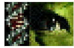
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Mutation spectrum of *FOXC1* and clinical genetic heterogeneity of Axenfeld-Rieger anomaly in India

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Purpose: Axenfeld-Rieger anomaly (ARA) is a form of anterior segment dysgenesis of the eye, mainly caused by mutations in the *FOXC1* gene. We had earlier reported a novel mutation in the wing region of FOXC1 in an autosomal dominant family. The present study was aimed to identify the spectrum of mutations in the *FOXC1* gene in a cohort of Indian ARA patients from different ethnic backgrounds, and to understand its role in the disease pathogenesis.
Methods: Two new autosomal dominant families and seven sporadic cases of ARA from different ethnic backgrounds were screened for mutations by direct sequencing of the coding region of the *FOXC1* gene. Another autosomal dominant ARA family that was previously reported by us was also included for comparative analysis of clinical genetic parameters. The segregation of the mutations in the autosomal dominant families was analyzed by haplotype and restriction analysis. Genotype-phenotype correlation were also undertaken to study the role of *FOXC1* in phenotypic manifestation in the patient cohort.
Results: Three of the nine ARA cases harbored mutations in *FOXC1*, of which two novel nonsense mutations Q2X and Q123X, resulted in haploinsufficiency of the gene product. The missense mutation (M161K) that we previously reported in an autosomal dominant family was also found in another family. Haplotype analysis of these two families suggested multiple founders in the same ethnic group. The mutations resulted in variable expressions of phenotype among the patients as assessed from their prognosis based on visual outcomes.
Conclusions: Significant genetic heterogeneity of *FOXC1* was observed in a multi-ethnic population studied in this region of India resulting in variable ARA phenotypes. The different visual outcome seen in the patients suggest a variable expression of FOXC1 and also provide some insight for understanding the gene functions in this population.

Anterior segment anomalies of the eye constitute a complex spectrum of disorders resulting from malformations of endothelial tissues, due to the disruption of migration and differentiation processes in the neural crest [1-3]. Axenfeld-Rieger phenotypes constitute various forms of anterior segment dysgenesis based on the presence of ocular and extra-ocular symptoms. Axenfeld anomaly, Rieger anomaly and Rieger syndrome comprise a series of overlapping phenotypes with systemic signs that include umbilical, facial and dental anomalies and are collectively grouped as Axenfeld-Rieger syndrome [1,2,4]. Axenfeld-Rieger anomaly (ARA) on the other hand does not manifest any systemic abnormalities and is associated with clinical symptoms that include a prominent anteriorly displaced Schwalbe's line, insertion of iris processes in the stroma, hypoplasia of the iris, corectopia and pseudopolycoria [4]. The disorder carries with it a 50% risk of developing glaucoma [1,5,6].

Three loci on chromosomes 4q25, 6p25, and 13q14 have been mapped for various Axenfeld-Rieger phenotypes [6]. Of these, mutations in the forkhead winged/helix transcription factor gene *FOXC1* (earlier known as the *FKHL7* gene) on chromosome 6p25 have been implicated in the causation of Axenfeld-Rieger phenotypes, particularly ARA [3,7,8]. Different mutations have been observed in *FOXC1* causing various Axenfeld-Rieger anomalies ranging from frameshift mutations due to deletions resulting in premature termination of translation in the forkhead domain, missense mutations reducing transactivation and protein interactions, and nonsense mutations causing haploinsufficiency of the gene product [1,4,8]. Structure-function analyses of mutations in *FOXC1* have indicated a reduced stability of FOXC1 in DNA binding, transactivation of target genes and transcriptional regulation [9].

Four mutations in *FOXC1* have so far been identified in ARA, of which a novel mutation in the wing region of this gene was reported by us, previously in an autosomal dominant ARA family in India [7]. We now report *FOXC1* gene mutations in a series of varied ARA cases and demonstrate the spectrum of clinical genetic heterogeneity with respect to the disease phenotype in the Indian scenario.

