Role of \textit{CYP1B1, MYOC, OPTN and OPTC} genes in adult-onset primary open-angle glaucoma: predominance of \textit{CYP1B1} mutations in Indian patients

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\textbf{Purpose:} Mutations in the \textit{CYP1B1, MYOC, OPTN}, and \textit{WDR36} genes result in glaucoma. Given its expression in the optic nerve, it is likely a mutation in the \textit{OPTC} gene is also involved in initiating glaucoma. This study was designed to evaluate the involvement of the \textit{CYP1B1, MYOC, OPTN}, and \textit{OPTC} genes in the etiology of adult-onset primary open-angle glaucoma (POAG) found in 251 Indian patients.

\textbf{Methods:} Blood samples were obtained from individuals for DNA isolation. A combination of polymerase chain reaction-single strand conformation polymorphism, allele-specific PCR, and DNA sequencing techniques were used to detect mutations in four genes. Four microsatellite markers from the \textit{CYP1B1} candidate region and three intragenic \textit{CYP1B1} single nucleotide polymorphisms (SNPs) were used to determine the origin of the most common \textit{CYP1B1} mutations.

\textbf{Results:} Three previously known mutations (Pro193Leu, Glu229Lys, and Arg368His) and one novel (Met292Lys) mutation were found in the \textit{CYP1B1} gene. Frequencies of the most common mutations, Glu229Lys and Arg368His, in patients were 5.12\% and 3.98\%, respectively. The Glu229Lys and Arg368His mutations were also found in normal controls at frequencies of 5\% and 2\%, respectively, suggesting that these mutations might be polymorphic variants in our population. The absence of allele sharing for D2S177, D2S1346, D2S2974, and D2S2331 markers and three intragenic \textit{CYP1B1} SNPs in patients suggested multiple origins for the Glu229Lys and Arg368His variants. Two of 251 (0.8\%) patients had the Gln48His mutation in \textit{MYOC}. There was no difference in the frequency of a \textit{MYOC} -83G>A promoter polymorphism between patients and controls. A novel \textit{OPTN} mutation, Thr202Arg, was detected in one of 251 (0.4\%) patients. The \textit{OPTN} variant Met98Lys was detected in similar frequencies in patients and controls. No mutation was detected in \textit{OPTC}. Taken together, 3.59\% (9/251) of our POAG patients had mutations in the \textit{CYP1B1, MYOC}, and \textit{OPTN} genes.

\textbf{Conclusions:} This is the first report to document the involvement of the \textit{CYP1B1, MYOC, and OPTN} genes in the etiology of POAG in the same set of Indian patients. Our study shows that mutations in these genes are rare in Indian POAG patients.

Glaucoma is the leading cause of irreversible blindness and the second leading cause of blindness after cataract, affecting 66 million people worldwide [1]. Glaucoma is a heterogeneous group of progressive optic neuropathies characterized by an excavation of the optic disc and progressive alteration of the visual field [2]. High intraocular pressure (IOP), defined as being above 21 mmHg, in both eyes and a positive family history for glaucoma are commonly associated risk factors. Based on the age of onset and other clinical features, glaucoma has been classified into primary congenital glaucoma (PCG), juvenile-onset open-angle glaucoma (JOAG), and adult-onset primary open-angle glaucoma (POAG). PCG manifests at birth or in early infancy (up to 3 years of age). Its phenotype is characterized by elevated IOP, corneal edema, enlargement of the globe (buphthalmos), epiphora, photophobia, and blepharospasm. PCG occurs as a result of developmental anomalies of the anterior chamber angle that prevent drainage of the aqueous humor, thereby elevating IOP. PCG is most commonly inherited as an autosomal recessive trait. JOAG is characterized by early onset (at 10-35 years of age) and autosomal dominant inheritance with high penetrance. POAG occurs after the age of 35 years and is the most common form of glaucoma.

In a majority of cases, glaucoma does not follow a clear-cut inheritance pattern. However, clustering of multiple affected individuals within families suggests that it has a genetic basis. Linkage analyses have identified 23 loci (GLC1A-GLC3L, GLC3A-GLC3B, 2p14, 2q33-q34, 5q21.1-q32, 10p12-p13, 14q11, 14q21-q22, 17p13, 17q25, and 19q12-q14) for different forms of glaucoma [3-10]. However, only four genes (\textit{MYOC}, \textit{TIGR}, \textit{CYP1B1}, \textit{OPTN}, and \textit{WDR36}) have been identified so far [4,11-13]. The myocilin/trabecular meshwork-induced glucocorticoid response protein (\textit{MYOC/TIGR}) gene, located at the GLC1A locus on chromosome 1q24.3-q25.2, has been shown to cause glaucoma in 2-4\% of POAG cases [14]. The cytochrome P450 (\textit{CYP1B1}) gene, located at the GLC3A locus on chromosome 2p22-p21, has been shown to cause PCG, JOAG, and POAG [2,12,15]. In a large family in
which MYOC-linked POAG segregated, a heterozygous mutation in CYP1B1 was associated with early onset of the disease, indicating that a CYP1B1 mutation might behave as a modifier of the MYOC gene [15]. The optineurin (OPTN) gene, located at the GLC1E locus on chromosome 10p14-p15, has been shown to cause normal tension glaucoma (NTG), a subtype of POAG [13]. The WD repeat-containing protein 36 (WDR36) gene, located at the GLC1G locus on chromosome 5q21.3-q22.1, has been shown to be mutated in POAG [4].

