MOLECULAR
GENETIC ANALYSIS OF INTRAOCULAR PRESSURE (IOP) - RELATED GLAUCOMAS IN INDIAN POPULATION

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2012

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN)

CERTIFICATE

This is to certify that the thesis entitled **"Molecular Genetic Analysis of Intraocular Pressure (IOP) - Related Glaucomas in Indian Population"** which is submitted by Kollu Nageswara Rao, ID No. **2007PHXF031P**, for award of Ph.D. degree of the institute, embodies original work done by him under my supervision.

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DEDICATED TO MY FAMILY, SUBHO SIR AND INDERJEET MADAM

ACKNOWLEDGEMENTS

This thesis is a part of the research being done since many years by Chakrabarthi's group and I take this opportunity to thank all those with whom I got a chance to work. I learned many new things during this period that will be helpful in shaping my future endeavors

First and foremost, I would like to express my deepest gratitude to my Supervisor Dr. Subhabrata Chakrabarti, Associate Director for Research, LVPEI for guiding me through the PhD program. I truly thank him for the concern and care he extended regarding my family's health. I sincerely appreciate your efforts to correct my research proposals and dissertation and this helped me to improve my writing skills. Sir, the list to thank you is endless.

I owe great thanks to Dr. Inderjeet for helping me trouble-shoot my experiments. She has been with me and supported me in crucial stages and was always there to counsel me to take correct decisions. She is a good mentor who displays a blend of strictness and gives liberty to do the work with finesse. She interacts with us in a friendly manner and at the same time takes active participation in Laboratory related activities.

I extend my sincere thanks to Dr.Balasubramanian, Director for Research, LVPEI who is my inspiration and showed me how to enjoy research in true sense. He helped me to get travel grant for ARVO. I thank you for all the encouragement and the space you provided for me to work at LV Prasad Eye Institute. I sincerely thank Dr. G.N. Rao and Dr. G. Chandrashekar for giving me the opportunity to pursue doctoral studies.

I appreciate the support from the research and consultancy division of BITS Pilani for taking care of my PhD related matters. It was very kind of Prof Ashish Das for helping me and clarifying my doubts whenever I needed. I am especially thankful to

Sharad Srivastava and Dr. Dinesh for taking care of my PhD related matters and providing prompt and valuable feedback from time to time.

I am grateful to other faculty members, Dr. Chitra Kannabiran, Dr. Indumathi Mariappan, Dr. Geeta K Vemuganti, and Dr. Savitri Sharma for providing me valuable comments and suggestions during research seminars.

I am indebted to my seniors Afia, Anees, Aparna, Guru, Hardeep, Joveetha, Kalyan, Kiran, Purushottam, Rajeshwari, Sagar, Saritha, Srilatha, Venu and Vidya.

I am thankful to my friends and colleagues-Farheen, Ganesh, Hameed, Harish, Jameela, Lakshmi, Mangalangi, Meha, Neeraja, Nishika, Praveen, Praveen, Pulla Rao, Rachana, Ramesh Babu, Saika, Santhosh, Sonika, Srinivas, Subba Rao, Shubha, Subhash, Vasundhara and Veena.

I am fortunate to have my roommates Chetan, Director Srinivas, Gowtham, Kiran, Naresh, Naveen, Pradeep, Sateesh, Sateesh and Suresh who always helped throughout my PhD.

I truly thank Savitri M for giving me moral support in many aspects during my Ph.D programme.

I would like to thank Dr. Mandal, Dr Sirisha, Dr Harsha, Dr Murali, and Dr. Sangwan for providing me the samples for my research work.

I would like to sincerely acknowledge Mr.Naidu, Rambabu and Sridhar who helped me in IHC related techniques.

I also thank Srinivas, Venkataswamy for helping in getting the eye balls.

I thank Jai Ganesh, Moiddin and Preeti for providing me administrative support.

I thank my Institution, LVPEI for providing me a very good infrastructure, resources for laboratory work, informative lectures and seminars and a chance to meet eminent scholars from various fields. The support and co-operation from the institute's communication (Ashok, Kartheek, Sangarsh and Sarika), ISD (Shiva, Mahindra), purchase (Jagadesh, Ragavulu), stores (Aparajitha and Rangababu) and maintenance departments (Dattu,Ravi, Krishna) was immense and I would like to thank all these people.

I truly thank Banu and Sudhakar for helping me get all the books, journals and photocopies needed for my research work. I also thank the people in the canteen for providing tea which helped me to get refreshed at regular intervals.

I thank my Parents, brothers, sisters, sister-in-laws and all my well wishers for their immense support without which all this would not have been possible.

Last but not the least I whole heartedly appreciate my wife Haritha for her love, care and understanding and making my life beautiful. I would like to thank my in-laws for kind wishes.

Kollu Nageswara Rao

SYNOPSIS

Glaucoma comprises a group of ocular disorders characterized by progressive degeneration of optic nerve head and retinal ganglion cells (RGCs) leading to visual field defects (Kwon *et al.,* 2009). Glaucoma is one of the leading causes of blindness affecting over 70 million people world wide (Quigley *et al.,* 2006). In India glaucoma is estimated to affect 12 million people and causes 12.8% of the total blindness (Dandona *et al.,* 2001). Based on gonioscopic findings, glaucomas can be classified into primary open angle glaucoma (POAG) and primary angle closure glaucoma (PACG). There are several risk factors for POAG, such as elevated intraocular pressure (IOP), family history, race, high myopia, thin cornea, systemic hypertension and diabetes (Tielsch *et al.,* 1994, Rudnicka *et al.,* 2006, Newman-Casey *et al.,* 2011, Marcus *et al.,* 2011). Elevated IOP is the common risk factor for POAG and experimental elevation of IOP have resulted in glaucoma in animal models (Levkovitch-Verbin *et al*., 2002). POAG is a complex disease and the exact mechanism leading to disease pathogenesis remains unknown.

Based on gene mapping studies, there are greater than 30 loci linked to POAG of which, 17 have been named from *GLC1A- GLC1Q.* Among them only five, namely, *GLC1A* (1q24.3-25.2), *GLC1E* (10p15-14), *GLC1G* (5q22.1), *GLC1O* (19q13.3) and *GLC1F* (7q35-36) have been characterized to harbor mutations in myocilin (*MYOC*), optineurin (*OPTN*), WD repeat domain 36 (*WDR36*), neurotrophic factor 4 (*NTF4*) and ankyrin repeat and SOCS box containing 10 (*ASB10*), respectively (Stone *et al.,* 1997, Rezaie *et al.,* 2002, Monemi *et al.,* 2005, Pasutto *et al.,* 2009, Fernández-Martínez *et al.,* 2011, Paustto *et al*., 2012). Cytochrome P450, Subfamily I, Polypeptide 1 (*CYP1B1; GLC3A* [2p22-p21]) is a candidate gene for primary congenital glaucoma

(PCG), and has also been implicated in POAG (Vincent *et al*., 2002). Recently, it has been shown that sequence variants in Retinitis Pigmentosa GTPase Regulator-Interacting Protein 1 (*RPGRIP1;* 14q11) may be associated with POAG in a European population (Fernandez-Martinez *et al*., 2011). Although POAG has a significant heritability, the linkage studies were not successful in identifying the genes with significant effect sizes. Lack of such findings suggests that glaucoma is a complex disease with multiple molecular mechanisms underlying its pathogenesis.

So far, six genome wide association studies (GWAS) have been conducted in POAG and several genetic variations have been associated with the disease. These studies revealed that the variations in *LOXL1, CDKN2BAS1, TMCO1, SIX1, GAS7* genes and those in proximity to *ZP4, PLXDC2, DKFZp762A217, CAV1* and *CAV2* were associated with POAG in certain populations (Thorleifsson *et al.,* 2007, Nakano *et al*., 2009, Thorleifsson *et al.,* 2010, Burdon *et al*., 2011, van Koolwijk *et al*., 2012, Wiggs *et al*., 2012)

Recent studies have shown that quantitative clinical traits in POAG, including optic nerve head parameters, central corneal thickness (CCT) and IOP were influenced by different genes. Based on this approach, genomic regions have been linked to raised IOP (regions on chromosomes 2, 5 and 19), CCT (*AKAP13, COL5A1, AVGR8, ZNF469, FOXO1, RXRA, COL8A2, CHSY1, IBTK, FAM53B* and *COL8A2),* optic disc area (*ATOH7, CDC7/TGFBR3, CARD10* and *SALL1*) and vertical cup to disc ratio (*CDKN2B, SIX1, SCYL1/LTBP3, CHEK2, ATOH7* and *DCLK1*) (Vitart *et al.,* 2010, Lu *et al.,* 2010, Macgregor *et al.,* 2010, Ramdas *et al*., 2010, Cornes *et al.,* 2011, Khor *et al.,* 2011, Vithana *et al.,* 2011). Various gene expression and animal models have

revealed that multiple pathways are involved in regulation of IOP and RGC death (Almasieh *et al*., 2012, Yang *et al*., 2011).

Overall, the genes identified so far have contributed to less than 10% of all POAG cases and the genetic determinant for majority of these cases is yet to be unidentified. However, the different genes identified through studies in humans, animals, and *in vitro* evidences are involved in regulation of IOP and RGC death and the exact role of these genes in causing POAG in humans is limited. Thus, a comprehensive approach is essential to identify these multiple genes predisposing to POAG, which would provide further insights into the disease pathogenesis and help in better management.

The aim of the current study was therefore, to identify the candidate genes involved in IOP- related glaucomas viz. POAG and PACG and to explore their possible involvement in the disease pathogenesis.

Five candidate genes, namely the Connective Tissue Growth Factor (*CTGF),* Neurotrophic Factor 4 (*NTF4),* Mitochondrially encoded NADH Dehydrogenase 1 (*mtND1*)*,* Bcl2 Associated X protein (*BAX*)*,* Fibulin 5 *(FBLN5*) and recently identified gene variants in Lysyl Oxidase Like 1 (*LOXL1)* and those in proximity to Zona Pellucida glycoprotein 4 (*ZP4)*, Plexin Domain Containing protein 2 (*PLXDC2)*, *DKFZp762A217, VAV2, VAV3,* Caveolin 1 and 2 (*CAV1* and *CAV2)* were analyzed in clinically well characterized IOP related glaucoma cases comprising of POAG (n=196), PACG (n=111) and ethnically matched normal controls (n=292). The variants in *LOXL1* and those located near *CAV1* and *CAV2* were further analyzed in a cohort of well characterized secondary glaucoma cases comprising of Pigmentary Glaucoma (PG; n=44) and Pigment Distersion Syndrome (PDS; n=34), as well as 108 ethnically

matched normal controls of Caucasian origin. Further, an ECM (extracellular matrix) associated gene *FBLN5* and an AMD (Age Related Macular Degeneration) associated *CFH* (Complement Factor H) variants were functionally characterized in POAG.

Screening was undertaken by direct sequencing and confirmed by PCR based restriction digestion. The localization of FBLN5 protein in the TM and other ocular tissues from normal cadaver eye balls were undertaken by immunohistochemistry. The expression of CFH and FBLN5 were analyzed by ELISA and western blotting. The allele and genotype frequencies were calculated using gene counting method. The association of SNPs with the disease was determined by calculating chi square (χ^2) test. HWE (Hardy-Weinberg equation) was estimated for the normal controls. Haplotype frequencies were calculated using the Haploview software (version 4.2). Linkage disequilibrium (LD) between the variants were calculated by using the LD plot function of this software.

Resequencing of *CTGF* (OMIM: 121009*)* led to the identification of three novel variants in the coding region (R22P, P36S, P102S), along with an intronic variation (g.325G>A) and two polymorphisms in the promoter region (g.-444G>C, g.-251delA) in POAG and only the promoter variants in PACG. These variants were rarely associated either with POAG or PACG. Although, *CTGF* is involved in the regulation of IOP (Chudgar *et al.,* 2006, Junglas *et al*., 2012), we were unable to identify significantly associated genetic variants with either POAG or PACG. Among the multiple genes involved in the regulation of ECM in TM, the present data indicated a limited involvement of *CTGF* in POAG and PACG.

NTF4 (OMIM: 162662) was recently identified candidate gene in POAG and mutations in this gene were shown to be responsible for altered neurotrophin signaling

(Pasutto *et al*., 2009). However, variations in *NTF4* were not associated with POAG and PACG in present study. A non synonymous variant (A88V), a silent variation (P151P) and two changes in the 3'UTR region along with a known polymorphism (rs11669977) were observed in cases of POAG whereas PACG cases exhibited only the A88V and rs11669977 variants. The mutations which were predominant in POAG in the European population were observed more in normal controls in the present Indian population (Rao *et al*., 2010). Similar findings from an American cohort supported the lack of involvement of *NTF4* in POAG (Liu *et al*., 2010). These observations indicated that unlike universal involvement of *MYOC* and *CYP1B1*, the *NTF4* does not seem to be a likely candidate for POAG.

Thirteen non-synonymous *mtND1* (OMIM: 516000) variations were observed of which six were exclusively present in POAG. However, these variations were neither pathogenic nor associated with POAG. Recent studies have demonstrated that mitochondrial abnormalities are present in POAG (Abu-Amero *et al*., 2006, Izzotti *et al*., 2011). Mutations in mitochondrial DNA are responsible for cell death by mitochondrial mediated pathways. Mutations in *mtND1* are responsible for promoting *BAX* dependent cell death (Perier *et al*., 2005). In the present study several *mtND1* gene variations were identified in POAG, but the frequency of these variants were statistically not significant and most of them were non pathogenic.

Resequencing of *BAX* (OMIM: 600040) did not reveal any major disease causing variation that could be associated with in POAG. Seven variations were observed, which include a single missense variation (G39W), two synonymous variations (D98D, V111V) and four known polymorphisms, but, none of these variations were associated with POAG. Studies in animal models have also shown that

BAX deficiency leads to IOP reduction and protection against RGC death in mice suggesting it as a candidate gene in glaucoma (Libby *et al.*, 2005). Despite the phenotypic similarity between *Dba2/J* mice model and glaucoma, the results of the present study did not suggest *BAX* to be a major candidate gene in POAG.

By using GWAS, Thorleifsson *et al.*, 2007 identified three variants in *LOXL1* gene on chromosome 15q22 were shown to be significantly associated with exfoliation syndrome/ exfoliation glaucoma (XFS/XFG). Although POAG and PACG share similar clinical features like optic disc damage and visual field loss with XFG, screening of XFG associated *LOXL1* gene variants in POAG and PACG indicated that these variants were not associated with primary glaucomas (Chakrabarti *et al* ., 2008). These results indicated that POAG was a relatively more complex disorder than XFG. Further screening of these variants in another secondary glaucoma indicated that the *LOXL1* variants were not associated either with PG or PDS (Rao *et al*., 2008). These observations indicated that *LOXL1* is specific to XFG and were not associated with primary and other secondary glaucomas. A recent meta-analysis further suggested the lack of involvement of *LOXL1* in POAG (Chen *et al*., 2010).

Study by Nakano *et al*., (2009) demonstrated significant association of six different variants located on three loci on chromosomes 1 (proximity to *ZP4*), 10 (proximity to *PLXDC2*) and 12 (*DKFZp762A217*) in two Japanese populations with both low and high pressure glaucomas. Even though these variants were not linked to a particular gene, all these exhibited significant associations with POAG. The screening of POAG associated susceptible loci in POAG and PACG indicated that these variations were not associated with pressure related glaucomas (Rao *et al*., 2010). The failure to replicate this association in our cohort may be due to inclusion of cases only with

elevated IOP, unlike mixture of both high and low pressure glaucomas in Japanese cohort. The differences in the allele frequencies did not exceed 5% (except for rs2499601) between our POAG cases and controls compared to the discovery cohort in the previous study. The allele frequencies among the normal controls in two Japanese cohorts were much higher at all the six loci compared to the normal controls from India, indicating perhaps a different genetic profile. These differences were unlikely due to the effect of population structuring because enrolled cases and controls from the same ethnic and geographic backgrounds from Southern India. Further, experimental errors were avoided because the screening was undertaken by resequencing in our cohort. Similar findings have been observed in Icelandic population indicating that the variants present near to *ZP4, PLXDC2* and *DKFZp762A217* were not associated with POAG with elevated IOP (Thorleifsson *et al* ., 2010).

Animal models have suggested that *VAV2* and *VAV3* are candidate genes for IOP regulation. *VAV2* and *VAV3* are involved in regulation of the cytoskeleton in the TM and its deficiency lead to buphthalmos, iridocorneal changes and elevated IOP with corresponding RGC death (Fujikawa *et al*., 2010). Variants in these genes are significantly associated with POAG in a Japanese population. However, the present study revealed that these variants were not associated either with POAG or PACG in the Indian cohort (Rao *et al*., 2010).

A recent study by Thorleifsson *et al*., (2010) identified significant association of variants between the *CAV1* and *CAV2* genes on 7q31 in an Icelandic population. Our study was unable to detect any significant association of *CAVI/CAV2* SNPs in POAG and PACG. These results were similar to Caucasian populations with POAG that did not exhibit any association to these variants (Kuehn *et al*., 2011, Wiggs *et al*., 2011).

The association of rs4236601 with POAG was widely varied across multiple cohorts. Our meta-analysis of these studies have shown a moderate association of rs4236601 with POAG.

Even though complement system is activated in POAG (Khalyfa *et al.*, 2007, Tezel *et al.*, 2010), the analysis of AMD associated *CFH* variant Y402H was not associated with POAG in the present study. Functionally we also confirmed that *CFH* expression was not altered in POAG cases harboring the risk genotype that was strongly associated with AMD.

Screening of an ECM gene in POAG revealed that variants in *FBLN5* were significantly associated with POAG and functional analysis showed that *FBLN5* was expressed in TM, retina and cornea indicating that variants present in this gene may have a pathogenic role in POAG.

We concluded that the present study provided information on the role of different candidate genes (*CTGF, NTF4, mtND1*, *BAX* and *FBLN5*) and also the role of various recently identified genetic variations in IOP related glaucomas in Indian population. The present data did not indicate the involvements of specific candidate genes in POAG and PACG indicating the complex molecular mechanisms underlying IOP regulation. We did not find association of the recently identified gene variations indicating genetic and clinical heterogeneity of POAG. However, we detected a significant association of variants in an ECM related gene *FBLN5,* thus suggesting its possible role in IOP regulation. Expression of *FBLN5* in TM and RGC further supports this observation. Overall, our data provided a better understanding of the underlying molecular mechanisms involved in IOP associated glaucomas, which may further help in devising molecular diagnostics for predictive testing in the future.

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ABSTARCT

The glaucomas comprise a group of progressive heterogeneous optic neuropathies that affects 70 million people worldwide. It is characterized by damages in the optic nerve head and corresponding visual field defects leading to irreversible blindness. It is estimated that in India glaucoma affects 12 million people and accounts for 12.8% of the blindness in the country. Primary open angle glaucoma (POAG) is the most common form of glaucoma with multiple molecular mechanisms underlying its pathogenesis. Being a complex disease, several chromosomal loci have been linked to POAG on different chromosomes, of which 17 have been named, from *GLC1A* to *GLC1Q*. But, only five loci namely, *GLC1A* (1q24.3-25.2), *GLC1E* (10p15-14), *GLC1G* (5q22.1), *GLC1O* (19q13.3) and *GLC1F* (7q35-36) have been characterized to harbor mutations in myocilin (*MYOC*), optineurin (*OPTN*), WD repeat domain 36 (*WDR36*), neurotrophic factor 4 (*NTF4*) and ankyrin repeat and SOCS box containing 10 (*ASB10*), respectively. Genome wide association studies (GWAS) have identified several variants to be associated with POAG in certain populations. These studies have revealed a large number of polymorphisms located in *LOXL1*, *CDKN2BAS1* and *TMCO1* and those flanking *ZP4*, *PLXDC2, DKFZp762A217, CAV1 and CAV2* to be associated with POAG.

Another line of studies have been initiated to identify genetic determinants for ocular quantitative traits, intraocular pressure (IOP), optic nerve parameters and central corneal thickness (CCT) through GWAS. A number of genomic regions have been identified that are susceptible for raised IOP (regions on chromosomes 2 and 19), CCT (*AKAP13, COL5A1, AVGR8, ZNF469, FOXO1, FOXO1* and *COL8A2),* optic disc area (*ATOH7, CDC7/TGFBR3* and *SALL1*) and vertical cup to disc ratio (*CDKN2B, SIX1,*

SCYL1/LTBP3, CHEK2, ATOH7 and *DCLK1*). Animal models and gene expression studies have revealed several pathways that are involved in regulation of IOP and RGC death. However, mutations in the identified candidate genes have contributed to less than 10% of POAG cases. Thus, there are different genes involved in POAG as evident from studies in humans, animals and *in vitro* results. However, their role in causing POAG in humans is limited. Thus, a comprehensive approach is needed to identify these multiple genes predisposing to POAG, which would provide further insights into the disease pathogenesis and help in better management

Hence, the aim of the current study was to identify the candidate genes involved in IOP related glaucomas viz. POAG and Primary Angle Closure Glaucoma (PACG) and to explore their possible involvement in disease pathogenesis.

Beginning with candidate gene analysis, 3 novel variants were identified in *CTGF* in the coding (R22P, P36S, P102S) and intronic (g.325G>A) regions and two polymorphisms in the promoter region (g.-444G>C, g.-251delA) of POAG and PACG. The present study indicated that *CTGF* variations were unlikely to be involved as a major candidate due to its limited involvement in these diseases. Our study also demonstrated that heterozygous variations in *NTF4* did not play a significant role in the pathogenesis of POAG and PACG. Turning to the mitochondria, 13 non-synonymous *mtND1* variations were observed of which eight were exclusively observed in POAG. But none of these were pathogenic and did not exhibit any association with POAG. Resequencing of *BAX* that was convincingly demonstrated to be associated in a mice model of glaucoma, did not reveal any disease-associated variations in POAG. All the 7 variations observed in *BAX* including a missense (G39W), two synonymous (D98D, V111V) and four known polymorphisms, were uninformative.

None of the pseudo exfoliation glaucoma (XFG) associated SNPs in *LOXL1,* nor the haplotypes generated with these risk variants were significantly associated with POAG or PACG in the present cohort. The lack of association of *LOXL1* with Pigmentary glaucoma (PG) or Pigmentary Disepersion Syndrome (PDS) further supports the fact that these variants may be specific to XFG and not involved with other primary or secondary glaucomas. Among the GWAS-associated SNPs in other populations, there was no association of the recently identified variations located in the proximity of *ZP4*, *PLXDC2* and *DKFZp762A217* in POAG or PACG. Similarly, neither the common variants near *CAV1* and *CAV2,* nor the risk alleles of *VAV2* and *VAV3* in the Japanese population, were found to be associated with POAG or PACG in the present cohort.

Among genes involved in innate immune response, the AMD associated *CFH* variant was not associated with POAG. Further functional analysis revealed that the expression of *CFH* was similar in both the cases and controls harboring the risk genotype. However, screening of an extracellular matrix (ECM)-related gene, revealed variations in *FBLN5* that were strongly associated with POAG. Further functional analysis showed that *FBLN5* was expressed in the trabecular meshwork (TM), retina and cornea, suggesting that it could be a candidate gene for IOP regulation and RGC death.

