Identification of the first intragenic deletion of \textit{PITX2} gene causing an Axenfeld-Rieger-Syndrome.

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ABSTRACT

Background: Axenfeld-Rieger syndrome (ARS) consists of bilateral congenital abnormalities of the anterior segment of the eye associated with abnormalities of the teeth, midface, and umbilicus. Most cases of ARS occur as a result of mutations in the genes encoding PITX2. We describe here a new family affected by an unusual form of ARS.

Case presentation We describe here a family with two members (father and daughter) affected by a severe form of Axenfeld-Rieger Syndrome (ARS). Both patients presented typical ARS and developed severe glaucoma, but the ocular phenotype was much more severe in the daughter than in the father. Unexpectedly, MRI detected an aggressive form of meningioma in the father. We studied PITX2 gene by exon screening but no mutation was found. Secondly, we carried out quantitative genomic PCR and we detected an intragenic deletion, which we characterized in detail.

Conclusion: These data provide the pathogenic role of the first intragenic deletion of the PITX2 gene. This work also demonstrates a possible explanation for the low rate of mutation detection in this gene.
Background

Rieger syndrome is an autosomal dominant disorder consisting of bilateral congenital abnormalities of the anterior segment of the eye associated with abnormalities of the teeth, midface, and umbilicus. These defects may include microdontia, hypodontia, anodontia and maxillary hypoplasia. Classic ocular features of ARS include iridocorneal synechiae, iris hypoplasia, corectopia, polycoria, and/or prominent Schwalbe’s line displaced to the anterior (posterior embryotoxon). Glaucoma develops in approximately 50% to 60% of patients with Axenfeld-Rieger syndrome (ARS). Originally described as separate clinical entities, Axenfeld's and Rieger's anomalies and various abnormalities of the anterior chamber of the eye are now considered to be variations of the same spectrum of developmental disorders, or even as the same developmental disorder: Axenfeld Rieger Syndrome (ARS).

ARS has been linked to five chromosomal loci (4q25, 6p25, 11p13, 13q14, 16q24) [1][2, 3]. Disease-causing mutations have been identified in three transcription factor genes. Two of these genes — PITX2 and FOXC1, which map to chromosomes 4q25 and 6p25, respectively — are the most frequently affected. ARS due to deletion of the paired-box transcription factor PAX6, which maps to 11p13, has been reported in only one case [4]. The causal genes at the 13q14 and 16q24 loci have yet to be identified.

The PITX2 gene (OMIM: 601542) was first identified by positional cloning of the 4q25 locus and has been shown to be involved in ARS pathogenesis. The PITX2 gene consists of seven exon [5] and encodes a member of the bicoid/paired-like homeodomain family [6]. However, only 40% of patients diagnosed with classical
Rieger syndrome have PITX2 mutations [3]. PITX2 haploinsufficiency may cause this syndrome [7]. PITX2 clearly plays an important role in embryonic and fetal development. PITX2 is also required for the normal development of neurons in the mouse midbrain [8, 9]. These results, together with the major role played by PITX2 in pituitary gland development provide ample justification for brain MRI in patients with ARS.

Several new PITX2 mutations causing ARS have recently been reported. For more information, see web databases especially:


We investigated the molecular basis of Axenfeld-Rieger Syndrome in one family in which no mutation of PITX2 or FOXC1 was found. In this report, we identified a large intragenic deletion of PITX2 by quantitative genomic PCR as the cause of an unusual form of ARS.
Cases presentation

We analyzed one family affected by ARS. Two patients in this family suffered from an unusual form of ARS. The man (I-2) was 51 years old and presented bilateral Axenfeld-Rieger syndrome, with chronic glaucoma monitored clinically and surgically for at least 10 years. During this period of follow-up, he underwent bilateral trabeculectomy. He presented the characteristic features of ARS: hypodontia, maxillary hypoplasia and redundant periumbilical skin.

MRI (magnetic resonance imaging) was carried out to complement this clinical analysis. On MRI, carried out in June 2005 (figure 1A), we observed a left frontal meningioma 4 mm in diameter and atrophy of the two optic nerves in intra-orbital sections, with marked dilatation of the myelin sheaths (figure 1B). A second MRI scan was carried out in January 2006 and showed that the meningioma had doubled in size in seven months.

This man also presents severe thyroid problems with goiter consisting of bulky nodules of up to 3 cm in diameter on both the right and the left. This man was also found to present hyperthyroidism not associated with pituitary adenoma or functional pituitary hormonal abnormalities.

