CYP1B1 and *MYOC* variants in neonatal-onset versus infantile-onset primary congenital glaucoma

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ABSTRACT

Objective To compare *CYP1B1* and *MYOC* variants in a cohort of neonatal-onset (NO) and infantile-onset (IO) primary congenital glaucoma (PCG).

Methods This prospective observational study included 43 infants with PCG (14 NO and 29 IO) presenting between January 2017 and January 2019 with a minimum 1-year follow-up. *CYP1B1* and *MYOC* genes were screened using Sanger sequencing with in-silico analysis of the variants using Polymorphism Phenotyping v.2 and Protein Variation Effect Analyser platforms. Allelic frequency was estimated using Genome Aggregation Database (gnomAd). Disease presentation and outcome were correlated to the genetic variants in both groups.

Results Babies with *CYP1B1* mutations had more severe disease at presentation and worse outcomes. Six of 14 (42.8%) NO glaucoma and 5 of 29 (17.2%) IO harboured *CYP1B1* mutations. Five of six babies in the NO group and three of five in the IO group harboured the variant c.1169G>A, [p.R390H]. They required more surgeries and had a poorer outcome. On in-silico analysis c.1169G>A, [p.R390H] scored very likely pathogenic. Two patients in the IO group who had the c.1294C>G, [p.L432V] variant had a good outcome. Five of 14 NO-PCG and 8 of 29 IO-PCG harboured the variant c.227G>A, [p.R76K] in the *MYOC* gene, which was scored benign by in-silico analysis, and was also found in 2 of 15 normal controls.

Conclusions Patients with *CYP1B1* pathogenic variants had a poorer outcome than those without. We found more NO PCG babies with *CYP1B1* mutations compared with IO PCG. This may be one of the reasons for NO PCG having a poorer prognosis compared with IO PCG.

INTRODUCTION

Primary congenital glaucoma (PCG) is a potentially blinding disease, which, if untreated, would result in a lifetime of blindness. It mainly presents as a developmental abnormality in the trabecular meshwork (TM), resulting in raised intraocular pressure (IOP).[1] PCG has a varied prevalence (1:10 000 in the western world to 1:1250 among the Slovak Gypsies).[2] Indian data from Andhra Pradesh reported a prevalence of 1:3300 births.[3] PCG has an autosomal recessive mode of transmission. Cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*, OMIM 601771)[4 5] is the most common identifiable cause of PCG worldwide. Other genes implicated are the latent transforming growth factor-beta binding protein 2,[5] heterozygous alleles in the angiopoietin receptor-encoding gene (*TEK*)[6] and *MYOC* (Myocilin) gene.[7 8]

PCG usually presents between 2 and 9 months of age (infantile-onset glaucoma (IO-PCG)),[9] but may be less commonly present at birth (neonatal-onset glaucoma (NO-PCG)). Glaucoma in a newborn indicates a very early developmental arrest in utero, and has more severe disease and worse surgical prognosis than IO-PCG.[9 10] This difference in babies with the onset at different periods of life leads to the question of whether the molecular biology of PCG presenting at birth is different from that which presents later. Panicker *et al*[11] identified certain mutations in the *CYP1B1* gene associated with a more severe prognosis. The presence of CYP1B1 mutations has been reported in younger children with PCG.[12] *MYOC* variants are less commonly reported with PCG, and the significance is uncertain.[7 13]

The purpose of this paper was to analyse variants in *CYP1B1* and *MYOC* genes in a cohort of NO-PCG and IO-PCG.

MATERIALS AND METHODS

Non-related infants with PCG were prospectively recruited when they presented to the Paediatric Glaucoma Clinic of the Advanced Eye Centre, Postgraduate Institute of Medical Education and Research, Chandigarh, India, between January 2017 and January 2019.

At presentation, all patients underwent an examination with torchlight, and those with any of the following features underwent a detailed examination under anaesthesia (EUA) using sevoflurane. (1) Large size and/or ground glass appearance of the cornea. (2) If the optic disc was visible, any cup-disc ratio >0.40.[14] (3) Epiphora and photophobia were considered corroborating factors. If the media was too hazy to permit visualisation, ocular ultrasonography was done to look for optic disc cupping and posterior segment evaluation. The following baseline data were recorded for each eye. (1) Corneal clarity graded 0–4 (0—normal lustre; 1—iris crypts visible; 2—iris crypts not visible; 3—iris not visible). (2) Corneal diameter. (3) IOP using Perkins applanation tonometer (PAT). (4) Anterior chamber details, gonioscopy, optic-disc evaluation if possible. Family history of glaucoma was recorded for each patient.

