Autosomal dominant NSHL. Four patients in our cohort carried *KCNQ4* variants (4/259). In Patient 62, a novel splicing variant (c.1876-1G>A) was inherited from the mother, who also had unilateral HL. A maternal truncated mutation in *GJB3* (p.W44X) was identified in patient 249, who exhibited bilateral progressive mild hearing impairment. This variant was absent in the gnomAD database and was predicted to be damaging by 20 prediction tools (REVEL 0.9). Additionally, a *de novo* missense variant c.439C>T (p.R147C) was discovered in *ACTG1*, which encodes γ1 actin, in a 6-month-old boy who presented with bilateral moderate HL (right ear: 60 dB, left ear: 65 dB).

Autosomal recessive NSHL. A total of 3 novel variants were identified in common genes. One patient exhibited a homozygous deletion of exon 1-3 in *SLC26A4*, while another had a non-frameshift variant (c.416_418delGAC) in trans with a known pathogenic splicing variant (c.919-2A>G) in *SLC26A4*. These two individuals experienced bilateral HL, with left-side thresholds of 77 dB and 95 dB and right-side thresholds exceeding 99 dB and 85 dB. In *MYO15A*, 13 variants were identified in 8 patients with HL (8/259, 3.1%), including 8 novel pathogenic or likely pathogenic variants. Patient 207 inherited a novel splicing variant in *MYO15A* (c.9534dupC, p.E3179Rfs*43) from his mother, and both exhibited bilateral HL. Patient 76 carried a novel missense variant (c.9163C>T, p.L3055F) inherited from his father and a novel splicing variant (c.3862delC, p.P1289Rfs_23) in *MYO15A* from his mother. Patient 218 had two novel missense variants (c.5254G>A, p.Val1752Ile and c.5644C>T, p.Pro1882Ser) in *PTPRQ*. This patient exhibited gradually reduced hearing, accompanied by slurred speech and a tendency to fall while walking. *PTPRQ* is associated with recessive deafness (DFNB84A), and the compound heterozygous variants, in this case were the etiology of HL. Patient 252 had two compound heterozygous variants in *LHFPL5* (c.26A>G, p.E9G; c.200A>G, p.Y67C) and *LOXHD1* (c.4741-1G>A, splicing; c.4180G>A, p.V1394M), exhibiting profound HL (Right: >95 dB; Left: >100 dB). Novel compound heterozygous *OTOA* mutations c.1486A>G (p.K496E) and c.1764delC (p.Q589Rfs*55) were reported in patient 35, who had bilateral moderate deafness. A novel nonsense mutation (c.126C>A, p.Y42X) and a previously reported nonsense mutation (c.975G>A, p.W325X) were identified in two boys who had both mutations inherited from their mother. Both exhibited bilateral mild or moderate deafness.

Syndromic HL. *USH2A* is associated with autosomal recessive Usher syndrome Type II (USH1; OMIM 276901), which is characterized by moderate to severe HL and blindness. W A total of 13 pathogenic variants were found in *USH2A* across 6 probands (6/259, 2.31%), including one novel variant (p.H2591Lfs*7). One patient presented with three variants in *USH2A* (p.H2591Lfs*7, p.R1870W, p.G1526R), exhibiting bilateral moderate deafness at the age of 1.5 years. USH1 is the most severe form, characterized by congenital profound

Table I. Variants detected within the genes associated with hearing loss and in silico prediction of their pathogenicity.

