Identification of four novel cytochrome P4501B1 mutations (p.I94X, p.H279D, p.Q340H, and p.K433K) in primary congenital glaucoma patients

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Purpose: Primary congenital glaucoma (PCG) is an autosomal recessive eye disorder that is postulated to result from developmental defects in the anterior eye segment. Mutations in the cytochrome P4501B1 (CYP1B1) gene are a predominant cause of congenital glaucoma. In this study we identify CYP1B1 mutations in PCG patients.

Methods: Twenty-three unrelated PCG patients and 50 healthy controls were enrolled in the study. CYP1B1 was screened for mutations by PCR and DNA sequencing.

Results: DNA sequencing revealed a total of 15 mutations. Out of these, four (p.I94X, p.H279D, p.Q340H, and p.K433K) were novel mutations and five were known pathogenic mutations. Five coding single nucleotide polymorphisms and one intronic single nucleotide polymorphism (rs2617266) were also found. Truncating mutations (p.I94X and p.R355X) were associated with the most severe disease phenotype. It is possible that patients with two null alleles with no catalytic activity may present with a more severe phenotype of the disease compared to patients with one null allele (heterozygous). The disease phenotype of patients with CYP1B1 mutations was more severe compared with the clinical phenotype of patients negative for CYP1B1 mutations.

Conclusion: Mutations in CYP1B1 are a major cause for PCG in our patients. Identifying mutations in subjects at risk of developing glaucoma, particularly among relatives of PCG patients, is of clinical significance. These developments may help in reducing the disease frequency in familial cases. Such studies will be of benefit in the identification of pathogenic mutations in different populations and will enable us to develop simple and rapid diagnostic tests for analyzing such cases.

Primary congenital glaucoma (PCG; OMIM 231300) is an autosomal recessive disorder of the eye. In this disease the trabecular meshwork (TM) and anterior chamber of the eye are affected, leading to impairment in the aqueous drainage, increased intraocular pressure (IOP), and optic nerve damage. PCG occurs during the neonatal or early infantile period [1]. The term PCG is reserved for those cases in which the only anatomic defect observed is isolated trabeculodysgenesis. This increased IOP results in ocular enlargement (buphthalmos), corneal clouding, and rapid optic nerve cupping. Progressive degeneration of the retinal ganglion cells (RGCs) results in the characteristic optic nerve atrophy and visual field defects found in glaucoma. Most cases of PCG are sporadic, but familial cases have also been reported. PCG is the most common type of pediatric glaucoma and accounts for 55% of such cases. Its expression and penetrance vary from 40–100%. Its incidence varies substantially from one population to another. It is estimated to occur in 1 in 10,000 births in Europe and 1 in 3,300 births in Andhra Pradesh, India [2,3].

Recently a putative PCG locus, GLC3A, was linked to markers on the short arm of chromosome 2 in 11 Turkish families [4]. Six other families did not show linkage to this locus, suggesting locus heterogeneity for this disease. Another PCG locus, GLC3B, was localized on chromosome 1p36 in some families but did not show linkage to chromosome 2 markers [5]. Other subsets of families that did not show linkage to these two loci provide evidence for at least a third of the unmapped loci [5]. Recently Stoilov et al. [6] identified three different mutations in the cytochrome P4501B1 (CYP1B1) gene in five unrelated Turkish families in which the disease had been linked to the 2p21 locus [6]. Even though three different loci have been mapped for PCG, mutations in CYP1B1 (GLC3A) are the most predominant cause of disease and are reported in various ethnic backgrounds [6-15]. Further, it is estimated that all the known loci/genes of glaucoma account for a minority of the total cases of glaucoma [4,5], and hence many other genes remain to be identified.

CYP1B1 is located on chromosome 2 and consists of three exons and two introns. The coding region of CYP1B1 starts at the 5’ end of exon 2 and ends within exon 3. It codes for a 543-amino acid protein and is expressed in the ocular tissues, such
TABLE 1. THE PRIMERS USED FOR PCR AMPLIFICATION.

| Primer sequence | Product size (bp) |
|-----------------|------------------|
| 1F-5′-TCTCCAGAGACTCGCTCCG-3′ | 786 |
| 1R-5′-GGGTCCGCTGGCTGATAG-3′ | 786 |
| 2F-5′-ATGCTTCCGACCACACT-3′ | 787 |
| 2R-5′-GATCTGGTGGTTGAGGGGT-3′ | 885 |
| 3F-5′-TCTCCAGAAATATTTAGTACGTG-3′ | 885 |
| 3R-5′-TATGGAGCAGACCTCACCTG-3′ | 885 |

as the anterior chamber, and in several nonocular tissues [16]. CYP1B1 is a member of the cytochrome P450 superfamily of drug-metabolizing enzymes. It catalyzes several oxidative reactions, some of which are biosynthetic, producing necessary hormones or compounds of intermediary metabolism in most living organisms and substrates, including many xenobiotics, vitamins, and steroids [17]. CYP1B1 also metabolizes vitamin A in two steps to all-trans-retinal and all-trans-retinoic acid. The latter is a potent morphogen and regulates in utero development of tissue growth and differentiation. CYP1B1 is involved in the metabolism of endogenous and exogenous substrates that take part in early ocular differentiation [18-20]. In the present study we screened all coding exons of CYP1B1 in 23 unrelated congenital glaucoma patients.

