• Basic Research •

A Chinese family with Axenfeld-Rieger syndrome: report of the clinical and genetic findings

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Abstract

• AIM: To describe a Chinese family affected by a severe form of Axenfeld-Rieger syndrome (ARS) and characterize the molecular defect in *PITX2* in the family.

• METHODS: Patients presented with typical ARS from a Chinese family were investigated. We performed genome-wide linkage scan and exome sequencing to identify the pathogenic mutations. Candidate mutations were verified for co-segregation in the whole pedigree using Sanger sequencing. Real-time polymerase chain reaction (RT-PCR) and Western blotting were performed to verify the expression of the pathogenic gene.

• RESULTS: Genome-wide linkage and exome sequencing analyses showed *PITX2* as the disease candidate gene. A>G substitution at position -11 of 3'ss of exon 5 (IVS5-11A>G) that co-segregated with the disease phenotype was discovered in the family. The *PITX2* messenger ribonucleic acid and protein levels were about 50% lower in patients with ARS than in unaffected family members in the family.

• CONCLUSION: Our findings implicate the first intronic mutation of the *PITX2* gene in the pathogenesis of a severe form of ARS in a Chinese family. This study highlights the importance of a systematic search for intronic mutation in ARS cases for which no mutations in the exons of *PITX2* have been found.

• KEYWORDS: Axenfeld-Rieger syndrome; exome sequencing; linkage analysis; *PITX2*; intronic mutation

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INTRODUCTION

Axenfeld-Rieger syndrome (ARS; OMIM 180500) is a rare hereditary multi-system disease with a morbidity rate of 1:200 000. The genetic pattern of ARS is autosomal dominant characterized by complete penetrance[1]. ARS has several overlapping phenotypes, including Axenfeld anomalies, Rieger anomalies, and Rieger syndrome[2].

The phenotype of the anterior segment of ARS has significant heterogeneity, developmental anomalies involving the cornea, iris, lens and angle[3]. The clinical phenotypes of the eye in ARS patients include iridogoniodysgenesis, posterior embryotoxon, polycoria, corectopia, iris stromal hypoplasia, and iris strands bridging the iridocorneal angle to the trabecular meshwork[4]. Anterior segmental dysplasia is the main cause of increased ocular pressure, and about half of ARS patients will occur secondary glaucoma[5].

ARS-related candidate pathogenic genes include *PITX2* (paired-like homeodomain transcription factor 2)[6-8] and *FOXC1* (forkhead box C1)[9-11]. Further study has found that chromosome site 13q14 is also associated with ARS. The deletion of the 16q23-q24 region[12], the deletion of the PAX6[13], and the missense variant in the PRDM5[14] gene have been found in ARS patients.

ARS in China is relatively rare. Our purpose is to describe the clinical phenotype and genetic characterization of a northeastern Chinese family affected by ARS.

SUBJECTS AND METHODS

Study Population A Han Chinese family with dominant ARS, and 200 matched, healthy controls were included. We performed ophthalmic examinations for all participants, including vision, ocular pressure measurement, corneal measurement, mirror microscopy, ultrasound A/B scanning and ultrasound biomicroscopy, and used the slit lamp to take pictures of the eyes. The study received written informed consent from all participants and carried out in accordance with the Declaration of Helsinki.

Genome-wide Linkage Analysis Using the genomic DNAs from the ARS family, the whole genome linkage analysis was performed using Illumina HumanOmniZhongHua-8 BeadChip. The specific steps were as follows, the genomic DNA of each sample was amplified, fragmented, precipitated and resuspended in a hybridization buffer. The denatured samples were hybridized on Illumina HumanOmniZhongHua-8 BeadChip. Then the BeadChip oligonucleotides were extended and detected on an Illumina Bead Array Reader by fluorescence imaging. We used 872 261 SNPs to conduct linkage analysis after quality control filtering. It was assumed that the disease allele frequency was 0.0001 with dominant inheritance by a penetrance rate of 1. We performed multipoint parametric linkage analysis in Merlin.

