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

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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

Axenfeld-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, micrrodontia, umbilical abnormalities, hearing defects, and congenital cardiac or kidney abnormalities.[1] 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.[2] Until now, four genetic loci have been associated with AR, including the genes *FOXC1* and *PITX2* located on 6p25 and 4q25, respectively.[3,4] A third locus was mapped to 13q14, but the gene has not yet been identified.[5] In addition, an isolated case of Rieger syndrome has been reported to harbor a deletion in the *PAX6* gene.[6]

FOXC1 belongs to the forkhead family of transcription factors which comprises at least 43 members[7] that act as critical regulators of embryogenesis, cell migration, and cell differentiation.[8,9] Mutations of the *FOXC1* gene have been identified as the underlying cause in a variety of anterior segment disorders.[3,10–20] 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.[13,21,22]

PITX2 encodes a bicoid-like homeodomain transcription factor and is expressed very early during tooth development.[23] 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,[24] and it has been shown that expression of *PITX2* in the neural crest is also necessary for optic stalk and anterior segment development.[25] To date, 30 mutations of the *PITX2* gene have been associated with AR[4,26–33] and other cases of anterior segment malformations, such as iridogoniodysgenesis,[34] iris hypoplasia,[35] and Peters' anomaly[36] (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.[37]

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

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

References	Nucleotide Change	Protein Change
Nishimura et al.[3]	c.153-163del	Nonsense
	c.335T→C	Missense (F112S)
	c.378C→G	Missense (I126M)
	c.392C→T	Missense (S131L)
Mears et al.[10]	c.93-102del	Nonsense
	c.245G→C	Missense (S82T)
	c.261C→G	Missense (I87M)
Swiderski et al.[11]	c.210delG	Nonsense
Mirzayans et al.[12]	c.67C→T	Nonsense (Q23X)
Nishimura et al.[13]	c.99-108del	Nonsense
	c.116-123del	Nonsense
	c.1512del	Nonsense
	c.265insC	Nonsense
	c.26-47ins	Nonsense
	c.236C→T	Missense (P79L)
Kawase et al.[14]	c.286insG	Nonsense
	c.272T→G	Missense (I91S)
	c.380G→A	Missense (R127H)
Suzuki et al.[15]	c.235C→A	Missense (P79T)
Panicker et al.[16]	c.482T→A	Missense (M161K)
Saleem et al.[17]	c.255GC→TT	Missense (L86F)
Komatireddy et al.[18]	c.4C→T	Nonsense (Q2X)
	c.367C→T	Nonsense (Q123X)
Mortemousque et al.[19]	c.272T→C	Missense (I91T)
Murphy et al.[20]	c.494G→C	Missense (G165R)
	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 *Nci*I restriction site. Screening for the P64R sequence variant in *PITX2* was performed by amplification of a PCR fragment using mismatch primers that introduce an *Eco*O109I restriction site in the mutant allele. The T→C transition at codon 115 (Y115S) of *FOXC1* results in the gain of an *Sma*I restriction site, whereas the G→C transversion at codon 149 (G149D) leads to the loss of a *Tse*I site. The A→G transversion at codon 161 (M161V) results in the loss of an *Nla*III recognition site. Screening for the P79R missense change in *FOXC1* was performed by amplification of a PCR fragment using mismatch primers that introduce an *Nla*IV 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.[4]	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.[34]	c.789G→A	Missense (R69H)
Alward et al.[35]	c.833C→T	Missense (R84W)
Doward et al.[36]	IVS3(−2)A→T	Splice site
Perveen et al.[26]	c.845A→G	Missense (K88E)
	c.851C→T	Missense (R90C)
	IVS2(−1)G→C	Splice site
	c.1083insC	Nonsense
	c.868-869delAA	Nonsense
	c.939delA	Nonsense
	c.1235-1236TA→AAG	Nonsense
Priston et al.[27]	c.830G→C	Missense (V83L)
	c.713-733dupl	Nonsense
Borges et al.[28]	c.1272delG	Nonsense
Philips[39]	c.774C→T	Missense (P64L)
	c.852G→C	Missense (R90P)
	c.896C→G	Missense (L105V)
	c.906A→C	Missense (N108T)
Wang et al.[29]	c.717-720delACTT	Nonsense
Brooks et al.[30]	c.1261delT	Nonsense
Lines et al.[31]	c.697delG	Nonsense
	IVS3(−1)G→T	Nonsense
	c.998delC	Nonsense
Saadi et al.[32]	c.959delC	Nonsense
Idrees et al.[33]	c.710C→T	Missense (R43W)