Inclusion criteria

Primary congenital glaucoma

Patients were diagnosed with PCG if they fulfilled the following characteristics. (1) Cornea

diameter >12.0 mm and/or presence of Haab's striae with or without IOP>18 mm Hg. (2) Cup-disc ratio >0.4,[14] or optic disc cupping on ocular ultrasonography. (3) No other anterior segment dysgeneses such as Axenfeld Rieger's syndrome, aniridia and so on. (4) No secondary cause of glaucoma viz retinoblastoma; trauma and so on. Eligible patients were divided into NO-PCG and IO-PCG, depending on whether the abnormality was noted within 1 month of age or between 1-month to 24 months of age, respectively (Childhood Glaucoma Research Network classification).[15] Postoperatively patients underwent a EUA at 6 weeks, 3 months, 6 months, and then as and when required depending on the clinical condition. Patients completing a minimum postoperative follow-up of 1 year were included.

Normal controls

Normal controls comprised healthy volunteers ethnic to the geographical areas from where the patients presented, with no family history of glaucoma. After a complete ophthalmological examination, those with open angles, normal IOP and clinically healthy optic disc (cup-to-disc ratio <0.7, no rim notch, C/D ratio asymmetry) were included.

Genetic analysis

Genomic DNA isolation and quantification

DNA was isolated from the blood using the QIAamp DNA Blood Midi kit (QIAGEN GmbH, QIAGEN Strasse 1, 40724, Hilden, Germany) following the manufacturer's instructions. DNA concentration was estimated using UV–Vis Spectrophotometer (QUAWELL, Q5000 San Jose, California, USA), and the 260/280 ratio between 1.8 and 2.0 was considered adequate for subsequent PCR.

PCR amplification of *CYP1B1* and *MYOC* genes

PCR was performed on a conventional PCR system (Eppendorf AG 22331, Hamburg), using the following programme: 94°C for 5 min 1 cycle, 94°C for 30 s; 55°C for 30 s; 72° for 1 min (35 cycles), 72°C for 5 min (1 cycle). The coding region of the *CYP1B1* gene starts from exon II. The 485 bp upstream regulatory region of *CYP1B1* (2748 bp to 3233 bp) and the coding region from exons 1, II, III of the *MYOC* gene were amplified using the following primers:

CYP1B1	**Primers**
Exon 1	5′AGCGGCCGGGGCAGGTTGTACC3′ 5′ATTGGGATGGGGACGGAGAA3′
Exon 2	5′TCTCCAGAGAGTCAGCTCCG3′ 5′GGGTCGTCGTGGCTGTAG3′
Exon 3	5′GATGCGCAACTTCTTCACG3′ 5′CTACTCCGCCTTTTTCAGA3′
MYOC	**Primers**
Exon 1	5′-TCACCAAGCCTCTGCAATG-3′ 5′-TGAACTCAGAGTCCCCCCAC-3′
Exon 2	5′-TGTCATCCTCAACATAGTCA-3′ 5′-TTCTGTTCCTCTTCTCCTC-3′
Exon 3	5′-GCTGTCACATCTACTGGCTC-3′ 5′-ATCTCCTTCTGCCATTGCCT-3′

The reference sequences for the genes are as follows:

CYP1B1: NM_000104.3 for the c position and NP_000095.2 for the p. position.

MYOC: NM_000261.2; for the c. position and NP_000252.1 for the p. position.

Agarose gel electrophoresis

The amplified products were run on 2% agarose gel, along with a 100 bp DNA ladder to serve as a DNA molecular weight marker. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN GmbH, QIAGEN Strsass 1, 40724, Hilden, Germany).

Gene sequencing

All purified PCR products were quantified before automated DNA sequencing was done bidirectionally using the 3730XL DNA Analyser (Applied Biosystems, USA). Finch TV software was used to view sequencing results. The results were blasted in the PubMed database with the reference gene and protein sequence for any mutations in the coding region or the genes' regulatory region.

In-silico functional assessment of identified variants

Functional analysis of the observed variants was analysed using two publicly available bioinformatic tools: PROVEAN (Protein Variation Effect Analyser),[16] and PolyPhen-2 (Polymorphism Phenotyping v.2).[17] PROVEAN is a web-based software tool (http://provean.jcvi.org), which predicts whether an amino acid substitution or deletion impacts biological function, and the PolyPhen-2 web interface, available at http://genetics.bwh.harvard.edu/pph2/, predicts the effect of a single-residue substitution on protein structure and function.

