Publications and Presentations
LIST OF PRESENTATIONS

1. Presented a poster entitled, “Involvement of Myocilin gene across different glaucoma phenotypes among Indian patients” at Asia-ARVO meeting, 2007 held at Singapore.

2. Presented a poster entitled, “Analysis of Methylene tetrahydrofolate reductase polymorphisms in Indian Primary open angle glaucoma patients,” at the 15th Annual meeting of Indian Eye research group, 2006, Hyderabad.


4. Presented a poster entitled, “Analysis of Interleukin-1 gene polymorphisms in Indian Primary open angle glaucoma patients,” at the 14th Annual meeting of Indian Eye research group 2005, Hyderabad.

5. Presented a poster entitled, “Involvement of Myocilin gene across different glaucoma phenotypes among Indian patients” at the 73rd annual meeting of Society for Biological Chemists India (SBCI), held at the GB Pant Agricultural University, Pantnagar, India, 2004.

LIST OF PUBLICATIONS


Glaucoma-Associated CYP1B1 Mutations Share Similar Haplotype Backgrounds in POAG and PACG Phenotypes

Subhabrata Chakrabarti, Koilkonda R. Devi, Sreelatha Komatireddy, Kiranpreet Kaur, Rajul S. Parikh, Anil K. Mandal, Garudadri Chandrasekhar, and Ravi Thomas

PURPOSE. To understand the involvement of the CYP1B1 gene in cases of primary open-angle (POAG) and primary angle-closure (PACG) glaucomas and obtain the haplotype background of these mutations.

METHODS. The entire coding region of CYP1B1 was screened by resequencing in 224 unrelated cases of POAG (n = 154) and PACG (n = 90) and 200 ethnically matched normal control subjects from Indian populations. Six intragenic single nucleotide polymorphisms (SNPs) in CYP1B1 (−13T>G, R486G, A1198, V432L, D449D, and N453S) were used to generate haplotype data for the cases and controls and linkage disequilibrium (LD) and haplotype analysis were performed with Haploview software, which uses the EM (expectation-maximization) algorithm.

RESULTS. The frequency of CYP1B1 mutations was higher among POAG (18.6%; 95% CI, 12.9–26.1) than PACG (11.1%; 95% CI, 6.1–19.3) cases. There was a marked allelic heterogeneity, and the Arg368His was the most prevalent mutation across both the phenotypes. The spectrum of CYP1B1 mutations was largely similar across different POAG populations. Haplotype groups with single nucleotide substitutions indicated the CC-G-G-T-A to be a risk haplotype associated with CYP1B1 mutations in POAG (P = 0.006) and PACG (P = 0.045), similar to that observed in cases of primary congenital glaucoma worldwide.

CONCLUSIONS. The results demonstrate an involvement of CYP1B1 in a proportion of POAG and PACG cases that should be explored further. The similar haplotype background of these mutations is indicative of their common origin across multiple glaucoma phenotypes. (Invest Ophtalmol Vis Sci. 2007;48:5439–5444) DOI:10.1167/iovs.07-0629

Glaucoma comprises a group of clinically and genetically heterogeneous optic neuropathies characterized by a progressive loss of vision and is the second leading cause of irreversible blindness worldwide. Based on gonioscopic findings, primary glaucomas are classified as primary open-angle (POAG) and primary angle-closure glaucoma (PACG). Both have characteristic optic nerve head changes, degeneration of retinal ganglion cells, and visual field loss, but PACG also has a closed angle or peripheral anterior synchia (PAS) on gonioscopy. Both of these conditions may be associated with elevated intraocular pressure (IOP) due to the obstruction of outflow. POAG affects 33 million worldwide and is more common in the West, whereas PACG is relatively more common among Asian populations.

Mutations Share Similar Haplotype Backgrounds in POAG and PACG Phenotypes

Glucoma being a complex disease would be attributed to multiple gene variants with various magnitudes of effect. Although the human cytochrome P450 gene CYP1B1 (OMIM 601771) has been implicated in primary congenital glaucoma (PCG; OMIM 231300) worldwide, it has been relatively less explored in POAG and not at all in PACG. An initial study implicated the involvement of CYP1B1 with MYOC through a digenic mechanism in a family with juvenile-onset open angle glaucoma (JOAG) and suggested that CYP1B1 is a modifier of MYOC expression. It was also observed that affected subjects harboring a mutant CYP1B1 allele in this family had an earlier age at onset than those with only a mutant MYOC allele. These findings led to the screening of CYP1B1 as a candidate gene among the patients with POAG and large among those with JOAG. The frequency of CYP1B1 mutations varied in patients from Canada (5.0%), France (4.6%), Spain (10.9%), Eastern India (4.5%), and Southern India (10.8%). The differences in mutation frequency could be partly explained by the definition of disease used in these studies. The Canadian patients had JOAG whereas the French patients had POAG, but elevated IOP was not an inclusion criterion, similar to studies from Eastern and Southern India. The results from these studies indicate a minor involvement of CYP1B1 among JOAG and late-onset POAG cases and suggest a possible role of this gene in glaucoma pathogenesis.

