Identification of four new PITX2 gene mutations in patients with Axenfeld-Rieger syndrome

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Purpose: Axenfeld Rieger syndrome (ARS) is an autosomal dominant inherited disorder affecting development of the ocular anterior chamber, abdomen, teeth and facial structures. The PITX2 gene is a major gene encoding a major transcription factor associated with ARS.

Methods: ARS patients were collected from six unrelated families. Patients and their families were ophthalmologically phenotyped and their blood was collected for DNA extraction. We screened the coding region of human PITX2 gene by direct sequencing. The consequences of the mutations described were investigated by generating crystallographic representations of the amino acid changes. In order to better understand the occurrence of glaucoma in ARS patients, we studied the PITX2 gene expression in human embryonic and fetal ocular tissue sections.

Results: We identified four novel PITX2 genetic alterations in four unrelated families with ARS. These mutations included two nonsense mutations (E55X and Y121X), an eight nucleotides insertion (1251 ins CGACTCCT) and a substitution (F58L), in familial and sporadic cases of ARS. We also showed for the first time that PITX2 is expressed at early stages of the human embryonic and fetal pericocular mesenchyme, as well as at later stages of human development in the fetal ciliary body, ciliary processes, irido corneal angle and corneal endothelium. The human fetal eye PITX2 gene expression pattern reported here for the first time provides a strong basis for explaining the frequent occurrence of glaucoma in patients affected by PITX2 gene mutations.

Conclusions: Two mutations identified affect the homeodomain (E55X and F58L). The E55X nonsense mutation is likely to alter dramatically the DNA-binding capabilities of the PITX2 homeodomain. Furthermore, there is a complete loss of the carboxy-terminal part of the PITX2 protein beyond the site of the mutation. The phenylalanine F58 is known to contribute to the hydrophobic network of the homeodomain. The crystallographic representations of the mutation F58L show that this mutation may change the conformation of the helical core. The F58L mutation is very likely to modify the homeodomain conformation and probably alters the DNA binding properties of PITX2. The other mutations (Y121X and the eight-nucleotide insertion (1251 ins CGACTCCT) CGACTCCT, at position 224 in PITX2A) result in partial loss of the C-terminal domain of PITX2. Pitx2 synergistically transactivates the prolactin promoter in the presence of the POU homeodomain protein Pit-1. Pitx2 activity is regulated by its own C-terminal tail. This region contains a highly conserved 14-amino-acid element involved in protein-protein interactions. The C-terminal 39-amino-acid tail represses DNA binding activity and is required for Pitx2 interactions with other transcription factors, for Pitx2-Pit-1 interaction and Pit1 syner gism. Pit-1 interaction with the Pitx2 C terminus masks the inhibitory effect and promotes increased DNA binding activity. Thus, the partial or complete loss of the C terminus tail can lead to decreased or absent DNA binding activity and trigger severe ARS phenotypes. Our in situ hybridization results obtained on human embryonic and fetal ocular tissue sections constitute the first molecular histological data providing an explanation for the occurrence of precocious glaucoma in human patients affected by ARS caused by PITX2 mutations. Further structural and biochemical studies are needed for understanding the wide spectrum of clinical phenotypes caused by the increasing number of new PITX2 mutations found in ARS affected patients.

Rieger syndrome displays complete penetrance, but variable expressivity. Despite originally being described as separate clinical entities, Rieger syndrome, Axenfeld syndrome, Axenfeld anomaly and Rieger anomaly are now considered to be variations of a single developmental disorder, Axenfeld-Rieger syndrome (ARS). Rieger syndrome is typically char-
Patrick E. Poole, John L. Vichinsky, and S. Rex comfortably of defects of the eyes, teeth and abdomen. The ocular disorders consist of bilateral congenital abnormalities of the anterior segment of the eye [1]. In contrast, Rieger’s anomaly consists exclusively of a congenital cleavage syndrome of the ocular anterior chamber. Ocular findings in Rieger’s syndrome include the prominent anterior displacement of Schwalbe’s line (posterior embryotoxon), peripheral iris strands extending to Schwalbe’s line, and iris thinning with atrophic holes. If the iris is normal, the condition is described as Axenfeld’s anomaly. If iris defects are present (including polyphoria) it is classified as Rieger’s anomaly. Iris findings in Rieger’s anomaly may range from mild hypoplasia to full-thickness hole formation. Glaucoma develops in approximately 50% to 60% of patients with Axenfeld-Rieger syndrome [2,3]. Dental abnormalities are a second characteristic feature of Rieger’s syndrome including hypodontia, microodontia or anodontia. Patients also present a flat midface, due to maxillary hypoplasia. Rieger’s syndrome may also be associated with excessive periumbilical skin and protrusion of the umbilical stump.

