Identification of novel *USH2A* mutations in patients with autosomal recessive retinitis pigmentosa via targeted next-generation sequencing

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Abstract. Retinitis pigmentosa (RP) is a group of inheritable blindness retinal diseases characterized by the death of photoreceptor cells and a gradual loss of peripheral vision. Mutations in Usher syndrome type 2 (*USH2A*) have been reported in RP with or without hearing loss. The present study aimed to identify causative mutations in a cohort of families with RP from China. A cohort of 62 non-syndromic families with RP and 30 sporadic cases were enrolled in this study. All affected members underwent a complete ophthalmic examination, including fundus photography, visual-field test and optical coherence tomography examination. Next-generation sequencing-targeted sequencing of 163 genes involved in inheritable retinal disorders was performed on the probands. Stringent bioinformatics data analysis was applied to identify potential candidate variants. In total, 6 novel mutations and 2 known mutations of *USH2A* were identified in 4 families with RP. A stop-gain mutation (c.C1731A) and a missense mutation (c.G8254A) were identified in RP family RP-2148. In another RP family, RP-2150, a known mutation (c.G802A) and a novel frameshift insertion mutation (c.12086dupA) were discovered. A novel stop-gain mutation (c.G11754A) and a missense mutation (c.G13465A) were identified in family rpz05. A novel missense mutation (c.C9328G) and a known missense mutation (c.G8232C) were also identified. These mutations were subsequently confirmed by Sanger sequencing. All 6 novel mutations affected highly conserved amino acid residues, and were absent in 1,000 ethnically matched controls. Taken together, the present study has reported on 6 novel *USH2A* mutations in 4 families with RP, and has expanded the mutation spectrum of *USH2A* in autosomal recessive RP in the Chinese population, thus providing important information for the molecular diagnosis and screening of RP.

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Introduction

Retinitis pigmentosa (RP; OMIM:226800) is an inherited retinal dystrophy and a genetically heterogeneous disease, with a worldwide prevalence of ~1:4,000 (1). The main features of RP are vision loss, tubular vision, night blindness, retinal 'bone-spicule' pigmentation, reduced vascular atrophy and certain complications of deafness (2,3). The genetic pattern of RP is diverse: The majority of the genetic mutations are autosomal dominant or autosomal recessive (ARRP), some are X-linked (4), and other modes of inheritance, including digenic or mitochondrial inheritance, have also been reported (5,6). The affected patients generally suffer poor vision under dim light and progressive visual loss due to the death of rod cells (7). To date, there is no effective way to treat this disease.

RP is one of the most genetically heterogeneous disorders. To date, >90 genes have been associated with ARRP (RetNet; https://sph.uth.edu/retnet/, accessed July 2019). In addition, mutations in a single gene can be associated with a broad phenotypic spectrum, and a specific phenotype can be caused by mutations in multiple genes (8). At present, known mutations can explain ~60% of RP cases (9,10). Therefore, identifying additional disease genes involved in RP is important for genetics diagnosis. Next-generation sequencing (NGS) has become one of the most important tools for the identification of disease-causing genes (11-18). In previous studies, a targeted NGS method has been used successfully to systematically screen coding regions of known retinal genes to identify pathological mutations (19-21).

The most common syndromic RP is Usher syndrome, which accompanies RP with hearing loss, with a prevalence of ~1:20,000 (22). In total, 16 genes have been identified as causative genes for Usher syndrome (https://sph.uth.edu/retnet/sum-dis.htm). The gene for Usher syndrome type 2 (USH2), *USH2A*, was mapped using linkage analysis by Kimberling *et al* (23) and Lewis *et al* (24) to chromosome 1 with 72 exons. It encodes usherin, a basement membrane protein with laminin epidermal growth factor and fibronectin type III domains (25,26) that is found in numerous tissues, including capillary and structural basement membranes in the retina and inner ear. Mutations in the *USH2A* gene are the most common cause of Usher syndrome (29% of all cases) and one of the most common reasons for ARRP (19-23%) (27-29). Mutations in *USH2A* were reported to be responsible for 7% of RP cases in North America (30). Jiang *et al* (31) identified 40 *USH2A* mutations in 32 patients with USH2, and reported that *USH2A* mutation severity determined patient clinical phenotypes.

The aim of the present study was to apply targeted NGS to a cohort of 62 families with RP and 30 sporadic cases from China to identify mutations underlying the disease. The current study focused on mutations in *USH2A*. In total, 6 novel mutations and 2 known mutations were identified.