We have reported the extent of CYP1B1 mutations along with their structural properties in PCG. We have also demonstrated a global clustering of these mutations on specific haplotype backgrounds, irrespective of geographic location, that could be useful in predictive testing. Herein, we report an extensive screening of the CYP1B1 gene in a cohort of patients with POAG or PACG from India, to determine its mutation spectrum and understand the haplotype backgrounds of these mutations.
METHODS

Clinical Details of the Subjects

The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board. The cohort comprised unrelated, consecutive patients with JOAG (n = 30), POAG (n = 104), PACG (n = 90), and 200 normal control subjects, who were seen at the L. V. Prasad Eye Institute (Hyderabad, India) between January 2002 and March 2007. The diagnoses of POAG and PACG were independently confirmed by two surgeons based on the following inclusion and exclusion criteria.

POAG (Including JOAG)

The diagnosis of POAG was based on open angles on gonioscopy, an IOP >21 mm Hg, and characteristic optic disc changes and corresponding visual field defects in patients >35 years of age. Visual field defects were considered to be glaucomatous if they were consistent with optic disc damage and met at least two of the criteria laid out by Anderson and Patella. The presence of a visual field defect required confirmation by a repeatable field performed within 2 weeks of the first reliable visual field result showing the defect. The field defects were further classified as mild, moderate, or severe. Such findings in patients between 5 and 35 years of age were labeled as JOAG. As the visual acuity ranged from 20/20 to 20/40, and their IOP was controlled, clinicopathologic diagnosis of glaucoma was made.

Primary Angle-Closure Glaucoma

PACG was defined as the presence of optic disc and visual field changes characteristic of glaucoma, along with appositional or synechial primary angle-closure (PAC) in patients older than 18 years. The visual field defects as defined in POAG. PAC (appositional) was defined as increased IOP (≥21 mm Hg) associated with nonvisibility of the filtering trabecular meshwork for more than 180°, in the absence of PAS, disc damage, or field changes. PAC (synechial) was defined as the presence of PAS with nonvisibility of the filtering trabecular meshwork for more than 180°, with or without increased IOP (>21 mm Hg), without disc damage or demonstrable field defects. The presence of even a single PAS in an angle with more than 180° of nonvisibility of trabecular meshwork was considered diagnostic of PAC. Other causes of synechiae were excluded.

Ocular hypertension, normal-tension glaucoma, lens-induced glaucoma, neovascular and pseudoexfoliation glaucoma, and secondary open-angle glaucoma were excluded. Other ocular diseases that can lead to secondary glaucoma were also excluded.

Normal adult individuals without any signs or symptoms of glaucoma and other systemic diseases served as control subjects. Their visual acuity ranged from 20/20 to 20/40, and their IOP was <21 mm Hg. Clinical examination on stereo biomicroscopy did not reveal any changes in the optic disc suggestive of glaucoma. The patients and controls were matched with respect to their ethnicity and geographical region of habitat.

Molecular Analysis

Peripheral blood samples (5–10 mL) were collected from each subject by venipuncture, with prior informed consent. DNA was extracted by standard protocols and the entire coding region of CYP1B1 was amplified using appropriate oligonucleotide primers and PCR protocols, as published earlier. The amplicons were purified (SigmaSpin columns; Sigma-Aldrich, St. Louis, MO) and bidirectionally sequenced using dye termination chemistry (BigDye on a 3100 DNA Analyzer; Applied Biosystems, Inc. [ABI], Foster City, CA), according to the manufacturer’s protocol. Sequencing analysis software was used to read the individual sequences. Six mutations (G61E, Y81N, Q144R, P193L, E229K, and R368H) were further confirmed by restriction digestion of the amplicon with appropriate restriction enzymes as published earlier, whereas the remaining five mutations were verified by resequencing. Multiple sequence alignment of the human CYP1B1 protein was performed along with other CYP1 protein across different families, to check for the conservation of the residues.

The SIFT (sorting tolerant from intolerant) homology tool (http://blocks.fhcrc.org/sift/SIFT.html) provided in the public domain by the Fred Hutchinson Cancer Research Center, Seattle, WA) was used to assess the effect of the substituted amino acid on the CYP1B1 protein, and a threshold score of less than 0.05 was considered to be deleterious to the protein.