Whole Exome Capture Total genomic DNA was isolated from the venous blood of participants with the DNA isolation kit (Tiangen, Beijing, China). Whole exome capture was performed by the Human All Exon (50 Mb) target enrichment system (Agilent Technologies, Santa Clara, CA, USA). Massive parallel sequencing was performed on the HiSeq 2500 Sequencing System (Illumina Inc., San Diego, CA, USA). Consensus Coding Sequence Region (CCDS) database provided the gene sequences in this array (http://www.ncbi.nlm.nih.gov/projects/CCDS/). Berry Genomics Co., Ltd. (Beijing, China) conducted the exome sequencing for the 3 individuals (III3, III4, and IV2).

Variant Analysis The sequencing data were aligned with the human reference genome NCBI Build 36.3. The duplicated data was delected, and SOAPaligner was used to compare the sequences of the 3 individuals. The SOAPsnp set was used to call the SNPs with the default parameters. SNPs and short indels were provided as sequence variants. SNPs and short indels were filtered against 1000 genome project (http://www.1000genome.org/), HapMap 8 (http://hapmap.ncbi.nlm.nih.gov/) database, the Single Nucleotide Polymorphism database (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi/), and YH database[15]. In view of familial ARS was rare, its pathogenic mutations was believed to be rare too. Therefore, only less common and novel variants were involved in the study. Less common variants meaned MAF (minor allele frequency) was less than 0.05 in the 1000 Genomes Project.

Verification of Variants Amplification primers for candidate genes were designed and synthesized according to the genome sequence of the human genome (hg18/build36.3). The target genes were amplified by polymerase chain reaction (PCR) and the target gene sequences were obtained by Sanger sequencing. In addition, the co-segregated mutantions were sequenced in 200 normal controls.

Ribonucleic Acid Extraction and Real-time Polymerase Chain Reaction RNA isolation kit (Tiangen, Beijing, China) was used to prepare total RNA from venous blood of all the family members. Total RNAs were reverse transcribed into cDNA. SYBR Premix Ex Taq kit (Tiangen, Beijing, China) was used in real-time PCR. The primer sequences for *PITX2* were 5'-TTC ACA TCT GGC TCC ATG TT-3' and 5'-GGG TTG CAT AGG CAG GTT AT-3'. The glyceraldehyde-3-phosphate dehydrogenase gene was assessed with the primers 5'-ATG CTG GCG CTG AGT ACG T-3' and 5'-AGC CCC AGC CTT CTC CAT-3'.

Western Blotting and Antibodies The buffy coat of the blood were harvested and lysed in RIPA buffer. The protein concentration determined by the bicinchoninic acid (BCA) protein reagent (Beyotime). Equal amounts protein samples were run on 10% SDS-PAGE gels, and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were probed with primary antibodies. After incubated with the secondary antibodies (HRP-conjugated goat anti-rabbit IgG), the signal bands were visualized by enhanced chemiluminescence (ECL) Western blotting detection reagent. GAPDH was served as the loading control.

RESULTS

Clinical Phenotypes and Assessment We described a Han Chinese family that had ARS with a dominant inheritance mode l (Figure 1). Five of 21 individuals in the four-generation family were identified to be affected with ARS. Most eye phenotypes were bilateral and similar in severity. But the severity degree was different among the patients. Ocular abnormalities of II2, III1 and III4 included bilateral buphthalmos, corectopia, iris atrophy, and corneal opacity (Figure 2A, 2C). Secondary glaucoma appeared during adolescence and caused blindness in II2, III1 and III4. III3 had bilateral corectopia, iris atrophy, and cataract (Figure 2B, Figure 3), and her IOP was 17 mm Hg in right eye and 19 mm Hg in left eye. IV2 had bilateral corectopia and iris atrophy (Figure 2D, Figure 4), and his IOP was 15 mm Hg in right eye and 16 mm Hg in left eye.

Five patients in the family had similar typical facial features of ARS, such as a broad nasal bridge, a broad forehead, flattening of the midface, a thin upper lip with a long philtrum, a protruding lower lip, hypodontia, and microdontia (Figure 2E-2H). The five patients had no hearing impairment and cardiovascular defects.

