Exome sequencing identifies procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 mutations in primary congenital and juvenile glaucoma

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Purpose: To report the association of procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) mutations with bilateral primary congenital glaucoma (PCG) in monozygotic twins and with nondominant juvenile-onset primary open-angle glaucoma (JOAG). **Methods:** We utilized family-based whole-exome sequencing to detect disease-causing mutations in a pair of monozygotic twins with de-novo PCG and compared its existence in 50 nonfamilial cases of JOAG and 30 healthy controls. To validate the identified mutations, direct Sanger sequencing was performed. For further evaluation of gene expression in the ocular tissues, we performed whole-mount *in situ* hybridization in zebrafish embryos. **Results:** We identified a novel missense mutation (c.1925A>G, p.Tyr642Cys) in the *PLOD2* gene in the monozygotic twin pair with PCG and another missense mutation (c.1880G>A, p.Arg627Gln) in one JOAG patient. Both mutations identified were heterozygous. Neither the parents of the twins nor the parents of the JOAG patient harbored the mutation and it was probably a de-novo change. The zebrafish *in situ* hybridization revealed expression of the *PLOD2* gene during embryogenesis of the eye. **Conclusion:** We observed an association of *PLOD2* mutations with PCG and with nonfamilial JOAG. This new gene needs to be further investigated for its role in pathways associated with glaucoma pathogenesis.

Key words: Congenital glaucoma, glaucoma, juvenile glaucoma, *PLOD2*

The first genetic site associated with primary congenital glaucoma (PCG) GLC3A was discovered at 2p21.[1] The *CYP1B1* gene at this locus encoding cytochrome P4501B1 is found to be most commonly associated with PCG[2] and cases of juvenile-onset open-angle glaucoma (JOAG).[3-5] Akarsu *et al.*[6] identified the 1p36 locus (GLC3B) linked to PCG; later, two other loci (GLC3C and GLC3D) were discovered, including the *LTBP2* gene.[7-9]

Among JOAG patients, the Myocilin (*MYOC*) gene is the most commonly known to be implicated with its prevalence varying from 5% to 35% among JOAG patients depending on the ethnic group studied.[4,10-13] *MYOC* mutations have also been shown to be associated with PCG,[14-16] emphasizing the genetic overlap between the two subtypes of glaucoma. Apart from *MYOC*, *CYP1B1*,[3,17] *LTBP2*[18,19] and *CPAMD8*[20] genes are also found to be associated with JOAG.

Among cases of congenital glaucoma, in addition to *CYP1B1* and *LTBP2*, *TEK*, *GPATCH*, and *PRSS56* mutations have also been associated;[21-24] however, together they explain only 10%–50% of those affected, depending on the population studied.[9,25,26] Genetic heterogeneity in nonsyndromic congenital and juvenile glaucoma[27,28] is the reason why efforts are being made to identify novel variants associated with them.

Next-generation sequencing enables exome-wide identification of novel variants and is currently the method of choice for identifying novel variants especially for diseases with genetic heterogeneity.[29] We undertook this study to analyze genetic variant(s) that could be the cause of PCG in a pair of monozygotic twins with the help of whole exome sequencing (WES).

Methods

Clinical evaluation

The parents of monozygotic twins presented to our hospital with complaints of the children having watering from their eyes since birth. Both the children were healthy males aged 8 months. The twins had similar facial features, body habitus, and blood group typing [Fig. 1]. They were of Indian ethnicity belonging to the Jat community in Haryana. This was a nonconsanguineous family and the parents had three children [Fig. 2].

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The twins were examined under anesthesia. Child B had horizontal corneal diameters of 14.5 and 15 mm in the right eye (RE) and left eye (LE), respectively. Corneas were hazy with the presence of circumferential Haab striae. The anterior chamber was deep with normal iris pattern and clear lens. Fundus examination revealed a cup disc ratio of 0.9:1 with a thin neuroretinal rim in both eyes. His IOP with Perkins tonometer was 28 and 30 mm Hg on timolol 0.25% eye drop in the RE and LE, respectively. Trabeculotomy and trabeculectomy with the use of mitomycin-C were performed in both eyes. The other twin (Child C) also had horizontal corneal diameters of 14 and 14.5 mm in the RE and LE, respectively. His fundus examination also revealed a cup disc ratio of 0.9:1 with a thin neuroretinal rim in both eyes. The IOP was 24 mm Hg on timolol 0.25% eye drop in both eyes. Both eyes of this child were also operated for glaucoma. The postoperative course in both children remained uneventful. Follow-up over 10 years post-surgery showed an IOP of <18 mm Hg in both eyes of both twins with a single anti-glaucoma drug.

