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 Madurai, 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-1β (-511 C>T and +3953 C>T), IL-1α (-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 assessed 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 \textit{MMP-9} -1590 C/T in the promoter and codon-6 SNP Q279R through a case control study in the PACG patients.

Mutations in \textit{MYOC} and \textit{CYP1B1} have been implicated in POAG and PCG but its involvement in PACG has not yet been demonstrated. The precise role of \textit{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 \textit{et al.}, 2005). The present study is an attempt to analyze the molecular genetics of late age onset primary glaucomas with the following aims,

\textbf{Objectives:}

1. To screen \textit{MYOC} and \textit{CYP1B1} in POAG, PACG and PAC cases.
2. To screen single nucleotide polymorphisms (SNPs) in candidate genes, \textit{IL-1\textbeta} (-511C>T and +3953 C>T), \textit{IL-1\textalpha} (-889C>T), \textit{MTHFR (Ala222Val, C>T)}, \textit{p-21 (Arg31Ser, C>A)} and \textit{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 $IL-1\beta$ (-511 C>T and +3953 C>T), $IL-1\alpha$ (-889 C>T), p-21 (Ser31Arg), $MTHFR$ C677T did not show any significant association to POAG. Similarly, the SNPs in $IL-1\beta$ (-511C>T and +3953 C>T), $IL-1\alpha$ (-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.