Axenfeld-Rieger syndrome (OMIM 180500) is an autosomal dominant genetic heterogeneous disorder. Rieger’s syndrome has been linked cytogenetically and/or genetically to five chromosomal loci: 4q25 (OMIM 601562) [4], 6p25 (OMIM 601090) [5], 13q14 (OMIM 601499) [6], 16q [7] and 11p13 (OMIM 607108) [8]. Positional cloning of the 4q25 region identified the PITX2 gene [1]. The 6p25 locus contains the FOXC1 gene (forkhead-like transcription factor), mutations of which also cause ARS [5]. For the third locus 11q13, a small deletion in the gene encoding PAX6 has been identified in one ARS patient [8]. Many disorders sharing the clinical signs with ARS have been associated with the chromosomal 16q24 region [7] but the gene has yet to be identified. However, approximately 40% of ARS cases cannot be attributed to chromosomal aberrations or gene mutations known to be associated with ARS [9,10]. Consequently, it is possible that several genes of which the mutation may cause ARS remain to be discovered.

The PITX2 gene consists of seven exons [11] and encodes a protein of the bicoid-like homeobox transcription factor family. PITX2 is a 33 kDa protein containing a 60-amino acid homeodomain with a lysine at residue 50 characteristic of the bicoid-related proteins [1,12]. Four human PITX2 isoforms have been described. PITX2A, PITX2B and PITX2C differ at the N-terminus but contain the full 60 amino acid homeodomain while the PITX2D isoform has a truncated nonfunctional homeodomain [11]. PITX2A and PITX2B are generated by alternative splicing mechanisms, whereas PITX2C and PITX2D use an alternative promoter. All isoforms include a protein of the bicoid-like homeobox transcription factor family.

METHODS

Patients: Blood samples were collected from six unrelated patients and their families after informed consent forms were signed, according to the Bioethics Laws of the European Union and France, and according to the Helsinki Declaration. The six patients came from four unrelated families. The families were recruited in the département d’ophtalmologie, centre hospitalier Necker-Enfants Malades and the Institut de Recherches en Ophtalmologie (Suisse). This research project was approved by a CCPRB and by the “Direction de la Recherche Clinique de l’Assistance Publique-Hôpitaux de Paris” in the framework of a National Hospital Program of Clinical Research on Inherited Genetic Diseases of the Eye. All affected patients presented the four cardinal features of ARS: ocular anterior dysgenesis, excessive protrusion of the periumbilical skin, dental abnormalities, and facial malformations. Hormonal tests to assess the functions of the diverse populations of pituitary cells were performed in all the patients included in this study. These tests consisted in the measurements of the plasmatic concentrations of adrenocorticotropic-hormone (ACTH), cortisol, prolactin, growth hormone (GH), thyroid-stimulating hormone (TSH), tri-iodothyronin (T3) and thyroxin (T4), follicle-stimulating hormone (FSH), luteinizizing hormone (LH), estradiol, progesterone, and various androgen derivatives. These tests were performed in all family members, patients and participating family included in this study. No patient included in this study presented any pituitary, heart, lung, nor central nervous system abnormality. Only one patient (BI11) displayed anal atresia. We also screened a control group of 100 unrelated ethnically matched individuals who had no form of ARS and who had no family history of congenital eye abnormality.

Mutation analysis: Genomic DNA was isolated from peripheral blood lymphocytes by proteinase K extraction protocol [13], and each of the exons of the PITX2 gene was amplified by PCR, using specific oligonucleotide primers (available upon request). Exons 1 and 6 were subdivided into two overlapping fragments. PCR was performed in a final volume of 20 µl, containing 50 ng of genomic DNA, 20 pmol of each primer; 10 mM Tris-HCl pH 8.3, variable MgCl2 concentration, depending on the exon amplified, 50 mM KCl, 1.5 units of Taq DNA polymerase (Gibco BRL, Gaithersburg, MD), 200 mM of each dNTP and 0.001% gelatin. The reaction mixture was subjected to 30 cycles of PCR in an automated thermal cycler (PTC 225). PCR products were purified with the Concert kit, from NucleoFast 96 PCR plates, and directly sequenced with the same primers as used for PCR, using the Big Dye Terminator method on an automatic ABI PRISM 310 sequencer.

