Correlations of Genotype with Phenotype in Indian Patients with Primary Congenital Glaucoma

Shirly G. Panicker,[1] *Anil K. Mandal,*[2] *Aramati B. M. Reddy,*[1] *Vijaya K. Gothwal,*[3] *and Seyed E. Hasnain*[4]

Purpose. To establish the genotype–phenotype correlations of various *CYP1B1* (human cytochrome P450) mutations in patients in India with primary congenital glaucoma (PCG).

Methods. The study cohort comprised 146 patients with PCG from 138 pedigrees. Patients were analyzed for six distinct *CYP1B1* mutations by sequencing and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) methods. A severity index for grading various PCG phenotypes was constructed based on clinical parameters.

Results. Six mutations were identified in 45 patients analyzed and genotype–phenotype correlations were established for 43 of them. The percentages of severe phenotypes associated with various mutations in at least one eye were: frameshift, 100%; G61E, 66.7%; P193L, 62.5%; E229K, 80%; R368H, 72%; R390C, 83.3%. The frameshift mutation resulted in blindness. Based on the severity index, the disease severity was graded from normal to severe and the prognosis from good to very poor (blind). De novo mutation was identified in one family.

Conclusions. This is the first study to attempt to devise a severity index for grading various PCG phenotypes and to use genotype as an indicator to predict the prognoses of the disorder. This index may help guide therapy and counseling of the afflicted family regarding the progression of the disorder. All patients with severe phenotypes showed poor prognoses ($r = 0.976$; $P < 0.0001$). The data derived from this study could be used as an added clinical tool in disease management. Integrated management of PCG that makes use of a genetic approach could yield better results than medical, surgical, and rehabilitation interventions alone. (*Invest Ophthalmol Vis Sci.* 2004;45:1149–1156) DOI:10.1167/iovs.03-0404

From the [1]Kallam Anji Reddy Molecular Genetics Laboratory, Brien Holden Eye Research Centre, Hyderabad Eye Research Foundation, Hyderabad, India; the [2]Jasti V. Ramanamma Children's Eye Care Centre, Hyderabad, India; the [3]Centre for Sight Enhancement, Vision Rehabilitation Centres, L.V. Prasad Eye Institute, Hyderabad, India; and the [4]Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India.

Presented in part at the annual meeting of the American Academy of Ophthalmology, Orlando, Florida, October 2002.

Supported in part by grants from the Department of Biotechnology (DBT), Government of India; the Indian Council of Medical Research (ICMR); the Hyderabad Eye Research Foundation; and the i2 Foundation, Dallas, Texas. ABMR is the recipient of a Senior Research (CSIR) Fellowship award from the Council of Scientific and Industrial Research.

Submitted for publication April 23, 2003; revised October 12, 2003; accepted November 10, 2003.

Disclosure: **S.G. Panicker**, None; **A.K. Mandal**, None; **A.B.M. Reddy**, None; **V.K. Gothwal**, None; **S.E. Hasnain**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Anil Kumar Mandal, Jasti V. Ramanamma Children's Eye Care Centre, L.V. Prasad Eye Institute, L.V. Prasad Marg, Banjara Hills, Hyderabad 500-034, Andhra Pradesh, India; mandal@lvpeye.stph.net.

Primary congenital glaucoma (PCG) is a severe form of childhood blindness caused by developmental defect(s) in the trabecular meshwork and anterior chamber angle of the eye. These abnormalities cause the obstruction of outflow of aqueous humor, which in turn results in raised intraocular pressure (IOP). If PCG is left untreated, it results in optic nerve damage and subsequent loss of vision. It is usually seen in the age group of birth to 3 years. Clinical manifestations include elevated IOP, enlargement of the globe, edema, and opacification of the cornea, with rupture of Descemet's membrane, photophobia, blepharospasm, anomalously deep anterior chamber and excessive tearing. It is mostly inherited as an autosomal recessive disorder and, in a few cases, parent-to-child transmission (pseudodominance) of the disease also occurs.[1] Inbred populations show a higher incidence of the disease. It is seen in 1 in 10,000 cases in the West,[2] 1 in 3300 in the state of Andhra Pradesh in India,[3] 1 in 2500 in Saudi Arabia,[4] and 1 in 2500 in the Slovakian Romany population.[5] Using linkage analysis, PCG (gene symbol *GLC3*) has been mapped to three different loci, GLC3A (at 2p21), GLC3B (at 1p36), and GLC3C (at 14q24.3) (Stoilov IR, et al. *IOVS* 2002; 43:ARVO E-Abstract 3015).[6,7] Although these three loci have been linked to PCG, only the gene, *CYP1B1* (Online Mendelian Inheritance in Man [OMIM] 601771, a member of the cytochrome P450 supergene family) at the GLC3A locus has been identified to date.

Approximately 45 mutations in the coding region (exons II and III) of this gene (GenBank accession no. U56438; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) have now been implicated in the pathogenesis of PCG. These include deletion, insertion, point mutation, missense, nonsense, frameshift, and chain terminator mutations. Membrane-bound cytochromes such as CYP1B1 have a transmembrane domain, which is located at the amino 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 molecule. The carboxyl terminal region has highly conserved core structures (CCSs) and is required for the proper heme-binding ability of the *CYP1B1* molecule. Those mutations at the N-terminus hinge region or C terminus CCSs are expected to interfere with fundamental properties of the cytochrome P450 molecule, such as proper folding, heme binding, and formation of stable hemoprotein complex, substrate accommodation, and interaction with the redox partner, and to decrease significantly the enzyme's metabolism.[1,8,9] Frameshift mutations causing premature stop codons in the open reading frames would result in functional null alleles.[1,10] Several *CYP1B1* mutations would cause conformational changes in the DNA which in turn affect the structure function relationship of *CYP1B1.*[11,12] This conformational change could result in disease manifestation. Though a wide spectrum of the aforementioned mutations in *CYP1B1* were reported in various ethnic populations,[1,10–27] scant information is available on the genotype–phenotype correlations of this devastating childhood blinding disorder.[10,22] Genotype–phenotype correlations could play a significant role in managing the disease. We have screened 146 patients with