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	−	Hypertelorismus, 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, taurodontism	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	−	Hypertelorismus, 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.[38]

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

Patient	Nucleotide Change	Predicted Amino Acid Alteration	Present in 100 Controls	Reported Before
FOXC1				
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
PITX2				
8	c.774C→T	P64L	No	Philips[39]
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[39] 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.

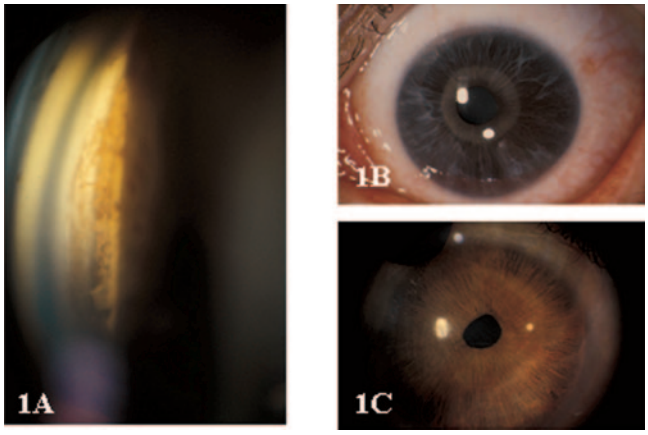

FIGURE 1. Typical ocular phenotypes of patients described in the study. (**A**) Gonioscopic appearance of iridocorneal adhesions extending from a prominent Schwalbe's line to the peripheral iris in patient 3. (**B**) Patient 8.5 showed a hypoplastic iris revealing the underlying pupillary sphincter muscle.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

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[16] 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[13] reported a patient with Rieger syndrome harboring a C→T transition at nucleotide position 236, resulting in an 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.[15] 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 forkhead 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.[20] 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,[13,15] impair protein localization

