Structural Assessment of *PITX2*, *FOXC1*, *CYP1B1*, and *GJA1* Genes in Patients with Axenfeld-Rieger Syndrome with Developmental Glaucoma

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PURPOSE. Axenfeld-Rieger (AR) is an autosomal dominant disorder with phenotypic heterogeneity characterized by anterior segment dysgenesis, facial bone defects, and redundant periumbilical skin. The *PITX2* gene, on chromosome 4q25, and the *FOXC1* gene, on chromosome 6p25, have been implicated in the different phenotypes of the syndrome through mutational events. Recently, the *CYP1B1* gene was found to be associated with Peters’ anomaly, and the gene associated with ocudental digitalis dysplasia syndrome, which presents some similarities with AR, was identified (connexin 43 – *GJA1* gene). The purpose of this study was to evaluate *PITX2*, *FOXC1*, *CYP1B1*, and *GJA1* gene mutations in Brazilian families with AR.

METHODS. Eight unrelated patients affected by AR (all eight with glaucoma and three with systemic manifestations) and their families were ophthalmologically evaluated and their blood was collected for DNA extraction purposes. The coding regions of *PITX2*, *FOXC1*, *CYP1B1*, and *GJA1* genes were completely evaluated through direct sequencing.

RESULTS. The frequency of mutations in the *FOXC1*, *GJA1*, *PITX2*, and *CYP1B1* genes in this study were 25%, 12.5%, 0% and 0%, respectively. In the *FOXC1* gene, two GGC triplet insertions (GGC375ins and GGC447ins) defined as a polymorphism, and two new mutations—a deletion (718 to 719delCT) and a nonsense mutation (Trp152STOP)—were identified. One polymorphism (Ala253Val) was identified in the *GJA1* gene in the same family presenting the Trp152STOP mutation in the *FOXC1* gene. In this family harboring both structural alterations, two patients who carried the *GJA1* (Ala253Val) and *FOXC1* (Trp152STOP) mutations developed less severe glaucoma compared with family members presenting the *FOXC1* (Trp152STOP) mutation alone.

CONCLUSIONS. Two new structural alterations in the *FOXC1* gene and a polymorphism in the *GJA1* gene were first described in Brazilian patients with AR and developmental glaucoma. A polymorphism in the *GJA1* gene (Aa253Val), for the first time identified in association with AR, raises the possibility of its participation as a modifier gene. (Invest Ophthalmol Vis Sci. 2006;47:1803–1809) DOI:10.1167/iovs.05-0979

Developmental anomalies of the ocular anterior chamber angle may lead to an incomplete development of the structures that form the conventional aqueous outflow pathway. Thus, disorders that present with such dysfunction tend to be associated with glaucoma. Among them, Axenfeld-Rieger (AR) malformation is a rare clinical entity with an estimated prevalence of one case in every 200,000 individuals.¹ AR represents a spectrum of disorders involving ocular and, in some cases, extracranial structures caused by disruption of the migration and differentiation of neural crest cells. The ocular structures involved in AR include the cornea (posterior embryotoxon), the iridocorneal angle (peripheral iridocorneal adherences and ultrastructural abnormalities of the trabecular meshwork), and the iris (peripheral adhesions between the iris and the cornea, and stroma atrophy).²

The association between AR and developmental glaucoma occurs in approximately 50% of the cases.³ Glaucoma may appear during childhood, but it is more common during adolescence or at the beginning of adulthood.² Glaucoma secondary to AR is difficult to manage and may result in severe damage to the optic disc and visual field.³

Systemic manifestations more commonly associated with AR include dental abnormalities (microdontia, hypodontia, and oligodontia) and facial malformations (hypoplasia of the maxillary bones).³ Redundant periumbilical skin, hypospadia, as well as other less frequent systemic alterations, may also be observed in AR.¹⁴⁻⁶

