Identification of R368H as a Predominant *CYP1B1* Allele Causing Primary Congenital Glaucoma in Indian Patients

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Purpose. To investigate the predominant mutation in the *CYP1B1* gene in patients in India with primary congenital glaucoma (PCG), using PCR-restriction fragment length polymorphism (RFLP) methods and to characterize the molecular defect in two generations of an affected family.

Methods. DNA samples from 146 patients with PCG from 138 pedigrees were analyzed for several distinct mutations in *CYP1B1* by PCR-RFLP.

Results. PCR-RFLP screening revealed that 30.8% of patients were positive for any one of the six mutations (376insA, 528G→A, 923C→T, 959G→A, 1449G→A, and 1514C→A), and 17.8% of the patients were found to have the rarely reported mutation R368H (1449G→A). All mutations were confirmed by DNA sequencing.

Conclusions. The results suggest extensive allelic heterogeneity in the Indian patients with PCG, with the predominant allele being R368H among the 146 Indian patients tested. It appears possible to use this approach for carrier detection in pedigrees with a positive family history and in population screening. The approach also offers a method for rapid screening of potential carriers and affected individuals. (*Invest Ophthalmol Vis Sci.* 2003;44:4200–4203) DOI:10.1167/iovs.02-0945

Primary congenital glaucoma (PCG) manifests at birth or in early infancy. The phenotype is characterized by elevated intraocular pressure, resulting in photophobia, corneal clouding, and enlargement of the globe, which, if left untreated, results in optic nerve damage and subsequent permanent loss of vision. The incidence of PCG varies geographically. Its incidence is as low as 1 in 10,000 persons in Western countries,[1] and as high as 1 in 1,250 in the Slovak population.[2] In Saudi Arabia, the reported incidence is 1 in 2,500,[3] whereas in the state of Andhra Pradesh in India, the incidence is estimated to be 1 in 3,300.[4] The high incidence in the Eastern populations is thought to be due to consanguineous marriage within these communities. Because PCG is mainly a congenital disorder, early and reliable diagnosis of the disease is vital, so that appropriate and prompt medical and surgical interventions can be initiated in time. This could in turn prevent unwanted visual loss, hence saving the vision in the child.

An autosomal recessive mode of inheritance pattern is well documented for PCG. Even though three different loci have been mapped for PCG[5,6] (Stoilov IR, et al. *IOVS* 2002;43:ARVO E-Abstract 3015), mutations in the *CYP1B1* gene (GLC3A locus[5]) is the most predominant[7] and is reported in various ethnic backgrounds.[7–20] An additional PCG locus, GLC3B,[6] has been mapped to the short arm of chromosome 1, region 36, and a third locus, GLC3C (Stoilov IR, et al. *IOVS* 2002;43:ARVO E-Abstract 3015), to 14q24.3, but the genes have not been identified in these two loci. Recently, we showed the association of *CYP1B1* with PCG in the Indian population[18] and detected five distinct mutations.

Although genetic heterogeneity has been shown for PCG, homogeneity in phenotype as well as genotype (E387K) has been reported in the Slovakian Romany people, and common haplotypes (G61E, D374N, R469W) have been associated with the Saudi Arabian population.[11,12] Inbreeding and consanguinity are prevalent in these communities, as in India. Thus, it is of interest to determine which haplotypes are present in the Indian patients. Against this background, we now describe the results of screening for the known mutations in a cohort of 138 pedigrees of 146 patients, by using PCR-RFLP–based simple diagnostic methods.

Materials and Methods

Selection and Evaluation of Study Subjects

The study protocol adhered to the tenets of the Declaration of Helsinki. After receiving due informed consent and appropriate clearance from the institutional review board, we recruited 146 patients for the study who were members of 138 pedigrees. The patients were completely unbiased with respect to sex, consanguinity, and familial incidence of the disease. All were clinically evaluated, and diagnosis of PCG was determined by examination with slit lamp biomicroscopy and gonioscopy, measurement of intraocular pressure, and perimetry, in some cases. Blood samples were collected over a period of 2 years in the Children's Eye Care Centre at the Institute. Seventy ethnically matched normal individuals served as control subjects.

