

# Identification of Novel Mutations Causing Familial Primary Congenital Glaucoma in Indian Pedigrees

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**PURPOSE.** To determine the possible molecular genetic defect underlying primary congenital glaucoma (PCG) in India and to identify the pathogenic mutations causing this childhood blindness.

**METHODS.** Twenty-two members of five clinically well-characterized consanguineous families were studied. The primary candidate gene *CYP1B1* was amplified from genomic DNA, sequenced, and analyzed in control subjects and patients to identify the disease-causing mutations.

**RESULTS.** Five distinct mutations were identified in the coding region of *CYP1B1* in eight patients of five PCG-affected families, of which three mutations are novel. These include a novel homozygous frameshift, compound heterozygous missense, and other known mutations. One family showed pseudodominance, whereas others were autosomal recessive with full penetrance. In contrast to all known *CYP1B1* mutations, the newly identified frameshift is of special significance, because all functional motifs are missing. This, therefore, represents a rare example of a natural functional *CYP1B1* knockout, resulting in a null allele (both patients are blind).

**CONCLUSIONS.** The molecular mechanism leading to the development of PCG is unknown. Because *CYP1B1* knockout mice did not show a glaucoma phenotype, the functional knockout identified in this study has important implications in elucidating the pathogenesis of PCG. Further understanding of how this molecular defect leads to PCG could influence the development of specific therapies. This is the first study to describe the molecular basis of PCG from the Indian subcontinent and has profound and multiple clinical implications in diagnosis, genetic counseling, genotype-phenotype correlations and prognosis. Hence, it is a step forward in preventing this devastating childhood blindness. (*Invest Ophthalmol Vis Sci*. 2002;43:1358-1366)

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The glaucomas, a heterogeneous group of optic neuropathies, if untreated, lead to optic nerve atrophy and permanent loss of vision. Glaucoma accounts for 15% of blindness worldwide.<sup>1</sup> One severe form of glaucoma, which occurs at birth or in early infancy (up to 3 years of age), is primary congenital glaucoma (PCG), which is mainly inherited as an autosomal recessive disorder. In contrast to a prevalence of 1:10,000 in the West,<sup>2</sup> prevalence is as high as 1:1250 among the Romany population of Slovakia,<sup>3</sup> and 1:2500 in the Middle East,<sup>4</sup> where inbreeding occurs, suggesting a genetic etiology. In the Indian state of Andhra Pradesh, the prevalence is 1:3300, and the disease accounts for 4.2% of all childhood blindness.<sup>5,6</sup> However, the genetic defect of this disorder was unknown, and this prompted us to undertake the investigation.

Genetic linkage studies by Sarfarazi et al.<sup>7</sup> and Akarsu et al.<sup>8</sup> mapped PCG to two different loci, *GLC3A* (at 2p21)<sup>7</sup> and *GLC3B* (at 1p36),<sup>8</sup> in which mutations within the *CYP1B1* gene (encoding the cytochrome P450 enzyme at *GLC3A*) were associated with the disease.<sup>9</sup> Several *CYP1B1* mutations in various ethnic backgrounds have been implicated in the pathogenesis.<sup>10-20</sup> To determine the possible genetic defect underlying PCG in India, molecular analyses of five families were undertaken, and the *CYP1B1* coding region was screened for mutations. Herein, we describe the pathogenic mutations (some of which are novel), including a natural *CYP1B1* functional knockout, their genotype-phenotype correlations, structure-function relationship, and the simple diagnostic methods developed for identifying these mutations.

## METHODS

### Clinical Evaluation and Patient Selection

The study protocol adhered to the tenets of the Declaration of Helsinki. After providing informed consent, five consanguineous PCG families were recruited for the study. These families were selected because all family members were available for the investigation. Patients and family members were evaluated by a glaucoma specialist (AKM) and were followed up for 10 years. The clinical data of the patients are described in Table 1. Ophthalmic examinations included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP), and perimetry in some cases. Clinical manifestations included elevated IOP, enlargement of the globe, edema, opacification of the cornea with rupture of the Descemet's membrane, thinning of anterior sclera and atrophy of the iris, anomalously deep anterior chamber, photophobia, blepharospasm, and excessive tearing.

### Mutation Screening and Sequence Analyses

Because mutations in *CYP1B1* are the predominant cause of PCG, the entire coding region (1.6 kb organized in exons II and III)<sup>21</sup> was screened for mutations. Only these two exons were screened, because both contain the mutational hot spots of the gene and all pathogenic mutations reported so far are harbored in exons II and III. DNA was extracted from the peripheral leukocytes of patients, family members and control subjects. Using three sets of overlapping primers, the *CYP1B1* gene was amplified from patients and control subjects (Table

