Mutation screening of the USH2A gene reveals two novel pathogenic variants in Chinese patients causing simplex usher syndrome 2

Chenhao He 1,2,3†, Xinyu Liu 1,2,3†, Zilin Zhong 1,2,3,4 and Jianjun Chen 1,2,3,4,5*

Abstract

Background: Usher syndrome (USH) is the most prevalent cause of the human genetic deafness and blindness. USH type II (USH2) is the most common form of USH, and USH2A is the major pathogenic gene for USH2. For expanding the spectrum of USH2A mutations and further revealing the role of USH2A in USH2, we performed the USH2A gene variant screening in Chinese patients with USH2.

Methods: Genomic DNA was extracted from peripheral blood of unrelated Chinese USH2 patients, we designed specific primers for amplifying the coding region (exons 2–72) of the USH2A gene. Sanger sequencing was used to study alleles. Silico prediction tools were used to predict the pathogenicity of the variants identified in these patients.

Results: Five heterozygous pathogenic variants were detected in four patients. Two patients were found to have two-mutations and two patients only have one. Two novel variants c.4217C > A (p.Ser1406X) and c.11780A > G (p.Asp3927Gly)) were predicted deleterious by computer prediction algorithms. In addition, three reported mutations (c.8559-2A > G, c.8232G > C and c.11389 + 3A > T) were also found in this study.

Conclusions: We identified five heterozygous pathogenic variants in the USH2A gene in Chinese patients diagnosed with Usher syndrome type 2, two of which were not reported. It expands the spectrum of USH2A variants in USH.

Keywords: Usher syndrome, USH2A, Mutations, Sequence variants

Background

Usher syndrome (USH), an autosomal recessive disorder, is a clinically and genetically heterogeneous disease. USH is characterized by retinitis pigmentosa (RP), bilateral sensorineural hearing impairment and intact vestibular responses [1]. It is the most prevailing cause of the human hereditary deafness and blindness. In worldwide, the general prevalence of USH approximately ranges from 3.3 to 6.4 per 100,000 individuals [2]. Up to now, it is unavailability of a therapy for the USH.

Clinically, according to the severity and progression of vision and hearing loss of patients, USH classified into USH type I (USH1), USH type II (USH2), and USH type III (USH3) [3]. Besides, approximately 20–30% cases are categorized as atypical USH. USH1 is the most serious form in the three types, patients with USH1 have congenital profound hearing loss and begin to lose their vision early in life. Different from the USH1 patients defined as having congenital deafness and blindness within the first decade of life, patients with USH2 exhibit congenital mild-moderate hearing and vision loss in the second decade of life, and generally show normal vestibular function in all their lives. USH2 is the most
common form of USH and USH2 patients account for
more than 50% of all USH patients [2, 4]. USH3 patients
are not born deaf and blind. They usually show a gradual
loss of their hearing and vision.

Up to now, 16 genes have been identified that may
cause USH [https://sph.uth.edu/retinet/sum-dis.htm],
three genes of them (USH2A (usherin) [5], ADGRV1
(Adhesion G Protein-Coupled Receptor V1) [6] and
DFNB31 (autosomal recessive deafness 31) [7]) are
the USH2 genes. USH2A gene is the major patho-
genic gene for USH2 and responsible for more than
74% USH2 cases [8]. USH2A gene is located on
chromosome 1q41 and has two alternatively spliced
isoforms. The shorter USH2A isoform was first identi-
fied in 1998 [5] and the much longer USH2A isoform
b was identified by van Wijk et al. in 2004 [9]. To
date, all 72 exons of USH2A isoform b have been car-
ried out plenty of mutational analyses and found
many pathogenic mutations (including splicing muta-
tions at splice sites) [10, 11]. The protein usherin,
encoded by the isoform b of USH2A, is presumed
with 5202 amino acids and anchored on the cell
membrane [12]. In mammalian photoreceptors, the
usherin are expressed specifically in the connecting
cilia and involved in the cargo delivery from the inner
segment to the outer segment [13]. Previous research
has shown that mutations of USH2A could
cause nonsyndromic recessive RP [14, 15]. What is
more, USH2A gene was also related to tactile sensitivity
and acuity [16].

In this study, five deleterious variants and 14 non-
pathogenic variants in the USH2A gene were identi-
fied in four Chinese USH2 patients by mutation
screening. Two of the pathogenic variants we detected
were novel.