Causal Regions Identified by Genome-wide Linkage The study identified eight susceptibility loci, rs1107550-rs4660192 (Chr1: 32048052-Chr1: 41842350; 9.8 Mb), kgp1419269-kgp12301015 (Chr4: 83673890-Chr4: 133385086; 49.7 Mb), rs877741-rs11744690 (Chr5: 148196737-Chr5: 158687153; 10.5 Mb genomic region), rs10904561-rs583227 (Chr10: 135656-Chr10: 13166875; 13 Mb), kgp5435398-rs1711782 (Chr13: 46707064-Chr13: 60101738; 13.4 Mb), rs332233-

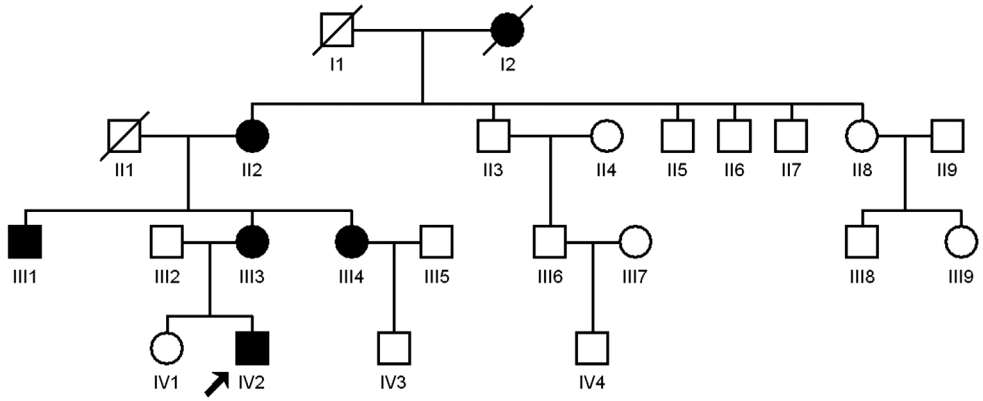

Figure 1 Pedigrees of the Chinese Axenfeld-Rieger syndrome family The filled square represents a male patient, and the filled circle indicates a female patient. An empty symbol represents a normal individual. Forward slash represents the deceased person.

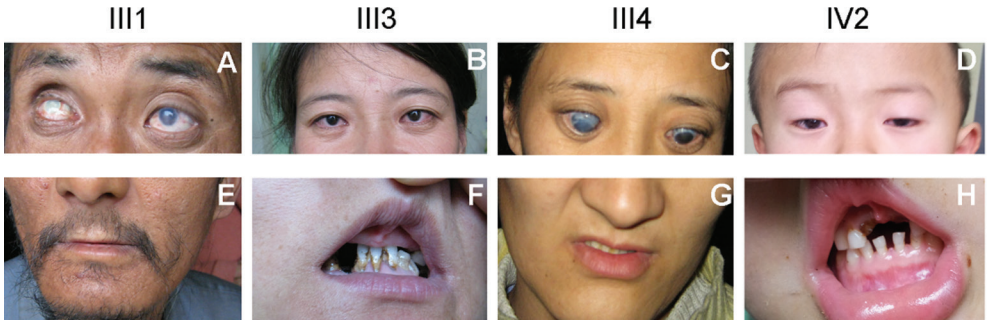

Figure 2 Clinical photographs of III1, III3, III4 and IV2 affected with Axenfeld-Rieger syndrome The patients in the family show similar clinical phenotypes, including a flattened midface, telecanthus, hypodontia or microdontia.

Table 1 Single nucleotide variants (SNV) and indels indentified in III3, III4, and IV2 through exome resequencing

Filter[1]	Genetic variants					
	SNV: exon	SNV: splicing site	SNV: intron	SNV: UTR	Indel	Total
III3	925	31	738	150	2227	4071
III4	1069	50	823	163	2308	4413
IV2	909	24	658	141	2332	4064
Heterozygous shared by five affected individuals non-synonymous in the linkage regions	12	0	3	2	29	46

[1]Not in 1000 Genomes Project (MAF≥0.05), the dbSNP, HapMap 8, YH database.

rs6495314 (Chr15: 64367745-Chr15: 78960529; 14.6 Mb), rs8044984-rs2037174 (Chr16: 23361853-Chr16: 27126459; 3.8 Mb), and rs12982096-rs3760961 (Chr19: 288738-Chr19: 3013374; 2.7 Mb), with a LOD score of 1.505.

