Title: Identification of *PITX3* mutations in individuals with various ocular developmental defects

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Running title: *PITX3* variants in eye disorders

ABSTRACT

Background: Congenital cataract displays large phenotypic (syndromic and isolated cataracts) and genetic heterogeneity. Mutations in several transcription factors involved in eye development, like *PITX3*, have been associated with congenital cataracts and anterior segment mesenchymal disorders.

Materials and methods: Targeted sequencing of 187 genes involved in ocular development was performed in 96 patients with mainly anophthalmia and microphthalmia. Additionally, Sanger sequencing analysis of *PITX3* was performed on a second cohort of 32 index cases with congenital cataract and Peters anomaly and/or sclereocornea.

Results: We described five families with four different *PITX3* mutations, two of which were novel. In family 1, the heterozygous recurrent c.640_656dup (p.Gly220Profs*95) mutation cosegregated with eye anomalies ranging from congenital cataract to Peters anomaly. In family 2, the novel c.669del (p.(Leu225Trpfs*84)) mutation cosegregated with dominantly inherited eye anomalies ranging from posterior embryotoxon to congenital cataract in heterozygous carriers and congenital sclereocornea and cataract in a patient homozygous for this mutation. In family 3, we identified the recurrent heterozygous c.640_656dup (p.Gly220Profs*95) mutation segregating with congenital cataract. In family 4, the *de novo* c.582del (p.(Ile194Metfs*115)) mutation was identified in a patient with congenital cataract, microphthalmia, developmental delay and autism. In family 5, the c.38G>A (p.Ser13Asn) mutation segregated dominantly in a family with Peters anomaly, which is a novel phenotype associated with the c.38G>A variant compared with the previously reported isolated congenital cataract.

Conclusions: Our study unveils different phenotypes associated with known and novel mutations in *PITX3*, which will improve the genetic counselling of patients and their families.

Keywords: PITX3, cataract, Peters anomaly, anterior segment mesenchymal disorder, sclereocornea.

INTRODUCTION

Congenital cataract affects 72 per 100,000 newborns in developed countries [1]. Congenital cataract can be presented as the only clinical feature (isolated) or in association with other ocular or extraocular abnormalities. It displays large phenotypic and genetic heterogeneity. Mutations in genes coding for proteins essential for the development and integrity of the lens, such as connexins and crystallins, can cause isolated cataract [2,3]. In contrast, mutations in different transcription factors involved in gene regulation during eye development such as *PAX6* [4], *FOXE3* [5], *PITX2* [6], *FOXC1* [7], *MAF* [8], *EYA1* [9] and *PITX3* [10] are associated with congenital cataract and anterior segment mesenchymal disorders (ASMD), such as Peters anomaly.

PITX3 codes for the paired-like homeodomain transcription factor 3 and is a member of the RIEG/PITX homeobox gene family [11]. PITX3 appears to have a conserved role in ocular development throughout vertebrates. In mouse models, a recessive mutation in *Pitx3* (*aphakia* mouse) results in microphthalmia and absent lenses [12,13]. Similarly, morpholino knockdown of *pitx3* in zebrafish results in abnormalities in the development of the retina and lens [14].

The PITX3 protein has two different domains, a N-terminal homeodomain and a C-terminal *otp*, *aristaless*, and *rax* (OAR) domain, which are also characteristic of the other members of RIEG/PITX homeobox gene family [11]. To date, five frameshift mutations, all located N-terminal of the OAR domain, have been described in 16 index cases displaying eye anomalies with variable expressivity ranging from mild conditions, such as posterior embryotoxon, to more severe ones such as Peters anomaly [10,15-20]. In contrast, only one heterozygous missense mutation located upstream of the homeodomain (p.Ser13Asn), and associated with congenital cataract and glaucoma, has been identified [10,15,17-20]. Only three patients from two families

with homozygous *PITX3* mutations have been reported so far. They presented with a more severe ocular phenotype (ASMD, microphthalmia) than the heterozygous family members which was, besides, occasionally accompanied by neurological features [15,17].

In this study, we describe phenotypes associated to new and known heterozygous mutations in *PITX3* as well as we present the third family ever described with a homozygous mutation in this gene.

MATERIALS AND METHODS

Patients, targeted and Sanger sequence analyses

The current cohort study was approved by the Cambridgeshire 1 Ethics Committee 04/Q0104/129 and by the French Ethics Committee "*Comité de Protection des Personnes (CPP) Sud-Ouest et Outre-Mer II*". Written informed consent was obtained from all participating subjects.

The medical history was taken from all participants. All patients were assessed by an ophthalmologist and a geneticist/paediatrician.

The first patient cohort together with the molecular and analysis methods used for targeted sequencing were previously described by Chassaing *et al.* (2016) [21]. Shortly, this cohort consisted of 96 patients with mainly anophthalmia and microphthalmia (AM), with or without other ocular or systemic anomalies, for whom previous molecular screening of four of the main AM genes (*SOX2*, *OTX2*, *RAX*, and *VSX2*) did not reveal any positive diagnosis. They were subsequently targeted sequenced for 186 known and candidate genes involved in ocular development, including *PITX3* (Table S1).

Due to the involvement of *PITX3* mutations in families with congenital cataract and AM along with Peters anomaly and/or sclereocornea, a second patient cohort of 32 index cases with congenital cataract and Peters anomaly and/or sclereocornea (and/or a family history thereof) was screened for mutations in *PITX3* by Sanger sequencing.

Primers for amplification and sequencing of exons and exon-intron boundaries of *PITX3* (ENST00000370002) are shown in Table S2. Amplification by PCR was performed on 25 ng of genomic DNA with Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA). PCR fragments were purified with a gel extraction kit (Neo Biotech CliniSciences, Nanterre, France) in accordance with manufacturer's protocol. Sequence analysis was performed with the 3500xL sequencer (Applied Biosystems, Foster city, CA, USA).

