

# Novel Mutations of *FOXC1* and *PITX2* in Patients with Axenfeld-Rieger Malformations

Nicole Weisschuh,<sup>1</sup> Paul Dressler,<sup>2</sup> Frank Schuettauf,<sup>3</sup> Christiane Wolf,<sup>1</sup> Bernd Wissinger,<sup>1</sup> and Eugen Gramer<sup>4</sup>

**PURPOSE.** To determine the prevalence of *FOXC1* and *PITX2* mutations and to assess clinical phenotypes in a cohort of German patients with Axenfeld-Rieger malformations.

**METHODS.** All coding exons of the *FOXC1* and *PITX2* genes were amplified by PCR from genomic DNA and subjected to direct DNA sequencing. Analysis of mutations in control subjects was performed by restriction fragment length polymorphism (RFLP) analysis.

**RESULTS.** Sequence variants were identified by DNA sequencing in 15 of 19 cases. Mutation screening identified four potentially pathogenic *FOXC1* mutations causing amino acid substitutions (P79R, Y115S, G149D, and M161V) that were not present in 100 control subjects. In addition, two different 1-bp deletions causing a frameshift and subsequent premature stop codon were identified in two subjects. One patient harbored a *FOXC1* nonsense mutation (S48X). Mutation screening also identified two potentially pathogenic *PITX2* mutations (P64L and P64R) in two index patients that were excluded in 100 healthy control subjects.

**CONCLUSIONS.** The findings in the present study clearly demonstrate that *FOXC1* and *PITX2* mutations are responsible for a significant proportion of Axenfeld-Rieger malformations in Germany. (*Invest Ophthalmol Vis Sci.* 2006;47:3846–3852) DOI:10.1167/iovs.06-0343

Axtenfeld-Rieger (AR) malformations comprise a series of clinically and genetically heterogeneous conditions. Affected individuals display a spectrum of classic ocular anomalies such as iris hypoplasia; a prominent Schwalbe line; adhesion of iris and cornea, microcornea, and corneal opacity, and increased intraocular pressure (IOP). In addition to the ocular phenotype, systemic features may also be associated with the disorder, including maxillary hypoplasia, hypodontia, microdontia, umbilical abnormalities, hearing defects, and congenital cardiac or kidney abnormalities.<sup>1</sup> These syndromic features are seen with incomplete penetrance and variable expressivity. Because of the severe changes in eye morphology, glaucoma develops in roughly half of all patients. The mode of inheritance is autosomal dominant and the incidence of the disease is estimated to be approximately 1:200,000.<sup>2</sup> Until now, four

genetic loci have been associated with AR, including the genes *FOXC1* and *PITX2* located on 6p25 and 4q25, respectively.<sup>3,4</sup> A third locus was mapped to 13q14, but the gene has not yet been identified.<sup>5</sup> In addition, an isolated case of Rieger syndrome has been reported to harbor a deletion in the *PAX6* gene.<sup>6</sup>

*FOXC1* belongs to the forkhead family of transcription factors which comprises at least 43 members<sup>7</sup> that act as critical regulators of embryogenesis, cell migration, and cell differentiation.<sup>8,9</sup> Mutations of the *FOXC1* gene have been identified as the underlying cause in a variety of anterior segment disorders.<sup>3,10–20</sup> The mutation spectrum comprises frameshift and nonsense as well as missense mutations in the forkhead domain (for an overview, see Table 1). The observation of interstitial duplications and deletions of the *FOXC1* gene in patients with anterior segment dysgenesis indicates the importance of a stringent control of *FOXC1* expression and function.<sup>13,21,22</sup>

*PITX2* encodes a bicoid-like homeodomain transcription factor and is expressed very early during tooth development.<sup>23</sup> From experimental data it seems likely that the molecular basis of tooth anomalies in AR is the inability of mutant *PITX2* to activate genes involved in tooth morphogenesis,<sup>24</sup> and it has been shown that expression of *PITX2* in the neural crest is also necessary for optic stalk and anterior segment development.<sup>25</sup> To date, 30 mutations of the *PITX2* gene have been associated with AR<sup>4,26–33</sup> and other cases of anterior segment malformations, such as iridogoniodysgenesis,<sup>34</sup> iris hypoplasia,<sup>35</sup> and Peters' anomaly<sup>36</sup> (Table 2). Very recently, *FOXC1* and *PITX2* were shown to interact physically, and this interaction may be an explanation for the similar phenotypes caused by mutations of the two genes.<sup>37</sup>

The purpose of this study was to determine the prevalence of *FOXC1* and *PITX2* mutations in a cohort of German patients with AR malformations.

## MATERIALS AND METHODS

### Ascertainment of Patients and Clinical Evaluation

Written informed consent was obtained from all subjects, and the study was approved by the ethics committees of the University Hospital Tübingen and the University Hospital Würzburg and conducted in accordance with the Declaration of Helsinki.

Ophthalmic examinations included slit lamp biomicroscopy, gonioscopy, and measurement of intraocular pressure (IOP), visual acuity, and visual fields. Diagnosis of hypodontia was based on panoramic radiographs. Other diagnoses were obtained from the patients' attending specialists.

