Comprehensive Molecular Screening in Chinese Usher Syndrome Patients

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**PURPOSE.** Usher syndrome (USH) refers to a group of autosomal recessive disorders causing deafness and blindness. The objectives of this study were to determine the mutation spectrum in a cohort of Chinese patients with USH and to describe the clinical features of the patients with mutations.

**METHODS.** A total of 119 probands who were clinically diagnosed with USH were recruited for genetic analysis. All probands underwent ophthalmic examinations. A combination of molecular screening methods, including targeted next-generation sequencing, Sanger–DNA sequencing, and multiplex ligation probe amplification, was used to detect mutations.

**RESULTS.** We found biallelic mutations in 92 probands (77.3%), monoallelic mutations in 5 patients (4.2%), and 1 hemizygous mutation in 1 patient (0.8%), resulting in an overall mutation detection rate of 78.2%. Overall, 132 distinct disease-causing mutations involving seven USH (ABHD12, CDH23, GPR98, MYO7A, PCDH15, USH1C, and USH2A) genes; 5 other retinal degeneration genes (CHM, CNGA1, EYS, PDE6B, and TULP1); and 1 nonsyndromic hearing loss gene (MYO15A) were identified, and 78 were novel. Mutations of MYO15A were responsible for 60% of USH1 families, followed by PCDH15 (20%) and USH1C (10%). Mutations of USH2A accounted for 67.7% of USH2 families, and mutation c.8559-2A>G was the most frequent one, accounting for 19.1% of the identified USH2A alleles.

**CONCLUSIONS.** Our results confirm that the mutation spectrum for each USH gene in Chinese patients differs from those of other populations. The formation of the mutation profile for the Chinese population will enable a precise genetic diagnosis for USH patients in the future.

Keywords: Usher syndrome, mutation screening, next generation sequencing

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 53% to 44% of USH cases. USH3 is rare in most populations, affecting 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.
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SUBJECTS AND METHODS

Patients

In total, 119 unrelated individuals who were clinically diagnosed with USH1 (20 cases) and USH2 (99 cases) were enlisted 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 calorictest. The clinical diagnosis of USH1 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.51 Based on the previously reported clinical diagnostic criteria,21 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 45, 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_020693).

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, CLRN1, DFNB31, 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.55 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://broadinstitute.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
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![Image of a graph showing the coverage of each USH gene.](http://arvojournals.org/)

**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.

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 (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.5573-8544A>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.
Additionally, we carried out Sanger sequencing in insufficiently covered regions of MYO7A in four patients when only monoallelic mutation or no mutation was identified by TES.

**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 covered regions of 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 ABDH12 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 5% (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. Cosegregation 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 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.I407DfsTer3) of TULP1—an RP-causing gene in the proband. Cosegregation 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 MYO15A in the proband and her brother (Fig. 4A, Supplementary Table S4).

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<th>Demography and Mutation Screening Results in This Study</th>
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* 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).
Molecular Diagnosis in USH2 Families and Clinical Profile

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 \(\text{p.L89FfsTer4 of CNGA1}\); patient 019415 carried mutations of \(\text{EYS}\); and proband 019524 had a mutation of \(\text{CHM}\). 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\(^6,10,11,26–28\); however, it is still about 15% lower than the 92.7% rate reported recently by Bonnet et al.\(^12\) 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 diagnose. In contrast, the clinical diagnosis of UHS2 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 ototoxic 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.\(^3\) In contrast, the most frequent USH2A mutations (p.Glu767Serfs*21, p.C3267R, and p.T3571M) in European patients were not detected in the current study.\(^6\) 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 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 USH, whereas missense mutations caused nonsyndromic deafness.\(^10\) 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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