Co-segregation of each mutation was performed by Sanger sequencing in all available family members.

Potential mosaicism was assessed in an asymptomatic heterozygous carrier in Family 5 by means of Digital Droplet PCR (ddPCR) assays with the Droplet Digital PCR QX200 System (Bio-Rad Laboratories, Hercules, USA) using a commercial TaqMan SNP Genotyping assays (ID: C_1007168_10; Thermo Fisher, Foster city, CA, USA) to genotype the previously known *PITX3* variant (c116C>G; p.(Thr39Arg)).

RESULTS

Description of *PITX3* mutations

Four mutations in *PITX3* were identified in five different families (Figures 1 and 2): two by screening a panel of 187 genes (families 1 and 4), and the other two by Sanger sequencing *PITX3* (families 2, 3 and 5). These mutations were associated with various ocular developmental disorders within the five families (comprising 12 affected individuals in total). Three of them (c.582del, p.(Ile194Metfs*115)), c.640_656dup (p.(Gly220Profs*95), and c.669del (p.(Leu225Trpfs*84)) were frameshift mutations and one was a missense mutation (c.38G>A, p.Ser13Asn). The c.669del and c.582del variants were novel.

Except for the previously reported c.38G>A variant, which was present in 1/78742 alleles in the ExAC database and in 1/225438 alleles in the gnomAD database, the other ones have not been reported before in the NCBI dbSNP138 database, the NHLBI Exome Sequencing Project, the 1000 genomes project, the gnomAD, the GME and the ExAC databases. As depicted in Figure 3, all frameshift mutations identified in *PITX3* were N-terminal of the OAR domain while the missense mutation (p.Ser13Asn) was N-terminal of the homeodomain.

All mutations presented in this manuscript were submitted to the Leiden Open Variant Database with the following IDs: 00001632110, 0000163211, 00001632112, 0000163213 and 0000163214.

Phenotypes of subjects harboring *PITX3* mutations

Mutations in *PITX3* were identified in five families (12 affected cases).

*Family 1, heterozygous c.640_656dup (p.Gly220Profs*95) mutation*: A large French family (Figure 1A) with autosomal dominant cataract with a large intra-familial variability ranging from congenital cataract to Peters anomaly, was ascertained. The index case (II:5) presented with Peters anomaly in one eye and cataract in the other eye. His mother (I:3) was not affected, however, his deceased father (I:4) had presented with posterior embryotoxon and congenital cataract. The index case had an older brother (II:4) who presented with congenital bilateral cataract that were operated at the ages of 30 (right eye) and 33 (left eye). The II:4 case had a seven months old child (III:3) with Peters anomaly and posterior embryotoxon. The younger sister (II:7) presented with congenital cataract operated at the age of 14 years old. The younger brother (II:6) was unaffected. A paternal cousin was identified by history with unilateral congenital cataract. General physical examination and history did not reveal any additional ocular or extra-ocular abnormalities.

*Family 2, homozygous c.669del (p.(Leu225Trpfs*84)) mutation*: A consanguineous family from Iraq (Figure 1B) was ascertained due to the proband, II:1, having bilateral congenital sclerocornea and ASMD, identified on neonatal screening. No ocular abnormalities were detected in her dizygotic twin, II.2. They were born prematurely (27 weeks of gestation, 960 g) by caesarean section. Transthoracic, transfontanelle and abdominal ultrasound examinations did not reveal anomalies. No infection was reported during pregnancy. At 14 months of age, the proband was unable to sit unaided. At that time, her length was 72.5 cm (-0.75 SD), weight was 8.85 kg (-

188 0.5 SD) and OFC was 44.5 cm (-0.1 SD). She had plagiocephaly, metopic ridge and a thin upper
189 lip, as well as broad thumbs and clinodactyly of the 5th finger. At two years of age, the index case
190 developed bilateral buphthalmos. At that time, wearing glasses, she could follow the movement
191 of bright objects. Her mother, I.1, presented with nasal and temporal posterior embryotoxon in
192 the right eye and temporal posterior embryotoxon in the left eye. Her father, I:2, presented with
193 congenital bilateral cataract associated with nasal posterior embryotoxon in the left eye. Her twin
194 brother had nasal posterior embryotoxon in the left eye. Her father reported that his two siblings
195 presented early onset cataract-like symptoms. The index's parents were first cousins.
196
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198 *Family 3, c.640_656dup (p.Gly220Profs*95) heterozygous mutation*: A small family from
199 France (Figure 1C) was ascertained as the index case (II:2) presented with Peters anomaly at
200 three months of age. The karyotype and array analyses of this patient were normal. General
201 physical examination showed short stature (-3SD), weight (-1.2 SD) and OFC (-0.7 SD), facial
202 features (prominent forehead, short nose, short columella, long philtrum, thin upper lip, full
203 cheeks), *genu valgum*, lax elbow joints and short hands with tapering fingers. He had a
204 psychomotor delay; he sat at 12 months and walked at 21 months of age. Because of the
205 diagnosis of Peters plus syndrome, molecular sequencing for *B3GLCT* was performed and it did
206 not reveal any mutations [22]. His mother, I.2, had a family history of juvenile bilateral cataract
207 and had cataract surgery at the age of 18. His father, I.1, was unaffected. His mother reported that
208 more family members on her side were affected with congenital or juvenile cataract; however, we
209 did not have access to their clinical data.
210