### Mutation Detection by Direct Sequencing

Patient DNA was extracted from peripheral blood lymphocytes using a standard salting-out procedure. Individual exons of the *PITX2* gene were amplified by polymerase chain reaction (PCR) using appropriate amplification protocols. Amplification of the single *FOXC1* exon was performed with a set of four overlapping primers. Primer pairs for amplification and sequencing are available on request. PCR fragments were purified (ExoSAP-IT enzyme cleanup; USB, Cleveland, OH) and

From the <sup>1</sup>Molecular Genetics Laboratory and <sup>3</sup>Department II, University Eye Hospital, Tübingen, Germany; the <sup>2</sup>Department of Prosthodontics, School of Dental Medicine, Julius-Maximilians University, Würzburg, Germany; and the <sup>4</sup>University Eye Hospital, Würzburg, Germany.

Submitted for publication March 29, 2006; revised May 3, 2006; accepted June 26, 2006.

Disclosure: N. Weisschuh, None; P. Dressler, None; F. Schuettauf, None; C. Wolf, None; B. Wissinger, None; E. Gramer, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Nicole Weisschuh, University Eye Hospital, Molecular Genetics Laboratory, Roentgenweg 11, D-72076 Tübingen, Germany; nicole.weisschuh@uni-tuebingen.de.

TABLE 1. Summary of *FOXC1* Mutations Reported to Date

References	Nucleotide Change	Protein Change
Nishimura et al. <sup>3</sup>	c.153-163del	Nonsense
	c.335T→C	Missense (F112S)
	c.378C→G	Missense (I126M)
Mears et al. <sup>10</sup>	c.392C→T	Missense (S131L)
	c.93-102del	Nonsense
	c.245G→C	Missense (S82T)
	c.261C→G	Missense (I87M)
	c.210delG	Nonsense
Swiderski et al. <sup>11</sup>	c.67C→T	Nonsense (Q23X)
Mirzayans et al. <sup>12</sup>	c.99-108del	Nonsense
Nishimura et al. <sup>13</sup>	c.116-123del	Nonsense
	c.1512del	Nonsense
	c.265insC	Nonsense
	c.26-47ins	Nonsense
	c.236C→T	Missense (P79L)
Kawase et al. <sup>14</sup>	c.286insG	Nonsense
	c.272T→G	Missense (I91S)
	c.380G→A	Missense (R127H)
Suzuki et al. <sup>15</sup>	c.235C→A	Missense (P79T)
	c.482T→A	Missense (M161K)
Panicker et al. <sup>16</sup>	c.255GC→TT	Missense (L86F)
Saleem et al. <sup>17</sup>	c.4C→T	Nonsense (Q2X)
Komatireddy et al. <sup>18</sup>	c.367C→T	Nonsense (Q123X)
	c.272T→C	Missense (I91T)
Mortemousse et al. <sup>19</sup>	c.494G→C	Missense (G165R)
Murphy et al. <sup>20</sup>	c.506G→C	Missense (R169P)

sequenced with dye-termination chemistry (Big Dye Termination chemistry; Applied Biosystems [ABI], Weiterstadt, Germany), and the products were separated on a DNA capillary sequencer (3100 Genetic Analyzer; ABI).

### Cloning of the *FOXC1* Gene

In two patients, we observed a heterozygous 1-bp deletion of the *FOXC1* gene. The corresponding PCR fragments of the *FOXC1* gene were cloned in a cloning vector (TA; Invitrogen, Carlsbad, CA) to confirm the nature of the mutation. Ligation and cloning were performed in accordance with the manufacturer's protocol.

### Detection of Nucleotide Variants by PCR/RFLP

Missense mutations detected in this study were assessed by analysis of 100 normal control subjects (200 chromosomes) applying PCR/restriction fragment length polymorphism (RFLP) assays. The respective fragments harboring the missense mutations were amplified from the affected patients and from control subjects. An aliquot of each amplicon was digested with the appropriate restriction enzyme (New England Biotechnology, Beverly, MA). All restriction digests were analyzed on a 4% agarose gel.

The C→T transition at codon 64 (P64L) of *PITX2* results in the loss of a *NciI* restriction site. Screening for the P64R sequence variant in *PITX2* was performed by amplification of a PCR fragment using mismatch primers that introduce an *EcoO109I* restriction site in the mutant allele. The T→C transition at codon 115 (Y115S) of *FOXC1* results in the gain of an *SmaI* restriction site, whereas the G→C transversion at codon 149 (G149D) leads to the loss of a *TseI* site. The A→G transversion at codon 161 (M161V) results in the loss of an *NlaIII* recognition site. Screening for the P79R missense change in *FOXC1* was performed by amplification of a PCR fragment using mismatch primers that introduce an *NlaIV* restriction site in the mutant allele. Detailed PCR and RFLP protocols are available on request.

## RESULTS

A total of 19 patients within 13 families with anterior segment dysgenesis were screened for *FOXC1* and *PITX2* mutations. All

patients exhibited abnormal ocular findings demonstrating phenotypes including diagnoses of Rieger and Axenfeld anomalies. In several of the patients a wide variety of nonocular manifestations were noted, including dental and cardiac defects. The clinical features and mutations are summarized in Tables 3 and 4 and examples of the anterior segment phenotypes are shown in Figure 1.

Complete sequencing of the coding exons revealed seven sequence alterations in the single *FOXC1* exon as well as two nucleotide changes in exon 4 of *PITX2*. All the changes were present in the heterozygous state. Eight of the nine mutations we found are described for the first time (Table 4, Fig. 2).