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METHODS

Clinical diagnoses and selection of cases: The study consisted of ten unrelated ARA families belonging to various ethnic backgrounds, and from different geographical locales of India, presenting at our Institute at Hyderabad, between 1997

and 2001. Of these, nine were new families, and one was an autosomal dominant family (ARA3) that we had previously reported [7]. The rationale for including the ARA3 family was to have a comparative analysis of the clinical and genetic traits with respect to all the families so far studied at our center. The guidelines of the Declaration of Helsinki were strictly followed and clearance was obtained from the Institutional Review Board prior to undertaking this study. Cases manifesting typical ARA were selected after a thorough clinical evaluation by our ophthalmologist (AKM). All the cases exhibited symptoms of iridocorneal tissue and angle anomaly, a prominently raised Schwalbe's line, corectopia, iris hypoplasia, polycoria, and were without any systemic abnormalities or other extraocular manifestations. Ophthalmic examination included slit-lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP), and visual acuity testing. Each of these patients had a regular follow-up every three months and their detailed clinical findings were recorded. Of the ten families, three had an autosomal dominant mode of inheritance while the rest were sporadic cases. Except for two probands of sporadic cases, the other probands had a congenital onset of the disease. As Axenfeld-Rieger phenotypes normally present overlapping clinical features, differential diagnosis were avoided by restricting the clinical examination and phenotypic assessment of the patients to the same ophthalmologist (AKM). Based on clinical manifestations, 5-10 ml of blood was drawn by venipuncture from the probands, their affected and normal relatives, and controls (after prior informed consent). A total of 72 ethnically matched normal individuals without any prior history of the disease or other systemic illness served as controls.

Mutation screening by sequencing: The human *FOXC1* gene (Genbank accession number AF078096) spans 1.6 kb and its coding region consists of a single exon encoding a 553 amino acid protein [3]. Direct sequencing of this exon in the ARA families screened for mutations. A set of four overlapping primers covering the entire coding region of the *FOXC1* gene (designed by us in the previous study [7]) were used to amplify the DNA samples. Genomic DNA (about 100 ng) were amplified using 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10% DMSO, 0.5 mM of each primer, and 1 unit of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) in a 25 µl reaction mixture. PCR was carried out in a PTC 200 thermal cycler (MJ Research, Waltham, MA) and the cycling conditions were as reported in our previous study [7]. The amplicons were purified prior to sequencing using Amicon Microcon PCR columns (Millipore, Bedford, MA). Bi-directional cycle sequencing was performed using the BigDye terminator kit (PE-Applied Biosystems, Foster city, CA) on an automated DNA sequencer ABI 3700 (PE-Applied Biosystems). The sequences were compared with the normal *FOXC1* gene sequence from the database. The segregation of the M161K mutation was analyzed using the restriction enzyme *Nla* III (New England Biolabs, Beverly, MA), as described earlier [7].

Genotyping and Haplotype analysis: In order to understand the founder effect of common mutations and segregation of the disease phenotype, microsatellite markers flanking the *FOXC1* gene locus on chromosome 6p25 were screened in members of three autosomal dominant families. Three markers D6S1574, D6S309 and D6S470 on 6p25 spanning 9.5 cM were selected from the ABI Linkage mapping set MD-10 (Version 2.5). The markers were amplified following the manufacturer's protocol, electrophoresed on an automated DNA sequencer ABI 377, and analyzed by GENESCAN software (PE-Applied biosystems, Version 2.1). Individuals were genotyped using the GENOTYPER software (PE-Applied biosystems, Version 2.0) and haplotypes were constructed from the genotype data. Multiple repetitions of experiments were done to exclude the possibility of sample contamination.