METHODS

Clinical evaluation and patient selection: Primary congenital glaucoma cases presenting at the Dr. R. P. Centre for Ophthalmic Sciences (AIIMS, New Delhi, India), were enrolled for this study. Six patients were female and 17 were male. Mean age of presentation was 15.17 months (range 1.5 – 132 months). After ethical approval of the Institutional Review Board (IRB00006862; All India Institute of Medical Sciences, New Delhi, India), 23 PCG cases were enrolled in this study. The diagnosis involved clinical ocular and systemic examination. Inclusion criteria of the patients were increased corneal diameter (>12.0 mm) and raised IOP (>21 mmHg) with presence/absence of Haab’s striae and optic disc changes (where examination was possible). Symptoms of epiphora and photophobia were the additional inclusion factors. The age of onset ranged from birth to 3 years. Detailed family histories up to three generations were taken, and pedigree charts were constructed. The history of ocular or other hereditary disorders was recorded. Glaucoma cases other than PCG were excluded. Fifty ethnically matched normal individuals without any ocular disorders were enrolled as controls. Peripheral blood samples were collected from patients and controls by venipuncture after informed consent. Blood samples were collected in EDTA vacutainer and stored in -80 °C until DNA isolation.

Mutation screening and sequence analysis: Genomic DNA was isolated from peripheral blood by the phenol chloroform method. The entire coding region, including exon–intron boundaries of CYP1B1, from patients and controls was amplified and screened for mutations by using three sets of overlapping primers (Table 1) [7,21]. The primers used were set I (1F–1R, 786 bp) [12], set II (2F–2R, 787 bp) [13], and set III (3F–3R, 885 bp) [12]. PCR amplifications for primer sets I and II were performed in a 40 µl volume containing 1.0 µl of 20 mM stock solution for each primer, 100 ng of genomic DNA, 1 unit of Taq polymerase (Banglore Genei), 0.1 mM of each dNTP, 4 µl of 10X PCR buffer (with 15 mM MgCl2) and 4 µl of dimethyl sulphoxide (Sigma), by means of 35 cycles of amplification, each consisting of 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 1 min extension at 72 °C [12], while conditions for set III were initial denaturation at 94 °C for 3 min followed by 30 cycles each at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

Amplified PCR products were purified using a gel/PCR DNA fragments extraction kit (number DF100; Geneaid Biotech Ltd., Sijhih City, Taiwan). Purified PCR products were sent for sequencing to MCLAB (Molecular Cloning Laboratories, South San Francisco, CA). DNA sequences were analyzed against the CYP1B1 reference sequence ENSG00000138061 using ClustalW2 (multiple sequence alignment program for DNA; European Molecular Biology Laboratory (EMBL) – European Bioinformatics Institute (EBI)).

Computational assessment of missense mutations: Two homology-based programs PolyPhen (polymorphism phenotyping; Division of Genetics, Department of Medicine, Brigham and Women’s Hospital/Harvard Medical School, Boston, MA) and SIFT (sorting intolerant from tolerant; the J. Craig Venter Institute Rockville, MD and La Jolla, CA) were used to predict the functional impact of missense changes identified in this study. PolyPhen structurally analyzes an amino acid polymorphism and predicts whether that amino acid change is likely to be deleterious to protein function [22-24]. The prediction is based on the position-specific independent counts (PSIC) score derived from multiple sequence alignments of observations. PolyPhen scores of >2.0 indicate the polymorphism is probably damaging to protein function. Scores of 1.5–2.0 are possibly damaging, and scores of <1.5 are likely benign. SIFT is a sequence homology-based tool that sorts intolerant from
tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect [25-28]. SIFT is based on the premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment. Positions with normalized probabilities <0.05 are predicted to be deleterious and those ≥0.05 are predicted to be tolerated.

RESULTS
All cases were found to be sporadic in origin. A total of 15 nucleotide changes were observed in this study. Out of these, five were previously reported coding single nucleotide polymorphisms (SNPs) and one was already reported as an intronic SNP; five were known pathogenic CYP1B1 mutations. Four novel nucleotide changes (two nonsynonymous, one frameshift, and one synonymous mutation) were also found in this study. Details of all nucleotide changes are presented below.

Identification of four novel mutations:

Isoleucine94stop (p.I94X) mutation—In this mutation a single base guanine (G) deletion (Figure 1) was observed at genomic position 38302285, coding nucleotide number c.247. This caused a frameshift after codon 82 and introduced a stop codon (TAG) at position 94 in the protein. This mutation produced a truncated CYP1B1 protein of 93 amino acids. This change was identified as a homozygous mutation in the patient (P55).

Histidine279aspartic acid (p.H279D) mutation—In this mutation a single base cytosine (C) was replaced by G (Figure 2) at genomic position 38301697, coding nucleotide number c.835. This resulted in a codon change from CAC to GAC and an amino acid change from histidine to aspartic acid (p.H279D), a nonsynonymous mutation in the CYP1B1 protein. This mutation was identified in one patient (P61) and was heterozygous.