Glaucoma is a treatable disease if detected early. Therefore, development of an accurate test for the detection of presymptomatic carriers at risk is important for the management of glaucoma. To this end, a few studies have been carried out to assess the roles of the MYOC [16-19], CYP1B1 [20], and OPTN [21-22] genes in the etiology of Indian POAG patients from different parts of the country. We have reported the genetic analysis of glaucoma in a large south Indian pedigree [18]. Moreover, there is no comprehensive study published to date that assesses the roles of three known glaucoma-causing genes (CYP1B1, MYOC, and OPTN) in the same set of Indian POAG patients. In this study, we report mutation analysis of the CYP1B1, MYOC, and OPTN genes in 251 Indian POAG patients from the south Indian state of Karnataka.

The OPTC (optcin) gene encodes a protein that is a member of the small leucine-rich repeat protein (SLRP) family, and is located on chromosome 1q31-q32 within an age-related macular degeneration (AMD) susceptibility locus [23]. Because of its protein profile in different parts of the eye, such as the iris, trabecular meshwork/ciliary body, retina, vitreous, and optic nerve, Friedman et al. [23] screened OPTC for mutations in individuals with POAG, NTG, and AMD. They failed to find any mutation in this gene. To rule out the OPTC gene as a glaucoma gene, we screened this gene in our POAG data set. The results of our analysis are presented herein.

**METHODS**

**Patients:** A total of 251 patients with adult-onset POAG were evaluated at the Minto Ophthalmic Hospital, and Bangalore West Lions Superspecialty Eye Hospital. Both hospitals are located in the city of Bangalore, Karnataka. The patients were natives of Karnataka and spoke the south Indian Kannada language. They ranged in age from 45 to 65 years. Of the 251 patients, 116 patients received the diagnosis of POAG based on the presence of characteristic glaucomatous optic neuropathy and defects in visual fields. The remaining 135 patients were given the diagnosis of glaucoma based on glaucomatous optic neuropathy only, as visual field defects could not be assessed in these patients due to an advance stage of glaucoma. IOP was more than 21 mmHg in 198 patients. The remaining 53 patients had NTG with IOP below 21 mmHg and significant optic disc damage and visual field defects at the time of diagnosis. All patients had open-angles (Shafer grade greater than III) on gonioscopy and no other eye or systemic abnormalities. Patients with secondary causes of glaucoma (e.g., uveitis, steroid-induced, or trauma) were excluded from this study. We recruited 100 normal controls, also from Karnataka and with the same linguistic background, through several eye care camps. Their ages ranged from 45 to 75 years. All participants went through a detailed clinical examination and were found to have no signs or symptoms of glaucoma or any other eye disease. Controls did not have any family history of glaucoma. Informed consent was obtained from each patient. This study followed the guidelines of the Indian Council of Medical Research, New Delhi.

**Molecular study:** Three to five ml of peripheral blood was collected from each individual in Vacutainer EDTA™ tubes (Beckton-Dickinson, Franklin Lakes, NJ). Genomic DNA was isolated from blood samples using a Wizard® genomic DNA extraction kit (Promega, Madison, WI). Mutation analyses of the four genes were carried out using a combination of PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) and DNA sequencing techniques. For PCR-SSCP, primer sets were designed for the CYP1B1, MYOC, OPTN, and OPTC genes, which covered their entire coding regions and intron-exon junctions. The reference mRNA sequences for the CYP1B1, MYOC, OPTN, and OPTC genes used were U03688, NM_000261, AF420371, and NM_014359, respectively. The genomic sequences for these genes were retrieved from the UCSC Genome Bioinformatics site. Primer details are shown in Table 1 Since abnormalities in WDR36 alone are not sufficient to cause POAG [24], we have not screened this gene for mutations in our POAG samples. In the future, we plan to screen this gene for mutations in our samples. PCR was carried out in a 25 µl reaction volume containing 50-100 ng of genomic DNA, 50 ng of each primer, 0.25 µl of alpha P³²-dCTP (3,000 Ci/mmmole; PerkinElmer, Wellesley, MA), 0.2 mM of each dNTP, and 1 U of Taq DNA polymerase (Bangalore Genei™, Bangalore, India) in a standard buffer supplied by the vendor. PCR was carried out in a Thermal Cycler PTC150 (MJ Research, Watertown, MA) under the following conditions: an initial denaturation at 95 °C for 2 min was followed by 35 cycles at 95 °C for 30 s, 55-72 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 5 min. Following PCR-SSCP, the gels were dried and subjected to Phosphor Image analysis (Fuji, Kanagawa, Japan). For sequencing, PCR was carried out as aforesaid but without including alpha P³²-dCTP in the reaction mixture. PCR amplified products were purified using Auprep™ PCR Purification Columns (Invitrogen, Delhi, India) and sequenced on an ABI PRISM A310 automated sequencer (PE Biosystems, Foster City, CA). Since we did not identify the MYOC Gln48His mutation reported from India earlier [19] in our samples by PCR-SSCP analysis, we used allele-specific PCR to screen the samples for the presence of this mutation. The sequence of the common forward primer for allele-specific PCR is as follows: 5′-TGC AAT GAG GAT CTT CTG TGC ACG-3′. The sequences of the reverse allele-specific primers are as follows: 5′(mutant allele)-GAC TGG CCA CAC TGA AGG TAT AA-3′ and 5′(wild-type allele)-GAC TGG CCA CAC TGA AGG TAT AC-3′. A 190 bp amplicon was generated with common forward and wild-type/mutant allele primers. Following detection of this mutation in patient samples, PCR products were sequenced to confirm the presence of the mutation. The MYOC-83G>A promoter poly-
Results & Discussion