TABLE 1. Clinical Data Ranges Observed in PCG Study Subjects

Clinical Parameters	Ranges Observed
Age of onset	By birth–3 years
Age of diagnosis	By birth–30 years
IOP (mm Hg)	24–55
Cup-to-disc ratio of the optic nerve	0.3:1 (total cupping)
Corneal diameter (mm)	11–17
Last recorded vision	6/6-NPL (normal-blind)
Corneal changes	Corneal scar, Haab's striae, edema, buphthalmos, megalocornea
Treatments (*n*)	Medical-surgical (1–3)

PCG from 138 pedigrees and reported six distinct *CYP1B1* mutations from 45 patients with PCG from India.[10,27] These include four novel mutations: ins 376A or Ter@223(frameshift), P193L, E229K, R390C, and two known mutations, G61E and R368H.

Herein, we describe the results of genotype–phenotype correlations of 43 Indian patients with PCG, its implications in disease prognoses and the de novo mutation identified. In addition, we report the severity index developed for grading various congenital glaucoma phenotypes that occur in India.

METHODS

Selection and Evaluation of Study Subjects

This investigation followed the tenets of the Declaration of Helsinki. The Institute's Ethics Committee approved the research. After obtaining informed consent, both consanguineous and nonconsanguineous subjects ($n = 146$) from 138 pedigrees were recruited. All subjects (both familial and sporadic cases) were clinically evaluated by a glaucoma specialist (AKM) and diagnosed with PCG by slit lamp biomicroscopy, gonioscopy, measurement of IOP, and perimetry wherever possible. About half (51.5%) of the families recruited were of a nonconsanguineous group; sporadic cases accounted for 80%. All subjects enrolled were followed up for several years, and samples were collected over 2 years in the Children's Eye Care Centre at the Institute. The various clinical parameters of PCG subjects and the ranges observed are given in Table 1. The quantitative clinical data of PCG study subjects are given in Table 2.

Mutation Screening of *CYP1B1* Gene and Direct Sequencing

Genomic DNA was prepared from peripheral lymphocytes. The coding regions (1.6 Kb) spanning exons, II and III of *CYP1B1* gene[28] (GenBank accession no. U56438) were amplified from genomic DNA of patients and control samples using three sets of primers as described earlier.[21] The amplicons were sequenced directly, and the patient and control sequences were compared, to identify the mutations.

PCR-RFLP Analysis

All six mutations identified earlier resulted in restriction site changes and, based on this polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method were developed[10,27] for further screening of the samples. All the PCR-RFLP–positive samples were sequenced again to confirm the respective mutations using an automated DNA sequencer (Prism 3700; Applied Biosystems, Foster City, CA) using dye terminator chemistry sequencing (Big Dye; Applied Biosystems). Seventy healthy volunteers without any history of eye disorders were used as normal control subjects.

Statistical Analysis

Because there was asymmetric phenotype between both eyes of several patients analyzed, severe phenotype exhibited in at least one eye was considered for calculating the percentage of severity of disease against each mutation. Correlation between severity and prognosis was estimated using Spearman's rank correlation coefficient, and $P < 0.05$ was considered to be statistically significant.

Microsatellite Analysis

Microsatellite analysis was performed to assess paternity in pedigree 0017 using 11 highly polymorphic short tandem repeat (STR) markers from the X- and Y-chromosomes and from the autosomes. The markers used were from a PCR amplification kit (AmpF*l*STR Profiler Plus; Applied Biosystems, Foster City, CA). This kit coamplifies the repeat regions of the following 11 short tandem repeat loci and their respective chromosomal locations are given in parentheses: D3S1358 (3p), vWA (12p12-pter), FGA (4q28), D8S1179 (8), D21S11 (21), D18S51 (18q21.3), D5S818 (5q21-31), D13S317 (13q22-31), D7S820 (7q11.21-22), and (X:p22.1-22.3; Y:p11.2). A segment of the X-Y homologous gene amelogenin was also amplified for gender identification. One primer of each locus-specific primer pair was labeled with either the 5-FAM, JOE, or NED NHS-ester dye, which was detected as blue, green, and yellow, respectively, on the sequencer (Prism 3700; Applied Biosystems).

RESULTS

Identification of De Novo Mutation

It is notable that de novo mutation was identified in one of the families. Mutation in family 0017 was confirmed by sequencing, and cosegregation of mutant alleles with disease phenotype was ascertained by PCR-RFLP analysis (Fig. 1). An interesting instance was observed in family 0017, in which the affected male child (proband-II.1) was homozygous for R368H, whereas their mother (I.2) was heterozygous (carrier) for the same mutation. No sequence change was detected in the father (I.1), after several rounds of sequencing and PCR-RFLP analyses. He was found homozygous for the wild-type allele (normal) and the proband (II.1) had both carrier (II.2) and normal (II.3) siblings. Usually, for the manifestation of an autosomal recessive disease, both parents are expected to be carriers, but in this family only one of the parents (the mother, II.2) was found to be a carrier, and the first male child (proband II.1) was affected by PCG. Hence, we reasoned that the absence of mutation in the father could be due to nonpaternity or occurrence of paternal de novo mutation in the germline.

A similar instance of a paternal de novo homozygous germline mutation (G365W) was reported in an American family.[11] Their line of evidence also corroborates our findings. Paternity in this nonconsanguineous family was established by analyzing 11 highly polymorphic short tandem repeat (STR) markers (AmpF*l*STR Profiler Plus Loci Kit; applied Biosystems) from the X and Y-chromosomes and from the autosomes (data not shown). For the Y-linked markers, the father and affected son (proband II.2) shared an identical haplotype, whereas, for the X-linked markers, the father and daughter shared another haplotype. Also, none of the autosomal markers showed any evidence of incompatibility in this pedigree. Therefore, no evidence for nonpaternity was found by our investigation. Moreover, the genomic DNA used for screening was obtained from peripheral leukocytes; hence, we interpreted this as a case of de novo mutation in the germline.