Statistical Analysis

The maximum-likelihood estimates of allele frequencies, Hardy-Weinberg equilibrium, and haplotype frequencies were estimated from the genotype data at six single-nucleotide polymorphism (SNP) loci using haplovview software, which uses the EM (expectation-maximization) algorithm. Pair-wise linkage disequilibrium (LD) between the individual SNPs was calculated using the LD-plot function of this software. The odds ratios were calculated, to assess the risk of the individual genotypes at all six SNP loci. Clinical parameters, such as IOP at presentation, cup-to-disc ratio, and visual field defects for the worst eye were considered when correlating the genotype with the phenotype. All calculations were performed with commercial software (SPSS ver. 14; SPSS, Chicago, IL).

RESULTS

Mutation Screening of CYP1B1 in POAG and PACG

The study cohort conformed to Hardy-Weinberg equilibrium. A total of 11 CYP1B1 mutations were observed, of which 4 (Q144R, W434R, F445C, and g.8148-8152del5bp) were novel. The overall spectrum of CYP1B1 mutations observed in POAG and PACG is demonstrated in Figure 1 (the electropherograms...
of all 11 mutations are provided in Supplementary Fig. S1, online at http://www.iovs.org/cgi/content/full/48/12/5439/DC1. The frequency of mutations was higher in POAG (18.6%; 25/134) than in PACG (11.1%; 10/90). Arg368His was the most prevalent mutation across both the phenotypes, similar to earlier studies of PCG from India.6,22 Further details are provided in Table 1. Allelic heterogeneity was relatively more in POAG than in PACG. SIFT scores indicated a deleterious effect for all the mutations except E229K.

The cosegregation of the heterozygous mutant allele was observed in only three families for the three mutations (G61E, Q144R, and P193L). DNA samples were unavailable from the relatives of the probands in the remaining cases harboring CYP1B1 mutations. Except for the Q144 residue, multiple sequence alignment indicated a strong conservation of the wild-type residues for all amino acids across multiple CYP1 families (Supplementary Fig. S2, http://www.iovs.org/cgi/content/full/48/12/5439/DC1).

Homozogosity of the mutant allele was noted in a JOAG case with G61E and in a POAG case with P193L mutations. There was only a single JOAG case with a compound heterozygous mutation (G61E and R368H). All other mutations were observed in the heterozygous state in JOAG (5/7), POAG (17/18), and all PACG.

The CYP1B1 mutation frequencies were different across all the studies performed on POAG in Indian populations.6,20 Of interest, the investigators in the study from Southern India found a carrier rate of 6.4% and 0.7% for the E229K and the R368H mutations, respectively, in their control populations21 that was not observed in the cohorts from Eastern India20 or in the present study.

Table 2 provides a comparison of JOAG, POAG, and PACG cases. As is evident from the table, JOAG cases had a higher prevalence of CYP1B1 mutations than did POAG cases. There was no significant difference in age at onset among JOAG cases with (20.1 ± 8.78 years) and without (20.9 ± 8.31 years) CYP1B1 mutations (P = 0.781). JOAG cases had a significantly higher mean IOP at presentation than did POAG cases, with and without CYP1B1 mutations (P < 0.001). The mean IOPs were similar among the JOAG and PACG cases with and without mutations. CYP1B1 mutations did not seem to be associated with disc changes (P = 0.192) and severe visual field defects (P = 0.417) in any of these phenotypes.

**Linkage Disequilibrium and Haplotype Analysis at the CYP1B1 Locus**

Six intragenic SNPs were typed at the CYP1B1 locus, to generate haplotypes among the cases and controls. Pair-wise LD analysis indicated strong LD (D" = 1) at two clusters, between three SNPs (−13T>G, C48R, and A119S) and between the two SNPs V432L and D449D (data not shown). The measure of LD between the other SNPs was similar to that in an earlier study.55

Four different haplotypes (with frequency >5%) were generated with these six SNPs in cases and controls. There were no significant differences in the haplotype frequencies when all POAG and PACG cases were compared with the controls (Tables 3, 4). Reanalysis of the cases with respect to their mutation status indicated a significantly higher frequency of the C-C-G-G-T-A haplotype in both POAG (P = 0.006) and PACG (P = 0.043) cases with CYP1B1 mutations (CYP1B1") than controls. However, there was no observable difference in frequencies of the other haplotypes among cases and controls. The significantly higher frequency of the C-C-G-G-T-A haplotype in POAG (P = 0.001) and PACG (P = 0.020) cases with CYP1B1 mutations was consistent, even when compared with cases without (CYP1B1") mutations (data not shown).