Crystallographic views: The consequences of the missense mutations described here were investigated by generating crystallographic representations of the amino acid change. All crystallographic views (Figure 1) were obtained and de-
derived from the structural model obtained by X-ray diffraction of the Drosophila engraviled homeodomain protein (pdb: 1HDD). The substituted residues were generated using SwissProt PDB viewer and the figures were generated with Rasmol software. These crystallographic representations enabled the interpretation of the physicochemical and steric...
changes and their consequences on the side-chain of a specific residue. These views provide supporting evidence concerning the effects of particular mutations in the corresponding region of PITX2.

Tissue preparation: Five embryonic and fetal eyes (from embryos aged 7 weeks (Carnegie stage 18), 9 weeks and 15 weeks) were obtained from the Fetal Pathology Unit of the Department of Histology-Embryology and Cytogenetics of “Hôpital Necker-Enfants-Malades” in Paris (France). These eyes were collected in strict accordance with French and European Union bioethics regulations as well as the Helsinki declaration. The eyes were stored at -80 °C and cryostat tissue sections (15 μm) were mounted on slides coated with 2% 3-aminopropyltriethoxysilane in acetone. Sections were fixed by incubation for 30 min in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated in ethanol, air dried and stored at -80 °C.

DNA probes for in situ hybridization: In situ hybridization was carried out as previously described [14].

RESULTS

We screened individuals from six unrelated families. We found only four mutations in the coding sequence of the PITX2 gene in patients from four families. Patients from the others two families did not carry any mutation in the PITX2 exons. PITX2 mutations were not found in either the unaffected family members or in the control patients. Table 1 summarizes the results of our PITX2 mutational screening.

Family A, consisting of four members, had only two children AII1 and AII2 affected by ARS. We identified a F58L missense mutation in these patients but not in their parents (Figure 2) or in the control patients. These patients are heterozygous. AII1 and AII2 presented with bilateral anterior chamber dysgenesis with bilateral corectopia, iridocorneal synechiae and prominent Schwalbe’s line. Both affected patients developed severe glaucoma during infancy. Both displayed abnormal, misshaped teeth (hypodontia, microodontia) and a protruding excessive periubilical stump with no other extraocular clinical manifestations. Their parents (AI1 and AI2) had a low intraocular pressure, but did not present any abnormality of their iridocorneal angle. They did not display any extraocular abnormality.

We investigated consequences of this missense mutation by generating crystallographic representations of the amino acid change. This amino acid is noted Phe20 or Leu20 because of its position in the homeodomain. The crystallographic views (Figure 1A,B) showed that the side chain of the leucine 20 residue (position in the homeodomain) is shorter and has different steric properties than the phenylalanine. As a consequence, the distances between atoms should be longer in the case of the mutant protein F58L. Nevertheless, the hydrophobic nature of the side chain was conserved. However, the local views of the environment of the residue Phe20, position within the homeodomain (Figure 1C,D) showed that the mutation F58L does not change the nature of the potential interactions between this residue and the residues Phe49, Trp48 in helix 3.

Family B, consisted of five members and had only one member (patient BII1) affected by ARS. We identified an E55X nonsense mutation in this patient only (Figure 2). Patient BII1 is also heterozygous. Anterior chamber abnormalities were observed in both eyes of this patient, but the clinical aspects of his two eyes were different. The right eye presented with complete aniridia associated with goniostreates, whereas the left eye displayed major corectopia associated with partial aniridia, goniostreates, and iris hypoplasia (Figure 3A,B). Both eyes developed precocious glaucoma, requiring several surgical interventions. This child had hypodontia, a prominent periubilical stump and had been treated at birth for anal atresia. His parents did not present any abnormality of their iridocorneal angle nor any extra-ocular abnormality.

Family C consisted of five members, three of whom were affected by ARS (CII1, CIII1, and CIII1). The Y121X nonsense mutation was identified in these three affected patients who are heterozygous (Figure 2). CII1, CIII1, and CIII1 presented with bilateral congenital glaucoma with buphthalmia associated with circumferential goniostreates. They also had dental abnormalities and excessive periubilical skin. CIII1 also presented a congenital cataract and underwent appropriate surgical procedures early on, enabling him to retain a minimal visual acuity of 2/20.