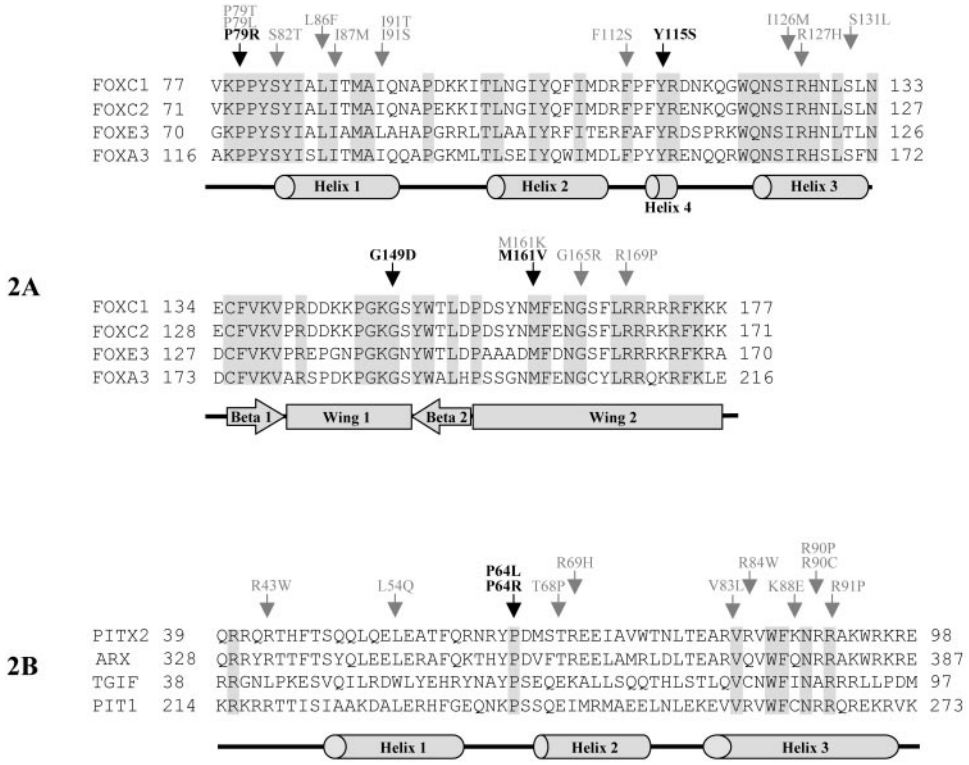

FIGURE 2. Protein sequence alignments of FOXC1 and PITX2 transcription factor family members and location of missense mutations identified in the DNA-binding domains. (**A**) Forkhead domains of human FOXC1 and related human FOX protein sequences. (**B**) Homeodomain alignment of human PITX2 and related 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.[40] 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.[39] 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.[4,26,34,35] 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.[41] Amino acid position 64 is located in the loop between helices 1 and 2 of the homeodomain (Fig. 2B) and is conserved considerably

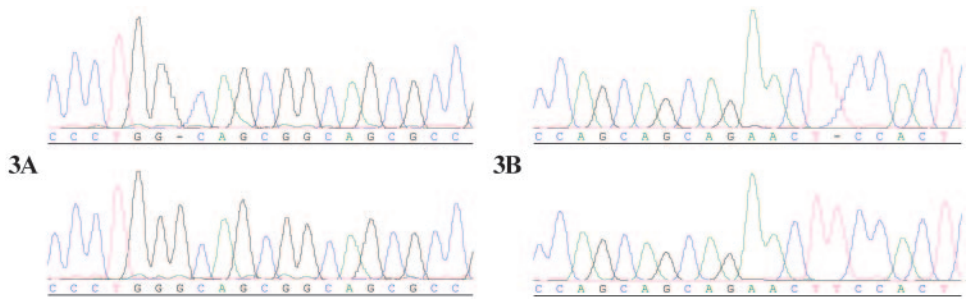

FIGURE 3. Sequence analysis of patients with *FOXC1* deletions (*top row*: mutant allele, *bottom row*: wild-type allele). (**A**) c.738delG (**B**) c.1511delT.

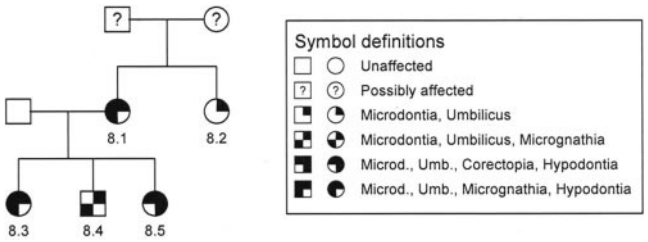

FIGURE 4. Phenotypic variability in a family with the *PITX2* missense change P64L (patients 8.1 to 8.5). All family members exhibited posterior embryotoxon, iris hypoplasia, and iridocorneal adhesions.

between homeodomain-containing proteins.[42] 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),[43] TGIF (holoprosencephaly),[44] and PIT1 (pituitary hormone deficiency).[45] 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.[46] 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.[3,4] Earlier studies have shown that PITX2 activates the *DLX2* gene which is required for tooth and craniofacial development.[24]

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[47–49] and in turn apparently interacts with FOXC1.[37] 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.[50] 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.

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