TABLE 1. Clinical Data of Subjects with Primary Congenital Glaucoma

Pedigree	Age of Onset	Age of Diagnosis	Presence of Haab's Striae	Corneal Diameter (mm) and Clarity at Diagnosis (OD; OS)	IOP at Diagnosis (mm Hg OD; OS)	Last C/D Ratio (OD; OS)	Last Visual Acuity (OD; OS)	Treatments (OD; OS)
PCG 4 Proband	By birth	2 wk	Present in OU	12; 12.5 Buphthalmos OU; hazy cornea and edema	36; 38	0.9; NA	NPL OU	Medical and 1× Trab/Trab OU; 1× PK* OD
Affected sibling	By birth	3 mo	NA OU	NA; Buphthalmos OU; hazy cornea and atrophic	NA OU	NA OU	NPL OU	Medical and 1× Trab/Trab treatments at 3 mo
PCG 11, Proband	By birth	2 wk	Absent OU	12; 12.5 Corneal edema OU	30 OU	NA OU	Fixing and following light OU	Medical and 1× Trab/Trab OU; 2× Trab/Trab OS
PCG 1 Proband	By birth	~5 y	Absent OU	NA; clear OU	24 OU	0.8; 0.6	20/25 OU	Medical treatment OU
Affected mother	Late onset in OD; >3 years	30 y	Absent OD; present OS	NA; Clear OD; hazy OS	34; 50	0.8; 0.9	20/20; NPL	Medical treatment OD
PCG 2, Proband	By birth	2 wk	Present OU	13 OU Buphthalmos OU; hazy cornea OU	NA OU	0.9 OU	20/30; PL	3× Trab/Trab OU; retinal reattachment surgery OS†; medical treatment OD
PCG 6 Proband	By birth	9 mo	Absent OU	13; 12.5 Corneal edema OU	26; 30	0.3 OU	20/40; 20/200	1× Trab/Trab OU
Affected sibling	By birth	3 mo	Absent OU	15 OU Corneal edema and scarring OU	32 OU	NA OU	PL; HM	Medical and 1× Trab/Trab OU

IOP, intraocular pressure; OD, right eye; OS, left eye; OU, both eyes; C/D, cup/disc ratio of the optic nerve; NPL, no perception of light; PL, perception of light; HM, hand motion; NA, not available; X, Times; Trab/Trab, combined trabeculectomy and trabeculectomy; PK\*, penetrating keratoplasty performed but resulted in graft failure; OS† left eye became atrophic.

2). Amplicons were sequenced directly, and the patient and control sequences were compared to identify all mutations. The primers used were as follows: set I (1 forward [F]/1 reverse [R], 786 bp),<sup>12</sup> set II (2F/2R, 648 bp),<sup>22</sup> and set III (3F/3R, 885 bp).<sup>12</sup> All PCRs were performed for only 30 cycles, and conditions for sets I and II were as reported earlier<sup>12</sup>; conditions for set III are given in Table 2. Twenty-five- to 50- $\mu$ L polymerase chain reactions (PCR) were performed with the following: 50 to 100 ng genomic DNA, 1 $\times$  PCR buffer with 1.5 to 2.0 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5  $\mu$ M of each primer, and 1 U *Taq* polymerase (Bangalore Genei, Bangalore, India), with or without 10% dimethyl sulfoxide (DMSO). Primer sets I and II had 10% DMSO and 1.5 mM MgCl<sub>2</sub>, whereas set III had only 2.0 mM MgCl<sub>2</sub>. The same sets of primers were used for PCR and bidirectional sequencing. The three amplicons were purified (pre-PCR sequencing kit; USB, Cleveland, OH), terminator cycle sequencing was performed (BigDye kit; PE-Applied Biosystems, Foster City, CA), and sequencing reactions were performed on an automated DNA sequencer (ABI model 377; PE-Applied Biosystems).

### PCR-Restriction Fragment Length Polymorphism Analyses and Cosegregation of Mutant Alleles with Disease Phenotype

In all cases, mutations resulted in either loss or gain of recognition sites (Table 2). For determining the cosegregation of mutant alleles with disease phenotype in the family, the respective fragment harboring the mutation was amplified from all family members, and an aliquot of amplicons was digested with the corresponding restriction enzymes (Table 2; MBI Fermentas, Vilnius, Lithuania). The fragments were separated on 8% polyacrylamide gel, stained with ethidium bromide and visualized to distinguish the wild type and mutant alleles. Seventy volunteer donors without history of eye disorders served as control subjects.

## RESULTS

### Identification of Pathogenic Mutations

#### Novel Frameshift Mutation and Functional Null Allele.

In family PCG4 (an uncle-to-niece marriage) two patients showed a homozygous insertion (Figs. 1A, 1B; Table 2) of a nucleotide A at cDNA position 376 (376insA). This novel mutation, not previously reported,<sup>10–20</sup> resulted in a frameshift that truncated the open reading frame (ORF) by creating a premature stop codon (TGA), 636 bp downstream from this insertion. Consequently, a truncated 222-amino-acid (aa) protein missing 321 aa from the C terminus was generated (Fig. 1C). This also abolished the restriction site *Eco*130I in exon II. Both the wild-type and the mutant proteins contained just 10 aa at the N terminus, which is similar in both, and the frameshift eliminated all CYP1B1 domains, resulting in a functional null allele. All unaffected members in family PCG4 were heterozygous for this mutation (Fig. 2A).