Family D consisted of five members but only two affected patients (DI1 and DI11). We identified an eight-nucleotide insertion, CGACTCTT at position 224 in PITX2A (Figure 2). Patients DI1 and DI11, who are heterozygous, presented with bilateral anterior chamber dysgenesis, marked by a posterior embryotoxon, inner goniostreates, outer iridocorneal synechiae, and corectopia. Both patients were hypermetropic. They developed early and severe bilateral glaucoma, associated with bilateral cataracts. However, the narrowness of the anterior chambers and their major abnormalities were likely the prin-

### Table 1. Summary of mutations in PITX2 gene detected in families with Axenfeld-Rieger syndrome

<table>
<thead>
<tr>
<th>Patient</th>
<th>Aminoacid change (isofrom a)</th>
<th>Nucleotide change (isofrom a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AII1 and AII2(family A)</td>
<td>F58L</td>
<td>753C&gt;G</td>
</tr>
<tr>
<td>BII1(family B)</td>
<td>E55X</td>
<td>742G&gt;T</td>
</tr>
<tr>
<td>CII1, CIII1, and CIII1 (family C)</td>
<td>Y121X</td>
<td>942C&gt;A</td>
</tr>
<tr>
<td>DI1 and DI11 (family D)</td>
<td>*</td>
<td>1251 ins</td>
</tr>
</tbody>
</table>

Shown are four novel mutations: the missense mutation 742C>G identified in patients AII1, AII2 (family A) that replaces a phenylalanine by a leucine, the mutation 753G>T identified in patient BII-1 (family B), and the mutation 942 C>A identified in patients CII1, CIII1, CIII1 (family C) that result both in a premature stop codon, and a insertion 1251 Ins (CGA CTC CT) identified in patients DI1 and DI11 (family D).
Figure 2. Four novel PITX2 mutations identified by DNA sequencing in four unrelated families with Axenfeld-Rieger syndrome. Pedigrees of four families with Axenfeld-Rieger syndrome are shown in the right column. Males and females are represented by squares and circles, respectively, and affected family members are darkened symbols. In the left column, partial DNA sequence chromatographs of PITX2 from affected individuals in each family are shown. In the center, partial DNA sequence chromatographs of PITX2 from corresponding unaffected family members are shown. The exact mutations in families were labeled next to each sequence according to the nomenclature.
pical factor accounting for the precocious opacification of their lenses.

Glaucoma develops in approximately 50-60% of patients with ARS [2]. However, all patients included in this study presented with a severe glaucoma. To better understand this ocular phenotype of ARS patients, we studied the expression of PITX2 in human embryonic and fetal ocular tissue sections.

We detected PITX2 transcripts in the periorcular mesenchyme, by in situ hybridization, at seven weeks of gestation (embryonic, Carnegie stage 18; Figure 4A,B) and at nine weeks of gestation (fetal; Figure 4C,D). At both stages, the labeling of PITX2 mRNA in the ocular region was exclusively confined to the anterior and posterior parts of the periorcular mesenchyme. No PITX2 hybridization signal was detected in any intraocular structure. The PITX2 in situ hybridization signals detected in the periorcular mesenchyme at both stages was completely specific as verified by the absence of PITX2 hybridization signals detected using two different 60 bp sense oligonucleotidic probes. The developing retinal pigment epithelium (RPE) is autofluorescent and did not display any in situ hybridization signal. In the three remaining fetal eyes (obtained at 15 weeks of gestation), PITX2 mRNA was present in the corneal endothelium and stroma (Figure 5A,B) and in large amounts in the iridocorneal angle (Figure 5C,D) as well as in the ciliary body (Figure 5E,F). Although there is an autofluorescence, produced by the pigmented cell layer of the ciliary processes, a specific PITX2 signal corresponding to PITX2 gene expression is unambiguously detected in the ciliary body and especially in the nonpigmented cell layer of the ciliary processes and in the developing ciliary muscles. This was checked during the analysis of the bright-field aspects of the RPE at the highest magnification using a DNRB photonic microscope (Leica, Rueil-Malmaison France; data not shown).