Materials and methods

Subjects and clinical assessments. The present study was approved by the Ethics Committee of the Hospital of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, Sichuan, P.R. China; approval no. SCPH-2017-076). All patients (age range, 17-65; 55% male patients and 45% female patients) from 62 unrelated Han Chinese families and 30 sporadic cases, as well as 1,000 healthy Han Chinese control individuals were recruited from the Sichuan Provincial People's Hospital between January 2014 and December 2016. All the patients, family members and controls involved in the study signed written informed consent for the collection of samples for sequencing and the publication of patient images and data. The patients and family members were clinically diagnosed at Sichuan Provincial People's Hospital. Peripheral blood samples were collected from probands and their family members. Genomic DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Inc.) according to the manufacturer's protocols.

Targeted NGS and genetic analysis. A targeted-NGS sequencing method described by Wang *et al* (19) was adapted in the present study. Briefly, genomic DNA samples were randomly fragmented into 300-500 bp fragments whose ends were end-repaired using polynucleotide kinase (Thermo Fisher Scientific, Inc.) and Klenow (New England Biolabs, Inc.). Subsequently, extra 'adenine' bases were added to the 3'end of the DNA fragments. Illumina Y-shaped index adapters (Illumina, Inc.) were then applied to generate DNA paired-end libraries. Subsequently, each captured library was loaded on to Illumina HiSeq 2000 (Illumina, Inc.) for quantification and sequencing. Using Burrows-Wheeler Aligner (version bwa-0.7.15), sequencing reads were aligned to the reference human genome (hg19 UCSC assembly; genome.ucsc.edu) (32). SAMtools (version 1.3) was applied to identify single-nucleotide variants and insertions/deletions (33). Filtrations and annotations of the variants were conducted according to a previously described protocol (34) based on autosomal recessive inheritance pattern using the following databases: i) NCBI CCDS (www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi); ii) RefSeq (www.ncbi.nlm.nih.gov/RefSeq); iii) Ensembl (www.ensembl.org); and iv) ENCODE (genome.ucsc.edu/ENCODE).

To ensure the accuracy and efficacy of the candidate mutations, the synonymous, intergenic and intronic variants were first filtered out, and mutations in any of the following databases were excluded: i) dbSNP138 (www.ncbi.nlm.nih.gov/projects/SNP); ii) 1000 genomes Project (ftp.1000genomes.ebi.ac.uk/vol1/ftp); iii) YH database (yh.genomics.org.cn); iv) HapMap Project (ftp://ftp.ncbi.nlm.nih.gov/hapmap); and v) an 'in house' database generated from 1,800 samples sequenced by whole exome sequencing (34).

According to the bioinformatics pipeline, low-quality variants and variants predicted to be benign by online tools (SIFT (sift-dna.org), PROVEAN (provean.jcvi.org) and Ensembl Variant Effect Predictor (grch37.ensembl.org/info/docs/tools/vep) were excluded (10).

Sanger sequencing analysis. DNA sequencing was performed using the dideoxy Sanger method by fluorescent automated sequencing on the ABI 3130xl Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sanger sequencing analysis was used to verify whether the remaining variants co-segregated with the RP phenotypes in the families. PCR primers (Table I) were designed using the Primer3 online tool (SourceForge.Net) and synthesized by Shanghai Sangong Pharmaceutical Co., Ltd. to amplify genomic DNA fragments. The PCR products were purified using FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Inc.) and sequenced using a BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 28 cycles of 96˚C for 15 sec, 50˚C for 10 sec and 60˚C for 4 min; followed by maintenance at 4˚C. The sequencing data were analyzed using Sequencing Analysis ABI Software v5.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Clinical diagnosis. Ophthalmic examinations were conducted, including visual acuity, intraocular-pressure, ocular-motility, pupillary-reaction, slit-lamp, visual field test, dilated-fundus, optical coherence tomography examination and visual electrophysiological tests.

Results

Clinical characteristics of the families with RP. In the present study, the two recruited families who were initially diagnosed with RP followed the pattern of autosomal recessive inheritance. Ophthalmic examinations identified 2 affected members in family RP-2148, and 1 affected member in RP-2150. Representative fundus photographs are shown in Fig. 1, which indicate an obvious waxen appearance of the discs, bone-spicule pigment deposit in the midperiphery, attenuation of the retinal arteries (Fig. 1A), waxy-pale disc and bone-spicule pigment (Fig. 1B). The patients' parents and other unaffected family members did not show any RP features. A visual field test revealed loss of peripheral vision in both eyes

Table I. Primers used for mutation analysis.

