Discussion
In this study major emphasis has been given on molecular characterization of the underlying complexities in POAG. Towards that goal, I attempted to elucidate the potential involvement of MYOC among the nuclear genes and the entire mitochondrial genome in glaucoma pathogenesis.

Analysis of MYOC gene in eastern Indian POAG patients (n=450) led to the identification of 17 changes including 10 novel variations, 3 reported mutations and 4 reported polymorphisms. The identified mutations included 5 nonsynonymous changes, 2 deletions and 1 nonsense mutation. The analysis revealed mutations in this gene in approximately ~2.7% of POAG cases, which is consistent with the world average regarding the involvement of MYOC in 2-4% of the disease. Most of the mutations (5/8) are located in exon 3 which codes for the olfactomedin domain, reported to be important for the functionality of the protein (Aroca-Aguilar et al. 2005).

To date, involvement of MYOC in Indian POAG cases has been examined mostly in eastern and southern Indian patients. The report by Chakrabarti et al. included 13.5% patients from western India and 23.5% patients from northern India in a cohort of 251 patients, but no POAG causing mutation in MYOC was identified in these two groups (Chakrabarti et al. 2005). A complete list of mutations in the MYOC gene identified in the Indian population is listed in the Indian Genetic Disease Database (http://www.igdd.iicb.res.in/IGDD/home.aspx) (Pradhan et al. 2011). Among the identified genetic defects, Gln48His is the most prevalent mutation detected in Indian population. Till date, this change has been reported both in POAG and PCG cases, only in patients with Indian origin with severe glaucoma phenotypes (Mukhopadhyay et al. 2002; Chakrabarti et al. 2005).

Two single-base deletion mutations (R125SfsX158 and D273DfsX34), which would cause termination of olfactomedin domain of myocilin, were detected in familial cases along with other potential disease causing variants. To date, small deletion accounts for 2.3% of the mutations identified in myocilin and all of those have been reported as glaucoma causing mutations (Hewitt et al. 2008).

Among the polymorphisms in MYOC, identified in the present study, high heterozygosity of two polymorphisms (-83G>A & c.227G>A) present in complete LD would be useful as markers for testing involvement of myocilin in suspect familial cases of POAG.

To date, studies done on MYOC suggest that haploinsufficiency is not a critical mechanism for POAG in individuals with mutations in the gene. There has been a lack of discernable phenotype in both Myoc-heterozygous (+/-) and Myoc null
mice (-/-) (Kim et al. 2001). Also, absence of POAG in carriers with MYOC homozygous mutations, in contrast to disease phenotype in carriers of heterozygous mutations, (Morissette et al. 1998) points towards the fact that disease-causing mutations in humans likely act by gain of function. The C terminal domain of myocilin is of functional importance. Calpain binds in the olfactomedin domain of myocilin (Sanchez-Sanchez et al. 2007) and proteolytic cleavage of myocilin occurs between amino acids Arg226 and Ile227 (Aroca-Aguilar et al. 2005). Mutation or absence of the olfactomedin domain of myocilin results in a misfolded protein product, which suppresses the proper secretion of the protein (Caballero et al. 2001; Jacobson et al. 2001; Kanagavalli et al. 2007). This misfolded protein accumulates in the endoplasmic reticulum, (Joe et al. 2003; Liu et al. 2004) causing ER stress and subsequently resulting in cell death (Joe et al. 2003; Liu et al. 2004; Yam et al. 2007). This proves that mutations in the olfactomedin domain or deletion mutations resulting in truncated protein with absent or partial olfactomedin domain are expected to give rise to possible pathogenic glaucoma phenotypes. This study on MYOC screening of POAG patients, including previously reports from this group, is the largest investigation to date carried out in a single cohort (n=765) in the Indian population. The identification of MYOC mutations in probands and preclinical diagnosis of the individuals at risk will help clinicians in better management of the disease.

