Comprehensive Molecular Screening in Chinese Usher Syndrome Patients

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Usher syndrome (USH) is an autosomal recessive disorder characterized by visual impairment due to retinitis pigmentosa (RP), sensorineural hearing loss, and variable vestibular dysfunction. This disorder affects 3.5 to 6.2 out of 100,000 people and accounts for approximately 50% of hereditary deaf-blind individuals. Clinically, USH is usually divided into three subtypes based on the onset age, the severity of retinal and hearing defects, and whether or not the disorder is accompanied by vestibular dysfunction. Usher syndrome type 1 (USH1) is characterized by congenital profound hearing impairment, early-onset night blindness, and balance dysfunction. Usher syndrome type 2 (USH2) patients present with moderate to severe nonprogressive hearing loss without vestibular dysfunction and usually develop RP in the second or the third decade of their life. Usher syndrome type 3 (USH3) patients show progressive hearing defects, a variable onset age of RP, and variable involvement of vestibular function. Of the three clinical subtypes, USH2 is the most prevalent one, affecting 56% to 67% of all USH patients, while USH1 is the most severe form, accounting for 33% to 44% of USH cases. USH3 is rare in most populations, affecting about 1% to 6% of USH patients, except in Finnish and Ashkenazi Jewish populations, in which approximately 40% of USH patients are classified as having USH3. Moreover, some USH patients show phenotypic variability, so they could not be classified under any of the three subtypes. These patients are finally diagnosed with atypical USH.

Furthermore, USH is genetically heterogeneous, and 13 genes have been reported to be associated with USH, based on RetNet (https://sph.uth.edu/Retnet/sum-dis.htm, in the public domain). Disease-causing genes of USH1 include CDH23, CIB2, MYO7A, PCDH15, USH1C, and USH1G. Among these genes, the most common one is MYO7A, which was reported to account for 15% to 53.2% of USH1 patients. Mutations of USH2A, GPR98, and DFNB31 are responsible for USH2 patients, and USH2A bears the main responsibility for this subtype (more than 60% of USH2 patients). The only USH3 gene is CLRN1. Recently, mutations of ABHD12, CEP250, and HARS have been described as causing atypical USH. Additionally, PDZD7 has been proposed as a modifier gene for patients with mutations of USH2A and a contributor of digenic inheritance with GPR98. What makes it more complicated is that mutations of USH2A may lead to RP without hearing loss, and mutations of all six USH1 genes may cause non-syndrome deafness. Such complexity poses challenges to the molecular diagnosis for USH patients.

Although traditional Sanger sequencing of all exons of the USH gene can solve more than 80% of USH families, it is both time consuming and expensive due to the large sizes of most USH genes (435 coding exons), especially for a large study...
cohort. An array-based mutation screening (arrayed primer extension technology, APEX) is an efficient and rapid technique of identifying previously reported mutations; however, it results in a lower mutation detection rate due to the high proportion of the family-specific pathogenic mutations. When the USH genotyping microarray is used, the mutation detection rate is about 33%, both in a large Spanish cohort and in an Italian cohort. Moreover, several studies have demonstrated that copy number variants (CNVs) of some USH genes account for certain proportions of mutations.

Most of these CNVs are heterozygous and cannot be detected by Sanger sequencing or APEX. These kinds of genomic DNA rearrangements must be detected by other techniques, such as real-time quantitative polymerase chain reaction (q-PCR), multiplex ligation-dependent probe amplification (MLPA), or comparative genomic hybridization (a-CGH). Next-generation sequencing (NGS) technologies developed in recent years can perform whole genome; whole exome; and targeted exome sequencing (TES). Several studies have proven TES as a high-efficiency method of molecular diagnosis of USH, with a detection rate of around 70%. Targeted NGS can detect both point mutations and large genomic DNA rearrangements; its ability to detect CNVs is related to the coverage depth of TES.

In one Spanish study, six different CNVs of USH2A, CDH23, PCDH15, and GPR98 were detected by their own capture panel TES, with a mean coverage of 1334x. In another study, two large deletions of USH2A were identified by TES, with a mean coverage of 300x. In contrast, no CNV was identified in a cohort that included 14 patients with only one mutation identified after their TES analysis, with a mean coverage of 77x.

