Novel compound heterozygous mutations in *CYP1B1* identified in a Chinese family with developmental glaucoma

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Abstract. Developmental glaucoma, a subset of glaucoma, is associated with trabeculodysgenesis and/or anterior segment dysgenesis. It is one of the major causes of childhood blindness. Understanding its genetic background is important to diagnose, and identify potential therapeutic targets, of this disease. The present study aimed to detect the molecular origin of developmental glaucoma in a Chinese pedigree and its association with glaucomatous phenotypes. A three-generation pedigree with developmental glaucoma was analyzed in the current study; a thorough ocular examination was performed on the proband and other individuals in the family. Genomic DNA was extracted from the peripheral blood of each individual, and possible disease-causing genes were screened for mutations using a candidate gene panel. Exons and adjacent regions of the target genes were captured and enriched by probe hybridization. The enriched genes were sequenced on an Illumina high-throughput sequencer. Variations were verified in other family members using Sanger sequencing. Disease causing mutations were analyzed by comparing the sequences and the structures of wild-type and mutated cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) proteins using PyMOL software. The proband was diagnosed with developmental glaucoma and his parents and other relatives were asymptomatic. Novel compound heterozygous mutations, c.3G>A (p.M1I) and c.1310C>T (p.P437L), in *CYP1B1* were detected in the proband, with the former inherited from his father and the latter from his mother. The c.3G>A (p.M1I) change is a novel mutation that disrupts the ATG start codon in exon one of *CYP1B1* and therefore interferes with the translation start site. In conclusion, the findings of the present study suggested that the aforementioned compound heterozygous mutations in *CYP1B1* may have caused developmental glaucoma in this Chinese family. The c.3G>A mutation in *CYP1B1* is a novel mutation, and this study expands the gene mutation spectrum of *CYP1B1*.

Introduction

Developmental glaucoma, also known as congenital glaucoma, is an ocular defect and is often associated with the abnormal development of the anterior chamber angle (1). Glaucomatous phenotypes including elevated intraocular pressure (IOP) are generally displayed at birth, but the onset may be delayed to adolescence (2). For the latter, the patients only present with elevated IOP, trabeculodysgenesis and glaucomatous optic neuropathy, but without the tearing, photophobia, blepharospasm, enlarged cornea and Haab's striae that is generally observed in congenital glaucoma (3).

Developmental glaucoma is an inherited disorder predominantly transmitted in an autosomal recessive manner (3). Although the pathogenic mechanism has not been completely elucidated in all cases, genetic defects appear to be the most crucial risk factor in developmental glaucoma (3). Several disease-associated loci have been previously identified, including glaucoma 3, primary congenital, A (*GLC3A*) (4), glaucoma 3, primary infantile, B (*GLC3B*) (5), glaucoma 3, primary congenital, C (*GLC3C*) and glaucoma 3, primary congenital, D *GLC3D* (6); in addition, two genes located in these genetic loci, cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) and latent-transforming growth factor

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β-binding protein 2 (*LTBP2*), have been implicated in the mechanisms underlying developmental glaucoma. *CYP1B1* mutations are the most commonly identified genetic defect causing developmental glaucoma (6,7). To date, >150 *CYP1B1* mutations have been identified in patients with developmental glaucoma worldwide (8). Of these, 43 variants have been reported in Chinese patients, including p.R390H, which is a common mutation identified in patients with primary congenital glaucoma (PCG) from all ethnic groups, and p.L107V, which is unique to Chinese patients with PCG (9).

The current study aimed to investigate the genetic background of developmental glaucoma in a Chinese family. Compound heterozygous mutations in the *CYP1B1* gene were identified in a patient, of which one of the mutations (c.3G>A) has not been previously reported, to the best of our knowledge.

Patients and methods

Patients. A three-generation pedigree with developmental glaucoma was recruited to this study Fig. 1, including the 14-year-old male proband, his parents and four other family members (the proband's three grandparents and his aunt). The study was conducted in accordance with the Declaration of Helsinki and the experimental protocol was approved by the Ethics Committee of The Shenzhen Eye Hospital (Shenzhen, China). The parents provided consent for participation and also provided consent for the minor patient.

Detailed ophthalmic examinations, including visual acuity tests, measurement of IOP, slit lamp biomicroscopy, corneal size and measurement of cup/disc ratio were performed on each of the individuals; additional examinations, including a Humphrey visual fields test and anterior-segment optical coherence tomography, were exclusively performed on the proband.