Two different 1-bp deletions were detected in *FOXC1*: one at nucleotide position 738 (patient 1) and the other at position 1511 (patient 2). Both deletions represent frameshift mutations that are predicted to result in premature translation termination after another 68 and 15 amino acids, respectively. In both cases, the corresponding PCR fragment of *FOXC1* was cloned to obtain appropriate sequencing results of wild-type and mutant alleles (Fig. 3). One patient (patient 3) was found to harbor a C→A transversion at nucleotide position 143. This nucleotide change causes a nonsense mutation and thus a truncated protein (S48X) lacking the forkhead DNA-binding domain.

In addition, four patients were found to harbor mutations in the *FOXC1* gene leading to amino acid substitutions: a C→G transversion at nucleotide position 236, leading to a substitution of proline with arginine (P79R, patient 4); a T→C transition at nucleotide position 339, leading to a substitution of tyrosine with serine (Y115S, patients 5.1 and 5.2); a G→A transition at nucleotide position 446 that replaces glycine with aspartate (G149D, patient 6); and a G→A transition at nucleotide position 481 that replaces methionine with valine (M161V, patients 7.1 and 7.2). None of these four missense mutations was observed in 100 ethnically matched control subjects ( $n = 200$  chromosomes).

TABLE 2. Summary of *PITX2* Mutations Reported to Date

References	Nucleotide Change	Protein Change	
Semina et al. <sup>4</sup>	c.744T→A	Missense (L54Q)	
	c.785A→C	Missense (T68P)	
	c.855G→C	Missense (R91P)	
	c.981G→A	Nonsense (W133X)	
	IVS3(-11)A→G	Splice site	
	IVS3(+5)G→C	Splice site	
	Kulak et al. <sup>34</sup>	c.789G→A	Missense (R69H)
		c.833C→T	Missense (R84W)
	Alward et al. <sup>35</sup>	IVS3(-2)A→T	Splice site
	Doward et al. <sup>36</sup>	c.845A→G	Missense (K88E)
c.851C→T		Missense (R90C)	
Perveen et al. <sup>26</sup>	IVS2(-1)G→C	Splice site	
	c.1083insC	Nonsense	
	c.868-869delAA	Nonsense	
	c.939delA	Nonsense	
	c.1235-1236TA→AAG	Nonsense	
Priston et al. <sup>27</sup>	c.830G→C	Missense (V83L)	
	c.713-733dupl	Nonsense	
	c.1272delG	Nonsense	
Borges et al. <sup>28</sup>	c.774C→T	Missense (P64L)	
	c.852G→C	Missense (R90P)	
	c.896C→G	Missense (L105V)	
	c.906A→C	Missense (N108T)	
	c.717-720delACTT	Nonsense	
Wang et al. <sup>29</sup>	c.1261delT	Nonsense	
	c.697delG	Nonsense	
Brooks et al. <sup>30</sup>	IVS3(-1)G→T	Nonsense	
	c.998delC	Nonsense	
Lines et al. <sup>31</sup>	c.959delC	Nonsense	
	c.710C→T	Missense (R43W)	
Saadi et al. <sup>32</sup>			
Idrees et al. <sup>33</sup>			

TABLE 3. Clinical Data and Phenotypes of Subjects/Pedigrees with AR

Patient	Family History	IOP Max [mm Hg] (OD;OS)	Excavation of Optic Disk	BCVA (OD;OS)	Visual Field Defect* (OD;OS)	Corneal Changes	Ocular Findings	Dental Anomalies	Other Findings	Mutation
1	Glaucoma	23;23	-OU	20/20;20/20	0;0	-	PE OU, iridocorneal adhesions OU	-	Hypertelorism, umbilicus	FOXC1:L246fsx68
2	Glaucoma, AR	45;52	+OU	20/25;HM	3;4	Corneal opacity OS	IH OU	-	-	FOXC1:N503fsx15
3	-	50;44	+OD	20/60;20/20	3;0	Microcornea OD	PE OU, iridocorneal adhesions OU, corectopia OU	-	-	FOXC1:S48X
4	Glaucoma	40;55	+OU	20/80;20/200	4;2	-	Iridocorneal adhesions OU, IH OU	-	Micrognathia	FOXC1:P79R
5.1	Glaucoma, AR	50;60	+OU	PL;20/25	3;4	-	PE OU, IH OU, iridocorneal adhesions OU	-	Middle-ear deafness	FOXC1:Y115S
5.2	Glaucoma, AR	30;41	+OU	20/16;20/25	1;2	Megalocornea OU	PE OU, IH OU, iridocorneal adhesions OU	-	-	FOXC1:Y115S
6	AR	33;28	-OU	20/40;20/30	NA	-	PE OU, iridocorneal adhesions OU, corectopia OU	-	Heart defect, hypospadia	FOXC1:G149D
7.1	Glaucoma, AR	16;16	NA	NA	0;0	-	PE OU, IH OU, iridocorneal adhesions OU, corectopia OU	-	Umbilicus, middle-ear deafness	FOXC1:M161V
7.2	Glaucoma, AR	17;18	NA	20/20;20/20	0;0	Megalocornea OU	PE OU, IH OU, iridocorneal adhesions OU	-	-	FOXC1:M161V
8.1	Glaucoma, AR	36;20	-OU	20/20;20/30	2;0	-	PE OU, IH OU, iridocorneal adhesions OU	Hypodontia, microdontia	Micrognathia, umbilicus	PITX2:P64L
8.2	Glaucoma, AR	30;22	+OS	20/20;20/20	NA	-	PE OU, IH OU, iridocorneal adhesions OU	Microdontia	Umbilicus	PITX2:P64L
8.3	Glaucoma, AR	35;35	-OU	NA	0;0	-	PE OU, IH OU, iridocorneal adhesions OU	Hypodontia, microdontia	Micrognathia, umbilicus	PITX2:P64L
8.4	Glaucoma, AR	15;16	-OU	20/20;20/20	0;0	-	PE OU, IH OU, iridocorneal adhesions OU	Microdontia	Micrognathia, umbilicus	PITX2:P64L
8.5	Glaucoma, AR	16;16	-OU	20/20;20/20	0;0	-	PE OU, IH OU, iridocorneal adhesions OU, corectopia OU	Hypodontia, microdontia	Umbilicus	PITX2:P64L
9	-	33;20	+OU	HM;20/40	NA;0	-	IH OU	Hypodontia, microdontia	Micrognathia, umbilicus	PITX2:P64R
10	-	30;32	+OU	PL;20/50	NA	-	PE OU, iridocorneal adhesions OU, corectopia OU	-	Hypertelorism, heart defect, middle-ear deafness	None
11	AR	21;21	-OU	20/40;20/50	NA	Corneal opacity OU	PE OU, iridocorneal adhesions OU	-	-	None
12	Glaucoma	28;28	+OU	20/20;20/30	0;0	-	PE OU, IH OU	Hypodontia	-	None
13	Glaucoma	NA	+OU	20/16;20/16	0;2	-	Iridocorneal adhesions OU, IH OU	Hypodontia	-	None