The present study provided information on the role of different candidate genes (*CTGF, NTF4, mtND1*, *BAX* and *FBLN5*) and other genetic variants in IOP related glaucomas in an Indian population cohort. The lack of association to majority of these genes, indicated the complex molecular mechanism underlying IOP-associated glaucomas. It also suggested the genetic and clinical heterogeneity of the condition. However, we found a functional association of an ECM related gene *FBLN5* suggesting

its possible role in disease pathogenesis. Overall, the present data provided further insights into the molecular mechanisms involved in IOP-associated glaucomas that may help in better understanding and management of the disease in the Indian scenario.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: REVIEW OF LITERATURE 13

2.1. Glaucoma 13

2.2. Etiology of glaucoma 13

2.3. Classification of glaucoma 14

2.3.1. Primary glaucomas 14

2.3.2. Secondary glaucomas 16

2.4. Prevalence of POAG 16

2.4.1. Prevalence of POAG in India 16

2.5. Risk factors for POAG 17

2.5.1. Elevated IOP as a risk factor in POAG 17

2.5.2. Age 17

2.5.3. Family history 17

2.5.4. Race or ethnicity 18

2.5.5. Myopia 18

2.5.6. Central corneal thickness 18

2.5.7. Diabetes 18

2.5.8. Hypertension 19

2.5.9. Gender 19

2.6. Genetics of POAG 19;

2.6.1. Candidate loci in POAG 20

2.6.2 Candidate genes linked to POAG 22

2.6.2.1. *MYOC* 22

2.6.2.2. *OPTN* 27

2.6.2.3. *WDR36* 30

2.6.2.4. *NTF4* 33

2.6.2.5. *RPGRIP1* 33

2.6.2.6. *ASB10* 34

2.6.2.7. *CYP1B1* 34

2.6.3. Association studies for POAG and PACG 36

2.6.4. Genome wide association studies 41

2.6.5. Quantitative trait loci for central corneal thickness, intraocular Pressure and cup to disc ratio 45

2.6.6. Interaction of genes and its influence on IOP 49

2.7. Molecular mechanism of IOP elevation 49

2.7.1. Structures involved in aqueous outflow resistance 50

2.7.2. Biological properties of aqueous humor and its outflow 50

2.7.3. Factors involved in IOP elevation 52

2.7.3.1. Age 53

2.7.3.2. Extracellular matrix and its role in elevation of IOP 53

2.7.3.3. Role of Oxidative Stress in IOP elevation 56

2.7.3.4. Role of mitochondria as a risk factor in IOP 57

2.7.4. Animal models in IOP regulation 57

2.8. Mechanism of pressure induced ONH degeneration and RGC death 59
2.8.1. Neurotrophin deprivation 59
2.8.2. Astrocyte mediated RGC death 59
2.8.3. Immune system mediated RGC death 60
2.8.4. Oxidative stress mediated RGC death 61
2.8.5. Mitochondria mediated RGC death 62
2.8.6. Glutamate mediated excitotoxicity 62
2.9. Gene expression studies in glaucoma 63
CHAPTER 3: METHODOLOGY 64
3.1. Enrolment of cases and controls 64
3.2. Clinical examination and data collection 65
3.2.1 Confirmation of diagnosis 65
3.2.2 Inclusion and exclusion criteria for POAG 65
3.2.2.1. The inclusion criteria for POAG 65
3.2.2.2. Exclusion criteria for POAG 66
3.2.3. Inclusion and exclusion criteria for PACG 67
3.2.3.1. The inclusion criteria for PACG 67
3.2.3.2. Exclusion criteria for PACG 67
3.2.4. Inclusion and exclusion criteria for Controls 67
3.2.4.1. Inclusion criteria for controls 67
3.2.4.2. Exclusion criteria for controls 68
3.3. Molecular genetic analysis of candidate genes and gene variants 68

3.3.1. Designing of primers 69

3.3.2. Amplification of candidate genes and gene variants by polymerase chain reaction (PCR) 75

3.3.3. Confirmation of the amplified PCR products by agarose gel Electrophoresis 75

3.3.4. Mutation screening by resequencing 76

3.3.4.1. Sequencing reaction 76

3.3.4.2. Purifying Extension Products 76

3.3.4.3. Electrophoresis 77

3.3.4.4. Interpreting Sequencing Results 77

3.3.5. Prediction of amino acid substitutions observed in candidate genes 78

3.3.5.1. Multiple sequence alignment 78

3.3.5.2. SIFT analysis of mutations 78

3.3.6. Screening of gene variations by PCR based restriction digestion 78

3.3.6.1. Methodology for restriction digestion 79

3.4. Illumina GoldenGate Genotyping Assay principle and protocol 80

3.4.1. Sample preparation and quantitation Picogreen Assay for golden gate 82

3.4.2. Confirmation of GoldenGate Genotyping Assay 84

3.5. Statistical analysis for gene variants 84

3.6. Functional analysis of ECM genes 84

3.6.1. Collection of AqH samples 84

3.6.1.1. Inclusion criteria for POAG 84

3.6.1.2. Exclusion criteria for POAG 85

3.6.1.3. Inclusion criteria for controls 85

3.6.1.4. Exclusion criteria for controls 85

3.6.2. Western blotting for the detection of FBLN5 in the aqueous humor and CFH in serum 86

3.6.2.1. Preparation of gel 86

3.6.2.2. Preparation of samples 87

3.6.2.3. Loading of samples 87

3.6.2.4. Transfer of proteins from SDS PAGE gels to PVDF membrane 88

3.6.2.5. Detection 89

3.6.3. ELISA for CFH 90

3.6.4. Immunohistochemistry for localization of FBLN5 in Human eye 91

3.6.4.1. Embedding 91

3.6.4.2. Blocking endogenous peroxidase activity 92

3.6.4.3. Antigen retrieval 92

3.6.4.4. Haematoxylin and Eosin 93

CHAPTER 4: RESULTS 94

4.1 Distribution of study cohort 94

4.2. Analysis of candidate genes 95

4.2.1. Analysis of *CTGF* gene 95

4.2.2. Analysis of the *NTF4* gene 101

4.2.3. Analysis of *mtND1* in glaucoma 104

4.2.4. Analysis of *BAX* gene in POAG patients 110

4.3. Analysis of gene variants (single nucleotide polymorphisms, SNPs) in POAG, PACG cases and controls 113

4.3.1. Analysis of *LOXL1* SNPs in POAG and PACG 113

4.3.2. Analysis of *LOXL1* SNPs in PDS and PG 117

4.3.3. Analysis of three susceptible loci in POAG and PACG 120

4.3.4. Analysis of *VAV2* and *VAV3* SNPs in POAG and PACG 124

4.3.5. Analysis of *CAV1* and *CAV2* polymorphisms in Indian POAG and PACG patients 127

4.3.6. Analysis of *CAV1* and *CAV2* SNPs in PG and PDS 131

4.3.7. Analysis of complement factor H (*CFH*) variant (Y402H; rs1061170, T > C) 134

4.3.8. Analysis of *FBLN5* SNPs in POAG 134

4.4. Functional analysis of *CFH* and *FBLN5* 136

4.4.1. Functional analysis of *CFH* by immunoblotting and ELISA 136

4.4.2. Analysis of *FBLN5* in aqueous humor by immunoblotting 138

4.4.3. Immunohistochemical localization FBLN5 in the eye 139

CHAPTER 5: DISCUSSION 143

CHAPTER 6: CONCLUSIONS 162

CHAPTER 7: CONTRIBUTIONS OF THE PRESENT STUDY 163

CHAPTER 8: LIMITATIONS OF THE PRESENT STUDY 165

CHAPTER 9: FUTURE SCOPE 166

LIST OF TABLES

Table No.	Title	Page No
Table 2.1. List of candidate loci identified in POAG		21
Table 2.2. Distribution of *MYOC* mutations across the world		25
Table 2.3. Distribution of *OPTN* mutations across the world		29
Table 2.4. Distribution of *WDR36* mutations across the world		32
Table 2.5. *CYP1B1* in adult onset POAG worldwide		36
Table 2.6. Status of candidate gene association with glaucoma in different studies		37
Table 2.7. Quantitative trait and their associated candidate genes		48
Table 3.1. Gene and gene variants studied in the present study		70
Table 3.2. Primer sequences used to amplify candidate genes in the present study		71
Table 3.3. Primer sequences used for screening of gene variants		73
Table 3.4. Reagents used in PCR reaction		75
Table 3.5. The thermal cycling conditions were as follows		75
Table 3.6. Reagents used in PCR reaction		76
Table 3.7. The thermal cycling conditions were as follows		76
Table 3.8. Reagents used for Restriction digestion		79
Table 3.9. Reagents for preparing resolution gel for SDS PAGE		86
Table 3.10. Reagents for preparing 5% stacking gel for SDS PAGE		87
Table 4.1. Age and gender distribution among POAG, PACG cases and normal individuals		95
Table 4.2. *CTGF* variants observed in POAG, PACG patients and controls		97
Table 4.3. Clinical features of patients with variations in the coding region of *CTGF* at presentation		99
Table 4.4. Distribution of heterozygous variations in *NTF4* observed in primary glaucomas and controls in the Indian cohort		102
Table 4.5. Clinical features of patients with A88V variation at presentation in Indian cohort		103

Table 4.6. Non synonymous *mtND1* Sequence Changes in POAG and controls 105

Table 4.7. Clinical features of patients carrying *mtND1* nonsynonymous variations 108

Table 4.8. Clinical features of controls containing *mtND1* variations 109

Table 4.9. Synonymous *mtND1* Sequence Changes in Patients with POAG and controls 109

Table 4.10. Variations observed in the coding region of *BAX* gene 111

Table 4.11. Clinical features of patients with variations in coding region of *BAX* at presentation 111

Table 4.12. Reported polymorphism in the *BAX* gene 113

Table 4.13 Distribution of Allele Frequencies and Their Odds Ratios for the *LOXL1* SNPs across POAG and PACG Cases in the Present Cohort 115

Table 4.14. Distribution of Genotype Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs in POAG and PACG 115

Table 4.15. Estimated *LOXL1* Haplotype Frequencies across POAG and PACG Cohorts 116

Table 4.16. Distribution of Allele Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs across PG and PDS Cases in the Present Cohort 118

Table 4.17. Distribution of Genotype Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs in PG and PDS 118

Table 4.18. Estimated *LOXL1* haplotype frequencies of PG/PDS patients and controls 119

Table 4.19: Distribution of Allele Frequencies and Their Odds Ratios for the six SNPs across POAG and PACG Cases in the Present Cohort 121

Table 4.20. Distribution of Genotype Frequencies and Their Odds Ratios for the 6 SNPs in POAG and PACG 122

Table 4.21. Haplotype frequencies in POAG and PACG cases and normal controls in an Indian population 123

Table 4.22. Distribution of Allele Frequencies and Their Odds Ratios for the *VAV2* and *VAV3* SNPs across POAG and PACG Cases in the Present Cohort 125

Table 4.23. Distribution of Genotype Frequencies and Their Odds Ratios for the *VAV2* and *VAV3* SNPs across POAG and PACG Cases in the Present Cohort 125

Table 4.24. Distribution of haplotype frequencies at the *VAV2* and *VAV3* loci in POAG, PACG and normal controls in the Indian cohort 126

Table 4.25. Distribution of Allele Frequencies and Their Odds Ratios for the *CAV1*, *CAV2* SNPs across POAG and PACG Cases in the Present Cohort 128

Table 4.26. Distribution of Genotype Frequencies and Their Odds Ratios for the rs1052990 and rs4236601 across POAG and PACG Cases in the Present Cohort 128

Table 4.27. Allele frequency and association of rs1052990 and rs4236601 with PG and PDS 133

Table 4.28. Distribution of Genotype Frequencies and Their Odds Ratios for the rs1052990 and rs4236601 across PG and PDS Cases in the Present Cohort 133

Table 4.29. Allele frequency and its association of rs1061170 between cases and controls 134

Table 4.30. Genotype frequency and its association of rs1061170 between cases and controls 134

Table 4.31. Allele frequency and its association of *FBLN5* with POAG 135

Table 4.32. Distribution of haplotype frequencies of *FBLN5* SNPs in POAG and normal controls in the Indian cohort 136

Table 5.1. Distribution of A88V variation in different populations 145

Table 5.2. Distribution of Allele Frequencies and Their odds Ratios for the *LOXL1* SNPs across POAG Cases of different populations 150

Table 5.3. Distribution of Estimated Haplotype Frequencies and Their Odds ratiosfor the Two SNPs (rs1048661 and rs3825942) across POAG and PACG in the Present Cohort and Other Populations 152

Table 5.4. The distribution of three chromosomal loci implicated in the Japanese population in the Indian POAG and PACG cohort 155

Table 5.5. Distribution of allele frequencies of rs4236601 with POAG in different populations 157

LIST OF FIGURES

Figure No	Title	Page No
Figure 2.1	Diagrammatic representation of the drainage structures in POAG	15
Figure 2.2	Diagrammatic representation of the drainage structures in PACG	15
Figure 3.1	Diagrammatic representation of GoldenGate genotyping assay	82
Figure 4.1	Schematic representation of the different domains of *CTGF* and the identified variations	96
Figure 4.2	Electropherograms representing *CTGF* variants	100
Figure 4.3	Multiple sequence alignment of *CTGF* protein across different species	100
Figure 4.4	Electropherograms representing the *NTF4* variations	102
Figure 4.5	Schematic representation of *mtND1* and location of observed variations	106
Figure 4.6	Conservation of M31V across different species	107
Figure 4.7	Chromatogram showing the g.856G>T (G39W)	111
Figure 4.8	Multiple sequence alignment for G39W variation	111
Figure 4.9	LD plot for all the 7 SNPs	129
Figure 4.10	Summary of meta- analysis plot (random effects)	130
Figure 4.11	Summary of meta- analysis plot (fixed effects)	131
Figure 4.12	Immuno blotting analysis of CFH in human POAG and control serum samples	137
Figure 4.13	Immuno blotting analysis of CRP in human POAG and control serum samples	137
Figure 4.14	Analysis of CFH in different dilutions of serum samples by ELISA in patients and controls carrying the risk genotype	138
Figure 4.15	Western blot analysis for FBLN5 in aqueous humor of POAG and normal subjects.	139
Figure 4.16	Immuno histochemical analysis of FBL5 in human cornea and anterior segment	140
Figure 4.17	Immunohistochemical analysis of FBLN5 in human retina	141

LIST OF ABBREVIATIONS

DPX	Distyrene-plasticizer-xylene
ABI	Applied Biosystems, Inc
BDT	Big Dye Terminator
bp	Basepair
CCT	Central Corneal Thickness
CDR	cup-to-disc ratio
D'	Coefficient of linkage disequilibrium
DAB	3, 3' diamino benzidine
DMSO	Dimethylsulphoxide
dNTPs	deoxy nucleotide triphosphates
EDTA	Ethylenediaminetetraaceticacid
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IOP	Intraocular pressure
JOAG	Juvenile open angle glauocma
kb	kilobase
kDa	Kilodalton
LD	Linkage Disequilibrium
mL	milliliter
M	Molar
mL	microliter
mM	millimolar
mmHg	millimetres of mercury
mtDNA	mitochondrial DNA
ng	nano gram
OD	right eye
OS	left eye

PACG	Primary angle closure glaucoma
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDS	Pigment dispersion syndrome
PG	Pigmentary Glaucoma
pm	pico molar
POAG	Primary open angle glaucoma
RFLP	Restriction fragment length polymorphism
RGC	retinal ganglion cells
Rpm	revolutions per minute
SDS	Sodium dodecylsulphate
SIFT	Sorting Intolerant From Tolerant
TAE	Tris acetate EDTA
TM	Trabecular meshwork
Tris	Tris(hydroxymethyl)aminomethane
Mg	Microgram
Ml	Microlitre
μM	Micromolar
χ^2	Chi-square

CHAPTER 1: INTRODUCTION

Glaucoma comprises a group of clinically and genetically heterogeneous optic neuropathies characterized by optic nerve head (ONH) degeneration, gradual and progressive loss of visual fields leading to irreversible blindness (Kwon *et al*., 2009). It affects around 70 million people and is the second leading cause of blindness worldwide (Quigley *et al*., 2006). It was estimated that by the year 2020 this number would reach 80 million, of which majority of the cases would be attributed to primary open angle glaucoma (POAG). In India glaucoma affects 12 million people and accounts for 12.8% of the total blindness, of which 6.48 million are estimated to be POAG (George *et al*., 2010). POAG being a complex disease, the underlying molecular mechanisms are still unclear and it exhibits a multifactorial etiology.

Glaucomas can be divided into two subtypes based on the resistance to aqueous humor outflow *viz*. primary and secondary glaucomas. In primary glaucomas, the initial events lead to aqueous outflow obstruction along with an elevated intraocular pressure (IOP) and usually not associated with any other ocular or systemic conditions. Elevated IOP develops as a result of high resistance to the drainage of aqueous humor. The normal level of IOP in the general population has been estimated to be between 10 to 21mmHg (Kanski *et al*., 2011). Secondary glaucomas on the other hand, are characterized by the involvement of other ocular or systemic disease resulting in alterations to the aqueous outflow (Shields *et al*., 2005). Further, primary glaucomas are of two types based on the anatomy and mechanisms i.e. POAG and primary angle closure glaucoma (PACG). Based on age of onset, POAG is further classified as juvenile open angle glaucoma (JOAG, that occurs before 35 years of age) and adult onset glaucoma. Generally, POAG is associated with elevated IOP (>21mmHg), but in

some cases, glaucomatous cupping is seen even with IOP <21mmHg, a condition referred to as normal tension glaucoma (NTG) (Shields *et al*., 2005). Along with elevated IOP, the other common risk factors for POAG are age, race, family history, thin cornea, myopia, diabetes, hypertension and oxidative stress (Tielsch *et al.,* 1994, Rudnicka *et al.,* 2006, Newman-Casey *et al.,* 2011, Marcus *et al.,* 2011).

Since, family history plays an important role in POAG, several studies have been conducted to understand the genetic mechanisms underlying POAG. So far, 30 chromosomal loci have been mapped by linkage analysis of which, 17 have been designated from *GLC1A* to *GLC1Q* by HUGO (Human genome organization) genome nomenclature committee and mutations in five genes have been characterized. These genes are myocilin (*MYOC; GLC1A*), optineurin (*OPTN; GLC1E*), WD repeat domain36 (*WDR36; GLC1G*), neurotrophic factor 4 (*NTF4; GLC1O*) and ankyrin repeat and SOCS box containing 10 (*ASB10; GLC1F*) (Stone *et al*., 1997, Rezaie *et al*., 2002, Monemi *et al*., 2005, Pasutto *et al*., 2009, Paustto *et al*., 2012).

MYOC was the first candidate gene mapped in POAG and identified on 1q21-q31 (Stone *et al*., 1997). Mutations in *MYOC* have been reported in different ethnic groups and accounts for 2-5% of POAG patients worldwide. Around ~72 mutations have been reported and the Gln368Stop mutation was the most common mutation observed across multiple populations except Japanese and Indian population (Gong *et al*., 2004). In India, a low frequency of *MYOC* mutations were observed and Gln48His was the predominant mutation observed in cases of JOAG, POAG and PCG (Kanagavalli *et al*., 2003, Chakrabarti *et al*., 2005). *In vitro* and *in vivo* studies have shown that *MYOC* mutations may act by gain of function in POAG (Kim *et al*., 2001, Gould *et al*., 2006). Mutations in *MYOC* may interfere with protein folding as it forms

protein aggregates in the endoplasmic reticulum (ER) leading to the activation of an unfolded protein response (UPR) in the trabecular meshwork ([TM]; Yam *et al*., 2007). Animal models have also shown that the expression of mutant *MYOC* induces ER stress and cell death resulting in elevation of IOP and retinal ganglion cell (RGC) death (Zode *et al*., 2011).

OPTN was the second candidate gene identified on 10p15-p14 (Rezaie *et al.,* 2002). However, only few mutations in this gene have been implicated in NTG (Alward *et al*., 2003, Aung *et al*., 2003, Xiao *et al*., 2009). In India mutations in *OPTN* had a minimal involvement in glaucoma (Mukhopadhyay *et al*. 2005; Sripriya *et al*., 2006). Among the several mutations, E50K located in the putative bZIP motif (basic region-leucine zipper motif) appeared to be strongly associated with NTG. *OPTN* serves many cellular functions by interacting with several proteins (Anborgh *et al*., 2005, Park *et al*., 2006, Zhu *et al*., 2007). *OPTN* is a negative regulator of tumor necrosis factor-alpha **(***TNFα)* wherein, it inhibits the *TNFα* mediated *NFkB* (Nuclear factor kB) activity thereby inhibiting the cell death (Sahlender *et al*., 2005, Nagabhushana *et al*. 2010).

WDR36 was the third candidate gene identified on 5q22.1 (Monemi *et al*., 2005). However, subsequent reports have shown that mutations in the *WDR36* may not be directly involved in POAG and it may act as a modifier gene (Hauser *et al*., 2006, Pasutto *et al*., 2008). *WDR36* was not associated with POAG in the Indian population (Mookherjee *et al*., 2011). Recent reports have shown that the *WDR36* is involved in the biogenesis of ribosomes by processing 18S rRNA and defects in *WDR36* induces the cell death by activation of *p53* mediated stress response pathway (Gallenberger *et al*., 2011).

NTF4 was recently identified as a candidate gene in POAG on 19q13.33 and mutations have been identified in 1.7% of European populations (Pasutto *et al*., 2009). *In vitro* studies have shown that these mutations impair neurotrophin signaling by reducing the binding affinity to tyrosine kinase B (*TrkB*) receptor. However, later studies have demonstrated the lack of involvement of *NTF4* in Caucasian, Indian and Chinese population (Liu *et al*., 2010, Rao *et al*., 2010, Vithana *et al*., 2010).

Study by Wiggs *et al*., (2000) have reported several loci, including regions on chromosome 2, 14, 17 and 19 in POAG by linkage analysis. Fernandez-Martinez *et al*., (2011) mapped a POAG locus on 14q11 and demonstrated mutations in the retinitis pigmentosa GTPase regulator interacting protein 1 (*RPGRIP1*) that was associated with an increased risk of POAG, JOAG and NTG. Several nonsynonymous variants identified in *RPGRIP1* were distinct from variants associated with photoreceptor dystrophies and were predominantly present in the *RPGR*-interacting domain. *In vitro* studies have shown that mutations in C2 domain of *RPGRIP1* altered its interaction with nephrocystin.

Recently, Pasutto *et al*., (2012) identified the causative gene for *GLC1F* region. Several variations in *ASB10* gene were identified in 6% of the POAG cases as compared to 2.8% of normal controls (p=0.008). However, the exact physiological function of the *ASB10* remains unclear.

Cytochrome P450, Subfamily I, Polypeptide 1 (*CYP1B1)*, a candidate gene for primary congenital glaucoma (PCG) has been mapped on the *GLC3A* locus at 2p21 (Stoilov *et al*., 1997). Animal models have demonstrated that *CYP1B1* deficiency leads to the abnormality in TM that are similar to those observed in human PCG (Libby *et al*., 2003). Mutations in *CYP1B1* were involved in JOAG, POAG and PACG in various

ethnic groups ranging from 2.2-22.3% and the mutation spectrum was largely similar across all the glaucoma phenotypes (Vincent *et al*., 2002, Melki *et al*., 2004, Chakrabarti *et al*., 2007).

By using whole genome linkage analysis and single nucleotide polymorphism (SNP) mapping, Jiao *et al*., (2009), identified a locus on chromosome 2 associated with POAG. Case control analysis later led to the identification of a strong association of the rs12994401 variant with POAG. However, this variant could not be replicated with POAG in the Japanese population (Mabuchi *et al*., 2010).

The complex nature of POAG implies that both genetic and environmental factors are likely to be involved in its pathophysiology. Apart from mutations in the candidate genes, SNPs in several genes were found to be associated in POAG. Several studies indicated variants in Atrial natriuretic peptide *(ANP), p53,* Tumor necrosi factor α *(TNFα),* Apolipoprotein E *(APOE),* Glutathione S-transferase *(GST),* Endothelial nitric oxide synthase *(eNOS)*, Interleukins and Optic atrophy type 1 (*OPA1)* were associated with POAG (Tunny *et al*., 1996, Juronen *et al*., 2000, Vickers *et al*., 2002, Lin *et al*., 2003, Powell *et al*., 2003, Lin *et al*., 2003, Ressiniotis *et al*., 2004). However, the associations of these genes were not consistent across all populations and thus their role in the disease pathogenesis still remains unclear.

As linkage studies were unsuccessful in identifying the causal genes in POAG, genome wide association studies (GWAS) were used for the identification of genes and genetic variants that may predispose to this disease. By using these methodologies, several gene variants in Lysyl oxidase-like 1 (*LOXL1),* and those in close proximity to Zona pellucida glycoprotein 4 (*ZP4),* Plexin domain containing 2 (*PLXDC2),* Transmembrane and tetratricopeptide repeat containing 2 *(TMTC2 or*

DKFZp762A217), Caveolin-1 *(CAV1),* Caveolin-2 *(CAV2),* Transmembrane and coiled-coil domains 1 (*TMCO1)* and Cyclin-dependent kinase inhibitor 2B antisense RNA 1 (*CDKN2B-AS1)* were identified to be genetically associated with POAG in different populations.

LOXL1 was first candidate gene identified in exfoliation syndrome/ exfoliation glaucoma (XFS/XFG) through GWAS (Thorleifsson *et al*., 2007). Three variants rs1048661 (R141L), rs3825942 (G153D) located on exon 1 and rs2165241 located in intron 1 of *LOXL1* were shown to be strongly associated with XFS/XFG while the rs2165241 was also associated with POAG. *LOXL1* is a member of lysyl oxidase family of proteins that are involved in the regulation of extracellular matrix (ECM) organization in the TM. However, replications of these variants in POAG in other populations revealed that these variants were not associated with POAG, PACG and pigmentary glaucoma (PG), thereby indicating specific associations of these variants with XFS/XFG (Chakrabarti *et al*., 2008, Rao *et al*., 2008).

Nakano *et al*., (2009) identified six intergenic variations near *ZP4* (rs547984, rs540782, rs693421, rs2499601), *PLXDC2* (rs7081455) and intronic region of *DKFZp762A217* (rs7961953) to be moderately associated with POAG through a 2-stage study in the Japanese population. However, both the discovery and replication cohorts comprised of low and high pressure glaucomas and the association of these variants were not specific to the IOP-associated POAG. Screening of these variants did not reveal any associations to POAG in the Indian and Icelandic populations with raised IOP (Rao *et al*., 2009, Thorleifsson *et al*., 2010).

Another GWAS conducted in the Icelandic population identified several variants associated with POAG of which, rs4236601 and rs1052990 located near to *CAV1* and *CAV2,* respectively, on 7q31 were strongly associated with POAG in the

discovery cohort from Iceland and across multiple replication cohorts from the UK, Australia and China (Thorleifsson *et al*., 2010). *CAV1* and *CAV2* comprise groups of scaffolding proteins and are involved in the regulation of *eNOS* and transforming growth factor-beta (*TGFβ)* signaling (Garcia-Cardena *et al*., 1997, Razani *et al*., 2001). However, screening of these variants in the Caucasian populations revealed that these were not strongly associated with POAG (Kuehn *et al*., 2011 and Wiggs *et al*., 2011)

A recent study by Burdon *et al*., (2011), identified two variants located near the *TMCO1* (rs4656461) and *CDKN2B-AS1* (rs4977756) genes to be significantly associated with POAG in the Australian population. *CDK2BAS1* encodes an RNA that modulates the *CDKN2B,* which regulates the cell cycle by activating retinoblastoma tumor suppressor pathway (Gonzalez *et al*., 2006). Recently it has been demonstrated that variants in *CDKN2B* influence the optic disc parameters and are significantly associated with POAG (Ramdas *et al*., 2011, Fan *et al*., 2011). *TMCO1* is a transmembrane protein involved in regulation of ER and mitochondrial activities (Zhang *et al*., 2010). Since ER and mitochondrial pathways are involved in POAG, *TMOC1* could play a role in pathogenesis of POAG (Kong *et al*., 2009, Zode *et al*., 2011). However, recently it has been shown that these variants are associated with NTG and not with HTG (Burdon *et al*., 2012, Nakano *et al*., 2012, Mabuchi *et al*., 2012).