The genetic and clinical characteristics of USH patients have been reported in many Caucasian case series; however, the mutation spectrum in Chinese patients is limited. In this paper, we report the results of a comprehensive molecular screening of 119 probands with USH, using a combination of techniques comprising TES, Sanger sequencing, and real-time q-PCR or MLPA analysis.

SUBJECTS AND METHODS

Patients

In total, 119 unrelated individuals who were clinically diagnosed with USH1 (20 cases) and USH2 (99 cases) were enrolled at the Genetics Laboratory of the Beijing Institute of Ophthalmology, Beijing Tongren Ophthalmic Center. This cohort included 87 sporadic patients and 32 cases with family histories. All molecular screening procedures were prospectively reviewed and approved by the ethics committee of Beijing Tongren Hospital and carried out under the institutional instructions of the Beijing Tongren Hospital Joint Committee on Clinical Investigation and according to the tenets of the Declaration of Helsinki. Each patient underwent a standard instructions of the Beijing Tongren Hospital Joint Committee on Clinical Investigation and according to the tenets of the Declaration of Helsinki. Each patient underwent a standard instructions of the Beijing Tongren Hospital Joint Committee on Clinical Investigation and according to the tenets of the Declaration of Helsinki.

The hearing loss was divided by the pure-tone average (PTA) over 500, 1000, 2000, and 4000 Hz: normal hearing, <20 dB; mild, moderate, severe, and profound hearing defects corresponding to 21 to 40 dB, 41 to 70 dB, 71 to 90 dB, and >91 dB, respectively. Based on the previously reported clinical diagnostic criteria, the patients in the cohort were further classified into subtypes based on their clinical histories, ophthalmologic examinations, the severity of their hearing loss, and the vestibular function assessment (delay in motor development or unsteady gait). Peripheral blood samples of all probands and their relatives were collected, and genomic DNA was isolated from leukocytes by means of the genomic DNA extraction and purification kit (Vigorous Whole Blood Genomic DNA extraction Kit; Vigorous Technology Development Co. Ltd, Beijing, China), according to the manufacturer's protocol. The DNA was quantified with a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Waltham, MA, USA).

PCR-Based Sequencing of the USH2A Gene

Our previous study indicated that exons 43, 13, and 2 of USH2A were mutational hot spots, so these exons were first sequenced for all patients diagnosed with USH2. As long as a heterozygous pathogenic/likely pathogenic mutation was detected, the remaining 68 coding exons, including the intron-exon boundary of USH2A, were sequenced to screen for the other pathogenic mutations. Overall, coding regions (exons 2–72) of USH2A were amplified by PCR in 29 USH2 patients (including six previously described cases). The PCR amplifications were done with standard reaction mixtures, and purified amplicons were sequenced using a DNA sequencer (ABI Prism 373A; Applied Biosystems, Foster City, CA, USA). The sequencing results were compared with the published cDNA sequence of USH2A (GenBankNM_206933).

TES and Bioinformatics Analysis

A TES panel was developed to capture 188 known inherited retinal degeneration (IRD) genes using custom enrichment kits (GenCap; MyGenostics, Beijing, China) and following the manufacturer's instructions, as described previously. The panel comprises 2894 exons with a total size of 550 kilobases (kb). Of the 2894 exons, 410 belong to 11 USH genes and one possible USH modifier gene, which include ABHD12, CDH23, CIB2, CIRN1, DENB31, GPR98, HARS, MYOTA, PCDH15, USH1C, USH2A, and PDZD7. A list of the target genes of our panel is summarized in Supplementary Table S1. Genomic DNA (1–3 μg) was fragmented into approximately 300–450 base pairs by endonuclease digestion and used to capture the targeted genomic sequences. Illumina library preparation and the capture experiments were performed as previously reported. The enrichment libraries were sequenced on a commercial device (NextSeq 500: Illumina, Inc., San Diego, CA, USA) as 100-bp paired end reads, according to the manufacturer's protocol. Raw sequencing data were processed for subsequent bioinformatics analysis (Fig. 1A). First, the Illumina sequencing adapters and low-quality reads were removed by using fastq_mcf software. Then, the duplicated reads were removed by using Picard tools (http://broad institute.github.io/picard/, in the public domain), and the high-quality reads were aligned with the reference human genome (hg19) by the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/, in the public domain). Finally, the single nucleotide polymorphisms (SNPs) and insertions or deletions (InDels) were called using the Genomic Analysis Toolkit Haplotype Caller. The variants were further annotated using ANNOVAR and associated with multiple databases, including
Variant annotations were described according to the following criteria: pathogenic or likely pathogenic variants and VUSs were verified by Sanger sequencing. Segregation analysis was performed in patients with presumed pathogenic mutations. Monoallelic variants were considered only when they were classified as pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign.