Patient ID	Genes	cDNA change, protein change	nucleotide	refLocal	Exon	Zygosity	Known/ Novel	ACMG Class	Inherit	Inherited or *de novo*	OMIM ID
P6	MITF	c.859G>A, p.E287K	chr3:70013998	NM_000248	exon9	Het	Novel	VUS	AD	Maternal	OMIM: 193510
P6	MYO15A	c.3026C>A, p.P1009H	chr17-18025140	NM_016239	exon2	Het	Known	VUS	AR	Maternal	OMIM: 600316
P6	MYO15A	c.9559C>T, p.R3187C	chr17-18065940	NM_016239	exon57	Het	Known	VUS	AR	Paternal	OMIM: 600316
P7	MYH9	c.5780C>T, p.P1927L	chr22:36678817	NM_002473	exon41	Het	Novel	Likely pathogenic	AD	*De novo*	OMIM: 603622
P8	SLC26A4	c.919-2A>G, splicing	chr7:107323898	NM_000441	exon8	Het	Novel	Pathogenic	AR	Maternal	OMIM: 600791
P12	SLC26A4	c.416_418del, p.139_140del	chr7:107314594-107314611	NM_000441	exon5	Het	Novel	Likely pathogenic	AR	Maternal	OMIM: 600791
P20	SLC26A4		chr7:107301080-107306560		exon-3	Hom	Novel	Pathogenic	AR	Unverified	OMIM: 600791
P11	FGFR3	c.931-2A>G, splicing	chr4:1804639	NM_001163213	exon8	Het	Novel	Likely pathogenic	AD	Paternal	OMIM: 610474
P23	EYA4	c.978C>G, p.F326L	chr6:133802608	NM_004100	exon12	Het	Novel	Likely pathogenic	AD	Maternal	OMIM: 601316
P24	MYO7A	c.2762G>A, p.R921Q	chr11:76892493	NM_000260	exon23	Het	Novel	VUS	AR	Paternal	OMIM: 276900 OMIM: 600060
P173	MYO7A	c.617G>A, p.R206H	chr11-76867932	NM_000260	exon7	Het	Known	VUS	AR	Unverified	OMIM: 276900 OMIM: 600060
P173	MYO7A	c.1133G>A, p.R378H	chr11-76871261	NM_000260	exon11	Het	Novel	VUS	AR	Unverified	OMIM: 276900 OMIM: 600060
P27	POLR1D	c.299T>C, p.L100P	chr13:28197284	NM_015972	exon2	Het	Novel	Likely pathogenic	AD	*De novo*	OMIM: 613717
P35	OTOA	c.1486A>G, p.K496E	chr16:21726471	NM_144672	exon13	Het	Novel	VUS	AR	Paternal	OMIM: 607039

Table I. Continued.

Patient ID	Genes	cDNA change, protein change	nucleotide	refLocal	Exon	Zygosity	Known/ Novel	ACMG Class	Inherit	Inherited or *de novo*	OMIM ID
P35	OTOA	c.1764delC, p.Q589Rfs*55	chr16:21730782-21730783	NM_144672	exon16	Het	Known	Likely pathogenic	AR	Maternal	OMIM: 607039
P62	KCNQ4	c.1876-1G>A, splicing	chr1:41303982	NM_004700	exon14	Het	Novel	Likely pathogenic	AD	Maternal	OMIM: 600101
P71	SALL1	c.2796dupC, p.N933Qfs*13	chr16:51173336-51173336	NM_002968	exon2	Het	Novel	Pathogenic	AD	*De novo*	OMIM: 107480
P76	MYO15A	c.3862delC, p.P1289Rfs*23	chr17-18029765-18029766	NM_016239	exon5	Het	Novel	Pathogenic	AR	Maternal	OMIM: 600316
P76	MYO15A	c.9163C>T, p.L3055F	chr17-18062595	NM_016239	exon53	Het	Novel	VUS	AR	Paternal	OMIM: 600316
P83	USH2A	c.4576G>A, p.G1526R	chr1:216348645	NM_206933	exon21	Het	Known	Likely pathogenic	AR	Maternal	OMIM: 276901
P83	USH2A	c.5608C>T, p.R1870W	chr1:216246607	NM_206933	exon28	Het	Known	VUS	AR	Paternal	OMIM: 276901
P83	USH2A	c.7772_7781 delACCCATA CAC, p.H2591 Lfs*7	chr1:216062209-216062219	NM_206933	exon41	Het	Novel	Likely pathogenic	AR	Paternal	OMIM: 276901
P108	MITF	c.641_643del GAA, p.214_ 215delRRinsR	chr3:70005611-70005614	NM_000248	exon7	Het	Known	Pathogenic	AD	*De novo*	OMIM: 193510
P112	ACTG1	c.439C>T, p.R147C	chr17:79478577	NM_001614	exon4	Het	Known	Likely pathogenic	AD	*De novo*	OMIM: 604717
P140	MITF	c.1177-1G>A, splicing	chr3:70013997	NM_001354605	exon10	Het	Known	Pathogenic	AD	*De novo*	OMIM: 193510
P144	PCDH15	c.398T>G, p.V133G	chr10:56128956	NM_033056	exon5	Het	Novel	VUS	AR	*De novo*	OMIM: 609533 OMIM: 602083
P154	PTPRQ	c.6087-3T>G, splicing	chr12:81066945	NM_001145026	exon40	Het	Known	VUS	AR	*De novo*	OMIM: 613391
P155	POU3F4	c.126C>A, p.Y42X	chrX:82763458	NM_000307	exon1	Hemi	Novel	Likely pathogenic	XLR	Maternal	OMIM: 304400
P173	MYO7A	c.617G>A, p.R206H	chr11:76867932	NM_000260	exon7	Het	Known	VUS	AR	*De novo*	OMIM: 276900 OMIM: 600060

Table I. Continued.