Allelic frequency analysis using Genome Aggregation Database

The observed *CYP1B1* and *MYOC* variants were assessed for rarity using a public database curated by the Genome Aggregation Database (gnomAD; http://gnomad-old.broadinstitute.org/).[18] The variants were annotated by allele frequency (AF), and assessed whether the AF was above the threshold frequency noted in the gnomAD database.

Outcome measures

Clinical outcome

Success rates were noted depending on postoperative IOP control.

- Complete success (good outcome): IOP ≤16 mm Hg using the PAT under anaesthesia without medications.
- Qualified success (fair outcome): same IOP criteria with two topical medications.
- Unfavourable (poor outcome): IOP >16 mm Hg under anaesthesia with >2 topical medications, requirement of repeat surgery or sight-threatening complications.

Correlation with the observed genotype

Genetic variants predicted as pathogenic or non-pathogenic by PolyPhen-2 and PROVEAN were correlated to the disease presentation and outcome, emphasising on unfavourable outcomes.

Statistical methods

Statistical analysis was done using SPSS V.20, IBM. The occurrence of mutations and outcomes were compared between the two groups using Fisher's exact test.

RESULTS

Eighty-six eyes of 43 babies with PCG (14 NO and 29 IO) were studied. Fifteen healthy subjects comprised the control group. Demography at presentation is depicted in table 1. The gender

Table 1 Demography and characteristics at presentation of neonatal-onset and infantile-onset PCG and normal ethnically matched controls

S No	Baseline characteristics	Neonatal onset PCG (n=28 eyes of 14)	Infantile onset PCG (n=58 eyes of 29)	P value	Normal controls
1	Gender (M: F)	4: 10	24: 5		4:11
2	Age at presentation	10.2±5.5 days	11.4±6.7 months		27 .9±2.1 years
3	Baseline IOP (mm Hg)	20.7±4.9	23.0±9.9	p=0.63	13.9±2.3 mm Hg
	CYPIBI mutations (n=22)	22.2±3.1 (n=12)	23.5±7.5 (n=10)	p=0.33	
	No *CYP1B1* mutations (n=64)	20.3±6.1 (n=16)	20.8±9.9 (n=48)	p=0.49	
4	P value	p=0.97	p=0.44		
5	Corneal clarity (grade)	2.7±1.3	1.5±1.1	p=0.002	Grade 0 in all
	CYPIBI mutations (n=22)	2.8±0.9 (n=12)	2.2±1.3 (n=10)	p=0.28	
	No *CYP1B1* mutations (n=64)	2.1±1.3 (n=16)	1.3±0.9 (n=48)	p=0.02	
6	P value	p=0.29	p=0.02		
7	Corneal diameter (mm)	12.1±1.1	13.2±1.1		Not applicable in adults
8	Axial length (mm)	19.3±2.1	22.5±1.9		
Follow-up					
1	Number of surgical or laser procedures required	3.6	2.5		
2	Outcome 1 year after surgery	Poor (n=11	Poor (n=5)	p=0.002	
		Fair (n=13)	Fair (n=13)		
		Good (n=4)	Good (n=40)	p<0.0001	

IOP, intraocular pressure; PCG, primary congenital glaucoma.

ratio was reversed in the two groups. 4/14 (28.5%) of NO babies were male compared with 24/29 (82.8%) in the infantile group. 6 of 14 (42.8%) NO-PCG harboured *CYP1B1* pathogenic variants compared with 5 of 29 (17.2%) of IO-PCG (p=0.016).

NO-PCG eyes had worse corneal clarity at presentation than the IO-PCG group (2.67±1.3; p=0.002; figures 1 and 2; table 1). However, this difference was significant (p=0.02) only in those without *CYP1B1* variants (table 1). There was no significant difference (p=0.28) in corneal clarity at presentation between NO-PCG and IO-PCG in those who harboured pathogenic *CYP1B1* variants. Within the groups, in the NO-PCG group, the presence of *CYP1B1* mutations did not affect baseline corneal clarity as much as it did in the IO-PCG group (table 1).

There was no significant difference in the baseline IOP between NO-PCG and IO-PCG (p=0.63). The IOP was marginally higher in the *CYP1B1* subset in both NO and IO groups, which did not reach statistical significance (p=0.9 and p=0.44, respectively).

Table 2 summarises mutations observed in both groups. Six of 14 (42.8%) NO-PCG and 5 of 29 (17.2%) IO-PCG patients harboured mutations in the *CYP1B1* gene (p=0.13). On comparing eyes of NO-PCG or IO-PCG, 12 of 28 (42.8%) of NO eyes harboured *CYP1B1* mutations compared with 10 of 58 (17.2%) eyes with IO glaucoma (p=0.016).