Figure 1: Monozygotic twins at 8 months of age

The parents and the sister of the twins were carefully examined to rule out raised IOP or glaucoma over the 10-year follow-up and were found to have a normal anterior segment and no evidence of glaucoma.
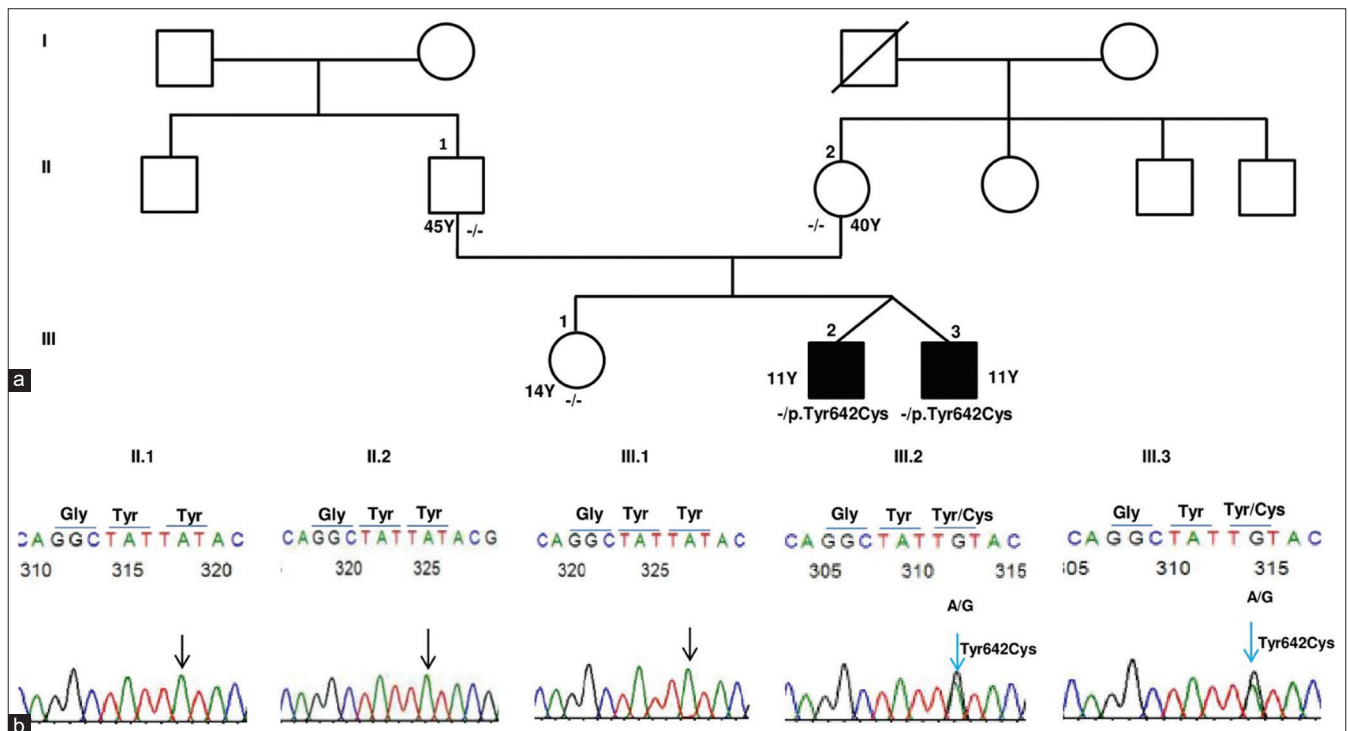
Genetic evaluation

The study was carried out in accordance with the tenets of the Declaration of Helsinki. Ethical approval was obtained from our institute's ethics committee and written informed consent was provided by the parents for blood collection and genetic evaluation.

