Myocilin Variants in Indian Patients With Open-angle Glaucoma

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Objective: To identify and evaluate *MYOC* variant alleles among patients with primary open-angle glaucoma (POAG) and age-matched control subjects in an Indian population.

Methods: Three hundred fifteen patients with POAG and 100 unrelated control subjects from the same ethnic background were enrolled in the study. The coding sequence of *MYOC* was amplified by polymerase chain reaction using genomic DNA, followed by sequencing of the polymerase chain reaction products. Four single nucleotide polymorphisms were genotyped in different Indian subpopulations comprising 1466 individuals using SEQUENOM's homogeneous MassEXTEND assay.

Results: One novel mutation (Gly399Asp), 6 reported mutations (Gln48His, Thr256Met, Thr353Ile, Gln368Stop, Pro370Leu, and Ala427Thr), and 6 single nucleotide polymorphisms were identified in *MYOC*.

Ala427Thr was identified in a patient with POAG and Parkinson disease. Four single nucleotide polymorphisms genotyped in control subjects were highly heterozygous and displayed a similar pattern of linkage disequilibrium among all linguistic groups.

Conclusions: *MYOC* mutations account for 2.2% of POAG cases. The Gln368Stop mutation (common among persons of the white race) found in 2 families does not seem to be of white race origin. Identification of a *MYOC* mutation (Ala427Thr) in a patient with POAG and Parkinson disease is interesting with respect to reported interaction of myocilin with synucleins.

Clinical Relevance: Studying the genetics of POAG is helpful for preclinical identification and for better disease management.

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GLAUCOMA IS A HETEROGEneous group of optic neuropathies with a complex genetic basis. It is a multifactorial optic disc neuropathy in which there is a characteristic acquired loss of retinal ganglion cells and atrophy of the optic nerve.[1] Primary open-angle glaucoma (POAG) (Online Mendelian Inheritance in Man [OMIM] 137760) is the most prevalent of the glaucoma subtypes. The disease is known to be transmitted as a monogenic disease and as a complex disease. Adult-onset POAG is inherited as a nonmendelian trait, whereas juvenile-onset POAG exhibits autosomal dominant inheritance.[2] Among 11 implicated loci and 3 identified candidate genes, mutations in the myocilin gene (*MYOC*) have been most widely studied. Although the pathophysiology is unknown, it has been suggested that mutant *MYOC* obstructs the outflow of the aqueous humor through the trabecular meshwork, resulting in increased intraocular pressure, which is frequently associated with glaucoma.[1]

MYOC, located on chromosome 1 (at 1q24.3) and spanning a genomic DNA region of approximately 17 kilobases (kb), contains 3 exons and is expressed as a 2.3-kb transcript with the translated product of 504 amino acids.[3] Most of the identified *MYOC* mutations are located in exon 3.[1]

About 1.5 million people are reported to have blindness because of glaucoma in India.[4] Previous studies[5] among Indian populations were based on small samples of patients with POAG. In this study, we evaluated a larger subset of patients to investigate the molecular basis of POAG among eastern Indian patients by screening *MYOC* for causal variants. Because POAG is a complex disease, it is likely that it may be caused by an interplay of multiple genes and environmental factors.

From that perspective, the role of the *p53* codon 72 polymorphism and the apolipoprotein E ε allele in *APOE*[6] have been examined for their association with POAG.[7,8] In the Indian population, we examined genomic variation in *MYOC*, which could be tested for its association with POAG and could serve as markers to examine cosegregation of *MYOC* alleles with POAG in familial cases. The Indian population comprises 4693 communities, with several thousand endogamous groups, 325 functioning languages, and 25 scripts. Some geographical regions of India practice consanguinity. Few populations outside of Africa have the degree of genetic diversity found in the Indian subcontinent.[9] The study subjects consisted of 4 major linguistic groups that define genetic variation in the population based on studies conducted on mitochondrial[10] and autosomal[9,11] genes.