Assessment of pathogenicity was based on the guideline reported by American College of Medical Genetics and Genomics. According to this guideline, we categorize variants into pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign. Monoallelic variants were considered only when they were classified as pathogenic or likely pathogenic variants. Presumed pathogenic or likely pathogenic variants and VUSs were verified by Sanger sequencing. Segregation analysis was performed in patients when DNA samples of their family members were available.

The coverage of target regions was normalized and compared with the average normalized data of all other samples of the same run to obtain the ratio of the relative coverage in order to detect deletions and duplications in the patients' genome sequences, when only monoallelic mutation or no mutation was identified by TES. The regions where the ratio was below 0.7 were considered putative deletions, while the regions where the ratio rose above 1.3 were speculated as putative duplications. Real-time q-PCR was carried out to validate putative CNVs of MYO7A, PCDH15, and USH1C in six patients. The q-PCR reactions were done on a commercial instrument (Rotor-Gene 6000; Corbett Research, Mortlake, NSW, Australia) in a 10-μl final volume, including 1-μl (100-ng) genomic DNA and 300-nM primers and a master mix (Eva Green PCR; Bio-Rad Laboratories, Hercules, CA, USA), as we previously described. Each assay was performed in triplicate. The human GAPDH gene was used as an internal control. The relative quantification (RQ) of the target gene was accomplished by using the RQ manager software (Bio-Rad Laboratories) and was calculated with the 2^ΔΔCT method. The threshold for the normal value was set at 0.8 to 1.3. The ranges of the RQ values for deletions and duplications were set at 0.45 to 0.74 and 1.6 to 1.8, respectively. The MLPA assay was performed to validate the putative CNVs of USH2A with a SALSA MLPA probe mix P361-A2/P362-A2 USH2A (Amsterdam, The Netherlands), following the manufacturer's protocol; this kit contains one probe for each exon of the USH2A gene.

**Supplementary PCR-Based Sequencing**

We performed Sanger sequencing of the reported intronic mutation c.-48G>A of MYO7A in USH1 patients and c.5573-854A>G, c.7595-2144A>G, c.8845+628C>T, and c.9959-4159A>G of USH2A in patients with USH2A when only monoallelic mutation or no mutation was identified by TES.
Disease-Causing Genes

For the 12 USH genes, the average coverage ranged from 230.8x (CLRN1) to 660.49x (PCDH15) (Figs. 1B, 1C). Among the 2894 targeted exons, about 174 (6.0%) were covered less than 30x. These poorly covered regions included 30 exons of MYO7A, CDH23, MYO7A, and CLRN1, a n d G P R 9 8 , A B H D 1 2 , D F N B 3 1 , C I B 2 , C I B 2 , C L R N 1 , and H A R S .

Targeted Exome Sequencing of Inherited Hearing Loss Genes

We performed TES of inherited hearing loss genes in proband 019791 and his brother (019791 II:1). The panel includes 199 hereditary hearing loss genes and comprises 4688 exons with a total size of 641 kb. A list of the targeted genes is provided in the supporting material (Supplementary Table S2). The detailed procedure of library construction, capture, sequencing, and bioinformatics analysis was just the same as described above.