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**Table 1. Details of CYP1B1 Mutations Observed in Cases of POAG and PACG in the Present Study and Other Populations**

<table>
<thead>
<tr>
<th>Amino Acid Change</th>
<th>Location</th>
<th>Nucleotide Change</th>
<th>Exon</th>
<th>Number of Patients with the Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.3987G</td>
<td>G</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.4046T</td>
<td>T</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.4236A</td>
<td>A</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.4381C</td>
<td>C</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.4491G</td>
<td>G</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.7940G</td>
<td>G</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.8147C</td>
<td>C</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.8171T</td>
<td>G</td>
<td>0/30</td>
</tr>
<tr>
<td>—</td>
<td>Exon II</td>
<td>g.8148-8152del5bp</td>
<td></td>
<td>0/30</td>
</tr>
</tbody>
</table>

**Table 2. Mutations in POAG and PACG 5441**

<table>
<thead>
<tr>
<th>Locus, Mutation</th>
<th>Previous Location, Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.3987G➔A</td>
<td>Saudi Arabia,30,32 Kuwait34</td>
</tr>
<tr>
<td>g.4046T➔T</td>
<td>Spain,19 France18 Germany35</td>
</tr>
<tr>
<td>g.4236A➔C</td>
<td>India22 Brazil37 Turkey90</td>
</tr>
<tr>
<td>g.4381C➔T</td>
<td>Spain,19 France18 Germany35</td>
</tr>
<tr>
<td>g.4491G➔G</td>
<td>Spain,19 France18 Germany35</td>
</tr>
<tr>
<td>g.7940G➔T</td>
<td>India22 Brazil37 Turkey90</td>
</tr>
<tr>
<td>g.8147C➔T</td>
<td>Saudi Arabia,30,32 India22</td>
</tr>
<tr>
<td>g.8171T➔G</td>
<td>Saudia Arabia,30,32 Egypt23</td>
</tr>
<tr>
<td>g.8148-8152del5bp</td>
<td>Total 7/30</td>
</tr>
</tbody>
</table>
TABLE 2. Distribution of Mean Ages at Onset and IOPs at Presentation among JOAG, POAG, and PACG Groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>JOAG Cases with CYP1B1 Mutations (n = 7)</th>
<th>JOAG Cases without CYP1B1 Mutations (n = 23)</th>
<th>POAG Cases with CYP1B1 Mutations (n = 18)</th>
<th>POAG Cases without CYP1B1 Mutations (n = 86)</th>
<th>PACG Cases with CYP1B1 Mutations (n = 10)</th>
<th>PACG Cases without CYP1B1 Mutations (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Frequency of Cases (95% CI)</td>
<td>23.3 (11.8–40.9)</td>
<td>76.7 (59.1–88.2)</td>
<td>17.3 (11.2–25.7)</td>
<td>82.7 (74.3–88.7)</td>
<td>11.1 (6.1–19.2)</td>
<td>88.9 (80.7–93.8)</td>
</tr>
<tr>
<td>Age at Onset (mean years ± SD)</td>
<td>20.1 ± 8.78</td>
<td>20.9 ± 8.31</td>
<td>51.3 ± 12.22</td>
<td>54.1 ± 10.56</td>
<td>57.4 ± 12.43</td>
<td>54.4 ± 11.18</td>
</tr>
<tr>
<td>IOP at Presentation (mean mm Hg ± SD)</td>
<td>37.3 ± 8.45</td>
<td>34.1 ± 8.33</td>
<td>24.8 ± 3.04</td>
<td>27.1 ± 6.26</td>
<td>32.6 ± 16.73</td>
<td>32.9 ± 10.11</td>
</tr>
</tbody>
</table>

DISCUSSION

The present study provides a mutation spectrum of the CYP1B1 gene in POAG and PACG. The involvement of CYP1B1 highlights its role as a potential candidate in disease pathogenesis that should be explored further. We observed a higher proportion of mutations in POAG in the present cohort than in other populations (Table 5). The spectrum of mutations observed in the present cohort was largely similar to that in the POAG populations in France, Spain, and India18–21 except for the four novel mutations observed in this study, all other variants were observed earlier in patients with PCG in India and other countries (Table 1).

To the best of our knowledge, this is also the first study to report the involvement of CYP1B1 in PACG. Although there are differences in the mutation frequencies of CYP1B1 across JOAG, POAG, and PACG, the 95% CI of these frequencies overlap (Table 2). It would also be interesting to investigate the role of CYP1B1 mutations in PAC, where there is no damage to the disc and visual fields, as opposed to PACG, where disc and fields are affected. Although that would be the subject for further study, we have screened 16 PAC cases, and none of them had CYP1B1 mutations (95% CI, 0–17.57). Although this was not significantly different from PACG cases with CYP1B1 mutations (as 95% CI overlaps; Table 2), the sample size was too small to draw any conclusion. Further studies on a larger sample are needed to determine CYP1B1 involvement in PAC.