BCVA, best corrected visual acuity; +, present; -, absent; PE, posterior embryotoxon; IH, iris hypoplasia; PL, perception of light; HM, hand movements; NA, not available.  
 \* According to Aulhorn classification.<sup>38</sup>

TABLE 4. Summary of *FOXC1* and *PITX2* Mutations

Patient	Nucleotide Change	Predicted Amino Acid Alteration	Present in 100 Controls	Reported Before
<i>FOXC1</i>				
1	c.738delG	L246fsx68	NA	No
2	c.1511delT	N503fsx15	NA	No
3	c.143C→A	S48X	NA	No
4	c.236C→G	P79R	No	No
5	c.339T→C	Y115S	No	No
6	c.446G→A	G149D	No	No
7	c.481A→G	M161V	No	No
<i>PITX2</i>				
8	c.774C→T	P64L	No	Philips <sup>39</sup>
9	c.774C→G	P64R	No	No

NA, not analyzed.

Mutation screening also revealed two sequence variants in the *PITX2* gene. Five members of a family with AR (patients 8.1 to 8.5) were found to harbor a C→T transition at nucleotide position 774. This missense mutation P64L in the homeodomain of *PITX2* has already been described before<sup>39</sup> and was found to segregate in the family (Fig. 4). Of note, we also identified another missense mutation at the same nucleotide position, but in this case (patient 9) we observed a C→G substitution that causes a proline-to-arginine transversion (P64R). Both mutations were excluded in 100 ethnically matched control subjects. In patients 10 to 13 sequencing revealed no changes either in the *FOXC1* or in the *PITX2* gene.

## DISCUSSION

Mutations in dominantly inherited disorders may be pathogenic either due to haploinsufficiency or because of dominant-negative effects. Nonsense or frameshift mutations result in truncated and therefore nonfunctional proteins. The disease-causing effects of such mutations are readily understandable. The effect of missense mutations, in contrast, depends on their location and the functional effect of the exchange. That may range from subtle effects to functionally null mutations, but may be even more deleterious than nonsense or frameshift mutations by provoking a dominant-negative effect in case the affected protein does oligomerize or interact with DNA or

other proteins. In our screening of patients with AR we were able to identify nonsense and frameshift as well as missense mutations. Altogether, we found nine different mutations in *FOXC1* and *PITX2*, eight of which were novel and are described for the first time.

The two 1-bp deletions and the stop codon mutation found in the *FOXC1* gene are predicted to result in truncated proteins and are consistent with haploinsufficiency as the underlying cause of AR, whereas the four missense mutations of highly conserved amino acids could impair DNA binding and, possibly, nuclear localization of the *FOXC1* protein. Two of the four missense mutations—Y115S and M161V—were shown to segregate in the corresponding families: Patient 5.1 has two daughters, one of them affected (patient 5.2). The latter also carries the Y115S mutation, whereas the unaffected daughter is homozygous for the wild-type allele (data not shown). Patient 7.1 has an affected daughter (patient 7.2) harboring the same mutation (M161V). The possible pathogenicity of this mutation is supported by the former report of an Indian family<sup>16</sup> showing a T→A transversion at nucleotide position 482 with subsequent replacement of methionine with lysine at position 161.

For the P79R and G149D missense changes identified in our study, a family history was reported, but other family members were not available for clinical or genetic analysis. Of note, a former study<sup>13</sup> reported a patient with Rieger syndrome harboring a C→T transition at nucleotide position 236, resulting in a substitution of lysine for proline at codon 79. Another patient in an independent report was found to demonstrate a C→A transversion at nucleotide position 235, subsequently replacing the proline residue with threonine.<sup>15</sup> This is, to the best of our knowledge, the first reported instance of a *FOXC1* mutation being found with three different mutation events at the same codon position.

