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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Supported in part by grants from the Department of Biotechnology, Government of India to the L. V. Prasad Eye Institute and the Centre for DNA Fingerprinting and Diagnostics; the Hyderabad Eye Research Foundation; and the i2 Foundation, Dallas, Texas.

Submitted for publication October 1, 2001; revised December 20, 2001; accepted January 11, 2002.

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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.1 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,2 prevalence is as high as 1:1250 among the Romany population of Slovakia,3 and 1:2500 in the Middle East,4 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.5,6 However, the genetic defect of this disorder was unknown, and this prompted us to undertake the investigation.

Genetic linkage studies by Sarfarazi et al.7 and Akarsu et al.8 mapped PCG to two different loci, GLC3A (at 2p21) and GLC3B (at 1p36), in which mutations within the CYP1B1 gene (encoding the cytochrome P450 enzyme at GLC3A) were associated with the disease. Several CYP1B1 mutations in various ethnic backgrounds have been implicated in the pathogenesis.10–20 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)21 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

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

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 trabeculotomy and trabeculectomy; PK*, penetrating keratoplasty performed but resulted in graft failure; OS† left eye became atrophic.
In another family (PCG1; marriage be-
tween first cousins), parent-to-child transmission of the disease was noted. This is an interesting pedigree in which the mother showed asymmetric severity and manifestations. The proband and the father were carriers (Fig. 3A). Two affected generations showed varying severity and manifestations. The proband had a novel mutation (Fig. 2A) that truncated the ORF by creating a premature stop codon (TGA), 636 bp downstream from this position. Consequently, an open reading frame (ORF) is translated from this point instead of the normal termination codon. The second mother showed asymmetric severity and manifestations (left eye blind, right eye mildly affected), whereas the proband displayed a uniform manifestation in both eyes. The proband had a novel compound heterozygous mutation (Table 2) within exon II. The compound heterozygous mutation was noticed. This is an interesting pedigree in which the mother showed asymmetric manifestation (left eye blind, right eye mildly affected), whereas the proband displayed a uniform manifestation in both eyes. The proband had a novel compound heterozygous mutation (Table 2) within exon II. The second mother showed asymmetric severity and manifestations (left eye blind, right eye mildly affected), whereas the proband displayed a uniform manifestation in both eyes.

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Exon</th>
<th>Mutation Position in cDNA (bp)</th>
<th>Hetero-/homozygous</th>
<th>Codon Change</th>
<th>Mutation Type</th>
<th>Restriction Site Change</th>
<th>Diagnostic Method Developed</th>
<th>Primers Used for Amplification (5'−3')</th>
<th>Novel or Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCG4</td>
<td>II</td>
<td>376insA</td>
<td>Homozygous</td>
<td>Ter@223</td>
<td>Frameshift</td>
<td>Eco130I digestion</td>
<td>PCR followed by Eco130I digestion</td>
<td>1F-tctccagagagtcagctccg (3676–3695)</td>
<td>Novel*</td>
</tr>
<tr>
<td>PCG11</td>
<td>II</td>
<td>528G−→A</td>
<td>Homozygous</td>
<td>G61E</td>
<td>Missense</td>
<td>TaqI digestion</td>
<td>PCR followed by TaqI digestion</td>
<td>2F-gatgcgaacttcttcacg (4258–4276)</td>
<td>Refs. 10, 12, 15</td>
</tr>
<tr>
<td>PCG1</td>
<td>II</td>
<td>923C−→T</td>
<td>Heterozygous</td>
<td>P193L</td>
<td>Missense</td>
<td>Eco81I digestion</td>
<td>PCR followed by Eco81I digestion</td>
<td>1F-tctccagagagtcagctccg (3676–3695)</td>
<td>Novel*</td>
</tr>
<tr>
<td>PCG2 and 6</td>
<td>III</td>
<td>1449G→A</td>
<td>Heterozygous</td>
<td>E29K</td>
<td>Missense</td>
<td>Eaam1101I digestion</td>
<td>PCR followed by Eaam1101I digestion</td>
<td>2R-eaam1101tctctctctctctctct (8095–8987)</td>
<td>Novel*</td>
</tr>
<tr>
<td>PCG2 and 6</td>
<td>III</td>
<td>1449G→A</td>
<td>Homozygous</td>
<td>R368H</td>
<td>Missense</td>
<td>TaqI digestion</td>
<td>PCR followed by TaqI digestion</td>
<td>3F-tcaccacatattgattctgccacagcct (7740–7765)</td>
<td>Novel* diagnostic method</td>
</tr>
</tbody>
</table>

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

*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 1 min) × 30 cycles. Final extension was at 72°C for 10 minutes.
one mutant allele (Fig. 3A). The presumed second mutant allele was homozygous or heterozygous state. The unaffected sibling (III.2) had only one heterozygous mutant allele (III.1), one from each parent. In this recessive mode of inheritance, the proband 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: 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 a arginine-to-histidine change at aa 368 (R368H) in CYP1B1 and a loss of restriction site TaqI 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 TaqI in exon II (Table 2).

**Nonpathogenic CYP1B1 Single Nucleotide Polymorphisms**

In addition to pathogenic mutations five other single nucleotide polymorphisms (SNPs; Table 5) were identified in the less conserved region of CYP1B1. Because PCG6 had two different homozygous missense mutations, the highly conserved residue (R368H, reported earlier) 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 the normal versus all truncated protein known in exon II is also shown (arrow). Sequences that are common between normal and wild-type protein; (B) mutant protein. The length of truncated protein and its references are shown on the right.

**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; (B) mutant protein. The length of truncated protein and its references are shown on the right.
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 the eyes of the patients (mother in family PCG1), whereas the same mutation (R368H) exhibited interfamily (families PCG2 and -6) as well as intrafamily (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. Unknown developmental defects of the trabecular meshwork and anterior chamber angle of the eye cause this disorder. 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).
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, 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 (unaffected 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

![Figure 3](image_url) (A) Pedigree showing segregation of alleles in PCG1 family. (B) 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. Arrows: mutated bases.

![Figure 4](image_url) 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.
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 925C→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,23 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,10,15 whereas in these Indian families, both are fully penetrant. R368H, reported earlier,15 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.23 Therefore, any homozygous impairment of this domain could lead to a severe phenotype. The other highly conserved G61E mutation12 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.9,10,15,23

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.25 Typical trabecular meshwork can be found only in humans and higher primates, whereas lower species have only a reticular meshwork.24 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.25 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-
etrance 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.

Acknowledgments

The authors thank the families for their participation in this study; the Clinical Biochemistry Services and the Jasti V. Ramanamma Children’s Eye Care Center staff at L. V. Prasad Eye Institute for their assistance in sample collection; Chitra Kannabiran for suggestions, Gullapalli N. Rao for encouragement and support, and Ata-Ur Rasheed for help in extracting DNA from some control samples.

References


