Molecular Genetic Studies on Indian Glaucoma patients

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THE DEPARTMENT OF BIOCHEMISTRY
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Dedicated to my beloved
Parents, Dr.A.K.Sarala and to all
the Patients

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DECLARATION

The research work embodied in this thesis entitled, "**Molecular Genetic Studies on Indian Glaucoma Patients",** has been carried out by me at the L. V. Prasad Eye Institute, Hyderabad, under the guidance of Prof. D. Balasubramanian and Prof. T. Suryanarayana. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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CERTIFICATE

This is to certify that this thesis entitled, "**Molecular Genetic Studies on Indian Glaucoma Patients**", submitted by **Koilkonda Rajeshwari Devi** for the degree of **Doctor of Philosophy** to the University of Hyderabad is based on the work carried out by her at the L. V. Prasad Eye Institute, Hyderabad, under our supervision. This work has not been submitted for any diploma or degree of any other University or Institution.

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ABBREVIATIONS

μg:	Microgram
μl:	Microlitre
μM :	Micromolar
A:	Adenine
bp:	Basepair
C:	Cytosine
cDNA:	Complementary DNA
DMSO:	Dimethylsulphoxide
dNTPs:	deoxy nucleotide triphosphates
dup:	Duplication
G:	Guanine
Ins:	Insertion
IOP:	Intraocular pressure
JOAG:	Juvenile open angle glauocma
kDa:	Kilodalton
PAC	Primary angle closure
PACG	Primary angle closure glaucoma
PAGE:	Polyacrylamide gel electrophoresis
PCG:	Primary congenital glaucoma
PCR:	Polymerase chain reaction
POAG:	Primary open angle glaucoma
RFLP:	Restriction fragment length polymorphism
rpm:	Revolutions per minute
SNP:	Single nucleotide polymorphism
T:	Thymine
TM:	Trabecular meshwork
EDTA:	Ethylenediaminetetraaceticacid
TAE:	Tris acetate EDTA
SDS:	Sodium dodecylsulphate
Tris:	Tris(hydroxymethyl)aminomethane
PBS:	Phosphate buffered saline

Chapter 1
Introduction

1. INTRODUCTION

Glaucoma is a complex disease leading to irreversible blindness worldwide. It involves the loss of retinal ganglion cells (RGCs), visual field defects, and degeneration of the optic nerve head (Ritch *et al.,* 1989). According to the WHO report on global burden of visual impairment, the number of persons with visual impairment worldwide based on the best-corrected visual acuity, is around 161 million: of these 37 million are blind and 124 million have low vision (Resnikoff *et al.,* 2004). Cataract is the leading cause of blindness (47.8%), followed by glaucoma (12.3%) and age-related macular degeneration (8.7%) (Resnikoff *et al.,* 2004). A recent report by Dandona *et al,* (2006) estimates that, the number of persons with visual impairment, based on uncorrected refractive error globally, is around 259 million (65% higher than the WHO estimate). This included 42 million persons who were blind and 217 million with less severe visual impairment.

According to a recent prevalence report, it is estimated that by the year 2010 around 60.5 million people worldwide will be affected with glaucoma, and this includes both primary open angle and angle closure glaucoma (POAG and PACG, respectively), which will rise to 79.6 million by the year 2020. Of this, 74% is predicted to have open angle glaucoma (Quigley *et al.,* 2006). Glaucoma is highly prevalent in India with POAG being the most common form (Thomas *et al.,* 2003). The prevalence of POAG in South Indian population has been reported to be 0.41%-4.29% (Jacob *et al.,*1998; Dandona *et al.,* 2000; Vijaya *et al.,* 2005).

Glaucomas are classified into primary and secondary based on their etiology and aqueous humor dynamics (Shields, 1998). Anatomically, based on the alteration in the anterior chamber angle leading to raised IOP, there are two forms of glaucoma: primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) glaucoma. In POAG, there is an increase in resistance to the outflow of aqueous humor due to obstruction at the trabecular meshwork. On the other hand, PACG is an anatomical disorder of the anterior segment of the eye characterized by permanent closure of part of the filtration angle as a result of the iris apposition to the trabecular meshwork (Ritch *et al.,* 1996).

POAG represents a common form of primary glaucoma and is characterized by loss of peripheral visual function and damage of the optic disc (Quigley *et al.,*1996). Thirteen chromosomal loci have been mapped by linkage in POAG. These are *GLC1A* (1q24.3-q25.2; Sheffield *et al.,* 1993), *GLC1B* (2cen-q13; Stoilova *et al.,* 1996), *GLC1C* (3q21-q24; Wirtz *et al.,* 1997), *GLC1D* (8q23; Trifan *et al.,* 1998), *GLC1E* (10p15-p14; Sarfarazi *et al.,* 1998), *GLC1F (*7q35-q36; Wirtz *et al.,* 1999), *GLC1G* (5q22.1; Samples *et al.,* 2004), *GLC1H* (2p16.3-p15; Suriyapperuma, *et al.,* 2007), *GLC1I* (15q11-q13; Allingham *et al.,* 2005), *GLC1J* (9q22; Wiggs *et al.,* 2004), *GLC1K* (20p12; Wiggs *et al.,* 2004), *GLC1L* (3p21-22; Baird *et al.,* 2005) and *GLC1M (*5q22.1-q32; Fan *et al.,* 2007*)*. Three of these, *viz- GLC1A*, *GLC1J* and *GLC1K* contribute to JOAG, while the rest are involved in adult onset POAG.

Three genes, namely, *MYOC (GLC1A)* encoding myocilin, *OPTN (GLC1E),* encoding optineurin and *WDR*36 (*GLC1G*) have been identified to harbour mutations causing POAG. Myocilin (*MYOC*, MIM 601652) was initially known as the trabecular meshwork-inducible glucocorticoid response (*TIGR*) gene and is the first gene to be identified in POAG (Stone *et al.,* 1997). The *MYOC* gene comprises three exons of which the first exon resembles the myosin-like domain and the third exon resembles the olfactomedin-like domain. So far, more than 73 mutations are known and most of these are missense mutations (Gong *et al.,* 2004); >90% (63) of the mutations were located in the third exon (Gong *et al.,* 2004; Yen *et al.,* 2007) suggesting that this is a functionally important domain (Adam *et al.,* 1997). The precise role of *MYOC* is poorly understood, however it is hypothesized that mutant *MYOC* is not secreted out from the rough endoplasmic reticulum, thereby leading to trabecular meshwork (TM) dysfunction and increase in aqueous outflow resistance, finally resulting in glaucoma (Zilling *et al.,* 2005).

A wide spectrum of mutations have been reported in *MYOC* in different populations, which accounts for 2 - 5% of all POAG cases worldwide (Pang *et al.,* 2002; Wiggs *et al.,* 1998; Yoon *et al.,* 1999; Aldred *et al.,* 2004). The most common *MYOC* mutation observed across different populations is the Gln368Stop (1.6%). This mutation was not observed in the Japanese (Fingert *et al.,* 1999). The Pro370Leu was found to be associated with juvenile onset open angle glaucoma (JOAG), high IOP and poor response to medical treatment

(Taniguchi *et al.,* 1999). A recent report from Taiwan indicated the mutation frequency to be 12.5% and suggested the Arg46Stop mutation to be a predominant mutation (6.25%) in patients with JOAG (Yen *et al.,* 2007). Haplotype analysis studies have indicated that the Gln368Stop and the Asn480Lys mutation carriers shared a similar haplotype background indicating common founder effect (Fingert *et al.,* 1999, Adam *et al.,* 1997; Brezin *et al.,* 1998). In India, mutations in *MYOC* account for 0.8-7.14% of all POAG cases (Kumar *et al.,* 2007; Mukhopadhyay *et al.,* 2002; Kanagavalli *et al.,* 2003; Sripriya *et al.,* 2004) and Q48H is the most prevalent mutation (Chakrabarti *et al.,* 2005)

Aung *et al.,* (2005), screened 106 Chinese PACG patients and found the disease causing variations in the normal controls as well. Besides many reports on the mutation frequency of *MYOC* in POAG and the recent demonstration of its involvement in primary congenital glaucoma or PCG (Kaur *et al.,* 2005), very little is known about its involvement in PACG. The commonality of some clinical features in these phenotypes like high IOP might indicate a common molecular mechanism due to the involvement of similar gene(s). Based on these evidences along with the ethnic variations in populations, we have investigated the involvement of *MYOC* in patients suffering from PACG and POAG in the Indian population.

OPTN (GLC1E) gene has been associated to normal tension glaucoma (NTG) (Sarfarazi *et al.,* 1998) and its expression was observed in different tissues (Rezaie *et al.,* 2002). *OPTN*, also called

as the NRP (NF-Kappa-B essential modulator [NEMO] related protein) (Schwamborn *et al.,* 2000), is found to interact with adenovirus E3-14.7K (Li *et al.,* 1998), Huntingtin (Faber *et al.,* 1998), transcription factor IIIA (Moreland *et al.,* 2000), and RAB8 (Hattula *et al.,* 2000). According to the earlier reports, sequence alterations in *OPTN* were found in 16.7% of 54 families from a predominantly NTG population (Rezaie *et al.,* 2002). The mutation E50K that is located in the putative bZIP motif, was observed in 7 of 54 families (Rezaie *et al.,* 2002). Later this mutation was observed in a patient with positive family history of NTG in a larger study comprising of 1048 mixed glaucoma patients (Alward *et al.,* 2003). Individuals with this mutation manifested the phenotype at an earlier age and had advanced optic disc cupping and progressing visual field loss when compared to those individuals without E50K mutation (Aung *et al.,* 2005). The variation R545Q that was initially reported as mutation, was later observed in normal controls in the Chinese (Leung *et al.,* 2003) and Japanese populations (Toda *et al.,* 2004, Funayama *et al.,* 2004). The M98K mutation, which was reported as associated risk factor (Rezaie *et al.,* 2002), was not associated with disease phenotype in other studies on POAG patients (Mukhopadhyay *et al.,* 2005, Alward *et al.,* 2003, Wiggs *et al.,* 2003) however Rakhmanov *et al,* (2005) had reported the same variation as an associated polymorphism in POAG. An earlier study from India did not implicate *OPTN* as a candidate gene in POAG (Mukhopadhay *et al.,* 2005) however a possible role of SNPs rather than mutations in

OPTN is suggested to be implicative in POAG pathology (Sripriya *et al.,* 2006).

The third candidate gene in POAG, *WDR36* mapped onto chromosome 5q22.1 (*GLC1G*) (Monemi *et al.,* 2005) comprises of 23 exons and codes for a 951 amino acid residue protein containing a WD40 domain with several conserved residues, including a Trp-Asp at the C-terminal end. These domains are involved in protein-protein interactions (Monemi *et al.,* 2005) and is co-regulated with *IL*-12. Mutations in *WDR36* have been reported in 17% of POAG cases in earlier reports (Hauser *et al.,* 2006) but some studies could not replicate its association to POAG (Fingert *et al.,* 2007).

While mutations in *MYOC* and other candidate genes have been identified in some POAG cases, the underlying molecular mechanism remains unknown (Stone *et al.,* 1997; Fingert *et al.,* 1999; Rezaie *et al.,* 2002; Monemi *et al.,* 2005). Mutations in the *CYP1B1* gene have been associated with autosomal recessive primary congenital glaucoma (PCG) (Stoilova *et al*.,1997; Bejjani *et al*, 1998; Stoilova *et al*, 1998; Plasilova *et al*, 1999; Bejjani *et al*, 2000; Martin *et al*, 2000). This disease is produced by an improper development of the trabecular meshwork and anterior chamber angle, which increases resistance to aqueous humor outflow leading to raised intraocular pressure (IOP). *CYP1B1* (MIM 601771) is located on chromosome 2p22-21 at the *GLC3A* locus (MIM 231300) and is composed of three exons of which the first is non-coding. The putative open reading frame starts in the second exon and is 1629bp in length (Tang *et al.,*1996). It encodes a

543 - amino acid dioxin inducible member of the cytochrome p450 gene superfamily, subfamily I. *CYP1B1* protein is a membrane-bound monomeric mixed function monooxygenase. It is proposed that this cytochrome participates in the iridocorneal angle development (Libby *et al.,* 2003). Mutations in this gene have been observed in different populations, accounting for 20-100% of all PCG patients (Mashima *et al.,* 2001; Sitorus *et al.,* 2003; Reddy *et al.,* 2004; Bejjani *et al.,* 2000; Plasilova *et al.,* 1999).

In a large family with digenic inheritance of *MYOC* and *CYP1B1,* it has been suggested that *CYP1B1* might be a modifier of *MYOC* expression (Vincent *et al.,* 2002). So far, there are four reports on the involvement of *CYP1B1* in POAG from different populations. Melki *et al,* (2004) observed a *CYP1B1* mutation frequency of 4.6% among French POAG patients, while Lopez-Garrido *et al,* (2006) reported it to be 10% among the Spanish patients. Two studies on Indian POAG patients observed a mutation frequency of 4.5% in Eastern India (Acharya *et al,* 2006) and 11.5% in Southern India (Kumar *et al.,* 2007). Another study reported the association of the common polymorphism N453S in *CYP1B1* with optic disc cupping and visual field changes in POAG (Melki *et al.,* 2005) but this could not be replicated in other cohorts. These reports indicated that *CYP1B1* was not only a major candidate gene in PCG, but also involved in POAG through an unknown molecular mechanism. Similar to *MYOC*, its involvement in PACG has not yet been demonstrated. The present study attempts to

screen the molecular genetic defects in *CYP1B1* among POAG and PACG cases.

As glaucoma is a complex disease, both genetic and environmental factors are involved in its pathophysiology. Apart from mutations in the candidate genes, SNPs in 16 candidate genes were found to be associated in POAG (Fan *et al.,* 2006). In the present study, SNPs in *IL1, MTHFR*, *p21* and *MMP9* genes are analysed in POAG and PACG in case-control cohorts.

Since glaucoma is also considered as a disease of cellular stress, the molecular events resulting in optic atrophy have been postulated as a causal mechanism (Wax, 2000), This involves the death of retinal ganglion cells due to apoptosis (Lin *et al.,* 2003) occurring as a result of the up-regulation of cell adhesion molecules, that are implicated in vascular diseases (Gimbrone *et al.,* 1997). One such molecule, endothelial leukocyte adhesion molecule-1 (ELAM-1) is found to be present in all glaucomatous tissues including the trabecular meshwork (TM). The expression of ELAM-1 is mediated by inflammatory cytokines such as interleukin-1 (*IL1*) that is regulated by the NF-κB family of dimeric DNA-binding complexes (Barnes *et al.,* 1997).

Interleukin 1 (*IL1*) is an important cytokine involved in the control of the inflammatory response. Two structurally distinct forms of IL1: *IL1*α (acidic form), and *IL1*β (neutral form) exist (Frutani, 1986) and genetic polymorphisms within the interleukin gene cluster have been hypothesized to enhance the production the interleukin protein (Emad

et al., 2000). Two polymorphisms in *IL1β*, -511C>T (promoter) and the +3953C>T (exon 5) and one in the promoter of *IL1α*, -889C>T, have been studied in multiple populations. A significant association of *IL1β,* +3953C>T with POAG was noted in a cohort of 58 POAG patients and 105 controls from a Chinese population (Lin *et al.,* 2003). Another Chinese study on 156 POAG patients and 167 controls demonstrated an association of the *IL1α*, -889C>T polymorphism in POAG ($p<0.05$) (Wang *et al.,* 2006). The same group tested the association of *IL1α*, -889 C>T polymorphism among the NTG cases as well and could not find an association; this was attributed to the fact that factors other than IOP are responsible for glaucomatous optic atrophy in individuals with NTG. A recent study by How *et al,* on 194 POAG cases (94 NTG and 100 HTG), 125 PACG and 79 control individuals, did not observe the association of the Interleukin-1 polymorphisms, *IL1β*-511C>T (rs16944), *IL1β* +3953C>T (rs1143634) and *IL1α* -889C>T (rs1800587) (How *et al.,* 2007). Based on the above results of *IL1* polymorphisms, the present study was taken up to look for the association of *IL1* SNPs among the Indian POAG and PACG patients.

Another mechanism leading to apoptotic death of RGCs in POAG and pseudoexfoliation glaucomas (PEXG), is related to elevated serum homocysteine levels, which can induce vascular injury (McCully *et al.,* 1996), alterations in ECM remodeling, and contribution to neuronal cell death by inducing apoptosis or excitotoxicity (Moore *et al.,* 2001). The enzyme 5-Methylenetetrahydrofolate reductase (*MTHFR*) catalyses methylation of 5,10-methylenetetrahydrofolate to 5-

methyltetrahydrofolate, which contributes a methyl group in the conversion of homocysteine to methionine. The latter is converted to 5-adenosylmethionine, the lone donor of $-CH_3$ to cytosine and lysine residues, respectively, in DNA and histones. *MTHFR* also has a role in *de novo* nucleotide biosynthesis (Kim, 1999) A polymorphism in the exon of *MTHFR*, 677C>T resulting in the substitution of alanine 222 to valine residue is responsible for the synthesis of a thermolabile form of *MTHFR* thereby decreasing the activity of the enzyme (Frosst *et al.,* 1995). This polymorphism lies in the binding site for the *MTHFR* co-factor flavin adenine dinucleotide (FAD). An *in vitro* study demonstrated that individuals with the *MTHFR* "TT" genotype had 30% *MTHFR* activity as compared to the wild-type (CC), whereas those with the heterozygous genotype (CT) were found to have 60% activity (Frosst *et al,* 1995). Hence, the individuals homozygous for the mutation have significantly elevated plasma homocysteine levels, which have been documented in glaucoma patients (Frosst et al., 1995). Thus, the 677C>T SNP in the *MTHFR* gene may be a risk factor in glaucoma.

Junemann *et al,* (2005) studied the association of 677C>T SNP in *MTHFR* in POAG and PEXG patients and reported a significant association in POAG. They concluded that the *MTHFR* C677T variant leading to moderate hyperhomocysteinemia might play a role in the pathogenesis of POAG (Junemann *et al.,* 2005). However, this association could not be replicated in any other studies on different populations (Fingert *et al.,* 2006; Mabuchi *et al.,* 2006; Mossbock *et al.,*

2006; Turacli *et al.,* 2005). We checked for the association of the *MTHFR* polymorphism among the Indian POAG patients.

Various checkpoints maintain the genetic integrity of cells by arresting the cell cycle that allows for genetic errors to be repaired. This is mediated by a transcription factor p53 at the G1/S checkpoint, in response to DNA damage (Cox, 1997). It acts by binding to a p53-specific DNA consensus sequence in responsive genes leading to the up-regulation of *p*21, which is an important component in the apoptotic pathway (Levine, 1997). A single nucleotide polymorphism at codon 31 position, transversion of C to A of *p21* gene, results in a Ser to Arg amino acid substitution which can alter the protein's stability. This polymorphism encodes a probable DNA-binding zinc-finger domain (Lori *et al.,* 1996) and may change the transcription function and thereby the expression of its protein (Tsai *et al.,* 2004). Gene expression studies by Su *et al.,* had demonstrated that individuals with heterozygous genotype (CA) showed 38% decrease in *p21* expression. A recent study on a Chinese cohort indicated that the frequency of the Arg allele of the *p21* codon 31 was more in POAG patients (Tsai *et al.,* 2004). In a similar study on a Caucasian population, this association was not evident (Ressinoitis *et al.,* 2005). In the present study, the association of codon $31^{ser\text{-}arg}$ SNP of the WAF-1/CIP-1/p-21 gene has been evaluated in a cohort of POAG patients.

Matrix metalloproteinases (MMPs), a family of zinc-dependent proteases, play an important role in ECM remodeling. More than 28 members of the MMP family have been identified, which are broadly

divided into five groups based on common structural domains: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (Liesi *et al.,* 1988; Brinckerhoff *et al.,* 2002; Chintala *et al.,* 1999; Nagase *et al.,* 1992; Woessner, 1998; Woessner 1999; Woessner 2002). The two most closely related MMPs, 72 kDa MMP2 (gelatinase A) and 92 kDa MMP9 (gelatinase B), degrade gelatin, type IV collagen, collagen XVIII, fibronectin, laminin and several proteoglycans present in the basement membranes (Matrisian *et al.,* 1994). It is observed that there is an upregulation of MMP9 followed by the degradation of ECM protein laminin in the nerve fiber layer, that results in the progressive loss of RGCs (Chintala *et al.,* 2006).

These results suggest that hyper-stimulation of glutamate receptors due to glutamate excitotoxicity might lead to *MMP9* induction, and in turn mediate RGC loss (Chintala *et al.,* 2006). The mechanisms involved in the pathophysiology and development of PACG are complicated and involve the anatomy of the angle, iris and the lens (Hung, 1990). PACG is characterized by increased lens thickness during aging with a shallow anterior chamber and scleral changes caused due to the ECM remodeling. The SNPs in the ECM regulating proteins like *MMP9* are of interest as they could lead to a possible mechanism in the manifestation of acute PACG. An earlier report on Taiwanese patients showed the association of an intragenic polymorphism Q279R (exon 6) with acute primary angle closure glaucoma (Wang *et al.,* 2006). In the present study, two polymorphisms

in *MMP9* –1590C>T (promoter) and Q279R (exon 6) have been studied for their association to PACG.

While majority of these studies have been conducted in other populations, there are very few reports on these candidate genes in Indian populations. In view of Thus, the present study was designed with the following objectives to address some of these issues pertaining to the molecular genetics of POAG and PACG:

1. To screen *MYOC* and *CYP1B1* in POAG, PACG and PAC cases.
2. To screen single nucleotide polymorphisms (SNPs) in candidate genes, *IL1*β (-511C>T and +3953C>T), *IL1*α (-889C>T), *MTHFR* (Ala222Val), *p21* (Arg31Ser) *MMP-9* (-1590C>T, and Gln279Arg) in POAG and PACG cases and controls.
3. To understand the association of genotype with phenotype in each category

Chapter 2
Review of Literature

2. REVIEW OF LITERATURE

2.1 GLAUCOMA

Glaucoma refers to a group of diseases that differ in their pathophysiology, clinical presentation and treatment (Shields, 2005). Elevated intraocular pressure (IOP) induces physical changes at the optic nerve head (ONH), visualized clinically as optic disc cupping (Figure 1), which causes optic nerve axonal compression at the lamina cribrosa, blockage of axoplasmic flow, and interference in retrograde neurotrophin transport to retinal ganglion cells (RGCs), leading to cell death and atrophy of the optic nerve head (Guo *et al.,* 2005). This manifests as characteristic visual field defects, most commonly the functional loss of peripheral vision (Thylefors and Negrel, 1994) as shown in Figure 2. In addition, there is also a marked loss of the supporting glial cells and blood vessels (Flammer, 2003). The visual field of an eye comprises all the areas that can be seen at any given moment while starting at a fixed point. Defects in the field are called “scotomas” or “blind spots” that appear as “holes” in one’s visual field (Flammer, 2003). These scotomas usually develop in the peripheral field initially leading to peripheral vision loss, gradually leading to central vision loss.

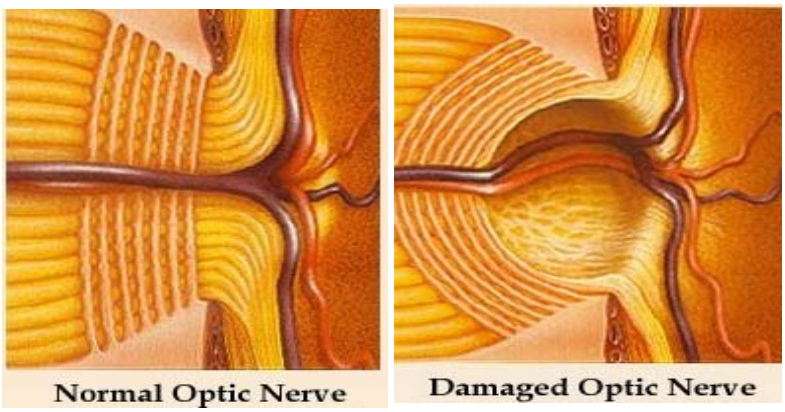


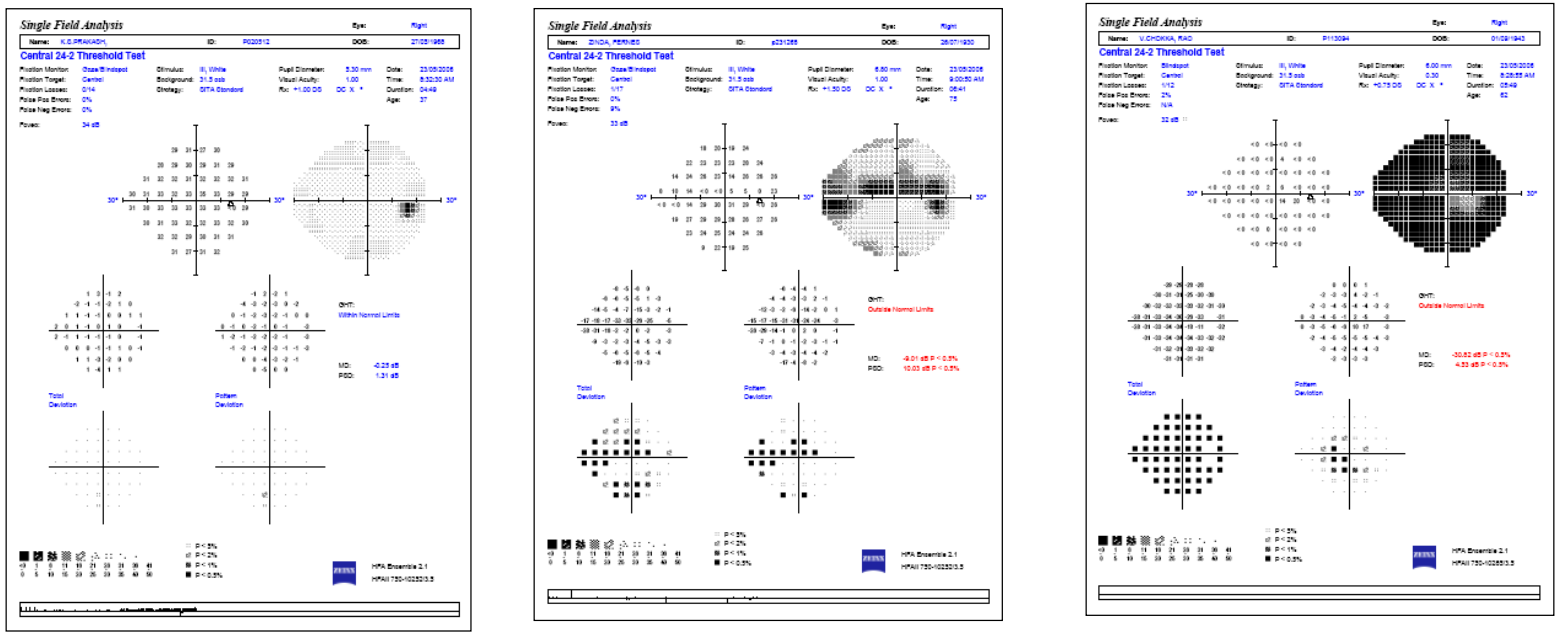
Figure 1. Optic nerve cupping in normal and glaucomatous optic disc (www.e-sunbear.com/glauc_path.html)

Cupping or excavation is the morphological alteration of the optic nerve head while the visual loss, which is irreversible, is considered the functional alteration (Flammer, 2003). The World Glaucoma association held at Singapore, from July 18-21, 2007, which represented 70 of the world's principal glaucoma societies, gave a consensus glaucoma definition which is referred to as a group of diseases that have in common a characteristic optic neuropathy with associated visual field loss for which elevated intraocular pressure (IOP) is one of the primary risk factors*(http://one.aao.org). The commonly accepted range for normal IOP in the general population is 10–22 mm Hg. and recognized that elevated IOP is the leading risk factor for glaucoma progression. The panel aimed to define clearly the relationship between IOP and the optic nerve to minimize permanent damage due to blindness.

The regulation of IOP is a complex physiologic trait that depends on production of aqueous humor, resistance to aqueous humor outflow, and

episcleral venous pressure. Aqueous humor inflow is related to the rate of aqueous humor production, while outflow depends on the resistance to the flow of aqueous from the eye and the pressure in the episcleral veins. Raise in IOP resulting from obstruction of aqueous humor outflow pathway contributes to the damage of optic nerve (Shields, 2005).

An elevated IOP is considered to be a major risk factor rather than symptom for glaucoma. The different forms of early and late onset glaucomas have raised IOP as a common feature. In cases of ocular hypertension, the IOP is >21 mm Hg without evidence of optic nerve damage or visual field abnormalities characteristic of glaucoma (Shields, 2005). Hence, it is important to first understand the different factors that control intraocular pressure (IOP) and the dynamics of aqueous humor outflow and inflow pathways.



a) No field defect b) Early glaucoma defect c) Advanced glaucoma defect

Figure 2. Humphrey visual field (24-2) analysis representing (a) normal visual fields (b) early glaucomatous field defects (c) advanced glaucomatous field defects

2.2 INTRAOCULAR PRESSURE (IOP)

Intraocular pressure is defined as the pressure of the fluid inside the eye, which varies among individuals. From a functional standpoint, a “normal’ intraocular pressure is one that does not result in glaucomatous optic nerve damage (Ritch *et al.,* 1996). Recently, leading experts from the World Glaucoma Association (WGA) held at Singapore, launched the group's first consensus on IOP. As it is the leading risk factor for glaucoma progression, accurate measurement of IOP plays a key role in assessing glaucoma risk and disease management.

In total, seven consensus points were evaluated to help determine how IOP should be measured and used, in order to understand better its overall role in glaucoma: 1) determinants of IOP, 2) measurement of IOP, 3) IOP variation, 4) epidemiology of IOP, 5) IOP as a risk factor for glaucoma development and progression, 6) clinical trials and IOP and 7) target IOP in clinical practice. This consensus report was based on more than two decades of groundbreaking research, which confirmed that IOP is a primary modifiable risk factor for glaucoma. Lowering IOP has been shown to be the only approach demonstrated to prevent and delay glaucoma progression.

Target IOP: The panel emphasized that IOP should be evaluated on an individual basis depending on where the patient is along the glaucoma disease continuum. The target levels for IOP should be defined as the estimated range where the risk of progressive disease is

unlikely to affect the patient's quality of life. Clinicians should consider the amount of glaucoma damage that has already occurred, the IOP at which the initial optic nerve damage occurred, life expectancy, the status of the fellow eye and family history of glaucoma. **IOP Measurement:** The panel also considered ways to measure IOP accurately. In particular, the measurement of central corneal thickness (CCT) was identified as crucial. The time of day, how long people have been awake and contact lens use all affect the precision of measurement of CCT.

2.2.1 Factors involved in the regulation of IOP

1. Genetics

IOP within the general population appears to be under hereditary influence, possibly though a polygenic, multifactorial mode (Shields, 2005). In addition, it tends to be higher in individuals with an enlarged cup-disc ratio (David *et al.,* 1987) and those who have relatives with open-angle glaucoma (Armaly, 1965; Seddon, 1983). Evidence of quantitative trait loci (QTL) for IOP on 5q22 and 14q22 has been reported on African American population by linkage analysis, where a reported POAG locus already exists (Rotimi *et al.,* 2006).

2. Age

IOP increases with age and the mean IOP has been reported to be 11.4 ± 2.4 mm Hg in newborns (Shields, 2005) and 8.4 ± 0.6 mm Hg in infants less than 4 months of age (Shields, 2005). In the adult population, IOP in individuals over 41 years is 15.4 mm Hg, with a

standard deviation of 2.65 (Shields, 2005). The upper limit of normal IOP in humans is usually 21 mm Hg. However an IOP of <21 mm Hg will not necessarily cause injury to the eye, or a pressure of >21 mm Hg may not be free of chronic type of optic nerve and other damage (Crick and Khaw, 2003). The positive correlation of IOP to age can be related to reduced facility of aqueous outflow since aqueous production actually appears to decrease slightly with increasing age (Gartner *et al.,* 1971).

3. Sex

Although men and women have the same IOP levels, the different forms of glaucoma occur more often in one gender than in the other (Flammer, 2003). Women suffer more frequently from angle closure glaucoma and normal tension glaucoma as their optic nerve head is more sensitive to IOP, while men suffer frequently with pigment dispersion glaucoma (Flammer, 2003).

4. Refractive Error

Hyperopic eyes are at higher risk to develop angle-closure glaucoma, while the myopic eyes are more frequently involved in pigmentary-dispersion glaucoma and are more sensitive to the effects of IOP (Flammer, 2003). An association between refractive error, glaucoma damage and IOP in a large population was observed and, glaucoma was four times more common in myopic than in hyperopic eyes at different age groups ranging from 55-79 years with IOP <15 mm Hg (Flammer, 2003). This overrepresentation of glaucoma in myopic eyes declined gradually with increasing IOP, and there was no relationship

between refraction and glaucoma damage in eyes with IOP > 31 mm Hg.

5. Race

Blacks have been reported to have higher pressures than Whites (Klein, 1981) and persons born in Africa or Asia were found to have higher mean IOPs than those born in Europe or America (David *et al.,* 1987).

Apart from the above factors, a few other factors exert short term influence, associated with a rise or fall in IOP, lasting from seconds to months and those are as follows:

6. Diurnal variation

IOP is subject to cyclic fluctuations from day to day and hour to hour. Normal diurnal variation is usually 4 mm Hg and is highest in the mornings and gradually falls during the first half of the day (Shields, 2005). The mechanism of diurnal IOP variation is uncertain; however, a relationship between adrenocortical steroids and diurnal IOP variation has been suggested (Shields, 2005).

7. Postural variation

Most studies show that IOP increases when changing from sitting to supine position, with average pressure differences of 0.3-6.0 mm Hg (Anderson *et al.,* 1973). This variation is greater in eyes with glaucoma (Shields, 2005).

8. Exertional influences

Exertion may lead to either lowering or elevation of IOP, depending on the nature of activity. Both prolonged exercise, such as running or

cycling as well as brief exercises have been reported to lower IOP in a study from Brazil (Shields, 2005). The mean reduction in IOP immediately after such brief and moderate exertion was 1.9 +/- 0.3 mmHg, compared with 0.8 +/- 0.2 mm Hg in the control group that was statistically significant. It has been suggested that reduction in IOP after a brief, moderate physical exertion may be due to sympathetic activity (Orgul *et al.,* 1994).

9. Lid and eye movement

Blinking has been shown to increase the IOP by 10 mm Hg, while hard lid squeezing may raise it to as high as 90 mm Hg (Shields, 2005). Repeated lid squeezing often leads to a slight reduction in IOP in normal eyes and very little in glaucomatous eyes (Shields, 2005).

10. Intraocular conditions

Some intraocular conditions like anterior uveitis result in slight reduction in IOP due to decrease in aqueous humor formation. Similarly, rhegmatogenous retinal detachment result in reduction in IOP due to reduced aqueous flow as well as a shunting of aqueous from the posterior chamber (Shields, 2005).

11. Systemic conditions

IOP might increase in response to adrenocorticotropic harmone, glucocorticoids and growth hormone and decrease in response to progesterone, estrogen, chorionic gonadotropin, and relaxin (Shields, 2005). IOP is lower in patients with hypothyroidism and higher in patients with hyperthyroidism (Shields, 2005) Patients with acromegaly have slight IOP elevation due to the central corneal thickness (Smith *et*

*al.,*1992). Also, diabetic hypertensive patients with retinopathy have been reported to have a higher IOP (Shields, 2005).

2.3 AQUEOUS HUMOR

Aqueous humor is produced from the ciliary body and flows out through the limbal region, which includes trabecular meshwork, the principal site of aqueous humor outflow. It has multiple physiologic functions throughout the various ocular structures.

2.3.1 Formation of aqueous humor

Aqueous is derived from plasma within the capillary network of the ciliary processes by three mechanisms:

a) Diffusion - Lipid-soluble substances are transported through the lipid portions of the cell membrane proportional to a concentration gradient across the membrane (Shields, 2005).

b) Ultrafiltration - Water and water-soluble substances, limited by size and charge, flow through micropores in the protein of the cell membrane in response to an osmotic gradient or hydrostatic pressure, influenced by IOP, blood pressure in the ciliary capillaries, and plasma oncotic pressure (Shields, 2005).

c) Active transport (secretion) - Water-soluble substances of larger size or greater charge are actively transported across the cell membrane, requiring the expenditure of energy. It accounts for the majority of aqueous production (Glabelt *et al.,* 2003). According to Solomon (2002), the rate of aqueous humor formation is 2.0 - 3.0

μl/min; volume of anterior chamber is 250 μl while that of the posterior chamber is 60 μl and turnover of aqueous occurs every 1.5 - 2 hours.

2.3.2 Regulation of aqueous humor production

Aqueous humor is a dynamic fluid that is vital to the health of the eye. The ciliary body extends from the base of the iris to become continuous with the choroids at the ora serrata. The ciliary body includes two parts, the pars plicata (corona ciliaris) and the pars plana (orbicularis ciliaris). The pars plicata contains the ciliary processes and pars plana is the posterior, flat part of the ciliary body measuring 4 to 4.5 mm in length. The precise localization of aqueous humor production appears to be in the anterior portion of the pars plicata along the tips or crests of the ciliary processes. The circulating aqueous humor enters the posterior chamber and flows around the lens and through the pupil into the anterior chamber (Figure 3).

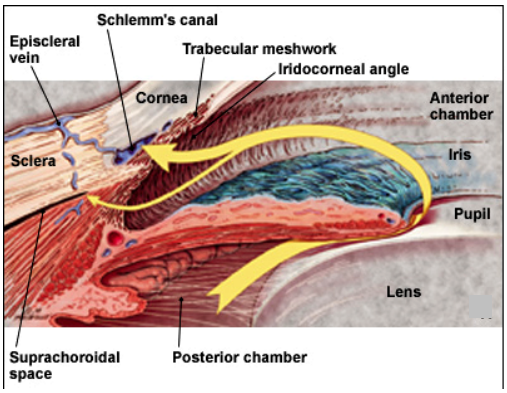


Figure 3. Normal outflow of aqueous humor through trabecular meshwork (large arrow) and uveoscleral routes (small arrow) (http://www.aafp.org/afp/990401ap/1871.html).

2.3.3 Aqueous humor outflow pathways

There are two different pathways of aqueous humor outflow, both located in the iridocorneal angle of the eye (Figure 4).

1. Trabecular or conventional outflow pathway: It is the main outflow route through trabecular meshwork (TM), accounting for ~80% of the aqueous outflow. After crossing the TM, aqueous humor reaches Schlemm's canal from where it drains directly to the aqueous veins. This pathway is IOP dependent. The ligamentous insertions of the ciliary muscle in the TM modulate the permeability of this tissue for aqueous humor. When the ciliary muscle contracts, its insertions widen the intercellular spaces in the TM and the permeability of the tissue increases (Llobet *et al.,* 2003).

2. Uveoscleral or non-conventional pathway: It is the minority outflow pathway and accounts for ~ 20% of the total flow (Llobet *et al.,* 2003). In this pathway the aqueous humor leaves the anterior chamber by diffusion through intercellular spaces among ciliary muscle fibers and is pressure independent (Bill *et al.,* 1975). When the ciliary muscle relaxes, the intercellular spaces of the TM become narrower and the trabecular outflow is subsequently reduced correspondingly, the uveoscleral outflow is increased (Llbet *et al.,* 2003). This pathway is the target for delivering specific anti-glaucoma drugs. The prostaglandin derivatives and related hypotensive lipids primarily lower IOP by enhancing the outflow

through this pathway. After binding and activating the prostaglandin F receptors in the ciliary smooth muscle, the precise mechanism by which prostaglandins improve uveoscleral outflow is not fully understood. Two possible mechanisms have been studied that is the relaxation of the ciliary muscle and remodeling the extracelular matrix of the ciliary muscle (Shields, 2005).

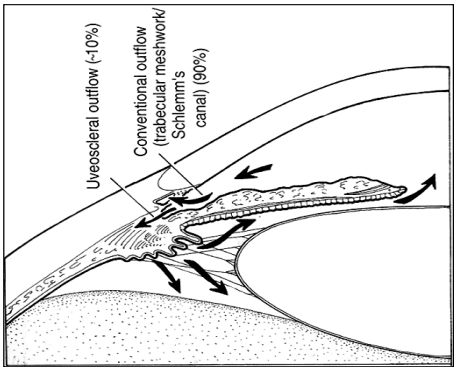


Figure 4. Schematic representation of aqueous humor outflow pathways (Epstein. Glaucoma. 4th ed. Baltimore, MD: Williams & Wilkins; 1997:p.19)

2.3.4 Composition of aqueous humor

Relative to plasma, aqueous humor is slightly hypertonic and acidic (pH 7.2 in AC) (Shields, 2005) with excess of ascorbate (15 times greater than arterial plasma) (Shields, 2005), less of protein (0.02% in aqueous vs. 7% in plasma), slight excess of chloride and lactic acid, slightly less sodium, bicarbonate, carbon dioxide, and glucose. Protein and antibodies in aqueous equilibrate with those in serum. Albumin/globulin ratio is similar to plasma, although there is less gamma globulin (Freddo *et al.,* 1990).

2.3.5 Functions of aqueous humor

The anterior and posterior chambers of the eye are filled with aqueous humor and the main functions are as follows:

1. Maintaining proper IOP which is important in early ocular development as well as in maintaining globe integrity throughout life.
2. Providing substrates and removing metabolites from the cornea, lens, and trabecular meshwork and in delivering high concentrations of ascorbate.
3. Participating in local paracrine signaling and immune responses.
4. Providing a colorless and transparent medium as a part of the eye's optical system
5. Providing substrates like glucose, oxygen and electrolytes for metabolic requirements of avascular cornea and lens.
6. Removing metabolic products like lactate, pyruvate and carbon dioxide.
7. Also having a possible role in metabolism of vitreous and retina (Shields, 2005).

2.4 TRABECULAR MESHWORK (TM)

In humans, the majority of the aqueous humor exits the eye via the trabecular meshwork (TM). It is a three-dimensional set of diagonally crossing collagen fibers contained within the scleral sulcus converting the latter into a circular channel, called Schlemm's canal, and consists of a connective tissue core surrounded by endothelium (Figure 5). Anatomically, TM can be divided into two parts: non-filtering and filtering TM (Ritch *et al.,* 1996)

2.4.1 Non-filtering TM

It is the anterior most portion, which lies adjacent to limbus, posterior to Schwalbe's line. It has no contact with Schlemm's canal and is therefore called as non-filtering TM. It consists of 3-5 trabecular beams covered by small trabecular cells, derived from corneal endothelial cells, characterized by high amounts of carbonic anhydrase, often lie closely together, forming elongated bands or rows. (Ritch *et al.,* 1996).

2.4.2 Filtering TM

This covers the inner wall of Schlemm's canal and consists of three morphologically and functionally different portions (from outward to inward). The juxtacanalicular tissue is the outermost layer with a network of fibrils, with cells embedded within, representing preferential aqueous pathways. The corneoscleral meshwork comprises the main portion of TM, consists of lamellae, covered by a single layer of endothelial lining supported by basement membrane. The uveal meshwork, which is anchored at the inner layers of the corneal stroma, or the corneoscleral meshwork (Figure 5).

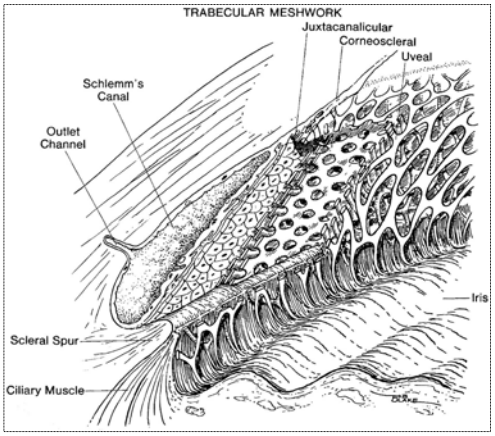


Figure 5. Three layers of the trabecular meshwork
(Shields. *Textbook of glaucoma*. 4th ed. Baltimore, MD: Williams & Wilkins;1998:p.16)

2.5 OPTIC NERVE HEAD IN GLAUCOMATOUS PATIENTS

The optic nerve head or optic disc is a round/oval 'plughole', comprising of more than a million nerve fibres, descending through a sieve-like sheet, lamina cribrosa. These fibres are then bundled together behind the eye as the optic nerve, which continues towards the brain. In glaucomatous condition, there is a progressive compression and cupping of the optic nerve (Ritch *et al.,* 1996). The retinal nerve fibres which spread unevenly across the surface of the retina, has a 'feathery' appearance, best visible immediately above and below the disc (Figure 6a). The nerve fibres converge on the edge of the disc they pour onto the scleral ring and then down its inner surface. This dense packing of nerve fibres just inside the scleral ring is visualised as the neuroretinal rim. The cup is the area central to the neuroretinal rim. The cup edge, where it meets the neuroretinal rim is best seen by the bend in small and medium-sized blood vessels as they descend into the cup (Figure 6b).