Severity Index for Grading PCG

Several cases of PCG with varying severity and manifestations have been identified in India. Hence, a severity index was constructed for grading various phenotypes. The phenotypes were graded from normal to severe, using the clinical parameters given in Table 3. A phenotype was graded "very severe" when the last recorded vision ranged between less than 20/400 and no percep-

TABLE 2. Quantitative Clinical Data of PCG Subjects Used for Genotype–Phenotype Correlations

Serial Number	Pedigree ID	Age of Onset	Age of Diagnosis	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)
1	*0004p*	*By birth*	*5 mo*	*12; 12.5* *Buphthalmos, Haab's striae, hazy cornea, and edema OU*	*36; 38*	*0.9; NA*	*NPL OU*	*Medical and 1× Trab/Trab OU; 1× PK* OD*
2	*0004s*	*By birth*	*3 mo*	*NA OU* *Buphthalmos, hazy cornea, and atrophic OU*	*NA OU*	*NA OU*	*NPL OU*	*Medical and 1× Trab/Trab OU*
3	0093p	By birth	By birth	15.5; 13 Hazy cornea and edema OU	35; 30	NA OU	20/160; 20/80	1× Trab/Trab OU
4	0093s	By birth	By birth	14.5; 16 Hazy cornea and edema OU	26; 30	0.5 OU	20/130; 20/960	Medical and 1× Trab/Trab OU
5	*0011p*	*By birth*	*2 wk*	*12; 12.5* *Corneal edema OU*	*30 OU*	*0.3; 0.6*	*20/50 OU*	*Medical and 1× Trab/Trab OU; 2× Trab/Trab OS*
6	0058p	By birth	By birth	13 Hazy cornea and corneal scar OU	32 OU	0.5; 0.6	20/360	1× Trab/Trab OU
7	*0001p*	*By birth*	*~5 y*	*NA* *Clear OU*	*24 OU*	*0.8; 0.6*	*20/25 OU*	*Medical treatment OU*
8	*0001m*	*Late onset in OD; >3 years*	*30 y*	*NA* *Clear OD, hazy OS*	*34; 50*	*0.8; 0.9*	*20/20; NPL in OD*	*Medical treatment OD*
9	0069p	By birth	By birth	14 OU Hazy cornea and edema OU	26; 24	0.6 OU	20/300; 20/120	Medical treatment OU; 1 × Trab/Trab OU
10	0024p	By birth	By birth	11.5 OU Hazy cornea OU	28; 31	NA OU	20/80 OU	Medical treatment OU; 1× Trab/Trab OD
11	0037p	1 y	5 y	14 OD; enucleated OS megalocornea, corneal scar OU, edema OD	26; NA	NA OU	PL in OD	3× Trab/Trab OD
12	0047p	By birth	By birth	NA OU Corneal scar OU	25; 32	0.4; 0.8	20/126; PL	3× Trab/Trab OU and 3× molteno implants
13	0125p	1 wk	1 wk	13.5; 14 Haab's striae and hazy cornea OU	20; 28	0.6; 0.4	20/670 OU	1× Trab/Trab OU; 1× Trab OD
14	*0002p*	*By birth*	*2 wk*	*13 OU* *Haab's striae, buphthalmos and hazy cornea OU*	*NA OU*	*0.9 OU*	*20/30; PL*	*3× Trab/Trab OU; retinal reattachment surgery OS, and medical treatment OD*
15	*0006p*	*By birth*	*9 mo*	*13; 12.5* *Megalocornea, corneal edema OU, and hazy cornea OS*	*26; 30*	*0.3 OU*	*20/40; 20/300*	*1× Trab/Trab OU*
16	0017p	By birth	By birth	13.5 OU Megalocornea, corneal edema OU and hazy cornea OS	26; 24	0.9 OU	NPL; 20/160	1× Trab/Trab OS
17	0040p	By birth	By birth	13; 12 Buphthalmos OU, corneal scar OD and hazy cornea OS	32; 26	0.6 OU	Fixing and following light	1× Trab/Trab OU
18	0076p	By birth	By birth	12 OU Buphthalmos and hazy cornea OU	26; 30	0.2 OU	20/400; 20/30	1× Trab/Trab OU; 1× Trab/Trab OS
19	0079p	4 mo	4 mo	13.5; 13 Corneal edema OD, Haab's striae OS, and buphthalmos OU	22; 28	NA OU	Fixing and following light	1× Trab/Trab OU
20	0130p	By birth	2.6 y	12; 13.5 Megalocornea OS	12; 28	0.3; 0.9	Fixing and following light	1× Trab/Trab OS
21	0137p	By birth	1 mo	14.5 OU Corneal scar OD and hazy cornea OS	26; 28	NA; 0.5	20/60 OU	1× Trab/Trab OU
22	0144p	By birth	By birth	11 OU Corneal scar and edema OD	36; 30	NA; 0.5	20/260 OU	1× Trab/Trab OU
23	*0006s*	*By birth*	*3 mo*	*15 OU* *Corneal edema and scar OU*	*32 OU*	*NA OU*	*PL; HM*	*Medical and 1× Trab/Trab OU*

TABLE 2 (*continued*). Quantitative Clinical Data of PCG Subjects Used for Genotype–Phenotype Correlations