Analysis of the entire coding region of the CYP1B1 gene in POAG patients revealed four different mutations (c.578C>T/Pro193Leu, c.685G>A/Glu229Lys, and c.1103G>A/Met292Lys), and c.1103G>A/Met292Lys) in 10.76% (27/251) of patients with mutations in our samples, 13 patients (5.2%) had the Glu229Lys mutation and 10 patients (4.6%) (11/236) of French patients with early-onset POAG had the Arg308His mutation (Table 2). In order to determine in an east Indian/Guyanese family with early-onset glaucoma, 97 controls were not screened for mutations as they are non-coding. AT, annealing temperature.

Note that primer sets CY2AF/CY2AR, OPTN4aF/OPTN4aR, and OPTN7F/OPTN7R work with 5% DMSO and 1.5 mM magnesium chloride. Primer sets CY2BF/CY2BR, CY2CF/CY2CR, CY2DF/CY2DR, and CY2EF/CY2ER work with 5% DMSO and 1.5 mM magnesium chloride. Primer set TG1BF/TG1DR works with 1 mM magnesium chloride. The rest of the primer sets work with 1.5 mM magnesium chloride. Exon 1 of CYP1B1, exons 2 and 3, and OPTC, and exon 1 of OPTC were not screened for mutations as they are non-coding. AT, annealing temperature.

Table 1. Details of primer sets used in the PCR-SSCP analysis of the CYP1B1, MYOC, OPTN, and OPTC genes.

| Gene | Exon | Primer sequence (5'-3') | Amplification size (bp) | AT (°C) |
|------|------|-------------------------|------------------------|--------|
| CYP1B1 | 2 | CY2AF: TGGTCTTGACCGCCTGTCCCTGCA | 264 | 67 |
| | 2 | CY2AR: GAGCTCTCCGGGCTGGAACCTCCTGCA | 264 | 67 |
| | 2 | CY2BF: TGCTGAGGACCGAAGGGAACCTCCTGCA | 211 | 68 |
| | 2 | CY2BR: GACACAGGCTGAGGGAAGGGAACCTCCTGCA | 262 | 67 |
| | 2 | CY2CF: TCTGAGGACCGAAGGGAACCTCCTGCA | 254 | 64 |
| | 2 | CY2CR: TCTGAGGACCGAAGGGAACCTCCTGCA | 261 | 64 |
| | 2 | CY2DF: TCTGAGGACCGAAGGGAACCTCCTGCA | 267 | 66 |
| | 2 | CY2DR: TCTGAGGACCGAAGGGAACCTCCTGCA | 255 | 60 |
| | 2 | CY2EF: TCTGAGGACCGAAGGGAACCTCCTGCA | 277 | 60 |
| | 2 | CY2ER: TCTGAGGACCGAAGGGAACCTCCTGCA | 291 | 60 |
| | 3 | CY3BF: TACCTCTCCGTCCCTGAGTAGTGA | 248 | 60 |
| | 3 | CY3AR: CACCTCTCCGTCCCTGAGTAGTGA | 255 | 64 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 212 | 60 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 256 | 62 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 244 | 60 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 270 | 66 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 267 | 60 |
| | 3 | CY3AF: TACCTCTCCGTCCCTGAGTAGTGA | 214 | 64 |
| OPTN | 16 | OPTN9R: CACAG ATTTGAATTCAGTGGCTGGA | 223 | 64 |
| | 16 | OPTN10R: CAATTCTGTATAAAAAGGCGATTCTCC | 264 | 60 |
| | 16 | OPTN11R: CAATCTGTATAAAAAGGCGATTCTCC | 223 | 58 |
| | 16 | OPTN12R: GAAGGTTGGGAGGCAAGACTATAAG | 224 | 58 |
| | 16 | OPTN13R: CAGGGCTGGCCTCGCTCAGCTGG | 252 | 60 |
| | 16 | OPTN14R: TGCATTCATCTAGGTACTAAGTTCTG | 229 | 60 |
| | 16 | OPTN15R: GAATCCATTGTAGAGAATGAAGTGGAA | 243 | 60 |
| | 16 | OPTN16R: CAAGTGAAACAAACACAACTGCCTG | 227 | 60 |
| | 16 | OPTN9F: TTCTCTTAAAGCCAAAGAGAAAGTAAC | 232 | 55 |
| | 15 | OPTN13F: CAGGCAGAATTATTTCAAAACCATTTCTAG | 252 | 60 |
| | 15 | OPTN14F: TGCATTCATCTAGGTACTAAGTTCTG | 229 | 60 |
| | 15 | OPTN15F: TCTTGCTGTATCTAGGTACTAAGTTCTG | 243 | 60 |
| | 15 | OPTN16F: CACTTTCCTGGTGTGTGACTCCATC | 280 | 64 |
| | 15 | OPTN9F: TTCTCTTAAAGCCAAAGAGAAAGTAAC | 286 | 60 |
| | 15 | OPTN10F: GCGCTATCTCCTACTGTTTGAA | 284 | 60 |
| | 15 | OPTN11F: CGTAAAGGAGCATTGTTTATCCTCA | 264 | 60 |
| | 15 | OPTN12F: GAAGGTTGGGAGGCAAGACTATAAG | 280 | 64 |
| | 15 | OPTN13F: CAGGGCTGGCCTCGCTCAGCTGG | 235 | 55 |
| | 15 | OPTN14F: TGCATTCATCTAGGTACTAAGTTCTG | 229 | 60 |
| | 15 | OPTN15F: TCTTGCTGTATCTAGGTACTAAGTTCTG | 243 | 60 |
| | 15 | OPTN16F: CACTTTCCTGGTGTGTGACTCCATC | 280 | 64 |
| | 15 | OPTN9F: TTCTCTTAAAGCCAAAGAGAAAGTAAC | 223 | 55 |
| | 15 | OPTN10F: GCGCTATCTCCTACTGTTTGAA | 284 | 60 |
| | 15 | OPTN11F: CGTAAAGGAGCATTGTTTATCCTCA | 264 | 60 |
| | 15 | OPTN12F: GAAGGTTGGGAGGCAAGACTATAAG | 280 | 64 |
| | 15 | OPTN13F: CAGGGCTGGCCTCGCTCAGCTGG | 235 | 55 |
| | 15 | OPTN14F: TGCATTCATCTAGGTACTAAGTTCTG | 229 | 60 |
| | 15 | OPTN15F: TCTTGCTGTATCTAGGTACTAAGTTCTG | 243 | 60 |
| | 15 | OPTN16F: CACTTTCCTGGTGTGTGACTCCATC | 280 | 64 |
| OPTC | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 198 | 64 |
| | 2 | OP2bR: CTCCCAGTGTCATGCAGGGAATGTA | 193 | 72 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 280 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 284 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 275 | 65 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 286 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 232 | 55 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 223 | 58 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 226 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 252 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 229 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 227 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 227 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 236 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 236 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 230 | 60 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 265 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 296 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 209 | 64 |
| | 2 | OP2aR: GAGGGATGGCATTTCTTGCAGGCCCA | 276 | 64 |
Table 2. Previously known and novel mutations detected in the CYP1B1, MYOC, and OPTN genes in primary open-angle glaucoma patients

