Seven novel mutations in the long isoform of the *USH2A* gene in Chinese families with nonsyndromic retinitis pigmentosa and Usher syndrome Type II

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**Purpose:** To describe the clinical and genetic findings in one Chinese family with autosomal recessive retinitis pigmentosa (arRP) and in three unrelated Chinese families with Usher syndrome type II (USH2).

**Methods:** One family (FR1) with arRP and three unrelated families (F6, F7, and F8) with Usher syndrome (USH), including eight affected members and seven unaffected family individuals were examined clinically. The study included 100 normal Chinese individuals as normal controls. After obtaining informed consent, peripheral blood samples from all participants were collected and genomic DNA was extracted. Genotyping and haplotyping analyses were performed on the known genetic loci for arRP with a panel of polymorphic markers in family FR1. In all four families, the coding region (exons 2–72), including the intron-exon boundary of the *USH2A* (Usher syndrome type –2A protein) gene, was screened by PCR and direct DNA sequencing. Whenever substitutions were identified in a patient, a restriction fragment length polymorphism (RFLP) analysis, single strand conformation polymorphism (SSCP) analysis, or high resolution melt curve analysis (HRM) was performed on all available family members and on the 100 normal controls.

**Results:** The affected individuals presented with typical fundus features of retinitis pigmentosa (RP), including narrowing of the vessels, bone-spicule pigmentation, and waxy optic discs. The electroretinogram (ERG) wave amplitudes of the available probands were undetectable. Audiometric tests in the affected individuals in family FR1 were normal, while indicating moderate to severe sensorineural hearing impairment in the affected individuals in families F6, F7, and F8. Vestibular function was normal in all patients from all four families. The disease-causing gene in family FR1 was mapped to the *USH2A* locus on chromosome 1q41. Seven novel mutations (two missenses, one 7-bp deletion, two small deletions, and two nonsenses) were detected in the four families after sequencing analysis of *USH2A*.

**Conclusions:** The results further support that mutations of *USH2A* are also responsible for non-syndromic RP. The mutation spectrum among Chinese patients might differ from that among European Caucasians.

Retinitis pigmentosa (RP) is a heterogeneous group of retinal dystrophies, characterized by progressive degeneration of the photoreceptors. Clinical features include progressive night blindness, constriction and gradual loss of the peripheral visual field, and eventual loss of visual acuity. With an incidence of 1 in 3,500, RP can be inherited as an autosomal recessive (arRP), an autosomal dominant (adRP), or an X-linked recessive (xLRP) pattern [1,2].

RP can be classified as syndromic and nonsyndromic RP, based on whether or not extra-ocular diseases exist. Nonsyndromic arRP is caused by the mutations of 32 identified genes [1,2]. Syndromic RP includes more than 30 different syndromes [1,2]. The most common syndrome is Usher syndrome (USH), which is also an autosomal recessive disorder characterized by sensorineural hearing loss, variable vestibular dysfunction, and visual impairment due to retinitis pigmentosa [2,3]. Clinically, USH is subdivided into three types: USH type I (USH1), USH type II (USH2), and USH type III (USH3). USH1 is the most severe form of this disease and is characterized by congenital profound hearing loss, prepuberal onset of RP, and vestibular dysfunction. Patients with USH2 experience congenital moderate to severe hearing impairment, and postpuberal onset of RP with intact vestibular function. Patients with USH3 show progressive postlingual hearing loss, later onset of RP, and variable vestibular dysfunction. Of the three clinical types, USH2, which accounts for more than half of all patients with USH, is the most common form of USH [2-4]. To date, reports indicate that three genes (*USH2A* [Usher syndrome type –2A protein], *GPR98* [G-protein coupled receptor 98], and *DFNB31* [CASK-interacting protein CIP98 isoform 1]) are responsible for USH2, and most USH2 patients have mutations in *USH2A* [3-9].