Statistical Analysis

Differences in the onset age of night blindness and hearing loss were compared with statistical software (SPSS version 20.0; SPSS, Inc., Chicago, IL, USA), and ANOVA was used to compare the differences among groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of Target Gene Capture and Sequence Depth

We applied our capture panel, which included 188 known retinal degeneration genes, to 90 USH patients in our study and achieved high-quality results. On average, the mean coverage of the target regions was 448.3x. For each sample, more than 99.5% of the target regions were covered, and 90.96% of the target regions were sequenced with more than 30x coverage. For the 12 USH genes, the average coverage ranged from 230.8x (CLRN1) to 660.49x (PCDH15; Figs. 1B, 1C). Among the 2894 targeted exons, about 174 (6.0%) were covered less than 30x. These poorly covered regions included 30 exons of nine USH genes (CDH23, MYO7A, GPR98, ABHD12, DFNB31, PCDH15, CIB2, CLRN1, and HARS).

USH Mutation Detection Rates and Involving Disease-Causing Genes

We identified biallelic disease-causing mutations in 92 probands (77.3%); monoallelic mutations in 5 probands (4.2%); one hemizygous mutation in one patient (0.8%); and no disease-causing allele in 21 unrelated patients (17.6%), achieving an overall mutation detection rate of 78.2% (93/119; Table 1). In total, we identified 132 distinct disease-causing mutations involving 13 genes in this cohort of patients (Supplementary Table S3). Mutations in USH1 genes were detected in 19.3% (23/119) of the probands in this cohort (Fig. 2A, Supplementary Table S4). Mutations in USH2 genes were identified in 58% (69/119) of the probands (Fig. 2A, Supplementary Table S4). Mutations in the atypical USH gene ABHD12 were identified in only 1% (1/119) of the probands (Fig. 2A, Supplementary Table S4). No mutation was detected in CIB2, DFNB31, and CLRN1. Mutations in non-USH IRD genes (CHM, CNGA1, and EYS) were detected in 3% (4/119) of the probands (Fig. 2A, Supplementary Table S4). Of the 152 putative disease-causing mutations, 78 were novel, including three CNVs (Fig. 3). Additionally, 11 VUSs involving five USH genes were detected in 10 patients (Supplementary Table S5).

Molecular Diagnosis in USH1 Families and Clinical Profile

Of the 20 unrelated patients who were clinically diagnosed with USH1, two disease-causing alleles were found in 17 patients and a single mutant allele was found in two probands. Co-segregation analyses were done in 16 of the 17 unrelated patients (94.1%) with biallelic mutations (Table 1 and Supplementary Table S4). Mutations of MYO7A were the most common in our cohort, accounting for 12 of the 20 probands with USH1 (60%), followed by mutations of PCDH15 and USH1C identified in 4 (20%) and 2 patients (10%), respectively (Fig. 2B). Table 2 summarizes the proportions of different kinds of mutations for the 36 mutant alleles. All USH1 patients had different extents of defects in visual acuity (0.1–0.9), profound hearing loss in their early childhood, and a history of motor developmental delay (Supplementary Table S4). The majority of the patients (14/19) underwent cochlear implantation in their early childhood. The mean onset age of the hearing defect of the patients was 0.5 years (range, 0–3 years), much earlier than the mean onset age of their visual defect, which was 5.07 years (range, 1–12 years; Table 3). Patient 019791 is worth describing in detail. This 7-year-old proband was diagnosed with USH1 based on her clinical phenotype and underwent cochlear implantation at around 3 years old. Her brother started to have a hearing impairment at around 11 years old. However, his audiograms showed a relatively mild downsloping hearing loss; he did not have any nyctalopia complaint, and his fundus was normal (Figs. 4A–C). We detected compound heterozygous VUSs (p.R356Q/p.R1604S) of PCDH15 and compound heterozygous mutations (p.R419Q/p.I407DfsTer3) of TULP1—an RP-causing gene in the proband. Co-segregation analysis showed that her brother (II:1) only carried one heterozygous mutation of TULP1 and one VUS of PCDH15. As the aforementioned results could not fully explain the hearing loss of the proband and her brother, we performed TES of inherited hearing loss in these two patients and detected compound heterozygous mutations (c.1128C>T, p.Y376X/c.6177+1G>?, c.1128C>T, p.Y376X/c.6177+1G>?) of MYO7A in the proband and her brother (Fig. 4A, Supplementary Table S4).