Genetic analysis. A total of 2 ml peripheral blood samples were collected from of each subject. Genomic DNA was extracted using a Massive Whole Blood Genomic DNA Extraction kit (Tiangen Biotech Co., Ltd.; cat. no. DP348-03). Second-generation sequencing was used to screen the candidate glaucoma causal genes in the proband's genome as previously described (10) (data not shown). The gene panel used in the present study was obtained from Shanghai Wickhams Bio-Pharmaceutical Technology Co., Ltd. The panel comprises 2,067 genes known to be involved in 2,486 types of ocular-related hereditary diseases, including corneal abnormalities, cataracts, glaucoma, nystagmus, optic nerve abnormalities, retinitis pigmentosa, strabismus and refractive errors. The 2,067 genes were investigated using targeted capture and high-throughput sequencing technologies (Illumina, Inc.) as previously described (10). The average sequencing depth was >150X, and 30X coverage was achieved for >98.5% of the genes screened. The test contents and classification criteria were based on authoritative disease phenotype databases Online Mendelian Inheritance in Man (omim.org/, updated 2020.05), DECIPHER (deciphergenomics. org/, updated 2019.04), Orphanet (orpha.net/, updated 2020.01) and Human Phenotype Ontology (hpo.jax.org/, updated 2020.01) and literature reports (3-9). The panel contained 95 congenital glaucoma-related genes, including TEK receptor tyrosine kinase (*TEK*), *CYP1B1*, and a number of other genes related to

glaucoma and other ocular diseases, such as neurotrophin 4, optineurin, ankyrin repeat- and SOCS box-containing 10, WD repeat domain 36, myocilin, OPA1 mitochondrial dynamin-like GTPase, paired box 6, forkhead box C1 and paired-like homeodomain 2.

PCR products were electrophoresed on 2% agarose gels, visualized following staining with GoldView (Beijing Solarbio Science & Technology Co., Ltd.) and photographing using a Tanon-1600 gel camera (Tanon Science and Technology Co., Ltd.). The PCR products were used to verify the results of the panel screening in the whole pedigree. Intronic primers flanking the exons (Table I) were designed based on gene sequences of *CYP1B1* (GenBank: U56438) and synthesized by BGI Genomics. DNA fragments were amplified by PCR using a MyCycler thermocycler (Bio-Rad Laboratories, Inc.). The PCR reaction was performed in a 25 μl reaction mixture containing 0.1 μg genomic DNA, 40 μmol/l forward and reverse primers, 3 mmol/l magnesium chloride and 2X Taq Master Mix (Sino Biological, Inc.). The following thermocycling conditions were used for the PCR: Initial denaturation step at 95˚C for 5 min; followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec; then a final extension step of 10 min at 72˚C.

The PCR products were subjected to 1% agarose gel electrophoresis, and the target PCR fragments were extracted using the QIAquick Gel Extraction kit (Qiagen China Co., Ltd.). The products were sequenced using an ABI 377XL automated DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sequencing data were compared with the published *CYP1B1* sequences.

Bioinformatics analysis. The wild-type CYP1B1 protein sequence was downloaded from the RCSB Protein Data Bank (https://www.rcsb.org) and saved as a PDB file. The amino acids in the CYP1B1 protein were changed manually using ICM-Pro (molsoft.com/icm_pro.html, v3.5) software, and the PDB files were uploaded into PyMOL (pymol.org/2/, v2.5.1) and Swiss Model online (swissmodel.expasy.org, updated 2021.07) to generate the 3D structure of the protein (the unaltered wild-type protein was used as a template and the PDB ID is Q16678). The tertiary structure of protein was predicted by the Swiss Model (swissmodel.expasy.org, updated 2021.07). The altered amino acids and key ligands were labeled in different colors. The functional effects of these two mutations were predict by PolyPhen-2 online software (genetics.bwh.harvard.edu/pph2/, updated 2016.01.05).