Another study by van Koolwijk *et al*., (2012) identified the genetic variants in *GAS7* (rs11656696) and *TMCO1 (*rs7555523) to be associated with IOP. Interestingly these two variants were also marginally associated with POAG. *GAS7* and *TMCO1* are expressed in tissues like TM, celiary body and ONH implicating its role in POAG. Biochemical protein interactions with known glaucoma disease genes

(*OPTN*, *WDR36*, *CAV1*, *NOS2*, *FOXC1*, *APOE*), as well as functional data support the involvement of these genes in aqueous humor dynamics and glaucomatous neuropathy.

Following GWAS in the glaucomatous groups that were inconsistently associated across several populations, studies were undertaken on specific quantitative traits such as IOP, cup to disc ratio (CDR) and central corneal thickness (CCT) in POAG as these were considered to be highly heritable and controlled by multiple genes (Klein *et al*., 2004, van Koolwijk *et al*., 2007). Several genes have been identified of which, variants in *ZNF469, COL5A1, COL8A2, AVGR8* and *AKAP13* were shown to influence CCT and those in *ATOH7, CDC7/TGFβR3, SALL1, CDKN2B, SIX1, SCYL1, LTBP3, CHEK2, RFTN1* and *DCLK1* influenced CDR (Abu *et al*., 2008, Desronvil *et al*., 2010, Ramdas *et al*., 2011, Fan *et al.,* 2011). However, studies have shown that except *CHEK2*, variants in *ATOH7, CDKN2B, SIX1, RFTN1* and *DCLK1* were associated with NTG and not with HTG (Burdon *et al*., 2012, Mabuchi *et al*., 2012, Nakano *et al*., 2012).

Overall the genes identified by conventional linkage analysis, case control association studies and GWAS contributed to a small fraction of POAG. Hence, there are several causal genes in POAG that are yet unidentified.

Gene expression studies have shown elevated levels of several ECM molecules (fibronectin, collagens, elastin) in the TM and aqueous humor of POAG compared to the normal subjects without glaucoma (Tamm *et al*., 2007, Acott *et al*., 2008). The elevated levels in the ECM may block the TM outflow, thereby resulting in the aqueous outflow resistance and elevation of IOP. Several regulatory molecules have been identified of which, the connective tissue growth factor (*CTGF)* is highly expressed in the TM (Tomarev *et al*., 2003). Higher expression of *CTGF* has also been observed in response to the *TGFβ*, IOP and mechanical stress (Chudgar *et al*., 2006, Fuchshofer *et*

al., 2009). It was demonstrated that recombinant *CTGF* induced the expression of ECM (fibronectin and collagens) in TM cells indicating it to be a possible candidate gene for IOP regulation (Junglas *et al*., 2009). Study by Junglas *et al*., (2012) have revealed that transgenic overexpression of *CTGF* in the mouse eye causes an increase in IOP and a continuous decline in the number of optic nerve axons. The elevated IOP in mouse eyes with *CTGF* overexpression is caused by molecular and structural changes in the TM outflow pathways. These changes did not involve closure of the iridocorneal angle (eg, due to iridocorneal adhesions) but occured in the presence of an open iridocorneal angle, a situation similar to POAG.

Mitochondrial abnormalities have been demonstrated in the TM of POAG across some studies (Izzotti *et al*., 2011). These may induce oxidative stress that might affect the cellularity of the TM. Additionally it was shown that mitochondrial DNA damage could be correlated with the elevation of IOP (Saccà *et al*., 2005). Increased lipid peroxidation, decreased antioxidant potential further supporting the role of oxidative stress in POAG (Yildirim *et al*., 2005). A study by Abu- Amero *et al.,* (2006) has shown that several mitochondrial DNA variations are present exclusively in POAG patients with increased mtDNA content and reduced mitochondrial respiration activity (MRA), further supporting the role of mitochondrial variations in POAG pathogenesis.

Studies on animal models have shown that *BCL2*-associated X protein (*BAX), VAV2, VAV3* and secreted protein acidic and rich in cysteine (*SPARC)* are important candidate genes for the regulation of IOP (Libby *et al*., 2005, Haddadin *et al*., 2009, Fujikawa *et al*., 2010). Mice deficient in two copies of *Bax* (*Bax*$^{-/-}$) had lower IOP than *Bax*$^{+/-}$ and *Bax*$^{+/+}$ indicating that apoptotic death of cells are involved in the aqueous humor outflow, which may further contribute to IOP elevation (Libby *et al*., 2005). *SPARC* is involved in the regulation of ECM in TM and *Sparc* deficient mice had lower

IOP than the wild type (Haddadin *et al*., 2009). *SPARC* is a matricellular protein that promotes the synthesis of ECM molecules. It is also known to express in the lens, cornea, TM and retinal pigement epithelium (RPE) and it is one of the most upregulated gene in response to elevated IOP and mechanical stretching (Rhee *et al*., 2003, Haddadin *et al*., 2009). Similarly, *VAV2* and *VAV3,* which are involved in cytoskeleton arrangements have shown that *Vav2/Vav3* deficient mice exhibited spontaneous glaucoma resulting in iridocorneal changes and elevated IOP (Fujikawa *et al*., 2010).

Among the several mechanisms proposed for RGC death in POAG, neurotrophin deprivation, glial cell activation, ECM remodulation, mitochondrial abnormalities and complement system activation have been demonstrated *in vitro*. The elevated IOP induces the stress on the lamina cribrosa and may prevent the transfer of neurotrophins from the brain to the eye and this results in the neurotrophin deprivation and RGC death (Lieven *et al*., 2006). Elevated IOP also activates astrocytes and this in turn become reactive and induces the synthesis of several ECM molecules, which remodulates the ONH (Johnson *et al*., 2007). Reactive astrocytes also synthesize several inflammatory molecules like *TNFα,* which further induces RGC death (Nakazawa *et al.,* 2006). Recent studies have shown the role of the complement system in RGC, wherein, elevated expression of certain complement factors (*C1q, C3*) and down regulation of complement factor H (*CFH*) have been observed (Khalyfa *et al*., 2007, Tezel *et al*., 2010).

Mitochondrial abnormalities have also been implicated in RGC death in Leber's hereditary optic neuropathy (LHON) in which, variations in the mitochondrially encoded NADH dehydrogenase 1 (*mtND1)* were associated with the disease and further it interacted with *BAX* in the cytosol to induce cell death (Perier *et al*., *et al*., 2005). In

animal models it has been shown that elevated IOP triggers the mitochondrial fission in RGC, which in turn releases *OPA1* and cytochrome c causing subsequent RGC death (Ju *et al*., 2008).

Animal models have also shown that *BAX* is one of the major candidate for RGC death. Mice carrying both $Bax^{+/-}$ and $Bax^{-/-}$ were protected from RGC death compared to mice with $Bax^{+/+}$ indicating that *BAX* could be candidate gene for susceptibility to RGC death (Libby *et al*., 2005).

The different genes identified through studies in humans and animal models and also through *in vitro* evidences have contributed to only a small fraction of POAG and the role of these genes in disease pathogenesis is yet unknown. Several candidate genes identified in animal models are yet to be replicated in the human system (Bidinost *et al*., 2006). Thus, specific candidate gene screening involved in the pathologies leading to POAG would be helpful in identifying genetic factors that may predispose to this disease. In addition, characterization of these genes would provide further insights into the disease pathogenesis, which would eventually helps in a relatively better management of the disease.

In order to address these issues, the following study was undertaken to identify candidate genes involved in IOP- related primary glaucomas *viz*. primary open angle glaucoma (POAG) and primary angle closure glaucoma (PACG) and to understand their possible role in the disease pathogenesis. The main objectives were

1. To screen candidate genes like *CTGF, NTF4, BAX,* and *mtND1* that were suggested to be involved in IOP regulation and RGC death in POAG.
2. Replication of genetic associations of the recently identified variants in *LOXL1* and those in proximity to *ZP4*, *PLXDC2*, *DKFZp762A217, VAV2, VAV3, CAV1* and *CAV2*.

3. Screening of the Age related Macular Degeneration (AMD) associated *CFH* variant (Y402H) followed by functional analysis of *CFH* in the human serum samples.
4. Screening of a genetically associated ECM gene followed by functional analysis of the protein using immunoblotting and immunohistochemistry (IHC).

CHAPTER 2: REVIEW OF LITERATURE

2.1. Glaucoma

Glaucoma is a complex, heterogeneous disease characterized by progressive degeneration of optic nerve head (ONH) and visual field defects (Kwon *et al.,* 2009). It affects around 70 million people and is the second leading cause of blindness worldwide (Quigley *et al.,* 2006). It was estimated that by the year 2020 this number would rise to 80 million people worldwide (Quigley *et al.,* 2006). Elevated intraocular pressure (IOP) is a major risk factor for glaucoma, and experimental evidences have shown that elevated IOP causes glaucoma in animal models (Levkovitch-Verbin *et al.,* 2002). The other common risk factors in glaucoma include age, race (Rudnicka *et al.,* 2006), family history (Tielsch *et al.,* 1994), thin cornea (Francis *et al.,* 2008), myopia (Marcus *et al.,* 2011), diabetes, hypertension (Newman-Casey *et al.,* 2011) and oxidative stress (Sacca *et al.,* 2008).

The clinical symptoms of glaucoma are usually not manifested in the earlier stages and sometimes vision loss is the first sign of the disease, because of which glaucoma is often called as a "silent thief of sight" (Shields *et al.,* 2005). Clinically, glaucoma is characterized by thinning of the retinal nerve fibre layer (RNFL), ONH cupping, and eventual loss of visual fields. Cupping of ONH occurs due to the loss of retinal ganglion cells (RGC) axons in combination with posterior bowing and collapse of the connective tissue sheets in the lamina cribrosa (Quigley *et al.,* 2011).

2.2. Etiology of glaucoma

Elevated IOP (>21mmHg) is a risk factor for POAG (Kooner *et al.,* 2008). This usually results from obstruction of the aqueous humor outflow due to alterations in conventional outflow pathway (Llobet *et al.,* 2003). The exact mechanism of IOP induced ONH degeneration is not yet clear. Recent studies have shown that elevated

IOP puts stress on optic nerve, leading to the degeneration of ONH. ONH cupping is the clinical feature of POAG and is caused by the degeneration of ONH leading to loss of ganglion cell axons resulting in characteristic peripheral visual field loss. At the end stage of POAG, loss of central visual acuity occurs and leads to complete irreversible blindness (Quigley *et al.,* 2011).

2.3. Classification of glaucoma

Based on etiology, glaucomas can be classified into primary and secondary glaucoma (Shields *et al.,* 2005).

2.3.1. Primary glaucomas: The initial events lead to the outflow obstruction and IOP elevation. These are confined to the anterior chamber angle or conventional outflow pathway, with no apparent contribution from other ocular or systemic disorders. Primary glaucomas can be sub classified into primary open angle glaucoma (POAG) and primary angle closure glaucoma (PACG).

a. Primary open angle glaucoma (POAG): POAG is a common form of glaucoma in which the iridocorneal angle is normal and obstruction of the outflow is located within the conventional outflow drainage system, mainly due to abnormal function of the TM leading to elevation of IOP (figure 2.1). POAG can occur with or without elevated IOP. Patients having IOP>21mmHg with glaucomatous cupping and progressive visual field loss is considered as HTG (high tension glaucoma). Individuals with IOP<21mmHg and with ONH cupping and visual field loss in the presence of open angle is considered as NTG (normal tension glaucoma). Based on the age of onset, POAG can be further sub classified into JOAG (occurs before 35 years of age) and adult onset (occurs above 35 years).

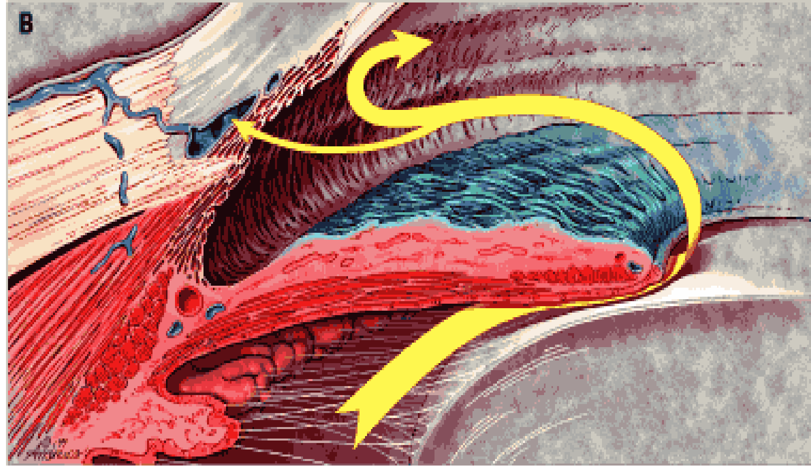

Figure 2.1. Diagrammatic representation of the drainage structures in POAG
(Source: http://www.aafp.org/afp/2003/0501/p1937.html)

b. Primary Angle closure glaucoma (PACG)

In PACG, the peripheral iris occludes the anterior chamber angle, thereby blocking the aqueous outflow (figure 2.2). Due to the shallow anterior chamber and a narrow filtration angle, a functional block occurs. This results in the accumulation of aqueous humor in the posterior chamber leading to the elevation of IOP resulting in ONH degeneration and progressive visual field loss.

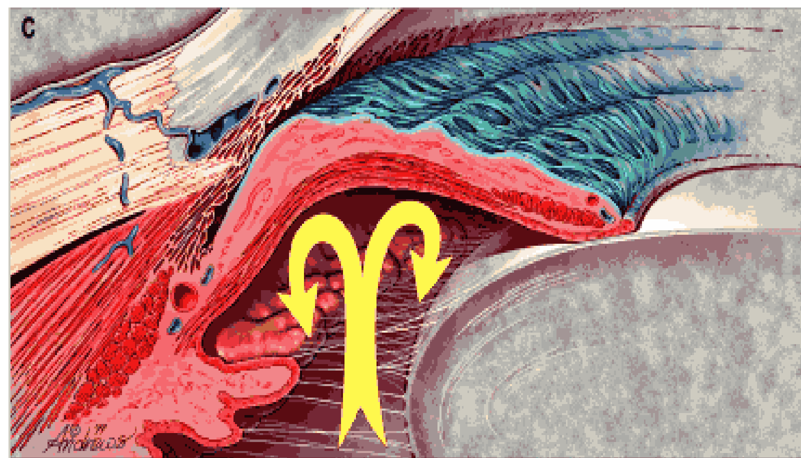

Figure. 2.2. Diagrammatic representation of the drainage structures in PACG
(http://www.aafp.org/afp/2003/0501/p1937.html)

2.3.2. Secondary glaucomas: These are characterized by the involvement of other ocular or systemic diseases (uveitis, trauma and diabetes) resulting in the alteration of aqueous humor dynamics, and abnormal elevation of IOP. Examples of secondary glaucoma include exfoliation glaucoma (XFG), neovascular glaucoma (NVG), and pigmentary glaucoma (PG).

2.4. Prevalence of POAG

The prevalence of POAG is varied across different populations. In the white population it ranged from 0.4 to 1.6% in 40 to 69 years age group whereas it is 3.3 to 10.8% in 70 to 95 years age group. In the black population the prevalence was 1.8 to 7.2% in those between 40 to 69 years and 11.2 to 22.5 in those aged 70 to 95 years. Asian population, the prevalence ranged from 0.4 to 1.6% in those aged between 30 to 69 years whereas it was 2.5 to 3.8% in those aged between 70 to 90 years (Rudnicka *et al.,* 2006).

2.4.1. Prevalence of POAG in India

Glaucoma is the third most common cause of blindness in India (Dandona *et al.,* 2001). It is estimated that glaucoma affects around 11.2 million people aged above 40 years and is responsible for 12.8% of the total blindness in the country (Dandona *et al.,* 2001). Of these, POAG was estimated to affect 6.48 million (George *et al.,* 2010). Over the last decade several population based studies like Vellore Eye Study (VES), Andhra Pradesh Eye Disease Study (APEDS), Aravind Comprehensive Eye Survey (ACES), Chennai Glaucoma Study (CGS), and West Bengal Glaucoma Study (WBGS) have been conducted (Jacob *et al.,* 1998, Dandona *et al.,* 2001, Ramakrishnan *et al.,* 2003, Raychaudhuri *et al.,* 2005, Vijaya *et al.,* 2006). The prevalence of POAG differs among these studies and this could be due to the difference in the age of the participants, definition of POAG and methodology used by each study. The VES reported less prevalence of POAG with 0.41% (95%CI, 0.08-0.81), because this study included

patients with the age ranging between 30-60 years. The prevalence of POAG in different studies in people above 40 years of age was 2.65% in APEDS, 1.29% in ACES, 1.85% in CGS (rural), 4.24% in CGS (urban) and 1.81% in WBGS.

2.5. Risk factors for POAG

POAG is associated with several risk factors like elevated IOP, age, family history, race, myopia, central corneal thickness (CCT), diabetes, hypertension and gender.

2.5.1. Elevated IOP as a risk factor in POAG: Elevated IOP is considered as a most important risk factor in treatment of POAG. Epidemiological studies have shown that the prevalence of glaucoma increases with elevated IOP. The Baltimore eye survey has shown that people with IOP>30mmHg were 38 times more likely to have POAG than people with lower IOP (15mmHg) (Sommer *et al*., 1996). The Blue mountain eye study has shown that people with IOP>21mmHg are at 4.7 times more risk for developing POAG than individuals with lower IOP (Mitchell *et al.,* 1999). Cases with uncontrolled IOP between 14 to 17mmHg after treatment have more visual field loss compared to those with IOP less than 14mmHg (Heijl *et al.,* 2002). Patients with lower IOP (<17mmHg) are less likely to progress visual field loss compared to patients with greater levels of IOP and they have higher rates of RNFL (Retinal Nerve Fiber Layer) changes (Medeiros *et al.,* 2010). Studies by Inatani *et al.,* (2008), have shown that POAG eyes with mean IOP levels of 21mmHg have significantly worse visual fields when compared to those with a mean IOP of <16mmHg. These observations indicate that elevated IOP is an independent risk factor for the progression of POAG.

2.5.2. Age: The prevalence of POAG increases with age. People older than 40 years of age are at increased risk for POAG across different populations (Rudnicka *et al.,* 2006).

2.5.3. Family history: Population based studies supported an association between a positive family history and POAG. In Baltimore Eye Study, the risk of having POAG

was approximately 3.7 fold higher for individuals who had a sibling with POAG (Tielsch *et al.,* 1994), whereas in Rotterdam Eye Study, the risk of having POAG was 9.2 fold higher for individuals who have first degree relative with POAG (Wolfs *et al.,* 1998).

2.5.4. Race or ethnicity: Several studies have demonstrated that African Americans are at four times higher risk of developing POAG when compared to Caucasians (Rudnicka *et al.,* 2006). Mechanism responsible for increased risk of POAG in black population is not known, but, it may be due to the genetic difference, presence of larger optic disc, thinner corneas, and increased oxidative stress (Seider *et al.,* 2009, Siegfried *et al*., 2011).

2.5.5. Myopia: Myopic patients are two to three times at a higher risk for POAG than normal individuals (Marcus *et al.,* 2011), because, high myopic patients have long axial length which is responsible for stretching of the optic disc leading to thinning of lamina cribrosa (Leung *et al.,* 2007).

2.5.6. Central corneal thickness (CCT): CCT was recently identified as a risk factor for POAG. Patients with CCT less than 555 microns in thickness are at greater risk for developing POAG compared to those whose cornea is more than 588 microns thick (Jonas *et al.,* 2005). However, the exact mechanism by which CCT is related to POAG pathogenesis is not yet known.

2.5.7. Diabetes: There were contradictory reports on association of POAG with diabetes. Few studies have shown that diabetes is significantly associated with POAG (Klein *et al.,* 1994). Recently, it has been demonstrated that diabetes alone contributes to 35% increased risk of developing POAG (Newman-Casey *et al.,* 2011). The exact mechanism as to how diabetes is related to POAG is not known. However, it has been shown recently that hyperglycemia delays axonal degeneration and RGC death in

hyperglycemic rats, indicating that diabetes protects from developing POAG (McKinnon *et al.,* 2011).

2.5.8. Hypertension: A number of studies have shown that there is a correlation between hypertension and development of POAG. However, this is again not consistently seen in all reports (Dielemans *et al.,* 1994, Leske *et al.,* 1995). A recent study by Newman-Casey *et al*., (2011), has shown that systemic hypertension alone is responsible for 17% of increased risk of developing POAG.

2.5.9. Gender: There was a conflicting result for gender and POAG. Baltimore Eye Survey and the Beaver Dam Eye study showed no difference in prevalence by sex (Klein *et al.,* 1992). Framingham Eye study and Rotterdam study showed an increased prevalence among men, whereas, the Blue Mountains Eye study showed an increased prevalence among women (Kahn *et al*., 1980, Dielemans *et al*., 1994, Mitchell *et al.,* 1996). Female sex hormones (especially estrogen) might provide some protective effect to the optic nerve by increasing ocular blood flow. But, after menopause these hormone levels go down and women may lose this protective effect (Vajaranant *et al.,* 2010).

2.6. Genetics of POAG

POAG being a complex disease and genetic predisposition represents a major risk factor as the rate glaucoma is much higher in first degree of relatives of patients as compared to normal population (Wolfs *et al.,* 1998). POAG exhibits complex mode of inheritance with incomplete penetrance and variable expressivity (Fan *et al.,* 2010). These features suggest that POAG has a multi-factorial etiology and is likely to involve interaction of several genes, majority of which are yet to be identified. So far, 30 chromosomal loci have been identified by using linkage and GWAS, out of which 17 have been designated from *GLC1A* to *GLC1Q* by HUGO genome nomenclature committee (www.gene.ucl.ac.uk/nomenclature) and mutations in five genes have been

identified to cause POAG. These include *MYOC, OPTN*, *WDR 36*, *NTF4* and *ASB10.* The genes and loci identified in POAG are summarized in Table- 2.1. Although these genes harbor POAG associated mutations, they exhibit a high degree of allelic heterogeneity with variable penetrance and expressivity.

2.6.1. Candidate loci in POAG

Sheffield *et al.,* (1993) demonstrated the first evidence of linkage in a JOAG on lq21-q31 which was called *GLC1A* (*GLC* stands for glaucoma, '1' for primary open-angle, and 'A' for the first loci discovered). Later, *MYOC* was identified as a candidate gene on this locus (Stone *et al.,* 1997). The location of a second gene on 10p15-p14 was reported in a large British family with normal pressure glaucoma and this locus was named as *GLC1E* (Sarfarazi *et al.,* 1998). Rezaie *et al.,* (2002), characterized *OPTN* on this locus and mutations have been identified in 16.7% families with hereditary POAG of which most of them had normal tension glaucoma. Based on the genome wide scan, Monemi *et al.,* (2005), mapped the third locus on 5q21.3 (*GLC1G)* and mutations in *WD636* on this locus have been identified in both NTG and POAG. Studies by Pasutto *et al.,* (2009), identified mutations in *NTF4* located on 19q13.3 representing *GLC1O* and this impairs the neurotrophin signaling in POAG. Pasutto *et al*., (2012) mapped *ASB10* gene on *GLC1F* region and the variants in *ASB10* are associated with POAG. In a recent report, Fernández-Martínez *et al.,* (2011), have shown that mutations in *RPGRIP1* located on 14q11 contributed to the risk for POAG in European population. Along with these, additional studies have been conducted and several candidate loci identified, but, the genes which are located on these loci are yet to be explored (Table 2.1).

Table 2.1. List of candidate loci identified in POAG

Chromosomal location	Phenotype	Locus name	Candidate gene	Reference
1q24.3–q25.2	JOAG, Adult onset	*GLC1A*	*MYOC*	Sheffield *et al.,* 1993, Stone *et al.,* 1997
2cen–q13	Adult onset	*GLC1B*		Stoilova *et al.,* 1996
3q21–q24	Adult onset	*GLC1C*		Wirtz *et al.,* 1997
8q23	Adult onset	*GLC1D*		Trifan *et al.,* 1998
10p15–p14	Adult onset, NTG	*GLC1E*	*OPTN*	Sarfarazi *et al.,* 1998 Rezaie *et al.,* 2002
7q35–q36	Adult onset	*GLC1F*	*ASB10*	Wirtz *et al.,* 1999, Pasutto *et al*., 2012
5q22.1	Adult onset	*GLC1G*	*WDR36*	Monemi *et al.,* 2005
2p16.3–p15	JOAG, Adult onset	*GLC1H*		Suriyapperuma *et al.,* 2007 Lin *et al.,* 2008,
15q11–q13	Adult onset	*GLC1I*		Allingham *et al.,* 2005
9q22	JOAG	*GLC1J*		Wiggs *et al.,* 2004
20p12	JOAG	*GLC1K*		Wiggs *et al.,* 2004
3p21–p22	Adult onset	*GLC1L*		Baird *et al.,* 2005
5q22.1-q32	JOAG	*GLC1M*		Pang *et al.,* 2006
15q22-q24	JOAG	*GLC1N*		Wang *et al.,* 2006
19q13.3	POAG &NTG	*GLC1O*	*NTF4*	Ip *et al.,* 1992, Pasutto *et al.,* 2009
12q14	NTG	*GLC1P*		Fingert *et al.,* 2011
4q35.1-q35.2	POAG	*GLC1Q*		Porter *et al.,* 2011
2q33.1-q33.3	POAG			Nemesure *et al.,*2003
10p12.33–p13.3	POAG			Nemesure *et al.,* 2003
2p14	POAG			Wiggs *et al.,* 2000
14q11	POAG		*RPGRIP1*	Wiggs *et al.,* 2000, Fernandez-Martinez *et al.,* (2011)
14q21–q22	POAG			Wiggs *et al.,* 2000
17p13	POAG			Wiggs *et al.,* 2000
17q25	POAG			Wiggs *et al.,* 2000
19q12-14	POAG			Wiggs *et al.* 2000
1p 32	POAG			Charlesworth *et al.,* 2005
10q 22	POAG			Charlesworth *et al.,* 2005
2p14	Elevated IOP			Duggal *et al.,* 2007
19q12–q14	Elevated IOP			Duggal *et al.,* 2007

2.6.2 Candidate genes linked to POAG

So far mutations in six genes have been identified in POAG. These genes are *MYOC (GLC1A), OPTN (GLC1E), WDR36 (GLC1G) NTF4 (GLC1O) ASB0 (GLC1F)* and *RPGRIP1* (14q11).