Table 1. Demography and Mutation Screening Results in This Study

<table>
<thead>
<tr>
<th>USH1 Patients, n</th>
<th>USH2 Patients, n</th>
<th>All Patients, n</th>
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<tbody>
<tr>
<td>USH/IRD Genes</td>
<td>USH/IRD Genes</td>
<td>USH/IRD Genes</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Biallelic mutation</td>
<td>16/1</td>
<td>72/4*</td>
</tr>
<tr>
<td>Monoallelic mutation</td>
<td>2/0</td>
<td>3/0</td>
</tr>
<tr>
<td>No mutation</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>99</td>
</tr>
</tbody>
</table>
FIGURE 2. Summary of proportion of patients with mutations of involved genes in this study. (A) Proportion of patients with mutations of each involved gene identified in all patients. (B) Proportion in USH1 patients. (C) Proportion in USH2 patients. The areas of slanted lines indicate patients with monoallelic mutations, and the grid areas indicate patients with mutations of two genes.

FIGURE 3. Colored fundus (CF) photographs and results of CNVs of the probands in families 019101, 019248, and 019511. (A–C, left) CF photographs show attenuation of retinal blood vessels and pigment proliferation. (A) Bar chart of readcount (middle) of proband 019101 showing two suspicious areas of CNVs (red squares) of USH1C. The real-time quantitative PCR result (right) verified the deletion of exon 7 (red square). (B) Electroretinogram (middle) of proband 019101 showing no responses in all five standard reactions. The real-time quantitative PCR result (right) show the deletion of exon 19 of MYO7A. (C) Bar chart of readcount (right superior) showing a suspicious area of CNV (red square) of USH2A identified in proband 019511. The result of multiplex ligation dependent probe amplification verified the gross deletion from exon 47 to exon 51 (red square).
Molecular Diagnosis in USH2 Families and Clinical Profile

Of the 99 unrelated patients who were clinically diagnosed with USH2, two disease-causing alleles were revealed in 75 patients, a single mutant allele in 3 probands, and a hemizygous mutation in one patient (Fig. 2C). Cosegregation analyses were done in 59 of the 75 unrelated patients (78.6%; Supplementary Table S4). Mutations of USH2A were the most frequent in our cohort, representing 67 of the 99 USH2 patients. Of the 99 unrelated patients who were clinically diagnosed with USH2, two disease-causing alleles were revealed in 75 patients (78.6%; Supplementary Table S4). The most common mutation was c.8559-2A>G, with an allele frequency of 19.1% (26/136), followed by mutation p.C934W (6.6%, 9/136), mutation p.R347fsTer41 (4.4%, 6/136); mutations p.A2249PfsTer30 and p.W2744C (each 2.9%, 4/136); and mutations p.G268R, p.R5143C, and p.R626X (each 2.2%, 3/136; Supplementary Table S3). The mutation c.8559-2A>G was detected in 23 unrelated patients; 3 patients had homozygous mutations, while the remaining 20 were compound heterozygotes (Supplementary Table S4). The most frequent missense mutation (p.C934W) was identified in nine probands, and all were in a heterozygous state. Approximately 90% of the mutant alleles of the three USH1 genes identified in the five USH2 patients were missense mutations (Table 2). Patient 019691 carried two compound heterozygous mutations (p.D428N/p.G2190D) and p.H557Y/p.G576S of USH2A, compound mutations of USH2A; Spl, splicing mutations. Miss, missense mutations; Non, nonsense mutations; CNV, copy number variation. Non-USH gene mutations: with non-USH gene mutations, with multiple genes mutations, with USH1 gene mutations, with other USH gene mutations. Values in bold represent the number and percentage of the mutant alleles identified in the USH1 patients. Values with no bold represent the number and percentage of the mutant alleles identified in the USH2 patients. Fs, frameshift mutations; Mis, missense mutations; Non, nonsense mutations; Spl, splicing mutations.