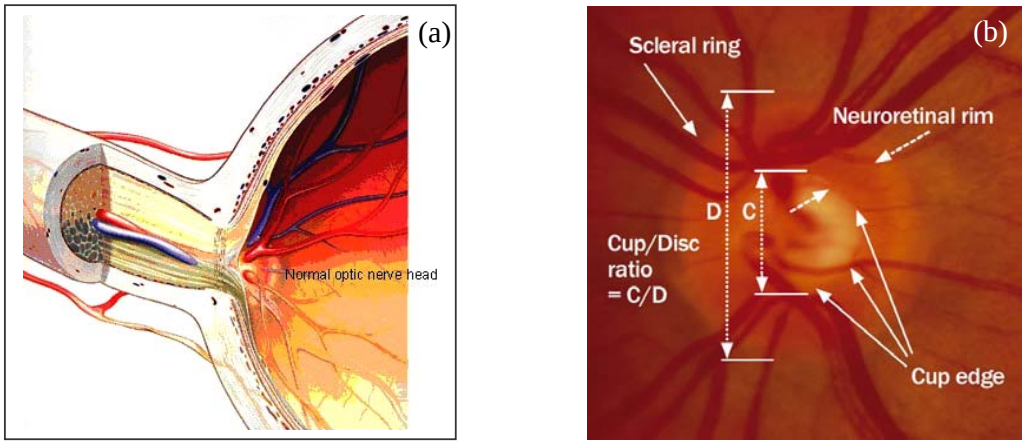


Figure 6: (a) Normal optic nerve head showing the retinal nerve fibres (www.merckmedicus.com/.../glaucoma/diagnosis.jsp).
(b) Optic nerve head showing the cup to disc ratio (www.jceh.co.uk/extra/19_59_044_f05.html)

2.5.1 Cup to disc ratio

Glaucoma is associated with an increase in the absolute size of the optic cup and thereby in the cup-disc ratio. Patients with a decrease in the number of nerve fibers leaving the eye, due to loss of axons or a larger sized optic disc with all of the axons intact, will both have cup to disc (C/D) ratios larger than normal (Jonas *et al.,* 1990). In a recent study by Varma *et al,* (1994) on 4877 normal individuals, the average C/D ratio was found to be 0.47 in Caucasians and 0.57 in African-Americans.

2.5.2 Retinal nerve fiber layer defect

Defects in the thickness and appearance of retinal nerve fiber layer are the earlier signs of glaucoma. Atrophy of the nerve fiber layer in glaucoma usually begins with preferential thinning of this layer in the superior or inferior arcuate regions, or both and can be visualised as a loss of the nerve fiber layer's striate pattern. This occurs due to the loss of axons resulting in the decrease of the neural tissue in the neural rim region. This is accompanied by or leads to increased visibility of the retinal vessels (Ritch *et al.,* 1996).

2.6 SYMPTOMS AND SIGNS OF POAG AND PACG

1. Raised IOP

The IOP is determined by a balance between aqueous production inside the eye and aqueous drainage out of the eye through the

trabecular meshwork. Normal IOP is 10-21 mm Hg, but it can exceed to 70 mm Hg in some glaucomatous conditions. The rate at which raised IOP causes optic nerve damage depends on factors, such as early or advanced glaucomatous damage is. In general, pressures of 20-30 mm Hg usually cause damage over several years, but pressures of 40-50 mm Hg can cause rapid visual loss and also precipitate retinovascular occlusion. High pressures may cause constant pain and visual blurring. As in any form of glaucoma, long term presure elevation can ultimately lead to blindness (Khaw *et al.,* 2004).

2. Haloes around lights and cloudy cornea

The cornea is kept transparent by the continuous removal of fluid by the endothelial cells. In acute angle closure glaucoma when the pressure rises quickly, the cornea becomes waterlogged, causing a fall in visual acuity and creating haloes around lights (like looking at a light through frosted glass) (Khaw *et al.,* 2004).

3. Pain

Pain is not a characteristic feature of primary open angle glaucoma. In acute angle closure glaucoma due to high IOP, severe pain with reddened eyes, headache and vomiting result (Khaw *et al.,* 2004).

4. Visual field loss

Pressure on the nerve fibres and chronic ischaemia at the optic nerve head cause damage to the retinal nerve fibres and usually result in characteristic patterns of field loss. However, central vision is spared initially, and the patient does not notice the defect. Vision may still be

6/6, even at the terminal stage of glaucomatous field loss (tunnel vision) (Khaw *et al.,* 2004).

5. Optic disc changes

The optic disc marks the exit point of the retinal nerve fibres from the eye. With a sustained rise in IOP the nerve fibres atrophy, leaving the characteristic sign of chronic glaucoma - the cupped, pale optic disc (Khaw *et al.,* 2004).

2.7 CLASSIFICATION OF GLAUCOMAS

Glaucomas can be classified based on many systems. The most common are mainly based on (a) the etiology underlying the disorder that leads to an alteration in aqueous humor dynamics and (b) the mechanism due to specific alteration in the anterior chamber angle leading to a rise in IOP.

2.7.1 Based on etiology:

1. Primary Glaucomas:

The glaucomas in which the initial events lead to outflow obstruction and IOP elevation are called primary glaucomas, are confined to the anterior chamber angle or conventional outflow pathway and IOP elevation, with no apparent contribution from other ocular or systemic disorders. These conditions are bilateral and may have a genetic basis. Example: Primary open angle glaucoma (POAG), Primary angle closure glaucoma (PACG) (Shields, 2005).

2. Secondary Glaucomas:

Secondary glaucomas are characterized by the involvement of predisposing ocular or systemic events resulting in alteration of

aqueous humor dynamics, and abnormal increase in IOP. The causes of secondary glaucoma are many and include inflammation, hemorrhage, neovascularization of the iris and adhesion. In secondary glaucoma, the anterior chamber angles may be open or closed.

Sometimes the trabecular meshwork may be torn by the contusion force, with the resultant loss of drainage function, producing a situation similar to the open angle type of glaucoma. Myopia is another condition, which can increase the occurrence of glaucoma (Shields, 2005). Diabetes mellitus is another condition wherein neovascularization in various tissues of the body may occur, notably in the retinal and renal tissues. It is not uncommon to find these new vessels in iris stroma and chamber angle meshwork in eyes of diabetics, giving rise to a form of chronic glaucoma (Hung, 1980). These conditions may be unilateral or bilateral, and some may have a genetic basis whereas others are acquired. Example: Pigmentary glaucoma, Pseudoexfoliation glaucoma.

The concept of primary and secondary classification of glaucomas represent a lack of understanding of the pathophysiological mechanisms underlying glaucoma, Furthermore glaucomas caused by developmental anomalies do not fit into either category (Shields, 2005).

2.7.2. Based on mechanism

Barkan, first recognized the distinction between open angle glaucoma and angle closure forms of glaucoma which was the basis for the mechanistic classification of the glaucoma (Barkan, 1938). A third

group, which did not fit into either of the two types, are developmental glaucomas (Shields, 2005).

1) Primary open angle glaucoma (POAG)

In open angle glaucoma, the anterior chamber angle structures, TM, scleral spur, and ciliary body are visible by gonioscopy (Shields, 2005). The elements obstructing aqueous outflow may be located on the anterior side of the TM (pre-trabecular mechanisms) or distal to the meshwork in Schlemm's canal (post-trabecular mechanism).

a) Pretrabecular mechanisms: a translucent membrane extends across the open iridocorneal angle, leading to the obstruction of aqueous outflow.
b) Trabecular mechanisms: the obstruction to the aqueous outflow is located within the trabecular meshwork.
c) Post trabecular mechanisms: the obstruction to the aqueous outflow results from increased resistance in Schlemm's canal due to collapse or absence of clogging of the canal (Shields, 2005).

Figure 7A shows the schematic representation of the obstruction of aqueous outflow through the trabecular meshwork.

2) Primary angle closure glaucoma (PACG)

In angle closure glaucoma, the peripheral iris is in apposition to the TM or peripheral cornea. As a result the peripheral iris may either be pulled (anterior mechanisms) or pushed (posterior mechanisms) into this position. In the anterior mechanisms of angle-closure glaucoma, an abnormal tissue bridges the anterior chamber angle and subsequently undergoes contraction, pulling the peripheral iris into the iridocorneal

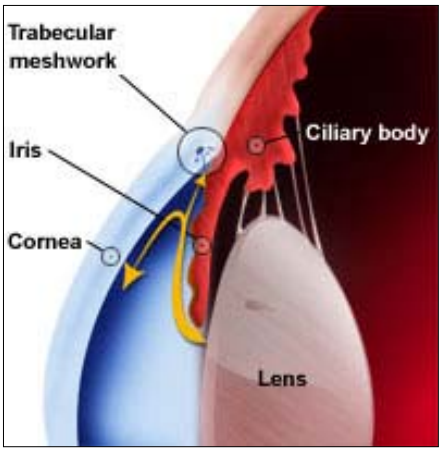
angle. In the posterior mechanisms, pressure behind the iris, lens, or vitreous causes the peripheral iris to be pushed into the anterior chamber angle, which occurs with or without the pupillary block (Shields, 2005). Due to the shallow anterior chamber and a narrow filtration angle, a functional block resulting in the build-up of aqueous in the posterior chamber occurs and this leads to a forward shift of the peripheral iris and closure of the anterior chamber angle (Ritch *et al.,* 1996) (Figure 7B).

3) Developmental Glaucomas

These glaucomas are not readily separated into open-angle and angle-closure mechanisms, but typically represent incomplete development of structures in the conventional aqueous outflow pathway. Clinically recognized developmental defects include a high insertion of the anterior uvea, as in congenital glaucoma (Shields, 2005).

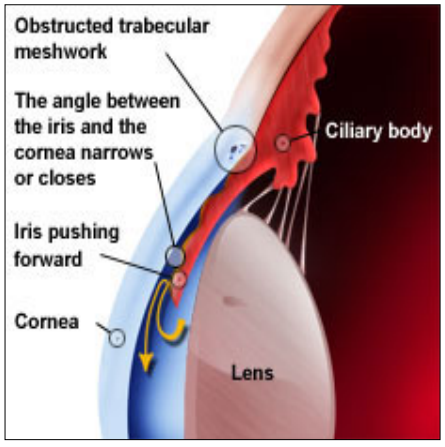
One disadvantage of this classification is that it ignores the pressure independent causative factors. In addition, many glaucomas have more than one mechanism of outflow obstruction at different times in the course of disease (Shields, 2005).

Figure 7. Aqueous humor flow in open angle and angle closure glaucomas



A) Open angle glaucoma

(http://www.merckfrosst.ca/images/en/patients/diseases/glaucoma/glauc_open.jpg)



B) Angle closure glaucoma

(http://www.merckfrosst.ca/images/en/patients/diseases/glaucoma/glauc_closed.jpg)

2.8 RISK FACTORS FOR OPEN ANGLE AND ANGLE CLOSURE GLAUCOMA

1. Age:

The depth and volume of the anterior chamber diminish with age (Fontana *et al.,* 1980) that may result from a thickening and forward displacement of the lens (Markowtz *et al.,* 1984; Okabe *et al.,* 1992). Also there is a progressive loss of nerve fibers with the increasing age over long period and is mostly noticed only in the late phase (Flammer 2003).

2. Race:

In general, the prevalence of PACG is increased in various populations of Inuit (Drance 1973) and individuals from Asia. Acute angle closure glaucoma is less common among the black populations and American Indians. The prevalence of POAG is highest in Black populations, intermediate in Whites, Hispanics, and southern Asian populations (Singapore, Chinese, Indian) and lowest in northern Asian populations (Mongolia, Inuit) (Shields, 2005). However, in the Advanced Glaucoma Intervention Study (AGIS), black race was not a risk factor for progression (AGIS investigators, 2002)

3.Gender:

Women are always at a higher risk of developing PACG due to the presence of a shallower anterior chamber angle (Drance, 1973; Fontana *et al.,* 1980). Also women have normal-tension glaucoma more frequently than men (Flammer, 2003).

4. Refractive error:

Myopic patients are at a higher risk of developing PACG, possibly indicating a spherical or anteriorly displaced lens or an increase in corneal curvature (Cherny *et al.,* 1992). An association of Myopia to open angle glaucoma has been documented in various prevalence studies (Shields, 2005)

5. Family History:

Family history is generally considered an important risk factor for PACG and POAG (Shields, 2005) In one study, 20% of 95 relatives of angle-closure glaucoma patients were thought to have potentially occludable angles (Shields, 2005).

6. Systemic Disorders:

The prevalence of open angle glaucoma increases two fold in patients with diabetes (Shields, 2005)**.** One study demonstrated an inverse correlation between type 2 diabetes or an abnormal glucose-tolerance test and the anterior chamber depth (Mapstone *et al.,* 1985).

2.9 EPIDEMIOLOGY OF GLAUCOMA

Glaucoma is the second leading cause of irreversible blindness worldwide after cataracts (Resnikoff *et al.,* 2004). According to a recent prevalence report, it is estimated that by the year 2010 around 60.5 million people worldwide will be afflicted with glaucoma, and this includes both open angle and angle closure glaucoma, and will rise to 79.6 million by the year 2020. Of this, 74% will have open angle glaucoma. Women will comprise 55% of OAG, 70% of ACG, and 59% of all glaucoma in 2010 (Quigley *et al.,* 2006).

Bilateral blindness would account for 4.5 million people with OAG and 3.9 million people with PACG in the year 2010, that would rise to 5.9 and 5.3 million people by 2020 respectively. It is estimated that by 2010, 74% of those with glaucoma will be POAG cases and 26% will be ACG. The mean prevalence for OAG worldwide in 2010 is estimated to be 1.96%, while that for ACG, 0.69% (Quigley *et al.,* 2006).

The prevalence of glaucoma varies greatly between racial and ethnic groups, significantly higher in Blacks (4.7%) than in Whites (1.3%) (Ritch *et al,* 1996). The incidence of glaucoma is also strongly influenced by age and race. The different prevalence studies on glaucoma worldwide, lack consistency in defining the disease (Forster *et al.,* 2002). These differences make it difficult to compare the prevalence findings of different studies. In India, different groups have studied the prevalence of glaucomas. The definition of glaucoma as defined by the different study groups is as follows, Table 1 summarizes the prevalence percentages of different glaucomas as reported by the different epidemiological studies from India.

2.9.1 Andhra Pradesh eye disease study (APEDS) (Dandona *et al.,* 2000)

The definition of POAG in APEDS was more modern and did not include IOP. The definitions of glaucomas were as mentioned below,

1. POAG was defined as the presence of glaucomatous optic disc damage along with visual field loss consistent with the disc findings, in the presence of open angle.

2. Suspected POAG was defined as suspected glaucomatous optic disc damage without definite visual field loss in the presence of an open angle.
3. Ocular hypertension (OHT) was defined as an IOP of 22 mm Hg or more without glaucomatous optic disc damage or visual field loss in the presence of open angle.

The mean IOP in participants 30 years of age or older was 15.36 mm Hg, with standard deviation of 3.33 mm Hg giving an upper limit of the 95% confidence interval (CI) for the distribution as 21.89 mm Hg. Glaucomatous optic disc damage or an IOP of 22 mm Hg or more secondary to an obvious cause and with an open-angle was defined as secondary open-angle glaucoma (Dandona *et al.,* 2000).

2.9.2 Aravind comprehensive eye study (ACES) (Ramakrishnan *et al.,* 2003)

The following definitions were used to classify persons into specific diagnostic categories

1. Ocular Hypertension was defined as intraocular pressure >21 mm Hg without evidence of optic nerve damage or visual field abnormalities characteristics of glaucoma; open and normal-appearing anterior chamber angle by gonioscopy.
2. POAG was defined as anterior chamber angles open and normal appearing by gonioscopy, typical features of glaucomatous optic disc as defined earlier, and visual field defects corresponding to the optic disc changes.

3. PACG was defined as those with at least two of the following criteria: glaucomatous optic disc damage or glaucomatous visual field defects in combination with anterior chamber angle partly or totally closed, appositional angle closure or synechiae in angle, absence of signs secondary angle closure (example uveitis, intumuscent or dislocated lens; micropherphakia; evidence of neovascularization in the angle; or congenital angle anomalies).
4. Secondary glaucoma cases with glaucomatous optic nerve damage and/or visual field abnormalities suggestive of glaucoma coupled with ocular disorders that contribute to a secondary elevation in IOP, such as neovascularization, injury, hypermature or dislocated lenses and uveitis.
5. Absolute glaucoma was defined as the end-stage glaucoma without adequate evidence regarding the primary insult or cause contributing to glaucomatous optic nerve damage.

2.9.3 West Bengal Glaucoma study (WBGS) (Raychoudhuri *et al.,* 2005)

The West Bengal Glaucoma study followed the criteria set by International society for geographic and epidemiological ophthalmology (ISGEO), for glaucoma

1. A visual cup to disc ratio (VCDR) of 0.7 or greater or asymmetry between the right and left VCDRs of 0.2 or more, and a visual field defect consistent with glaucoma (an abnormal 68 point field test)

2. A VCDR of 0.9 or greater in either eye or asymmetry between the right and left VCDRs of 0.3 or more, and a reliable field test result could not be obtained
3. An IOP greater than 26 mm Hg and visual acuity worse than 3/60, or evidence of previous glaucoma filtering surgery, when the optic disc could not be examined because of media opacity.

Open angle glaucoma was defined as those with open anterior chamber angles, and following 1 or 2 of the above criteria unless there was any other sign of retinal or optic nerve diseases like diabetes mellitus, branch or central retinal vein occlusion, or signs of pseudoexfoliation, trauma or pigment dispersion. If any of the later signs were present, a diagnosis of secondary open angle glaucoma was made. A diagnosis of suspected POAG was made in the presence of an open angle of the anterior chamber, a VCDR of 0.7 or more, or asymmetry between the right and left VCDRS of 0.2 or more without an associated definite visual field abnormality.

Angle closure glaucoma was defined as presence of occludable angle along with any of the above-mentioned criteria. An angle in which the pigmented trabecular meshwork was not visible throughout three quarters or more of the angle circumference in the primary position was classified as occludable. In the absence of any other cause for angle closure, patients with an occludable angle meeting any of the criteria for glaucoma described above were diagnosed as having chronic PACG. Patients were diagnosed as having acute PACG if they had signs of past attack of acute angle closure on iris and lens surfaces, or

if they reported a clear history of seeing a rainbow halo around light, sudden or intermittent attacks of painful red eye, and dimness of vision. If there were characteristic disc changes but no field changes in the presence of an occludable angle, a diagnosis of suspected PACG was made. Angle closure glaucoma associated with signs of other primary causes was classified as secondary angle closure glaucoma. In addition to applying the ISGEO criteria (Forster *et al.,* 2002) described above to clinical findings, optic disc photographs and visual field assessments were reviewed by three ophthalmologists.

Table 1. Prevalence studies of Glaucoma in Indian population

Region	Name of the study	Study results							
		POAG (%)	Suspected POAG (%)	OHT (%)	PACG (%)	Occ angles	Sec. glaucoma (%)	Cataract (%)	Blind (%)
South India	Andhra Pradesh Eye Disease Study (APEDS) - Urban population (n = 2522)	2.56 (95% CI, 1.22-3.91)	1.11 (95%CI, 0.43-1.78)	0.42 (95%CI, 0.11-1.12)	-		-	-	-
	Aravind Comprehensive Eye Study (ACES) - Rural population (n = 5150)	1.7 (95%CI, 1.3-2.1)	-	-	0.5 (95%CI, 0.3-0.7)-		0.3 (95%CI, 0.2-0.5)	-	-
	Chennai Glaucoma Study (CGS) (n = 3924) 753 subjects were studied	3.79 (including macular scars)	-	-	-		-	74.6%	132 (3.36%)
	Vellore eye study (VES) (n = 1521) 972 subjects were studied	0.41		3.08	4.32	1.03			
East India	West Bengal Glaucoma Study (WBGS)- Rural population (n=1269)	2.7 (95%CI 1.7-3.7)-50-59yrs 6.5 (95%CI 0.1-14.1)-80yrs	-	-	-		-	-	-
North India	n = 2425	37 %	-	-	63 %		-	-	-

2.10 GENETICS OF POAG AND PACG

POAG is genetically heterogeneous, and 17 chromosomal loci have been identified as shown in the Table 2. Only four of them, GLC1A, GLC1J, GLC1K and GLC1M, contribute to JOAG, while the others contributed to adult onset POAG. Other loci, which are also associated to glaucoma, are also shown in the Table 2. Three causative genes have been described: myocilin (*MYOC*/*GLC1A*), optineurin (*OPTN*/*GLC1E*), and WD repeat domain 36 (*WDR36*/*GLC1E*) in POAG and around 16 POAG-associated genes, have been identified till date from association studies (Fan *et al.,* 2005). However, the locus/loci involved in PACG is not identified till date. Table 2 lists the loci and the mapped candidate genes in POAG, JOAG and NTG patients by the different groups. Apart from these, at least 15 glaucoma candidate genes have been suggested from association studies. Although most of them are reported in single studies, a couple of genes have been investigated in multiple cohorts (Table 3). However, the role of these genes in the etiology of POAG is not known.

In the present study the two glaucoma candidate genes *MYOC* and *CYP1B1* were screened for mutations in POAG, PAC and PACG phenotypes. Also, we looked at the association of single nucleotide polymorphisms within the candidate genes, *IL1*α, *IL1*β, *p21*, methylenetetrahydrofolate reductase (*MTHFR*) and matrix metalloproteinase (*MMP9*) genes in POAG, PAC and PACG cases. These genes are hence discussed in greater details.

Table 2. Candidate Loci/genes involved in Glaucoma

CANDIDATE LOCI	GENE	PHENOTYPE	POPULATION	REFERENCE
GLC1A (1q24.3-q25.2)	Myocilin (*MYOC*)	JOAG	USA	Stone *et al.,* 1997
GLC1B (2cen-q13)	-	POAG	USA	Stoilova *et al.,* 1996
GLC1C (3q21-q24)	-	POAG	USA	Wirtz *et al.,* 1997
GLC1D (8q23)	-	POAG	USA	Trifan *et al.,* 1998
GLC1E (10p15-p14)	Optic neuropathy inducing protein (*OPTN*)	NTG	USA	Rezaie *et al.,* 2002
GLC 1F (7q35-q36)	-	POAG	USA	Wirtz *et al.,* 1999
GLC 1G (5q22.1)	WD repeat-containing protein 36 (*WDR 36*)	POAG	USA	Monemi *et al.,* 2005
GLC 1H (2p16.3-p15)	-	POAG	USA	Suriyapperuma *et al.,* 2007
GLC 1I (15q11-q13)	-	POAG	USA	Allingham *et al.,* 2005
GLC 1J (9q22)	-	JOAG	USA	Wiggs *et al.,* 2004
GLC 1K (20p12)	-	JOAG	USA	Wiggs *et al.,* 2004
GLC 1L (3p21-22)	-	POAG	Australia	Baird *et al.,* 2005
GLC 1M (5q22.1-q32)	-	JOAG	Philippine	Fan *et al.,* 2007

Table 2 contd..

CANDIDATE LOCI	GENE	PHENOTYPE	POPULATION	REFERENCE
GLC 1N (15q22-q24)	-	POAG	USA	Wang *et al.,* 2006
GLC3A (2p21)	Cytochrome P450 1B1 (*CYP1B1*)	PCG	USA	Stoilov *et al.,* 1997
GLC3B (1p36.2-p36.1)	-	PCG	USA	Akarsu *et al.,* 1996
GLC3C (14q24.3-q31.1)	-	PCG	USA	Stoilov, 2002
2p14	-	POAG	USA	Wiggs *et al.,* 2000
2q33-q34	-	POAG	USA	Nemesure *et al.,*2003
3p21-p22	-	POAG	Australia	Baird *et al.,* 2005
10p12-p13	-	POAG	USA	Nemesure *et al.,*2003
14q11	-	POAG	USA	Wiggs *et al.,* 2000
14q21-q22	-	POAG	USA	Wiggs *et al.,* 2000
17p13	-	POAG	USA	Wiggs *et al.,* 2000
17q25	-	POAG	USA	Wiggs *et al.,* 2000
19q12-q14	-	POAG	USA	Wiggs *et al.,* 2000

Table 3. Other candidate loci/genes involved in glaucoma

CANDIDATE GENES	GEN BANK Acc. No.	SNP	dbSNP ID	PHENOTYPE	POPULATION	REFERENCE
GSTM1	NM_000561	-	-	POAG	Estonian	Juronen *et al.,* 2000
MTHFR	NM_005957	c.677 C>T	rs1801133	POAG	German	Junemann *et al.,* 2005
ACP1	NM_177554			POAG		Abecia *et al.,* 1996
OPA1	NM_015560	IVS 8 +4C>T IVS 8 +32C>T	rs166850 rs10451941	NTG	Singapore	Aung *et al.,* 2002
CDKNA1	NM_000389	S31R c.328 C>A	rs1801270	POAG	Taiwan	Tsai *et al.,* 2004
TNF-α	NM_000594	-308 G>A	rs1800629	NTG		Funayama *et al.,* 2004
NOS	NM_000603			POAG/NTG	UK	Logan *et al.,* 2005
IGF2	NM_000612	ex 9 *Apa I* A>G	rs630	POAG	Taiwan	Tsai *et al.,* 2003
APOE	NM_000041			POAG	French	Copin *et al.,* 2002
AGTR2	NM_000686	3123 C>A	rs11091046	POAG	Japanese	Hashizume, 2001
EDNRA	NM_001957	A>C +70G	rs5335	POAG/NTG	Japanese	Ishikawa *et al.,* 2005
IL1B	NM_000576	-511 C>T F105F C>T	rs16944 rs1143634	POAG	Taiwan	Lin *et al.,* 2003
NPPA	NM_006172			POAG	Australian	Tunny *et al.,* 1996
OCLM	NM_022375			POAG	Japan	Fujiwara *et al.,* 2003
TAP1	NM_000593	NA	NA	POAG	Taiwan	Lin *et al.,* 2004
TP53	NM_000546	72 codon G>C 16 bp dup	rs1042522 rs17878362	POAG	Taiwan	Lin *et al.,* 2002

2.10.1 Myocilin (*MYOC*)

MYOC was first identified by Stone *et al.,* in 1997 as trabecular meshwork-induced glucocorticoid response protein (TIGR), which mapped to 1q where the locus, *GLC1A*, was mapped for JOAG by linkage analysis. The genomic structure of *MYOC* consists of 3 exons, exon 1 codes for a myosin-like domain and exon 3 codes for an olfactomedin-like domain (Figure 8a). The promoter region (~5kb) includes the putative sequences for consensus TATA and CAAT box sequences; multiple hormone and cell signaling response elements including 7 glucocorticoid response elements (GREs) and 3 estrogen response elements (EREs); elements that could correlate to oxidative damage, DNA damage and heat shock responses including one *NF-κB* and four shear stress response elements (SSRE) and two heat shock protein response elements (HSPRE). The promoter region also contains a 13 GT and 6 CA dinucleotide repeat units (Nguyen *et al.,* 1998). The *MYOC* gene codes for a glycoprotein of 504 amino acids.

2.10.1.1 Global spectrum of *MYOC* mutations:

Mutations were identified in the *TIGR* gene in glaucoma patients. *MYOC* mutations exist in approximately 3% of late-onset POAG patients and a greater proportion of JOAG patients (Alward *et al.,* 2002). Till date more than 73 mutations are known and most of these are misense mutations. Majority (63) of the mutations were found in the olfactomedin-like domain (Gong *et al.,* 2004) suggesting that this is a functionally important domain

(Adam *et al.,* 1997). The distribution of the *MYOC* mutations in the different populations is as shown in Table 4.

Stone *et al,* (1997) first identified three mutations in *MYOC*, which was initially associated with juvenile open angle glaucoma (*GLC1A*) (Sheffield *et al.,* 1993). Further numerous other researchers worked on the mutation screening in glaucoma. Wiggs *et al,* (1998) reported 8% mutation frequency, while Shimizu *et al,* had reported a 36% of mutation frequency in JOAG. Alward *et al,* (2002) reported 6.38% of mutations in JOAG cases.~3% of the POAG patients from Midwestern America harboured the *MYOC* mutations (Stone *et al.,* 1997; Alward *et al.,* 1998). These studies were followed by a larger study on 1703 glaucoma patients from five different populations including three Caucasian populations from Iowa, Australia and Canada and a group of African-American patients from New York city and a group of Asian patients from Japan. The overall frequency of myocilin mutations was ~2-4% (Fingert *et al.,* 1999).

The most common *MYOC* mutation observed was Gln368Stop in 1.6% of glaucoma probands and was found in all groups except the Japanese group (Fingert *et al.,* 1999). The second most common mutation Arg46Stop was shared only by Asian populations. Many mutations were found only in specific regions like the Asn480Lys mutation was found in two geographically close European countries but not anywhere else (Brezin *et al.,* 1998). The Gln48His mutation was found only in Indian population (Mukhopadhyay *et al.,* 2002) which is also the finding of the

present study and the Cys433Arg mutation only in Brazil (Vasconcellos *et al*, 2000).

In a larger study by Alward *et al,* (1998), mutations were observed in 4.6% of POAG cases and among all the mutations Q368X was the predominant mutation found in 2% of the cases. The same mutation was found in 0.16% of the controls. The individuals with Y437H and I477N mutations manifested the disease four decades earlier than that of individuals with Q368X. The same group screened 779 individuals affected with POAG, JOAG, normal tension glaucoma, ocular hypertension and secondary conditions like pigmentary glaucoma and cortico-steroid induced glaucoma and found 3% of cases to harbour disease causing variations in *MYOC* and the Q368X mutation was observed in 2.9% of the cases.

A gain of function mechanism caused by the mutant MYOC protein is proposed as a possible mechanism, through a study by Wiggs and Vollrath on a 29 year old women patient who had a complex deletion of the maternal copy of chromosome 1 that included the entire *TIGR/MYOC* gene and had only a single functional copy of *MYOC*. The patient however did not show any evidence of glaucoma on clinical examination which suggests that haploinsufficiency of MYOC protein is not the cause of early-onset glaucoma, associated with mutations in *MYOC*. Instead it is more likely that these mutations result in a gain of function or cause a dominant negative effect. Mutant protein may form a complex with wild

type protein and prevent its normal action, creating a dominant negative effect of the mutant MYOC protein. This might also have an effect on trabecular function of secretion and/ or processing of other proteins (Wiggs and Vollwrath, 2001).

Among the Chinese population mutations were observed in 1.5% of the POAG cases (Pang *et al.,* 2002). The Arg46Stop mutation was observed with similar frequency in both patients and controls indicating that the truncation mutation does not pose any risk for glaucoma (Pang *et al.,* 2002). One of the controls was homozygous for the same mutation without showing symptoms of glaucoma. Aung *et al.,* (2005), screened 106 PACG patients and found the disease causing variations to be present in the control individuals as well. The Arg46Stop change was found in 2.2% of the controls. Among the Japanese population mutations were found in 4% (Suzuki *et al.,* 1997) and 2.9% (Kubota *et al.,* 2000) of POAG patients. The Pro370Leu was found to associate with juvenile onset glaucoma, high IOP and poor response to medical treatment (Taniguchi *et al.,* 1999). A recent report from Taiwan had reported the mutation frequency to be 12.5% In addition, they suggested that Arg46Stop mutation was the predominant mutation with a frequency of 6.25% and is a hot spot in Taiwanese patients with JOAG (Yen *et al.,* 2007).

2.10.1.2 Haplotypes associated with *MYOC* mutations

Haplotype-sharing studies by Fingert *et al*, among the subjects including African American individuals showed that all the POAG probands with the Gln368Stop mutation shared common 100% closely linked markers suggesting a close genetic relationship among the subjects (Fingert *et al.,* 1999). *Faucher et al,* showed that *MYOC* mutation carriers share similar haplotypes over a long stretch of DNA in five (including Gln368Stop) of the six mutations examined. These findings suggest that Gln368Stop is exclusively European in origin and is a relatively young mutation (Faucher *et al.,* 2002). Asn480Lys is the other mutation, which comes from a single founder (Adam *et al.,* 1997; Brezin *et al.,* 1998). In a recent study by Hewitt *et al.,* the *MYOC* mutation Thr377Met had a common haplotype in genealogically independent pedigrees. They concluded that the Australian families originated from Greece and the FYROM (former Yugoslavian Republic of Macedonia) have a common founder, however, the British, Finnish, and Indian families have a distinct haplotype from the Greek suggesting that Thr377Met mutation has occurred *de novo* more than once (Hewitt *et al.,* 2007).

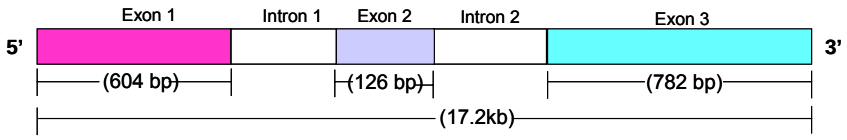
2.10.1.3 *MYOC* mutations reported in India

Mutations in *MYOC* account for 0.8-7.14% of the POAG cases (Kumar *et al.,* 2007; Mukhopadhyay *et al.,* 2002; Kanagavalli *et al.,* 2003; Sripriya *et al.,* 2004). Mukhopadhyay's group screened 56 POAG patients and found 7.14% of the cases to harbour *MYOC* mutations, and Q48H was the most prevalent mutation observed in 5.3% of cases (Mukhopadhyay *et al.,* 2002). Among the studies on the south Indian populations, the Q48H mutation has been detected in 2% and 2.5% of POAG and PCG cases from our centre (Chakrabarti *et al.,* 2005), five of the eight glaucoma patients, harboured the mutation in a large three generation Asian family, consisting of 24 members, with 15 congenital microcoria patients (Ramprasad *et al.,* 2005). Another study by Kanagavalli *et al,* on South and North Indian polpulations , reported a frequency of 2% for Q48H mutation. It has not been reported from any other populations so far, suggesting that this could be unique to India.

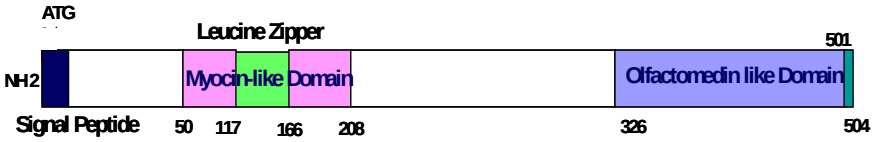
Another novel mutation Pro274Arg was found in a four-generation autosomal dominat family with members affected with JOAG and POAG. The mutation segregated with the disease phenotype, with one severely effected patient being homozygous for the mutation (Markandaya *et al.,* 2004). The earlier reported mutations Gly367Arg and Thr377Meth were also observed in Indian population (Kanagavalli *et al.,* 2003). A recent report from the Kanyakumari district of South India reported a novel

mutation Ser331Thr along with earlier reported mutation Pro370Leu in 2 POAG (2%) cases (Rose *et al.,* 2007).

a)



(b)



Transcript length – 2,083 bps; Translation product – 504 residues

Figure 8: Schematic representation of the *MYOC* gene and its protein

Table 4. Distribution of *MYOC* mutations in different populations

S.No.	c. DNA POSITION	AMINO ACID CHANGE	MUTATION TYPE	PHENOTYPE	POPULATION	Control frequen	REFERENCE
1	c.34 GGG>CGG	Gly12Arg	Missense	POAG, PACG	China China China	8/388 2/49 4/132	Aung *et al.,* 2005 Pang *et al.,* 2002 Lam *et al.,* 2000
2	c.47 CCA>CTA	Pro16Leu	Missense	POAG	China	1/388	Pang *et al.,* 2002
3	c.49 GCT>TCT	Ala17Ser	Missense	POAG	China	1/291	Pang *et al.,* 2002
4	c.73 TGC>CGC	Cys25Arg	Missense	POAG/JOAG	Italy	-	Bruttini *et al.,* 2003
5	c.136 CGA>TGA	Arg46Term	Nonsense	POAG NTG POAG PACG	Korea Japan China China	1/100 4/132 9/402	Yoon *et al.,* 1999 Mabuchi *et al.,* 2001 Lam *et al.,* 2000 Aung *et al.,* 2005
6	-	17 bp dup 56-72	Frameshift	POAG	USA	-	Fingert *et al.,* 1999
7	c.144 CAG>CAT	Gln48His	Missense	POAG and PCG	India	-	Mukhopadhyay *et al.,* 2002 Sripriya *et al.,* 2004 Chakrabarti *et al.,* 2005 Ramprasad *et al.,* 2005 Kaur *et al.,* 2005
8	c.244 CGC>TGC	Arg82Cys	Missense	POAG	USA/Australia	-	Alward *et al.,* 1998
9	c.271 CGA>TGA	Arg91Term	Ninsense	POAG	China	-	Lam *et al.,* 2000
10	c.376 CGG>TGG	Arg126Trp	Missense	POAG	Canada	-	Faucher *et al.,* 2002
11	c.473 CGA>CAA	Arg158Gln	Missense	POAG	Japan	-	Kubota *et al.,* 2000
12	c.624 GAC>GAG	Asp208Glu	Missense	POAG Exfoliative glaucoma	China Japan USA (Iowa) China	- 1/100 - 3/388	Lam *et al.,* 2000 Kubota *et al.,* 2000 Alward *et al.,* 2002 Pang *et al.,* 2002

Table 4 contd . .

13	c.736 GGA>AGA	Gly246Arg	Missense	POAG	France		Adam *et al*, 1997
14	c.754 GGA>AGA	Gly252Arg	Missense	POAG POAG	USA Australia		Rozsa *et al*, 1998 Hewitt *et al.*, 2007
15	c.781GAA>AAA	Glu261Lys	Missense	POAG	Spain		Vazquez *et al*, 2000
16	c.814 CGA>GGA	Arg272Gly	Missense	POAG	USA		Shimizu *et al*, 2000
17	c.821 CCC>CGC	Pro274Arg	Missense	JOAG	India		Markandaya *et al*, 2004
18	c.854 ACG>ATG	Thr285Met	Missense	POAG	Sweden		Jansson *et al*, 2003
19	c.856 TGG>CGG	Trp286Arg	Missense	POAG	Iowa	-	Fingert *et al*, 1999
20	c.878 ACG>AAG	Thr293Lys	Missense	Pigmentary glaucoma POAG POAG	Dutch USA/Australia Canada	 - -	Vincent *et al*, 2002 Alward *et al*, 1998 Faucher *et al*, 2002
21	c.898 GAA>AAA	Glu300Lys	Missense	POAG	China	-	Pang *et al*, 2002
22	c.967 GAA>AAA	Glu323Lys	Missense	POAG	Barbados		Rozsa *et al*, 1998
23	c.1010 CAG>CGG	Gln337Arg	Missense	JOAG	USA (Edinburg)		Stoilova *et al*, 1997
24	c.1009 CAG>GAG	Gln337Glu	Missense	POAG	Spain		Vazque *et al*, 2000
25	c.1021TCC>CCC	Ser341Pro	Missense	POAG	Korea		Kee *et al*, 1997
26	c.1025 AGA>AAA	Arg342Lys	Missense	POAG	Ghana		Challa *et al*, 2002

Table 4 contd . .

27	c.1035 ATA>ATG	Ile345Met	Missense	POAG/NTG	Germany		Michels-Rautenstrauss *et al*, 2002
28	c.1054GAG>AAG	Glu352Lys	Missense	POAG POAG	USA/Canada USA (Iowa)	-	Fingert *et al.,* 1999 Alward *et al.,* 2002
29	c.1058 ACA>ATA	Thr353Ile	Missense	POAG	USA/Australia Japan China Korea	- - - 6/388	Fingert *et al.,* 1999 Fingert *et al.,* 1999 Pang *et al.,* 2002 Yoon *et al.,* 1999
30	c.1079 ATC>AAC	Ile360Asn	Missense	POAG	Japan		Kubota *et al.,* 2000
31	c.1081 CCT>TCT	Pro361Ser	Missense	POAG	USA/Australia	-	Fingert *et al.,* 1999
32	c.1087 GCT>ACT	Ala363Thr	Missense	POAG	Japan		Kubota *et al.,* 2000
33	c.1091 GGC>GTC	Gly364Val	Missense	POAG	USA/Australia	-	Fingert *et al.,* 1999
34	c.1099 GGA>AGA	Gly367Arg	Missense	JOAG JOAG POAG POAG	Germany Italy/France Canada Japan		Michels-Rautenstrauss *et al.,* 2002 Vincent *et al.,* 2002 Faucher *et al.,* 2002 Suzuki *et al.,* 1997
35	c.1102 CAG>TAG	Gln368Term	Nonsense	POAG JOAG POAG POAG/ NTPOAG/OHT	USA/Australia N. America Spain Sweden Swiss Canada England Germany	-	Fingert *et al.,* 1999 Wiggs *et al.,* 1998 Vazque *et al.,* 2000 Jansson *et al.,* 2003 Mataftsi *et al.,* 2001 Faucher *et al.,* 2002 Vincent *et al.,* 2002 Michels-Rautenstrauss *et al.,* 2002

Table 4 contd . .

36	c.1109 CCG>CTG	Pro370Leu	Missense	POAG JOAG JOAG	Japan USA N. America Brazil India Germany Sweden Greece		Taniguchi *et al.,* 1999 Wiggs, 1998 Vasconcellos *et al.,* 2000 Mukhopadhyay *et al.,* 2002 Michels-Rautenstrauss *et al.,* 2002 Jansson *et al.,* 2003 Vincent *et al.,* 2002
37	c.1130 ACG>AAG	Thr377Lys	Missense	JOAG	Ireland/Scotland	-	Vincent *et al.,* 2002
38	c.1130 ACG>ATG	Thr377Met	Missense	POAG POAG	Australia USA Morocco N. America		Fingert *et al.,* 1999 Shimizu *et al.,* 2000 Melki *et al.,* 2003 Wiggs *et al.,* 1998
39	c.1139 GAC>GCC	Asp380Ala	Missense	JOAG	Ghana		Kennan *et al.,* 1998
40	c.1138 GAC>GGC	Asp380Gly	Missense	POAG	USA (Iowa)		Alward *et al.,* 1998
41	c.1138 GAC>AAC	Asp380Asn	Missense	POAG	Ghana (West Africa)		Challa *et al.,* 2002
42	c.1179 AGC>AGA/C	Ser393Arg	Missense	POAG	USA/Australia	4/91	Fingert *et al.,* 1999
43	c.1178 AGC>AAC	Ser393Asn	Missense	POAG	Germany		Michels-Rautenstrauss *et al.,* 2002
44	c.1193 AAA>AGA	Lys398Arg	Missense	POAG	US	4/91	Shimizu *et al.,* 2000

Table 4 contd . .

45	c.1196 GGT>GTT	Gly399Val	Missense	POAG/JOAG	E.India		Vincent *et al.,* 2002
46	c.1265 CGT>CAT	Arg422His	Missense	POAG	USA (Iowa)	-	Alward *et al.,* 1998
47	c.1267 AAG>GAG	Lys423Glu	Missense	POAG	France		Adam *et al.,* 1997
48	c.1276 GTC>TTC	Val426Phe	Missense	POAG	Spain		Mansergh *et al.,* 1998
49	c.1279 GCC>ACC	Ala427Thr	Missense	POAG	Canada (Quebec)		Faucher *et al.,* 2002
50	c.1297 TGT>CGT	Cys433Arg	Missense	POAG	Brazil		Vasconcellos *et al.,* 2000
51	c.1300 GGC>AGC	Gly434Ser	Missense	Preperimetric OAG	Germany		Michels-Rautenstrauss *et al.,* 2002
52	c.1309TAC>CAC	Tyr437His	Missense	POAG POAG/JOAG	USA/Australia N. America USA	-	Fingert *et al.,* 1999 Wiggs *et al.,* 1998 Alward *et al.,* 2002
53	c.1313 ACC>ATC	Thr438Ile	Missense		France		Melki *et al.,* 2003
54	c.1334 GCA>GTA	Ala445Val	Missense	OHT POAG	France Canada Australia	-	Vincent *et al.,* 2002 Faucher *et al.,* 2002 Fingert *et al.,* 1999
55	c.1342 ACC>CCC	Thr448Pro	Missense		Japan		Yokoyama *et al.,* 1999
56	c.1348 AAC>GAC	Asn450Asp	Missense	Preperimatric OAG	Germany		Michels-Rautenstrauss *et al.,* 2002
57	-	1 bp del codon 453	Frameshift	POAG	USA	-	Fingert *et al.,* 1999
58	c.1373 GGT>GAT	Gly458Asp	Missense	POAG	Japan		Ikezoe *et al.,* 2003

Table 4 contd . .

