Evaluation and understanding of myocilin mutations in Indian primary open angle glaucoma patients

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Purpose: To screen for mutations in the MYOC gene of patients with Primary Open Angle Glaucoma (POAG) in India and to better understand the mutations using a possible model of myocilin.

Methods: We analyzed DNA for mutations in 107 subjects with POAG and 90 normal control subjects. The exonic sequences of the MYOC gene from all subjects were amplified by Polymerase Chain Reaction (PCR). We carried out Single Strand Conformation Polymorphism (SSCP) for all the PCR products. The DNA samples which showed mobility shift in the banding pattern in SSCP gel were sequenced. We also analyzed the presence of the common mutation Gln368Stop using a specific restriction enzyme Taq I. The mutations observed here and elsewhere have been mapped onto a possible model built for myocilin using a knowledge-based consensus modeling approach.

Results: Two heterozygous mutations Gly367Arg (1099G>A) and Thr377Met (1130C>T) were identified in exon3 of the MYOC gene of probands 40-1 and 51-1 respectively, from material obtained from the 107 unrelated subjects with POAG. These two mutations were not present in the normal controls studied. We identified a Single Nucleotide Polymorphism (SNP) Gly122Gly (366C>T) in exon1 of proband 57-1 as a non-disease causing variation. The common mutation Gln368Stop found in the Western population was not observed in the POAG cases screened in Indian population. The possible structural model for myocilin suggests a predominantly β-strand rich C-terminal region (181-504) which is connected by the α-helical mid-region (111-180) to the N-terminal region (34-110) which has low secondary structure content. Both the mutations, Gly367Arg and Thr377Met identified in our study, map on to the C-terminal region. These mutations disfavor burial of this region during oligomer formation due to the charged or bulky nature of the mutants. Most of the other mutations known for myocilin also are surface exposed on the C-terminal region.

Conclusions: Our findings indicate that the mutation frequency of the MYOC gene is 2% in the Indian population affected with POAG, which is not a well-studied ethnic group of the Asian continent. The variations identified in our study have been previously reported in the Western population. The nonsense mutation Gln368Stop was not observed in the present study and thereby suggests that it may not be a common disease-causing mutation in the Indian population. Amongst other Asian populations, studies in Japan also didn’t report this nonsense mutation. The location of these mutations suggest that a plausible mode of action could be by disruption of dimer or oligomer formation by the C-terminal region allowing greater chances of nucleation of aggregation by the N-terminal region.

Glaucoma is a heterogenous group of ocular diseases with a characteristic optic neuropathy and visual field loss, often associated with elevated intraocular pressure (IOP). Primary open angle glaucoma (POAG) is the most common variant of glaucoma comprising nearly half of the estimated 67 million people with glaucoma worldwide [1]. POAG is one of the leading causes of irreversible blindness in the world. In India, POAG is the most common form of glaucoma and about 1.5 million people are blind due to glaucoma [2]. The manifestation of this group of eye conditions could start at birth or may appear after the age of 80, depending on the type of glaucoma present in an individual. Juvenile onset open angle glaucoma (JOAG), a form of POAG may manifest clinically between the ages of 3 and 30 [3-6]. The late onset form of this condition usually manifests clinically before the age of 40 and is the most prevalent type [7-12]. Besides, differences in age of onset, there are other features that may help differentiate between these two subgroups of POAG. The disease is more severe in JOAG and subjects with significantly higher intraocular pressures (IOP) may be more refractory to treatment with medicines [4-6]. In contrast, the late onset form usually has a milder presentation with progressive development, moderate elevation of IOP and satisfactory outcomes with medical treatment [7,8,13-15]. Considerable evidences suggest that POAG has a significant genetic basis and therefore, molecular genetic methods are to be used to further investigate the pathophysiologic mechanisms of the disease. Polansky et al. discovered a protein that was markedly increased when the trabecular meshwork cells were exposed to corticosteroids. They named the protein Trabecular Meshwork Inducible Glucocorticoid Receptor (TIGR) [16,17]. The gene for the TIGR protein was viewed as an attractive candidate gene for glaucoma. The finding by Stone et al. [18] that the TIGR gene was in the

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interval containing GLC1A locus of chromosome 1, further increased interest in this gene. The TIGR protein was later assigned the name myocilin because it shared the homology with the protein myosin [19] and it is now referred to as MYOC.