Amplicon	Primer	Temperature (˚C)	Size (bp)
p.G2752R	F: ACATTCTGAGGTACGGTGGG	59	543
	R: TGTCCTTCAGAACACCCACA	60	
p.C577X	F: ATAGAAGCACACAGGCCTCC	59	427
	R: ACCCTACCATGCCCGTAAAT	60	
p.G268R	F: GTCCTACAGTGTCCATGGAGA	58	464
	R: TCCTCAAGAGTAGCACTAGTGA	57	
p.H4029fs	F: CTCTGCTGTAGTGTTTGCGC	59	462
	R: ACAGGCTGTGAAGGGAGTTT	59	
p.G4489S	F: CCACCTGCAGCCTTACTCTC	60	330
	R: AAAATACCCCCTTGGCTGTT	59	
p.W3918X	F: GTGTGCAGCTGTCACTGGTT	59	312
	R: AATGCCATTGGGAGATTCTG	59	
p.P3110A	F: AATGAGGGAAGGTGGGATTC	60	501
	R: TATGCTCCGCAAAAGGATTC	60	
p.W2744C	F: CAAACCCAGGAAACAGCATT	60	310
	R: TGCGGAAGTCACATTGGTTA	60	

Primers were designed with Primer3web.

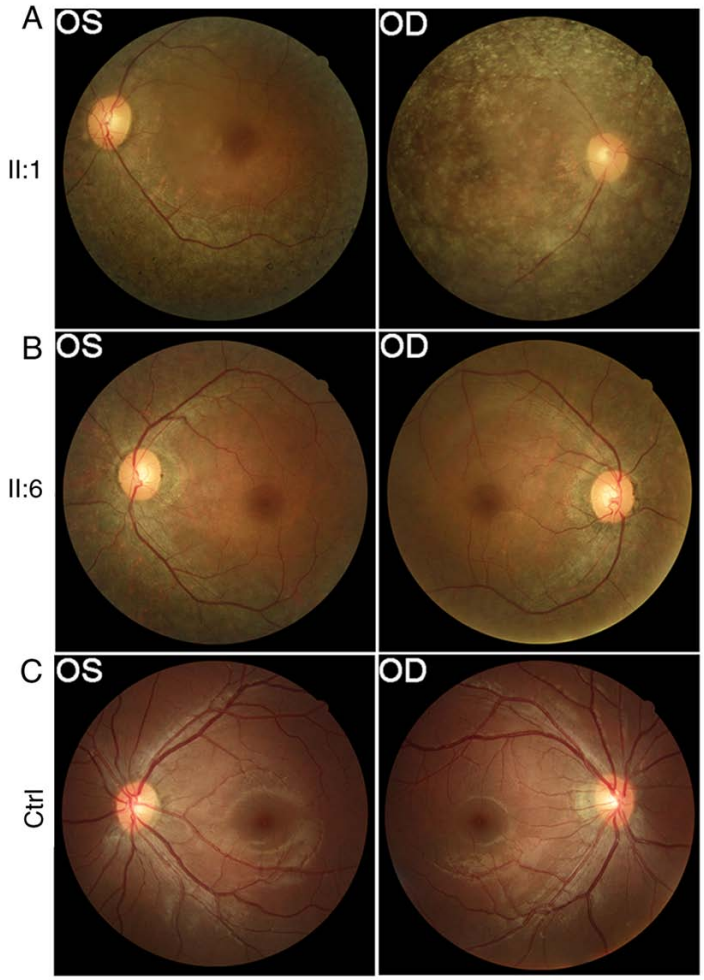

Figure 1. Fundus photographs of patients and an unaffected individual in RP-2148. (A) Fundus photographs show typical changes (waxy-pale disc, arteriolar attenuation and bone-spicule pigment) of fundus in the OS and OD. (B) Fundus photographs show waxy-pale disc and bone-spicule pigment in patient II:6. (C) Fundus photographs of the unaffected control III:1 show normal retina. OS, *oculus sinister*/left eye; OD, *oculus dexter*/right eye; Ctrl, control.