| Patient (Age of onset/diagnosis) | Location of mutation | MYOC | CYP1B1 | OPTN | IOP (RE:LE) | CD (RE:LE) ratio and other clinical details |
|----------------------------------|----------------------|------|--------|-------|-------------|---------------------------------------------|
| 4 (35/55)                        | Arg508His/+          | -    | -      | -     | 13/12       | 0.90:0.8 RE-involved because of poor vision  |
| 7 (49/50)                        | Arg368His/+          | -    | -      | -     | 22/23       | 0.60:0.7 RE-appearing defect, LE-loss of sensitivity in the lower arcuate region  |
| 27 (56/57)                       | Glu229Lys/+          | -    | -      | -     | 14/38       | 0.50:0.5 RE-involved sensitivity upper arcuate defect  |
| 30 (52/53)                       | -                    | Thr202Arg*/+ | 28/22 | 0.80:0.6 RE-involved defect with nasal step, LE-loss of sensitivity in the lower arcuate region  |
| 37 (47/49)                       | Arg368His/+          | -    | -      | -     | 38/26       | 0.50:0.5 RE-involved defect, LE-density upper and lower arcuate defect going in for tubular vision  |
| 38 (46/46)                       | Arg368His/+          | -    | -      | -     | 22/22       | 0.50:0.5 LE-loss of sensitivity in the lower arcuate region  |
| 44 (63/70)                       | Gln48His/+           | -    | -      | -     | 43/48       | 0.90:0.9 RE-involved because of poor vision  |
| 54 (55/56)                       | Glu229Lys/+          | -    | -      | -     | 19/20       | 0.80:0.7 NTG, BE-upper arcuate defect  |
| 61 (43/45)                       | Glu229Lys/+          | -    | -      | -     | 17/18       | 0.50:0.6 NTG, BE-involved upper arcuate defect  |
| 66 (48/53)                       | Glu229Lys/+          | -    | -      | -     | 22/28       | 0.80:0.8 RE-involved upper and lower arcuate scotoma going in for tubular vision, LE-PL-ve  |
| 68* (64/65)                      | Gln48His/+           | -    | -      | -     | 28/32       | 0.60:0.5 RE-involved developing arcuate scotoma, LE-redced sensitivity in lower arcuate region  |
| 69 (48/55)                       | Arg368His/+          | -    | -      | -     | 21/32       | 0.90:0.9 RE-involved because of poor vision  |
| 73 (47/52)                       | Glu229Lys/+          | -    | -      | -     | 25/23       | 0.80:0.8 RE-involved because of poor vision  |
| 79 (45/46)                       | Glu229Lys/+          | -    | -      | -     | 28/32       | 0.50:0.6 RE-involved developing scotoma in the superior arcuate region  |
| 111 (50/53)                      | Arg368His/+          | -    | -      | -     | 28/27       | 0.80:0.7 RE-involved arcuate scotoma, LE-redced sensitivity in upper arcuate region  |
| 112 (62/64)                      | Glu229Lys/+          | -    | -      | -     | 42/39       | 0.90:0.6 RE-density upper and lower arcuate scotoma going in for tubular vision  |
| 119 (55/57)                      | Arg368His/ Met921Lys* | -    | -      | -     | 48/32       | 0.70:0.7 RE-involved because of poor vision, LE-density tubular scotoma sparing the central vision  |
| 120 (55/63)                      | Met921Lys*+/+        | -    | -      | -     | 43/48       | 0.80:0.5 RE-involved because of poor vision  |
| 121 (52/60)                      | Met921Lys*+/+        | -    | -      | -     | 48/52       | 0.90:0.5 RE-involved because of poor vision  |
| 122 (55/57)                      | Met921Lys*+/+        | -    | -      | -     | 42/38       | 0.80:0.6 RE-involved because of poor vision  |
| 131 (57/58)                      | Glu229Lys/+          | -    | -      | -     | 48/323      | 0.80:0.8 RE-involved due to poor vision, LE-isolated dense scotoma in the inferior arcuate region with nasal stepping  |
| 168 (45/47)                      | Glu229Lys/+          | -    | -      | -     | 28/32       | 0.90:0.5 RE-involved upper and lower arcuate scotoma going in for tubular vision, LE-PL-ve  |
| 170 (48/49)                      | Arg368His/+          | -    | -      | -     | 38/32       | 0.80:0.5 RE-involved upper and lower arcuate scotoma going in for tubular vision, LE-isolated dense scotoma in lower arcuate region with nasal stepping  |
| 179 (50/52)                      | Glu229Lys/ Pro193Leu | -    | -      | -     | 28/32       | 0.70:0.7 RE-involved upper arcuate scotoma, LE-PL-ve  |
| 182 (59/79)                      | Pro193Leu/+          | -    | -      | -     | 26/24       | 0.60:0.7 RE-involved upper arcuate defect  |
| 199 (55/57)                      | Glu229Lys/+          | -    | -      | -     | 38/22       | 0.80:0.9 RE-density upper and lower arcuate scotoma going in for tubular vision with macular splitting  |
| 202 (50/53)                      | Arg368His/+          | -    | -      | -     | 16/14       | 0.60:0.7 NTG, BE-involved lower arcuate defect  |
| 226 (69/72)                      | Arg368His/+          | -    | -      | -     | 10/14       | 0.70:0.6 NTG, BE-involved upper and lower arcuate defect  |
| 235 (62/63)                      | Glu229Lys/+          | -    | -      | -     | 18/12       | 0.70:0.4 NTG, RE-double arcuate scotoma with nasal stepping,  |
| 237 (69/70)                      | Glu229Lys/+          | -    | -      | -     | 28/26       | 0.90:0.7 RE-density double arcuate scotoma going in for tubular vision  |