59	c.1395 ATC>ATG	Ile465Met	Missense	POAG	USA/Australia Japan	-	Fingert *et al.,* 1999
60	c.1408 CGC>TGC	Arg470Cys	Missense	POAG/NTG	USA/Australia USA Germany	-	Fingert *et al.,* 1999 Alward *et al.,* 1998 Michels-Rautenstrauss *et al.,* 2002
61	c.1412 TAT>TGT	Tyr471Cys	Missense	POAG	China	-	Pang *et al.,* 2002
62	c.1430 ATT>AAT	Ile477Asn	Missense	POAG	USA/Australia USA	-	Fingert *et al.,* 1999 Alward *et al.,* 1998
63	c.1430 ATT>AGT	Ile477Ser	Missense	POAG	France	-	Adam *et al.,* 1997
64	c.1440 AAC>AAA	Asn480Lys	Missense	POAG	The Netherlands France		Brezin *et al.,* 1998
65	c.1442 CCC>CGC	Pro481Arg	Missense		Sweden	-	Jansson *et al.,* 2003
66	c.1442 CCC>CTC	Pro481Leu	Missense	POAG	Canada	-	Fingert *et al.,* 1999
67	c.1441CCC>ACC	Pro481Thr	Missense	POAG	USA/Australia	-	Fingert *et al.,* 1999
68	c.1441CCC>TCC	Pro481Ser	Missense	POAG	Japan	-	Mabuchi *et al.,* 2001
71	c.1447GAG>TAG	Glu483Term	Nonsense	POAG	USA/Australia Canada	-	Fingert *et al.,* 1999 Faucher *et al.,* 2002
72	_	1515+20G/A	-	POAG	Japan		Mabuchi *et al.,* 2001
73	_	1544ins489Stop	Frameshift	POAG	Spain		Vazquez *et al.,* 2000
74	c.1496 ATC>AGC	Ile499Ser	Missense	Mixed POAG	USA		Shimizu *et al.,* 2000
75	c.1495 ATC>TTC	Ile499Phe	Missense	POAG	French	-	Adam *et al.,* 1997
76	c.1504TCC>CCC	Ser502Pro	Missense	JOAG	British		Stoilova *et al.,* 1998

Despite the importance of *MYOC* in glaucoma, its normal function(s) in aqueous humor physiology remains unknown. It has been postulated that *MYOC* facilitates aqueous humor outflow through the TM. Other suggestions include a protective role against stress (Johnson *et al.,* 2000). However, early truncating or deletion alleles are not pathogenic in people and mice with null alleles do not develop high IOP or glaucoma. Furthermore, histological analysis of these mice detected no drainage structure abnormalities, even at the ultrastructural level (Kim *et al.,* 2001). Together, these data suggest that *MYOC* is not necessary for normal IOP homeostasis and that pathogenesis of *MYOC* mutations is not due to loss of function.

Different groups have shown *in vitro* that mutant *MYOC* forms insoluble aggregates that are not secreted and accumulate intracellularly (Caballero *et al.,* 2000; Gobeil *et al.,* 2004, Jacobson *et al.,* 2001). A recent study shows that mutant *MYOC* molecules induce ER stress and cell death when TM cells are grown at normal body temperature (Liu, 2004). However, when cells are grown at a lower temperature that promotes protein folding and secretion, the cells survive. This strongly suggests that accumulation of misfolded proteins is pathogenic. TM cells appear more susceptible than another cell types and this may be due to cell type–specific processing of mutant molecules (Liu *et al.,* 2004).

An increased susceptibility of TM cells raises the possibility that protein folding abnormalities and ER stress may contribute to glaucoma

caused by other genes. Such a toxic mechanism also suggests that the normal functions of other glaucoma genes may have nothing to do with physiological IOP homeostasis. These insights suggest that treatments that promote protein folding and secretion may have beneficial effects for IOP. Because ER stress often induces apoptosis (Rao *et al.,* 2004), antiapoptotic treatments may also be beneficial by reducing cell death. However, it is not yet clear that TM cell death is important for IOP elevation *in vivo*.

Because glucocorticoids induce *MYOC* in TM cells and cause steroid-induced glaucoma, increased *MYOC* production was suggested to increase resistance to aqueous humor outflow and thus raise IOP (Fautsch *et al.,* 2000; Nguyen *et al.,* 1998; Polansky *et al.,* 1997). In possible agreement with this are culture studies where low-level overexpression of wild-type *MYOC* alters cellular properties and/or renders TM cells more prone to apoptosis (Liu *et al.,* 2004, Wentz-Hunter *et al.,* 2004). In other culture studies, TM cells are relatively insensitive to wild-type MYOC unless it is expressed at high levels (Sohn *et al.,* 2002). With the caveat that it is not yet certain that mice will develop *MYOC*-induced glaucoma, 15-fold overexpression of *MYOC* in mice did not cause TM cell abnormalities, elevated IOP, or glaucoma, even after extensive aging (Gould *et al.,* 2004). These *in vivo* mouse experiments, along with the lack of null allele phenotypes, suggest that alterations in *MYOC* level are not sufficient to alter IOP or induce pathology. If true, promoter

variants, in the absence of coding mutations, are unlikely to have an influence on disease progression. *MYOC* mutations were not found in human steroid glaucoma patients (Alward *et al.,* 2002; Fingert *et al.,* 2001).

In a French-Canadian family, patients heterozygous for a specific mutation developed glaucoma, whereas homozygotes had no phenotype (Morissette *et al.,* 1998). Thus far, no biochemical assays have revealed anything unique about this allele (Gobeil *et al.,* 2004, Joe *et al.,* 2003, Liu *et al.,* 2004). It is possible that the mutant chromosome carries a dosage-sensitive, linked modifier gene that abrogates the phenotype when present on both chromosomes. There is further evidence for a modifier gene in another study (Vincent *et al.,* 2002). In this study, a specific *MYOC* mutation was associated with POAG, but the age of onset was substantially earlier when patients were also heterozygous for a mutation in another glaucoma gene, *CYP1B1* (see below). The *CYPIBI* allele was suggested to modify the glaucoma phenotype. However, it is not clear if heterozygosity for the mutation in this enzyme-encoding gene truly affects the phenotype or if it simply acts as a marker for a linked modifier gene. More detailed analysis is required to resolve this.

2.10.2 Cytochrome P450 1B1 *(CYP1B1)*

The *CYP1B1* gene (Cytochrome P450, family 1, subfamily b, polypeptide 1; (Figure 9) encodes an enzyme that participates in iridocorneal angle development.It consists of three exons and two introns Its open reading

frame starts in the second exon and consists of 1629 bp and codes for a protein of 543 amino acids. The *CYP1B1* gene is transcriptionally activated by polycyclic aromatic hydrocarbons (Ah), which act via Ah receptor complex (Murray *et al.,* 2001). It is inducible by 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD or dioxin) (Sutter *et al.,* 1994). The 5' regulatory region consistes of dioxin-responsive elements and basal regulatory sequences (Wo *et al.,* 1997). *CYP1B1* lacks the consensus TATA box in the promoter region and contains nine TCDD- responsive enhancer core-binding motifs (Tang *et al.,* 1996).

In the developing mouse eye, *CYP1B1* is reported to be most highly expressed in the ciliary body after birth (Bejjani *et al.,* 2002). CYP1B1-/- mice are grossly normal, but have focal malformations of the iridocorneal angle (Libby *et al.,* 2003). In affected regions, malformations may include hypoplastic trabecular meshwork, abnormally located basal lamina in the trabecular meshwork and iridocorneal adhesions. Recessive mutations in *CYP1B1* associate with human congenital glaucoma (Stoilov *et al.,* 1997; Bejjani *et al.,* 1998; Bejjani *et al.,* 2000; Belmouden *et al.,* 2002; Panicker *et al.,* 2002; Reddy *et al.,* 2003; Panicker *et al.,*2004; Reddy *et al.,* 2004; Chakrabarti *et al.,* 2006; Achary *et al.,* 2006). Developmental malformations in some children with congenital glaucoma resemble those observed in *CYP1B1*-/- mice (Allen *et al.,* 1955, Maumenee *et al.,* 1958, Libby *et al.,* 2003). Table 5 lists as many as 79 mutations in *CYP1B1* associated with PCG and also POAG.

CYP1B1 belongs to a family of monomeric, mixed function monooxygenases (Sutter *et al.,* 1994). Its expression can be induced by aromatic hydrocarbons acting as ligands for a nuclear receptor complex (Denison *et al.,* 1989; Shehin *et al.,* 2000). The receptor complex consists of two basic helix-loop-helix proteins, the aryl hydrocarbon receptor (AHR) and the aryl hydrocarbon nuclear translocator (ARNT) (Reyes *et al.,* 1992). It is hypothesized that *CYP1B1* is involved in metabolism of signaling molecules important in ocular development (Sarfarazi and Stoilov 2000). It is possible that in the absence of *CYP1B1*, a key signaling molecule is not produced/activated or alternatively not degraded/deactivated. One possibility is that *CYP1B1* influences anterior segment development through a mechanism involving retinoic acid signaling. *CYP1B1* oxidizes all- trans-retinol to all- trans-retinal, which is a rate-limiting step in retinoic acid biosynthesis (Chen *et al.,* 2000). However, the exact role of *CYP1B1* in ocular development is not known. CYP1B1-/- mice are a valuable tool to address these possibilities. In a study by Choudhary *et al.,* who localized the CYP1B1 protein in the mouse eye during development by immunohistochemistry, found that the enzyme might play important roles in normal eye development and function as in humans, and that the mouse may prove to be an excellent model for determination of the roles of CYP1B1 in human eye development and function (Choudhary *et al.,* 2007).

According to a recent report by Chambers *et al, CYP1B1* alone plays an important role in the oxidation of retinol to retinal and subsequently to retinoic acid (RA), but unlike the CYP26s, *CYP1B1* cannot participate in the breakdown of RA. They also showed the role of *CYP1B1* in regulating dorsoventral patterning of the hindbrain and spinal cord in a manner that is consistent with both its endogenous expression and its RA-synthetic properties. They also reported *CYP1B1*'s contribution for AP patterning and regulation of epibranchial placode neurogenesis. They also reported a novel finding of an retinal dehydrogenase (RALDH) independent RA-signaling mechanism, which operates during early embryogenesis (Chambers *et al.,* 2007).

2.10.2.1 Involvement of *CYP1B1* PCG cases

Stoilov *et al*. (1997) identified *CYP1B1* as a candidate gene for Primary congenital glaucoma (PCG) which manifests as an autosomal recessive disorder, resulting from the maldevelopment of trabecular meshwork and anterior chamber angle leading to the obstruction of aqueous humor outflow and raised IOP Apart from *CYP1B1*, which maps to 2p21 (*GLC3A*) (Sarfarazi *et al.,* 1995) two other loci are linked to PCG, 1p36 (*GLC3B*) (Akarsu *et al.,* 1996) and 14q24.3 (*GLC3C*) (Stoilov *et al*, 2002).

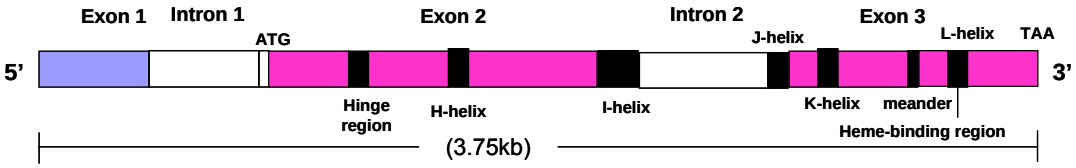
Many pathogenic mutations were identified in *CYP1B1 in PCG* phenotype, and the mutation frequency varied ethnically which ranged from ~20% in Japanese (Mashima *et al.,* 2000), ~33.3% in Indonesians (Sitorus *et al.,* 2003), ~37.5% in Indians (Reddy *et al.,* 2004), ~50%

among the Brazilians (Stoilov *et al.,* 2002) to almost 100% among the Saudi Arabians (Bejjani *et al.,* 2000) and Slovakian Gypsies (Plasilova *et al.,* 1999). G61E is the predominant mutation in the Soudi Arabian population (Bejjani *et al.,* 2000) and R368H the Indian population (Reddy *et al.,* 2003). Haplotype analysis based on five *CYP1B1* SNPs (R48G, A119S, V432L, D449D and N453S), showed that the 'C-G-G-T-A' is the most common haplotype associated with different *CYP1B1* mutations (Chakrabarti *et al.,* 2006).

2.10.2.2 Involvement of *CYP1B1* in POAG/JOAG cases

Recently a report by Vincent *et al.,* (2002) showed *CYP1B1* to be involved in a di-genic mechanism in JOAG, along with *MYOC* (Vincent *et al.,* 2002). In this study, individuals harboring mutations both in *MYOC* and *CYP1B1* gene manifested JOAG phenotype and individuals with only *MYOC* mutations showed late-onset POAG phenotype suggesting that *CYP1B1* might be a modifier of *MYOC* expression and these two genes might be acting through a common biochemical pathway. Individuals carrying both *MYOC* and *CYP1B1* mutations had mean age of onset of 27 years and individuals only with *MYOC* mutations had a mean age of onset of 51 years. *CYP1B1* mutations were also identified in early onset open angle glaucoma patients in French population with 4.6% mutation frequency (Melki *et al.,* 2004). The median age of diagnosis of these patients was 40 years (range from 13 - 52 years), which was significantly lesser than *CYP1B1* non-carriers. *CYP1B1* mutations were also reported in Spanish

POAG population with 10.9% frequency and in HTG cases with 8.1% frequency (Lopez-Garrido *et al.,* 2006). Involvement of *CYP1B1* was also reported in Indian POAG population (Acharya *et al.,* 2006), where it accounted for 4.5% of the cases similar to that of French population. This group identified heterozygous *CYP1B1* mutations in POAG while in juvenile onset POAG the mutations were observed to be in homozygous state suggesting a possible monogenic association in juvenile onset POAG. A recent report by Kumar *et al.,* (2007) who screened all the four candidate genes, *CYP1B1*, *MYOC*, *OPTN* and *OPTC* in adult onset POAG cases, found a higher frequency of 10.76% cases harboring *CYP1B1* mutations.



Transcript length – 5,122bps; Translation product – 543 residues

Figure 9. Schematic representation of *CYP1B1* gene with different regions marked

Table 5. Distribution of *CYP1B1* mutations across the different populations

S.NO	c.DNA POSITION	AMINO ACID CHANGE	MUTATION TYPE	PHENOTYPE	POPULATION	CON FREQ	REFERENCE
1	c.348 ATG>ACG	Met1Thr	Missense	Peters' anomaly	France	-	Vincent *et al.,* 2001
2	c. 376 ins A	^ 9 codon	Frameshift	PCG	India		Panicker *et al.,* 2002
3	c.401 CAG>TAG	Gln19Term	Nonsense	PCG	Brazil	-	Stoilov *et al.,* 2002
4	c.429 TCG>TGG	Ser28Trp	Missense	POAG	Spain	-	Lopez-Garrido *et al.,* 2006
5	c. 506 del C	^ 53 codon	Frameshift	PCG	Turkey		Mashima *et al.,* 2001
6	c.513 GCG>GTG	Ala56Val *	Missense	PCG	India		-
7	c.516 TGG>TAG	Trp57Term	Nonsense	Peters' anomaly POAG	Brazil France India	-	Vincent *et al.,* 2001 Acharya *et al.,* 2006
8	c.517 TGG>TGC	Trp57Cys	Missense	PCG	Hispanic		Stoilov *et al.,* 1998
9	c.520 del A	^ 57 codon	Frameshift	PCG	France		Colomb *et al.,* 2003

Table 5 contd . . . .

10	c.528 GGA>GAA	Gly61Glu	Missense	PCG POAG	India Saudi Arabia Ecuador Turkey Gypsies Spain	- - -	Panicker *et al.,* 2002 Bejjani *et al.,* 1998 Lopez-Garrido *et al.,* 2006
11	c.576 CTG>CCG	Leu77Pro	Missense	PCG	Saudi Arabia India	- -	Bejjani *et al.,* 2000 Reddy *et al.,* 2004
12	c.587 TAC>AAC	Tyr81Asn	Missense	POAG	France Spain	-	Melki *et al.,* 2004 Lopez-Garrido *et al.,* 2006
13	c.596 GTT>TTT	Val84Phe	Missense	PCG POAG	India Spain	-	- Lopez-Garrido *et al.,* 2006
14	c.624 del C	^ 92 codon	Frameshift	PCG	Costa Rica		Soley *et al.,* 2003
15	c.689 GCC>CCC	Ala115Pro	Missense	PCG	India	-	Reddy *et al.,* 2004
16	c.741 ATG>AGG	Met132Arg	Missense	PCG	India	-	Reddy *et al.,* 2004
17	c.777 CAG>CGG	Gln144Arg	Missense	PCG, POAG	India Spain		Chakrabarti *et al.,* 2003 Lopez-Garrido *et al.,* 2006
18	c.777 CAG >CCG	Gln144Pro	Missense	PCG	India	-	Reddy *et al.,* 2004
19	c.779-788 del CGGCGCGCAG	^ 144 codon	Frameshift	PCG	Saudi Arabia		Bejjani *et al.,* 2000
20	c.779 CGG>TGG	Arg145Trp	Missense	POAG	Spain	-	Lopez-Garrido *et al.,* 2006

Table 5 contd . . . .

21	c.847 ins T	^ 166 codon	Frameshift	PCG	Turkey		Stoilov *et al.,* 1998
22	c.881 del G	^ 178 codon	Frameshift	PCG	Morocco		Belmoulden *et al.,* 2002
23	c.888 CTG>CCG	Leu181Pro	Frameshift	PCG	India		-
24	c.911 GCC>CCC	Ala189Pro	Missense	OHT	Spain	-	Lopez-Garrido *et al.,* 2006
25	c.921 GAC>GTC	Asp192Val	Missense	PCG	Japan		Mashima *et al.,* 2001
26	c.924 CCG>CTG	Pro193Leu	Missense	PCG	India	-	Panicker *et al.,* 2002
27	c.938 GTC>ATC	Val198Ile	Missense	PCG	Japan		Mashima *et al.,* 2001
28	c.990 AGC>ATC	Ser215Ile	Missense	PCG	Indonesia		Sitorus *et al.,* 2003
29	c.1031 GAA>AAA	Glu229Lys	Missense	PCG POAG PCG POAG POAG	Lebanon France India Spain India	- 6.4% - 5%	Michels-Rautenstrauss *et al.,* 2001 Colomb *et al.,* 2003 Panicker *et al.,* 2002 Reddy et ., 2003 Lopez-Garrido *et al.,* 2006 Acharya *et al.,* 2007 Kumar *et al.,* 2007
30	c.1040 GGG>CGG	Gly232Arg	Missense	PCG	France		Colomb *et al.,* 2003

Table 5 contd . . . .

31	c.1061 AGC>CGC	Ser239Arg	Missense	PCG	India		Reddy *et al.,* 2004
32	c.1088 CAG>TAG	Gln248Term	Nonsense	PCG	France		Colomb *et al.,* 2003
33	c.1212 ins C	^ 288 codon	Frameshift	PCG	Turkey	-	Stoilov *et al.,* 1997
34	c.1186 TGC>TGA	Cys280Term	Nonsense	PCG	India Japan		Mashima *et al.,* 2001
35	c.1143-1151 del GCAACTTCA	^ 268 codon	Inframe deletion	PCG	Saudi Arabia		Bejjani *et al.,* 2000
36	c.1187 GAA>TAA	Glu281Term	Nonsense	PCG	Turkey		Stoilov *et al.,* 1998
37	c.1221 ATG>AAG	Met292Lys	Missense	PCG	India		-
38	c.1304 GTA>TTA	Val320Leu	Missense	PCG	Japan		Mashima *et al.,* 2001
39	c.1318-1319 ins AT	^ 323	Frameshift	PCG	Japan		Ohtake *et al.,* 2000
40	c.1334-1335 del GC	^ 329	Frameshift	PCG	Japan		Mashima *et al.,* 2001
41	c.1334 GCC>ACC	Ala330Thr	Missense	PCG	India		-
42	c.1334- c.1335 GCC>TTC	Ala330Phe	Missense	PCG	Japan		Mashima *et al.,* 2001

Table 5 contd . . . .

43	c.1334 GCC>TCC	Ala330Ser	Missense	OHT	Spain	-	Lopez-Garrido *et al.,* 2006
44	c.1379 CTC>TTC	Leu345Phe	Missense	POAG	Canada (Toronto)		Vincent *et al.,* 2002
45	c.1407-1418 del CTCGAGTGCAG G	^ 353 codon	Inframe deletion	PCG	Indonesia Europe		Sitorus *et al.,* 2003
46	c. 1407-1422 del CTCGAGTGCAG GCAGA	^ 354 codon	Frameshift	PCG	Turkey	-	Stoilov *et al.,* 1997
47	c.1409 CGA>TGA	Arg355Term	Nonsense	PCG	India Japan		Michels-Rautenstrauss *et al.,* 2001
48	c.1436 GTG>ATG	Val364Met	Missense	PCG	Japan Indonesia		Ohtake *et al.,* 2000 Sitorus *et al.,* 2003
49	c.1439 GGG>TGG	Gly365Trp	Missense	PCG	USA		Stoilov *et al.,* 1998
50	c.1449 G>A CGT>CAT	Arg368His	Missense	PCG POAG	Saudi Arabia Brazil USA India India	- - - - 0.7% 2.13% (1/47) 2%	Bejjani *et al.,* 2000 Stoilov *et al.,* 2002 Sena *et al.,* 2004 Panicker *et al.,* 2002 Reddy *et al.,* 2003 Acharya *et al.,* 2006 Melki *et al.,* 2004 Kumar *et al.,* 2007
51	c.1458 TGT>TTT	Cys371Phe*	Missense	PCG	India		-
52	c.1466 GAC>AAC	Asp374Asn	Missense	PCG	Saudi Arabia		Bejjani *et al.,* 1998

Table 5 contd . . . .

53	c.1482 CCC>CTC	Pro379Leu	Missense	PCG	Turkey		Stoilov *et al.,* 1998
54	c.1505 GAA>AAA	Glu387Lys	Missense	PCG	Brazil USA France Canada Gypsy Hispanic		Stoilov *et al.,* 1998
55	c.1515 CGC>CAC	Arg390His	Missense	PCG POAG	Pakistan India France	- -	Stoilov *et al.,* 1998 Reddy *et al.,* 2004 Melki *et al.,* 2007
56	c.1514 CGC>AGC	Arg390Ser	Missense	PCG	India Saudi Arabia France		Bejjani *et al.,* 2000
57	c.1514 CGC>TGC	Arg390Cys	Missense	PCG	India Ecuador	-	Reddy *et al.,* 2003 Curry *et al.,* 2004
58	c.1542 ATT>AGT	Ile399Ser	Missense	PCG	France		Colomb *et al.,* 2003
59	c.1546-1555 dup TCATGCCACC	^ 400	Frameshift	PCG	Turkey	-	Stoilov *et al.,* 1998
60	c.1556-1565 ins TCATGCCACC	^ 403	Frameshift	PCG	Turkey		Stoilov *et al.,* 1998
61	c.1571 GTC>TTC	Val409Phe	Missense	POAG	Spain	-	Lopez-Garrido *et al.,* 2006
62	c.1613 AAC>TAC	Asn423Tyr	Missense	PCG	France		Colomb *et al.,* 2003

Table 5 contd . . . .

63	c.1621 ins G	^ 424	Frameshift	PCG	Japan		Kakuchi *et al.,* 1999
64	c.1656 CCG>CTG	Pro437Leu	Missense	PCG	Turkey Brazil India	- - -	Stoilov *et al.,* 1998 Stoilov *et al.,* 2002 Reddy *et al.,* 2004
65	c.1671 CCA>CGA	Pro442Arg	Missense	PCG	India		Sitorus *et al.,* 2003
66	c.1674 GCT>GGT	Ala443Gly	Missense	PCG OHT POAG	India Brazil Spain France	- -	Michels-Rautenstrauss *et al.,* 2001 Lopez-Garrido *et al.,* 2006 Melki *et al.,* 2007
67	c.1677 CGA>CAA	Arg444Gln	Missense	PCG	Japan		Mashima *et al.,* 2001
68	c.1676 CGA>TGA	Arg444Term	Nonsense	PCG	France		Colomb *et al.,* 2003
69	c.1680 TTC>TGC	Phe445Cys	Missense	POAG	India		Chakrabarti *et al.,* 2003
70	c.1691 del G	^ 448 codon	Frameshift	PCG	-		Stoilov *et al.,* 1998
71	c.1723-1724 del AG	^458 codon	Frameshift	PCG	Brazil		Stoilov *et al.,* 2002
72	c.1736 TCA>CCA	Ser464Pro *	Missense	PCG	India		-
73	c.1743 GGC>GAC	Gly466Asp	Missense	PCG	India	-	Reddy *et al.,* 2004

Table 5 contd . . . .

74	c.1751 CGG>TGG	Arg469Trp	Missense	PCG	India Soudi Arabia Turkey UK		Bejjani *et al.,* 1998
75	c.1842 GAG>GGG	Glu499Gly	Missense	PCG	Japan		Mashima *et al.,* 2001
76	c.1890 TCA>TTA	Ser515Leu	Missense	POAG	India	-	Acharya *et al.,* 2006
77	c.1902 AAT>AGT	Asn519Ser*	Missense	PCG	India		-
78	c.1914 AGA>ACA	Arg523Thr	Missense	POAG	India	-	Acharya *et al.,* 2006
79	c.1935 GAT>GGT	Asp530Gly	Missense	POAG	India	-	Acharya *et al.,* 2006

* Not yet reported

2.11 SINGLE NUCLEOTIDE POLYMORPHISMS IN GLAUCOMA

Glaucoma is a complex disorder and both genetic and environmental factors play a role in its etiology. Apart from the mutations in the candidate genes, SNPs in 16 other candidate genes listed in Table 3 were found to be associated to POAG (Fan *et al.,* 2006). Of these, SNPs in the *MTHFR*, p-21, *IL-1* and *MMP9* genes were evaluated in POAG and PACG in the present study.

2.11.1 Methylenetetrahydrofolate reductase (*MTHFR*)

The enzyme 5-Methylenetetrahydrofolate reductase (*MTHFR*) catalyses methylation of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which contributes a methyl group in the conversion of homocysteine to methionine. The latter is converted to 5-adenosylmethionine, the lone donor of -CH3 to cytosine and lysine residues, respectively in DNA and histones. *MTHFR* also has a role in de novo nucleotide biosynthesis (Kim, 1999). The human *MTHFR* gene is localized to 1p36.3 and is composed of 11 exons (Goyette *et al.,* 1998). The promoter region of the *MTHFR* gene does not have a TATA box but contains CpG islands, multiple potential SP-1 binding sites, and binding sites for several other transcription factors (Gaughan *et al.,* 2000).

Frosst identified a 677C>T substitution in the exon 4, that converts an alanine 222 to a valine (A222V) residue and is responsible for the synthesis of a thermolabile form of *MTHFR*. The mutation in the heterozygous or homozygous state correlated with reduced enzyme

activity and increased thermolability in lymphocyte extracts (Frosst, 1995). Individuals homozygous for the mutation had significantly elevated plasma homocysteine levels, which have been documented in glaucoma patients. Thus, the 677C>T mutation may represent genetic risk factor in glaucoma. The 677C>T SNP in the *MTHFR* gene decrease the activity of the enzyme, leading to hyperhomocysteinemia, particularly in folate-deficient states.

Junemann *et al.,* estimated the prevalence of the 677C>T single-nucleotide polymorphism in the *MTHFR* gene in POAG and pseudoexfoliation open-angle glaucoma (PEXG). The authors found significant evidence of a higher prevalence of C677T in POAG (9% homozygotes, 49% heterozygote, 42% wildtype, P = 0.01, OR = 2.38) than in PEXG (9% homozygotes, 41% heterozygote, 50% wildtype, P = 0.09, OR 1.78) compared to controls (3% homozygotes, 34% heterozygote, 63% wildtype). They concluded that the *MTHFR* C677T variant leading to moderate hyperhomocysteinemia might play a role as a genetic risk factor in the pathogenesis of POAG (Junemann *et al.,* 2005). Similar studies on association of *MTHFR* to POAG, NTG and PEXG were conducted by different groups across different populations in the world and the genotype frequencies of these studies are as shown the Table 6. All the four reports showed no association of the SNP to any of the above glaucoma phenotypes (Fingert *et al.,* 2006; Mabuchi *et al.,* 2006; Mossbock *et al.,* 2006; Turacli *et al.,* 2006).

The finding of Junemann *et al.,* might have resulted due to several possible reasons. One reason being a false positive association resulting because of a small cohort size, ethnic differences in the *MTHFR* allele frequencies, which is earlier reported (Rady *et al.,* 2002; Esfahani *et al.,* 2003). In European populations, the prevalence of homozygosity for the *MTHFR c.*677 T-allele has been reported to be between 4.0 to 26.4% with a greater prevalence in southern Europe (Wilcken *et al.,* 2003). Hence, ethnic variation could be a possible explanation. Another alternative explanation may be a linkage disequilibrium with that of a nearby polymorphism (Mabuchi *et al.,* 2006).It is hypothesized that homocysteine is toxic to RGCs, and induces apoptotic cell death in RGCs by the over stimulation of N-methyi-D-aspartate (NMDA) receptors and caspase-3 activation. Hence elevated homocysteine and glutamate contribute to RGC death (Moore *et al.,* 2001).

POPUL-ATION	Germany (Junemann *et al.*, 2005)			USA (Fingert *et al.*, 2006)			Japan (Mabuchi *et al.*, 2006)			Australia (Mossbock *et al.*, 2006)			Turkey (Turacli *et al.*, 2006)	
PHEN-OTYPE	**PEXG** (n=71) (%)	**POAG** (n=76) (%)	**CONT** (n=71) (%)	**PEXG** (n=45) (%)	**POAG** (n=178) (%)	**CONT** (n=166) (%)	**NTG** (n=131) (%)	**POAG** (n=133) (%)	**CONT** (n=106) (%)	**PEXG** (n=138) (%)	**POAG** (n=204) (%)	**CONT** (n=211) (%)	**PEXG** (n=76) (%)	**CONT** (n=34) (%)
CC	50	42	63	26.7	40.4	75	41.2	51	45.3	52.2	58.3	49.5	51.3	52.9
CT	41	49	34	65.6	43.3	73	44.3	55	36.8	36.2	34.8	40.8	40.8	35.4
TT	9	9	3	8.9	16.3	10.8	14.5	27	19	11.6	6.9	9.5	7.9	11.8
p-value	= 0.01	> 0.05		= 0.046	> 0.05		> 0.05	> 0.05		> 0.05	> 0.05		> 0.05	

Table 6. Genotype frequencies of *MTHFR* SNP across different populations: PEXG indicates pseudoexfoliation glaucoma; POAG indicates primary open angle glaucoma; NTG indicates normal tension glaucoma CONT indicates controls; n is the total sample size

2.11.2 The DNA repair gene *WAF-1 / CIP-1/ p-21*

The apoptotic mechanism consists of multiple interacting pathways, some of which are still unclear. It appears that following DNA damage, cells can either proceed to apoptosis or enter a transient arrest cycle, allowing time for DNA repair. *p21* gene, also known as wildtype p53-activated fragment 1 (*WAF1)* or cyclin dependent kinase interacting protein 1 (*CIP1)*, is a key component of this pathway. It can be up-regulated either by activated wild type p53, which acts as a transcription factor (Levine, 1997), or independently, by various factors such as TGF β, vitamin D, TPA and nerve growth factor. *p21* expression results in the inhibition of cyclin-dependent kinases (Cdks), that are essential for cell division. Consequently, cell cycle is arrested at the G1 phase, until genomic repair is established. An unstable or altered p21 protein, therefore, could significantly affect the activity of Cdks, modifying the cellular response to genomic injury and abolishing the protective effect of p21. A single nucleotide polymorphism at codon 31 position of p21 gene, following a C to A transverse change, which results in a Serine/Arginine amino acid substitution, can alter the protein's stability. This polymorphism encodes a probable DNA-binding zinc-finger domain (Lori *et al.,* 1996). The p21 codon 31 polymorphism may change the transcription function and change the expression of its protein (Tsai *et al.,* 2004). Gene expression studies by Su *et al.,* had demonstrated that the individuals with heterozygous genotype showed an approximate 38% decrease in *p21*

expression. There is evidence from a recent case-control study on a Chinese cohort that the Arg allele of the *p21* codon 31 polymorphism is more common amongst POAG patients (Tsai *et al.,* 2004). In a similar study the hypothesis of a possible association between the *p21* codon 31 polymorphism and POAG on a Caucasian population was also tested (Ressinoitis *et al.,* 2005) (see Table 7).

In our cohort, we have not detected any statistically significant difference of the Ser and Arg allelic frequencies between the POAG group and the healthy individuals. The contrasting results among the Chinese and Caucasian studies can be attributed to the sampling bias, as the Chinese cohorts were smaller (58 POAG and 59 control subjects) or the ethnic disparity is another possible explanation. The Arg allele is considerably more common in Chinese, with reported frequency of 0.5. Our findings correlate well with previous studies on Swedish and French populations, which identified a low Arg allele frequency of 0.05.

Table 7. Distribution of p-21 SNP across the different populations

POPULATION	**Taiwan (Tsai *et al.,* 2004)**		**Caucasians (Ressinoitis *et al.,* 2005)**	
PHENOTYPE	**POAG** (n=58) (%)	**CONTROLS** (n=59) (%)	**POAG** (n=140) (%)	**CONTROLS** (n=73) (%)
Ser/Ser	21	42	91.4	83.6
Ser/Arg	45	44	7.1	10.3
Arg/Arg	34	14	1.5	0.9
p-value	= 0.00782		> 0.05	

2.11.3 Interleukin-1 (*IL-1*)

Glaucoma and genetic polymorphisms of the immune system were not perceived as related but their relationship has been suspected and investigated recently. The involvement of immune system in optic atrophy has been postulated as one of the possibility (Wax, 2000). Small heat shock antibodies or an immune mimicry mechanism to rhodopsin antibodies might cause optic nerve damage. The production of TNF-α increased when glial cells were exposed to ischemia condition and elevated hydrostatic pressure (Tezel *et al.,* 2000). There was an increase in the serum autoantibody against glutathione S-transferase in patients with glaucoma (Yang *et al.,* 2001). It is therefore hypothesized that glaucoma might also arise from an immune disorder and the ganglion cells ultimately die due to apoptosis (Hui-Ju Lin *et al.,* 2003).

Interleukin 1 (*IL1*) is an important cytokine involved in the control of the inflammatory response. There are two structurally distinct forms of IL1: *IL1* (α), which is the acidic form, and *IL1* (β), the neutral form (Figure 9). Both are 17-kD proteins coded by separate genes. The *IL1*α gene has 10,206 bp with 7 exons and 6 introns (Furutani, 1986). *IL1*β gene also has 7 exons and both *IL1*α and *IL1*β are located on chromosome on 2q13-q21 (Lafage *et al.,* 1989).

The IL1α and IL1β proteins are synthesized by a variety of cell types including activated macrophages, keratinocytes, stimulated B-

lymphocytes, and fibroblasts, and are potent mediators of inflammation and immunity (Lord *et al.,* 1991). Interleukin 1 is involved in apoptosis (cell death) and both the alpha and the beta forms are released as a consequence of cell injury regardless of the insult (Hogquist *et al.,* 1991).

Genetic polymorphisms of the *IL1*α and *IL1*β genes may be associated with severity of glaucoma. Different polymorphisms have been described in the *IL-1*β gene and atleast two of them could influence the protein production: One polymorphism was referred to as *IL1*β +3953 C>T in the exon 5 and the other in the promoter region that is – 511 C>T. *IL1*α gene polymorphism in the promoter region was referred to as *IL-1A* -889. It is reported that the neurotoxic mediators of inflammation, such as interleukin-1 are expressed at abnormally high levels by glial cells in inflammatory diseases like Alzheimer disease (AD) and may lead to neuronal injury. The polymorphisms of interleukin1, specifically, the promoter polymorphism at position *IL1*α -889C>T showed an association of the 'T' allele to AD (Du *et al.,* 2000; Grimaldi *et al.,* 2000; Nicoll *et al.,* 2000). Table 8 lists the SNP distribution in *IL1* across the Chinese population, associated with predisposition to glaucoma.

Fluorescent microscopic studies pertaining to over-expression of the precursor form of IL1α in different cell types, in the presence of saturating concentrations of IL1 receptor antagonist (IL1RN), lead to the observation that the precursor form of IL1A was initially present in the cytoplasm of

resting cells, then it is translocated to the nucleus after activation by an endotoxin, a Toll-like receptor 4 (TLR4) ligand. The IL1α precursor or its propiece, but not the C-terminal mature form, activated a GAL4 transcription system, probably through the N terminus where the nuclear localization signal resides. Over expressed precursor and propiece forms were also sufficient to activate Nuclear factor kappa B (NFKB) and AP-1 (Activated protein 1). Intracellular functions of IL1A may play a role in the genesis of inflammation by augmenting the transcription of proinflammatory genes (Werman *et al.,* 2004). IL1 has also been reported to increase outflow facility by stimulating the expression of matrix metalloproteinase enzymes, which in turn reduces extracelular resistance (Bradley *et al.,* 2000; Pang *et al.,* 2003; Bradley *et al.,* 1998; Kee *et al.,* 1997)

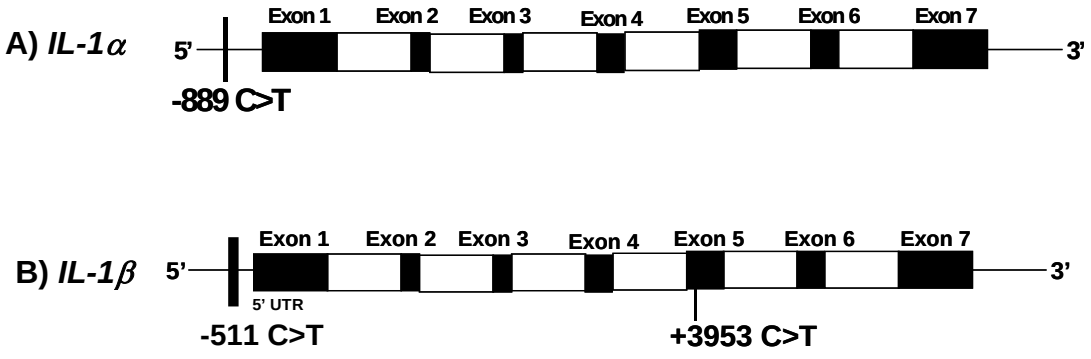


Figure 10. Schematic representation of IL-1α and IL-1β genes

Table 8: Distribution of genotype frequencies of IL -1 SNPs across the different populations

POPULATION		Taiwan (Lin *et al.*, 2003)		China (Wang *et al.*, 2006)		China (Wang *et al.*, 2007)		China (How *et al.*, 2007)			
PHENOTYPE		POAG (n=58) (%)	CONT (n=105) (%)	POAG (n=156) (%)	CONT (n=167) (%)	NTG (n=162) (%)	CONT (n=167) (%)	HTG (n=100) (%)	NTG (n=94) (%)	PACG (n=125) (%)	CONT (n=79) (%)
***IL-1β*-511**	CC	31.7	26.6	-	-	-	-	35	22.3	33.6	30.4
	CT	30	47.6	-	-	-	-	46	53.2	48.0	53.1
	TT	16.6	25.7	-	-	-	-	19	24.2	18.4	16.1
p-value: > 0.05				-	-	-	-	p-value: > 0.05			
***IL-1β*+3953**	CC	87.9	97.14	-	-	-	-	94	98.9	96	97.5
	CT	12.1	2.86	-	-	-	-	6	1.1	4	2.5
	TT	0	0	-	-	-	-	0	0	0	0
p-value: <0.05				-		-		p-value: > 0.05			
***IL-1α*-899**	CC	-	-	63	75	73	75	87	79.8	88	81.0
	CT	-	-	32	24	25	24	12	19.1	10.4	16.5
	TT	-	-	5	1	2	1	1	1.1	1.6	2.5
				p-value: <0.05		p-value: >0.05		p-value: > 0.05			

The results of our study are similar to a recently reported study by How *et al*, who studied the link between inflammation and genetics in relation to glaucoma (POAG and PACG) pathogenesis in Chinese patients. Similar to the results from our study population, this group also could not replicate the results of the previous reports (Table 8). The minor allele (T) frequency at the *IL1*α -889C>T studied by Wang *et al.,* in Whites was much higher among the POAG patients (Wang *et al.,* 2006) while the NTG patients did not show any such significant association (Wang *et al.,* 2007). These results are in contrast to the positive association of *IL1*β+3953 C>T reported by Lin *et al.,* on a small cohort of 58 POAG patients and 105 control samples from Taiwan. Taking these reports into account along with our findings we can say that the there are other factors in the role of IL1 in glaucoma that may include environmental, racial, and ethnic influences. Population diversity may also explain the differences in the results of these studies.

2.11.4 Matrix metalloproteinase 9 (*MMP9*)

Type IV collagenase (72- and 92-Kd) proteins are members of a group of secreted zinc metalloproteases which, in mammals, degrade the collagens of the extracellular matrix. Other members of this group include interstitial collagenase (MMP1) and stromelysin (MMP3). The 72-kD type IV collagenase (MMP2, or CLG4A) is secreted from normal skin fibroblasts, whereas the 92-kD collagenase (CLG4B) is produced by normal alveolar macrophages and granulocytes. The 92-kD type IV collagenase is also

known as 92-kD gelatinase, type V collagenase, or matrix metalloproteinase-9 (*MMP9*) (Nagase *et al.,* 1992).

MMP9, is involved in the degradation of extracellular matrix molecules, The release of *MMP9* might induce stem cell mobilization by cleaving matrix molecules to which stem cells are attached (Opdenakker *et al.,* 1998; Pruijt *et al.,* 1999). *MMP9* is involved as a mediator of the IL8 induced mobilization of hematopoietic progenitor cells (HPCs) (Laterveer *et al.,* 1996).

MMP9 -mediated N-terminal cleavage of IL8 potentiates IL8 activation of neutrophils, as measured by increased intracellular calcium, *MMP9* secretion, and neutrophil chemotaxis (van den Steen *et al.,* 2000). *MMP9* is predominantly expressed in neutrophils, macrophages, and mast cells, rather than in oncogene-positive neoplastic cells (Coussens *et al.,* 2000).

MMP9 gene has been localized to chromosome region 20q11.2-q13.1 through linkage (St Jean *et al.,* 1995). *MMP9* has 13 exons (Huhtala *et al.,* 1991). The 13 exons of *CLG4B* are 3 more than have been found in other members of this gene family. The extra exons encode the amino acids of the fibronectin-like domain, which has been found only in the 72- and 92-kD type IV collagenases.

A polymorphism (-1590C>T) in the promoter region of the *MMP9* gene has a functional effect on transcription and is associated with the severity of the atherosclerosis in patients with coronary artery disease

(Zhang *et al.,* 1999). Sequence variants were found in the 2.2-kb promoter sequence and all 13 exons (totaling 3.3 kb) of the *MMP9* gene. They identified a total of 10 variable sites, 4 in the promoter region, 5 in the coding region (3 of which altered the amino acid encoded), and 1 in the 3-prime untranslated sequence. Sequence inspection suggested that some of the variants would have a functional impact on either level of expression or enzymatic activity. Tight linkage disequilibrium was detected between variants across the entire length of the gene, and frequencies of different haplotypes were determined (Zhang *et al.,* 1999).

It is thus clear that a large number of genes are associated with various types of glaucoma, namely POAG, JOAG, PACG and PCG glaucomas. In addition genotype frequencies of SNPs of various glaucoma- associated genes are also described across different populations of the world. It is against this background that the present molecular genetic analysis of glaucoma, particularly POAG, in populations of India has been undertaken.

The candidate genes, *MYOC* and *CYP1B1* are involved in POAG and PCG but the involvement of these genes in PACG and PAC has not been demonstrated yet. The commonality of some clinical features like high IOP among the different glaucoma phenotypes, might indicate a common molecular mechanism due to the involvement of similar gene(s). Hence, these genes were screened for mutations and single nucleotide polymorphisms. Apart from these genes several candidate genes are

known to play a role indirectly in glaucoma pathogenesis. It is interesting to study these SNPs in relation to glaucoma pathogenesis. Among them the candidate genes involved in inflammation, apoptotic pathways resulting due to excitotoxic mechanism or improper cell cycle regulation form the major part. The SNPs within the cytokines involved in inflammation that is *IL1*α –889C>T, *IL1*β –511C>T, *IL1*β +3953C>T were screened in both POAG and PACG by case control studies. Also the SNPs in the candidate gene playing a role in the cell cycle regulation such as *p21* codon 31 polymorphism and the M*THFR* c.677C>T SNP which is involved in the excitotoxic conditions were screened in POAG cases. The SNPs within the *MMP9* matrix metalloproteinase 9 gene (-1590 and Q279R) were analysed for their involvement in PACG phenotype.

The importance of the genetic data thus obtained will help us in obtaining a mutation spectrum of candidate genes *MYOC* and *CYP1B1* in POAG and PACG in Indian scenerio. The baseline data can be used to develop a molecular diagnostic method for predictive testing and genotype-phenotype correlation would provide new avenues to explore therapeutic strategies.

Chapter 3
Materials and Methods

3. MATERIALS AND METHODS

3.1 ENROLLMENT OF STUDY SUBJECTS

The study was approved by the Institutional Review Board and followed the guidelines of the Declarations of Helsinki. A total of 273 glaucoma patients (143 POAG, 113 PACG and 17 PAC) presenting at the L. V. Prasad Eye Institute from 12 different states of India (Table 1) between January 2003-February 2006 were included. The geographic distribution of the patients is shown in Table 1.