Associated *PITX2* Mutation in the Axenfeld-Rieger Syndrome Family We focused on finding the pathogenic genes in these candidate regions by exome sequencing (III3, III4, and IV2). Through the exon subgroup sequencing, each sample produced an average of 7.41 billion base sequencing data. BWA was used to compare the data with the hg19 human reference genome[16]. The average sequencing depth of each sample was 67.54. An average of 86.74% of the exon sequences were sequenced at least 10X. Single nucleotide variants (SNV) and indels were filtered by GATK Unified Genotyper[17].

The filtered SNV and indels were annotated. Then they were filtered against 1000 genome project, HapMap 8 database, the SNP database, and YH database. In view of the synonymous variants were unlikely to be pathogenic. We screened out non-synonymous mutations (nonsense, missense and read-through), encoding indels, and variants of splice donor and acceptor sites. The filtered data was shown in Table 1.

Twelve non-synonymous SNVs, 3 introns, 29 indels and 2 UTRs were screened out by recommended filtering criteria. Only one SNV (chr4:110618699) was located in the linkage region and was co-segregated in the ARS pedigree. It lied in the upstream of the 3'ss of exon 5 of *PITX2* (NC_00000.12), an A>G change (IVS5-11A>G). The mutation didn't in the 200 control individuals (Figure 5).

***PITX2* Expression Analysis** We investigated the effect of the detected mutation on *PITX2* expression in the ARS family. Compared with normal individuals in the pedigrees, the expression level of *PITX2* mRNA was reduced by about 46% in patients (P<0.01; Figure 6). And the expression level

