Whole Exome Sequencing of Six Chinese Families with Hereditary Non-syndromic Hearing Loss

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Research article

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Abstract

Background:

Hereditary non-syndromic hearing loss (NSHL) has a high genetic heterogeneity with >152 genes identified as associated molecular causes. The present study aimed to detect the possible damaging variants of the deaf probands from six unrelated Chinese families.

Methods:

After excluding the mutations in the most common genes, GJB2 and SLC26A4, 12 probands with prelingual deafness and autosomal recessive inheritance were evaluated by whole-exome sequencing (WES). All the candidate variants were verified by Sanger sequencing in all patients and their parents.

Results:

Biallelic mutations were identified in all deaf patients. Among these six families, 10 potentially causative mutations, including 3 reported and 7 novel mutations, in 3 different deafness-associated autosomal recessive (DFNB) genes (MYO15A, COL11A2, and CDH23) were identified. The mutations in MYO15A were frequent with 7/10 candidate variants. Sanger sequencing confirmed that these mutations segregated with the hearing loss of each family.

Conclusions:

Next-generation sequencing (NGS) approach becomes more cost-effective and efficient when analyzing large-scale genes compared to the conventional polymerase chain reaction-based Sanger sequencing, which is often used to screen common deafness-related genes. The current findings further extend the mutation spectrum of hearing loss in the Chinese population, which has a positive significance for genetic counseling.

Background

Hearing loss is one of the most common sensorineural disorders with high heritability [1]. More than 5% of the world’s population has disabling hearing loss (432 million adults and 34 million children) (http://www.who.int/mediacentre/factsheets/fs300/en/, updated March 2018). In China, about 30,000 newborns suffer from congenital hearing impairment for every 20 million live births each year [1]. Approximately 70% of the cases are non-syndromic hearing loss (NSHL), which can be divided into autosomal dominant deafness (DFNA, 15–20%), autosomal recessive deafness (DFNB, 80%), X-linked deafness (DFNX, 1%), and mitochondrial deafness in 1% [3, 4].

Genetic factors account for an estimated 60% of profound hearing loss present at birth or during early childhood [3, 4]. Among various ethnic groups, the most common cause for DFNB is mutated Connexin 26, encoded by GJB2 gene (MIM 121011) [6, 7]. In the Chinese population, mutations in the SLC26A4
gene (14.5%) are the second common pathogenic cause of DFNB in addition to the GJB2 gene (17.9%) [8, 9]. Sanger sequencing to detect mutations in GJB2 and SLC26A4 is a routine method during genetic testing and counseling for deafness patients. However, for GJB2 or SLC26A4-negative patients, especially those from small-sized recessive families, the potential genetic causes for deafness are yet unclear.

The genetic heterogeneity of hearing loss, with about 152 deafness-associated genes, identified to be associated with NSHL [10], makes conventional polymerase chain reaction (PCR)-based Sanger sequencing impractical for large-scale gene detection because of its cost-inefficiency and time-consumption [11]. In recent years, next-generation sequencing (NGS) approach may make it possible to analyze multiple genes, including all the deafness-associated genes, in one test [12]. Whole exome sequencing (WES), a platform of NGS, offers powerful applications for diagnosis as well as identifying rare variants or new causative genes [13, 14].

In this study, we utilized WES to investigate the contributing genetic factors in patients from six unrelated Chinese families with autosomal recessive hearing loss but did not harbor GJB2 and SLC26A4 mutations.

Methods

Six unrelated Chinese families (code as HL01–06) were recruited from the outpatient clinic of the Department of Otolaryngology, Head and Neck Surgery, Xijing Hospital (Xi’an, Shaanxi, China) in 2019. These families presented an autosomal recessive pattern of hearing loss, with at least two affected siblings in each family but with unaffected parents. Physical and neurological examination excluded any putative environmental factors responsible for the condition or syndromic hearing loss.

This study was approved by the Ethics Committee of the Xijing Hospital. Written informed consent was obtained from all the study subjects or their guardians.

Clinical test

All affected individuals underwent a comprehensive auditory evaluation, including otoscope examination, tympanometry, pure-tone audiometry (PTA, at frequencies from 250 to 8000 Hz), auditory brainstem response (ABR), distortion product otoacoustic emissions (DPOAEs), or steady-state auditory response (ASSR) test in children aged <5 years. The level of hearing impairment was assessed by the World Health Organization (WHO)'s standard: “slight/mild (26–40 dB); moderate (41–70 dB); severe (71–90 dB), and profound (>90 dB)” [15]. Computed tomography (CT) scan and magnetic resonance imaging (MRI) was performed to detect normal development of the inner ear and auditory nerve.