*USH2A*, located on chromosome 1q41, has two alternatively spliced isoforms: a short *USH2A* isoform a, consisting of 21 exons, and a long *USH2A* isoform b, consisting of 51 additional exons at the 3′ end of *USH2A* [5, 9]. The protein usherin, encoded by USH2A isoform b, is a
| Primer | Forward sequence (5’-3’) | Reverse sequence (5’-3’) | Products (bp) | Tm (°C) |
|--------|--------------------------|--------------------------|---------------|---------|
| Exon 2 | GCCCTGGGATGAGCTTCAG      | GGTTTGGAAATCAGGCTTG      | 840           | 62      |
| Exon 3 | CACACCTGAACGTGACACATACC  | CTGCTGCAAGTTTTGGAGTACG   | 840           | 63      |
| Exon 4 | GTTCCTCCAGCTGAGAAAGAGTA  | GTGGTATTTTGGTCAGGCTCTAG  | 382           | 62      |
| Exon 5 | GTAAGTATTGCTGTTGTAACAG    | CAGCATTTACTCCTTCGCTTCC   | 173           | 62      |
| Exon 6 | CGTATGTCATTTGTTGAAGG     | GGCATTTTGGATTCAAAACCA    | 432           | 58      |
| Exon 7 | TTTGAAATCTAAATTTTCAAGTTG | TGTTGGTGAAGGGAGAAAGTCTC  | 372           | 64      |
| Exon 8 | CACCATTTGATTTGCTGCTGC    | GTGCTTTGCACTTTTGAATTTGC  | 370           | 62      |
| Exon 9 | CAACATGTGTTAGAATGTTGAG   | GTGTTGGTTGGAGTACAGTTGAG  | 367           | 62      |
| Exon 10| TGATATGTCCTTTACTCCTTCG   | GCATTGTAAGTAAAGCAGACACAG | 356           | 62      |
| Exon 11| TGTACGATTTGTTGAAAGG      | GCAATTGCTTTATTGCTGTTCA   | 371           | 62      |
| Exon 12| CCTGTCTTGTACCTAAATGAGC   | TTTCAGTTGGAATATGAGATGTA  | 323           | 58      |
| Exon 13| GCAAACCTGCTTTGCAAGAGCCC  | GTCAGATGGCAAAACGCAAAAC   | 816           | 58      |
| Exon 14| GGAAGTATTGTCCTTGTGATAG   | GAAATTTGATTCTTGACTGCTG   | 379           | 64      |
| Exon 15| AAGCCTGCTTACTCTGCAATGCT  | TTCTGATGTTGCAATATGAGGAG  | 360           | 58      |
| Exon 16| GAACTCCGCTTACAGAAAGACC   | CCACAACCAGCATTACATGCTC   | 354           | 64      |
| Exon 17| GAGAAGAAGGCAGTTACGAAATG  | GATTCATCATTGCACTTCTGACCA | 626           | 64      |
| Exon 18| AGATGAAAAACCCCTTGGGATGATG | GGAAGGATTGCACTTTAGHGAGG  | 378           | 62      |
| Exon 19| TCAGAAACATACAAAAGGAGTTGGA | TGCCCTGTCTTTACATCATAGAG  | 379           | 60      |
| Exon 20| TGGTGGTCTGTGCTACAAATCCC  | GAGTTATGGAAGGGGAGAGAACA  | 381           | 64      |
| Exon 21| AGCCATAAGATACCAGTACGAGCA | GCATCTAAAGGCGCTGTTAC    | 502           | 64      |
| Exon 22| CCTGCTTTGCTGTCAGGTTGC    | GCTGAGAAGGCTATCCAGTAC    | 451           | 62      |
| Exon 23| CAGGAAAGGCAGATTTGATGCG   | CCCAAAGGCAAACCAGTTAC    | 447           | 58      |
| Exon 24| CCTAAGGGAATGTTGGGACA     | CTTGGAAGGCTTATGGGACA     | 371           | 58      |