Glutamine340histidine (p.Q340H) mutation—In this mutation a single base G was replaced by thymine (T) (Figure 3) at genomic position 38301512, coding nucleotide number c.1020. This resulted in a codon change from CAG to CAT and an amino acid change from glutamine to histidine (p.Q340H), a nonsynonymous mutation in the CYP1B1 protein. This mutation was identified in one patient (P56) and was heterozygous and present with the p.R390H mutation in this patient.

Lysine433lysine (p.K433K) mutation—In this mutation a single base G was replaced with adenine (A) (Figure 4) at genomic position 38298198, coding nucleotide number c.1299. This resulted in a codon change from AAG to AAA and resulted in no amino acid change (lysine). This was a neutral mutation (p.K433K) in patient P69.

All four novel mutations p.I94X, p.H279D, p.Q340H, and p.K433K have been registered in GenBank with accession numbers GQ925803, GQ925804, GQ925805, and GQ925806, respectively.

Other previously reported pathogenic CYP1B1 mutations:

Glutamic acid229lysine (p.E229K) mutation—This mutation resulted in G being replaced with A at genomic position 38298187 (rs57865060), coding nucleotide number c.685. This resulted in a codon change from GAA to AAA and an amino acid change from glutamic acid to lysine (p.E229K), a nonsynonymous mutation in the CYP1B1 protein. This change was found in one patient (P65) and was heterozygous.

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Figure 1. DNA sequence chromatogram of CYP1B1 exon 2 equivalent to codon 81–85. A: The reference sequence derived from control is shown. B: Sequence derived from congenital glaucoma patient P55 shows the homozygous deletion of G at c.247, which caused a p.asp83thrfsX12 (p.I94X) mutation.

Figure 2. DNA sequence chromatogram of CYP1B1 exon 2 equivalent to codon 277–280. A: The reference sequence derived from control is shown. B: Sequence derived from congenital glaucoma patient P55 shows heterozygous c.835C>G, which predicts a codon change of CAC>GAC and a p.H279D mutation.
Arginine355stop (p.A355X) mutation—In this mutation a single base C was replaced by T (Figure 5) at genomic position 38298434, coding nucleotide number c.1063. This resulted in a codon change from CGA to TGA (p.R355X), a nonsense mutation in the CYP1B1 protein. This resulted in a truncated CYP1B1 protein of 355 amino acids. The p.R355X mutation was described only once in the literature [29]. This change was homozygous in one patient (P70).

Arginine368histidine (p.R368H) mutation—In this mutation a single base G was replaced by A at genomic position 38298394 (rs28936414), coding nucleotide number c.1103. This resulted in a codon change from CGT to CAT and an amino acid change from arginine to histidine (p.R368H), a nonsynonymous mutation. This change was homozygous in one patient (P68).

Arginine390cysteine (p.R390C) mutation—In this mutation a single base C was replaced by T at genomic position 38298329 (rs56010818), coding nucleotide number c.1168. This resulted in a codon change from CGC to TGC and an amino acid change from arginine to cysteine (p.R390C), a nonsynonymous mutation. This mutation was identified in one patient (P64) and was heterozygous.

Arginine390histidine (p.R390H) mutation—In this mutation a single base G was replaced by A (Figure 6) at genomic position 38298328, coding nucleotide number c.1169. This resulted in a codon change from CGC to CAC and an amino acid change from arginine to histidine (p.R390H), a nonsynonymous mutation. This mutation was identified in one patient (P56) and was heterozygous.

Nonpathogenic CYP1B1 single nucleotide polymorphisms:

In addition to these pathogenic mutations, six previously reported single nucleotide polymorphisms [8] were identified in a less conserved region of the CYP1B1 protein. Details of these polymorphisms are provided below.

Cytosine (C) to thymine (T) change in intron 1—In this mutation, C was replaced by T at genomic position 38302544, nucleotide position 780 in CYP1B1 (rs2617266) in intron I. This was observed in 13 patients but was absent in controls.
Arginine48glycine (p.R48G)—In this mutation, C was replaced by guanine (G) at genomic position 38302390 (rs10012), coding nucleotide number c.142. This resulted in a codon change from CGG to GGG and an amino acid change from arginine to glycine (p.R48G), a nonsynonymous mutation in the CYP1B1 protein. This change was also present in controls.

Alanine19serine (p.A119S)—In this mutation G was replaced by T at genomic position 38302177 (rs1056827), coding nucleotide number c.355. This resulted in a codon change from GCC to TCC and an amino acid change from alanine to lysine (p.A119S), a nonsynonymous mutation in the CYP1B1 protein. This change was found in patients P55 and P73 but absent in controls.

Leucine432valine (p.L432V)—In this mutation a single base C was replaced by G at genomic position 38298203 (rs1800440), coding nucleotide number c.1347. This resulted in a codon change from CTG to GTG and an amino acid change from leucine to valine (p.L432V), a nonsynonymous mutation in the CYP1B1 protein. This change was identified in four patients; it was homozygous in three patients (P52, P55, and P68) and heterozygous in one patient (P69) and was also present in controls.

