Multimodal Imaging and Genetic Characteristics of Chinese Patients with USH2A-Associated Nonsyndromic Retinitis Pigmentosa

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Abstract

Background To determine the clinical characteristics and molecular genetic background responsible for USH2A mutations associated with nonsyndromic retinitis pigmentosa (RP) in five Chinese families, a retrospective cross-sectional study was performed. Data of detailed history and comprehensive ophthalmological examinations were extracted from medical charts. Genomic DNA was sequenced by whole-exome sequencing. The pathogenicity predictions were evaluated by in silico analysis. The structural modeling of the wide-type and mutant USH2A proteins was displayed based on I-Tasser software.

Results The ultrawide-field fundus imaging showed a distinctive pattern of hyperautofluorescence in the parafoveal ring with macular sparing. Ten USH2A variants were detected, including seven missense mutations, two splicing mutations and one insertion mutation. Six of these variants have already been reported, and the remaining four were novel. Of the de novo mutations, the p.C931Y and p.G4489S mutations were predicted to be deleterious or probably damaging; the p.M4853V mutation was predicted to be neutral or benign; and the IVS22+3A>G mutation was a splicing mutation that could influence mRNA splicing and affect the formation of the hairpin structure of the USH2A protein.

Conclusions Our data further confirm that USH2A plays a pivotal role in the maintenance of photoreceptors and expand the spectrum of USH2A mutations that are associated with nonsyndromic RP in Chinese patients.

Background

Retinitis pigmentosa (RP) is the most common inherited retinal degeneration and occurs in approximately 1 in 4000 individuals worldwide [1]. Typical RP is heralded by primary rod degeneration leading to night blindness and the development of tunnel vision, while loss
of cone function leads to the deterioration of central vision [2, 3]. The typical clinical signs of RP include retinal arteriolar attenuation and a generalized and diffuse pattern of mottled and moth-eaten retinal pigment epithelium (RPE). However, the disease onset, progression, retinal characteristics, and visual prognosis may vary remarkably among patients, even within the same family [4, 5]. In addition, its early symptoms and signs can be atypical, which may result in misdiagnosis.

RP has various inheritance patterns, including an autosomal dominant (AD) pattern (30–40%), an X-linked pattern (10–15%), and an autosomal recessive (AR) pattern (50–60%) [5]. Sporadic cases account for approximately 40% of all cases, although these data vary between different populations [6].

To date, nearly 3100 pathogenic mutations have been reported in more than 80 genes associated with nonsyndromic RP, and 55 of these mutations have been related to arRP [7–9]. Although the functions of some of these genes have been studied extensively, many of them confer different phenotypes. It is therefore difficult to establish a clear genotype-phenotype correlation, since different genes cause distinct or partially overlapping clinical phenotypes [10].

Mutations in Usher syndrome 2A (USH2A) are a considerable cause of RP and can result in two distinct phenotypes: nonsyndromic RP and Usher syndrome type IIa. Patients with Usher syndrome type IIa experience both RP and congenital sensorineural hearing impairment, while patients with nonsyndromic RP are without extraocular symptoms [3].

In this study, we applied a whole-exome sequencing approach and copy number variation (CNV) analysis to test four thousand known monomeric inheritance diseases in 25 patients clinically diagnosed with RP. Of them, five unrelated patients (probands) were identified with ten USH2A mutations, including four novel heterozygous mutations in USH2A. With in silico and functional prediction, we further explored the potential
pathogenic effects of these mutations, which may represent possible mechanisms for nonsyndromic RP. This study is the first to model the three-dimensional structures of wild-type and mutant USH2A proteins, which is more intuitive to understand the possible effects of protein structural changes on protein functions.

In addition, we evaluated the retinal imaging features of patients with nonsyndromic RP resulting from USH2A mutations. With multimodal imaging, different stages of the disease among patients were compared, which may provide new insights into the natural progress of USH2A-associated nonsyndromic RP.