Exome sequencing was carried out in the family with the monozygotic twin pair concordant for PCG. For comparison, we selected from our cohort 50 other patients with non-familial JOAG and 30 healthy controls. Two ml of blood, drawn from the antecubital vein of cases and controls were collected in ethylene diamine tetraacetate (EDTA) vial, and DNA was isolated by salting out method. Exome capture was achieved using the Sure Select Human All Exon V5 (50.4 Mb) (Agilent Technologies, Santa Clara, California, USA) by following the manufacturer's instructions. Briefly, 1 μg of genomic DNA was fragmented (150–200 bp) and ligated to adapter primers and then PCR amplified. Further, biotinylated RNA capture probes (~120 bp) were used for hybridization of amplified DNA-fragment libraries. Hybridized DNA was recovered by streptavidin-coated magnetic bead separation (Dynal, Invitrogen, Carlsbad, CA). HiSeq2000System (Illumina, San Diego, CA). Captured DNA was eluted and then subject to flow-cell massively-parallel sequencing on a HiSeq2000System (Illumina, San Diego, CA). Hybrid-capture libraries were amplified to add the sequencing primers and identifying tags

Figure 2: (a) The pedigree of the monozygotic twins with *PLOD2* (p.Tyr642Cys) mutation. Affected status shaded in black is for PCG. -/-indicates homozygous wild genotype. -/+ indicates heterozygous mutation for p.Tyr642Cys. Y indicates age in Years at the last follow-up. (b) DNA Sanger sequencing traces revealed wild allele at c. 1925 of *PLOD2* in father (II.1) mother (II.2) and sibling (III.1) of the monozygotic twins and confirmed a heterozygous G allele [c.1925A>G(p.Tyr642Cys)] in the monozygotic twins (III.2, III.3)

and then subjected to paired-end (2 × 101 bp read length) multiplex sequencing. All samples were sequenced with a minimum of 100× coverage.

Exome variant analysis

All the FastQ files were assessed for the quality of raw sequence data. Raw reads with poor base quality and sequence adapters were trimmed by trimmomatic. The clean reads with Phred score >20 were mapped to the human reference genome build hg19/GRCh37 using Burrows–Wheeler Aligner (BWA). SAM tools were used for sorting of reads and duplicate reads were marked using Picard tools (http://picard.sourceforge. net/). Genome analysis tool kit (GATK) v2.7.2)[30] was used for calling variants. Further, the variant calling format files (VCF) were annotated using Golden Helix VarSeq Software v. 1.2.1 (Bozeman, MT). Target coverage and read depth were reviewed by the Integrated Genomics Viewer (IGV, http:// www. broadinstitute.org/igv/). Variant filtering was based on (a) read depth (DP) of >10, (b) genotype quality (GQ) score of >20, and (c) predicted missense or loss-of-function mutation. All exonic single nucleotide polymorphisms (SNPs) from the respective VCF files of the monozygotic twins were compared to determine the concordant and discordant variants; the variants were considered concordant if the genotypes were identical in both VCF files.

We prioritized the variants by following steps. (1) Rare or novel variants (having <1% minor allele frequency) including frameshift, splice, stop gain, stop loss, or missense predicted to be damaging by at least one of the pathogenic prediction software were filtered. Minor allele frequency (MAF) was accessed from public databases including 1000 Genome Project phase 3 (www. 1000genomes.org), dbSNP Common 151 (http:// ncbi.nlm.nih.gov/SNP/), ExomeVariantServer (EVS, http://evs. gs.washington.edu/EVS/); (2) potential disease-causing variants were further selected by including variants shared by affected twins and excluded variants shared by unaffected sibling; (3) included de-novo variants; and (4) only those variants that were potentially pathogenic and had an allele frequency <0.001 in the South Asian population. We then chose our genes based on other patients with JOAG that had a similar gene with an associated pathogenic or a variant with uncertain significance. From this analysis, we got the *procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2)* gene (also called *LH2*) with a de novo mutation present in both twins as well as in a JOAG patient. Further, the prioritized variants were validated via PCR amplification and by Sanger sequencing using an ABI 3730XL sequencer (Applied Biosystems/Life Technologies, Carlsbad, CA, USA).

Whole-mount *in situ* hybridization

For functional characterization of the gene, we looked for its expression in ocular tissues of the zebrafish embryo using whole-mount *in situ* hybridization (WISH). The zebrafish has advantages for studying the gene expression in ocular development because of its fast development, the optical clarity during embryogenesis, and the readily available genomic sequence. The details of the methodology for WISH are provided in the supplementary file.