Mutation Screening of *CYP1B1*

Genomic DNA was extracted from the peripheral leukocytes of all patients with PCG and control subjects. The translated region (1.6 kb) spanning exons II and III of the gene for cytochrome P4501B1 (*CYP1B1*)[21] from patients and control subjects were amplified by using three sets of primers, as described earlier.[18]

PCR-Restriction Fragment Length Polymorphism and Direct Sequencing

The PCR-RFLP methods described earlier[18] were followed, along with an *Hin*6I (MBI Fermentas, Vilnius, Lithuania) restriction enzyme–based RFLP for 1514C→T (R390C) mutation. DNA samples from 70 volun-

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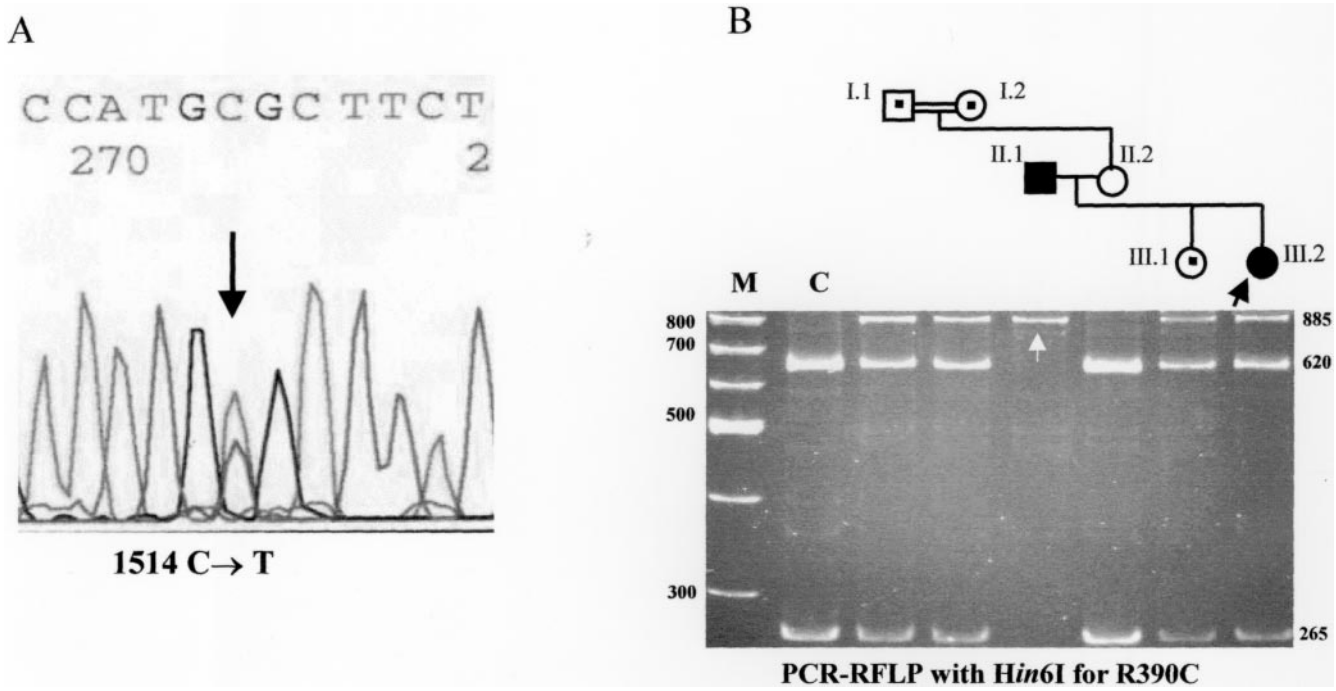

FIGURE 1. (**A**) Electropherogram of the sense strand of genomic DNA from PCG proband, with novel heterozygous missense mutation. Note the heterozygous change 1514C→T (R390C) in the mutant allele of the proband (*arrow*). (**B**) PCR-RFLP analysis of cosegregation of a mutant allele with a disease phenotype in a PCG pedigree. *Filled square*: affected individuals; *filled circle*: unaffected individuals; *arrow*: the proband. Males and females are shown by *squares* and *circles*, respectively. *Left*: DNA molecular weight marker in base pairs; *right*: allele sizes; *lane C*: control; *arrowhead*: mutant allele. Restriction site change and mutation (nucleotide as well as aa changes) are shown at the *bottom* of the gel. The 885-bp amplification product generated from primer pair 3F/3R[19] was cleaved by *Hin*6I into two fragments of sizes 620 and 265 bp in unaffected individuals. The C1514T mutation in the affected individuals abolished the *Hin* 6I site, and the resultant uncut 885-bp mutant allele segregated along with the disease phenotype. The "normal" *Hin*6I cleavage products present in affected individuals were generated from the nonmutated *CYP1B1* allele in these individuals.

tary donors, without a history of systemic and eye disorders, were used in control experiments. PCR-RFLP–positive samples were sequenced (for reconfirming the respective mutations) using an automated DNA sequencer (Big Dye Terminator cycle sequencing, ABI Prism 3700; Applied Biosystems, Foster City, CA).