DNA preparation

Genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (CwBio, Beijing, China), according to the manufacturer's protocol, and stored at 4 °C. DNA purity and concentration were
assessed by Nano Photometer® spectrophotometer (Implen, CA, USA). The absorbance (OD260/OD280) of the qualified sample was 1.8–2.0.

**Pre-screening of common deafness genes**

The pre-screening of common deafness genes, *GJB2* and *SLC26A4*, was performed by polymerase chain reaction (PCR) amplification (Bio-Rad, USA), and the exons and flanking splicing sites were further assessed by Sanger sequencing.

**WES**

A total of 12 patients from six families were analyzed by WES. Agilent SureSelect Human All Exon V6 DNA Kit (Agilent, Santa Clara, CA, USA) was used for whole-exome capturing. The qualified genomic DNA was randomly fragmented to an average size of 180–280 bp by Covaris S220 sonicator. Next, the DNA fragments were end-repaired and phosphorylated, followed by A-tailing and ligation at the 3’-ends with paired-end adaptors (Illumina) with a single “T” base overhang; the purification was carried out using AMPure SPRI beads from Agencourt. Then, the size distribution and concentration of the libraries were determined by Agilent 2100 Bioanalyzer and quantified using real-time PCR (2 nM). Finally, the DNA library was sequenced on Illumina HiSeq 4000 for paired-end 150-bp reads. The variants were filtered based on the monogenic autosomal recessive trait.

**Filter and Evaluation**

The variants obtained from previous steps were filtered with the minor allele frequency (MAF)<1% in the databases, including the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org) and the 1000 Genomes databases (http://browser,1000genomes.org). Only variants, such as missense, nonsense, splicing site (splicing junction 10 bp), in-frame indels, and frameshift indels, which can cause amino acid changes, were analyzed further. According to the principle of autosomal recessive inheritance, homozygous or compound heterozygous variants shared by affected siblings from the same family were evaluated. Searching the Deafness Variation Database (DVD; http://deafnessvariationdatabase.org), Online Mendelian Inheritance in Man database (OMIM; http://www.omim.org), ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/), and Human Gene Mutation Database (HGMD; Http://www.hgmd.cf.ac.uk/), allowed us to screen the reported pathogenic or putatively pathogenic variants. Furthermore, the novel pathogenic functional nonsynonymous variants were predicted by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and Mutation Taster (http://www.mutationtaster.org/), while conservation scores were calculated by PhyloP. At least two of these tools were required to show that the nonsynonymous variants were not benign. The American College of Medical Genetics and Genomics (ACMG) guidelines[16] were used to classify the pathogenic and likely pathogenic levels of these variants.

**Verification**
The variants identified to cause deafness in these families were verified by Sanger sequencing in all patients and their parents. The primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3/), and Genetool was used to analyze the sequencing data.

**Results**

**Clinical findings**

All the affected members of the six families presented bilateral symmetrical NSHL that began at birth or the critical age of language learning. Immittance test demonstrated normal and bone conduction values equal to the air conduction measurements, suggesting a sensorineural hearing impairment. The CT scan of the temporal bone in the proband excluded inner ear malformations. Among 12 deaf patients from six unrelated families, 10 showed bilateral symmetrical severe hearing loss except, while 2 had moderate hearing loss in the HL01 family. The members of the HL01 family belonged to Hui ethnicity, and the parents of the patients are remote cousins. The clinical information of the deaf probands is summarized in Table 1.

**Common deafness genes sequencing**

*GJB2* and *SLC26A4* genes were sequenced in the proband of every family, and no mutations were detected.