Results

Clinical findings. The best-corrected Snellen visual acuity of the proband was 20/25 in the right eye and 20/20 in the left eye, and the intraocular pressure without antiglaucoma medications was 42 mmHg for the right eye and 43 mmHg for the left eye. Both horizontal corneal diameters were 13 mm. The slit lamp examination showed a clear cornea, a deep anterior chamber, and a loose and atrophic iris in both eyes (Fig. 2A). Gonioscopic examination showed the presence of trabeculodysgenesis in both eyes (Fig. 2C). Fundoscopic examination of both eyes showed advanced optic disc cupping with a C/D ratio of 0.95 in the right and 0.85 in the left eye (Fig. 2D). B-scan ultrasonography

Table I. Primers used for the PCR amplification of the *CYP1B1* gene.

Exon	Primer sequence (5'→3')	Product size (bp)
CYP1B1	F: CATTTCTCCAGAGAGTCAGC	1,260
Exon 2	R: GCTTGCAAACTCAGCATATTC	
CYP1B1	F: ACCCAATGGAAAAGTCAGCC	927
Exon 3	R: GCTTGCCTCTTGCTTCTTATT	

CYP1B1, cytochrome P450 family 1 subfamily B member 1; F, forward; R, reverse.
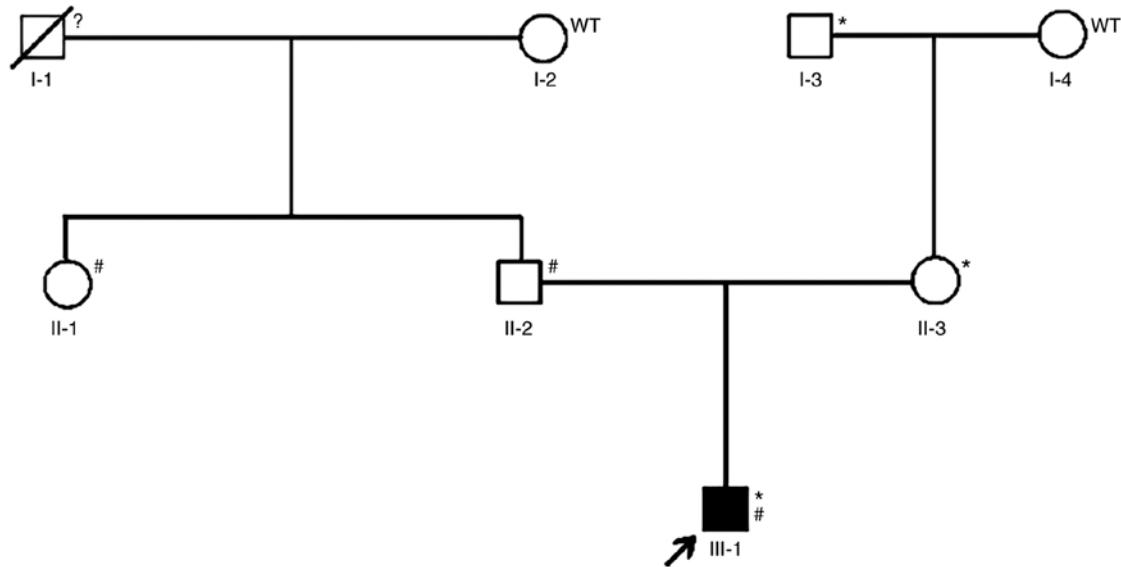

Figure 1. Pedigree of a Chinese family with developmental glaucoma. II-2 carries variant c.3G>A (p.M1I) of *CYP1B1*, and II-3 carries variant c.1310C>T (p.P437L). Squares represent males and circles represent females, and a filled symbol indicates an affected individual. The arrow indicates the proband, and genotypes are given by different symbols: #variant c.3G>A (p.M1I); *variant c.1310C>T (p.P437L); ?no genotype identified; and WT, wild-type.

showed that the axial length was 25.9 mm in the right eye and 26.7 mm in the left eye. A 360-degree circumferential trabeculotomy procedure was performed on the proband's right eye and controlled IOP was achieved. His left eye responded well to two antiglaucoma drugs, such as prostaglandin analogues and a β blocker. No significant ocular abnormalities were noticed in other family members and the relatives, except that loose irises were found in the proband's mother (Fig. 2B).