Five variants in the *CYP1B1* gene were observed, including one silent (c.1347T>C, [p.D449D]), three missense (c.1294C>G, [p.L432V], c.1169G>A, [p.R390H] and c.1358A>G, [p.N453S]) and one non-sense c.1158T>A, [p.Y386*]. The most common haplotype was the combination of c.1169G>A, [p.R390H] and c.1358A>G, [p.N453S]) seen in 7 of the 11 (63.6%) patients (four NO and three IO). Isolated variants c.1169G>A, [p.R390H] and c.1158T>A, [p.Y386*] were seen in one NO patient each. The variant c.1294C>G, [p.L432 V] was found in two IO patients only, who had mild disease.

Two variants, c.1347T>C, [p.D449D] in *CYP1B1,* and c.227G>A, [p.R76K] in *MYOC* were seen in the control group and were probably non-pathogenic. The c.1347T>C, [p.D449D] variant was seen in 4 of 14 NO-PCG, 9 of 29 IO-PCG and 10 of the 15 normal controls tested. Five of 14 NO-PCG and 8 of the 29 IO-PCG harboured the variant c.227G>A, [p.R76K] in the *MYOC* gene, which was also found in 2 of 15 normal controls.

Figures 3 and 4 show the chromatograms depicting the variants observed in both genes.

Phenotype-genotype correlations

Babies with *CYP1B1* mutations presented with more severe glaucoma and had worse outcomes than those without mutations (figures 1 and 2; table 3). Though the numbers were too small for meaningful statistical comparisons, patients with c.1169G>A, [p.R390H] had the worst corneal clarity at presentation (figure 1). Nine of 22 (41%) eyes in patients with *CYP1B1* mutations had a poor outcome compared with those without (7 of 64; 10.9%: p=0.004). Conversely, complete success was seen in 40 of 64 (62.5%) eyes in babies without mutations compared with 4 of 22 (18.1%) eyes with mutations (p=0.0004). The two IO patients harbouring c.1294C>G, [p.L432V] variant had a good outcome (figure 2). Forty of 58 (69%) of IO-PCG eyes had a complete success where the IOP was controlled without medications, compared with only four eyes (14.2%) in NO-PCG eyes (p<0.0001). The best outcomes were seen in babies with no mutations in *CYP1B1*.

Functional prediction using PolyPhen-2 and PROVEAN

The prediction results of the variants were similar on both PolyPhen-2 and PROVEAN (table 2). The missense mutation c.1169G>A, [p.R390H], was flagged to be most likely pathogenic, followed by c.1358A>G, [p.N453S], and were encountered only in PCG patients. The variant c.227G>A, [p.R76K] in *MYOC* was scored to be 'benign'. The variant c.1294C>G, [p.L432V] was also scored 'non-pathogenic', though it was not found in any of the healthy subjects screened.

Genome Aggregation Database

For the variant p.R390H, the AF in the population is 0.0001032l, with a 95% filtering AF of 0.0002 for genomes and 0.0003 for exomes. Our NO-PCG cohort showed an AF of 0.285 (homozygous), and 0.036 (heterozygous), both significantly higher