Aspartic acid449aspartic acid (p.D449D)—In this mutation a single base T was replaced by C at genomic position 38298139 (rs1056836), coding nucleotide position 5174 in the gene, coding nucleotide number c.1294. This resulted in a codon change from GCC to TCC and an amino acid change from asparagine to serine (p.N453S), a synonymous mutation in the CYP1B1 protein. The p.N453S mutation was present in two patients (P51 and P62) but absent in controls.

Asparagineserine453serine (p.N453S)—In this mutation a single base A was replaced by G at genomic position 38298139 (rs1800440), coding nucleotide number c.1358. This resulted in a codon change from AAC to AGC and an amino acid change from asparagine to serine (p.N453S), a nonsynonymous mutation in the CYP1B1 protein. The p.N453S mutation was present in two patients (P51 and P62) but absent in controls.

The clinical manifestations of PCG patients have been tabulated (Table 2), and the CYP1B1 sequence variants identified in the various studies to date have been summarized (Table 3). The clinical phenotype of the cases with pathogenic CYP1B1 mutations was more severe compared to cases without CYP1B1 mutations. The mean IOP of cases with pathogenic CYP1B1 mutations was 30.21 mmHg compared to 23.96 mmHg in mutation-negative cases; the difference is significant (p value <0.005). The mean corneal diameter in patients without the CYP1B1 mutations was 12.625×12.181 mm (left eye) and 12.406×12.781 mm (right eye), whereas it was 13.833×13.750 mm (left eye) and 13.416×15.50 mm (right eye) in mutation-negative cases. Haab’s striae were present in two cases (P56 and P61), which were positive for the CYP1B1 mutations.

DISCUSSION

Structural/functional implications of mutations:

p.I94X mutation—In the isoleucine94stop mutation (p. I94X) mutation a truncated protein of 93 amino acids is produced in which only the first 82 amino acids are the same as the wild-type CYP1B1 protein (Figure 7). This truncated protein lacks all functional domains of the CYP1B1 protein and is a nonfunctional protein [6,21,29,30].

p.H279D mutation—This histidine residue lies in the carboxyl terminal of the G helix in the CYP1B1 protein. Replacement of an aromatic, weak basic, amino acid histidine whose charge state depends upon its protonation state with an aliphatic, strong acidic, and negatively charged aspartic acid at this locus. This in turn affects the local charge distribution, and hence the structure of the protein is disturbed. Histidine is conserved at this locus in the CYP1A1 protein from 12 different species (Figure 8) and in the CYP1B1 protein from seven different species (Figure 9) analyzed, suggesting that histidine performs some important functions at this locus. No other known pathogenic mutation was present in the patient (P61), and the PSIC score of this mutation was 2.628, indicating that this change is probably damaging to the protein function. The SIFT score of p.H279D was 0.00 and is predicted to be deleterious for the protein function.