Table 1. Geographical distribution of POAG, PACG and PAC cases

State	No. of POAG* cases (n=143)	No. of PACG cases (n=113)	No. of PAC cases (n=17)
Andhra Pradesh	99 (69.2%)	103 (91.1%)	14 (82.3%)
West Bengal	28 (19.5%)	-	1 (5.8%)
Karnataka	5 (3.5%)	3 (2.65%)	-
Jharkhand	3 (2.1%)	1 (0.88%)	-
Maharashtra	3 (2.1%)	1 (0.88%)	-
Orissa	2 (1.3%)	1 (0.88%)	1 (5.8%)
Madhya Pradesh	1 (0.7%)	-	1 (5.8%)
Chattisgarh	1 (0.7%)	1 (0.88%)	-
Kerala	1 (0.7%)	-	-
Tamilnadu	-	1 (0.88%)	-
Bihar	-	1 (0.88%)	-
Assam	-	1 (0.88%)	-

*Includes 27 JOAG cases.

Majority of the glaucoma patients and the control individuals who participated in the study belonged to Andhra Pradesh.

3.1.1 Clinical examinations

The clinical examinations were performed by qualified ophthalmologists at the institute. All the patients were clinically evaluated by the following methods.

3.1.1.1 Applanation tonometry (Haag-Strait, Bern, Switzerland)

Applanation tonometry measures the intraocular pressure (IOP) by subjecting the eye to a force that flattens the cornea or the degree of corneal indentation produced by a fixed force (Ritch *et al.,* 1996).

Method:

1. A drop of topical anaesthetic is instilled into the conjunctiva of the patient followed by staining the tear film with fluorescein. The patient is then positioned at the slit lamp with the forehead firmly against the headrest and looking straight ahead.
2. The prism is cleaned by wiping with alcohol and a very bright beam projected obliquely at the prism using the cobalt blue filter, the centre of the prism was positioned in front of the apex of the cornea.
3. The dial is set between 10 and 20 mm Hg and is made to advance until it touches the apex of the cornea.
4. When the prism touches the apex of the cornea, two yellow semicircles are seen which represent the fluorescein-stained tear film touching the upper and lower halves of the prism, and the dial is adjusted such that the inner edges of the two semicircles just touch indicating the perfect flattening of the cornea.

5. The IOP is then determined by reading the number on the dial and multiplying by 10 (Kanski, 2003).

3.1.1.2 Gonioscopy (4M and 2M lens, Volk Optical Inc., Mentor, OH, USA)

Gonioscopy is used to view the anterior chamber angle structures and to estimate the width of the chamber. The angle of the anterior chamber cannot be visualized directly through an intact cornea because the light emitted from angle structures undergoes total internal reflection. A goniolens eliminates this by replacing the cornea-air interface with a new interface, which has a refractive index greater than that of the cornea and tears (Kanski, 2003).

3.1.1.3 Perimetry (Humphrey visual fields) (Carl Zeiss Meditec, Dublin, CA, USA)

Perimetry is a method to evaluate the visual fields. The visual field is described as an island of vision surrounded by a sea of darkness. The outer aspect of the visual field extends approximately 60° nasally, 90° temporally, 50° superiorly and 70° inferiorly (Kanski, 2003). The observer is instructed to maintain a fixation on a small target at a central location on a large screen. Then a small stimulus, typically spots of light, is presented at various positions on the screen and the observer is asked to report by pressing a button whenever the flash of light appears, without moving the eye from the central fixation point (Ronald, 2002). If the flash of light falls into a scotoma, it is not seen this will then be registered on the printout as a blind spot. Sequential

visual fields in a glaucoma patient can be used to determine the progression of the disease.

3.1.1.4 Ophthalmoscopy (Direct and indirect, Heine, Germany)

The diameter of the cup can be expressed as a fraction of the diameter of the disc both in the vertical and horizontal meridians. This is called the 'cup-disc ratio'. Normal cup to disc ratio (C:D ratio) is 0.3 or less, and a ratio greater than 0.3 is regarded with suspicion as patients with glaucoma are characterized with an abnormally high C:D ratio due to the optic nerve damage. The optic nerve head and retinal nerve fiber layer in glaucoma can be assessed by direct and indirect ophthalmoscopy, slit lamp techniques and also by fundus photography.

3.1.1.5 Newer techniques

The retinal nerve fiber layer (RNFL) thickness was earlier qualitatively assessed in glaucoma diagnosis by fundus photography, but newer techniques now allow quantitative evaluation. This includes high-resolution RNFL imaging devices like optical coherence tomography (OCT) and scanning laser polarimetry (SLP). The latest version of SLP is GDx VCC (Glaucoma diagnostics variable corneal compensation GDxVCC). This assesses the nerve fiber layer thickness and has the highest diagnostic accuracy, with a sensitivity and specificity of 91.7% and 95.0% (Nicolas *et al.,* 2007).

The clinical data on different parameters like IOP at the time of presentation and during subsequent follow-ups, cup to disc (CD) ratios, best corrected visual acuity measured through Snellen's chart, visual fields categorized into mild, moderate and severe as per Anderson's criteria (Anderson *et al.,* 1987a) along with the treatment modalities were documented from the medical records. Details pertaining to the

family history and the pedigree information were obtained from the proband and documented.

3.1.2 INCLUSION AND EXCLUSION CRITERIA

3.1.2.1 Inclusion criteria for POAG patients

Patients with the following characteristics were included in the study:

1. IOP > 21 mm Hg
2. Raised cup to disc ratio (>0.4:1)
3. Open angles on gonioscopy (Angle structures visible up to scleral spur)
4. Glaucomatous visual field defects (consistent and repeatable in 3 fields)
5. Age more than five years

Patients with the above criteria and having an onset before 30 years of age were classified as having juvenile open angle glaucoma (JOAG).

Exclusion criteria for POAG patients

Patients with the following characteristics were excluded from the study:

1. Primary open angle suspects
2. Angle closure suspects
3. Pseudoexfoliation glaucoma
4. Mechanical traumas or any ocular surgery leading to secondary glaucoma

3.1.2.2 Inclusion criteria for PACG and PAC patients

1. Age above 18 years

2. Diagnosis of PAC or PACG:

i) Primary angle closure (PAC) was classified as synechial or appositional.

a) Primary (appositional) angle closure was defined as raised IOP (>21 mm Hg) associated with non-visibility of the filtering trabecular meshwork for more than 180 degrees, in the absence of peripheral anterior synechiae, disc damage, or field changes.

b) Primary (synechial) angle closure was defined as presence of peripheral anterior synechiae with non-visibility of the filtering trabecular meshwork for more than 180 degrees, with or without a raised IOP (>21 mm Hg), without disc damage or demonstrable field defects. The presence of even a single synechiae was considered significant. Other causes of synechiae were excluded.

ii) Primary angle closure glaucoma (PACG): PACG was defined as presence of optic disc and visual field changes characteristic of glaucoma along with PAC (appositional or synechial).

3. Visual field defects were considered to be glaucomatous if they were consistent with optic disc damage and met at least two of the criteria laid out by Anderson (Anderson et al., 1987a). The criteria for glaucomatous field defects were:

i) Three or more non-edge points on the pattern deviation plot that have sensitivities expected in fewer than 5% of the normal population ($p<0.05$) and one of the points with a sensitivity expected in fewer than 1% of the normal population ($p<0.01$)

ii) Pattern standard deviation (PSD) should have a value that occurs in less than 5% of normal reliable fields ($p<0.05$)

iii) Abnormal results in a GHT (glaucoma hemi field test).

The presence of a visual field defect required confirmation by a repeat field; this was performed within 2 weeks of the first reliable field showing the defect.

Exclusion criteria for PACG and PAC patients:

1. Age less than 18 years.
2. Definite cases of only primary angle closure suspect (PACS).
3. Patients with lens induced secondary angle closure glaucoma, neovascular glaucoma.
4. Patients with pseudoexfoliation syndrome and angle closure glaucoma.
5. Presence of other ocular pathology, which can cause secondary angle closure glaucoma (Example: Central retinal vascular occlusion).

3.1.2.3 Inclusion criteria for controls

1. Older than 40 years of age
2. No family history of glaucoma
3. Normal optic disc (<0.4:1)

4. IOP < 18 mm Hg
5. Individuals without any systemic diseases

3.1.2.4 Sample size

Clinically diagnosed cases of POAG (n=109), JOAG (n=34), PACG (n=113) and PAC (n=17) along with ethnically matched unaffected normal controls (n=113) were included in the study. Of the POAG cases, only 48 cases were screened for mutations in the candidate genes *MYOC* and *CYP1B1* as the remaining were screened earlier. However all the cases were analysed for screening the SNPs in Interleukin-1β, Interleukin-1α, *MTHFR*, *p21* and *MMP-9* genes. All the subjects were diagnosed independently by two clinicians and cases that had a disagreement among observers were excluded.

3.1.2.5 Blood sample collection

Around 6-8 ml of peripheral blood was drawn from each of the subjects by venipuncture in heparin-coated vacutainer tubes (Vacuette, Greiner bio-one GmbH, Austria) with prior informed consent. The samples were then stored at -20^{o}C deep freezer for further use.

3.2 MOLECULAR ANALYSIS

3.2.1 Genomic DNA Extraction

The DNA was isolated by phenol-chloroform extraction method (Sambrook et al., 1989) with a slightly modified protocol. The composition of the stock solutions is given in Annexure-I. The stepwise procedures were as follows:

1. The frozen blood (~ 8ml) was brought to the laminar flow hood and kept for thawing at room temperature.
2. The blood, once thawed, was transferred into a 50 ml centrifuge tube. Equal volume of 1 X phosphate-buffered saline (PBS) was added, mixed well by inverting and centrifuged at 3,500 rpm for 10 min.
3. The supernatant was carefully removed with a Pastuer pipette and discarded.
4. To the brown pellet, a second wash was given with equal volumes of 1 X PBS to remove the remaining erythrocytes.
5. To the obtained blood Leukocyte pellet, 7.5 ml of Extraction buffer, 37.5 μl of Proteinase K (20 μg/ml) (Bangalore Genei, Bangalore, India), and 15 μl RNase (10 mg/ml) (Bangalore Genei, Bangalore, India) was added, vortexed in a cyclomixer (Remi Equipments, Mumbai, India) and incubated in a water bath at 37^{o} C overnight.
6. To the dissolved pellet equal volumes of equilibrated phenol (Ambion, Texas, USA) was added, mixed gently by inverting and centrifuged at 4,500 rpm for 10 min.
7. The upper aqueous layer was separated and taken into a fresh centrifuge tube and equal volumes of phenol-$CHCl_3$ (Qualigens Fine Chemicals, Mumbai, India) (1:1) was added, mixed well and centrifuged at 4,500 rpm for 10 min.
8. The upper aqueous layer was separated gently, taken into a fresh centrifuge tube and equal volumes of $CHCl_3$ was added, mixed well and centrifuged at 4,500 rpm for 10 min.

9. The aqueous layer was separated and 1.5 ml of 10 mM Ammonium Acetate (Qualigens Fine Chemicals, Mumbai, India) and 2-2.5 volumes of Absolute chilled ethanol (Qualigens Fine Chemicals, Mumbai, India) was added.
10. The precipitated DNA was removed into a 1.5 ml centrifuge tube and 70% ethanol was added to it and spun for 2 min to remove impurities.
11. The supernatant was discarded and the pellet dried and dissolved in an appropriate amount of fresh autoclaved Milli Q water and the DNA was stored at –20^{o} C for further applications.

3.2.2 DNA Quantification

The concentration and purity of DNA was estimated using a spectrophotometer (UV-1601, Shimadzu, Japan). DNA was diluted 100 times, that is 10 μl stock genomic DNA sample was dissolved in 990 μl of de-ionised MilliQ water. The sample was then transferred into a cleaned quartz cuvette and the OD at 260 nm and 280 nm absorbance recorded. An OD of 1 at 260 nm indicates 50 μg of DNA per ml. Hence, the quantity of DNA in the sample was calculated by using the formula.

Conc. of DNA (μg/ml) = OD at 260 nm X 50 X 100 (Dilution factor)

For estimating the quality of DNA, the ratio of the OD at 260 and 280 nm is recorded. A ratio of 260/280 is equal to 1.8, indicates pure genomic DNA without any contamination. A ratio >1.8 indicates RNA or phenol contamination and a ratio < 1.8 indicates protein contamination.

3.2.3 Candidate gene screening

The candidate genes were screened for mutations by a variety of techniques such as Single Strand Conformation Polymorphism (SSCP), restriction digestion with appropriate restriction enzyme and direct sequencing. Further details of the genes screened are provided in Table 2.

Table 2. Different methods used in candidate gene screening

S.No.	Gene	Gen Bank ID*	Region	Variation screened	Screening method
1	*MYOC*	BC029261	3 coding Exons	Entire Exons	SSCP and Sequencing
			Promoter	-1000C>G	Restriction digestion
2	*CYP1B1*	U56438	2 coding Exons	Entire Exons	Sequencing
3	Interleukin-1β	M15330	Promoter	-511C>T	Restriction digestion
4			Exon - 5	Phe105Phe	Restriction digestion
5	Interleukin-1α	M28983	Promoter	- 889C>T	Restriction digestion
6	*p-21*	U03106	Exon - 2	Ser31Arg	Restriction digestion
7	*MTHFR*	BC053509	Exon - 4	Ala222Val	Restriction digestion
8	*MMP-9*	NM_004994	Promoter	-1590C>T	Restriction digestion
9			Exon - 4	Gln279Arg	Restriction digestion

***** NCBI

3.2.4 Polymerase Chain Reaction (PCR)

The coding regions of the candidate genes were amplified with pre-designed primers in a thermal cycler PTC-200 (MJ Research, Watertown, MA, USA). Overlapping primer sets were designed around

the intron/exon boundaries using the Primer 3 software (http://frodo.wi.mit.edu/).

3.2.4.1 Amplification conditions

The primer sequences along with the annealing temperatures and $MgCl_2$ concentrations for each of the primer set are shown in Table 3.

Table 3. List of the primer sequences used for amplifying the candidate genes

Gene	Location	Primers for PCR	Amplicon size (bp)	$MgCl_2$ (mM)	Annealing temp (^{0} C)
MYOC	Promoter (-1000C>G)	MTF 5'-GCGAATAGAGCCATAAAC-3' MTR 5'-TCTGGGGAACTCTTCTCA-3'	296	1.5	56.5
	Exon -1	1F: 5'GGCTGGCTCCCCAGTATATA 3' 1R: 5' ACAGCTGGCATCTCAGGC 3'	180	1.5	56.5
	Exon -1	2F: 5' ACGTTGCTCCAGCTTTGG 3' 2R: 5' GATGACTGACATGGCCTGG 3'	196	1.5	56.5
	Exon -1	3F: 5'AGTGGCCGATGCCAGTATAC 3' 3R: 5'CTGGTCCAAGGTCAATTGGT 3'	189	1.5	56.5
	Exon -1	4F: 5'AGGCCATGTCAGTCATCCAT 3' 4R: 5'TCTCTGGTTTGGGTTTCCAG 3'	214	1.5	56.5
	Exon -1	5F: 5' TGACCTTGGACCAGGCTG 3' 5R: 5'CCTGGCCAGATTCTCATTTT 3'	200	1.5	56.5
	Exon -1	6F: 5'TGGAGGAAGAGAAGAAGCGA3' 6R: 5'CTGCTGAACTCAGAGTCCCC3'	185	1.5	56.5
	Exon - 2	7F: 5'AACATAGTCAATCCTTGGGCC3' 7R: 5'TAAAGACCATGTGGGCACAA3'	223	1.5	56.5
	Exon - 3	8F: 5'TTATGGATTAAGTGGTGCTTCG3' 8R: 5' ATTCTCCACGTGGTCTCCTG 3'	177	1.5	56.5
	Exon - 3	9F: 5' AAGCCCACCTACCCCTACAC 3' 9R: 5'AATAGAGGCTCCCCGAGTACA 3'	184	1.5	56.5
	Exon - 3	10F: 5' ATACTGCCTAGGCCACTGGA 3' 10R: 5' CAATGTCCGTGTAGCCACC 3'	192	1.5	56.5
	Exon - 3	11F: 5'TGGCTACCACGGACAGTTC 3' 11R: 5'CATTGGCGACTGACTGCTTA 3'	197	1.5	56.5
	Exon - 3	12F: 5' GAACTCGAACAAACCTGGGA 3' 12R: 5'CATGCTGCTGTACTTATAGCGG3'	195	1.5	56.5
	Exon - 3	13F: 5' AGCAAGACCCTGACCATCC 3' 13R: 5' AGCATCTCCTTCTGCCATTG 3'	179	1.5	56.5
CYP1B1	Exon - 2*	P1F: 5'TCTCCAGAGAGTCAGCTCCG 3' P1R: 5' GGGTCGTCGTGGCTGTAG 3'	786	2.5	56
	Exon - 2*	P2F: 5' GATGCGCAACTTCTTCACG 3' P2R: 5' CTACTCCGCCTTTTTCAGA 3'	648	2.5	56
	Exon - 3	P3F: 5' GCTCACTTGCTTTTCTCTCT 3' P3R: 5' AAATTTCAGCTTGCCTCTTG 3'	653	2.5	60

Table 3 contd . . .

Gene	Location	Primers for PCR	Amplicon size (bp)	$MgCl_2$ (mM)	Annealing temp (^{0}C)
INTERLEUKIN	1α-Promoter (-889C>T)	IL-1α (-889F): 5'GCATGCCATCACACCTAGTT' 3' IL-1α (-889R): 5'TTACATATGAGCCTTCCATG3'	194	1.5	51.0
	1β - Promoter (-511C>T)	IL-1β (-511F): 5'TGGCATTGATCTGGTTCATC3' IL-1β (-511R): 5'GTTTAGGAATCTTCCCACTT 3'	304	1.5	56.5
	Exon - 5	IL-1β (+3953F): 5'GTTGTCATCAGACTTTGACC3' IL-1β (+3953R):5'TTCAGTTCATATGGACCAGA3'	249	1.5	56.5
P-21	Exon - 2	p-21 FP: 5'GTCAGAACCGGCTGGGGATG3' p-21 RP: 5' CTCCTCCCAACTCATCCCGG3'	272	1.5	55.0
MTHFR	Exon - 4	MTHFR FP: 5'TGAAGGAGAAGGTGTCTGCGGGA 3' MTHFR RP: 5'CCTCACCTGGATGGGAAAGATCC3'	146	1.5	55.0
MMP – 9	Promoter (-1590C>T)	MMP9-1590 FP5'GCCTGGCACATAGTAGGCCC3' MMP9-1590 RP5'CTTCCTAGCCAGCCGGCATC3'	436	1.5	58.0
	Exon - 6	MMP9-6 FP 5'CGCCTTCTCCCCCTTTCCCACA 3' MMP9-6 RP 5'AGAGGGCGGGGCTGAACCT 3'	250	1.0	56.5

* 10X DMSO was used in these reactions

The PCR reagents were mixed in a 0.2 ml tube and placed in the thermal cycler. The optimized PCR reagents used in the reaction are shown in Table 4. The following PCR protocol was followed:

1. Initial denaturation 94^o C - 5 min
2. Denaturation 94^o C - 30 sec
3. Annealing X ^{o} C - 30 sec (X- as shown in Table 3)
4. Extension 72^o C - 30 to 60 sec

   Number of cycles 30 - 35 (steps 2 to 4)
5. Final extension 72^o C - 5 min
6. Final hold 4^o C - 15 min

Table 4: Reagents used in a standard PCR method

S.No.	Reagents	Stock Concentration	Working Concentration	Total volume for one reaction (μl)
1	Taq buffer	10X	1X	2.5
2	DNTP	2000 μM	200 μM	2.5
3	Forward primer	5 pm/μl	5-10 pm	2.0
4	Reverse primer	5 pm/μl	5-10 pm	2.0
5	Taq polymerase	3 U/μl	0.5-1U	0.2
6	Genomic DNA	50 ng/μl	50 – 100 ng	1 - 2
7	MilliQ water	-	Adjusted the reaction volume to 25μl	-

3.2.4.2 Confirmation of the PCR amplification by agarose gel Electrophoresis

The amplified products were checked on a 1.5% agarose gel, and the protocol was as follows:

1. The gel casting platform (Bangalore Genei, Pvt Ltd, Bangalore, India) and the gel comb were set up in the desired configuration.
2. An appropriate quantity of agarose was weighed into a 500 ml glass flask and 1XTBE was added to it.
3. The flask was then swirled once and heated for 2-3 min in a microwave oven to melt the agarose by intermittent shaking.
4. The solution was then cooled under the running water and ethidium bromide (USB, Amersham Biosciences, New Jersey, USA) was added (final concentration 1 μg/ml).
5. The flask was swirled and the material poured into the gel mold to cast the gel.

6. When the gel solidified completely (~45 min), the comb was removed.
7. The cast gel was then placed in the electrophoresis tank with 1XTBE buffer and 3-4 μl of the sample was loaded into the wells after mixing with 0.8-1 μl of 6X loading dye containing bromophenol blue (USB, Amersham Biosciences, NJ, USA) and xylene cyanol (USB, Amersham Biosciences, NJ, USA) along with a 100 bp DNA ladder (MBI fermentas, Lithuania).
8. The gel was run at 100V for 30-40 min until the desired separation of the bands was obtained.
9. The gel was then visualized in the gel doc system (Uvitec Ltd., Cambridge, UK), under the UV light and photographed for documentation.

3.2.5 Mutation screening

Mutation screening was done either by SSCP or resequencing.

3.2.5.1 Single Strand Conformation Polymorphism (SSCP)

SSCP detects mismatches in the base sequence of the ssDNA sample due to difference in the conformation compared to the wild type sequence, detected as a mobility shift due to a different band pattern. The sensitivity of SSCP analysis has been shown to be about 85-95% if fragments are shorter than 150 bp and if optimal running conditions such as appropriate concentration of glycerol in the gel and standardized temperature (4° C or room temperature) and voltage are used. Samples showing aberrant patterns are further characterized by

sequencing. In the present study, two different running conditions were used for each pair of primers. SSCP procedure involved the following:

Gel Preparation

1. The glass plates were cleaned with isopropanol and assembled for the gel.
2. The following reagents were added for an 8% non-denaturing polyacrylamide gel and mixed thoroughly.

   Acrylamide: Bisacrylamide (29:1) - 20 ml (8%)

   10 X TBE stock - 2.5ml (0.5X)

   Glycerol (Qualigens Fine Chemicals, Mumbai, India) -2.5 ml (5%).

   The final volume was made upto 50μl with MilliQ water.

3. Finally, 300μl of 10% APS (Sigma Chemical Co. St. Louis, MO, USA), and 30 μl of TEMED (USB, Amersham Biosciences, NJ, USA) were added to the above mix at the end for polymerization, mixed well and poured into the set glass plates.

Sample Preparation

1. PCR product (3 μl) was taken into a 1.5 ml eppendorf tube and 6 μl of the formamide dye containing bromophenol blue and xylene cyanol were added to it and mixed by vortexing.
2. The sample was then denatured on a dry bath at 95° C for 5 min.
3. The samples were then snap-chilled by placing on ice mixed with salt.

Electrophoresis

1. The gel plates were transferred to the electrophoresis tank with buffer (0.5 X TBE).
2. Polyacrylamide gels were pre-run for 15 min to remove impurities such as unpolymerised acrylamide followed by loading of the samples. The gels were run at 70-75 Volts at room temperature or 110-120 Volts at 4° C overnight.

Silver staining of the gels

1. The gels were removed from the glass plates and put into the fixative solution containing 10% ethanol and 0.5% glacial acetic acid and kept on a shaker for 45 min to 1 h.
2. The gels were then washed twice for 5 min in MilliQ water and transferred into the silver nitrate solution (0.2%) for 10 min.
3. The gels were then removed and washed twice for 5 minutes with MilliQ water.
4. The gels were then transferred into the developer solution (1.5% NaOH and 0.4% formaldehyde) and placed on a shaker till the bands could be visualized.
5. The gel picture was documented and the samples showing mobility shift were further characterized by sequencing.

3.2.5.2 DNA sequencing

Bi-directional cycle sequencing was performed on an automated DNA sequencer ABI 310 (Applied Biosystems, Foster city, CA, USA) using

BigDye terminator chemistry (Version 3.1). The different steps involved in bi-directional sequencing are as follows,

I) Purification of the PCR amplicons

The PCR product was purified using PCR purification columns (MO BIO Laboratories, Carlsbad, CA, USA) to remove excess dNTPs, Taq polymerase and primers. The following steps were involved:

1. To the PCR product, 5 volumes of the SpinBind solution was added, mixed well by pipetting and transferred to the spin filter unit.
2. It was next centrifuged for 10-30 sec at a minimum of 13,000 rpm in a tabletop microcentrifuge.
3. The spin filter basket was removed and the liquid flow- through was discarded from the tube by decanting
4. The spin filter basket was replaced in the same tube and 300 μl of Spin Clean buffer was added to the spin filter
5. It was then centrifuged for 10-30 sec at a minimum of 13,000 rpm.
6. The spin filter basket was removed, the liquid flow- through discarded by decanting, and the basket was replaced in the same tube.
7. The centrifugation step repeated as before.
8. The spin filter was transferred to the clean collection tube, which is provided.
9. 50 μl of elution buffer (10 mM Tris) solution was added directly onto the center on the white spin filter membrane
10. The centrifugation step repeated.

11. The spin filter basket was removed and the DNA sample, free of contamination, was collected into a microcentrifuge tube
12. For concentrating the DNA solution, 2 μl of 5M NaCl and 100 μl of chilled ethanol was added, mixed well and centrifuged at 14,000 rpm. Ethanol was then discarded and the DNA pellet was dissolved in 10-13 μl of autoclaved Milli Q water.

II) Dye terminator cycle sequencing

The purified PCR product was checked for quality by running on an agarose gel with standards of known quality. The primer stocks were diluted to a final concentration of 3.2 pm/μl and the cycle sequencing reaction was set up using the conditions shown in Table 5.

Table 5. Conditions used for cycle sequencing

S.No.	Reagent	Concentration
1	Big Dye terminator (Version 3.1)	4.0 μl
2	Forward/Reverse primer	1.0 μl (3.2 pm/μl)
3	Template	2.0 μl (50 -100 ng)
4	Milli Q water	3.0 μl
5	Total reaction volume	10 μl

The following steps were then followed.

1. Initial Denaturation	-	96°C - 1 min
2. Denaturation	-	96°C - 10 sec
3. Annealing	-	50°C - 5 sec
4. Extension	-	60°C - 4 min
No of cycles	-	25 (from 2 to 4)
5. Final hold	-	4^{o} C for 15 min

III) Precipitation

1. A master mix was made as follows:

   3M sodium acetate (pH 4.6) - 3 μl

   (Qualigens Fine Chemicals, Mumbai, India)

   Ethanol - 250 μl

2. The volume of the PCR product after cycle sequencing reaction was made up to 100 μl with autoclaved MilliQ water, transferred to 1.5 ml centrifuge tubes and 253 μl of the above master mix was added to each tube.
3. Contents of the tube were mixed thoroughly and centrifuged immediately at a speed of 12,000 rpm for 20 min at room temperature.
4. The orientation of the tubes was marked as a precautionary measure for the subsequent steps.
5. The supernatant was decanted and to each of the tube 250 μl of 70% ethanol was added.

6. The tubes were spun at 14,000 rpm on a tabletop centrifuge for 10 min and the supernatant was decanted.
7. The 70% ethanol wash step was repeated without inverting the tubes this time.
8. The supernatant was aspirated and the pellet was air dried.
9. The pellet was resuspended in 10μl of HiDi formamide (Applied Biosystems, Foster city, CA, USA) and vortexed.
10. The sample was then transferred to 0.5 ml 310 autosampler tubes and capped with septa.
11. The tubes were tapped once and spun down gently.
12. The contents of the tubes were checked for air bubbles.

IV) Electrophoresis

The samples were then prepared, using the stepwise procedure as follows:

1. The injection syringe was rinsed with distilled water.
2. The POP 6 (Applied Biosystems, Foster city, CA, USA) polymer was brought to room temperature and a small amount (~0.6 ml) of the polymer was gently drawn into the syringe for priming.
3. Matrix files were created for all the four bases, by running the matrix standards, to aid the correction of spectral overlap.
4. The capillary was first connected to the pump block and then positioned in the vertical track of the detector, by opening laser detector door and was arranged such that the central portion of the capillary window would rest over the laser detector window. The end

of the capillary was adjusted so that it was positioned directly below the opening of the glass syringe.

5. The capillary and the cathode were adjusted in such a way that the capillary protrudes beyond the electrode by a maximum of 0.5 mm (The anode was always kept immersed in the electrophoresis buffer while the cathode, located above the autosampler was placed in the sample during eletrophoresis).
6. The injection syringe was then loaded with polymer (~ 0.5 ml) carefully without introducing any air bubbles.
7. Meanwhile the 10X Genetic analyzer EDTA buffer (Applied Biosystems, Foster city, CA, USA) was diluted to 1X concentration with deionised water to use in the run, the anode buffer reservoir was filled with the 1X Genetic analyzer EDTA buffer and was placed on the pump block.
8. The autosampler consists of two 4 ml vials and an eppendorf tube (1.5 ml) with the lid clipped off. One of the 4 ml vial was filled with 1X Genetic analyzer EDTA buffer for the run and the other 4 ml vial was filled with deionised water for the purpose of washing. The wastes produced during the run were discarded into the eppendorf.
9. The samples were denatured at 95° C for 5 min and subsequently snap- chilled on ice.
10. The sample sheet was prepared according to the order of the samples loaded in the autosampler and the injection list contained information about order for running samples, injection number and the module and running conditions for each injection.

11. The run module parameters, which includes run time, temperature and voltage, injection time and voltage were set.
12. The Autosampler calibration was done after removing the sample tray and the calibration dots in the front and back of the tray platform was aligned with respect to the capillary end and the settings were saved.
13. The samples were then placed in the autosampler tray with respect to the order of the samples in the sample sheet.
14. The test CCD Sensitivity module was run to ensure a stable baseline.
15. The polymer was filled into the capillary from the run module in the programme.
16. The sample reactions were run for 60 to 120 min depending on the size of the amplicon and the run was monitored periodically.

V) Sequence analysis

1. The raw data of the sequences were obtained through the data collection software (Version 3.0).
2. The Sequence Analysis software (Version 3.7) was used to analyze the raw data that was obtained in the form of electropherograms.
3. The sequences were compared to the wild type gene sequence obtained from GENBANK (http://www.ncbi.nlm.nih.gov/).
4. Sequence alterations were noted and compared with the known sequence variations from the human gene mutation database (http://www.hgmd.cf.ac. uk/ac/index.php), or by reviewing the literature.

5. The CLUSTAL W software analysis (http://www.ebi.ac.uk/clustalw/) was used for multiple sequence alignment to see the conservation of the variation across different species.
6. The SIFT (sorting intolerant from tolerant) software http://blocks.fhcrc.org/sift/SIFT.html) was used to predict the effect of the variation on the protein's function, based on homology search and the physical properties of amino acids.
7. SIFT analysis was used to find if the mutation was tolerated by the protein suggestive of pathogenicity.

3.2.5.3 Screening for variations by PCR based restriction digestion method

PCR based restriction digestion method was used to confirm nucleotide variations in patients and controls. Variations resulting in the loss or gain of restriction site of a particular restriction endonuclease were chosen from the WEB cutter software (Version 2.0) (http://rna.lundberg.gu.se/cutter2/).

Procedure:

1. The DNA fragment of interest was amplified using the primers flanking the variation.
2. A master mix for 15-20 μl of total volume was made and aliquoted into 0.5 ml microfuge tubes. The reagents shown in Table 6 were added.

Table 6. Reagents used for restriction digestion

S.No.	Reagents	Stock Concentration	Working Concentration	Total volume for one reaction (μl)
1	Compatible Buffer	10X	1X	2.5
2	Enzyme	10U/μl	2-3U/μl	0.2-0.3
3	Template (PCR Product)	-	50-100ng	2-3
4	MilliQ water	-	Adjusted the Reaction volume to 10μl	Made up to the reaction volume

3. To the master mix, 2-3 μl of PCR product was added into the respective tubes, vortexed briefly and incubated overnight.
4. The digested samples were then mixed by vortexing with 3.5 μl of bromophenol blue dye, spun and loaded on an 8% polyacrylamide gel along with a 100 bp DNA ladder.
5. The sample was subjected to electrophoresis at 25 mA current and run for 2–3 h based on the size of the fragment.
6. The gels were then stained with ethidium bromide and visualized in the gel doc system. The genotypes were recorded directly from the gel.

   The details of the restriction enzymes used and the reaction conditions are listed in Table 7.

3.3 VALIDATION:

The entire gene screening was repeated twice. The genotypes were further cross-validated by a masked observer randomly. Any discrepancies with respect to genotyping were resolved by repeating the experiments with fresh samples that were subjected to further independent validatio.

Table 7. List of restriction enzymes used in PCR-RFLP to screen the patients and controls for the mutations and SNPs within the candidate genes

S.No	Gene	Variation	Position (c.DNA / g.DNA)	dbSNP ID / AccessionNumber	Restriction enzyme	Temp (°C)	Product Size (bp)	Allele sizes (bp)		
								WT	Het	Mut
1	*MYOC*	R33K	c.98 G>A	-	*Mva* I (-)	37	334	131, 100, 66, 20,17	131, 120,100, 66,20,17	131,120,66, 20,17
2		Q48H	c.144 G>T	CM023962	*Acc* I (-)	37	334	271, 63	334, 271, 63	334
3		P56T	c.166 C>A	-	*Rsa*I (+)	37	334	334	334, 286, 48	286, 48
4		T353I	c.1058 C>T	CM990909	*TspR1* (-)	65	190	94, 76, 18	94, 76, 18	170, 18
5		Q368X	c.1102 C>T	CM971023	*Taa*I (-)	65	339	182,83, 44, 30	226,182,83, 44, 30	226, 83, 30
6		-1000 C>G	-	rs12035719	*Cai* I (-)	37	295	261, 34	295, 261, 34	295
7		-83 G>A	-	rs2075648	*Eco* 88I (+)	37	334	299, 35	334, 299, 35	334
8		R76K	c.227G>A	rs2234926	*Bsm*AI (+)	37	189	92,97	189, 92,97	189
9	*CYP1B1*	G61E	c.528G>A	rs28936700	*Taq* I (+)	65	786	627, 84, 75	627, 309, 318, 84, 75	309, 318, 84, 75
10		Q144R	c.777A>G	HM030008	*Msp* A1I (-)	37	786	175, 161, 160, 153, 71, 66	313, 175, 161, 153, 71, 66	313, 175, 161, 71, 66
11		P193L	c.924 C>T	CM023063	*Eco* 8II (+)	37	786	786	786, 682, 104	682, 104

Table 7 contd….

S.No	Gene	Variation	Position (c.DNA / g.DNA)	dbSNP ID / AccessionNumber	Restriction enzyme	Temp (°C)	Product Size (bp)	Allele sizes (bp)		
								WT	Het	Mut
12	*CYP1B1*	E229K	c.1031 G>A	CM014173	*Eam* 11041(-)	37	648	648	586, 348,	586, 62
13		R368H	c.1449 G>A	CM000137	*Taa* I (-)	65	653	402, 152, 99	402, 251, 152, 99	402, 251
14	*IL-1β*	-511 C>T	-	rs16944	*Eco81*I (+)	37	304	190, 114	304, 190, 114	304
15		F105F	c.315 C>T	rs1143634	*Taq* 1 (-)	65	249	135, 114	249, 135, 114	249
16	*IL-1α*	-889 C>T	-	rs1800587	*Nco*1 (-)	37	194	178, 16	194,178, 16	194
17	*p-21*	S31R	c.328 C>A	rs1801270	*Blp* I (-)	37	272	183, 89	272,183, 89	272
18	*MTHFR*	A222V	c.677 C>T	rs1801133	*Hinf* 1 (+)	37	146	146	146, 121, 25	121, 25
19	*MMP-9*	-1590 C>T	-	rs3918242	*Sph* I (+)	37	436	436	436, 242, 194	242, 194
20		Q279R	c.855 A>G	rs2664538	*Msp* 1 (+)	37	250	177, 73	177, 129, 73, 48	129, 73, 48

(+) indicates creation of site; (-) indicates abolition of site; bold-mutations; unbold – SNPs; WT-Wild type, Het-Heterozygous, Mut-Mutant.

The allele and gene frequencies were calculated by the gene counting method and the difference in the distribution of allele and genotype frequencies was assessed using Chi-square test. Odds ratios with 95% confidence intervals were determined for specific alleles and genotypes in the candidate gene frequencies of the single nucleotide polymorphisms in the control and patient groups were estimated.

Chapter 4
Results

4. RESULTS

The primary objective of this study was to highlight and understand the molecular genetic defects underlying primary glaucomas (POAG, JOAG, PACG) and PAC cases by screening candidate genes like *MYOC*, *CYP1B1, IL-1*, *MTHFR*, *p-21* and *MMP*-9 for mutations and / or association to the disease phenotype. Several novel and known mutations were observed in *MYOC* and *CYP1B1*. A total of 143 primary open angle glaucoma (POAG) cases that also included 34 JOAG cases, 113 primary angle closure glaucoma (PACG) cases and 100 normal controls were included in the study. PAC cases (n=17) were also randomly enrolled while recruiting PACG cases and were analyzed separately.

The mean ages at onset in POAG was 52.7 $\pm$ 10.2 years (range 36-77 years) and in PACG was 53 $\pm$ 11 years (range 14 to 79 years). JOAG cases had a mean age at onset of 21.4 $\pm$ 9.2 years (range 6 to 35 years) and the controls had a mean age of 58.2 $\pm$ 9.87 years.

The family history and gender distribution among these cases are shown in table 1. Majority of the PACG cases were sporadic (78%), while the proportion of familial and sporadic cases among the POAG and JOAG cases were similar. There was a male preponderance in the JOAG and POAG cases that was not apparent in PACG.

Table 1. Family history and gender distribution among POAG, JOAG and PACG cases

Parameter	POAG (n=109)	JOAG (n=34)	PACG (n=113)
Familial	45 (41%)	15 (44%)	25 (22%)
Sporadic	64 (59%)	19 (56%)	88 (78%)
Male	96 (88%)	25 (74%)	60 (53%)
Female	13 (12%)	9 (26%)	53 (47%)

4. 1 MUTATION SCREENING

The candidate genes, *MYOC* and *CYP1B1* were screened for mutations among POAG and PACG cases by resequencing.

4.1.1 Analysis of the *MYOC* gene

MYOC screening revealed four different mutations in 4/48 (8.3%) cases of POAG. Three of these four mutations have been reported earlier (Gln48His, Thr353Ile and Gln368Stop), whereas the Arg33Lys mutation was novel. One hundred and one PACG patients were analyzed for the *MYOC* gene, and two different *MYOC* mutations were observed in 6/101 (5.94%) cases. One of the reported mutation, Gln48His, was seen in 5/101 (5%) PACG and 2/17 (11.76%) PAC cases, while the Pro56Thr mutation was novel. The mutations were heterozygous and absent in 100 control subjects. Electrophoregrams of

all the mutations are shown in Figures 2, and Table 3 summarizes the clinical features of patients harboring these mutations. The detailed description of the mutations in POAG and PACG is as follows:

4.1.1.1 MYOC mutations in POAG and PACG cases

1. Arg33Lys (*c.98AGG>AAG)*:

A novel change from Guanine to Adenine at c.98AGG>AAG resulted in the replacement of Arginine by Lysine at codon 33 in exon-1 in a sporadic POAG case (P-125). This substitution resulted in the abolition of the *Mva*I restriction site (Fig. 3a) and the change was not observed in the controls. Multiple sequence alignment across the different species indicated that the change was in the conserved region (Fig. 4) but SIFT analysis showed that the change is tolerated by the protein (Table 2). This patient manifested the disease at the age of 42 years with an IOP of 24 mm Hg in both eyes, along with a cup to disc ratio of 0.9:1. The visual acuity recorded with glasses was 20/40 and 20/100 in the right and left eye, respectively, along with severe visual field loss at the time of presentation (Table 3). On surgical treatment the IOPs lowered to 7 and 12 mm Hg in the right and left eye, respectively.

2. Gln48His (*c.144CAG>CAT)*:

A heterozygous change from Guanine to Thymine at c.144CAG>CAT resulted in the replacement of Glutamine by Histidine at codon 48 (Gln48His) in a POAG case (P-158). A sibling of the proband also harbored the change (Fig. 1) but was not clinically diagnosed at our centre. According to the family history given by the proband, her sibling did not manifest any signs or symptoms of glaucoma. The change

resulted in the abolition of the *Acc*I restriction site (Fig. 3b) and was not observed in the controls. Multiple sequence alignment across 9 different species showed that the residue is highly conserved (88.8%) (Fig. 4). SIFT score predicted that this amino acid substitution affects the protein's function (Table. 2). The patient harbouring the change manifested the disease at the age of 50 years and had IOPs of 19 (OD) and 17 (OS) mm Hg at presentation along with a cup to disc ratio of 0.8:1 and visual acuity recorded with glasses was 20/25 and 20/20 in the right and left eye, respectively (Table 3). Both eyes had mild visual field defect. On treatment the IOPs reduced to 15 and 16 mm Hg in the right and left eye, respectively.

Gln48His was also observed in 5/101 (5%) PACG and 2/17 (11.76%) PAC cases. Table 3 shows the clinical features of all the PACG and PAC patients harboring this mutation. All the PACG patients were heterozygous for the change and showed variable clinical phenotype. This mutation was also observed in a sibling of one proband (PACG-3), who was not available for diagnosis at the time of presentation. However, according to the information given by the proband, her sibling did not manifest the disease symptoms during her inclusion in the study. Gln48His was earlier reported to be a pathogenic mutation in POAG and primary congenital glaucoma patients in Indian populations (Mukhopadhyay *et al.,* 2002; Sripriya *et al.,* 2004; Chakrabarti *et al.,* 2005)**.** The distribution of the Q48H mutation across different studies is shown in Table 4.

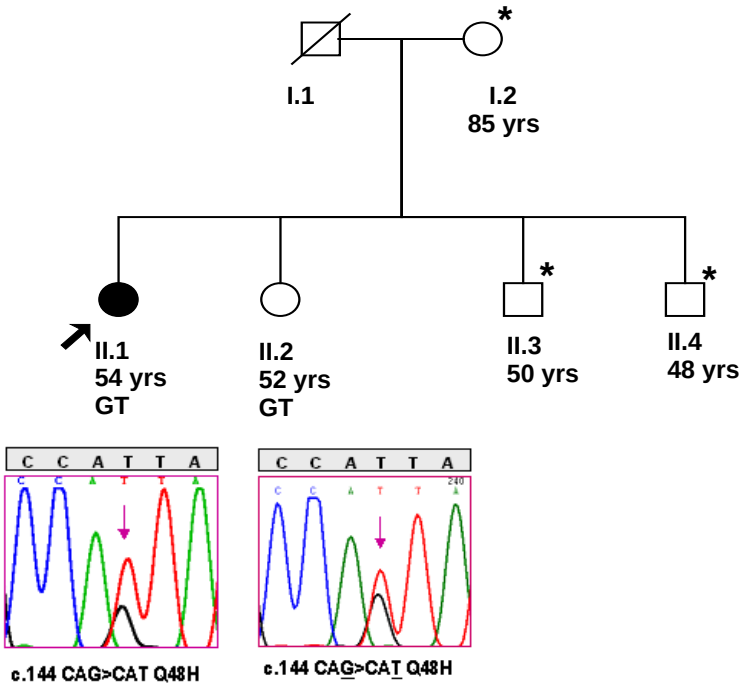


Figure 1. Segregation of Q48H (c.144G>T) in a POAG case (P-158). Darkened and clear symbols indicate the affected and normal individuals, respectively. The proband is indicated by an arrow. The age and genotypes of the individuals are indicated below the symbols. The individuals marked with asterisk (*) were not analysed, as their DNA was not available. The chromatograms of the Q48H mutation are shown below the individuals harboring the change. The wild type sequence is shown in the panel above the chromatogram.

3. Thr353Ile (*c.1058 ACA>ATA)*:

Another heterozygous mutation from Cytosine to Thymine at c.1058ACA>ATA resulted in the substitution of Threonine to Isoleucine in exon-3 in the patient (J-094) and this patient had an earlier manifestation (29 years) of the disease symptoms. The mutation was absent in the controls and conserved across 9 different species (Figure 4). SIFT scores indicated that the mutation affected the normal function of the protein (Table 2). The patient had severe IOPs of 50 and 42 mm Hg in the right and left eye, respectively, C:D ratio of 0.9:1 in both the eyes along with severe visual acuity in the right eye and

severe visual field defects. On surgical treatment IOPs reduced to 13 and 8 mm Hg in the right and left eye, respectively (Table 3).