*Family 4, heterozygous c.582del (p.(Ile194Metfs*115)) mutation (de novo)*: A family trio from North Ireland was ascertained (Figure 1D). The index case (II:1) was a 12 year-old boy born 10 days post term following a normal pregnancy (except for some early bleeding in pregnancy) with a birth weight of 3900 g. He was diagnosed with bilateral congenital cataract and microphthalmia on day 1 because of no red reflex. He had bilateral cataract surgery at three weeks of age and was fitted with contact lenses. He subsequently had a left broad iridectomy, with capsulotomy and vitrectomy at the age of four months, a left Ahmed valve insertion at seven months of age and right inferior oblique anteriorisation (squint surgery) at two years of age. At two years seven months of age, he had severe visual impairment with navigational vision. His early motor milestones were delayed: he sat independently at 11 months of age. However, he started walking at 12 months, and then steadily at 15 months. He had early behavioral issues with constant crying and was delayed in acquiring social skills. His first word was around one year. He needed speech therapy to improve clarity of speech. He was later diagnosed with autism which at the age of 12 years was severe. His growth was initially around the 90^{th} % for height and weight until recently when he became average height. There is a family history of autistic spectrum disorder in three male cousins and epilepsy in a female cousin, all on the maternal side. At age 12 years, he had a head circumference of 55.7 cm (50^{th}-75^{th} %), height 147.3cm (50^{th}%) and weight 36.9kg (9-25^{th}%). He had a double row of teeth, but no other dysmorphic features. He had bilateral microphthalmia, cloudy corneas with a corneal diameter of 9 mm bilaterally, was aphakic with a broad iridectomy on the left eye and a small pupil on the right. Parental eye examinations showed the mother had very slight enlargement of the optic cups, and the father had tilted myopic optic discs only. Array and chromosome analysis were both normal.

Family 5, heterozygous c.38G>A (p.Ser13Asn) mutation: A family from France (Figure 1E) with Peters anomaly was ascertained. The index case (III:1) presented bilateral Peters anomaly while his mother presented unilateral Peters anomaly. Neurological development of the index case (III:1) was within normal ranges. There was no facial dysmorphism presented by the index case as by the affected mother (II:2) apart from one palatal tooth for the index case. The length, weight and OFC of the index case were at +1 SD at age 10 years.

The grandmother (I:2), who carried the c.38G>A variant heterozygously, displayed bilateral nuclear and cortical cataract without any anterior segment anomaly. Because she only developed cataract at 70 years of age, we classified her as not affected (Figure 1E). We confirmed the mutation in the grandmother (I:2) on a new blood sample as well as on a saliva sample in which no mosaicism was further ascertained by ddPCR quantification (estimated fractional abundance of 50% for the mutated allele, Figure S1) in the analyzed tissues. By history, no other members from the mother's side had any ocular or extra-ocular features.

DISCUSSION

The aim of this study was to identify mutations in *PITX3* in patients with congenital cataracts accompanied by anterior segment dysgenesis or microphthalmia. We were able to identify four different mutations, including two novel ones, in five families (12 affected cases).

Except for the p.Ser13Asn, all mutations described by us and others introduce a frameshift [10,15,17-20]. The phenotypic variability associated to these mutations was large even within members of the same family. Most of them were dominantly inherited and associated to cataract accompanied by additional eye disorders ranging from embryotoxon to microphthalmia. In contrast, Aldahmesh et al. described the only *PITX3* mutation associated with autosomal

recessive inheritance to date [15]. The index case with a homozygous c.640_656del (p.(Ala214Argfs*42)) presented sclereocornea and microphthalmia and he was born from a healthy first cousin mating, both of whom were heterozygous for the *PITX3* mutation [15]. Homozygously mutated patients were identified in two other families (Bidinost et al. and this report) but these mutations lead to ocular disorders even in heterozygous carriers [17]. Thus, in the latter families, the homozygous patients have a double-dose of a dominant mutation, while in the one presented by Aldahmesh et al., the mutation seemed to be truly recessive. Independently of the mode of inheritance, homozygous mutated patients seem to have a more severe phenotype than heterozygous ones. Indeed, all have severe ocular phenotypes as sclerocornea associated with microphthalmia (3/4) or severe ASMD (1/4). In addition, two of them presented with developmental delay. Here, we also presented two patients with heterozygous *PITX3* mutations with developmental delay (Family 3, patient III:2) or autism (Family 4, patient II:1). The neurological involvement might be associated to the *PITX3* mutation, however, we could not rule out a different cause as one patient presented with additional features (short stature, facial features and finger abnormalities) that were not previously associated with *PITX3* mutations, and the other carrying a *de novo PITX3* mutation had a familial history of autistic features.

There is also a wide variability regarding the ocular involvement among heterozygous patients ranging from unilateral nasal posterior embryotoxon to congenital cataract and microphthalmia. This variability was evidenced even within patients from the same family, but also within the same individual as patient II:2 from family 5 presented with unilateral Peters anomaly with contralateral normal eye. This suggests that penetrance of *PITX3* mutations may be incomplete. This was already demonstrated in the large family described by Aldahmesh et al. in which 1/31 heterozygous patients was unaffected [15]. In Family 5, the penetrance of the p.Ser13Asn mutation was supposed to be incomplete as the asymptomatic grandmother, who presented with

late onset cataract, also carried the mutation. Besides, the hypothesis of mosaicism could not be demonstrated in the analyzed tissues (blood and saliva) as the fractional abundance of the mutated allele was similar to the expected for a fully heterozygous carrier in two different tissues (blood and saliva). In addition, the p.Ser13Asn mutation cosegregated with Peters anomaly in this family, a new phenotypic manifestation associated with this mutation as, to date, it has only been identified in a family with isolated congenital cataract [10]. One plausible explanation of phenotypic variability and incomplete penetrance is that stochastic effects, genetic background, and environmental factors during development might result in variable active protein available at different time points that can be crucial or detrimental during development [23-26].