Genetics and bioinformatics analyses. Compound heterozygous mutations for *CYP1B1* c.3G>A (p.M1I) and c.1310C>T (p.P437L) were identified by the panel screening. The novel heterozygous mutation c.3G>A (p.M1I) and a previously reported mutation (8), c.1310C>T (p.P437L), in *CYP1B1* were identified in individual III-1 (Fig. 1), with the former inherited from his father and the latter from his mother. The novel variant, c.3G>A (p.M1I), was found to alter the ATG start codon of exon 1 of *CYP1B1*, which disrupted the translation start site (Fig. 3). The two mutations were predicted to be 'possibly damaging' based on PolyPhen-2 analysis. Both P437 and M1 amino acid positions were found to be highly conserved in *CYP1B1*, from *Homo sapiens* through to *Danio rerio* (Fig. 4).

To predict the putative structural and functional impact of these mutations on the CYP1B1 protein, the protein structure was predicted using PyMOL (Swiss Model). The novel c.3G>A mutation was found to be located in the AUG start codon for methionine, changing it to AUA, which encodes isoleucine (p.M1I). The major effect of this change was predicted to be the elimination of the initiation codon of translation, with the next downstream AUG being at position 293 and encoding a truncated frameshifted peptide of 53 amino acids. The c.1310C>T (p.P437L) mutation in the *CYP1B1* gene is located in the meander region of the translated protein (Fig. 5). As shown in Fig. 5A and C, the substitution caused minimal distortion of the protein fold and did not result in regional crowding, but the change from a small hydrophilic amino acid with obligate torsion of the backbone to a medium-sized aliphatic residue would be expected to have significant effects on activity, as implied in the *in silico* analysis.

These findings indicated that both p.M1 and p.P437 are important amino acids required to maintain the normal function of *CYP1B1*, and both were implicated in the clinical glaucomatous phenotype of the proband.