To validate the suspect variants as disease causing mutations one needs to determine alteration in biological function of the mutant protein by cell-based or, even better, animal model studies. To decipher the molecular event, investigation at the cellular level can shed enough light to unfold the underlying story. In that context, subcellular fractionation indicated that intracellular MYOC in TM cells is associated not only with Endoplasmic Reticulum and Golgi apparatus, but also with mitochondria (Wentz-Hunter et al. 2002). The mitochondrial association was visualized by immune electron microscopy (Ueda et al. 2002). Studies show that level of MYOC imported into the mitochondria of TM cell is dramatically higher than into the mitochondria from corneal fibroblasts and mouse liver, consistent with the notion that MYOC processing and localization may be distinct in TM cells (Sakai et al. 2007). MYOC is predicted to contain mitochondrial transit peptide at its N-terminus (amino acid residues 1 - 47). Both N and C-terminus contain a Lysine and Arginine rich mitochondrial tethering domain (amino residues 33-46 and 460-504) (Sakai et al. 2007). These authors have shown depolarization of mitochondrial membrane potential (\( \Psi_m \)) in response to Dexamethasone treatment. In addition, Pro370Leu mutant MYOC is damaging for
mitochondrial function of HTM cells, as indicated by decline of $\Psi_m$, indicator of mitochondrial energy state. The latter is hypothesized to occur because of disruption of outer mitochondrial membrane (Wang et al. 2007) and facilitates the release of pro apoptotic factors like Bcl-2 family proteins from mitochondria (Sakai et al. 2007).

With this background, functional studies were undertaken with Gln48His and Pro370Leu MYOC mutants. Glutamine in 48th codon is highly conserved across species. In Gln48His residue, the highly polar uncharged glutamine is replaced by positively charged highly basic histidine. The other variant Pro370Leu, selected for the study is highly penetrant across generations in pedigrees harbouring the mutation and is reported in both juvenile and adult onset glaucoma with severe phenotype (Wiggs et al. 1998; Vincent et al. 2002; Mukhopadhyay et al. 2003).

Triton-X detergent solubility, characteristic of normal and mutant MYOC protein was used to distinguish between pathogenic and benign MYOC mutants (Zhou et al. 1999). Pro370Leu has been reported to form Triton insoluble aggregates and was used as a positive control (Zhou et al. 1999). In line with this observation, high level of protein aggregates was obtained in the Triton insoluble fraction for Gln48His mutant protein. Often proteins carrying mutations which affect the native folding are not efficiently degraded and form Russell bodies (Yam et al. 2007) in the ER or cytoplasmic aggregates (Kopito 2000). ER retention has been implicated in the pathogenesis of various diseases (Rutishauser et al. 2002). Thus it is possible that Gln48His forming protein aggregates similar to Pro370Leu might have a pathogenic role to play in POAG.

As it is already reported that myocilin mutants induces ER stress in TM cells, we checked for the level of ER stress marker, p-eIF2a in TM cells transfected with either wild type myocilin, Gln48His or Pro370Leu mutants and noticed overexpression of all three variants of myocilin induces ER stress as observed by significant level of phosphorylation of eIF2a compared to control. Overexpression of human myocilin in the eyes of Drosophila melanogaster results in distortion of ommatidia (The 'eye' of Drosophila) which is accompanied by fluid discharge (Borras et al. 2003) and also activation of unfolded protein response (Carbone et al. 2009). Thus, phosphorylation of the wild type protein was also observed when overexpressed in TM cells. The level of eIF2a phosphorylation was lower in Pro370Leu compared to wild type or Gln48His mutant. It is a well known phenomenon that this ER stress response activates Unfolded Protein Response (UPR), a self protecting mechanism. The direct effect is attenuation of protein
translation (Schroder et al. 2005). Pro370Leu downregulates ER stress response compared to wild type or Gln48His mutant, which might perturb the protective mechanism of the cell and increase the vulnerability of the TM cells. Such a mechanism is similar to the pathogenic role of mutant PS1, which is linked to familial Alzheimer Disease on the signalling pathway of unfolded protein response (Sato et al. 2001; Walter et al. 2005). This, observation corroborates with the clinical phenotype, where Gln48His has been seen to give milder phenotype compared to Pro370Leu. We suggest that the disturbed response may prolong ER stress and change the secretory function of HTM cells, leading to an altered extracellular environment and, ultimately, to HTM cell dysfunction and death.

Studies show that this ER stress triggers autophagy in cells, under a variety of pathophysiological conditions, including cancer (Kondo et al. 2000; Liang et al. 2011), renal failure (Kawakami et al. 2009) neurodegeneration (Hara et al. 2006; Komatsu et al. 2006; Scheper et al. 2011) and heart failure (Nakai et al. 2007). We identified that myocilin mutants inducing ER stress trigger autophagy in trabecular meshwork cells. In TM cells transfected with wild type myocilin, Gln48His and

![Figure 4.1: ER stress preferentially activates autophagy in neuronal cells.](image)