2.6.2.1. *MYOC*

MYOC (OMIM 601652) was the first candidate gene identified for POAG on lq21-q31 (*GLC1A*) (Stone *et al.,* 1997). This gene is also referred to as TM induced glucocorticoid receptor (*TIGR*) as its expression was induced by glucocorticoid (steroid) treatment to the TM cells (Clark *et al.,* 2001). *MYOC* consist of three exons and encodes for a transcript of about 2.5kb and is secreted as a 57 kDa glycoprotein (Kubota *et al.,* 1997, Nguyen *et al.,* 1998).

Mutations in *MYOC* accounts for 2-5% of POAG across different populations. A total of ~72 disease causing mutations have been reported till date, of which Gln368Stop was the most common mutation observed across multiple populations except the Japanese and Indian population (Fingert *et al.,* 1999, Gong *et al.,* 2004). Among the Indian population, the frequency *MYOC* mutations were low and Gln48His was the predominant mutation observed in cases of JOAG, POAG and PCG (Kanagavalli *et al.,* 2003, Chakrabarti *et al.,* 2005). A few polymorphisms have been identified in the promoter, of which, -1000C>G was shown to be strongly associated with POAG (Polansky *et al.,* 2003). The probable disease-causing and benign variations in *MYOC* are available in the Myocilin allele-specific phenotype database (htpp://www.myocilin.com/variants.php). Genotype phenotype correlation has been demonstrated with *MYOC* mutations and a strong correlation has been observed with specific mutations (Povoa *et al.,* 2006, Resch *et al.,* 2009). The distribution of *MYOC* mutations is provided in Table 2.2.

MYOC is a glycoprotein, consisting of two major domains: A myosin-like domain near the N terminal and an olfactomedin-like domain near the C terminal region. The N-terminal region of *MYOC* contains leucine zipper motifs within two coil-coil domains that are important for its interactions with several other proteins and these motifs are important for regulating the protein function (Fautsch *et al.,* 2001). The presence of >90% glaucoma-associated mutations in olfactomedin domain indicated its potential functional importance (Resch *et al.,* 2009). In humans the *MYOC* is expressed in a number of ocular and non ocular tissues including the TM that exhibiting the highest level of expression, followed by the sclera, ciliary body, choroid, cornea, iris, lamina cribrosa, retina, and optic nerve. The non ocular tissues include mammary gland, small intestine, thymus, prostate, testis, colon, stomach, thyroid, trachea, bone marrow, and brain (Knaupp *et al.,* 2004).

The normal physiological function of *MYOC* is not yet known. *In vitro* and *in vivo* studies have shown that wild type *MYOC* is not directly involved in IOP regulation, indicating that mutations in *MYOC* in humans may act by gain of function (Kim *et al.,* 2001, Gould *et al.,* 2006). *MYOC* is involved in the cytoskeletal organization and ECM remodulation by interaction with ECM proteins and Wnt signaling pathway (Fautsch *et al.,* 2006, Kwon *et al.,* 2009). The mutations in *MYOC* do not affect its expression, but, it affects the stability of the *MYOC* by modulating its folding (Yam *et al.,* 2007). Studies have shown that wild type myocilin is secreted as a multimeric glycoprotein and is synthesized by ER-Golgi pathway, whereas the mutant myocilin is not secreted from TM cells and it forms hetero multimers and this further interacts with wild type *MYOC* and inhibits its secretion. The accumulated *MYOC* forms insoluble aggregates in the ER leading to ER stress. Excessive and sustained ER stress triggers apoptotic cell death leading to the loss of TM cells, contributing to

elevated IOP (Shepard *et al.,* 2007). *MYOC* is also known to be associated with the mitochondrial pathway and it has been shown that overexpression of *MYOC* carrying P370L, Y437H mutations results in higher endogenous ROS (reactive oxygen species) production and down regulation of antioxidant status with increased signal for apoptosis and endoplasmic reticulum stress (He *et al.,* 2009, Joe *et al.,* 2010).

It has been shown in animal models that neither the absence of *MYOC* in the eye drainage structures (Kim *et al.,* 2001) nor its elevated levels of *MYOC* (Gould *et al.,* 2006) is responsible for elevation of IOP. These findings suggest necessity of expression of mutant protein for elevation of IOP. The PTS1R (peroxisomal target sequence) which is present in human *MYOC* is essential for mutant protein to induce cell death and cause elevation of IOP (Zode *et al.,* 2011).

Table 2.2. Distribution of *MYOC* mutations across the world

S.No	Country	Phenotype	Frequency of mutations (%)	% (Predominant mutation)	Reference
1	USA	POAG	3.9	2.7 (Q368X)	Stone *et al.,* 1997
2	Japan	POAG	5.7	3.8 (P370L)	Suzuki *et al.,* 1997
3	UK	POAG	4.7	2.3(Q368X)	Wiggs *et al.,* 1998
4	African-American	POAG	17.2	10.3(Q368X)	Allingham *et al.,* 1998
5	Japan	POAG	2.8	0.9(R45X,T353I,I465M)	
6	Canada	POAG	3.1	1.2 (E352K,(Q368X)	
7	Australia	POAG	2.8	2(Q368X)	
8	New York city	POAG	2.5	0.6(G352K)	
9	Iowa	POAG	4.2	2.2(Q368X)	Fingert *et al.,* 1999
10	Korea	POAG	4.4	2.2(R45SX),2.2(T353I)	Yoon *et al.,* 1999
11	Brazil	POAG	28.0	28(C433R)	Vasconcellos *et al.,* 2000
12	China	POAG	2.0	1(R46X) 1(D208E)	Lam *et al.,* 2000
13	Japan	POAG	2.9	R46X, R158Q, I360N, &A363T	Kubota *et al.,* 2000
14	Switzerland	POAG	4.27	4.27(Q368X)	Mataftsi *et al.,* 2001
15	Netherlands	POAG	2.2	2.2 (N480K)	Hulsman *et al.,* 2002
16	Queba	POAG	4.4	1.2 (Q368X)	Faucher *et al.,* 2002
17	Iowa	POAG	3.2	1.9(Q368X)	Alward *et al.,* 2002
18	China	POAG	1.4	0.5 (R91X,E300K,Y471C)	Pang *et al.,* 2002

19	West Africa	POAG	4.4	2.2(D380N, R342K)	Challa *et al.,* 2002
20	Germany	POAG	3.2	0.57 (Q368X)	Michels-Rautenstrauss *et al.,* 2002
21	India	POAG	7.1	5.3 (Q48H)	Mukhopadhyay *et al.,* 2002
22	France	POAG	7.5	5 (Q368X)	Melki *et al.,* 2004
23	Morocco	POAG	1.7	1.7 (T377M)	Melki *et al.,* 2003
24	India	POAG	1.8	0.9 (G367R,T77M)	Kanagavalli *et al.,* 2003
25	India	POAG	2.0	2 (Q48H)	Sripriya *et al.,* 2004
26	UK	POAG	1.4	1.4 (Q368X)	Aldred *et al.,* 2004
27	Japan	POAG	2.9	I360N, A363T, T448P,P369L	Ishikawa *et al.,* 2004
28	India	POAG/PCG	2.2	2.2 (Q48H)	Chakrabarti *et al.,* 2005
29	India	PCG	5.5	5.5 (Q48H)	Kaur *et al.,* 2005
30	Brazil	POAG	6.6	4 (C433R)	Povoa *et al.,* 2006
31	India	POAG	2.0	S331T and P370L	Rose *et al.,* 2007
32	India	POAG	0.8	0.8 (Q48H)	Kumar *et al.,* 2007
33	India	POAG	2.5	Q368X, Q48H, T256M, T353I, Q368X, P370L, G99D and A427T	Bhattacharjee *et al.,* 2007
34	Taiwan	POAG	12.5	6.25 (R46X)	Yen *et al.,* 2007
35	Spain	POAG	2.7	R346T, Q368X, A445V, Y479H	Lopez-Martinez *et al.,* 2007
36	China	PCG	2.5	E230K,R272X,S313F	Chen *et al.,* 2008
37	India	POAG	1.0	1 (G399D)	Acharya *et al.,* 2008
38	Japan	POAG	2.1	1.4 (A63T)	Mengkegale *et al.,* 2008
39	UK	POAG	2.2	1.5 (Q368X)	Ennis *et al.,* 2010

2.6.2.2. *OPTN*

OPTN (OMIM 602432) identified as the second gene in the *GLC1E* region (10p15-p14) (Sarfarazi *et al.,* 1998, Rezaie *et al.,* 2002). Initially, mutations in *OPTN* were reported in 16.7% families with hereditary POAG, of which, most of them had NTG. However, subsequent studies have shown that mutations in *OPTN* are rare and only a few mutations have been implicated in POAG and NTG (Table 2.3). Among the different mutations, E50K has been studied across different populations and is located on bZIP motif and is strongly associated with NTG. In case of Indian scenario, *OPTN* mutations could play a minor role in POAG (Mukhopadhyay *et al.,* 2005, Sripriya *et al.,* 2006). Recently, it has been shown that *OPTN* is also involved in amyotrophic lateral sclerosis (ALS) (Maruyama *et al.,* 2010). The distribution of *OPTN* mutations is provided in Table 2.3

OPTN contains three non coding (5'UTR) and 13 coding exons that codes for 577 amino acids and is expressed as ~66 kDa protein. *OPTN* contains several motifs: bZIP, two leucine zippers, coiled coil motifs and zinc finger domain. *OPTN* is expressed in both ocular and non ocular tissues that include TM, RGC, non pigmented ciliary epithelium, heart, brain, placenta, skeletal muscle and kidney (Rezaie *et al.,* 2002).

Optineurin is involved in regulation of exocytosis, vesicle trafficking from the Golgi to the plasma membrane, organization of the Golgi stacks, regulation of NF-κB activity, antiviral signaling, metabotropic glutamate receptor signaling and regulation of gene expression by interacting with different proteins like Rab8, Huntingtin, myosin VI and TBC1D17 (Anborgh *et al*., 2005, Zhu *et al*., 2007, Chalasani *et al*., 2007, Mankouri *et al*., 2010, Park *et al.,* 2010). Knockdown experiments have shown that lack of optineurin affects structure of the Golgi and endocytic trafficking (Sahlender *et al.,*

2005, Nagabhushana *et al*., 2010). The pathogenic mutant E50K impairs trafficking of transferrin possibly due to altered interactions with Rab8 and transferrin receptor. This impaired trafficking results in reduced uptake of transferrin leading to cell death in E50K expressing cells (Nagabhushana *et al.,* 2010). Another important function of optineurin is regulation of transcription factor NF-κB activity. NF-κB plays an important role in the expression of many genes involved in regulation of immune response, apoptosis, cell cycle and its abnormal activation is involved in the pathogenesis of many diseases (Camandola *et al.,* 2007). Studies have demonstrated that lack of *OPTN* induced the TNFα-mediated NF-κB activity, whereas, overexpression of optineurin inhibited the NF-κB activity. The negative regulation of NF-κB activity is due to the competition of optineurin with NEMO for binding to RIP (Receptor Interacting Protein) to prevent NF-κB activation (Zhu *et al.,* 2007). Cylindromatosis (CYLD) is another important interacting partner for *OPTN* and mutations in *OPTN* alters its interaction and inhibits the CYLD mediated inhibition of TNFα-induced NF-κB activation. These studies indicate that *OPTN* plays an important role in regulation of NF-κB mediated functions (Nagabhushana *et al.,* 2011). A study by Chalasani *et al.,* (2007), has shown that cells carrying the E50K mutation selectively induce RGC death by increasing the oxidative stress.

Table 2.3. Distribution of *OPTN* mutations across the world

S.No	Country	Phenotype	Frequency of mutations (%)	% (Predominant mutation)	Reference
1	USA	POAG/NTG	16.7	13.5 (E50K)	Rezaie *et al.*, 2002
2	UK	NTG	1.5	1.5 (E50K)	Aung *et al.*, 2003
3	Iowa and Japan	POAG	0.2	0.1 (E50K, E142P)	Alward *et al.*, 2003
4	China	POAG	1.6	0.8 (E103D,H486R)	Leung *et al.*, 2003
5	Japan	POAG	0.5	0.5 (H26D)	Funayama *et al.*, 2004
6	Japan	POAG/NTG	1.1 (POAG) 1.5 (NTG)	1.5 (R545Q)	Fuse *et al.*, 2004
7	India	POAG	3%	3 (R545Q)	Mukhopadhyay *et al.*,2005
8	Germany	NTG	1.8	0.9 (A336G, A377T)	Weisschuh *et al.*, 2005
9	India	POAG/NTG	4.1 (POAG) 6 (NTG)	4.1 (POAG) 6 (NTG) (M98K)	Sripriya *et al.*, 2006
10	China	POAG	Single family with 6 affected members	K322E	Xiao *et al.*,2009

2.6.2.3. *WDR36*

*WDR36 (*OMIM: 609669) is third candidate gene identified on *GLC1G* locus (5q22.1) by Monemi and coworkers in 2005. However, it was later demonstrated that *WDR36* is not associated with POAG and it may act as a modifier gene (Table 2.4). In the case of Indian population, *WDR36* is not associated with POAG (Mookherjee *et al.,* 2011). The distribution of *WDR36* mutations is provided in Table 2.4.

WDR36 is composed of 23 exons and is expressed predominantly as two transcripts (5.9 and 2.5 kb) and codes for 100 kDa protein (Chi *et al.,* 2010). *WDR36* is conserved across the species and contains four domains; WD repeat domain, Utp21 domain, AMP dependent synthetase and ligase domain and cytochrome cd1- nitrite reductase like domain (Skarie *et al.,* 2008). *WDR36* is widely expressed in several ocular tissues which include lens, iris, sclera, ciliary muscles, ciliary body, TM, retina, and ONH (Monemi *et al.,* 2005).

The exact role of *WDR36* in POAG is not yet known. Functionally, *WDR36* is homologous to yeast Utp1 and is involved in ribosome biogenesis by processing of 18S rRNA. Loss of *WDR36* result in reduced levels of rRNA and this further involved in activation of *p53* stress response pathway (Skarie *et al.,* 2008). The variations in the *WDR36* and *p53* increased the susceptibility to POAG (Blanco-Marchite *et al.,* 2011). The genetic variants present in *WDR36* alone do not produce any significant defects, but, when combined with the disruption of *STI1* (which synthetically interacts with *UTP21*, a homolog of human *WDR36*), resulted in altered cell viability due to alterations in the levels of pre-rRNA (Footz *et al***.,** 2009). *WDR36* acts as a modifier gene that requires variations in another gene or unknown risk factor for initiating POAG. Recently, it has been shown that *WDR36* plays an essential role in the preimplantation stages of embryonic development and *in vitro* experiments by using

human TM cells demonstrated that lack of *WDR36* induce apoptotic cell death by activation of *BAX, p53*, and *CDKN1A*. Based on these observations, it is likely that lack of *WDR36* disrupts 18S rRNA maturation and leads to activation of the *p53* stress response pathway which in turn results in cell death (Gallenberger *et al.,* 2011).

Table 2.4. Distribution of *WDR36* mutations across the world

S. No	Country	Phenotype	Frequency of mutations (%)	% (predominant mutation)	Reference
1	USA	POAG	6.9	3.85 (D658G)	Monemi *et al.,* 2005
2	USA	POAG	11.0	3.3 (M671V)	Hauser *et al.,* 2006
3	Japan	POAG	0.7	0.7 (S664L)	Miyazawa *et al.,* 2007
4	Germany	NTG	9.8	1.7 (A449T,D33E,A163V,H212P)	Weisschuh *et al.,* 2007
5	Germany	POAG/NTG	1.7	P31T,Y97C,D126N,T403A,H411Y,H411L,P487R	Pasutto *et al.,* 2008
6	China	POAG	1.8	1.8 (I713V)	Fan *et al* ., 2009
7	Italy	POAG	3.0	3 (D658G)	Frezzotti *et al.,* 2011

2.6.2.4. NTF4

NTF4 (GLC1O; OMIM 162662) is a recently identified candidate gene and mutations in this have been shown to be responsible for 1.7% of POAG in European population. Molecular modeling and *in vitro* studies have shown that mutations reduced the binding affinity of *NTF4* to its target receptor TrkB, thereby reducing the function of neurotrophins (Pasutto *et al.,* 2009). However, later studies have demonstrated the lack of involvement of *NTF4* in Indian, Caucasian and Chinese populations (Liu *et al.,* 2010, Rao *et al.,* 2010, Vithana *et al.,* 2010)

NTF4 consists of two non coding (5'UTR) and one coding exon and codes for 22 kDa protein. *NTF4* is known to express in both ocular and non ocular tissues including TM, retina, brain, lung, heart and pancreas (Lambert *et al.,* 2001).

Cells within the lamina cribrosa (LC) express neurotrophins (NTs) and Trk (tyrosine kinase) receptors (Lambert *et al.,* 2001). *NTF4* plays an important role in neuronal development, survival, and differentiation (Huang *et al.,* 2010). Deprivation of neurotrophins plays a major role in the axonal loss in IOP induced glaucoma (Pease *et al.,* 2000). Animal models have shown that elevated IOP and ischemia impairs the neurotrophin signaling thereby leading to RGC death (Johnson *et al.,* 2009).

2.6.2.5. RPGRIP1

RPGRIP1 (OMIM 605446) is located on 14q11.2 and this was reported previously as a candidate gene for LCA (Leber congenital amaurosis) (Dryja *et al.,* 2001). Fernandez-Martinez *et al.,* (2011), mapped a previously reported POAG locus (Wiggs *et al.,* 2000) on 14q11 and demonstrated that heterozygous mutations in *RPGRIP1* increased the risk of POAG, JOAG and NTG. Several non-synonymous heterozygous variants were identified in *RPGRIP1* that were distinct from the variants associated with photoreceptor dystrophies and were predominantly present in the *RPGR*-interacting

domain. *In vitro* studies have demonstrated that mutations in C2 domain of *RPGRIP1* altered its interaction with nephrocystin.

2.6.2.6. *ASB10*

Using linakge analysis Writz *et al*., (1999) identified *GLC1F* locus on chromosome 7q35-q36. Subsequently screeing of genes in 2.2Mb critical region on *GLC1F* region revealed identification of ankyrin repeat and SOCS box-containing protein 10 (*ASB10*) as a novel candidate POAG gene. Sequencing analysis of *ASB10* in two different cohorts (USA and Germany) found several variations in POAG (70/1172; 6.0%) as compared to normal controls (13/461; 2.8%; p=0.008). Molecular modeling suggested that missense variants change biophysical properties of *ASB10* and destabilize the ankyrin repeats. *ASB10* mRNA and protein were found to be strongly expressed in TM, RGCs and ciliary body. Silencing of *ASB10* transcripts in perfused anterior segment organ culture reduced outflow facility by approximately 50% compared to controls infected anterior segments (p=0.02).

2.6.2.7. *CYP1B1*

*CYP1B1 (*OMIM 601771) is the candidate gene for PCG and was mapped on *GLC3A (*2p21) (Sarfarazi *et al.,* 1995, Stoilov *et al.,* 1997). Many pathogenic mutations were identified in *CYP1B1* in PCG cases, and the mutation frequency varied ethnically from ~20%-100% (Stoilov *et al.,* 1997, Plasilova *et al.,* 1999, Bejjani *et al.,* 2000, Mashima *et al.,* 2001, Stoilov *et al.,* 2002, Sitorus *et al.,* 2003, Chakrabarti *et al.,* 2010). *CYP1B1* has also been implicated in juvenile and adult onset forms of glaucoma in various ethnic groups (Table 2.5). Vincent *et al.,* (2002), have shown that *CYP1B1* along with *MYOC* are involved in POAG through digenic mechanism in a glaucoma family from East Indian origin. Later, the association of *CYP1B1* has been reported across the world and mutations with adult onset glaucomas ranged from 2.2%-23.3%. In India,

mutations in *CYP1B1* have been reported in POAG (18.8%) and PACG (10%) (Chakrabarti *et al.,* 2007) (Table 2.5).

CYP1B1 is a member of *CYP450* super family consisting of three exons and spans 8.5 kb of genomic DNA and codes for a 61 kDa protein. *CYP1B1* is expressed in ocular tissues which include iris, TM, ciliary body and RGC as well as non ocular tissues (Doshi *et al.,* 2006).

It has been shown in animal models that *CYP1B1* deficiency leads to abnormality in TM similar to those observed in human PCG (Libby *et al.,* 2003). *CYP1B1* is involved in the metabolism of steroids, retinol, retinal, arachidonate and melatonin (Vasiliou *et al.,* 2008). Even though the exact role of *CYP1B1* in IOP regulation in adult glaucomas is not known, recent studies have shown that mutations reduced *CYP1B1* activity, thereby altering the metabolism of steroids (Chavarria-Soley *et al.,* 2008). *CYP1B1* is also involved in the metabolism of estrogen and thereby it further regulates the expression of *MYOC*. *MYOC* contains ERE (estrogen response elements) on the promoter and its expression can be induced by 17β estradiol treatment (Nguyen *et al.,* 1998). Due of reduced activity of the *CYP1B1* due to mutations, the conversion of 17β estradiol into 4-hydroxy estradiol is blocked and this results in activation of *MYOC* expression (Ray *et al.,* 2009). However, studies have shown that increased levels of myocilin are not responsible for IOP elevation indicating that *CYP1B1* and *MYOC* may interact by a different mechanism (Gould *et al.,* 2006).

Table 2.5. ***CYP1B1*** **in adult onset POAG worldwide**

S.No	Country	Phenotype	Frequency of mutations (%)	% (predominant mutation)	References
1	Canada	JOAG	5.0	R368H, 1546dup10, L345F	Vincent *et al.,* 2002
2	France	POAG	4.6	1.3 (A443G)	Melki *et al.,* 2004
3	India	POAG	4.5	2.0 (S515L)	Acharya *et al.,*2006
4	Spain	POAG	12.1	3.6 (Y81N)	Lopez-Garrido *et al.,* 2006
5	India	POAG	10.8	5.2 (E229K)	Kumar *et al.,* 2007
6	India	POAG	17.3	5.8 (R368H)	Chakrabarti *et al.,* 2007
		JOAG	23.3	6.6 (G61E)	
		PACG	11.1	5.6 (R368H)	
7	Iran	POAG	11.1	4.7 (R368H and G61E)	Suri *et al.,* 2008
		POAG	2.2		
8	Germany	JOAG	8.5	1.5 (Y81N)	Pasutto *et al.,* 2009
		NTG	4.1		

2.6.3. Association studies for POAG and PACG

POAG is a complex genetic disorder and it is likely that multiple genes and environmental factors contribute to the regulation of IOP and RGC death. Association studies have demonstrated that many genes are in involved in POAG (Table 2.6). However, most of these studies were not consistent in different populations. The variation of association could be due to racial differences, sample size, poorly characterized controls and clinical heterogeneity between different populations. The POAG associated genes include *ANP, p53, TNFα, APOE, GST, eNOS,* Interleukins and *OPA1* (Tunny *et al.,* 1996, Juronen *et al.,* 2000, Vickers *et al.,* 2002, Powell *et al.,* 2003, Lin *et al.,* 2003, Ressiniotis *et al.,* 2004).