Discussion

Even though most cases of developmental glaucoma seem to be sporadic, up to 40% of cases are considered to be genetically
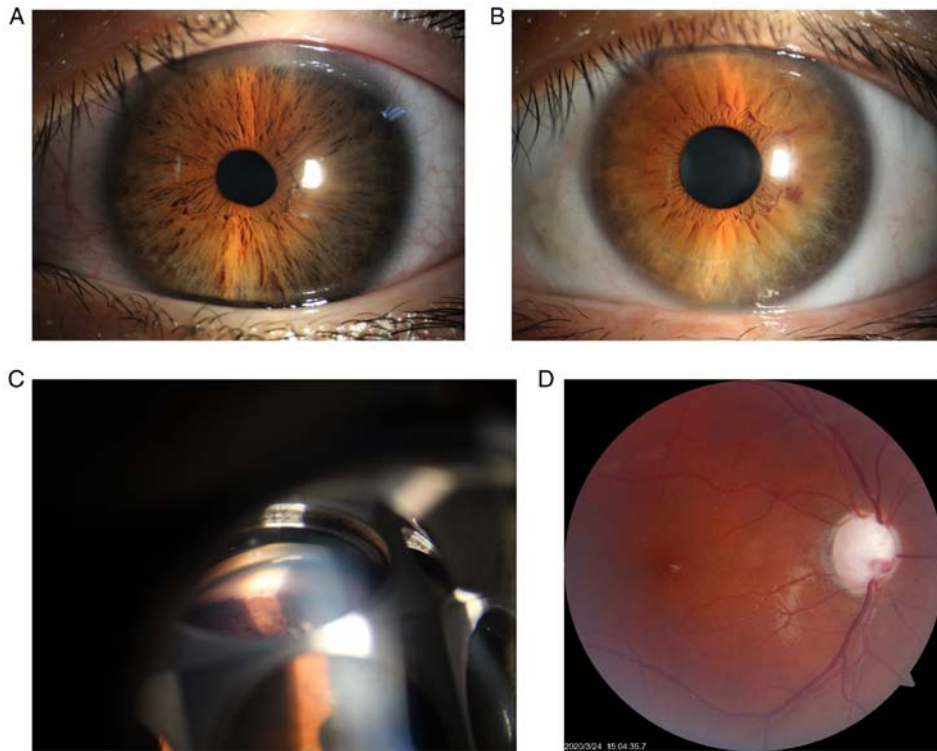
Figure 2. Clinical manifestations of the proband and their mother. (A) Loose and atrophic iris of the proband. (B) Loose iris of the proband's mother. (C) Trabeculodysgenesis and (D) glaucomatous cupping of the proband.
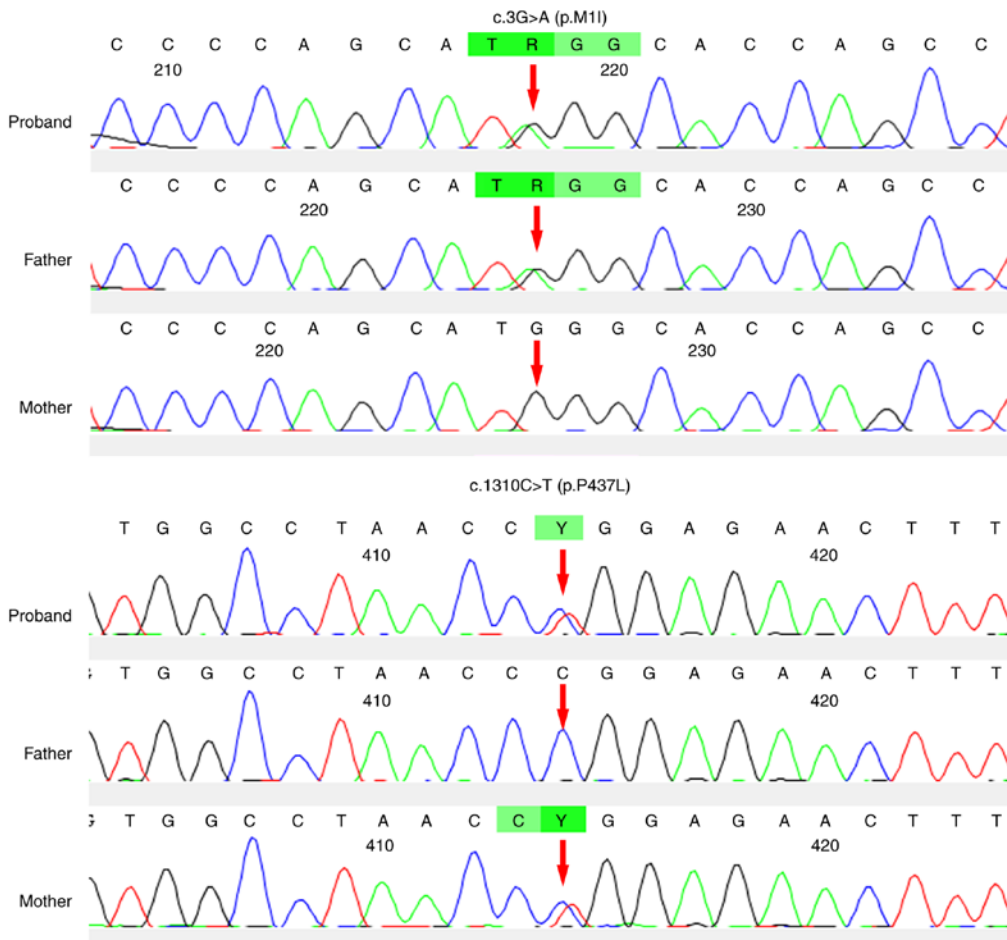