As previously mentioned, except for the (p.Ser13Asn), all *PITX3* mutations known to date lead to a frameshift. The p.Ser13Asn variant is very rare in the general population (1/225438 alleles in the gnomeAD database), and it was previously identified *de novo* in a patient with congenital cataract [10]. This variant affects a conserved amino acid and was predicted to be deleterious by different prediction softwares (Polyphen-2 and Mutation Taster), but tolerated by SIFT software. Functional analyses that studied the consequences of this mutation on PITX3 function have demonstrated only minor functional effects in comparison to the p.Gly220Profs*95 mutant which showed a partial loss of function of the protein activity [27]. These functional studies identified a slightly decrease in the DNA binding ability of the p.Ser13Asn mutant, however a 23% decrease in its ability to increase reporter activity was found which may support our premise that this variant is disease-causing [27]. Of note, no other deleterious variants were identified among the 187 genes screened in the index case carrying this mutation (case III:1, family 5).

The genetic pathway in which *PITX3* is involved is not elucidated yet. Knockdown of *foxe3* and *pitx3* in zebrafish by using morpholinos demonstrated that *pitx3* is genetically upstream of *foxe3* since in *pitx3* morphants the expression of *foxe3* was abolished, while in *foxe3* morphants, *pitx3*

expression was detected [28]. Also, *Pitx3* is downregulated in lenses of heterozygous *Pax6* mutant mice, implying that *Pax6* is genetically upstream of *Pitx3* [29]. Mutations in *PAX6* are associated with aniridia, congenital cataract, Peters anomaly and microphthalmia, amongst other ocular disorders; while mutations in *FOXE3* are also associated with Peters anomaly, cataract, congenital aphakia, sclerocornea and microphthalmia. This phenotypic overlap between mutations in *PAX6*, *PITX3* and *FOXE3* suggests that the three genes could be involved in the same genetic pathway in humans as well as in mice and zebrafish.

In conclusion, we have presented known and novel mutations in *PITX3* that are causative of congenital cataract, ASMD (including Peters anomaly) and microphthalmia in families that show large phenotypic variability. Further investigations are needed to elucidate the cause of this clinical variability as well as the molecular pathways that involve *PITX3* during ocular development.

URLs

ExAC database: http://exac.broadinstitute.org/

GnomAD database: http://gnomad.broadinstitute.org/

dbSNP138 database: https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=138

NHLBI Exome Sequencing Project: http://evs.gs.washington.edu/EVS/

1000 genomes project: http://phase3browser.1000genomes.org/index.html

Leiden Open Variant Database: http://www.lovd.nl/3.0/home

Polyphen-2: http://genetics.bwh.harvard.edu/pph2/

Mutation Taster: http://www.mutationtaster.org/

SIFT: http://sift.jcvi.org/

GME database: http://igm.ucsd.edu/gme/

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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REFERENCES

1. Thylefors B, Negrel AD, Pararajasegaram R, Dadzie KY. Global data on blindness. Bull World Health Organ. 1995; 73: 115-21.

2. Graw J. Congenital hereditary cataracts. Int J Dev Biol. 2004; 48: 1031-44.

3. Shiels A, Hejtmancik JF. Mutations and mechanisms in congenital and age-related cataracts. Exp Eye Res. 2017; 156: 95-102.

4. Hanson I, Churchill A, Love J, Axton R, Moore T, Clarke M, Meire F, van Heyningen V. Missense mutations in the most ancient residues of the PAX6 paired domain underlie a spectrum of human congenital eye malformations. Hum Mol Genet. 1999; 8: 165-72.

5. Semina EV, Brownell I, Mintz-Hittner HA, Murray JC, Jamrich M. Mutations in the human forkhead transcription factor FOXE3 associated with anterior segment ocular dysgenesis and cataracts. Hum Mol Genet. 2001; 10: 231-6.

6. Doward W, Perveen R, Lloyd IC, Ridgway AE, Wilson L, Black GC. A mutation in the RIEG1 gene associated with Peters' anomaly. J Med Genet. 1999; 36: 152-5.

7. Honkanen RA, Nishimura DY, Swiderski RE, Bennett SR, Hong S, Kwon YH, Stone EM, Sheffield VC, Alward WL. A family with Axenfeld-Rieger syndrome and Peters Anomaly caused by a point mutation (Phe112Ser) in the FOXC1 gene. Am J Ophthalmol. 2003; 135: 368-75.

8. Jamieson RV, Perveen R, Kerr B, Carette M, Yardley J, Heon E, Wirth MG, van Heyningen V, Donnai D, Munier F, Black GC. Domain disruption and mutation of the bZIP transcription factor, MAF, associated with cataract, ocular anterior segment dysgenesis and coloboma. Hum Mol Genet. 2002; 11: 33-42.

9. Azuma N, Hirakiyama A, Inoue T, Asaka A, Yamada M. Mutations of a human homologue of the Drosophila eyes absent gene (EYA1) detected in patients with congenital cataracts and ocular anterior segment anomalies. Hum Mol Genet. 2000; 9: 363-6.

372 10. Semina EV, Ferrell RE, Mintz-Hittner HA, Bitoun P, Alward WL, Reiter RS, Funkhauser C,
373 Daack-Hirsch S, Murray JC. A novel homeobox gene PITX3 is mutated in families with
374 autosomal-dominant cataracts and ASMD. Nat Genet. 1998; 19: 167-70.

375 11. Gage PJ, Suh H, Camper SA. The bicoid-related Pitx gene family in development. Mamm
376 Genome. 1999; 10: 197-200.