Shown are details of 30 primary open-angle glaucoma (POAG) patients with previously known and novel mutations in the CYP1B1, MYOC, and OPTN genes. Since the Glu229Lys and Arg368His mutations were found in normal controls with frequencies of 5% and 2%, respectively, these mutations might be polymorphic variants in our population. By excluding these mutations, the frequency of patients with mutations in three glaucoma-causing genes is 3.59% (9/251). In the table, * indicates a novel mutation; + identifies wild-type alleles; # indicates a family history of glaucoma; other cases are sporadic. The following abbreviations were used: normal tension glaucoma (NTG), right eye (RE), left eye (LE), both eyes (BE), perception of light negative (PL-ve), and cup to disc (CD). The age of onset and diagnosis are in years.
tion. The presence of the Arg368His mutation in controls is not surprising as Melki et al. [2] discovered 2.13% (1/47) of French controls with this mutation. The Glu229Lys and Arg368His mutations were the most common mutations in Indian PCG patients with frequencies of 16.22% (6/37) and 59.46% (22/37), respectively [28]. However, ethnically matched population screening of 140 chromosomes for the CYP1B1 mutations showed 6.4% and 0.7% carriers for the Glu229Lys and Arg368His mutations, respectively [28]. The other known mutation, Pro193Leu, was not present in 100 normal controls (data not shown). In order to determine whether the Glu229Lys variant in 13 patients could have a common origin, all patients were genotyped with four microsatellite markers (D2S177-D2S1346-CYP1B1-D2S2974-D2S2331) and three intragenic CYP1B1 SNPs. An absence of allele sharing for the markers in patients suggested that Glu229Lys has multiple origins (Table 3). A similar result was obtained for the Arg368His mutation (Table 3).

In addition to mutations, nine CYP1B1 population variants and polymorphisms were also identified in our samples, including two novel SNPs (Table 4). It is interesting to note that c.142C>G (Arg48Gly) and c.355G>T (Ala119Ser) occurred at a high frequency and were in complete linkage disequilibrium (Table 4). Similarly, c.1294G>C (Val432Leu) and c.1347T>C (Asp449Asp) also occurred at a high frequency and were in complete linkage disequilibrium (Table 4). Interestingly, c.142C>G (Arg48Gly) and c.355G>T (Ala119Ser) always occurred with c.1294G>C (Val432Leu) and c.1347T>C (Asp449Asp) in patients. Similar results were obtained in JOAG patients and normal controls from the east Indian state of West Bengal (Table 5) [20]. The significance of this phenomenon is not clear at present.

Analysis of the MYOC gene revealed that 2/251 (0.8%) patients had the Gln48His mutation in a heterozygous state. This mutation was not present in 100 normal controls (data not shown). No other mutation was detected in our samples. The Gln48His mutation has been detected in 4/200 (2%) POAG patients in a heterozygous state from West Bengal [19]. This mutation has also been detected in 5/200 (2.5%) PCG patients [19]. Previously, we detected a novel Pro274Arg mutation in a four-generation family with members affected with JOAG and POAG, and with one severely affected patient being homozygous for the mutation [18]. Overall, the frequency of MYOC mutations has been found to be 2-4% in different populations [14]. Kanagavalli et al. [17] noted 1.87% (2/107) of patients with POAG from the South Indian state of Tamilnadu who had mutations in the MYOC gene. However, Mukhopadhyay et al. [16] discovered a higher frequency (7.14% or 4/56) of POAG patients from West Bengal with MYOC mutations. This could be a statistical anomaly due to a small sample size or a true pattern representative of different geographic origins.