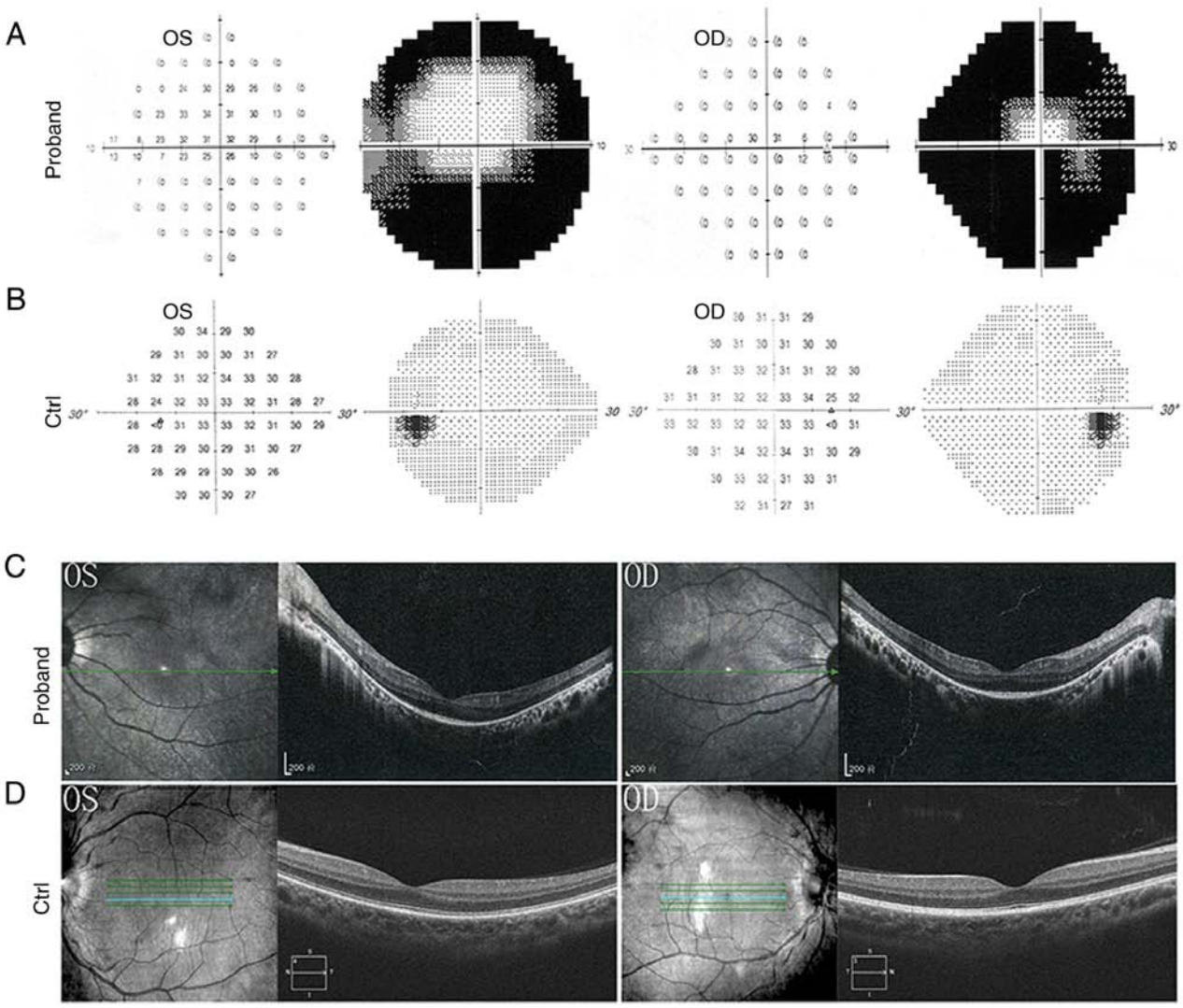

Figure 2. Visual field test and OCT pictures of the proband in RP-2150 and a healthy control. Loss of peripheral vision in both eyes was revealed by a visual field test in the (A) proband, as compared with that of the (B) control individual. (C) OCT examination of the proband revealed disorganized photoreceptor layers and thinner retina due to atrophy. (D) OCT examination result of a healthy control. OCT, optical coherence tomography; OS, *oculus sinister*/left eye; OD, *oculus dexter*/right eye; Ctrl, control.

(Fig. 2A and B). Optical coherence tomography examination, which provides an indirect measure of axonal and neuronal injury in the anterior visual pathways, revealed disorganized photoreceptor layers and thinner retina (Fig. 2C).

