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ARTICLE

Glaucoma spectrum and age-related prevalence of individuals with *FOXC1* and *PITX2* variants

Emmanuelle Souzeau*,[1], Owen M Siggs[1], Tiger Zhou[1], Anna Galanopoulos[2], Trevor Hodson[3],
Deepa Taranath[1], Richard A Mills[1], John Landers[1], John Pater[1], James E Smith[4,5,6], James E Elder[7,8],
Julian L Rait[9,10], Paul Giles[11], Vivek Phakey[12], Sandra E Staffieri[7,9,10], Lisa S Kearns[9,10], Andrew Dubowsky[13],
David A Mackey[14,15], Alex W Hewitt[9,10,15], Jonathan B Ruddle[7,9,10], Kathryn P Burdon[1,15] and Jamie E Craig[1]

Variation in *FOXC1* and *PITX2* is associated with Axenfeld-Rieger syndrome, characterised by structural defects of the anterior chamber of the eye and a range of systemic features. Approximately half of all affected individuals will develop glaucoma, but the age at diagnosis and the phenotypic spectrum have not been well defined. As phenotypic heterogeneity is common, we aimed to delineate the age-related penetrance and the full phenotypic spectrum of glaucoma in *FOXC1* or *PITX2* carriers recruited through a national disease registry. All coding exons of *FOXC1* and *PITX2* were directly sequenced and multiplex ligation-dependent probe amplification was performed to detect copy number variation. The cohort included 53 individuals from 24 families with disease-associated *FOXC1* or *PITX2* variants, including one individual diagnosed with primary congenital glaucoma and five with primary open-angle glaucoma. The overall prevalence of glaucoma was 58.5% and was similar for both genes (53.3% for *FOXC1* vs 60.9% for *PITX2*, $P = 0.59$), however, the median age at glaucoma diagnosis was significantly lower in *FOXC1* (6.0 ± 13.0 years) compared with *PITX2* carriers (18.0 ± 10.6 years, $P = 0.04$). The penetrance at 10 years old was significantly lower in *PITX2* than *FOXC1* carriers (13.0% vs 42.9%, $P = 0.03$) but became comparable at 25 years old (71.4% vs 57.7%, $P = 0.38$). These findings have important implications for the genetic counselling of families affected by Axenfeld-Rieger syndrome, and also suggest that *FOXC1* and *PITX2* contribute to the genetic architecture of primary glaucoma subtypes.
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INTRODUCTION

Anterior segment dysgenesis is a heterogeneous group of developmental disorders affecting the anterior structures of the eye.[1] Axenfeld-Rieger malformation (ARM) represents a subgroup of anterior segment dysgenesis and refers to congenital ocular features including abnormal angle tissue, iris stromal hypoplasia, pseudopolycoria (additional pupillary opening in the iris), corectopia (displaced pupil), posterior embryotoxon (thickened and centrally displaced Schwalbe's line) and/or peripheral anterior synechiae (irido-corneal adhesions).[2,3] A range of systemic features can also be present as part of the Axenfeld-Rieger syndrome (ARS), with the most common including facial dysmorphism, dental anomalies, periumbilical skin, cardiac defects and hearing loss.[3,4] The presence of anomalies in the anterior chamber angle and drainage structures of the eye contribute to a lifetime risk of developing glaucoma and can lead to irreversible blindness,[5] although the severity of the anomalies does not seem to correlate well with the age at diagnosis, the severity or the progression of glaucoma.[5] It is usually reported that ~50% of individuals with ARM will develop glaucoma, with an age of onset ranging from birth

to late in adulthood.[5] Guidelines for appropriate follow-up strategies for affected individuals without glaucoma lack a clear evidence base.

ARS is genetically heterogeneous. Deleterious sequence variants in the *FOXC1* (6p25.3, MIM 601090)[6,7] and *PITX2* genes (4q25, MIM 601542),[8] as well as two additional loci (RIEG2 at 13q14 and 16q24),[9,10] have been associated with ARS. In addition, several copy number variants and chromosomal rearrangements disrupting *FOXC1* or *PITX2* have been reported in association with ARS.[11–14] Deleterious variants in both genes account for approximately 40% of individuals with ARS,[11,15] with inter- and intra-familial variable expressivity frequently reported.[16,17] Although the ARM ocular phenotype associated with variants in *FOXC1* and *PIXT2* is thought to be highly penetrant and undistinguishable between both genes, there is a variable expressivity of systemic features. Variants in *PITX2* are more often associated with dental and/or umbilical anomalies, whereas individuals with *FOXC1* variants often have the ARM phenotype alone, or display a range of other systemic anomalies including heart anomalies, hearing defects, developmental delay and/or growth delay.[11,15,18] In addition, *FOXC1* has recently been identified as a

primary open-angle glaucoma (POAG) susceptibility locus[19] and an essential regulator of lymphangiogenesis.[20]

FOXC1 encodes a forkhead transcription factor encoded by a single exon, whereas *PITX2* is a member of a paired class of homeodomain transcription factors and encodes four alternative transcripts (*PITX2A, B, C* and *D*). Both are expressed during embryonic development and regulate downstream genes important for cell differentiation and migration via DNA binding. *FOXC1* and *PITX2* are co-expressed in the periocular mesenchyme and can physically interact with each other via their C-terminal domain and their homeobox domain, respectively.[21] *PITX2* can inhibit the transactivation activity of *FOXC1*, which is lost in the presence of *PITX2* loss-of-function variants.[21] The transcriptional activity of both genes requires tight regulation during embryogenesis for proper development of anterior segment tissues.

