Variant analysis of 92 Chinese Han families with hearing loss

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Abstract
Background: Hearing loss (HL) is the most frequent sensory deficit in humans, HL has strong genetic heterogeneity. The genetic diagnosis of HL is very important to aid treatment decisions and to provide prognostic information and genetic counseling for the patient’s family.

Methods: We undertook pedigree analysis in 92 Chinese non-syndromic HL patients by targeted next-generation sequencing and Sanger sequencing.

Results: Among the 92 HL patients, 18 were assigned a molecular diagnosis with 33 different variants in 14 deafness genes. Eighteen of the variants in 12 deafness genes were novel. Variants in TMC1, CDH23, LOXHD1 and USH2A were each detected in two probands, and variants in POU3F4, OTOA, GPR98, GJB6, TRIOBP, SLC26A4, MYO15A, TNC, STRC and TMPRSS3 were each detected in one proband.

Conclusion: Our findings expand the spectrum of deafness gene variation, which will inform genetic diagnosis of deafness and add to the theoretical basis for the prevention of deafness.

Keywords: Hearing loss, Genetic heterogeneity, Molecular diagnosis, Genetic counseling

Background
Hearing loss (HL) is the most frequent sensory deficit in humans, with a prevalence of approximately 1/1000 in newborns [1, 2]. Hearing loss in approximately 50% to 60% of individuals is caused by genetic factors [3]. Among these, approximately 70% are non-syndromic HL (NSHL), in which the hearing impairment is the only distinctive clinical feature, while 30% of HL patients are syndromic with other abnormalities [4]. NSHL also has strong genetic heterogeneity.

The genetic diagnosis of NSHL is very important to aid treatment decisions and to provide prognostic information and genetic counseling for the patient’s family [5, 6]. The genetic mode of NSHL inheritance can be autosomal recessive, autosomal dominant, mitochondrial, or X/Y-linked. The development of molecular diagnostic technology has greatly reduced the cost of testing, and next-generation sequencing (NGS) has become an effective way of providing comprehensive and efficient diagnosis for NSHL [7]. To date, 224 genes have been reported to be associated with hearing loss (https://morl.lab.uiowa.edu/genes-included-otoscope-v9). Sixty-six are autosomal dominant, 117 are autosomal recessive, 21 are autosomal dominant/autosomal recessive, 9 are mitochondrial, and 5 are X-linked. However, most of the variations in these genes are rare and have only been reported in one or a few families [8].

Molecular epidemiological studies have found that the three common deafness genes GJB2, SLC26A4, and mtDNA 12S rRNA accounted for 30–50% of congenital HL [9]. In China, nine variants in four genes are the most common causes of NSHL, including c.235delC (18.3%), c.299_300delAT (5.6%), c.176del16 (1.8%) and c.35delG (0.14%) of GJB2; c.919-2A>G (15.4%) and c.35delG (0.14%) of GJB2; c.919-2A>G (15.4%) and c.2168A>G (1.08%) of SLC26A4; m.1555A>G (1.76%)
and m.1494C>T (0.16%) of mtDNA 12S rRNA; c.538C>T (0.41%) of \textit{GJB3} [10–12]. A large neonatal cohort study in Beijing, China, showed that the heterozygous carrier rate of \textit{GJB2} gene was 2.3\%, the \textit{SLC26A4} was 1.6\%, the mtDNA 12S rRNA was 0.2\% and the \textit{GJB3} was 0.3\% [12].

Here, we recruited 92 Chinese Han NSHL families, who were confirmed not to carry the common HL variants in \textit{GJB2}, \textit{SLC26A4} and \textit{MT-RNR1}. Targeted NGS for known deafness genes was performed on the probands of each family to search for the genetic etiology of HL.

Methods

Recruitment of patients

92 patients with non-syndromic deafness were clinically diagnosed with bilateral sensorineural hearing loss at the Chinese People’s Liberation Army (PLA) General Hospital (Beijing). Audiological tests were performed in the hearing center of the Chinese PLA General Hospital. Tests included pure-tone audiometry (or behavioral audiometry) for patients > 4 years old and multiple-frequency auditory steady-state evoked response (ASSR) tests for patients \( \leq 4 \) years old [13]. All the probands were from non-consanguineous families. They were aged from 6 months to 54 years, and the age of onset ranged from birth to 22 years (Table 2).