This man’s daughter (II-1) was 21 years old at the time of the study. She presented the same characteristic features of ARS (figure 2A and 2B) but had developed them earlier. She had severe glaucoma resulting in early left eye removal and the fitting of prosthesis. She presented no abnormality of the optic nerves or retrochiasmatic visual pathways. She displayed no sign of meningioma or optic nerve abnormality on MRI, in sharp contrast to her father.
The father’s visual acuity was reduced to 20/200 in both eyes, with only narrow parts of the visual field unaffected. The daughter had poor visual acuity in her remaining eye, limited to fingers counting. Slit lamp examination and photographic imaging showed polycoria associated with irido-corneal synechia and iris hypoplasia in both father and daughter (figure 2B). Both slit lamp examination and anterior optical coherent tomography (figure 3) with an OCT3 Stratus machine defocused for anterior chamber examination showed polycoria. We also observed goniostands and, in some sectors of the anterior chamber, parts of the iris closely apposed to the cornea, severely reducing the irido-corneal angle and accounting for the persistent high intraocular pressure (40 mmHg minimum value), measured with a Goldmann aplanation tonometer, despite previous trabeculectomy and ongoing maximal anti-glaucomatous medical treatment. Both the father (I-2) and the daughter (II-1) were fully examined in the Cardiology, Pneumology and Endocrinology Departments of our hospital. They displayed no abnormalities other than the thyroid abnormalities of I-2, which were not of pituitary origin and were appropriately treated.

Genetics analysis

Characterization of a deletion in the PITX2 gene

We looked for PITX2 mutations in this family (figure 4A) that might account for this unusual phenotype. We screened the seven exons of this gene by PCR and sequencing but found no mutation. We then searched for an intragenic deletion in the PITX2 gene, by quantitative genomic PCR (table1 of figure 4). We used various sets of primers to amplify the entire gene. Using different primers pairs, corresponding to
exon 5 and exon 6, we showed that these two exons were each present as a single copy. We confirmed this result using two different endogenous DNA control genes (ALB and ERBB2). Only one copy of the PITX2 gene was found in each of the two patients affected by this unusual form of ARS. We identified, in both patients, an intragenic deletion corresponding to part of exon 5, intron 6 and part of exon 6.

We tried to amplify a larger DNA fragment as a means of determining more precisely the start and end points of this deletion. We used two primers, one binding just before exon 5, and the other, just after exon 6. We isolated a fragment of 272 base pairs (bp) from the patients suffering from Rieger syndrome by PCR (figure 4D). In control genomic DNAs from unaffected patients, we obtained a 3331 bp fragment.

We characterized this deletion by sequencing the amplified fragments of genomic DNA from controls and patients (figure 4B and figure 4C). We found that the deletion began after a sequence of six nucleotides in exon 5 (5’-CTCCAG-3’) and ended after the same repeated sequence in exon 6. This 3059 bp deletion concerned the nucleotides between nucleotides 18183 and 21242 within the PITX2 gene (gi|13183092|gb|AF238048.1|[13183092]). This deletion removes the end of exon 5, the whole of the following intron and the start of exon 6.

**The PITX2 mRNA**

We investigated the sequence of the PITX2 mRNA by RT-PCR analysis on total RNA from lymphoblastoid cell lines established for the two patients. We amplified a normal PITX2 cDNA fragment but no abnormal fragment (data not shown).
Discussion

We have analyzed a family suffering from an unusual form of ARS. The difference of the phenotype between father and daughter is typical of ARS. An interesting feature of the clinical differences between I-2 and II-1 is that a severe, acute increase in intraocular pressure had an effect similar to axotomy without optic nerve damage visible on MRI in II-1. By contrast, a chronic increase in intraocular pressure progressing over a long period of time in I-2 triggered the progressive disappearance of the axons in the optic nerve, with enlargement of the myelin sheath, suggesting that this enlargement may have provided a certain degree of neuroprotection.

Atrophy of the optic nerve observed on MRI of the father has never previously been reported in patients with ARS, but a recent study provides a possible explanation for this observation. An analysis of mice in which the pitx2 gene was specifically knocked out in the neural crest revealed atrophy of the optic nerve [12]. They strongly suggest that the PITX2 gene is involved in formation of the optic nerve and/or its susceptibility to high intraocular pressure. These results, combined with those of Ittner et al. and Berry et al, [13] [14] show that PITX2 may regulate levels of an extrinsic factor required for optic nerve development.

No other case of meningioma in association with ARS has ever been reported. The occurrence of both meningioma and ARS in I-2 may simply reflect an unlikely coincidence, with these two conditions occurring simultaneously in the same patient via different mechanisms. Patient II-1 who carried the same mutation, did not present any sign of meningioma when she was examined.
We need to determine whether this clinical observation results from deletion of the *PITX2* gene or whether it is an independent event. Some meningiomas have been reported to be associated with the translocation of part of chromosome 4 to chromosome 22 [15], but no direct relationship with *PITX2* gene was found. Moreover, PITX2A can function as a negative regulator of FOXC1 transactivity with its C-terminal domain [14]. FOXC1 was recently directly implicated in tumors originating from the mesenchyme: synovial sarcomas. In some cancer FOXC1 and these target gene involved in TGFβ pathway appear to be upregulated, as shown by microarray experiments and RT-qPCR [16]. We can note that the deletion identified in this family affected the C-terminal domain of PITX2 and may affect FOXC1 activity. This hypothesis concerning the role of PITX2 in human oncogenesis should be explored by furthers studies. It would be wise to monitor at least patient II-1, and potentially all patients with ARS, regularly by brain and spine MRI.