Patient ID	Genes	cDNA change, protein change	nucleotide	refLocal	Exon	Zygosity	Known/ Novel	ACMG Class	Inherit	Inherited or *de novo*	OMIM ID
P173	MYO7A	c.1133G>A, p.R378H	chr11:76871261	NM_000260	exon11	Het	Novel	VUS	AR	*De novo*	OMIM: 276900 OMIM: 600060
P178	KCNQ4	c.2014G>A, p.V672M	chr1:41304121	NM_004700	exon14	Het	Known	VUS	AD	*De novo*	OMIM: 600101
P191	OTOGL	c.141+1G>A, splicing	chr12:80605778	NM_173591	exon3	Het	Novel	Pathogenic	AR	*De novo*	OMIM: 614944
P191	OTOGL	c.6409G>T, p.E2137X	chr12:80761445	NM_173591	exon53	Het	Novel	Pathogenic	AR	*De novo*	OMIM: 614944
P207	MYO15A	c.4898T>C, p.I1633T	chr17:18041451	NM_016239	exon16	Het	Known	VUS	AR	Paternal	OMIM: 600316
P207	MYO15A	c.9534dupC, p.E3179Rfs*43	chr17:18065915-18065915	NM_016239	exon57	Het	Novel	Likely pathogenic	AR	Maternal	OMIM: 600316
P215	KCNQ4	c.827G>T, p.Trp276Leu	chr1:41285137	NM_004700.4	exon5	Het	Known	Pathogenic	AD	Paternal	OMIM: 600101
P217	PCDH15	c.4699_4715dup, p.Leu1573Glufs Ter18	chr10:55582770-55582770	NM_033056.4	exon33	Het	Novel	VUS	AR	Maternal	OMIM: 609533 OMIM: 602083
P217	PCDH15	c.2001G>A, p.Thr667=	chr10:55839181	NM_033056.4	exon17	Het	Novel	VUS	AR	Paternal	OMIM: 609533 OMIM: 602083
P218	PTPRQ	c.5254G>A, p.Val1752Ile	chr12:81025994	NM_0011450-26.2	exon36	Het	Novel	VUS	AR	Unverified	OMIM: 613391
P218	PTPRQ	c.5644C>T, p.Pro1882Ser	chr12:81046598	NM_0011450-26.2	exon40	Het	Novel	VUS	AR	Unverified	OMIM: 613391
P219	MYO1A	c.893-2A>C, splicing	chr12:57437144	NM_005379.4	intron 10	Het	Novel	Likely pathogenic	AD	Maternal	OMIM: 607841
P242	MITF	c.397G>T, p.E133X	chr3:69990438	NM_000248	exon4	Het	Novel	Likely pathogenic	AD	Unverified	OMIM: 193510
P242	MYH14	c.5384G>A, p.R1795H	chr19:50796859	NM_001145809	exon39	Het	Novel	Likely pathogenic	AD	*De novo*	OMIM: 600652

Table I. Continued.

Patient ID	Genes	cDNA change, protein change	nucleotide	refLocal	Exon	Zygosity	Known/ Novel	ACMG Class	Inherit	Inherited or *de novo*	OMIM ID
P247	KCNQ4	c.140T>C, p.L47P	chr1:41249905	NM_004700	exon1	Het	Known	VUS	AD	Paternal	OMIM: 600101
P249	GJB3	c.131G>A, p.W44X	chr1:35250494	NM_024009	exon2	Het	Novel	Likely pathogenic	AD	Maternal	OMIM: 612644
P252	LHFPL5	c.26A>G, p.E9G	chr6:35773473	NM_182548	exon1	Het	Novel	VUS	AR	Maternal	OMIM: 610265
P252	LHFPL5	c.200A>G, p.Y67C	chr6:35773647	NM_182548	exon1	Het	Known	Likely pathogenic	AR	Paternal	OMIM: 610265
P252	LOXHD1	c.4741-1G>A, splicing	chr18:44104565	NM_144612	exon31	Het	Novel	Likely pathogenic	AR	Paternal	OMIM: 613079
P252	LOXHD1	c.4180G>A, p.V1394M	chr18:44114330	NM_144612	exon27	Het	Novel	Likely pathogenic	AR	Maternal	OMIM: 613079