Genetic findings. To reveal pathogenic genetic factors, targeted NGS was applied to a cohort of patients with RP. Sanger sequencing was performed to verify the identified mutations (Fig. 3). In the proband of RP-2148, a stop-gain mutation (c.C1731A:p.C577X) was identified in exon 10 and 1 missense mutation (c.G8254A:p.G2752R) was identified in exon 42 (Table II and Fig. 3A). The proband RP-2148 II:1 carried compound heterozygous mutations: One allele (c.G8254A) came from the mother, whereas the other allele (c.C1731A) came from the father. Both his mother and father were heterozygous carriers. In RP-2150, the present study identified a missense mutation (c.G802A:p.G268R) and a frameshift insertion mutation (c.12086dupA:p.H4029fs) in the proband of RP-2150 II:1, which carried compound heterozygous mutations (Table II and Fig. 3B). One allele (c.12086dupA) came from the proband's father. Unfortunately, his mother was not available for genotyping. The missense mutation p.G268R has been previously reported in patients with RP (35). Sequence alignment analysis showed that these mutations affect highly conserved amino acid residues (Fig. 4). Furthermore, the mutation c.G8254A:p.G2752R was predicted to be damaging or deleterious by SIFT or PROVEAN software. None of the aforementioned mutations were present in 1,000 ethnically matched controls.

In an attempt to identify additional *USH2A* mutations, 3 novel mutations and 1 known mutation were detected in two unrelated families. A stop-gain mutation c.G11754A [p.W3918X] and a missense mutation c.G13465A [p.G4489S] were identified in family rpz05 (Fig. S1). In family rpz06, a novel missense mutation c.C9328G [p.P3110A] was detected in the proband (Fig. S2). The proband also carried a known missense mutation, c.G8232C:p.W2744C (36). Both c.G8254A [p.G2752R] and c.C9328G [p.P3110A] affected highly conserved amino residues (Fig. 4), and were predicted to be damaging or deleterious by SIFT or PROVEN software (Table II). In addition, none of the

Table II. *USHA2* mutations identified in patients with RP.

Family ID	Nucleotide change	Allele state	Amino acid change	dbSNP	Type	SIFT	PROVEAN
2148-II:1	c.C1731A	Het	p.C577*	Novel	Stop-gain	NA	NA
2148-II:1	c.G8254A	Het	p.G2752R	Novel	Missense	Damaging	Deleterious
2150-II:1	c.G802A	Het	p.G268R	Known	Missense	Damaging	Deleterious
2150-II:1	c.12086dupA	Het	p.H4029fs	Novel	Frameshift	NA	NA
rpz05-II:1	c.G13465A	Het	p.G4489S	Novel	Missense	Damaging	Deleterious
rpz05-II:1	c.G11754A	Het	p.W3918*	Novel	Stop-gain	NA	NA
rpz06-II:1	c.C9328G	Het	p.P3110A	Novel	Missense	Damaging	Deleterious
rpz06-II:1	c.G8232C	Het	p.W2744C	Known	Missense	Damaging	Deleterious

USHA2, Usher syndrome type 2; RP, retinitis pigmentosa; dbSNP, the Single Nucleotide Polymorphism database; PROVEAN, Protein Variation Effect Analyzer; SIFT, Sorting Intolerant From Tolerant; NA, not available.