Methods
Sample collection and ethics statement
Unrelated Chinese patients diagnosed with USH2
were included in this study. Two hundred unrelated
normal individuals were recruited in this study as
healthy controls. All patients underwent careful clin-
ical examinations in Shanghai Tenth People’s Hospital
and Clinical diagnosis of Usher syndrome were based
on examination of optical coherence tomography
(OCT) and electroretinogram (ERG), the typical RP
fundus appearance, intact vestibular function, and
sensorineural hearing impairment. The reference se-
quence from NCBI served as controls. This study was
granted approval by the Declaration of Helsinki and
approved by the institutional review board (IRB) of
Tongji Eye Institute of Tongji University School of
Medicine (Shanghai, China). Written informed con-
sent was obtained from all participants.

The grading system for severity of hearing impairment
and evaluation of vestibular function
The severity of hearing impairment can be assessed
according to the pure tone hearing threshold: mild hearing loss:
26–40 dB HL, moderate hearing loss: 41–80 dB HL, severe
hearing loss: > 80 dB HL. Vestibular function tests include
position tests and hot and cold water tests. (1) Position
test: Dix-Hallpike technique was used to induce dizziness.
Keeping the patient horizontal with his head pressed
down by 30°. The head and eyes of the patient first turn to
the right and then to the left, and repeated it several times
to observe the severity and duration of nystagmus and diz-
iness. (2) Hot and cold water test: otoscopy should be
performed before the test, and it can be performed with-
out tympanic membrane perforation. The patient lies on
his back and raises his head 30°to keep the lateral semi-
circular canal becomes upright. Each external ear canal was
filled with cold or warm water for 40 s. Discomfort from
warm water is usually lighter than cold water. In normal
patients, cold water stimulates the nystagmus of the slow-
phase stimulus side and the fast phase deviates from the
stimulus side; warm water stimulus has the opposite re-
response; in patients with vestibular cochlear nerve and ves-
tibular nucleus disease, irrigation on the lesion side
cannot induce nystagmus or nystagmus appears healthy
slightly slower or shorter duration.

Sample preparation and variant screening
Peripheral blood samples from all the participants were
collected in EDTA tubes. Standard protocols of Relax-
Gene Blood DNA System (TianGen, Beijing, China) were
used to extract Genomic DNA according to the manufac-
turer’s instructions. DNA samples were stored at −80°C
environment before used. Using the Primer3 software
(http://primer3.sourceforge.net/) designed specific primers
comprising USH2A exons 2 to 72 (Table S1) (including
the intron-exon boundary). The coding region was
amplified by polymerase chain reaction (PCR) and using
Sanger sequencing which was performed using ABI3730
Automated Sequencer (PE Biosystems, Foster City, CA,
USA) study alleles. Nucleotide sequences assayed by
Sanger sequencing were compared with the published
DNA sequence of the USH2A gene (NCBI Reference
Sequence: NM_206933.3(http://genome.ucsc.edu/cgi-bin/hg/hg?hsid=785073911_5XSAy4TZHHazDdeKszSK5wYZ4
AfE&g=htcCdnaAli&i=NM_206933&c=chr1&l=21579623
2&r=216596790&o=215796232&aliTable=refSeqAli)). The
cDNA numbering +1 position corresponds to A in the
ATG translation initiation codon for USH2A.

Predictions of the pathogenic effect of missense
variations and splice-site
We used several different computer algorithms: SIFT and
PROVEAN (http://provean.jcvi.org/genome Submit_2.
Fig. 1 Representative clinical examination of the USH2 patients. 

a The appearance of the fundus of patient No.003 shows typical retinal degeneration including irregular pigment clumps in the retina and attenuation of the retinal vessels. 

b OCT of left eye of patient No.002. 

c Result of pure tone audiogram testing of patient No.002 indicated bilateral hearing loss, cross or circle labels indicate air-conduction hearing, and right angle labels indicate bone-conduction hearing. 

d Tympanogram of patient No.003 demonstrated limited sound system activity of the middle ear. 

e The results of ERG of patient No.003 displayed indistinguishable wave amplitude.