Table 3. Correlations Between Onset Age of Patients With USH and Their Carrying Mutations

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Onset Age of NB, y</th>
<th>Onset Age of HL, y</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Total USH1*</td>
<td>17</td>
<td>4.87 ± 3.81</td>
<td>1–12</td>
</tr>
<tr>
<td>With MYO7A mutations*</td>
<td>10</td>
<td>3.65 ± 2.20</td>
<td>2–8</td>
</tr>
<tr>
<td>With other USH gene mutations</td>
<td>6</td>
<td>6 ± 4.43</td>
<td>2–12</td>
</tr>
<tr>
<td>With multiple genes mutations</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total USH2*</td>
<td>71</td>
<td>17.21 ± 9.47</td>
<td>2–49</td>
</tr>
<tr>
<td>With USH2A mutations*</td>
<td>60</td>
<td>18.1 ± 9.28</td>
<td>2–49</td>
</tr>
<tr>
<td>Three mutations</td>
<td>4</td>
<td>16.75 ± 3.59</td>
<td>14–22</td>
</tr>
<tr>
<td>Mis/Mis mutations</td>
<td>12</td>
<td>21.42 ± 9.98</td>
<td>5–38</td>
</tr>
<tr>
<td>Mis/Del mutations</td>
<td>20</td>
<td>17.6 ± 7.08</td>
<td>3–30</td>
</tr>
<tr>
<td>Del/Del mutations</td>
<td>24</td>
<td>17.08 ± 11.08</td>
<td>2–49</td>
</tr>
<tr>
<td>With GPR98 mutations</td>
<td>2</td>
<td>16 ± 8.49</td>
<td>10–22</td>
</tr>
<tr>
<td>With USH1 gene mutations</td>
<td>4</td>
<td>9.67 ± 5.86</td>
<td>3–14</td>
</tr>
<tr>
<td>With multiple genes mutations</td>
<td>1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>With non-USH gene mutations*</td>
<td>3</td>
<td>13.3 ± 14.57</td>
<td>5–30</td>
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Del, deleterious mutations (including frameshift mutations, splicing mutations, nonsense mutations, and gross deletion); HL, hearing loss; NB, night blindness.

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Table 2. Number and Percentage of the Mutant Alleles Identified in Each USH Gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>ABHD12, n</th>
<th>CDH23, n</th>
<th>GPR98, n</th>
<th>MYO7A, n (%)</th>
<th>PCDH15, n (%)</th>
<th>USH1C, n</th>
<th>USH2A, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mis</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>8 (36)</td>
<td>2 (25)</td>
<td>2</td>
<td>56 (41)</td>
</tr>
<tr>
<td>Non</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>4 (18)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spl</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7 (32)</td>
<td>0</td>
<td>1 (12.5)</td>
<td>34 (25)</td>
</tr>
<tr>
<td>Fs</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2 (9)</td>
<td>1 (17)</td>
<td>5 (62.5)</td>
<td>28 (21)</td>
</tr>
<tr>
<td>CNV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values in bold represent the number and percentage of the mutant alleles identified in the USH1 patients. Values with no bold represent the number and percentage of the mutant alleles identified in the USH2 patients. Fs, frameshift mutations; Mis, missense mutations; Non, nonsense mutations; Spl, splicing mutations.
Unfortunately, the patient was unwilling to undergo a pure-tone audiometry examination. Of the four patients carrying mutations of other IRD-causing genes, patients 019512 and 019650 harbored the same homozygous mutation (p.L89FfsTer4 of \textit{CNGA1}); patient 019415 carried mutations of \textit{EYS}; and proband 019524 had a mutation of \textit{CHM}. In our further medical history review and cosegregation analysis, we found that patient 019650 had a history of plural streptomycin injection when he was 1 year old, while his twin brother (without the usage of ototoxic drugs) who carried the same mutations did not complain of any hearing defect (Figs. 4A, 4E). Patients 019512, 019415, and 019524 all complained about hearing loss since their middle age, but patient 019415 pure-tone audiometry examination showed normal results after his molecular testing.