Several chromosomal loci have been identified with POAG, but the mutations in the MYOC gene of chromosome 1/GLC1A have been specifically associated with the development of late onset and JOAG. There has been close to fifty point mutations and several single nucleotide polymorphisms identified in multiple ethnic groups associated with late onset and juvenile glaucomas worldwide. Overall, 90% of the mutations were located in exon 3, which contains the olfactomedin homology. The myocilin mutations have been identified in 2-4% of individuals with primary open angle glaucoma, in the various populations studied [20-27]. Mutations in the MYOC gene have been studied in several populations from Asia, including Japan [28-30], China [31], and Korea [32]. Recently, Mukhopadhyay et al. [33] have, reported mutations and single nucleotide polymorphisms in the MYOC gene in Indian population with POAG. The candidate MYOC gene was studied for the prevalence of mutations in individuals with primary and juvenile open angle glaucoma in order to broaden our understanding of the molecular basis of this disease in an ethnically diverse Indian population. To understand the structural basis of the mutations identified in our study and elsewhere [34] were mapped on the model for visualization and interpretation.

METHODS

Enrollment of index cases: Patients with primary open angle glaucoma (POAG) of Indian ethnic origin were recruited from the Glaucoma Services of the Aravind Eye Hospital, Madurai, South India. The nature of the study was discussed and informed consent was obtained from all patients willing to participate. The Institutional Review Board and Ethics Committee of the Aravind Eye Hospital, Aravind Medical Research Foundation, approved the study and tenets of Declaration of Helsinki on Human trials were adhered to strictly.

Clinical diagnosis involved a detailed workup for medical and family history of glaucoma and ocular diseases. Ophthalmic evaluation included best-corrected Snellen visual acuity, measurement of intraocular pressures by Goldmann applanation tonometry, anterior chamber angle evaluation by Goldmann two-mirror Gonioscope and optic disc and retinal nerve fiber examination by 90-diopter indirect lens. Humphrey autoperimeter was used to evaluate the patient’s visual fields. The inclusion criteria were individuals diagnosed with POAG based on optic disc changes typical of glaucoma and matching visual field defects by autoperimetry and open iridocorneal angles on gonioscopy. Glaucoma due to angle closure, trauma, inflammation and other secondary causes were excluded by clinical examination and gonioscopy.

The controls (without any history of glaucoma) for the study were recruited from the general Ophthalmology Clinic of the Aravind Eye Hospital, who were detected to have no ocular or systemic diseases, other than simple refractive errors. The control population was chosen to match the ethnic and geographic background of the patients with primary open angle glaucoma.

DNA preparation: We collected peripheral blood from the POAG patients and normal controls. Genomic DNA was isolated from whole blood using the salt precipitation method as described by Miller et al. [35]. DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for Polymerase Chain Reaction (PCR) analysis.

Polymerase chain reaction: PCR was carried out for all the coding sequences of the MYOC gene using a Thermocycler (MJ Research, MA, USA). Genomic DNA samples (50-100 ng) were amplified using the QIAGEN PCR Kit (QIAGEN, Hilden, Germany) in a 10 μl reaction containing 0.2 mM dNTPs, 5X Q solution (an innovative additive from QIAGEN) with 1X concentration of PCR mix and 0.25 U Taq polymerase. Each exon was amplified using primers reported by Alward et al. [34] under the following conditions; an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s with a final extension step at 72 °C for 10 min.

Single strand conformation polymorphism (SSCP): Amplified products of all the samples were analyzed for mutation by SSCP analysis. PCR products were diluted with the denaturing dye (95% formamide (S.d.fine-Chem Ltd., Mumbai, India), 10 mM NaOH (Qualigens, Mumbai, India), 0.05% Bromophenol blue (HiMedia, Mumbai, India), 0.05% Xylene cyanol (LOBA Chemie, Mumbai, India) and denatured for 5 min at 96 °C. PCR products were resolved on 6% polyacrylamide gel. The electrophoresis was carried out at 800 V for 5 to 7 h. Silver staining was done by fixing the gel in 40% methanol-35% formalin solution (S. D. Fine-Chem Ltd., Mumbai, India), rinsed twice with water for 5 min then soaked the gel in 0.02% Na2S2O3 (Ranbaxy, Delhi, India) for one min, washed the gel with distilled water, immersed in 0.1% AgNO3 (Merck, Mumbai, India) solution for 10 min, and finally the gel was rinsed and developed with 3% Na2CO3 (S. D. Fine-Chem Ltd.) till the bands appeared. All the reagents were of analytical grade.