Genomic DNA preparation

Blood samples (1–2 mL) were collected from the probands and their parents. Genomic DNA was extracted using a Tiangen DNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions and quantified spectrophotometrically by NanoDrop 2000 manufacturer (ThermoScientific, USA).

Targeted -NGS and Sanger sequencing

Targeted capture of candidate disease genes (Table 1) was performed using the GenCap™ Custom Enrichment kit (MyGenostics, Beijing, China). Data analysis and bioinformatics analysis were performed according to method described by previous study [6]. Candidate variants were confirmed in the proband’s parents in each family by Sanger sequencing. The PCR products were bi-directionally sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI 3500DX Genetic Analyzer (Applied Biosystems, USA) after purification of the products in 2% agarose gels by using a Tiangen Midi Purification kit (Tiangen Biotech, Beijing, China).

Bioinformatics analysis

Variants are described according to the nomenclature recommended by the Human Genome Variation Society (www.hgvs.org/). Variants were annotated using ANNOVAR (https://annovar.openbioinformatics.org/en/) and filtered according to their predicted effects and allele frequencies in the public database, gnomAD (http://gnomad.broadinstitute.org/). Novel variants were checked in the Human Gene Variant Database (HGMD; www.hgmd.cf.ac.uk/), ClinVar database (www.ncbi.nlm.nih.gov/clinvar/) and gnomAD database. We use PolyPhen2 (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph2) and PROVEAN (http://provean.jcvi.org/index.php) tools to assess the possible functional role of the novel variant. The conservativeness of the novel site is evaluated on the UCSC website (https://genome.ucsc.edu/). InterVar (http://wintervar.wglab.org/) was used to evaluate the pathogenicity of all

Table 1 Genes in the hearing loss target-NGS panel

| Gene                  | ACTG1 | CLPP | FOX1 | KRT9 | PCDH9 | SOX3 |
|-----------------------|-------|------|------|------|-------|------|
| ALX3                  |       |      |      |      |       |      |
| ATP6V0A1              |       |      |      |      |       |      |
| ATP6V0A2              |       |      |      |      |       |      |
| ATP6V0A4              |       |      |      |      |       |      |
| ATP6V0B               |       |      |      |      |       |      |
| ATP6V0C               |       |      |      |      |       |      |
| ATP6V0D               |       |      |      |      |       |      |
| ATP6V0E1              |       |      |      |      |       |      |
| ATP6V0E2              |       |      |      |      |       |      |
| ATP6V1A               |       |      |      |      |       |      |
| ATP6V1C               |       |      |      |      |       |      |
| ATP6V1D               |       |      |      |      |       |      |
| ATP6V1F               |       |      |      |      |       |      |
| ATP6V1G               |       |      |      |      |       |      |
| ATP6V1G2              |       |      |      |      |       |      |
| ATP6V1G3              |       |      |      |      |       |      |
| ATP6V1H               |       |      |      |      |       |      |
| BCL2L2                |       |      |      |      |       |      |
| BSDN                  |       |      |      |      |       |      |
| C19orf83              |       |      |      |      |       |      |
| CABP2                 |       |      |      |      |       |      |
| CAT                   |       |      |      |      |       |      |
| CCDC50                |       |      |      |      |       |      |
| CDC23                 |       |      |      |      |       |      |
| CDH23                 |       |      |      |      |       |      |
| CEACAM16              |       |      |      |      |       |      |
| CHD7                  |       |      |      |      |       |      |
| CIB2                  |       |      |      |      |       |      |
| CKMT1A                |       |      |      |      |       |      |
| CLDN14                |       |      |      |      |       |      |