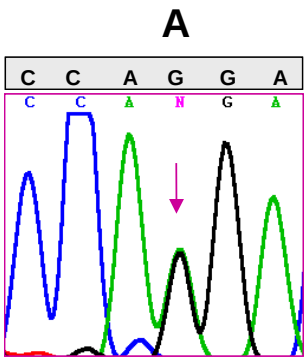
4. Gln368Stop (*c.1102 CAG>TAG)*:

A substitution of Cytosine to Thymine at c.1102CAG>TAG, resulted in the termination mutation of Glutamine residue at codon 368 in a POAG patient (P-134). Gln368Stop is a predominant *MYOC* mutation observed in different populations worldwide (Fingert *et al.,* 1999 Wiggs *et al.,* 1998; Vazque *et al.,* 2000; Jansson *et al.,* 2003; Mataftsi *et al.,* 2001; Faucher *et al.,* 2002; Vincent *et al.,* 2002; Michels-Rautenstrauss *et al.,* 2002). The mutation resulted in the abolition of *Taa*I restriction site (Fig. 3d), was absent in the controls and multiple sequence alignment revealed conservation of the residue across different species (Fig. 4). The patient harboring the change manifested the disease at 52 years and had IOPs of 23 and 22 mm Hg in the right and left eye, respectively, C: D ratio of 0.9:1 with visual acuity of 20/20 and mild visual field defects in both the eyes (Table 3). On medical treatment the IOP reduced to 14 mm Hg in both the eyes.

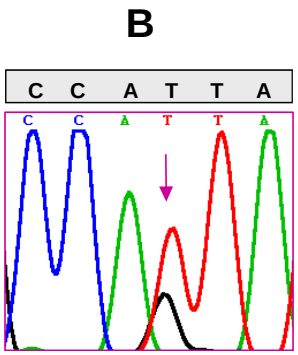
5. Pro56Thr (*c.166 CCC>ACC)*:

A novel change from Cytosine to Adenine at c.166CCC>ACC resulting in the replacement of Proline with Threonine at codon 56 was observed in a PACG patient (A-044). This variation resulted in the creation of *Rsa*I restriction site (Fig. 3c) and was absent in the control subjects. Multiple sequence alignment showed that the residue lay in the conserved region of the gene (Fig. 4). SIFT scores indicated that the mutation affected the protein's function (Table 2). The proband

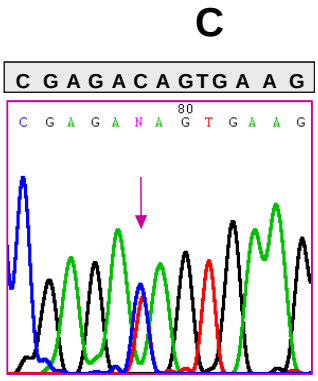
manifested the disease at 55 years of age and had IOPs of 41 and 22 mm Hg along with a C:D ratios of 0.9:1 and 0.8:1 in the right and left eye, respectively. The visual acuity was 20/2400 in the right eye and a 20/50 in the left eye. Visual field defects were severe in the right eye and on surgical treatment the IOP reduced to 14 mm Hg in both eyes.



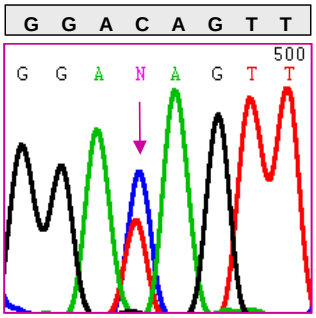
***c.98 AGG>AAG* : Arg33Lys**



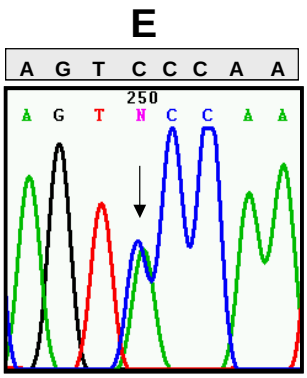
***c. 144 CAG>CAT* : Gln48His**



***c.1058 ACA>ATA* : Thr353Ile**



c.1102 CAG>TAG : Gln368Stop



***c.166 CCC>ACC* : Pro56Thr**

Figure 2. Electropherograms representing the *MYOC* mutations. The wild type sequence is shown in the box above the chromatogram and the arrow point indicates the heterozygous peak of the respective mutation.

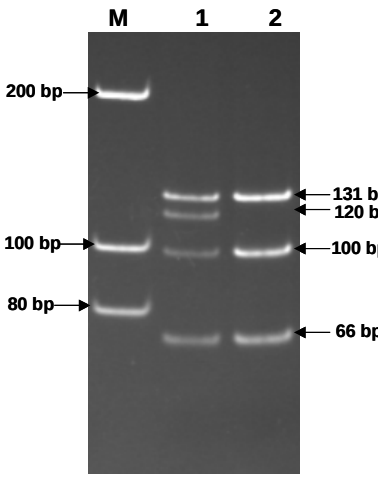
(A) A heterozygous substitution of AGG>AAG at c.98 resulting in the Arg33Lys mutation.

(B) A heterozygous substitution of CAG>CAT at c.144 resulting in the Gln48His mutation

(C) A heterozygous substitution of ACA>ATA at c.1058 resulting in the Thr373Ile mutation.

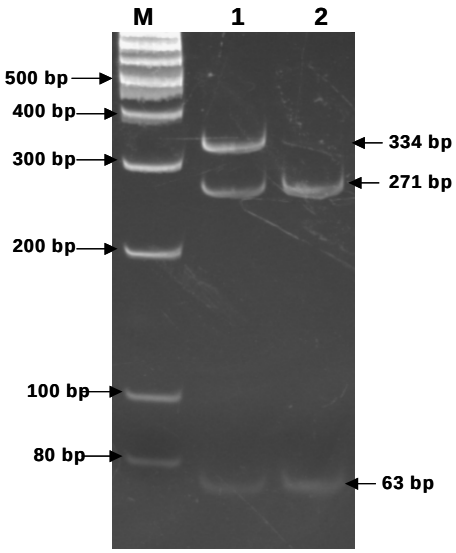
(D) A heterozygous substitution of CAG>TAG at c.1102 resulting in Gln368Stop mutation

(E) A heterozygous substitution of CCC>ACC at c.166 resulting in the Pro56Thr mutation



a) Restriction Digestion for R33K mutation using *Mva*I enzyme

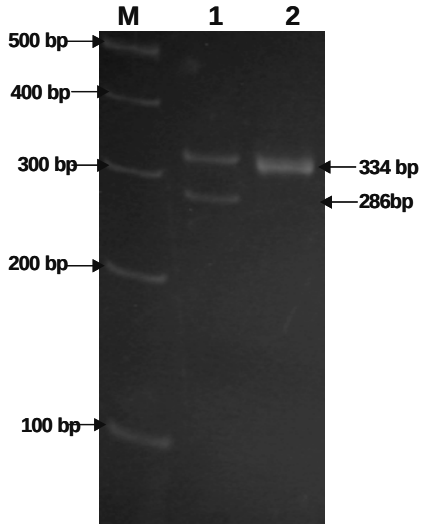
The R33K mutation (AGG>AAG) abolishes a restriction site for *MvaI*, thereby generating six fragments of 131, 120, 100, 66, 20 and 17 base pairs in the PCR amplicon (334 bp) in a patient heterozygous for the mutation (GA) as shown in lane 1. The amplicon on digestion is cleaved to five fragments (131, 100, 66, 20 and 17 base pairs) in a normal individual with the wild type alleles (GG; lane 2). The lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania). The 20 and 17 bp fragments are not shown in the gel.



b) Restriction Digestion for Q48H mutation using *Acc*I enzyme

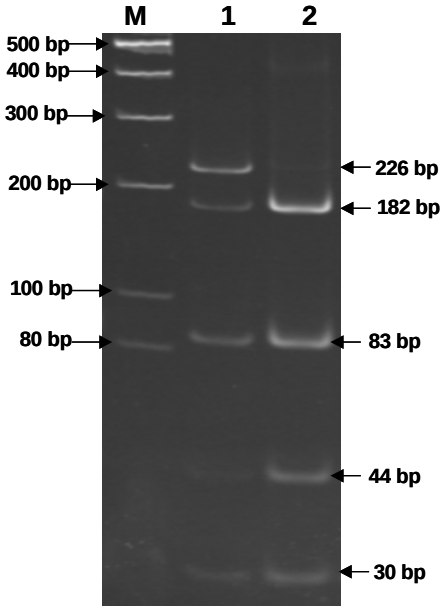
The Q48H mutation (CAG>CAT) abolishes a restriction site for *AccI*, thereby generating three fragments of 334, 271 and 63 base pairs in the PCR amplicon (334bp) in a patient heterozygous for the mutation (GT) as shown in lane 1. The amplicon on digestion is cleaved to two fragments (271 and 63 bp) in a normal individual with the wild type alleles (GG; lane 2). The lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

Figure 3. PCR based restriction digestion analysis of *MYOC* mutations on 8% PAGE gels in POAG and PACG cases



c) Restriction Digestion for P56T mutation using *RsaI* enzyme

The P56T mutation (CCC>ACC) creates a restriction site for *RsaI*, thereby generating three fragments of 334, 286 and 48 base pairs in the PCR amplicon (334 bp) in a patient heterozygous for the mutation (CA) as shown in lane1. The amplicon remains intact in a normal individual with the wild type alleles (CC; lane 2). The lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania). The 48 bp fragment is not shown in the gel.



d) Restriction Digestion for Q368X mutation using *TaaI* enzyme

The Q368X mutation (CAG>TAG) abolishes a restriction site for *TaaI*, thereby generating six fragments of 226, 182, 83, 44 and 30 base pairs in the PCR amplicon (339 bp) in a patient heterozygous for the mutation (CT) as shown in lane 1. The amplicon on digestion is cleaved to four fragments (182, 83, 44 and 30 base pairs) in a normal individual with the wild type alleles (CC; lane 2). The lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

Figure 3 contd ..

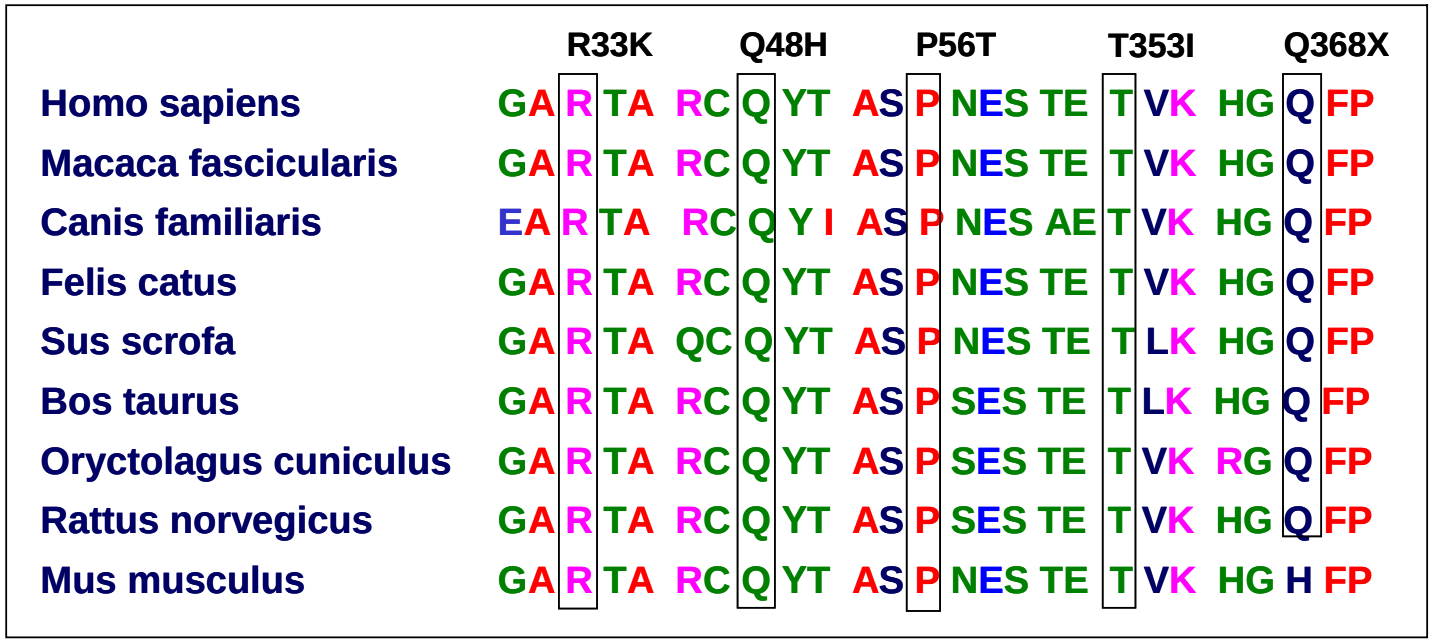


Figure 4. Multiple sequence alignment of the Myocilin protein across nine different species. The wild type residue is boxed across the species

Table 2. Distribution of *MYOC* mutations in POAG, JOAG, PACG and PAC cases

Sl. No.	*MYOC* mutations	Exon	cDNA position	POAG (n = 38)	JOAG (n = 10)	All POAG$^{\Psi}$ (n=48+88)	PACG (n = 101)	PAC (n = 17)	SIFT Score	Effect on the protein's function
1	Arg33Lys $^{+}$	I	c.98 G>A	1	-	1	-	-	0.4	Tolerated
2	Gln48His	I	c.144 G>T	1	-	2	5	2	0.01	Affect Protein Function
3	Pro56Thr $^{+}$	I	c.166 C>A	-	-	-	1	-	0.01	Affect Protein Function
4	Thr353Ile	III	c.1058C>T	-	1	1	-	-	0.0	Affect Protein Function
5	Gln368Stop	III	c.1102 C>T	1	-	1	-	-	-	-
6	Pro370Leu	III	c.1109C>T	-	-	2	-	-	0.06	Tolerated
7	Asp395Asn	III	c.1183G>A	-	-	1	-	-	1.0	Tolerated
8	Tyr479His	III	c.1457T>C	-	-	1	-	-	0.0	Affect Protein Function
	Total			3 (7.8%)	1 (10%)	9 (6.61%)	6 (5.9%)	2 (11.76%)		

$^{\Psi}$ Overall frequency of *MYOC* mutations, includes the data from the previous study (n = 88) from our center.
$^{+}$ Novel mutations

Table 3. Clinical features of POAG, JOAG, PACG and PAC patients with *MYOC* mutations at presentation

S. No.	PATIENT ID	PHENOTYPE	AGE AT ONSET	MUTATION	IOP[+] (OD; OS)	CD RATIOS[++] OD;OS	VISUAL ACUITY[ξ] (OD; OS)	VISUAL FIELDS[&] (OD; OS)	TREATMENT MODALITY[Θ]
1	J - 094	JOAG	29	T353I	50; 42	0.9 ; 0.9	20/400; 20/25	3; 3	Surgical Trab (OU)
2	P -125	POAG	42	R33K	24; 24	0.9 ; 0.9	20/40; 20/100[#]	3; 3	Surgical Trab (OU)
3	P -158	POAG	50	Q48H	19; 17	0.8 ; 0.8	20/25; 20/20[#]	1; 1	Medication
4	P - 134	POAG	52	Q368X	23; 22	0.9 ; 0.9	20/20; 20/20	1; 1	Medication
5	A - 060	PAC	52	Q48H	22; 16	0.8 ; 0.9	20/160; 20/1200	3; 3	Surgical YPI (OU)[ξ]
6	A - 113	PAC	50	Q48H	20; 22	0.6 ; 0.6	20/25; 20/25	1; 1	Surgical YPI (OU)
7	A - 003	PACG	60	Q48H	28; 18	0.7 ; 0.9	20/20; 20/400	1; 3	Surgical YPI (OU)
8	A - 014	PACG	50	Q48H	17; 18	0.3 ; 0.8	20/25; 20/20	1; 2	Surgical YPI (OU)
9	A - 058	PACG	58	Q48H	23; 20	0.9 ; 0.9	20/1200; 20/80	3; 3	Surgical YPI (OU)
10	A - 064	PACG	50	Q48H	20; 42	0.9 ; 0.9	20/25; AE	3; 3	Surgical (TSCPC, YPI
11	A - 129	PACG	69	Q48H	29; 27	0.9 ; 0.9	20/200; 20/25	2; 2	Surgical YPI (OU)
12	A - 044	PACG	55	P56T	41; 22	0.9 ; 0.8	20/2400; 20/50	3; 1	Surgical YPI (OU)

[+] Intraocular pressure (IOP), OU = both eyes, OD = right eye, OS = left eye; [++]Cup to disc (CD) ratios; [ξ]Visual acuity at presentation; [&]Visual field defects : 1 = Early defect, 2 = Moderate, 3 = Severe; AE - Absolute eye; [Θ]Treatment modality : YPI = Yag peripheral iridectomy, TSCPC=Transscleral cyclo photocoagulation, Trab = Trabeculectomy

Table 4. Distribution of Gln48His in Indian populations

S. NO	GEOGRAPHIC LOCATION	POAG	PCG	PAC	PACG	CONTROLS	REFERENCE
1	East India	3/56 (5.3%)	-	-	-	0/51	Mukhopadhyay *et al.,* (2002)
2	South and North India	2/100 (2%)	-	-	-	0/50	Sripriya *et al.,* (2004)
3	South India	-	4/72 (5.5%)	-	-	0/100	Kaur *et al.,* (2005)
4	South, East India	4/200 (2%)	5/200 (2.5%)*	-	-	0/300 *	Chakrabarti *et al.,* (2005)
5	South India	1/24 (4.1%)	-	-	-	0/100	Ramprasad *et al.,* (2005)
6	South India	1/48 (2.1%)	-	2/17 (11.7%)	5/113 (4.42%)	0/100	**Present study**

* Includes the data of Kaur *et al.,* (2005)

4.2 MYOC screening for Single Nucleotide Polymorphisms

Non-synonymous and synonymous codon changes, earlier reported as polymorphisms, were found in the promoter region, exon I and III of *MYOC*. The promoter region showed two different polymorphisms, -1000C>G (mt.1) and -83G>A. These polymorphisms were screened by PCR based restriction digestion. The *MYOC* mt.1 and the –83 G>A polymorphism resulted in the abolitions of restriction sites for *Cai*I (Fig. 4a) and *Eco88I* (Fig. 4b), respectively. Another coding region SNP (R76K) was screened by direct sequencing.

The allele and genotype frequency distributions of the polymorphisms at -1000C>G, –83G>A and R76K among POAG and PACG cases are shown in Tables 5, and 6. There were no statistically significant differences in the allele and genotype frequencies of these SNPs among the cases and controls (p>0.05). The -83G>A and R76K SNPs, were in linkage disequilibrium similar to most studies from Chinese, Japanese, Indian and Philippine populations (Lam *et al.,* 2000; Mabuchi *et al.,* 2001; Ray *et al.,* 2003; Wang *et al.,* 2004).

In addition, four different silent changes were observed in 6 probands with PACG and a proband with POAG (Table 7). Three of these synonymous codon changes were reported earlier (Fingert *et al.,* 1999); one of the silent changes (Gly375Gly) in a PACG case was novel.

Table 5. Allele frequencies of *MYOC* polymorphisms in POAG and PACG cases

Alleles / Subjects	MYOC mt.1 (rs12035719)			-83 G>A (rs2075648)			R76K (rs2234926)		
	C (n)	G (n)	p-value	C (n)	G (n)	p-value	G (n)	A (n)	p-value
POAG	85	11	0.2024	52	44	0.089	52	44	0.089
Controls	184	20		119	91		119	91	
PACG	211	25	0.817	143	93	-	143	93	-
Controls	184	20		119	91		119	91	

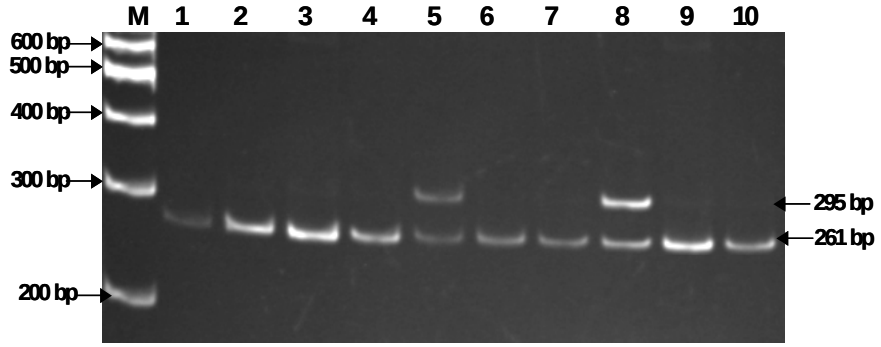


Figure 4a. Restriction Digestion for *MYOC* –1000 C>G polymorphism using *Cai*I enzyme: The –1000 C>G polymorphism abolishes a restriction site for *Cai*I, thereby generating an intact fragment of 295 base pairs in the PCR amplicon of the subject homozygous (GG) for the SNP The amplicon on digestion is cleaved to three fragments (295, 261 and 34 base pairs) in heterozygous (CG) subject (lanes 5 and 8) and into two fragments (261 and 34 base pairs) in the subject with the wild type (CC) alleles (lanes 1, 2, 3, 4, 6, 7, 9, 10). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

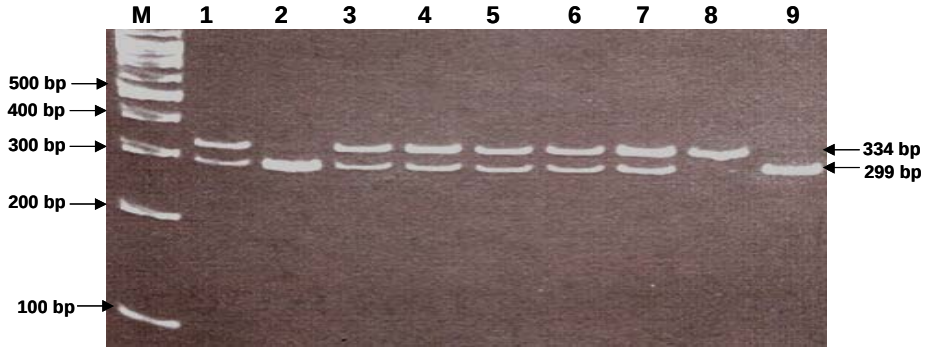


Figure 4b. Restriction Digestion for *MYOC* –83 G>A polymorphism using *Eco88I* enzyme:

The –83 G>A polymorphism abolishes a restriction site for *Eco*881, thereby generating an intact fragment of 334 base pairs in the PCR amplicon of the subject homozygous (AA) for the SNP (lane 8). The amplicon on digestion is cleaved to three fragments (334, 299 and 35 base pairs) in heterozygous (GA) subject (lanes 1, 3, 4, 5, 6, 7) and into two fragments (334 and 299 base pairs) in the subject with the wild type (GG) alleles (lanes 2 and 9). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

Table 6. Distribution of genotype frequencies of *MYOC* polymorphisms in POAG and PACG cases

SNP	dbSNP ID	Gene Position*	Location	Genotype	POAG (n=48) (%)	PACG (n=118) (%)	Control (n=105) (%)	Odds Ratio (95% CI) (POAG Vs Controls)	p value	Odds Ratio (95% CI) (PACG Vs Controls)	p value
MYOC mt.1	rs12035719	g.169,889,372	Promoter	CC	37 (77.1)	93 (78.8%)	83 (81.3)	1	-	1	-
				CG	11 (22.4)	25 (21.1%)	18 (17.6)	1.37 (0.58-3.2)	0.463	1.23 (0.63-2.4)	0.53
				GG	0	0	1 (0.98)	-	-	-	-
-83 G>A	rs2075648	g.169,888,457	Promoter	GG	16 (33.3)	42 (35.6%)	38 (36.1)	1	-	1	-
				GA	20 (41.6)	59 (50%)	43 (40.9)	1.1 (0.5-2.42)	0.805	1.24 (0.68-2.2)	0.47
				AA	12 (25.0)	17 (14.4%)	24 (22.8)	1.2 (0.47-2.9)	0.71	0.64 (0.2-1.4)	0.25
R76K	rs2234926	g.169,888,148	Exon-1	GG	16 (33.3)	42 (35.6%)	38 (36.1)	1	-	1	-
				GA	20 (41.6)	59 (50%)	43 (40.9)	1.1 (0.5-2.42)	0.805	1.24 (0.68-2.2)	0.47
				AA	12 (25.0)	17 (35.6%)	24 (22.8)	1.2 (0.47-2.9)	0.71	0.64 (0.2-1.4)	0.25

*Gene Bank ID - BC029261

Table 7. Synonymous codon changes in *MYOC* among the POAG and PACG cases

S. No.	*MYOC* variation	Location	c. DNA position	POAG (n=48)	PACG (n=106)	CONTROL (n=100)
1	Gly122Gly	Exon-I	c.366 C>T	-	1	-
2	Tyr347Tyr	Exon-III	c.1041 T>C	1	3	1
3	Thr351Thr	Exon-III	c.1053 C>T	-	1	-
4	Gly375Gly *	Exon-III	c.1183 C>A	-	1	-

Gen Bank ID: BC029261

* Novel

4.3 Mutational analysis of *CYP1B1* in POAG cases

The entire coding region of *CYP1B1* was screened for mutations by resequencing among 48 POAG, 90 PACG and 15 PAC cases. Among the POAG cases four different mutations in 6 cases (12.5%) were observed and all these mutations were missense (Gly61Glu, Pro193Leu, Ser239Arg and Arg368His) (Table 9). Three different missense mutations (Gln144Arg, Glu229Lys, Arg368His) were observed in 11 PACG cases (12.2%), in heterozygous condition. PAC cases did not show any mutations in *CYP1B1* gene. Electropherograms of all these mutations are shown in the Fig. 8. The description of the mutations found in POAG and PACG is given below and a summary of the clinical features of the patients harboring these mutations is shown in Table 10.

4.3.1. CYP1B1 mutations detected in POAG and PACG cases

1. Gly61Glu *(c.528 GGA>GAA):*

A homozygous transition from Guanine to Adenine at c.528 GGA>GAA resulted in the replacement of Glycine by Glutamic acid at codon 61 in a familial JOAG case (J-162) with a history of consanguinity. The homozygous mutation was also detected in a JOAG affected sibling of the proband (Fig. 5). The residue was highly conserved (Fig. 9) and SIFT scores indicated that the mutation affected the protein's function. This mutation resulted in the creation of a restriction site for *Taq*I (Fig. 6). The patient with the mutation had an onset at 23 years. The IOPs

recorded at the time of presentation were 46 and 18 mm Hg along with C:D ratios of 0.9:1 and 0.6:1 and visual acuity of 20/1200 and 20/20 in the right and left eyes, respectively. The manifestation of the disease was asymmetric with the right eye being severely affected than the left eye and gradually there was a complete loss of vision in the right eye. The patient was on medication for the other eye.

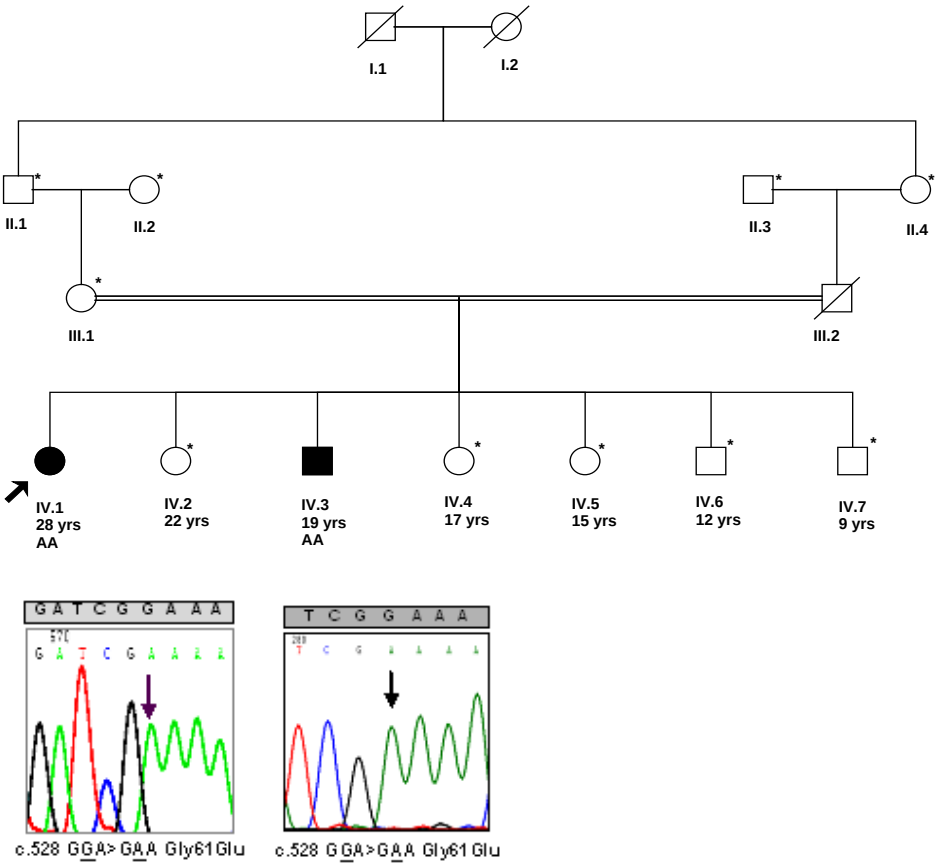


Figure 5. Segregation of the Gly61Glu mutation (c.528GGA>GAA) in a JOAG family (J-162). Blackened symbol indicates JOAG cases while the clear symbol denotes the unaffected status. Slash marks through the symbols denote deceased individuals; asterix indicates the family members whose DNA samples were unavailable for analysis. The genotype for the mutation and the age at presentation of both the proband and the affected sibling, are indicated below the pedigree. The electropherograms show the homozygous substitution at c.528G>A resulting in the Gly61Glu mutation in the proband and one of the affected sibling.

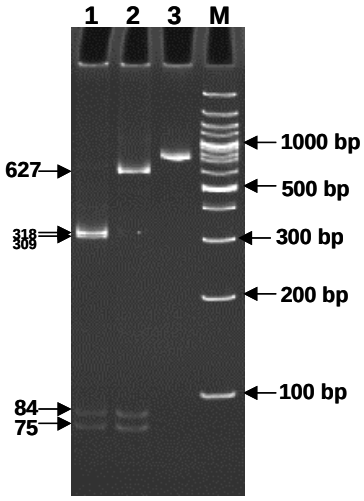


Figure 6. Restriction Digestion for G61E mutation using *Taq*I enzyme :
The G61E mutation (GGA>GAA) abolishes a restriction site for *Taq*I, thereby generating four fragments of 318, 309, 84 and 75 base pairs in the PCR amplicon (627 bp) in a patient homozygous for the mutation (AA) as shown in lane 1. The amplicon on digestion is cleaved to three fragments (627, 84 and 75 base pairs) in a normal individual with the wild type alleles (GG; lane 2). Lane 3 shows the uncut sample and lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

2. Pro193Leu (*c.924CCG>CTG):*

A change from Cytosine to Thymine at c.924CCG>CTG resulted in the replacement of Proline by Leucine at codon 193 in three POAG patients (P-076, P-089, P-128). Two of these POAG patients exhibited the mutation in heterozygous condition while one of the proband (P-076) was homozygous for the change and manifested the disease at the age of 45 years. Multiple sequence alignment showed that the residue was conserved across the different species (Fig. 9). But, SIFT analysis indicated that the amino acid change is tolerated by the protein (Table 8). The IOPs of the patient (P-076) harboring both mutant alleles were 28 and 18 mm Hg in the right and left eye, respectively, cup to disc ratios were 0.9:1 in both the eyes and visual acuity recorded with glasses was 20/2400 in the right eye and 20/30 in the left eye. Mild visual field defects were observed at the time of presentation. The patient had undergone trabeculectomy in both the eyes and had reduced IOPs of 19 and 12 mm Hg in the right and left eyes, respectively. The other two patients who exhibited the mutation in heterozygous state had relatively less severe phenotype with respect to the presenting IOP and visual acuity (Table 9).

3. Ser239Arg *(c.1061 AGC>GGC):*

A heterozygous change from Adenine to Guanine at c.239AGC>GGC resulted in the replacement of Serine with Arginine in a proband with POAG (P-123). Multiple sequence alignment of CYP proteins across different species showed that the residue was not conserved (Fig. 9) but, SIFT scores indicated that the mutation affected the protein's function (Table 8). The proband, aged 38 years at the time of presentation showed asymmetric manifestation of the disease symptoms, with IOPs of 25 and 16 mm Hg along with a C:D ratio of 0.9:1 (OD) and 0.8:1 (OS), and visual acuity of 20/200 and 20/20 in the right and left eye, respectively. Visual field defects were severe in the right eye (Table 10). On surgical treatment, the IOP came down to 10 mm Hg, and the visual acuity improved to 20/25 in the right eye.

4.Arg368His (*c.1449CGT>CAT):*

A heterozygous change resulted in the substitution from Guanine to Adenine, at c.1449CGT>CAT, replacing the Arginine residue at codon 368 by Histidine in a POAG proband (P-134). The residue was highly conserved (Fig. 9) and SIFT analysis predicted an affect on the protein's function (Table 8). The mutation resulted in the abolition of restriction site for *Taa*I (Fig. 7). The patient harboring the change manifested the disease at 52 years and had IOPs of 23 mm Hg and 22 mm Hg in the right and left eyes, respectively, C: D ratio of 0.9:1, visual acuity of 20/20 and mild visual field defects in both the eyes (Table 9). This patient was also heterozygous for a *MYOC* mutation (Gln368Stop)

thus inheriting two mutant alleles. This case is described with additional details in section 4.5 of this chapter.

The R368H mutation was also observed in five PACG patients (PACG-055, PACG-059, PACG-081, PACG-095, PACG-115). The variable degrees of clinical features exhibited by these patients are shown in Table 9. All these cases were sporadic and manifested the mutation in the heterozygous condition.

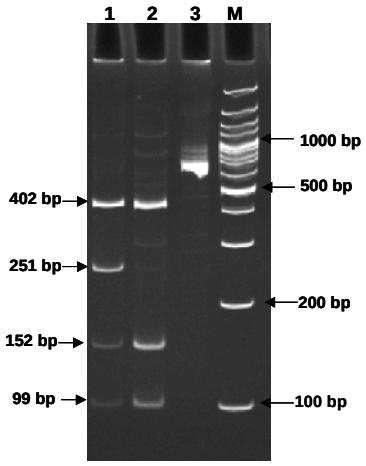


Figure 7. Restriction Digestion for R368H mutation using *Taa*I enzyme

The R368H mutation (CGT>CAT) abolishes a restriction site for *Taa*I, thereby generating four fragments of 402, 251, 152 and 99 base pairs in the PCR amplicon (653 bp) in a patient heterozygous for the mutation (GA) as shown in lane 1. The amplicon on digestion is cleaved to three fragments (402, 152 and 99 base pairs) in a normal individual with the wild type alleles (GG; lane 2). The lane 3 shows the uncut fragment and lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

5) *Gln144Arg (c.777CAG>CGG):*

A heterozygous change from Adenine to Guanine at c.777CAG>CGG resulted in the replacement of Glutamine with Arginine at codon 144 in a PACG proband (A - 017). The mutation was absent in the controls. Multiple sequence alignment showed a relatively lower (15.8%) conservation of the residue across the CYP-450 proteins in different species (Fig. 9) and SIFT analysis indicated an affect on the protein's function (Table 8). The patient manifested the disease at the age of 61 years and exhibited IOPs of 33 and 15 mm Hg in the right and left eye respectively, C: D ratio documented post YAG PI was 0.8:1 in both the

eyes and visual acuity was 20/50 and 20/200 in the right and left eyes, with advanced visual field defect documented in the right eye (Table 9). On surgical treatment the IOP reduced to 17 mm Hg in the right eye.

6) *Glu229Lys (c.1031GAA>AAA):*

A heterozygous change from Guanine to Adenine at c.1031GAA>AAA resulted in the replacement of Glutamic acid with Lysine at codon 229 in 5 sporadic PACG cases (PACG-058, PACG-061, PACG-066, PACG-079, PACG-137). Multiple sequence alignment showed that the residue was relatively less conserved across different species (Fig. 9) and SIFT analysis, indicated that the mutation was tolerated by the protein (Table 8). In the present study, this mutation was present in patients with variable degrees of severity (Table 9). One of this patients (A-058) was also heterozygous for the *MYOC* mutation Gln48His. This case is described with additional details in section 4.5 of this chapter.

The patients (PACG and POAG) with *MYOC* and *CYP1B1* gene mutations exhibited heterogeneity in the clinical features. The PACG patients with mutations exhibited a mean age at onset of 56.7 ± 10.9 (SD) years while the adult-onset POAG patients had a mean age of onset of of 50.45 ± 11.08 (SD). There was no statistically significant difference among these two groups (p=0.09). The mean IOPs at presentation among the PACG, POAG and JOAG cases with mutations were 31.7 ± 14.1 (SD), 32.48 ± 9.57(SD), 36.27 ± 9.166 (SD), respectively. There was no significant difference when IOPs were compared among the POAG and PACG cases (p=0.833), and among

JOAG and PACG patients (p=0.3441) with mutations. The phenotypic manifestation of the individuals with mutations in both *MYOC* and *CYP1B1* was heterogeneous as shown in Tables 3 and 9. The frequency of *MYOC* mutations was 8.3% in POAG, 5.94% in PACG and 11.7% in PAC cases. The Arg33Lys and Pro56Thr mutations were the novel mutations observed in a case of POAG and PACG. The Q48H mutation, which is the most prevalent mutation in POAG in Indian populations (Chakrabarti *et al.,* 2005) was also predominant in PACG, occurring with a frequency of 5%. The mutation frequency was similar to earlier studies from India (Kumar *et al.,* 2007; Mukhopadhyay *et al.,* 2002; Kanagavalli *et al.,* 2003; Sripriya *et al.,* 2004). The promoter (-1000C>G, -83G>A) and coding region (R76K) polymorphisms and the synonymous codon changes (Gly122Gly, Tyr347Tyr, Thr351Thr) did not exhibit any association to POAG and PACG.

The frequency of *CYP1B1* mutations was 12.5% in POAG and 12.2% in PACG. PAC cases did not harbor any *CYP1B1* mutations. The R368H mutation, which was reported as the most prevalent mutation in PCG (Reddy *et al.,* 2003) and more recently in POAG (5.76%) and JOAG (3.3%) from our centre (Chakrabarti *et al.,* 2007) was also the prevalent mutation among the PACG cases (5.5%) along with E229K (5.5%).

Two sporadic cases with POAG and PACG were found to harbor heterozygous mutant alleles of both *MYOC* and *CYP1B1*. The POAG case exhibited the Q368X (*MYOC*) and R368H (*CYP1B1*) while the PACG patient had Q48H (*MYOC*) and E229K (*CYP1B1*) mutation.

Unlike an earlier report (Vincent *et al.,* 2002) the phenotypes of these patients did not manifest severity with respect to intraocular pressure and C:D ratio (Tables 3 and 9). Both the patients had the disease manifested in the fifth decade of their life.

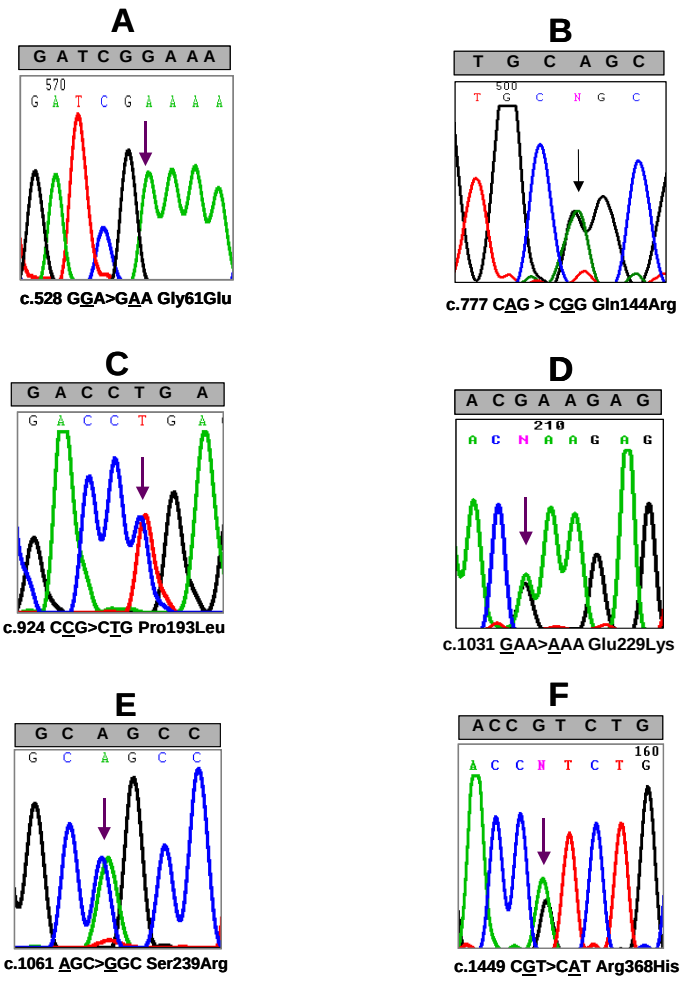


Figure 8. Electropherograms of *CYP1B1* mutations: The wild type sequence is shown in the box above the chromatogram and the arrow point indicates the heterozygous or homozygous peak of the respective mutation.

(A) A homozygous substitution of GGA>GAA at c.528 resulting in the Gly61Glu mutation

(B) A heterozygous substitution of CAG>CGG at c.777 resulting in the Gln144Arg mutation

(C) A heterozygous substitution of CCG>CTG at c.924 resulting in the Pro193Leu mutation

(D) A heterozygous substitution of GAA>AAA at c.1031 resulting in the Glu229Lys mutation

(E) A heterozygous substitution of AGC>GGC at c.1061 resulting in the Ser239Arg mutation

(F) A heterozygous substitution of CGT>CAT at c.1449 resulting in the Arg368His mutation

Table 8. Distribution of *CYP1B1* mutations in POAG and PACG cases

Sl. No.	*CYP1B1* Mutations	Exon	c.DNA position	Open Angle Glaucoma		All POAG[+] (n = 48+86)	PACG (n = 90)	SIFT Score	Effect on the protein Function
				POAG (n = 38)	JOAG (n = 10)				
1	Gly61Glu	II	c.528 G>A	-	1	2	-	0.0	Affect Protein Function
2	Tyr81Asn	II	c.587 T>A	-	-	1	-	0.0	Affect Protein Function
3	Gln144Arg	II	c.777 A>G	-	-	3	1	0.01	Affect Protein Function
4	Pro193Leu	II	c.924 C>T	3	-	3	-	1.00	Tolerated
5	Glu229Lys	II	c.1031 G>A	-	-	5	5	0.10	Tolerated
6	Ser239Arg	II	c.1061 A>G	1	-	1	-	0.0	Affect Protein Function
7	Arg368His	III	c.1449 G>A	1	-	7	5	0.0	Affect Protein Function
8	Trp434Arg	III	c.1646 T>C	-	-	1	-	0.0	Affect Protein Function
9	Pro437Leu	III	c.1656 C>T	-	-	1	-	0.0	Affect Protein Function
10	del 5bp	III	c.1657 del 5bp	-	-	1	-	-	-
11	Phe445Cys	III	c.1680 T>G	-	-	1	-	0.0	Affect Protein Function
	Total frequency			5 (10.4%)	1 (10%)	25 (18.65 %)	11 (10.4%)		

[·] Gen bank ID - U56438

[+] Overall frequency of *CYP1B1* mutations, includes the data from the previous study (n = 88) from our center.