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Table 2.6. Status of candidate gene association with glaucoma in different studies

S.No	Chromosomal location	Gene name	Phenotype	Country	Reference	phenotype	Country	References
			Association			No association		
1	1p36.21	*ANP*	POAG	Australia	Tunny *et al.,* 1996	POAG NTG	Australia Korea	Richardson *et al.,*1997 Jeoung *et al.,* 2007
2	Xq22-q23	*AGTR2*	NTG	Japan	Hashizume *et al.,* 2005			
3	17q23.3	*ACE*	POAG POAG	Turkey UK	Ozkur *et al.,* 2004 Bunce *et al.,* 2005			
4	6p21.2	*P21*	POAG	China	Tsai *et al.,* 2004	POAG POAG	Caucasian Turkey	Ressiniotis *et al.,* 2005 Saglar *et al.,* 2009
5	9q32-q33	*TLR4*	NTG	Japan	Shibuya *et al.,* 2008	NTG	Korea	Suh *et al.,* 2011
6	19q13.2	*XRCC1* and *XPD*	POAG	Pakistan	Yousaf *et al.,* 2011	POAG	Turkey	Guven *et al.,* 2007
7	7q21.3-q22	*PAI1*				POAG	Austria	Mossbock *et al.,*2008
8	20q11.2-q13.1	*MMP9*	PACG PACG PACG	China China Australia	Wang *et al.,* 2006 Cong *et al.,* 2009 Awadalla *et al.,* 2011	PACG POAG	Singapore Austria	Aung *et al.,* 2008 Mossbock *et al.,* 2010
9	11q23	*MFRP*				PACG PACG	China China	Aung *et al.,* 2008 Wang *et al.,* 2008
10	5q31.1-q31.2	*Hsp70*	POAG PACG	Japan Pakistan	Tosaka *et al.,* 2007 Ayub *et al.,* 2010			
11	14q32.1	*CYP46A1*	POAG	France	Fourgeux *et al.,*2009	POAG	Austria	Mossböck *et al.,* 2011

12	5q31-q32	*B2AR*				POAG	Turkey	Gungör *et al.,* 2003
13	6p21.3	*TNF alpha*	POAG POAG POAG POAG POAG	Taiwan Japan Iran China Turkey	Lin *et al.,* 2003 Funayama *et al.,* 2004 Razeghinejad *et al.,* 2009 Fan *et al.,* 2010 Bozkurt *et al.,* 2011	POAG	Austria	Mossbock *et al.,* 2006
14	17p13.1	*P53*	POAG POAG POAG NTG	China UK USA China	Lin *et al.,* 2002 Ressiniotis *et al.,* 2004 Daugherty *et al.,* 2009 Fan *et al.,* 2010	POAG HTG,NTG POAG POAG,NTG OHT, POAG	India Tasmania Brazil Japan Turkey	Acharya *et al.,* 2002 Dimasi *et al.,* 2005 Silva *et al.,*2009 Mabuchi *et al.,*2009 Saglar *et al.,* 2009
15	3q28-q29	*OPA1*	NTG NTG NTG&HTG NTG	Singapore UK Japan UK	Aung *et al.,* 2002 Powell *et al.,* 2003 Mabuchi *et al.,* 2007 Yu-Wai-Man *et al.,* 2010	POAG NTG NTG&POAG POAG	UK Korea West Indies Caucasian, African-American, and Ghanaian	Aung *et al.,* 2002 Woo *et al.,*2004 Yao *et al.,*2006 Liu *et al.,* 2007
16	1p36.3	*MTHFR*	POAG POAG PACG NTG	Germany Germany Pakistan Korea	Junemann *et al.,* 2005 Junemann *et al.,* 2005 Micheal *et al.,* 2009 Woo *et al.,* 2009	POAG POAG&NTG POAG NTG, POAG	Sweden Japan Austria Japan	Zetterberg *et al.,*2007 Mabuchi *et al.,* 2006 Mossbock *et al.,* 2006 Mabuchi *et al.,* 2006

17	2q14 2q14	*ILIβ* *IL1α* (-889)	POAG POAG	China China	Lin *et al.,*2003 Wang *et al.,* 2006			
		IL1A (-889C/T)				POAG, NTG	China	Wang *et al.,* 2006, 2007
		IL1A (-889C/T), IL1B (+3953C/T), and IL1B (-511C/T)				NTG	China	How *et al.,*2007
		IL1B (+3953C/T) (-511C/T) IL1A and IL1B	POAG	India	Mookherjee *et al.,* 2010	NTG	China	Wang *et al.,* 2007
18	22q11.23& 1p13.3	*GSTM1* *GSTM1* *GSTM1&T1* *GSTM1&T1* *GSTM1*	POAG POAG PACG POAG POAG	Estonia Turkey Turkey Saudi Arabia Brazili	Juronen *et al.,* 2000 Yilmaz *et al.,* 2005 Unal *et al.,* 2007 Abu-Amero *et al.,* 2008 Rocha *et al.,* 2011	POAG	Sweden	Jansson *et al.,* 2003

19	7q36	*eNOS*	POAG POAG, PACG POAG	Australia Pakistan China	Tunny *et al.,* 1998 Ayub *et al.,* 2010 Liao *et al.,* 2011			
20	19q13.2	*APOE*	POAG NTG POAG POAG	France Tasmania China Saudi Arabia	Copin *et al.,* 2002 Vickers *et al.,* 2002 Lam *et al.,* 2006 Al-Dabbagh *et al.,* 2009	NTG POAG POAG POAG	UK UK Sweden Turkey	Lake *et al.,* 2004 Ressiniotis *et al.,*2004 Zetterberg *et al.,* 2007 Saglar *et al.,* 2009
21	11p15.5	*IGF2*	POAG	China	Tsai *et al.,* 2003			
22	7q21.1	*HGF*	PACG	Nepalese	Awadalla *et al.,* 2011			
23	11q22-q23	*MMP1*	POAG	Polish	Majsterek *et al.,* 2011	POAG	Austria	Mossbock *et al.,* 2010
24	6p12	*SRBD1,* *ELOVL5*	POAG	Japan	Mabuchi *et al.,* 2011			
25	9p21/ 14q23/ 14q23	*CDKN2B,* *SIX1/SIX6*	POAG	Caucasian	*Fan et al., 2011*			
26	2q31-q32	*CALCRL*	PACG	China	Cao *et al.,* 2009			

2.6.4. Genome wide association studies

Classical linkage studies were unsuccessful in identifying the potential candidate genes in POAG. The previously identified genes *MYOC, OPTN*, and *WDR36* contributed less than 10% of the POAG cases. Due to its late onset and unavailability of large affected families, the identification of genes by linkage studies were not possible. GWAS represent a powerful approach for gene mapping in large cohorts using high density markers like single nucleotide polymorphisms (SNPs) on microarray platforms. These methodologies have been successful in identification of several loci associated with complex diseases like cancer, diabetes, rheumatoid arthritis, Parkinson disease, Alzheimer's disease, and age-related macular degeneration (AMD) (Hirschhorn *et al.,* 2005, Klein *et al.,* 2005, Edwards *et al.,* 2005, Easton *et al.,* 2007, Plenge *et al.,* 2007, Frayling *et al.,* 2007).

Such methodologies were first employed in cases of secondary open angle glaucoma, XFG. XFG is an age related disorder characterized by accumulation of fibrillar exfoliative material in anterior segment leading to elevated IOP and ONH degeneration. Lysyl Oxidase Like 1 *(LOXL1;* 15q24.1) was the first candidate gene identified for XFG/XFS (Thorleifsson *et al.,* 2007). Three genetic variations have been identified of which two nonsynonymous coding SNPs, rs1048661 (R141L) and rs3825942 (G153D), were located in exon 1, and one intronic SNP (rs2165241), located in intron 1. These variants were significantly associated with p values 2.3×10^{-12} to 1.0×10^{-27} and OR of 2.46 (1.91–3.16) to 3.62 (2.87–4.55). The intronic variant was also shown to be associated with POAG (rs2165241; $\boldsymbol{p} = 0.04$ OR 1.36 (1.01–1.83) in the Icelandic population, but were not associated in Swedish population ($p = 0.18$). These findings have subsequently been replicated in XFS/XFG in numerous populations globally (Chen *et al.,* 2010). *LOXL1* is a member of the lysyl

oxidase family of proteins that catalyzes the tropoelastin polymerization to form the mature elastin. Elastin fibers are a major component of many ocular structures such as TM and the lamina cribrosa. Both POAG and XFG are complex disorders that share a similar clinical phenotype that include elevated IOP and glaucomatous optic neuropathy (Ritch *et al.,* 1996). Abnormal synthesis of extracellular matrix in the TM was also responsible for elevated IOP in POAG (Tamm *et al.,* 2007). Since genetic variants in *LOXL1* are significantly associated with XFG/XFS, these variants were analyzed in POAG in different populations. These variants were not associated with POAG from India (Chakrabarti *et al.,* 2008), Caucasian, African-American, Ghanaian (Liu *et al.,* 2008), Chinese (Gong *et al.,* 2008) and Japanese populations (Mabuchi *et al.,* 2008), Saudi Arabian (Abu-Amero *et al.,* 2011). Further screening of these SNPs in Indian PACG and Caucasian, European PG patients indicate that their exclusive involvement with XFS/XFG only (Chakrabarti *et al.,* 2008, Rao *et al.,* 2008, Wolf *et al.,* 2010).

The next GWAS in POAG was done in Japanese population that demonstrated a significant association of six variants located on three different chromosomes, viz. 1 (*ZP4*), 10 (*PLXDC2*) and 12 (*DKFZp762A217*). It is a two stage study in which GWAS was conducted and 255 variants were identified, that are associated with POAG. These variants were further analyzed in a second cohort with customized genotyping. Six variants located on three different chromosomes were associated with both high and low pressure glaucomas with combined p values from two stages ranging from $1.0x0^{-5}$ to $9.0x10^{-5}$ with an OR of 1.33 - 1.49 (Nakano *et al.,* 2009). However, replication of these variants in Indian population did not reveal association to IOP-associated primary glaucoma (POAG and PACG) (Rao *et al.,* 2009). This lack of association was further supported by a recent study on different population from Iceland (Thorleifsson *et al.,* 2010) indicating that these associations were not a universal phenomenon.

Recently, Thorleifsson *et al*., (2010) have conducted another GWAS study on POAG in Iceland and identified several variants on 7q31 of which rs4236601 was significantly associated with an OR= 1.36 (1.23-1.50) and p= 5.0 x 10^{-10}. These results were replicated in independent POAG cohorts from Sweden UK, Australia with OR = 1.18, p = 0.0015 and Chinese population with OR = 5.42, p = 0.0021. The risk variants identified in this study are located close to *CAV1* and *CAV2*, both of which are expressed in the TM and RGCs. Caveolins are a group of scaffolding proteins that serves an important regulatory signaling function in endothelial cells. Loss of caveolin-1 function can cause increased expression of *NOS3* suggesting that caveolin-1 could modulate IOP through eNOS (Garcia-Cardena *et al.,* 1997). Along with *eNOS, CAVs* are also involved in the regulation of *TGFβ* signaling by interacting with *TGFβ* type I receptor (Razani *et al.,* 2001). Both *eNOS* and *TGFβ* are involved in POAG pathogenesis and interactions of these with cveolins indicate that caveolins could play a pathogenic role in POAG (Neufeld *et al.,* 1997, Fleenor *et al.,* 2006). However, a recent study has shown that POAG risk allele at the 7q31 locus (rs4236601) was not associated with IOP related glaucomas (POAG) in the Iowa population (Kuhen *et al.,* 2011). Similarly in Caucasian population the rs4236601 was not associated with POAG (OR=1.17, p 0.1995), whereas rs1052990 was associated with NTG (OR 1.27, p 0.0392) (Wiggs *et al*., 2011). A later study by Burdon *et al.,* (2011) showed that *CAV1* SNP (rs4236601) was not significantly associated with POAG among Australian population (OR=1.07; p=0.17). Overall these findings indicate that *CAVs* may not be involved in the pathogenesis of POAG.

A recent study by Burdon *et al.,* (2011), identified POAG associated loci at *TMCO1* (rs4656461) with OR = 1.68, p = 6.1 × 10^{-10} and *CDKN2B-AS1* (rs4977756) with OR = 1.50, p = 4.7 × 10^{-9} in Australians of European descent. Later, this was

replicated in an independent cohort of cases with advanced OAG and two additional cohorts of relatively less severe OAG. *CDKN2B-AS1* (*CDKN2B* antisense RNA 1) and *TMCO1* (transmembrane and coiled-coil domains 1) are expressed in retina, ONH, iris and ciliary body. The rat ocularhyper tension model has shown a strong upregulation of *Cdkn2a* and *Cdkn2b* expression in the retina. *CDKN2B-AS1* is located on the 9p21 and associated with cardiovascular disease, diabetes, intracranial aneurysm and glioma (Helgadottir *et al.,* 2007, Scott *et al*., 2007, Bilguvar *et al*., 2008, Shet *et al*., 2009). The antisense RNA encoded by *CDKN2B-AS1* regulates neighboring genes at 9p21, particularly *CDKN2B*. CDKN2B (cyclin-dependent kinase inhibitor 2B) protein is a cyclin dependent kinase that activate retinoblastoma tumor suppressor pathway thereby regulating cell growth and also interacts with TGFβ (Gonzalez *et al.,* 2006). Recently, it has been shown that variants in the *CDKN2B* influence the optic disc parameters and is significantly associated with POAG (Ramdas *et al.,* 2010, Fan *et al.,* 2011). Mutations in the *TMCO1* have been reported in craniofacial dysmorphism, skeletal anomalies, and mental retardation (Xin *et al.,* 2010). *TMCO1* is a transmembrane protein with coiled coil domain and localizes to Golgi, endoplasmic reticulum and mitochondria (Xin *et al.,* 2010). Both endoplasmic reticulum and mitochondrial abnormalities are involved in POAG and variations in *TMCO1* could play a significant role in POAG (Joe *et al.,* 2003, Kong *et al.,* 2009). How ever, recent studies have shown that the variants in *CDKN2B-AS1* are associated with NTG and not with HTG (Nakano *et al*., 2012, Burdon *et al*., 2012, Wiggs *et al*., 2011).

Recently, Carbone *et al.,* (2011) identified two POAG associated variants by using comparative genomics to analyze the genes involved in UPR (Unfolded Protein Response), ubiquitination, proteolysis, and oxidative stress pathways. These variants were located in *BIRC6* (rs2754511, Baculoviral IAP Repeat Containing 6) and *PDIA5*

(rs11720822, Protein Disulfide Isomerase Family A, member 5). BIRC6 is an ubiquitin carrier protein involved in the regulation of apoptosis, while PDIA5 is an ER chaperone that is induced during ER stress (Liu *et al.,* 2007, Ni *et al.,* 2007). Both apoptosis and ER stress are involved in TM, RGC death indicating that these genes could play an important role in POAG (Joe *et al.,* 2003, Libby *et al.,* 2005).

By using whole genome wide linkage and SNP mapping, Jiao *et al.,* (2009) identified a locus on Chromosome 2 to be associated with POAG. The region was characterized on 2p by performing linkage analyses in 146 multiplex families from Barbados Family Study of Glaucoma (BFSG) cohort. Case control analysis in independent groups from BFSG participants identified a strong association of rs12994401 with POAG. This region overlapped with previous linkage studies in Chinese and African families, indicating that this locus could be a significant cause of glaucoma in the Chinese and Europeans. Replication of these SNPs did not reveal any association with POAG in Japanese population (Mabuchi *et al.,* 2010) and is marginally associated with POAG from Indian population (Balasubbu *et al*., 2012).

2.6.5. Quantitative trait loci for central corneal thickness, intraocular pressure, and cup to disc ratio

POAG is a complex disorder with phenotypic variability with age of onset, IOP and CDR among the patients and identifying the genes responsible for this complex disease is difficult. The complexity of POAG can be reduced by separately studying the quantitative features of phenotypes including CCT, IOP and CDR rather than studying the entire complex phenotype. Studies have shown that CCT, IOP and CDR are the quantitative traits for POAG that are highly heritable (Klein *et al.,* 2004, van Koolwijk *et al.,* 2007). These quantitative traits are possibly controlled by multiple genes which may further help in dissecting the genetic basis of POAG. Mapping genes

responsible for IOP, CCT and CDR has several important advantages, including objective definitions of the phenotype and possible reduction of the underlying molecular heterogeneity. The genes which are responsible for these quantitative traits are likely to contribute to the overall risk of developing POAG.

Several studies have been conducted to identify the quantitative trait loci (QTLs) that influence IOP. Duggal *et al.,* (2007) have identified the regions on chromosomes 6, 13 and 19p linked to IOP in populations form USA. Later, the regions of 10q22 and 5q22 were shown to be associated with IOP (Charlesworth *et al.,* 2005, Rotimi *et al.,* 2006). Recently, study by van Koolwijk *et al*., (2012) has demonstrated that IOP was significantly associated with rs11656696, located in *GAS7* (Growth arrest-specific protein 7) at 17p13.1 ($p = 1.4 \times 10^{-8}$), and with rs7555523, located in *TMCO1*at 1q24.1 ($p = 1.6 \times 10^{-8}$). These variants were also marginally associated with POAG. *GAS7* and *TMCO1* are expressed in ocular cells and tissues implicated in glaucoma (TM as well as in the lamina cribrosa, optic nerve, and retina). Biochemical protein interactions with known glaucoma disease genes (*OPTN*, *WDR36*, *CAV1*, *NOS2*, *FOXC1* and *APOE*) supports the involvement of these genes in aqueous humor dynamics and glaucomatous neuropathy.

Recently, four large scale GWAS have been conducted to identify the genetic factors that influence the magnitude of CCT. Several genes have been identified of which *ZNF469* was identified as an important quantitative trait locus for CCT in all these studies (Table 2.7). *ZNF469* is an excellent candidate for regulating CCT and mutations in this gene have been previously linked with brittle cornea syndrome (Abu *et al.,* 2008). Other genetic factors that influence CCT are collagen genes (*COL5A1* and *COL8A2*), autogenous vein graft remodeling associated protein 8 (*AVGR8*), and A-kinase anchor protein 13 (*AKAP13*). Collagens have been shown to be

expressed in ocular tissues, such as TM, RGC, and ONH (Tripathi *et al.,* 1994, Sawaguchi *et al.,* 1999). Elevated expression of *COL8A2* has also been observed in the TM cells in response to dexamethasone treatment (Rozsa *et al.,* 2006). Missense changes in the *COL8A2* have been observed in a group of POAG patients with very thin CCT (Desronvil *et al.,* 2010). These results indicate that CCT is an important quantitative trait that may help to identify casual gene in POAG.

Change in cup to disc ratio (CDR) is another important quantitative trait for POAG and it is measured as the vertical cup disc ratio (VCDR) (Chang *et al.,* 2005). Furthermore, it has been shown that CDR is a heritable trait and larger optic discs suffer more from IOP related stress and individuals of African descent generally have larger disc diameters compared to people in Asian and European population (Seider *et al.,* 2009). Upto now three GWAS has been conducted in different populations to identify the genetic factors influencing the optic disc area and VCDR. Several loci have been identified, which include *ATOH7, CDC7/TGFBR3* and *SALL1* for optic disc area, and *CDKN2B, SIX1, SCYL1/LTBP3, CHEK2, ATOH7* and *DCLK1* for VCDR (Table 2.7). Meta- analysis showed that three common variants (*CDKN2B, ATOH7* and *SIX1*) were significantly associated with optic disc parameters (Ramdas *et al.,* 2011). Recently it has been shown that SNPs associated with VCDR (rs1063192, (*CDKN2B)* and rs10483727 (*SIX1/SIX6)*) were also associated with POAG (p = 0.0006 and p = 0.0043 for rs1063192 and rs10483727, respectively) (Fan *et al.,* 2011, Osman *et al*., 2012). These results indicate that genetic variants influencing optic disc area can significantly contribute to POAG. The genes and loci identified for quantitative trait are provided in Table 2.7.

Table 2.7. Quantitative trait and their associated candidate genes

Quantitative trait	Chromosome location	Gene	p value	Reference
CCT	15q25.3	*AKAP13*	1.4×10^{-8}	Vitart *et al.,* 2010
	9q34.2	*COL5A1*	1.1×10^{-6}	Vitart *et al.,* 2010
	13q12.11	*AVGR8*	3.5×10^{-9}	Vitart *et al.,* 2010
	16q24.2	*ZNF469*	7.1×10^{-9}	Vitart *et al.,* 2010
	13q14.1	*FOXO1*	3.5×10^{-9}	Vitart *et al.,* 2010
	16q24.2	*ZNF469*	8.9×10^{-11}	Lu *et al.,* 2010
	13q14.1	*FOXO1*	4.6×10^{-10}	Lu *et al.,* 2010
	10q26.13	*FAM53B*	7.0×10^{-9}	Lu *et al.,* 2010
	1p34.3	*COL8A2*	5.4×10^{-13}	Vithana *et al.,* 2011
	9q34.2	*COL5A1*	3.0×10^{-9}	Vithana *et al.,* 2011
	16q24.2	*ZNF469*	1.6×10^{-16}	Vithana *et al.,* 2011
	13q12.11	*RXRA*	5.0×10^{-8}	Vithana *et al.,* 2011
	6q14.1	*IBTK*	2.1×10^{-11}	Cornes *et al.,* 2011
	15q26.3	*CHSY1*	7.6×10^{-11}	Cornes *et al.,* 2011
	7q11.2	*NA*	8.8×10^{-9}	Cornes *et al.,* 2011
	9p23	*NA*	3.4 x 10-8	Cornes *et al.,* 2011
	16q24.2	*ZNF469*	-	Cornes *et al.,* 2011
	1p34.3	*COL8A2*	-	Cornes *et al.,* 2011
	15q25.3	*AKAP13*	-	Cornes *et al.,* 2011
	9q34.2	*COL5A1*	-	Cornes *et al.,* 2011
	13q12.11	*RXRA*	-	Cornes *et al.,* 2011
Optic disc area	1p22	*CDC7/ TGFBR3*	1.8×10^{-2}	Ramdas *et al*., 2010
	10q21.3–22.1	*ATOH7*	2.0×10^{-32}	Ramdas *et al*., 2010
	10q21.3–22.1	*SALL1*	6.4×10^{-8}	Ramdas et al., 2010
	1p22	*CDC7/ TGFBR3*	7.5×10^{-17}	Khor *et al.,* 2011
	10q21.3–22.1	*ATOH7*	2.0×10^{-15}	Khor *et al.,* 2011
	22q13.1	*CARD10*	2.7×10^{-12}	Khor *et al.,* 2011
	10q21.3–22.1	*ATOH7*	1.3×10^{-10}	Macgregor *et al.,* 2010
VCDR	9p21	*CDKN2B*	1.9×10^{-14}	Ramdas *et al*., 2010
	14q22–23	*SIX1*	9.3×10^{-11}	Ramdas *et al*., 2010
	11q13	*SCYL1*	4.4×10^{-9}	Ramdas *et al*., 2010
	10q21.3–22.1	*ATOH7*	2.0×10^{-8}	Ramdas *et al*., 2010
	13q13	*DCLK1*	4.8×10^{-8}	Ramdas *et al*., 2010
	17q23	*BCAS3*	3.1×10^{-7}	Ramdas *et al*., 2010

2.6.6. Interaction of genes and its influence on IOP

POAG is a multifactorial disease in which a single gene may not contribute to the disease progression. The first genetic interaction was observed between *APOE* (g.–491T) with a variant on *MYOC* promoter (g.–1000G) in POAG patients with increased IOP and these patients were highly resistant to IOP lowering drugs indicating that *APOE* is a modifier gene for *MYOC* (Copin *et al.,* 2002*)*. It was also shown that variant in the *TNF α* (g.-863A/C) interact with *OPTN* (M98K) and increases an individual's susceptibility to POAG. Patients carrying these variants had significantly worse visual field scores (p = 0.026) than those only with *OPTN* /603A (or Lys98) (Funayama *et al.,* 2004). It was demonstrated *in vitro* that TNF *α* induces the expression of *OPTN* through *NFkB* (Zhu *et al.,* 2007). Study by Fan *et al.,* (2005) has shown that polymorphisms in *MYOC*, *OPTN,* and *APOE* interactively contribute to POAG and it has been demonstrated that expression of *MYOC* is induced by *OPTN* (Park *et al.,* 2007). Variants in *OPTN,* Noelin 2 and Olfactomedin interact and increase the susceptibility to POAG (Funayama *et al.,* 2006). A recent study by Blanco-Marchite *et al.,* (2011), demonstrated that the interaction between the variations in *WDR36* and *p53* polymorphisms leads to an increased susceptibility to POAG.

2.7. Molecular mechanism of IOP elevation

Elevated IOP is a major risk factor and reducing IOP is the only treatment option for POAG. Under normal conditions, the secretion and drainage of aqueous humor is in a balanced state and once this is lost, it leads to an elevation of IOP. Several ocular tissues are involved in aqueous humor dynamics and these are ciliary body, TM, and the uveoscleral pathway. Abnormality in conventional outflow is an identified mechanism in the elevation of IOP. However, the factors which are responsible for abnormal function of TM are not yet unknown.

2.7.1. Structures involved in aqueous outflow resistance

In POAG, the aqueous resistance occurs in TM and is the major outflow pathway in humans (Llobet *et al.,* 2003). TM contains three differentiated layers of which the outermost part which is near to the anterior chamber, is the uveal meshwork and is formed by prolongations of the connective tissue from iris and ciliary body stromas. The intracellular spaces present in the uveal meshwork are larger and this layer does not cause much resistance to aqueous humor outflow. The corneal meshwork present next to the uveal meshwork is characterized by the presence of lamellae covered by endothelium like cells. The lamellae are composed of glycoproteins, collagens, hyaluronic acid and elastic fibers. Compared to uveal meshwork, this layer consists of small intercellular spaces and is responsible for increase in outflow resistance. The third layer which is in direct contact with schlems canal is called juxtacanalicular or cribriform meshwork. This is characterized by a layer of cells embedded in dense ECM proteins. The intercellular spaces are very narrow and majority of the aqueous outflow resistance occurs in this layer. Endothelial cell layer is the last barrier that aqueous humor has to cross before exiting the eye and is called Schlemm's canal. Schlemm's canal is essentially an endothelium-lined tube, resembling lymphatic vessel. Due to the presence of high density of pores across its surface, it contributes to the generation of major aqueous humor outflow resistance (Llobet *et al.,* 2003).

2.7.2. Biological properties of aqueous humor and its outflow

Aqueous humor is a byproduct of blood and is secreted by ciliary epithelium in the posterior chamber. In normal physiological conditions, the total amount of aqueous humor in humans is around 250µL and an average rate of its formation is 2.75µL /minute (Brubaker *et al.,* 1991). Protein concentration in aqueous humor ranges from 0.05 to 0.15 g/L, whereas, in blood plasma it ranges from 60 to 70 g/L (Davson *et al.,*

1990). Eventhough the concentration differs, all the plasma proteins are present in aqueous humor indicating that aqueous humor proteins are derived from plasma rather than synthesized locally. The major components of the aqueous humor are organic and inorganic ions, carbohydrates, glutathione, urea, amino acids, proteins, oxygen, carbon dioxide, and water (Goel *et al.,* 2010). Anti-oxidant substances are also found in the aqueous humor, such as glutathione (derived by diffusion from the blood) and ascorbate (which helps to protect against light induced oxidative damage) (McLauchlan *et al.,* 1998). A number of ECM proteins and their metabolizing enzymes, several growth factors and their antagonists are present in the aqueous humor and they influence TM outflow, consequently regulating the IOP. Aqueous humor formation is a complex process and three mechanisms have been proposed for its formation (Johnson *et al.,* 2000). These are diffusion, ultrafiltration and active transport. Active secretion is the major contributor for aqueous humor formation and is responsible for 80 to 90% of the total aqueous humor formation.