Missense mutations that affect Y115 and G149 in the fork-head domain of *FOXC1* have not been reported before. However, a multiple sequence alignment of different FOX family members shows considerable conservation of these residues, indicating functional importance (Fig. 2A). For two of the missense mutations we identified—P79R and M161V—functional data of amino acid exchanges are available, although not for the same substitutions we observed. The biochemical analysis of a mutant *FOXC1* protein harboring a replacement of methionine with lysine at amino acid position 161 has revealed a reduction in the ability of DNA binding, suggesting an essential role of the methionine residue for normal DNA binding.<sup>20</sup> The functional consequences of substitutions of the proline residue at amino acid position 79 have been extensively studied. It has been shown that P79L and P79T, both having been observed in patients with AR,<sup>13,15</sup> impair protein localization

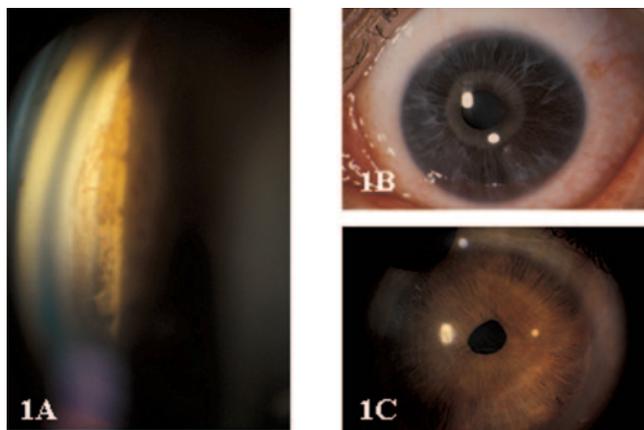
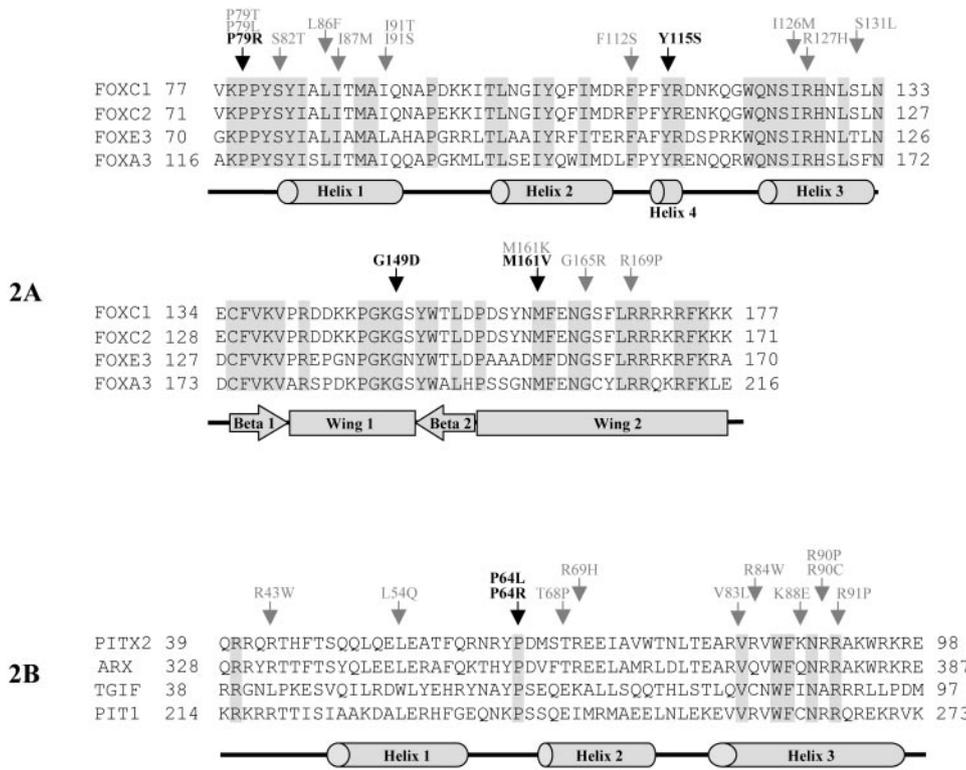


FIGURE 1. Typical ocular phenotypes of patients described in the study. (A) Gonioscopic appearance of iridocorneal adhesions extending from a prominent Schwalbe's line to the peripheral iris in patient 3. (B) Patient 8.5 showed a hypoplastic iris revealing the underlying pupillary sphincter muscle. (C) External view of mild corectopia and hypoplasia of the iridic stroma in patient 3.



**FIGURE 2.** Protein sequence alignments of FOXC1 and PITX2 transcription factor family members and location of missense mutations identified in the DNA-binding domains. (A) Forkhead domains of human FOXC1 and related human FOX protein sequences. (B) Homeodomain alignment of human PITX2 and related human homeodomain-containing proteins. *Shaded boxes:* conserved residues among protein family members. Amino acid substitutions identified in this study are shown in *bold*. Protein secondary structures were predicted by Swiss PdbViewer (<http://www.expasy.org/> provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland).