377 12. Semina EV, Murray JC, Reiter R, Hrstka RF, Graw J. Deletion in the promoter region and
378 altered expression of Pitx3 homeobox gene in aphakia mice. Hum Mol Genet. 2000; 9: 1575-85.

379 13. Rieger DK, Reichenberger E, McLean W, Sidow A, Olsen BR. A double-deletion mutation in
380 the Pitx3 gene causes arrested lens development in aphakia mice. Genomics. 2001; 72: 61-72.

381 14. Shi X, Bosenko DV, Zinkevich NS, Foley S, Hyde DR, Semina EV, Vihtelic TS. Zebrafish
382 pitx3 is necessary for normal lens and retinal development. Mech Dev. 2005; 122: 513-27.

383 15. Aldahmesh MA, Khan AO, Mohamed J, Alkuraya FS. Novel recessive BFSP2 and PITX3
384 mutations: insights into mutational mechanisms from consanguineous populations. Genet Med.
385 2011; 13: 978-81.

386 16. Ye X, Zhang G, Dong N, Meng Y. Human pituitary homeobox-3 gene in congenital cataract
387 in a Chinese family. Int J Clin Exp Med. 2015; 8: 22435-9.

388 17. Bidinost C, Matsumoto M, Chung D, Salem N, Zhang K, Stockton DW, Khoury A,
389 Megarbane A, Bejjani BA, Traboulsi EI. Heterozygous and homozygous mutations in PITX3 in a
390 large Lebanese family with posterior polar cataracts and neurodevelopmental abnormalities.
391 Invest Ophthalmol Vis Sci. 2006; 47: 1274-80.

18. Verdin H, Sorokina EA, Meire F, Casteels I, de Ravel T, Semina EV, De Baere E. Novel and recurrent PITX3 mutations in Belgian families with autosomal dominant congenital cataract and anterior segment dysgenesis have similar phenotypic and functional characteristics. Orphanet J Rare Dis. 2014; 9: 26.

19. Berry V, Francis PJ, Prescott Q, Waseem NH, Moore AT, Bhattacharya SS. A novel 1-bp deletion in PITX3 causing congenital posterior polar cataract. Mol Vis. 2011; 17: 1249-53.

20. Berry V, Yang Z, Addison PK, Francis PJ, Ionides A, Karan G, Jiang L, Lin W, Hu J, Yang R, Moore A, Zhang K, Bhattacharya SS. Recurrent 17 bp duplication in PITX3 is primarily associated with posterior polar cataract (CPP4). J Med Genet. 2004; 41: e109.

21. Chassaing N, Ragge N, Plaisancie J, Patat O, Genevieve D, Rivier F, Malrieu-Eliaou C, Hamel C, Kaplan J, Calvas P. Confirmation of TENM3 involvement in autosomal recessive colobomatous microphthalmia. Am J Med Genet A. 2016; 170: 1895-8.

22. Dassie-Ajdid J, Causse A, Poidvin A, Granier M, Kaplan J, Burglen L, Doummar D, Teisseire P, Vigouroux A, Malecaze F, Calvas P, Chassaing N. Novel B3GALTL mutation in Peters-plus Syndrome. Clin Genet. 2009; 76: 490-2.

23. Cvekl A, Tamm ER. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. Bioessays. 2004; 26: 374-86.

24. Perveen R, Lloyd IC, Clayton-Smith J, Churchill A, van Heyningen V, Hanson I, Taylor D, McKeown C, Super M, Kerr B, Winter R, Black GC. Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. Invest Ophthalmol Vis Sci. 2000; 41: 2456-60.

25. Gould DB, John SW. Anterior segment dysgenesis and the developmental glaucomas are complex traits. Hum Mol Genet. 2002; 11: 1185-93.

26. Zazo Seco C, Serrao de Castro L, van Nierop JW, Morin M, Jhangiani S, Verver EJ, Schraders M, Maiwald N, Wesdorp M, Venselaar H, Spruijt L, Oostrik J, Schoots J, Baylor-Hopkins Center for Mendelian G, van Reeuwijk J, Lelieveld SH, Huygen PL, Insenser M, Admiraal RJ, Pennings RJ, Hoefsloot LH, Arias-Vasquez A, de Ligt J, Yntema HG, Jansen JH, Muzny DM, Huls G, van Rossum MM, Lupski JR, Moreno-Pelayo MA, Kunst HP, Kremer H. Allelic Mutations of KITLG, Encoding KIT Ligand, Cause Asymmetric and Unilateral Hearing Loss and Waardenburg Syndrome Type 2. Am J Hum Genet. 2015; 97: 647-60.

27. Sakazume S, Sorokina E, Iwamoto Y, Semina EV. Functional analysis of human mutations in homeodomain transcription factor PITX3. BMC Mol Biol. 2007; 8: 84.

28. Shi X, Luo Y, Howley S, Dzialo A, Foley S, Hyde DR, Vihtelic TS. Zebrafish foxe3: roles in ocular lens morphogenesis through interaction with pitx3. Mech Dev. 2006; 123: 761-82.

29. Chauhan BK, Zhang W, Cveklova K, Kantorow M, Cvekl A. Identification of differentially expressed genes in mouse Pax6 heterozygous lenses. Invest Ophthalmol Vis Sci. 2002; 43: 1884-90.