DNA sequencing: PCR products, which showed mobility shift in SSCP were extracted from 1% agarose gel and column purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and direct sequencing was performed using ABI Prism 377 DNA sequencer (Applied BioSystems) with dye-termination chemistry. The nucleotide changes were detected by identifying the double peaks in the chromatogram due to the heterozygous nature of the variation.

Restriction digestion: From the restriction map of exon3 of MYOC using CUTTER, it was identified that a Gly367Arg mutation resulted in loss of a restriction site (Btg I) in exon3. The Gly367Arg mutation, which was identified by DNA sequencing was restriction digested with the enzyme, Btg I. The specific DNA fragment was amplified using specific primers (5’ ATA CTG CCT AGG CCA CTG GA 3’) and (3’ CAT TGG CGA CTG ACT GCT TA 5’) to produce a 387 bp product. The PCR product was restriction digested with Btg I under the conditions described by the manufacturer (New England Biolabs, Beverly, MA) in order to reconfirm the mutation. In
the same way, the common mutation from different populations in the world (Gln368Stop) produced loss of a restriction site for the enzyme Taa 1. The DNA samples for all 107 POAG patients were amplified using specific primers 5'TAC CGA GAC AGT GAA GGC TG 3' and 5'TGT AGC TGC TGA COG TGT AC 3' (a gift from Paul N. Baird, Centre for Eye Research, Melbourne, Australia). The PCR product of 255 bp was restriction digested with Taa 1 (MBI Fermentas, Vilnius, Lithuania) as described by Paul Baird et al. [36]. The presence of this common mutation was analyzed using Taa 1 enzyme in our study.

Model building: The model for myocilin was built using knowledge based consensus-modeling approach [37,38]. The details of the modeling will be published elsewhere. A brief description of the method used is given here. Fold recognition was done using the following Web-based software programs; (1) searches against the protein data bank (PDB) by PSI BLAST, (2) Conserved Domain Database, and (3) SUPFAM search with full length sequence and overlapping fragments. The SUPFAM search identified a significant match with a part of 1h70, DDAH (Dimethyl arginine Dimethyl aminohydrolase, also called Pentein) [39]. The myocilin region 180 to 433 is threaded on to A0 to A253 of 1h70 with gaps using the Insight 2000 software (Accelrys Software, Bangalore, India). The disulphide bond between Cys245-Cys437 identified by Naggy et al [40] was found to be feasible in the threading and was incorporated using Insight 2000 software. For other regions FASTA searches against PDB were done using overlapping fragments and significant matches were used as templates for modeling. Myocilin regions 1 to 61, 70 to 174, and 453 to 504 were modeled using 1B0K (445 to 505), 1I84 (S823-S923) and 1K8Q (A92 to A816) respectively. The helical segments (34 to 180) were put together taking into account the secondary structure packing. Consecutive fragments were joined using loop searches with Insight 2000 software. Splice repair was performed to optimize the peptide geometry using the homology module of Insight 2000 software. Energy minimization by conjugate gradient algorithm was done using consistent valence force field until minimum energy value was obtained. The model building and visualization was done using Insight 2000 software on the SGI-O2 machine. Mutations that were identified in this study and elsewhere [34] were mapped on to the model for visualization and interpretation.

RESULTS & DISCUSSION

Among the subjects studied, 51 were affected by JOAG, 56 were affected by adult onset POAG and 90 were normal controls (Mean age 38±12.4) known to be free of glaucoma. The POAG cases fell in the age group of 8 to 71 years (Mean age 36±14.8).

All the POAG patient samples and controls enrolled in this study were screened for mutations in the MYOC gene by SSCP analysis and DNA sequencing. Among the 107 patient samples two heterozygous mutations and one polymorphism were identified in three different probands. All the variants identified were missense mutations. The common mutation Gln368Stop found in the Western population was analyzed in our study, by Taa 1 restriction digestion. This analysis revealed

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the absence of Gln368Stop mutation in all the 107 patients screened. Among the Asian population studied for mutations in the MYOC gene the Japanese population also did not exhibit this mutation [23]. The mutant residues reported in our study and in other studies [34] are surface exposed in the predominantly β-strand rich C-terminal region (181-504) of the model.