p.Q340H mutation—This glutamine residue lies in the carboxyl terminal of the I helix. Replacement of a polar uncharged amino acid (glutamine) with a weak basic amino acid (histidine) may or may not alter the structure/function of the protein. Glutamine is not conserved at this locus in the CYP1A1 protein from 12 different species analyzed (Figure 9).
| Pt. ID | Age of onset of disease | Sex | Age at presentation/ sampling | Corneal Diameter (mm) OS/OD and clarity at diagnosis | Buphthalmos | IOP OS/OD (mmHg) At presentation | Haabs striae | Last Cup Disc ratio OS/OD | Photo-phobia | Mutations | Treatments |
|-------|-------------------------|-----|------------------------------|-----------------------------------------------|-------------|-------------------------------|-------------|-----------------|---------------|-----------|-------------|
| P51   | By birth                | F   | 36 months                    | 11x1.5/13x1.5; OU mild edema                   | OU; OD OS   | 22/28                         | no          | 0.8:1:0.9:1     | Yes           | —         | Medical and OU/Trab/Trab+MMC; OU trabeculectomy and mitomycin C treatment; |
| P52   | By Birth                | F   | 2 months                     | 12.5x12.5/13x13; OS mild edema                 | OU          | 20/20                         | no          | Hazy media/0.5:1 | Yes           | —         | Medical and OU/Trab/Trab+MMC |
| P53   | By birth                | F   | 4 months                     | 11x12/12x12; OU mild edema                     | OU; OD OS   | 40/25                         | no          | Hazy media      | Yes           | —         | Medical and OU/Trab/Trab+MMC |
| P54   | By birth                | M   | 9 months                     | 15x15/14.5x14.5; no edema                      | OU          | 26/26                         | no          | No glow         | No            | —         | Medical and OU/Trab/Trab+MMC |
| P55   | By birth                | M   | 8 months                     | Phthisic eye/12x12; OU severe edema            | OD; OS Phthisic eye | NA/37 | no | NA/0.9:1 | No | p.I94X (H) | Medical and OD/Trab/Trab+MMC |
| P56   | By birth                | F   | 12 months                    | 14x14/14.5x14.5; OU severe edema               | OU; OS OD   | 30/28                         | OU +ve      | Hazy media      | No            | p.Q364X (H) + p.R390H (H) | Medical and OU/Trab/Trab+MMC; |
| P57   | By birth                | M   | 3 months                     | 14x14/14.5x14.5; OU osphatic edema             | OU; OD OS   | 28/30                         | no | 0.7:1:0.7:1 | No            | —         | Medical and OD/Trab/Trab+MMC; |
| P58   | By birth                | M   | 15 months                    | 14x14/12.5x12.5; No edema                      | OU; OD OS   | 20/16                         | no | total cupping/0.5:1 | No            | —         | Medical and OU/Trab/Trab+MMC; |
| P59   | By birth                | M   | 10 months                    | 14x14/13.5x13.5; No edema                      | OU; OD OS   | 31/31                         | OS +ve      | Not available   | No            | —         | Medical and OD/Trab/Trab+MMC; |
| P60   | 7 months                | F   | 41 months                    | 14x14/13.5x13.5; No edema                      | OU; OD OS   | 24/18                         | no | 0.4:1:0.6:1 | Yes           | —         | Medical and OD/Trab/Trab+MMC; |
| P61   | By birth                | M   | 4 months                     | 15x15/14x14; OU mild edema                     | OU; OD OS   | 30/34                         | OU +ve      | Not visible/0.9:1 | No            | p.H297D (h) | Medical and OD/Trab/Trab+MMC; |
| P62   | By birth                | M   | 8 months                     | 15x15/14x14; OU mild edema                     | OU; OD OS   | 22/16                         | no | 0.6:1:0.6:1 | Yes           | —         | Medical and OD/Trab/Trab+MMC; |
| P63   | 3 months                | M   | 12 months                    | 12x12/11x12; No edema                          | OU; OD OS   | 22/23                         | no | 0.4:1:0.4:1 | No            | —         | Medical and OD/Trab/Trab+MMC; |
| P64   | By birth                | M   | 1 month                      | 12x12/11.5x11.5; OD severe edema               | OU; OD OS   | 28/24                         | no | Not visible/0.7:1 | Yes           | p.R390C (h) | Medical and OD/Trab/Trab+MMC; |
| P65   | By birth                | M   | 6 months                     | 13x12.5x12; OU edema                           | OU; OD OS   | 25/26                         | no | 0.7:1:0.7:1 | No            | p.E229K (h) | Medical and OD/Trab/Trab+MMC; |
| P66   | 3 months                | M   | 13 months                    | 13x12/12.5x12; No edema                        | OU; OD OS   | 22/24                         | no | 0.4:1:0.4:1 | no            | —         | Medical and OD/Trab/Trab+MMC; |
| P67   | 11 months               | M   | 132 months                   | 13x13/13x14; OU mild edema                     | OU; OD OS   | 21/24                         | no | Not visible    | Yes           | —         | Medical and OD/Trab/Trab+MMC; |
| P68   | By birth                | M   | 6 months                     | 13x13/13.5x14; OU severe edema                 | OU; OD OS   | 26/28                         | no | 0.8:1:0.8:1 | no            | p.R368H (H) | Medical and OD/Trab/Trab+MMC; |
| P69   | By birth                | M   | 4 months                     | 13x12.5x12; No edema                           | OU; OD OS   | 24/26                         | no | 0.5:1:0.6:1 | no            | —         | Medical and OD/Trab/Trab+MMC; |
| P70   | By birth                | M   | 45 days                      | 15x15/14x14; OU severe edema                   | OU; OD OS   | 30/40                         | Not visible | Not visible | no            | p.R355X (H) | Medical and OD/Trab/Trab+MMC; |
| P71   | By birth                | M   | 18 months                    | 12x2/12x2; No edema                            | OU          | 22/23                         | no | 0.4:1:0.4:1 | Yes           | —         | Medical and OD/Trab/Trab+MMC; |
| P72   | By birth                | M   | 45 days                      | 12x12/12x1.5; OU mild edema                    | OU; OD OS   | 20/24                         | no | No glow       | Yes           | —         | Medical and OD/Trab/Trab+MMC; |
| P73   | By birth                | M   | 2 months                     | 12.5x13/1x12; OU mild edema                    | OU; OD OS   | 22/22                         | no | Hazy media    | no            | —         | Medical and OD/Trab/Trab+MMC; |