Serial Number	Pedigree ID	Age of Onset	Age of Diagnosis	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)
24	*0022p*	*6 mo*	*8 mo*	*14 OU* *Megalocornea, corneal edema, scar and hazy OU*	*34; 35*	*NA*	*20/600 OU*	*1× Trab/Trab OU; 1× Trab/Trab OD*
25	0035p	By birth	28 d	11 OU Corneal edema and hazy OU	26 OU	NA OU	20/960 OU	2× Trab/Trab OU
26	0051p	By birth	4 mo	13 OU Haab's striae and hazy cornea OU	28; 26	0.3; 0.4	20/250 OU	1× Trab/Trab OU
27	0071p	By birth	By birth	11.5; 11 Corneal scar OD, corneal edema and hazy OU	28; 26	NA	20/200 OU	1× Trab/Trab OU
28	0071s	By birth	By birth	13 OU Corneal scar OD and hazy OU	28; 20	NA	Fixing and following light	1× Trab/Trab OU
29	0075p	By birth	By birth	14 OU Buphthalmos, corneal scar, and edema OU	34; 38	NA	20/2400 OU	1× Trab/Trab OU
30	0150p	By birth	3 mo	13 OU Megalocornea OU	28; 30	0.2 OU	20/200; 20/60	1× Trab/Trab OU
31	0136p	By birth	3 mo	13; 14 Haab's striae OS	32; 34	0.2 OU	20/180 OU	1× Trab/Trab OU
32	0067p	By birth	By birth	15; 14 Haab's striae OD, corneal scar, and edema OS	16; 12	NA	20/60 OU	1× Trab/Trab OU; 3× Trab OD; 7× Trab OS
33	0025p	By birth	10 y	16; 14 OU Haab's striae, buphthalmos OU, and megalocornea OD	50; 55	1.0 OU	20/200 OU	1× Trab/Trab OD
34	*0035m*	By birth	8 y	NA OU Megalocornea, corneal edema, scar and hazy OU	NA OU	NA OU	PL in OU	2× Trab/Trab OU; 1× PK OS; 1× Iridencleisis OU
35	0095p	1 y	1 y	12 OU Megalocornea and hazy cornea OU	38; 36	0.4; 0.3	PL in OU	1× Trab/Trab OU
36	0100p	By birth	4 mo	12; 12.5 OU Haab's striae, buphthalmos OU, and megalocornea OD	20 OU	NA	20/20; 20/200 OU	1× Trab/Trab OU; 1× Trab OD; 1× Trab OS; and medical treatment
37	0039p	By birth	By birth	NA OU NA OU	19; 18	0.4; 0.5	20/20; 20/25	Medical treatment OD; 1× Trab OS
38	0005p	By birth	By birth	12 OU Corneal edema OU	40; 42	0.3 OU	20/120 OU	1× Trab/Trab OU
39	0005f	By birth	By birth	NA OU Buphthalmos, megalocornea, and corneal edema OU	18 OU	NA OU	NPL OU	1× Trab/Trab OU; 1× Trab OD; iridencleisis OU
40	0012p	By birth	By birth	12; 12.5 Buphthalmos, corneal scar, and corneal edema OU	32; 34	NA OU	20/400 OU	1× Trab/Trab OU; 1× PK OD
41	0012s	By birth	By birth	12 OU Buphthalmos, megalocornea, corneal scar and hazy OU	28; 32	0.3 OU	CF in OU	1× Trab/Trab OU
42	0018p	By birth	By birth	13 OU Buphthalmos, megalocornea, and hazy OU	28; 30	NA OU	NA OU	1× Trab/Trab OU
43	0092p	By birth	By birth	12.5; 13 OU Buphthalmos, megalocornea, and hazy OU	22; 24	NA OU	20/600; 20/960	1× Trab/Trab OU

IOP, intraocular pressure; OD, right eye, OS, left eye, OU, both eyes; C/D, cupdisc ratio of the optic nerve; NPL, no perception of light, PL, perception of light, HM, hand motion; NA, not available; Trab/Trab, combined trabeculotomy and trabeculectomy; p, proband; s, sibling; m, mother; f, father. Quantitative data of the patients shown in *italics* were reported earlier by us,[10] but are included in the present study for comparison.

* PK, penetrating keratoplasty was performed but resulted in graft failure.

† OS, left eye became atrophic.