Under physiological conditions, aberrant ER proteins are removed by ERAD and degraded by the UPS. A disturbance of ER homeostasis resulting in activation of the UPR will activate the autophagy pathway, but not the UPS. This preference may relate to a decreased capability to export misfolded proteins from the ER lumen under ER stress conditions or to the inability of the proteasome to degrade aggregated proteins. The concerted action of UPS and autophagy is normally sufficient to restore ER homeostasis. In adult onset diseases, the autophagic capacity may not be sufficient due to age-related impairment of the autophagic-lysosomal system and/or increased demand for autophagy by (prolonged) activation of the UPR. UPS: ubiquitin proteasome system; UPR: Unfolded protein response. Adapted from Scheper et al, Autophagy, 7:8, 910-911; August 2011.
Pro370Leu mutants, conversion of unconjugated form of LC3-I to its lipidated form LC3-II was observed. LC3-II is found in the autophagosome membrane and is considered as the hallmark of autophagy. Also there was upregulation of another autophagy inducing protein Beclin-1 for both the myocilin mutants. These observations suggest for the first time that myocilin mutants are likely to triggers autophagy in trabecular meshwork cells. However, further studies are required to establish the involvement of autophagy in POAG pathogenesis.

Autophagy can also be activated for the purpose of cellular autolysis and self clearance (Levine et al. 2005), or as a mechanism to remove toxic, multimeric complexes that eventually promote cell death in neurodegenerative diseases (Martinez-Vicente et al. 2007). In addition, in many neurodegenerative disorders altered proteins are first degraded through either the ubiquitin-proteasome system, or via chaperone-mediated autophagy, and impairment of these mechanisms promotes protein aggregation (Figure 4.1) (Komatsu et al. 2007; Martinez-Vicente et al. 2007). POAG being a neurodegenerative disorder (Ray et al. 2009), Myocilin mediated POAG pathogenesis might function in similar fashion, where after prolonged recycling mechanism following UPR, there is a decreased rate of autophagy and finally might result in cell death.

In the course of study, attempt was made to show that the putative EREs in MYOC promoter are functionally active. It was observed from this lab prior to my participation in this part of the study that the EREs could induce transcription of MYOC in the presence of 17β estradiol (PhD dissertation of Dr. Suddhasil Mookherjee). To gain further support to the observation, I used 17β estradiol competitor and showed that the basal promoter activity of the MYOC promoter is inhibited in RPE cells, which further proves that the putative EREs of the MYOC promoter are active. In addition, a twofold higher expression of the endogenous myocilin was observed in Trabecular meshwork cell line upon treatment with 17β estradiol. This was further supported by previous observation from the lab that nuclear localization of estrogen receptor-alpha occurs in 17β estradiol induction. This transactivation was eventually brought about by binding of estrogen receptor and 17β estradiol complex on the putative EREs of the MYOC promoter, as evident from our ChIP experiment results.

As already stated over expression of human myocilin activates unfolded protein response (Carbone et al. 2009) in Droshophila. Over expression of wild type myocilin is also believed to be involved in glaucoma pathogenesis based on steroid
induced glaucoma. Myocilin over expression is known to compromise the adhesive property of cultured TM cells through activation of cAMP/PKA and inhibition of Rho kinase (Shen et al. 2008). On the contrary, BAC mediated myocilin over expression in mouse do not produce any glaucoma phenotype (Gould et al. 2004). This is not rare that mutations in homologues genes in mouse do not produce similar phenotype observed in human, which also varies depending on the mouse strain used in the specific study.

Now, shifting the focus from the nuclear genome to the mitochondrial genome, the involvement of mitochondria has remained much unexplored territory in glaucoma pathogenesis especially in Indian population. In the present study we evaluated 101 POAG patients and 71 controls to investigate the possible involvement of the mitochondrial genomic variants in POAG pathogenesis. Except for one patient, none of the individuals included in the study was found to harbor any pathogenic glaucoma causing MYOC mutation.

Analysis of the mitochondrial genome showed that in both patients and controls the ratio of transition to transversion (Tn/Tv) was higher in the coding region compared to the control region. This is a familiar pattern of mitochondrial genome which attests the accuracy of the sequencing (Pereira et al. 2009). A lower ratio of transition to transversion observed in the coding region in patients compared to controls, indicates the preponderance of transversion is higher among the patients than controls. The transversion changes alters the chemical structure drastically, the consequence of the change is more lethal than those of transition. In the control region, no selection pressure acts on the mutants, so the Tn/Tv ratio is comparable among patients and controls for this region.

