

Novel Mutation in *FOXC1* Wing Region Causing Axenfeld-Rieger Anomaly

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PURPOSE. To determine the possible molecular genetic defect underlying Axenfeld-Rieger anomaly (ARA) and to identify the pathogenic mutation causing this anterior segment dysgenesis in an Indian pedigree.

METHODS. The *FOXC1* gene was amplified from genomic DNA of members of an ARA-affected family and control subjects using four novel sets of primers. The amplicons were directly sequenced, and the sequences were analyzed to identify the disease-causing mutation.

RESULTS. A heterozygous novel missense mutation was identified in the coding region of the *FOXC1* gene in all three patients in this family. Consistent with the autosomal dominant inheritance pattern, the mutation segregated with the disease phenotype and was fully penetrant. The mutation was found in the wing region of the highly conserved forkhead domain of the *FOXC1* gene and resulted in a very severe phenotype leading to blindness.

CONCLUSIONS. This is the first study to demonstrate that a mutation in the *FOXC1* wing region can cause an anterior segment dysgenesis of the eye. This mutation resulted in blindness in the ARA-affected family, and the findings suggest that the *FOXC1* wing region has a functional role in the normal development of the eye. Moreover, this is the first study from India to report the genetic etiology of Axenfeld-Rieger anomaly. Genotype-phenotype correlations of *FOXC1* may help in establishing the disease prognosis and also in understanding the clinical and genetic heterogeneity associated with various anterior segment dysgenesis caused by this gene. (*Invest Ophthalmol Vis Sci.* 2002;43:3613-3616)

The glaucomas are a group of heterogeneous disorders and are a major cause of blindness worldwide. Axenfeld-Rieger anomaly (ARA) is a form of developmental glaucoma, caused by the maldevelopment of the anterior segment of the eye.¹ It is inherited in an autosomal dominant fashion, and glaucoma develops in 50% to 75% of the cases.² It consists of a spectrum of developmental defects of the anterior chamber of the eye, with wide variability in expression. Ocular features in ARA

include prominent anterior Schwalbe's line, abnormal angle tissue, hypoplastic iris, polycoria, corectopia, and glaucoma.³ One gene for this disorder has been mapped to chromosome 6 in the p25 region.² A few mutations in a forkhead/winged-helix transcription factor gene *FOXC1* (formerly known as *FREAC3* and *FKHL7*) have been implicated in the pathogenesis of this disorder.^{4,5}

Although several cases of this disorder with varying severity and manifestations have been identified in India, the genetic etiology was unknown. Therefore an Indian pedigree with multiple affected members in two generations was studied to identify the genetic defect. We herein report the identification of a novel wing mutation in the forkhead domain of the transcription factor gene *FOXC1* that causes the defect, the possible functional role, the diagnostic method developed, and the genotype-phenotype correlation of the mutation.

METHODS

Clinical Evaluation and Patient Selection

The study protocol adhered to the tenets of the Declaration of Helsinki. After providing informed consent, one clinically well-characterized nonconsanguineous ARA-affected family with four members was recruited for the study. This family was selected because it had three affected members in two generations, and all family members were available for the investigation. Patients and family members were evaluated by a glaucoma specialist (AKM) and were followed up for 10 years. Father and both children were affected; the mother was not affected. The clinical data and phenotypes of all three patients are described in Table 1. Ophthalmic examinations included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP) and visual acuity. This family did not exhibit any systemic abnormalities, and no extraocular manifestations were seen.