RESULTS

Mutation screening of the FOXC1 *gene:* Direct sequencing of the *FOXC1* coding sequence revealed a C->T nucleotide change at cDNA position 367, resulting in a nonsense mutation (Gln123Stop) in the ARA7 family, causing a truncation

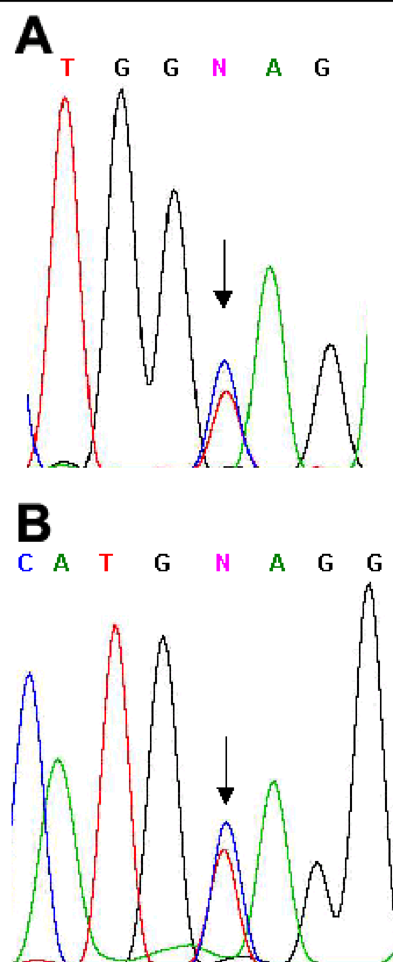

Figure 1. Novel mutations in ARA cases. **A**: Electropherogram of a sense strand of DNA from the proband of the ARA7 family (ARA7A) showing a C->T change at cDNA postion 367, resulting in a mutation from glutamine to a stop codon (Q123X). **B**: Electropherogram of a sense strand of DNA from the proband of ARA10 (ARA10A) family showing a C->T change at cDNA position 4, resulting in a mutation from glutamine to a stop codon (Q2X).

of the FOXC1 protein in the helix 3 region of the forkhead domain (Figure 1A). This mutation has been previously reported in the murine homolog of *FKHL7*, *Mf1*, in a mouse with congenital hydrocephalus phenotype [5]. This is perhaps the first report of the Q123X mutation in human ARA and is found to segregate with the disease phenotype in the three-generation ARA7 family. The unaffected grandmother (ARA7G) of the proband in this family was also found to harbor the same mutation and had the 1-1-1 affected haplotype (Figure 2A).

The M161K mutation in the wing 2 region of *FOXC1*, previously reported in family ARA3 [7], was also seen in family ARA8. The mutation segregated with the disease phenotype in the three-generation ARA8 family and was confirmed by restriction analysis with the enzyme *Nla* III. However, haplotype analysis indicated the segregation of different affected haplotypes, such as 1-3-1 and 3-2-2 in ARA3 and ARA8 families, respectively (Figure 2B).

A sporadic case (ARA10) exhibited a nucleotide change at cDNA position 4 (C->T), leading to the generation of a stop codon (Figure 1B). To our knowledge, this nonsense mutation is a novel one, leading to premature truncation of the protein considerably before the forkhead domain in the second codon (Gln2Stop). All the mutations observed in this study were absent in the normal controls. The other sporadic cases however did not show any mutations in the *FOXC1* gene.