Footnote: M- male; F- female; H-homozygous; h-heterozygous; X- times; Trab/Trab+MMC- combined trabeculotomy trabeculectomy and mitomycin C treatment; OD- right eye; OS- left eye; OU- both eyes; mutations in bold letters- novel mutations.
| S. No. | Patient number | Genomic location | Nucleotide change | Codon change | Type of mutation | Location in protein | Mutation identified | Observational history of mutations in different diseases | Origin (reference) |
|--------|----------------|------------------|-------------------|--------------|-----------------|-------------------|-------------------|-------------------------------------------------|-------------------|
| 1      | P51, P53, P55, P58, P65, P71, P72, P73 | g.38302544 | C>T | NA | Intronic | NA | NA | PCG | India, Saudia Arabia, Oman, Brazil [7,13,35,36] |
| 2      | P51, P53, P54, P57-P61, P63-P65, P71-P73 | g.38302390 | c.142 C>G | CGG>GGG | Missense | 48 | p.Arg48 Gly | PCG | Saudia Arabia, India, Japan [8,11,15] |
| 3      | P55 | g.38302285 | c.247del G | FS | FS | FS after 82 | p.188fsX12 | PCG | This study |
| 4      | P55, P73 | g.38302177 | c.355G>T | GCC>TCC | Missense | 119 | p.Arg355 Pro | PCG | Saudia Arabia, Japan, India [7,11,15] |
| 5      | P65 | g.38301847 | c.685G>A | GAA>AAA | Missense | 229 | p.Glu229 Lys | PCG, POAG | France, India, Germany [7,29,31] |
| 6      | P61 | g.38301697 | c.835C>G | CAC>GAC | Missense | 279 | p.His279 Asp | PCG | This study |
| 7      | P61 | g.38301512 | c.1020G>T | CAG>CAT | Missense | 340 | p.Glu340 Lys | PCG | This study |
| 8      | P70 | g.38298434 | c.1063C>T | GGA>TGA | Missense | 355 | p.Ala355 Stop | PCG | Germany, India [28] this study |
| 9      | P68 | g.38298394 | c.1103G>A | CGT>CAT | Missense | 368 | p.Arg368 His | PCG, PA, POAG | Saudia Arabia, India, France [7,15,32] |
| 10     | P64 | g.39298329 | c.1168C>T | CTC>TGC | Missense | 390 | p.Arg390 Lys | PCG, POAG | Pakistan, India, France [7,2,32] |
| 11     | P56 | g.38298328 | c.1169G>A | CGC>CAC | Missense | 390 | p.Arg390 His | PCG | Pakistan, India, Turkey [7,1,29] |
| 12     | P52, P55, P68, P69 | g.38298203 | c.1294C>G | CTG>GTG | Missense | 432 | p.Leu432 Val | PCG, PA | India, Japan, Turkey [7,1,29] |
| 13     | P69 | g.38298198 | c.1299G>A | AAG>AAA | Neutral | 433 | p.Lys433 Ser | PCG | This study |
| 14     | P51, P53-P67, P69-P71, P73 | g.38298350 | c.1347T>C | CAT>GAT | Neutral | 449 | p.Asn449 Asp | PCG | Japan, India [7,11] |
| 15     | P56, P67 | g.38298139 | c.1358A>G | AAC>GAC | Missense | 453 | p.Asn453 Ser | PCG | France, India [7,12] |

Footnote: PCG- Primary congenital glaucoma; POAG- Primary open angle glaucoma; PA-Peter’s anomaly; FS-frameshift; X- stop codon; NA- not applicable; mutations in bold letters- novel mutations.
8) but is conserved in the CYP1B1 protein from seven different species analyzed (Figure 9). The PSIC score of this mutation was 0.276, indicating that this change is benign to protein function. The SIFT score of p.Q340H was 0.05 and is predicted to be tolerated. The patient with the p.Q340H mutation also had a known pathogenic CYP1B1 mutation (p.R390H) and had a PSIC score of 2.799 and a SIFT score of 0.00. The p.R390H mutation has previously been reported [21] to adversely affect or damage protein function.

p.E229K mutation—The p.E229k mutation occurred in the carboxyl terminal of the F helix in the vicinity of the substrate-binding region in the CYP1B1 protein. Substitution of E to K leads to a change from a negatively charged residue to a positively charged side chain, and this in turn affects the local charge distribution. This disturbs an important cluster of salt bridges. In wild-type CYP1B1 protein, R-194::E-229, R-194::D-333, and D-333::K-512 form a triangle of ionic bond interactions, holding the I helix with the F helix and β-strand S3.2. As a result of this mutation, the R-194::E-229 interaction is lost, which has the potential to destabilize the other ionic interactions in the protein [30]. The SIFT score of the p.E229K mutation was 0.01 and is predicted to be deleterious for the protein function. The CYP1B1 protein with the p.E229K mutation shows 20–40% enzymatic activity compared to the wild-type CYP1B1 protein [31].

p.R368H mutation—This arginine residue lies between the J and K helix in an exposed loop [8,15]. In this mutation the positively charged amino acid arginine is replaced by histidine whose charge state depends upon its protonation state. Consequences of this change are not immediately apparent. In the wild type, arginine at position 368 interacts with G-365, D-367, V-363, and D-374. Because of the R368H mutation, interaction between D-367 and D-374 are weakened. The PSIC score of this mutation was 2.653, indicating that this change is probably damaging to protein function. The SIFT score of p.R368H was 0.00 and is predicted to be deleterious for the protein function. How p.R368H affects the conformation and functionality of the protein is still not clear [31].

p.R390H/C mutation—This arginine residue is located in the conserved α helix K [8]. It forms the consensus sequence GluXXArg, which is conserved among all members of the cytochrome P450 superfamily [21]. Arg390 and Glu387 are one helical turn apart and are predicted to form a salt bridge. The parallel orientation of their side chains is more transparent in the three-dimensional model. Conservation of this motif indicates that presence of arginine at this position is essential for the normal function of the P450 molecule. The PSIC scores of p.R390C and p.R390H were 3.474 and 2.799, respectively.
indicating that both these changes are probably damaging to protein function. The SIFT score of p.R390H/C was 0.00 and is predicted to be deleterious for the protein function.