Results

Clinical Features

Among 25 patients clinically diagnosed with RP, 10 eyes of 5 probands from 5 pedigrees with nonsyndromic RP resulting from USH2A mutations were included (Figure 1). There were two males and three females in the identified probands. The age at presentation ranged from 31 to 60 years, the age of onset ranged from 12 to 46 years. The median follow-up was 46 months (range: 40–50 months). In the better eye, two patients (40%) had normal visual acuity or mild visual loss (0.9-0.6), one patient (20%) showed moderate visual impairment (0.5-0.2), and the other two patients (40%) presented low vision (<0.2). The patients’ demographic features are summarized in Table 1.

Fifteen family members from five pedigrees participated in our study (including five probands), and all of them were tested for whole-exome sequencing or verified by Sanger sequencing. All participants denied hearing impairment or deafness or balance problems. Anterior segment examinations were unremarkable. The clinical and genetic details of each pedigree are described in the supplemental materials.

Multimodal Imaging and Disease Progression

In the early stage of RP, the pigment deposits of the RPE are punctate or bone spicule-
shaped (Figure 2, pedigree 1) in the ultrawide-field fundus autofluorescence (FAF) and progressively aggravated into a mottled appearance in the mid periphery of retina (Figure 2, pedigree 2) with the course advancing. In the advanced stage, a lobular RPE defect along retinal vessels could be seen in the mid periphery of retina (Figure 2, pedigree 3 and 4). In the late stage, patchy RPE defects confluent into a zonal area of atrophy in the mid and far periphery of the retina with macular sparing (Figure 2, pedigree 5).

Moreover, although the multimodal fundus appearances varied among different patients, they shared something in common. All the probands showed the distinctive pattern of diffuse and homogeneous hyperautofluorescence parafoveal ring with macular sparing, which could be observed in ultrawide-field FAF [11].

**Genetic and Bioinformatics Analyses**

In total, 17 sequence variants (including ten USH2A variants) were identified in five probands. Among the ten variants of the USH2A gene, seven were missense mutations, two were splicing mutations, and one was an insertion mutation. The c.2802T>G (p.934C>W) variant was recurrent and was found in 2/5 probands (40%), 2/10 USH2A variants (20%) in our study, but almost all of the patients carried private sequence variants. Six of the variants have already been reported in the literature [12–16], while the remaining four were novel (Table 2).

**Location of the novel mutations in USH2A:** The p.C931Y mutant is located within the laminin EGF-like domain (Lam EGF) of USH2A, and the p.G4489S and p.M4853V mutants are located within the fibronectin type III domain (FN3). Figure 4 (in the supplemental materials) shows the schematic diagram of the reported mutations along the USH2A protein domains (without showing the novel intronic mutation IVS22+3A>G).

**Allele frequency:** Allele frequency for the nine detected USH2A variants (except for IVS22+3A>G) in the general population ranged from 0% to 0.07825% in total. The allele
frequency for the three novel missense variants (p.C931Y, p.G4489S, p.M4853V) was 0.005771%, 0.000000%, and 0.005766%, respectively (Table 3).

**Conservative prediction:** A total of 127 homologous genes of USH2A taken from 127 species were used to construct the phylogenetic tree (Figure 5A). The human USH2A homolog located in a same sub clade together with that in the primate Bonobo and Chimpanzee, suggesting these USH2A genes share the most evolutionary similarity with each other. The Weblogo software draws the seqlogo diagram of the sequence around the amino acids corresponding to each mutation point. As shown in Figure 5B, the third amino acid in the diagram indicates the amino acid corresponding to each mutation point. The larger the letter, the more conservative the amino acid at this site is. The conservative calculation of amino acids corresponding to each mutation site in the phylogenetic tree can be found in Table 3. Among the nine available mutation sites on USH2A, seven had conservative values greater than 0.5, while the amino acids at the 34 and 4853 sites had conservative values less than 0.5.