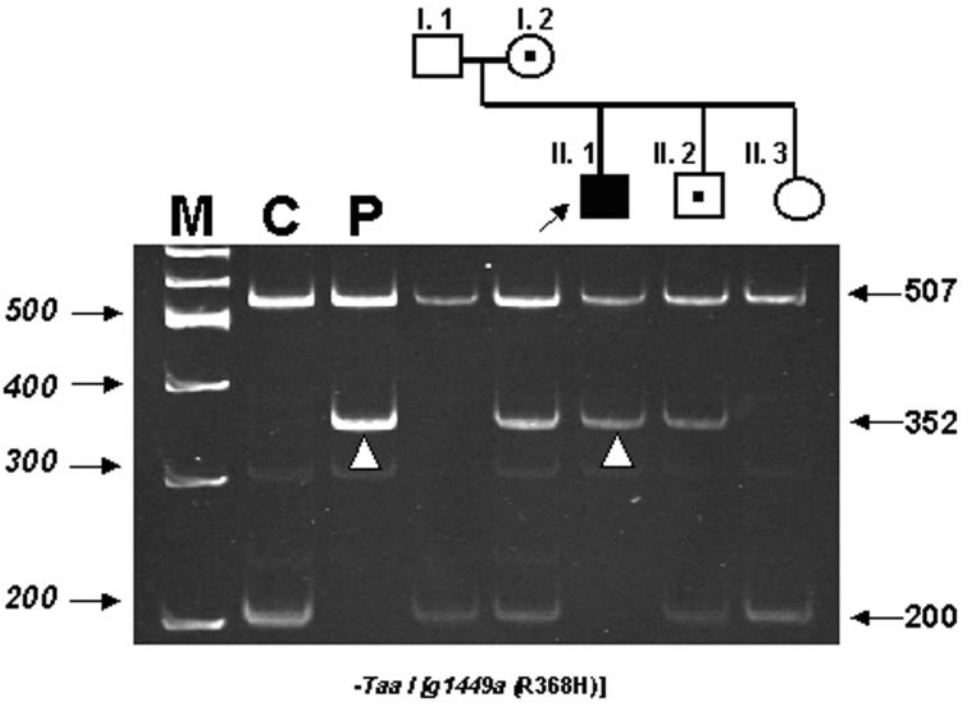

FIGURE 1. PCR-RFLP analyses of the cosegregation of mutant allele with the disease phenotype. *Filled square*: Affected individual; *arrow*: proband; *dot in open symbol*: carriers; DNA molecular weight marker (*lane M*) in base pairs (*left*); allele sizes (*right*); control (*lane C*); patient (*patient*); mutant allele (*arrowhead*). Restriction site changes and mutations (nucleotide as well as amino acid changes) are shown at the *bottom* of the gel. Restriction digestion of wild-type allele in the control generated 507- and 200-bp fragments (*lane C*). Mutation abolishes the *Taa*I site. In carriers, in addition to the wild-type allele, a mutant allele of 352 bp was present. In the disease phenotype (homozygous) a mutant allele of 352 bp was present. The father's DNA (I.1) was the same as the control DNA and he bore no mutant allele of 352 bp.

tion of light (NPL), or total blindness. The severe phenotypes associated with various mutations are given in Table 4.

Genotype–Phenotype Correlations

By direct sequencing and PCR-RFLP methods six distinct *CYP1B1* mutations were identified in 43 Indian patients with PCG.[10,27] The respective genotype–phenotype correlations are shown in Table 5. The six mutations identified comprise of four novel mutations: ins 376A or Ter@223 (frameshift), P193L, E229K, R390C, and two known mutations, G61E and R368H. All these patients had bilateral PCG. The prognosis of the disease was assessed for each patient based on his or her last recorded vision. The prognosis was graded into four categories: good, fair, poor, and very poor.

Depending on the combination of alleles, the genotype–phenotype correlations varied (Table 5). The worst phenotype was seen with frameshift mutation (0004p and 0004s) followed by R390C homozygous mutation (Table 5). All the patients with R390C homozygous mutation (0005f, 0012p, 0012s, 0018p, and 0092p) showed very severe phenotype and very poor prognosis compared with heterozygous mutation (0005p). Of all the mutations identified herein, the predominant one was R368H, which appeared in 25 patients. Six patients had the R390C mutation, followed by E229K, five; P193L, four; G61E, three; and frameshift, two (Table 4). With all mutations, severe phenotypes were observed in at least one eye of the patients. The percentages of severe phenotypes seen in at least one eye against various mutations were frameshift, 100%, G61E, 66.7%; P193L, 62.5%; E229K, 80%; R368H, 72%; and R390C, 83.3% (Table 4). All patients with frameshift and G61E had homozygous mutations, whereas all E229K-bearing patients were heterozygous. Patients with P193L, R368H, and R390C had both homozygous and heterozygous mutations. Thirty-two percent of patients with the R368H mutation showed asymmetric phenotypes between eyes (Table 5). Good correlation was found between the severity and prognosis of the disorder ($r = 0.976$; $P < 0.0001$).

DISCUSSION

We reported the direct association of *CYP1B1* mutations with PCG phenotypes from India.[10] Subsequently, in the current study, we screened by direct sequencing and PCR-RFLP analyses a large PCG cohort (146 subjects from 138 pedigrees) and identified six distinct mutations in 45 patients. We also found R368H to be the predominant mutation causing PCG in India.[27] This allele was earlier rarely reported from Middle East and Brazil,[17,22] but in India 16.2% of the patients screened had the mutation.[27] This indicates that the mutation frequency varies, depending on the geographical location as well as ethnic background. Though a spectrum of *CYP1B1* mutations from various ethnic backgrounds[1,10–27] have been implicated in the pathogenesis of PCG, only a very few studies have reported genotype–phenotype correlations.[21,22] In this investigation, we describe the genotype–phenotype correlations of 43 patients and their prognoses. Although PCG with varying phenotypes have

TABLE 3. Severity Index Used for Grading Various Indian PCG Phenotypes

Clinical Parameters Used for Grading	Normal	Mild	Moderate	Severe/Very Severe*
Corneal diameter (mm)	Up to 10.5	>10.5–12	>12–13	>13
IOP (mm Hg)	Up to 16	>16–20	>20–30	>30
C/D ratio	0.3–0.4	>0.4–0.6	>0.6–0.8	>0.8
Last recorded visual acuity	20/20	<20/20–20/60	<20/60–20/200	<20/200–20/400, <20/400–NPL (blind)*
Corneal clarity	No edema	Mild edema	Severe edema	Severe edema and Haab's striae

* Very severe phenotype.

TABLE 4. Severe Phenotypes Associated with *CYP1B1* Mutations in Indian PCG Patients

Serial Number	Pedigrees/Patients (*n*)	Mutations Identified	Eyes with Severe Phenotypes (*n*) (Eyes Evaluated, %)	Eyes with Very Poor Prognoses (*n*) (Eyes Assessed; *n,* %)
1	1 (2)	Ter@223	4/4 (100)	Very poor (100)*
2	2 (3)	G61E	4/6 (66.7)	Very poor (66.7)
3	3 (4)	P193L	5/8 (62.5)	Very poor (1/8, 12.5)*
				Very poor (4/8, 50)
4	5 (5)	E229K	8/10 (80)	Very poor (80)
5	22 (25)	R368H	36/50 (72)	Very poor (72)
6	4 (6)	R390C	10/12 (83.3)	Very poor (2/12, 16.6)*
				Very poor (8/12, 66.6)

* Blind.

been identified, this is first severity index for grading the various PCG phenotypes that has been developed. It enables grading of PCG from normal to very severe phenotypes (Table 3).

Severe phenotypes were associated with all six mutations described in this study, but the percentage of severity varied with each mutation. For the different mutations, the associated percentages of severe phenotypes in at least one eye of the patient were: G61E, 66.7%; P193L, 62.5%; E229K, 80%; R368H, 72%; R390C, 83.3%; and frameshift, 100% (Table 4).