Genotype-phenotype correlations: Variable phenotypic manifestations were seen in all of the ten families with or without *FOXC1* mutations (Table 1). In family ARA3, early intervention in the offspring (ARA3A and ARA3B) had a better prognosis than their affected father (ARA3C) [7]. In contrast, we found that the proband (ARA8A) of ARA8 family harboring the same mutation did not to show a similar prognosis, perhaps due to her advanced secondary glaucoma at presentation. She underwent transscleral cyclophotocoagulation and attained a visual acuity of 20/260 along with a cup to disc ratio of 0.4 in her right eye. Although her IOP was controlled, her left eye had a strabismus with a dense corneal scar, and she could only gain a visual acuity of “fixes and follows” light (Table 1). Her affected father (ARA8C) and grandfather (ARA8G) also had severe glaucoma and had developed monocular phthisis bulbi at presentation. After medical intervention their visual acuity was “no light perception.” However, in their better eye, their IOPs were controlled, corneas were clear, and they had a visual acuity of 20/30. The father (ARA3C) of the proband in ARA3 had severe secondary glaucoma and was blind in his right eye at presentation. In spite of surgical intervention, he had a poor visual outcome with almost total cupping (0.9), corneal haze and edema and diminished vision in the left eye [7]. We also noticed that all the affected members in family ARA8 had a severe loss of vision in one of their eyes, which was not recovered even after surgical intervention and medical treatment (Table 1).

Early intervention in the proband with the nonsense mutation (Q123X; ARA7A) resulted in a relatively fair prognosis with controlled IOP, normal cup to disc ratio, and visual acuity of 20/670 in both eyes, better than his affected mother (ARA7B) and uncle (ARA7U, Table 1). These two relatives