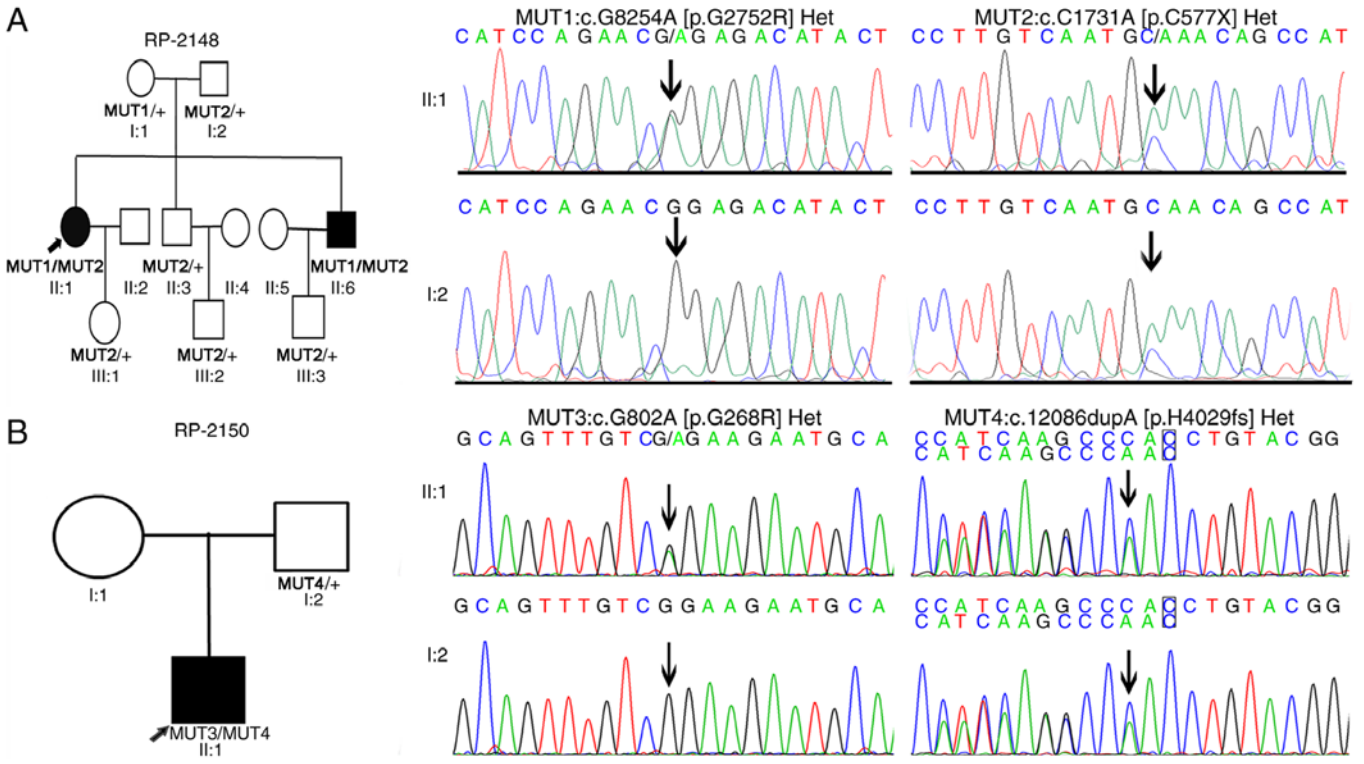

Figure 3. Pedigree of the families and *USH2A* mutations identified by Sanger sequencing analysis. (A) Diagram shows the segregation of compound heterozygous mutations c.G8254A, p.G2752R (MUT1) and c.C1731A, p.C577X (MUT2). Genotypes are presented as follows: MUT1/MUT2 represents the proband II:1 and affected individual II:6 carrying both mutations as compound heterozygous; MUT1/+ and MUT2/+ indicate heterozygous carriers. The proband's mother carried the heterozygous MUT1 mutation, and his father carried heterozygous MUT2 mutations. (B) Diagram showing a missense mutation MUT3:c.G802A [p.G268R] and a frameshift insertion mutation MUT4:c.12086dupA [p.H4029fs] in the proband of RP-2150 II:1. His unaffected father I:2 carried heterozygous MUT4. Black circles represent affected females. Black squares represent affected males. White circles represent unaffected females. White squares represent unaffected males. Arrows indicate the proband. *USH2A*, Usher syndrome type 2.

aforementioned mutations were present in 1,000 ethnically matched controls. These novel mutations data provide valuable information for the genetic diagnosis of *USH2A*-related RP.

Discussion

Mutations in the *USH2A* gene are the most common cause of Usher syndrome (29% of all cases) and one of the most common causes underlying ARRP (19-23%) (25,26). In North America, *USH2A* mutations account for 7% of RP cases (30). In a multi-ethnic cohort, Jiang *et al* (31) identified 40 *USH2A* mutations in 32 patients with USH2. Huang *et al* (37) reported 8 mutations in *USH2A* in 4 patients from 75 patients with non-syndromic RP, and 2 mutations in a family with Usher syndrome by Sanger sequencing screening. The present study applied targeted NGS analysis, and identified 8 *USH2A* mutations in a cohort