Table1. Patients with HL from six Chinese families analyzed by WES
<table>
<thead>
<tr>
<th>Family and patients code</th>
<th>Hearing status</th>
<th>Year of birth</th>
<th>Sex</th>
<th>Ethnicity</th>
</tr>
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<tr>
<td>HL01:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI-1</td>
<td>congenital moderate sensorineural HL</td>
<td>2008</td>
<td>female</td>
<td>Hui</td>
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<tr>
<td>VI-2</td>
<td>congenital moderate sensorineural HL</td>
<td>2011</td>
<td>male</td>
<td>Hui</td>
</tr>
<tr>
<td>HL02:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1</td>
<td>congenital profound sensorineural HL</td>
<td>2003</td>
<td>male</td>
<td>Han</td>
</tr>
<tr>
<td>II-2</td>
<td>congenital profound sensorineural HL</td>
<td>2015</td>
<td>male</td>
<td>Han</td>
</tr>
<tr>
<td>HL03:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>II-1</td>
<td>congenital profound sensorineural HL</td>
<td>2004</td>
<td>female</td>
<td>Han</td>
</tr>
<tr>
<td>II-2</td>
<td>congenital profound sensorineural HL</td>
<td>2007</td>
<td>female</td>
<td>Han</td>
</tr>
<tr>
<td>HL08:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>congenital profound sensorineural HL</td>
<td>2001</td>
<td>male</td>
<td>Han</td>
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<tr>
<td>II-2</td>
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<td>2006</td>
<td>male</td>
<td>Han</td>
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<td>HL09:</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>congenital profound sensorineural HL</td>
<td>2006</td>
<td>male</td>
<td>Han</td>
</tr>
<tr>
<td>II-2</td>
<td>congenital profound sensorineural HL</td>
<td>2009</td>
<td>male</td>
<td>Han</td>
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<tr>
<td>HL15:</td>
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<td>II-1</td>
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<td>2015</td>
<td>male</td>
<td>Han</td>
</tr>
<tr>
<td>II-2</td>
<td>congenital profound sensorineural HL</td>
<td>2018</td>
<td>female</td>
<td>Han</td>
</tr>
</tbody>
</table>

HL- hearing loss; WES – whole exome sequencing.

**Candidate pathogenic variants identified by whole-exome sequencing**
Among all the patients from the six families showing NSHL with autosomal recessive inheritance, we screened 76 genes recorded in the Heretical Hearing Loss Homepage database (http://hereditaryhearingloss.org/. Accessed Aug 2020), and these were related to non-syndromic autosomal recessive hearing loss.

Consistent with the recessive inheritance, 10 possible causative variants in 3 deafness-related genes (MYO15A, COL11A2, and CDH23) were detected in homozygotes (two families) or compound heterozygotes (four families, Additional file 1). Although the genes previously known to be associated with hearing loss, except for 3 variants (p.T323Hfs*19 in COL11A2, p.3417delS and c.5964+3G>A in the MYO15A gene) associated with non-syndromic deafness in previous studies [17-21], 7/10 variants were identified in this study for the first time; these included 4 missense variants (p.C1297F and p.K2217E in CDH23, p.G1315R and p.F2861S in MYO15A), 2 frame-shifting (p.Q2749Efs*93 and p.S3474Pfs*43 in MYO15A), and 1 splicing site (c.4875+1G>T in MYO15A). Moreover, c.4875+1G>T at the conserved donor splice site of MYO15A might affect the correct translation of the protein. c.8240-8241delAC (p.Q2749Efs*93) and c.10419–10423delCAGCT (p.S3474Pfs*43) in MYO15A are predicted to generate a premature termination codon. For 4 missense variants, c.3943G>A (p.G1315R) and c.8582T>C (p.F2861S) in MYO15A, c.3890G>T (p.C1297F) and c.6649A>G (p.K2217E) in CDH23, evolutionary conservation prediction showed that the amino acid substitutions were highly conserved across vertebrates. The first 3 missense variants interrupt the normal protein function, as predicted by the bioinformatic programs (Polyphen2, SIFT, and Mutation Taster). However, p.K2217E in CDH23 was a benign variant predicted by Polyphen2 but damaging by other programs; albeit it has not been recorded in 1000 Genome database and has an extremely low frequency in the ExAc database. In addition, p.K2217E was found only in patients from HL02 family with another missense mutation (p.C1297F); the members were cosegregated with hearing loss, suggesting that this was likely pathogenic rather than a benign variant.

**Parental genotyping of the candidate variants**

Parental genotypes were assessed using Sanger sequencing to determine whether these candidate variations followed the recessive inheritance pattern or de novo pattern. All the 10 candidate variants were biallelic and showed co-segregation with the deaf phenotype of the families (Fig. 1).