| Exon 25| TGTGAATCAATAGGCTTTCGAG   | TGTGGCCTTGTGAGAATGAC     | 459           | 58      |
| Exon 26| GGTCTTCGCTTTGCTCAGTTGC   | TTTCAGTTGCACTTTGAGTGGT   | 722           | 62      |
| Exon 27| TGCTTCGAGGAACTGCAATGTT   | GGTGCTCTGGTATTGGCTGAG    | 497           | 62      |
| Exon 28,29| TGCTGCAAGAGGCAAAATGGA   | GCTTCAGGGTAAATGTCCCTCC   | 579           | 62      |
| Exon 30| TGCGGCCATAAAGGTGAAG      | TGACGCTTTCCACTCATTTAG    | 434           | 58      |
| Exon 31| GCAGAAAGGGGAGAAATGGCAG   | CAAATTTAGGGTGGGTTGCTG    | 392           | 64      |
| Exon 32| TGATTTTCTTGTGTGGCTCTG    | GCATTCTGTTAATATTTTGAGC   | 357           | 58      |
| Exon 33| TGAAAGCCATATTTGATTATGAC  | CCTGCTTGATGAATCTAC       | 392           | 58      |
| Exon 34| ATTTCCCTTTGGCCCTCCAG     | AGGATGGGAGAGGATTCTTCAG   | 512           | 56      |
| Exon 35| TTGGGAGAAATGAAAGGATGAC   | CCAATTTCCTCCCAACTAGAG    | 431           | 62      |
| Exon 36| AAAATCAACTCAAGAGTGCTTGCC | CCTGCGTTGAAAGGCTAGTGGC   | 352           | 62      |
| Exon 37| TGTGCTTTGAGTACCTGCTG     | AAGCAGACCTGTTGATCAAGG    | 434           | 60      |
| Exon 38| TTGTAGGCGAGTCACCACTGAG   | TGTGAGCTGTTGATGAAGCAGC   | 614           | 64      |
| Exon 39| CAGAGCTTCAAGGAAATGGCAAG  | AAGTTCCATGCGGAAGAGAACCTC | 526           | 54      |
| Exon 40| TGAGATCCTTACGTGATGCGAGA  | GGCATTCTCCTTGTTGCTG     | 373           | 58      |
| Exon 41| TGTGCTTTACCAAGTGTTGCA    | AAGGCGAATAACCCAGTTTCT    | 890           | 58      |
| Exon 42| GCAAAATTTCTAGGCGCTTCTG   | AAAGGCTCTTTGATTCTTCAC    | 492           | 62      |
| Exon 43| ATGCGCAGAACAGCCGTAAG     | AGCCGTCGCAAAGGCAATAG     | 469           | 62      |
| Exon 44| TTTTGTAGAGGGGGTGAAGG     | TGTGACATGGGGGAGGTTG      | 367           | 58      |
| Exon 45| CATTTCCAAAAACAAAGGCTCCTC | TTAGCCCTACCCCTTCTC      | 464           | 58      |
| Primer | Forward sequence (5′-3′) | Reverse sequence (5′-3′) | Products (bp) | Tm (°C) |
|--------|-------------------------|--------------------------|---------------|---------|
| Exon 46 | TCATCATATCCACTTGGTCAC   | CCCTCTCTCTTTCCCTTCC     | 599           | 54      |
| Exon 47 | AGGGAAGGTGGGATTCTAGAC   | TGTCATGGCTGAGGATACCC    | 280           | 59      |
| Exon 48 | CCTCACTGATGGATGGTATTTC  | CTTCTCTTTCCGTTGGAATTC  | 530           | 54      |
| Exon 49 | TCCGATAGCTCCTGAAAATACA  | TTGTGAGAGGAGGGTGTTTG    | 432           | 56      |
| Exon 50 | ACCGTGTAGTGATGGATGTG    | TTGGAAAGAACATGTTTTTCC  | 678           | 54      |