At least five loci associated with the AR phenotype have been described and two of them have its gene already identified. The *PITX2* gene (paired-like homeodomain transcription factor 2–MIM 601542—*RIEG 1*) was positionally cloned by Semina et al.⁷ in 1996. This gene is located on chromosome 4q25 and belongs to the paired-bicoid family of homeodomain transcription factor (paired homeobox gene). The homeobox genes regulate the expression of other genes during embryonic development,⁷ and *PITX2* is involved in the differentiation of the ocular mesenchyma, the dental lamina, and the umbilical cord.⁷ The second gene to be mapped was the *FOXC1* (forkhead box C1–MIM 601090), which, like the *PITX2* gene, acts as a transcription factor and is situated on a cluster of loci for anterior segment disorders associated with glaucoma on chromosome 6p25.⁹ Furthermore, there is enough evidence to suggest that the *PAX6* gene (paired box gene 6—MIM 607108) on chromosome 11p13¹⁰ and the *MAF* gene (v-MAF avian musculoaponeurotic fibrosarcoma oncogene homolog—MIM 177075) on chromosome 16q24¹¹,¹² may also be involved in AR. Another locus in the 13q14 region (RIEG2—MIM 601499) has been shown to be associated with AR, but the gene has not been identified yet.¹⁵
In 1997, Stoilov et al.\textsuperscript{14} described structural alterations in the \textit{CYP1B1} gene (MIM 601771), a member of the cytochrome P450 enzyme family, in patients with congenital glaucoma linked to the GLC3A locus. Mutations in the \textit{CYP1B1} gene have been reported to be associated with other forms of glaucomas, including Peters' anomaly.\textsuperscript{15-17} and to act as a modifier gene in juvenile open-angle glaucoma.\textsuperscript{18} Considering that Peters' anomaly is characterized by anterior ocular malformation because of defective migration of neural crest cells, it is possible to hypothesize that this gene could play a yet noninvestigated role in the pathogenesis of AR.

Finally, Paznekas et al.\textsuperscript{19} identified mutations in the \textit{GJA1} gene (gap junction protein, alpha 1—MIM 121014), located on chromosome 6q21q23.2, in individuals with oculardontodigital dysplasia syndrome (ODDD). The \textit{GJA1} gene encodes the connexin 43 protein, one of the proteins that form gap junctions, responsible for controlling the passage of ions and small molecules between adjacent cells. ODDD is an autosomal dominant syndrome with high penetrance and phenotypic variability. This syndrome is characterized by several clinical manifestations involving ocular (atrophy of the iris, iridocyclitis, cataract, glaucoma, optic nerve atrophy, optical disc hypervascularization, microphthalmia, microcornea, strabismus, nystagmus, ambyopia, palpebral fissure reduction, persistent pupillary membrane), dental (microdontia, hypoplasia, enamel hypoplasia, hypodontia), and bone abnormalities (clinodactyly; synactyla of fingers and toes, and aplasia or hypoplasia of the middle phalanges and tubular bones; hypo- or hypertelorism; nasal tapering; palatine cleft; microcephalus; cranial and mandibular hyperostosis). Because ODDD is characterized by ocular and facial malformations that are similar to those found in AR, the \textit{GJA1} gene could be considered a possible candidate in the complex pathophysiology of this disease.

An assessment of the \textit{PITX2} and \textit{FOXC1} genes, as well as other candidate genes (\textit{GJA1} and \textit{CYP1B1}), in individuals and families with or without extraocular manifestations of AR may help to improve the characterization of the disease from a molecular and clinical standpoint.

The purpose of this study was to determine the frequency of mutations in the \textit{PITX2}, \textit{FOXC1}, \textit{CYP1B1}, and \textit{GJA1} genes in a Brazilian population with AR.

**Materials and Methods**

Eight patients (probands) with AR malformation and their family members were consecutively evaluated at the Glaucoma Service of the University of Campinas, Brazil. The inclusion criterion adopted for probands was the presence of any ocular anomaly found in AR associated with developmental glaucoma, characterized by intraocular pressure (IOP) higher than 21 mmHg, measured on at least three occasions.

Four structural alterations were found in the \textit{PITX2} and \textit{FOXC1} genes, as well as other candidate genes (\textit{GJA1} and \textit{CYP1B1}), in individuals and families with or without extraocular manifestations of AR.