Mutation Screening and Sequence Analyses

Because mutations in *FOXC1* are known to cause ARA, the entire coding region (1.6 kb organized in one exon)⁴ was screened for mutations. DNA was extracted from the peripheral leukocytes of the three patients, the mother, and the control subjects. Previous studies had used either 9 or 12 sets of overlapping primers to amplify this highly GC-rich gene.^{4,6,7} For mutation screening, these studies had used either single-strand conformation polymorphism (SSCP) followed by sequencing or direct sequencing of polymerase chain reaction (PCR) products, with these sets of overlapping primers. In the present study, mutation screening strategy was simplified by four novel sets of overlapping primers developed by us. Using these primers (spanning the entire exon), we amplified the *FOXC1* gene in patients and control subjects (Table 2). Amplicons were sequenced directly, and patient and control sequences were compared, to identify all sequence variations. The primers used and PCR conditions are described (Table 2). The same sets of primers were used for PCR and bidirectional sequencing.

All PCR reactions were done (PTC 200; MJ Research, Watertown, MA), using 100 ng genomic DNA in 25- μ L reactions containing 1 \times PCR buffer, 200 μ M of the dNTPs, 0.5 μ M of each primer, 10% dimethyl sulfoxide (DMSO), and 1 U *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). All PCR products were purified on separation columns

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Supported in part by grants from the Department of Biotechnology, Government of India to L. V. Prasad Eye Institute and Centre for DNA Fingerprinting and Diagnostics; the Hyderabad Eye Research Foundation; and the i2 Foundation, Dallas, Texas.

Submitted for publication April 1, 2002; revised June 19, 2002; accepted July 8, 2002.

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TABLE 1. Clinical Data and Phenotypes of Subjects with ARA

Pedigree/ Subjects	Age at Onset	Age at Diagnosis	Corneal Diameter at		IOP at Diagnosis (mm Hg) (OD; OS)	Last C/D Ratio (OD; OS)	Last Recorded Vision (OD; OS)	Presence of Schwalbe's Line and Abnormal Angle Tissue (OU)	Presence of Corectopia, Hypoplastic Iris, and Polycoria (OU)	Corneal Changes/ Clarity/ Diagnosis (OU)	Age at Surgical Intervention	Treatments (OD; OS)
			(OD; OS)	(OU)								
ARA I.1 (affected father, proband)	By birth	24 y	NA OU	42; 35	NA, total cupping	NPL OD; CF 1 m OS	+	+	Megalocornea, Hazy, Edema	1 × Trab OS; Medical OS	24 y	
ARA II.1 (affected child)	By birth	2 wk	11; 11.5	24; 26	0.2:0.3	20/360 OU*	+	-	Megalocornea, Hazy, Edema	1 × Trab/Trab OU	2 mo	
ARA II.2 (affected sibling)	By birth	2.5 mo	12 OU	30; 28	0.4:0.3	20/80 OU†	+	-	Megalocornea, Hazy, Edema	1 × Trab/Trab OU	2.5 mo	

IOP, intraocular pressure; OD, right eye; OS, left eye; OU, both eyes; C/D, cup-to-disc ratio of the optic nerve; NPL, no perception of light (blind), +, present; -, absent; CF, counting fingers; NA, not available; X, Times; Trab, Trabeculectomy; Trab/Trab, combined trabeculectomy and trabeculectomy. 1 m, one meter; y, year; wk, week; mo, month.

* Best spectacle corrected visual acuity.

† Unaided vision.

(Amicon Microcon PCR; Millipore, Bedford, MA), terminator cycle sequencing was performed (BigDye kit; Applied Biosystems, Foster City, CA), and sequencing reactions were performed on an automated DNA sequencer (ABI Prism 377; Applied Biosystems).

PCR-Restriction Fragment Length Polymorphism Analysis and Cosegregation of Mutant Alleles with Disease Phenotype

The novel mutation identified in this study resulted in loss of the *Nla*III recognition site. For determining the cosegregation of mutant alleles with disease phenotype in the family, the respective fragment harboring the mutation was amplified from all family members, by using set II of the primers (Table 2), and an aliquot of amplicon was digested with *Nla*III restriction enzyme (New England Biolabs, Beverly, MA). The fragments were separated on 8% polyacrylamide gel, stained with ethidium bromide, and visualized to distinguish the wild-type and the mutant alleles. Sixty-one ethnically matched volunteer donors without history of eye disorders served as control subjects.