**Novel Compound Heterozygous Mutations and Pseudodominance.** In another family (PCG1; marriage between first cousins), parent-to-child transmission of the disease was noticed. This is an interesting pedigree in which the daughter (proband) and mother were affected with bilateral PCG and the father was a normal carrier (Fig. 3A). Two affected generations showed varying severity and manifestations. The mother showed asymmetric manifestation (left eye blind, right eye mildly affected), whereas the proband displayed a uniform milder manifestation in both eyes. The proband had a novel compound heterozygous missense mutation (Table 2) within exon II. The first mutation (Fig. 3B) was a C $\rightarrow$ T substitution at 923 bp, resulting in a proline-to-leucine change at aa 193 (P193L) and a gain of the restriction site *Eco*81I. The second

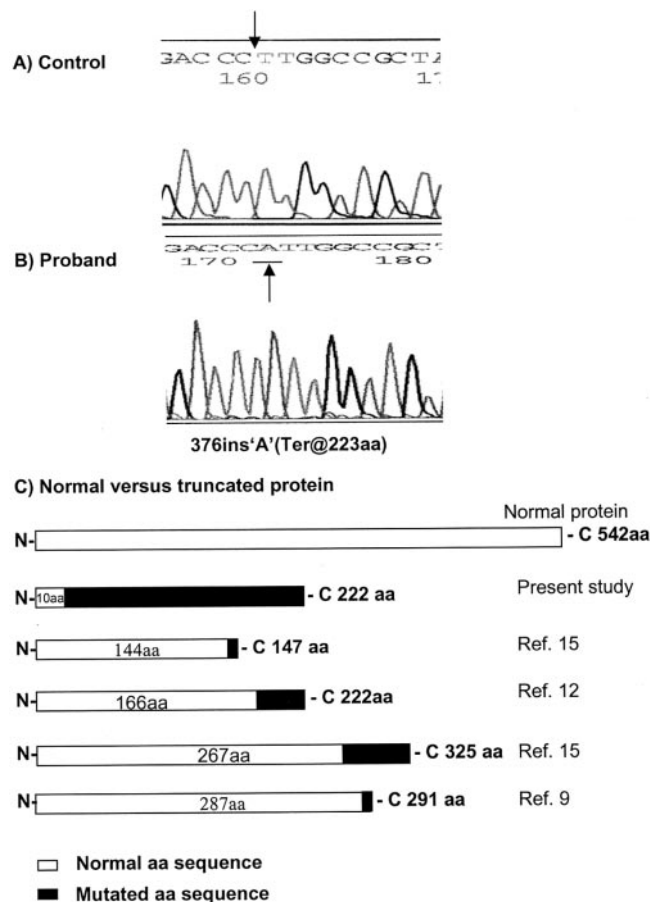
TABLE 2. Mutations Causing PCG Phenotype

Pedigree	Exon	Mutation Position in cDNA (bp)	Hetero-/homozygous	Codon Change	Mutation Type	Restriction Site Change	Diagnostic Method Developed	Primers Used for Amplification (5'-3')	Novel or Reported
PCG4	II	376insA	Homozygous	Ter@223	Frameshift	- <i>Eco</i> 130I	PCR followed by <i>Eco</i> 130I digestion	1F-ictcagagagcagctccg (3676-3695) 2FR-agttagggccgaagccat (4199-4217)	Novel*
PCG11	II	528G $\rightarrow$ A	Homozygous	G61E	Missense	+ <i>Taq</i> I	PCR followed by <i>Taq</i> I digestion	1F-ictcagagagcagctccg (3676-3695) 1R-gggctcgtcggctgtag (4461-4444)	Refs. 10, 12, 15
PCG1	II	923C $\rightarrow$ T	Heterozygous	P193L	Missense	+ <i>Eco</i> 81I	PCR followed by <i>Eco</i> 81I digestion	2R-gatgcgaactcttcacg (4258-4276) 2R-ctactccgccttttcaga (4905-4887)	Novel*
PCG2 and 6	II	959G $\rightarrow$ A	Heterozygous	E229K	Missense	- <i>Eam</i> 1104I	PCR followed by <i>Eam</i> 1104I digestion	2F-gatgcgaactcttcacg (4258-4276) 2R-ctactccgccttttcaga (4905-4887)	Novel*
	III	1449G $\rightarrow$ A	Homozygous	R368H	Missense	- <i>Taq</i> I	PCR followed by <i>Taq</i> I digestion	13F-iccagaataattagcaacg (7740-7765) 3R-tatggagcacctcaacctg (8624-8605)	Novel* diagnostic method

Gain and loss of restriction sites are indicated by + or - signs, respectively. Nucleotide numbering is based on sequence reported by Tang et al.<sup>21</sup>

\* Reported for the first time in this study.

† PCR conditions for set III primers are initial denaturation of 94°C for 3 min followed by (94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 72°C for 1 min)  $\times$  30 cycles. Final extension was at 72°C for 10 minutes.



**FIGURE 1.** Electropherogram of the sense strand of genomic DNA from the proband in family PCG4, showing a novel homozygous frameshift mutation. Note the homozygous insertion of a nucleotide A (376insA) in the mutant allele of the proband (B), which is absent in the control (A). The mutation, which is *underlined* and shown by an *arrow*, results in premature termination at aa 223. The comparison of normal versus all truncated protein known in exon II is also shown (C). (□) Sequences that are common between normal and wild-type protein; (■) mutant protein. The length of truncated protein and its references are shown on the *right*.

mutation (Fig. 3B) was a G→A substitution at 959 bp, resulting in replacement of glutamic acid with lysine at aa 229 (E229K) and loss of restriction site *Eam*1104I (Fig. 2C).

