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; Suriyaperuma, 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 *WDR36* (*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; Srirpriya 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 (Acharyya 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: IL1α (acidic form), and IL1β (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 \textit{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 \textit{et al}., 1995). This polymorphism lies in the binding site for the $MTHFR$ co-factor flavin adenine dinucleotide (FAD). An \textit{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 \textit{et al}., 1995). Hence, the individuals homozygous for the mutation have significantly elevated plasma homocysteine levels, which have been documented in glaucoma patients (Frosst \textit{et al}., 1995). Thus, the 677C>T SNP in the $MTHFR$ gene may be a risk factor in glaucoma.

Junemann \textit{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 \textit{et al}., 2005). However, this association could not be replicated in any other studies on different populations (Fingert \textit{et al}., 2006; Mabuchi \textit{et al}., 2006; Mossbock \textit{et al}.,
2006; Turacli et al., 2005). We checked for the association of the \textit{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 \(p21\), 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\textsuperscript{ser-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 \textit{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 \textit{MYOC} and \textit{CYP1B1} in POAG, PACG and PAC cases.
2. To screen single nucleotide polymorphisms (SNPs) in candidate genes, \textit{IL1}β (-511C>T and +3953C>T), \textit{IL1}α (-889C>T), \textit{MTHFR} (Ala222Val), \textit{p21} (Arg31Ser) \textit{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.