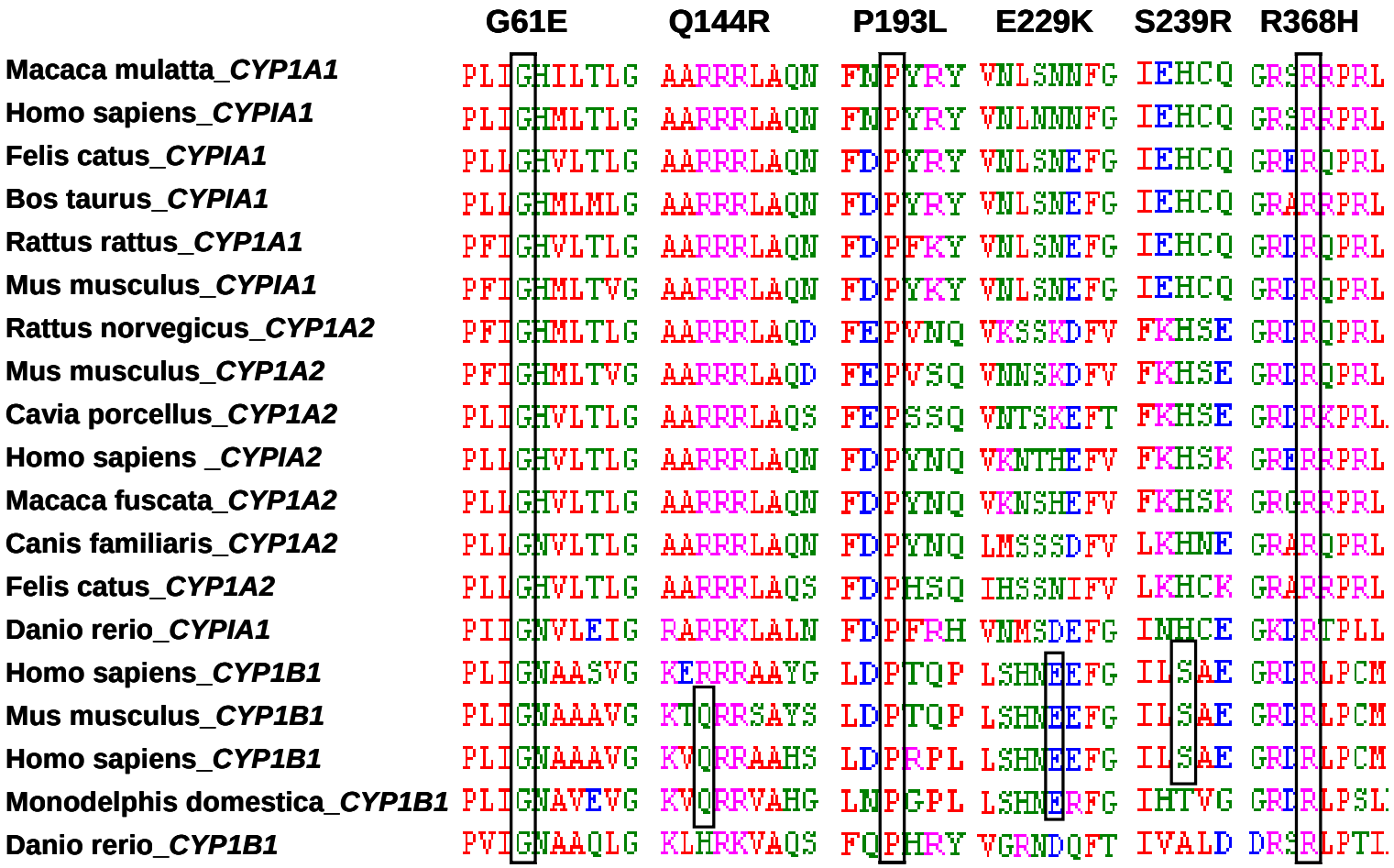


Figure 9 Multiple sequence alignment of the cytochrome P-450 1B1 protein across different species. The wild type residue is boxed across species

Table 9. Clinical features of POAG, JOAG and PACG patients with *CYP1B1* mutations

S. No.	PATIENT ID	PHENO TYPE	AOO	MUTATION	IOP AT PRESENTATION[+] (OD : OS)	CD RATIO[++] (OD ; OS)	VISUAL ACUITY[ξ] (OD ; OS)	VISUAL FIELDS[Ψ] (OD ; OS)	TREATMENT MODALITY[Θ]
1	J - 162	JOAG	23	G61E (H)	46 ; 18	0.9 ; 0.6	20/1200 ; 20/20	3 ; 1	Medication
2	P - 76	POAG	45	P193L (H)	28 ; 18	0.9 ; 0.9	20/2400 ; 20/30	1 ; 1	Surgical Trab (OU)
3	P - 89	POAG	55	P193L (h)	14 ; 14	0.5 ; 0.5	20/60 ; 20/30	1 ; 1	Medication
4	P -128	POAG	41	P193L (h)	19 ; 25	0.9 ; 0.9	20/25 ; 20/25	1 ; 3	Surgical Trab (OU)
5	P - 123	POAG	38	S239R (h)	25 ; 16	0.9 ; 0.8	20/200 ; 20/20	3 ; 1	Surgical Trab (OU)
6	P -134	POAG	52	R368H (h)	23 ; 22	0.9 ; 0.9	20/20 ; 20/20	1 ; 1	Medication
7	A - 17	PACG	61	Q144R (h)	33 ; 15	0.8 ; 0.8	20/50 ; 20/200	3 ; 1	Surgical YPI (OU)
8	A - 58	PACG	58	E229K (h)	23 ; 20	0.9 ; 0.9	20/1200 ; 20/80	3 ; 3	Surgical YPI (OU)
9	A - 61	PACG	55	E229K (h)	17 ; 24	0.8 ; 0.9	20/40 ; 20/1200	3 ; 3	Surgical YPI (OU)
10	A - 66	PACG	60	E229K (h)	69 ; -	0.9 ; -	20/2400 ; -	3 ; -	Surgical Trab (OD)
11*	A - 79	PACG	33	E229K (h)	42 ; 42	0.3 ; 0.9	20/20 ; 20/506	1 ; 3	Surgical YPI (OU)

Table 9 (Contd..)

S. No.	PATIENT ID	PHENO TYPE	AOO	MUTATION	IOP AT PRESENTATION[+] (OD : OS)	CD RATIO[++] (OD ; OS)	VISUAL ACUITY[ξ] (OD ; OS)	VISUAL FIELDS[Ψ] (OD ; OS)	TREATMENT MODALITY[Θ]
12	A - 137	PACG	49	E229K (h)	24 ; 16	0.5 ; 0.5	20/30 ; 20/200	2 ; 3	Surgical YPI (OU)
13	A - 55	PACG	38	R368H (h)	12 ; 26	0.4 ; 0.9	20/20; 20/60	1 ; 3	Surgical Trab (OS)
14	A - 59	PACG	50	R368H (h)	26 ; 6	0.6 ; 0.6	20/380 ; 20/760	1 ; 3	Surgical Trab (OD ; YPI)
15	A - 81	PACG	64	R368H (h)	15 ; 49	0.4 ; 0.8	20/60 ; 20/1200	1 ; 3	Surgical YPI (OU)
16	A - 95	PACG	70	R368H (h)	12 ; 24	0.6 ; 0.8	20/25 ; 20/50	1 ; 3	Surgical YPI (OS)
17	A - 115	PACG	70	R368H (h)	10 ; 10	0.4 ; 0.6	20/100 ; 20/200	1 ; 1	Surgical YPI (OU)

[+] Intraocular pressure (IOP), OU = both eyes, OD = right eye, OS = left eye; [++]Cup to disc (CD) ratios; [ξ] Visual acuity at presentation; [Ψ]Visual field defects : 1 = Early defect, 2 = Moderate, 3 = Severe; AE - Absolute eye; [Θ]Treatment modality : YPI = Yag peripheral iridectomy, TSCPC=Transscleral cyclo photocoagulation, Trab = Trabeculectomy; h - Heterozygous mutation.

4.4. Single Nucleotide Polymorphisms in *CYP1B1*

Six different polymorphisms were also observed in *CYP1B1* gene viz., –13T/C (intron-I), R48G (C>G), A119S (G>T) in exon II and V432L (G>C), D449D (T>C) and N453S (A>G) in exon III. The details of these SNPs are given in Table 10. The SNPs R48G and A119S in exon II, V432L and D449D in exon III were found to be in linkage disequilibrium. The allele and genotype distribution of these polymorphisms in POAG and PACG are shown in Tables 11 and 12. Odds ratios along with 95% confidence intervals were determined for finding the disease susceptibility of specific genotypes.

Table 10. List of *CYP1B1* polymorphisms observed in POAG and PACG

S.No	dbSNP ID*	Location	Nucleotide Change	Amino acid change
1	rs2617266	Intron I	T>C	-
2	rs10012	Exon II	CGG>GGG	R48G
3	rs1056827	Exon II	GCC>TCC	A119S
4	rs1056836	Exon III	GTG>CTG	V432L
5	rs1056837	Exon III	GAT>GAC	D449D
6	rs1800440	Exon III	AAC>AGC	N453S

- Gen Bank ID for *CYP1B1* - U56438

Table 11. Allele frequencies of the *CYP1B1* polymorphisms in POAG and PACG cases and controls

S. No	SNP	GENOTYPE	POAG (%) (n = 48)	PACG (%) (n = 105)	CONTROLS (%) (n = 100)	ODDS RATIO POAG Vs CONT (95% CI)	p value	ODDS RATIO PACG Vs CONT (95% CI)	p value
1	-13T>C	T	32	45	51	1	0.0064	1	0.395
		C	68	55	49	2.21 (1.24-3.9)		1.27 (0.72-2.21)	
2	R48G	C	65	61	57	1	0.246	1	0.565
		G	35	39	43	0.71 (0.4-1.26)		0.84 (0.48-1.48)	
3	A119S	G	65	60	57	1	0.246	1	0.667
		T	35	40	43	0.71 (0.4-1.26)		0.88 (0.5-1.55)	
4	V432L	G	35	24	20	1	0.017	1	0.494
		C	65	76	80	0.46 (0.24-0.88)		0.79 (0.4-1.54)	
5	D449D	T	35	24	20	1	0.017	1	0.494
		C	65	76	80	0.46 (0.24-0.88)		0.79 (0.4-1.54)	
6	N453S	A	83	86	87	1	0.428	1	0.836
		G	17	14	13	1.37 (0.62-2.99)		1.08 (0.48-2.45)	

As evident from Table 11, the –13C allele showed a significant difference among the POAG cases and controls (p value = 0.0064; OR = 2.21, 95%CI, 1.24-3.9).Also the odds ratio for the heterozygous genotype (TC) was 2.84 (0.9-8.4) and for the homozygous genotype (CC) was 3.25 (1.07-9.84) (Table 12). However, there was no statistically significant difference of the –13 C allele among the PACG cases and controls. The allele and genotype frequencies of the coding SNPs within *CYP1B1* did not show any significant difference (p>0.05) among the POAG and PACG cases in comparision to the controls (Table 12).

Table 12. Distribution of genotype frequencies of *CYP1B1* polymorphisms in POAG and PACG cases

S. No	SNP	GENOTYPE	POAG (%)	PACG (%)	CONTROLS (%)	ODDS RATIO POAG Vs Controls (95% CI)	p value	ODDS RATIO PACG Vs Controls (95% CI)	p value
1	-13T>C	TT	10.4	20	26	1	-	1	-
		TC	47.9	42.8	42	2.84 (0.9-8.4)	0.052	1.34 (0.6-2.8)	0.437
		CC	41.6	37.1	32	3.25 (1.07-9.84	0.032	1.5 (0.7-3.22)	0.312
2	R48G	CC	41.6	37.1	33	1	-	1	-
		CG	47.9	43.8	42	0.9 (0.42-1.91)	0.793	0.95 (0.5-1.82)	0.884
		GG	10.4	19	25	0.33 (0.1-1.0)	0.044	0.68 (0.31-1.48)	0.333
3	A119S	GG	41.6	36.1	32	1	-	1	-
		GT	47.9	43.8	43	0.85 (0.4-1.81)	0.685	0.93 (0.48-1.78)	0.828
		TT	10.4	20	25	0.32 (0.1-0.97)	0.039	0.72 (0.33-1.57)	0.408
4	V432L	GG	12.5	4.76	4	1	-	1	-
		GC	27	37.1	33	0.26 (0.06-1.08)	0.055	0.82 (0.23-3.34)	0.787
		CC	60.4	58	63	0.3 (0.08-1.17)	0.072	0.64 (0.16-2.53)	0.530
5	D449D	TT	12.5	4.76	4	1	-	1	-
		TC	27	36.1	33	0.26 (0.06-1.08)	0.055	0.848 (0.2-3.43)	0.817
		CC	60.4	59	63	0.3 (0.08-1.17)	0.072	0.634 (0.16-2.48)	0.512
6	N453S	AA	68.7	72.3	75	1	-	1	-
		AG	29.1	23.8	22	1.44 (0.65-3.17)	0.356	1.08 (0.54-2.14)	0.816
		GG	2.08	3.8	3	0.75 (0.07-7.5)	0.812	1.13 (0.22-5.82)	0.878

4.5. Mutant alleles of *MYOC* and *CYP1B1* in probands of POAG and PACG

One of the POAG proband (P-134) exhibited heterozygous mutations in *MYOC* (Gln368Stop) and *CYP1B1* (Arg368His). The segregation of these could not be studied, as the DNA samples from other members were not available for analysis. The pedigree of the proband along with the chromatograms of the mutations is shown in Figure 10. The proband harboring the mutations manifested the disease at the age of 52 years. He had an IOP of 23 and 22 mm Hg in the right and left eyes, respectively, and a C: D ratio of 0.9:1 in both the eyes. The visual acuity was 20/20 in both the eyes and he was under medical treatment.

Another case of PACG (A-058) exhibited heterozygous mutations in *MYOC* (Gln48His) and *CYP1B1* (Glu229Lys). Similar to the previous case, the segregation of these mutations could not be studied, as the DNA samples from other members were not available for analysis. The proband harboring the mutations manifested the disease at the age of 58 years. He had an IOP of 23 and 20 mm Hg in the right and left eyes, respectively, and a C: D ratio of 0.9:1 in both the eyes. The visual acuity was 20/1200 and 20/80 in the right and left eye, respectively. The patient had undergone surgical treatment in both the eyes.

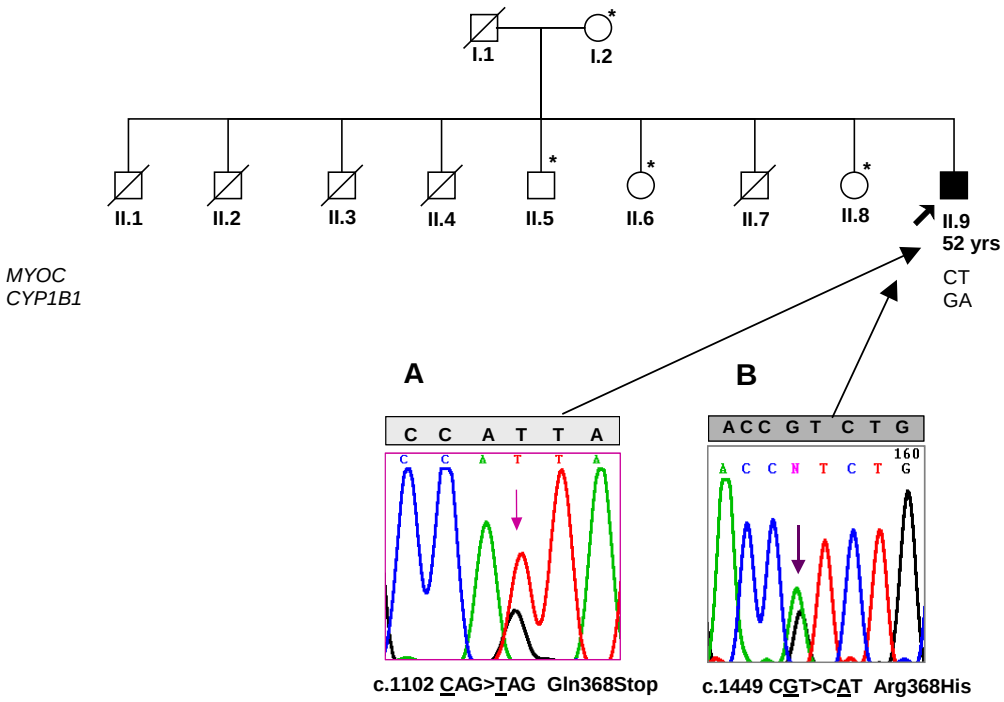


Figure 10. Pedigree of a proband (P-134) showing the involvement of *MYOC* (Gln368Stop) and *CYP1B1* (Arg368His). Blackened symbol indicate affected individual with POAG while the clear symbols denote the unaffected status. Slash marks through the symbols denote deceased individuals, asterix indicates the family members whose DNA samples were not available. The genotypes for the mutations and the age at onset, are indicated below the proband's symbol. The chromatograms of *MYOC* and *CYP1B1* mutations are shown below the pedigree. The wild type sequence is shown in the box above and the arrow point indicates the point of mutation. A) Gln368Stop (*MYOC*)
B) Arg368His (*CYP1B1*).

4.6 SCREENING FOR SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

4.6.1. Analysis of single nucleotide polymorphisms (SNPs) in POAG cases and controls

4.6.1.a. INTERLEUKIN-1 (*IL-1*):

One hundred and two controls and 106 POAG patients were screened for the polymorphisms in Interleukin -1β (-511 C>T SNP in the promoter and +3953 C>T in exon-5) and Interleukin -1α gene (-889 C>T SNP in the promoter). The restriction digestion patterns, on an 8% polyacrylamide gel for the *IL-1* SNPs is shown in Figures 11a, 11b and 11c. There was no statistically significant difference in the allele and genotype frequencies between POAG cases and controls ($p > 0.05$). Odds ratios (95% CI) were calculated for all the three SNPs. The mutant genotype TT at –511 position of IL -1β showed an odds ratio [OR] of 1.99 (95%CI-1.1-3.6), thus contributing to a weak association to POAG. The same polymorphisms were screened among a cohort of 88 PACG patients and there was no significant difference in the allele and genotype frequencies between the cases and controls ($p>0.05$) (Tables 13 and 14).

The genotypes of the IL-1 polymorphisms were compared with the visual field defects [Mild, moderate and severe (Hodapp *et al.,* 1993)] in the worst eye. Also the genotypes were compared among the cases with and without *MYOC* and *CYP1B1* mutations. There was no statistically significant difference among the *IL-1* genotypes with

respect to the visual fields (p>0.05) and also among the cases with and without *MYOC* and *CYP1B1* mutations (p>0.05) (Tables 15, 16, and 17).

Table 13. Allele frequencies of Interleukin-1 polymorphisms in POAG and PACG cases and controls

SNP	Allele	POAG (%) (n=106)	PACG (%) (n=88)	Controls (%) (n=102)	p-value POAG Vs Controls	p-value PACG Vs Controls
IL-1β (–511C>T)	C	55	58	68	0.059	0.143
	T	45	42	32		
IL-1β (+3953C>T)	C	83	85	85	0.702	-
	T	17	15	15		
IL-1α (-889C>T)	C	69	72	66	0.651	0.36
	T	31	28	34		

Table 14. Genotype distribution of Interleukin-1 polymorphisms in POAG and PACG cases and controls

SNP	Genotypes	POAG (%)	PACG (%)	Controls (%)	OR (95% CI) POAG Vs Controls	p value	OR (95% CI) PACG Vs Controls	p-value
IL-1β (–511C>T)	CC	30.1	34	46	1	-	1	-
	CT	53.7	51.1	41.1	1.99 (1.1-3.63)	0.024	1.69 (0.9-3.12)	0.101
	TT	16	14.7	12.7	1.92 (0.82-4.49)	0.130	1.56 (0.64 – 3.8)	0.323
IL-1β (+3953C>T)	CC	68.8	71.5	71.5	1	-	1	-
	CT	29.2	26.1	22.5	1.34 (0.71-2.52)	0.352	1.16 (0.59-2.26)	0.666
	TT	1.88	2.27	5.88	0.33 (0.06-1.7)	0.168	0.38 (0.075-1.9)	0.238
IL-1α (-889C>T)	CC	47.1	52.2	44.1	1	-	1	-
	CT	43.3	36.3	42.1	0.96 (0.53-1.7)	0.898	0.73 (0.39-1.34)	0.311
	TT	9.4	11.3	13.7	0.64 (0.25-1.59)	0.337	0.69 (0.28-1.73)	0.439

Table 15. Association between *IL-1β* –511 genotypes and visual fields in POAG and PACG cases

Genotypes	VFD IN POAG$^{\varphi}$			p-value	VFD IN PACG$^{\varphi}$			p-value	POAG		p-value	PACG		p-value
	Mild	Mod	Sev		Mild	Mod	Sev		*Cases with mutations	Cases without mutations		*Cases with mutations	Cases without mutations	
CC	4	6	22	0.291	5	0	25	0.1006	7	25	0.83	4	26	0.98
CT	5	4	48		4	5	36		14	43		6	39	
TT	1	3	12		1	1	11		3	14		2	11	

*****Cases with *MYOC* or *CYP1B1* mutations

$^{\varphi}$ VFD-Visual Field defect (Mild, Mod-moderate and Sev-Severe)

Table 16. Association between *IL-1β* +3953 genotypes and visual fields in POAG and PACG cases

Genotypes	POAG$^{\varphi}$			p-value	PACG$^{\varphi}$			p-value	POAG		p-value	PACG		p-value
	Mild	Mod	Sev		Mild	Mod	Sev		*Cases with mutations	Cases without mutations		*Cases with mutations	Cases without mutations	
CC	6	8	58	0.068	7	4	52	0.183	17	56	0.587	8	55	0.317
CT	3	4	24		3	1	19		6	25		3	20	
TT	1	1	0		0	1	1		1	1		1	1	

* Cases with *MYOC* or *CYP1B1* mutations

$^{\varphi}$ VFD-Visual Field defect (Mild; Mod-moderate and Sev-Severe)

Table 17. Association between *IL-1α* -889 genotypes and visual fields in POAG and PACG cases

Genotypes	φVFD IN POAG			p-value	φVFD IN PACG			p-value	POAG		p-value	PACG		p-value
	Mild	Mod	Sev		Mild	Mod	Sev		*Cases with mutations	Cases without mutations		*Cases with mutations	Cases without mutations	
CC	3	4	43	0.155	5	3	38	0.1028	12	38	0.287	7	39	0.8847
CT	5	9	31		2	2	28		8	38		4	28	
TT	2	0	8		3	1	6		4	6		1	9	

* Cases with *MYOC* or *CYP1B1* mutations

φ VFD-Visual Field defect (Mild; Mod-moderate and Sev-Severe)

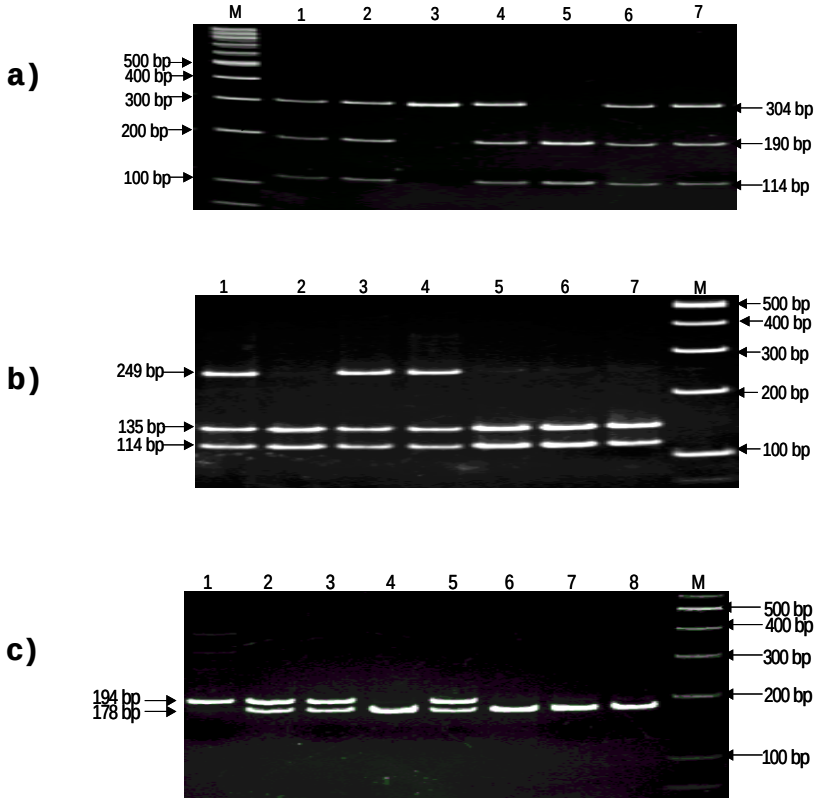


Figure 11. Restriction Digestion for *IL-1β* and *IL-1α* polymorphisms

a) *IL-1β* –511 C>T: The *IL-1β* –511 (C>T) polymorphism abolishes a restriction site for *Eco8I*1, thereby generating an intact fragment of 304 base pairs in the PCR amplicon in the subject homozygous (TT) for the SNP as shown in the lane 3. The amplicon on digestion is cleaved to three fragments (304, 190 and 114 base pairs) in heterozygous (CT) subject (lanes 1, 2, 4, 6 and 7) and into two fragments (190 and 114 base pairs) in the subject with the wild type (CC) alleles (lane 5). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

b) *IL-1β* +3953 C>T: The *IL-1β* +3953 (C>T) polymorphism abolishes a restriction site for *Taq*I, thereby generating an intact fragment of 249 base pairs in the PCR amplicon in the subject homozygous (TT). for the SNP. The amplicon on digestion is cleaved to three fragments (249, 135 and 114 base pairs) in heterozygous (CT) subject as shown in the lanes 1, 3 and 4 and into two fragments (135 and 114 base pairs) in the subject with the wild type (CC) alleles (lanes 2, 5, 6 and 7). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

c) *IL1α* -889 C>T: The *IL1α* -889 (C>T) polymorphism abolishes a restriction site for *Nco*1 site, thereby generating an intact fragment of 194 base pairs in the PCR amplicon in the subject homozygous (TT) for the SNP as shown in the lane 1. The amplicon on digestion is cleaved to three fragments (194, 178 and 16 base pairs) in heterozygous (CT) subject (lanes 2,3 and 5) and into two fragments (178 and 16 base pairs) in the subject with the wild type (CC) alleles (lanes 4, 6, 7 and 8). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

4.6.1.b. *MTHFR* [c.677C>T]:

110 POAG patients and one hundred and thirteen controls and were screened for the c.677C>T (A222V) polymorphism in the *MTHFR* gene. The restriction digestion pattern, on an 8% polyacrylamide gel for the *MTHFR* (c.677C>T) SNP is shown in Figure 12. There were no significant differences in the allele and genotype frequency distributions between the POAG cases and controls (p>0.05) (Tables 18 and 19).

Table 18. Allele frequencies of the *MTHFR* polymorphism (c.677C>T) among the POAG cases and controls

Subjects	C (%)	T (%)
Patients (n =110)	190 (86)	30 (14)
Controls (n = 113)	194 (86)	32 (14)

Table 19. Genotype distribution of the *MTHFR* polymorphism (c.677C>T) among the POAG cases and controls

Genotypes	Patients (%) (n=110)	Controls (%) (n=113)	Odds Ratio (95% CI)	p value
CC	74.5	73.4	1	-
CT	23.6	24.7	0.93 (0.5-1.73)	0.843
TT	1.8	1.76	1.01 (0.13-7.35)	0.99

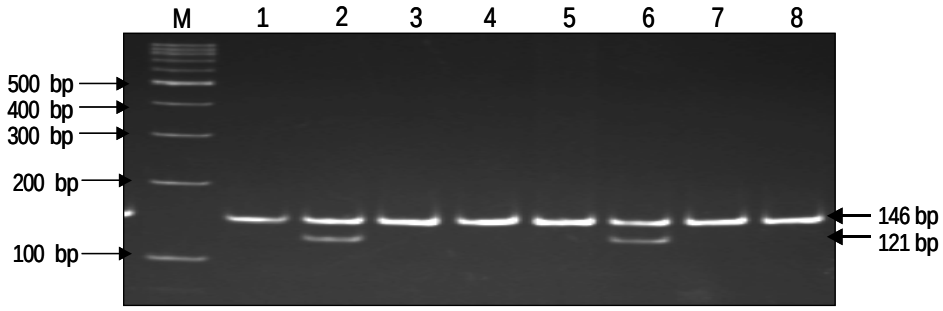


Figure 12. PCR based restriction digestion analysis of *MTHFR c.677C>T* polymorphism

A change of C>T at c.677 region of *MTHFR* results in the creation of a restriction site using *Hinf*I. The 146 bp PCR amplified fragment with the wild type genotype (CC) is shown as an intact band of 146 bp (lanes 1, 3, 4, 5, 7 and 8), the homozygous mutant genotype (TT) generates two bands of sizes 121 bp and 25 bp and the heterozygous genotype (CT) generates three bands of sizes 146 bp, 121 bp and 25 bp (lanes 2 and 6). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

4.6.1.c. p-21 (C/A) Ser31Arg polymorphism:

A cohort of one hundred and six POAG patients and 100 controls were screened for the polymorphism in p-21 (C/A Ser31Arg). The restriction digestion pattern, on an 8% polyacrylamide gel for the p-21 (C/A) Ser31Arg SNP is shown in Fig 13. There was no significant difference in the distribution of allele and genotype frequencies between the cases and controls (p>0.05) (Table 20 and 21).

Table 20. Allele frequencies of p-21 (C/A) Ser31Arg polymorphism among the POAG cases and controls

Subjects	C (%)	A (%)	p value
Patients (n=106)	180 (84)	32 (16)	0.547
Controls (n=100)	176 (87)	24 (13)	

Table 21. Genotype distribution of p-21 (C/A) Ser31Arg polymorphism among the POAG cases and controls

Genotypes	Patients (%) (n=106)	Controls (%) (n=100)	Odds Ratio (95% CI)	p value
Ser/Ser (CC)	70.7	76	1	-
Ser/Arg (CA)	28.3	24	1.26 (0.67-2.36)	0.457
Arg/Arg (AA)	0.94	0	-	-

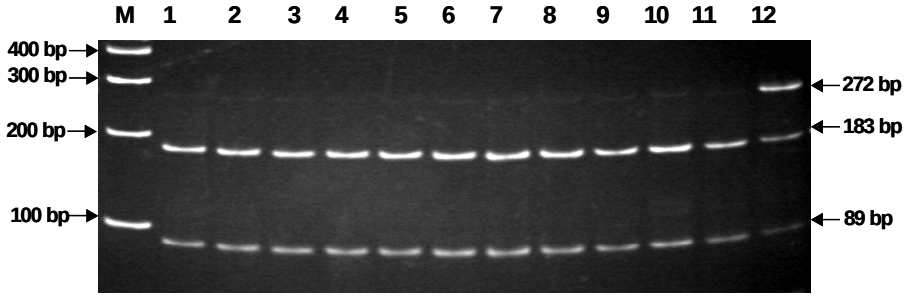


Figure 13. PCR based restriction digestion analysis of p-21 C>A polymorphism

A change of C>A resulting in S31R of *p-21* results in the abolition of restriction site using *Blp*I. The 272 bp PCR amplified fragment with the wild type genotype (CC) shows two bands of sizes 183 bp and 89 bp (lanes 1-11), the homozygous mutant (AA) genotype gives rise to a single band of size 272 bp and heterozygous genotype (CA) generates three bands of sizes 272 bp, 183 bp and 89 bp (lane 12). Lane M contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

4.6.2.b. *MMP9* promoter (–1590C>T) and coding region polymorphism Gln279Arg (A>G)

A cohort of one hundred and ten PACG patients and 100 controls were screened for the polymorphism in the promoter region (-1590 C>T) of *MMP9*. We also screened 114 PACG cases and 106 controls for the polymorphism in *MMP-9* gene at c.855 (A>G) resulting in Gln279Arg codon change. The restriction digestion pattern, on an 8%

polyacrylamide gel for the *MMP9* promoter (–1590C>T) and coding region Gln279Arg (A>G) polymorphisms is shown in Figure 14. There were no significant differences in the allele and genotype frequencies between the cases and controls (p>0.05) (Tables 22 and 23).

Table 22. Allele frequencies of *MMP9* polymorphisms in PACG cases and controls

SNP	Alleles	Patients (%)	Controls (%)	p-value
MMP-9 –1590C>T	C	139 (75) (n=94)	149 (75) (n=100)	-
	T	49 (25) (n=94)	51 (25) (n=100)	
MMP-9 Gln279Arg (A>G)	A	85 (46) (n=98)	95 (45) (n=109)	0.887
	G	111 (54) (n=98)	123 (55) (n=109)	

Table 23. Genotype distribution of *MMP9* –1590 (C/T) promoter and Q279R (A/G) polymorphisms among the PACG cases and controls

SNP	Genotypes	Patients (%)	Controls (%)	Odds Ratio	p value
MMP9 -1590 C>T	CC	56.3	56	1	-
	CT	35.1	37	0.94 (0.52-1.72)	0.847
	TT	8.5	7	1.21 (0.4-3.56)	0.732
MMP9 Gln279Arg (A>G)	AA	21.4	20.1	1	-
	AG	43.8	46.7	0.88 (0.43-1.82)	0.736
	GG	34.6	33	0.98 (0.46-2.11)	0.978

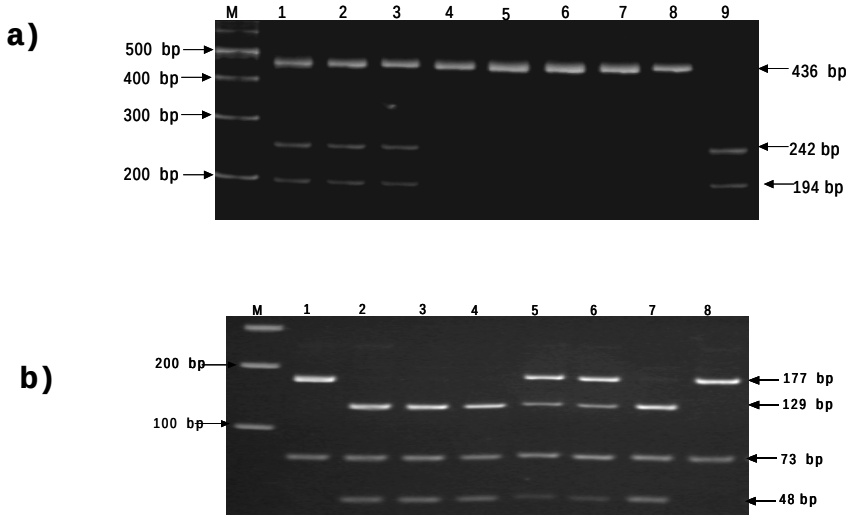


Figure 14. PCR based restriction digestion analysis of *MMP-9* polymorphisms a) A change of C>T at –1590 promoter region of *MMP-9* resulted in the creation of restriction site for *Sph*I. The 436 bp fragment with the wild type genotype (CC) remains intact as shown in the lanes 4, 5, 6, 7 and 8, while the substitution of C toT at –1590 position generates two bands of sizes 242 bp and 194 bp (lane 9). The heterozygous genotype (CT) is shown by three bands of sizes 436 bp, 242 bp and 194 bp (lanes 1, 2 and 3). Lane 'M' contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

b) A change of A>G resulting in Q279R of *MMP-9* gene resulted in the creation of restriction site for *Msp*I. The 250 bp fragment with the wild type genotype (AA) generates two fragments of sizes 177 bp and 73 bp (lanes 1, and 8) while with the homozygous mutant (GG) genotype is cleaved into three bands of sizes 129 bp, 73 bp and 48 bp (lanes 2,3,4, and 7). The heterozygous genotype (AG) is cleaned into four bands of sizes 177 bp, 129 bp, 73 bp and 48 bp (lanes 5, 6 and 8). Lane M contains the 100 bp DNA ladder (MBI fermentas, Lithuania).

Chapter 5
Discussion

5. DISCUSSION

Glaucoma comprises a group of progressive disorder of the optic nerve head and a leading cause of visual field defects (Wilson and Marton *et al.,* 1996). It comprises a group of clinically and genetically heterogenous disorders of the eye resulting in irreversible loss of vision. It is a complex disorder (Lander and Schork *et al.,* 1994), and exhibits locus heterogeneity, phenocopies and incomplete penetrance (Gong *et al.,* 2004). In addition to the inherited factors, non-genetic factors like hypertension, diabetes and cigarette smoking are also implicated in its pathophysiology (Fan *et al.,* 2006). The etiology of glaucoma is yet unknown but the disease is often found to cluster in families (Benedict *et al.,* 1842; Alward *et al.,* 1998; Stokes, 1940). The juvenile form of POAG (JOAG) is inherited as an autosomal dominant trait, whereas the inheritance of adult onset POAG is complex (Wiggs *et al.,* 1998).

Usually the first-degree relatives have a 7-10 fold risk of developing POAG compared to the general population (Wilson *et al.,* 1987; Tielsch *et al.,* 1991). The molecular genetics of POAG has been studied by linkage analysis and whole genome scans but the underlying molecular mechanism is not yet understood. So far seventeen chromosomal loci (*GLC1A* - *GLC1N* and *GLC3A* - *GLC3C*) have been mapped in POAG/JOAG and PCG cases. Of these three genes *MYOC* at *GLC1A*, *OPTN* at *GLC1E* and *WDR*36 at *GLC1G* have been characterized and were found to harbor mutations (Stone *et*

al., 1997; Rezaie *et al.,* 2002; Monemi *et al.,* 2005). *CYP1B1* at *GLC3A* was found to be predominantly involved in primary congenital glaucoma (PCG) cases (Stoilov *et al.,*1997). While the genetic basis of POAG has been studied extensively through out the world, there has been lacunae in understanding the genes involved in PACG, ever since it was reported to be transmitted by a single, dominant gene in 1953 (Tornquist *et al.,* 1953). This can be attributed to the late age of onset of the disease thereby limiting the presence of large families segregating the disease phenotype (Aung *et al,* 2005).

Myocilin (*MYOC*), was the first gene to be identified in POAG (Stone *et al.,* 1997). Since its identification many disease causing variants and polymorphisms have been reported in *MYOC*. Mutations in *MYOC* exist in approximately 3% of late-onset POAG patients and a greater proportion (6.38%) of JOAG patients (Alward *et al.,* 2002). *OPTN* gene was found associated to normal tension glaucoma (NTG) (Sarfarazi *et al.,* 1998). It codes for a protein hypothesized to play a neuroprotective role in the optic nerve (Vittitow *et al.,* 2002) and its expression was observed in many different tissues (Rezaie *et al.,* 2002). *WDR36* is the third candidate gene (*GLC1G*) for POAG and it codes for a protein with multiple G-beta WD40 repeats and is involved in T-cell activation and highly coregulated with interleukin-2 (IL2) (Monemi *et al.,* 2005). Mutations in *WDR36* have been reported by Monemi's group, however there were contradicting reports on its actual involvement in POAG by many studies (Fingert *et al.,* 2007; Weisschuh*et al.,* 2007). A recent report by Miyazawa and his group in

a Japanese NTG and HTG cohorts indicated that the gene is associated to HTG (Miyazawa *et al.,* 2007).

The *CYP1B1* gene at *GLC3A* locus was initially identified as candidate gene for primary congenital glaucoma (PCG) (Stoilov *et al.,* 1997) and mutations in *CYP1B1* were first identified in PCG patients from ethnically mixed populations (Heon *et al.,* 2000; Kakiuchi-Matsumoto *et al.,* 2001). A report by Vincent's group showed digenic mechanism in a JOAG patient who harboured heterozygous mutations in *CYP1B1* and *MYOC* genes, The involvement of *CYP1B1* as a candidate gene in PACG disease pathogenesis is not yet demonstrated in India and elsewhere.

Apart from mutations in the candidate genes, single nucleotide polymorphisms (SNPs) in the associated genes namely *IL1*α*, IL1*β*, MTHFR*, *MMP9*, *p21*, *p53*, *APOE* and *TNF*α, etc., have also been implicated in POAG and PACG patients (Junemann *et al.,* 2005; Lin *et al.,* 2003; Wang *et al.,* 2006; Tsai *et al.,* 2004; Fan *et al.,* 2006). SNPs within these genes might play important role in apoptotic death of RGCs and contribute to the progressive glaucomatous phenotype, however the results from case-control studies conducted in ethnically and geographically different populations are not the same. The association of most of these SNPs to the POAG or PACG disease pathogenesis could not be replicated across different populations; however, they provide significant insights in glaucoma pathogenesis.

In the present study we tried to investigate the role of *MYOC* and *CYP1B1* in two different forms of glaucoma cohorts, POAG and

PACG from Indian population, as an attempt to generate genetic data which can be of considerable help for the management of the disease. Also the role of SNPs in the promoter and intragenic regions of *IL1*α, *IL1*β, *MTHFR*, *p21* and *MMP9* genes has been investigated.

In the present study, the mutations in *MYOC* accounted for 7.9% (3/38) in POAG, 10% (1/10) in JOAG, 5.94% (6/101) in PACG and 11.76% (2/17) in PAC cases. The mutation frequency of open angle glaucoma was similar to the earlier reports from different populations worldwide. The largest study on 1703 glaucoma patients from five different populations observed the overall frequency of *MYOC* mutations ranging from 2 - 4% (Fingert *et al.,* 1999). The most common *MYOC* mutation Gln368Stop was observed in 1.6% of all POAG except the Japanese (Fingert *et al.,* 1999). The second most common mutation Arg46Stop was shared only by Asian populations. The frequency of this mutation was 6.25% and it was a hot-spot in Taiwanese patients with JOAG (Yen *et al.,* 2007). *MYOC* mutations accounted for 1.5% of the POAG cases among the Chinese population (Pang *et al.,* 2002). Aung *et al,* (2005) screened 106 PACG patients and observed the disease causing variants in the controls as well. *MYOC* mutations ranged from 2.9 - 4% (Suzuki *et al.,* 1997; Kubota *et al.,* 2000) among the Japanese POAG patients. The Pro370Leu was found to be associated with juvenile open angle glaucoma (JOAG), high IOP and poor response to treatment (Taniguchi *et al.,* 1999; Mukhopadhyay *et al.,* 2003). This mutation was observed to segregate in autosomal dominant JOAG families in the French (Adam *et al.,*

1997), Japanese (Suzuki *et al.,* 1997), English (Wiggs *et al.,* 1998), North American (Shimizu *et al.,* 2000), Brazilian (Vasconcellos *et al.,* 2000) and Indian JOAG patients (Mukhopadhyay *et al.,* 2002). In addition, the Arg46Stop mutation was the predominant mutation and the haplotype analysis have indicated that the Gln368Stop and Asn480Lys mutation carriers shared a similar haplotype background, possibly due to a common founder effect (Fingert *et al.,* 1999, Adam *et al.,* 1997; Brezin *et al.,* 1998). Overall frequency of *MYOC* mutations, including the data from the previous study (n = 88) from our center was 9% and was higher to other reports from India that is 7% from Eastern India (Mukhopadhyay *et al.,* 2002), 2% from Southern India (Kanagavalli *et al.,* 2003), 2% from Southern and Northern India (Sripriya *et al.,* 2004).

Herein, we report for the first time the presence of Q48H in 5% (5/101) of PACG cases, 11.76% (2/17) of PAC cases and a single POAG case 2.63% (1/38). An initial screening of 200 POAG and 200 PCG cases from different parts of India along with 300 ethnically matched normal controls indicated that the Q48H mutation was equally distributed among POAG and PCG phenotypes (Chakrabarti *et al.,* 2005). Earlier this mutation was reported from two other studies from Eastern (Mukhopadhyay *et al.,* 2002), Southern and Northern (Sripriya *et al.,* 2004) India. At the level of protein this mutation imparts an additional positive charge on the protein and is prone to protein-protein interactions. According to a report by Sripriya, *et al,* Q48H mutation was found to remove the extended sheets in the glycosaminoglycan

(GAG) initiation site (amino acid 42-45) and was found to show a difference in the core-surface ratio when compared with the wild type protein (Sripriya *et al.,* 2004).

The phenotype associated with Q48H mutation across different Indian populations including the present study was heterogeneous (Table 1). Among the angle closure cases, age at onset was from 50-69 years, with the documented IOPs at presentation of 18-42 mm Hg. Their cup to disc ratios ranged from 0.8 to 1 and most of them underwent YAG peripheral iridectomy for treatment. The single POAG case with Q48H mutation exhibited an IOP of 19 and 17 mm Hg, with a C:D ratio of 0.8:0.8, and is 50 yrs old. In an earlier study from our centre the patient with Q48H mutation was 35 yrs old with an IOP of 22 mm Hg in both the eyes and a C:D ratio of 0.9:0.8 (Table 1). The observed heterogeneity in the manifestation of the disease may be due to the presence of genetic modifiers along with the influence of the environmental factors. Comparision of the phenotypes among the glaucoma patients harboring Q48H mutation from India showed that this mutation manifested with JOAG, POAG, PACG and PAC phenotypes with ages of onset ranging from 17-70 years, IOPs of 20-42 mm Hg (Sripriya *et al.,* 2004, Mukhopadhayay *et al.,* 2002; Chakrabarti *et al.,* 2005, Bhattacherjee *et al.,* 2007). Their cup to disc ratios ranged from 0.6 to 0.9 and most of the patients underwent surgical treatment. A marked severity was noted with respect to visual field defects among the POAG cases from different cohorts (Table 1). However a founder effect could not be determined for this mutation due

to the lack of haplotype data (Mukhopadhyay *et al.,* 2002, Sripriya *et al.,* 2004). Since Gln48His mutation is a common mutation in different forms of glaucomas in the Indian population, this might be useful to develop screening methods for identifying asymptomatic individuals who may be at risk of developing the disease, similar to the Q368X mutation in different Caucasian populations (Fingert *et al.,* 1999, Alward *et al.,* 2002, Alward *et al.,* 1998).