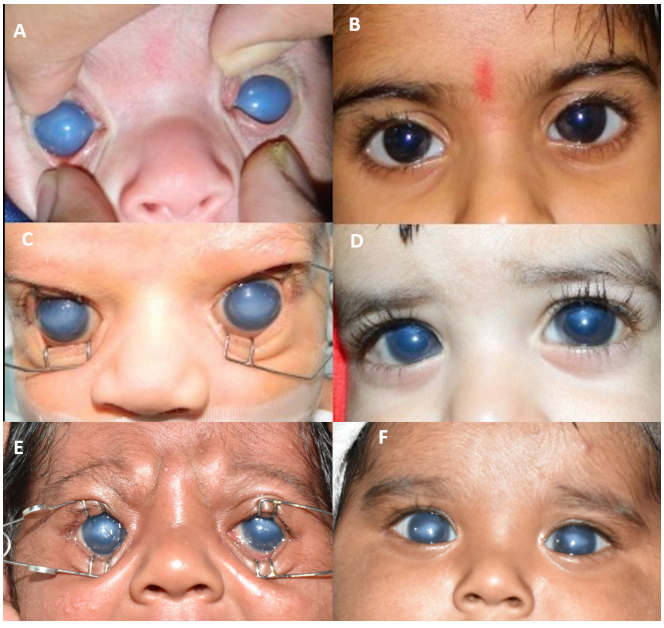

Figure 1 Presentation and follow-up in babies with neonatal-onset glaucoma. Top: neonatal-onset glaucoma with no *CYP1B1* variant, presented 1 day after birth. (A) Diffuse corneal haze at presentation. (B) Three years postoperative picture showing clear cornea and controlled intraocular pressure (IOP) after one surgery (combined trabeculotomy and trabeculectomy in each eye). Middle: neonatal-onset glaucoma with c.1158T>A, [p.Y386*] *CYP1B1* variant, presented at 10 days. (C) Diffuse corneal oedema with central scarred Haabs' striae. (D) Eighteen months postoperative follow-up showing resolution of diffuse corneal oedema but persistence of central corneal opacity due to the scarred Haabs striae. Required three topical drugs for IOP control. Bottom: neonatal-onset glaucoma with c.1169G>A, [p.R390H], and c.1358A>G, [p.N453S] *CYP1B1* variants, presented 3 days after birth. (E) Diffuse corneal oedema with scarred Haabs' striae. (F) Two years postoperative follow-up showing resolution of diffuse corneal oedema but persistence of dense scar in the cornea due to the scarred Haabs striae. Required two surgeries in each eye and two topical medications for IOP control.

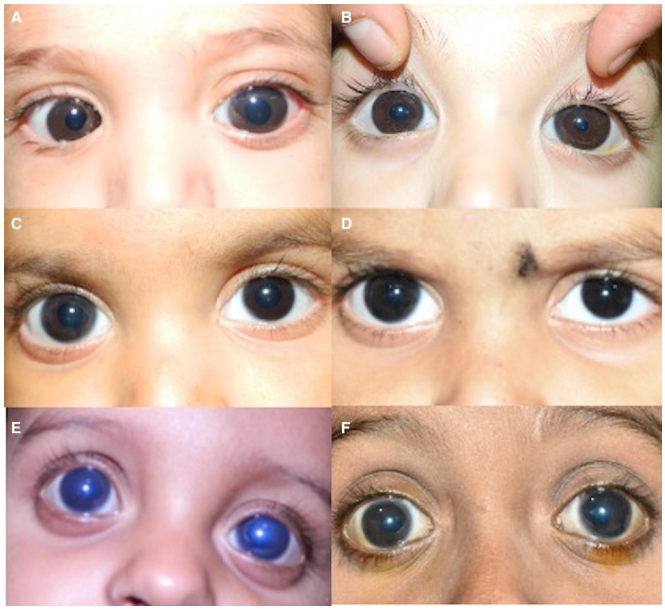

Figure 2 Presentation and follow-up in babies with infantile-onset glaucoma. Top: infantile-onset glaucoma with no *CYP1B1* variant, who presented at 4 months. (A) Mild corneal haze in both eyes at presentation, with left eye buphthalmos. (B) Thirty months postoperative picture showing clear cornea and controlled intraocular pressure (IOP) after one surgery in each eye. Middle: Infantile-onset glaucoma with c.1294C>G, [p.L432 V] variant *in CYP1B1*, presented at 6 months. (C) Mild corneal haze in both eyes with right eye buphthalmos. (D) Sixteen months postoperative follow-up showing resolution of the corneal oedema. IOP was controlled with one surgery in both eyes. Bottom: infantile-onset glaucoma with c.1169G>A, [p.R390H], and c.1358A>G, [p.N453S] variants in *CYP1B1*, presented at 3 months of age. (E) Diffuse corneal oedema with left scarred Haabs' striae. (F) Three years postoperative follow-up showing resolution of diffuse corneal oedema but persistence of dense scar in the cornea of the left eye due to the scarred Haabs striae. Required three surgeries in the left eye and two surgeries in the right eye and is on one topical medication for IOP control.

than the 95% filtering AF ($p<0.00001$). The variant N453S variant occurs at a population AF of 0.1495; the 95% confidence filtering AF 0.2233 for exomes and 0.1647 for genomes. In our study, the NO-PCG set showed an AF of 0.285 for the variant N453S; above both filtering AF values. For the variant p.L432V, the 95% filtering AFs are 0.88 and 0.84 for exomes and genomes, respectively. In our study, the AF for this variant was 0.069, much below the values of 0.88/0.84.

The p.Y386* variant is not listed in the gnomAD database.

DISCUSSION

Pathogenic variants in *CYP1B1* may result in the arrest of normal development of the TM and outflow system.[19 20] Approximately 147 mutations in *CYP1B1* in PCG have been reported in various ethnic groups,[21] from 20% in Japanese, 44% among Indians, to 100% among the Saudi Arabians and Slovakian Gypsies.