| Patient ID | Sex | Age | Age of diagnoses | Gene | Inheritance | NM Transcript | Nucleotide change | Amino acid change | Variant type | gnomAD allele frequency | Reported or not | Disease | Pedigree |
|------------|-----|-----|------------------|------|-------------|---------------|------------------|------------------|--------------|-----------------------|----------------|---------|---------|
| 12471      | M   | 5.5Y| 2.5y             | POU3F4 | XLD         | NM_000307     | c.881A>G        | p.E294G         | Missense     | --                     | Novel          | X-linked deafness-2| Nuclear family |
| 12601      | F   | 33Y | 33Y              | CDH23 | AR          | NM_00110219   | c.228delC       | p.W77Gs         | Frameshift   | 0.00000725          | Novel          | Autosomal recessive deafness-3B | Nuclear family |
| 12792      | F   | 24Y | 6Y               | LOXHD1| AR          | NM_138691     | c.741+2T>C      | p.A2277D        | Missense     | --                     | Novel          | Autosomal recessive deafness-7 | Sporadic case |
| 12834      | M   | 31Y | 31Y              | TMCI  | AR          | NM_138691     | c.741+2T>C      | p.A2119fs       | Frameshift   | 0.0000065            | Novel          | Autosomal recessive deafness-77 | Nuclear family |
| 12852      | M   | 4Y  | 1Y               | LOXHD1| AR          | NM_144612     | c.5888delG      | p.G1963fs       | Frameshift   | 0.0000065            | Novel          | Autosomal recessive deafness-77 | Nuclear family |

Table 2 Variants analysis of the 18 HL patients in this study
| Patient ID | Sex | Age | Age of diagnoses | Gene | Inheritance | NM Transcrip | Nucleotide change | Amino acid change | Variant type | gnomAD allele frequency | Reported or not | Disease | Pedigree |
|------------|-----|-----|------------------|------|-------------|--------------|------------------|------------------|--------------|------------------------|----------------|---------|----------|
| 12918      | M   | 54Y | 54Y              | TNC  | AD          | NM_002160    | c.1641C>A       | p.C547X         | Nonsense      | 0.000004               | Novel          | Autosomal dominant deafness-56 | Sporadic case |
| 12929      | F   | 3Y  | 3Y               | TMC1 | AR          | NM_138691    | c.100C>T        | p.R34X          | Nonsense      | 0.000004               | [24, 24]       | Autosomal recessive deafness-7 | Nuclear family |
| 12932      | M   | 11Y | 10Y              | STRC | AR          | NM_153700    | c.4778C>T      | p.A1593V       | Missense      | 0.000004               | Novel          | Autosomal recessive deafness-16 | Nuclear family |
| 12933      | M   | 3Y  | 3Y               | TMPRSS3 | AR | NM_024022 | c.916G>A       | p.A306T        | Missense      | 0.000145               | [29, 30]       | Autosomal recessive deafness-8 | Nuclear family |
|            |     |     |                  | TMPRSS3 | AR | NM_024022 | c.271C>T       | p.R91X         | Nonsense      | 0.000004               | [27]           |                     |          |

M, male; F, female; Y, year's old; M, month; –, not included in the gnomAD database
variants according to the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) [14].

Results

Variant analysis

Among 92 probands analyzed, we determined a genetic diagnosis in 18, and all the 92 probands were from non-consanguineous families. Three modes of inheritance were observed, including 15 autosomal recessive cases, 2 autosomal dominant cases, and 1 X-linked recessive case (Table 2). Fourteen deafness gene variants were detected. Those in TMC1, CDH23, LOXHD1 and USH2A were each detected in two probands, and those in 10 other deafness genes were each detected in one proband (Table 2). The 18 probands carried 33 different variants (Table 2), of which 18 were novel, accounting for 54.5% of the total variants (18/33). These 33 variants included variants in 14 deafness genes (Table 2). We identified 18 novel variants in 12 deafness genes, which were not previously reported in ClinVar or HGMD. According to the ACMG guidelines and InterVar software, 10 variants of them were categorized as “pathogenic” variant, and 8 were categorized as “likely pathogenic” variants (Table 3).