| Exon 51 | ATCCCAGCAACTGCTTAGAC    | AAAGCTTCTCCCTGAGACAG    | 552           | 62      |
| Exon 52 | TGCCGAGCTGCGAAAATCTG   | GCCCTCAAAGTATGAGAAATTG | 564           | 54      |
| Exon 53 | TCCTCCTCTGCTACTCT      | GGTGAGTGCATAGGGAATTT   | 500           | 56      |
| Exon 54 | ATGCTATTTTCTTCAGAACC   | TCTCCTCCCTCCAGCATAGG   | 428           | 54      |
| Exon 55 | AAGGGAAATAGCTCTCCTCAAG | CCCCCCAAACAAATACTCAG    | 396           | 62      |
| Exon 56 | AGCCCTTTAGAGGTCTCAGACC | CAAGCCCTGAAAGATAGGAC   | 422           | 54      |
| Exon 57 | GGGGATGGTGTTGACTTTTG   | ATGGCCAAATAGGGAGAAG     | 382           | 56      |
| Exon 58 | GCCAAAGGTCTTGGCAATTTTG | TTTATCCAGGAGACCCACTATG  | 399           | 62      |
| Exon 59 | GACCACTATTGTCTGGCCATCT  | GCCGACTGTTGATTTTTCTGG  | 488           | 54      |
| Exon 60 | TGCAAGAGCCAGAGTTAAA    | GATTCTCCTGGTTGGAGCA    | 343           | 54      |
| Exon 61 | TGCACCAGGAAAGAAGACAGC  | TTAATCCCGTGACTACATTGC  | 638           | 54      |
| Exon 62 | TGTGGCACATGAGGTCTCAGAG | TGAAGGGAGTTTTCCACAG    | 417           | 60      |
| Exon 63-A | AGTGTTAAAAAGGGGCTAAGT | GGAATCTACAAGGTTGGAGAGA | 600           | 54      |
| Exon 63-B | ATTCATGGTATGGATGCTTG | CCAATTCTCCAGGCAATTTATTT | 591           | 59      |
| Exon 63-C | GAATGGAGGTGTTGCTACAGCTA | GCCTGAGCCATAGAAAAAGGTC | 600           | 58      |
| Exon 64 | AACATCTGCGCTACGGCAAG   | AGTGCCTTTTCAAATTTGTC   | 602           | 62      |
| Exon 65 | TGTCTTTTGTTGCGGCAATTTC | ACCGTAGGCAACTGAGAAGACG | 440           | 58      |
| Exon 66 | TGAGGAGGGTGACTTCTTG    | CTTGAGGAGTCAGGAGTAG    | 445           | 60      |
| Exon 67 | GAGCAGTCTCTGCAAATTG    | TCCCCAAGAAATCTCTCT     | 579           | 56      |
| Exon 68 | GTTTGAGATGGTGCTTCTTG   | GTTGAAGCTGGGGAACAGA    | 344           | 60      |
| Exon 69 | CGTCATACTTGTCTTGGGAATTC | CAACACCTTGGCAACATTTCTC | 338           | 60      |
| Exon 70 | ATCCAAATAGCAGGGGCAAG   | CCTCTCTGGTCCTCCACAC    | 462           | 60      |
| Exon 71 | GCTGCTAATCTCTGTAGGTTGACA | TAAGTGCTAGGGAGGAGTGTG | 499           | 56      |
| Exon 72 | TGAGGCTTCTGAGGCTTAC    | CTGCCAACAGAACCAGAAGTG  | 651           | 58      |
transmembrane protein, which has 5,202 amino acids [9]. The usherin is transiently expressed in the stereocilia of cochlear hair cells, suggesting an important role in their maturation [4,9-11]. In mammalian photoreceptors, the usherin is expressed specifically in the connecting cilia, which links the inner and outer retinal segments; this would appear to indicate that it is crucial for the long-term maintenance of photoreceptors [9-11].