Table 1 The clinical information of the patients

| Patient number | Gender | Diagnosis | Inheritance pattern | Onset age | ERG  | Fundus appearance | Hearing impairment | Vestibular function | Night blindness |
|----------------|--------|-----------|---------------------|-----------|------|-------------------|-------------------|-------------------|-----------------|
| 001            | M      | USH2      | autosomal recessive | 21        | no reaction | RP                | Severe            | Normal            | Yes             |
| 002            | M      | USH2      | autosomal recessive | 21        | no reaction | RP                | Moderate          | Normal            | Yes             |
| 003            | F      | USH2      | autosomal recessive | 19        | no reaction | RP                | Moderate          | Normal            | Yes             |
| 004            | F      | USH2      | autosomal recessive | 23        | no reaction | RP                | Severe            | Normal            | Yes             |
| Patient number | Exon/Intron | Nucleotide change | Amino acid change | Type | MutationTaster | SIFT | PROVEAN | PolyPhen-2 | HSF | Ref. |
|----------------|-------------|-------------------|-------------------|------|----------------|------|----------|-------------|-----|------|
| 001            | IVS42       | c.8559-2A > G     | –                 | Heterozygous | Disease causing | –    | –        | –           |     | [17] |
| 002            | EX19        | c.4217C > A       | p.Ser1406X        | Heterozygous | Disease causing | Damaging | Deleterious | –           |     | Novel |
| 002            | EX61        | c.11780A > G      | p.Asp3927Gly      | Heterozygous | Disease causing | Tolerated | Deleterious | Possibly damaging [Score 0.911] | –   | Novel |
| 003            | IVS58       | c.11389 + 3A > T  | –                 | Heterozygous | Disease causing | –    | –        | –           |     | [17] |
| 004            | EX42        | c.8232G > C       | p.Trp2744Cys      | Heterozygous | Disease causing | Damaging | Deleterious | Possibly damaging [Score 1.000] | –   | [18] |
| 004            | IVS42       | c.8559-2A > G     | –                 | Heterozygous | Disease causing | –    | –        | –           |     | [17] |
php), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://www.mutationtaster.org/) to predict the pathogenic effect of missense variants. Human Splicing Finder (HSF) (http://www.umd.be/HSF3/) was used to predict the pathogenicity of splicing-site. Evolutionary conservation across species was evaluated through the alignment of orthologous USH2A protein sequences (including Mouse, Troglodyte, Bovine, Chicken, Mulatta and Zebradise) with the human USH2A protein sequence, using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Results
Clinical characteristics of the USH2 patients
According to the data of their families, all the recruited patients followed the pattern of autosomal recessive inheritance. Representative fundus photographs indicated typical RP features (Fig. 1a), and representative OCT imaging suggested significantly diminished retinal thickness in patients (Fig. 1b). Moreover, most patients have moderate-to-severe hearing impairment, and analysis of pure tone audiogram testing demonstrated bilateral decrease of air-conduction and bone-conduction auditory (Fig. 1c). The tympanograms were showed type A, which means limited activity of the middle ear transmission system (Fig. 1d). The ERG wave amplitude of patients were undetectable (Fig. 1e). Those features indicate the diagnosis of USH2, and detailed clinical information of the patients is summarized in Table 1.

Pathogenicity analysis of the USH2A variants
In this study, we found 19 changes among four USH2 patients by exon sequencing of the USH2A gene. According to the result of computer algorithm, five of them were predicted to be pathogenic variants (Table 2). All the other 14 variants predicted non-pathogenic are listed in the Table S2.

These five heterozygous mutations include a nonsense mutation (c.4217C>A (p.Ser1406X)), two splice site mutations (c.8559-2A>G and c.11389+3A>T), and two missense mutations (c.8232G>C (p.Trp2744Cys) and c.11780A>G (p.Asp3927Gly)). All of these can be predicted as harmful by the computer prediction tool.

In the five pathogenic variants, two of them (c.4217C>A (p.Ser1406X) and c.11780A>G (p.Asp3927Gly)) were novel (can not be found in the variants in publicly available human genome aggregation data sets) and three (c.8559-2A>G, c.8232G>C (p.Trp2744Cys)) were shown in the Table 2.

![Fig. 2](image)

**Fig. 2** Direct sequencing analysis of the pathogenic variants in USH2A identified in this study. **a** Sequence shown the heterozygous nonsense variant c.4217C>A (p.Ser1406X) and the corresponding wild-type sequence. **b** Sequence shown the heterozygous missense variant c.8232G>C (p.Trp2744Cys) and the corresponding wild-type sequence. **c** Sequence shown the heterozygous one-base-substitution variant c.8559-2A>G and the corresponding wild-type sequence. **d** Sequence shown the heterozygous one-base-substitution variant c.11389+3A>T and the corresponding wild-type sequence. **e** Sequence shown the heterozygous missense variant c.11780A>G (p.Asp3927Gly) and the corresponding wild-type sequence. Arrows indicate the position of variants.
744Cys) and c.11389 + 3A > T) were reported. All the variants predicted to be pathogenic were absent in 200 Chinese unrelated healthy controls.