The maternal grandfather (I.1), mother (II.1), and proband (III.1) in PCG1 were heterozygous for 923C→T, whereas the father (II.2), proband (III.1), and unaffected sibling (III.2) were heterozygous for 959G→A (Figs. 3A, 3B). Consistent with the recessive mode of inheritance, the proband had inherited two heterozygous mutant alleles (III.1), one from each parent. Sequence analysis of the affected mother (II.1) revealed only one mutant allele (Fig. 3A). The presumed second mutant allele has yet to be identified. For recessive disease to develop, the patient should have two mutant alleles either in the heterozygous or homozygous state. The unaffected sibling (III.2) had inherited one paternal heterozygous mutant allele, and it may be that she also inherited the unknown mutant allele from the mother. Repeated sequencing of the *CYP1B1* coding region in the mother (II.1) and unaffected sibling (III.2) failed to identify the other heterozygous mutation, suggesting its possible presence either in the promoter or in some other regulatory region. Although we presume that this unaffected sibling (III.2) had inherited two mutant alleles, at 8 years of age she had not yet

shown any symptoms of glaucoma and hence she could be considered as a glaucoma suspect. The first mutation (P193L) maps to a region highly conserved among various types of cytochromes, whereas the E229K mutation is conserved only among the *CYP1B1* types (Fig. 4). Screening of 70 control subjects by PCR-restriction fragment length polymorphism (RFLP) not only confirmed the absence of this compound heterozygous mutation in the normal population, but also supports that it is likely to be pathogenic. However, a few control subjects (12.8%) were heterozygous for the 923C→T (E229K) mutation, but none for the 959G→A (P193L) mutation.

**Homozygous Missense Mutations.** Three families were identified with two known homozygous missense mutations<sup>10,15,23</sup>: two with the R368H homozygous mutation and one with the G61E homozygous mutation (Table 2). Both are highly conserved across various members of the cytochrome P450 superfamily (Fig. 4). These mutations were found to segregate with four patients (families PCG2 and PCG11, one patient each; PCG6, two patients) in three unrelated consanguineous families (PCG2 and PCG6, first-cousin marriage; PCG11, uncle-to-niece marriage). Consistent with recessive inheritance, mutant alleles segregated with disease phenotypes in all families.

Patients in families PCG2 and PCG6 showed the same homozygous mutation: G→A substitution at 1449 bp. This resulted in an arginine-to-histidine change at aa 368 (R368H) in *CYP1B1* and a loss of restriction site *Taa*I in exon III (Table 2). In PCG11, substitution of a nucleotide G→A at cDNA position 528 resulted in a glycine-to-glutamic acid replacement at aa 61 (G61E) of *CYP1B1* and a gain of the restriction site *Taq*I in exon II (Table 2).

### Nonpathogenic *CYP1B1* Single Nucleotide Polymorphisms

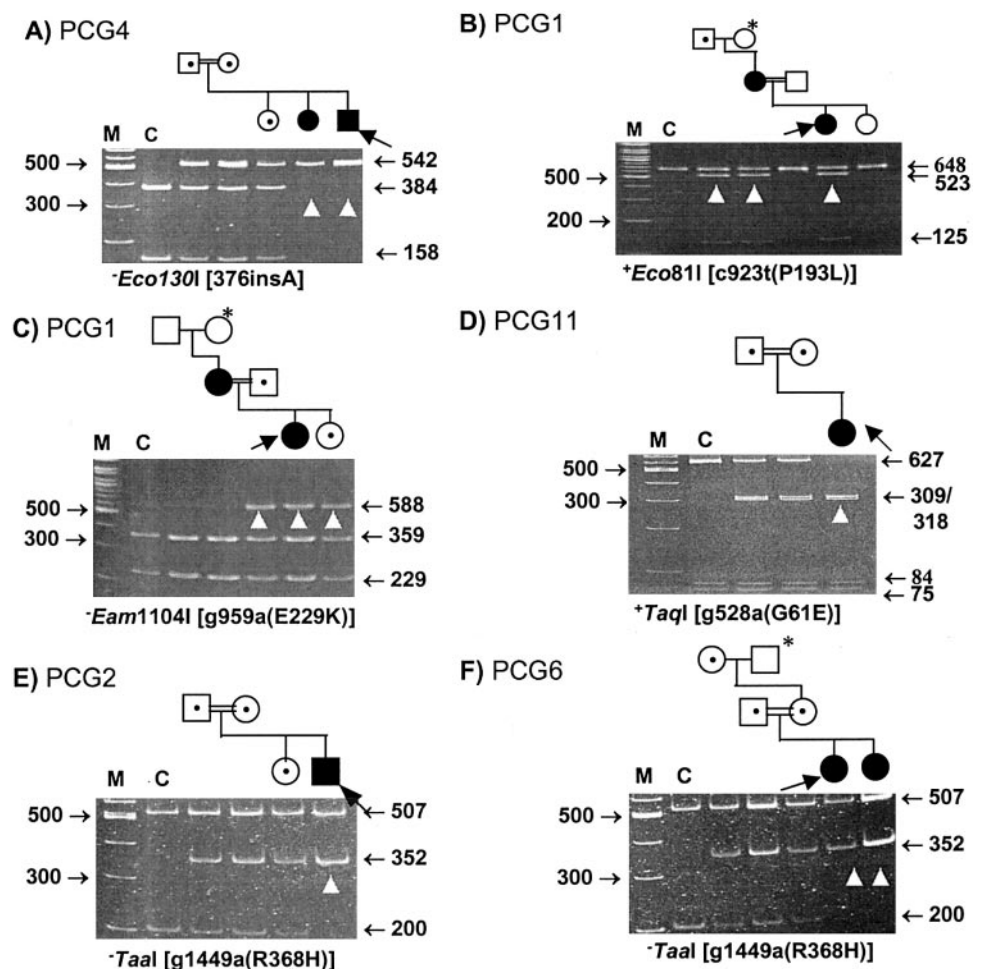
In addition to pathogenic mutations five other single nucleotide polymorphisms (SNPs; Table 3) were identified in the less conserved region of *CYP1B1*. Because PCG6 had two different homozygous missense mutations, the highly conserved residue (R368H, reported earlier)<sup>15</sup> was considered to be a pathogenic mutation, whereas the less conserved one (G184S) was taken to be a novel polymorphism (Fig. 4).