TITLES AND LEGENDS TO FIGURES

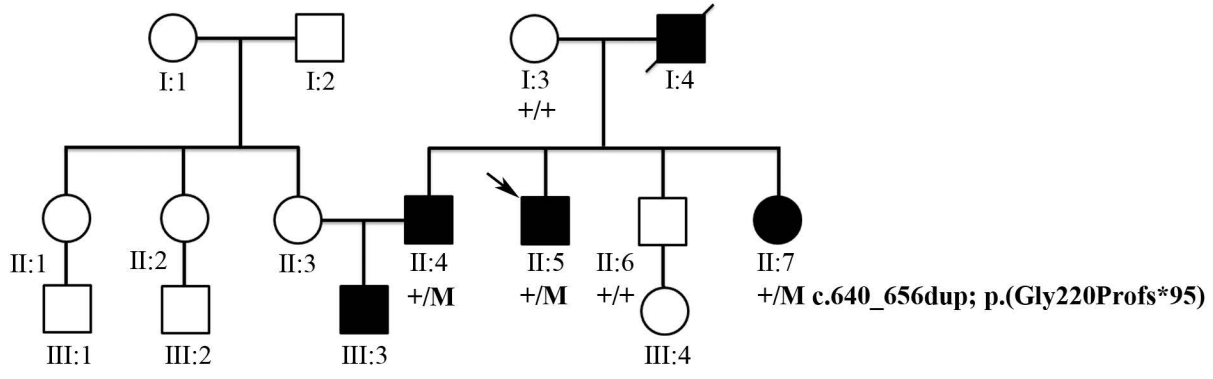
Figure 1: Families affected by *PITX3* mutations. "M" indicates the corresponding mutation and "+" the wild-type allele. The arrows indicate the index case. Dark-filled symbols indicate the individual was affected with an ocular developmental disorder, more details are shown in Table 1. Grey filled symbols in Family 2 show an unclear affection status (these patients presented only embryotoxon, a common clinical manifestation).

Figure 2: Electropherograms showing the genetic defects in *PITX3* in the different families.

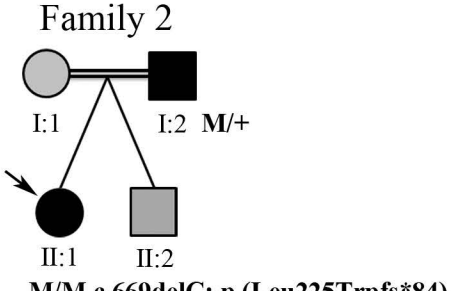
Figure 3: Schematic representation of PITX3 protein domains with the mutations associated with congenital cataract. In dark gray, the homeodomain, and in light grey, the OAR domains are indicated [27]. The mutations reported in this study are underlined [10,15-18]. Novel mutations are indicated with the superscript 'N'.

Table 1: Genotype and detailed phenotypic description of the affected individuals described here.

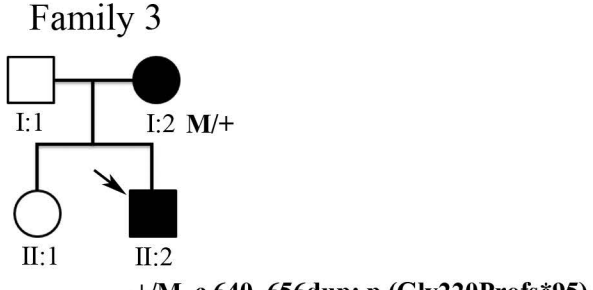
A.


Family 1
I:1
I:2
I:3
+/+
I:4
II:1
II:2
II:3
II:4
+/M
II:5
+/M
II:6
+/+
II:7
+/M c.640_656dup; p.(Gly220Profs*95)
III:1
III:2
III:3
III:4

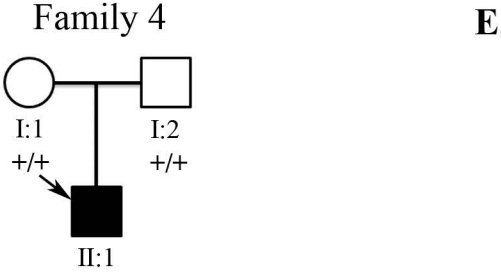
B.


Family 2
I:1
I:2 M/+
II:1
II:2
M/M c.669delC; p.(Leu225Trpfs*84)

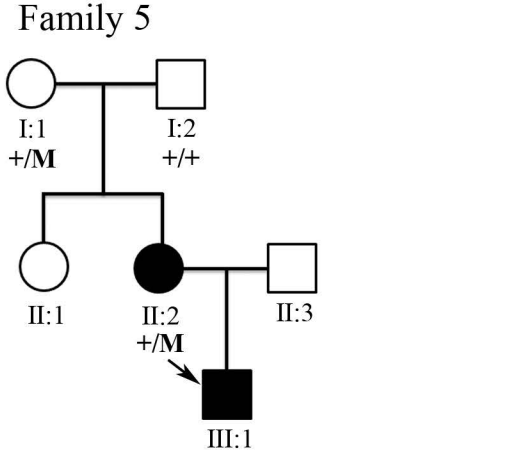
C.


Family 3
I:1
I:2 M/+
II:1
II:2
+/M c.640_656dup; p.(Gly220Profs*95)

D.

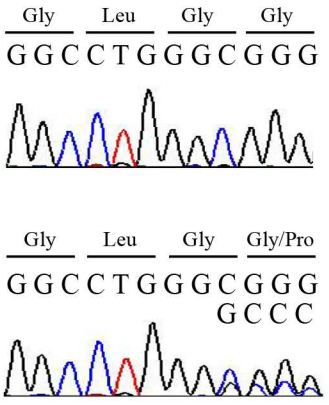

Family 4
I:1
+/+
I:2
+/+
II:1
M/+ c.582delC; p.(Ile194Metfs*115)

E.