**Discussion**

In this study, WES was applied to search for molecular etiology in 12 patients from six unrelated Chinese families with non-syndromic deafness. These families showed obvious autosomal recessive heritance based on at least two affected individuals with normal parents. After excluding the common GJB2 and SLC26A4 mutations, we analyzed the WES results of 76 non-syndromic autosomal recessive HL-related genes. Overall, 3 reported and 7 novel variants in 3 distinct deafness genes (COL11A2, CDH23, and MYO15A) were identified and cosegregated with HL. The corresponding amino acid
changes included 4 missense mutations, 3 frameshift indel mutations, 1 in-frame mutation, and 2 splicing-site mutations.

In family HL01, the patients carried a reported homozygous variant c.966dupC (p.T323Hfs*19) in the COL11A2 gene, and both parents were carriers of the heterozygous variant. Remarkably, this was also the only family to present moderate bilateral deafness. The COL11A2 encoding type XI collagen is critical for the integrity and development of the skeleton [22]. Mutations in COL11A2 (OMIM 120290) are associated with several conditions that include autosomal dominant (DFNA13, OMIM 601868) or recessive NSHL (DFNB53, OMIM 609706), as well as Stickler syndrome (OMIM 184840), otospondylomegaphyseal dysplasia (OMIM 215150), fibrochondrogenesis (OMIM 614524), and Weissenbacher-Zweymuller syndrome (OMIM 277610). Hitherto, 61 variants have been reported to be pathogenic or likely pathogenic, of which, 12 are associated with sensorineural hearing loss (http://www.hgmd.cf.ac.uk/ac/). In addition, c.966dupC (p.T323Hfs*19) has also been detected in the probands from consanguineous Iranian families that show profound [17] or moderate [18] sensorineural hearing loss. COL11A2 is considered a “rare gene” responsible for deafness, especially in China. Only a few cases of variants have been reported in the hearing-loss patients from China. Ji et al. [23] reported 4 copy number variations (CNVs) in 11/79 sporadic patients clinically diagnosed with sensorineural hearing loss. Among these, only 1 patient carried two types of CNVs, while the remaining 10 had only one heterozygous form of CNV without any other form of variation in the COL11A2 gene. Chen et al. [24] detected p.Pro445Ser in 116 Chinese patients with hereditary hearing loss using next-generation sequencing that examined 60 genes relevant to hearing loss. Thus, COL11A2 gene is not the main cause of deafness and could be easily missed by the conventional sequencing approach.

Homozygous or compound heterozygous variants in MYO15A gene were identified in the affected members from families HL02, HL04, HL05, and HL06, who presented similar phenotypes showing a bilateral congenital severe hearing loss. Compound heterozygous c.8240_8241delAC (p.Q2749Efs*91)/c.10419_10423delCAGCT (p.S3474Pfs*41), compound heterozygous c.4875+1G>T/c.3943G>A (p.G1315R), homozygous c.8582C>T (p.F2861S), and c.5964+3G>A/c.10245-10247delCTC (p.3417delS) were identified as causative pathogenic etiology in the patients from HL02, HL04, HL05, and HL06, families, respectively. MYO15A is the corresponding gene of DFNB3 deafness (OMIM: 600316) [25]. Moreover, the variants of MYO15A gene are the third or the fourth most common causes of autosomal recessive hereditary hearing loss [26]. To date, 244 MYO15A pathogenic or likely pathogenic mutations contribute to hearing loss (http://www.hgmd.cf.ac.uk/ac/). Such mutations are identified in the sequence encoding all the domains of myosin XV protein [27]. In this study, we identified 7 variants in MYO15A gene, including 2 reported pathogenic mutations: c.5964+3G>A and c.10245-10247delCTC (p.3417delS) [19-21]. Five novel variants included 2 missense mutations (p.G1315R and p.F2861S), 2 protein-truncating mutations (p.Q2749Efs*93 and p.S3474Pfs*43), and 1 canonical + 1 splicing-site mutation (c.4875+1G>T). p.G1315 and c.4875+1G>T, occurring in exons 7 and 15, respectively, located within the motor domain, the most important functional unit in myosin XV protein for the dysfunction of the motor domain leads to short stereocilia with a severe deafness phenotype [28]. p.Q2749Efs*93 and p.S3474Pfs*43, leading to early termination of its coding peptide chain, might affect
the normal formation of the FERM domain of myosin XVA. p.F2861S variant is also harbored within the conserved FERM domain. The substitution of phenylalanine with serine changed the hydrophobicity and altered the structure of the protein. Earlier findings suggested that mutations, which affect both isoforms of MYO15A, would result in profound deafness [29, 30]. According to ACMG, we hypothesized that these variants occurring in the above exons were the causative pathogenic variants.