**DISCUSSION**

We identified four different mutations not described until now in families who were affected by autosomal dominant ARS. For each patient, we also described the clinical phenotype. The previously unidentified missense substitution F58L, detected in family A, alters the phenylalanine in position 58 in isoform PITX2a and changes it to a leucine, located at the end of helix 2 of the homeodomain. This first mutation probably resulted from a parental mosaicism. The mutation probably arose de novo in the germline of one parent. Each parent did not carry the mutation found in the two affected children. This mutation affects the homeodomain of PITX2. Interestingly, most of the PITX2 mutations described in patients with Rieger’s syndrome occur in the homeodomain [15,16]. The modified phenylalanine is a highly conserved residue in several species (human, rat, mouse, chick, zebrafish, *Xenopus*) and is conserved in homeodomain proteins (Bicoid, Antennapedia and Engrailed) suggesting a conserved function (Figure 6). The homeodomain is well-conserved in this family of proteins, both in terms of amino acid residues and secondary and tertiary structures. The homeodomain comprises three helices; helix 3 is the most important, because it is the site of DNA recognition and binding. The compact structure of the homeodomain is due to a network of specific intramolecular interactions between these three helices. The residues involved in DNA binding are primarily and specifically in positions 47, 50, 51, and 53, with specificity highest for residue 50 [17]. These results were established for various structural models of the Engrailed protein obtained by X-ray diffraction (PDB id: 1HDD) [18]. As the Engrailed and PITX2 proteins present 35% absolute sequence identity in this region, and because of similarities at structural level, the structural model obtained for Engrailed can be used as a crystallographic support for studying or predicting the effects of this mutation on the PITX2 protein [19]. The conformation of the homeodomain is stabilized by hydrophobic interactions between the three helices, via residue Ala54, for example. Recognition and binding base contacts are mediated by the residues Val47, Lys50 and Asn51 from helix 3. DNA phosphate groups also interact with Trp48, which interacts with Phe20 and Phe49 in the hydrophobic network of the helical core [20] (Figure 1C). These interactions stabilize the conformation of the PITX2 homeodomain and any mutation affecting this hydrophobic network is likely susceptible to affect the conformation of this core and the recognition as well as binding of DNA. The structural consequences of the F58L mutation identified in the patients are unclear, but Phe20 has been implicated in the network ensuring the correct conformation of the homeodomain of PITX2 [20]. Thus, the F58L mutation probably affects conformation of the homeodomain. We propose the hypothesis that the potential modification of the homeodomain conformation caused by the F58L mutation...
might affect the DNA recognition as well as DNA binding activities of the PITX2 protein (Figure 1D). These changes in the binding of PITX2 to its target genes may account for the observed phenotype of the affected patients. However, the functional consequences of this mutation cannot be predicted from the crystallographic representations used. Thus, our hypothesis concerning the consequences of the F58L mutation on the structure requires a confirmation by biochemical assays. Recently, the solution structure of a complex containing the K50 class homeodomain pituitary homeobox protein 2 (PITX2) bound to its consensus DNA site (TAATCC) has been reported [21]. Previous studies have suggested that residue 50 is an important determinant of differential DNA-binding specificity among homeodomains. Although structures of several homeodomain-DNA complexes have been determined, this is the first structure of a native K50 class homeodomain. The only K50 homeodomain structure determined previously is an X-ray crystal structure of an altered specificity mutant, Engrailed Q50K (EnQ50K) [17]. Analysis of the NMR structure of the PITX2 homeodomain indicates that the lysine at position 50 makes contacts with two guanines on the antisense strand of the DNA, adjacent to the TAAT core DNA sequence, consistent with the structure of EnQ50K. The evidence obtained by this study suggests that this side chain may make fluctuating interactions with the DNA, which is complementary to the crystal data for EnQ50K [21]. It is important to emphasize that the structure previously determined for the EnQ50K mutant [17] provided some interesting insights into the possible role of lysine at position 50 [21]. This leaves clearly open the possibility that the crystallographic structures of both Engrailed and EnQ50K might provide additional interesting insights on other (mutated or not mutated) aminoacid residues of PITX2 localized in a region strongly similar to the one existing in Engrailed and containing several identical aminoacids. A model of the PITX2 homeodomain structure was created previously by threading analysis, which allowed predictions to be made regarding the role of Rieger syndrome mutations in PITX2 dysfunction, although this model was not necessarily an indication of the true PITX2 homeodomain molecular structure [19,21]. This model was based on the crystallographic structure of the Engrailed homeodomain in complex with its target DNA [19]. The DNA-binding functions of some PITX2 residues R46, K50, R52, and R53 have been previously discussed. Mutating these residues would disrupt many favorable interactions with the DNA, and biochemical studies have indicated that these mutations interfere with DNA binding. Overall, these results are similar to the threading analysis [19,21]. The PITX2 structure recently determined on the basis of NMR spectroscopic analysis opens new avenues for future studies which will be focused on analyzing the mutant proteins by NMR spectroscopy [21]. At the present time, no crystallographic structure of the PITX2