### Structural Implications of Mutant Proteins

It is interesting to note that, of the four amino acid mutations (excluding insertion mutation), three occur in the less-conserved N-terminal domain of the protein. An alignment of the amino acid sequence with a homologue of known three-dimensional structure (Protein Data Bank [PDB] code: 1DT6) revealed that all the mutation sites are away from the heme-binding pocket and therefore probably do not affect directly the binding of the heme. However, these sites seem to be important in maintaining the structural integrity of the protein. The conserved glycine residue at position aa 61 is in a left-hand helical conformation and is in a very unique position where the peptide chain takes a sharp turn. Position aa 193 forms the N-capping region of the helix (aa 173–210) and is most suited for proline, which is also highly conserved. Any amino acid change at this position may disrupt the helical structure. The same is probably true for the position E229, which is in the middle of the helix (aa 218–234). R368 is probably less important structurally, because the site is in the loop region, which is on the surface of the protein and is probably necessary for protein-protein interactions.

Our examination of the translated product of the frameshift mutation (376insA) revealed that the amino acid sequence of

**FIGURE 2.** PCR-RFLP analyses of the cosegregation of different mutations with disease phenotypes. *Filled squares and circles:* Affected individuals; *arrow:* probands; *dot in open symbol:* carriers; *double line:* consanguinity. DNA molecular weight marker (*lane M*) in base pairs (*left*); allele sizes (*right*); control (*lane C*); mutant allele(s) (*arrowheads*). Restriction site changes and mutations (nucleotide as well as amino acid changes) are shown at the *bottom* of each panel. (\*) Sample for analysis unavailable. (A) Wild-type allele amplification and restriction digestion of amplicon from control DNA generated 384- and 158-bp fragments (*lane C*). Mutation abolishes the *Eco*130I site. In heterozygous individuals (carriers) in addition to the wild-type allele, a mutant allele of 542 bp was present. In the disease phenotype (homozygous) only a mutant allele of 542 bp was evident. (B) C→T substitution in PCG1 results in a gain of an *Eco*811 site, which is evident from the cleavage of the 648-bp fragment (*lane C*) into 523-bp and 125-bp fragments. In carriers, in addition to the wild-type allele a mutant allele of 648 bp was present. (C) Restriction digestion of the wild-type allele in the control generated 359- and 229-bp (*lane C*) fragments and abolished the *Eam*1104I site. In carriers, in addition to the wild-type allele 588, mutant alleles of 359 and 229 bp were present. (D) Restriction digestion of the wild-type allele in the control showing undigested fragment of 627 bp (*lane C*). Mutation creates a *Taq*I site. In carriers, in addition to the wild type allele, mutant alleles of 318 and 309 bp were present. In the disease phenotype (homozygous) only mutant alleles of 318 and 309 bp were present. (E, F) Restriction digestion of wild type allele in the control generated 507- and 200-bp fragments (*lane C*). Mutation creates a *Taal* site. In carriers, in addition to the wild-type allele, a mutant allele of 352 bp was present. In the disease phenotype (homozygous) only mutant alleles of 507 and 352 bp were present.



the new ORF does not show an appreciable match with any of the known protein sequences in the PDB. A secondary structure prediction of the sequence showed that the translated product is mostly made of coiled regions.

### Genotype-Phenotype Correlations

Correlation between genotype and phenotype based on this study was evident from a comparison of the different mutations associated with varying manifestations and prognoses of the disease (Table 4). The PCG phenotypes associated with various mutations showed varying severity and manifestations. In some cases, there was asymmetric manifestation between eyes of the patients (mother in family PCG1), whereas the same mutation (R368H) exhibited interfamilial (families PCG2 and -6) as well as intrafamilial (family PCG6) variability (Tables 1, 4).