Peripheral blood was collected in a sterile flask containing 10% EDTA as anticoagulant and DNA was extracted by following the instructions of a DNA purification kit (GFX Blood DNA Purification Kit; Amersham Biosciences, Piscataway, NJ). The following reagents were used in the amplification of the \textit{PITX2}, \textit{FOXC1}, \textit{CYP1B1}, and \textit{GJA1} genes:

- 20 pmoles of each primer.
- 1× enzyme buffer (10× buffer = 20 mM Tris-HCl [pH 8.4], 50 mM KCl, 0.01% gelatin).
- 1.5 mM MgCl\textsubscript{2}.
- 200 \muM nucleotides (dATP, dCTP, dTTP, dGTP).
- 1 U of DNA polymerase (Taq DNA polymerase; Invitrogen, Carlsbad, CA)
- 0.5 \muM of genomic DNA, completing the volume to 50 \muL with sterile deionized H\textsubscript{2}O.

Formamide at a final concentration of 5% (v/v) was used for all the \textit{PITX2} gene regions, and dimethylsulfoxide, at a final concentration of 10%, was used for all the \textit{FOXC1} gene regions and for regions 1 and 2 of the \textit{CYP1B1} gene. The primer pairs for \textit{PITX2}, \textit{FOXC1}, \textit{GJA1}, and \textit{CYP1B1} gene amplification were designed based on sequences identified by the following access numbers: 601542; 601090; 121014; and 601771; respectively (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). PCR conditions for each gene are described in Table 1.

PCR reactions for the four genes were conducted in a thermal cycler (model 9600; Applied Biosystems, Foster City, CA). Three microtubes of the reaction products were separated through electrophoresis in 1.5% agarose gels stained with ethidium bromide and compared with a DNA standard (Low DNA Mass Ladder standard; Invitrogen). PCR products were purified with exonuclease I and alkaline phosphatase (Fermentas Inc., Hanover, MD), after which they were subjected to sequencing reactions.

PCR products were automatically sequenced by using the sequencing reaction kit (Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1; Applied Biosystems), according to manufacturer's instructions, and electrophoresed in a DNA automatic analyzer (ABI PRISM 310; Applied Biosystems). The sequences were searched for similarities by using the BLAST search algorithm available at http://www.ncbi.nlm.nih.gov/blast.

To investigate mutations found among probands and family members in the studied genes in the control group, specific restriction enzymes were used to assess their frequency in the general population.

This study adhered to the tenets of the Declaration of Helsinki and received the approval of Ethics Committee of the University of Campinas. All patients signed an informed consent.

**Results**

From March 2001 to December 2002, eight families (82 individuals) with at least one member having AR and developmental glaucoma were examined at the Glaucoma Service of the University of Campinas (Table 2).

The mean age of the eight affected individuals (probands) was 17.8 ± 9.8 years (range, 8–38 years). The mean age at glaucoma diagnosis was 10.6 ± 10.7 years (range, birth to 33 years), and the mean duration of glaucoma was 7.5 ± 4.5 years (range, 2–16 years). Four of eight cases (50%) had previously undergone antiglaucoma surgery in at least one eye. Clinical manifestations of AR were restricted to the eyes in 5 probands (62.5%).

Mutations were not found in the coding or splicing regions of the \textit{PITX2} gene in any of the patients with AR.

Structural analysis of the \textit{CYP1B1} gene revealed five polymorphisms among the families. Four of these polymorphisms had already been described in Brazilian patients: one in the second exon (R48G), and three in the third exon (L432V, D494D, and N453S).\textsuperscript{20} Another polymorphism, previously reported in the Japanese population (V243Y—exon 2),\textsuperscript{21} was identified in heterozygosis in two families (families 1 and 5).

Four structural alterations were found in the \textit{FOXC1} gene in unrelated individuals with AR, consisting of two polymorphisms in two separate GGC repeats within the \textit{FOXC1} coding region (after the forkhead domain, in the second activation domain), a two base-pair deletion, and a nonsense point mutation.

The first polymorphism was an insertion of an extra GGC triplet (GGC\textsubscript{375}ins), found in three of the probands. In two of the patients, this change was present in homozygosis (6/7) and in one in heterozygosis (6/7). The second one was downstream of the first repeat (codon 447) and consisted of a variation from 6 to 8 GGC repeats (the most frequent allele has 7 glycine

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\textsuperscript{4} Cella et al.\textsuperscript{1804}
One proband presented the allele 8 in homozygosis (8/8) and one presented the allele 6 in heterozygosis (6/7).