Since identification of USH2A, several studies have indicated that mutations of this gene can cause a significant proportion of non-syndromic recessive RP [12-20].

This study investigated a Chinese family with nonsyndromic arRP. After haplotyping analysis, the disease-causing gene was mapped to the USH2A region. Mutations screening of the USH2A gene, corresponding to the USH2A isoform b, was then performed in this nonsyndromic RP family and in three USH2 families. Seven novel mutations were identified.

**METHODS**

Clinical data and sample collection: This study adhered to the tenets of the Declaration of Helsinki for research involving human subjects. The Beijing Tongren Hospital Joint Committee on Clinical Investigation approved the study. One Chinese family with nonsyndromic RP and three unrelated Chinese families with USH were referred to Beijing Tongren Hospital. After informed consent was obtained, each participant underwent careful ophthalmologic examinations, including best-corrected visual acuity testing using E decimal charts, slit-lamp biomicroscopy, fundus examination with dilated pupils, visual field testing, and electroretinogram (ERG) examination. Three probands from the three families

| Primer | Sequence (5'-3') |
|--------|-----------------|
| U11 SSCP | F: TGATGCAGGAAGGAAGCTGTG |
| U11 SSCP | R: CCTGGCAATATGAGTCTTC |
| U32 HRM | F: ATCCCTTCCAGTTCTTTG |
| U32 HRM | R: CAGATAGGAAACCGCTGGAT |
| U38 SSCP | F: AATTGGCCAGTCAACTCG |
| U38 SSCP | R: GCACCAAAGGGTTGTCTC |
| U48 PAGE | F: TGGATCCATGCCGCTAAAAC |
| U48 PAGE | R: CACTTGGAGTCTTGAGTAGA |

Abbreviations: U represents USH2A; the number represents the name of the exon; F represents forward; R represents reverse.
with USH underwent audiometric testing, including otoscopy and standard pure-tone audiometry, and vestibular tests. The patients with nonsyndromic arRP were given audiometric tests after the disease gene was mapped to chromosome 1q41, where the USH2A gene is located. Clinical diagnosis of USH2 was based on the clinical history, typical RP fundus appearance, sensorineural hearing impairment, and intact vestibular function. Peripheral blood was obtained by venipuncture, and genomic DNA was extracted according to standard phenol protocols.

Genotyping and haplotyping analysis: Genotyping was performed with 50 microsatellite markers from autosomes for the known arRP loci in family FR1 (Appendix 1). Then, genotyping and haplotyping analysis was performed with another six microsatellite markers - D1S237, D1S419, D1S556, D1S229, D1S227, and D1S2860 - around the USH2A gene. The fine mapping primer sequences were obtained from the Human Genome Database (GDB). Pedigree and haplotype maps were constructed using Cyrillic V. 2.0 software.

Mutation screening of the USH2A gene: Mutation screening was performed in all four families using direct DNA sequence analysis. The coding region (exons 2–72) and the exon-intron boundaries of USH2A were amplified by PCR in the probands of the four families. For direct sequencing, amplicons were purified (Shenneng Bocai PCR purification kit; Shenneng, Shanghai, China). An automatic fluorescence DNA sequencer (ABI, Prism 373A; Perkin Elmer, Foster City, CA), used according to the manufacturer’s instructions, sequenced the purified PCR products in both the forward and reverse directions. Nucleotide sequences were compared with the published cDNA sequence of the USH2A gene (GenBank NM_206933.2). For USH2A, cDNA numbering +1 corresponds to A in the ATG translation initiation codon in RefSeq (AY481573.1).

Restriction fragment length polymorphism analysis: Variations (c.2802T>G, c.8232G>C, c.3788G>A, and c.14403C>G) found in the sequencing were confirmed with the restriction endonucleases Hinc II (TaKaRa, Dalian, China), HpyCH4V, BsaI, and SpeI (New England Biolabs, Ipswich, MA), respectively, which were used in all available family members and in the 100 normal controls.

Single strand conformation polymorphism: To validate the variations (c.1876C>T and c.7123delG) found in the sequencing, a single strand conformation polymorphism (SSCP) analysis was performed in all available family members and in the 100 normal controls. As the PCR fragments used in SSCP analysis were between 150 and 300 bp, two pairs of specific primers were designed for detecting mutations in exon 11 and exon 38 (Table 2).