**Identification of MYOC mutations at Exon3:** Among 107 POAG patients screened, Gly367Arg and Thr377Met were found in exon3 of the MYOC gene from probands 40-1 and 51-1 respectively. Both of them were identified as heterozygous transition mutations, Gly367Arg (1099G>A) and Thr377Met (1130C>T, Table 1). This is the first report of the two mutations in POAG patients of the Indian population.

**Heterozygous Gly367Arg mutation at Exon 3:** Gly367Arg has been earlier reported in other populations by Taniguchi et al. [41], Suzuki et al. [28], and Cobb et al. [42]. In this study we found Gly367Arg in exon 3 of the MYOC gene in proband 40-1. This was identified by SSCP analysis (Figure 1A), which showed a mobility shift in the bands. This was followed by DNA sequencing. The forward sequence shows heterozygous transition mutation where allele A is present instead of allele G at nucleotide position 1099 as indicated in chromatogram (Figure 1C). The normal sequence is seen in Figure 1B. Normal controls did not exhibit this allelic variation. The heterozygous mutation was reconfirmed by restriction digestion analysis with Btg I. The wild type (normal control) sequence revealed two distinct bands of 220 bp and 167 bp. (Figure 1D). The digestion of DNA sample with Gly367Arg mutation, showed three distinct bands of 387 bp (mutant allele) and 220 bp and 167 bp, the latter being same as that of the normal control.

**Clinical description of the patient with the heterozygous mutation Gly367Arg:** A 32-year-old female reported with history of defective vision in both eyes noticed three months prior to her hospital visit. She had been diagnosed to have glaucoma by her ophthalmologist and had been on pilocarpine and timolol in both eyes, a month prior to her visit. She had no systemic diseases and no h/o glaucoma in the family. Anterior segment evaluation was remarkable for an afferent pupil defect in the right eye and anterior chamber angles were open by gonioscopy. Her corrected acuity was 6/6 in the left eye and light perception in the right eye. Ocular pressures by applana-

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All probands with MYOC mutations had moderate to severe glaucomatous disc damage and field loss, high intraocular pressure on presentation and required filtering surgery to normalise IOP. LE indicates the left eye and RE indicates the right eye.
tion tonometry were 50 and 40 mm Hg. Posterior segment evaluation revealed advanced glaucomatous optic nerve damage with totally excavated discs in the right eye and a cup to disc ratio of 0.9 in the left eye. She had trabeculectomy with adjunctive mitomycin in the left eye, which had controlled her ocular pressures in the mid teens.

**Heterozygous Thr377Met mutation at Exon 3:** SSCP analysis revealed the mobility shift in the banding pattern (Figure 2A) in proband 51-1 and was sequenced to identify the allele specific variation. This variation (Thr377Met) found in exon3 of the MYOC gene is a heterozygous transition mutation where allele T is present instead of allele C at nucleotide position 1130 as indicated in chromatogram (Figure 2C). The normal sequence is seen in Figure 2B. We also propose that Thr377Met might be a disease causing mutation since none of the control subjects studied showed the mutation. Thr377Met mutation has been reported by Fingert et al. [23], Shimizu et al. [43] and Wiggs et al. [44].

**Clinical description of the patient with the heterozygous mutation Thr377Met:** A 52-year-old male with complaints of (c/o) defective vision and colored halo was on treatment for glaucoma since 6 months before he was evaluated at our Center. His corrected acuity was 6/6 in each eye and had high ocular pressures in spite of medical therapy (44 and 36 mm Hg on applanation tonometry). He reported a positive family history of glaucoma and visual fields by Humphrey’s autoperimetry, was characteristic for arcuate scotomas of moderate severity in both eyes. His anterior segments of both eyes were within normal limits and fundus evaluation revealed moderate optic disc excavation with a cup to disc ratio of about 0.7. Anterior chamber angles were normal with open iridocorneal angles. Ocular pressures were controlled by trabeculectomy in both eyes.