Although the morphological ocular anomalies associated with *FOXC1* and *PITX2* variants have been well described, the spectrum and prevalence of glaucoma in mutation carriers have not been well characterised. In this study, we delineated the glaucoma prevalence and phenotype in a cohort of patients diagnosed with ARM and their family members with *FOXC1* and *PITX2* variants.

METHODS

Participants' recruitment

Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee. The study was conducted in accordance with the revised Declaration of Helsinki. Individuals were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG) as described previously.[22] Informed written consent and a blood or saliva sample for DNA extraction purposes were obtained. Clinical information was collected by the patient's usual treating ophthalmologist. The feedback of results and genetic counselling was provided to the participants.

Individuals with ARM recruited in the ANZRAG were tested for the *FOXC1* and *PITX2* genes. Family members of individuals with variants in these genes were offered genetic testing. The cohort included all ARM probands and their family members identified as having a deleterious variant in the *FOXC1* or the *PITX2* genes. The diagnoses of ARM and glaucoma were made by the treating specialist who referred the patient to the ANZRAG. Individuals with ARM were recruited in the ANZRAG regardless of their glaucoma status. Advanced glaucoma was defined as visual field loss in the worse eye related to glaucoma with at least two out of the four central fixation squares having a pattern standard deviation $<0.5\%$ on a reliable Humphrey 24-2 field, or a mean deviation of <-22 dB. Ocular hypertension was defined by an intraocular pressure $\geqslant 21$ mm Hg.

Genetic testing

FOXC1 and *PITX2* genetic testing was performed through the NATA (National Association of Testing Authorities) accredited laboratories of SA Pathology at the Flinders Medical Centre (Bedford Park, SA, Australia). Venous blood specimens were collected into EDTA tubes; genomic DNA was prepared from a 200 μl sample of blood and extracted by a QIAcube automated system using QIAamp DNA Blood Mini kit (Qiagen, Chadstone, VIC, Australia) reagents according to the manufacturer protocols. Saliva specimen were collected into an Oragene•DNA Self-Collection Kit (DNA Genotek Inc., Ottawa, ON, Canada) and the DNA was isolated from 500 μl of sample and extracted as described in the manufacturer's instructions. The entire coding sequence and intron exon boundaries of *FOXC1* and *PITX2* were amplified as overlapping fragments in separate reactions with primer pairs (Supplementary Table 1).

Each polymerase chain reaction (PCR) was prepared using 100 ng of purified genomic DNA as template, 0.5 μM of each primer and a 1× concentration of AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Scoresby, VIC, Australia); 2.5 μl of 360 GC Enhancer (Thermo Fisher Scientific) was added to each *PITX2* reaction mix, and 5 μl to each *FOXC1* reaction mix. All reactions were adjusted to a final volume of 25 μl with deionised water. Gene template targets were amplified in a Veriti thermal cycler (Thermo Fisher Scientific)

using separate conditions for each gene; *FOXC1*: Step 1, 95 °C for 10 min; Step 2, 95°C for 30 s, 55 °C for 30 s, 72 °C for 30 s, repeated for 40 cycles; Step 3, 72 °C for 7 min. *PITX2*: Step 1, 94 °C for 5 min; Step 2, 94 °C for 30 s, 61 °C for 30 s (decreasing after five cycles by 1 °C every five cycles), 72 °C for 1 min, repeated for 15 cycles; Step 3, 94 °C for 30 sec, 58 °C for 50 s, 72 °C for 1 min, repeated for 35 cycles; Step 4, 72 °C for 10 min.

PCR amplified products were prepared for DNA sequencing by the 'ExoSAP' method using a 10 μl sample of each PCR reaction treated with 5 U of Exonuclease I (Biolabs, Ipswich, MA, USA) and 1 U of shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA) to remove residual primers and dNTPs. Bi-directional BigDye Terminator Cycle Sequencing (Thermo Fisher Scientific) reactions of the appropriate template and *FOXC1* and *PITX2* PCR primers were resolved and base called on an Applied Biosystems 3130*xl* Genetic Analyser (Thermo Fisher Scientific).

Detection of sequence variants was performed with the aid of the Mutation Surveyor v4.0 (SoftGenetics LLC, State College, PA, USA) programme; all forward and reverse sequence trace files were assembled by the programme against the *FOXC1* (NM_001453.2) and *PITX2* (NM_153427.2) GenBank reference. Significant differences in the relative peak heights of the sequence traces observed between that of the patient sample and a normal control were automatically called as a sequence variant by Mutation Surveyor; all such calls were visually inspected for confirmation. The Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org) and PolyPhen-2 HumVar (http://genetics.bwh.harvard.edu/pph2) software programmes were used to predict the potential impact of amino acid substitutions on the protein. Homologene (www.ncbi.nlm.nih.gov/homologene) was used to assess the conservation among mammalian species. Allelic frequencies were compared with the population frequencies from the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS) and the Exome Aggregation Consortium v0.3.1 (ExAC, http://exac.broadinstitute.org). All variants are publically available at the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/; accession numbers SCV000494251-SCV000494276).

FOXC1 and *PITX2* were analysed for copy number variation by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P054-B2 FOXL2-TWIST1 probemix (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. The copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130*xl* Genetic Analyzer, and the data analysed using Peak Scanner v2.0 (Thermo Fisher Scientific). Breakpoints of the genomic deletions and duplications that have been further characterised in Clinical Genetics using SNP arrays have been added in Tables 1 and 2.

Statistical analysis

PASW Statistics, Rel. 18.0.1.2009 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as median ± standard deviation. Fisher's exact test and Mann–Whitney *U*-test were used for the assessment of differences in non-parametric data.