### DISCUSSION

This is the first genetic study from India to describe the molecular defect underlying the PCG phenotype and demonstrates the direct association of the *CYP1B1* mutations with this devastating childhood blindness.<sup>22</sup> Unknown developmental defects of the trabecular meshwork and anterior chamber

angle of the eye cause this disorder.<sup>10,21,24</sup> In our investigation of five consanguineous PCG-affected Indian families, five pathogenic mutations (including three novel ones) were identified in eight affected members. These include a novel homozygous frameshift mutation resulting in a functional null allele and compound heterozygous missense and known missense mutations (Table 2). That all are disease-causing mutations is shown by the fact that all mutant alleles cosegregate with the disease phenotype and are absent in the normal population and that the mutated residues are highly conserved across various members of the cytochrome P450 superfamily (Fig. 4). In addition, five SNPs were found in the affected families. These were either observed in the general population and/or were found to affect poorly conserved amino acid residues exclusively (Fig. 4). This study also indicates that *CYP1B1* could be the predominant cause of PCG in the Indian ethnic background, because all families analyzed so far have had mutations in this gene.

Pseudodominant inheritance was seen in one family, whereas all others showed autosomal recessive inheritance with full penetrance. All patients inherited two mutant alleles, whereas unaffected members were heterozygous (carriers) for a single mutant allele segregating in that particular family, except in the pseudodominant family (Fig. 2).

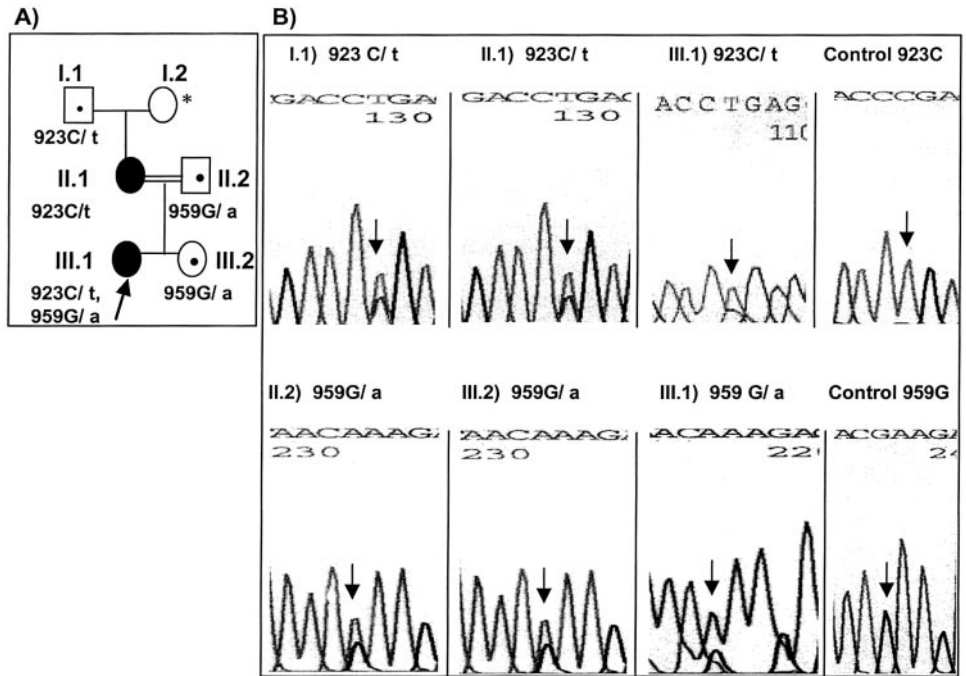


FIGURE 3. (A) Pedigree showing segregation of alleles in PCG1 family. (\*) Grandmother's DNA was not available for analysis. Heterozygous alleles are denoted by a slash separating wild type in uppercase letters and mutant residues in lowercase letters. (B) Electropherogram of the sense strand of various members of family PCG1. Residues are numbered based on cDNA sequence. Arrows: mutated bases.

Of all mutations identified herein, the frameshift mutation resulted in the most severe phenotype. Only the first 10 aa of the 543-aa CYP1B1 protein remain unchanged by the frameshift, whereas the remainder of the protein was replaced by an out-of-frame polypeptide of 222 aa. Despite maximum medical and prompt surgical treatments, both patients in family PCG4 exhibited a most devastating phenotype and were blind (Fig. 5).

In all PCG-pseudodominant families reported so far,<sup>10,12,15</sup> the affected parent has been homozygous and the other a normal carrier; but analysis of the present pseudodominant family (PCG1) indicates that the affected parent (II.1) is a compound heterozygote. Moreover, an interesting observation is that probably there are three compound heterozygous individuals (II.1, III.1, and III.2) in this family, all segregating with different combinations of mutant alleles (Fig. 3) with varying expression, of which one exhibits normal phenotype (unaf-

ected sibling [III.2]—a glaucoma suspect). The exact age of onset of the disease in this case was difficult to ascertain because the affected status of the mother (II.1) was revealed through her daughter (the proband [III.1]). The presence of Haab's striae in the left eye of the affected mother (Table 1) suggests that she had PCG in that eye before 3 years of age, whereas the right eye had late-onset PCG. An asymmetric manifestation of PCG was seen in the affected mother (the left eye became blind at 21 years, whereas IOP in the right eye is under control with medication).