**Pathogenicity prediction:** The three novel missense variants (p.C931Y, p.M4853V, and p.G4489S) could not be found in the 1000 Genomes Database. They were predicted to be deleterious (p.G4489S and p.C931Y), and neutral (p.M4853V) by Provean; to be probably damaging (p.G4489S and p.C931Y) and benign (p.M4853V) by PolyPhen-II; to affect protein function (p.C931Y) and to be tolerated (p.G4489S and p.M4853V) by SIFT; and to be radical (p.C931Y), moderately conservative (p.G4489S) and conservative (p.M4853V) by Grantham scores. The bioinformatics analyses of the USH2A variants are summarized in Table 3.

Additionally, IVS22+3A>G is a novel splicing variant that could be considered pathogenic. The prediction and analysis of the secondary structure of DNA around the intron mutation IVS22+3A>G showed that the mutation position formed a hairpin structure (Figure 6 in the
supplemental materials, circled in red). Hairpin loop structures are an important structural motif in nucleic acids that have been shown to play important roles in many biological processes [17].

**Protein structure modeling and analysis:** Three-dimensional structural modeling of the wild-type and mutant USH2A proteins were cut in four regions according to the principle of covering the mutation point to the greatest extent (Table 4 in the supplemental materials). Region 1 (amino acids 1-1468) covers code-shifting mutations caused by insertions at bases 99-100: p.R34Sfs (c.99_100insT); region 2 (amino acids 747-2239) covers three missense mutations, including p.C931Y (c.2792G>A), p.C934W (c.2802T>G), and p.G1861S (c.5581G>A); region 3 (amino acids 3774-5202) covers four missense mutations, including p.G4489S (c.13465G>A), p.Y4673H (c.14017T>C), p.M4853V (c.14557A>G), and p.R5143C (c.15427C>T); and region 4 (amino acids 1869-3369) covers the splicing mutation in exon 42: IVS22+3A>G. Figure 7 shows the three-dimensional structural modeling of the wild-type and mutant USH2A proteins and their alignments in these four regions.

**Protein function prediction in mutation point:** The amino acid positions 922 and 931 as well as 934 and 948 were found to form disulfide bonds (Figure 8 in the supplemental materials). The predicted results showed that the reliability of disulfide bond formation is 1. In our study, the amino acids at positions 931 and 934 of the USH2A protein were the sites containing the gene mutations, and it can be assumed that the mutations at amino acids 931 and 934 will lead to disulfide bond breaking and thereby affect the structure of the USH2A protein.

**Discussion**

The USH2A gene is located on chromosome 1q41 and encodes the usherin protein, which is expressed in the supportive tissue in the inner ear and retina. The usherin protein plays a
critical role in the development of cochlear hair cells and the long-term maintenance of photoreceptors [18, 19]. In addition, it is functionally connected with the other proteins in the interactome of the retina [20].

However, it remains unknown why some mutations in USH2A result in Usher syndrome type IIa while others develop nonsyndromic RP [3]. A growing number of studies have shown that the total loss of function of the USH2A protein predisposes patients to Usher syndrome type IIa but that remnant protein function can lead to RP with or without hearing loss, indicating that mutations in the USH2A gene are relatively less harmful in nonsyndromic RP than in Usher syndrome type IIa. A multicenter research performed in the Netherlands and Belgium noted that truncating mutations are restricted to the Usher syndrome phenotype [3].

Nonsyndromic USH2A retinopathy is a primary cause of adult-onset recessive retinal degeneration [21]. In our study of nonsyndromic RP, we also found that the severity of the disease (an earlier visual decline) is related to the severity of the gene mutation as follows: insertion > splicing > missense mutation (Provean prediction: deleterious > neutral). The proband in pedigree 5, in whom there was 1 insertion mutation, began to suffer from visual impairment when she was 12 years old (Tables 1 and 2). In addition, our study shows that the severity of visual impairment observed in nonsyndromic RP patients with USH2A mutations is related to the progression of the disease (Table 1). Most USH2A-associated RP patients developed severe visual impairment [best-corrected visual acuity (BCVA) \( \leq 0.05 \), legally blind] around the age of 50 years old, consistent with previous reports [3, 21].

