

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

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30 Running title: *PITX3* variants in eye disorders

31 **ABSTRACT**

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

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

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

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

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

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71 INTRODUCTION

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

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

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

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

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

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102 **MATERIALS AND METHODS**

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104 **Patients, targeted and Sanger sequence analyses**

105 The current cohort study was approved by the Cambridgeshire 1 Ethics Committee
106 04/Q0104/129 and by the French Ethics Committee “*Comité de Protection des Personnes (CPP)*
107 *Sud-Ouest et Outre-Mer IP*”. Written informed consent was obtained from all participating
108 subjects.

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

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

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

123
124 Primers for amplification and sequencing of exons and exon-intron boundaries of *PITX3*
125 (ENST00000370002) are shown in Table S2. Amplification by PCR was performed on 25 ng of
126 genomic DNA with Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA). PCR
127 fragments were purified with a gel extraction kit (Neo Biotech CliniSciences, Nanterre, France)
128 in accordance with manufacturer's protocol. Sequence analysis was performed with the 3500xL
129 sequencer (Applied Biosystems, Foster city, CA, USA).

130
131 Co-segregation of each mutation was performed by Sanger sequencing in all available family
132 members.

133
134 Potential mosaicism was assessed in an asymptomatic heterozygous carrier in Family 5 by means
135 of Digital Droplet PCR (ddPCR) assays with the Droplet Digital PCR QX200 System (Bio-Rad
136 Laboratories, Hercules, USA) using a commercial TaqMan SNP Genotyping assays (ID:
137 C_1007168_10; Thermo Fisher, Foster city, CA, USA) to genotype the previously known *PITX3*
138 variant (c116C>G; p.(Thr39Arg)).

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141 **RESULTS**

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143 **Description of *PITX3* mutations**

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

152 Except for the previously reported c.38G>A variant, which was present in 1/78742 alleles in the
153 ExAC database and in 1/225438 alleles in the gnomAD database, the other ones have not been
154 reported before in the NCBI dbSNP138 database, the NHLBI Exome Sequencing Project, the
155 1000 genomes project, the gnomAD, the GME and the ExAC databases. As depicted in Figure 3,
156 all frameshift mutations identified in *PITX3* were N-terminal of the OAR domain while the
157 missense mutation (p.Ser13Asn) was N-terminal of the homeodomain.

158 All mutations presented in this manuscript were submitted to the Leiden Open Variant Database
159 with the following IDs: 00001632110, 0000163211, 00001632112, 0000163213 and
160 0000163214.

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164 **Phenotypes of subjects harboring *PITX3* mutations**

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

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

180

181 *Family 2, homozygous c.669del (p.(Leu225Trpfs*84)) mutation:* A consanguineous family
182 from Iraq (Figure 1B) was ascertained due to the proband, II:1, having bilateral congenital
183 sclerocornea and ASMD, identified on neonatal screening. No ocular abnormalities were detected
184 in her dizygotic twin, II.2. They were born prematurely (27 weeks of gestation, 960 g) by
185 caesarean section. Transthoracic, transfontanelle and abdominal ultrasound examinations did not
186 reveal anomalies. No infection was reported during pregnancy. At 14 months of age, the proband
187 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.

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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

211 *Family 4, heterozygous c.582del (p.(Ile194Metfs*115)) mutation (de novo):* A family trio
212 from North Ireland was ascertained (Figure 1D). The index case (II:1) was a 12 year-old boy born
213 10 days post term following a normal pregnancy (except for some early bleeding in pregnancy)
214 with a birth weight of 3900 g. He was diagnosed with bilateral congenital cataract and
215 microphthalmia on day 1 because of no red reflex. He had bilateral cataract surgery at three
216 weeks of age and was fitted with contact lenses. He subsequently had a left broad iridectomy,
217 with capsulotomy and vitrectomy at the age of four months, a left Ahmed valve insertion at seven
218 months of age and right inferior oblique anteriorisation (squint surgery) at two years of age. At
219 two years seven months of age, he had severe visual impairment with navigational vision. His
220 early motor milestones were delayed: he sat independently at 11 months of age. However, he
221 started walking at 12 months, and then steadily at 15 months. He had early behavioral issues with
222 constant crying and was delayed in acquiring social skills. His first word was around one year.
223 He needed speech therapy to improve clarity of speech. He was later diagnosed with autism
224 which at the age of 12 years was severe. His growth was initially around the 90th % for height and
225 weight until recently when he became average height. There is a family history of autistic
226 spectrum disorder in three male cousins and epilepsy in a female cousin, all on the maternal side.
227 At age 12 years, he had a head circumference of 55.7 cm (50th-75th %), height 147.3cm (50th%)
228 and weight 36.9kg (9-25th%). He had a double row of teeth, but no other dysmorphic features. He
229 had bilateral microphthalmia, cloudy corneas with a corneal diameter of 9 mm bilaterally, was
230 aphakic with a broad iridectomy on the left eye and a small pupil on the right. Parental eye
231 examinations showed the mother had very slight enlargement of the optic cups, and the father had
232 tilted myopic optic discs only. Array and chromosome analysis were both normal.