RESULTS

The cohort consisted of 53 individuals from 24 families with heterozygous *FOXC1* or *PITX2* variants. The pedigrees are depicted in Figure 1. There were 25 males (47.2%) and 28 females (52.8%). The majority were Caucasian (94.3%). The mean age of the participants was 35.7 ± 20.4 years. Twenty probands (83%) had a family history of ARM and/or glaucoma.

FOXC1 variants were present in 30 individuals (56.6%) compared with 23 (43.4%) in *PITX2*. We identified 19 sequence variants across both genes (Tables 1 and 2 and Figure 2), of which 15 were considered novel (not reported in the literature and not present in the EVS or the ExAC database). Four nonsense, eight frameshift and one splice site sequence variants were considered to affect function. Among six missense sequence variants identified, *PITX2* p.(Pro64Leu) has previously been reported among different families as deleterious.[23–26] The other five missense sequence variants have not been reported before and were absent from the EVS and the ExAC database. Sequence

Table 1 Summary of *FOXC1* variants identified and associated phenotype

ID	Age	Sex	Genomic location (NC_000006.12, hg38)	Nucleotide change (NM_001453.2)	Amino acid change (NP_001444.2)	Ocular features	Systemic features	Glc	Age glc dx
1A	70	M	g.1610545_1610554del	c.100_109del10	p.(Gly34ThrfsTer8)	PAS, PE	Nil	Yes	3
1B	41	F	g.1610545_1610554del	c.100_109del10	p.(Gly34ThrfsTer8)	Haab's striae, mild PAS	Nil	Yes	4
2A	25	M	g.1610561_1610568del	c.116_123del8	p.(Ala39GlyfsTer41)	PE, IH, corectopia	Nil	No	—
2B	34	M	g.1610561_1610568del	c.116_123del8	p.(Ala39GlyfsTer41)	IH, PE, PAS, corectopia	Nil	No	—
3A	37	M	g.1610701C>T	c.256C>T	p.(Leu86Phe)	Corectopia	Nil	Yes	33
3B	6	F	g.1610701C>T	c.256C>T	p.(Leu86Phe)	Corectopia	Nil	OHT	5
4	56	F	g.1610713G>A	c.268G>A	p.(Ala90Thr)	PE, PAS, ectropion uvea	Nil	Yes	33
5A	41	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	IH, corectopia, Haab's striae	Nil	No	—
5B	13	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	PE, PAS	Pulmonary stenosis	No	—
5C	42	M	g.1610761C>T	c.316C>T	p.(Gln106Ter)	IH, PE, PAS	Nil	Yes	37
5D	10	F	g.1610761C>T	c.316C>T	p.(Gln106Ter)	PE, PAS	Nil	OHT	6
6A	37	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	Haab's striae, mild corectopia, cataracts	Short stature	Yes	0
6B	54	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	PE, very mild PAS	Hearing Loss	Yes	12
6C	4	M	g.1610902A>C	c.457A>C	p.(Thr153Pro)	Corneal haze, PE, mild corectopia	Nil	No	—
6D	17	F	g.1610902A>C	c.457A>C	p.(Thr153Pro)	PE, PAS, mild corectopia	Intellectual disability, heart defect, hearing Loss, short stature, hydrocephalus	No	—
7	26	F	g.1611044_1611062del	c.599_617del19	p.(Gln200ArgfsTer109)	PE, PAS, corneal oedema, ectropion uveae	Nil	Yes	0
8A	1	M	g.1611111_1611126del	c.666_681del16	p.(Ile223ProfsTer87)	PE	Heart defect	Yes	0
8B	28	M	g.1611111_1611126del	c.666_681del16	p.(Ile223ProfsTer87)	NA	Nil	Yes	8
9A	46	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	Corectopia, pseudopolycoria, ectropion uveae, Haab's striae	Nil	Yes	1
9B	44	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	PE, IH, corectopia	Nil	No	—
9C	69	F	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	PE, amblyopia	Club foot	No	—
9D	76	M	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	IH, ectropion uveae	Club foot	No	—
9E	50	F	g.1611370_1611394del	c.925_949del25	p.(Ser309CysfsTer84)	NA	Nil	No	—
10A	29	M	g.1611710C>A	c.1265C>A	p.(Ser422Ter)	PE, corectopia, megalocornea	Nil	OHT	2
10B	33	M	g.1611710C>A	c.1265C>A	p.(Ser422Ter)	NA	Nil	Yes	8
11A	41	F	g.1611936C>G	c.1491C>G	p.(Tyr497Ter)	Mild PAS, PE	Hearing Loss	Yes	15
11B	6	M	g.1611936C>G	c.1491C>G	p.(Tyr497Ter)	PE, PAS	Nil	No	—
12	7	F	g.(?_165632)_(3549058_35532219)del	c.(?_-1)_(*1_?)del	—	PE, PAS, corectopia	Fine motor skills delay	Yes	2
13A	43	M	g.?_?insNC_000006.12:g.(947938_951144)_(1832702_1833922)dup	c.(?_-1)_(*1_?)dup	—	PAS	Nil	Yes	18
13B	64	F	g.?_?insNC_000006.12:g.(947938_951144)_(1832702_1833922)dup	c.(?_-1)_(*1_?)dup	—	Nil	Nil	Yes	34

Abbreviations: Glc, glaucoma; IH, iris stromal hypoplasia; OHT, ocular hypertension; PAS, peripheral anterior synechiae; PE, posterior embryotoxon; NA, not available.