A two base-pair deletion (CT) was detected in the family 8 proband (patient WDC) at nucleotides 718 and 719 (718 to 719delCT) located after the forkhead domain, more specifically, in amino acid 240 of the inhibition exon domain (Fig. 1A). The deletion was present in heterozygosis, and only the proband had the syndrome and a structural alteration of the FOXC1 gene, characterizing a de novo mutation (Fig. 1B).

A nonsense point mutation was detected in six individuals of family 6. The mutation involved the substitution of the amino acid tryptophan (TGG) for a termination codon (TGA) in position 152 (Trp152STOP) located in the forkhead domain of the FOXC1 gene (Fig. 2).

Family 6, composed of three generations, had 10 of its members evaluated. All the 6 affected members with the Trp152STOP mutation had extraocular manifestations of the syndrome and developmental glaucoma. Overall, these individuals had low stature, maxillary hypoplasia, and dental abnormalities. Ocular alterations, such as posterior embryotoxon, peripheral iridocorneal adherences, and iris stromal hypoplasia were present in all of them. With the exception of 2 second-generation members (including the proband), all the other patients had poor vision and needed surgery to reduce IOP. The nonsense mutation (Trp152STOP) detected in family 6 presented concomitant segregation with the AR phenotype, suggesting that the syndrome had an autosomal dominant transmission in this family.

### Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences 5'-3'</th>
<th>Fragment Size (bp)</th>
<th>PCR Cycles (annealing temperature and time)</th>
</tr>
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<tr>
<td>PITX2-exon 2</td>
<td>S-GGGGCAGTAAAGCCAGACT</td>
<td>289</td>
<td>94°C-1' 60°C-1' 72°C-1'</td>
</tr>
<tr>
<td></td>
<td>AS-GAGCTAAGGCAAGATCTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PITX2-exon 3</td>
<td>S-GGATGGTTGAGGAGGAGGAAG</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS-CTGTAACCTGCAACACTTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PITX2-exon 4 (region 1)</td>
<td>S-CACCTGGGCCATCTCTTCTGCT</td>
<td>324</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS-GAGGAGATAGCTGACAGGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PITX2-exon 4 (region 2)</td>
<td>S-TATGAAGGTGAACACCTCTGTTT</td>
<td>400</td>
<td></td>
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<tr>
<td></td>
<td>AS-CCATCGGCCGAAAGTCTCTA</td>
<td></td>
<td></td>
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<tr>
<td>FOXC1-exon 1 (region 1)</td>
<td>S-CCCGGACTCGGGACTCGGGGC</td>
<td>429</td>
<td>94°C-30' 62°C-30' 72°C-45'</td>
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<tr>
<td></td>
<td>AS-AAGGCGTCCATGTAGAAGCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXC1-exon 1 (region 2)</td>
<td>S-CCTAGAGCCAGATGGTGAAGC</td>
<td>710</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS-CTGAAAGCCGCTGCTAGGTT</td>
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<td></td>
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<td>FOXC1-exon 1 (region 3)</td>
<td>S-ATCAAGAGCGGAGAAGGTAGG</td>
<td>635</td>
<td>94°C-30' 58°C-30' 72°C-45'</td>
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<td></td>
<td>AS-TGGACGGGAGCGAAGAGTA</td>
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<td></td>
</tr>
<tr>
<td>FOXC1-exon 1 (region 4)</td>
<td>S-TACCAGCTGCAACTGTCGAGG</td>
<td>517</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS-GGTTTGAGTTAGTTTGCGGT</td>
<td></td>
<td></td>
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<tr>
<td>GJA1-exon 2 (region 1)</td>
<td>S-GATCGTTTTCTGGTGGTGCC</td>
<td>925</td>
<td>94°C-1' 60°C-1' 72°C-1'</td>
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<td></td>
<td>AS-CCTTTTTGCCTTAAGCGGG</td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td>AS-GGCTAGAAAGGCTTTACCTT</td>
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<tr>
<td>CYPIB1-exon 2 (region 1)</td>
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<tr>
<td></td>
<td>AS-GGATGCTGCTGGCTGCTAG</td>
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<tr>
<td>CYPIB1-exon 2 (region 2)</td>
<td>S-ATGGCTTTCGGCCACTCT</td>
<td>787</td>
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<td></td>
<td>AS-GATCTTGGTTTTGGGATGT</td>
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<tr>
<td>CYPIB1-exon 3</td>
<td>S-TCCCAAGAATATTAATTTGAATGTCACTG</td>
<td>885</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS-TATGGAGGACACACCTACCTG</td>
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<td></td>
</tr>
</tbody>
</table>