The MYOC promoter polymorphism at -83G>A was initially reported from Western countries ([14,29] Table 5). This polymorphism has been also observed in Hong Kong and the Philippines [30,31]. Alward et al. [29] suggested that it is unlikely to be a disease-causing mutation. Since this polymorphism was detected in many countries, we wanted to determine its prevalence in our patient and control samples. We did not find any significant difference in the frequency of -83G>A between POAG samples and controls (76.72% in POAG versus 76.53% in controls; Table 4). This is similar to the earlier report from West Bengal by Mukhopadhyay et al. [16]. Interestingly, -83G>A is in linkage disequilibrium with Arg76Lys.
both in patients and controls (Table 4 and Table 5), suggesting that the -83G>A polymorphism is not a risk factor for developing glaucoma in Indians. Taken together, our observations suggest that mutations in MYOC gene may not be a major factor in the appearance of the POAG phenotype in India.

Mutation analysis of the entire OPTN gene revealed that one patient (0.40%) was heterozygous for a novel mutation, Thr202Arg (c.915C>G) in exon 7 (Figure 1B, Table 2). This mutation fulfills the criteria of a mutation because it is not present in 93 controls (data not shown) and the threonine residue is conserved in human, macaque, mouse, and rat (Figure 1B). Sripriya et al. [22] recently screened the OPTN gene in 100 high tension glaucoma patients from another south Indian state, Tamilnadu, and did not detect any mutation. Mukhopadhyay et al. [21] screened the entire coding region of this gene and detected 6/200 (3%) POAG patients from West Bengal, who were heterozygous for the Arg545Gln mutation in exon 16. As observed in other populations [32-36], the present observation also suggests that OPTN mutations are rare in POAG patients. A total of eight mutations (Glu50Lys, c.691-692insAG, Arg545Gln, Ala336Gly, Ala377Thr, His26Asp, His486Arg, and Glu104Asp) have been reported in the OPTN gene from different populations, including the His486Arg mutation in a JOAG patient [13,21,32-36]. Interestingly, one of the three mutations, Arg545Gln (c.1944G>A), reported by Rezaie et al. [13], has been detected in similar frequencies in Japanese glaucoma patients and control subjects [37]. Alward et al. [38] commented that the Arg545Gln variation is likely to be a nondisease-causing polymorphism. With the Thr202Arg mutation identified in this study, the total number of OPTN mutations, excluding Arg545Gln, reaches eight.

Rezaie et al. [13] initially reported that Met98Lys (c.603T>A) is a risk-associated alteration (risk factor) for developing glaucoma. They found this variant in both affected individuals and controls, but it was more common in affected individuals than in controls (13.6% versus 2.1%). Alward et al. [38] and Fuse et al. [33] showed a significant association between Met98Lys and glaucoma in Japanese patients. On the other hand, Toda et al. [37] found similar frequencies of Met98Lys in Japanese glaucoma patients and controls. Interestingly, Met98Lys has been shown to be a polymorphic variant in German, French, and Moroccan patients [35,39]. Sripriya et al. [22] did not detect Met98Lys in 100 controls, although it

| Patient | Variant | D2S177 | D2S1346 | c.142C>G | c.355G>T | c.1294G>T | D2S2974 | D2S2331 |
|--------|---------|--------|---------|-----------|-----------|-----------|---------|---------|
| 27     | Glu229Lys | 1      | 5       | C         | G         | G         | T       | C       | 12      | 24      |
| 54     | Glu229Lys | 4      | 4       | G         | G         | T         | T       | C       | 12      | 45      |
| 61     | Glu229Lys | 4      | 4       | G         | G         | A         | T       | C       | 22      | 36      |
| 66     | Glu229Lys | 5      | 4       | C         | G         | G         | T       | C       | 22      | 34      |
| 73     | Glu229Lys | 5      | 2       | G         | C         | G         | T       | C       | 22      | 47      |
| 79     | Glu229Lys | 4      | 5       | C         | C         | T         | T       | C       | 22      | 47      |
| 112    | Glu229Lys | 4      | 5       | C         | C         | G         | T       | C       | 12      | 78      |
| 131    | Glu229Lys | 4      | 5       | C         | G         | T         | G       | C       | 22      | 70      |
| 168    | Glu229Lys | 5      | 7       | C         | C         | C         | C       | G       | 22      | 47      |
| 179    | Glu229Lys | 3      | 3       | C         | C         | G         | C       | T       | 22      | 38      |
| 199    | Glu229Lys | 5      | 4       | C         | G         | G         | C       | G       | 22      | 34      |
| 235    | Glu229Lys | 7      | 9       | C         | G         | C         | C       | C       | 22      | 37      |
| 237    | Glu229Lys | 3      | 4       | C         | C         | G         | C       | G       | 23      | 37      |
| 4      | Arg368His | 6      | 8       | C         | G         | G         | T       | C       | 12      | 45      |
| 7      | Arg368His | 2      | 3       | C         | G         | G         | C       | T       | 12      | 45      |
| 37     | Arg368His | 3      | 3       | C         | G         | G         | C       | T       | 12      | 45      |
| 38     | Arg368His | 4      | 4       | C         | G         | G         | C       | T       | 22      | 45      |
| 69     | Arg368His | 3      | 4       | C         | G         | G         | C       | T       | 33      | 45      |
| 111    | Arg368His | 5      | 5       | C         | G         | G         | C       | T       | 22      | 45      |
| 119    | Arg368His | 4      | 5       | C         | G         | C         | C       | T       | 22      | 45      |
| 170    | Arg368His | 3      | 4       | C         | G         | C         | C       | T       | 22      | 45      |
| 202    | Arg368His | 3      | 4       | C         | G         | C         | C       | T       | 12      | 56      |
| 226    | Arg368His | 3      | 4       | C         | G         | C         | C       | T       | 22      | 56      |