Table 2 Summary of *PITX2* variants identified and associated phenotype

ID	Age	Sex	Genomic location (NC_000004.12, hg38)	Nucleotide change (NM_153427.2)	Amino acid change (NP_00316.2)	Ocular features	Systemic features	Glc	Age glc/OHT
14A	38	M	g.110621211_110621232del	c.184_205del22	p.(Arg62AlafsTer86)	Corectopia	Dental, periumbilical skin, WPW syndrome	Yes	14
14B	66	F	g.110621211_110621232del	c.184_205del22	p.(Arg62AlafsTer86)	PAS, IH	Dental, periumbilical skin	Yes	12
15A	82	M	g.110621225G>A	c.191C>T	p.(Pro64Leu)	Nil	Dental, periumbilical skin	Yes	48
15B	55	F	g.110621225G>A	c.191C>T	p.(Pro64Leu)	IH, PE, PAS	Dental, umbilical hernia	Yes	25
15C	42	M	g.110621225G>A	c.191C>T	p.(Pro64Leu)	Mild IH	Dental, periumbilical skin	OHT	20
15D	20	F	g.110621225G>A	c.191C>T	p.(Pro64Leu)	IH, PE	Dental, periumbilical skin	OHT	18
16	42	M	g.110621163C>T	c.252+1G>A	—	IH	Dental, umbilical hernia	Yes	20
17A	20	M	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PE, PAS, pseudopolycoria	Dental, periumbilical skin	Yes	9
17B	70	M	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PAS, pseudopolycoria, corectopia	Dental, umbilical hernia, absent left vas deferens	Yes	28
17C	26	F	g.110618670G>C	c.271C>G	p.(Arg91Gly)	PE, PAS, corectopia	Dental, periumbilical skin	Yes	26
18A	49	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Corectopia, pseudopolycoria, PAS	Dental, periumbilical skin, epilepsy	Yes	19
18B	22	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Corectopia, pseudopolycoria, PAS, PE, IH	Dental, umbilical hernia	Yes	21
18C	16	F	g.110618667C>G	c.274G>C	p.(Ala92Pro)	Peters, PAS, IH, cataract	Dental, umbilical hernia	OHT	15
19	7	M	g.110618315_110618316del	c.487_488delTC	p.(Ser163HisfsTer35)	Rieger anomaly	Dental, periumbilical skin, cleft palate	Yes	1
20A	59	F	g.110618365_110618386del	c.555_576del22	p.(Thr186SerfsTer4)	IH, PE	Dental, periumbilical skin	Yes	26
20B	37	F	g.110618365_110618386del	c.555_576del22	p.(Thr186SerfsTer4)	IH, PAS, PE	Dental, periumbilical skin	No	—
21A	24	M	g.110618293A>T	c.648T>A	p.(Cys216Ter)	PAS, PE, IH, ectropion uvea, pseudopolycoria	Dental, periumbilical skin	Yes	3
21B	18	F	g.110618293A>T	c.648T>A	p.(Cys216Ter)	PE	Dental, periumbilical skin	No	—
22A	45	F	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, PAS, IH	Dental, periumbilical skin	No	—
22B	20	M	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, IH, corectopia, pseudopolycoria	Dental, periumbilical skin, fine motor skills delay	No	—
22C	17	F	g.(?_110618049)_(110622472_?)del	c.(?_47-1103)_(*76_?)del	—	PE, IH	Dental, periumbilical skin, imperforate anus	OHT	17
23	36	M	g.(111426357_111528916)_(111888401_111990971)del	c.(?_47-1103)_(*76_?)del	—	PAS, corectopia, pseudopolycoria	Dental, umbilical hernia	Yes	13
24	28	F	g.(?_110618124)_(110632999_?)del	c.(?_1)_(*1_?)del	—	PE, PAS, IH, corectopia	Dental, periumbilical skin, epilepsy	OHT	6

Abbreviations: Glc, glaucoma; IH, iris stromal hypoplasia; OHT, ocular hypertension; PAS, peripheral anterior synechiae; PE, posterior embryotoxon; WPW, Wolff–Parkinson–White.
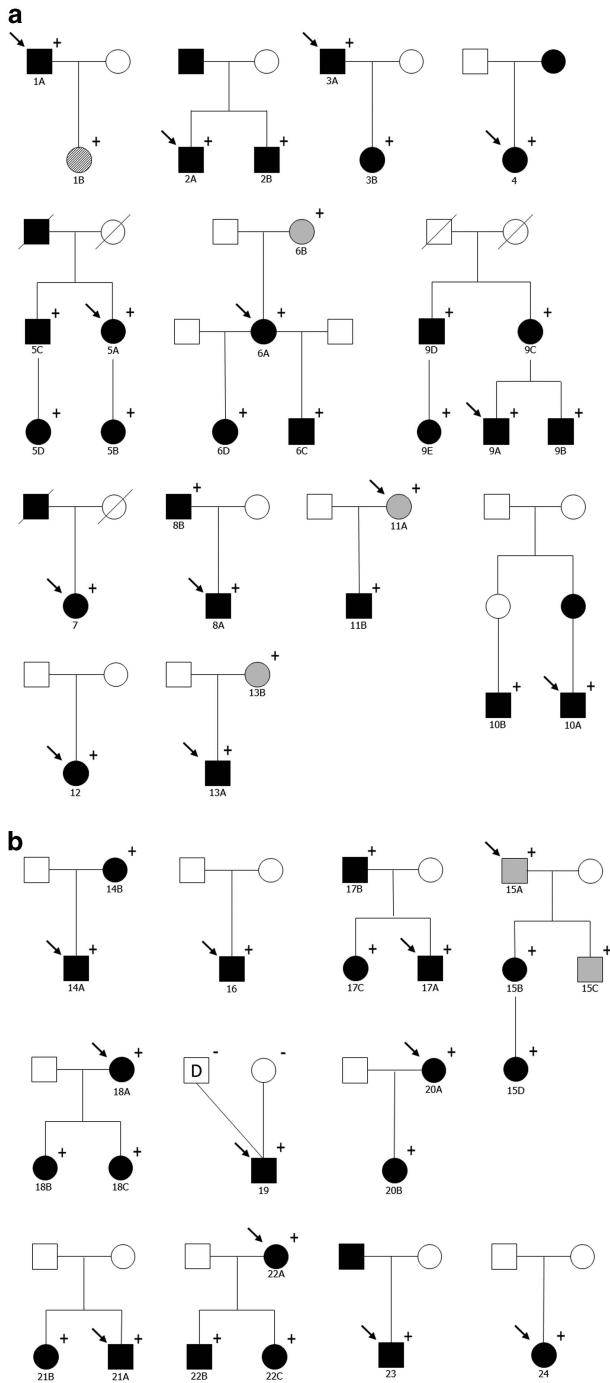
Figure 1 Pedigrees of the families. Round symbols indicate females; square symbols, males; black symbols, Axenfeld-Rieger Syndrome; grey symbols; primary open-angle glaucoma, dashed symbols; primary congenital glaucoma, unfilled symbols, unaffected; diagonal line, deceased; arrow, proband; D, sperm donor; +/−, presence/absence of the gene variant. (**a**) Families with *FOXC1* variants. (**b**) Families with *PITX2* variants.