233

234 *Family 5, heterozygous c.38G>A (p.Ser13Asn) mutation:* A family from France (Figure
235 1E) with Peters anomaly was ascertained. The index case (III:1) presented bilateral Peters
236 anomaly while his mother presented unilateral Peters anomaly. Neurological development of the
237 index case (III:1) was within normal ranges. There was no facial dysmorphism presented by the
238 index case as by the affected mother (II:2) apart from one palatal tooth for the index case. The
239 length, weight and OFC of the index case were at +1 SD at age 10 years.
240 The grandmother (I:2), who carried the c.38G>A variant heterozygously, displayed bilateral
241 nuclear and cortical cataract without any anterior segment anomaly. Because she only developed
242 cataract at 70 years of age, we classified her as not affected (Figure 1E). We confirmed the
243 mutation in the grandmother (I:2) on a new blood sample as well as on a saliva sample in which
244 no mosaicism was further ascertained by ddPCR quantification (estimated fractional abundance
245 of 50% for the mutated allele, Figure S1) in the analyzed tissues. By history, no other members
246 from the mother's side had any ocular or extra-ocular features.

247

248 **DISCUSSION**

249

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

253 Except for the p.Ser13Asn, all mutations described by us and others introduce a frameshift
254 [10,15,17-20]. The phenotypic variability associated to these mutations was large even within
255 members of the same family. Most of them were dominantly inherited and associated to cataract
256 accompanied by additional eye disorders ranging from embryotoxon to microphthalmia. In
257 contrast, Aldahmesh et al. described the only *PITX3* mutation associated with autosomal

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

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

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

291 As previously mentioned, except for the (p.Ser13Asn), all *PITX3* mutations known to date lead to
292 a frameshift. The p.Ser13Asn variant is very rare in the general population (1/225438 alleles in
293 the gnomAD database), and it was previously identified *de novo* in a patient with congenital
294 cataract [10]. This variant affects a conserved amino acid and was predicted to be deleterious by
295 different prediction softwares (Polyphen-2 and Mutation Taster), but tolerated by SIFT software.
296 Functional analyses that studied the consequences of this mutation on *PITX3* function have
297 demonstrated only minor functional effects in comparison to the p.Gly220Profs*95 mutant which
298 showed a partial loss of function of the protein activity [27]. These functional studies identified a
299 slightly decrease in the DNA binding ability of the p.Ser13Asn mutant, however a 23% decrease
300 in its ability to increase reporter activity was found which may support our premise that this
301 variant is disease-causing [27]. Of note, no other deleterious variants were identified among the
302 187 genes screened in the index case carrying this mutation (case III:1, family 5).

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

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

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

318

319 **URLs**

320 ExAC database: <http://exac.broadinstitute.org/>

321 GnomAD database: <http://gnomad.broadinstitute.org/>

322 dbSNP138 database:

323 [https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&](https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=138)
324 [build_id=138](https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=138)

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

326 1000 genomes project: <http://phase3browser.1000genomes.org/index.html>

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

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

329 Mutation Taster: <http://www.mutationtaster.org/>

330 SIFT: <http://sift.jcvi.org/>

331 GME database: <http://igm.ucsd.edu/gme/>

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337

338 **DECLARATION OF INTEREST**

339 The authors report no conflicts of interest. The authors alone are responsible for the content and
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436 **TITLES AND LEGENDS TO FIGURES**