Sequence Alignment

Multiple sequence alignment was performed by submitting various forkhead protein sequences to the European Bioinformatics Institute server. Alignment was performed with Clustal W software (provided in the public domain by the European Bioinformatics Institute, Hinxton, UK, and available at <http://www2.ebi.ac.uk/clustalw>).

RESULTS

Identification of a Novel Pathogenic Mutation in the *FOXC1* Wing Region

All three patients (the father and two children) in an ARA-affected family had a heterozygous missense mutation (T→A substitution) at 482 bp (cDNA position) in the highly conserved forkhead domain of the *FOXC1* transcription factor gene. This mutation resulted in the change of amino acid methionine to lysine at 161 amino acid position (M161K) in *FOXC1* and also abolished the *Nla*III recognition site in the DNA (Fig. 1). PCR-restriction fragment length polymorphism (RFLP) analysis showed that mutant alleles segregated only with the disease phenotype (Fig. 2) and not with the unaffected mother and the control subjects analyzed. This mutation was also absent in 61 ethnically matched control subjects (data not shown). The mutated methionine residue has been conserved across various species during evolution (Fig. 3).

The forkhead domain contains three α helices and two wing regions. The respective amino acid positions in *FOXC1* are α helix-1: 83 to 93; α helix-2: 101 to 110; α helix-3: 119 to 132; wing-1: 143 to 151 and wing-2: 155 to 176.^{8,9} Because the amino acid change occurred at position 161, it is in the wing-2 region.

Genotype-Phenotype Correlations

Variable expression of the disease phenotype was noticed between two affected generations of this family (Table 1). This mutation resulted in a very severe phenotype in the father, which, without prompt and early surgical intervention, led to blindness. In contrast, the same mutation with early surgical intervention in the children resulted in moderate severity and reasonably good prognoses (Fig. 4).

DISCUSSION

This is the first study to demonstrate that a mutation in the wing region of the forkhead/winged-helix transcription factor gene *FOXC1* can result in an anterior segment dysgenesis of the eye, ARA. Therefore, the mutation reported herein is a

TABLE 2. Novel sets of *FOXC1* Primers and Conditions Used for Amplification

Primer Sets Used for Amplification (5'-3')/Size	Position in cDNA (bp)	Fragment Size (bp)	MgCl ₂ Used (mM)	Annealing Temperature (°C)
ARA1F - CCCGGACTCGGACTCGGC - 18 mer ARA1R - AAGCGGTCCATGATGAACTGG - 21 mer	-93 to -76 335-315	429	1	62
ARA2F - CCCAAGGACATGGTGAAGC - 19 mer ARA2R - CTGAAGCCCTGGCTATGGT - 19 mer	217-235 926-908	710	1	58
ARA3F - ATCAAGACCGAGAACGGTACG - 21 mer ARA3R - GTGACCGGAGGCAGAGAGTA - 20 mer	676-696 1310-1291	635*	1	58
ARA4F - TACCACTGCAACCTGCAAGC - 20 mer ARA4R - GGGTTCGATTTAGTTCGGCT - 20 mer	1177-1196 1693-1674	517	1.25	58

PCR conditions for all primer sets: initial denaturation at 94°C for 3 minutes followed by (94°C for 30 seconds, annealing for 30 seconds), 72°C for 45 seconds for 35 cycles, with final extension at 72°C for 7 minutes.

* Because of a nonspecific fragment, set 3 PCR fragment was eluted, diluted 1:10, and reamplified for sequencing, with 25 cycles.

novel one. Moreover, this is the fourth mutation in this gene known to cause the ARA phenotype and the first study from the Indian subcontinent to describe the molecular basis of ARA. So far, only three *FOXC1* mutations have been reported to cause this disorder. These include a 10-bp deletion in the upstream of the forkhead domain,⁴ a missense mutation (Ile87Met),⁴ and a single nucleotide insertion⁵ in the $\alpha 1$ helix region in the forkhead domain of this gene. Consistent with the autosomal dominant inheritance, all three affected members had one mutant allele segregating with the disease phenotype, and the mutation was fully penetrant in the family.

