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)[9,10]. 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 ultrasonic 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.
**Axenfeld-Rieger syndrome in a Chinese family**

**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 detected, 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/), dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/), 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 meanted 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 mutations 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 GCC 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 I (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-
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. 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. 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’s 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
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\cite{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.
PITX2 is expressed in neural crest cells and is essential for the normal development of optic stalk and formation of the anterior segment structures\textsuperscript{[19]}. There are at least four different PITX2 transcription isforms, PITX2a, PITX2b, PITX2c and PITX2d. They have different biological properties respectively\textsuperscript{[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\textsuperscript{[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\textsuperscript{[21]}. Up to now, several intron mutations have been reported to cause ARS\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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 4 General pictures (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.
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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