The PSIC scores of the nonpathogenic single nucleotide polymorphisms were <2 for p.R48G, p.A119S, p.N453S, and p.L432V, indicating that all these changes were benign to protein function. The SIFT scores of the nonpathogenic single nucleotide polymorphisms were >0.05 for p.R48G, p.L432V, p.K433K, and p.D449D, indicating that all these changes were tolerated in the protein.

PCG is a clinically and genetically heterogeneous disorder. More than 50 different mutations have been reported in the entire coding region of CYP1B1 from various populations. We screened the entire coding region of CYP1B1 in 23 congenital glaucoma patients by using primers described elsewhere [8]. Of all mutations identified herein, the frameshift mutation (c.247delG) and nonsense mutation (c.1063C>T) resulted in the most severe disease phenotype.

The patient (P55) with the p.I94X (homozygous) mutation is a male child of a consanguineous marriage without any family history of glaucoma; he presented at 8 months of age. He was born at full term through a normal vaginal delivery. He had severe bilateral corneal edema at birth. At the age of 2 months he had congestion with discharge in the left eye and was diagnosed to have a left corneal ulcer and was treated with antibiotics; the left eye consequently developed phthisis. The right eye dimensions increased, and he was diagnosed as having buphthalmos at the age of 8 months. Combined trabeculotomy and trabeculectomy with mitomycin C was performed in his right eye. He was diagnosed as having 100% blindness at 8 months. His parents were also screened for CYP1B1 mutations by DNA sequencing but were found to be negative for any pathogenic CYP1B1 mutations.

Patient P70 has a p.R355X (homozygous) mutation and is a male offspring of a non-consanguineous marriage; he presented at 45 days. At birth, he had bilateral congenital glaucoma, and his left eye was diagnosed to have a corneal ulcer and was treated with antibiotics. The left eye then developed phthisis. The right eye dimensions increased, and he was diagnosed as having buphthalmos at the age of 8 months. Combined trabeculotomy and trabeculectomy with mitomycin C was performed in his right eye. He was diagnosed as having 100% blindness at 8 months.

Figure 8. Multi sequence alignment of the human CYP1B1 protein with the CYP1A1 protein from different species. Red Underlined amino acids shows the conserved residues in human CYP1B1 and different CYP1A1 protein from different species (when mutated) causing primary congenital glaucoma phenotype. While Red letter shows amino acid conserved in different CYP1A1 protein from different species but not present in human CYP1B1 protein.

Figure 9. Multisequence alignment of the human CYP1B1 protein with the CYP1B1 protein from different species. Underlined red amino acids show the conserved residues (when mutated) causing the primary congenital glaucoma phenotype. Red colored amino acid shows the non-conservation of glutamic acid at this locus in Zebrafish CYP1B1. Blue-colored amino acids show the less conserved residues in CYP1B1 protein from different species.
glaucoma and had IOPs of 30 and 40 mmHg in his left and right eye, respectively. He had severe corneal clouding in both eyes, at birth, and therefore the fundus was not visualized. Combined trabeculotomy and trabeculectomy with mitomycin C was performed in both eyes. He had no light perception and was visually blind since 45 days of age. His parents were also negative for the pathogenic CYP1B1 mutations. The absence of mutations in the parents of P55 and P70 could be due to a parental germline mutation, which cannot be tested by using peripheral leukocytes.

Patient P56 is a female child of a non-consanguineous marriage; she presented at the age of 1 year. She has p.Q340H (heterozygous) and p. R390H (homozygous) mutations. She had bilateral congenital glaucoma since birth. She had a corneal diameter of 14.0×14.0 mm (right eye) and 14.5×14.5 mm (left eye) and IOPs of 28 and 30 mmHg in the right and left eye, respectively. She had Haab’s striae in both eyes, and the fundus was not visible. Combined trabeculotomy and trabeculectomy with mitomycin C was performed in both eyes.

Patient P61 is a male child of a non-consanguineous marriage; he presented at the age of 4 months and has a p.H279D (heterozygous) mutation. He had bilateral congenital glaucoma at birth. At presentation corneal diameter and IOPs of his left and right eye were 15.5×15.0 mm and 14.0×14.0 mm and 30 mmHg and 34 mmHg, respectively. The cup to disc ratio of the left eye was not visible due to the hazy media and that of the right eye was 0.9:1. He had Haab’s striae in both eyes. Combined trabeculotomy and trabeculectomy with mitomycin C was performed in both eyes.