Two novel variants (c.4217C > A (p.Ser1406X) (Fig. 2a) in exon 19 and c.11780A > G (p.Asp3927Gly) (Fig. 2e) in exon 61) were found in patient No.002. In the family of patient No.002, c.4217C > A (p.Ser1406X) was found in his mother (Figure S1 B) and c.11780A > G (p.Asp3927Gly) was found in his father (Figure S1 C). Parents of patient No.002 are normal. Reported intron sequence variant c.8559-2A > G (Fig. 2c) and missense variant c.8232G > C (p.Trp2744Cys) (Fig. 2b) in exon 42 were found in patient No.004. Interestingly, intron sequence variant c.8559-2A > G also found in patient No.001 and his unaffected father (Figure S1 A). Finally, an intron sequence variant c.11389 + 3A > T (rs753886165) (Fig. 2d) was found in patient No.003. However, in patient No.001 and No.003, we do not find the allelic variant in the USH2A gene. The pedigrees of the four patients with variants in USH2A are shown in the Fig. 3.

For the exon pathogenic variants identified in this study, we examined the location of them along the usherin. Finally, we identified functional domains the exon variants located within (Fig. 4a). Additionally, we aligned USH2A sequences among different species, including Human, Troglodyte, Mulatta, Bovine, Chicken, Mouse and Zebrafish for each of the two novel missense variants by Clustal Omega. The results of the conservative analysis of amino acid sequences were shown in Fig. 4.

**Discussion**

Currently, 16 genes associated with USH have been identified, and three are USH2-causing gene. The USH2A gene causes 30–40% of USH2 cases and 10–15% of recessive RP cases [19]. Usherin is localized to a spatially restricted membrane microdomain in mammalian photoreceptors [13]. Previous researches have shown that congenital usherin protein mutations can induce the connecting cilium disorder and eventually lead to blindness [20].

Up to now, mutation screening in Chinese patients was revealed 25 mutations in previous researches [15, 18, 21–24]. In Southern population of China, 8.47% of sporadic RP patients are belong to USH [21]. In this study, we identified two novel variants (a missense variant and a nonsense variant) in the USH2A gene of four Chinese patients diagnosed with USH2 and found three reported mutations.

Isoform b of USH2A consists 8 domains, including N-terminal signal peptide (SP), laminin G-like domain (Lam GL), laminin N-terminal (Lam NT), laminin-type EGF-like domain (EGF Lam), fibronectin Type III (FN3), laminin G domain (LamG), transmembrane region (TM), and a PDZ-binding motif (PBM) at its C-terminal end [9]. By the PBM interacted with the PDZ domain of harmonin and whirlin, USH2A integrated into the USH protein network [25].

All of the two novel pathogenic variants are located in the FN3 domain (Fig. 4a). c.4217C > A (p.Ser1406X) is located on exon 19, and leads to a subsequent loss of 3796 amino acids, which make the protein usherin to lose more than 70% of its amino acids including 30 TM domains, 2 LamG domains, TM domain, and PBM domain. Therefore, heterozygous nonsense variant c.4217C > A (p.Ser1406X) causing a premature stop codon at 1406 is located on exon 19, and leads to a subsequent loss of 3796 amino acids, which make the protein usherin to lose more than 70% of its amino acids including 30 TM domains, 2 LamG domains, TM domain, and PBM domain. Therefore, heterozygous nonsense variant c.4217C > A (p.Ser1406X) affecting the structure and function of the protein usherin have a great possibility of causing the USH2. Novel missense variant p.Asp3927Gly (c.11780A > G) replaces a polaraspartic acid with a non-polar hydrophobic glycine at codon 3927. Amino acid substitutions caused by reported missense variant p.Trp2744Cys (c.8232G > C) occur at highly conserved sites among the tested species. Interestingly, sites of

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Fig. 3 Pedigree of the Chinese Usher syndrome type II patients’ family. The black filled shapes mean individuals diagnosed with USH2 and the unfilled mean unaffected ones. Males are represented by squares, females circles. Patient number is below the individuals' symbol. Individuals with available DNA samples were marked with asterisk. Question mark means the unknown allelic variant. M1: c.4217C > A (p.Ser1406X); M2: c.8559-2A > G; M3: c.8232G > C; M4: c.11389 + 3A > T; M5: c.11780A > G (p.Asp3927Gly)
novel missense variant p.Asp3927Gly (c.11780A > G) in the Human, Troglodyte, Mulatta, Chicken, Zebrafish and Bovine are conserved while the Mouse not.