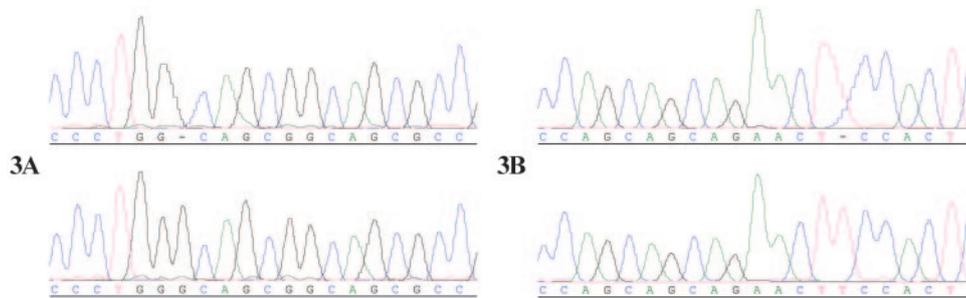
and transactivation capability of the protein whereas a P79K substitution had a less severe effect in vitro, reaching two thirds of the wild-type level in a transactivation assay.<sup>40</sup> However, as this mutation has not been reported in patients with Axenfeld-Rieger malformation, no clinical data are available to judge this particular substitution. The proline-to-arginine substitution found in patient 4 in our study also introduces a positively charged amino acid residue at this site, implying a similar effect. Yet, because this patient shows a phenotype rather typical for Axenfeld-Rieger malformation it seems that this proline-to-arginine substitution has a more deleterious effect than was implied in the in vitro assay with the similar proline-to-lysine substitution.

Taken together, we have several facts that indicate the pathogenicity of the four missense mutations we found in the *FOXC1* gene: Two mutations (Y115S and M161V) were shown to segregate in the corresponding families; two altered amino acid positions (79 and 161) had been reported to be essential for proper protein activity; each of the four is located in the DNA binding domain and affects conserved residues and furthermore was excluded in 100 ethnically matched control subjects.

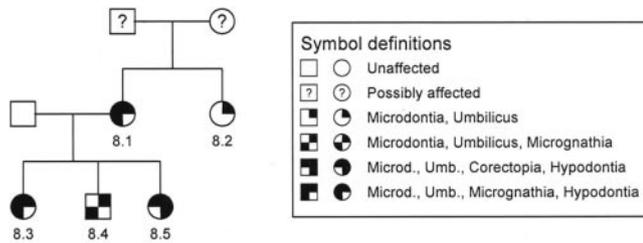
Sequence analysis of the *PITX2* gene demonstrated two sequence changes within the homeodomain. Patients 8.1 to 8.5 from one pedigree exhibited a C→T transition at nucleotide

position 774, changing proline to leucine in codon 64 of the protein. This sequence change has been reported before in a pedigree with Axenfeld-Rieger.<sup>39</sup> In our pedigree, the five affected mutation carriers show a certain degree of phenotypic variability (Fig. 4), only the Axenfeld phenotype and the umbilical abnormalities seem to be highly penetrant. On the contrary, the dental phenotype varies among the family, with only some members showing hypodontia or micrognathia, although all members exhibit microdontia. It is also noteworthy that two of the younger family members show intraocular pressures that are within the lower range of the spectrum. The reason for this intrafamilial variation is unclear, but phenotypic variability has been described for *PITX2* mutations.<sup>4,26,34,35</sup> One might speculate that the phenotypic outcome in patients with Axenfeld-Rieger is also dependent on additional genetic and environmental influences.

Of note, we found another individual (patient 9) to harbor a nucleotide transversion at nucleotide position 774, but in this case a C→G transversion, causing the predicted missense change P64R. This particular change has not been reported yet. Experimental data show that mutant PITX2 proteins that harbor missense mutations in their homeodomain demonstrated reduced or even abolished DNA binding.<sup>41</sup> Amino acid position 64 is located in the loop between helices 1 and 2 of the homeodomain (Fig. 2B) and is conserved considerably



**FIGURE 3.** Sequence analysis of patients with *FOXC1* deletions (*top row:* mutant allele, *bottom row:* wild-type allele). (A) c.738delG (B) c.1511delT.



**FIGURE 4.** Phenotypic variability in a family with the *PITX2* missense change P64L (patients 8.1 to 8.5). All family members exhibited posterior embryotoxon, iris hypoplasia, and iridocorneal adhesions.

between homeodomain-containing proteins.<sup>42</sup> Amino acid changes at this particular residue have been shown to have a disease-causing effect in the case of the homeodomain transcription factors ARX (x-linked myoclonic epilepsy),<sup>43</sup> TGIF (holoprosencephaly),<sup>44</sup> and PIT1 (pituitary hormone deficiency).<sup>45</sup> Because of their rigid structure, proline residues provoke the orientation of helices and are therefore often found in loops. It may be that a replacement of this proline residue with leucine or arginine changes the orientation of the first and third helices and subsequently alters the stability of the homeodomain.<sup>46</sup> Because the P64L missense mutation has been reported before in a pedigree with Axenfeld-Rieger and was not present in 100 ethnically matched control subjects, we consider it to be pathogenic, as well as the P64R mutation. It was striking that all our patients harboring *PITX2* mutations exhibited a dental phenotype, but none of the patients who carried a *FOXC1* mutation had dental anomalies. This result is consistent with those of previous studies showing that mutations in the *PITX2* gene appear to be more strongly associated with dental findings than mutations in the *FOXC1* gene.<sup>3,4</sup> Earlier studies have shown that *PITX2* activates the *DLX2* gene which is required for tooth and craniofacial development.<sup>24</sup>

In several cases (e.g., patients 2 and 5.1), significant differences were noted in visual acuity between the two eyes due to unilateral glaucoma. This asymmetry may reflect the fact that *PITX2* has been shown to be associated with genes involved in lateralization<sup>47-49</sup> and in turn apparently interacts with *FOXC1*.<sup>37</sup> It remains to be established whether this left-right difference is related to the functional importance of *PITX2* in lateralization processes during development. Mutations in either of both genes may be responsible for the differences between the two sides in ocular development; however, a functional connection between the two gene products and laterality in the eye has not been reported.