USH2A-associated RP patients show variable clinical characteristics (different ages of onset, disease progression, and fundus appearance), and it was therefore difficult to establish reliable genotype-phenotype correlations [20]. In addition, most patients are
compound heterozygotes as a result of carrying different mutations on the maternal and paternal allele, making it even more difficult to predict the effect of each of these mutations on the phenotype and complicating attempts to evaluate a possible allelic hierarchy [22].

Ultrawide imaging may provide more information about the contrast between the posterior pole and mid-periphery of the retina, so it can better assist in clinical diagnosis. Increased posterior pole autofluorescence occurs with increased accumulation of lipofuscin in the RPE or loss of rod outer segments [23, 24]. And the enhancement of parafoveal autofluorescence in FAF corresponds to the thinning of the outer retina in optical coherence tomography (OCT). Therefore, hyperautofluorescence parafoveal ring with macular sparing may be an imaging biomarker for genotype association in retinal dystrophies and RP. Different fundus manifestations (phenotypes) may be due to the different stages of RP, rather than to different genotypes.

Besides, angiographically silent cystoid macular edema presented in pedigree 1 has been previously reported in other cases [25]. Cystoid macular edema secondary to retinal degenerative diseases can easily lead to misdiagnosis, and this type of macular edema has a poor response to anti-VEGF therapy. Therefore, it is helpful to diagnose RP by grasping distinctive patterns, combining with clinical symptoms, electroretinogram (ERG) and visual field changes.

As for USH2A gene mutation and its effect on protein function, Caroline C.W. Klaver et al studied the USH2A genotypes and found that less than 19% of mutations were homozygous and 69% were private, which suggests that the heterogeneity is very large and that novel mutations may occur frequently [3]. The high prevalence of de novo variants and the poor rate of variants common in other ethnic groups indicate a unique mutational spectrum of the USH2A gene in Chinese patients with nonsyndromic RP [20].
In our study, interestingly, the proband (II:2) and her affected big brother (II:3) in pedigree 1 were found to be heterozygous carriers of M1 and M2 mutations (on the same chromatid) in the USH2A gene, associated with retinitis pigmentosa without hearing loss. Although there are high prevalence of carriers in inherited retinal dystrophies mutations [26], such a carrier state is hard to cause retinitis pigmentosa independently. Liquori A et al. have pointed out that deep intronic mutations in USH2A are underestimated. And unfortunately, analyzing USH2A transcripts is challenging and for 1.8–19% of Usher syndrome type II individuals who carry a single USH2A recessive mutation, a second mutation is yet to be identified [27]. The probability of an additional retinal dystrophy gene mutation conferring a compound heterozygote state may also elucidate the presence of extensive retinal degeneration in this case [28]. Other possible mutations associated with retinal dystrophy found in the comprehensive genetic testing of the proband in Pedigree 1 are referred to supplemental materials (Table 5 in the supplemental materials). However, whether these mutations are deleterious requires further family members Sanger sequencing and functional experiments to verify.

Other studies have also illustrated this possibility [20, 25, 29–32]. A study based in Italy found that one patient received the USH2A variant from the unaffected father and the MYO7A variant from the unaffected mother, which was heterozygous for mutations in both USH genes [20]. Similar findings in other studies support the hypothesis of a possible digenic/oligogenic inheritance of the syndrome/nonsyndromic RP or the possible joint impact of variants of different genes on the clinical phenotype of the patient.

Unrecognized mutations could present in the promoter, regulatory regions, and deep intronic areas, usually not tested during conventional mutation screening [20]. Our study has several limitations. It is a retrospective study that does not provide enough information on time-dependent longitudinal changes. In addition, the number of patients
with mutations in the same gene places restrictions on quantitative and statistical analysis, as well as further delineation of genotype-phenotype correlation. Moreover, although in silico prediction, protein modeling and disulfide bond function prediction of de novo mutations have been carried out, there is still a lack of further cellular and animal functional validation partly due to the difficulty of large protein synthesis and point mutation.