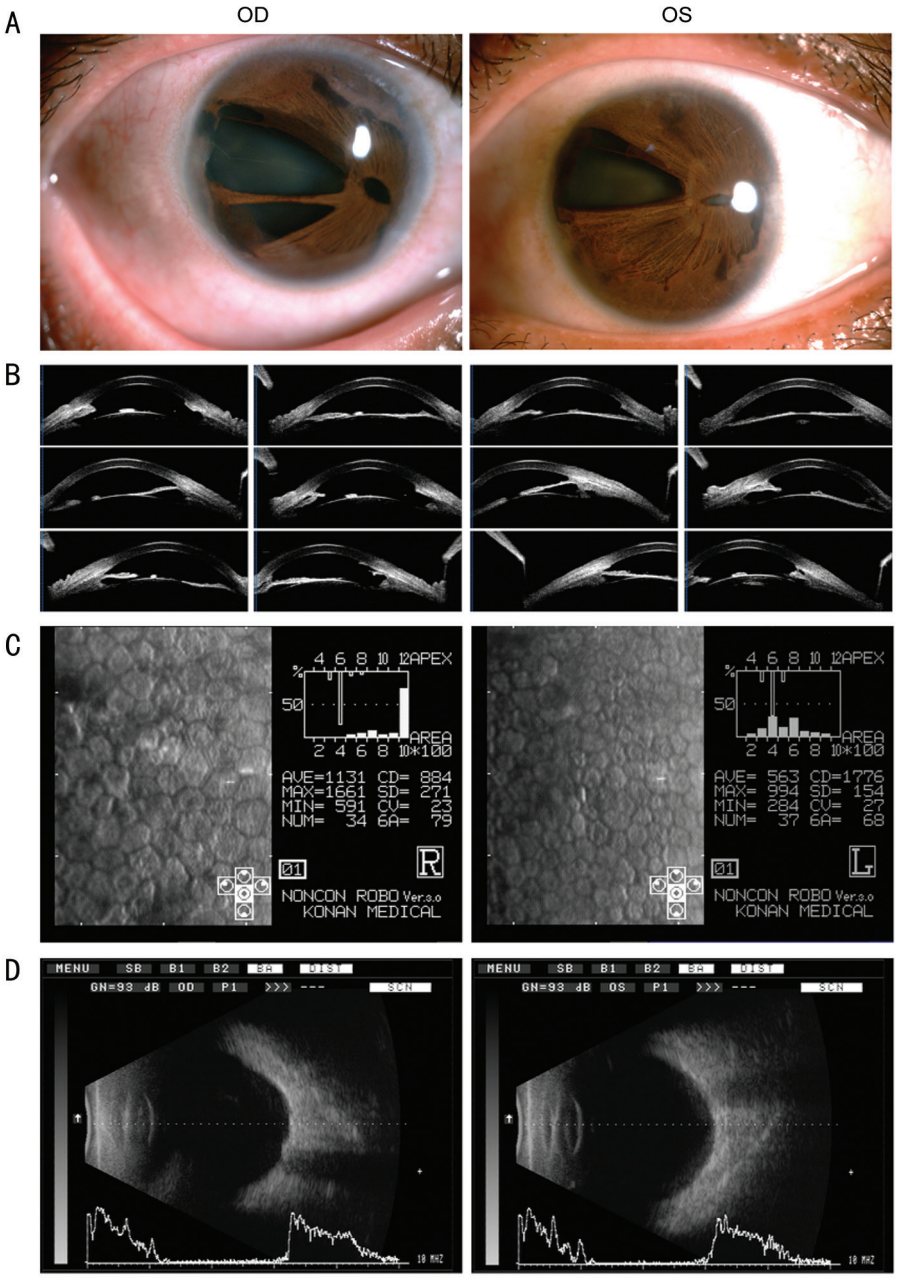

Figure 3 General pictutes (A), photographs of optical coherence tomography (B), photographs of specular microscopy (C), and ultrasound biomicroscopy (D) of patient III3 OD represents the right eye and OS represents left eye.

of *PITX2* protein in patients decreased by about 60% (P<0.01; Figure 6).

DISCUSSION

ARS patients usually have a variety of systemic abnormalities, including facial and dental deformities. The main abnormalities of the face include prominent forehead, a broad and flat nasal bridge, maxillary hypoplasia with flattening of the midface, and telecanthus. Dental abnormalities include hypodontia or microdontia. In the abdomen, abnormal skin evolution caused by the accumulation of skin around the navel. In addition, in some ARS patients, there are various clinical phenotypes, such as growth retardation, pituitary abnormalities, anal stenosis, and hypospadias unspecified. However, exceptions to the above mentioned phenotypic abnormalities are not considered to be the classical features of ARS[18].

High permeability in eye morphogenesis of ARS patients are associated with the development of glaucoma. About 50% of ARS patients develop secondary glaucoma. Glaucoma may occur in childhood, but it is more common in adolescence or adulthood. In some cases it may occur even after middle age. Treatment of secondary glaucoma is usually tricky and can lead to severe disc damage and visual field defects. In our study, secondary glaucoma appeared during adolescence and caused blindness in II2, III1 and III4. Bilateral eye became buphthalmos and suffered devastating damage. But III3 didn't have secondary glaucoma at the age of 40.

PITX2 belongs to the family of bicoid-like homeobox transcription factor. The members of homeobox gene family play important roles in the development of the individual, especially in the pattern formation and cell fate decisions.