PCR conditions for all primer sets. Initial denaturation at 94°C for 5 min, followed by 35 cycles at above temperatures and time, and final extension at 72°C for 7 min. S, sense; AS, anti-sense; bp, base pairs.

### Table 2.

<table>
<thead>
<tr>
<th>Families (probands’ initials)</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Duration of Glaucoma (y)</th>
<th>Previous Filtrating Surgery</th>
<th>Topical Hypotension Drugs (n)</th>
<th>Extraocular Manifestations</th>
<th>Family Members Evaluated (μ)</th>
<th>Family Members Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (DS)</td>
<td>F</td>
<td>9</td>
<td>2</td>
<td>Yes</td>
<td>2</td>
<td>No</td>
<td>12</td>
<td>None</td>
</tr>
<tr>
<td>2 (ESO)</td>
<td>F</td>
<td>19</td>
<td>11</td>
<td>No</td>
<td>1</td>
<td>3</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>3 (FAVS)</td>
<td>M</td>
<td>8</td>
<td>7</td>
<td>No</td>
<td>2</td>
<td>Yes</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>4 (UP)</td>
<td>M</td>
<td>17</td>
<td>3</td>
<td>No</td>
<td>2</td>
<td>No</td>
<td>14</td>
<td>None</td>
</tr>
<tr>
<td>5 (MCV)</td>
<td>M</td>
<td>16</td>
<td>16</td>
<td>Yes</td>
<td>2</td>
<td>Yes</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>6 (OGS)</td>
<td>M</td>
<td>38</td>
<td>5</td>
<td>No</td>
<td>1</td>
<td>Yes</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>7 (VS)</td>
<td>F</td>
<td>25</td>
<td>5</td>
<td>Yes</td>
<td>1</td>
<td>No</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>8 (WDC)</td>
<td>M</td>
<td>11</td>
<td>8</td>
<td>Yes</td>
<td>2</td>
<td>No</td>
<td>29</td>
<td>None</td>
</tr>
</tbody>
</table>
A polymorphism was found in the GJA1 gene in three members of family 6. The GJA1 polymorphism was characterized by the substitution of cysteine by thymine in codon 253 (GGC -> GTG), resulting in the substitution of the amino acid alanine by valine (Ala253Val) in heterozygosis (Fig. 2A). One member carried the Ala253Val polymorphism and had a normal coding sequence of the FOXC1 gene, presenting normal phenotype and no associated glaucoma (Fig. 2B). The other two members harbored structural alterations in the GJA1 gene (Ala253Val), as well as in the FOXC1 gene (Trp152STOP). Both patients had AR and developmental glaucoma, but the IOP was under control with topical antiglaucoma medications. The onset of glaucoma in these two individuals was delayed in comparison with the other family members with AR; the optic disc set of glaucoma in these two individuals was delayed in comparison with the other family members with AR; the optic disc in these two individuals was delayed in comparison with the other family members with AR.

FIGURE 1. (A) Direct sequencing of the PCR product encompassing exon 1 of the FOXC1 gene. Above: Normal control. Below: Patient WDS presenting a two base-pair deletion (CT) between nucleotides 718 and 719 indicated by the arrow. (B) Pedigree of family 8 showing the proband indicated by an arrow. He is the only carrier of the syndrome, associated with glaucoma.

A nonsense mutation (Trp152STOP) was found in six members of family 6. The FOXC1 nonsense mutation was characterized by the substitution of thymine by glycine in codon 178 (GGC -> CCG), and results in the substitution of the amino acid tryptophan by proline (Trp152Pro) in heterozygosis (Fig. 1A). The mutation was detected in the family members with AR and in the control group through SSCP analysis. The mutation was not found in the family members with AR and in the control group through SSCP analysis. The mutation was not found in the family members with AR and in the control group through SSCP analysis.