**Conclusions**

In conclusion, this study identified four novel mutations of the USH2A gene using a whole-exome sequencing approach, which further confirms that USH2A plays a pivotal role in the maintenance of photoreceptors and expands the spectrum of USH2A mutations that are associated with nonsyndromic RP in Chinese patients. Furthermore, we are the first to construct the tertiary structure of wild-type and mutant USH2A proteins by using three-dimensional protein structural modeling and analysis, which makes the changes in protein structure caused by different mutations observed directly. Nevertheless, for some mutations with uncertain pathogenicity, further functional experiments are needed to validate.

Through the evaluation and analysis of ultrawide-field fundus images, we found that nonsyndromic RP caused by USH2A mutation has the same distinctive pattern of hyperautofluorescence parafoveal ring with macular sparing. Meanwhile, we clarified that different fundus appearances are only the characteristics of different stages of RP, not different phenotypes caused by different genotypes. Better knowledge of the molecular genetic background underlying nonsyndromic RP in specific populations may contribute to more efficient diagnostic strategies and future therapeutic approaches.

**Methods**
Study Population and Clinical Examination

This study was approved by the medical ethics committee of Shanghai General Hospital, Shanghai Jiao Tong University (No. 2019KY044) and conducted in accordance with the Declaration of Helsinki. Patients assessed at Shanghai General Hospital with an established diagnosis of RP were included. Except for visual problems, these patients did not have other health-related issues.

All patients underwent a comprehensive ophthalmologic evaluation including BCVA, a slit-lamp examination, ultrawide-field color fundus photography (Heidelberg Engineering, Heidelberg, Germany), ultrawide-field FAF (Heidelberg) imaging, a spectral-domain (SD) OCT scan (Heidelberg), and an ERG (Electrophysiological Diagnostic Unit Retimax, Roland Consult, Brandenburg, Germany). Ultrawide-field fluorescein angiography (FFA; Heidelberg) was performed on all participants who agreed to collaborate. The diagnosis of RP was based on clinical manifestations, typical biomicroscopic findings in the fundus, FAF, FFA, OCT and ERG. Atypical presentations of RP were also included. After enrollment, the diagnosis was further confirmed by genetic testing. We included siblings, parents and offspring of the probands in our cohort. ERG was not a focus of this study, but 5 probands did undergo ERGs performed according to an International Society for Clinical Electrophysiology of Vision (ISCEV) protocol [33, 34].

Genetic Analysis

Genomic DNA was extracted from surplus peripheral blood leukocytes isolated from previous samples collected from diagnosed individuals using QIAamp DNA Blood Midi Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

To obtain a molecular diagnosis, all probands were submitted to whole-exome sequencing using a xGen Exome Research Panel v1.0 (Integrated DNA Technologies, USA) on an
Illumina Novaseq6000 platform (Illumina, USA). The xGen Exome Research Panel v1.0 consists of 429,826 individually synthesized and quality-controlled xGen Lockdown® Probes. The Exome Research Panel spans a 39-Mb target region (19,396 genes) of the human genome and covers 51 Mb of end-to-end tiled probe space. All probes in the panel are manufactured according to GMP standards. Mass spectrometry and OD measurements are taken for each probe to ensure appropriate representation of the correctly manufactured probes in the pool (https://sg.idtdna.com/pages/products/next-generation-sequencing/hybridization-capture/lockdown-panels/xgen-exome-research-panel). More than 95% of the targeted sequences were covered plenarily for high-confidence variant calling (>20 X coverage; mean coverage depth of over 100X). Paired-end alignment was performed using BWA aln to the 1000 genomes hg19/GRCh37 reference genome. SAM files were sorted and converted to BAM, and duplicates were marked with Picard. GATK was applied for local realignment and base quality score recalibration, and variants were called jointly in all samples using the GATK’s HaplotypeCaller in the “GENOTYPE_GIVEN_ALLELES” mode [35]. CNV mutation frequency information was obtained by searching the Exome Aggregation Consortium (ExAC) website (http://exac.broadinstitute.org/), which includes data obtained from 60706 unrelated individuals sequenced as part of various disease-specific and population genetic studies. Through ExAC, the allele frequencies of the variants can be determined and filtered by a 0.01 standard.