Genetics of PCG has been reported mainly from Southern India,[22 23] and from Delhi,[24] the capital city of India, home to multiple ethnicities. Our institute caters to mainly the north-west Indian population, which is ethnically more homogenous. In contrast to South India, consanguinity in Northwest India is culturally less acceptable. In our cohort, *CYP1B1* mutations were found in 42% NO-PCG and 17.2% IO-PCG. The difference may be clinically meaningful. Lower age at onset with the occurrence of any *CYP1B1* mutation has been reported in Indian,[11] South Korean[12] and Lebanese[25] cohorts. The proportion of *CYP1B1* mutations in a PCG population is thus likely to depend on the cohort's age being studied.

The mutation spectrum in our study differed from that published in India. Tanwar *et al*[24] reported *CYP1B1* mutations in 46% of PCG with Ter@223 mutation in 18%, p.R390H in 16% and p.R368H in 8% of patients. Two studies from South India reported mutations in 37.5% and 36.3%, respectively,[17 23] with p.R368H[18] as the predominant mutation.

Given the proximity of North-Western India to Pakistan, the historical reality of both countries' shared ethnicity is evident from the nearly identical mutations found in our two populations of PCG. Our predominant variant was the combination of c.1169G>A, [p.R390H], and c.1358A>G, [p.N453S] seen in 7 of the 11 (63.6%) patients. c.1169G>A, [p.R390H] has been described as a founder mutation in Pakistan.[26] c.1358A>G, [p.N453S] has been reported previously as a non-pathogenic variant in studies on Pakistani,[26] Iranian[27] and Brazilian patients,[28 29] but not reported from India previously. The significance of this novel mutation in Indian subjects co-occurring with the more severe c.1169G>A, [p.R390H] mutation is not

Table 2 Summary of all variants found in neonatal-onset and infantile-onset primary congenital glaucoma and the control group

Gene sequence variants	PolyPhen prediction	PROVEAN score	Neonatal onset (6 of 14 patients; (42.8%)	Infantile onset (5 of 29 patients; (17.2%)	Controls (n=15)
Variants in CYP1B1					
c.1169G>A (p.R390H) + c.1358 A>G (p.N 453S) (Homozygous)	Probably damaging Possibly damaging	−4.850 deleterious −3.452 deleterious	4/14 (28.5%)	3/29 (10.3%)	0
c.1169 G>A (p.R390H) Heterozygous	Probably damaging	−4.850 deleterious	1/14 (7.1%)	0	
c.1158 T>A (p.Y386*) Heterozygous	Premature stop codon. deleterious		1/14 (7.1%)	0	
c.1294C>G (p.L432V) Homozygous	Benign	0.418 neutral	0	2/29 (6.9%)	
Silent variant in CYP1B1					
c.1347T>C (p.D449D)	No amino acid change		8/14 (57.1%)	9/29 (31.0%)	10/15 (66.7%)
Variants in MYOC (scored benign by in-silico analysis)					
c.227G>A, (p.R76K) Heterozygous	Benign	−0.868 neutral	5/14 (35.7%)	8/29 (27.6%)	2/15 (13.3%)

PolyPhen, Polymorphism Phenotyping; PROVEAN, Protein Variation Effect Analyser.

clear. We found c.1294C>G, [p.L432V], flagged as 'benign' by in-silico analysis, in two patients with IO-PCG. A Pakistani cohort,[26] and Della Paolera *et al*[29] also reported this variant as non-pathogenic. We found heterozygous variants in two patients, as has been previously reported.[30] Kabra *et al*[31] reported mutations in the *TEK* gene, co-occurring in patients with heterozygous

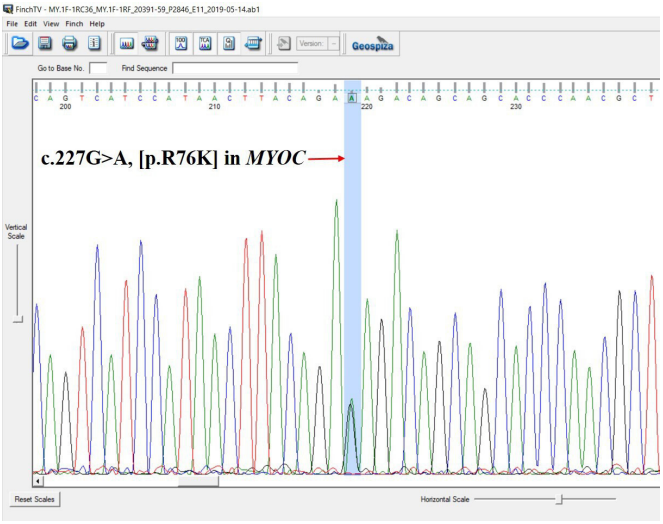

Figure 4 Representative chromatogram of the benign variant in *MYOC*.