The most common cause of ARS is a mutation in the *PITX2* gene or the *FOXC1* gene. However, analyses of genomic DNA for mutations by exon-by-exon screening may fail to detect a mutant allele. This is illustrated by our case. Mutation screening of the seven exons and adjacent intronic regions of the *PITX2* gene detected no molecular change. We considered that either a large DNA rearrangement masked by the presence of the wild-type *PITX2* normal allele or an undetected intronic mutation were the most probable explanations for the apparent absence of any PITX2 gene alteration. The absence of clinical abnormalities in the previous generation suggests that this alteration may be a *de novo* genomic alteration.

Following the detection by genomic quantitative PCR of a large genomic deletion, we developed a PCR protocol for amplifying exon 5, intron 6 and exon 6, to
look for a large DNA re-arrangement. Fine mapping of the deletion breakpoint by direct sequencing of the long PCR products showed that the deletion had removed a 3059 bp region extending from the end of exon 5 to the start of exon 6. The junctions at the beginning and end of the deletion contained a short direct 6 bp repeat (CTCCAG). This is not the first time that quantitative genomic PCR has been used to detect PITX2 deletions. However to date, only microdeletions and gross deletions without refined molecular characterization have been reported [17]. The deletions previously reported were not molecularly characterized and, in most cases, might correspond to contiguous syndromes [17]. Some involved the loss of several genes and resembled the interstitial microdeletions detectable by FISH. This is the first report of an entirely intragenic PITX2 deletion, accurately characterized, that does not lead to complete PITX2 gene deletion. There are several possible explanations for this large deletion. All the mechanisms proposed to account for deletions or other rearrangements in human genetic disorders — including genomic disorders, a subset of genetic disorders associated with large deletions and/or duplications — are related to the presence of repetitive sequences. One of these mechanisms is the slipped mispairing hypothesis, which was first proposed by Streisinger [18] and was first observed in yeast. The CTCCAG of exon 5 could bind to the corresponding GAGGTC sequence in exon 6 on the other strand. This would result in the formation of a single-stranded loop that could be excised by a DNA repair enzyme, such as RAD [19]. This mechanism seems quite likely for this deletion (data not shown). However, we cannot yet rule out the possibility of non homologous end joining (NHEJ) as the causal mechanism for this deletion.

The intragenic deletion in this family closely matches what is known about the haploinsufficiency effects of most PITX2 mutations [20]. Our inability to detect a
PITX2 cDNA after RT-PCR with total RNA extracted from lymphoblastoid cell lines or from white blood cells does not mean that the mutated PITX2 allele is not expressed in vivo.

**Conclusion:**

Mutational status has been reported for many patients with ARS. However, the overall detection rate for mutations is low. This is the first time that a large intragenic deletion of the PITX2 gene has been identified, with a PCR protocol designed to amplify larger genomic fragments than those usually amplified for direct sequencing. The detection and the characterization of such a deletion was made possible by initial data provided by quantitative genomic PCR. The advent of new Taq polymerases (TaKaRa La Taq enzyme Cambrex) for long-range PCR allows in conjunction with quantitative genomic PCR to refine PITX2 mutation analysis and ARS molecular diagnosis as well as to increase the sensitivity of genomic mutational screening.
Acknowledgments

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Figures legends

**Figure 1**: MRI scan for patient I-2

A: Left frontal meningioma measuring 4 mm in diameter.

B: atrophy of the two optic nerves in their intraorbital portions (before the intracanal portion) with dilatation of the sheaths.

**Figure 2**: Phenotypic analysis of patient II-1

A: Panoramic dental X ray showing hypodontia, B: Eye imaging showing polycoria

**Figure 3**: OCT examination of patient II-1

A: Partial iris apposition to the cornea, decreasing the irido-corneal angle of the right eye, presence of abnormal goniostands

B: Abnormal goniosynechiae associated with goniostands in a supratemporal position in the right eye. These structures significantly decrease the irido-corneal angle and provide a clear explanation for the very high intraocular pressure measured in this remaining eye.

C: Polycoria with a heterogeneous iris and abnormal structure of the cornea. An irido-corneal synechia is visible between the corneal endothelium and the inner part of iris. It seems to be the remnant of iris hypoplasia and contributes to polycoria.

D: Visualization of abnormal goniostands and of an abnormal remnant iris bridge affected by partial hypoplasia
Dosage of the *PITX2* gene was determined by genomic quantitative PCR (table 1). We used ALB and ERBB as endogenous DNA control genes. *PITX2* is expressed from only one copy in patients I-2 and II-1, in contrast to what was observed for other members of the family (I-1 and II-2) and unrelated cases (856, 23128, 31964).

A: Genealogy of the family

B: Chromatography sequence of PITX2 in patient I-2

C: Normal chromatography sequence of PITX2 (patient I-1)

D: Agarose gel showing the deletion: the abnormal fragment detected only in patients I2 and II1 has a size of a 272bp
Table 1

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A

Family Z

B

Start of the deletion

C

D

DNA ladder

Patient I-2

Patient I-1

272 bp

500 bp

100 bp