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

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

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

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

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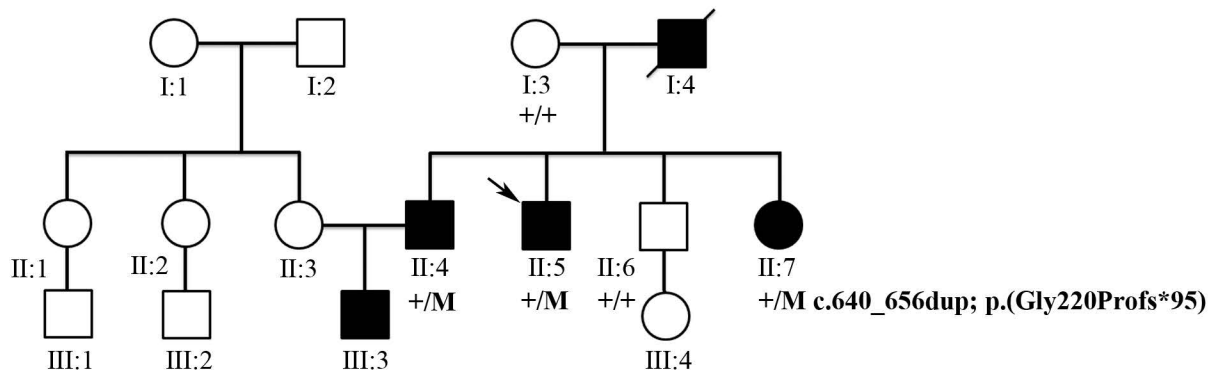
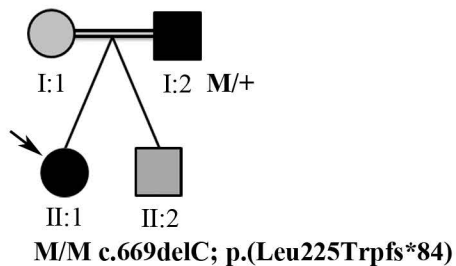
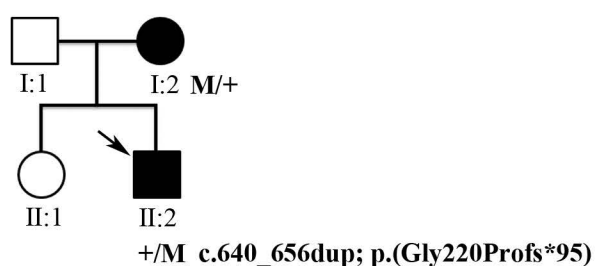
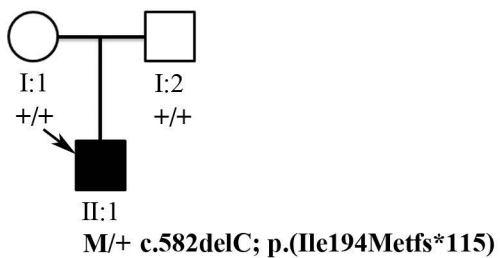
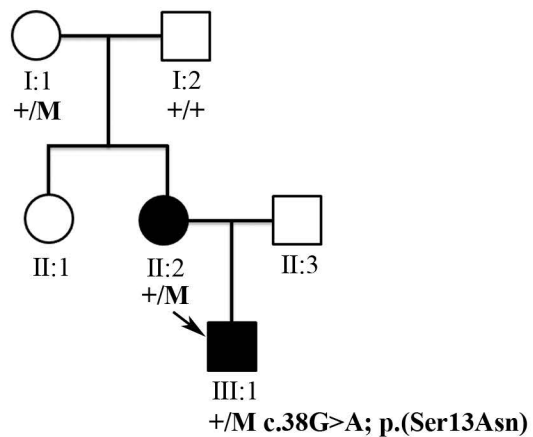
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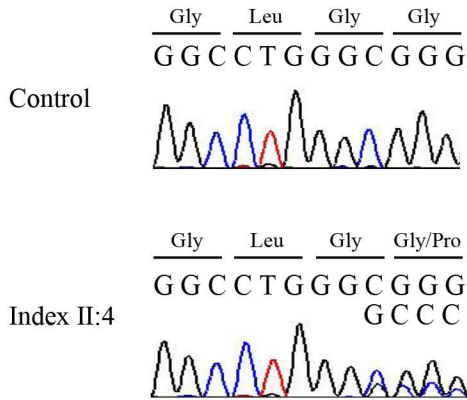
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A.**Family 1****B.****Family 2****C.****Family 3****D.****Family 4****E.****Family 5**