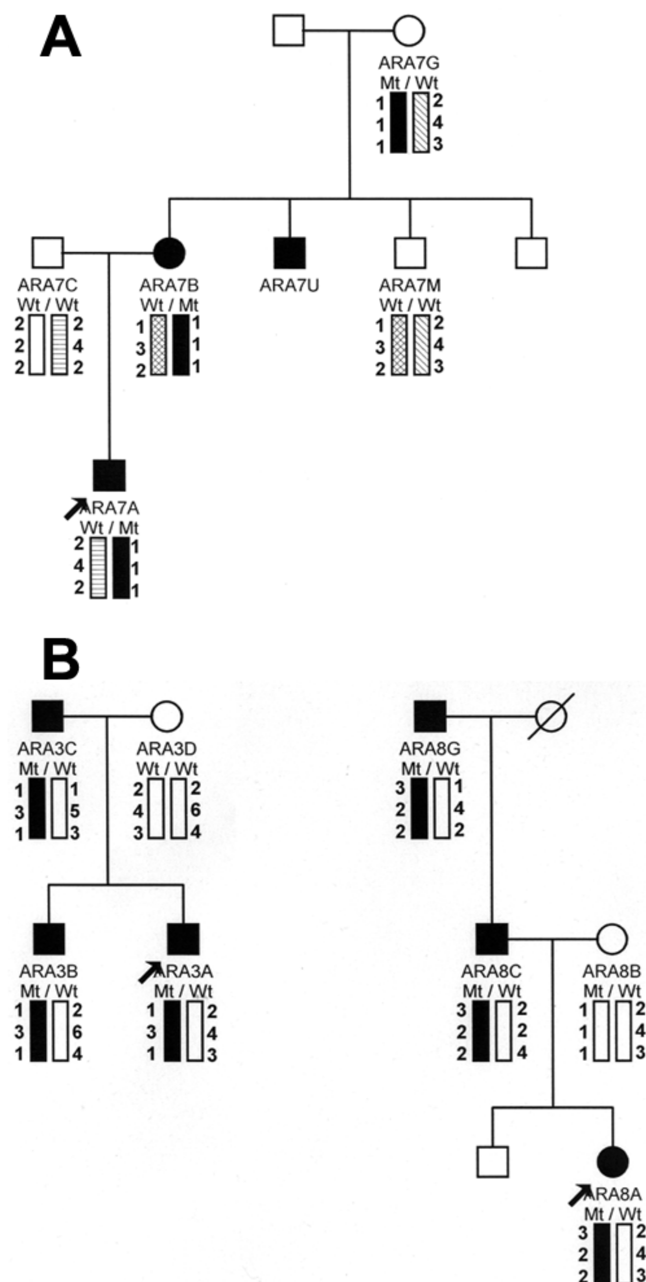

Figure 2. Haplotypes of ARA families. **A**: Haplotype of family ARA7 exhibiting the Q123X mutation. Darkened bars indicate the affected haplotype. The order of the markers from telomere to centromere and their inter-marker distances are: D6S1574-4.7 cM-D6S309-4.8 cM-D6S470. Note that the affected haplotype 1-1-1 is segregating in this family, and the grandmother (ARA7G) is also carrying the same haplotype. “Mt” and “Wt” represent the mutant and wild type chromosomes, respectively. **B**: Haplotype of family ARA3 and ARA8 exhibiting the M161K mutation. Darkened bars indicate the affected haplotype. The order of the markers from telomere to centromere and their inter-marker distances are: D6S1574-4.7 cM-D6S309-4.8 cM-D6S470. Note the affected haplotypes 1-3-1 and 3-2-2 segregating in ARA3 and ARA8 family, respectively. “Mt” and “Wt” represent the mutant and wild type chromosomes, respectively.

were diagnosed with severe secondary glaucoma at 10 and 8 years of age, respectively, and were undergoing medical treatment. They, too, had developed phthisis bulbi in one of their eyes at presentation at a later age. In spite of surgical intervention, they had raised IOPs, total cupping, and very little vision in their relatively better eye. The grandmother of the proband (ARA7G) in this family also harbored the same mutation, but did not show any symptoms of ARA or glaucoma and had a normal IOP (14 mm Hg in both eyes), along with visual acuity of 20/30 in both eyes.

The proband of a sporadic case (ARA10A) with the other nonsense mutation (Q2X) presented at 10 years of age and did not have any signs of secondary glaucoma. Although his cup to disc ratio was on the borderline, his cornea was clear and his IOP was controlled. On medical treatment, he exhibited good prognosis along with a visual acuity of 20/20 in both the eyes. Five of the six probands of sporadic cases, who did not show any mutation in the *FOXC1* gene, were intervened very early (between 2-9 months) and had fair prognosis with relatively better visual acuity (Table 1). One (ARA4A) had a very high cup to disc ratio in both the eyes (0.9) and in spite of late surgical intervention at 19 years of age exhibited a fair prognosis. The IOP in these probands are under control and they are being regularly monitored for development of glaucoma.

DISCUSSION

Mutations in the forkhead transcription factor gene *FOXC1* have been associated with anterior segment dysgenesis of the eye with various phenotypes [1,4,10]. Four mutations in different regions of *FOXC1* have been implicated in ARA [7]. The present study has identified three different mutations, two of which are novel, and points out the clinical genetic heterogeneity of the *FOXC1* gene in Indian ARA populations (Table 2).

The nonsense mutation (Q123X) in the forkhead region of *FOXC1*, we believe, has been identified for the first time in a human ARA family. The segregation of this mutation with the disease phenotype, its absence in normal individuals, and conservation of its normal residue across species implies its pathogenic nature. This mutation in the third helix is likely to disrupt the sequence-specific contacts with the major groove of the core target sequence [1]. Intriguingly, the normal grandmother (ARA7G) of the proband also carried the same mutation and the affected haplotype (Figure 2A). We wonder whether she might have inherited a modifier locus along with the mutation, which prevents the expression of the phenotype. However, she has transmitted the mutation through her germline, which resulted in the ARA phenotype in subsequent generations. As this mutation truncates the protein in the forkhead domain, it must be lethal for the expression of the phenotype. We therefore presume that she must be a somatic mosaic for the modifier locus that suppresses the expression of the phenotype. Alternately, the modifier locus may be non-penetrant, as a rare dominant locus may result in 50% of clinically unaffected individuals carrying the affected genotype. A

Table 2. Different mutations observed in ARA families

Family ID Number	Nucleotide change	cDNA position	Mutation	Type of mutation
ARA8	T->A	482	M161K	Missense
ARA7	C->T	367	Q123X*	Nonsense
ARA10	C->T	4	Q2X*	Nonsense

Spectrum of mutations in ARA families, along with their locations and associated changes. Mutations marked with an asterisk ("*") are novel.