METHODS

SELECTION OF STUDY SUBJECTS

Indian patients with POAG inhabiting West Bengal (eastern India), speaking Bengali, and belonging to the Indo-European linguistic group, regardless of any family history of POAG, were recruited from the Regional Institute of Ophthalmology, Kolkata, and from the Dristipradip Eye Clinic, Kolkata. Diagnoses were obtained from clinical ocular and systemic examinations. Ocular examinations included measurement of intraocular pressure using applanation tonometry (Goldmann tonometer) or noncontact air-puff tonometry. A Goldmann 3-mirror gonioscope was used to assess the angles of the anterior chamber and the optic disc. The optic disc was also evaluated using a +78-diopter lens in some patients. Automated threshold field analysis was performed using a Humphrey (Carl Zeiss Meditech Inc, Dublin, Calif) or Medmont (Medmont Internation Pvt Ltd, Victoria, Australia) version 6 field analyzer. The retinal nerve fiber layer was investigated by scanning laser polarimetry with the GDx Vcc (Carl Zeiss Meditech Inc).

For this study, the inclusion criteria were (1) optic disc cupping or visual field changes in patients with an intraocular pressure of greater than 20 mm Hg and (2) optic disc cupping and visual field changes in patients with an intraocular pressure of less than 21 mm Hg. Patients with a history of ocular inflammation, ocular trauma, or angle closure in any quadrant were excluded. Control subjects were chosen to match the age, race/ethnicity, geographic region, and linguistic background of the patients. Individuals were subjected to routine eye examinations, followed by retinal nerve fiber layer analysis using scanning laser polarimetry.

To study single nucleotide polymorphisms (SNPs) in the Indian population, 36 ethnic subgroups, classified on the basis of the 4 major linguistic groups and comprising 1466 individuals, were included in this study. The linguistic groups are Indo-European, Austro-Asiatic, Tibeto-Burman, and Dravidian.[9] These individuals did not undergo any eye examination as part of this study.

COLLECTION OF BLOOD SAMPLES AND GENOMIC DNA PREPARATION

Ten milliliters of peripheral blood was collected with EDTA from the patients with POAG and from controls with their written consent. The internal review committee on research approved the project per regulations of the Indian Council of Medical Research. For evaluation of the intragenic SNPs in *MYOC* in different linguistic groups of the Indian subcontinent, identification of populations and collection of samples were carried out with the help of trained anthropologists, social workers, and community health workers. Endogamy of the populations was established by taking extensive information about the marriage patterns, gathered through pedigrees and interviews with family members of the donor and through review of the published literature. It was ensured that individuals were unrelated at least to the first-cousin level, and attempts were made to collect blood samples from male and female subjects in equal numbers.

Genomic DNA was prepared from fresh whole blood using the conventional phenol-chloroform method, followed by ethanol precipitation. DNA was then dissolved in 10mM Tris-hydrochloride and 0.1mM EDTA (pH 8.0).[12]

POLYMERASE CHAIN REACTION, DNA SEQUENCING, RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS, AND GENOTYPING

Polymerase chain reaction was carried out in a total volume of 25 µL containing 50 to 100 ng of genomic DNA to amplify *MYOC* exons and adjoining splice junctions as described previously.[5] The polymerase chain reaction products, free of contaminating bands because of nonspecific amplification, were subjected to bidirectional sequencing using a DNA sequencer with dye termination chemistry (ABI 3130XL; Applied Biosystems, Foster City, Calif) to identify any alteration of sequence from the normal variant.

For detection of Gln368Stop in the family members of the proband, restriction fragment length polymorphism analysis using *Taa*I was performed as previously described.[13] An *Ava*I restriction fragment length polymorphism assay was used to detect the –83G>A polymorphism in the *MYOC* promoter, as described by Lam et al.[14]

Probands and affected family members were genotyped using 2 short tandem repeat polymorphic markers (D1S2815 and D1S2790) that closely flank *MYOC*.[3] The frequencies of the selected SNPs in different linguistic groups of the Indian population were identified using the homogeneous MassEXTEND assay, an effective genotyping method run on the MassARRAY system performed using the SEQUENOM (SEQUENOM Inc, San Diego, Calif) platform at the Center for Genomic Application (New Delhi, India). Commercially available software (Assay Design 2.0; SEQUENOM Inc) was used to design the polymerase chain reaction and homogeneous MassEXTEND primers for each SNP to be investigated.