Aqueous humor leaves the eye by two different mechanisms and both are located at the anterior chamber angle (Goel *et al.,* 2010). In conventional outflow (also called TM outflow), the aqueous humor passes through the TM, from there into the inner wall of Schlemm's canal and enters in to the aqueous veins where it subsequently mixes with blood in the episcleral veins. Aqueous humor outflow via the trabecular pathway is IOP dependent, usually measured as outflow facility, and expressed in millimeters of mercury (mm of Hg). Approximately 80% of aqueous humor is removed by the conventional TM pathway, and the remaining is removed by the uveoscleral pathway (Millar *et al.,* 1995). In uveoscleral or nonconventional pathway, the aqueous humor leaves the anterior chamber by diffusion through intercellular spaces among ciliary muscle fibers. Although this pathway is responsible for minor amount of

aqueous humor outflow in humans, the uveoscleral or non- conventional pathway is the target for several antiglaucoma drugs (prostaglandin analogs) and is involved in reduction of IOP in POAG (Millar *et al.,* 1995).

Circulation of aqueous humor to the anterior segment of the eye serves a number of important functions (Brubaker *et al.,* 1991)

1. It supplies nutrients and oxygen and removes metabolic wastes from the cornea, crystalline lens, and TM.
2. It inflates the globe and creates an IOP to maintain the optical functions of the eye.
3. It transports the ascorbic acid into the anterior chamber of the eye where it acts as an antioxidant by scavenging the free radicals.
4. It provides local immune response during inflammation.

2.7.3. Factors involved in IOP elevation

The secretion and regulation of aqueous humor outflow is a physiologically important process required for the normal functioning of eye. In a healthy eye, aqueous humor generates an average IOP of around 15mmHg (Millar *et al.,* 1995). This amount of pressure is required to inflate the eye ball thereby helping to maintain the proper shape and optical properties of the eye. An increase in IOP (>21mmHg) is a risk factor for POAG and is responsible ONH damage resulting in visual field loss (Kwon *et al.,* 2009). The exact mechanisms responsible for increased outflow resistance in POAG are yet to be identified. Several hypotheses have been postulated to explain as to why TM alters its function in POAG and how this contributes to outflow resistance. The factors involved in TM abnormal function and outflow resistance are age, increased ECM and Oxidative stress.

2.7.3.1. Age

Age is one of the risk factors for the development of POAG (Rudnicka *et al.,* 2006). It has been shown that with increase in age, there is an increased expression of ECM in TM which in turn may contribute to the reduction of aqueous humor outflow resulting in elevation of IOP. It is well known that in patients with glaucoma and with increased age, there is a significant loss of TM cells (Gabelt *et al.,* 2005). The exact mechanism by which the cell population is reduced in normal and glaucomatous human TM tissue is not yet known. TM serves several functions and this cell depletion leads to the disintegration and loss of the trabecular beams resulting in loss of phagocytotic capacity of the TM cells for effective cleaning, thereby leading to blocking of the conventional outflow pathway and development of elevated IOP in POAG (Gabelt *et al.,* 2005).

2.7.3.2. Extracellular matrix and its role in elevation of IOP

TM cells secrete several ECM molecules and regulation of these molecules is crucial for the regulation of outflow facility. A variety of ECM molecules have been identified in TM and these include glycosamino glycans (GAGs), proteoglycans, collagens, elastin fibrils, fibronectin and laminin (Tamm *et al.,* 2007). GAGs are the most abundant ECM molecules in TM and are major contributors for the outflow resistance. Studies have shown that enzymes which selectively degrade these GAGs reduce the outflow resistance. Several GAGs have been identified in the TM and these include hyaluronic acid, chondratin sulfate, dermatan sulfate, keratin sulfate and heparin sulfate. TM cells express several proteoglycans like decorin, lumican, versican and biglycan (Tamm *et al.,* 2007). Collagens are another major contributors for outflow resistance and TM cells expresses several collagens like collagen type I to type VI. SPARC and thromobospondins are highly expressed matricellular proteins in TM and these are involved in ECM modulation (Tamm *et al.,* 2007, Acott *et al.,* 2008).

In addition to structural components of ECM, TM cells also secrete a variety of proteolytic enzymes which are involved in the metabolism of ECM. These enzymes are MMPs (Matrix Metallo Proteinases) and their inhibitors, TIMPs (Tissue Inhibitors of Metalloproteinases) (Acott *et al.,* 2008). Several MMPs have detected in the TM, and these include MMP1, MMP2, MMP3 and MMP9 and their inhibitors TIMP1 and TIMP2. TM cells also synthesizes several other enzymes like tissue transglutaminase (tTgase), lysyl oxidase (LOX) and lysyl oxidase like (LOXL) and are involved in cross linking the several ECM molecules. tPA (tissue plasminogen activator) is another important ECM regulator involved in the activation of MMPs (Tamm *et al.,* 2007, Acott *et al.,* 2008).

In addition to these, TM cells also secrets several regulatory molecules which are involved in the regulation of ECM synthesis. They are transforming growth factor β (*TGFβ*), connective tissue growth factor (*CTGF*), interleukin 1 (IL1) and tumor necrosis factor α (*TNFα*) (Acott *et al.,* 2008). Multiple reports have shown higher levels of TGFβ2 in the aqueous humor collected from POAG eyes compared to aqueous humor from patients who underwent cataract surgery (Tripathi *et al.,* 1994, Inatani *et al.,* 2001, Picht *et al.,* 2001). The factors which are responsible for elevation of *TGFβ2* in the aqueous humor of POAG patients are still not clear. Treatment of cultured human TM cells with *TGFβ2* stimulates the expression of several ECM genes, including versican, elastin, collagens, fibrillin, laminin, and fibulin (Zhao *et al.,* 2004). In addition to ECM, *TGFβ2*-treated human TM cells induces the expression of *PAI-1* thereby inhibiting the activity of MMPs. *PAI-1* inhibits the activity of both tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Both tPA and uPA catalyze the conversion of plasminogen into plasmin, a key protein involved in the activation of MMPs (Fuchshofer *et al*., 2003). These results indicate that *TGFβ2* is directly and indirectly

involved in the accumulation of ECM in TM. In addition to abnormal ECM synthesis, *TGFβ2* induces the expression of transglutaminase (*TGM2*) (Welge-Lussen *et al.,* 2000) and *LOX* family enzymes (Sethi *et al.,* 2011) and both are involved in the cross linking of ECM proteins and this cross linked proteins are more resistant to degradation by proteolytic enzymes. Recently, adenoviral transfer of *TGFβ2* has been shown to reduce the outflow facility and elevate the IOP by inducing the synthesis of several ECM molecules (Shepard *et al.,* 2010). TM also expresses bone morphogenic proteins (*BMPs*) which counteract the activity of *TGFβ* (Wordinger *et al.,* 2002). However, increased expression antagonists will bind to BMPs (gremlin) and is not available to counteract the TGFβ2 activity (Wordinger *et al.,* 2007). Connective tissue growth factor (*CTGF*) is an essential downstream mediator of *TGFβ2* for ECM synthesis. *CTGF* is a member of the *CCN* (*CTGF*, cysteine-rich angiogenic protein 61, and nephroblastoma overexpression gene) family of regulatory proteins. In the eye, the expression of *CTGF* has been found in TM (Tomarev *et al.,* 2003), iris, ciliary muscle, and retina. *CTGF* expression has also been detected in the aqueous humor (Wunderlich *et al.,* 2000) and recent studies have reported that the concentration of *CTGF* was increased in the aqueous humor of patients with XFS (Browne *et al.,* 2011). Similar to ECM, higher expression of *CTGF* in TM has also been observed on treatment with TGFβ2 and in presence of elevated IOP (Fuchshofer *et al.,* 2009). Study by Chudgar *et al.,* 2006 has shown that TM also expresses higher amounts of *CTGF* following mechanical stretch. Junglas *et al.,* (2009) demonstrated that recombinant *CTGF* is as potent as *TGFβ2* to induce expression of ECM in TM cells and did not affect the proteolytic enzymes and their inhibitors. Study by Junglas *et al*., (2012), has shown that adenoviral over expression of *CTGF* in mice causes a phenotype that shares similar features that observed in patients with POAG, such as open iridocorneal angle, high IOP, and

degeneration of optic nerve axons. Although the increase in CTGF correlates with an increase of fibronectin within the TM, the mechanism that is responsible for the increase in IOP appears due direct action of CTGF on the TM actomyosin cytoskeleton.

Recently, gene expression analysis has shown the increased expression of several ECM molecules in the TM of POAG patients and these include matrix Gla protein, chitinase 3-like 1 (glycoprotein 39), TIMP2, fibronectin, cartilage link protein, MAGP 2, collagens type I, III, V, VI, XI, XII and XIV, thrombospondin 3, periostin, MMP-1, -3 and -10, integrin α9, cochlin (Bhattacharya *et al.,* 2005, Liton *et al.,* 2006). However, the exact role of these molecules in the elevation of IOP is not yet known.

2.7.3.3. Role of Oxidative Stress in IOP elevation

Evidence from both *in vitro* and *in vivo* and studies over the past few years strongly supports the presence of oxidative stress in the elevation of IOP. Free radicals which are generated during the oxidative stress affect the cellularity of TM. Oxidative damage to the DNA of TM cells is significantly higher in POAG subjects, as indicated by increased 8-hydroxy-20-deoxy guanosine (8-OH-dG) (Izzotti *et al.,* 2003). Additional studies reported significant correlation among 8-OH-dG levels in the TM, increased IOP, and visual field damage (Saccà *et al.,* 2005). The importance of oxidative damage in POAG has been further supported by a significant depletion of total antioxidant potential in the aqueous humor (Ferreira *et al.,* 2004), increase in serum antibodies against glutathione-S-transferase (GST) (Yang *et al.,* 2001), decrease in glutathione levels (Gherghel *et al.,* 2005), increase in lipid peroxidation (Yildirim *et al.,* 2005), and increased expression of endothelial-leukocyte adhesion molecule (ELAM-1) (Wang *et al.,* 2001) in patients as compared to normal individuals. These findings suggest that oxidative stress plays an important role in elevation of IOP in POAG.

2.7.3.4. Role of mitochondria as a risk factor in IOP

The association of genetic variations in mitochondrial genome with POAG supports the role of mitochondria in POAG (Abu-Amero *et al.,* 2006). POAG patients carrying the *GSTM1* deletion have increased TM cell death as compared to POAG patients without deletion in *GSTM1*(Abu-Amero *et al.,* 2008). A study by Abu-Amero *et al.,* (2006), has shown that several mitochondrial abnormalities were present exclusively in POAG patients with increased mtDNA content and reduced MRA (mitochondrial respiration activity). A study by Izzotti *et al.,* (2011), has shown that there is a significant correlation between the amount of mitochondrial DNA deletion and level of oxidative damage in TM cells. Above observations indicate that mitochondrial abnormalities could play an important role in elevation of IOP.

2.7.4. Animal models in IOP regulation

As availability of human tissues is limited, animal models have been created to understand the molecular mechanism involved in the elevation of IOP. Studies by Libby *et al.,* (2005) have shown that mice carrying $Bax^{-/-}$ has lower IOP compared to mice with $Bax^{+/-}$ and $Bax^{+/+}$. These results suggest that cell death of cells involved in aqueous humor outflow may contribute to IOP elevation. Previous studies have demonstrated that loss of TM cells more in POAG patients when compared age matched normal individuals. Together, these findings indicate that cell death occurs in TM and *BAX* is one of the candidates for TM cell death.

A study by Haddadin *et al.,* (2009), has shown that *SPARC* deficient mice have lower IOP than the wild type mice. *SPARC* is a matricellular protein that promotes the synthesis of ECM molecules. *SPARC* is known to express in lens, cornea, TM, and RPE and is one of the most upregulated gene in response to elevated IOP and mechanical stretching. Furthermore an elevated level of SPARC has been observed in POAG and

PACG and the *SPARC* gene is located on 5q31.3-q32 within *GLC1M* locus (Pang *et al.,* 2006, Chua *et al.,* 2008). These observations indicate that *SPARC* is one of the possible candidates for IOP regulation. Inoue- Mochita *et al.,* (2009), have shown that mice deficient in G-protein signaling 2 (*RGS2)* has lower IOP than wild type mice. *RGS2* is known to be involved in vascular smooth muscle relaxation and blood pressure homeostasis. Interestingly, the contractile status of TM and ciliary muscle (CM) regulates the aqueous humor outflow, with contraction of the TM leads to decreased aqueous humor outflow, and contraction of CM leading to increased aqueous humor drainage (Wiederholt *et al.,* 2000). Hence *GAS2* may play an important role in regulation of IOP by modulating the contraction of TM and CM.

Study by Fujikawa *et al.,* (2010) demonstrated that *Vav2/Vav3* deficiency is associated with buphthalmos, progressive iridocorneal angle changes, and elevation of IOP with selective loss of RGCs and progressive ONH cupping in mice. The expression of *VAV2* and *VAV3* was demonstrated in iridocorneal angle and also in retina in both mouse and human eyes. In addition, association of *VAV2* and *VAV3* variants with POAG of Japanese population indicated that these proteins may play an important role in pathogenesis of POAG. *VAVs* are a family of guanine nucleotide exchange factors (GEFs) that activates Rho guanosine triphosphatases (GTPases) in a phosphorylation dependent manner (Bustelo *et al.,* 2001). Rho GTPases controls the cell behavior via regulating the specific filamentous actin structures involved in migration, adhesion, and morphogenesis (Schmidt *et al.,* 2002). Studies have shown that cytoskeleton present in the TM plays an important role in the regulation of aqueous humor outflow and Rho GTPases modulates these cytoskeletal dynamics (Filla *et al.,* 2006). Hence, *VAV*s could be involved in activation of Rho GTPases and thereby involved in regulation of cytoskeleton arrangements in TM and this further helps in regulation of IOP.

Bestrophin is another important candidate gene and mice with *Best2*$^{-/-}$ significantly have lower IOP than their wild type counterparts (Bakall *et al.,* 2008).

2.8. Mechanism of pressure induced ONH degeneration and RGC death

Elevated IOP is a major risk factor for POAG and is involved in the formation of mechanical stress on ONH. Loss of RGCs and their axons results in characteristic ONH cupping and visual field defects in POAG. The exact molecular mechanism involved in IOP induced RGC death is not clear. Several hypotheses have been proposed to explain how elevated IOP trigger RGC death in POAG. These include loss of neurotrophic support, astrocyte mediated ONH remodeling, nitric oxide induced RGC death, immune system activation, mitochondrial mediated RGC death and glutamate excitotoxicity.

2.8.1. Neurotrophin deprivation: In normal physiological conditions, the RGC axons exit the eye through the lamina cribrosa and survival of these cells depend on neurotrophic support provided by the brain (Johnson *et al*., 2009). Elevated IOP increases the mechanical stress on the lamina cribrosa causing decreased transport of neurotrophins from the brain resulting in neurotrophic deprivation (Vrabec *et al.,* 2007). It has been shown in animal models that elevated IOP causes blockage of BDNF (brain derived neurotrophic factor) and may contribute to the RGC death (Pease *et al.,* 2000). Supplementation of neurotrophins (BDNF, NGF and BDNF producing mesenchymal stem cells) transiently protected the RGC from pressure induced cell death and these observations indicating that neurotrophin deprivation is involved in RGC death in IOP induced primary glaucomas (Lambiase *et al.,* 2009, Harper *et al.,* 2011).

2.8.2. Astrocyte mediated RGC death: Under normal physiological conditions glial cells provide the neurotrophic support by insulating the neurons, providing the growth factors and synthesizing ECM molecules (Johnson *et al.,* 2009). In humans, majority of glial cells are astrocytes in the retina and ONH (Johnson *et al.,* 2009). In response to

elevated IOP, these astrocytes in the ONH becomes activated and synthesize molecules (*MMP9*) that are involved in the remodeling of ECM and these changes can affect the biomechanical properties of ONH which inturn increases stress on RGCs (Chintala *et al.,* 2002). Activated astrocytes are also involved in mediating the inflammation by inducing the synthesis *TNFα*. Increased expression of TNFα and its receptors has been observed in ONH of both human and animal models (Tezel *et al.,* 2001). Intravitreal injection of *TNFα* induces the RGC death by activating apoptotic pathway (Nakazawa *et al.,* 2006). Nitric oxide (NO) is another important cytotoxic molecule involved in astrocyte mediated RGC death. An increased expression of nitric oxide synthase 2 (*NOS2*) in reactive astrocytes of glaucoma patients and animal models has been observed (Liu *et al.,* 2000). NO is involved in several biological functions, including regulation of vascular tone, neurotransmitter release and synaptic plasticity (Moncada *et al.,* 2006). But an excess level of NO has been shown to induce RGC death in animal models (Cho *et al.,* 2011). These observations suggest that in response to elevated IOP, astrocytes become activated and secrete elevated levels of TNFα and nitric oxide, both of which are cytotoxic to RGC.

2.8.3. Immune system mediated RGC death: Increasing evidence from both *in vivo* and *in vitro* studies over the past few years strongly supports presence of immune system in pathogenesis of POAG. Increased expression of antibodies for retinal and optic nerve head proteins have been observed in POAG patients. These antibodies are against crystallins, vimentin, HSP27, HSP60, HSP70, glutathione *S*-transferase, myelin basic protein, α-fodrin, and γ-enolase (Tezel *et al*., 1998, Maruyama *et al.,* 2000, Yang *et al.,* 2001, Grus *et al*., 2006, Joachim *et al*., 2007, Joachim *et al*., 2008). Direct application of these antibodies at a similar concentration to RGCs induced cell death in both *in vivo* and *in vitro* conditions (Tezel *et al.,* 1998). Recent studies have also shown

that complement activation is involved in glaucomatous neurodegeneration. It has been shown in animal models with elevated IOP modulates the immune system by increasing the expression of several complement factors (C1qa, C1qb, C1qg, C1s, C1r, C3, and C4) (Stasi *et al.*, 2006, Howell *et al.,* 2011). Recently, it was demonstrated that elevation of C3 and MAC (membrane attack complex) were observed in the retina of eyes with elevated IOP and depletion of complement system leading to reduced RGC death accompanied by decreased levels of MAC, active caspase-8 and active caspase-9 (Jha *et al.,* 2011). CFH, a regulator of alternative complement pathway is down regulated in RGC, while several complement factors are upregulated (C3 and C1s), indicating an abnormal activation of complement system in RGC death (Khalyfa *et al.,* 2007).

2.8.4. Oxidative stress mediated RGC death: Activation of oxidative stress by elevated IOP is another important pathway for glaucomatous neurodegeneration. Study by Ferreira *et al.,* 2010 have demonstrated the decreased antioxidant levels, increased lipid peroxidation and nitrite concentration in the ONH of pressure induced glaucoma models. Liu *et al* (2007) have shown that oxidative stress is an early event in IOP induced neuronal damage. An oxidative protein modification has been observed in both human and animal models and reduced activity of these proteins contributed to glaucomatous neurodegeneration (eg. GAPDH and HSP) (Tezel *et al.,* 2005). The activities of several enzymes which are involved in protecting the cells against oxidative stress (SOD, GPx, and CAT, GSH, thiobarbituric acid-reactive substances (TBARS), and melatonin) were down regulated in glaucoma patients (Ghanem *et al.,* 2010). Oxidative stress also modulates the immune system by down regulating the expression of CFH (Tezel *et al.,* 2010).

2.8.5. Mitochondria mediated RGC death: Mitochondrial dysfunction is another important mechanism for RGC death (Kong *et al.,* 2009). Under normal physiological conditions, mitochondria play an important role in several physiological functions including generation of ATP and regulation of apoptosis. Recently, it was demonstrated that elevated IOP triggers mitochondrial fission, OPA1 and cytochrome c release leading to RGC death (Ju *et al.,* 2008). Mitochondrial dysfunction leads to RGC death through caspase-dependent and caspase-independent pathways, initiated by the loss of mitochondrial membrane potential, release of cell death mediators, and oxidative stress (Tezel *et al.,* 2004). This mitochondria mediated cell death is controlled by Bcl2 family of proteins. In glaucoma, the proapoptotic *Bcl2* family members *Bad, Bax,* and *Bid* have been implicated in RGC death (Libby *et al.,* 2005).

2.8.6. Glutamate mediated excitotoxicity: Glutamate toxicity is another potential mechanism of IOP mediated RGC loss in glaucoma (Lotery *et al.,* 2005). Elevated levels of extracellular glutamate have been reported in vitreous of human glaucomatous eyes and in monkeys with elevated IOP (Dreyer *et al.,* 1996). Glutamate is a neuro transmitter and is converted to glutamine mediated by glutamine synthetase. Glutamine is then released by the glial cells and taken up by neurons, where it is hydrolyzed by glutaminase to form glutamate again, completing the retinal glutamate- glutamine cycle (Moreno *et al.,* 2005). To date, five excitatory amino-acid transporters (EAAT1–5) have been identified in the retina and play significant role in the clearance of glutamate in the nervous system. EAAT1&2 were significantly reduced in an experimental pressure induced glaucoma model and human glaucoma patients (Naskar *et al.,* 2000). Elevated IOP also effects the expression of two key glial proteins, glutamate aspartate transporter (GLAST) and glutamine synthetase (GS), both of which are required for regulating extracellular glutamate levels and glutamate metabolism (Ishikawa *et al.,* 2010). Animal

models have also shown that elevated IOP induces the expression of several glutamate receptors (NMDA; N-methyl D-aspartate) which mediate the excitotoxicity (Dyka *et al.,* 2004).

2.9. Gene expression studies in glaucoma

The metabolic activity of the TM, RGC, and astrocytes are dependent on the expression of several genes. However, genes responsible for abnormal function of these cells in the POAG are not known. Gene expression analysis provides a way to identifying the mechanisms that could be involved in the pathophysiology of POAG. Over the past few years several gene expression studies have been conducted on TM, RGC and astrocytes. Majority of the studies analyzed the aqueous humor and TM samples from human POAG patients by using different methodologies like 2D gel electrophoresis, Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF), Microarray, RNA Differential Display (RDD). The genes which are identified in these studies are largely involved in the regulation of ECM and its remodeling, cell death and oxidative stress (Tomarev *et al*., 2003, Borrás *et al*., 2003, Bhattacharya *et al*., 2005, Vittal *et al*., 2005, Liton *et al*., 2006). Due to unavailability of RGC and astrocytes from POAG patients, animal models have been created to mimic the disease condition. The expression studies have been conducted on RGC, astrocytes and whole retina by using genome wide expression profile technologies. Several genes have been identified in response to elevated IOP and these are involved in neurotrophin signalling, immune system activation, remodeling of ECM in ONH, cell death, inflammation and oxidative stress (Khalyfa *et al*., 2007, Hernandez *et al*., 2000, 2002, Miyahara *et al*., 2003, Charles *et al*., 2005, Johnson *et al*., 2007, Yang *et al*., 2007, Tezel *et al*., 2010). These findings support the existence of several mechanisms involved in abnormal function of TM as well as in ONH in POAG.

CHAPTER 3: METHODOLOGY

The present study was carried out to identify the underlying genetic mechanism in IOP associated POAG and PACG cases. Additionally, some of the GWAS results were confirmed in cases of PG and PDS. Further, the results of the association were compared by functional analysis.

3.1. Enrolment of cases and controls

The study was performed according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB). Informed consent was taken from each patient while collecting the blood samples. The subjects included in this study were recruited from the VST Glaucoma Center at the L.V. Prasad Eye Institute, Hyderabad, India. This included an earlier cohort what collected between January 2002 and March 2007. A total of 252 primary glaucoma patients (141 POAG and 111 PACG) and 219 age matched normal controls and additional cases (n=55) and controls (n=73) collected between March, 2010 and March, 2011. Thus the total sample size available for this study was 599 subjects that included the cases of POAG (n=196), PACG (n=111) and ethnically matched normal controls (n=292). Along with primary glaucomas, 78 unrelated patients with pigmentary glaucoma (PG, n=44) and pigmentary dispersion syndrome (PDS, n=34) and 108 age matched controls which were obtained from the New York Eye and Ear Infirmary (NYEE), New York, USA were included in the present study.

For functional analysis aqueous humor samples from POAG cases (n=27) and controls (n=30) were collected during surgery and stored at -80°C refrigerator for further use. Immunohistochemistry (IHC) analysis done with the eye balls collected from the Ramayamma International Eye Bank (RIEB) and immediately fixed in 10% buffered formalin.

3.2. Clinical examination and data collection

1. All the subjects answered a predesigned questionnaire that included their personal, demographic and other systemic and clinical details.

2. The participants underwent a detailed clinical examination by a qualified ophthalmologist at the institute. The clinical examination included:

- Applanation tonometry using goldmann applanation tonometer (Haag-Strait, Bern, Switzerland) to monitor IOP
- Goniscopy (4M and 2M lens, Volk Optical Inc., Mentor, OH, USA) to examine the iridocorneal angle
- Ophthalmoscopy (Direct and indirect, Heine, Germany) to measure the cup-to-disc ratio and
- Automated static perimetry (Humphreys visual field analyzer, Carl Zeiss Meditec, Dublin, CA, USA) to monitor the visual fields

3. Systemic examination includes diabetes, long term hypertension and head or eye injury was evaluated.

4. Blood sample were collected from each subject by venipuncture with prior informed consent.

3.2.1 Confirmation of diagnosis

Each subject was independently evaluated by at least two glaucoma specialists based on preset inclusion criteria. Inter observer agreement for diagnosis was done by kappa statistics.

3.2.2 Inclusion and exclusion criteria for POAG

The POAG cases were collected based on a stringent inclusion and exclusion criteria.

3.2.2.1. The inclusion criteria for POAG

a. IOP >21mmHg

b. Open angles on gonioscopy

c. Characteristic glaucomatous optic nerve head damage (defined by raised CDR >0.4:1)

d. Glaucomatous visual field defects

e. The presence of a visual field defect required confirmation by a repeatable field; this was performed within 2 weeks of the first reliable visual field result showing the defect. The field defects were further classified as mild, moderate and severe

f. Visual field defects were considered to be glaucomatous if they were consistent with optic disc damage and met at least two of the Anderson criteria and these were as follows

    i. A Glaucoma Hemifield test outside normal limits

    ii. A cluster of three or more non-edge points in a location typical for glaucoma, all of which are significantly lower on pattern deviation plot at $p<0.05$ and one that is lower at a $p<0.01$

    iii. A corrected pattern standard deviation that is significant at $p < 0.05$.