In our cohort of patients with AR malformations, we were able to identify the potential disease-causing mutation in 70% of the index cases. This result is above the common calculation that mutations in *FOXC1* and *PITX2* are responsible for approximately 35% of cases.<sup>50</sup> Taking into account that our screening protocol only applied sequencing, which does not reveal gross insertions or duplications, the prevalence of mutations in both genes may be even higher in our patient cohort. Nevertheless, the high prevalence of mutations in both genes may be accidental due to the relatively small number of patients; therefore, it seems likely that additional genes with yet unknown function in anterior segment development contribute to the spectrum of AR malformations.

### Acknowledgments

The authors thank Ulrich Schiefer and all participating assistant doctors of the glaucoma special ambulance unit of the University Eye Hospital Tübingen for substantial help with sample collection.

### References

- Shields MB, Buckley E, Klintworth GK, Thresher R. Axenfeld-Rieger Syndrome: a spectrum of developmental disorders. *Surv Ophthalmol.* 1985;29:387-409.
- Gorlin RJ, Pindborg J, Cohen MM. Syndromes of the head and neck. In: *Syndromes with Unusual Dental Findings*. New York: McGraw-Hill; 1976:649-651.
- Nishimura DY, Swiderski RE, Alward WL, et al. The forkhead transcription factor gene *FKHL7* is responsible for glaucoma phenotypes that map to 6p25. *Nat Genet.* 1998;19:140-147.
- Semina EV, Reiter R, Leysens NJ, et al. Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, *RIEG*, involved in Rieger syndrome. *Nat Genet.* 1996;14:392-399.
- Phillips JC, del Bono EA, Haines JL, et al. A second locus for Rieger syndrome maps to chromosome 13q14. *Am J Hum Genet.* 1996;59:613-619.
- Riise R, Storhaug K, Brondum-Nielsen K. Rieger syndrome is associated with *PAX6* deletion. *Acta Ophthalmol Scand.* 2001;79:201-203.
- Katoh M, Katoh M. Human *FOX* gene family. *Int J Oncol.* 2004;25:1495-1500.
- Sasai N, Mizuseki K, Sasai Y. Requirement of *FoxD3*-class signaling for neural crest determination in *Xenopus*. *Development.* 2001;128:2525-2536.
- Kaufmann E, Knochel W. Five years on the wings of fork head. *Mech Dev.* 1996;57:3-20.
- Mears AJ, Jordan T, Mirzayans F, et al. Mutations of the forkhead/winged-helix gene, *FKHL7*, in patients with Axenfeld-Rieger anomaly. *Am J Hum Genet.* 1998;63:1316-1328.
- Swiderski RE, Reiter RS, Nishimura DY, et al. Expression of the *Mf1* gene in developing mouse hearts: implication in the development of human congenital heart defects. *Dev Dyn.* 1999;216:16-27.
- Mirzayans F, Gould DB, Heon E, et al. Axenfeld-Rieger syndrome resulting from mutation of the *FKHL7* gene on chromosome 6p25. *Eur J Hum Genet.* 2000;8:71-74.
- Nishimura DY, Searby CC, Alward WL, et al. A spectrum of *FOXC1* mutations suggests gene dosage as a mechanism for developmental defects of the anterior chamber of the eye. *Am J Hum Genet.* 2001;68:364-372.
- Kawase C, Kawase K, Taniguchi T, et al. Screening for mutations of Axenfeld-Rieger syndrome caused by *FOXC1* gene in Japanese patients. *J Glaucoma.* 2001;10:477-482.
- Suzuki T, Takahashi K, Kuwahara S, Wada Y, Abe T, Tamai M. A novel (Pro79Thr) mutation in the *FKHL7* gene in a Japanese family with Axenfeld-Rieger syndrome. *Am J Ophthalmol.* 2001;132:572-575.
- Panicker SG, Sampath S, Mandal AK, Reddy AB, Ahmed N, Hasnain SE. Novel mutation in *FOXC1* wing region causing Axenfeld-Rieger anomaly. *Invest Ophthalmol Vis Sci.* 2002;43:3613-3616.
- Saleem RA, Murphy TC, Liebmann JM, Walter MA. Identification and analysis of a novel mutation in the *FOXC1* forkhead domain. *Invest Ophthalmol Vis Sci.* 2003;44:4608-4612.
- Komatireddy S, Chakrabarti S, Mandal AK, et al. Mutation spectrum of *FOXC1* and clinical genetic heterogeneity of Axenfeld-Rieger anomaly in India. *Mol Vis.* 2003;9:43-48.
- Mortemousse B, Amati-Bonneau P, Couture F, et al. Axenfeld-Rieger anomaly: a novel mutation in the forkhead box C1 (*FOXC1*) gene in a 4-generation family. *Arch Ophthalmol.* 2004;122:1527-1533.
- Murphy TC, Saleem RA, Footz T, Ritch R, McGillivray B, Walter MA. The wing 2 region of the *FOXC1* forkhead domain is necessary for normal DNA-binding and transactivation functions. *Invest Ophthalmol Vis Sci.* 2004;45:2531-2538.
- Lehmann OJ, Ebenezer ND, Jordan T, et al. Chromosomal duplication involving the forkhead transcription factor gene *FOXC1* causes iris hypoplasia and glaucoma. *Am J Hum Genet.* 2000;67:1129-1135.
- Lehmann OJ, Ebenezer ND, Ekong R, et al. Ocular developmental abnormalities and glaucoma associated with interstitial 6p25 duplications and deletions. *Invest Ophthalmol Vis Sci.* 2002;43:1843-1849.