An intriguing finding that apparently does not match a typical recessive pattern of inheritance is the presence of a heterozygous CYP1B1 mutation in PCG patients. This situation has been previously reported [7,29]. A heterozygous p.Y81N mutation has also been described in PCG patients from Germany, and a heterozygous p.E229K mutation has been identified in unrelated French and Indian patients [7,32]. Few heterozygous CYP1B1 mutations were associated with the milder, primary, open-angle glaucoma phenotypes in patients from Spain, France, and India. The presence of a heterozygous CYP1B1 mutation in PCG suggests the possibility of other loci, yet undetected, that may be involved in anterior chamber formation. Recently the presence of double heterozygote variants CYP1B1 and FOXC1 has been described in two PCG cases, although the role of possible digenic inheritance in disease causation is yet to be established [33]. Defective variants of modifier genes and/or environmental factors have an additive effect with loss-of-function CYP1B1 alleles to produce the disease phenotype. However further work is required to understand this mechanism.

Previous studies have reported that the age of disease onset in PCG patients with CYP1B1 mutations is younger than in patients without CYP1B1 mutations [34]. Our data show that the onset age in three patients (P60, P66, and P67) was 7, 3, and 11 months, while the rest of the patients presented at birth. In these 20 patients there is no significant difference in the age of disease onset in CYP1B1 mutation-positive and mutation-negative cases, although clinical phenotypes of patients (P55, P56, P61, P68, and P70) with homozygous CYP1B1 mutations were more severe compared to patients (P64 and P65) who were heterozygous for the CYP1B1 mutations (Table 1). It is possible that patients with two null alleles with no catalytic activity may present with a more severe phenotype of the disease compared to patients with one null (heterozygote) allele. The disease phenotype of patients with homozygous/heterozygous CYP1B1 mutations was more severe compared to the clinical phenotype of patients negative for the CYP1B1 mutations.

We also observed a higher mean IOP in a group of patients with CYP1B1 mutations. In accordance with the idea of associating the severe phenotypes with the null CYP1B1 allele, the percentage of severe phenotypes in at least one eye has been reported to be associated with various mutations ranging 80-100% for a frameshift mutation (e.g., c.376insA) and truncating mutations [11]. Three different truncation mutations (p.C280X, p.E281X, and p.R355X) producing a truncated protein of 279, 280, and 354 amino acids, respectively, have also been associated with more severe disease phenotypes [11,21,29]. In patient P55 with a homozygous p.I94X mutation, a truncated protein of 93 amino acids is produced that has the first 82 amino acids similar to the wild-type CYP1B1 protein. The disease phenotype of this patient is severe with a left phthisic and a right buphthalmic eye with a cup to disc ratio of 0.9:1. He is visually blind. Another patient (P70) with a p.R355X mutation had bilateral buphthalmos with severe corneal edema and a corneal diameter of 15.0×15.0 mm and 15.0×14.5 mm in the left and right eye, respectively. He was blind at the age of 45 days. Patient P61 with a p.H279D mutation had bilateral buphthalmos with mild edema in both eyes and a corneal diameter of 15.5×15.5 mm and 14.0×14.0 mm in the left and right eye, respectively. He was blind at the age of 4 months. The range of percentages of severe phenotypes in at least one eye is 62–83% for different mutations, such as p.G61E, p.E229K, p.R368H, and p.R390C [9].

Membrane-bound cytochromes, such as CYP1B1, have a molecular structure containing a transmembrane domain located at the N-terminal end of the molecule. This is followed by a proline-rich “hinge” region, which permits flexibility between the membrane-spanning domain and the cytoplasmic portion of the protein molecule. The COOH-terminal ends are highly conserved among different members of the cytochrome P450 superfamily [17]. This family contains a set of conserved core structures responsible for the heme-binding region of these molecules. The heme-binding region is essential for the normal function of every P450 molecule. Between the hinge region and the conserved core structure lies a less conserved
substrate-binding region. The cytochrome P450 protein functions like any classical enzyme molecule [18,19]. Mutations affecting such enzymes generally produce recessive phenotypes because in heterozygous subjects the normal allele is capable of compensating for the mutant allele. Mutations in the CYP1B1 protein interfere with the integrity of the CYP1B1 protein as well as its ability to adopt a normal conformation and to bind heme; for example, induced mutations in the hinge region have previously been reported to interfere with the heme-binding properties of the cytochrome P450 molecules.

Thus mutations of CYP1B1 are a major cause of PCG in our study as well as various other studies [6-15,35-37]. This study confirms genetic heterogeneity of the disease. We identified four novel mutations in this study in addition to one previous reported [7]. Studies of pathogenic sequence variants of CYP1B1 in different populations will contribute to a better understanding of the pathogenesis of PCG and will aid in analyzing the structure–function relationship of different CYP1B1 mutations. Identifying mutations in subjects at risk of developing glaucoma, particularly among relatives of PCG families, is of clinical significance. Monitoring vision in these families would be helpful. These developments may help in reducing the disease frequency in familial cases. Such studies will also help in understanding the pathogenic mutations in our patient populations and enable us to develop simple and rapid diagnostic tests for analyzing such cases. This may lead to the development of novel therapies in the management of congenital glaucoma.

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