Sanger sequencing was subsequently applied to further validate the identified mutation(s). Linkage analysis was performed by direct sequencing among the available family members.

**Bioinformatics analyses**
Conservative Prediction: Information about the structure of the *USH2A* gene was acquired by searching the Ensembl database (http://asia.ensembl.org), and a phylogenetic tree of the gene was drawn using the Genetree tool. The sequence of multiple alignment of the USH2A homologous family was extracted from the phylogenetic tree data, and the evolutionary conservativeness of amino acids corresponding to each mutation was calculated. The seqlogo map of the amino acid sequence around the mutation site was drawn by Weblogo (http://weblogo.berkeley.edu/), and the conservativeness of the amino acid was determined from the map [36].

Pathogenicity Prediction: The sequence of protein O75445, which corresponds to the *USH2A* gene, was obtained from the UniProt database (http://www.uniprot.org/). The protein sequence was used as the input sequence, and the amino acid location of the mutation was selected. The pathogenicity of the mutation site was predicted using the PolyPhen-2 database (http://genetics.bwh.harvard.edu/pph2/) [37]. Additionally, the chemical dissimilarity of codon replacements was predicted by Grantham scores, which are categorized into four classes: conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151) according to the classification proposed by Li et al [38, 39]. Moreover, the possible functional impact of an amino acid change was predicted by Provean (http://provean.jcvi.org/genome_submit_2.php) and SIFT (http://sift.jcvi.org/).

Prediction and analysis of the secondary structure of DNA around the intron mutation: The secondary structure prediction software Mfold (http://unafold.rna.albany.edu/) was used to predict the secondary structure of DNA around the intron splice site mutation IVS22+3A>G. The left 200 bp-long and right 200 bp-long sequence of the mutation point was selected as the input sequence, and the folding temperature parameter was set to 37 degrees for the calculation [40].
Protein structure modeling and analysis: The USH2A protein sequence was retrieved from the UniProt database, and the domain of the sequence was cut by the principle of covering the mutation point to the greatest extent. I-Tasser software (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to simulate the protein structure before and after mutation. The wide-type and mutant protein structure was then analyzed using TM-align (https://zhanglab.ccmb.med.umich.edu/TM-align/) [41-43].

Mutation Point Protein Function Prediction: The functional characteristics of amino acids in the USH2A protein were searched using the UniProt database. The disulfide bonds of USH2A were predicted by DISULFIND software (http://disulfind.dsi.unifi.it/) [44].

Abbreviations

AD = autosomal dominant; AR = autosomal recessive; BCVA = best-corrected visual acuity; CNV = copy number variation; E = exon; ERG = electroretinogram; ExAC = Exome Aggregation Consortium; FAF = fundus autofluorescence; FFA = fluorescein angiography; FN3 = fibronectin type III domain; Lam EGF = laminin EGF-like domain; Lam G: Laminin G domains; Lam GL: Laminin G-like domain; Lam NT: Laminin N-terminal; ISCEV = International Society for Clinical Electrophysiology of Vision; M = mutation; PBM: PDZ-binding motif; PolyPhen-2: polymorphism phenotyping v2; Provean = protein variation effect analyzer; RP = retinitis pigmentosa; RPE = retinal pigment epithelium; SD-OCT = spectral-domain optical coherence tomography; SIFT = sort intolerant from tolerant; TM = transmembrane region; USH2A = Usher syndrome 2A.