BIOINFORMATIC ANALYSIS OF EXPERIMENTAL DATA

Novel changes were evaluated for their potential effect on the protein product using the SIFT (sorting intolerant from tolerant) score (http://blocks.fhcrc.org/sift/SIFT.html). The SIFT software predicts the potential of a substituted amino acid to be deleterious in a protein sequence. A score less than 0.05 is the benchmark of deleterious change.[15]

Evaluation of nucleotide changes for alteration in splicing was performed using Web-based software (ESEfinder [http://rulai.cshl.edu/tools/ESE] and RESCUE-ESE [relative enhancer and silencer classification by unanimous enrichment; http://genes.mit.edu/burgelab/rescue-ese]). Determination of linkage disequilibrium among SNPs in *MYOC* was performed using Haploview 3.2 software (http://www.hapmap.org/cgi-perl/gbrowse/hapmap/).

Table 1. Mutations in *MYOC* in Eastern Indian Patients With Primary Open-angle Glaucoma

Nucleotide Change	Exon	Amino Acid Change	Genotype	SIFT Score	No. of Changes	
					Patients (n = 315)	Control Subjects (n = 100)
c.144G>T	1	Gln48His*†	Heterozygous	0.09	3	0
c.767C>T	3	Thr256Met*	Heterozygous	0.15	1	0
c.1058C>T	3	Thr353Ile*	Heterozygous	0.01	1	0
c.1102C>T	3	Gln368Stop	Heterozygous	. . .	2	0
c.1109C>T	3	Pro370Leu*†	Heterozygous	0.03	1	0
c.1196G>A	3	Gly399Asp*‡	Homozygous	0.00	1	0
c.1279G>A	3	Ala427Thr*	Heterozygous	0.02	2	0

Abbreviation: SIFT, sorting intolerant from tolerant.
*Conserved in human, mouse, rat, and pig.
†Already published.[5]
‡Novel change.

Table 2. Single Nucleotide Polymorphisms in *MYOC* in Eastern Indian Patients With Primary Open-angle Glaucoma

Nucleotide Change	Location	Amino Acid Change	Source	No. of Changes	
				Patients (n = 315)	Controls (n = 100)
−83G>A	Promoter	Not applicable	Alward et al,[17] 1998	220	69
c.227G>A	Exon 1	Arg76Lys*	Alward et al,[17] 1998	220	69
c.855G>T	Exon 3	Thr285Thr†	. . .	1	0
c.952C>T	Exon 3	Leu318Leu†	. . .	2	0
c.1041T>C	Exon 3	Tyr347Tyr	Fingert et al,[3] 1999	8	0
c.1182C>T	Exon 3	Thr394Thr†	. . .	1	0

*Already published.[5]
†Novel change.

RESULTS

The patient pool (probands) consisted of subjects with juvenile-onset POAG (57 individuals aged 10-35 years) and subjects with adult-onset POAG (258 individuals). The age at diagnosis ranged from 10 to 84 years, with a mean±SD age of 55.69±16.78 years. The patients were not known to have any other eye disorder. The mean±SD age of the 100 unrelated control subjects enrolled in the study was 53.86±8.92 years (age range, 40-80 years).[16] The nucleotide changes identified in the patients are given in **Table 1** and **Table 2** with the genotype of the patients, evaluation of the variant alleles, and potential downstream effect on the protein. Clinical information regarding the patients harboring *MYOC* mutations is given in **Table 3**.

IDENTIFICATION OF MUTATIONS IN *MYOC*

Five mutations (Gln48His, Thr353Ile, Gln368Stop, Pro370Leu, and Ala427Thr) were detected in the heterozygous state in *MYOC* in patients with POAG (Table 1 and Table 2). Among these, Gln48His (c.144G>T), located in exon 1 of *MYOC* and identified in 3 patients with juvenile-onset POAG, has been reported previously by our laboratory.[5] The Thr353Ile (c.1058C>T) mutation was identified in a patient with sporadic juvenile-onset POAG. Pro370Leu (c.1109C>T), associated with a severe glaucoma phenotype worldwide, was detected in an 18-year-old woman. The mutant allele was found to segregate within the family in all 3 members affected with POAG but was not detected in any family member without POAG.[5] Ala427Thr (c.1279G>A) was identified in 2 patients with sporadic POAG. One of the patients with the mutation also has Parkinson disease. The other patient with the mutation was diagnosed as having POAG at age 46 years. Vision in the left eye was greatly reduced at that time and was reported as finger counting.