It was also observed that the clustering of variants in the coding region of mitochondrial genome was significantly higher among the patients than in controls. The non-synonymous variants result in more profound effect by altering the biological activity of the coded protein. Analysis of the coding region showed that the non-synonymous changes in Complex I of mtDNA are more frequent in patients compared to control; same was the case for Complex III. These observations points towards the involvement of the Complexes in POAG pathogenesis. Among the complex I genes, ND5 represents the potential locus to be involved in POAG pathogenesis where 48% of the changes clustered in patients compared to only 31% in controls (p<0.0001). In addition, in the RNA genes, differences in clustering were discernable when observed at individual gene level. The frequency of changes in

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12SrRNA was statistically higher (p<0.0001) in patients than in control.

Mitochondrial Complex I is the entry port of the respiratory chain. It is an 850kDa supra molecular complex composed of more than 40 polypeptides and contains a flavin mononucleotide (FMN) molecule and eight iron-sulphur clusters as redox active centers, embodied in the extra-membrane in the peripheral arm. Seven polypeptides of Complex I are encoded by mitochondrial genes (ND 1, 2, 3, 4, 4L, 5, and 6). Complex I catalyses transfer of electrons from NADH via FMN to the metal redox pathway and subsequently to ubiquinone (UQ), located in the membrane embedded part of the complex (Brandt 2006). Thus Complex I produces significant level of $O_2^-$ by molecular oxidation of $O_2$, thereby accounting for most of the constitutive ROS generated by the mitochondrial respiratory chain (Chance et al. 1979; Boveris et al. 2000; Turrens 2003). The rate of $O_2$ production is increased by electron transfer inhibition with rotenone, a hydrophobic pesticide (Boveris et al. 1973). Depending upon the physiological conditions or specific pathological modifications of the Complex I, each of the redox active centres contribute to ROS generating activity. However, the exact function of each ND subunit is incompletely understood. Biochemical studies till date suggests the involvement of ND5 and ND6 in the proton pumping activity (Kao et al. 2005) as well as binding of ubiquinone (Carelli et al. 1999; Cardol et al. 2002).

Over the years, variants in Complex I have been linked to several mitochondrial and neurological diseases. A similar study on mitochondrial variations in POAG patients by Abu Amero et al, also reported clustering of mitochondrial variants in Complex I in a Saudi Arabian POAG cohort (Abu-Amero et al. 2006). Similar observation has been made in other optic neuropathies like LHON (Wallace et al. 1988; Brown et al. 1992), mitochondrial diseases viz. MERRF (Naini et al. 2005) and MELAS (Valente et al. 2009), where most of the mutations till date have been found in different Complex I genes. Defects in mitochondrial complex I are known to be involved in increased production of ROS and are linked to several degenerative disorders (Kwong et al. 2006). Complex I deficiencies are also reported in devastating neurological disorders like Parkinson disease (Greenamyre et al. 2001; Keeney et al. 2006), Huntington disease (Arenas et al. 1998) and Wilson disease (Schapira 2002).

Interestingly, a higher level of endogenous ROS has been observed in glaucomatous trabecular meshwork cells and these cells were also found to be more sensitive to inhibition of Complex I activity by Rotenone (ROT) compared to the
normal tissue. On the other hand, inhibition of Complex II and III had little effect on the TM cells (He et al. 2008). The rate of O₂ production by Complex I is increased by electron transfer inhibition with rotenone (Boveris et al. 1973). Inhibition with ROT resulted in further increase in ROS production, the release of cytochrome C, decrease in ATP level and Ψm in glaucomatous TM cells, leading to apoptosis. This effect was found to be reversed with the use of antioxidants (He et al. 2008). Thus inhibition of Complex I activity in glaucomatous TM might be correlated with the accumulation of higher number of mtDNA variants in POAG. Such phenomenon of mitochondrial dysfunction and reduced complex I activity has been observed in other neurological disorder like Parkinson's disease (Navarro et al. 2010). Reduced Complex I activity in Parkinson's disease experimental animals intoxicated with Complex I inhibitors reproduce the clinical symptoms of Parkinson's disease in humans (Navarro et al. 2010). Thus this observation is likely to be of pathogenic importance even in case of POAG and might be associated with TM cell degeneration.

Though Complex I mutations have been identified in multiple instances, this study for the first time identifies ND5, the largest subunit of Complex I, as hotspot of variation in POAG patients. In Complex I, mutation in ND5 gene has been consistently found in several disorders like LHON, MERRF, MELAS (DiMauro et al. 2005). A recent study in E. Coli has shown that ND5 is involved in the proton pumping mechanism (Nakamaru-Ogiso et al. 2010). Thus variation in ND5 gene is expected to perturb the equilibrium of the respiratory chain, leading to inhibition of the overall Complex I activity. Thus, downstream functional assays to determine the pathogenicity of the variants identified may help in better understanding of the role of ND5 in POAG pathogenesis.