**Genotype-Phenotype Correlation:** Mutations at Gly367Arg and Thr377Met correlate with the phenotypic expression of affected probands. Alteration of amino acid sequence by nucleotide change in the MYOC gene, which was not present in the normal controls, is the only support on MYOC mutations for its pathogenicity. The IOP and cup disc in the POAG population studied here ranged from 10-58 mm Hg, 0.6-0.95 with the mean of 33.32±11.22 and 0.88±0.08 respectively. In probands 40-1 (Gly367Arg) and 51-1 (Thr377Met), the phenotype was most severe in terms of elevated IOP and cup disc ratio (Table 2). Mutations leading to premature termination codons (i.e., nonsense mutations) are well known to lead to a phenomenon known as nonsense-mediated decay of

![Figure 3. A biallelic SNP Gly122Gly in exon1 of MYOC. A: SSCP analysis. Lane 1 is the normal control, Lane 2 is one of the probands not showing mobility shift, and Lane 3 is proband 51-1 showing mobility shift as indicated by the arrow. B: Normal reverse sequence. C: Reverse sequence derived from proband 51-1 showing heterozygous transition change at nucleotide 368 (368C>T) resulting in a Glycine to Glycine change, which is a non-disease causing polymorphism at codon 122.](image-url)

![Figure 4. Taa1 restriction digestion to screen the Gln368Stop mutation. Lane 1 is the 100 bp ladder, Lane 3-9 are some of the probands among the 107 probands showing 211 and 44 bp products. Lane 11 is the positive control for Gln368Stop mutation showing 255, 211 and 44 bp products.](image-url)
mRNA [45,46]. This means that the mRNA carrying such a mutation is rapidly degraded in the cell before it can be translated into a truncated protein. One such nonsense mutation is Gln368Stop. This mutation is predicted to lead to accelerated decay of the mutant mRNA relative to the normal mRNA produced by the normal allele. Hence, it is likely that there is more normal protein compared to mutant protein. In contrast, missense mutations such as Gly367Arg do not lead to nonsense mediated mRNA decay since they do not produce a premature stop codon. In these patients the normal and mutant mRNA are likely to be present in equal amounts. Thus, according to Cobb et al. [42], in patients carrying missense mutations such as Gly367Arg and Thr377Met are likely to have equal ratio of mutant and normal protein resulting in the severity of the phenotype.

**Identification of SNP at Exon 1 of MYOC:** A biallelic (C/T) SNP was identified in the coding sequence of MYOC in proband 57-1 (Table 1). This polymorphism has been previously reported by Alward et al. [34]. The mobility shift in banding pattern is given in Figure 3A. The allelic change occurred in exon1 at nucleotide position 368 and the SNP has been assigned as Gly122Gly (366C>T, Figure 3C). The normal sequence is seen in Figure 3B. The protein product is not altered as a result of this SNP, which implies non-pathogenicity. None

![Figure 5: A possible structural model for myocilin](image-url)

**Figure 5.** A possible structural model for myocilin. A: The secondary structural elements in the model are colored as follows; Helix (Red), β-strand (Yellow), Turns (Blue) and random coil (green). The N-term and C-term are marked. The N-term region has low secondary structure content and the C-term region is more compact. The long helix in front of the C-term region is the coiled coil region. B: Two orthogonal views of the CPK space-filling model are shown. The view at 90 is equivalent to viewing the model shown in A from behind the page. The color scheme is indicated in the figure.
of the normal individuals studied expressed this polymorphism. This SNP has been reported in the Western population as non-disease causing polymorphism. Thus suggesting that some other factor(s) apart from MYOC might play a role in the pathogenicity of POAG.

Clinical description of the patient with Gly122Gly polymorphism: We evaluated a 43-year-old male with h/o prior treatment for open angle glaucoma for failed medical therapy. He had been on medical therapy for glaucoma for the preceding ten years, but without adequate control of IOP. His corrected central acuity was 6/6 in each eye with mild degree of myopic astigmatism. Slit lamp biomicroscopy revealed normal anterior segments with open iridocorneal angles on gonioscopic evaluation. Ocular pressures on applanation tonometry were 32 and 37 mm Hg in the right and left eyes respectively in spite of using maximal medical therapy. Posterior segment evaluation was remarkable for advanced glaucomatous optic disc damage with a cup to disc ratio of about 0.9. Humphrey’s autoperimetry was characteristic for advanced glaucomatous visual field loss sparing only the central 5° of the visual fields in either eye. Ocular pressures were controlled by trabeculectomy with adjunctive mitomycin in both eyes.