CYP1B1 mutations. Two of our patients harboured a heterozygous *CYP1B1* mutation. Though we did not test for *TEK* mutations, it is a possibility to keep in mind.

Some studies[7 32 33] have reported *MYOC* mutations as a contributor to PCG. No pathogenic *MYOC* variant was found in our study. The variant c.227G>A, [p.R76K] observed in two control subjects was deemed benign by in-silico analysis.

Large exome-sequencing databases, including the Exome Aggregation Consortium and the gnomAD, have been recently used to filter out variants unlikely to be pathogenic. In our study, the NO-PCG subset showed a significantly higher AF for the variant c.1169G>A, [p.R390H] and c.1358A>G, [p.N453S].

We found one non-sense variant c.1158T>A, [p.Y386*]. Loss of function mutants reported for *CYP1B1* comprise four classes.[34–36] The causal connection with pathogenicity is more

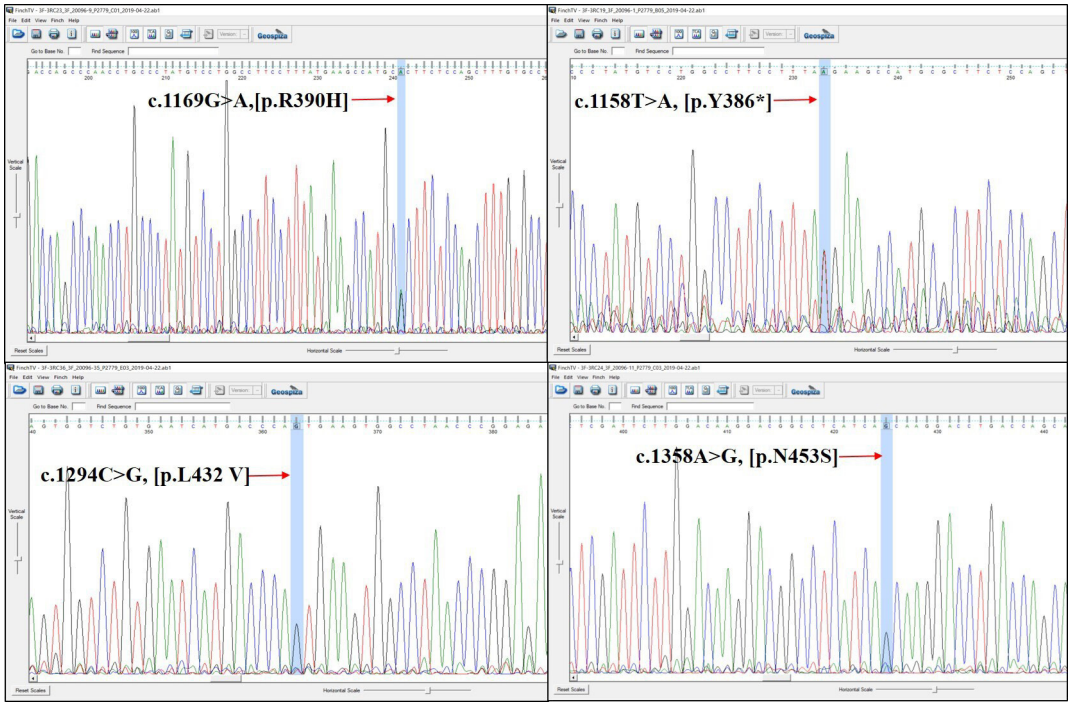

Figure 3 Representative chromatograms of the *CYP1B1* variants.