We also observed that POAG patients harbor higher number of variations in 12S rRNA gene. However, no study reports the association of this gene in any eye disorder. Multiple studies have reported the association of variations in 12SrRNA with aminoglycoside induced and non-syndromic hearing loss (Zhao et al. 2005; Lu et al. 2010; Guan 2011) and also in carcinoma (Han et al. 2005). Aminoglycoside binds to 12SrRNA, causing mistranslation or premature termination of protein synthesis (Davies et al. 1968; Noller 1991). Individuals harboring 12S rRNA variants are susceptible to aminoglycoside induced hearing loss. Thus it will be interesting to examine whether, exposure of aminoglycosides, used in certain eye drops, can exacerbate the disease condition in POAG patients harboring variations in 12S rRNA
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16S rRNA has been reported to code for a functional peptide, Humanin, which is neuroprotective in nature and is used as a potential therapeutic agent in Alzheimer's disease and Diabetes Mellitus (Muzumdar et al. 2009). Variation in this gene is found to be less in patients. Glaucoma being a neurodegenerative disease (Ray et al, 2009), a selection pressure is expected to act in the patients for changes happening in this region to keep a protective mechanism active.

Mitochondrial tRNAs play an important role in mtDNA translation because the nuclear tRNA is not transported from cytoplasm into mitochondria in human (Tarassov et al. 2007; Salinas et al. 2008). The mutations in mt-tRNAs can change the secondary structure and alter the tertiary structure, thus finally affecting the translation of mtDNA encoded genes. Many pathogenic mutations in mt-tRNA genes, especially on the stem portions (McFarland et al. 2004), have been reported to be associated with human diseases but the molecular bases for the proposed association has not been extensively investigated (McFarland et al. 2004; Zifa et al. 2007; Scaglia et al. 2008; Elson et al. 2009). Over the last few years, increasing number of human neurodegenerative disorders have been found to be correlated with point mutations in mitochondrial tRNA. Both the number of mutations (>70) and widely variable phenotypes (e.g. cardiopathies, myopathies, encephalopathies, diabetes, deafness) render the understanding of the genotype to phenotype relationships very complex (Florentz 2002). From this perspective, study regarding the structural change occurring due to each private mutation in POAG patients is worth pursuing.

In our study, a possible association between mtDNA haplogroups and POAG was investigated, but no such evidence was detected. Lack of association of mitochondrial haplogroup with POAG pathogenesis, may be correlated with the observation that it is not a particular haplotype that confers susceptibility to POAG in this population. The significantly greater susceptibility to POAG in subjects with African ancestry compared to those with Eurasian ancestry, in other geographical areas, has been well documented (Racette et al. 2003). A recent study done by Abu-Amero in Saudi Arabian POAG patients and controls showed a higher preponderance of haplogroup L in the patients, i.e. there is POAG predisposition in Saudi individuals with mtDNA of African ancestry (Abu-Amero et al. 2011). A previous study from the same group, did not find any haplogroup association with the POAG patients (Abu-Amero et al. 2008). They correlated this observation to
unknown racial risk factors for this disease or unexpected population substructure in Saudi-Arabia. Our results are congruent with the lack of differences in the haplogroup distribution between POAG patients and healthy controls in a study performed on white people from England (Andrews et al. 2006). Our study population belongs to the Indo-European ethnic group who do not have any African ancestry. In India most of the population belongs to M macro haplogroup (Babizhayev et al. 1993), which is primarily of Eurasian ancestry. Thus, our observation is consistent with the published literature on similar population groups.

Thus analysis of the mitochondrial genome further suggests that, contrary to the common disease-common variant hypothesis, pathogenic mutations are largely population-specific, which is evident by the occurrence of higher number of novel mutations, and different SNPs may be associated with the same disease in different populations. Therefore, to obtain a comprehensive picture of the deleterious variability in the human population, as well as to improve the diagnostics of individuals carrying susceptible disease loci, it is necessary to survey different populations independently.

This study thus provides information on the potential defects in MYOC and mitochondrial genome in POAG that might be linked to the disease pathogenesis in eastern Indian patients and also investigates the underlying molecular basis for the malfunction of the mutant myocilin through autophagy – an important cellular event not yet linked to glaucoma.