XLR, X-linked recessive; AD autosomal dominant; AR, autosomal recessive; VUS, variant with uncertain significance.

hearing impairment, early retinitis pigmentosa and constant vestibular areflexia. A total of 9 variants associated with USH1 were identified in 5 patients, involving the *MYO7A*, *PCDH15* and *CDH23* genes. In *MYO7A*, two patients exhibited four compound heterozygous mutations, including two novel variants (p.R378H and p.R921Q). Patient 144 carried a novel compound heterozygous mutation in *PCDH15* (c.398T>G, p.V133G), which led to bilateral profound deafness. The second allele contained a novel missense mutation (c.398T>G) located in exon 5, within the EC1 domain of *PCDH15,* which is highly conserved across species. Patient 58 carried two reported missense variants (c.137C>A, p.T46K; c.9904G>A, p.E3302K) in *CDH23* and was diagnosed with bilateral moderate HL at the age of 1 year.

Discussion

HL is one of the most etiologically heterogeneous disorders, influenced by age and genetic factors (11-14). Investigating environmental and genetic contributions to age-related HL remains challenging. However, advancements in NGS have significantly improved the identification of monogenic HL. Numerous novel genetic variants have been identified that contribute to HL. A total of 253 pediatric-onset patients with HL were successfully sequenced and 203 variants were identified in 130 genes (with a diagnostic rate of 41.11%), suggesting that HL panel-based NGS is an effective diagnostic tool for determining the genetic etiology of HL.

To date, ~150 genes have been associated with non-syndromic and syndromic HL (Hereditary HL Homepage). Genetic screening projects for neonatal deafness in China have typically targeted 9-20 loci, utilizing methods such as targeted DNA sequencing or whole exome sequencing. The diagnostic rate of known deafness gene-targeted sequencing is between 30-50%, depending on the population and genes analyzed (13). A gene list curated from the HL GCEP was applied and known mitochondrial DNA loci were included (11). Of the 130 genes in our targeted panel, 84 genes were classified as having definitive or strong evidence of gene-disease relationship, while the remaining genes had limited or moderate evidence. Positive results were identified in 37 of 84 definitive/strong HL genes (44.04%), a diagnostic rate notably higher than that observed when including genes with limited or moderate evidence (41 out of 130 genes, 31.54%). This finding focuses on genes with strong or definitive evidence for achieving high diagnostic yields. Genes with limited or moderate evidence included *COL4A6* (limited), *DIAPH3* (moderate), *MYH14* (Moderate), *P2RX2* (moderate) and *TNC* (moderate). Overall, the diagnostic rate of this study (42.5%) surpassed that report for exome sequencing alone in pediatric cases of mild-moderate sensorineural HL in a pediatric group (38.4%) (14), which indicates that this strategy is cost effective for monogenic causes of pediatric onset HL.

Consistent with previous studies, the most common cause for NSHL in our cohort were *GJB2* and *SLC26A4* (6,7). Pathogenic variants associated with syndromic HL were identified, including Usher syndrome (9/259) and CHARGE syndrome (3/259). Pathogenic variants in *USH2A* are the most common cause of type II Usher syndrome and *CDH23* and *MYO7A* genes for type I Usher syndrome.