Table 3 Phenotype correlation with each genotype in both groups

	Neonatal onset (n=14; 28 eyes)				Infantile onset (n=29; 58 eyes)		
	CYP1B1 mutations				*CYP1B1* mutations		
	c.1169 G>A (p.R390H) c.1358 A>G (p.N453S)	c.1169 G>A (p.R390H)	c.1158T>A (p.Y386 Stop)	No mutation	c.1169 G>A (p.R 390H) c.1358 A>G (p.N453S)	c.1294C>G (p.L432V)	No mutation
No. of eyes and patients	8 eyes of 4 patients	2 eyes of 1 patient	2 eyes of 1 patient	16 eyes of 8 patients	6 eyes of 3 patients	4 eyes of 2 patients	48 eyes of 24 patients
Age at presentation	12.5±7.5 days	14 days	11 days	6.7±3.3 days	13.6±9.6 months	9.0±4.2 months	12.1±9.4 months
Presenting IOP (mm Hg)	20.2±2.7	22.0	25.0	19.7±5.6	25±7.9	24±5.1	23.8±9.5
Corneal clarity	2.5±1.1	3	1	1.9±1.4	2.0±0.9	1.3±0.5	0.7±0.8
Outcome	Poor 4/8 (50%) Fair 4/8 (50%)	Poor 2/2	Fair 2/2	Poor 5/16 (31.2%) Fair 7/16 (43.7%) Good 4/16 (25%)	Poor 3/6 (50%) Fair 3/6 (50%)	Good 4/4 (100%)	Poor 2/48 (4.1%) Fair 10/48 (20%) Good 36/48 (75%)
Overall outcome in neonatal vs infantile PCG with and without mutations	**NO-PCG with mutations (n=12)**		**NO-PCG no mutations (n=16)**		**IO-PCG with mutations (n=10)**	**IO-PCG no mutations (n=48)**	
	Poor 6/12 (50%) Fair 6/12 (50%) Good 0		Poor 5/16 (31.2%) Fair 7/16 (43.7%) Good 4/16 (25%)		Poor 3/10 (30 %) Fair 3/10 (30 %) Good 4/10 (40%)	Poor 2/48 (4.1%) Fair 10/48 (20%) Good 36/48 (75%)	
Poor outcome	Eyes in patients with *CYP1B1* mutations (n=22)		Eyes in patients without *CYP1B1* mutations (n=64)		P value		
	9/22 (41%)		7/64 (10.9%)		0.004		

IO, infantile-onset; IOP, intraocular pressure; NO, neonatal-onset; PCG, primary congenital glaucoma.

difficult to propose since there is no evidence that there is a loss of normal function from truncation of *CYP1B1* at residue 386. The variant c.1294C>G, [p.L432V] occurred in 2/29 IO-PCG cases, predicted as 'benign' on PolyPhen-2. We did not find this variant in any of our controls. Phenotypically, the patients with this variant were similar to those who harboured no mutations.

Though we found no statistically significant difference in the IOP of NO-PCG and IO-PCG, it must be remembered that the normal IOP is in the low teens in neonates. For this subset, a mean IOP of 20.7 mm Hg is likely to indicate a more severe disease. Patients with *CYP1B1* mutations required a greater number of surgeries compared with those without mutations. Al-Shahrani and Khan[37] also reported that *CYP1B1*-related PCG appears to be a more severe disease that responds poorly to initial angle surgery.

Though early-onset glaucoma in our cohort had a more severe disease, it must be kept in mind that there is a U-shaped association of age at presentation and prognosis. Children with severe disease present early, but children with missed diagnoses present late with advanced disease. We are unsure whether the late presentations would have been less severe glaucoma had they presented early and were managed in time.

While studying the underlying genetic variability of NO-PCG and IO-PCG, we made an interesting observation regarding gender. We observed that the IO cohort had a significantly male preponderance (82.8%), while the NO cohort was predominantly female (28.5% male). Mokbel *et al*[38] from Egypt reported 72% of newborn glaucoma were female compared with 30% in the infantile group. Mandal *et al*[39] from South India reported 64% of newborn glaucoma were female. It is possible that since the frequency of IO-PCG is so much greater than NO-PCG, the female preponderance of the latter gets lost in the overall numbers of PCG. However, we also noted a significant male predominance in patients with *CYP1B1* mutations in both groups. Our observations were at variance with Suri *et al*,[40] who found that male predominance was statistically significant in patients without *CYP1B1* mutations, but not in those with *CYP1B1* mutations.

One limitation of our study was calculating the prevalence of *CYP1B1* mutations in the NO-PCG and IO-PGG groups. The difference did not reach significance using the number of individuals (p=0.13), but the difference was significant when recalculated using the numbers of eyes (p=0.016). Since we were comparing outcomes in babies' eyes with and without *CYP1B1* mutations, we compared the eyes also. Asymmetry in presentation and the asymmetric outcome is well known in PCG. We wished to analyse the outcome in eyes belonging to babies harbouring CYP1B1mutations. The greater proportion of *CYP1B1* mutations in eyes with NO-PCG may be one of the underlying reasons for the more severe phenotype observed in this subset.

Our study's results strongly suggest that NO-PCG should be considered a separate entity of PCG with distinct genotypic and phenotypic characteristics. Whole exome sequencing may provide answers to many unsolved questions and pave the beginning of the search for a genetic cure for this potentially permanently blinding disease.

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Competing interests None declared.

Patient consent for publication Parental/guardian consent obtained.

Ethics approval Informed consent was taken from parents of all infants to participate in the study and to use their pictures for educational purposes; the study adhered to the tenets of the Declaration of Helsinki. Ethics clearance was obtained from the Institute Ethics Committee (INT/IEC/2017/1219).

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