High-resolution melt curve analysis: To confirm the variation (c.6249delT) found in the sequencing, a high-resolution melt curve analysis (HRM) was performed in the available family members and in the 100 normal controls. Primer sequences

Figure 2. The appearance of the fundus in two patients with non-syndromic retinitis pigmentosa (RP) or Usher syndrome type II (USH2). A: Fundus appearance of patient 077066 from family one (FR1), shows typical retinal degeneration with attenuation of the retinal vessels, irregular pigment clumps in the retina, and waxy pallor of the optic nerve head. B: Fundus appearance of patient 019092 from family F8.
| Family number | Proband | Best corrected visual acuity (R/L) | Onset age of night blindness (year) | Fundus appearance | Onset age of hearing loss (year) | Hearing impairment | Cataract | Visual field | ERG | Vestibular function |
|---------------|---------|-----------------------------------|----------------------------------|------------------|-------------------------------|-------------------|----------|--------------|-----|-------------------|
| FR1          | 077006  | 0.4/0.4                           | 25                               | RP               | Normal hearing                | Normal            | Both eyes | N/A          | Wave undetectable | Normal |
| F6           | 073001  | 0.5/0.4                           | 13                               | RP               | 5                             | Moderate (sp)     | No       | N/A          | N/A | Normal |
| F7           | 019082  | 1.0/1.0                           | 17                               | RP               | 1                             | Moderate (sp)     | No       | 10°          | N/A | Normal |
| F8           | 019092  | 0.6/0.6                           | 12                               | RP               | 8                             | Moderate (sp)     | No       | 10–15°       | Wave undetectable | Normal |

Abbreviations: R represents right eye; L represents left eye; SP represents slight progressive; N/A represents data not available.
| DNA change               | Exon | Protein change | Type of nucleotide change | Family number | Frequency | Source       |
|-------------------------|------|----------------|---------------------------|---------------|-----------|--------------|
| c.2802T>G               | 13   | p.C934W        | Heterozygous              | FR1           | 2/200     | This study   |
| c.8232G>C               | 42   | p.W2744C       | Heterozygous              |               | 0/200     | This study   |
| c.1876C>T               | 11   | p.R626X        | Heterozygous              | F6            | 0/190     | [24]         |
| c.6249delT              | 32   | p.I2084fs      | Heterozygous              |               | 0/200     | This study   |
| c.3788G>A               | 17   | p.W1263X       | Heterozygous              | F7            | 0/200     | This study   |
| c.9492_9498delTGATGAT   | 48   | p.D3165fs      | Heterozygous              |               | 0/200     | This study   |
| c.7123delG              | 38   | p.G2375fs      | Heterozygous              | F8            | 0/200     | This study   |
| c.14403C>G              | 66   | p.Y4801X       | Heterozygous              |               | 0/200     | This study   |

The “Frequency” column, shows the number of chromosomes.
Figure 3. A direct sequencing analysis of the coding region of the Usher syndrome type IIA (USH2A) gene. A: Sequence presents the heterozygous missense mutation c.2802T>G (p.C934W) detected in patient 077006. B: Sequence shows the heterozygous missense mutation c.8232G>C (p.W2744C) identified in patient 077006. C: Sequence presents the heterozygous nonsense mutation c.1876C>T (p.R626X) identified in patient 073001. D: Sequence shows the heterozygous nonsense mutation c.3788G>A (p.W1263X) detected in patient 019082. E shows the heterozygous, one-base-deletion mutation c.6249delT (p. I2084fs) in patient 073001; F is the corresponding wild-type sequence. G presents a heterozygous 7 bp deletion mutation c.9492_9498del TGATGAT (p. D3165fs) in patient 019082; H shows the corresponding wild-type sequence. I presents the heterozygous, one-base-deletion mutation c.7123delG (p. G2375fs) in patient 019092; J shows the corresponding wild-type sequence. K: Sequence shows the heterozygous nonsense mutation c.14403C>G (p. Y4801X) detected in patient 019092.
were designed to obtain the best HRM performance, avoiding hairpin and primer–dimer formation as much as possible, and keeping the amplicon length under 250 base pairs. One pair of specific primers was designed for detecting a mutation in exon 32 (Table 2). The 10 μl reaction mixture consisted of 5 μl SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 1 μl genomic DNA (10–150 ng/μl), 0.5 μl forward primer (10 pmol/μl), 0.5 μl reverse primer (10 pmol/μl), and 3 μl double distilled water. PCR cycling and an HRM analysis were performed on the Rotor-Gene 6000™ (Corbett Research, Mortlake, NSW, Australia) [22].