Of all the mutations studied, frameshift and R390C homozygous mutations were found to be associated with very severe phenotypes and very poor prognoses. Even though multiple surgical interventions were performed in two patients (0004p and 0004s) with frameshift mutations, both eventually became blind (Table 5). This could be because the frameshift mutation resulted in a functional null allele lacking all domains of *CYP1B1.*[10] Whether surgery was performed at 1 week or 4 weeks, all five patients (0005f, 0012p, 0012s, 0018p, and 0092p) with R390C homozygous mutations exhibited uniformly very severe phenotypes and had very poor prognoses (Table 5). This indicates that probably clinical interventions in these patients had limited value. However, another study in a group of patients shows that early and prompt surgical interventions resulted in better prognosis.[29,30] Probably these patients had mutations that were different from those reported in the current study, and they may be less severe. This study gives the genotype–phenotype correlations of a large number of patients with PCG (25 patients) with R368H mutation. It was found that 72% of them had severe phenotype in at least one eye. Both R368H and R390C residues are highly conserved across various members of the cytochrome P450 superfamily (data not shown). These residues map to helix K, which is one of the highly conserved core structures (CCSs) and is thought to be involved in proper protein folding and in active heme binding. Therefore, these mutations could lead to severe phenotypes.[1,10,17]

The highly conserved glycine residue at position 61 is in a left-handed helical conformation and is in a very unique position, where the peptide chain takes a sharp turn.[10] The G61E mutation[12] is adjacent to the N-terminal proline-rich region of CYP1B1, is also likely to affect proper protein function, and hence results in disease manifestation. The proline-proline-glycine-proline motif may serve to join the membrane-binding N terminus to the globular region of P450 protein.[13,14,18,24] Mutations in the hinge region have been reported to interfere with the proper folding and heme-binding properties of cytochrome P450 molecules.[1,8] It has been shown that this mutation significantly reduces the enzyme's metabolism.[9]

P193 and E229 amino acid (aa) residues are also conserved among various members of the cytochrome P450 superfamily.[10] A molecular simulation study has shown that P193L and E229K mutations could bring conformational changes in the protein (Achary MS, et al., unpublished observation, 2002). The P193 aa residue in CYP1B1 comes in the N-capping region of the helix E (aa 173-210) and is most suited for proline. The replacement of proline with leucine at this position (P193L) could disrupt the helical structure and cause severe conformational change in the mutant protein. Similarly, E229 is in the middle of the helix F (218 to 234) and the replacement of this residue at position 229 could cause conformational change. This is also associated with the premature termination of the F helix at this position[10] (Achary MS, et al., unpublished observation, 2002). Hence, it is possible that the conformational changes caused by P193L and E229K mutations impairs the structure–function relationship of CYP1B1 and in turn results in manifestation of disease.

Mutational analyses of *CYP1B1* coding exons revealed homozygous mutations in 30 of 43 Indian patients described in this study. Two patients (0001 and 0035) showed compound heterozygous mutations, whereas in 11 patients, only single heterozygous mutations were detected. Because we could not identify the second mutation in 11 heterozygous patients, we conclude that it could be due to mutations in (1) *CYP1B1* promoter or control region; (2) genes linked to other PCG loci such as GLC3B and GLC3C; (3) other glaucoma genes such as *FOXC1* and *MYOC*, resulting in digenic inheritance; or (4) some other unknown genes causing glaucoma. Mutations in the forkhead transcription factor gene *FOXC1* (formerly called *FKHL7*) could also contribute to the development of PCG.[31] Hence, it is possible that PCG can be due to mutations in multiple genes (such as *CYP1B1* and *FOXC1*, *CYP1B1* and *MYOC*, genes linked to GLC3B and C or some other loci). Digenic inheritance in glaucoma has been shown recently in two instances, such as in early-onset glaucoma in humans and also in mice with PCG.[32,33] *CYP1B1* and *MYOC* mutations were identified in early-onset glaucoma in humans,[32] whereas mutations in *CYP1B1* and *FOXC1* were detected in mice with PCG.[34] This points to the fact that mutations in genes other than *CYP1B1* can cause PCG, because all these genes could contribute to the development of anterior chamber angle. PCG is caused by unknown developmental defect(s) in trabecular meshwork and anterior chamber angle of the eye.[1] Angle structures are mainly derived from the neural crest cells; hence, defects in genes expressed in neural crest cells could also contribute to PCG.

The genotype/phenotype correlation varies, depending on the combination of alleles. The PCG phenotypes associated with heterozygous mutations varied from mild to severe, and this variation could be due to the various combinations of alleles (Table 5). The phenotypic heterogeneity of this disorder seen in India could reflect the underlying genetic heterogeneity of the disorder. We screened 146 well-characterized patients with PCG for *CYP1B1* mutations and detected mutations in only 45 of them. This indicates that mutations in non-*CYP1B1* genes in other loci could also

TABLE 5. Genotype–Phenotype Correlations of *CYP1B1* Mutations in Indian PCG Patients