Figure 4. PITX2 gene expression at seven and nine weeks of gestation in ocular tissue sections. Bright-field aspect of PITX2 gene expression in seven weeks of gestation human embryonic ocular tissue sections (A) and corresponding dark-field sections (B). Bright-field (C) and dark-field (D) aspects of PITX2 gene expression pattern in nine weeks of gestation human fetal ocular tissue sections. White arrows mark the specific signals. PITX2 transcripts are detected in the periocular mesenchyme (PM) at both stages. Autofluorescence is shown in the retinal pigment epithelium (RPE; B and D).
homeodomain in complex with its core target DNA sequence is available. However, crystal structures of several different homeodomains in complexes with DNA have revealed that the overall fold and DNA docking arrangements are well conserved [22]. These crystallographic studies are in good agreement with NMR results [22-26]. We propose a model of the F58L mutation that causes an ARS syndrome based on the only crystallographic data related to PITX2 available and still relevant for now. Our goal is to gain molecular insight into the pathophysiology of a particular form of an ARS syndrome caused by a specific PITX2 substitution. Our model might not be true. Only additional experiments will allow us to get the definitive answer. The comparison of our proposed structural representation of the F58L mutation based on indirect crystallographic data with the one based on the NMR structure of the PITX2-core target DNA complex will obviously bring a deeper understanding of the mechanisms of this disease and will shed additional light on PITX2 normal structure and function. Time domain NMR spectroscopic techniques are used to probe molecular dynamics in solutions. While they provide invaluable data, they do not answer all questions about the pathophysiology of a genetic disease at the molecular and even at the atomic level. Molecular dynamics (MD) analysis of the engrailed homeodomain-DNA recognition have recently been reported [27]. Recent surveys of structural data on protein-protein and protein-DNA recognition sites have indicated that water is present in abundance at the interface, and the analysis of different high resolution structures allowed the identification of at least as many water-mediated interactions as direct hydrogen bonds or salt bridges [28]. In particular, water can play an important role in mediating protein DNA interaction due to the polarity of the surface characterized by the presence of phosphate groups on the DNA side and by the frequent abundance of positively charged groups on the protein side. Since then, many examples have been found, suggesting that the description of protein DNA interactions is incomplete without an atomic description of water molecules at the interface [28,29]. High X-ray and neutron diffraction studies of

![Figure 5. PITX2 gene expression at fifteen weeks of gestation in fetal ocular tissue sections. On the left side Brightfield (A, C, and E) and in the right side the corresponding Dark Field aspects (B, D, and F) of PITX2 gene expression at fifteen weeks of gestation. The specific signals are shown by white arrows (B, D, and F). PITX2 mRNAs were detected in corneal endothelium (CE) and stroma (A and B). PITX2 mRNAs were also strongly detected in irido-corneal angle (ICA; C and D) and in the ciliary body especially in the non pigmented cell layer of the ciliary processes (CP) and in the developing ciliary muscles (CM; E and F). Autofluorescence is shown in the pigmented cell layer of the ciliary processes (D).](http://www.molvis.org/molvis/v12/a163/)
crystals can provide some information about macromolecular hydration, but they are not able to provide a dynamical description. This information on water behavior is provided in inelastic neutron scattering and NMR spectroscopy [30,31], or by accurate molecular dynamics (MD) simulation procedures. The latter approach is a powerful tool for examining the macromolecule solvation, because it can provide a description at atomic level and at the appropriate time scale. In the last years, MD simulation has been used to investigate the molecular basis of protein-DNA or RNA recognition [32-35]. Structures of macromolecular complexes are necessary for a mechanistic description of biochemical and cellular processes. They can be solved by experimental methods, such as X-ray crystallography, NMR spectroscopy and electron microscopy, as well as by computational protein structure prediction, docking and bioinformatics. Recent advances and applications of these methods emphasize the need for hybrid approaches that combine a variety of data to achieve better efficiency, accuracy, resolution, and completeness for the best description of both normal and mutated macromolecules [36]. Thus, it is clear that the pathophysiology of any disease caused by alterations of macromolecular structures can be grasped in depth by the implementation of several techniques, especially because in physiological conditions macromolecules are in solution.