Table 1. Comparison of phenotypes of JOAG/POAG/PACG patients with Q48H mutation in Indian populations

Region	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual field defects	Reference
North India	JOAG	17	33; 26	0.6; 0.4	Generalized reduction of sensitivity	Sripriya *et al.,* 2004
East India	POAG	70	24; 36	0.9; 0.5	Diffuse depression with a scotoma OS	Mukhopadhyay *et al.,* 2002
	JOAG	20	20; 14	0.9; 0.9	Glaucomatous	
	JOAG	32	28; 38	0.5; 0.7	Glaucomatous	
South India	POAG	65	21; 23	0.6; no view	Consistently unreliable	Sripriya *et al.,* 2004
	POAG	35	22; 22	0.9; 0.8	Severe ; Mild	Chakrabarti *et al.,* (2005); Bhattacherjee *et al.,* 2007
	JOAG	20	20,14 PO	0.9:1	Glaucomatous	
	POAG	70	24:36	0.9:0.5	Diffused depression with scotoma in superotemporal quadrant	
	JOAG	32	28:38	0.5:0.7	Glaucomatous field changes	

Region	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Surgery
South India	JOAG	32	28:38	0.5:0.7	Glaucomatous field changes	Bhattacherjee *et al.,* 2007
	POAG	50	19; 17	0.8 ; 0.8	Mild; Mild	Present study
	PAC	52	22; 16	0.8 ; 1	Severe; Severe	
	PAC	50	20; 22	0.6 ; 0.6	Mild; Mild	
	PACG	60	28; 18	0.7 ; 0.9	Mild; Severe	
	PACG	50	17; 18	0.3 ; 0.8	Mild; Moderate	
	PACG	58	23; 20	0.9 ; 0.9	Severe; Severe	
	PACG	50	20; 42	0.9 ; 1	Severe; Severe	
	PACG	69	29; 27	0.9 ; 0.9	Moderate ; Moderate	

In the present study, we observed a single PACG case with Q48H mutation that also harbored a mutant *CYP1B1* allele (E229K). However, the patient did not manifest severe glaucomatous condition as was observed in an earlier study from our centre, which demonstrated the same mutation along with the mutant allele (P437L) in *CYP1B1* in a sporadic POAG case that exhibited marked severity of the disease condition. Vincent *et al,* (2002) for the first time reported a similar digenic inheritance in a large pedigree where the mutant alleles in both *MYOC* (Gly399Val) and *CYP1B1* (Gln368His) augmented the disease severity and the patient had a JOAG phenotype with an age at onset of 27 years, while the patients with mutation only in *MYOC* had a POAG phenotype, had mean age at onset of 51 years. They reported that *CYP1B1* mutation R368H alone may be a cause of a mild phenotype and proposed that it may act as a modifier of *MYOC* expression. It was also suggested that these two genes may interact through a common biochemical pathway (Vincent *et al.,* 2002). In an earlier study from our centre a similar digenic inheritance with mutations Gln48His (*MYOC*) and Pro437Leu (*CYP1B1*) was observed in a POAG case, where the patient manifested the disease at the age of 35 years. Another study from our centre reported the involvement of these two genes in a case of primary congenital glaucoma (PCG). This proband harbored heterozygous mutant alleles Gln48His (*MYOC*) and Gln368His (*CYP1B1*) and showed severity with respect to IOP while his asymptomatic parents were carriers for the mutations (Kaur *et al.,* 2005).

Among the *MYOC* mutations Arg33Lys and Pro56Thr in single cases of POAG and PACG were the novel mutations. The Arg33Lys mutation might not be pathogenic as it results in the substitution of biochemically similar amino acid, however the mutation Pro56Thr results in the conversion of a non-polar hydrophobic amino acid to a polar hydrophilic amino acid and thus might make the protein sticky and in turn make it more accessible to interact with non-specific proteins.

The mutation Thr353Ile in *MYOC* was found in a sporadic case of JOAG who manifested a severe phenotype with respect to IOP, C:D ratios, visual fields and visual acuity. However, earlier studies from Korean and Chinese populations have shown comparatively less severe phenotype among the POAG cases who harboured the same mutation (Yoon *et al.,* 1999; Lam *et al.,* 2000) (Table 2). The origin of this mutation could not be determined as haplotype data is not available from any of the earlier studies. Biochemically the substitution results in the replacement of a polar amino acid with a non-polar amino acid Threonine at 353 residue. This site is a putative phosphorylation site by protein kinase C. Thus, the phosphorylation of *TIGR*/*MYOC* may play a role in the regulation of IOP in trabecular-meshwork (TM) cells (Yoon *et al.,* 1999). However this mutation was found to be non-pathogenic by a Chinese group as it was found in control population as well (Pang *et al.,* 2002).

Table 2. Comparision of phenotypes of JOAG/POAG/PACG patients with T353I mutation among the various populations

Population	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Korean	POAG	59	26:24	0.8:0.6	Severe (OU)	Yoon *et al.,* 1999
Chinese	POAG	67	26:28	0.3:0.4	NA	Lam *et al.,* 2000
Indian	JOAG	32	16:12	0.9:0.5	Superior and inferior arcuate scotoma in RE, isolated paracentral scotoma in LE	Bhattacherje e *et al.,* 2007
Indian	JOAG	29	50; 42	0.9; 0.9	Severe: Severe	Present study

One of the sporadic cases of POAG had Gln368Stop *MYOC* mutation in a heterozygous condition. This mutation codes for a premature truncation of 135 amino acids at the C-terminus of myocilin or approximately half of the olfactomedin domain. A comparision of the phenotypes of the glaucoma patients harboring this mutation from different populations is shown in the table 3. The proband with this mutation also had a heterozygous *CYP1B1* mutation (Arg368His). This patient also did not manifest a severe phenotype and this could be attributed to the fact that unlike the missense mutations, the truncated proteins are easily recognized by the control mechanisms in the endoplasmic reticulum and, if so, are rapidly eliminated, while the subtly altered myocilin may escape these control mechanisms thereby augmenting the phenotype (Tamm, 2002). The other family members could not be analyzed due to the non-availability of the DNA samples. The phenotypic manifestation of the patients with Gln368Stop mutation was heterogenous. The age of the patients with this mutation ranged from 30-86 years with a mean of 60 $\pm$ 13.4 years and the IOPs ranged from 9-50 mm Hg with a mean IOP of 28 $\pm$ 7.76 mm Hg (Table 3).

The prevalence of Gln368Stop mutation in our patient cohort was 2.63% (1/38) in POAG and the prevalence in other populations ranged from 0% in Japan, 2.2% in Iowa, USA, to 4.3% in Switzerland (Fingert et al., 1999; Mataftsi et al., 2001). Hence this mutation was found at least once in all populations except the Japanese. The distribution of this mutation was helpful in the analysis pertaining to population history, movement, and diversity analysis. The origin of the

mutation is from a common ancestor (Fingert et al., 1999) and was present only in European descendents (with one exception of an African American). Gong *et al.,*(2004) reported that Gln368Stop arose after the divergence of Asians and Caucasians because it was not common to both the populations.

Table 3. Comparison of phenotypes of JOAG/POAG/PACG patients with Q368X mutation among the various populations

Population	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Caucasian	POAG	53	31:31	0.7:0.7	NA	Allingham *et al.,* 1998
Caucasian	POAG	75	28:24	0.9:0.9	NA	Allingham *et al.,* 1998
Caucasian	POAG	56	34:34	1.0:1.0	NA	Allingham *et al.,* 1998
Spanish	POAG	72	30	NA	Early defect	Vazquex *et al.,* 2000
Caucasian	POAG	56	16.16	NA	NA	Baird *et al.,* 2005
Caucasian	POAG	72	9.9	NA	NA	Baird *et al.,* 2005
Caucasian	JOAG	37	31	NA	NA	Shimizu *et al.,* 2000
Caucasian	POAG	41	38	NA	NA	Shimizu *et al.,* 2000
Australian	POAG	70	33	NA	NA	Craig *et al.,* 2001
Australian	POAG	70	40	NA	NA	Craig *et al.,* 2001
Australian	POAG	75	29	NA	NA	Craig *et al.,* 2001
Australian	POAG	64	26	NA	NA	Craig *et al.,* 2001
Australian	POAG	60	24	NA	NA	Craig *et al.,* 2001

Table 3 contd..

Population	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Australian	POAG	60	28	NA	NA	Craig *et al.,* 2001
Australian	POAG	63	28	NA	NA	Craig *et al.,* 2001
English	JOAG	39	30	NA	NA	Vincent *et al.,* 2002
Caucasian	POAG	86	26 : 24	0.5 : 0.5	NA	Faucher *et al.,* 2002
Caucasian	ACG	46	25 : 17	0.9 : 1	NA	Faucher *et al.,* 2002
Caucasian	POAG	71	25 : 24	0.7 : 0.7	NA	Faucher *et al.,* 2002
Caucasian	POAG	72	50 : 38	1.0 : 1.0	NA	Faucher *et al.,* 2002
Caucasian	POAG	62	29 : 29	0.9 : 0.8	NA	Faucher *et al.,* 2002
USA	JOAG	30	30 : 30	NA	NA	Willoughby *et al.,* 2004
Caucasian	POAG	61	28 : 28	0.6:0.5	Glaucomatous visual field defect	Hewitt *et al.,* 2006
Indian	POAG	55	21 : 21	0.9 :0.8	Superior arcuate defect	Bhattacharjee *et al.,* 2007
Indian	POAG	62	18 :14	0.7 :0.8	Superior arcuate defect with gross constriction in left eye	Bhattacharjee *et al.,* 2007
Indian	**POAG**	**52**	**23 ; 22**	**0.9; 0.9**	**Mild: Mild**	**Present Study**

However, the later reports were suggestive of Gln368Stop mutation to be a modifier of the POAG phenotype and not a disease causing mutation (DCM) (Jansson, *et al.,* 2003) as the Gln368Stop was also observed among the normal controls (Jansson *et al.,* 2003; Faucher *et al.,* 2002). The distribution of *MYOC* mutations indicate a wide geographical spread of these variants over time. Certain mutations are common across geographically and ethnically diverse populations indicating possible founder effects (Baird *et al.,* 2003).

Mutations in *MYOC* resulted in a gain of function, thus accounting for dominant inheritance. It was reported by Morissette *et al,* (1998) in a large French Canadian family that glaucoma developed in three heterozygous siblings harboring missense mutations at codon 423 whereas homozygous mutant siblings did not manifest the disease, indicating that the POAG results only when there is one wild-type copy and one mutant copy of *MYOC*. Tamm , (2002) proposed a model of ‘‘homoallelic complementation’’, in which mutated Lys423Glu myocilin has a dominant-negative effect that forms defective heterodimers or multimers with native myocilin, but forms functional homodimers with itself.

In addition to the potential disease causing mutations, several SNPs and synonymous codon changes were observed in the promoter [*MYOC*.mt1–1000C>G, –83G>A] and coding regions (Arg76Lys, Gly122Gly, Tyr347Tyr, Thr351Thr and Gly375Gly) in POAG and PACG. There was no significant association of *MYOC*.mt1 –1000C>G to either of the phenotypes. The –83 G>A and R76K variations were

not associated with the disease phenotype in both PACG and POAG phenotypes. Earlier, these SNPs where studied in association to POAG phenotype by different groups and there are contradicting reports. The results were in accordance with the earlier studies (Mukhopadhyay *et al.,* 2002, Pang *et al.,* 2002). An earlier report by Colomb *et al,* (2001) suggested the association of the *MYOC*.mt1 minor allele to high IOP and severe visual field loss in their cohort. Another report associated the *MYOC*.mt1 variant to increase in the worsening of the optic disc and visual defects (Polansky *et al.,* 2003). The lack of association in the present study could be attributed to the ethnic and geographic variation. It can also be related to the difference in the inclusion criteria of the respective studies.

CYP1B1 screening in POAG and PACG showed mutations that were mostly in heterozygous condition, previously associated to PCG in homozygous condition (Chakrabarti *et al.,* 2007). The mutation frequency was 13.1% (5/38) in POAG, 10% (1/10) in JOAG and 12.2% (11/90) in PACG patients, which was relatively higher compared to the previous studies (Melki, *et al.,* 2004, Lopez-Garrido *et al.,* 2006, Acharya *et al,* 2006, Kumar *et al.,* 2007). All the mutations were missense mutations (G61E, P193L, S239R and R368H) in POAG and (Q144R, E229K and R368H) in PACG cases. Most of the times the mutations were in heterozygous state however, two of the POAG probands showed mutations in homozygous condition. The patients with sequence alterations showed heterogeneity with respect to the glaucomatous phenotypic characteristics like intraocular pressure, cup

to disc ratio, visual acuity and visual field defects. The frequency of *CYP1B1* mutations in geographically different populations was 4.6% among the French JOAG patients (Melki, *et al.,* 2004), 10.9% and 8.1% among the Spanish POAG and ocular hypertension (OHT) cases. The criteria for a diagnosis of OHT was pressure greater than 21 mm Hg on two or more occasions in the absence of a field defect and open angles on gonioscopy, with no history of angle closure and absence of any ocular disease contributing to the elevation of pressure (Lopez-Garrido *et al.,* 2006). The mutation frequency was 4.5% from Eastern India (Acharya *et al,* 2006) and more recently, 10.76% from Southern India (Kumar *et al.,* 2007). The overall frequency of *CYP1B1* mutations from our centre was 18.6% (25/134) which includes 17.3% (18/104) of POAG and 7/30 (23.3%) of JOAG cases (Chakrabarti *et al.,* 2007). Four mutations were novel (Q144R, W434R, c.1657 del 5bp and F445C). The R368H mutation and the E229K mutations were the most common mutations in both POAG and PACG cases. R368H mutation occurred with a frequency of 5.76% (6/104) and 3.3% (1/30) respectively, in POAG and JOAG cases while the E229K mutation had a frequency of 3.84 (4/104) and 3.3% (1/30) in POAG and JOAG cases. Among the PACG cases both the mutations had equal frequency of 5.5% (5/90). These mutations were also predominant in Indian PCG patients (Reddy *et al.,* 2003).

In our cohort, we found a JOAG proband who harbored homozygous G61E mutation who manifested relatively severe phenotype with an age at onset of 23 years (Chakrabarti *et al.,* 2007).

The G61E lies within highly conserved region affecting the hinge region (Stoilov *et al.,* 1998; Yamazaki *et al.,* 1993) of the protein replacing a neutral amino acid with a negatively charged amino acid. The delayed onset of the phenotype in comparision to the primary congenital glaucoma might be due to the additional modifier locus. One of the affected sibling of the proband also showed the co-segregation of G61E in homozygous condition. This mutation was reported earlier in PCG cases from different populations (Belmouden *et al.,* 2002; Stoilov *et al.,* 1998; Panicker *et al.,* 2002; Reddy *et al.,* 2004; Panicker *et al.,* 2004) and has been observed in heterozygous condition in POAG from Morocco (Belmouden *et al.,* 2002).

Three sporadic POAG cases had Pro193Leu mutation. One of the proband was homozygous for the mutation and showed severe phenotype with respect to the IOP and C:D ratio, however the patient had a late age of onset. This mutation was earlier reported to be a pathogenic mutation among Indian PCG cases (Panicker *et al.,* 2002). The mutation resulted in the replacement of a biochemically similar amino acid but comparative modeling and simulation data have shown that this site lies away from the functionally important regions (FIRs) and is in the N-cap position of E-helix. Replacement of Proline might affect the stability of the E-helix, thereby affecting the packing in this region (Achary *et al.,* 2006).

A sporadic PACG case exhibited the heterozygous Gln144Arg mutation. Earlier this mutation was found to segregate along with the affected phenotype in a large four-generation autosomal dominant

POAG family from our centre (Chakrabarti *et al.,* 2007). However two asymptomatic individuals also harbored this mutation indicating incomplete penetrance of the *CYP1B1* gene and this is well documented in PCG belonging to Saudi Arabian and Indian populations (Bejjani *et al.,* 2000; Panicker *et al.,* 2004). The Glutamine residue is not highly conserved in the CYP1 families across species and is present only in human and mouse *CYP1B1* proteins. It is replaced by arginine in rest of the species as shown in the multiple sequence alignment. In an earlier report a change from glutamine to proline at this codon position was documented in a PCG case (Reddy *et al.,* 2004). This mutation resulted in the replacement of a neutral amino acid arginine to a positively charged glutamine and occurs in the middle of the C-helix very close to heme binding region which is the junction interconnecting helix I, helix C and the B'/C-helix (Achary *et al.,* 2006). Hence substitution of Gln144Arg may result in the disruption in the interactions within the protein.

In the present study, R368H mutation was the prevalent mutation, among the POAG and PACG cases. A comparision of the phenotypes of JOAG, POAG and PACG patients harboring the R368H mutation is shown in the table 4. The age range among PACG patients harboring this mutation was 38-70 years with a mean of 58.4 $\pm$ 14 years. The IOPs ranged from 10-49 mm Hg with a mean of 27 $\pm$ 14 mm Hg; cup to disc ratios ranged from 0.6-0.9. The varied phenotype of the patients harbouring the mutation could be attributed to the role of other factors in the causation of the disease. The R368H mutation was

earlier reported to be the most predominant mutation in PCG (Reddy *et al.,* 2003, Reddy *et al.,* 2004). It was also observed in POAG population from India (Acharya *et al.,* 2006, Chakrabarti *et al.,* 2007) but not in French (Melki *et al.,* 2004) and Spanish (Lopez-Garrido *et al.,* 2006) populations. This was also reported to be a pathogenic mutation among PCG patients in Brazil (Stoilov *et al.,* 2002) and Saudi Arabia (Bejjani *et al.,* 2000). This suggests a wide prevalence of this mutation across multiple glaucoma phenotypes. Another predominant mutation in POAG and PACG was E229K. According to a previous report from our centre, the E229K mutation was the second most predominant mutation among the POAG patients 3.84% (4/104) (Chakrabarti *et al.,* 2007).

It was observed in all POAG populations screened so far suggesting that this could be a predominant *CYP1B1* mutation in POAG. The frequency of this mutation was 0.4% (1/236) in French (Melki *et al.,* 2004), 0.5% (1/200) in Eastern India (Acharya *et al.,* 2006) and 2.4% (2/82) in Spanish (Lopez-Garrido *et al.,* 2006) populations with mixed glaucomas. A comparision of the phenotypes of JOAG, POAG and PACG patients harboring the E229K mutation is shown in the table 5.

Table 4. Comparision of phenotypes of JOAG/POAG/PACG patients with R368H mutation among the various populations

Population	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
English	JOAG	39	30	NA	NA	Vincent *et al.,* 2002
Indian	JOAG	37	16 : 22	0.8: 0.7	NA	2006
	JOAG	37	16 : 22	0.8 :0.9	Upper arcuate scotoma more in right eye than in the left eye	Acharya *et al.,* 2006
	POAG	53	13 : 12	0.9: 0.9	NA	Kumar *et al.,* 2007
	POAG	49	22 : 23	0.5: 0.5	NA	Kumar *et al.,* 2007
	POAG	47	38 : 26	0.5: 0.8	NA	Kumar *et al.,* 2007
	POAG	46	22 : 22	0.5 : 0.6	NA	Kumar *et al.,* 2007

Table 4 (contd..)

Population	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Indian	POAG	48	21 : 32	0.9 : 0.9	NA	Kumar *et al.*, 2007
	POAG	50	28 : 27	0.8 : 0.7	NA	Kumar *et al.*, 2007
	POAG	55	48 : 32	0.7 : 0.7	NA	Kumar *et al.*, 2007
	POAG	48	38 : 32	0.8: 0.9	NA	Kumar *et al.*, 2007
	POAG	**52**	**23 ; 22**	**0.9; 0.9**	**Mild: Mild**	**Present Study**
	PACG	**38**	**12 ; 26**	**0.4; 0.9**	**Mild :Severe**	**Present Study**
	PACG	**50**	**26 ; 12**	**0.6; 0.6**	**Mild :Severe**	**Present Study**
	PACG	**64**	**15 ; 49**	**0.4; 0.4**	**Mild :Severe**	**Present Study**
	PACG	**70**	**12 ; 24**	**0.6; 0.8**	**Mild :Severe**	**Present Study**
	PACG	**70**	**10 ; 10**	**0.4; 0.6**	**Mild : Mild**	**Present Study**

A recent study by Kumar *et al,* (2007) observed the Glu229Lys mutation in 5.2% and the R368H in 3.98% of POAG. They also observed these in controls indicating these variations to be polymorphisms. Previously, R368H was reported in 2.13% of the French population (Melki *et al.,* 2004). Among Indian PCG cases the mutation frequencies of Arg368His and Glu229Lys were 59.46% (22/37) and 16.22%(6/37) respectively (Reddy *et al.,* 2003).

Table 5. Comparison of phenotypes of JOAG/POAG/PACG patients with E229K mutation among the various populations

Population	Pheno type	Age at Onset	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Indian	JOAG	17	24: 32	0.7 : 0.8	Glaucomatous field changes	Acharya *et al.,* 2006
Spanish	POAG	66	18 : 18	0.5 : 0.4	Mild : normal	Lopez Garrido *et al.,* 2006
	POAG	48	20 : 21	0.7 : 0.7	Mild : mild	
French	POAG	35	25 : 21	NA	NA	Melki *et al.,* 2007
Indian	POAG	56	14 : 38	0.5 : 0.5	NA	Kumar *et al.,* 2007
	POAG	48	22 :28	0.8 :0.9	NA	
	POAG	47	25 : 23	0.8 : 0.9	NA	
	POAG	45	28 : 32	0.5 : 0.6	NA	
	POAG	62	42 : 39	0.9 : 0.9	NA	
	POAG	57	48 : 32	0.8 : 0.8	NA	

Table 5 (contd..)

Population	Pheno type	Age at Onset	IOP at presentation	CD ratio OD; OS	Visual fields	Reference
Indian	POAG	45	28 : 32	0.9 : 0.9	NA	Kumar *et al.,* 2007
	POAG	50	28 : 32	0.7 : 0.7	NA	Kumar *et al.,* 2007
	POAG	55	38 : 22	0.8 : 0.9	NA	Kumar *et al.,* 2007
	POAG	69	28 : 26	0.9 : 09	NA	Kumar *et al.,* 2007
	PACG	58	23 ; 20	0.9 ; 0.9	Severe : Severe	Present Study
	PACG	55	17 ; 24	0.8 ; 1.0	Severe : Severe	Present Study
	PACG	60	69 ; -	0.9 ; -	Severe: -	Present Study
	PACG	33	42 ; 42	0.3 ; 1.0	Mild : Severe	Present Study
	PACG	49	24 ; 16	0.5 ; 0.5	Moderate : Severe	Present Study

In addition to the above-mentioned disease causing mutations, five intragenic polymorphisms and one polymorphism in the first intronic region were also observed. These SNPs were not significantly associated with POAG and PACG. Of the six SNPs, two SNPs were found in the exon 2, that is R48G and A119S and two in exon 3, V432L and D449D, which were most of the time observed together in patients and controls.

Single nucleotide polymorphisms (SNPs) within the candidate genes might play an important role in apoptotic death of RGCs but pathway is not yet clearly understood. Both positive and negative associations of the SNPs to POAG disease pathogenesis are being reported in different populations and this discrepancy might result due to several reasons. Mechanical stress to the retinal neurons caused due to high IOP, ischemia and excessively excited amino acids might activate the immune mechanisms resulting in RGC apoptosis (Lin *et al.,* 2003). Cytokines are the key proteins, which play a major role in the immune mechanisms, and their levels show variation in glaucomatous condition (Yang *et al.,* 2001). Of the many cytokines *IL1* is a key mediator of immune and inflammatory responses (Steinkasserer *et al.,* 1992). In our study, the distribution of *IL1*β –511C>T, +3953C>T and *IL1*α –889C>T polymorphisms were evaluated in a cohort of POAG and PACG cases and controls and no significant difference in the allele and genotype frequencies was found between the cases (POAG and PACG) and controls. Also there was no

association of the interleukin polymorphisms to the glaucomatous visual field defects in both POAG and PACG patients.

Lin *et al,* (2003) showed a significant association of *IL1β* +3953C>T polymorphism to POAG in a cohort of 58 POAG patients and 105 controls belonging to the Chinese population. Another Chinese study on 156 POAG patients and 167 controls observed an association of *IL1α* -889 polymorphism ($p<0.05$) (Wang *et al.,* 2006). The same group tested the association of *IL1α* -889C>T polymorphism among the NTG cases as well. This was based on the fact that the factors other than IOP were responsible for glaucomatous optic atrophy in individuals with NTG. However, there was no significant association of *IL1α* -889 promoter SNP to the NTG cases (Wang *et al.,* 2007). In contrast to the earlier studies on Chinese population in a recent study by How *et al,* on 194 POAG cases (94 NTG and 100 HTG), 125 PACG and 79 control individuals, observed that the *IL1β*-511C>T, *IL1β*+3953C>T and *IL1α*-889C>T polymorphisms did not indicate any significant association to POAG or PACG. In addition, none of the common haplotypes showed any significant difference between the HTG, NTG, PACG and normal control subjects (How *et al.,* 2007) and the results of the present study are in accordance with the results of this study.

The present study evaluated Ser to Arg polymorphism in a cohort of POAG cases and controls. There was no significant difference in the distribution of allele and genotype frequencies among

patients and controls in the present study. Association of Arg form of *p21* codon 31 polymorphism to POAG was first reported in a Chinese cohort of 58 POAG cases and 59 control samples (Tsai *et al.,* 2004). Our results are similar to a study on Caucasian population, which included 140 POAG patients and 73 controls (Ressiniotis *et al.,* 2005). The reason for not finding any association in our study could be attributed to the ethnic variation. More studies from other populations with larger sample size are warranted to determine the role of this SNP in the glaucoma pathology.

Plasma homocysteine levels are elevated in Pseudoexfoliation glaucoma (PEXG) and POAG (Bleich *et al.,* 2004). Homozygosity at 677C>T polymorphism in the *MTHFR* gene results in moderately elevated homocysteine levels thereby this SNP is considered a potential risk factor for POAG (Weisberg *et al.,* 2001). The present study, evaluated 677C>T in a cohort of POAG cases and controls and found that there was no significant difference in the allele and genotype frequencies of the 677C>T polymorphism among the POAG cases and controls. A previous study on 76 Caucasian POAG and 71 PEXG patients and 71 control subjects, observed an association of 677C>T SNP to POAG but not to PEXG (Junemann *et al.,* 2005). Subsequently, studies from populations of Turkey (Turacli *et al.,* 2005), Japan (Mabuchi *et al.,* 2006), Iowa (Fingert *et al.,* 2006) and Austria (Mossbock *et al.,* 2006) could not replicate the association of 677C>T to POAG and PEXG. This study on POAG supports the previous findings.

MMP9 is a secreted multidomain enzyme that is important for the remodeling of the ECM and the migration of normal and tumor cells. It aids in the cleavage of denatured collagen (gelatins) and type IV collagen present in basement membranes. Activation of *MMP9* via neuronal nitric oxide synthase contributes to N-methyl-D-aspartate (NMDA) induced RGC death (Manabe *et al.,* 2005). The -1590C>T polymorphic site in the promoter region is an important regulatory element that appears to be the binding site for the transcription repressor protein (Zhang *et al.,* 1999). In the present study, the role of *MMP9* gene polymorphisms as a possible risk factor in PACG pathogenesis was determined. There was no significant difference in the allele and genotype frequencies of the *MMP*9 promoter and intragenic polymorphisms. Our study results were in contrast to the Chinese report who while studying the SNPs of extracellular matrix, MMPs, tissue inhibitors and other glaucoma-associated genes observed a significant association of Q279R (rs2664538) SNP of the *MMP9* gene to PACG (Wang *et al.,* 2006).

To summarize, *MYOC* was involved in 8.3% of POAG (including JOAG) cases, which was similar to the global frequency (Gong *et al.,* 2004). This is the first report to detect *MYOC* mutations in PACG cases. The mutation T353I was associated with a sporadic JOAG case with high IOPs and poor response to medical treatment. No association was found with the coding and promoter SNPs of *MYOC* to POAG and PACG. The *CYP1B1* showed involvement in 12.5% of the cases of POAG (that included a JOAG case), which was relatively higher

compared to those in French (4.6%) (Melki *et al.,* 2004), Indian (4.5% and 11.5%) (Acharya *et al.,* 2006; Kumar *et al.,* 2007) and Spanish (10%) (Lopez-Garrido *et al.,* 2006) patients. The overall mutation frequency in *CYP1B1* from our center was 18.65% (25/134)(including JOAG) (Chakrabarti *et al.,* 2007). This is the first report to show the involvement of *CYP1B1* in PACG cases (10.4%). All the *CYP1B1* mutations resulted in varying degrees of disease severity. The intragenic polymorphisms in *CYP1B1* did not show any association to POAG and PACG. The single nucleotide polymorphisms in *IL1*α*, IL1*β*, MTHFR*, *p-21* and *MMP9* were not associated to POAG and PACG. More studies with larger sample sizes are warranted to determine the role of these SNPs in POAG and PACG.

The genetic data thus obtained from the current study provides an overview of the mutation spectrum in candidate genes in POAG and PACG. The data indicates a clinical and genetic heterogeneity of glaucoma. This may help in developing methods for predictive testing and a reliable molecular diagnostics for screening in populations. Further the genotype-phenotype correlation would provide new avenues to explore the therapeutic strategies.

SUMMARY

Background

Glaucoma is a complex disease leading to irreversible blindness worldwide. It involves loss of retinal ganglion cells (RGCs), visual field defects, and degeneration of optic nerve head (Ritch *et al.,* 1989) leading to cupping of the optic nerve. According to the report on global burden of visual impairment, the total number of persons with visual impairment worldwide, including that due to the uncorrected refractive error, was estimated as 259 million (65% higher than the WHO estimate based on the best-corrected visual acuity), and this includes 42 million persons with blindness and 217 million persons with less severe visual impairment (Dandona *et al.,* 2006). According to the WHO report, cataract is the leading cause of blindness (47.8%), followed by glaucoma (12.3%) and age-related macular degeneration (8.7%). A greater prevalence of visual impairment is present among women than in men in every region of the world: the ratios range from 1.5 to 2.2. (Resnikoff *et al.,* 2004). Glaucoma is highly prevalent in India with POAG being the most common form (Thomas *et al.,* 2003). According to a study on urban population in Hyderabad, Andhra Pradesh, the prevalence of POAG was found to be twice that of PACG (Dandona *et al.,* 2000 and Dandona *et al.,* 2000). A comprehensive survey report in Madhurai, also in Tamil Nadu, reported the prevalence of POAG to be three times that for PACG (Ramakrishnan

et al., 2003). The prevalence of POAG in South Indian population has been reported to be 1.62% (Dandona *et al.,* 2000; Vijaya *et al.,* 2005). According to a recent prevalence report, it is estimated that by the year 2010 around 60.5 million people worldwide will be afflicted with glaucoma, and this includes both POAG and primary angle closure glaucoma (PACG), and this will rise to 79.6 million by the year 2020. Of this, 74% will have open angle glaucoma (Quigley *et al.,* 2006).

The glaucomas are classified into primary and secondary based on events leading to the etiology underlying the disorder that leads to an alteration in aqueous humor dynamics (Shields, 1998). Gonioscopically, based on the alteration in the anterior chamber angle leading to a rise in IOP, there are two main forms of glaucoma: POAG and PACG. In POAG, there is an increase in resistance to the outflow of aqueous humor due to obstruction at the trabecular meshwork. On the other hand, PACG is an anatomical disorder of the anterior segment of the eye characterized by permanent closure of part of the filtration angle as a result of the iris apposition to the trabecular meshwork (Ritch *et al.,* 1989).

POAG represents a common form of primary glaucoma and is characterized by loss of peripheral visual function and damage of the optic disc. (Quigley *et al.,*1996). Thirteen chromosomal loci, viz., *GLC1A* - *GLC1M* have been mapped in POAG/normal tension glaucoma (NTG) patients by linkage. Of these, genes at *GLC1A* (*MYOC)*, *GLC1E* (*OPTN*) and *GLC1G* (*WDR36*) have been cloned and characterized (Stone *et al.,*

1997, Rezaie *et al.,* 2002, Monemi *et al.,* 2005). Myocilin (*MYOC*) initially known as the trabecular meshwork-inducible glucocorticoid response (*TIGR*) gene, is the first gene to be identified in POAG (Stone *et al.,* 1997). Mutations in *MYOC* have been reported from almost all parts of the world. The *OPTN* gene is primarily associated with NTG. The *CYP1B1* gene at *GLC3A* locus was initially identified as candidate gene for primary congenital glaucoma (PCG) (Stoilov *et al.,* 1997). But recent studies have indicated its involvement in juvenile open angle glaucoma (JOAG) through a digenic mechanism along with mutant alleles in *MYOC* (Vincent *et al.,* 2002, Melki *et al.,* 2004) and proposed that *CYP1B1* may act as a modifier of *MYOC* expression and that these two genes may interact through a common pathway. Faucher *et al* (2002) reported two mutations in *MYOC* in two of the PACG cases, one each with Pro481Leu and Gln368Stop after screening 17 PACG cases. Another report by Vincent *et al* (2002) reported Gly399Val in a patient with mixed POAG-PACG phenotype. Though the studies had a limitation of small sample size, they provided initial evidence of the involvement of *MYOC* as a candidate gene in PACG. Aung *et al* (2005) screened *MYOC* in a cohort of PACG cases in the Chinese population and reported that *MYOC* might not be a candidate gene in their cohort as the sequence alterations were identified among the normal Chinese subjects as well. An earlier study from our centre had reported the involvement of mutant alleles in *MYOC* and *CYP1B1* in a single POAG case (Chakrabarti *et al.,* 2005). In the present study

involvement of mutant alleles in the genes was found in a PACG case as well.

MYOC mutations exist in approximately 3% of late-onset POAG patients and a greater proportion (6.38%) of JOAG patients (Alward *et al.,* 2002). Till date more than 73 mutations are known and most of these are missense mutations. Majority (63) of the mutations were found in the olfactomedin-like domain (Gong *et al.,* 2004) suggesting that this is a functionally important domain (Adam *et al.,* 1997). A larger study on 1703 glaucoma patients from five different populations observed the overall frequency of myocilin mutations ranged from 2 - 4% (Fingert *et al.,* 1999). The most common *MYOC* mutation was Gln368Stop observed in 1.6% of glaucoma probands and in all the groups except Japanese (Fingert *et al.,* 1999). The second most common mutation Arg46Stop was shared only by Asian populations. The Gln48His mutation was found only among Indian population (Mukhopadhyay *et al.,* 2002; Sripriya *et al.,* 2004; Chakrabarti *et al.,* 2005) which is also the finding of the present study.

MYOC mutations accounted for 1.5% of the POAG cases among the Chinese population (Pang *et al.,* 2002). Aung *et al* (2005) screened 106 PACG patients and observed the disease causing variants in the controls as well. *MYOC* mutations ranged from 2.9 - 4% (Suzuki *et al.,* 1997; Kubota *et al.,* 2000) among the Japanese POAG patients.

The Pro370Leu was found to be associated with juvenile open angle glaucoma (JOAG), high IOP and poor response to treatment

(Taniguchi *et al.,* 1999). A recent report from Taiwan had reported the mutation frequency of *MYOC* to be 12.5% (Yen *et al.,* 2007). In addition, they suggested that Arg46Stop mutation was the predominant mutation with a frequency of 6.25% and is a hot-spot in Taiwanese patients with JOAG (Yan *et al.,* 2007). Haplotype analysis have indicated that the Gln368Stop and Asn480Lys mutation carriers shared a similar haplotype background, possibly due to a common founder effect (Fingert *et al.,* 1999, Adam *et al.,* 1997; Brezen *et al.,* 1998).

In India, mutations in *MYOC* accounted for 0.8-7.14% of all POAG cases (Kumar *et al.,* 2007; Mukhopadhyay *et al.,* 2002; Kanagavalli *et al.,* 2003; Sripriya *et al.,* 2004) and Q48H was the most prevalent mutation (Chakrabarti *et al.,* 2005). The phenotype associated with Q48H mutation across different Indian populations (Sripriya *et al.,* 2004; Mukhopadhyay *et al.,* 2002) was heterogeneous. The mutation manifested both in JOAG and POAG phenotypes with ages at onset ranging from 17 – 70 years along with IOPs of 21 - 38 mm Hg. Their cup to disc ratios ranged from 0.4 to 0.9. Four of 6 patients underwent trabeculectomy. A marked severity was noted with respect to visual field defects in individuals with POAG than JOAG, there was no other significant difference in the clinical presentation in patients with Q48H.

In addition to the potential disease causing mutations, several SNPs and synonymous codon changes were observed in the promoter [MYOC.mt1 (–1000C>G), –83G>A] and coding regions of *MYOC*

(Arg76Lys, Gly122Gly, Tyr347Tyr, Thr351Thr) in POAG (Fingert *et al.,* 1999). While there was no association of the MYOC.mt1 polymorphism with the disease phenotype in the Turkish population (Ozgul *et al.,* 2005), using the Cox proportional hazards model, Polansky *et al* (2003) showed that MYOC.mt1 (+) variant accelerates the worsening of both optic disc and visual field defects. Although the allele frequencies were not different between French patients and controls, MYOC.mt1 carriers exhibited poor IOP control on medication and greater degree of visual field loss in their cohort (Colomb *et al.,* 2001). Also another report associated the MYOC.mt1 variant to increase in the worsening of the optic disc and visual defects (Polansky *et al.,* 2003). The –83 G>A and R76K variations were not associated with the disease phenotype in both POAG and PACG phenotypes (Mukhopadhyay *et al.,* 2002, Pang *et al.,* 2002; Aung *et al.,* 2005).

CYP1B1 mutations were identified in JOAG patients in French population with a mutation frequency of 4.6% (Melki *et al.,* 2004), in Spanish POAG and HTG cases with a frequency of 10.9% and 8.1% (Lopez-Garrido *et al.,* 2006). Among the Indian POAG cases mutations were found in 4.5% of cases (Acharya *et al.,* 2006). A recent report by Kumar *et al.,* (2007) who screened all the four candidate genes, *CYP1B1*, *MYOC*, *OPTN* and *OPTC* in adult onset POAG cases, found a higher mutation frequency of 10.76% within *CYP1B1*. Screening for the *CYP1B1* gene in our cohort had shown a mutation frequency of 20.2% (10 different

mutations, in 22 probands with OAG). Four mutations were novel (Q144R, W434R, c.1657 del 5bp and F445C). The R368H mutation and the E229K mutations were most common with frequency of 7.3% and 5.5% respectively, in POAG cases. These mutations were also predominant in Indian PCG patients (Reddy *et al.,* 2003). However, there are no reports on the involvement of *CYP1B1* among PACG cases.

Monemi *et al.,* (2005) mapped the third candidate gene in POAG onto 5q (*GLC1G*) known as *WDR36* which comprises the T-cell activation WD repeat-containing protein and is highly co-regulated with *IL*-12 (Mao *et al.,* 2004). Mutations in *WDR36* have been reported with a frequency of 17% in an earlier report on a patient cohort from the United States (Hauser *et al.,* 2006) but subsequent reports concluded that the *WDR36* may not be a candidate gene in POAG as the pathogenic mutations were also observed in the control individuals (Fingert *et al.,* 2007). This could be attributed to a geographic and ethnic heterogeneity among the patient cohorts in different studies (Kramer *et al.,* 2006; Fingert *et al.,* 2007).

The role of SNPs in the promoter and intragenic regions of *IL-1*β (-511 C>T and +3953 C>T), *IL-1*α (-889 C>T), p-21 (Ser31Arg), *MTHFR* C677T and *MMP-9* (-1590 C>T and Q279R) have been evaluated in association to POAG and PACG cases in different populations across the world by association studies. The involvement of immune system in optic atrophy has been postulated as one of the possible mechanism (Wax, 2000). It is hypothesized that glaucoma might result due to the death of

ganglion cells by apoptosis due to the aberrant immune mechanisms (Lin *et al.,* 2003). A study on screening of *IL-1* polymorphisms in a Chinese cohort of POAG patients showed a significant association of *IL*-1β +3953 C/T polymorphism to POAG (Lin *et al.,* 2003). Subsequently, another study on Taiwanese cohort showed a significant association of C/T polymorphism at position *IL*-1β (-889) to POAG patients (Wang *et al.,* 2006) and the same group did not find the association to NTG cases (Wang *et al.,* 2007). Recently, How *et al* (2007) reported no significant association of Interleukin-1 polymorphisms, *IL*-1β (-511) C/T, *IL*-1β (+3953) C/T and *IL*-1β (-889) C/T to either POAG or PACG phenotypes. On the basis of these reports, the association of these SNPs were assesed in our cohort of POAG and PACG cases**.**

p21 gene is an important component in the apoptotic pathway (Levine, 1997) and an SNP at codon 31 position, a C to A transversion change, results a Ser to Arg amino acid substitution. Association of Arg form of p21 to POAG was first reported by a Chinese group (Tsai *et al.,* 2004). This study was followed by another study on Caucasian POAG population that did not find an association (Ressiniotis *et al.,* 2005). In the present study the Ser/Arg polymorphism was evaluated in our cohort of POAG cases.

The enzyme 5-Methylenetetrahydrofolate reductase (*MTHFR*) catalyses methylation of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which contributes a methyl group in the conversion

of homocysteine to methionine. (Kim, 1999). Frosst, (1995) identified a 677C>T substitution in the exon 4, that converts the alanine 222 to a valine (A222V) residue and is responsible for the synthesis of a thermolabile form of *MTHFR*. Junemann *et al* (2005) evaluated the prevalence of the 677C>T single-nucleotide polymorphism in the *MTHFR* gene in POAG and pseudoexfoliation open-angle glaucoma (PEXG) and found a significant association of *MTHFR* SNP to the POAG cases. They concluded that the *MTHFR* C677T variant leading to moderate hyperhomocysteinemia might play a role as a genetic risk factor in the pathogenesis of POAG. Similar studies on association of *MTHFR* to POAG, NTG and PEXG were conducted by different groups across different populations in the world. These reports showed no association of the SNP to any of the above glaucoma phenotypes (Fingert *et al.,* 2006; Mabuchi *et al.,* 2006; Mossbock *et al.,* 2006). The *MTHFR* C677T variant therefore was evaluated among the POAG cases.

MMP-9 is associated with the leaking glaucoma filtering blebs (Chintala *et al.,* 2005) and NTG (Golubnitschaja *et al.,* 2004). The -1590 C/T polymorphic site in the promoter region is an important regulatory element that appears to be the binding site for the transcription repressor protein (Zhang *et al.,* 1999). Also, a transition from A to G at nucleotide 855 in exon 6 resulted in the substitution of glutamine for arginine at codon 279 and was found to be significantly associated to PACG (Wang *et al.,* 2006). Based on these reports we investigated the sequence

variations of *MMP-9* -1590 C/T in the promoter and codon-6 SNP Q279R through a case control study in the PACG patients.

Mutations in *MYOC* and *CYP1B1* have been implicated in POAG and PCG but its involvement in PACG has not yet been demonstrated. The precise role of *MYOC* is poorly understood but the commonality of some clinical features like raised IOP among these phenotypes might indicate a common molecular mechanism due to the involvement of similar gene(s). While the molecular genetics of POAG is well documented in different populations including the Indian population, there is only one report on molecular genetics of PACG (Aung *et al*., 2005). The present study is an attempt to analyze the molecular genetics of late age onset primary glaucomas with the following aims,

Objectives:

1. To screen *MYOC* and *CYP1B1* in POAG, PACG and PAC cases.
2. To screen single nucleotide polymorphisms (SNPs) in candidate genes, *IL-1*β (-511C>T and +3953 C>T), *IL-1*α (-889C>T), *MTHFR (Ala222Val, C>T), p-21* (Arg31Ser, C>A) and *MMP-9* (-1590, C>T, and Gln279Arg, A>G) in POAG and PACG cases.
3. To understand the association of genotype with phenotype in each category.

Methodology

The study protocol was approved by Institutional Review Board and adhered to the guidelines of Declaration of Helsinki. Based on the pre-defined inclusion criteria, clinically diagnosed cases (independently confirmed by two clinicians) of POAG (n=109), JOAG (n=34), PACG (n=113) and PAC (n=17) were included in the study. Of the POAG cases, only 48 cases were screened for mutations in the candidate genes *MYOC* and *CYP1B1* as the remaining were screened earlier. One hundred and thirteen ethnically matched normal subjects who satisfied the inclusion criteria were enrolled for the study. Blood samples were collected from patients and controls with prior informed consent. Genomic DNA was extracted by phenol-chloroform method and the coding regions of the candidate genes were amplified by the polymerase chain reaction (PCR). Mutation screening was performed by single strand conformation polymorphism (SSCP) in case of *MYOC* and direct sequencing in *CYP1B1*. Variants observed in SSCP were characterized by resequencing. Association studies of SNPs in *IL-1α, IL-1β, p-21*, *MTHFR* and *MMP-9* were performed by PCR based restriction digestion method. Gene-counting method was used to estimate the allele and gene frequencies. The observed genotypes were compared with phenotypic traits like, IOP at presentation, visual field defects and CD ratio of the severe most eye. The Chi square test of significance was used to

determine the statistical significance in the distribution of allele and genotype frequencies between patients and controls. Odds ratios along with 95% CI were calculated for the variant genotypes of the candidate SNPs.