Serial Number	Pedigree ID	Age at Intervention	Mutations Identified	Severity by Eye	Prognoses by Eye
1	*0004p*	5 mo	Ter@223	Very severe OU	Very poor OU*
2	*0004s*	3 mo	Ter@223	Very severe OU	Very poor OU*
3	0093p	1 mo	G61E	Severe OU	Poor OU
4	0093s	2 mo	G61E	Severe OD	Poor OD
				Very severe OS	Poor OD
5	*0011p*	2 wk	G61E	Mild OU	Good OU
6	0058p	1 wk	P193L	Severe OU	Poor OU
7	*0001p*	ND	P193L (h)	Mild OU	Good OU
			E229K (h)		
8	*0001m*	ND	P193L (h)	Normal OD	Good OD
				Very severe OS	Very poor OS*
9	0069p	1.6 y	P193L (h)	Very severe OU	Very poor OU
10	0024p	1 mo	E229K (h)	Very severe OU	Very poor OU
11	0037p	5 y	E229K (h)	Very severe OU	Very poor OU
12	0047p	10 y	E229K (h)	Very severe OU	Very poor OU
13	0125p	3 mo	E229K (h)	Severe OU	Poor OU
14	*0002p*	35 d	R368H	Mild OD	Good OD
				Severe OS	Poor OS
15	*0006p*	8 mo	R368H	Mild OD	Good OD
				Severe OS	Poor OS
16	0017p	9 y	R368H	Very severe OD	Very poor OD*
				Severe OS	Poor OS
17	0040p	5 mo	R368H	Very severe OD	Very poor OD
				Severe OS	Poor OS
18	0076p	1 y	R368H	Severe OD	Poor OD
				Moderate OS	Fair OS
19	0079p	3 mo	R368H	Very severe OD	Very poor OD
				Severe OS	Poor OS
20	0130p	3 y	R368H	Severe OD	Poor OD
				Very severe OS	Very poor OS
21	0137p	1 mo	R368H	Severe OD	Poor OD
				Moderate OS	Fair OS
22	0144p	1 mo	R368H	Severe OD	Poor OD
				Moderate OS	Fair OS
23	*0006s*	4 mo	R368H	Severe OU	Poor OU
24	0022p	6 mo	R368H	Very severe OU	Very poor OU
25	0035s	28 d	R368H	Very severe OU	Very poor OU
26	0051p	5 mo	R368H	Very severe OU	Very poor OU
27	0071p	2 wk	R368H	Severe OU	Poor OU
28	0071s	2 wk	R368H	Severe OU	Poor OU
29	0075p	1 wk	R368H	Very severe OU	Very poor OU
30	0150p	1 mo	R368H	Moderate OU	Fair OU
31	0136p	2 mo	R368H	Moderate OU	Fair OU
32	0067p	2 mo	R368H	Severe OU	Poor OU
33	0025p	10 y	R368H (h)	Very severe OU	Very poor OU
34	0035p	8 y	R368H (h)	Very severe OU	Very poor OU
35	0095p	1.2 y	R368H (h)	Very severe OU	Very poor OU
36	0100p	1.3 y	R368H (h)	Moderate OD	Fair OD
				Severe OS	Poor OS
37	0039p	21 d	R368H (h)	Moderate OU	Fair OU
38	0005p	29 d	R368H (h)	Moderate OU	Fair OU
			R390C (h)		
39	0005f	2 mo	R390C	Very severe OU	Very poor OU*
40	0012p	4 mo	R390C	Very severe OU	Very poor OU
41	0012s	2 mo	R390C	Very severe OU	Very poor OU
42	0018p	1 wk	R390C	Very severe OU	Very poor OU
43	0092p	29 d	R390C	Very severe OU	Very poor OU

Genotype–phenotype correlations of patients shown in italics were reported earlier by us,[10] but are included in the present study for comparison. (h), heterozygous mutation.

*Blind.

cause this disorder and also highlights the genetic complexity of PCG in India.

This is the first study to describe the genotype–phenotype correlations of a large number of patients with PCG. A severity index for grading congenital glaucoma has been developed for the first time. This is the second report demonstrating the occurrence of *de novo* mutation in *CYP1B1* gene causing PCG. This study also indicates that probably genotype could be used as an indicator in predicting the prognosis of the disease—for instance, in the case of frameshift and R390C mutations described in this study. Because PCG results in high life-long morbidity, genetic counseling and rehabilitation of the patient are very important in reducing the burden of the afflicted family, and may improve the quality of life. An integrated management of PCG using genetic approach along with medical, surgical, and rehabil-

itation interventions could yield better results in tackling this devastating blinding disease of childhood. In sum, the data derived from this study could be used as an added clinical tool in managing the disease better.

Establishing genotype–phenotype correlations of PCG may aid in knowing the prognosis of the disease, in guiding therapy and in counseling the afflicted families. Therefore, further studies involving large number of families from various ethnic backgrounds would be required in establishing the genotype–phenotype correlations of this blinding disorder in children.

Furthermore, the molecular consequences of the mutations found to date, provide a framework for genotype–phenotype correlation and suggest future studies in light of results of investigation of normal and mutant CYP1B1.

Acknowledgments

The authors thank the patients and their 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 (LVPEI) for their assistance in sample collection; Dorairajan Balasubramanian and Gullapalli N. Rao, LVPEI, for encouragement and support; Rishita Nutheti, International Center for Advancement of Rural Eye Care (ICARE), for assistance in statistical analysis; Hampapathalu A. Nagarajaram and colleagues, Center for DNA Fingerprinting and Diagnostics, Hyderabad, for the microsatellite analysis and for their unpublished data on molecular dynamics, respectively; and the anonymous reviewers for their constructive comments.