Among the 18 probands who received a genetic diagnosis, 15 were autosomal recessive, two were autosomal dominant, and one was X/Y-linked. Yang et al. [23] recruited 190 NSHL patients, and after excluding the common GJB2, SLC26A4 and MT-RNR1 variants, 33 probands were determined to have rare HL variants, 28 were autosomal recessive, four were autosomal dominant, and one was mitochondrial. The number of autosomal recessive patients was much lower than in our study, which might be caused by regional differences. In our study, variants in TMC1, CDH23, LOXHD1 and USH2A were each detected in two probands, while variants in POLJS4, OTOA, GPR98, GJB6, TRIOBP, SLC26A4, MYO15A, TNC, STRC and TMPRSS3 were each detected in one proband. Among the 33 rare HL cases reported by Yang et al., the most frequently detected variant was in MYO15A (four times), then in TMC1, USH2A, PCDH15, and GPR98 (three times each) [23]. Although the detection rates of the TMC1 and USH2A variants were high in both this study and that of Yang et al., we detected an MYO15A variant in only one case, while Yang et al. did not detect any LOXHD1 variants, which we detected twice in our patients. These differences may be caused by regional differences between north and south China. Of course, this may also be caused by the sample size not being large enough.

Some deafness gene screening techniques can screen for hot-spot variants in SLC26A4. However, targeted screening tests might miss rare variants of SLC26A4. In patient 12751, we detected a compound heterozygous variant, c.589G>A/ c.1238A>G, which was not in the variant hot-spots of SLC26A4. Therefore, for patients with deafness, it is best not to use deafness gene screening technology. Targeted sequencing technology or whole exome sequencing technology should be used for diagnosis.

CNV is one of the main forms of structural genome variation, and is a cause of many genetic diseases. NGS is increasingly used to test for CNVs in many diseases. In patient 12932, we detected a CNV (a heterozygous deletion) in STRC, which has been previously reported [27, 28]. STRC CNV is common in HL patients [31] and 72 types of deletion and 35 duplications of STRC are included in the ClinVar database. Targeted-NGS methods to detect CNVs in HL patients can still be improved, for example specificity and sensitivity can be enhanced; however, whole exome sequencing (WES) or whole-genome

Discussion

In this study, we performed a variant analysis of 92 unrelated Chinese NSHL patients. We determined a molecular diagnosis in 18 probands, with 33 different variants in 14 deafness genes (Table 2). We identified
sequencing (WGS) are recommended to detect CNV in HL patients.

In our study, we identified 18 novel variants in 12 deafness genes. These variants included eight missense variants, four nonsense variants, five frameshift variants and 3 splice site variants (Table 3). The nonsense variants and frameshift variants caused the peptide chain to terminate prematurely, which shortened the length of the peptide chain, and then affected the function of the gene. We use PolyPhen2 and PROVEAN tools to assess the possible functional role of the eight novel missense variant. The missense variants c.805C>T, c.5957T>C and c.6830C>A of CDH23, c.774A>C of OTOA were assessed as probably damaging by PolyPhen2, however, these four missense variants were assessed as neutral by PROVEAN. Then we checked the conservative of these four missense variants, all the four variant were highly conserved in different species. Combined with ACMG guidelines and InterVar software, we speculated that these four missense variants were "likely pathogenic" variants.

Conclusions

We used targeted-NGS for genetic diagnosis of 18 NSHL probands. We identified 18 novel variants in 12 deafness genes, which enlarged the variant spectrum of deafness genes in the Han Chinese population. These findings help inform the genetic diagnosis of deafness and add to the