Bioinformatics analysis: Garnier-Osguthorpe-Robson (GOR) software was used to predict the effect of the mutation on the secondary structure of USH2A [23]. This method infers the secondary structure of a sequence by calculating the probability for each of the four structure classes (helix, sheet, turn, and loop), based on the central residue and its neighbors from the calculated matrices [23].

RESULTS

Clinical findings: This study identified one Chinese family, consisting of four patients and one unaffected relative, diagnosed with non-syndromic RP, and three unrelated Chinese families, including four patients and six unaffected relatives diagnosed with USH2. The inheritance pattern in the families was autosomal recessive (Figure 1). All the patients had experienced night blindness and vision acuity impairment. The patients with USH2 had hearing impairment in early childhood. Ophthalmoscopic examination demonstrated attenuation of the retinal vessels, bone-spicule pigmentation in the fundus, and waxy pallor of the optic nerve head (Figure 2). The wave amplitudes of the ERG of the probands were indistinguishable from the baseline. Audiometric tests indicated moderate to severe sensorineural hearing impairment in the patients with USH2; in contrast, the results from the patients with non-syndromic arRP were normal. Vestibular functions of all the patients were normal. The detailed clinical information for each family’s proband is summarized in Table 3.

Genotyping results: Family FR1 was genotyped with 50 polymorphic markers around the known arRP loci. The mapping results excluded the other known arRP loci with the exception of the USH2A. Further genotyping and haplotyping analysis for the six markers (D1S237, D1S419, D1S556, D1S229, D1S227, and D1S2860) suggested that the USH2A
gene might be the disease-causing gene in this family (Figure 1).

**Mutation analysis:** Sequencing of the USH2A gene revealed 17 sequence variants in this study, eight of which were pathogenic mutations (Table 4). All eight pathogenic mutations were heterozygous; seven of them were first detected in the current study (Figure 3 and Table 4). Using RFLP, SSCP, or HRM analysis, the eight mutations co-segregated with the affected individuals and carriers, but not with the unaffected individuals and normal controls (two bands). Analyses did not detect the other seven mutations in the 100 normal controls, with the exception of p.C934W, which was identified in its heterozygous state in two individuals among the 100 normal controls (Table 4).

Four different combinations of heterozygous mutations were detected in the four families. In family FRI (non-syndromic arRP), two missense mutations, c.2802T>G (p.C934W) and c.8232G>C (p.W2744C), were detected in different alleles of patient 077006 (Figure 4, Figure 5, Figure 6). Using the GOR method, the results for secondary structure prediction suggested that p.C934W replaced two β sheets “E” with two coils “C” at amino acids 935 and 940, respectively. Mutation p.W2744C substituted a β sheet “E” and two turn sheets “T” for three coils “C” at amino acids 2745, 2747, and 2748, respectively (Figure 7). For the three USH2 families (F6, F7, and F8), one allele carried nonsense mutations, c.1876C>T (p.R626X), e.3788G>A (p.W1263X), and c.1876C>T (p.R626X), e.3788G>A (p.W1263X), and c.
14403C>G (p. Y4801X), respectively, while the other allele harbored deletion mutations c.6249delT (p. I2084fs), c.9492_9498delTGATGAT (p. D3165fs), and c.7123delG (p. G2375fs), respectively (Figure 1, Figure 3, Figure 4, Figure 5, Figure 6).

In addition to the eight pathogenic mutations detected in this study, nine nonpathogenic sequence variants were also identified. Table 5 summarizes these variants based on their nature and frequency.

**DISCUSSION**

This study detected eight different mutations of the USH2A gene isoform b in one non-syndromic arRP family and in three USH2 families. Scandinavian, French, European, and Canadian studies [12,14,16,24-26] previously reported the nonsense mutation p.R626X. The remaining seven mutations were first identified in this study.