Each number under a microsatellite marker column represents an allele. As can be seen from the genotypes at four microsatellite marker loci, some patients with either of the variants did not share alleles. For example, patients 168 and 179 with Glu229Lys did not share alleles at D2S177, D2S1346, and D2S2331. Although single nucleotide polymorphisms (SNPs) are less polymorphic than microsatellite markers, patients 54 and 73 did not share alleles at c.142C>G. Similarly, patients 7 and 38 with Arg368His did not share alleles at D2S177, D2S1346, and D2S2331. However, alleles at three SNP loci were not useful to determine nonsharing of alleles for Arg368His. Overall, the results suggest multiple origins for both variants.
was present in 7/170 (4.1%) POAG and 3/50 (6%) NTG patients from Tamilnadu. However, a statistical analysis did not show any significant correlation with clinical parameters [22]. In another study by Mukhopadhyay et al. [21], the frequency of Met98Lys was found to be 11% and 5.5% in POAG and controls from West Bengal, respectively. Sripriya et al. [22] found Met98Lys in 6% of NTG patients, whereas Mukhopadhyay et al. [21] failed to find it in NTG patients. In our dataset, the frequency of this variant was found to be similar in our POAG samples and controls (7.97% in POAG versus 7.29% in controls; Table 4), suggesting that it may not be a risk factor for developing glaucoma in Indian populations.

Mutation analysis of the OPTC gene did not detect any mutation in our POAG samples. Screening of the OPTC gene was carried out in POAG cases by Friedman et al. [23]. The Leu268Pro polymorphism, identified by Friedman et al. [23] in 6/87 (6.9%) of POAG/NTG patients and in 8/55 (14.55%) of controls (Table 5), was found in 31/251 (12.35%) of our POAG samples (Table 4). Another synonymous codon change Leu270Leu, identified by Friedman et al. [23] in 2/55 (3.66%) of controls (Table 5), was also detected in 5/251 (1.99%) of our POAG samples (Table 4).

In summary, 3.59% (9/251) of our POAG patients had mutations in the CYP1B1, MYOC, and OPTN genes. Two previously known CYP1B1 mutations, Glu229Lys and Arg368His, were found in similar frequencies in POAG patients and controls, suggesting that these mutations might be polymorphic variants in our population. A similar situation exists for the CYP1B1 Ala443Gly mutation, first reported by Melki et al. [2] in French patients, and was found to be a polymorphic variant in an Ethiopian population with a frequency of 7% [40]. No association was found between the OPTN Met98Lys

Table 4. Polymorphisms and population variants observed in the CYP1B1, MYOC, OPTN, and OPTC genes in primary open-angle glaucoma patients and controls

| Gene    | Polymorphism/population variant | Exon | Intron | POAG patients | Controls |
|---------|--------------------------------|------|--------|---------------|----------|
| CYP1B1  | c.IVS1-12C>T                    | 1    |        | 2/251 (0.80%) | -        |
|         | c.IVS1-14-15delTC**             | 1    |        | 2/251 (0.80%) | -        |
|         | c.142C>G (Arg48Gly)             | 2    |        | 165/251 (65.74%) | -        |
|         | c.355G>T (Ala119Ser)            | 2    |        | 165/251 (65.74%) | -        |
|         | c.729G>C (Val243Val)            | 2    |        | 2/251 (0.80%) | -        |
|         | c.1294G>C (Val432Leu)           | 3    |        | 228/251 (90.84%) | -        |
|         | c.1347T>C (Asp449Asp)           | 3    |        | 228/251 (90.84%) | -        |
|         | c.1358A>G (Asn453Ser)           | 3    |        | 116/251 (46.22%) | -        |
|         | c.1446C>G (Leu482Leu)**         | 3    |        | 1/251 (0.40%)  | 0/93 (0.0%) |
| MYOC    | -83G>A (Promoter region)#       |      |        | 89/116 (76.72%) | 75/98 (76.53%) |
|         | c.227G>A (Arg76Lys)**           | 1    |        | 89/116 (76.72%) | 74/97 (76.29%) |
|         | c.366C>T (Gly122Gly)            | 1    |        | 1/251 (0.40%)  | -        |
|         | c.1041T>C (Tyr347Tyr)           | 3    |        | 9/251 (3.59%)  | -        |
|         | c.1303C>T (Gly434Gly)**         | 3    |        | 1/251 (0.40%)  | -        |
| OPTN    | c.412G>A (Thr94Thr)**           | 4    |        | 29/251 (11.55%) | 23/50 (46%) |
|         | c.603T>A (Met98Lys)             | 5    |        | 20/251 (7.97%)  | 7/96 (7.29%) |
|         | c.712C>T (Ala134Ala)            | 6    |        | 1/251 (0.40%)  | -        |
|         | IVS7-5T>C**                     | 7    |        | 83/251 (33.07%) | 11/50 (22%) |
|         | IVS7-10G>A**                    | 7    |        | 1/251 (0.40%)  | 0/50 (0.0%) |
|         | IVS7+24G>A                      | 7    |        | 35/251 (14.34%) | -        |
|         | c.1866G>A (Ser519Ser)**         | 15   |        | 2/251 (0.80%)  | 11/50 (22%) |
| OPTC    | c.486C>T (Phe162Phe)**          | 4    |        | 1/251 (0.40%)  | 0/50 (0.0%) |
|         | c.803T>C (Leu268Pro)            | 6    |        | 31/251 (12.35%) | -        |
|         | c.810G>A (Leu270Leu)            | 6    |        | 5/251 (1.99%)  | -        |
|         | c.859G>A (Val287Met)**          | 7    |        | 1/251 (0.40%)  | 0/50 (0.0%) |
|         | IVS2-15C>A**                    | 2    |        | 1/251 (0.40%)  | -        |