RESULTS

Identification of a Novel Pathogenic Mutation

Direct sequencing of the complete coding region of a proband's DNA from two generations of an affected pseudodominant family (005) revealed a compound heterozygous missense mutation. The first one was a known mutation,[12,18,20] a G→A substitution at base pair position 1449, leading to the amino acid (aa) change arginine to histidine at position 368 (R368H), whereas the second mutation was a novel sequence variation, a C→T substitution at base pair 1514 (Fig. 1A), causing the change arginine to cysteine at position 390 (R390C). The father (Fig. 1B, II.1) in the same family, also affected by PCG, was homozygous for the novel mutation 1514C→T. The grandparents (I.1 and I.2) as well as the unaffected sibling (III.1) were heterozygous (carriers) for the mutation. Both mutations, R368H and R390C were found in exon III and resulted in loss of restriction sites *Taa*I and *Hin*6I respectively. The cosegregation of mutations in the family was ascertained by using the PCR-RFLP method. In this pedigree, the grandparents (I.1 and I.2) had a consanguineous marriage, whereas the parents (II.1 and II.2) were nonconsanguineous (Fig. IB).

PCR-RFLP Analyses of Six Pathogenic Mutations

PCR-RFLP analyses were performed for all six mutations: 376insA, 528G→A, 923C→T, 959G→A, 1449G→A, and 1514C→T. Of the 146 patients in 138 pedigrees, 45 patients in 37 pedigrees were positive for one of these six mutations. All the PCR-RFLP–positive samples were subsequently sequenced to confirm the mutation. More than 30% of the patients were carriers of the respective mutation, as revealed later by sequencing. Among the six mutations, R368H was the predominant PCG allele in this cohort, and 17.8% of the patients were found to be either homozygous or heterozygous for this mutation.

DISCUSSION

PCG is a clinically and genetically heterogeneous disorder, mainly inherited as an autosomal recessive disease, that occurs among various populations of the same ethnic background. Recent molecular genetic studies in various ethnic groups, such as Turkish, Hispanic, Saudi Arabian, Romanian, Brazilian, Canadian, Japanese, Pakistani, German, Lebanese, and Indian, revealed several mutations in the coding region of *CYP1B1*. All these studies have so far reported approximately 44 different mutations in the entire coding region of *CYP1B1*,[17–20] and the mutations' heterogeneity varies with the population.

The homogeneity–heterogeneity pattern varies with ethnic groups, as does the phenotypic uniformity of the condition. Whereas the Slovak Romany cases showed allelic homogeneity and phenotypic uniformity,[11] other population studies reported high clinical and allelic heterogeneity. Among these groups, higher homogeneity was present in the Saudi Arabian population (with 72% having the G61E allele and 12% the R469W allele[12]), whereas other populations demonstrated increased genetic heterogeneity. The homogeneity reflects the higher rate of inbreeding in this population. Our PCR-RFLP

TABLE 1. Distribution of Six Mutations in Consanguineous and Nonconsanguneous, Pedígrees

Pedigree ID	Mutation	Consanguineous/ Nonconsanguineous
0001 (2)	P193L (h)	Consanguineous
	E229K (h)	
0002	R368H (H)	Consanguineous
0004 (2)	376Ins A H)	Consanguineous
0005 (2)	R368H (h)	Nonconsanguineous
	R390C (h)	
0006 (2)	R368H (H)	Consanguineous
0011	G61E (H)	Consanguineous
0012 (2)	R390C (H)	Consanguineous
0017	R368H (H)	Nonconsanguineous
0018	R390C (H)	Consanguineous
0022	R368H (H)	Consanguineous
0024	E229K (h)	Consanguineous
0025	R368H (h)	Nonconsanguineous
0035 (2)	R368H (H)	Consanguineous
0037	E229K (h)	Consanguineous
0039	R368H (h)	Nonconsanguineous
0040	R368H (H)	Consanguineous
0051	R368H (H)	Nonconsanguineous
0057	E229K (h)	Consanguineous
0058	G61E (H)	Consanguineous
0067	R368H (H)	Consanguineous
0069	P193L (h)	Nonconsanguineous
0071 (2)	R368H (H)	Nonconsanguineous
0075	R368H (h)	Nonconsanguineous
0076	R368H (H)	Consanguineous
0079	R368H (H)	Nonconsanguineous
0092	R390C (H)	Nonconsanguineous
0093 (2)	G61E (H)	Consanguineous
0095	R368H (h)	Nonconsanguineous
0100	R368H (h)	Consanguineous
0116	E229K (h)	Consanguineous
0125	E229K (h)	Nonconsanguineous
0130	R368H (H)	Nonconsanguineous
0136	R368H (H)	Nonconsanguineous
0137	R368H (H)	Nonconsanguineous
0143	R390C (H)	Nonconsanguineous
0144	R368H (H)	Nonconsanguineous
0150	R368H (H)	Nonconsanguineous

h, Heterozygous mutation; H, homozygous mutation; (2) two patients in the same pedigree.

screening, for six distinct alleles, in a cohort of 146 patients in 138 pedigrees showed a frequency of 16.21% for allele R368H.