Figure 3. Mutations of the *CYP1B1* gene in a Chinese family with developmental glaucoma. Compound heterozygous mutations c.3G>A (p.M1I) and c.1310C>T (p.P437L) in *CYP1B1* were identified in the proband. The variant c.3G>A was inherited from the father and c.1310C>T was inherited from the mother.
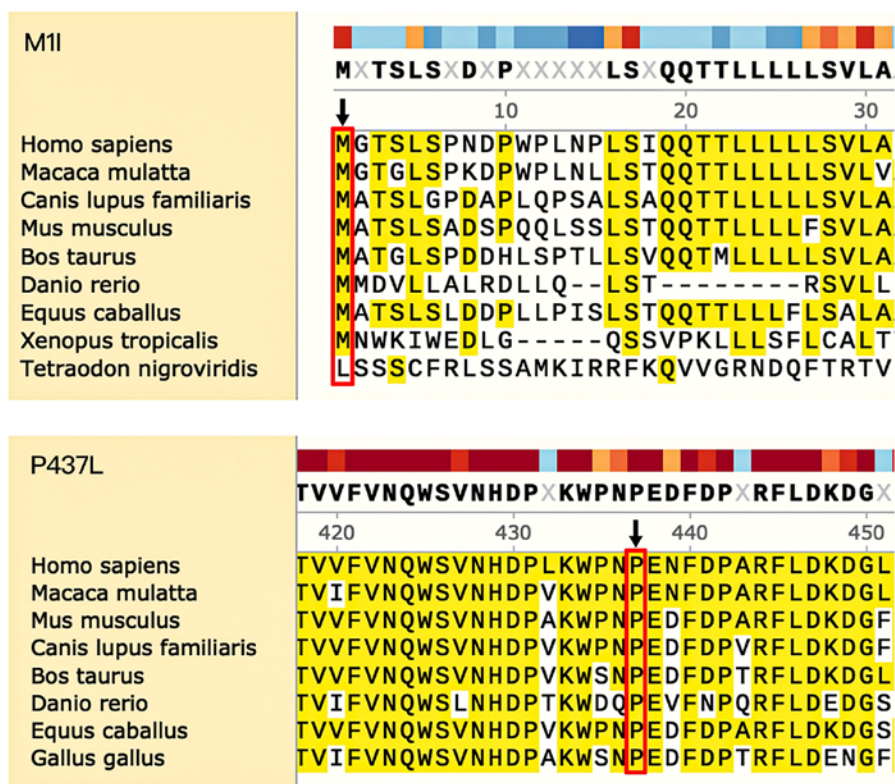
Figure 4. *CYP1B1* mutations lead to altered amino acid residues p.M1I and p.P437L. Multiple alignments of p.M1I and p.P437L of CYP1B1 protein from different species. Both variants occurred on the conserved residues of the CYP1B1 protein.
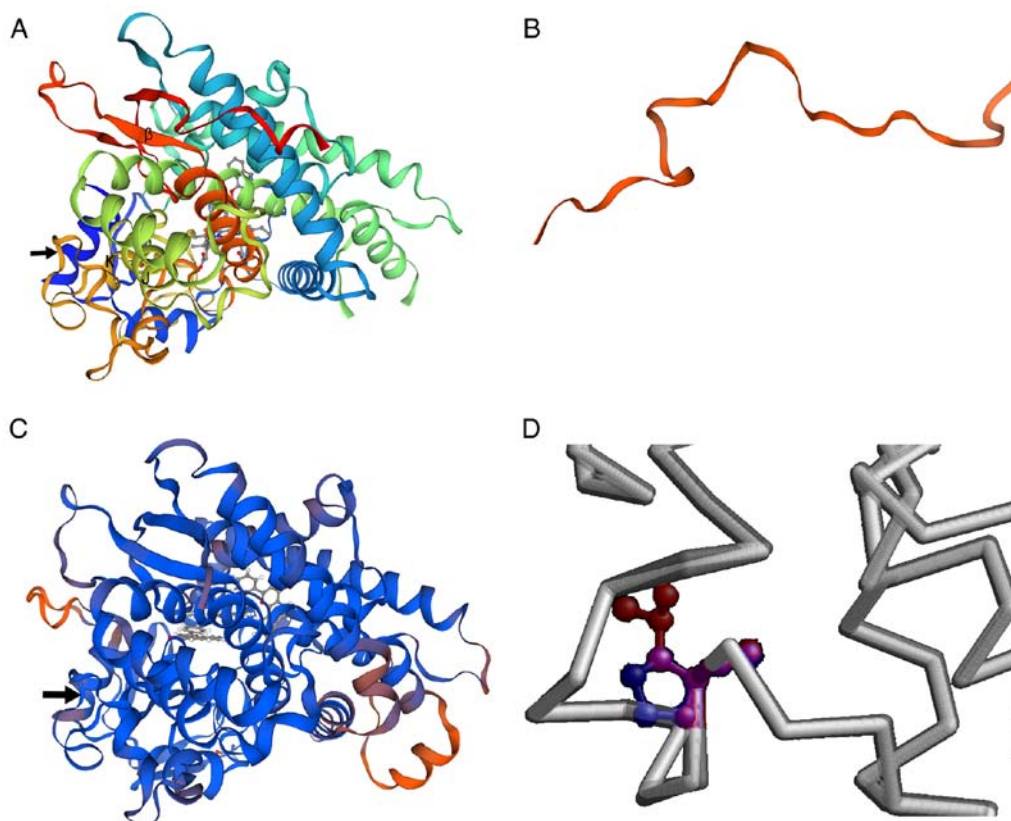