Among the 203 variants identified in this cohort of Chinese individuals, there were 13 *de novo* variants, accounting for 8.5%. Maternal variants comprised 25 (16.34%), while paternal variants accounted for 21 (13.73%). The total number of inherited variants, including both maternal and paternal, was 55 (35.95%), and there were 39 (25.49%) variants classified as unknown. These findings indicate that inherited variants are a common etiology for pediatric onset deafness, and carrier screening for deafness-related genes could reduce the incidence of HL, particularly for *GJB2, SLC26A4* and *MYO15A*. The mutation frequencies of GJB2, SLC26A4 and MYO15A exhibit significant regional differences. GJB2 mutations are highly prevalent in Chinese, Japanese and Korean populations, with the 235delC variant being the most common, whereas in European populations, the 35delG mutation is the most frequently observed. By contrast, the incidence of GJB2 mutations is relatively low in African populations. In the Han Chinese population, the allele frequency of this mutation is 13.9% (15), which is comparable to the 13.4% detection rate (34/253) found in the present study. The pathogenic effects of GJB2 mutations are typically associated with early-onset HL, and in some cases, hearing impairment may worsen with age (16). SLC26A4 mutations are highly prevalent in Chinese and Japanese populations. A study in China identified IVS7-2A>G and H723R as the most common mutation types (17). In European and American populations, the mutation spectrum of SLC26A4 is more diverse, and the mutation frequency is relatively lower. HL caused by SLC26A4 mutations can manifest in childhood and may progressively worsen with age (18). Additionally, CNVs and mitochondrial DNA mutations account for 2.7% (7/259) of HL cases. Incorporating CNV calling and other methodologies could enhance the diagnostic yield by 4%. Due to increased awareness of predisposition to aminoglycoside ototoxicity associated with MT-RNR1 and MT-TS1, there has been a decrease in mitochondrial HL cases as a precaution to avoid aminoglycoside ototoxicity in China.

A total of 19 novel pathogenic or likely pathogenic variants and 14 novel uncertain variants were identified across 130 HL-related genes. *KCNQ4*, which is expressed in the inner ear and involved in the central auditory ion channel, is associated with autosomal dominant deafness-2A. To date, 94 *KCNQ4* pathogenic or likely pathogenic variants have been reported in ClinVar, of which 79 are missense mutations. A 6-year-old boy inherited a novel splicing variant (c.1876-1G>A) from his father, who has unilateral HL. Wasano *et al* (19) suggested that *KCNQ4* may also exhibit autosomal recessive inheritance and presents early in onset compared with the autosomal dominant pattern of loss of function variants. This boy exhibited unilateral moderate HL (50 dB). Additionally, a maternal truncated mutation in *GJB3* (p.W44X) was identified in a 1-year old boy with unilateral mild hearing impairment (50 dB). It has been previously reported that *GJB3* is responsible for bilateral high-frequency HL (19,20).

MYO15A is among the third or fourth most frequent causes of autosomal recessive (ARNSHL). Patients with *MYO15A* variants typically present with profound congenital sensorineural deafness and also milder auditory phenotype as well. Zhang *et al* (21) reported 28 *MYO15A* variants, of which 14 were novel, highlighting its increasing prevalence in East Asia. A total of 13 variants in *MYO15A* were identified in 8 patients with HL (8/259, 3.1%) and 8 of 13 were novel pathogenic or likely pathogenic variants in the present study. The frequency of MYO15A mutations is higher in Asian and South Asian populations, while it is lower in European and African populations (21). MYO15A mutations typically result in congenital or infant-onset severe HL, and unlike GJB2 or SLC26A4 mutations, MYO15A-related HL does not progressively deteriorate with age (22).

All variants were compound heterozygous variants inherited from father and mother. Among the 8 patients, 3 cases had moderate mild HL and 5 cases had severe HL. All of them had bilateral deafness, and no unilateral deafness was found. *POU3F4* encoded a transcription factor associated with X-linked recessive HL. A total of two novel nonsense mutations were found in two boys, both inherited maternally. One mutation (p.Y42X) in patient 155 resulted in absence of the POU domain and Homeodomain. Another patient 12 affected patient was bilateral mild or moderate with the mutation p.W325X which disrupted Homeodomain of *POU3F4*.

Digenic inheritance has been reported for certain combinations of mono-allelic variants in HL genes, particularly those associated with Usher syndrome (23-25). This suggests digenic or oligogenic inheritance pattern might play roles on etiology of HL. However, evidence supporting this pattern remains limited, and further studies in large cohorts are needed. *USH2A* resulted in an autosomal recessive Usher syndrome Type IIA (MIM: 276901) characterized by moderate to severe HL and blindness. The present study found that six patients carried *USH2A* gene mutations; they were all diagnosed with bilateral mild or moderate deafness. *USH2A* gene mutations were not found in patients with unilateral deafness. The USH2A mutation is the most common genetic cause of Usher syndrome type 2 in Europe and North America and has also been frequently detected among Usher syndrome patients in China, Japan and Iran (26). USH2A mutations typically lead to progressive retinitis pigmentosa and HL, which manifest during adolescence or adulthood. The mutation type and disease severity are closely associated with age-related factors (22).