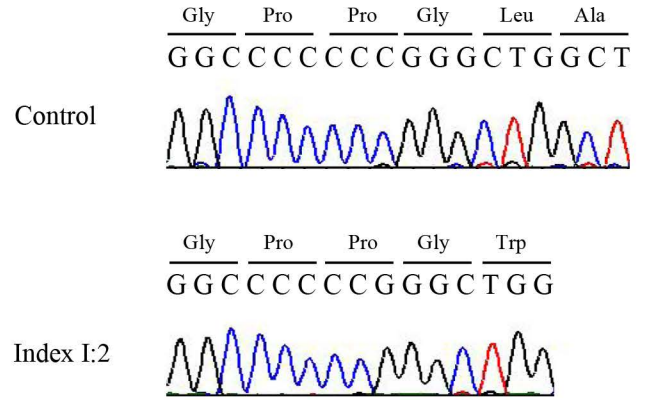
A. Family 1

Heterozygous c.640_656; p.Gly220Profs*95



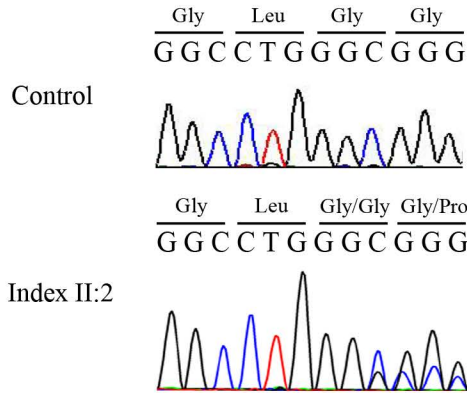
B. Family 2

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



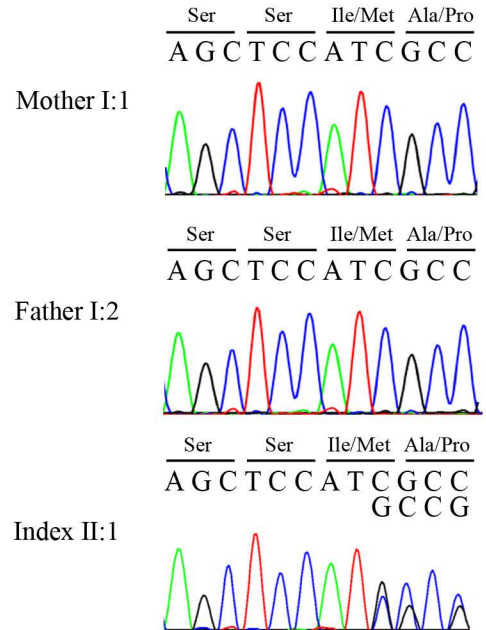
C. Family 3

Heterozygous c.640_656; p.Gly220Profs*95



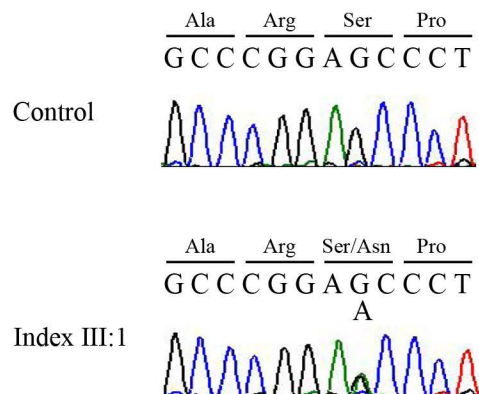
D. Family 4

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



E. Family 5

Heterozygous c.38G>A; p.Ser13Asn



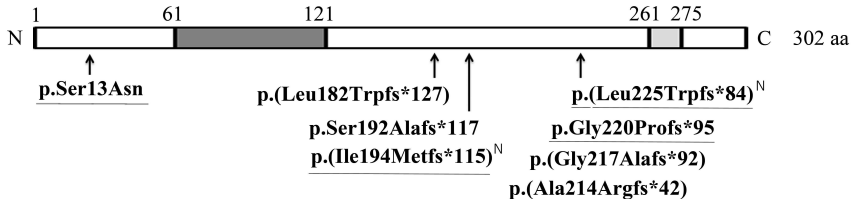


Table 1: Genotype and detailed phenotypic description of the affected individuals described here.

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	
B3GALT1	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		CGGCCGAGGCATAAGGGCAG GA		
SEQ-PITX3-EXON4.2-F		CCATCGCCGCCTCCATGGT	c.597_c.*70	423
SEQ-PITX3-EXON4.2-R		GGGCGGGAGCAAGCCAGTCA A		