Family 5
I:1
+/M
I:2
+/+
II:1
II:2
+/M
II:3
III:1
+/M c.38G>A; p.(Ser13Asn)

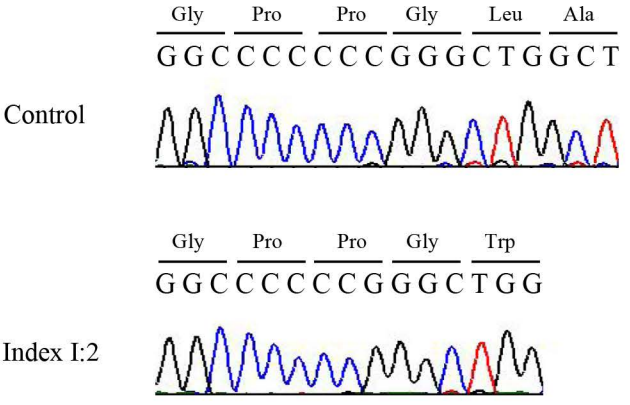
A. Family 1

Heterozygous c.640_656; p.Gly220Profs*95



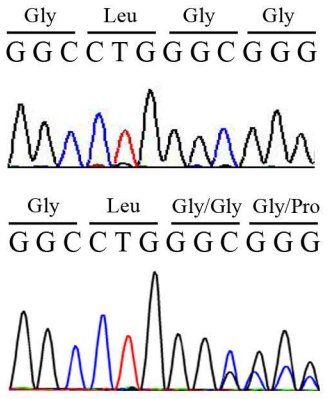
B. Family 2

Homozygous c.669delC; p.(Leu225Trpfs*84)



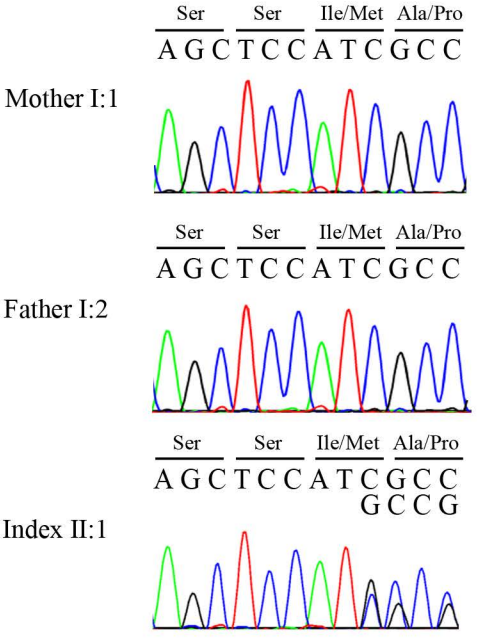
C. Family 3

Heterozygous c.640_656; p.Gly220Profs*95



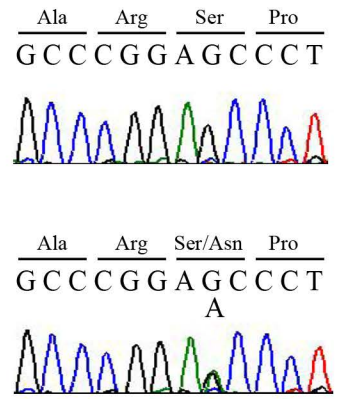
D. Family 4

De novo c.582delC; p.(Ile194Metfs*115)



E. Family 5

Heterozygous c.38G>A; p.Ser13Asn



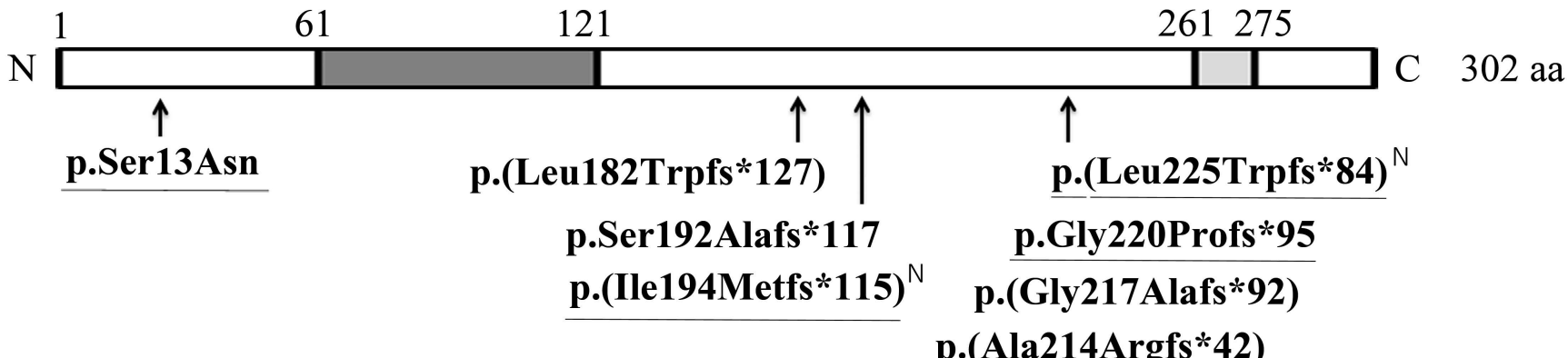

1
61
121
261
275
N
C
302 aa
p.Ser13Asn
p.(Leu182Trpfs*127)
p.Ser192Alafs*117
p.(Ile194Metfs*115)N
p.(Leu225Trpfs*84)N
p.Gly220Profs*95
p.(Gly217Alafs*92)
p.(Ala214Argfs*42)

Table 1: Genotype and detailed phenotypic description of the affected individuals described here.