highly conserved across different species (Supplementary Figure 1) and predicted deleterious by Polyphen-2 and SIFT algorithms. As a result, these five missense variants were also considered likely to be disease-causing. Finally, we identified five copy number variants: one deletion and one duplication of the *FOXC1* gene, and three deletions of the *PITX2* gene. All encompassed the entire gene, apart from one *PITX2* deletion, which spanned the last two coding exons.

In this cohort, 31 individuals (58.5%) had glaucoma, 6 (11.3%) had ocular hypertension and 16 (30.2%) showed no sign of glaucoma (Tables 1 and 2 and Supplementary Tables 2 and 3). Among the individuals with glaucoma, 48.4% (15/31) had advanced glaucoma as defined by the ANZRAG visual field criteria. The prevalence of glaucoma did not differ between *FOXC1* (16/30, 53.3%) and *PITX2* carriers (14/23, 60.9%, $P = 0.59$), with the median age of glaucoma diagnosis at 13.5 ± 12.2 years (range 0–48 years) for the entire cohort. Median age at diagnosis was significantly lower in *FOXC1* compared with *PITX2* carriers (6.0 ± 13.0 years (range 0–37) *vs* 18.0 ± 10.6 years (range 1–48), $P = 0.04$). The penetrance at 10 years of age was 29.4% for the whole cohort and was significantly higher for *FOXC1* carriers (42.9%) than *PITX2* carriers (13.0%, $P = 0.03$). Penetrance at 25 years was 63.8% for the whole cohort and was similar between both genes (57.7% for *FOXC1* carriers *vs* 71.4% for *PITX2* carriers, $P = 0.38$). The median age of individuals who did not have glaucoma was 29.5 ± 21.2 years (range 5–77 years) and was not significantly different between *FOXC1* (34.0 ± 24.2 years, range 5–77) and *PITX2* carriers (20.0 ± 13.1 years, range 17–46, $P = 0.74$).

As shown in Tables 1 and 2, there was inter- and intra-familial phenotypic variability in ocular morphology and glaucoma type, although glaucoma prevalence was not different between loss-of-function variants, missense variants and copy number variants ($P = 0.72$). Among the 53 individuals included in this study, 47 (88.7%) had classic ocular features of ARM (Figure 3). One family member of an individual with ARM was diagnosed with primary congenital glaucoma (PCG) and had mild features of ARM on re-examination after genetic testing (1B). Five family members of individuals with ARM had been diagnosed with POAG and had only mild or no features of ARM on re-examination after genetic testing (Figure 4). Four of the POAG cases had systemic features consistent with ASD, three carried *FOXC1* variants (11A, 6B, 13B) and two had *PITX2* variants (15A, 15C).

All individuals with *PITX2* variants had dental and umbilical anomalies including microdontia, hypodontia, redundant periumbilical skin or umbilical hernia. Additional systemic features were present in seven individuals and included Wolff–Parkinson–White syndrome, cleft palate, imperforate anus, fine motor skill delay, unilateral vas deferens and epilepsy. In comparison with *PITX2*, the majority of *FOXC1* carriers (21/30, 70.0%, $P < 0.001$) had no systemic features. Among the *FOXC1* carriers who did have systemic features, a range of abnormalities were reported including hearing loss (3/30, 10.0%), heart anomalies (3/30, 10.0%), short stature (2/30, 6.7%), club foot (2/30, 6.7%), hydrocephalus (1/30, 3.3%), intellectual disability (1/30, 3.3%) and fine motor skills delay (1/30, 3.3%).