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Declarations

**Ethics approval and consent to participate**

This study was approved by the medical ethics committee of Shanghai General Hospital, Shanghai Jiao Tong University (No. 2019KY044) and conducted in accordance with the Declaration of Helsinki. The data presented in this study was procured through retrospective chart review and details are not identifiable to any individual patient.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
Competing interests

The authors declare no conflict of interest. The funding organizations had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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Authors’ contributions

CC and QS contributed equally to this study and are listed as co-first authors. SY and XX had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conception and design: SY, CC

Data collection: CC, QS, TQ, DL, SY

Analysis and interpretation: CC, QS, MG, KL, XX, SY

Drafting the manuscript: CC, SY

Critical revision of the manuscript: SY, XX, MG, QS, KL

Supervision: XX, SY
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Tables

Table 1. Clinical characteristics of probands with nonsyndromic retinitis pigmentosa

| Pedigree No. | Proband No. | Gender | Age | Onset | Inheritance | BCVA OD/OS | Follow-up (months) |
|--------------|-------------|--------|-----|-------|-------------|------------|-------------------|
| 1            | 1-II:2      | Female | 47  | 46    | Unknown     | 0.25/0.3   | 50                |
| 2            | 2-II:4      | Female | 37  | 37    | AR          | 1.0/0.9    | 46                |
| 3            | 3-II:1      | Male   | 60  | 45    | AR          | 0.03/0.05  | 44                |
| 4            | 4-II:1      | Male   | 31  | 31    | AR          | 1.0/0.9    | 46                |
| 5            | 5-II:1      | Female | 54  | 12    | AR          | 0.03/0.01  | 40                |

OD: oculus dexter; OS: oculus sinister; AR: autosomal recessive.

Table 2. Identified mutations in USH2A in patients with nonsyndromic retinitis pigmentosa

| Pedigree No. | Mutation No. | Gene | Exon | Type   | cDNA | Amino acid | Genotype | Report |
|--------------|--------------|------|------|--------|------|------------|----------|--------|
| 1            | M1           | USH2A| 13   | Missense | c.2792G>A | C931Y | Hetero | Novel |
|              | M2           | USH2A| 64   | Missense | c.14017T>C | Y4673H | Hetero | Report |
| 2            | M3           | USH2A| 42   | Splicing | IVS42-2A>G | R2853G | Hetero | Report 7 |
|              | M4           | USH2A| 13   | Missense | c.2802T>G | C934W | Hetero | Report |
| 3            | M5           | USH2A| 66   | Missense | c.14557A>G | M4853V | Hetero | Novel |
|              | M6           | USH2A| Intron | Splicing | IVS22+3A>G | Splicing | Hetero | Novel |
| 4            | M7           | USH2A| 71   | Missense | c.15427C>T | R5143C | Hetero | Report 8 |
|              | M8           | USH2A| 28   | Missense | c.5581G>A | G1861S | Hetero | Report 7 |
|              | M9           | USH2A| 63   | Missense | c.13465G>A | G4489S | Hetero | Novel |
|              | M10          | USH2A| 2    | Insertion | c.99_100insT | R34Sfs | Hetero | Report |

Hetero: Heterozygous;
| USH2A Mutation | Protein domain | Allele frequency | Conservative | PolyPhen-2 | Provean | Grantham score | SIFT |
|----------------|----------------|-----------------|--------------|------------|---------|----------------|------|
| R34Sfs         | Signal peptide | 0.000000%       | 0.244        | NA         | NA      | NA             | NA   |
| C931Y          | Lam EGF        | 0.005771%       | 0.858        | Probably damaging (0.999) | Deleterious | Radical (194) | Dam≥ (0.00) |
| C934W          | Lam EGF        | 0.001649%       | 0.858        | Probably damaging (0.999) | Deleterious | Radical (215) | Dam≥ (0.00) |
| G1861S         | FN3            | 0.000831%       | 0.638        | Probably damaging (1.000) | Deleterious | Radical (159) | Dam≥ (0.00) |
| R2853G         | FN3            | 0.000000%       | 0.701        | Probably damaging (0.999) | Deleterious | Moderately radical (125) | Dam≥ (0.01) |
| G4489S         | FN3            | 0.000000%       | 0.685        | Probably damaging (1.000) | Deleterious | Moderately conservative (56) | Toler≤ (0.06) |
| Y4673H         | FN3            | 0.000000%       | 0.591        | Possibly damaging (0.754) | Deleterious | Moderately conservative (83) | Toler≤ (0.14) |
| M4853V         | FN3            | 0.005766%       | 0.134        | Benign (0.010) | Neutral | Conservative (21) | Toler≤ (0.42) |
| R5143C         | TM             | 0.07825%        | 0.646        | Benign (0.031) | Deleterious | Radical (180) | Dam≥ (0.02) |
| IVS22+3A>G     | Intron         | NA              | NA           | NA         | NA      | NA             | NA   |