Segregation of mutant alleles with the disease phenotype, absence of mutant alleles in control subjects, and a high degree of conservation of mutated residue across species during evolution imply that the mutation we report is pathogenic. Although various mutations in the *FOXC1* gene have been implicated in the pathogenesis of a spectrum of ocular disorders such as Axenfeld anomaly, Axenfeld-Rieger syndrome, Rieger anomaly, Iris hypoplasia, Peter's anomaly, iridogoniodygenesis type 1, ARA, and congenital glaucoma, none was found to be in the wing region.^{4-8,10} Hence, this is the first wing mutation known to cause any anterior segment dysgenesis related to the *FOXC1* gene.

FOXC1 is a member of the forkhead/winged-helix family of transcription factors. These transcription factors contain a monomeric, 110-amino-acid DNA binding domain (forkhead domain) and have been conserved throughout evolution from yeast to human.^{11,12} This DNA-binding motif is a variant of the helix-turn-helix motif and consists of three α helices and two

large loops that form wing structures, W1 and W2.^{8,9,12} Amino acids 155 to 176 of the forkhead domain constitute the wing-2 region.^{8,9} The mutation in this study was found in amino acid position 161, within the wing-2 region (see the Results section). So far, no other mutation has been reported in this region. Therefore, this is the first mutation identified in the wing-2 region of the *FOXC1* transcription factor. The forkhead/winged-helix family of transcription factors are essential in a variety of developmental processes, including embryogenesis and tissue specific cell differentiation.^{11,12} It has been reported that the W2 region of HNF- γ (a member of the forkhead family of transcription factors) contacts with the minor groove of DNA.⁹ Because *FOXC1* is a transcription factor, the W2 mutation could possibly affect the DNA-protein interaction. Haploinsufficiency of forkhead transcription factors has been shown to cause aberrant ocular development.^{8,13} Considering these facts, it is tempting to speculate that this mutation may affect the migration and/or differentiation of the mesenchymal cells that contribute to the anterior segment of the eye.¹³

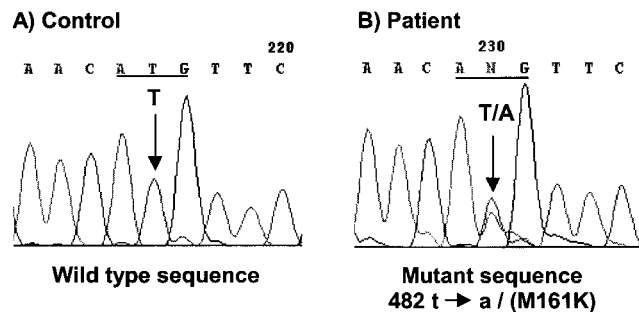


FIGURE 1. Electropherogram of the sense strand of genomic DNA from the ARA proband, showing a novel heterozygous missense mutation. The heterozygous change 482 t \rightarrow a (M161K) was present in the mutant allele of the proband (B) and absent in the control (A). The mutation (underscore and arrow) results in a change in amino acid at codon 161.