Results

No pathogenic variants (including copy number variations) were detected that cosegregated with the disease in the PCG twins at any of the known loci for Mendelian forms of POAG (GLC1A, GLC1B, GLC1D-Q) or PCG (GLC3A-D), including the known causative genes, namely *MYOC* (GLC1A), *CYP1B1* (GLC3A), *WDR36* (GLC1G), *ASB10* (GLC1F), *OPTN* (GLC1E), *NTF4* (GLC1O), *TBK1* (GLC1P), *LTBP2* (GLC3C), *PAX6, TEK, ANGPT1,* and *FOXC1*.

We identified a heterozygous, nonsynonymous, missense deleterious mutation in the *PLOD2* gene on exon 17 (chr3:145 789071T>CGRch37;NM_000935.2:c.1925A>G; p.Tyr642Cys) in the twins. The allele frequency of this variant is not reported in the dbSNP database nor in the 1000 Genomes database. This de-novo heterozygous mutation was confirmed in both the twins as well as its absence in both the parents and sister by Sanger sequencing [Fig. 2]. The mutation was predicted to be deleterious (0.807) by the Condel program.[31] One JOAG patient was found to harbor another heterozygous nonsynonymous, missense mutation in the *PLOD2* gene on exon 17 (chr3: 145789116 C>TGRch37; NM_000935.2:c.1880G>A; p.Arg627Gln) (rs748652746). The allele frequency of this variant in the South Asian population is reported to be 0.000556 [Table 1]. The SIFT and Polyphen score of this variant were 0.46 and 0.59, respectively. The patient had been diagnosed with JOAG since the age of 31 years with an untreated IOP of 28 mm Hg and a 0.7:1 cupping in BE. His anterior segment was within normal limits (normal corneal size). Gonioscopy revealed a featureless angle that was probably due to the compaction of trabecular beams, suggesting an immature trabecular meshwork. The variation was validated by Sanger sequencing [Fig. 3]. The parents of this JOAG patient were unaffected and did not harbor the mutation. His siblings were unavailable for examination. Both these *PLOD2* variants are highly conserved in the evolutionary process with a genomic evolutionary rate profiling (GERP) value of >5 [Fig. 4]. Table 2 gives the variants that segregated and those that did not segregate with the disease, including those variants associated with glaucoma, for both the monozygotic twins as well as the JOAG patient.

We also explored the expression dynamics of the *plod2* gene in the vertebrate model, zebrafish. The *plod2* gene expression analysis was performed during zebrafish embryonic development using reverse transcription-polymerase chain reaction (RT-PCR) [Fig. 5a]. The presence of *plod2* gene much before zygotic transcription started, indicates the essential requirement of *plod2* during early embryonic development. The *plod2* gene was cloned before making a DIG-labeled antisense mRNA probe for using *in situ* hybridization. Embryos were harvested at 24 and 48 hours of development for mRNA *in situ* hybridization [Fig. 5b-d]. The expression analysis revealed that the *plod2* gene is induced in the regions such as the head and anterior segment of the eye during embryonic development suggesting the possibility of involvement of *plod2* in the structural and functional integrity of zebrafish eye.