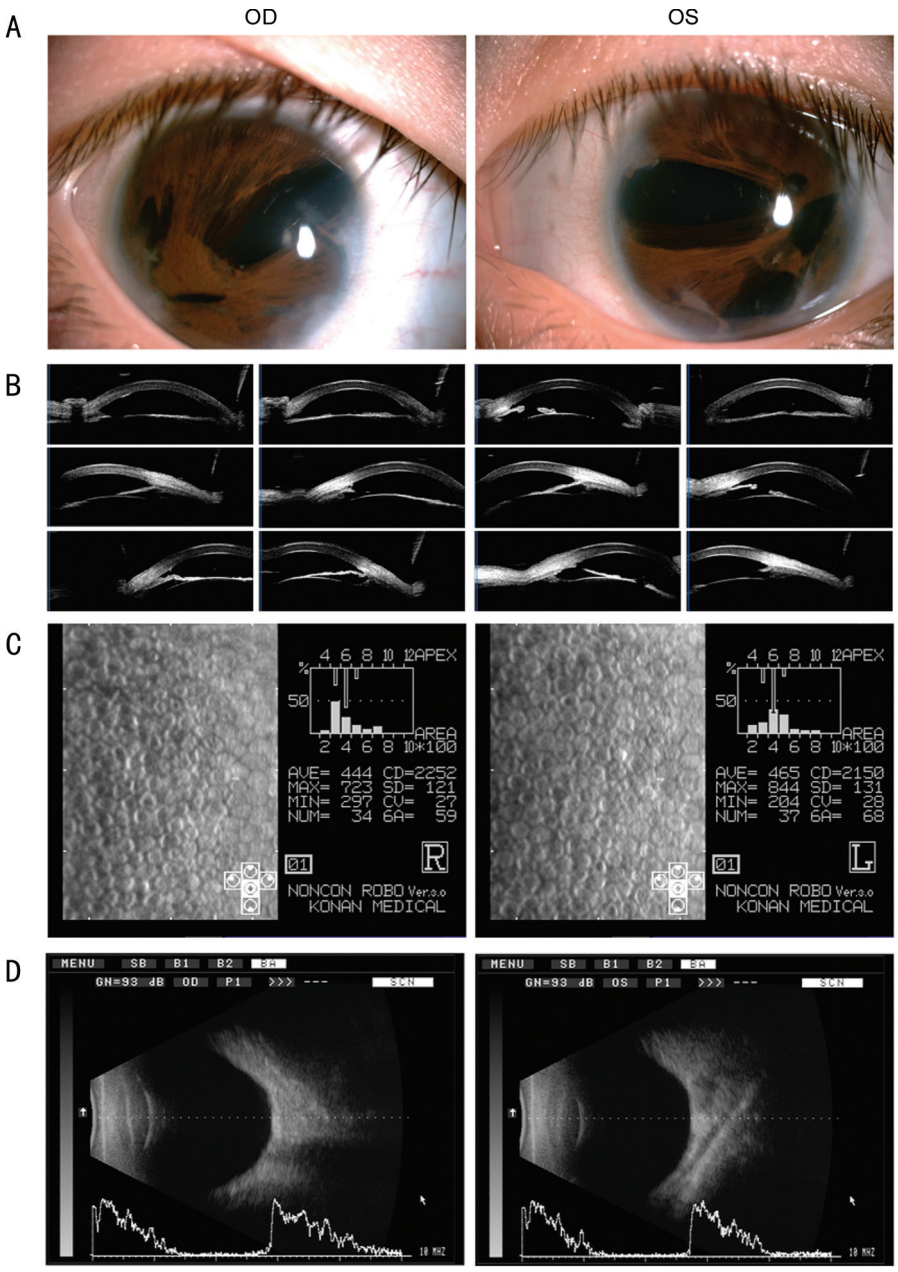

Figure 4 General pictutes (A), photographs of optical coherence tomography (B), photographs of specular microscopy (C), and ultrasound biomicroscopy (D) of patient IV2 OD represents the right eye and OS represents left eye.

PITX2 is expressed in neural crest cells and is essential for the normal development of optic stalk and formation of the anterior segment structures[19]. There are at least four different *PITX2* transcription isforms, PITX2a, PITX2b, PITX2c and PITX2d. They have different biological properties respectively[20]. PITX2a, PITX2b and PITX2c contain the same homologous domain and COOH-terminus, but there are differences in structure at the NH2-terminus. PITX2d is truncated, resulting in a nonfunctional homology domain[20]. Although the effect and specific mechanism of PITX2 in the pathogenesis of ARS are still unclear, the lack of normal expression of PITX2 protein is considered to be one of the main molecular mechanisms of ARS development[21]. Up to now, several intron mutations have been reported to cause ARS[22-23], but the vast majority of ARS-induced mutations are mostly located in the COOH terminal domain and homologous domain. Through the integration of Wnt and signal retinoic acid, it is found that PITX2 is the key molecule in the anterior segment model[24]. Mutations within the HD domain can hinder the ability of the protein to bind to the homologous DNA target sequence, leading to abnormal regulation of the target genes.

Our study implicated the intronic mutation of the *PITX2* gene associated with the pathogenesis of ARS in China. It revealed that this intron mutation described in the previous literature may affect the expression of PITX2 protein and trigger the pathogenesis of ARS. An A>G change 11 nt upstream of the 3'ss of exon 5 (IVS5-11A>G) of *PITX2* was co-segregated with the disease phenotype in the ARS family[25]. The polypyrimidine was located between the branch point and the splice point. Polypyrimidines played an important role in the strength of 3'ss.