Thr256Met was detected in the heterozygous state in a 49-year-old man with no family history of glaucoma. The patient was diagnosed as having the disease at age 46 years, when his visual field analysis showed minimum enlargement of a blind spot in both eyes. Gly399Asp was detected in a patient with sporadic juvenile-onset POAG in the homozygous state (Table 3). Both residues were found to be conserved based on *MYOC* sequences in human, mouse, rat, and pig, and the changes were absent in controls. The Thr256Met change has been previously reported in 1 patient from a study[18] of normal-tension glaucoma. This study did not provide a detailed clinical description of that patient and characterized the variant as a polymorphism. However, the substitution of a polar (uncharged) hydrophilic residue by a nonpolar residue suggests this variant may be disease causing.

Table 3. Clinical Features of Patients With Primary Open-angle Glaucoma (POAG) Carrying *MYOC* Mutations

Patient	Age at Diagnosis, y	Mutation	POAG Subtype	RE, LE Intraocular Pressure, mm Hg	RE:LE Cup/Disk Ratio	Visual Field Changes	Comments
GL14*	20	Gln48His	Sporadic juvenile	20, 14 PO	0.9:1	Glaucomatous field changes	. . .
GL41*	70	Gln48His	Familial	24, 36	0.9:0.5	Diffused depression with scotoma in superotemporal quadrant	Early cataract in LE, trabeculectomy in LE
GL92*	32	Gln48His	Sporadic juvenile	28, 38	0.5:0.7	Glaucomatous field changes	. . .
GL452	46	Thr256Met	Sporadic	27, 30	0.5:0.5	Minimum enlargement of blind spot in BE	Spondylosis
GL178	32	Thr353Ile	Sporadic juvenile	16, 12	0.9:0.5	Superior and inferior arcuate scotoma in RE, isolated paracentral scotoma in LE	. . .
GL34*	18	Pro370Leu	Familial juvenile	18, 24 PO	0.8:0.7	Superior and inferior arcuate scotoma in RE, scotomatous defect in supranasal quadrant in LE	. . .
GL145*	55	Gln368Stop	Familial	21 BE, PO	0.9:0.8	Superior arcuate defect	Left eye has myopic chorioretinopathy and squint, cataract in BE
GL429	62	Gln368Stop	Familial	18, 14	0.7:0.8	Vision loss in RE, superior arcuate defect with gross constriction in LE	Retina detachment occurred in RE
GL52	20	Gly399Asp	Familial juvenile	30, 27	0.9:0.8	Superior and inferior arcuate scotoma in BE	. . .
GL211	46	Ala427Thr	Sporadic	30, Not measured†	. . .	Central tubular vision in RE	Cataract in BE
GL437	76	Ala427Thr	Sporadic	14, 14	. . .	Arcuate zone depression in RE, enlarged blind spot in LE	Parkinson disease

Abbreviations: BE, both eyes; LE, left eye; PO, postoperative; RE, right eye.
*Already published.[5]
†Not visible because of dense cataract.

Gln368Stop (c.1102C>T), a common mutation among persons of white race with variable penetrance,[19] was detected in 2 familial cases of POAG. The change was identified in family 1 (**Figure 1**A) and is the first report of this mutation among Asians.[20] In addition to the proband, the mutation was present in III:8, III:9, IV:1, and IV:4. However, none of the mutation carriers in the family showed any sign of disease onset, which indicates possible incomplete penetrance in the family. On screening other candidate genes (*OPTN*, *CYP1B1*, and *OPTC*) for a potential digenic form of the disease in the proband, no suspected variant allele was detected. The mutation was also detected in the heterozygous state in 3 of 4 individuals in family 2 (Figure 1B). Field analysis of the proband, diagnosed at the age of 62 years, showed complete diminution of vision in the right eye and a superior arcuate defect with gross field constriction in the left eye. Trabeculectomy was performed in both eyes. Two of her daughters (II:2 and II:5) were heterozygous for the mutation and were found to be glaucomatous by retinal nerve fiber layer analysis, while the genotypically normal son (II:3) had no aberration on retinal nerve fiber layer analysis. Haplotype analysis based on 2 short tandem repeat polymorphic markers (D1S2815 and D1S2790) and an SNP marker (–83G>A) suggested that the mutant chromosome in the 2 families does not share a common origin (Figure 1). Allele sizes for the short tandem repeat polymorphic markers in the patients with POAG of white race (241 base pair [bp], 245 bp for D1S2790 and 220 bp, 233 bp for D1S2815) were different from those of 2 Indian patients (250 bp, 244 bp for D1S2790 and 222 bp, 224 bp for D1S2815), suggesting the absence of any founder mutation.