DISCUSSION

In this study, we investigated the clinical phenotype of 53 ARM probands and their family members with variants in *FOXC1* or *PITX2*. Glaucoma was present in 59% and ocular hypertension in an additional 11%. Shields[5] reported glaucoma in 58% of individuals with a purely clinical diagnosis of ARM (with or without systemic features), and glaucoma prevalence among ARS patients with *FOXC1* or *PITX2* variants has previously been estimated at between 35% and
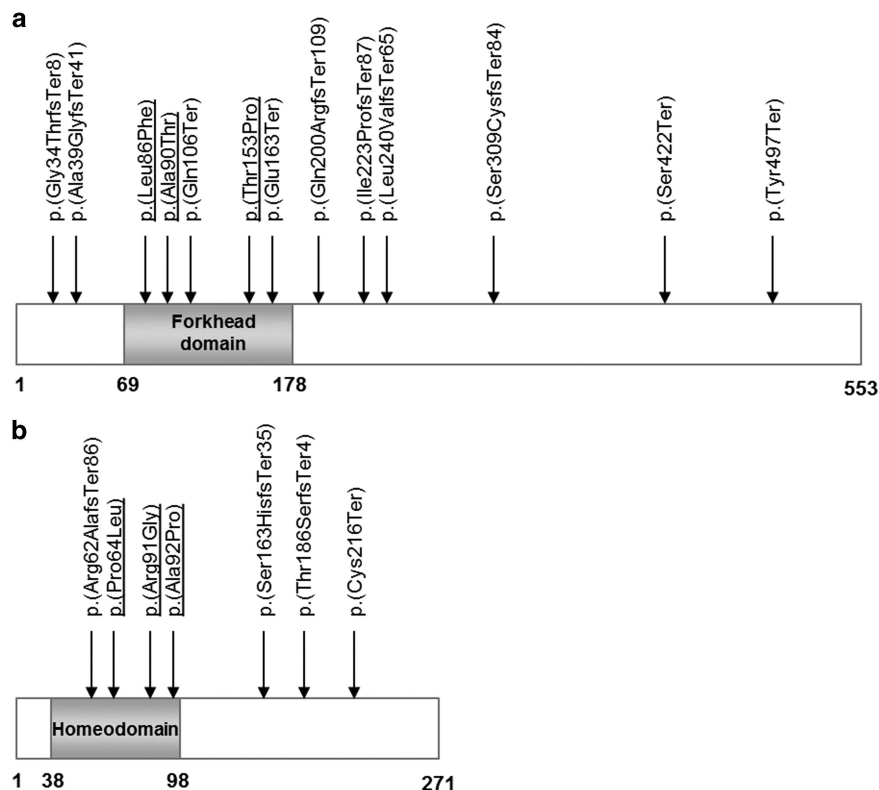
variants p.(Leu86Phe), p.(Ala90Thr) and p.(Thr153Pro) are located in the forkhead domain of the *FOXC1* gene which is important for DNA binding. Both Polyphen-2 and SIFT predicted these sequence variants to be damaging to the protein, with all three residues highly conserved across different species (Supplementary Figure 1). Similarly, *PITX2* variants p.(Arg91Gly) and p.(Ala92Pro) are located in the homeobox domain, which is essential for binding to *FOXC1*,[21] with both residues

a

p.(Gly34ThrfsTer8)
p.(Ala39GlyfsTer41)
p.(Leu86Phe)
p.(Ala90Thr)
p.(Gln106Ter)
p.(Thr153Pro)
p.(Glu163Ter)
p.(Gln200ArgfsTer109)
p.(Ile223ProfsTer87)
p.(Leu240ValfsTer65)
p.(Ser309CysfsTer84)
p.(Ser422Ter)
p.(Tyr497Ter)

Forkhead domain

1   69   178   553

b

p.(Arg62AlafsTer86)
p.(Pro64Leu)
p.(Arg91Gly)
p.(Ala92Pro)
p.(Ser163HisfsTer35)
p.(Thr186SerfsTer4)
p.(Cys216Ter)

Homeodomain

1 38   98   271

Figure 2 Distribution of the identified variants in the FOXC1 (**a**) and PITX2 proteins (**b**). Missense variants are underlined.

75%.[11,15,18] This is the first study to investigate the age-related glaucoma prevalence of each gene, along with the associated glaucoma phenotype.

We found a similar prevalence of glaucoma between *FOXC1* and *PITX2* carriers. Previous studies found a higher prevalence of glaucoma among *FOXC1* carriers than for *PITX2* carriers.[11,15,18] These differences can be explained by the age at diagnosis of affected individuals and the age of unaffected carriers reported. Although we have identified similar rates of glaucoma between both genes, the median age at diagnosis was significantly younger for *FOXC1* carriers than for *PITX2* carriers. Moreover, the prevalence at 25 years old was similar between both genes but the prevalence at 10 years old was significantly higher for *FOXC1* carriers than for *PITX2* carriers. Our results suggest that young unaffected individuals can still develop glaucoma at a later age. As a result, previous studies that were skewed toward *PITX2* carriers[15] or had young unaffected individuals[11,15] had a lower glaucoma prevalence than studies including a high proportion of *FOXC1* carriers.[18] Our findings showed that although the prevalence of glaucoma is similar between both genes, patients with *FOXC1* variants are likely to develop glaucoma at a younger age than *PITX2* carriers. In addition, our cohort included all available family members who were found to carry the suspected deleterious variant, which reduced the risk of recruitment bias.

In our study, one individual was initially diagnosed as PCG and five as POAG by their referring ophthalmologist. On re-examination, the individual with PCG and two individuals diagnosed with POAG had mild irido-corneal adhesions, whereas one individual with POAG had mild iris stromal hypoplasia. Two of the four *FOXC1* carriers had hearing loss and the other two *PITX2* carriers had dental and/or umbilical anomalies, none of which had been recorded before the molecular diagnosis. Individuals with variants in *FOXC1* or *PITX2* but

without ocular features of ARS have been reported before,[15,27,28] and in some individuals ARM can be so mild that it results in a clinical diagnosis of PCG or POAG. The variable expressivity associated with *FOXC1* and *PITX2* variants can make clinical diagnosis of ARS challenging, especially in the absence of ARM. However, all individuals were part of families with ARM and four out of six had systemic features consistent with ARS, emphasising the importance of delineating the systemic features associated with each gene to assist clinicians in reaching a differential diagnosis. Our findings confirmed that *PITX2* variants are strongly associated with dental and umbilical anomalies, whereas the majority (70%) of *FOXC1* carriers lack systemic features; however, our cohort is biased toward participants with ocular features.