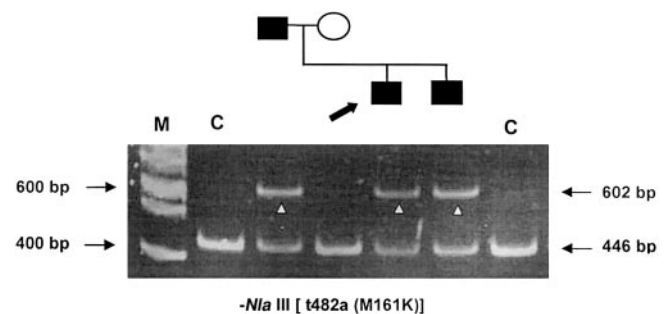


FIGURE 2. PCR-RFLP analysis of cosegregation of the mutant allele with the disease phenotype in an ARA pedigree. (■) Affected male individuals; (○) unaffected female individual; arrow: proband. DNA molecular weight marker (lane M) in base pairs (left); allele sizes (right); control (lane C); mutant allele (small arrowheads), restriction site change and mutation (nucleotide as well as amino acid changes) are shown below the gel. The 710-bp *FOXC1* amplification product generated from the *FOXC1* primer pair 2F/2R (Table 3) was cleaved by *Nla*III into five fragments of sizes 8, 40, 60, 156, and 446 bp in unaffected individuals. The T482A mutation in the affected individuals abolished the *Nla*III site between the 156- and 446-bp fragments, and the resultant 602-bp mutant allele segregates along with the disease phenotype. The normal *Nla*III cleavage products present in affected individuals were generated from the wild-type *FOXC1* allele in these individuals. Fragments less than 446 bp are not shown in this 8% polyacrylamide gel.

The abnormal eye development and the devastating phenotype (blindness in the father) in this family indicates that the *FOXC1* wing region has an important functional role in the normal development of the eye (Fig. 4; Table 1). The genotype-phenotype correlation indicates that this mutation resulted in blindness in the father (very severe phenotype and very poor prognosis). The father had asymmetric manifestations in both eyes (the right eye became blind in childhood; the left eye had very poor vision), whereas the children had uniform manifestations. Late surgical intervention in the father's left eye did not restore vision. Hypoplastic iris, polycoria, and corectopia were present in the father, which indicated an advanced stage of the disease. These manifestations were absent in the children (Table 1). The differences in clinical manifestations between the two affected generations—late surgery in the father (24 years of age) and early surgery in the children (2–2.5 months)—or to the late diagnosis of the disease in father (Table 1).

Several anterior segment dysgenesis show overlapping clinical features and many are due to different mutations in the *FOXC1* gene.^{1,3–8,10} Hence, the clinical and genetic heterogeneity of anterior segment disorders can be understood better with the accumulation of genotype data of the *FOXC1* gene. It may also help in the classification of these disorders as well as in understanding the prognosis of the disease.

In sum, this study adds one novel mutation to the existing spectrum of mutations that cause anterior segment dysgenesis and also provides insight into the functional dissection of the *FOXC1* gene. The novel sets of primers used in this study have simplified the mutation screening strategy of the *FOXC1* gene. This investigation also suggests that the *FOXC1* gene is possibly defective in other Indian ARA-affected families. However, further analysis of ARA families is needed to establish the genotype-phenotype correlations of this ocular disorder.

Acknowledgments

The authors thank the family for participating in this study; the Clinical Biochemistry Services and Jasti V. Ramanamma Children's Eye Care Center staff at the L. V. Prasad Eye Institute for their assistance in

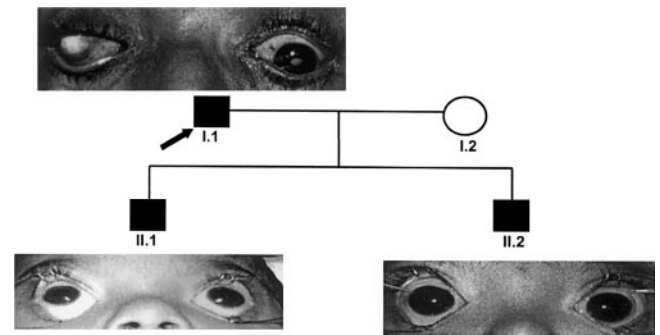


FIGURE 4. Phenotypes of an ARA pedigree after surgery showing varying prognosis. I.1 was the affected father with late surgical intervention showing a very severe phenotype and a very poor prognosis (blind in the right eye and very poor vision in the left eye). II.1 and II.2 were the affected children with early surgical intervention showing clear corneas and reasonably good prognoses. Corneal edema completely cleared after surgery. Follow-up periods after surgery were I.1: 10 years, II.1: 4 years, and II.2: 3 years. For ages of surgical interventions in affected members, refer to Table 1.

