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\(^1\) and accounts for approximately 50% of hereditary deaf-blind individuals.\(^1\) 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.\(^1\),\(^2\) 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.\(^2\) 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.\(^3\),\(^4\) 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.\(^5\)

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.\(^6\)–\(^8\) 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).\(^6\)–\(^12\) The only USH3 gene is CLRN1.\(^1\) Recently, mutations of ABHD12, CEP250, and HARS have been described as causing atypical USH.\(^12\) Additionally, PDZD7 has been proposed as a modifier gene for patients with mutations of USH2A and a contributor of digenic inheritance with GPR98.\(^13\) What makes it more complicated is that mutations of USH2A may lead to RP without hearing loss,\(^1\) and mutations of all six USH1 genes may cause non-syndrome deafness.\(^15\)–\(^20\) 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,\(^5\),\(^11\) 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.21 When the USH genotyping microarray is used, the mutation detection rate is about 3%, both in a large Spanish cohort and in an Italian cohort.21,22 Moreover, several studies have demonstrated that copy number variants (CNVs) of some USH genes account for certain proportions of mutations.23,24 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).25 Next-generation sequencing (NGS) technologies developed in recent years can perform whole genome; whole exome; and targeted exome sequencing (TES).26 Several studies have proven TES as a high-efficiency method of molecular diagnosis of USH, with a detection rate of around 70%.10,26–29 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.10,26 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 135×.10 In another study, two large deletions of USH2A were identified by TES, with a mean coverage of 500×.20 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 77×.30 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 ophthalmic evaluation consisting of best-corrected visual acuity (BCVA); slit-lamp biomicroscopy; and a fundus examination. Most of the patients also underwent optical coherence tomography (OCT); visual field; and electroretinogram (ERG) examinations. More than half of the probands (67 probands) had auditory examinations, including otoscopic exploration; pure-tone audiometry; and otoacoustic emission (OAE) tests conducted by otolaryngologists. Three patients had detailed vestibular tests, including videonystagmography (VNG) and a vestibular calorice test. The clinical diagnosis of USH was based on the existence of RP and sensorineural hearing loss (proven by either pure-tone audiometry or self-reported hearing loss). For those patients who had audiometric data, the severity of 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.31 Based on the previously reported clinical diagnostic criteria,32 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).33,34 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_206933.2).

**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.35 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, DNB31, GPR98, HARS, MYOTA, PCDH15, USH1C, USH2A, and PDD27. 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.35 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...
1000 genome (http://phase3browser.1000genomes.org/index.html, in the public domain); ESP6500 (http://evs.gs.washington.edu/EVS/, in the public domain); dbSNP (https://www.ncbi.nlm.nih.gov/snp, in the public domain); ExAC (http://exac.broadinstitute.org/, in the public domain); and an in-house variant database (My Genostics). The alternative alleles with less than five reads and/or a frequency less than 30% were removed to exclude likely false-positive variants. The pathogenicity of each variant was predicted by the PolyPhen2 (http://genetics.bwh.harvard.edu/pph/, in the public domain); Mutation Taster (http://www.mutationtaster.org/, in the public domain); and SIFT (http://sift.jcvi.org/, in the public domain) programs. Those variants that may involve the splicing effect were analyzed with the NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/, in the public domain); Human Splice Finder (HSF, http://www.umd.be/HSF3/, in the public domain); and Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html, in the public domain). Then, we carried out further analysis to identify the putative pathogenic mutations in each patient. Two databases, Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php, in the public domain) database and the Leiden Open (source) Variation database (http://www.lovd.nl/, in the public domain), were used to search for reported pathogenic mutations.

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.

**CNVs Analysis and Validation**

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 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.

**FIGURE 1.** Strategy of TES and sequence depth in this study. (A) Analytical pipeline for variant identification. (B) Mean coverage of each USH gene. (C) Mean coverage depth obtained for each USH gene.
Mutations Spectrum of Chinese Patients With USH

**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 was 448.3x. For each sample, more than 30x coverage was achieved on average. The poorly covered regions included 30 exons of the 2894 targeted exons, about 174 (6.0%) were covered less than 30x. These poorly covered regions included 30 exons of 132 distinct disease-causing mutations, 78 were novel, identified in 44 (20%) and 2 patients (10%), respectively (Fig. 2A). 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 down-sloping hearing loss; he did not have any nystagmus 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.L407D/S613Ter3) 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>T) of MYO7A in the proband and her brother (Fig. 4A, Supplementary Table S4).