Although we observed a higher mutation frequency of CYP1B1 in POAG than in other populations, our results are not very different from those in a Spanish population,19 when we look at the confidence intervals in these two studies (Table 5). The frequency differs, however, from those in French and other Indian populations. These differences may be partially attributable to the definitions of POAG used in these studies.18,20,21 In contrast to the French and other Indian studies, we used raised IOP (> 21 mm Hg) in the definition of POAG and PACG, as it was our inclusion criterion. It is well known that CYP1B1 is a major candidate gene in PCG that is associated with increased IOP.14–16 Hence, this could partially explain the higher frequency of CYP1B1 mutations in our patient cohort. The report on the Spanish patients with POAG19 also included increased IOP (> 21 mm Hg) as a major inclusion criterion, and, as just noted, their mutation rates are not very different from ours (Table 5).

It is interesting to note that the prevalent mutation was different across all previously reported POAG populations (Table 5). Also, the frequency of heterozygous mutations was similar across these studies. Although the R368H mutation was common in patients in both the Indian and Canadian studies, it...
was noted in only 2 of the 60 patients with JOAG in the Canadian report. One of the Canadian patients with the R368H mutation had an East Indian/Guyanese ancestry, but we were not able to determine whether this patient shared a common haplotype background with the Indian patient due to unavailability of data.

The median age at onset of the patients with POAG in the present cohort was similar to that of the French sample, but was significantly lower than that of the Spanish patients with POAG. The median age of the Canadian patients was significantly lower, as no cases older than 40 years were enrolled. Another study on patients with POAG from Eastern India reported a mutation frequency (4.5%) similar to that of the French population but a higher mean age (52.43 ± 19.33 years) than that of our cohort. Of interest, this mutation was also found on the G-T-C-C-A haplotype, similar to that observed among the PCG patients from Ecuador, Saudi Arabia, and Morocco. The E229K mutation that was observed on the G-T-C-C-A haplotype in cases of POAG and PACG with CYP1B1 mutations was not possible due to the unavailability of data from other populations. Based on the present analysis we speculate that the presence of specific CYP1B1 mutations on specific haplotype backgrounds in PCG worldwide and in patients with POAG and PACG in the present cohort is indicative of common founders. The mutations on these haplotypes would have migrated across different geographical regions due to population movements as reported in PCG in our previous study. Thus, glaucoma-associated CYP1B1 mutations share a similar haplotype background across POAG, PACG, and PCG.

Another interesting observation was the presence of CYP1B1 mutations on specific haplotypes that was earlier observed in PCG. We noted that C-G-G-T-A was the risk haplotype in cases of POAG and PACG with CYP1B1 mutations. These results were consistent (even after reanalyzing the data set) based on a five-locus haplotype (i.e., C-G-G-T-A), similar to previous studies in different PCG populations worldwide. On the other hand, the G-T-C-C-A haplotype that was largely associated with the unaffected controls and PCG cases without CYP1B1 mutations was similar in frequency in the POAG and PACG cases with CYP1B1 mutations and the controls (Tables 3, 4). In tune with our previous study on PCG, most of the mutations observed in the POAG and PACG clustered on the C-G-G-T-A haplotype. The R368H mutation, which was the prevalent mutation in POAG and PACG in the present study, similar to PCG in India, was found on the background of the C-G-G-T-A haplotype across all these phenotypes. Of interest, this mutation was also found on the same haplotype in Saudi Arabian and Brazilian PCG patients. The G61E mutation in POAG was also found on the G-G-T-A haplotype, similar to that observed among the PCG patients from Ecuador, Saudi Arabia, and Morocco. The E229K mutation that was observed on the G-T-C-C-A haplotype among patients with PCG in India and Germany was also seen to harbor the same mutation in POAG and PACG cases. Another striking similarity was the presence of the Y81N mutation in a case of POAG on the G-T-C-C-A haplotype. This mutation was also found on the same haplotype among German patients with PCG.

Similar to our earlier hypothesis on the evolution of CYP1B1 mutations, we confirm that there is a strong clustering of these mutations on specific haplotype backgrounds, irrespective of geographical location. A larger proportion of mutations were seen on the C-G-G-T-A haplotype and a smaller proportion on the G-T-C-C-A haplotype, further confirming the former to be an ancient haplotype and the latter to be a recent haplotype. A formal haplotype comparison among other POAG cases with CYP1B1 mutations was not possible due to the unavailability of data from other populations. Based on the present analysis we speculate that the presence of specific CYP1B1 mutations on specific haplotype backgrounds in PCG worldwide and in patients with POAG and PACG in the present cohort is indicative of common founders. The mutations on these haplotypes would have migrated across different geographical regions due to population movements as reported in PCG in our previous study. Thus, glaucoma-associated CYP1B1 mutations share a similar haplotype background across POAG, PACG, and PCG.