The mutation E55X in family B probably affects the DNA binding domain and the carboxy-terminal part of the PITX2 protein located beyond it. It introduces a premature stop codon in the PITX2 coding sequence and might result in a truncated protein which lacks a part of the homeodomain and all the carboxy-terminal part of PITX2 beyond the homeodomain. PITX2 mRNAs have a short half-life and are unstable. The stabilization is due to a reduced interaction of PITX2 3' UTR with some destabilizing ARE-BP (ARE binding proteins) as well as to an increased interaction with a stabilizing ARE-BP-HuR [37]. Consequently, we suggest that the mutation E55X change the PITX2 RNA stability, which might cause the precocious degradation of PITX2 mRNAs, through nonsense-mediated mRNA decay. Further experiments are necessary to confirm this hypothesis. In the case of truncated mRNAs being translated, a truncated protein lacking most of the DNA-binding domain and especially the helix 3 which binds DNA might be produced. The truncated protein might result in a shortened nonfunctional PITX2 protein. This mutation identified is probably a sporadic form of ARS resulting from a de novo mutation which precociously arose in the patient, or it may be a familial form resulting from a de novo mutation in the germline of one parent. The Y121X nonsense mutation in family C affects the C-terminal part of the PITX2 protein. This mutation might lead to the production of a truncated protein with partial loss of the C-terminal domain. The eight-nucleotide insertion, 1251InsCGACTCCT in family D, creates a frameshift of reading and introduces a stop codon at position 240 in PITX2a. This insertion might also lead to the production of a truncated protein lacking the C-terminal amino acids. These three last mutations identified lead to a truncated C-terminal tail of PITX2. Interestingly, in other Rieger patients, PITX2 was also described with a truncated C-terminal tail [38-40]. This C-terminal region contained a highly conserved 14-amino acid element called the OAR domain [1]. This domain is strongly conserved in all PITX2 proteins, across a wide range of species, suggesting a conserved function. The C-terminal tail of homeodomain proteins, such as PITX2, has been shown to be involved in autoregulation of their activity by inhibiting DNA binding and is involved in protein-protein interactions [41]. Indeed, the C-terminal 39-amino acid tail represses DNA-binding activity and is required for PITX2-Pit-1 (POU homeodomain protein) interaction and PIT-1 synergism [42]. Pit-1 interaction with the PITX2 C-terminus masks the inhibitory effect of this domain and promotes DNA binding and transcriptional activity, without affecting binding specificity [41]. Moreover, PITX2 synergistically transactivates the prolactin promoter in the presence of the Pit-1 in the pituitary. Therefore, the three mutant proteins, which lack the C-terminal domain, may fail to interact with Pit-1 and to transactivate the prolactin gene, thereby affecting pituitary gland development.

Figure 6. Amino acid sequence alignment of human PITX2 homeodomain with others homeodomain proteins and with PITX2 proteins from diverse species. A: Alignment of sequence from homeodomain showing the conservation of the aminoacid residue F58 of the homeodomain. The phenylalanine is conserved in the family of homeodomain proteins in terms of aminoacid residue. B: Alignment of PITX2 sequence in diverse species. The phenylalanine is well conserved residue in PITX2 proteins from several species (human, rat, mouse, chicken, zebrafish, Xenopus).
These results led us to search for pituitary endocrine abnormalities in patients affected by ARS in families B, C and D. We found no pituitary dysfunctions in any of our affected patients, despite hormonal explorations. The absence of pituitary endocrine dysfunction in our patients may be accounted by a yet to be defined compensatory mechanism as yet undefined at the molecular level and involving PITX1 gene expression in the pituitary glands of our affected patients [43-45]. In addition, PITX2 is also required for eye and tooth development, suggesting that the activation of PITX2 may result from the interaction of factors with the C-terminal tail, increasing DNA-binding and transcriptional activities in these specific tissues [46]. In conclusion, the three truncated proteins lacking the C-terminal tail may be unable to interact with specific cellular factors for the synergistic activation of transcription, resulting in the developmental defects observed in ARS patients. In vertebrates, PITX2 gene has been implicated in the control of left-right asymmetry of mesoderm-derived organs such as heart, gut and lungs [47-51]. The mutations observed in our cohort affected three PITX2 isoforms, due to their location in a region common to the isoforms A, B, and C. However, no isomerism or abnormal asymmetry was detected in our affected patients who had the genetic alterations. ARS syndrome caused by heterozygous PITX2 mutations result in developmental defects within the ocular anterior segment in structures derived from the pericocular mesenchyme, including the cornea, iris, as well as outflow tract and a high incidence of early onset glaucoma [1,2,52,54].

The mouse Pitx2 gene is expressed in both the neural crest and the mesoderm-derived precursors of the pericocular mesenchyme. Murine Pitx2 is expressed throughout the pericocular mesenchyme, including not only the structures of the anterior segment but also the sclera, ocular vasculature, and extracocular muscles [1,55]. Complete loss of function in mice results in agenesis or severe disruption of pericocular mesenchyme structures and extrinsic defects in early optic nerve development. Pitx2-deficient mice did not allow to determine the specific requirements for Pitx2 in neural crest versus mesoderm throughout mouse eye development. Only roles of PITX2 gene expression in the initial stages of eye development could be assessed due to early embryonic lethality [56-60].