	RP-2148 p.G2752R	RP-2148 p.C577X	rpz05 p.G4489S	rpz05 p.W3918X
H. sapiens	VTWKPPLIQNGDILSYEIHMP	VYAFNCKPCQCNSHSKSCHYN	LRRDGTIVYTGLETRYRDFTL	GIEEESVLFVWSEGALEFMDE
P. troglodytes	VTWKPPLIQNGDILSYEIRMP	VYAFNCKPCQCNSHSKSCHYN	LRRDGTIVYTGLETRYHDFTL	GIEEESVLFVWSEGALEFMDE
M. mulatta	VTWKPPLIQNGDLLSYEIRMP	VHAFNCKRCQCNSHSKSCYYN	LRRDGTIVYTGLETRYHDFTL	GIEEESLLFIWSEGALEFTDE
C. lupus	VTWKPPLIQNGDMLSYEIRMP	VNAFNCKPCQCNNHSRSCHYN	LRRDGTIVYTGLETRYHDFTL	GIEEESLLFVWSEGALEFTDA
B. taurus	VTWKPPLILNGDILNYEIRMP	VHAFNCKPCECNSHSRSCHYN	LRRDGTIVYTGLETRYHDFTL	GIEEESLVFVWSEGALEFIDD
M. musculus	VSWKAPLMQNGDILSYEIRMP	VNAFNCKPCQCHGHASSCHYD	LRRDGAIVYVGLETRYHDFTL	DTEEESLLFVWSEGALEFTDD
R. norvegicus	VTWKAPLIRNGDIVSYEIRMP	VHAFNCKPCQCHGHASSCHYD	LRRDGAIVYIGLETRYHDFIL	DTEEESLLFVWSEGALEFTDD
G. gallus	VSWKPPLIQNGEILNYEIRMP	VHAYNCKPCQCYSHAVSCHYD	LRRDDVLVYSGQETRYLDFTL	GSQEDLLLFIWAEGALEFIDA
D. rerio	VTWAAPLIPNGEIERYEIRMP	IQAFNCRPCQCYGHASSCHYN	LRRNGSLIYTGTDTRYHDFTL	GTQEELLVFIWTEGPLEFIDA

	RP-2150 p.G268R	RP-2150 p.H4029fs	rpz06 p.P3110A	rpz06 p.W2744C
H. sapiens	QSLNGLEQFVGRMQDFRLYQV	FTVKGTSHQAHLYGLEPFTTY	GTQITTVEDTPSDIPTPTIRG	VLEPDAVQVTWKPPLIQNGDI
P. troglodytes	QSLNGLEQFVGRMQDFRLYQV	FTVKGTSHQAHLYGLEPFTTY	GTQITTVEDTPSDIPTPTIRG	VLEPDAVQVTWKPPLIQNGDI
M. mulatta	QSLNGLEQFVGRMQDFRLYQV	FTVMGTSHQAHLFGLEPFTTY	GTQITTVEDTPSDIPTPTIRG	VLGPDAAQVTWKPPLIQNGDL
C. lupus	QSLNGLEQFVGRMQDFRLYQV	FTVMGTSHQAHLFGLEPFTTY	GTQFTTVEDTPSDIPTPTIHG	VLGPHTAEVTWKPPLIQNGDM
B. taurus	QSLNGSEQFVGRMQDFRLYQV	FTVMGTSYQAHLFGLEPFTTY	RTQITTVEDTPSDIPTPTIHG	VLGPDAAKVTWKPPLILNGDI
M. musculus	QSLNGSEQFVGRMQDFRLYNV	FTVTGTSRQAHLFGLEPFTTY	GTQVSTAEDTPSDISIPVIRG	VLAPDTVEVSWKAPLMQNGDI
R. norvegicus	QSLNGSELFVGRMQDFRLYNV	FTVKGSSRQAHLFGLEPFTTY	GTQVSTAEDTPSGISIPIIRD	VLGPDAVEVTWKAPLIRNGDI
G. gallus	QSFTGLEQFVGRMQDFRFYPV	LTVTGGKHQAHLFGLKPFTTY	GTQITTVEDEPEQLSAPVIHV	VQGPHSVKVSWKPPLIQNGEI
D. rerio	RSSNGSNQFIGRMQDFRFYPK	LTVLGNVFQAYVFGLEPYTTY	STQVTTVEDTPAEISTPHIQV	VLGPESVQVTWAAPLIPNGEI

Figure 4. Protein sequence alignment of USH2A protein across different species. The amino acid residues affected by the identified mutations are conserved in 9 species. The orthologs are *Homo sapiens*, *Pan troglodytes*, *Macaca mulata*, *Canis lupus*, *Bos taurus*, *Mus musculus*, *Gallus gallus*, *Danio rerio* and *Xenopus tropicalis*. Orthologous alignments of the detected mutations in the present study suggest their evolutionarily important functions. USH2A, Usher syndrome type 2.