MYOC SNPs IN THE INDIAN POPULATION

We detected 6 nucleotide variants, 4 of which were coding SNPs (c.855G>T, c.952C>T, c.1041T>C, and c.1182C>T); these nucleotide variants represented silent changes and rare variants and were identified only among patients (Table 1 and Table 2). Among these, the silent change (Thr394Thr [c.1182C>T]) was predicted to affect splicing of the gene by in silico analysis but needs to be validated experimentally. Two variants (–83G>A and c.227G>A) were detected in patients and controls in similar frequencies. Based on the distribution of the alleles and the genotypes between the patients and the controls, neither of the polymorphisms was found to be associated with the POAG.

We had intended to evaluate linkage disequilibrium among heterozygous SNPs and haplotype diversity in the *MYOC* locus using healthy individuals. This information might be useful to evaluate *MYOC* variant alleles (using suspect SNPs) for their association with POAG in nonfamilial cases (case-control studies). In this context, it is noteworthy that the HapMap project (http://www.hapmap.org /cgi-perl/gbrowse/gbrowse/hapmap) does not include the Indian subcontinental population, representing one sixth of the world's population. Therefore, we selected 4 SNPs— c.227G>A (rs2234926), intron 1 C>A (rs171002), in-

tron 2 A>G (rs235873), and intron 2 G>T (rs235869)—in *MYOC* to determine the heterozygosity and pairwise linkage disequilibrium between these markers in the Indian population, as part of the effort undertaken by the Indian Genome Variation consortium.[9] The 4 SNPs were found to have a heterozygosity value of 0.3 or higher in all linguistic groups except the Tibeto-Burman one (**Figure 2**A). Only 2 SNPs (rs171002 and rs235869) of the 4 SNPs examined were included in the HapMap project and were observed to have similar heterozygosity among 4 world populations (Figure 2B). Estimation of pairwise linkage disequilibrium (r^2) between the markers indicated the presence of the following 3 groups: (1) markers 1 (reference sequence [rs] 235869), 3 (rs171002), (2) marker 4 (rs2234926), and (3) marker 2 (rs235873) as shown in Figure 2. The pattern was mostly similar in the 4 linguistic groups of India, as shown in Figure 2B. An estimation of haplotype diversity among these linguistic groups indicates 4 common predominant haplotypes in addition to a few rare ones found in low frequencies.

COMMENT

The present study carried out among 315 patients with POAG identified 7 mutations in 11 patients. Therefore, our study revealed *MYOC* mutations in 2.2% of POAG cases, which is consistent with the reported involvement of *MYOC* in 2% to 4% of patients with the disease. We, and other investigators,[20] identified a single mutation in exon 1 (Gln48His), compared with 6 *MYOC* disease-causing variants identified in exon 3 among Indian patients with POAG. *MYOC* exon 3 codes for the olfactomedin domain, which is important with respect to the functionality of the protein.[21]

Of these novel variants identified, Gly399Asp was found in the proband in the homozygous state. The subject's heterozygous parents (aged 48 and 40 years) did not manifest glaucoma. This suggests that this variant is not truly pathogenetic or that the likely disease mechanism for this particular mutation is unlike other exon 3 *MYOC* mutations.[22,23] However, mutation of the same codon with Val instead of Asp has been reported to cause late-onset POAG (mean age at onset, 51 years) in a Guyanese family and juvenile-onset POAG when the mutation is present with a *CYP1B1* mutation (Arg368His).[24] Therefore, it is likely that in the Indian family of the proband, the heterozygote parent might manifest POAG at a later age and that the homozygous genotype for the mutation resulted in juvenile-onset POAG in the proband. We are investigating defects in other candidate genes in the proband for a potential digenic form of the disease. Thr256Met and Gly399Asp, being nonconservative substitutions in the functional olfactomedin domain of *MYOC*, are expected to have deleterious effects on protein function.[21]