Table 1. Clinical phenotypes of ARA patients

Patient ID Number	Age at onset	Age at diagnosis/ intervention	Corneal diameter OD	Corneal diameter OS	IOP at diagnosis OD	IOP at diagnosis OS	IOP at treatment OD	IOP at treatment OS	Last C/D ratio OD	Last C/D ratio OS	Last recorded vision OD	Last recorded vision OS	Corneal changes/ clarity (OU)	Treatments
ARA1A	Birth	3 months	13	14	32	26	14	14	NA	0.3	PLPR	20/160	Megalocornea, haze, edema	Trab+ OU
ARA2A	2 years	2 years	13	13	31	27	14	14	0.4	0.3	20/125	20/125	Megalocornea, haze, edema	Trab OU
ARA3A	Birth	2.5 months	12	12	30	28	12	14	0.4	0.3	20/80	20/80	Megalocornea, haze, edema	Trab OS
ARA3B	Birth	2 weeks	11	11.5	24	26	11	12	0.2	0.3	20/80	20/80	Megalocornea, haze, edema	Trab+ OU
ARA3C	Birth	24 years	NA	NA	42	35	14	14	NA	0.9	NLP	CF-1m	Megalocornea, haze, edema	Trab OS; Medical OS
ARA4A	15 years	19 years	12	12	54	54	09	13	0.9	0.8	20/20	20/20	Clear cornea	Trab+ OU
ARA5A	Birth	3 months	10.5	10.5	12	12	10	10	0.2	0.2	FF	FF	Clear cornea	Observation
ARA6A	Birth	9 months	10.5	10.5	14	16	14	14	0.3	0.3	FF	FF	Clear cornea	Observation
ARA7A	Birth	18 days	12	12.5	28	30	20	18	0.2	0.4	20/670	20/670	Haze, edema	Trab+ OU
ARA7B	Birth	10 years	NA	12	NA	NA	PB	30	NA	0.9	NLP	20/200	Clear cornea	Trab OS
ARA7U	Birth	8 years	13	NA	NA	NA	36	PB	NA	NA	LP	NLP	Clear cornea	Trab OD
ARA8A	Birth	3 days	13	12	32	36	12	13	0.4	NA	20/260	FF	Megalocornea, haze, edema	Trab+ OD; TsCPC OS
ARA8C	Birth	28 years	12.5	NA	21	NA	18	PB	0.7	NA	20/30	NLP	Clear cornea	Medical OD
ARA8G	Birth	54 years	NA	12.5	NA	16	PB	16	NA	0.3	NLP	20/30	Clear cornea	Observation
ARA9A	Birth	3 months	12.5	12.5	32	30	12	14	0.3	0.3	20/130	20/130	Megalocornea, haze, edema	Trab+ OU
ARA10A	Birth	10 years	12	12	12	12	12	12	0.5	0.6	20/20	20/20	Clear cornea	Medical OU

Abbrevations: IOP: Intraocular pressure; C/D ratio: cup to disc ratio; PLPR: Light perception with projection; Trab: Trabeculotomy; Trab+: Trabeculotomy/Trabeculectomy; NLP: No light perception; LP: Light perception; CF: Counts fingers; FF: Fixes and follows light; PB: Phthisis bulbi; TsCPC: Transscleral cyclophotocoagulation; NA: Not available; OD: Right eye; OS: Left eye; OU: Both eyes

similar situation of non-penetrance has been observed in diseases like primary congenital glaucoma with normal individuals carrying a pathogenic *CYP1B1* gene mutation [11]. No extra-ocular tissue involvement was observed in the ARA7 family, unlike the congenital hydrocephalus phenotype in the mouse that first showed the Q123X mutation in its *Mf1* gene [5,12,13]. It may be speculated that the Q123X mutation exhibits heterogeneity in variable expression of phenotype across species. Since this is the first report on the presence of the Q123X mutation in humans, this has not allowed any further comparisons on its phenotypic manifestations. Because of early surgical intervention, the proband (ARA7A) had a relatively better visula outcome than his mother (ARA7B) and uncle (ARA7U, Table 1).