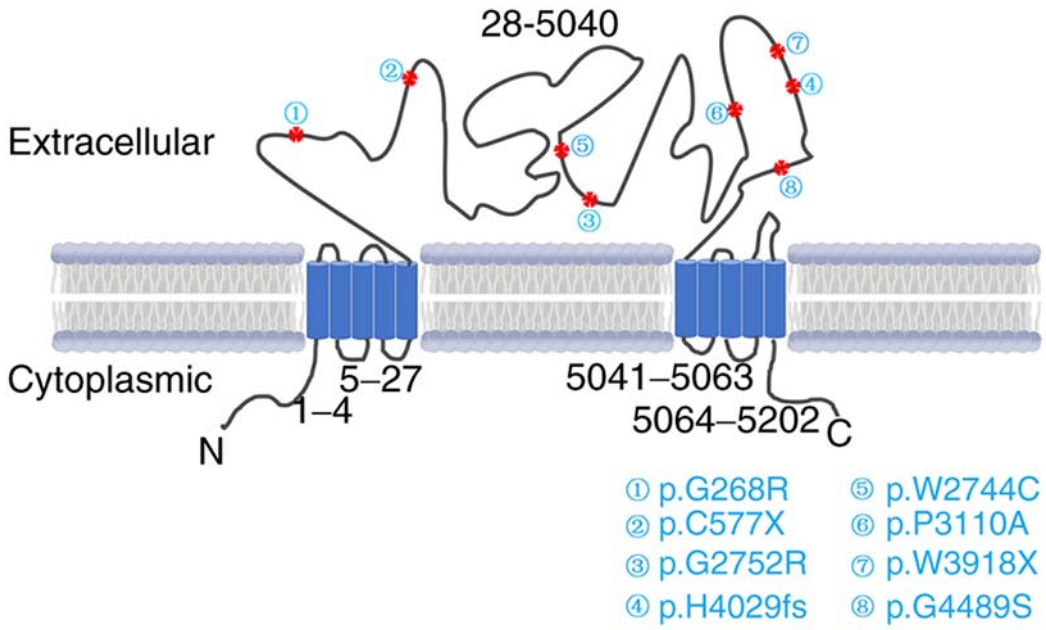

Figure 5. Diagram showing the location of the identified *USH2A* mutations in the current study. *USH2A* encodes a large transmembrane protein, and all 8 mutations are located in the exocytoplamic region. *USH2A*, Usher syndrome type 2.

of 62 patients with non-syndromic RP and 30 simplex cases in northern and western China. In total, 6 of the identified mutations were novel and absent from ethnically matched controls. Initially, no hearing problems were noted in these patients when they were enrolled in this study, and therefore hearing tests were not performed. Further follow-up hearing tests on these patients, however, could provide valuable information regarding the effect of these mutations on hearing.

Human *USH2A* encodes a large protein, usherin, containing 5,202 amino acids. In mammalian photoreceptors, the usherin protein is localized to the apical inner segment, in the amphibious photoreceptor (38). Deficiency in the *USH2A* gene in mice causes progressive photoreceptor degeneration and moderate hearing impairment, mimicking the visual and auditory deficits in patients with *USH2A* mutations (38). In the present study, all the 8 mutations identified were located in the exoplasmic functional domains of USH2A, and are predicted to be damaging (Fig. 5).

Taken together, in the present study 8 mutations have been identified in *USH2A* in a cohort of patients with non-syndromic RP from northern and western China. A total of 6 mutations were novel, of which 3 were stop-gain or frameshift insertions that disrupted the protein's function. In addition, all 3 novel missense mutations were predicted to be harmful, as determined by prediction software. These data have provided valuable information for the genetic diagnosis of RP cases caused by *USH2A*. Due to the limited scope of the current study, biological functions of these mutant proteins, however, were not assessed. Therefore, the effect of these missense mutations warrants further investigation. In summary, these data expand on the mutation spectrum of patients with non-syndromic RP in the Chinese population.

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Availabilty of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XZ, SL, KS and XJZ analyzed and interpreted the data. XZ, XL, WT and YY performed the sample sequencing and data analysis. XZ and XJZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Hospital of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, China; approval no. SCPH-2017-076). All the patients, family members and controls involved in the study signed written informed consent for the collection of samples for sequencing.

Patient consent for publication

All the patients, family members and controls involved in the study signed written informed consent for the publication of patient images and data.

Competing interests

The authors declare that they have no competing interests.

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