The role of CYP1B1, particularly in retinoic acid synthesis is pivotal during embryonic development. Recent studies on chick embryogenesis have demonstrated its importance in the dorsoventral patterning of the neural tube that is consistent with its endogenous expression. Several in vitro and in vivo studies in lower organisms have demonstrated the sites of expression of CYP1B1 at different stages of development in the anterior retina and anterior segment of the eye. Although these studies have provided convincing evidence of the possible role of CYP1B1, its actual molecular mechanism leading to glaucoma in humans has to be deciphered. Although the functions of CYP1B1 mutations leading to POAG and PACG remain to be characterized, it is nevertheless an important candidate gene that should be screened in patients with glaucoma worldwide, to establish its involvement in the disease’s pathogenesis.

**Acknowledgments**

The authors thank all the patients and volunteers for their participation in this study.

**References**


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**Table 5. Characteristics of CYP1B1 Mutations across Different POAG Populations**

<table>
<thead>
<tr>
<th>Populations (cases)</th>
<th>Median Age at Onset (Years Range)</th>
<th>% Frequency of CYP1B1 Mutation (95% CI)</th>
<th>% Frequency of Heterozygous CYP1B1 Mutation (95% CI)</th>
<th>Prevalent CYP1B1 Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada (n = 60)17</td>
<td>23.6 (8-36)</td>
<td>5.0 (1.7-13.7)</td>
<td>66.6 (20.8-93.8)</td>
<td>R368H</td>
</tr>
<tr>
<td>France (n = 236)18</td>
<td>40 (13-52)</td>
<td>4.6 (2.6-8.2)</td>
<td>90.9 (62.3-98.4)</td>
<td>A443G</td>
</tr>
<tr>
<td>Spain (n = 82)19</td>
<td>59.9 (48-77)</td>
<td>11.0 (5.9-19.6)</td>
<td>100.0 (70.1-100.0)</td>
<td>Y81N</td>
</tr>
<tr>
<td>Eastern India (n = 200)20</td>
<td>NA*</td>
<td>4.5 (2.4-8.3)</td>
<td>88.9 (56.5-98.1)</td>
<td>S515L</td>
</tr>
<tr>
<td>Southern India (n = 251)21</td>
<td>NA*</td>
<td>10.7 (7.5-15.2)</td>
<td>92.5 (76.6-97.9)</td>
<td>E229K</td>
</tr>
<tr>
<td>Present study; India (n = 134)</td>
<td>46 (10-80)</td>
<td>18.6 (12.9-26.1)</td>
<td>88.0 (80.2-93.0)</td>
<td>R368H</td>
</tr>
</tbody>
</table>

* Not available.

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CYP1B1 Mutations in POAG and PACG 5443

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**Notes:**


ELECTROPHEROGRAMS OF CYP1B1 MUTATIONS

1. Y81N (c.587 TAC>AAC)
2. E229K (c.1031 GAA> AAA)
3. Q144R (c.777 CAG>C GG)
4. P193L (c.923 CCG>CTG)
5. G61E (c.528 GGA>G AA)

**Mutations:***
- Y81N: G to A at position 587
- E229K: A to T at position 1031
- Q144R: A to G at position 777
- P193L: G to C at position 923
- G61E: A to G at position 528
Supplementary Figure 1. Electropherograms of the eleven different CYP1B1 mutations observed in POAG and PACG. The wildtype sequences are provided on the panel above the boxes. The arrows indicate the point of substitution for panels 1-10 and the points of deletion in panel 11.
Gln48His is the prevalent myocilin mutation in primary open angle and primary congenital glaucoma phenotypes in India

Subhabrata Chakrabarti,1 Kiranpreet Kaur,1 Sreelatha Komatireddy,1 Moulinath Acharya,2 Koilkonda R. Devi,1 Arijit Mukhopadhyay,2 Anil K. Mandal,3 Seyed E. Hasnain,4 Garudadri Chandrasekhar,1 Ravi Thomas,3 Kunal Ray2

1Kallam Anji Reddy Molecular Genetics Laboratory and 2VST Centre for Glaucoma Care, L. V. Prasad Eye Institute, Hyderabad, India; 3Indian Institute of Chemical Biology, Kolkata, India; 4Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India

Purpose: Myocilin gene defects have been originally implicated in primary open angle glaucoma (POAG). Based on multiple reports for the occurrence of Gln48His mutation (c.144G>T; HGMD accession number CM023962) among Indian POAG patients, we wanted to estimate the prevalence of this mutation in primary open angle and primary congenital glaucoma (PCG) in India and assess its role in the causation of the disease.