The mother had glaucoma diagnosed at age 30 (Table 1) and had ocular features indicating that disease may have begun in one eye before age 3. However, because the second CYP1B1 mutation has not been identified in the mother (II.1), and this missing allele, as passed on to her 8-year-old daughter (III.2), has resulted in a normal phenotype (Fig. 3A), this seems to be a complex situation, for which various plausible explanations

	R48G	G61E	A119S	G184S	P193L	E229K	R368H	V432L	D449D	
	†	*	†	†		*	*	†	†	
CYP1A1 (g pig)	L K S ... L I	<b>G</b> H M L ... D L Y ... L M A ... F D	P Y R ... L N N E F ... R E	R Q P ... K L W ... D G T ... D11043						
CYP1A1 (human)	L K N ... L I	<b>G</b> H M L ... D L Y ... L M A ... F N	P Y R ... L N N N F ... R S	R R P ... K L W ... D G A ... NM_000499						
CYP1A1 (mouse)	L K T ... F I	<b>G</b> H M L ... D L Y ... V M A ... F D	P Y K ... L S N E F ... R D	R Q P ... E L W ... S G T ... NM_009992						
CYP1A1 (trout)	L K R ... I I	<b>G</b> N M L ... D L Y ... V M D ... F D	P F R ... M S D E F ... M I	R T P ... E L W ... D G T ... U62796						
CYP1A2 (human)	L K S ... L L	<b>G</b> H V L ... D L Y ... L M A ... F D	P Y N ... L A Q E G ... Q V	R R P ... E L W ... D G T ... AF182274						
CYP1A2 (trout)	L K R ... I I	<b>G</b> N V L ... D L Y ... V M D ... F D	P F R ... M S D E F ... M I	R T P ... E L W ... D G T ... U62797						
CYP1B1 (human)	R S A ... L I	<b>G</b> N A A ... A F A ... G S A ... L D	P R P ... H N E E F ... R D	R L P ... V K W ... D G L ... U03688						
CYP1B1 (mouse)	W S S ... L I	<b>G</b> N A A ... P F A ... R C A ... L D	P T Q ... H N E E F ... R D	R L P ... A K W ... D G F ... U03283						
CYP1B1 (rat)	W S S ... L I	<b>G</b> N A A ... P F A ... R C A ... L D	P T Q ... H N E E F ... R D	R L P ... A K W ... D G F ... U09540						
CYP2A7 (human)	S R G ... F I	<b>G</b> N Y L ... E Q A ... E S G ... I D	P T F ... M M L G I ... K N	R Q P ... R F F ... K G Q ... U22029						
CYP2B6 (human)	T H D ... L L	<b>G</b> N L L ... K I A ... E A Q ... M D	A T F ... L F Y Q T ... P H	R P P ... H Y F ... N G A ... AF182277						
CYP2C8 (human)	R R R ... I I	<b>G</b> N M L ... N S P ... E A H ... C D	P T F ... R F N E N ... R H	R S P ... K E F ... N G N ... NM_000770						
CYP2D6 (human)	A R Y ... G L	<b>G</b> N L L ... P V P ... E A A ... F R	P N G ... L A Q E G ... Q V	R R P ... A V W ... Q G H ... NM_000106						
CYP2E1 (human)	S S W ... I I	<b>G</b> N L F ... D L P ... E A H ... F D	P T F ... L F N E N ... P S	R I P ... Q E F ... N G K ... AF182276						
CYP2F1 (human)	D K G ... I L	<b>G</b> N L L ... D Y P ... E G S ... F D	P T F ... L I N D N ... R A	R L P ... S Q F ... N Q S ... J02906						
CYP3A5 (human)	R L G ... L L	<b>G</b> N V L ... A I S ... E A E ... V	- - - P Q D P F ... N K	A P P ... K Y W ... K K D ... NM_000777						
CYP4B1 (human)	R R T ... L F	<b>G</b> H A L ... G L L ... K A R ... F	- - - R D S S Y ... D Q	D F F ... A V W ... N A S ... NM_000779						

FIGURE 4. Multiple sequence alignment of various members of the cytochrome P450 superfamily. Bold letters with asterisk and shading: conserved residues (when mutated) causing PCG phenotype. †Polymorphic residues. Right: sequence accession numbers. The human CYP1B1 sequence is underlined.

TABLE 3. Single Nucleotide Polymorphisms Identified in PCG-Affected Families

Pedigree	Intron/Exon	Sequence Change (genomic/cDNA position in bp)	Codon Change	Mutation Type	Novel* or Reported
PCG1, PCG2, PCG6, and PCG11	Intron I	3793 T→C (-13 bp)†	Not applicable	—	Refs. 12, 15
PCG4	Exon II	488 C→G	R48G	Missense	Refs. 12, 15, 19, 18
PCG6	Exon II	896 G→A	G184S	Missense	Novel*
PCG2	Exon III	1640 G→C	V432L	Missense	Refs. 9, 12, 15, 18, 19
PCG1	Exon III	1693 T→C	D449D	Silent	Refs. 12, 15, 19, 18

\* Reported for the first time in this study.