In the course of our study, we identified one mutation (Pro370Leu) associated worldwide with a severe glaucoma phenotype and another mutation (Gln48His) as a potential risk factor for glaucoma among patients of Indian origin.[25] We also identified the Gln368Stop mutation, which has to date been predominantly found among persons of white race/ethnicity.
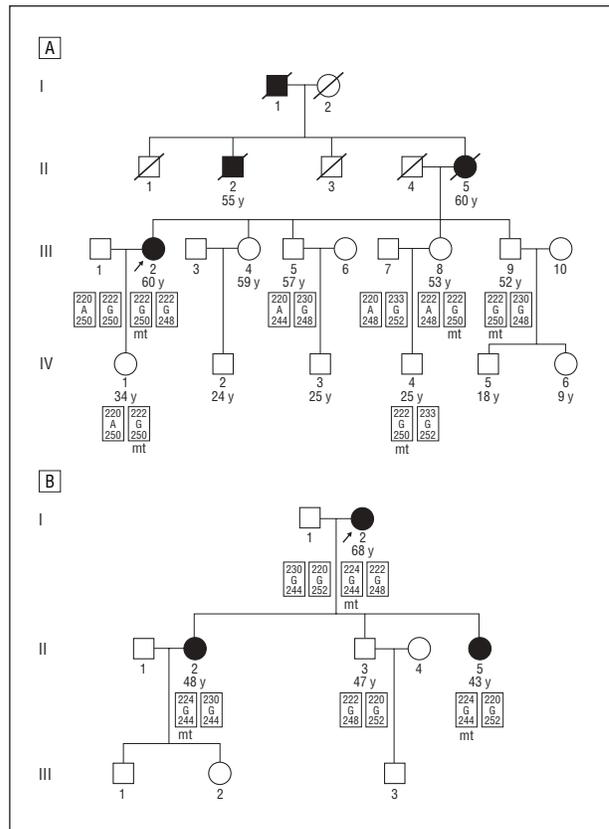

Figure 1. Pedigrees of eastern Indian families with a Q368X mutation in *MYOC*. Haplotypes are shown based on 2 microsatellite markers and an intragenic single nucleotide polymorphism marker (−83G>A) in the order of D1S2815 (1q24.4), −83G>A (intragenic), and D1S2790 (1q24.3). A, Family 1. B, Family 2. mt indicates mutant haplotype; squares, men; circles, women; filled symbols, individuals manifesting primary open-angle glaucoma; virgule, deceased; and arrow, proband.

Identification of the Ala427Thr mutation in *MYOC* in a patient with Parkinson disease represents an interesting case. Recently, it has been shown that γ-synuclein overexpression inhibits secretion of myocilin from cells to cultured media in the mouse HT22 hippocampal cell line.[26] γ-Synuclein and α-synuclein are expressed in the human trabecular meshwork, and mutations in α-synuclein cause Parkinson disease and other neurodegenerative disorders.[17,26] This information points toward a possible connection between α-synuclein and *MYOC* and invokes a putative involvement of α-synuclein with *MYOC* mutations in POAG. However, the observation may be coincidental and would require further evidence to substantiate the potential interaction between these 2 genes.

Analysis of 4 intragenic SNPs in the Indian population revealed 3 SNPs (SNP1 [rs235869], SNP2 [rs235873], and SNP4 [rs2234926] in Figure 2) that could serve as markers to examine cosegregation of *MYOC* alleles with POAG in familial cases. The lower pairwise linkage disequilibrium (r^2) for SNP2, relative to the other 3 SNPs, suggests late arrival of SNP2 in the Indian population.