This mutation has so far been reported in only a very few PCG families from Saudi Arabia and Brazil and at a very low frequency.[12,20] In the present study, however, based on the mutation screening, we found it to be a predominant allele associated with PCG in India. This is the highest reported frequency of this mutation of all ethnic backgrounds studied so far, indicating that the frequency of the mutation could vary based on the ethnic origin as well as geographical location. Sequence analysis of the remaining families negative for these six mutations should to be performed to determine whether there are any other predominant alleles in Indian patients with PCG. The possibility of locus heterogeneity in Indian patients with PCG also should be explored further.

Ethnically matched population screening of 140 chromosomes for these six mutant alleles showed 6.4% and 0.7% carriers for E229K and R368H, respectively. The present data are unlikely to be due to a possible founder effect for the predominant R368H allele, because patients were from ethnically as well as geographically diverse groups in India. Also, these mutations are equally distributed in both consanguineous and nonconsanguineous pedigrees (Table 1). Of the total families recruited, 51.5% belonged to the nonconsanguineous group. Sporadic cases accounted for 80%, and bilateral 88%. Males accounted for 57% of the affected individuals.

Mutations at codon 390, where arginine is changed to either histidine or serine, have been reported.[10,12] This is the first report of arginine changing to cysteine at the same codon. *Hin*6I-based PCR-RFLP can be therefore used to detect any of these mutations at this codon. Although mutations at codons 368 and 390 have been reported earlier, our report of this combination of mutations is novel, as is the sequence variation in codon 390. This two-generation affected family is also interesting in that whereas the affected father (II.1) had a homozygous mutation (R390C), the mother (II.2) was a carrier of one of the alleles (R368H; data not shown), and the proband (affected child 111.2) had a compound heterozygous mutation (R390C and R368H). Consistent with the autosomal mode of inheritance, the affected individuals (II.1 and III.2) in this pedigree had two mutant alleles from their respective parents; the mutations were completely penetrant.

Consanguineous marriages and marriages within a distinctive caste or community increase the predisposition and incidence of recessively inherited and multifactorial diseases in the population. It is important to know the carrier status of unaffected members in the families with a positive history to identify the at-risk individuals in such families. Earlier studies have reported that 30% to 35% of blind children in India show a history of hereditary disorder.[22] In the higher socioeconomic levels of developed countries, 22% to 55% of children with genetic disease show an autosomal recessive mode of inheritance.[23] Hence, development of techniques such as PCR-RFLP, the amplification refractory mutation system (ARMS)-PCR, allele-specific oligonucleotide (ASO) blot analysis, and other methods are important for segregation analysis in families with a positive history and for possible prenatal diagnosis and genetic counseling. Moreover, because this disease carries high and life-long morbidity, development of strategies that are noninvasive, rapid, and cost-effective are very useful in screening populations with a high incidence of this disease. This could in turn help in identifying individuals at risk and also assist in preventing unwanted visual loss in the afflicted families. An earlier study on thalassemia major in a Sardinian population showed that genetic screening and counseling helped to reduce the incidence from 1 in 250 live births to 1 in 4000.[24]

Similarly, the molecular diagnostic methods used in the current study could be used as an added clinical tool in decreasing the incidence of the devastating binding disorder PCG in the afflicted families.

Moreover, our clinical experience in PCG has shown that early diagnosis, along with prompt medical and surgical interventions, result in better prognosis.[25] We thus see the use of the PCR-RFLP molecular diagnosis described in this study as a tool to identify the disease early and to initiate appropriate and prompt treatments, especially in patients with late manifestation and positive family history. Based on this study, we suggest that PCG mutation screening in India should be performed based on the prevalence of the mutation.

Our study shows that 31% of the patients studied had one of the six mutations that we sought in the screening. Whereas only direct screening or methods such as denaturing HPLC can identify all mutations in *CYP1B1,* R368H appears to be the predominant mutant allele causing PCG in the population studied herein. Given this lead, we believe that screening for this mutation should be given priority, and subsequently the other reported mutations should be screened for in the order of prevalence. Thus, the data derived from this study highlight the use of a rapid screening system for mutations that could assist the medical community in the management of this devastating condition

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