In ARS, glaucoma is often challenging to treat and the intraocular pressure difficult to control, often requiring incisional surgery (trabeculectomy or glaucoma drainage implant) and in some cases repeated surgical procedures.[5,18] Standard medications and surgical procedures often have a lower success rate than in non-ARS glaucoma patients.[29] In our study, 48% of individuals with glaucoma had advanced visual field loss and all had incisional surgery. This highlights the importance of early molecular diagnosis for effective monitoring and treatment options.

Although the exact mechanism by which ARS causes glaucoma is not fully understood, it is hypothesised that an arrested development of the anterior chamber angle structures during gestation (characterised by an incomplete maturation of the trabecular meshwork, an absent or poorly developed Schlemm's canal and/or a high insertion of the iris) alters the aqueous humour flow, thereby increasing intraocular pressure and resulting in glaucoma.[5] Broad phenotypic variability associated with *FOXC1* and *PITX2* variants has been reported before,[11,14,17,18] as it has in this study, although Shields[5] previously reported that the severity of ocular defects did not correlate with the
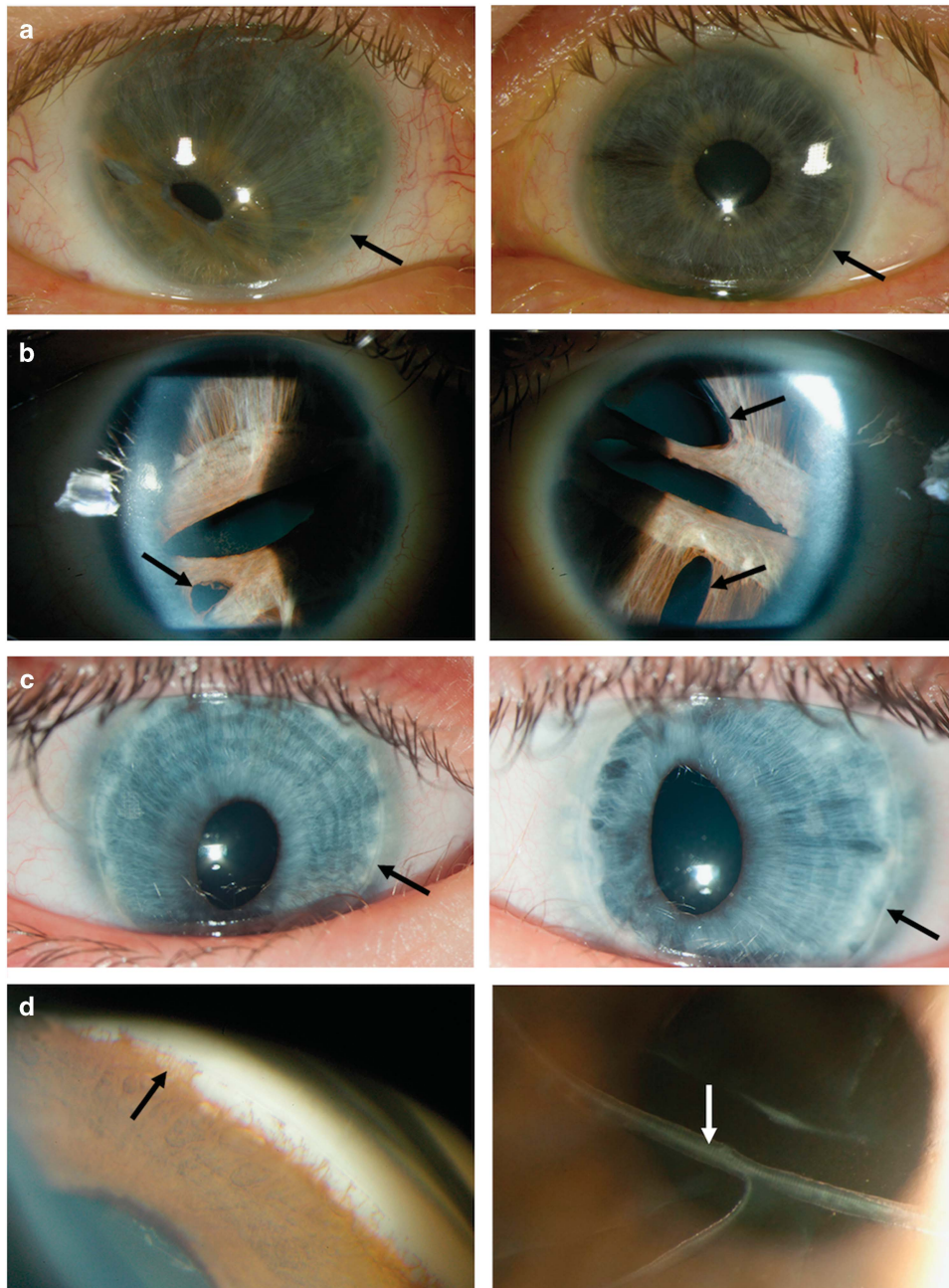
Figure 3 Clinical photographs of individuals with ocular features of Axenfeld-Rieger Malformation. Photographs in (**a–c**) showing the right eye (left panel) and the left eye (right panel). (**a**) Slit lamp photos showing corectopia in the left panel, iris stromal hypoplasia in both eyes and posterior embryotoxon (black arrows) in both eyes (individual 9B). (**b**) Slit lamp photos showing corectopia, pseudopolycoria (black arrows) and iris stromal hypoplasia in both eyes (individual 18B). (**c**) Slit lamp photos showing corectopia and posterior embryotoxon (black arrows) in both eyes (individual 12). (**d**) Gonioscopy showing irido-corneal adhesions (black arrow, left panel) and photo showing the presence of breaks in the Descemet's membrane (Haab's striae, white arrow, right panel; individual 5A).

development of glaucoma. *FOXC1* and *PITX2* both encode developmental transcription factors expressed in a tightly regulated temporal and spatial manner during development. Therefore, it is likely that disruption to the protein expression or activity level might not be well tolerated. However, inter- and intra-familial variability is often reported,[16,17,30] and is reflected by the variability in glaucoma phenotype within the families described here. Clinical heterogeneity of *FOXC1* and *PITX2* variants is likely to be explained by genetic modifiers, as suggested by the differing ocular phenotypes of *Foxc1*-deficient mice on different genetic backgrounds.[31] However, these modifiers remain to be identified.