Figure 5. CYP1B1 protein structure was predicted using swissmodel.expasy.org online and PyMOL software. (A) Wild-type protein. (B) Truncated protein caused by the c.3G>A (p.M1I) mutation. (C) CYP1B1 protein with the c.1310C>T (p.P437L) variant. Black arrows in parts indicate the 437P or 437L positions in A and C, respectively; red dots indicate ligands. (D) Structures of the wild-type and p.P437L CYP1B1 protein variants were overlaid to show the minimal distortion of the protein fold, but with some effects of the constraints of the proline residue and opening of the structured near the leucine side chain. Purple, red and blue indicate the mutated amino acid residue; J, K and β mark the J-helix, K-helix and β-sheet, respectively. *CYP1B1*, cytochrome P450 family 1 subfamily B member 1.

inherited, demonstrating an autosomal recessive inheritance pattern with variable penetrance (11,12). Mutations in the genes, *CYP1B1*, *LTBP2* and *TEK* have been reported in patients with developmental glaucoma, and among these, mutations in *CYP1B1* are the most commonly reported (13-15). *CYP1B1* is a drug-metabolizing enzyme of the cytochrome P450 gene family, which is expressed in a wide spectrum of tissues, including the iris, trabecular meshwork (TM), ciliary body and anterior uveal tract of the eye (16). *CYP1B1* serves a crucial catalytic role in the synthesis of cholesterol, steroids and other lipids (17-19). These metabolic reactions and products are important for the differentiation and growth of multiple tissues including liver, cardiovascular and eye (19-21). In PCG, *CYP1B1* mutations interfere with metabolism of retinol, a key metabolite required for TM development (22,23). Another major cause of TM pathogenesis is abnormal oxidation status owing to improper *CYP1B1* gene activity. The presence of oxidative stress during early development can cause TM hypoplasia, leading to developmental glaucoma (24). Teixeira *et al* (23) reported that *Cyp1b1*$^{-/-}$ mice had progressively decreased levels of collagen in the TM, increased TM atrophy, an elevated IOP and increased glaucomatous lesions, with the latter very similar to the manifestations of human PCG.

The structure of the CYP1B1 protein comprises four conserved helix bundles, including the J- and K-helices, β-sheets, a meander region and the heme-binding region (25). In addition, the N-terminal hinge region and C-terminal conserved core structures (CCSs) are known to be important regions for maintaining enzymatic activity (2,26). Notably, clinical reports on a variety of different ethnic groups have suggested that patients with developmental glaucoma with *CYP1B1* mutations in the N-terminal hinge region or CCSs, such as the missense mutations c.517G>C (26) and c.1439G>T (27), present with more severe glaucomatous phenotypes.

To date, >150 mutations in the *CYP1B1* gene have been described in cases of developmental glaucoma, with some predominantly associated with severe glaucomatous pathology (7,28). In the current study, a novel heterozygous mutation, c.3G>A (p.M1I), within the *CYP1B1* gene of a Chinese family with developmental glaucoma was identified. This heterozygous mutation caused the loss of the primary AUG start codon for methionine, which was replaced by a triplet AUA encoding isoleucine (p.M1I) and was predicted to affect the initiation of translation, as the initial methionine codon surrounded by a Kozak sequence is crucial for ribosomal recognition (29). The next conserved Kozak sequence where translation could be initiated is at the c.293 AUG and ends at the 452 UAA position on the mRNA. The new ORF shifted protein is predicted to be shortened to only 53 amino acids and would include different amino acids from the wild-type CYP1B1 protein as it starts in a new open reading frame. Translation can be initiated at non-AUG codons, including ACG, but this is extremely rare and requires a favorable mRNA secondary structure (29). In the current study, this mutation in the proband was inherited from his father, who was clinically asymptomatic, indicating the probable functional null nature of this mutation.