| Table 3 | Pathogenicity analysis of novel variants |
|---|---|---|---|---|---|
| **Gene** | **Nucleotide change** | **Amino acid change** | **PolyPhen2 Result (Score)** | **PROVEN Result (Score)** | **Pathogenicity** | **Conservative** | **ACMG evidence** |
| CDH23 | c.805C>T | p.R269W | PD(1.000) | N (−2.154) | LP | Yes | PM1, PM2, PP1, PP3 |
| CDH23 | c.5994delG | p.V1998fs | – | – | P | Yes | PM1, PM2, PP1, PP3 |
| CDH23 | c.5957T>C | p.L1986P | PD(1.000) | N (−0.743) | LP | Yes | PM1, PM2, PP1, PP3 |
| CDH23 | c.6830C>A | p.A2277D | PD(0.999) | N (−2.146) | LP | Yes | PM1, PM2, PP1, PP3 |
| GJB6 | c.228delC | p.W77Gfs | – | – | P | Yes | PV51PM2, PV51PM4 |
| GPR98 | c.12640C>T | p.Q4214X | – | – | P | Yes | PV51, PV52, PV53 |
| LOXHD1 | c.2295G>A | p.W76SX | – | – | P | Yes | PV51, PV52, PV53 |
| LOXHD1 | c.134A>C | p.Y45S | PD(0.999) | D (−5.352) | LP | Yes | PM1, PM2, PP1, PP3 |
| LOXHD1 | c.635delG | p.A2119fs | – | – | P | Yes | PV51, PV52, PV54 |
| MYO15A | c.6611G>A | p.R2204H | PD(1.000) | D (−4.955) | LP | Yes | PM1, PM2, PP1, PP3 |
| OTOA | c.774A>C | p.L258F | PD(1.000) | N (−2.150) | LP | Yes | PM2, PM3, PP1, PP3 |
| OTOA | c.881A>G | p.E294G | PD(1.000) | D (−7.000) | LP | Yes | PM1, PM2, PP1, PP3 |
| STRC | c.4778C>T | p.A1593V | PD(0.999) | D (−3.044) | LP | Yes | PM2, PM3, PP1, PP3 |
| TMCI | c.741+2T>C | splicing | – | – | P | Yes | PV51, PV52, PV54 |
| TNC | c.1641C>A | p.S547X | – | – | P | Yes | PV51, PV52, PV53 |
| TRIOBP | c.1960C>T | p.R654X | – | – | P | Yes | PV51, PV52, PV53 |
| TRIOBP | c.5968delT | p.F1990fs | – | – | P | Yes | PV51, PV52, PV54 |
| USH2A | c.3791delC | p.S1264fs | – | – | P | Yes | PV51, PV52, PV54 |

Table 4 Primer sequences for RT-PCR

| Primer’s name | Primers | Length |
|---|---|---|
| STRC | Forward AGTAAGTCTCCTTACCTCAG 81 bp | Reverse TCCAGCATACAGCTTATGAT |
| GAPDH | Forward CGGAGTCAGCGATATCGTCTAT 308 bp | Reverse AGCCTTTCATGTTGAAGAC |

Fig. 1 The real time PCR verification of the STRC copy number variant
theoretical basis for the prevention of deafness. However, 74 patients in our cohort did not receive a clear genetic diagnosis; therefore, further WES or WGS testing is needed to identify mutations in other HL-causing genes or to discover new disease-causing genes for these patients.

Abbreviations
ACMG: American College of Medical Genetics and Genomics; ASSR: Auditory steady-state evoked response; CNV: Copy number variation; HGMD: Human Gene Variant Database; HL: Hearing loss; NSHL: Non-syndromic hearing loss; NGS: Next-generation sequencing; WES: Whole exome sequencing; WGS: Whole-genome sequencing.

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Authors’ contributions
XHJ, PD, HFG and XM designed the study and write the manuscript. XHJ, SSSH, LSA and CZ performed the molecular tests. All authors contributed to the editing of the manuscript and the scientific discussions. All authors read and approved the final manuscript.

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Availability of data and materials
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2021), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human:HR001546) that are publicly accessible at https://bigd.big.ac.cn/gsahuman/browse/HR001546.

Declarations

Ethics approval and consent to participate
This study was undertaken according with the tenets of the Declaration of Helsinki 1975 and its later amendments. This study was approved by the Ethics Committee of the Chinese People's Liberation Army (PLA) General Hospital (Beijing) (reference number S2016-120-02). Written informed consent was obtained from all of the adult participants and written informed consent of patients younger than 16 years old was obtained from their parents.

Consent for publication
Written consent was obtained from all the participants and for those younger than 18 years old, obtained from their parents.

Competing interests
The authors declare no competing interests.

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