23. Hjalt TA, Semina EV, Amendt BA, Murray JC. The Pitx2 protein in mouse development. *Dev Dyn*. 2000;218:195-200.
24. Espinoza HM, Cox CJ, Semina EV, Amendt BA. A molecular basis for differential developmental anomalies in Axenfeld-Rieger-Syndrome. *Hum Mol Genet*. 2002;11:743-753.
25. Evans AL, Gage PJ. Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. *Hum Mol Genet*. 2005;14:3347-3359.
26. Perveen R, Lloyd IC, Clayton-Smith J, et al. Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. *Invest Ophthalmol Vis Sci*. 2000;41:2456-2460.
27. Priston M, Kozlowski K, Gill D, et al. Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. *Hum Mol Genet*. 2001;10:1631-1638.
28. Borges AS, Susanna R Jr, Carani JC, et al. Genetic analysis of PITX2 and FOXC1 in Rieger Syndrome patients from Brazil. *J Glaucoma*. 2002;11:51-56.
29. Wang Y, Zhao H, Zhang X, Feng H. Novel identification of a four-base-pair deletion mutation in PITX2 in a Rieger Syndrome family. *J Dent Res*. 2003;82:1008-1012.
30. Brooks BP, Moroi SE, Downs CA, et al. A novel mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome. *Ophthalmic Genet*. 2004;25:57-62.
31. Lines MA, Kozlowski K, Kulak SC, et al. Characterization and prevalence of PITX2 microdeletions and mutations in Axenfeld-Rieger malformations. *Invest Ophthalmol Vis Sci*. 2004;45:828-833.
32. Saadi I, Toro R, Kuburas A, Semina E, Murray JC, Russo AF. An unusual class of PITX2 mutations in Axenfeld-Rieger syndrome. *Birth Defects Res A Clin Mol Teratol*. 2006;76:175-181.
33. Idrees F, Bloch-Zupan A, Free SL, et al. A novel homeobox mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome associated with brain, ocular, and dental phenotypes. *Am J Med Genet B Neuropsychiatr Genet*. 2006;141:184-191.
34. Kulak SC, Kozlowski K, Semina EV, Pearce WG, Walter MA. Mutation in the RIEG1 gene in patients with iridogoniodysgenesis syndrome. *Hum Mol Genet*. 1998;7:1113-1117.
35. Alward WL, Semina EV, Kalenak JW, et al. Autosomal dominant iris hypoplasia is caused by a mutation in the Rieger syndrome (RIEG/PITX2) gene. *Am J Ophthalmol*. 1998;125:98-100.
36. Doward W, Perveen R, Lloyd IC, Ridgway AE, Wilson L, Blank GC. A mutation in the RIEG1 gene associated with Peters' anomaly. *J Med Genet*. 1999;36:152-155.
37. Berry FB, Lines MA, Oas JM, Footz T, Underhill DA, Gage PJ, et al. Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Hum Mol Genet*. 2006;15:905-919.
38. Aulhorn E, Karmeyer H. Frequency distribution in early glaucomatous visual field defects. *Doc Ophthalmol Proc Ser*. 1977;14:75-83.
39. Phillips JC. Four novel mutations in the PITX2 gene in patients with Axenfeld-Rieger syndrome. *Ophthalmic Res*. 2002;34:324-326.
40. Saleem RA, Banerjee-Basu S, Murphy TC, Baxevanis A, Walter MA. Essential structural and functional determinants within the forkhead domain of FOXC1. *Nucleic Acids Res*. 2004;32:4182-4193.
41. Kozlowski K, Walter MA. Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. *Hum Mol Genet*. 2000;9:2131-2139.
42. Chi Y-I. Homeodomain revisited: a lesson from disease-causing mutations. *Hum Genet*. 2005;116:433-444.
43. Stromme P, Sundet K, Mork C, Cassiman JJ, Fryns J-P, Claes S. X linked mental retardation and infantile spasms in a family: new clinical data and linkage to Xp11.4-Xp22.11. *J Med Genet*. 1999;36:374-378.
44. Gripp KW, Wotton D, Edwards MC, et al. Mutations in TGIF cause holoprosencephaly and link NODAL signalling to human neural axis determination. *Nat Genet*. 2000;25:205-208.
45. Pernasetti F, Milner RDG, Al Ashwal AAZ, et al. Pro239ser: a novel recessive mutation of the Pit-1 gene in seven Middle Eastern children with growth hormone, prolactin, and thyrotropin deficiency. *J Clin Endocr Metab*. 1998;83:2079-2083.
46. Chaney BA, Clark-Baldwin K, Dave V, Ma J, Rance M. Resolution structure of the K50 class homeodomain PITX2 bound to DNA and implications for mutations that cause Rieger syndrome. *Biochemistry*. 2005;44:7497-7511.
47. Yoshioka H, Meno C, Koshiha K, et al. Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell*. 1998;94:299-305.
48. Logan M, Pagan-Westphal SM, Smith DM, Paganessi L, Tabin CJ. The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell*. 1998;94:307-317.
49. Piedra ME, Icardo JM, Albajar M, Rodriguez-Rey JC, Ros MA. Pitx2 participates in the late phase of the pathway controlling left-right asymmetry. *Cell*. 1998;94:319-324.
50. Walter MA. PITs and FOXes in ocular genetics: the Cogan lecture. *Invest Ophthalmol Vis Sci*. 2003;44:1402-1405.