sample collection; Dorairajan Balasubramanian and Gullapalli N. Rao for encouragement and support; and Koteswar Rao, Information Systems Department, L. V. Prasad Eye Institute, for assistance in preparation of the graphics.

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		M161K																					
FOXE3 (human)	P	A	A	A	D	M	F	D	N	G	...	AAF82793.1											
FOXE1 (human)	P	N	A	E	D	M	F	E	S	G	...	AAC51294.1											
FOXD2 (human)	P	E	S	A	D	M	F	D	N	G	...	AAC15421.1											
FOXD4 (human)	P	A	S	Q	D	M	F	D	N	G	...	AAA92040.1											
FOXD3 (rat)	P	Q	S	E	D	M	F	D	N	G	...	AAA41319.1											
FOXD3 (zebrafish)	P	Q	S	E	D	M	F	D	N	G	...	AAC06366.1											
FOXD1 (human)	P	E	S	A	D	M	F	D	N	G	...	AAC50661.1											
<u>FOXC1 (human)</u>	<u>P</u>	<u>D</u>	<u>S</u>	<u>A</u>	<u>N</u>	<u>M</u>	<u>F</u>	<u>E</u>	<u>N</u>	<u>G</u>	...	<u>AAC18081.1</u>											
FOXC1 (mouse)	P	D	S	Y	N	M	F	E	N	G	...	AAC24209.1											
FOXC1 (zebrafish)	P	D	S	Y	N	M	F	E	N	G	...	AAG44241.1											
FOXC2 (human)	P	D	S	Y	N	M	F	E	N	G	...	CAA69400.1											
FOXC2 (mouse)	P	D	S	Y	N	M	F	E	N	G	...	CAA63244.1											
FOXC2 (rat)	P	D	S	Y	N	M	F	E	N	G	...	AAA41320.1											
FKHL18 (human)	P	D	C	H	D	M	F	E	H	G	...	AAC15420.1											
Croc (drosophila)	P	D	S	Y	N	M	F	D	N	G	...	AAB35643.1											
FOXL1 (human)	P	R	C	L	D	M	F	E	N	G	...	AAG40312.1											
FOX11 (mouse)	P	R	C	L	D	M	F	E	N	G	...	CAA63243.1											
FOXA1 (zebrafish)	P	D	S	G	N	M	F	E	N	G	...	AAC06367.1											
FOXA2 (zebrafish)	P	D	S	G	N	M	F	E	N	G	...	CAA80443.1											
Fkh (drosophila)	P	D	S	G	N	M	F	E	N	G	...	AAA28535.1											
FOXF1 (human)	P	A	S	E	F	M	F	E	E	G	...	AAC50399.1											
FOXF2 (human)	P	A	S	E	F	M	F	E	E	G	...	AAD19875.1											
FOXG1C (human)	P	S	S	D	V	F	I	G	G	...	CAA52241.1												
FOXG1B (human)	P	S	S	D	V	F	I	G	G	...	CAA52239.1												
FOXG1A (human)	P	S	S	D	V	F	I	G	G	...	CAA55038.1												
FOXJ1 (human)	P	Q	Y	A	E	R	L	L	S	G	...	CAA67729.1											

FIGURE 3. Multiple sequence alignment of human *FOXC1* and related FOX protein sequences. **Bold letter with asterisk (*)** and **shading** represent the conserved residue methionine M161K (when mutated) that causes the ARA phenotype. **Right:** sequence accession numbers. The human *FOXC1* sequence is **underscored**.