**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 DFNB31 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 down-sloping hearing loss; he did not have any nystagmus 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.L407D/S613Ter3) 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>T) 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>Gene</th>
<th>USH1 Patients, n</th>
<th>USH2 Patients, n</th>
<th>All Patients, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>USH/IRD Genes</td>
<td>%</td>
<td>USH/IRD Genes</td>
<td>%</td>
</tr>
<tr>
<td>Biallelic mutation</td>
<td>16/1</td>
<td>72/4*</td>
<td>88/5*</td>
</tr>
<tr>
<td>Monoallelic mutation</td>
<td>2/0</td>
<td>3/0</td>
<td>5/0</td>
</tr>
<tr>
<td>No mutation</td>
<td>1/0</td>
<td>20/0</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>20/1</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* One patient with a hemizygous mutation.
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).
Table 2. Number and Percentage of the Mutant Alleles Identified in Each USH Gene

<table>
<thead>
<tr>
<th>AHBD12, 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>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>3</td>
<td>4 (18)</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spl</td>
<td>0</td>
<td>0</td>
<td>7 (32)</td>
<td></td>
<td>0</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Fs</td>
<td>0</td>
<td>1</td>
<td>2 (9)</td>
<td>1 (17)</td>
<td>5 (62.5)</td>
<td>0</td>
</tr>
<tr>
<td>CNV</td>
<td>0</td>
<td>0</td>
<td>1 (5)</td>
<td></td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>22</td>
<td>6</td>
<td>4</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.

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 (67.7%). The most frequent missense mutations (Table 3). The mean onset age (of both visual and hearing defect) of the patients carrying deleterious mutations (either compound heterozygous or homozygous) was statistically younger than that of the patients harboring two missense alleles (ANOVA \( P = 0.019 \)) or the patients with one missense coupled with one deleterious allele (ANOVA \( P = 0.024 \); Table 3). In contrast, no statistically significant difference was observed in the onset age of the visual defect among the patients with different kinds of mutations (Table 3). The mean onset age (of both visual and hearing defects) of the four patients with the mutations of USH1 genes was earlier than that of the patients with USH2A mutations (Table 3). Patient 019691, who carried two compound mutations of CDH23 (p.D428N/p.G2190D) and PDE6B (p.H557Y/p.G576S), was a 45-year-old male who had suffered from night blindness since he was 25 years old. He had complained of a mild hearing defect over the past 4 years.

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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</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 ± 4.45</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>3</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>3–30</td>
</tr>
</tbody>
</table>

Del, deleterious mutations (including frameshift mutations, splicing mutations, nonsense mutations, and gross deletion); HL, hearing loss; NB, night blindness.

\* Exclude patients with history of ototoxic drugs usage.
\† \( P = 0.024 \).
\‡ \( P = 0.019 \).
His fundus showed a typical RP appearance (Fig. 4D); 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\textsuperscript{6,10,11,26–28}; however, it is still about 15% lower than the 92.7% rate reported recently by Bonnet et al.\textsuperscript{12} 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 diagnosis. In contrast, the clinical diagnosis of USH2 is relatively challenging due to different
degrees of hearing loss (from mild to severe). Hearing impairment may be related to many nongenetic factors, such as excessive noise exposure in the working or living environment, trauma, the usage of otoxic drugs, and a combination with other diseases.\(^9\) In this cohort, more than half of the USH2 patients did not undergo a pure-tone audiometry examination, leading to the possibility of a misdiagnosis for some of these patients, just like patient 019415. This issue is one of the limitations of the current study.

Consistent with the previous study, MYO7A and USH2A were the most common mutated genes in the USH1 and the USH2 patients, accounting for 60% and 68% of mutations, respectively. However, the mutation spectrum observed in the current study differed from that found in the Caucasian population. More than half of the mutations of these two genes were first identified in the current study. Consistent with previous studies, the most common mutation of USH2A was c.8559-2A>G, which was only detected in Chinese and Japanese patients.\(^{30,31,33,34}\) In contrast, the most frequent mutations (p.Glu767Serfs*21, p.C3267R, and p.T571M) in European patients were not detected in the current study.\(^6,12,40\)

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.\(^6\) 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.\(^10\) 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. Additionally, 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 percentage of the missense mutations is much higher in the RP patients with mutations of USH2A.\(^{28}\)

Several previous studies indicated the existence of a genotype-phenotype correlation for the mutations of CDH23 and PCDH15, which were deleterious mutations resulting in Usher, whereas missense mutations caused nonsyndromic deafness.\(^{10,17,41}\) 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.\(^{28}\)

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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