Results and Discussion

MYOC:

MYOC screening revealed 8.3% (4/48) mutations in POAG, 5.94% (6/101) in PACG and 11.7% (2/17) of PAC cases. The Arg33Lys mutation was novel in POAG and Pro56Thr was novel in PACG. The overall frequency of MYOC mutations in POAG was similar to other reports from India (Mukhopadhyay et al., 2002, Kanagavalli et al., 2003, Sripriya et al., 2004), and abroad (Gong et al., 2004). The Q48H mutation was the most prevalent mutation in POAG in Indian populations (Sripriya et al., 2004, Mukhopadhyay et al., 2002) and was observed in 5% PACG cases. The Thr353Ile mutation in MYOC was observed in an autosomal dominant JOAG case and the proband presented with a severe phenotype with respect to the IOPs of 50 and 42 mm Hg in the right and left eye, C:D ratio of 0.9:1 in both the eyes along with severe visual acuity of 20/800 in the right eye and severe visual field defects in both the eyes. The mutation frequency in the present study is similar to the earlier studies from India (Kumar et al., 2007; Mukhopadhyay et al., 2002; Kanagavalli et al., 2003; Sripriya et al., 2004) and the Q48H was the most prevalent mutation among the PACG and POAG cases (Chakrabarti et al., 2005).

Two different sporadic cases in POAG and PACG were found to harbor heterozygous mutant alleles of both *MYOC* and *CYP1B1.* The POAG case exhibited the Q368X (*MYOC*) and R368H (*CYP1B1*) while the PACG patient had Q48H (*MYOC*) and E229K (*CYP1B1*) mutation. Unlike an earlier report (Vincent *et al.,* 2002) the phenotypes of these patients did not manifest severity with respect to intraocular pressure and C:D ratio. Both the patients had the disease manifested in the fifth decade of their life. The promoter (-1000C>G, -83G>A) and coding (R76K) polymorphisms and the synonymous codon changes (Gly122Gly, Tyr347Tyr, Thr351Thr) did not exhibit any association to POAG and PACG.

CYP1B1

CYP1B1 screening revealed 12.5% (6/48) mutation frequency in POAG, which is relatively higher compared to previous studies (Melki *et al.,* 2004, Lopez-Garrido *et al.,* 2006, Acharya *et al.,* 2006, Kumar *et al.,* 2007). This is perhaps the first study to screen *CYP1B1* in PACG cases that had a frequency of 12.2% (11/90). All the mutations were missense mutations in POAG (G61E, P193L, S239R and R368H) and in PACG (Q144R, E229K and R368H) cases. PAC cases did not exhibit any mutations in *CYP1B1*. All these mutations were earlier reported in primary congenital glaucoma cases (Stoilov *et al.,* 1997; Bejjani *et al.,* 1998; Bejjani *et al.,* 2000; Belmouden *et al.,* 2002; Panicker *et al.,* 2002; Reddy *et al.,* 2004).

The R368H mutation which was reported as the most prevalent mutation in PCG (Reddy *et al.,* 2003) and more recently in POAG (5.76%) and JOAG (3.3%) from our centre (Chakrabarti *et al.,* 2007) is also the prevalent mutation among PACG cases (5.5%) along with E229K (5.5%). Most of these mutations were observed in heterozygous condition, but two probands were homozygous for these mutations. Both the probands harboring these mutations (G61E and P193L) had relatively severe phenotypes. The proband with homozygous mutation G61E manifested the disease at an earlier age of 23 years and had an IOP of 46 mm Hg in the right eye and a cup to disc ratio of 0.9:1 in both the eyes, and a visual acuity of 20/1200 in the right eye. His affected sibling also harbored the homozygous mutation. The proband with homozygous mutation P193L manifested an IOP of 28 mm Hg in the right eye and had a C:D ratio of 0.9:1 in both the eyes and a visual acuity of 20/2400 in the right eye. Individuals harboring the same mutation exhibited variable phenotypes.

The mutation frequency in POAG was 12.5% compared to the French (4.6%; Melki *et al.,* 2004), Spanish (10%; Lopez-Garrido *et al.,* 2006), Eastern Indian (4.5%; Acharya *et al.,* 2006) and Southern Indian (11.5%; Kumar *et al.,* 2007) populations. While, there are a few reports on *CYP1B1* screening in POAG, the present study demonstrated its involvement in PACG as well. None of the SNPs in *CYP1B1* were significantly associated with the disease phenotype.

SNP screening in other candidate genes: The SNPs, in *IL-1β* (-511 C>T and +3953 C>T), *IL-1α* (-889 C>T), *p*-21 (Ser31Arg), *MTHFR* C677T did not show any significant association to POAG. Similarly, the SNPs in *IL-1β* (-511C>T and +3953 C>T), *IL-1α* (-889 C>T) and *MMP-9* (-1590 C>T and Q279R) were not associated to PACG. Earlier studies had reported a significant association of these SNPs with certain phenotypic traits such as raised IOP and worsening of visual fields in POAG (Junemann *et al.,* 2005; Lin *et al.,* 2003; Wang *et al.,* 2006; Tsai *et al.,* 2004). The association of most of these SNPs to POAG or PACG pathogenesis could not be replicated across different populations, which could be attributed to ethnic variations. However, they have provided some insights in glaucoma pathogenesis. Further studies from other populations are warranted to determine their role in glaucoma pathology.

Conclusion

The genetic data based on the results obtained from the current study provides information on the mutation spectrum of candidate genes in POAG and PACG cases in Indian population. The data highlights the complex molecular mechanism underlying glaucoma and the associated clinical and genetic heterogeneity. This data may be helpful for developing inexpensive molecular diagnostic methods for screening some common mutation in the predisposed families.

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COMPOSITIONS OF THE REAGENTS

I.1 DNA EXTRACTION

10XPBS:

Nacl (Qualigens Fine Chemicals, Mumbai, India) - 4gm

KCl (Qualigens Fine Chemicals, Mumbai, India) - 0.2gm

Na_2HPO_4 (Qualigens Fine Chemicals, Mumbai, India) - 1.44gm

KH_2PO_4 (Qualigens Fine Chemicals, Mumbai, India) - 0.24gm

Milli Q water - 80ml

The pH was adjusted to 7.4 with 1M HCl and the volume was made upto 100ml with autoclaved Milli Q water.

EXTRACTION BUFFER

1M Tris (pH 8.0) (Sigma chemicals Co. St.Louis, USA) - 1.0ml

0.5 M EDTA (Sigma chemicals Co. St.Louis, USA). - 20ml

10%SDS (SISCO research laboratories PVT LTD Bombay. India) - 5.0ml

The volume was made up to 100ml with autoclaved Milli Q water.

I.2 SILVER STAINING

Fixative (200ml):

Glacial Acetic acid (Qualigens Fine Chemicals, Mumbai, India) - 1ml

Absolute alcohol (Qualigens Fine Chemicals, Mumbai, India) - 20ml

The volume was made up to 200ml with autoclaved Milli Q water.

Silver stain (200ml):

Silver nitrate solution (0.2%)

(Qualigens Fine Chemicals, Mumbai, India) - 20ml

The volume was made up to 200ml with autoclaved Milli Q water.

Developer (200ml):

NaOH (1.5%) (Qualigens Fine Chemicals, Mumbai, India) - 5ml

Formaldehyde (0.4%) (Qualigens Fine Chemicals, Mumbai, India) - 600μl

The volume was made up to 200ml with autoclaved Milli Q water.

I.3 ACRLYLAMIDE-BIS-ACRLYLAMIDE SOLUTION (29:1) (for PAGE)

Acrlylamide (Sigma chemicals Co. St.Louis, USA) - 29gm

Bis-acrlylamide (Sigma chemicals Co. St.Louis, USA) - 1gm

The volume was made up to 100ml with autoclaved Milli Q water

I.4 ACRLYLAMIDE-BIS-ACRLYLAMIDE SOLUTION (19.5:0.5)

(for SSCP- PAGE)

Acrlylamide (Sigma chemicals Co. St.Louis, USA) - 19.5gm

Bis-acrlylamide (Sigma chemicals Co. St.Louis, USA) - 0.5gm

The volume was made up to 100ml with autoclaved Milli Q water

I.5 50X TAE AGAROSE GEL LOADING BUFFER (1lt)

Tris base (Sigma chemicals Co. St.Louis, USA) - 242 gm

Glacial acetic acid - 57.1ml

0.5M EDTA (pH-8.0) (Sigma chemicals Co. St.Louis, USA). - 100ml

I.6 10X TBE PAGE GEL LOADING BUFFER

Tris base (Sigma chemicals Co. St.Louis, USA) - 108gm

Boric acid (Sigma chemicals Co. St.Louis, USA) - 55gm

0.5M EDTA (pH-8.0) (Sigma chemicals Co. St.Louis, USA). - 40ml

I.7 6X SAMPLE LOADING BUFFER:

De-ionised water - 7.0ml

Sucrose (Sigma chemicals Co. St.Louis, USA) - 2.5gm

Bromophenol blue

(USB, Amersham Biosciences, New Jersey, USA) - 25.0mg

The volume was made up to 10ml with autoclaved Milli Q water

I.8 FORMAMIDE LOADING BUFFER

Bromophenol blue

(USB, Amersham Biosciences, New Jersey, USA) - 5.0mg

Xylene Cyanol

(USB, Amersham Biosciences, New Jersey, USA) - 5mg

Formamide (Qualigens Fine Chemicals, Mumbai, India) - 5ml

I.9 ETHIDIUM BROMIDE:

5mg of Ethidium bromide (USB, Amersham Biosciences, New Jersey, USA) was added in 1ml of autoclaved Milli Q water.

Publications and Presentations

LIST OF PRESENTATIONS

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

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

3. Presented a poster entitiled, "*Analysis of the Methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism in Indian Primary Open-angle Glaucoma patients,''* at the International symposium on "Human Genomics and public Health," 31st Annual conference of ISHG-2006 held at New Delhi.

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

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

6. Presented a poster entitled, *"Molecular Genetic Study of Nanophthalmos: Involvement of MYOC."* at the 10th Asian and Oceanian Biochemists and Molecular Biologists (FAOBMB) Congress, held at the IISc, Banglore, India, 2003.

LIST OF PUBLICATIONS

1. Subhabrata Chakrabarti, Kiranpreet Kaur, Sreelatha Komatireddy, Moulinath Acharya, **Koilkonda R. Devi**, Arijit Mukhopadhyay, Anil K. Mandal, Seyed E. Hasnain, Garudadri Chandrasekhar, Ravi Thomas, Kunal Ray. ***Gln48His is the prevalent myocilin mutation in primary open angle and primary congenital glaucoma phenotypes in India.*** **Mol Vis. 2005**;11:111-113.

2. Subhabrata Chakrabarti, **Koilkonda R. Devi,** Sreelatha Komatireddy, Kiranpreet Kaur, Rajul S. Parikh, Anil K. Mandal, Garudadri Chandrasekhar, and Ravi Thomas. **Glaucoma-Associated *CYP1B1* Mutations Share Similar Haplotype Backgrounds in POAG and PACG Phenotypes**. ***Invest Opht -almol Vis Sci,*** 2007;48:5439-5444.

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

Subhabrata Chakrabarti,[1] *Koilkonda R. Devi,*[1] *Sreelatha Komatireddy,*[1] *Kiranpreet Kaur,*[1] *Rajul S. Parikh,*[2] *Anil K. Mandal,*[2] *Garudadri Chandrasekhar,*[2] *and Ravi Thomas*[2]

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

Methods. The entire coding region of *CYP1B1* was screened by resequencing in 224 unrelated cases of POAG ($n = 134$) and PACG ($n = 90$) and 200 ethnically matched normal control subjects from Indian populations. Six intragenic single nucleotide polymorphisms (SNPs) in *CYP1B1* (−13T>C, R48G, A119S, V432L, D449D, and N453S) were used to generate haplotype data for the cases and controls and linkage disequilibrium (LD) and haplotype analysis were performed with Haploview software, which uses the EM (expectation–maximization) algorithm.

Results. The frequency of *CYP1B1* mutations was higher among POAG (18.6%; 95% CI, 12.9–26.1) than PACG (11.1%; 95% CI, 6.1–19.3) cases. There was a marked allelic heterogeneity, and the Arg368His was the most prevalent mutation across both the phenotypes. The spectrum of *CYP1B1* mutations was largely similar across different POAG populations. Haplotypes generated with intragenic SNPs indicated the C-C-G-G-T-A to be a risk haplotype associated with *CYP1B1* mutations in POAG ($P = 0.006$) and PACG ($P = 0.043$), similar to that observed in cases of primary congenital glaucoma worldwide.

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

Glaucoma comprises a group of clinically and genetically heterogeneous optic neuropathies characterized by a progressive loss of vision and is the second leading cause of irreversible blindness worldwide.[1,2] Based on gonioscopic findings, primary glaucomas are classified as primary open-angle glaucoma (POAG; OMIM 137750; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and primary angle-closure glaucoma (PACG). Both have characteristic optic nerve head changes, degeneration of retinal ganglion cells, and visual field loss, but PACG also has a closed angle or peripheral anterior synechia (PAS) on gonioscopy.[3] Both of these conditions may be associated with elevated intraocular pressure (IOP) due to the obstruction of outflow.[4] POAG affects 33 million worldwide and is more common in the West,[2,5,6] whereas PACG is relatively more common among Asian populations.[7,8]

Genetic heterogeneity is well documented in POAG, and 11 chromosomal loci (*GLC1A-GLC1K*) have been mapped by linkage analysis.[9] Of these, only three genes harboring *GLC1A* (Myocilin; *MYOC*; OMIM 601652),[10] *GLC1E* (Optineurin; *OPTN*; OMIM 602432),[11] and *GLC1G* (*WDR36*; OMIM 609669)[12] have been characterized. In addition, approximately 15 candidate genes have been identified by association studies that require a thorough replication in different populations.[9] Glaucoma being a complex disease would be attributed to multiple gene variants with various magnitudes of effect.[13]

Although the human cytochrome P450 gene *CYP1B1* (OMIM 601771) has been implicated in primary congenital glaucoma (PCG; OMIM 231300) worldwide,[14–16] it has been relatively less explored in POAG and not at all in PACG. An initial study implicated the involvement of *CYP1B1* with *MYOC* through a digenic mechanism in a family with juvenile-onset open angle glaucoma (JOAG) and suggested that *CYP1B1* is a modifier of *MYOC* expression. It was also observed that affected subjects harboring a mutant *CYP1B1* allele in this family had an earlier age at onset than those with only a mutant *MYOC* allele.[17] These findings led to the screening of *CYP1B1* as a candidate gene among the patients with POAG and largely among those with JOAG. The frequency of *CYP1B1* mutations varied in patients from Canada (5.0%),[17] France (4.6%),[18] Spain (10.9%),[19] Eastern India (4.5%),[20] and Southern India (10.8%).[21] The differences in mutation frequency could be partly explained by the definition of disease used in these studies. The Canadian patients had JOAG[17] whereas the French patients had POAG, but elevated IOP was not an inclusion criterion,[18] similar to studies from Eastern[20] and Southern[21] India. The results from these studies indicate a minor involvement of *CYP1B1* among JOAG and late-onset POAG cases and suggest a possible role of this gene in glaucoma pathogenesis.

We have reported the extent of *CYP1B1* mutations along with their structural properties in PCG.[22,23] We have also demonstrated a global clustering of these mutations on specific haplotype backgrounds, irrespective of geographic location, that could be useful in predictive testing.[16] Herein, we report an extensive screening of the *CYP1B1* gene in a cohort of patients with POAG or PACG from India, to determine its mutation spectrum and understand the haplotype backgrounds of these mutations.

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METHODS

Clinical Details of the Subjects

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

POAG (Including JOAG)

The diagnosis of POAG was based on open angles on gonioscopy, an IOP >21 mm Hg, and characteristic optic disc changes and corresponding visual field defects in patients >35 years of age. Visual field defects were considered to be glaucomatous if they were consistent with optic disc damage and met at least two of the criteria laid out by Anderson and Patella.[24] The presence of a visual field defect required confirmation by a repeatable field performed within 2 weeks of the first reliable visual field result showing the defect. The field defects were further classified as mild, moderate, or severe.[25] Such findings in patients between 5 and 35 years of age were labeled as JOAG. As the presence of visual field defects was one of the inclusion criteria, only patients older than 10 years were included in the study.

Primary Angle-Closure Glaucoma

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

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

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

Molecular Analysis

Peripheral blood samples (5–10 mL) were collected from each subject by venipuncture, with prior informed consent. DNA was extracted by standard protocols[26] and the entire coding region of *CYP1B1* was amplified using appropriate oligonucleotide primers and PCR protocols, as published earlier.[27] The amplicons were purified (SigmaSpin columns; Sigma-Aldrich, St. Louis, MO) and bidirectionally sequenced using dye termination chemistry (BigDye on a 3100 DNA Analyzer; Applied Biosystems, Inc. [ABI], Foster City, CA), according to the manufacturer's protocol. Sequencing analysis software was used to read the individual sequences. Six mutations (G61E, Y81N, Q144R, P193L, E229K, and R368H) were further confirmed by restriction digestion of the amplicon with appropriate restriction enzymes as published earlier,[18,22,27] whereas the remaining five mutations were verified by resequencing. Multiple sequence alignment of the human CYP1B1 protein was performed along with other CYP1 protein across different families, to check for the conservation of the residues. The SIFT (sorting tolerant from intolerant) homology tool (http://blocks.fhcrc.org/sift/SIFT.html/ provided in the public domain by the Fred Hutchinson Cancer Research Center, Seattle, WA) was used to assess the effect of the substituted amino acid on the CYP1B1 protein, and a threshold score of less than 0.05 was considered to be deleterious to the protein.[28]

Statistical Analysis

The maximum-likelihood estimates of allele frequencies, Hardy-Weinberg equilibrium, and haplotype frequencies were estimated from the genotype data at six single-nucleotide polymorphism (SNP) loci using Haploview software, which uses the EM (expectation–maximization) algorithm.[29] Pair-wise linkage disequilibrium (LD) between the individual SNPs was calculated using the LD-plot function of this software. The odds ratios were calculated, to assess the risk of the individual genotypes at all six SNP loci. Clinical parameters, such as IOP at presentation, cup-to-disc ratio, and visual field defects for the worst eye were considered when correlating the genotype with the phenotype. All calculations were performed with commercial software (SPSS ver. 14; SPSS, Chicago, IL).

RESULTS

Mutation Screening of *CYP1B1* in POAG and PACG

The study cohort conformed to Hardy-Weinberg equilibrium. A total of 11 *CYP1B1* mutations were observed, of which 4 (Q144R, W434R, F445C, and g.8148-8152del5bp) were novel. The overall spectrum of *CYP1B1* mutations observed in POAG and PACG is demonstrated in Figure 1 (the electropherograms

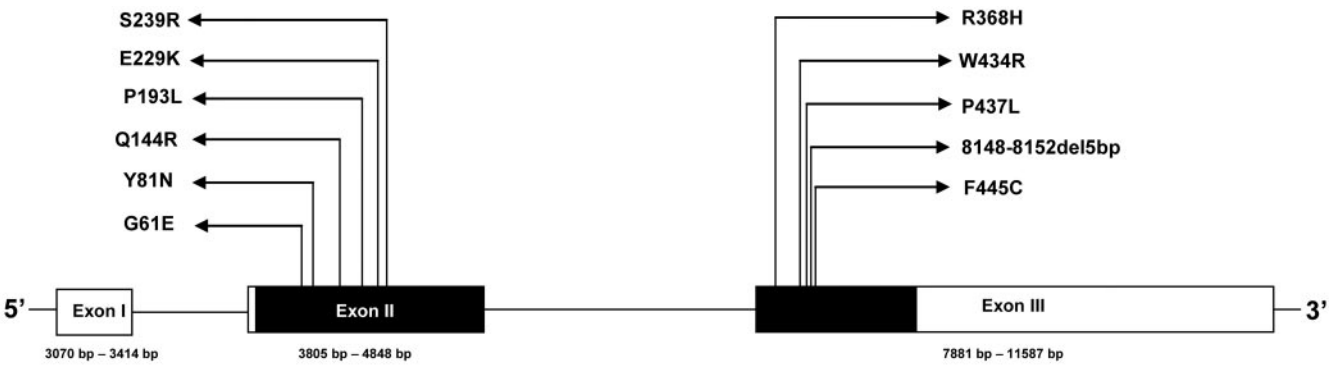

FIGURE 1. Schematic representation of the *CYP1B1* mutations observed in POAG and PACG. *Arrows*: location of the mutations within the *CYP1B1* gene. *Shaded regions*: coding regions in exon II and III of *CYP1B1* gene.

of all 11 mutations are provided in Supplementary Fig. S1, online at http://www.iovs.org/cgi/content/full/48/12/5439/DC1). The frequency of mutations was higher in POAG (18.6%; 25/134) than in PACG (11.1%; 10/90). Arg368His was the most prevalent mutation across both the phenotypes, similar to earlier studies of PCG from India.[16,22] Further details are provided in Table 1. Allelic heterogeneity was relatively more in POAG than in PACG. SIFT scores indicated a deleterious effect for all the mutations except E229K.

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

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

The *CYP1B1* mutation frequencies were different across all the studies performed on POAG in Indian populations.[20,21] Of interest, the investigators in the study from Southern India found a carrier rate of 6.4% and 0.7% for the E229K and the R368H mutations, respectively, in their control populations[21] that was not observed in the cohorts from Eastern India[20] or in the present study.

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

Linkage Disequilibrium and Haplotype Analysis at the *CYP1B1* Locus

Six intragenic SNPs were typed at the *CYP1B1* locus, to generate haplotypes among the cases and controls. Pair-wise LD analysis indicated strong LD ($D' = 1$) at two clusters, between three SNPs (−13T>C, R48G, and A119S) and between the two SNPs V432L and D449D (data not shown). The measure of LD between the other SNPs was similar to that in an earlier study.[35]

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

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

Nucleotide Change	Location	Amino Acid Change	Restriction Site Generated†	SIFT Score	Number of Patients with the Mutation			Controls with the Change	Mutation Previously Observed in Other Populations	
					JOAG	POAG	PACG		JOAG/POAG	PCG
g.3987G>A	Exon II	G61E	*TaqI* (+)	0.00	2/30	—	—	0/200	Spain[19]	India,[22] Turkey,[30] Ecuador,[31] Saudi Arabia,[30,32] Morocco,[33] Kuwait[34]
g.4046T>A	Exon II	Y81N	*HaeII* (−)	0.00	1/30	—	—	0/200	Spain,[19] France[18]	Germany[35]
g.4236A>C	Exon II	Q144R*	*MspA1I* (−)	0.01	1/30	1/104	1/90	0/200	—	—
g.4381C>T	Exon II	P193L	*Eco81I* (+)	0.01	—	3/104	—	0/200	India[21]	India[22]
g.4491G>A	Exon II	E229K	*Eam1104I* (−)	0.10	1/30	4/104	4/90	0/200	Spain,[19] France,[18] India[20,21]	India,[22] France[36]
g.4520A>C	Exon II	S239R	—	0.00	—	1/104	—	0/200	—	India[22]
g.7940G>A	Exon III	R368H	*TaaI* (−)	0.00	1/30	6/104	5/90	0/200	India[20,21]	India,[22] Brazil,[37] Saudi Arabia,[30,32] Kuwait[34]
g.8137T>C	Exon III	W434R*	—	0.00	—	1/104	—	0/200	—	—
g.8147C>T	Exon III	P437L	—	0.00	—	1/104	—	0/200	—	India,[22] Brazil,[37] Turkey[30]
g.8171T>G	Exon III	F445C*	—	0.03	—	1/104	—	0/200	—	—
g.8148-8152del5bp	Exon III	Frame shift*	—		1/30	—	—	0/200	—	—
Total					7/30	18/104	10/90	0/200		

* Novel mutation.

† Restriction enzymes that were used to confirm the different mutations: (+) gain and (−) loss of restriction sites.

TABLE 2. Distribution of Mean Ages at Onset and IOPs at Presentation among JOAG, POAG, and PACG Groups

Parameters	JOAG Cases with *CYP1B1* Mutations (n = 7)	JOAG Cases without *CYP1B1* Mutations (n = 23)	POAG Cases with *CYP1B1* Mutations (n = 18)	POAG Cases without *CYP1B1* Mutations (n = 86)	PACG Cases with *CYP1B1* Mutations (n = 10)	PACG Cases without *CYP1B1* Mutations (n = 80)
% Frequency of Cases (95% CI)	23.3 (11.8–40.9)	76.7 (59.1–88.2)	17.3 (11.2–25.7)	82.7 (74.3–88.7)	11.1 (6.1–19.2)	88.9 (80.7–93.8)
Age at Onset (mean years ± SD)	20.1 ± 8.78	20.9 ± 8.31	51.3 ± 12.22	54.1 ± 10.56	57.4 ± 12.43	54.4 ± 11.18
IOP at Presentation (mean mm Hg ± SD)	37.3 ± 8.43	34.1 ± 8.33	24.8 ± 3.04	27.1 ± 6.26	32.6 ± 16.73	32.9 ± 10.11

TABLE 3. Distribution of Estimated *CYP1B1* Haplotype Frequencies among POAG Cases and Controls

Haplotypes	POAG (All Cases)	Controls	P	POAG *CYP1B1*(+)†	Controls	P	POAG *CYP1B1*(−)‡	Controls	P
T-G-T-C-C-A	36.7%	44.8%	0.079	40.1%	44.7%	0.557	36.0%	44.7%	0.072
C-C-G-G-T-A	21.2%	20.1%	0.809	38.4%	20.1%	0.006*	17.9%	20.1%	0.535
C-C-G-C-C-A	22.2%	18.2%	0.287	9.9%	18.3%	0.143	24.3%	18.3%	0.132
C-C-G-C-C-G	17.4%	13.5%	0.252	7.5%	13.3%	0.253	20.6%	13.5%	0.058

* Significant.
† Cases harboring *CYP1B1* mutation.
‡ Cases without *CYP1B1* mutations.

TABLE 4. Distribution of Estimated *CYP1B1* Haplotype Frequencies among PACG Cases and Controls

Haplotypes	PACG (All Cases)	Controls	P	PACG *CYP1B1*(+)†	Controls	P	PACG *CYP1B1*(−)‡	Controls	P
T-G-T-C-C-A	36.8%	44.8%	0.099	44.9%	44.6%	0.978	37.3%	44.8%	0.130
C-C-G-G-T-A	20.8%	20.1%	0.861	39.9%	20.1%	0.043*	18.3%	20.1%	0.660
C-C-G-C-C-A	21.3%	18.5%	0.463	10.1%	18.4%	0.352	22.1%	18.5%	0.372
C-C-G-C-C-G	14.0%	13.3%	0.831	5.0%	13.3%	0.284	14.6%	13.3%	0.698

* Significant.
† Cases harboring *CYP1B1* mutation.
‡ Cases without *CYP1B1* mutations.

DISCUSSION

The present study provides a mutation spectrum of the *CYP1B1* gene in POAG and PACG. The involvement of *CYP1B1* highlights its role as a potential candidate in disease pathogenesis that should be explored further. We observed a higher proportion of mutations in POAG in the present cohort than in other populations (Table 5). The spectrum of mutations observed in the present cohort was largely similar to that in the POAG populations in France, Spain, and India.[18–21] Except for the four novel mutations observed in this study, all other variants were observed earlier in patients with PCG in India and other countries (Table 1).

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

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

It is interesting to note that the prevalent mutation was different across all previously reported POAG populations (Table 5). Also, the frequency of heterozygous mutations was similar across these studies. Although the R368H mutation was common in patients in both the Indian and Canadian studies, it

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

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

* Not available.

was noted in only 2 of the 60 patients with JOAG in the Canadian report.[17] One of the Canadian patients with the R368H mutation had an East Indian/Guyanese ancestry,[17] but we were not able to determine whether this patient shared a common haplotype background with the Indian patient due to unavailability of data.

The median age at onset of the patients with POAG in the present cohort was similar to that of the French sample,[18] but was significantly lower than that of the Spanish[19] patients with POAG. The median age of the Canadian patients was significantly lower, as no cases older than 40 years were enrolled.[17] Another study on patients with POAG from Eastern India[20] reported a mutation frequency (4.5%) similar to that of the French population but a higher mean age (52.43 ± 19.33 years) than that of our cohort. Of interest, the prevalent mutation in the Eastern Indian (S515L)[20] and Southern Indian (E229K)[21] cohort was also different from that in the present study (R368H).

Another interesting observation was the presence of *CYP1B1* mutations on specific haplotypes that was earlier observed in PCG.[16] We noted that C-C-G-G-T-A was the risk haplotype in cases of POAG and PACG with *CYP1B1* mutations. These results were consistent (even after reanalyzing the data set) based on a five-locus haplotype (i.e., C-G-G-T-A), similar to previous studies in different PCG populations worldwide.[16,32,33,35,37] On the other hand, the G-T-C-C-A haplotype that was largely associated with the unaffected controls and PCG cases without *CYP1B1* mutations[16] was similar in frequency in the POAG and PACG cases with *CYP1B1* mutations and the controls (Tables 3, 4). In tune with our previous study on PCG,[16] most of the mutations observed in the POAG and PACG clustered on the C-G-G-T-A haplotype. The R368H mutation, which was the prevalent mutation in POAG and PACG in the present study, similar to PCG in India,[16,22] was found on the background of the C-G-G-T-A haplotype across all these phenotypes. Of interest, this mutation was also found on the same haplotype in Saudi Arabian[32] and Brazilian[37] PCG patients. The G61E mutation in POAG was also found on the C-G-G-T-A haplotype, similar to that observed among the PCG patients from Ecuador,[31] Saudi Arabia,[32] and Morocco.[33] The E229K mutation that was observed on the G-T-C-C-A haplotype among patients with PCG in India[16] and Germany[35] was also seen to harbor the same mutation in POAG and PACG cases. Another striking similarity was the presence of the Y81N mutation in a case of POAG on the G-T-C-C-A haplotype. This mutation was also found on the same haplotype among German patients with PCG.[35]

Similar to our earlier hypothesis on the evolution of *CYP1B1* mutations, we confirm that there is a strong clustering of these mutations on specific haplotype backgrounds, irrespective of geographical location.[16] A larger proportion of mutations were seen on the C-G-G-T-A haplotype and a smaller proportion on the G-T-C-C-A haplotype, further confirming the former to be an ancient haplotype and the latter to be a recent haplotype.[16] A formal haplotype comparison among other POAG cases with *CYP1B1* mutations was not possible due to the unavailability of data from other populations. Based on the present analysis we speculate that the presence of specific *CYP1B1* mutations on specific haplotype backgrounds in PCG worldwide and in patients with POAG and PACG in the present cohort is indicative of common founders. The mutations on these haplotypes would have migrated across different geographical regions due to population movements as reported in PCG in our previous study.[16] Thus, glaucoma-associated *CYP1B1* mutations share a similar haplotype background across POAG, PACG, and PCG.

The role of CYP1B1, particularly in retinoic acid synthesis is pivotal during embryonic development. Recent studies on chick embryogenesis have demonstrated its importance in the dorsoventral patterning of the neural tube that is consistent with its endogenous expression.[38] Several in vitro and in vivo studies in lower organisms have demonstrated the sites of expression of CYP1B1 at different stages of development in the anterior retina and anterior segment of the eye.[38–41] Although these studies have provided convincing evidence of the possible role of CYP1B1, its actual molecular mechanism leading to glaucoma in humans has to be deciphered. Although the functions of *CYP1B1* mutations leading to POAG and PACG remain to be characterized, it is nevertheless an important candidate gene that should be screened in patients with glaucoma worldwide, to establish its involvement in the disease's pathogenesis.

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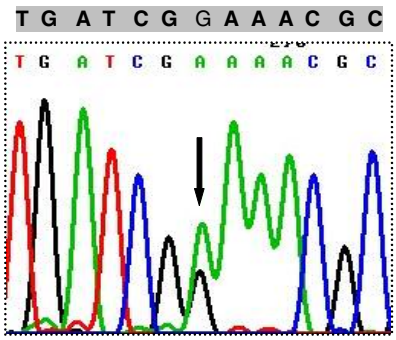
ELECTROPHEROGRAMS OF CYP1B1 MUTATIONS

1



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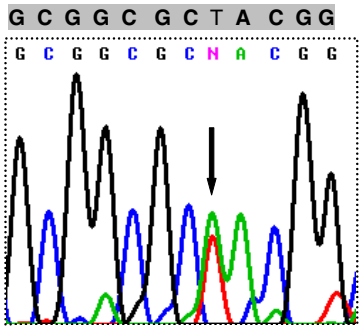

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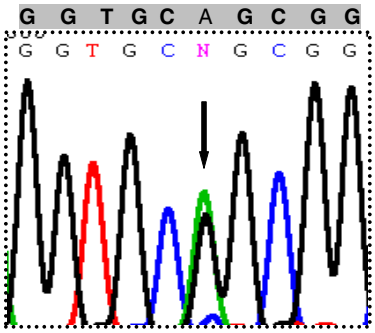
3



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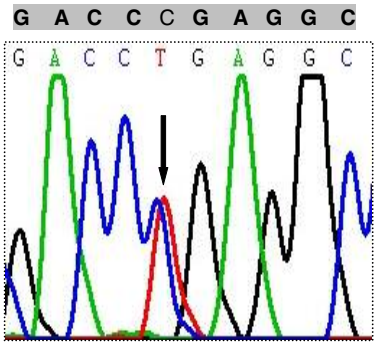

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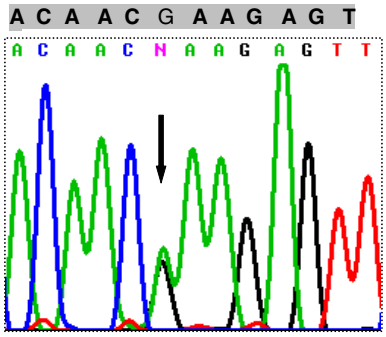

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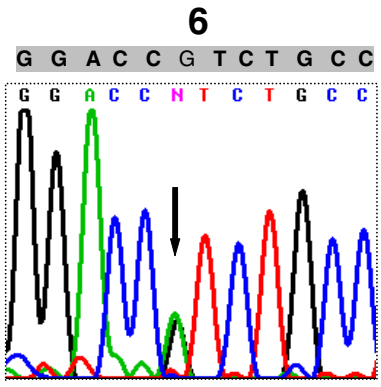

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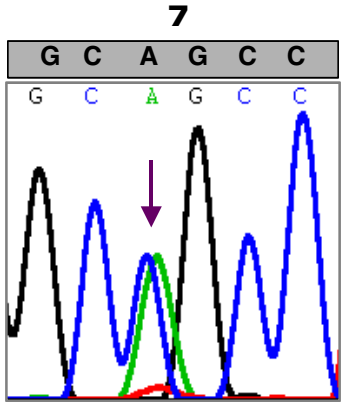

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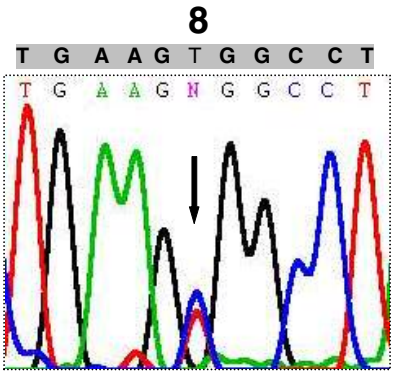

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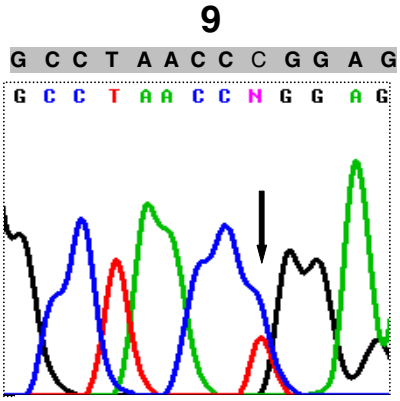

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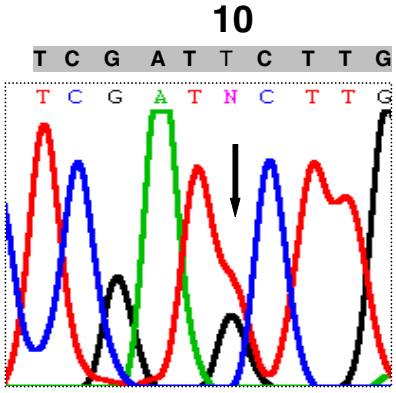

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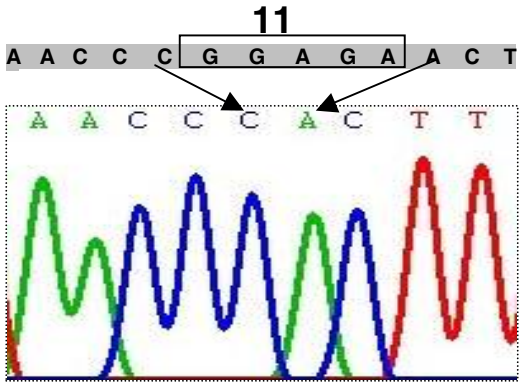
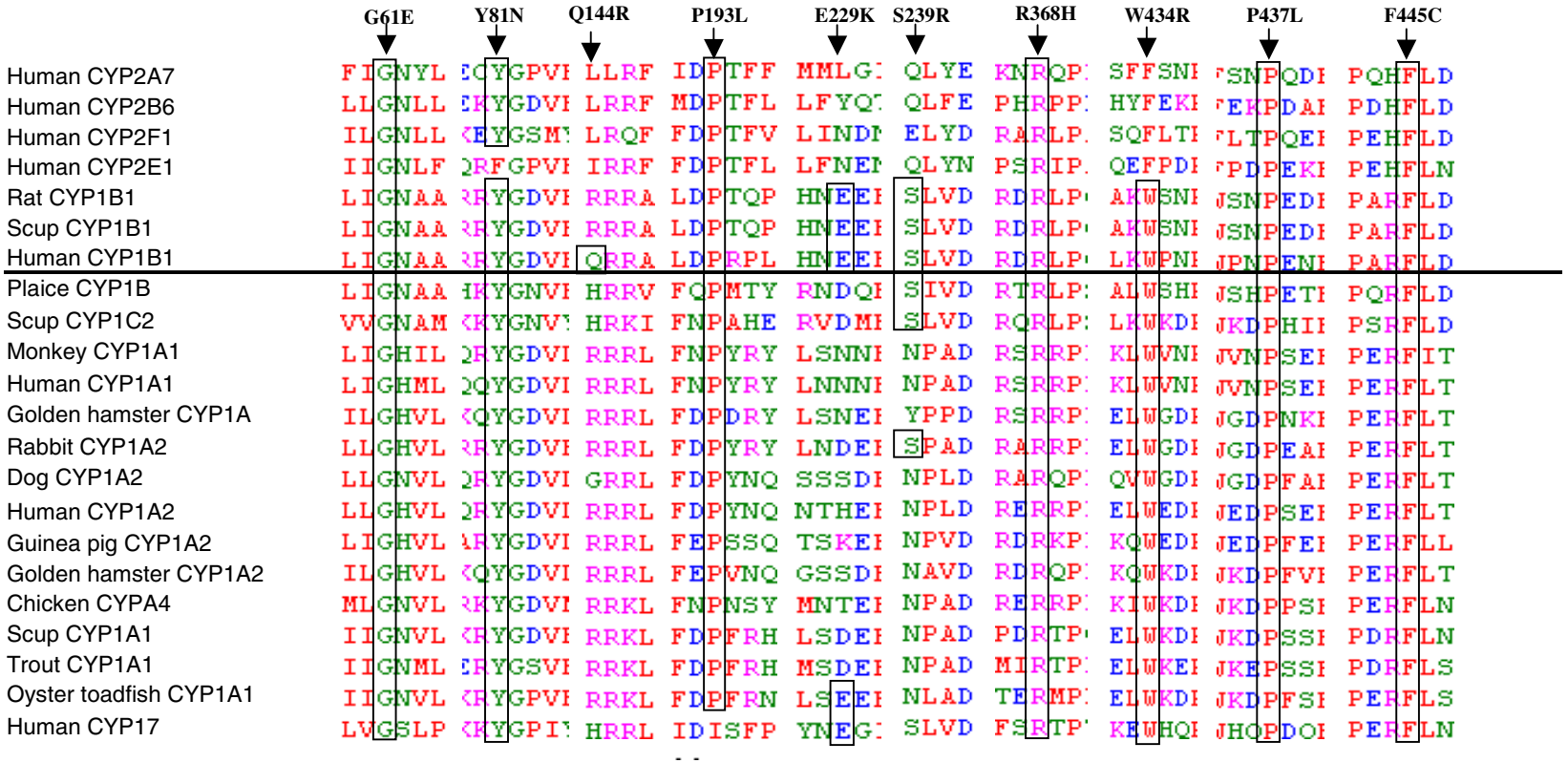


C.1657delGGAGA

Supplementary Figure 1. Electropherograms of the eleven different CYP1B1 mutations observed in POAG and PACG. The wildtype sequences are provided on the panel above the boxes. The arrows indicate the point of substitution for panels 1-10 and the points of deletion in panel 11.


G61E
Y81N
Q144R
P193L
E229K
S239R
R368H
W434R
P437L
F445C
Human CYP2A7
Human CYP2B6
Human CYP2F1
Human CYP2E1
Rat CYP1B1
Scup CYP1B1
Human CYP1B1
Plaice CYP1B
Scup CYP1C2
Monkey CYP1A1
Human CYP1A1
Golden hamster CYP1A
Rabbit CYP1A2
Dog CYP1A2
Human CYP1A2
Guinea pig CYP1A2
Golden hamster CYP1A2
Chicken CYPA4
Scup CYP1A1
Trout CYP1A1
Oyster toadfish CYP1A1
Human CYP17

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Gln48His is the prevalent myocilin mutation in primary open angle and primary congenital glaucoma phenotypes in India

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Purpose: Myocilin gene defects have been originally implicated in primary open angle glaucoma (POAG). Based on multiple reports for the occurrence of Gln48His mutation (c.144G>T; HGMD accession number CM023962) among Indian POAG patients, we wanted to estimate the prevalence of this mutation in primary open angle and primary congenital glaucoma (PCG) in India and assess its role in the causation of the disease.
Methods: Two hundred cases each of POAG and PCG were screened for the Gln48His mutation by RFLP (*Acc*I) analysis of the PCR amplicons followed by confirmation of the c.144G>T change by direct sequencing.
Results: The Gln48His mutation was detected in 9 different glaucoma patients (four POAG and five PCG). While all four POAG cases were heterozygous, among PCG cases, four were heterozygous and one exhibited homozygous genotype for the mutation. One each of POAG and PCG patients was detected to be heterozygous for *CYP1B1* mutation (c.1656C>T, Pro437Leu) and (c.1449G>A, Arg368His), respectively. None of the 300 ethnically matched normal controls contained either the *MYOC* or *CYP1B1* mutation(s).
Conclusions: The myocilin mutation, Gln48His, represents an allelic condition involving a spectrum of glaucoma phenotypes in Indian populations, and could be a potential risk factor towards disease predisposition among patients of Indian origin. The study also highlights the role of *MYOC* as a candidate in different glaucoma subtypes that needs to be investigated further.

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

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METHODS

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

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

Collection of blood samples and genomic DNA preparation, polymerase chain reaction (PCR), and the Gln48His

mutation screening by digesting the PCR amplicons with *Acc*I restriction enzyme were done as described earlier [4]. The loss of the *Acc*I site suggested presence of the mutation (c.144G>T) which was confirmed by direct sequencing. The patients containing the mutant *MYOC* allele was screened for the *CYP1B1* mutation by direct sequencing as described earlier [6].

RESULTS & DISCUSSION

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

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

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

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

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

Table 1. Distribution of myocilin mutation Gln48His among the Indian glaucoma patients

Phenotype	Number of individuals	Number of Gln48His mutations
POAG	200	4 (2.0%)
PCG	200	5 (2.5%)
Normal	300	0

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

Table 2. Genotype and phenotype of glaucoma patients with the *MYOC* Gln48His mutation

Patient Number	Phenotype	Age at symptom onset	Genotypes: MYOC (c.144)	Genotypes: CYP1B1 (c.1449/c.1656)
1	POAG	37 years	(G,'T')	(G,G)/(C,'T')
2	JOAG	-	(G,'T')	(G,G)/(C,C)
3	POAG	-	(G,'T')	(G,G)/(C,C)
4	JOAG	-	(G,'T')	(G,G)/(C,C)
5	PCG	at birth	('T','T')	(G,G)/(C,C)
6	PCG	at birth	(G,'T')	(G,G)/(C,C)
7	PCG	at birth	(G,'T')	(G,G)/(C,C)
8	PCG	4 months	(G,'T')	(G,G)/(C,C)
9	PCG	at birth	(G,'T')	(G,'A')/(C,C)

Among all the nine patients harboring the myocilin (NM_000261) mutation, two were also heterozygous for *CYP1B1* (NM_000104) mutations. Three of the POAG patients (patient 2, 3, and 4) have been described before [4]. The mutant alleles are enclosed by apostrophes. *MYOC* mutation: c.144G>T, Gln48His; and *CYP1B1* mutations: c.1449G>A, Arg368His and c.1656C>T; Pro437Leu.

gests that the *MYOC* mutation could be associated to different subtypes of glaucoma that need to be further investigated to better appreciate the role of *MYOC* in glaucoma pathogenesis.

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