For the Family # 2 and Family # 3, the following possibilities could be attributed to the unknown allelic variants: 1. Variants in deep-intronic regions of USH2A were not detected because this part of the genome was not covered in the screening. 2. Variants in regulatory elements except the USH2A gene cannot be excluded. 3. The duplication or deletion of other alleles may not be detected due to the absence of copy number variation analysis.

Because of unknown allelic variants in Family # 2 and Family # 3, we suppose that other pathogenic variants may exist in patients. Data from Family # 2 was supportive for the pathogenicity of the novel nonsense variant c.4217C > A (p.Ser1406X) and novel missense variant c.11780A > G (p.Asp3927Gly). The other three pathogenic variants are known pathogenic mutations that have been reported. However, sufficient biological and clinical evidence was required to reveal the relationship between the identified variants and the USH2. The detailed reasons of these pathogenic mutations leading to visual defects and hearing impairment have not been elucidated, and pending further function and mechanism investigations.

In all the three USH2-causing genes, USH2A gene is the most important causative gene, and the usherin which encoded by USH2A is crucial for the long-term maintenance of mammalian photoreceptors [13]. Accordingly, identification of the mutations in the USH2A gene will not only elucidate the role of USH2A in USH2, but also aid the clinical diagnosis and help to find effective treatments for USH2.

Conclusions
In conclusion, we have described five heterozygous variants may cause USH2 in USH2A in four Chinese patients with USH2, two of which were novel. The specific mechanism for these variants to induce USH2 needs further research to confirm. The findings in this study expand the spectrum of USH2A mutations in USH.
Additional file 1: Table S1. Primer information for the USH2A gene exons 2 to 72 sequencing. (XLS 31 kb)

Additional file 2: Table S2. variants predicted non-pathogenic in this study.

Additional file 3: Figure S1. Sequencing data of variants c.8559-2A>G, c.4217C>A (p.Ser1406X) and c.11780A>G (p.Asp3927Gly) identified in the father of patient No.001 and the parents of patient No.002.

Abbreviations
ADGRV1: Adhesion G protein-coupled receptor V1; DFN31: Autosomal recessive deafness 31; EGF: Laminin-type EGF-like domain; ERG: Electroretinogram; FN3: Fibronectin Type III; HSF: Human splicing finder; IRB: Institutional review board; Lam G: Laminin G-like domain; Lam NT: Laminin N-terminal; Lam G: Laminin G domain; OCT: Optical coherence tomography; PBM: PDZ-binding motif; PCR: Polymerase chain reaction; RP: Retinitis pigmentosa; SP: Signal peptide; TM: Transmembrane region; USH: Usher syndrome; USH1: Usher syndrome type I; USH2: Usher syndrome type II; USH2A: Usherin; USH3: Usher syndrome type III

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Authors’ contributions
CJJ and ZZL conceived and designed the experiments, HCH and LXY performed the experiments, CJJ and HCH analyzed the data, CJJ and LXY contributed reagents/materials/analysis tools. HCH drafted the manuscript. All authors have read and approved the final version of this manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
This study was approved by the institutional review board (IRB) of Tongji Eye Institute of Tongji University School of Medicine in Shanghai (registration number 2013YYX12), China. Written informed consent was voluntarily provided from all participating members.

Consent for publication
All patient’s written consent for their personal or clinical details along with any identifying images to be published in this study was obtained.

Competing interests
The authors declare that they have no competing interests.

Author details
1Birth defect group, Translation Research Institute of Brain and Brain-Like Intelligence, Shanghai Fourth People’s Hospital Affiliated to Tongji University School of Medicine, Shanghai 200081, China. 2Department of Medical Genetics, Tongji University School of Medicine, Shanghai 200092, China. 3Department of Pediatrics of Shanghai Tongli Ten People’s Hospital, Tongji University School of Medicine, Shanghai 200092, China. 4Department of Ophthalmology of Shanghai Tongli Ten People’s Hospital, and Tongji Eye Institute, Tongji University School of Medicine, Shanghai 200092, China. 5Birth defect group, Medical wing building, Tongji University School of Medicine, 1239 SipingRoad Yangpu District, Shanghai 200092, China.

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