Such patients fulfilling the inclusion criteria in the ages between below 35 years were included as JOAG (Juvenile Open Angle Glaucoma)

3.2.2.2. Exclusion criteria for POAG

Patients having following characters were excluded from the present study

a. IOP<21mmHg and cases ocular hypertension (OHT)

b. Open angle suspects

c. Angle closure suspects

d. Lens induced glaucoma (Phacomorphic glaucoma)

e. Neovascular glaucoma

f. Presence of any secondary form of glaucoma including exfoliation and PG

g. History of ocular and head injury

3.2.3. Inclusion and exclusion criteria for PACG

The PACG cases were collected based on a stringent inclusion and exclusion criteria

3.2.3.1. The inclusion criteria for PACG

a. Patients older than 18 years
b. Patients with IOP >21mmHg
c. Patients with anterior or posterior synechiae associated with non visibility of the filtering TM for more than 180 degrees.
d. Evidence of characteristic glaucomatous optic disc damage (defined by raised cup to disc ratio >0.4:1)
e. Characteristic glaucomatous visual field loss

3.2.3.2. Exclusion criteria for PACG

a. Age less than 18 years
b. Presence of ocular disease which can cause secondary angle closure glaucoma (lens induced secondary angle closure, neovascular glaucoma, and pseudo exfoliation glaucoma)
c. Angle closure suspects
d. Ocular or head injury
e. Presence of other eye pathology which can contribute to angle closure glaucoma

3.2.4. Inclusion and exclusion criteria for Controls

The normal adult individuals without any signs or symptoms of glaucoma and other systemic diseases were collected based on a stringent inclusion and exclusion criteria

3.2.4.1. Inclusion criteria for controls

a. Normal individuals older than 60 years
b. No signs and symptoms of glaucoma and other systemic diseases
c. Visual acuity from 20/20 to 20/40

d. IOP < 21mmHg

e. No family history of glaucoma

f. Normal gonioscopic findings with normal anterior chamber without any pigmentary and exfoliation materials.

g. Normal optic disc with C:D is <0.4:1 with asymmetry of cup-to-disc ratio ≤ 0.2:1 (corrected for size) and absence of beta zone peripapillary atrophy

h. No retinal abnormalities with normal pattern of neuroretinal rim and absence of notching or thinning of the rim, disc hemorrhage and nerve fiber layer defects.

3.2.4.2. Exclusion criteria for controls

a. Age less than 60 years

b. Subjects with any ocular disease except simple cataract

c. Subjects having systemic diseases (diabetes, long term hypertension)

d. IOP>21mmHg

e. CDR>0.4

f. Family history of glaucoma

g. Severe myopic patients

3.3. Molecular genetic analysis of candidate genes and gene variants

The screening of candidate genes and their variants done either by resequencing or PCR based restriction digestion. The following steps were included:

a. Designing primers

b. Amplification of candidate genes by polymerase chain reaction

c. Resequencing by automated DNA sequencing technologies

d. PCR based restriction digestion methodologies

3.3.1. Designing of primers

Primers for the respective genes and gene variants were designed using primer designing softwares (http://frodo.wi.mit.edu/cgi-in/primer3/primer3_www_slow.cgi and http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Rozen *et al*., 2000, Lipshultz *et al*., 2008). Primer-BLAST (Basic Local Alignment Search Tool) was used to identify the non specific binding. All the PCR primers were procured from Eurofins (http://www.eurofinsdna.com/) and Sigma Aldrich (http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna.html). To achieve 100 pm/µL working stock, lyophilized oligos were resuspended in required amount of autoclaved deionized water, vortexed gently and spin down briefly. For each PCR reaction, oligos were further diluted to 5 pm/µL with autoclaved deionized water. The following genes and gene variants were studied in the present study (Table 3.1). The primers used in this study are provided in Table 3.2 and 3.3.

Table 3.1. Gene and gene variants studied in the present study

Gene	Complete sequence	Gene variations	Method used
CTGF	√		Resequencing
BAX	√		Resequencing
mtND1	√		Resequencing
NTF4	√		Resequencing
LOXL1		rs1048661	Resequencing
		rs3825942	Restriction digestion
		rs2165241	Resequencing/ Restriction digestion
ZP4		rs547984	Resequencing
		rs540782	
		rs693421	Restriction digestion
		rs2499601	Resequencing/ Restriction digestion
PLXDC2		rs7081455	Restriction digestion
DKFZp762A217		rs7961953	Resequencing
VAV2		rs2156323	Resequencing
VAV3		rs2801219	Resequencing
CAV2		rs4730742	Resequencing
CAV2		rs8940	Restriction digestion
CAV2		rs1052990	Restriction digestion
CAV2		rs10258482	Resequencing
CAV1		rs10227696	Restriction digestion
CAV1		rs4236601	Resequencing
CAV1		rs926198	Resequencing
FBLN5		rs2244158	Resequencing
FBLN5		rs2160079	Resequencing

Table 3.2. Primer sequences used to amplify candidate genes in the present study

S. No	Primer Name	Primer sequence (5'—3')	Annealing temperature (°C)	Product size (bp)
1	*CTGF PF*	CAGGTAGGCATCTTGAG	56**	600
	CTGF PR	CACTGGCTGTCTCCTC		
2	*CTGF1F*	CGCAGTGCCAACCATGAC	56	500
	CTGF 2R	CCGAGCGGTTTCTTTTTCC		
3	*CTGF3F*	CTGACGCTCTGGATGTGAGA	60	364
	CTGF3R	TCACGACCCTGACTTAGAGGA		
4	*CTGF4F*	GTTTGTGTGCTCTGCTCTCG	60	300
	CTGF4R	CCCTAAGTTGGGTCCTATCCA		
5	*CTGF5F*	GGGTTGAAGAAAGCCACTCC	58	504
	CTGF5R	GGGAATCTTTTCCCCCAGT		
7	*BAX1F*	CTGGCACTTATCGGGAGATG	58	364
	BAX1R	CCCCTCAGTGCTTGGAGAT		
8	*BAX2F*	CCAGGTACCTCTTCCCTTCC	59	424
	BAX3R	GGGCCCAGACTCCTAGTTCT		
9	*BAX4F*	CTTGGGGCTCAGTCTCCTTA	58	357
	BAX4R	TCTCTCTCCATGCCCTCTGT		
10	*BAX5F*	GGTGGGGCAGTTGAGAGTAA	58	371
	BAX5R	CAGCCTGACCAATACGGAGT		
11	*BAX6F*	CAGTGGGGACAAGGTTCAGT	60	592
	BAX6R	AGCACTTTGAGAGGCTGAGG		
12	*BAX7F*	AGCGACTGATGTCCCTGTCT	58	479
	BAX7R	TCATCCCTAACCCACAGACC		
13	*mtND1IF*	GCCCGGTAATCGCATAAAAC	60	454
	mtND1IR	CGATCAGGGCGTAGTTTGAG		
14	*mtND1IIF*	GCCTAGCCGTTTACTCAATCC	60	477

	mtND1IIR	GGGAGGTTAGAAGTAGGGTCTTG		
15	*mtND1IIIF*	TCGCCCTATTCTTCATAGCC	60	470
	mtND1IIIR	ATGGGCCCGATAGCTTATTT		
19	*NT4E2F1*	CTTCTTTCCCCACTGAAGTTTTT	58	524
	NT4E2R1	CACCTTCCTCAGCGTTATCAG		
20	*NT4E2F2*	CCCCGAGTAGTCCTGTCTAGG	59	544
	NT4E2R2	CTCTCAGCATCCAGCTCTGTTAT		
21	*NT4E2F3*	ATGGATTCGAATTGACACTGC	59	494
	NT4E2R3	CATCTGCTTGCCTGTTCTAGTTT		
22	*NT4E2F4*	CCAGTTTGGGAACTCATCAAATA	58	537
	NT4E2R4	GATCACTTCTCATTCAACCTTGC		

** indicates 10% DMSO was used

Table 3.3. Primer sequences used for screening of gene variants

S. No	Primer name	Primer sequence (5'—3')	Annealing temperature (°C)	Product size (bp)
1	*LOXL1 IF*	GCAGGTGTACAGCTTGCTCA		
	LOXL1 IR	ACACGAAACCCTGGTCGTAG	60	464
2	*LOXL1 2F*	TAGGGCCCCTTGGAGAATAG		
	LOXL1 2R	GTCCCATTCCCCTCTCAATC	60	264
3	*ZP1F*	GGCTGGAAGCAAAAGACAGA		
	ZP1R	TGTTGCCTGATTACCCTGCT	60	564
4	*ZP2F*	TGCTTTTGCACCAACCAATA		
	ZP2R	AAACTGGGCATTTGCTTTGA	59	500
5	*ZP3F*	GCTTTGAAGGTCCTTGGTCTT		
	ZP3R	TGGCTTACCTCCATTAACACG	60	319
6	*PLXDC2F*	CATTGCAGCCTCCAGCTATT		
	PLXDC2R	TGCAAAGATTCCCAGATCCT	58	479
7	*TMTC2F*	TTGGTTTGCATTGCATGAAG		
	TMTC2R	ATCTTGGTCCAGCATTCGTT	60	396
8	*VAV2F*	TGTGACGGCTGCAAGGCAGG		
	VAV2R	TTCCAGTGCTCTCCCGGCCA	58	401
9	*VAV3F*	AAGCAATAGCAGGGCAGATG		
	VAV3R	GAGAACAGGCATGCTCCAAT	58	434
10	*CAVP1F*	CTTGGCCACTTCCTGAAAAC		
	CAVP1R	CATCACCCTGCTTACCCATT	58	504
11	*CAVP2F*	CCGCTGTGATCCAATTATCC		
	CAVP2R	TGAACAGAACAGTGGTGCTTT	58	475
12	*CAVP3F*	GCCATTGCAGCATCCTTAGA		
	CAVP3R	TCCGAGAAGTCTGCTCATCA	58	487

13	*CAVP4F*	CCAGAGCAAAGCGACATAGA		
	CAVP4R	CAGGTAACTTTGGGCTTGGA	58	417
14	*CAVP5F*	AAGCTCCCTCTGTGTTCCTG		
	CAVP5R	TGCAGTGGTGCATTCATAGG	58	562
15	*CAVP6F*	CAGGGATGACGGGATTTAAG		
	CAVP6R	TAAGAGTGAGGCAGGCATGA	58	466
16	*CAVP7F*	CCTATCCATTCCCAGGTTGA		
	CAVP7R	CTCACCAAGCACATCCTGAA	58	444
17	*FBLN1F*	ACCCTGATGCAGGAATGAAG		
	FBLN1R	TCTAGAGCCCACTGCCATCT	58	437
18	*FBLN2F*	GCCAAATGCTGCTAAGAAGG		
	FBLN2R	AGCCCCTTGTAGTTCAGCAA	58	502

3.3.2. Amplification of candidate genes and gene variants by polymerase chain reaction (PCR)

All the amplifications were setup in Veriti™ 96 well Thermal Cycler (Applied Biosystems, Inc. [ABI], Foster City, CA). 25 µL of each reaction was set up in 0.2 mL tubes (PCR-02-C, Axygen, CA, USA) and occasionally in 96 well plates (PCR-96-FLT-C, Axygen, CA, USA). The reagents and their concentrations are provided in Table 3.4. The programme for PCR amplification was provided in Table 3.5

Table 3.4. Reagents used in PCR reaction

S.No	Reagents	Final concentrations	Total volume
1	Genomic DNA	50 ng – 100 ng	1 µL – 2 µL
2	PCR buffer	10X	2.5 µL
3	$MgCl_2$	25 mM	1.5-2 µL
3	dNTP mix	2 mM	2.5 µL
4	Forward primer	5 pM	1 µL
5	Reverse primer	5 pM	1 µL
6	Taq polymerase (1U/µL) (Bangalore Genei Cat No 105919)	1 U/µL	1 µL
7	Autoclaved deionized water		Made up to the reaction volume of 25 µL

Table 3.5. The thermal cycling conditions were as follows

Step	Temperature (°C)	Time (minutes)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	0.45	35
Annealing	Provided in table (3.2 &3.3)	0.30	
Extension	72	0.45	
Final extension	72	10	1

3.3.3. Confirmation of the amplified PCR products by agarose gel electrophoresis

PCR amplicons were run on 2% agarose gel to determine their quality.

A negative control (without DNA) was run parallely which were run in the same gel to determine the specificity of the amplicon. A 100bp ladder (Fermentas, Hanover, MD) was used to determine the sizes of the amplified fragments.

3.3.4. Mutation screening by resequencing

The amplified PCR products were screened for detection of mutations and genetic variants by resequencing on automated DNA sequencing (ABI3130xl, Applied Biosystems, Inc. [ABI], Foster City, CA).

3.3.4.1. Sequencing reaction involved the following procedure:

All the amplifications were setup in Veriti™ 96 well thermal cycler (Applied Biosystems, Inc. [ABI], Foster City, CA).

10 µl of each reaction was set up in 0.2 ml tubes (PCR-02-C, Axygen, CA, USA) and occasionally in 96 well plates (PCR-96-FLT-C, Axygen, CA, USA). The reagents and their concentrations are provided in Table 3.6. The programme for PCR amplification was provided in Table 3.7.

Table 3.6. Reagents used in PCR reaction

S.No	Reagents	Final concentrations	Total volume
1	PCR amplicons	5 ng – 20 ng	0.5 µL – 1 µL
2	5X sequencing buffer	1X	2 µL
3	Primer	3.5 pM	0.7 µL
3	BDT	-	0.25 µL
4	Autoclaved deionized water	-	Made up to the reaction volume of 10 µL

Table 3.7. The thermal cycling conditions were as follows

Step	Temperature (°C)	Time (minutes)	No of cycles
Initial Denaturation	96	1	1
Denaturation	96	0. 10	25
Annealing	56	0.05	
Extension	60	4	

3.3.4.2. Purifying Extension Products

In order to remove background noise because of unused dyes, primers, and nucleotides, the resequencing PCR amplicons were precipitated by the following methods.

i. The 96-well reaction plate was briefly centrifuged at 100g for one minute.

ii. 1 µL of 125 mM EDTA (pH 8.0) was added to each well and briefly centrifuged

iii. 1 µL of 3M Sodium Acetate, pH 5.2 was added to each well and briefly centrifuged.

iv. 25 µL of 100% ethanol was added to each well and the plate was sealed with aluminum foil and was placed on a rota shaker (Remi RS-12) for five minutes for mixing.

v. The final reaction was incubated on ice for 15 minutes. After 15 minutes the plate was removed from the ice and centrifuged at 3000g for 30 minutes at 4°C

vi. Plate was inverted gently to remove the supernatant and kept on a tissue towel. It was then in an inverted position centrifuged at 185g for one minute to remove the excess supernatant.

vii. The precipitated DNA was washed with 35 µL of 70% ethanol by centrifugation at 3000g for 15 minutes.

viii. Plate was inverted gently to remove the supernatant and kept the plate on a tissue towel in an inverted position and recentrifuged at 185g for one minute to remove the excess supernatant.

ix. The plate was air dried to remove the entire ethanol for 5-10 minutes.

3.3.4.3 Electrophoresis

i. 10 µL of 50% HiDi was added to the each well and denatured at 95°C for 5 minutes and immediately snap chilled on ice for five minutes.

ii. The plate was loaded on automated DNA sequencer (Model 3130xl DNA Analyzer; ABI), according to the manufacturer's protocol.

iii. Before running the samples, the sample sheet was prepared by defining the plate name, sample name, result group, analysis protocol and instrument protocol.

3.3.4.4. Interpreting Sequencing Results

On completion of sequencing run the raw data was collected and analyzed by using Chromas sequencing software (version 2.33). The sequence data obtained was

compared with the reference sequence for the gene available at NCBI website (http://www.ncbi.nlm.nih.gov/). Any variation in the sequence was noted for further analysis.

3.3.5. Prediction of amino acid substitutions observed in candidate genes

The effect of observed amino acid substitutions in candidate genes were predicted by using following methods

3.3.5.1. Multiple sequence alignment

Multiple sequence alignment was done to determine the conservation of the residues of the mutated bases based on their homology across different species. Protein sequences for the different species were downloaded from the NCBI (National Center for Biotechnology Information) data base and CLUSTAL W software analysis (http://www.ebi.ac.uk/clustalw/) was used (Higgins *et al*., 1996)**.**

3.3.5.2. SIFT analysis of mutations

Sorting Intolerant From Tolerant (SIFT) program was used for predicting whether an amino acid substitution affects protein function, based on sequence homology and the physical properties of amino acids. The protein sequences were downloaded from the NCBI database in fasta format and submitted for SIFT analysis (http://blocks.fhcrc.org/sift/SIFT_seq_submit2.html) (Ng *et al*., 2003). SIFT substitutions with scores less than 0.05 was considered to be deleterious to the protein function.

3.3.6. Screening of gene variations by PCR based restriction digestion

PCR based restriction digestion was performed for selected gene variations in the subjects. Restriction enzymes were selected based on the location of sequence variant in a DNA that may either create or abolish the recognition site for particular restriction

enzyme. Restriction enzymes were selected by using NEB cutter (http://tools.neb.com/NEBcutter2/, Vincze *et al*., 2003).

3.3.6.1. Methodology for restriction digestion

i. The PCR amplifications were digested by adding the 2-3 µL of template to a master mix prepared by adding all the reagents according to the manufacturer's protocol (Table 3.8).

ii. The final volume was made to 10 µL for each reaction.

Table 3.8. Reagents used for Restriction digestion

S. No	Reagents	Stock concentration	Working concentration	Total volume for one reaction (uL)
1	Compatible buffer	10X	1X	1 U/ uL
2	Enzyme	10 U/ uL	3-4 U	0.3-0.4
3	Template (PCR product)	-	50-100 ng	2-4 uL
4	Autoclaved deionized water	-		Made up to the reaction volume (10 uL)

iii. The reaction was incubated overnight according to the manufacturer's guidelines.

iv. Following incubation, the 96 well plate or 0.5 mL tubes were removed from the incubator or dry bath and briefly centrifuged.

v. 2 µL of 6X loading dye was added to each reaction and mixed properly by vortexing followed by brief centrifugation.

vi. Electrophoresis was done using Hoefer SE600X Chroma Standard Vertical unit

vii. To prepare a 12% gel, the following was used for preparation of gel

30% Acrylamide (29% Acrylamide plus 1% N, N´- Methylenebisacrylamide)	16.000 mL
10X TBE buffer	4.000 mL
10% APS	0.280 µL
TEMED	0.014 µL

Water	19.706 mL
Total Volume	**40.000 mL**

viii. The gel solution was poured immediately between the assembled glass plates. Combs (1.5 mm thick) were inserted and the gel was left for about 30- 45 minutes to complete the polymerization.

ix. On polymerization the combs were removed and the wells were flushed with distilled water to remove any unpolymerized acrylamide.

x. Upper buffer chamber (cathode) was fixed to the top of the plates, which was then placed in the lower buffer tank (anode). Upper and lower buffer reservoirs were filled with 1X TBE buffer up to the level mark.

xi. Samples were loaded in the appropriate wells along with undigested sample and DNA ladder. Electrophoresis was performed to separate the DNA fragments at 25mA constant current up to 2-3 hours.

xii. On completion of electrophoresis, the plates were removed and the gel was transferred gently from the glass plate in to the tray containing Ethidium Bromide (0.5 μg/mL) staining solution and incubated for 15-20 minutes on shaker and photographed using an UV transilluminator (BioRad ChemiDoc™ XRS Gel Documentation System).

xiii. The genotypes were recorded directly from the gels by comparing with undigested control and 100 bp ladder

3.4. Illumina GoldenGate Genotyping Assay principle and protocol

The Illumina GoldenGate Genotyping Assay is a flexible, pre-optimized assay that uses a discriminatory DNA polymerase and ligase to analyze 96, 384 and 1,536 variants simultaneously. The DNA sample used in this assay is activated to bind to the paramagnetic particles, which are combined with the activated DNA for hybridization. Three oligonucleotides are designed for each variant of which two of them are specific

for allele of the variant site (allele specific ologonucleotide, ASO) and third oligo that hybridizes several bases downstream from the variant site (also called locus specific ologonucleotide, LSO). All three oligos contain regions of genomic complementarity and universal PCR primer sites and LSO contains unique address sequence that target a particular bead type. During the primer hybridization process, the assay oligos hybridizes to the genomic DNA which is bound to paramagnetic particle. Because hybridization occurs prior to the amplification and no amplification bias can be introduced into the assay. Following hybridization several washing steps are performed to reduce the noise by removing excess and mishybridized oligos. Extension of the appropriate ASO and ligation of extended product to the LSO joins the information about the genotype present at the variant site. Universal primers are Cy3 and Cy5 labeled. After downstream processing the single stranded, dye labeled DNA are hybridized to their complement bead type through their unique address sequences. The genotypes are determined by the relative fluorescence of the two labels observed for each bead (Figure 1).

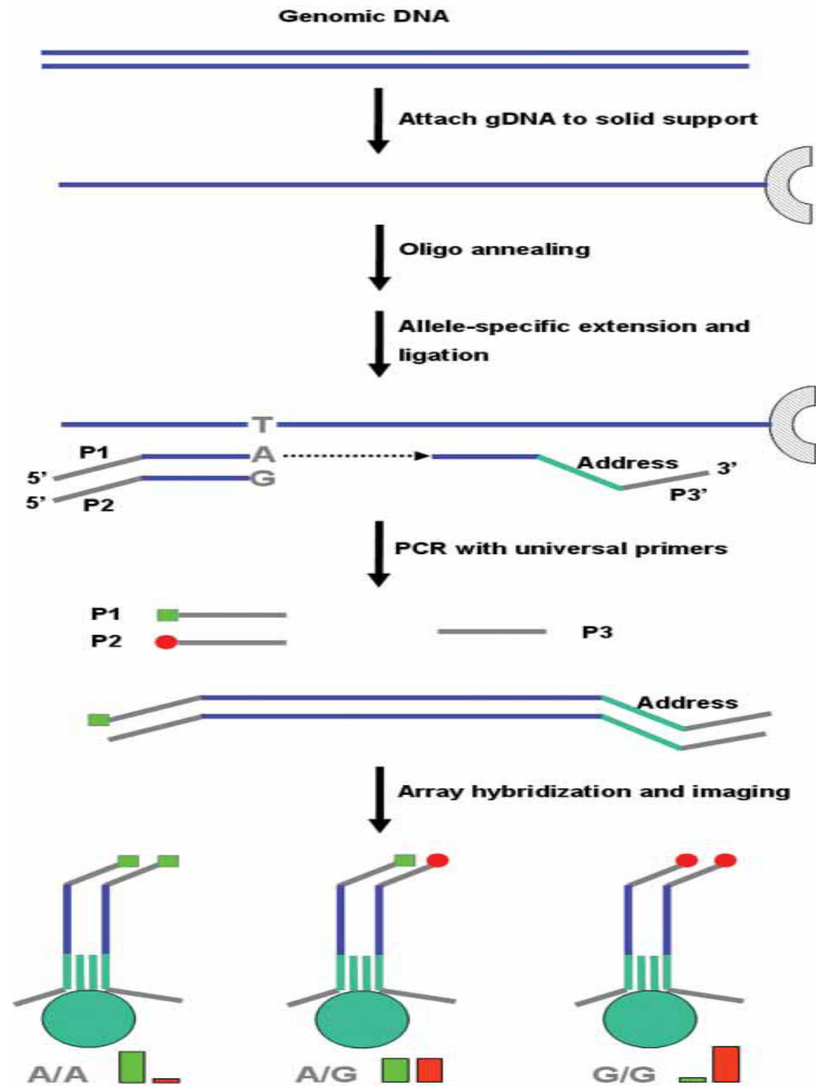


Figure 1. Diagrammatic representation of GoldenGate genotyping assay

Steps in Illumina GoldenGate Genotyping Assay is as follows

3.4.1. Sample preparation and quantitation Picogreen Assay for golden gate genotyping

Picogreen is a fluorochrome that selectively binds dsDNA and has characteristics similar to that of SYBR-Green. It has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm.

Preparation of standards

The stock λ DNA is 100 μg/ 1000 μL

Prepared 1 μg/ 1000 μL of λ DNA (10 μL of above stock and 990 μL of TE)

Done the serial dilution with TE to get 1000 pg/μL to 50 pg/μL

Preparation of Picogreen

Prepared 1:400 dilution of Picogreen (1:400; 1 μL of Picogreen and 399 μL of 1X TE), in a sterile 50 mL plastic container.

i. Added 25 μL of diluted samples or standards in to triplicate in 384 well plate.

ii. Using an 8-channel pipette, transfered 25 μL of Pico Green in each well of 384 well plate.

iii. Covered the plate with aluminum foil and briefly spun the plate.

iv. Incubated the plate for 5 minutes. After incubation read the plate in varioscan flash.

v. Standard curve was drawn and the quantity of the DNA was estimated. Normalized the DNA samples to 50 ng/μL with 1X TE. Using an 8-channel pipette, transfered 5 μL normalized DNA sample to each well of the plate.

Further steps involved in Golden Gate Genotyping assay is as follows

1. Activation of DNA
2. Precipitation of SUD Plate
3. PCR for binding
4. Immobilizing the double-stranded PCR products
5. Hybridization
6. Scanning

3.4.2. Confirmation of GoldenGate Genotyping Assay

The data for golden gate genotyping assay was further confirmed by screening the selected variants rs2244158, rs2160079 (*FBLN5*) by using PCR based automated DNA sequencing.