A limitation of the study is that not every family member was tested, although all first-degree relatives of mutation carriers were invited to participate. Therefore, it is possible that individuals with milder phenotypes exist in the population but were not included. In addition, recruitment was somewhat biased towards glaucoma since participants were part of an advanced glaucoma registry, although the registry includes participants with ARM irrespective of their glaucoma status. Finally, diagnoses were made by different treating specialists, which may have introduced some variation in the phenotypic descriptions.

In conclusion, 59% of *FOXC1* and *PITX2* carriers in our cohort had glaucoma. Variants in both genes were associated with a similar risk of
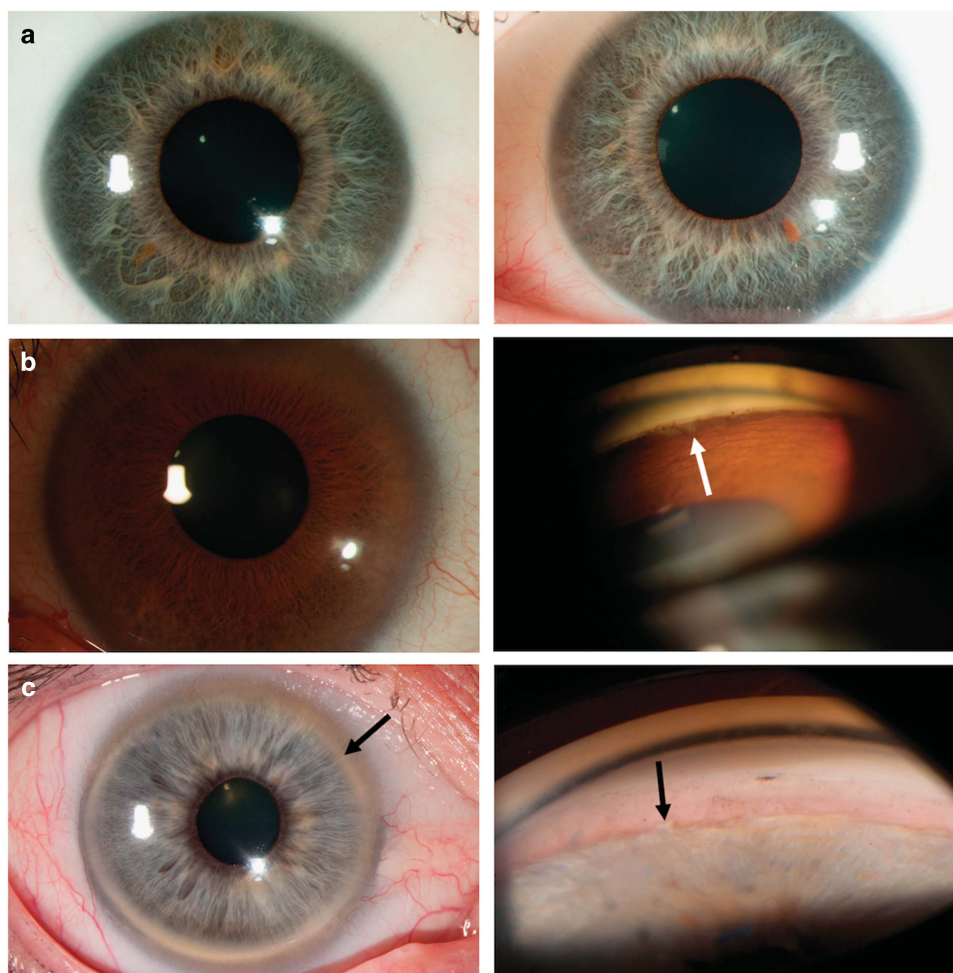
Figure 4 Clinical photographs of individuals with glaucoma and no or mild ocular features of Axenfeld-Rieger malformation after re-examination. Photographs in **a** showing the right eye (left panel) and the left eye (right panel). (**a**) Slit lamp photos showing iris stromal hypoplasia and diffuse posterior embryotoxon in both eyes (individual 15D). (**b**) Slit lamp photo showing the absence of iris anomalies and diffuse posterior embryotoxon in the left panel and gonioscopy showing mild irido-corneal adhesions (white arrow) in the right panel (individual 11A). (**c**) Slit lamp photo showing posterior embryotoxon (black arrow) in the left panel and gonioscopy showing mild irido-corneal adhesions (black arrow) in the right panel (individual 6B).

glaucoma, with *FOXC1* carriers displaying an earlier age of onset than *PITX2* carriers. These findings have implications when counselling individuals and their family members about their risk of developing glaucoma following genetic testing results. Furthermore, one family member was diagnosed with PCG and five with POAG, suggesting that variants in *FOXC1* and *PITX2* may also contribute to the genetic architecture of POAG and PCG. Further sequencing of large patient cohorts will be needed to determine the contribution of these genes to other glaucoma subtypes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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