To determine the specific roles of Pitx2 in the neural crest precursor pool, neural crest-specific Pitx2 knockout mice (Pitx2-ncko) were generated [61]. Because Pitx2-ncko mice are viable, Pitx2 gene function could be analyzed in later stages of eye development. Pitx2 is intrinsically required in the neural crest for specification of corneal endothelium, corneal stroma, and the sclera. These results are in excellent agreement with those we obtained in human fetal ocular tissues. The in situ hybridization results show for the first time that PITX2 is expressed in the human embryonic and fetal pericocular mesenchyme at early developmental stages. Our results are in accordance with previous reports in developing mice [55,61-65]. The pericocular mesenchyme makes a major contribution to the development of ocular anterior segment structures that may be affected in ARS. These results suggest that PITX2 is involved in early human eye development, particularly in the development of the anterior segment. Furthermore, the significant PITX2 expression in the human developing ciliary body, ciliary processes, iridocorneal angle, and corneal endothelium provides a strong molecular basis for explaining the frequent occurrence of glaucoma in patients with ARS, such as those caused by the novel PITX2 mutations reported in the present study.

All ARS-affected patients involved in this study had severe, early onset glaucoma. The disease’s underlying molecular mechanisms and why only 50-60% of affected individuals develop glaucoma are not known. Collectively, these observations suggest that more complex factors than simply elevated intraocular pressure (IOP) contribute to the etiology of glaucoma in affected patients. Indeed, Pitx2-ncko mice exhibit a unique optic nerve phenotype in which the eyes are progressively displaced toward the midline until they are directly attached to the ventral hypothalamus. As Pitx2 is not expressed in the optic stalk, an essential function of PITX2 protein in neural crest is to regulate an extrinsic factor(s) required for development of the optic nerve. The reduced number of Pax2-positive astrocyte precursors that invade the axons of the retinal ganglion cells (RGCs) in the Pitx2-ncko mice is particularly noteworthy. Astrocytes are critical for the maintenance and survival of the RGC axons and they have been implicated in the pathogenesis of glaucoma. If human PITX2 patients have a reduced number of astrocytes or altered astrocyte function, it could make their optic nerves more vulnerable to the effects of other factors like elevated IOP and lead to the RGC axon damage and death seen in glaucoma. The observed deficiency in astrocytes in Pitx2-ncko eyes provides additional evidence for defective retinal vessel development because astrocytes guide retinal vessel formation. It has not been clear whether defects in ocular blood flow are a cause or an effect of glaucoma. The recent results with Pitx2-ncko mice raise the possibility that PITX2 patients may have fewer ocular vessels, which would be another risk factor for developing glaucoma [61]. Moreover, several recent studies have started to unravel the increasing genetic complexity of susceptibility to glaucoma both in humans and mice [66,67]. From the study of this genetic complexity might arise the identification of modifier genes preventing the manifestation of the glaucomatous phenotype in at least 40% of ARS-affected patients.

Interestingly, we found no PITX2 mutations in some Rieger patients, although PITX2 is a major gene associated with ARS. The phenotype may be caused by mutations in another gene previously identified as being associated with ARS, such as FOXC1 or PAX6, or other unknown gene mutations. Approximately 40% of ARS cases cannot be attributed to chromosomal aberrations or gene mutations known to be associated with ARS [9,10]. Indeed, some patients have chromosomal translocations involving different regions of the genome, possibly indicating the involvement of other loci in this syndrome [68]. It is also possible that different forms of ARS might be caused by genetic alterations affecting other components of the PITX2 pathway.

Furthermore, in this study, we limited our search for hu-
man gene mutations to the coding region of PITX2. We have not studied possible mutations causing alternative splicing alterations since we did not have access to total mRNA extracted from lymphoblastoid cell lines or fibroblasts. Furthermore, mutations may occur in portions of the gene that are not routinely screened (5'UTR, distant intronic sequences, 3'UTR, promoters and intra- or extragenic regulatory sequences).

**Conclusion:** In summary, we identified four novel mutations in ARS patients that correspond to one missense mutation, two nonsense mutations and one insertion mutation. Particularly, we viewed the mutated homeodomain corresponding to the substitution by crystallographic representations. Our results suggest that this mutation probably affects the conformation of homeodomain which interacts with DNA. The functional consequences of this mutation on the PITX2 protein cannot be predicted from crystallographic data. The confirmation of our hypothesis requires the implementation of appropriate biochemical assays. Additionally, we show for the first time that the PITX2 gene is expressed during early stages of human eye development, especially in structures involved in the anterior chamber organogenesis. This ocular gene expression pattern can explain the frequent occurrence of glaucoma in a high percentage of patients affected by ARS caused by PITX2 mutations.

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