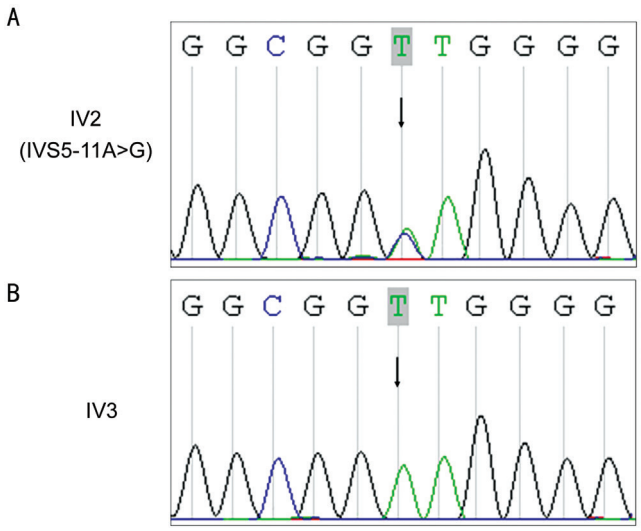

Figure 5 The sequence of the *PITX2* gene chr4: 110618699 in the ARS family A represents patient IV2; B represents the normal individual IV3. The arrow indicates chr4: 110618699.

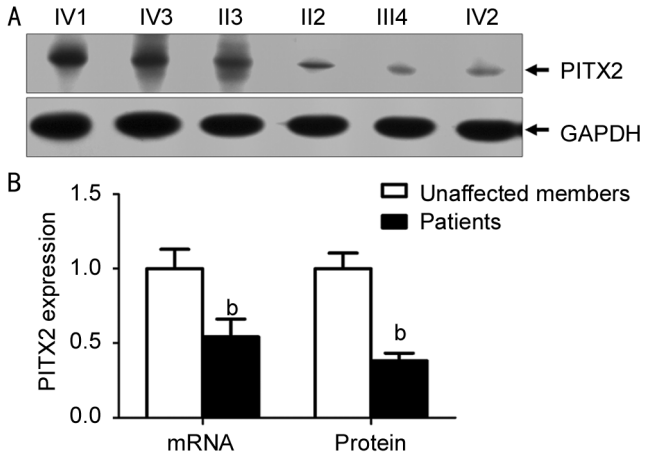

Figure 6 The effect of the detected mutation on *PITX2* expression in the ARS family A: The expression level of *PITX2* protein in patients and normal individuals deteceted by Western blotting; B: *PITX2* protein and mRNA expression levels were applied to the mean±SD. $^{b}P<0.01$ compared with normal individuals in the pedigrees.

The disruption of purine resulted in a 3'ss quality reduction. The strength of the 3'ss may not be altered by the IVS5-11A>G mutation, but a new "AG" dinucleotide was produced. It is assumed that the new "AG" could compete with the original one 11 nt downstream[26].

In conclusion, our study reported for the first time a mutation in the intron region that triggered the pathogenesis of a Chinese ARS family. The analysis of the expression level of *PITX2* further confirmed the possibility of development of ARS induced by *PITX2* haploid deficiency. At the same time, we summarized the variable phenotype in five patients in the ARS family and expanded the clinical phenotype profile of ARS in a different racial background.

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REFERENCES

1 Hjalt TA, Semina EV. Current molecular understanding of Axenfeld-Rieger syndrome. *Expert Rev Mol Med* 2005;7(25):1-17.

2 Alward WL. Axenfeld-Rieger syndrome in the age of molecular genetics. *Am J Ophthalmol* 2000;130(1):107-115.

3 Ito YA, Walter MA. Genomics and anterior segment dysgenesis: a review. *Clin Exp Ophthalmol* 2014;42(1):13-24.

4 Tumer Z, Bach-Holm D. Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *Eur J Hum Genet* 2009;17(12):1527-1539.

5 Idrees F, Vaideanu D, Fraser SG, Sowden JC, Khaw PT. A review of anterior segment dysgeneses. *Surv Ophthalmol* 2006;51(3):213-231.

6 Seifi M, Footz T, Taylor SA, Elhady GM, Abdalla EM, Walter MA. Novel PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. *Acta Ophthalmol* 2016;94(7):e571-e579.

7 Yin HF, Fang XY, Jin CF, Yin JF, Li JY, Zhao SJ, Miao Q, Song FW. Identification of a novel frameshift mutation in PITX2 gene in a Chinese family with Axenfeld-Rieger syndrome. *J Zhejiang Univ Sci B* 2014;15(1):43-50.