The second variant detected in the present study, c.1310C>T (p.P437L), was previously reported in different

ethnic groups worldwide (10,11,30-32). A report from Pakistan reported cases of PCG caused by variants in *CYP1B1*, including c.1310C>T. It was predicted that this mutation may alter the protein backbone conformation at this position, as the proline residue at position 437 is rigid and located on the folded protein's surface, and the substitution of proline with leucine at this position may influence the special conformation and distort the interactions with other molecules (33). The modeling results of the current study suggested that distortion of the protein fold, was minimal and would favor decreased CYP1B1 enzyme activity as a mechanism. A previous study using sequence analysis and homology modeling reported that PCG resulting from *CYP1B1* mutations disrupted either the hinge region or the conserved core structures of cytochrome P4501B1 (24). By contrast, the p.P437L variant may affect the meander region. The segregation of the mutant *CYP1B1* alleles was consistent with autosomal recessive inheritance of the disease in five pedigrees investigated (26). An analysis of *CYP1B1* in Brazilian patients with PCG showed that four of the nine mutations were present as compound heterozygotes, two in homozygotes and only one mutant allele was identified in three of the cases. In one patient, the c.8147C>T (p.P437L) and c.8182delG mutations were identified in a compound heterozygote, and clinical examination revealed a highly compromised phenotype with low visual acuity and difficultly controlling IOP (13). Screening of *CYP1B1* and *LTBP2* in Saudi families with PCG showed that PCG cases with *CYP1B1* variants, including p.P437L, had a more severe subepithelial haze in cornea and a greater C/D ratio compared with those cases with no identified mutation (32). Moreover, in a Pakistani family with PCG, two affected individuals carrying the c.1310C>T mutation in *CYP1B1* manifested PCG symptoms during the first year after birth and subsequently underwent bilateral trabeculectomy. One had an elevated IOP with bilateral megalocornea, with opacities and decreased visual acuity with the perception of light only; the other also had megalocornea with increased lacrimation and photophobia (34). In addition to the aforementioned reports, the c.1310C>T mutation in *CYP1B1* has also been previously reported in families with PCG in Spain and India (31,35), but not in China, to the best of our knowledge.

In autosomal recessive phenotypes, heterozygous carriers are generally asymptomatic. However, parents carrying the pathogenic variant in a heterozygous state may present a mild phenotype (36). In the present study, the father carrying one of the compound heterozygous mutations (c.3G>A) appeared asymptomatic, whereas the mother carrying the second mutation (c.1310C>T) presented with loose and mildly atrophic irises, similar to, but less severe than, the proband but without other developmental disorders, such as trabeculodysgenesis, suggesting that a single copy of this mutation may cause a relatively mild form of the disease. However, there is no evidence showing that one of these heterozygous mutations contributes more to the pathogenesis of the child.

Previous studies indicated an association between specific mutations and the severity of anterior chamber angle abnormalities (8). The C-terminus of the CYP1B1 protein includes a substrate binding region and CCS, whereas the N-terminal of the CYP1B1 protein includes a membrane-spanning domain and a hinge region (19).

Mutations leading to protein variants p.E229K (37) and p.S239R (38) have also been shown to disrupt the three-dimensional structures of the I-helix, and subsequently lead to severe glaucomatous phenotypes. By contrast, the CYP1B1 protein structure showed that p.P437L is located in the meander region (26,39). Taken together, these data indicate that in addition to the hinge region and CCSs, the meander region can also be important for maintaining the function of the *CYP1B1* gene.

In conclusion, the results of the present study revealed, for the first time to the best of our knowledge, the coexistence of the novel c.3G>A mutation alongside the c.1310C>T mutation in a Chinese pedigree of developmental glaucoma. In addition, our data indicated the importance of additional regions, such as the meander region, suggesting that they can also affect the structure of the CCSs and are crucial in regulating the function of *CYP1B1* and avoiding TM abnormalities. However, future studies are required to determine the influence of this mutation on metabolism and selection of key metabolites, which may lead to the development of potential therapeutic strategies for PCG.

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL and JFH conceptualized and designed the experiments and supervised the study. DZ, TW and MF collected the samples and clinical information. SC, YW and XJ performed the genetic analysis and bioinformatics evaluations. SC conducted the molecular biology experiments and drafted the manuscript. XL and JFH reviewed and edited the manuscript. All authors have read and approved the final manuscript. SC and XL confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and the experimental protocol was approved by the Ethics Committee of the Shenzhen Eye Hospital (Shenzhen, China). The parents provided consent for participation and also provided consent for the minor patient.

Patient consent for publication

The mother provided consent for the publication of images of her iris as well as consent for her child in Fig. 2.

Competing interests

The authors declare that they have no competing interests.

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