Methods: Two hundred cases each of POAG and PCG were screened for the Gln48His mutation by RFLP (AccI) analysis of the PCR amplicons followed by confirmation of the c.144G>T change by direct sequencing.

Results: The Gln48His mutation was detected in 9 different glaucoma patients (four POAG and five PCG). While all four POAG cases were heterozygous, among PCG cases, four were heterozygous and one exhibited homozygous genotype for the mutation. One each of POAG and PCG patients was detected to be heterozygous for CYP1B1 mutation (c.1656C>T, Pro437Leu) and (c.1449G>A, Arg368His), respectively. None of the 300 ethnically matched normal controls contained either the MYOC or CYP1B1 mutation(s).

Conclusions: The myocilin mutation, Gln48His, represents an allelic condition involving a spectrum of glaucoma phenotypes in Indian populations, and could be a potential risk factor towards disease predisposition among patients of Indian origin. The study also highlights the role of MYOC as a candidate in different glaucoma subtypes that needs to be investigated further.

Glaucoma, the second leading cause of blindness worldwide, represents a group of disorders with varied clinical symptoms [1]. The underlying molecular mechanism is still unknown although 7 chromosomal loci (GLC1A to GLC1G) have been mapped for primary open angle glaucoma (POAG) and 3 (GLC3A to GLC3C) for primary congenital glaucoma (PCG), of which only GLC1A (Myocilin), GLC1E (Optineurin) and GLC3A (CYP1B1) have been characterized [1-3]. The myocilin gene (MYOC) exhibits a wide spectrum of mutations and accounts for 2-5% cases of POAG [1]. Some pathogenic mutations (e.g., Gln368Stop) are widely prevalent while others are recurrent (Gly252Arg, Gly367Arg, and Pro370Leu) in varying frequencies in different populations [1-3]. The limited studies done on Indian POAG patients suggest that the Gln48His mutation in MYOC recurs in different ethnic groups but is restricted to the people of Indian origin according to the published literature [4,5]. In this context, we attempted to investigate the prevalence of the myocilin mutation (Gln48His) among Indian glaucoma patients comprising of POAG and PCG cases.

METHODS

The study protocols adhered to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board. Two hundred cases each of POAG and PCG were recruited from the southern (37.5%), eastern (35.5%), western (13.5%), and northern (23.5%) parts of India.

Cases were enrolled as POAG on the basis of an elevated intraocular pressure of >21 mm Hg and/or glaucomatous disc changes in the presence of typical field defects, along with an open angle on gonioscopy and no other secondary causes. Cases of ocular hypertension were excluded from this category. On the other hand, PCG cases were included on the basis of an increased corneal diameter (>12.0 mm) along with raised intraocular pressure (>21 mmHg) and/or presence of Haab’s striae, or optic disc changes (where examination was possible). The ages of onset ranged from 0-1 years and symptoms of epiphora, photophobia, and rupture in the Descemet’s membrane were the corroborating factors. Three hundred ethnically matched normal individuals without any signs or symptoms of glaucoma and other systemic diseases served as controls. Their visual acuity ranged from 20/20 to 20/40 and IOP was <21 mm Hg. Clinical examination on stereo biomicroscopy did not reveal any changes in the optic disc suggestive of glaucoma.

Collection of blood samples and genomic DNA preparation, polymerase chain reaction (PCR), and the Gln48His
mutation screening by digesting the PCR amplicons with AccI restriction enzyme were done as described earlier [4]. The loss of the AccI site suggested presence of the mutation (c.144G>T) which was confirmed by direct sequencing. The patients containing the mutant MYOC allele was screened for the CYP1B1 mutation by direct sequencing as described earlier [6].

RESULTS & DISCUSSION
Among 200 POAG cases we identified 4 individuals carrying the MYOC Gln48His mutation (Table 1), including 3 mutants reported earlier [4]. One of the patients was also heterozygous for a CYP1B1 mutation (c.1656C>T; Pro437Leu) suggesting a digenic inheritance, as shown in a JOAG family [2], which could not be investigated further because one of the proband’s parents and his siblings were deceased. In addition, other studies from India have reported two other POAG cases harboring the same mutation [5]. These observations clearly establish that Gln48His is a common mutation among Indian patients which, however, has not yet been reported from any other population.