In the two families ARA3 and ARA8, which belong to the same ethnic group, the mutation M161K was observed, however with two different sets of affected haplotypes (Figure 2B). This suggests two independent origins of the M161K mutation in these two families and suggests multiple founders for this mutation in the same ethnic group, as both were Hindu families belonging to the same caste group (Vaishyas) in the same geographical region. Early interventions led to a better prognosis in the probands of the ARA3 family, as opposed to the visual outcomes in ARA8 (Table 1). Moreover, the disease seems to have a severe unilateral manifestation among the affected individuals of the ARA8 family. It appears that the 3-2-2 haplotype for the M161K mutation might manifest a more pronounced phenotype in this family. As seen in an earlier study [8], this is another rare instance of a *FOXC1* mutation replicating in two unrelated ARA families.

The other nonsense mutation (Q2X), seen in the proband of family ARA10, appears to be novel and is the only mutation so far seen in a sporadic case of ARA. It is expected to produce a functionally null allele since it truncates the FOXC1 protein ahead of the forkhead domain. Although this mutation is expected to result in a null phenotype, the patient showed a relatively better visual outcome in spite of late medical intervention. This raises the possibility of a modifier locus, which can suppress the expression of the *FOXC1* gene (the situation in family ARA7). Alternately, this may represent a mutation with a variable phenotypic expression. This region of the FOXC1 protein contains poly-Ala repeats, which are seen in the activation domain regions of other transcription factors [14]. Hence it is likely that the Q2X mutation in the N-terminal transactivation domain might be terminating the activation of transcription of FOXC1 [9].

The other sporadic cases did not show any mutations in *FOXC1*, leading to the possibility of other genes responsible for ARA. Interestingly the proband in the ARA9 family shared the same affected haplotype with the proband of ARA10 (data not shown), unlike other probands of sporadic cases. There have been reports where *FOXC1* mutations are not observed in Axenfeld-Rieger families [3,15]. Recent studies have shown that chromosomal duplications of the 6p25 region have resulted in anterior segment anomalies due to an increased gene dosage of FOXC1 or some unknown genes within the duplicated segment [8,16].

We note that the *FOXC1* gene is involved in 3 of 9 new ARA cases in the present study population. However the frequency varies across populations with respect to Axenfeld-Rieger phenotypes and is particularly high in Axenfeld-Rieger syndromes [8,17]. The M161K mutation was seen in two cases and might represent a common *FOXC1* gene mutation in Indian ARA patients. Also, this is the first report that elucidates the presence of nonsense mutations in ARA that were earlier observed only in Axenfeld-Rieger phenotypes with systemic abnormalities [5]. Haploinsufficiency of transcription factors FOXC1 and FOXC2, responsible for maintaining the ocular drainage structures, are reported to result in anterior segment anomalies in mice with similar clinical abnormalities in different genetic backgrounds [2]. However, our results suggest that haploinsufficiency in *FOXC1* results in variable clinical manifestations in human ARA, as seen in ARA7 and ARA10 families.

Altered amounts of gene dosage of FOXC1 transcription factor have also been noted in deletions and duplications of 6p25 region in anterior segment abnormalities [18]. Disease causing mutations and chromosomal duplications modulate the levels of FOXC1, thereby hampering its regulatory control for efficient transcriptional activation [14]. The present study documents the heterogeneity of *FOXC1* in causing variable phenotypic manifestations and provides some insight to understanding the gene functions in Indian ARA populations.

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