In family HL03, we identified two novel deafness co-segregating variants c.G3890T (p.C1297F) and c.A6649G (p.K2217E) in CDH23 gene. The two affected sisters, carrying the heterozygous compound variants, showed bilateral congenital profound hearing loss. According to the parents, the individual II:3 had a normal hearing but was not available for testing, neither clinical nor genetic. Mutations in CDH23 are the pathogenic cause for Usher syndrome 1D (USH1D, OMIM: 601067), autosomal recessive NSHL (DFNB12, OMIM: 601386), and pituitary adenoma 5 (OMIM: 617540). A total of 373 disease-related forms of variations are included in the HGMD database, of which 351 were associated with deafness or Usher syndrome. More than half of the variants were nonsense, splice-site, and frameshift types in the patients with USH1D, causing severe sensorineural deafness, vestibular dysfunction, and retinitis pigmentosa, whereas individuals with DFNB12, presenting only as autosomal recessive moderate-severe sensorineural hearing loss, usually carried missense variants in any domain of CDH23 gene (data summarized from the website: http://www.hgmd.cf.ac.uk/ac/). However, only a few of these variants were detected in the Chinese population [31]. In the present study, two variants were identified in the CDH23 gene by WES in two patients with severe hearing loss. These two variants were reported for the first time and were contained in the highly conserved domain of the CDH23 gene. Other than deafness, neither of the patients had visual problems or vestibular dysfunction at the time of this report. Nonetheless, we cannot definitely rule out that they would not develop retinopathy later in their lives, since they are currently only 11- and 5-years-old, respectively. Thus, these two novel mutations expanded the CDH23 mutation spectrum.

Next, we identified 2 homozygous variants in the affected members from HL01 and HL05 families. HL01 family came from the Ningxia Hui Autonomous Region in China. All the members were of Hui nationality, and the parents of the patients were remote cousins. Homozygous variants accounted for a high rate of diagnoses in close and presumed consanguineous multiplex families with recessive inherited disease [32], suggesting us to consider the segregation of homozygous variants. The normal parents of HL05 were not consanguinely married and were of Han nationality from Shaanxi province in China. All the other members were normal, except the deaf offspring. The homozygous variant detected in MYO15A indicated its high prevalence in the Chinese population and suggested the necessity of screening MYO15A in the recessive inherited families with multiple affected individuals.

Conclusions

The current data confirmed the utility of WES as an effective supplement to traditional Sanger sequencing for analyzing potential damaging candidate variants. WES-based genetic testing might provide valuable insight into the genetic etiology of a heterogeneous disease, such as hearing loss. The mutations identified in the present study are thought to be pathological or likely pathological because
there are all located in functionally important and evolutionarily conserved domains, and the variants are cosegregated with the deafness phenotype; also, these were not observed in 100 unrelated controls. Moreover, our findings further extended the pathogenic variations of *MYO15A*, *CDH23*, and *COL11A2* genes leading to non-syndromic autosomal recessive deafness, which clarified the diagnosis and provided essential information for accurate genetic counseling and guidance for future gene-based therapies.

**Abbreviations**

NSHL: Hereditary non-syndromic hearing loss

WES: whole-exome sequencing

DFNB: deafness-associated autosomal recessive

DFNA: autosomal dominant deafness

DFNX: X-linked deafness

NGS: Next-generation sequencing

NSHL: non-syndromic hearing loss

PCR: polymerase chain reaction

PTA: pure-tone audiometry

ABR: auditory brainstem response

DPOAEs: distortion product otoacoustic emissions

ASSR: steady-state auditory response

ExAC: Exome Aggregation Consortium

DVD: Deafness Variation Database

OMIM: Online Mendelian Inheritance in Man database

HGMD: Human Gene Mutation Database

CNVs: copy number variations

**Declarations**

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Xijing Hospital. Written informed consent was obtained from all the study subjects or their guardians.

Consent for publication

The written informed consent for publication of the enrolled patients’ clinical data have been obtained from their parents.

Availability of data and materials

The data supporting the conclusions of this article are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors` contributions

Conceptualization and Funding acquisition, DJZ; Manuscript drafting, PFL; Exome sequencing and segregation analysis, FPC; Investigation, JW and JC; Ascertained families and obtained clinical data, SJW and QL; PCR and Sanger sequencing, PFL and WL; Supervision, DJZ. PFL and FPC contributed equally to this work. All authors read and agreed to the final version of the manuscript.

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