**DISCUSSION**

In this study, we performed comprehensive disease-causing mutation screening in 119 Chinese USH patients. Combined TES and Sanger–DNA direct sequencing determined that our overall mutation detection rate for the current cohort was 78.2%. This solving proportion is compatible with the reported rates in several previous studies using TES or Sanger–DNA direct sequencing; however, it is still about 15% lower than the 92.7% rate reported recently by Bonnet et al. The mutation detection rate is related to the accuracy of the patients' clinical diagnoses. In our study, the mutation detection rate (85%) for USH1 patients was higher than that (76.8%) for USH2 patients. The reason might be that the USH1 patients' relatively distinct phenotype makes it easier to achieve a precise clinical diagnose. In contrast, the clinical diagnosis of UHS2 is relatively challenging due to different clinical features.
genes were first identified in the current study. Consistent with population. More than half of the mutations of these two respectively. However, the mutation spectrum observed in the USH2 patients, accounting for 60% and 68% of mutations, in the current cohort. For MYO7A, no frequent mutation was observed in the current cohort, which might be related with the small number of patients with MYO7A mutations.

In this study, we first reported the three CNVs of three different genes (MYO7A, USH1C, and USH2A) in Chinese USH patients; however, the rate of CNVs (1.6%, 3/188) was much lower than that (approximately 9%) observed in Caucasian patients. Targeted NGS can detect a large genomic DNA arrangement; however, its capability to detect CNVs is related to the coverage depth. A recent study involving a Spanish cohort reported that the percentage of CNVs was 11.8%, and the researchers observed that the CNV analysis produced uncertain results in the target regions, with a coverage of less than 250x. In the current study, the mean coverage for the 12 USH genes was 386.7x, much lower than the 1334x reported in the Spanish study, so the rate of CNVs might be underestimated. The other possibility is that the rates of the CNVs in Chinese patients is really lower than that in Caucasian patients, which needs further research for future verification.

It is quite challenging to obtain a solid correlation between genotype and phenotype as most of the patients carry their private mutations. In this study, we observed that the patients with two null mutations of USH2A had an early onset age of hearing loss, implying the presence of a genotype–phenotype association. Personally, the patients harboring two null mutations of USH2A had more severe hearing loss than the patients carrying two missense mutations (Supplementary Table S4). This finding is consistent with the previous observation that the rate of the missense mutations in Chinese patients is really lower than that in Caucasian patients, which needs further research for future verification.

Several previous studies indicated the existence of a genotype–phenotype correlation for the mutations of CDH23 and PCDH15, which were deleterious mutations resulting in USH, whereas missense mutations caused nonsyndromic deafness. We did not observe this kind of genotype–phenotype correlation in the current cohort; however, we noted that the rates of missense mutations of MYO7A and PCDH15 found in the USH2 patients were much higher than those detected in the USH1 patients. Two patients (019791 and 019691) were found harboring the mutations of more than one genes—one RP-causing gene and one USH1 gene or one inherited hearing loss gene. Such results indicated that the visual defects (RP) and hearing loss of these two patients might co-occur and be caused by mutations in multiple genes. This kind of rare case was reported in a Chinese patient who was found harboring mutations of MYO7A and CNGA1.

In this cohort, five patients had only one mutation detected in USH2A or MYO7A; other unidentified mutations may be in the promoter region or in some deep intronic regions. Given the different mutation spectrum in Chinese patients, it is no surprise that the five mutations in the deep intronic regions of USH2A and MYO7A that were reported in European patients were not found in the current cohort. Of the 21 patients with no identified mutation, five patients were found carrying heterozygous variants of uncertain of the 5 USH genes (Supplementary Tables S4, S5); therefore, they might carry the second missing mutant allele in some deep intron regions or some CNVs of these five genes. For the remaining 16 patients, most acquired a hearing impairment after reaching 30 years of age and did not undergo a pure-tone audiometry examination, which could not exclude the previously mentioned misdiagnosis possibility. The other possibility is that they carried some mutations of other genes that were not included in our panel.

In conclusion, our results suggest that Chinese patients appear to have a different mutation spectrum for each USH gene. The formation of the mutation profile for the Chinese population will enable precise genetic diagnoses for USH patients, especially for atypical clinical cases in the future.

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