† Indicates -13 bp upstream of ATG or genomic DNA position 3793 bp.

can be considered: (1) The dramatic phenotypic variability observed between the two eyes of the affected mother is possibly the consequence of an as yet unknown mutation within the promoter region (perhaps a promoter deletion), and may indicate that *CYP1B1* is a dosage-sensitive gene. (2) The mother may simply be a carrier of congenital glaucoma who happens also to have an early-onset form of glaucoma caused by mutation at another locus or glaucoma of a nongenetic origin. (3) It may be possible that heterozygosity for the 923C→T mutation causes late-onset disease, although to our knowledge there are no reported instances of development of late-onset disease in carriers of the *CYP1B1* mutation. (4) If the mother has a new mutation and is mosaic for the mutation, she could have one eye more affected than the other, because of unequal representation of the defect in the two eyes. It is possible that she has an unaffected child who inherited that chromosome, because of the absence of the mutation in the germ line. Although various roles for *CYP1B1* in eye development have been proposed recently,<sup>23</sup> it is tempting to speculate that the likely role of *CYP1B1* is in the detoxification or elimination of a toxic metabolite, which may be harmful to the normal development of the eye.

Previous studies have indicated that the G61E and R368H mutations are not fully penetrant in Saudi families,<sup>10,15</sup> whereas in these Indian families, both are fully penetrant. R368H, reported earlier,<sup>15</sup> maps to helix K, which is one of the highly conserved core structures (CCSs). This homozygous mutation seen in three patients of two unrelated families (PCG2 and PCG6) shows a very severe phenotype, in either one or both eyes. The CCSs are suspected to be involved in

proper protein folding and in active heme binding.<sup>23</sup> Therefore, any homozygous impairment of this domain could lead to a severe phenotype. The other highly conserved G61E mutation<sup>12</sup> is adjacent to the N-terminal proline-rich region of *CYP1B1* and is also likely to affect the proper protein function and result in disease manifestation. The proline-proline-glycine-proline motif may serve to join the membrane-binding N terminus to the globular region of the P450 protein.<sup>9,10,15,23</sup>

Because the anterior chamber angle in humans has undergone some very recent evolutionary changes, this may be a problem in using animal models, especially the *CYP1B1* knockout mice, for studying PCG's pathogenesis.<sup>23</sup> Typical trabecular meshwork can be found only in humans and higher primates, whereas lower species have only a reticular meshwork.<sup>24</sup> Although it may be difficult to extrapolate the findings obtained from the *CYP1B1* null mice, the phenotype obtained in such mice need not be the same as that of the functional *CYP1B1* knockout identified in the present study. This view is in fact substantiated by a study, wherein it was demonstrated that *CYP1B1* null mice did not show any obvious blindness or evidence of glaucoma, as assessed by standard behavioral comparisons with wild-type mice in their response to light and dark.<sup>25</sup> Furthermore, a frequent observation in various knockout studies is that the phenotypes do not transfer identically across species.

The information derived from this study has both basic and clinical relevance. Genetic counseling can be provided to at-risk families that will aid in the prevention of PCG-related blindness. The characterization of *CYP1B1* and the spectrum of mutations with evidence of pathogenicity and high pen-

TABLE 4. Genotype/Phenotype Effect

Pedigree	Mutation	Laterality	Severity* (OD; OS)	Prognosis (OD; OS)
PCG4				
Proband	Ter@223 aa	Bilateral	OU very severe†	OU very poor
Affected sibling	Ter@223 aa	Bilateral	OU very severe†	OU very poor
PCG11, proband	G61E	Bilateral	OU mild	OU good
PCG1				
Proband	P193L and E229K	Bilateral	OU mild	OU good
Affected mother	P193L‡	Bilateral with late onset OD	OD normal OS very severe†	OD good OS very poor
PCG2 proband	R368H	Bilateral	OD mild OS very severe	OD good OS very poor
PCG6				
Proband	R368H	Bilateral	OD mild OS very severe	OD good OS very poor
Affected sibling	R368H	Bilateral	OU very severe	OU very poor

\* Severity of the disease is arbitrarily graded based on the corneal changes, IOP, cup-to-disc ratio, and last recorded visual acuity (20/20 is normal; <20/20-20/40 is mild; <20/40-20/200 is severe; <20/200-PL is very severe; NPL is blind; see Table 1).

† Affected individual is blind.

‡ Second mutation in PCG1 mother is unknown.



**FIGURE 5.** Photograph showing blind phenotype in the proband in family PCG4, who had a novel homozygous frameshift mutation (376insA).

entrance could have profound clinical implications in the management of PCG. This will facilitate prenatal diagnosis for this condition, which carries high life-long morbidity. Indeed, further screening of probands using the simple, fast, and inexpensive PCR-RFLP diagnostic methods developed in this study has enabled us to rapidly identify similar mutations in several other PCG-affected families (Reddy et al., manuscript in preparation). However, further analysis of more families with PCG is needed to determine the clinical correlation with the severity of the disease, if any.

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