NA: not applicable; Lam EGF: laminin EGF-like domain; FN3: fibronectin type III; TM: transmembrane region;

PolyPhen-2 (polymorphism phenotyping v2): “Probably damaging” (0.909-1, it is believed most likely to affect protein function or structure), “Possibly damaging” (0.447-0.908, it is believed to affect protein function or structure), “Benign” (0-0.446, most likely lacking any phenotypic effect).

Provean (protein variation effect analyzer): Variants with a score equal to or below -2.5 are considered "deleterious"; Variants with a score above -2.5 are considered "neutral". Grantham scores, which categorize codon replacements into classes of increasing chemical dissimilarity, were designated conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151) according to the classification proposed by Li et al.\textsuperscript{16,17}. 
SIFT (Sort Intolerant From Tolerant): Ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is \( \leq 0.05 \) and tolerated if the score is \( > 0.05 \).

Figures
Figure 1

The five Chinese pedigrees with nonsyndromic retinitis pigmentosa and mutations in the USH2A gene. Squares and circles indicate males and females, respectively; arrows symbolize the probands; Black and white denote the status of family members affected or unaffected, respectively; Question marks next to the square or circle indicates the questionable status of the family members. The genotype of each evaluated individual is listed below the individual’s symbol and identification number. W: wild type; M1: p.C931Y; M2: p.Y4673H; M3: p.R2853G (IVS42-2A>G); M4: p.934W; M5: p.M4853V; M6: IVS22+3A>G; M7: p.R5143C; M8: p.G1861S; M9: p.G4489S; M10: R34Sfs.
Figure 2
Multimodal images of the probands with USH2A mutations in 5 pedigrees. Ultrawide-field fundus photographs are shown to the left, ultrawide-field fundus autofluorescence images in the middle, and optical coherence tomographic images to the right. Only left eyes are shown, but findings in 5 probands presented a high degree of bilateral symmetry. The yellow arrows indicate the margin of the hyperautofluorescence in the parafoveal ring.
Figure 3

The phylogenetic tree of the USH2A gene and the conservative prediction of each mutation site. (A) The phylogenetic tree shows the sequence of multiple alignment of the USH2A homologous family. (B) The seqlogo map of amino acid sequences around the mutation point and their conservativeness. The third amino acid in the diagram belongs to the amino acid corresponding to each mutation point.

Wild-type
A. Location: 1-1468

Mutation
p.R34Sfs

Alignment
ARG-34
SER-34

B. Location: 747-2239

p.C931Y
p.C934W
p.G1861S

C. Location: 3774-5202

p.G4489S
p.M4853V

p.MET-1080
GLY-1115
CYS-1370
Figure 4

Three-dimensional structural modeling of wild-type and mutant USH2A protein in four regions. (A) Region 1 (1-1468 amino acid): p.R34Sfs (c.99_100insT); (B) Region 2 (747-2239): p.C931Y (c.2792G>A), p.C934W (c.2802T>G), p.G1861S (c.5581G>A); (C) Region 3 (3774-5202): p.G4489S (c.13465G>A), p.Y4673H (c.14017T>C), p.M4853V (c.14557A>G), p.R5143C (c.15427C>T); (D) Region 4 (1869-3369): p.R2853G (IVS42-2A>G).

Supplementary Files

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