We also screened 200 PCG cases for the MYOC Gln48His mutation and identified 5 cases harboring the mutant allele (Table 1), which also included one homozygote (Table 2). Among Indian PCG cases about 40% are CYP1B1 mutants [6]. Interestingly, 4 of the 5 PCG cases harboring MYOC mutation lacked any CYP1B1 defect. The presence of Gln48His in the homozygous state in one PCG case devoid of any CYP1B1 mutation, and absence of the Gln48His mutation in 300 ethnically matched normal controls strongly argue for the role of the mutant MYOC protein causing PCG. However, no study has yet described the functional mechanism for the involvement of MYOC in PCG; although an earlier study showed that heterozygous MYOC and CYP1B1 mutations cause JOAG through a digenic mechanism. It was also hypothesized that CYP1B1 may be a modifier of MYOC expression and these two genes might act through a common biochemical pathway [2]. It is worthwhile to mention here that there are examples of single gene defects (e.g., RDS/peripherin) manifesting clinically distinguishable eye diseases [7].

The PCG proband homozygous for the MYOC mutation (Gln48His) was born out of a consanguineous marriage, as evident by homozygous genotypes of markers in the patient (data not shown). However, we did not have the opportunity to investigate the segregation of the MYOC mutant alleles in this family because the parents and siblings were deceased. Clinically this patient had a relatively severe phenotype (Corneal diameter 14 mm, total cupping, and an IOP of 74 and 50 mm Hg in the right and left eyes, respectively) compared to other PCG patients with the heterozygous MYOC mutation [8]. The outcome in terms of vision and IOP control (on treatment) was also poor. Interestingly, it has been shown in a large French-Canadian family that homozygotes for a MYOC missense mutation (Lys423Glu) are asymptomatic while heterozygotes are affected with POAG suggesting a dominant negative effect in single dosage of the defective CYP1B1 rather than haploinsufficiency in this family [9]. Thus our observation in homozygous MYOC mutant (Gln48His) is remarkably different, which suggests that accumulation of much larger dataset and functional studies might shed more light to decipher the biology of pathogenesis of glaucoma.

It is possible that for the other 3 PCG cases lacking the CYP1B1 mutation, some other yet unidentified locus together with the MYOC Gln48His mutation might be involved in the causation of the disease. In one PCG patient having one mutant allele each for CYP1B1 (c.1449G>A; Arg368His) and MYOC (c.144G>T; Gln48His), the disease might be caused by digenic inheritance, as proposed for JOAG [2]. The father and the mother of this patient were heterozygous for the mutant MYOC and CYP1B1 alleles, respectively, and did not manifest any glaucomatous symptoms [8]. Although it has been hypothesized that CYP1B1 could be a modifier of MYOC expression and that these two genes might act through a common biochemical pathway [2], there are no functional evidences so far to support this point. The genotypes of all nine patients with Gln48His mutation are described in Table 2.

Although we cannot ascribe causality of all glaucoma phenotypes to the Gln48His mutation alone, it is likely to be a potential risk factor towards disease predisposition. Hence we recommend the screening for this MYOC mutation in all glaucoma patients of Indian origin. The study presented here sug-

| Table 1. Distribution of myocilin mutation Gln48His among the Indian glaucoma patients |
|-----------------|-----------------|-----------------|-----------------|
| Phenotype       | Number of       | Number of       | Number of       |
|                 | individuals     | Gln48His         | mutations       |
| POAG            | 200             | 4 (2.0%)         |                 |
| PCG             | 200             | 5 (2.5%)         |                 |
| Normal          | 300             | 0                |                 |

In addition to the data presented below, Sripriya et al. [5] reported a Gln48His mutation in two Indian POAG patients out of 100 screened for defects in MYOC.

| Table 2. Genotype and phenotype of glaucoma patients with the MYOC Gln48His mutation |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient Number  | Phenotype       | Age at symptom onset | MYOC (c.144G>T) | CYP1B1 (c.1449G>A) |
| 1               | POAG            | 37 years (G,’T’)    | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 2               | JOAG            | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 3               | POAG            | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 4               | JOAG            | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 5               | PCG at birth    | (’T’,’T’)           | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 6               | PCG at birth    | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 7               | PCG at birth    | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 8               | PCG at birth    | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |
| 9               | PCG at birth    | (G,’T’)             | (G,G)/(C,C)     | (G,G)/(C,C)     |

Among all the nine patients harboring the myocilin (NM_000261) mutation, two were also heterozygous for CYP1B1 (NM_000104) mutations. Three of the POAG patients (patient 2, 3, and 4) have been described before [4]. The mutant alleles are enclosed by apostrophes. MYOC mutation: c.144G>T; Gln48His; and CYP1B1 mutations: c.1449G>A; Arg368His and c.1656C>T; Pro437Leu.
gests that the MYOC mutation could be associated to different subtypes of glaucoma that need to be further investigated to better appreciate the role of MYOC in glaucoma pathogenesis.

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