Rivolta et al. first reported that about 4.5% of 225 patients from North America with non-syndromic recessive RP carried the missense mutation p.C759F [12]. Then, Bernal et al. found...
### Table 5. Presumed nonpathogenic variants of the Usher syndrome type II A (USH2A) gene found in this study.

| Exon | Nucleotide change | Codon | rs number | Family number | Allele frequency | Source |
|------|-------------------|-------|-----------|---------------|-----------------|--------|
| 2    | c.373A>G          | p.A125T| rs10779261| F6            | N/A             | [14]   |
| 3    | c.504A>G          | p.T168T| rs4253963  | F7            | 267/720         | [20]   |
| 21   | c.4457A>G         | p.K1486R| rs1805049 | F7            | 76/180          | [24]   |
| 28   | IVS27–34delC      |       | rs71556647 | FR1           | N/A             | [c]    |
| 32   | c.6317T>C         | p.L2106T| rs6657250 | FR1, F6, F7, F8| N/A             | [29]   |
| 34   | c.6506T>C         | p.L2169T| rs10864219 | FR1, F8       | 27/100          | [15]   |
| 48   | IVS48+78C>T       |       |           | FR1           | N/A             | This study |
| 52   | c.10232A>C        | p.E3411A| rs10864198| FR1           | 23/64*          | [27]   |
| 63   | c.12612G>A        | p.T4204T| rs2797235  | FR1, F8       | N/A             | [27]   |

Abbreviations: N/A represents data not available; the asterisk indicates that the allele frequency referred to patients.
that there was a similar detecting frequency (4.6%) for p.C759F in Spanish patients [13]. Two novel missense mutations, p.C934W and p.W2744C, were found in family FR1. Although p.C934W was identified (in a heterozygous state) in two individuals among the 100 normal controls, both mutations have been classified as deleterious-effect missense mutations with several lines of evidence. Both mutations co-segregated with the phenotype of family FR1 and both residues (C934 and W2744), located in the 8th Lam EGF domains and in the 14th FN3 repeat of the usherin, respectively, were highly conserved in different species (Figure 8). The results of GOR suggested that p.C934W and p.W2744C lead to secondary structure changes around residues 934 and 2744, which might interfere with the correct folding of the usherin (Figure 7). As the results of audiometric tests for the patients from family FR1 were normal, the two compound missense mutations might be responsible for RP without hearing loss.

Three different compound heterozygous mutations were identified in three families (F6, F7, and F8) with USH2 and all six mutations directly or indirectly resulted in premature termination of the USH2A translation. This is consistent with Dreyer et al. [25] previous observation that patients carrying compound heterozygous mutations (either two truncating or one truncating combined with one missense) in exon 22–72 presented the Usher type II phenotype. In contrast to the patients from the three USH2 families, the patients in FR1 carried two missense mutations. A recent study in a cohort of 272 Spanish patients with non-syndromic RP resulted in the identification of two mutant alleles of the USH2A gene in nine patients, with seven of them carrying either homozygous missense mutations or two heterozygous missense mutations [18]. In a large Chinese family, four patients carrying one truncating combined with one missense mutation (p.G1734R) exhibited RP with hearing loss, while the only person harboring the homozygous misense mutation (p.G1734R) presented RP without hearing loss [19]. However, this phenomenon was not observed in one Israeli family with three non-syndromic RP patients carrying one missense mutation and one truncating mutation [15].

As in our previous study [21], with the exception of one mutation (p.R626X), the other mutations identified in the current study were novel and were spread relatively evenly along the USH2A gene (Figure 9). These results indicate that the mutation spectrum for the USH2A gene among Chinese or Asian patients differs from the mutation spectrum among European Caucasians. The common mutations, p. E767fs for USH2 and p.C759F for arRP in Caucasians, are not detected in Chinese and Japanese patients [12-14,16,18,19,21,27-29].

In conclusion, our results further support previous indications that the mutations of the USH2A gene are also responsible for non-syndromic RP in Chinese patients. The
mutation spectrum among Chinese patients appears to differ from that among European Caucasians.

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Appendix 1. 50 markers used in the known arRP genotyping.

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