For three clinical types of USH, USH1 is the most severe form characterized by congenital profound impairment, early retinitis pigmentosa and constant vestibular areflexia. A total of five genes have been identified: myosin VIIA (*MYO7A*; USH1B), Usher syndrome 1C (*USH1C*; USH1C), cadherin-related 23 (*CDH23*; USH1D), protocadherin-related 15 (*PCDH15*; USH1F) and Usher syndrome 1G (*USH1G*; USH1G). A total of 9 variants associated with USH1 were identified in 5 patients, involving *MYO7A*, *PCDH15* and *CDH23* genes.

The *MYO7A* gene encodes Myosin VIIA. Mutations and defect of Myosin VIIA result in recessive Usher syndrome type 1B and DFNB2 or autosomal dominant HL (DFNA11). A total of >340 different mutations in *MYO7A* gene have been reported (http://www.umd.be/MYO7A). A total of four compound heterozygous *MYO7A* mutations were identified in two patients (2/259), including two novel mutations (p.R378H and p.R921Q). A total of 3 mutations were located in Motor domain and one of them in coiled-coil region of *MYO7A*. Both patients exhibited unilateral HL and one had

inner ear deformation. *CDH23* mutations cause USH1D, characterized by autosomal recessive congenital sensorineural HL, vestibular dysfunction and visual impairment (16,27). Patient 58 carried two reported missense variants (c.137C>A, p.T46K; c.9904G>A, p.E3302K) in the *CDH23* gene. This patient was diagnosed with bilateral moderate HL at 1 year of age. The earlier onset of hearing problem in the present study compared with the 3-13 years old onset in 10 Japanese patients. Since most patients with *CDH23* mutated allele reported to develop night blindness at 10-12 years old, genetic counselling might be warranted. *PCDH15* is a member of the cadherin superfamily, encode membrane proteins essential for calcium-dependent cell-cell adhesion and is crucial for normal retinal and cochlear function. Mutations in this gene result in HL and USH1F. A total of two patients (patient 144 and patient 217) with *PCDH15* gene mutations were identified. Patient 217 (4.1 years old) carried a novel compound heterozygous *PCDH15* mutations (c.4699_4715dup, p.Leu1573GlufsTer18; c.2001G>A, p.Thr667*), resulting in bilateral and profound deafness in the present study. The novel variantc.4699_4715dup (p.Leu1573GlufsTer18, NM_033056.4) may not undergo nonsense-mediated decay due to its location in the final exon, though the biological function of *PCDH15* isoforms (CD1, CD2 and CD3) remains unclear. Patient 144 (2.5 years old) carried a novel missense mutation (c.398T>G, p.V133G) and presented with bilateral and profound deafness.

NGS technologies have advantage to address the etiology of genetic heterogeneity such as HL which will be difficult to clinically distinguish. In the present study on pediatric HL, targeted exon-captured NGS approach focusing on a subset of known HL-associated genes proved to be both cost effective and high yield, especially for genetic screening. In this clinical scenario, it will benefit genetic counseling to face with challenge of causal link between variants of new genes to HL; however, limitations of targeted exon-captured NGS are needed to notice and even to recommend alternative genetic testing if necessary.

In conclusion, high-coverage targeted NGS was applied to elucidate the genetic profile of HL in the Chinese population. The results of the present study identified *GJB2, SCA26A4, MYO15A* and *USH2A* as the most common causative genes, with *GJB2* emerging as the predominant variant associated with early-onset deafness. Additionally, 33 novel variants were identified in known deafness-related genes, thereby expanding the variant spectrum of these genes. The current findings also provide insight into the age- and severity-related gene frequency patterns in HL. For patients with unresolved genetic cases, further investigations into the digenic model or other potential causes are warranted.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YF acquired funding and conducted project administration. ZZ and JD performed data curation, formal analysis, software analysis and visualization, and wrote the original draft. WC and JS provided resources and conducted investigation. FZ, XZ and PW conceptualized and supervised the study, and wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. JD and YF confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2024-IRB-0116-P-01) by the Ethics Committee of Children's Hospital Zhejiang University School of Medicine (Zhejiang, China).

Patient consent for publication

Written informed consent was obtained from patients and their parent or guardian for the publication of anonymized data and any accompanying images.

Competing interests

The authors declare that they have no competing interests.

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