The deletion of one allele of the PITX2 gene (Gln23STOP) was found in four members of family 6, inserting a termination codon at position 23 (refSNP ID: rs17653265). The Ala253Val structural alteration in the GJA1 gene, detected in heterozygosis, did not present concomitant segregation. Members of family 6 with the Trp152STOP mutation (in the FOXC1 gene) and the Ala253Val polymorphism (in the GJA1 gene) had a less severe glaucoma than the other affected relatives who were carriers of the Trp152STOP mutation alone.

DISCUSSION

Over the last several years, the identification of genes and loci involved in the different forms of glaucoma has led to a better understanding of the pathogenetic mechanisms of primary open-angle glaucoma, congenital glaucoma, and developmental glaucoma, such as the one associated with AR.

Although AR has an autosomal dominant inheritance, there have been reports of sporadic cases in the literature. Among the eight families evaluated in this study, only one (12.5%) showed affected family members. These data differ from that reported by Shields, in which affected family members were present in almost 40% of the cases, and indicates that the occurrence of isolated cases may be frequent.

The frequency of mutations in the PITX2 gene in patients with AR ranges from 9.4% to 42.2%. In Brazil, the only available report identified a 40% frequency of mutations among five unrelated families with AR associated with systemic manifestations. These variable results demonstrate the extensive genetic heterogeneity of AR. Several PITX2 gene mutations have been described in association with AR, mostly in patients with systemic manifestations. The present study was unable to identify any structural alteration in the coding region of PITX2, even though three of the eight families presented probands with ocular and systemic manifestations.

Our findings allow us to formulate the hypothesis that, despite the previously described association between AR systemic manifestations and PITX2 gene mutations, the proportion of mutations in this gene may be smaller when the cases are isolated. Moreover, the occurrence of hemizygosis, as proposed by Lines et al., may justify the low frequency of structural alterations of the PITX2 gene in this population. A more appropriate analysis would involve the use of the FISH methodology to investigate individuals of this group so that the deletion of one allele of the PITX2 gene is averted.

The presence of mutations in the FOXC1 gene in patients with AR was initially described in independent studies conducted by Nishimura et al. and Mears et al., which involved AR patients with abnormalities restricted to the ocular region. However, Mirzayans et al. identified a nonsense mutation (Gln23STOP) in the FOXC1 gene, just before the forhead domain, and suggested that the haploinsufficiency involving the FOXC1 gene may result in phenotypes accompanied by systemic alterations. Overall, FOXC1 gene mutations do not help in predicting the presence or the absence of systemic alterations in AR, different from the PITX2 gene, where it is possible to establish a determined genotype/phenotype correlation based on residual gene function.

In this study, four structural alterations were found in the FOXC1 gene, of which two (one deletion and one nonsense mutation) had not been previously described, to our knowledge, in the literature. The two GGC triplet insertions (GGC375ins and GGC447ins) that lead to a glycine incorporation had already been described as a polymorphism by Mears et al. in similar proportions between cases and controls.

The deletion found in this study (718 to 719delCT) was located in the FOXH1 inhibition domain and was present in only one individual (family 8 proband) with severe glaucoma and no systemic manifestation. Several other deletions have previously been described in patients with various phenotypes, but none of them was situated in the inhibition domain. These alterations were not present in the parental generation, characterizing a de novo mutation. This finding suggests that the absence of family members with AR does not exclude the possibility of associated structural alterations in the FOXC1 gene.

The nonsense mutation (Trp152STOP) was found in six members of family 6, inserting a termination codon at position 152. This mutation is located in the forhead domain, in an extremely conserved region among different species (FOXC1 amino acid alignment was performed by the ClustalW program at www.ebi.ac.uk/clustalw, and sequences were obtained from the National Center for Biotechnology Information (NCBI) En...
This alteration leads to protein truncation from this position, which may characterize haploinsufficiency. This is the second mutation found in association with extraocular manifestations of the syndrome. In this study, all the individuals with the Trp152STOP mutation presented with AR with ocular and systemic manifestations. This mutation had a concomitant segregation and an autosomal dominant pattern of inheritance with high penetrance. In fact, all the affected individuals presented the mutation, of which four showed severe phenotypes characterized by glaucoma refractory to clinical and surgical treatment, as well as reduced visual acuity.