References

1. Sarfarazi M, Stoilov I. Molecular genetics of primary congenital glaucoma. *Eye.* 2000;14:422–428.
2. Francois J. Congenital glaucoma and its inheritance. *Ophthalmologica*. 1972;181:61–73.
3. Dandona L, Williams JD, Williams BC, Rao GN. Population-based assessment of childhood blindness in Southern India. *Arch Ophthalmol*. 1998;116:545–546.
4. Jaffar MS. Care of the infantile glaucoma patient. In: Reineck RD, ed. *Ophthalmology Annual.* New York: Raven Press, 1988;15.
5. Genicek A, Genicekova A, Ferak V. Population genetical aspects of primary congenital glaucoma. I. Incidence, prevalence, gene frequency, and age of onset. *Hum Genet*. 1982;61:193–197.
6. Sarfarazi M, Akarsu AN, Hossain A. Assignment of a locus (GLC3A) for primary congenital glaucoma (buphthalmos) to 2p21and evidence for genetic heterogeneity. *Genomics*. 1995;30:171–177.
7. Akarsu AN, Turacli ME, Aktan SG, et al. A second locus (GLC3B) for primary congenital glaucoma (buphthalmos) maps to the 1p36 region. *Hum Mol Genet*. 1996;5:1199–1203.
8. Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Effect of two mutations of human CYP1B1, G61E and R469W, on stability and endogenous steroid substrate metabolism. *Pharmacogenetics*. 2001;11:793–801.
9 Mammen JS, Pittman GS, Li Y, et al. Single amino acid mutations, but not common polymorphisms, decrease the activity of CYP1B1 against (−) benzo[a]pyrene-7R-trans-7,8-dihydrodiol. *Carcinogenesis*. 2003;24:1247–1255.
10. Panicker SG, Reddy ABM, Mandal AK, et al. Identification of novel mutations causing familial primary congenital glaucoma in Indian pedigrees. *Invest Ophthalmol Vis Sci*. 2002;43:1358–1366.
11. Stoilov I, Akarsu AN, Alozie I, et al. Sequence analysis and homology modeling suggest that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. *Am J Hum Genet*. 1998;62:573–584.
12. Mashima Y, Susuki Y, Sergeev Y, et al. Novel cytochrome P4501B1 (*CYP1B1*) gene mutations in Japanese patients with primary congenital glaucoma. *Invest Ophthalmol Vis Sci.* 2001;42:2211–2216.
13. Stoilov I, Akarsu AN, Sarfarazi M. Identification of three different truncating mutations in cytochrome P4501B1 (*CYP1B1*) as the principal cause of primary congenital glaucoma (buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum Mol Genet*. 1997;6:641–647.
14. Bejjani BA, Lewis RA, Tomey KF, et al. Mutations in *CYP1B1*, the gene for cytochrome P4501B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia. *Am J Hum Genet*. 1998;62:325–333.
15. Plasilova M, Ferakova E, Kadasi L, et al. Linkage of autosomal recessive primary congenital glaucoma to the GLC3A locus in Roms (gypsies) from Slovakia. *Hum Hered*. 1998;48:30–33.
16. Plasilova M, Stoilov I, Sarfarazi M, Kadasi L, Ferakova E, Ferak V. Identification of a single ancestral *CYP1B1* mutation in Slovak Gypsies (Roms) affected with primary congenital glaucoma. *J Med Genet*. 1999;36:290–294.
17. Bejjani BA, Stockton DW, Lewis RA, et al. Multiple *CYP1B1* mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest frequent *de novo* events and a dominant modifier locus. *Hum Mol Genet*. 2000;9:367–374.
18. Martin SN, Sutherland J, Levin AV, Klose R, Priston M, Heon E. Molecular characterisation of congenital glaucoma in a consanguineous Canadian community: a step towards preventing glaucoma related blindness. *J Med Genet*. 2000;37:422–427.
19. Ohtake Y, Kubota R, Tanino T, Miyata H, Mashima Y. Novel compound heterozygous mutations in the cytochrome P450 1B1 (CYP1B1) in a Japanese patient with primary congenital glaucoma. *Ophthalmic Genet*. 2000;21:191–193.
20. Kakiuchi-Matsumoto T, Isashiki Y, Ohba N, Kimura K, Sonoda S, Unoki K. Cytochrome P4501B1 gene mutations in Japanese patients with primary congenital glaucoma. *Am J Ophthalmol*. 2001; 131:345–350.
21. Michels-Rautenstrauss KG, Nat Dr rer, Mardin CY, et al. Primary congenital glaucoma: three case reports on novel mutations and combinations of mutations in the GLC3A (*CYP1B1*) gene. *J Glaucoma*. 2001;10:354–357.
22. Stoilov IR, Costa VP, Vasconcellos JPC, et al. M. Molecular genetics of Primary Congenital Glaucoma in Brazil. *Invest Ophthalmol Vis Sci*. 2002;43:1820–1827.
23. Belmouden A, Melki R, Hamdani M, et al. A novel frameshift founder mutation in the cytochrome P450 1B1 (CYP1B1) gene is associated with primary congenital glaucoma in Morocco. *Clin Genet*. 2002;62:334–339.
24. Ohtake Y, Tanino T, Suzuki Y, et al. Phenotype of cytochrome P4501B1 gene (CYP1B1) mutations in Japanese patients with primary congenital glaucoma. *Br J Ophthalmol*. 2003;87:302–304.
25. Soley GC, Bosse KA, Flikier D, et al. Primary congenital glaucoma: a novel single-nucleotide deletion and varying phenotypic expression for the 1546-1555dup mutation in the GLC3A (CYP1B1) gene in 2 families of different ethnic origin. *J Glaucoma*. 2003;12:27–30.
26. Sitorus R, Ardjo SM, Lorenz B, Preising M. CYP1B1 gene analysis in primary congenital glaucoma in Indonesian and European patients. *J Med Genet*. 2003;40:e9.
27. Reddy ABM, Panicker SG, Mandal AK, et al. Identification of R368H as a predominant CYP1B1 allele causing primary congenital glaucoma in Indian patients. *Invest Ophthalmol Vis Sci.* 2003;44:4200–4203.
28. Tang YM, Wo YP, Stewart J, et al. Isolation and characterization of the human cytochrome P450 *CYP1B1* gene. *J Biol Chem*. 1996; 271:28324–28330.
29. Mandal AK, Naduvilathu TJ, Jaganadan A. Surgical results of combined trabeculotomy-trabeculectomy for developmental glaucoma. *Ophthalmology*. 1998;105:974–982.
30. Mandal AK, Gothwal VK, Bagga H, Nutheti R, Mansoori T. Outcome of surgery on infants younger than 1 month with congenital glaucoma. *Ophthalmology*. 2003;110:1909–1915.
31. Nishimura YD, Swiderski RE, Alward WLM, et al. The forkhead transcription factor gene *FKHL7* is responsible for glaucoma phenotypes which map to 6p25. *Nat Genet*. 1998;19:140–147.
32. Vincent LA, Billingsley G, Buys Y, et al. Digenic inheritance of early-onset glaucoma: CYP1B1, a potential modifier gene. *Am J Hum Genet*. 2002;70:448–460.
33. Libby RT, Smith RS, Savinova OV, et al. Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science*. 2003;299:1578–1581.