Shown are the frequencies of polymorphisms and population variants in the CYP1B1, MYOC, OPTN, and OPTC genes seen in primary open-angle glaucoma (POAG) patients and controls. In the table, * indicates a risk factor, ** notes a novel single nucleotide polymorphism (SNP), and # highlights two polymorphisms, -83G>A and Arg76Lys, that were detected in high frequencies in 116 patients and thus were not screened in the remainder of the 251 patients.
variant and glaucoma. Mutations in MYOC and OPTN are rare in Indian POAG patients. This is the first study to document the prevalence of mutations in three glaucoma-causing genes in the same set of Indian POAG patients. Our study suggests that mutations in these genes are rare in Indian POAG patients.

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We did not look for another MYOC promoter polymorphism at nt -1000 (-1000C>G; MYOC.mt1; asterisk) in our primary open-angle glaucoma (POAG) data set because Alward et al. [43] and Özgül et al. [44] have observed that it is not a risk factor for the development of glaucoma in patients from the U.S.A. or Turkey, respectively.

TABLE 5. REPORTED FREQUENCIES OF KNOWN POLYMORPHISMS AND POPULATION VARIANTS IN THE CYP1B1, MYOC, OPTN, AND OPTC GENES IN PRIMARY OPEN-ANGLE GLAUCOMA PATIENTS AND CONTROLS FROM DIFFERENT POPULATIONS

| Gene | Polymorphism/ population variant | Frequency in population |
|------|----------------------------------|-------------------------|
| CYP1B1 | c.IVS1-12C>T  | 23  | 22  | Eastern India  | Acharya et al. [20] |
| | c.142C>G (Arg48Gly) | 43.5 | 39  | Eastern India  | Acharya et al. [20] |
| | c.355G>T (Ala119Ser) | 43.5 | 39  | Eastern India  | Acharya et al. [20] |
| | c.729G>C (Val243Val) | 1.27 | 4.26 | France | Melki et al. [2] |
| | c.1294G>C (Val432Leu) | 51  | 59  | Eastern India  | Acharya et al. [20] |
| | c.1347T>C (Asp449Asp) | 51  | 60  | Eastern India  | Acharya et al. [20] |
| | c.1358A>G (Asn453Ser) | 16.5 | 14  | Eastern India  | Acharya et al. [20] |
| MYOC | -83G>A (Promoter region)* | 18  | 23  | U.S.A. | Alward et al. [29] |
| | | 30  | 39  | Japan | Suzuki et al. [41] |
| | c.227G>A (Arg76Lys) | 73.2 | 68.62 | Eastern India | Mukhopadhyay et al. [16] |
| | c.366C>T (Gly122Gly) | 19  | 18.7 | U.S.A. | Alward et al. [29] |
| | c.1041T>C (Tyr347Tyr) | 5.4  | 7.7  | U.S.A. | Alward et al. [29] |
| OPTN | c.603T>A (Met98Lys) | 28.6 | 24.6 | China | Leung et al. [36] |
| | | 13.6 | 2.1  | U.S.A. | Rezaie et al. [13] |
| | | 11  | 5.5  | Eastern India | Mukhopadhyay et al. [21] |
| | | 4.1  | 0.0  | South Indian state of Tamil Nadu | Sripriya et al. [22] |
| | c.712C>A (Ala134Ala) | 16.9 | 5  | Japan | Fuse et al. [33] |
| | | 6.25 | 7  | Germany | Weisschu et al. [35] |
| | | 4.64 | 4.54  | France | Melki et al. [39] |
| | | 10.7  | 8.33  | Morocco | Melki et al. [39] |
| | | 13.33  | 13.78  | Japan | Toda et al. [37] |
| | | 20.7  | 9.0  | Japan | Alward et al. [38] |
| | c.803T>C (Leu268Pro) | 1.75  | 0.92  | Afro-Caribbean Jewish/Scottish/African Somali | Willoughby et al. [42] |
| | c.810G>A (Leu270Leu) | 10.9  | 4 | China | Leung et al. [36] |
| OPTC | c.803T>C (Leu268Pro) | 6.9  | 14.55  | French-Canada | Friedman et al. [23] |
| | | 0.0  | 3.66  | French-Canada | Friedman et al. [23] |
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