Discussion

This study reports the novel association of *PLOD2* with glaucoma. The rare presentation of PCG in this pair of monozygotic twins gave us an opportunity to look for genetic mutations yet unknown in PCG. While rare cases of bilateral PCG in monozygotic twins are known, there is no genetic study reported for them.[32-34] Although discordance of glaucoma has been reported among monozygotic twins,[34] the twins in our study showed no discordance and presented
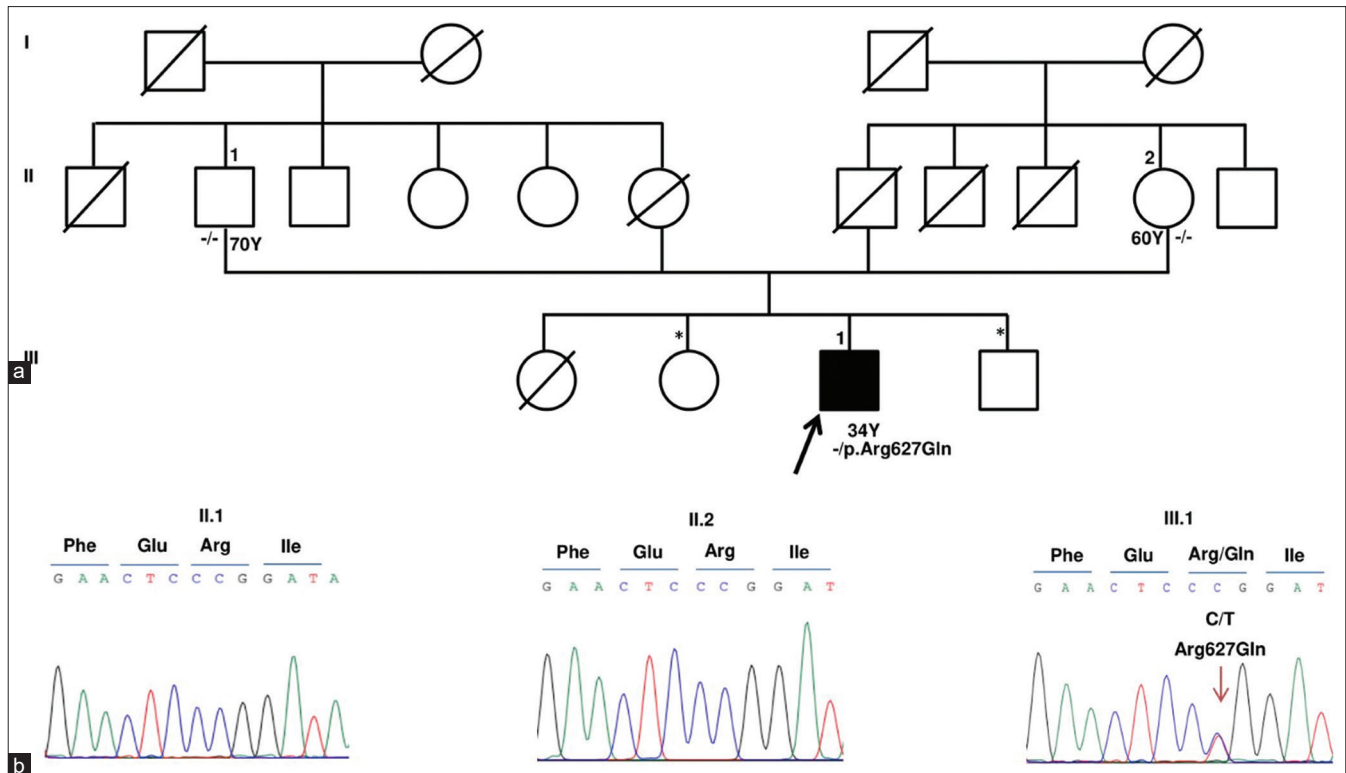
Figure 3: (a) The Pedigree of JOAG patient with *PLOD2* (p.Arg627Gln) mutation. Affected status is shaded in black. ↑ indicates proband. -/- represents homozygous wild genotype. -/+ indicates p. Arg627Gln heterozygous mutation. Siblings (*) were not available for the genetic study. Y indicates age in years at the time of study. (b) DNA Sanger sequencing traces revealed wild allele "C" at chr3:145789116 of *PLOD2* in father (II.1) and mother (II.2) and confirmed a heterozygous T allele [chr3: 145789116 C>T(p.Arg627Gln)] in the proband (III.1)
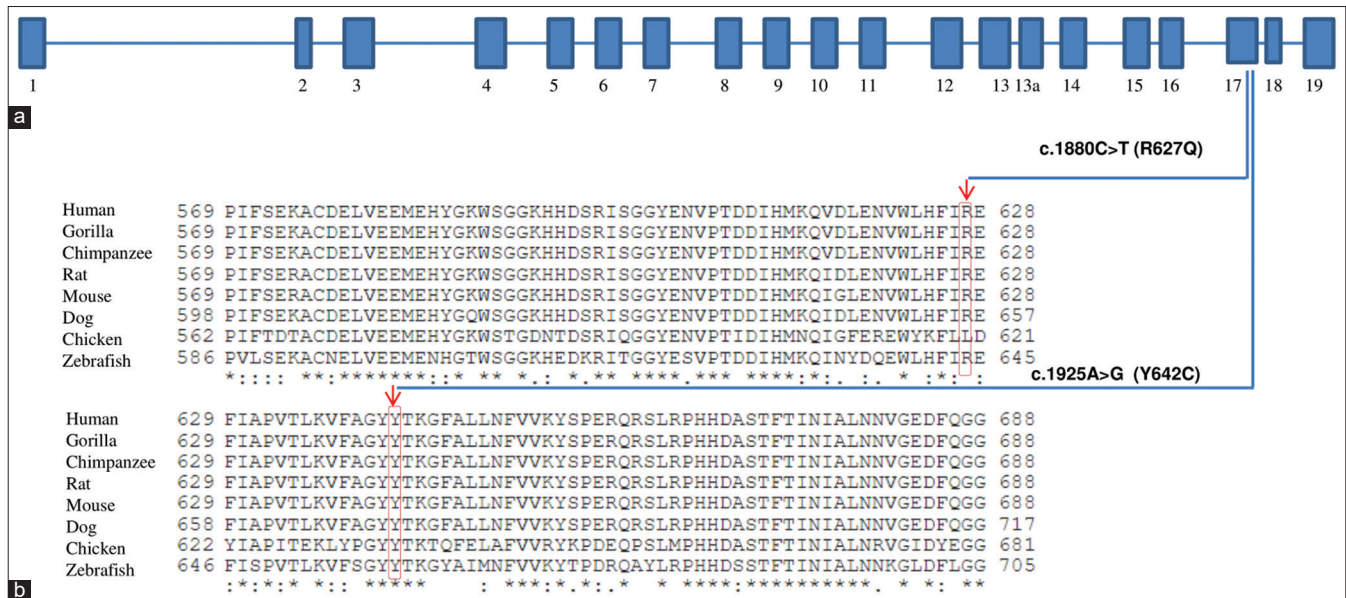
Figure 4: (a) Schematic exon organization of *PLOD2* and the exon location of R627Q and Y642C are indicated. (b) *PLOD2* protein sequence alignment across multiple species showing conservation of arginine (R627) and tyrosine (Y642) residues is seen