Our study on the role of *MYOC* in patients with POAG, so far the largest single study from India (to our knowledge), shows that the frequency of *MYOC* mutations among Indian patients is similar to that among other population
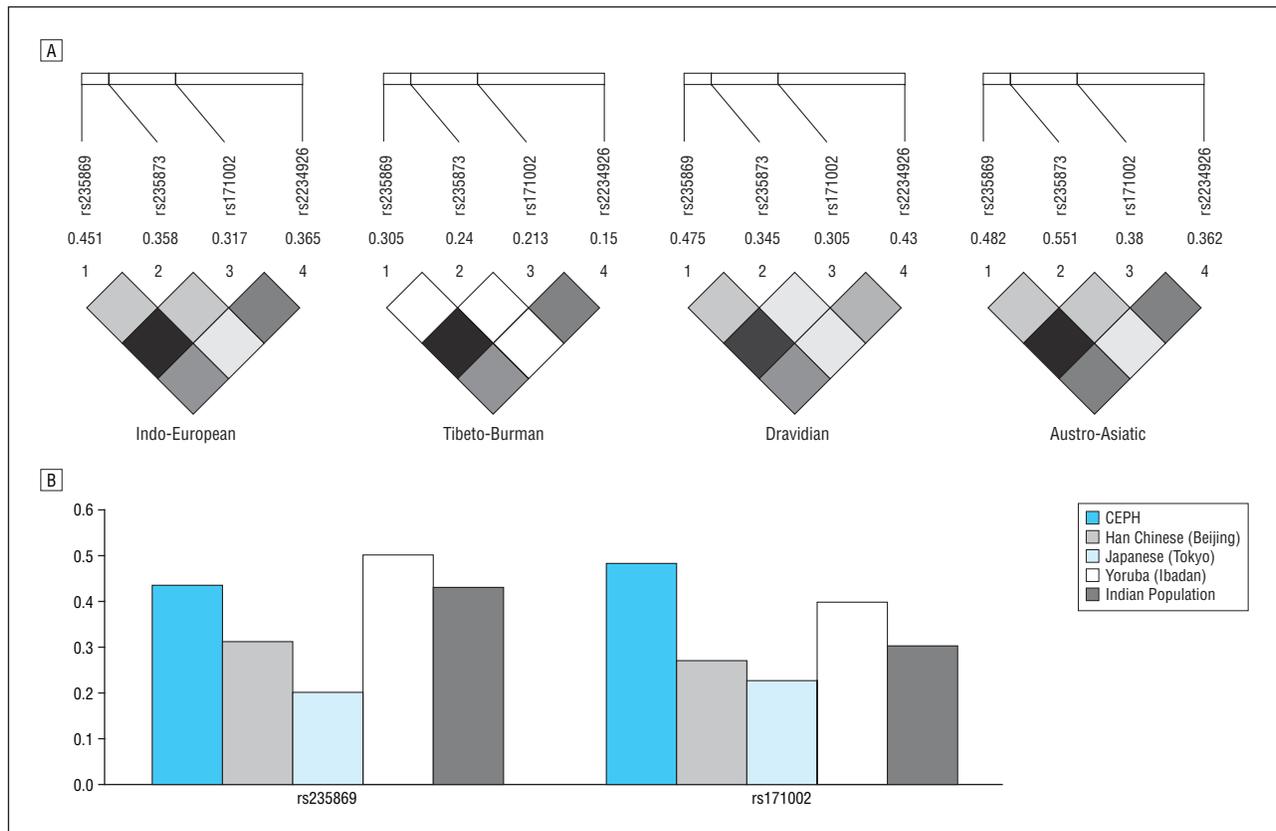
Figure 2. Evaluation of single nucleotide polymorphisms (SNPs) in *MYOC* across Indian and other populations. A, Linkage disequilibrium (LD) patterns of SNPs among the linguistic groups of India with their respective observed heterozygosities. The top bar shows the relative position of SNPs identified by dbSNP (SNP database) with their respective rs (reference sequence) numbers. The extent of LD decreases with lighter shades (calculated using Haploview 3.2 software with standard color schemes). B, Variation of SNPs in Indian and other worldwide populations. CEPH indicates a reference population collected by Centre d'Etude de Polymorphisme Humain, France, and is used worldwide for genetic studies.

groups. The identification of informative SNPs in the diverse population groups within India will provide wider opportunity to study cosegregation of *MYOC* alleles with juvenile-onset POAG in familial cases of the disease in India. In addition to direct mutation screening, linkage-based diagnosis of familial juvenile-onset POAG cases will assist clinicians in better management of the disease.

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From the Archives of the Archives

The most essentially different forms are alcohol and tobacco amblyopia on the one hand, and quinine amaurosis on the other.

In the former group there is the clinical picture of a retrobulbar partial optic neuritis with a central scotoma and normal limits of the field. The anatomical changes are limited to the papillomacular bundle of fibers.

The second group is characterized by the existence of vascular changes, diminution of the caliber of the vessels, and necrosis in consequence of ischemia and of the toxic effect on the nerve elements. To this group belong quinine, salicylic acid, male fern, pomegranate root. There is excessive contraction of the visual field, but never multiple peripheral neuritis.

Reference: Uhthoff B. Uhthoff (Breslau) discussed toxic optic neuritis. *Arch Ophthalmol.* 1901;30:70-80.