Family	Individual	Mutation	Ocular phenotype	Extraocular phenotype
1	II.5 (index)	p.[Gly220Profs*95];[=]	Peters anomaly in one eye and cataract in the other eye	None
	I.4	Not tested	Posterior embryotoxon and congenial cataract	None
	II.4	p.[Gly220Profs*95];[=]	Congenital bilateral cataract	None
	III.3	Not tested	Peters anomaly and posterior embryotoxon	None
	II.7	p.[Gly220Profs*95];[=]	Congenital cataract.	None
2	II.1 (index)	p.[(Leu225Trpfs*84)];[(Leu225Trpfs*84)]	Bilateral congenital sclereocornea and ASMD	Facial features Broad thumbs
	II.2	p.[(Leu225Trpfs*84)];[=]	Nasal posterior embryotoxon in the left eye	None
3	II.2 (index)	Not tested	Bilateral Peters anomaly	Short stature Facial features Short hands with tapering fingers Psychomotor delay
	I.2	Not tested	Juvenile bilateral cataract	None
4	II:1 (index)	p.[Gly220Profs*95];[=]	Bilateral congenital cataract and microphthalmia	Autism Double row of teeth
5	III:1(index)	p.[Gly220Profs*95];[=]	Bilateral Peters anomaly	None
	II:2	p.[(Ile194Metfs*115)];[=]	Unilateral Peters anomaly	None

SUPPLEMENTARY DATA

Table S1: List of the 186 known and candidate genes involved in ocular development that were part of the targeted sequencing panel.

ABCG5	CRABP2	GAS1	NAT1	SFRP2	FOXH1
ADAM17	RBP1	GBX2	NEUROD4	SHH	SCLT1
ADCY7	CREG1	GDF2	NOTCH1	SIX3	TBC1D32
AHR	CRYAA	GDF6	NOTCH4	SIX6	GJA8
AK9	CRYBA1	GJA1	TENM3	SLC4A7	CRYGC
ALDH1A1	CRYGA	GLI1	OLFM2	SMO	TBC1D20
ALDH1A2	CRYGD	GLI2	OTX2	SMOC1	RAB18
ALDH1A3	VCAN	GLI3	PAX2	SNX3	NAA10
ALDH1L1	CYP26A1	GLIS3	PAX3	SOX1	PRSS3
ALG2	CYP26B1	GPRC5C	PAX6	SOX10	CTBP2
ALKBH1	CYP26C1	GRIP1	PBX1	SOX14	MTCH2
ASXL1	DHH	HCCS	PDS5A	SOX2	
ATOH7	DHRS3	HHAT	PITX2	SOX21	
B3GALTL	DISP1	HHIP	PITX3	STRA6	
BCMO1	DKK1	HMGB3	PLCG1	SUFU	
BCOR	DMBX1	HMX1	POMT2	SV2C	
BEST1	DOCK2	HPGD	PRSS56	SYNE1	
BFSP1	EFHD1	IFT172	PTCH1	TADA3	
BFSP2	EN1	IGBP1	PTCH2	IL10	
BMP4	ENTPD2	IHH	RAB23	TMEM150A	
BMP7	EPSTI1	IKBKG	RAB3IL1	TMEM170A	
ZCCHC24	EYA1	IL1R1	RAB3GAP1	TMEM175	
GRCC10	EYA2	KIF3A	RAB3GAP2	TMEM67	
C1orf101	EYA3	KRT27	RARA	TRPV4	
C3orf52	FAT1	LGR4	RARB	TSHZ2	
CDH20	FAT4	LIM2	RARG	TUBGCP6	
CASC3	FBXW11	LRAT	RAX	VAX1	
CASP3	FGF19	MACF1	RBP4	VAX2	
CDON	FLNA	MAF	RDH10	VSX2	
CES5A	FNBP4	MAFB	CLVS1	ZIC2	
CHD7	FOXE3	MDH1B	RPP40	ZNF335	
CHRD	FOXN4	MEIS1	RXRA	NKX2.1	
CLDN19	FRAS1	MFRP	RXRB	NKX2.2	
COX7B	FREM2	MIR204	RXRG	IRX1	
CRABP1	FRS2	MITF	SCARB1	IRX2	

Figure S2. Absolute quantification of the allele abundance for the mutation c.38G>A, p.Ser13Asn in Family 5. Digital Droplet PCR (ddPCR) assays were performed using Taqman Genotyping assays. Fractional abundance of mutated allele, represented in percentage, was calculated for the FAM-positive droplets vs VIC- (wild type allele) positive droplets (FAM/FAM+VIC). Experiments were performed in quadruplicated. Blood and saliva samples were tested in the asymptomatic grandmother (I:2) and compared with the index case (III:1) as heterozygous symptomatic carrier and the unaffected grandfather (I:1) as wild-type individual.

Table S1: Primers sequences for the amplification and sequencing of *PITX3* exonic and splice site regions by Sanger sequencing are indicated.

PRIMERS	**EXON**	**PRIMER SEQUENCES**	**SEQUENCED REGIONS** (ENST00000370002)	**SIZE (bp)**
SEQ-PITX3-EXON2-F	2	GAAAGGCGCCAGGGAATTTA	c.-91_118+118	366
SEQ-PITX3-EXON2-R		CAAGCCAGCGCATATTCTC		
SEQ-PITX3-EXON3-F	3	CGGTGGGAGCCAGCGAGTG	c.119-74_c.321+46	362
SEQ-PITX3-EXON3-R		CTCCGGGTCGCAGGCTGAG		
SEQ-PITX3-EXON4.1-F	4	CCGCCCTTCAGCCGCTGGGA	c.322-41_c.704	467
SEQ-PITX3-EXON4.1-R		CGGCCGAGGCATAAGGGCAGGA		
SEQ-PITX3-EXON4.2-F		CCATCGCCGCCTCCATGGT	c.597_c.*70	423
SEQ-PITX3-EXON4.2-R		GGGCGGGAGCAAGCCAGTCAA		