with similar severity and onset of the disease. We relied on the facial similarity and blood group typing along with a 96% concordance in the Exome to establish monozygosity.

In the absence of the known Mendelian genes for POAG and PCG in our cases, as well as those that increase the risk susceptibility to glaucoma, we believe *PLOD2* could be an important gene associated with de-novo or sporadic cases of PCG and JOAG. The pathogenicity of the two variants is supported by their extremely low frequency in the general population, their absence in controls along with their evolutionary

Table 1: The characteristics of the two variants detected in the PCG and JOAG patients

Diagnosis	Chr. Position	Exon	Codon	Protein Change	gnomAD variant count	gnomAD total alleles	gnomAD Ethnically Matched alleles (South Asian)	Polyphen 2 (Score)
PCG	3:145789071	17	c.1925A>G	p.Tyr642Cys	Novel	-	-	Probably damaging (0.998)
JOAG	3:145789116	17	c.1880G>A	p.Arg627Gln	17	250598	30600	Probably damaging (0.598)

Table 2: Variants segregating and nonsegregating with the disease in the monozygotic twins and the JOAG patient
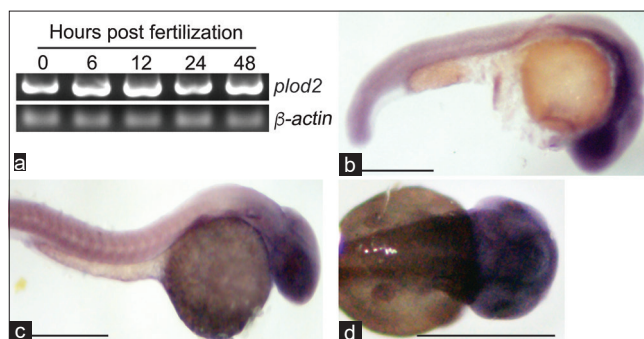
	Monozygotic twins	JOAG patient
Segregating with disease		
Total variants	n=548	n=695
Glaucoma associated	n=1 (NF1 gene)*	n=1 (PAX6 gene)**
Nonsegregating with disease		
Total variants	n=2174	n=1195
Glaucoma associated	n=0	n=1 (OPTN gene)***

*NF1 (neurofibromin 1) gene, rs191111884 an intronic variant (ClinVar: Benign). **PAX6 (paired box 6) gene, rs759391101 an inframe deletion (ClinVar: Benign). ***OPTN (optineurin) gene, rs71492279 an intronic variant (ClinVar: Benign)