8 Reis LM, Tyler RC, Volkmann Kloss BA, *et al*. PITX2 and FOXC1 spectrum of mutations in ocular syndromes. *Eur J Hum Genet* 2012; 20(12):1224-1233.

9 Micheal S, Siddiqui SN, Zafar SN, Villanueva-Mendoza C, Cortés-González V, Khan MI, den Hollander AI. A novel homozygous mutation in FOXC1 causes Axenfeld Rieger Syndrome with congenital glaucoma. *PLoS One* 2016;11(7):e0160016.

10 Yang HJ, Lee YK, Joo CK, Moon JI, Mok JW, Park MH. A family with Axenfeld-Rieger Syndrome: report of the clinical and genetic findings. *Korean J Ophthalmol* 2015;29(4):249-255.

11 Du RF, Huang H, Fan LL, Li XP, Xia K, Xiang R. A novel mutation of FOXC1 (R127L) in an Axenfeld-Rieger syndrome family with glaucoma and multiple congenital heart diseases. *Ophthalmic Genet* 2016;37(1): 111-115.

12 Werner W, Kraft S, Callen DF, Bartsch O, Hinkel GK. A small deletion of 16q23.1->16q24.2 [del(16)(q23.1q24.2).ish del(16)(q23.1q24.2) (D16S395+, D16S348-, P5432+)] in a boy with iris coloboma and minor anomalies. *Am J Med Genet* 1997;70(4):371-376.

13 Riise R, Storhaug K, Brondum-Nielsen K. Rieger syndrome is associated with PAX6 deletion. *Acta Ophthalmol Scand* 2001;79(2): 201-203.

14 Micheal S, Siddiqui SN, Zafar SN, Venselaar H, Qamar R, Khan MI,

den Hollander AI. Whole exome sequencing identifies a heterozygous missense variant in the PRDM5 gene in a family with Axenfeld-Rieger syndrome. *Neurogenetics* 2016;17(1):17-23.

15 Li G, Ma L, Song C, *et al*. The YH database: the first Asian diploid genome database. *Nucleic Acids Res* 2009;37(Database issue): D1025-D1028.

16 Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;25(14):1754-1760.

17 McKenna A, Hanna M, Banks E, *et al*. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010;20(9):1297-1303.

18 Burkitt Wright EMM, Spencer HL, Daly SB, *et al*. Mutations in PRDM5 in brittle cornea syndrome identify a pathway regulating extracellular matrix development and maintenance. *Am J Hum Genet* 2011;88(6):767-777.

19 Evans AL, Gage PJ. Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. *Hum Mol Genet* 2005;14(22):3347-3359.

20 Cox CJ, Espinoza HM, McWilliams B, Chappell K, Morton L, Hjalt TA, Semina EV, Amendt BA. Differential regulation of gene expression by PITX2 isoforms. *J Biol Chem* 2002;277(28):25001-25010.

21 Lines MA, Kozlowski K, Kulak SC, *et al*. Characterization and prevalence of PITX2 microdeletions and mutations in Axenfeld-Rieger malformations. *Invest Ophthalmol Vis Sci* 2004;45(3):828-833.

22 Maciolek NL, Alward WL, Murray JC, Semina EV, McNally MT. Analysis of RNA splicing defects in PITX2 mutants supports a gene dosage model of Axenfeld-Rieger syndrome. *BMC Med Genet* 2006;7:59.

23 de la Houssaye G, Bieche I, Roche O, *et al*. Identification of the first intragenic deletion of the PITX2 gene causing an Axenfeld-Rieger Syndrome: case report. *BMC Med Genet* 2006;7:82.

24 Gage PJ, Qian M, Wu D, Rosenberg KI. The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development. *Dev Biol* 2008;317(1):310-324.

25 Borges AS, Susanna R Jr, Carani JC, Betinjane AJ, Alward WL, Stone EM, Sheffield VC, Nishimura DY. Genetic analysis of PITX2 and FOXC1 in Rieger Syndrome patients from Brazil. *J Glaucoma* 2002;11(1):51-56.

26 Zeniou M, Gattoni R, Hanauer A, Stevenin J. Delineation of the mechanisms of aberrant splicing caused by two unusual intronic mutations in the RSK2 gene involved in Coffin-Lowry syndrome. *Nucleic Acids Res* 2004;32(3):1214-1223.