The frequency of FOXC1 gene mutations in patients with AR ranges from 20% to 30%.35,40 In Brazil, five families, with a total of 23 affected individuals, were molecularly assessed, and no mutation was found in the FOXC1 gene,27 supporting the hypothesis that AR with extraocular manifestations has a low frequency of FOXC1 mutations.36,41 Among the eight families investigated in our series, two structural alterations in the FOXC1 gene were found to be disease-causing mutations, which resulted in a frequency of 25%. Overall, eight individuals presented mutations in the FOXC1 gene and, of these, six belonged to family 6. Only one individual (family 8 proband) had AR manifestations restricted to the eye. Because the mutations found in the FOXC1 gene were in functionally important regions and the polymorphisms were previously discussed by Mears et al.,35 these structural changes were not evaluated in the control group.

Most of the polymorphisms in the CYP1B1 gene identified in this study were in heterozygosity and had already been reported in the Brazilian population.20 The V243V CYP1B1 polymorphism (previously described in a Japanese population21) was also present in heterozygosis in two probands (families 2 and 5), with no structural alterations in the other evaluated genes. Regarding the GJA1 polymorphism (Ala253Val), one of the affected individuals belonged to the first generation of family 6 and had a normal phenotype. The other two patients, with concomitant structural alterations in the GJA1 gene (Ala253Val) and FOXC1 gene (Trp152STOP), belonged to the second generation of family 6 and showed AR (presenting similar abnormalities in the anterior segment as the other family members) with less severe developmental glaucoma than the other relatives with the Trp152STOP mutation alone. The clinical characteristics and the family history excluded the possibility of ODDD. The structural alteration detected in the GJA1 gene leads to an alanine-valine (Ala253Val) substitution located in the cytoplasmatic domain, which characteristically presents a great variability, not only among the different connexins of the same species but also in the same connexin among several species (connexins amino acid alignment was performed by the ClustalW program at www.ebi.ac.uk/
clusal and sequences were obtained from the NCBI’s Entrez-Protein Web site (www.ncbi.nlm.nih.gov/entrez).

The presence of susceptibility alleles and modifier genes has been reported in other types of glaucoma.\textsuperscript{14,15,42,43} These genes could interfere with the phenotypes of glaucoma, increasing or decreasing the risk of the development of the disease, as well as modulating its severity. Although family 6 was not able to be tested statistically because of the small number of individuals, the possibility that the Ala253Val polymorphism in the \textit{GJA1} gene acts as a modifier gene, attenuating the glaucoma phenotype, could not be totally excluded. Another possible role of the Ala253Val polymorphism would be to act as a marker for another nearby gene. The “protective” mechanism could include modifications in the gap junctions at the level of the trabecular meshwork, allowing increased aqueous humor drainage and, thereby, reducing the severity of glaucoma. The role of the \textit{GJA1} gene and its product, the connexin 43 protein, may also participate in the pathophysiology of the glaucomatous optic disc damage. A support for this hypothesis comes from the observation of a loss of immunohistochemical labeling for connexin 43, a component of gap junctions between astrocytes, in response to IOP elevation (Johnson EC, et al. IOVS 2000;41:ARVO Abstract 4767).\textsuperscript{14,15} Polymorphisms in the \textit{CYP1B1} gene also appear to act as modifiers, especially on the MYOC gene (associated with primary open-angle glaucoma) through a not yet understood mechanism.\textsuperscript{16} Thus, functional studies with both \textit{GJA1} and \textit{CYP1B1} genes warrant further investigation.

This study revealed that, among eight families with AR and developmental glaucoma, structural alterations in the \textit{FOXC1}, \textit{GJA1}, \textit{PITX2}, and \textit{CYP1B1} genes were present in 25%, 12.5%, 0%, and 0% of the affected individuals, respectively. The Trp152Stop mutation in the \textit{FOXC1} gene had concomitant segregation and complete penetrance. Finally, the observation of concomitant structural alterations in the \textit{FOXC1} and \textit{GJA1} genes reported herein for the first time, resulted in a less severe phenotype than that presented in patients with the \textit{FOXC1} mutation alone within the same family.

\section*{References}