Taa 1 restriction digestion revealed absence of Gln368Stop mutation in Indian POAG patients: Among the various mutations identified in different populations worldwide, Gln368Stop is the common mutation present in exon 3 of the MYOC gene. This mutation produces a truncated form of myocilin protein. This mutation commonly occurs in adult onset POAG patients at the age of 40 years. It has been reported that this common mutation was found in 1.6% of glaucoma probands and was found in all groups except the Japanese [23]. The presence of this common mutation was analyzed in Indian population using the restriction enzyme Taa 1 whose recognition sequence is altered as a result of this mutation. We screened a total of 107 POAG probands to identify the presence of this mutation in the Indian population along with the positive control for this mutation. Figure 4 shows that all individuals participated in this study were negative for this mutation, which is indicated by two distinct bands at 211 bp and 44 bp (wild type alleles in both the copy of the gene). In positive control three distinct bands of sizes 255 bp (mu-tant allele), 211 bp and 44 bp (wild type allele) were observed because of heterozygous nature of the mutation.

Understanding mutations based on model: More recently the C-terminal Olfactomedin-like domain has been characterized by Circular Dichorism (CD) and has been found to be composed of predominantly of β-sheet [40]. Our model resulted in a β-strand rich C-terminal region (181-504) having the Cys245-Cys433-disulphide bond. The proposed model for myocilin (Figure 5A) suggests that the N-terminal region has less secondary structure content than the other regions. Moreover, the mid-region forms a set of disjointed helices, which can provide flexibility and inter-molecular interaction through the coiled coil helical region. The C-terminal region is quite compact and contains the olfactomedin-like region (245-504) [40] along with an adjacent 181-244 region, identified as the pentein fold of DDAH protein. Interestingly, this DDAH protein also has been reported to form dimers and oligomers like myocilin [39]. The mutations identified in this study and elsewhere (Figure 5B) are all surface exposed. There are no mutations in the mid-region. Both the mutations, Gly367Arg and Thr377Met identified in our study, map on to the C-terminal region. Both mutations disfavor burial of this region during oligomer formation due to the charged or bulky nature of the mutants. Most of the other mutations known for myocilin also are surface exposed on the C-terminal region. The location of these mutations and the truncation of the C-terminal region by Gln368Stop suggest that a plausible mode of action could be by disruption of dimer or oligomer formation by the C-terminal region. This would then allow for greater chances of nucleation of aggregation by the N-terminal region. Upon initiation of aggregation, the hinge region would allow the coiled coil region to become available for further interaction leading to a domino type of effect. Conformational changes of the N-terminal and hinge regions induced by the molecular environment in the normal protein could also favour aggregation. This would also explain the occurrence of POAG in the normal population as only 2-4% of POAG cases are caused by mutations.

The frequency of mutation in MYOC shows a low frequency of about 2% present in the Indian POAG population, which is not a well-studied ethnic group in the Asian continent. Understanding these mutations based on a plausible model suggests a possible induced conformation change mechanism for the aggregation, which is in agreement with the low frequency of mutations associated with POAG. It would be useful to study the impact of MYOC sequence and structural changes associated with POAG in various ethnic groups of India, in advancing the molecular genetics based diagnosis and evaluation of POAG.

ACKNOWLEDGEMENTS

We sincerely thank the members of the families for participation in this study. The Indian Council of Medical Research, New Delhi, India, financially supported this work. We gratefully acknowledge Dr. Edwin Stone, Department of Molecular Ophthalmology, University of Iowa, USA, for his contribution and support of this study. We thank Dr. V. R. Muthukkaruppan, Director Research for valuable suggestions and encouragement. We thank Dr. Reena Chandrashekhar, Senior Scientist, and Dr. Praveen Kumar Nirmalan, for critical evaluation of the manuscript. We thank Mr. G. Neethirajan, Senior Research Fellow and Ms. V. R. Muthukkaruppan, for their technical assistance and Mr. R. Jeya Krishnan, Mr. M. Rajkumar for the photographic work.

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