Figure 5: Expression of *plod2* gene during zebrafish embryonic development (a) RT-PCR analysis of the plod2 at various time points post-fertilization. (b and c) Bright-field microscopy lateral view images of zebrafish embryos at 24 hpf (b) and 48 hpf. (c) embryos expressing *plod2* gene as revealed by mRNA *in situ* hybridization. (d) Bright-field microscopy dorsal view image of the 48 hpf zebrafish head region showing *plod2* gene expression. Scale bar indicates 1 mm

conservation, and their effect on protein function. The *PLOD2* gene consists of 19 exons, codes for lysyl hydroxylase2 (LH2) and the LH2 enzyme is responsible for the hydroxylation of collagen telopeptide which directs cross-linking of collagen fibrils in the extracellular matrix, necessary to provide tensile integrity and stability.[35,36] *PLOD2* mutations are known to occur among patients with Brucks syndrome,[37] a rare form of osteogenesis imperfect characterized by bone fragility and congenital joint contractures. None of our patients had any orthopedic problems.

Though *PLOD2* mutations are not known to have an association with causing raised IOP or glaucoma, the *PLOD2* enzyme is known to have an association with the *PITX2* gene, the mutations of which cause Axenfeld Reiger (AR) syndrome.[38] The *PLOD2* gene is a potential downstream target for the *PITX2* gene.[39,40] Although none of the three cases reported here with *PLOD2* mutations had features of AR syndrome, we believe the pathways of trabecular meshwork (TM) development may have been altered due to the mutations, leading to the immaturity of the TM. The *PLOD* enzymes have been shown to be important in collagen cross-linking and hence may be postulated to have a role in TM development pathways.[35] The G protein-coupled receptor 48 (Gpr48) has been shown to be associated with anterior segment dysgenesis during development and deletion of the Gpr48 in mice has been shown to lead to decreased levels of both *PLOD1* and 2 enzymes.[41] We also found *PLOD2* expression in the ocular tissues during embryonic development of the eye as shown in our *in vivo* study using the zebrafish model.

The LH2 enzyme in mice has been found to be associated with embryogenesis[42] and its deficiency is related to endoplasmic reticulum stress and apoptosis.[43] In the evolutionary ladder of complex multicellular organisms, lysyl hydroxylation plays an important role in the initial steps

of development as embryonic lethality was associated with LH2-null mice.[44] However, *plod2* zebrafish mutants survive into adulthood despite complete loss of LH2 but with disturbed fibrillar organization.[45] It is possible that different disorders might develop, depending on the species and extent of the variation in the *PLOD2* gene. *PLOD2* mutations identified in Brucks syndrome2 cause a marked reduction in LH2 activity, whereas an elevated expression of LH2 has been recognized as a general phenomenon that promotes fibrosis and cancer progression.[43,46] Most *PLOD2* mutations associated with Brucks syndrome involve exons 17–19 encoding the C-terminal domain with LH activity[47], and the amino acid sequence in this region has shown high homology among LH1, LH2, and LH3 across species, suggesting the region being important for lysyl hydroxylase function. In our study, both *PLOD2* mutations were in exons 17 encoding the C-terminal domain for LH activity.

The *PLOD2* gene also lies in the 3q24 locus, which was originally described by Wirtz *et al.*[48] for its association with adult-onset POAG. Because PCG, JOAG, and adult-onset POAG form a continuum, especially with regards to the overlapping genetic variations described in these three phenotypes that are distinguished mainly by their age of onset, one could postulate that the *PLOD2* mutations might also play a role in adult-onset POAG. At this juncture, we can only hypothesize that the effects of *PLOD2* mutations could be related to angle maldevelopment in humans.

The earlier age of onset with c.1925A>G change leading to congenital glaucoma points to the greater pathogenicity of this mutant in comparison to the c.1880G>A, which resulted in delayed onset glaucoma in the JOAG patient. While the frequency of c.1880G>A change among South

Asians is 0.0005556, the c.1925A>G change was absent in South Asians.

Conclusion

In conclusion, novel missense mutations have been identified in a new gene locus, namely *PLOD2* by WES. A larger sample of congenital and juvenile glaucoma patients would be needed to replicate our results. There is also a need for further research as regards the cause and effect relationship of this gene with glaucoma.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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