3.5. Statistical analysis for gene variants

Allele and genotype frequencies were calculated by the gene counting method. The differences in frequencies in cases and controls were calculated by chi square method (χ^2). The odds ratios were calculated to assess the risk of the individual alleles and genotypes of the variants. HWE (Hardy-Weinberg equation) was estimated for the normal controls and haplotype frequencies were calculated using the Haploview software (version 4.2). Linkage disequilibrium (LD) between the variants were calculated by the LD plot function of this software (Barrett *et al*., 2005).

3.6. Functional analysis of ECM genes

The results obtained in the present study was confirmed by following functional analysis

3.6.1. Collection of AqH samples

100 μL of aqueous humor samples were collected from cataract and POAG patients prior to surgery. A total 27 POAG and 30 controls were included in the present study. The inclusion and exclusion criteria for cases and controls is as follows

3.6.1.1. Inclusion criteria for POAG is as follows

Age >50 years

Open angles on gonioscopy

IOP>21mmHg

CDR >0.5:1 with significant visual field changes

3.6.1.2. Exclusion criteria for POAG is as follows

Age <50 years

IOP<21 mmHg

CDR<0.5:1

Patients with Diabetes, Pseudoexfoliation, Retinal abnormalities, High myopia, No visual field changes, head or eye injuries, and previous surgeries in the eye were excluded from the present study

3.6.1.3. Inclusion criteria for controls is as follows

Simple cataract cases

Age >60 years,

IOP<21 mmHg

CDR <0.4:1 were included in the present study

3.6.1.4. Exclusion criteria for controls is as follows

Complex cataract

Patients with Age <60 years

IOP>21 mmHg

CDR>0.4:1

Patients with Diabetes, Pseudoexfoliation, Retinal abnormalities, High myopia, head or eye injuries, and subjects who underwent previous surgeries in the eye were excluded from the present study.

Aqueous humor was collected through a limbal paracentesis site using 27 gauge needle in a 1 mL syringe. Care was taken to avoid touching intraocular tissues and to prevent contamination of aqueous samples with blood. Samples were immediately placed on ice and centrifuged at 3500 g for one minute and stored at –80 °C refrigerator for further use.

3.6.2. Western blotting for the detection of FBLN5 in the aqueous humor and CFH in serum

Western Blotting was done by using mouse monoclonal FBLN5 (Abcam) and secondary antibody (Anti-Mouse IgG (whole molecule)-Peroxidase; Sigma Aldrich-. For CFH it was done by using mouse monoclonal antibody raised against purified human CFH and secondary Ab anti mouse peroxidase conjugate. The following protocol was followed

3.6.2.1. Preparation of gel

i. Assembled the glass plates according to the manufacturer's instructions

ii. An 8% resolving gel was prepared by adding following components:

Table 3.9. Reagents for preparing resolution gel for SDS PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

S.No	Components	Volume (mL)
1	Deionized water	4.6
2	30% acrylamide mix	2.7
3	Tris-Cl (1.5 M, pH 8.8)	2.5
4	10% SDS	0.1
5	10% ammonium persulfate	0.1
6	TEMED	0.006

iii. All the reagents were mixed properly and the acrylamide solution was poured between the glass plates leaving sufficient space for the stacking gel (Comb length plus 1cm). 200-300 µL of isobutanol was added on top of the gel to prevent oxidation.

iv. After polymerization is completed (15-20 minutes), the butanol was removed from the top of the gel and washed with water.

v. In a disposable plastic 15 mL tube, 5% stacking gel was prepared as indicated below (Table 3.10).

Table 3.10. Reagents for preparing 5% stacking gel for SDS PAGE

S.No	Components	Volume (mL)
1	Deionized water	2.1
2	30% acrylamide mix	0.5
3	Tris-Cl (1.0 M, pH 6.8)	0.38
4	10% SDS	0.03
5	10% Ammonium persulfate	0.03
6	TEMED	0.003

vi. The stacking gel was poured on to the polymerized resolving gel

3.6.2.2. Preparation of samples

The sample for western blotting was prepared in appropriate volume of gel loading buffer (for 20 µL of aqueous humor sample 20 µL of 2X sample buffer dye (2X Laemmli buffer). For CFH and CRP, 2 µL of serum was mixed with 18 µL of 1X PBS and added equal volume of 2X sample buffer dye to each samples. All the samples were boiled for 4-5 minutes to denature the proteins prior to loading on the gel.

3.6.2.3. Loading of samples

i. On polymerization the comb was removed and the wells were washed with water to remove any unpolymerized acrylamide.

ii. Prepared 1X TGS by adding 1 volume of 5X buffer with four volumes of deionized water

iii. The gel was mounted in the electrophoresis apparatus.

iv. Tris glycine electrophoresis buffer was added to the top and bottom reservoirs. Removed the air bubbles that were trapped in the gel

v. Samples were centrifuged and loaded in to the appropriate wells.

vi. Electrophoresis was done at 8 V/cm (75 V) and after the dye front moved in to the resolving gel, the voltage was increased to 15 V/cm (100 V) and the gel was run until the bromophenol blue reached the bottom of the resolving gel.

3.6.2.4. Transfer of proteins from SDS PAGE gels to PVDF membrane

i. Equal size (to the gel) of PVDF (Polyvinylidine Fluoride) membrane was prepared and it was soaked in methanol for 2-3 minutes then equilibrated in transfer buffer for 5 minutes.

ii. Equal sizes of six filter papers were prepared and these were equilibrated with transfer buffer for 5 minutes.

iii. The gel was first rinsed briefly in deionized water and equilibrated for 15 minutes in transfer buffer.

iv. The safety cover was removed from semi dry cell and a sandwich gel was prepared gel sandwich as follows:

Bottom: anode metal plate: Placed in this order:

Pre-wet filter paper (3 papers) -- Pre-wet PVDF -- Equilibrated PAGE gel -- Pre-wet filter paper (3 papers)

v. The air bubbles between layers were rolled out using glass rod or 13 mm plastic tube.

vi. The cathode plate was placed on top.

vii. The protein transfer was done at 15V for one hour

viii. The power supply was disconnected and the lid was opened and marked well positions and different lanes for the marker with a pencil on PVDF membrane. The PVDF membrane was removed and placed in a clean box containing 5% nonfat milk to block the unbound free spaces on the membrane.

ix. The membrane was incubated for two hours on a gel shaker at room temperature

x. After blocking, without washing, the membrane was placed in primary antibody (Mouse monoclonal to FBLN5, 1:1000 dilution, 2 μL of antibody was mixed with 2

mL of blocking solution) and incubated overnight at 4°C. For CFH 1:200 dilutions was used.

xi. After the primary antibody incubation, washed the blot three times with 1X PBS containing 0.1% of Tween20 for 3 times, five minutes each.

xii. The membrane was incubated for two hours in secondary antibody (Anti-Mouse IgG (whole molecule)-Peroxidase; 1:6000 dilution, 1 μL of antibody was mixed with 6 mL of blocking solution) and the blot was washed for three times with 1X PBS containing 0.1% of Tween20 for three times, five minutes each. For CFH 1:6000 dilution was used.

3.6.2.5. Detection

i. Added 500 μL of detection reagent A with 500 μL of detection reagent B in a ratio of 1:1 in a 1.5 mL tube (covered with aluminum foil) at room temperature

ii. Drained off the excess wash buffer from the washed PVDF membrane and placed protein side up on a sheet of saran wrap.

iii. Added the above mixed detection reagents on to the membrane.

iv. Incubated the reaction at room temperature for five minutes

v. Drained off excess detection reagent by holding the membrane gently with forceps and touching the edges against the tissue paper

vi. Placed the PVDF membrane side down on to fresh piece of saran wrap and closed the saran wrap around the membrane to form an envelope and gently removed air bubbles.

vii. Placed the wrapped blots, protein side up, in an x-ray film cassette and was subjected for autoradiography for one minute.

viii. The film was removed and replaced with a second sheet of unexposed film.

ix. The exposed film was placed in a tray containing developer for 2-3 minutes and then washed with water and transfered to another tray containing fixer solution and was incubated for 1-2 minutes

x. The film was air dried and scanned

3.6.3. ELISA for CFH

i. Different dilutions of serum samples were prepared. Samples were diluted to 1:500, 1:1000,1:2000,1:4000,1:6000, 1:8000, 1:10,000, 1:12,000 with 1X PBS.

ii. A polystyrene 96 well plate was coated with 100 µL of diluted serum samples.

iii. Plate was covered and incubated for overnight at 4°C.

iv. The plates were washed three times with 1X PBS-containing 0.005% of Tween 20.

v. The nonspecific binding sites were blocked with 100 µl (per well) of a 1X PBS containing 0.005% of Tween 20 (PBST) and 5% BSA (bovine serum albumin) for 2 hours at room temperature

vi. After washing with PBST for three times, 100 µL of mouse monoclonal antibody raised against purified human CFH (1:100 dilution in blocking buffer, Santa - Cruz Biotechnology, Inc) was added and incubated for 2 hours at room temperature

vii. After washing for 3 times with PBST, 100 µL of Horseradish peroxidase-conjugated antimouse immunoglobulin G (IgG; 1:40,000 dilution in blocking buffer, Sigma Aldrich) was added per well, and it was incubated for 1 hour at room temperature

viii. The plates were then washed three times in PBST and color development proceeded with the addition 100 µL of substrate (Tetramethyl Benzidine [TMB])

ix. After 20 minutes incubation in the dark at 37°C, the reaction was stopped by adding 50 µL of 1N H_2SO_4 per well. The optical densities (OD) were measured at 450 nm with a 620-nm reference filter.

3.6.4. Immunohistochemistry for localization of FBLN5 in Human eye

The Immunohistochemistry was done by using primary and secondary antibody to identify the expression of FBLN5 in the eye.

3.6.4.1. Embedding

i. Human cadaveric eye balls were collected and fixed in 10% buffered formalin for overnight at room temperature. After overnight fixation the eye balls were cut in to two halves and processed in an automated processor by following steps:

a. The eye ball was kept in the embedding cassette

b. It was dehydrated in 70% ethanol and changed twice for one hour followed by 95% ethanol for one hour at room temperature and 100% ethanol one hour at room temperature

c. Xylene was added and changed twice for one hour at room temperature

d. The eye ball was embedded in paraffin wax for one hour at 58-64°C.

ii. After embedding, the serial sections of the eyes were cut in the plane parallel to the ocular axis and mounted on silane-coated (3-aminopropyltriethoxysilane) glass slides.

iii. To remove the paraffin wax the slides were placed in a hot air oven for 10 minutes at 60-70°C.

iv. The slides were removed from oven and washed in Xylene for five minutes and parallely in three different chambers of IPA (isopropyl alcohol [100%, 90%, and 70%]).

v. The sections were rehydrated by washing in deionized water for 2-3 minutes followed by 1X PBS for two times five minutes each.

3.6.4.2. Blocking endogenous peroxidase activity

i. Prepared 3% Hydrogen peroxide (H_2O_2) and methanol (CH_3OH) solution (30 mL of 6% H_2O_2 and 30 mL of CH_3OH)

ii. Poured the 3% H_2O_2/CH_3OH solution in a clean coupling jar and slides were placed in the Jar and incubated for 30 minutes at room temperature.

iii. Removed the slides from coupling jar and washed with water for 5 minutes followed by 1X PBS for two times 5 minutes each.

3.6.4.3. Antigen retrieval

i. Preheated the required amount of Trypsin solution in incubator at 37°C

ii. Added 1-2 mL of preheated Trypsin solution on to the section and incubated for 30 minutes at 37°C.

iii. Reaction was stopped by keeping the slides in a jar containing tap water (for 2-3 minutes).

iv. Washed 3 times with 1X PBS (5 minutes each time) and blocked the nonspecific binding sites with 2.5% of BSA in 1X PBS for 30 minutes at room temperature.

v. Added primary Ab (Mouse monoclonal to FBLN5, 1:100 dilution) on to the section and incubated in humid chamber for overnight at 4°C

vi. Washed 3 times with 1X PBS (five minutes each time)

vii. Added secondary Ab complex (one step polymer-HRP, Biogenex, CA, USA) on to the section and incubated for 30 minutes in humid chamber at room temperature.

viii. Washed 3 times with 1X PBS (five minutes each time)

ix. Prepared the DAB (3, 3' diamino benzidine, Biogenex, CA, USA) by adding 36 µL to 964 µL of buffer

x. Added the above solution on the section and incubated for 5-10 minutes in dark at room temperature

xi. Removed the DAB and washed the slides under tap water

3.6.4.4. Haematoxylin and Eosin Staining

i. Stained the sections with haematoxylin and eosin (Sigma Aldrich) by keeping slides in a jar containing haematoxylin eosin stain for 2-3 minutes

ii. Washed the slides under tap water for 5 minutes followed by 1% acidic alcohol (single dip)

iii. Washed the sections in three different grades of alcohol (80, 90 and 100%) for 3 minutes each time

iv. Washed the sections in xylene in three different jars for three minutes each time

v. Removed the slides from the xylene and removed excess liquid by keeping on tissue towel

vi. Mounted the section with cover slip by using DPX

vii. Observed the sections at 10X and 40X magnification under light microscope (Olympus, Prog Res^R Tokyo, Japan).

CHAPTER 4: RESULTS

The primary objective of this present study was to understand the underlying genetic mechanism of IOP associated POAG and PACG cases in the Indian population. This was accomplished by screening candidate genes involved with specific functions in the outflow pathway (*CTGF, NTF4, mtND1* and *BAX)* and replication of genetic associations of the recently identified variants in *LOXL1*, and those in close proximity to *ZP4*, *PLXDC2, DKFZp762A217, VAV2, VAV3, CAV1* and *CAV2*. Additionally, the *LOXL1, CAV1* and *CAV2* variants were further confirmed in cases of secondary glaucomas viz. PG and PDS. While exploring genes involved in the outflow pathway, we also characterized a novel ECM-related gene and undertook functional analysis to further validate the results obtained from genetic association.

4.1 Distribution of study cohort

A total of 599 subjects were enrolled that included cases of POAG (n=196), PACG (n=111) and ethnically matched normal controls (n=292) in the present study. The demographic details of these subjects are provided in Table 4.1. The mean age at onset in POAG was 50.8± 15.4 years, while it was 53±11 in PACG and 60.9±9.5 for the normal subjects, respectively. Male subjects were relatively more among the POAG cases (83.7%) compared to the PACG cases (53.2%) and normal controls (55.1%). Majority of the PACG cases were sporadic (78%), while the proportion of familial and sporadic cases amongst POAG cases were equally distributed.

Table 4.1. Age and gender distribution among POAG, PACG cases and normal individuals

Phenotype	Age (yrs ± SD)	Male	Female	Familial	Sporadic
POAG	50.82±15.49	83.7%	16.3%	42%	58%
PACG	53±11	53.2%	46.8%	22%	78%
Controls	60.91±9.50	55.1%	44.9%	--	--

4.2. Analysis of candidate genes

The candidate genes *CTGF, NTF4, mtND1* and *BAX* were screened for identifying mutations among POAG and PACG cases by resequencing.

4.2.1. Analysis of *CTGF* gene

Screening of *CTGF* (OMIM 121009, 6q23.2) was carried out in a cohort of 332 subjects that comprised POAG (n=121), PACG (n=106) and controls (n=105). Six variations were observed in *CTGF,* which were located in the promoter (g.-444G>C, g.-251delA), coding (R22P, P36S, P102S) and intronic region 1 (g.325>A). A schematic representation of the location of these variations are provided in Figure 4.1. Except for the g.-444G>C, all other variations observed in this study were novel and have not been observed any other disease (Table 4.2). Other than the R22P and P102S variants, all others were observed in equal frequencies amongst the cases and controls (Table 4.2). In case of PACG, only two promoter variations were observed with frequencies similar to the controls. Electropherograms of these observed variations are provided in Figure 4.2.

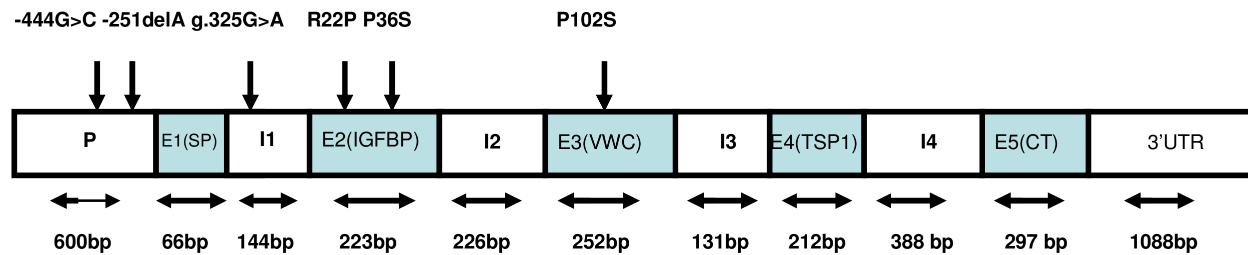


Figure 4.1. Schematic representation of the different domains of *CTGF* and the identified variations

The variations identified in the present study are indicated by arrows. P: Promoter; E: Exon; I: Intron; UTR: Untranslated region; SP: Signal peptide; IGFB: Insulin growth factor binding domain; VWC: Von Willebrand Factor Type C repeat domain; TSP 1: Thrombospondin type 1 repeat domain; CT: C-terminal cystine knot domain.

Table 4.2. ***CTGF*** **variants observed in POAG, PACG patients and controls**

S. No	Nucleotide change	Location	Amino acid change	SIFT score	No. of cases with the variation (%) POAG (n=121)	No. of cases with the variation (%) PACG (n=106)	No. of controls with variation (n=105)	Other diseases
1	g.-444 G>C	Promoter	-	-	5 (4.1)	3 (2.8)	8 (7.6)	Diabetic nephropathy, Ischemic heart disease Blom *et al*., 2001, McKnight *et al*., 2006
2	g.-251 del A	Promoter	-	-	5 (4.1)	3 (2.8)	3 (2.8)	Novel
3	g.325 GG>GA	1st intron	-	-	1 (0.8)	-	1 (0.9)	Novel
4	g.389 CGG >CCG	2nd exon	R22P	0.24	1 (0.8)	-	0	Novel
5	g.426 CCG >TCG	2nd exon	P36S	0.10	1 (0.8)	-	1 (0.9)	Novel
6	g.850 CCC >TCC	3rd exon	P102S	0.51	1 (0.8)	-	0	Novel

Gen bank ID for *CTGF* NT_025741

The details of the observed variations in *CTGF* are as follows

a) Arg22Pro

This missense heterozygous change was observed at position g.389 resulting in the replacement of Arginine (CGG) by Proline (CCG) at codon 22 (R22P) in the insulin growth factor binding domain (IGFBD) (Figure 4.1). The patient with this variation had JOAG with optic disc cupping of 0.9:1 in both eyes along with severe visual field defects (Table 4.3). The co-segregation of the mutant allele in this case could not be done due to the unavailability of DNA samples from other family members. The proline introduced by this mutation presents a very rigid residue that may abolish the required flexibility of the protein at this position. There was a difference in charge between the wild type (arginine, polar and basic) and the mutant amino acid (proline, non polar and hydrophobic). SIFT analysis showed that the substitution at this position was predicted to be well tolerated by the protein with a score of 0.21.

b) Pro36Ser

This heterozygous change was observed at g.426 position resulting in the replacement Proline (CCG) by Serine (TCG) at codon 36 (P36S). This variation was located on IGFBD (Insulin-like Growth Factor binding domain) domain (Figure 1). The patient with this variation had JOAG with optic disc cupping of 0.9 in the right and 0.6 in the left eye along with severe visual field defects (Table 4.3). Similar to the previous mutation, the co-segregation of the mutant allele in this case could not be done due to the unavailability of DNA samples from other family members. Prolines are known to be very rigid and therefore induce a special backbone conformation, which might be required at this position and this mutation would perhaps disturb this special conformation. The hydrophobicity of the wild type (proline, non polar, hydrophobic) and new mutant residue (Serine, polar and

uncharged) was different. But, SIFT analysis showed that this variation might be tolerated by the protein with a score of 0.10.

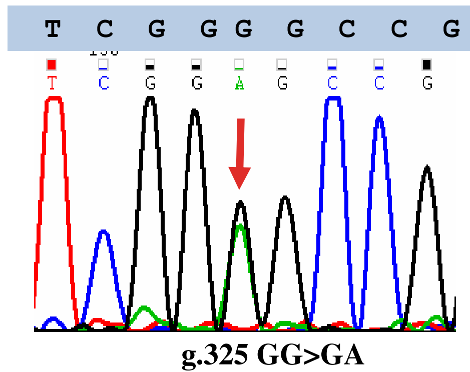
c) Pro102Ser

Another heterozygous variation observed at the g.850 position resulted in the replacement of Proline (CCC) by Serine (TCC) at codon 102 (P102S). This variation was located in the Von Willebrand Factor Type C repeat domain (VWC) (Figure 1) and was conserved across different species compared to the other variations (Figure 4.3). However, SIFT analysis did not predict an intolerance to the protein with a score of 0.51. Patient with this variation had optic disc cupping of 0.8:1 in both the eyes and severe visual field defects (Table 4.3). The co-segregation of this mutant allele also could not be done due to unavailability of DNA samples from the other family members. As proline is important for maintaining confirmation at this position, the creation of serine residue at this position due to the mutation could perhaps disturb this special conformation.

Table 4.3. Clinical features of patients with variations in the coding region of *CTGF* at presentation

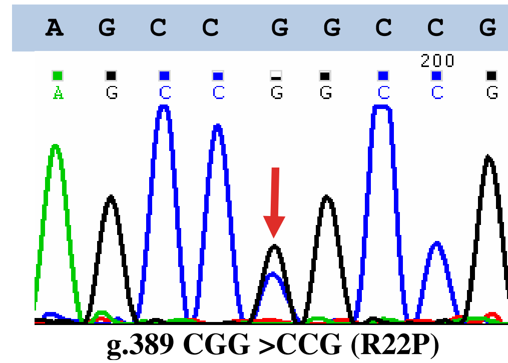
Patient ID	*CTGF* variation	Age at onset	C: D (OD; OS)	Visual field defects (OD:OS)
P145	R22P	27	0.9;0.9	Severe, severe
P162	P36S	23	0.9;0.6	Severe, mild
P62	P102S	25	0.8;0.8	Severe, severe

OD: right eye; OS: left eye; C: D ratio: cup to disc ratio; VFD as per Anderson's criteria (Hodapp *et al*., 1993).



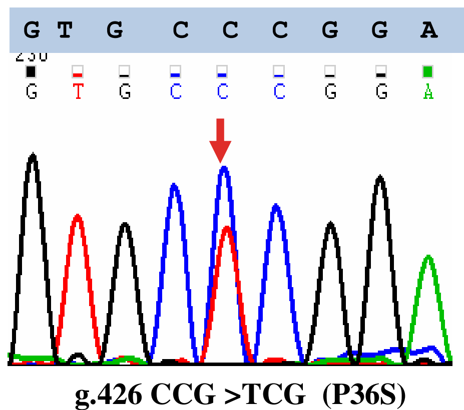
g.325 GG>GA

Figure A Electropherograms showing heterozygous intronic variation (g.325 GG>GA) in *CTGF*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.



g.389 CGG >CCG (R22P)

Figure B. Electropherograms showing heterozygous missense variation (g.389 CGG >CCG, (R22P)) in *CTGF*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.



g.426 CCG >TCG (P36S)

Figure C. Electropherograms showing heterozygous missense variation (P36S) in *CTGF*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.

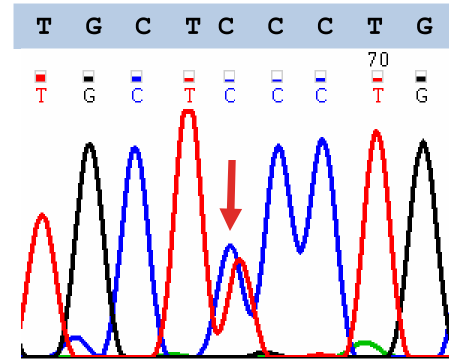


Figure D. Electropherograms showing heterozygous missense variation (P102S) in *CTGF*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow

Figure 4.2. Electropherograms representing *CTGF* variants

```
                                   R22P          P36S                               P102
Mouse   MLASVAGPISLALVLLA-LCT R PAMGQD-CSAQCQC A AEAAPHCPA .... PANRKIGVCTAKDGA P CVFGGSVYRSGESFQSS
Rat     MLASVAGPVSLALVLL--LCT R PATGQD-CSAQCQC A AEAAPRCPA .... PANRKIGVCTAKDGA P CVFGGSVYRSGESFQSS
Pig     MSATGLSPVRCAFVLLLALCS R PASGQD-CSGQCQC A AGKRRACPA .... PANRKIGVCTAKDCA P CVFGGTVYRSGESFQSS
Cow     MSATGLGPVRCAFVLLLALCS R PASSQDCCSAPCQC P AGPAPRCPA .... PTNRKIGVCTAKDGA P YIFGGTVYQSGESFQSS
Human   MTAASMGPVRVAFVVLLALCS R PAVGQN-CSGPCRC P DEPAPRCPA .... PANRKIGVCTAKDGA P CIFGGTVYRSGESFQSS
Xenopus-MSAG----KVTAVLLFALFC W VSDAQE-CNGECQC P -NKVPVCDP .... RVNRKIGVCTAREGA P CVFGGTVYRSGESFQSS
Newt    -MSAGMGALRLPLLLAVALLS W VSCAQD-CSGECRC P -NKPPECPA .... RVNKKIGVCTAKDGA P CVFGGMVYRSGESFQSS
```

Figure 4.3. Multiple sequence alignment of *CTGF* protein across different species (ClustalW)

4.2.2. Analysis of the *NTF4* gene

The *NTF4* gene (OMIM 162662, 19q13.33) was screened in a cohort of 537 subjects that included cases of POAG (n=141), PACG (n=111) and ethnically matched normal controls (n=285). Resequencing of entire coding region of *NTF4* in POAG revealed five different variations that included the most prevalent mutation observed in a previous study (A88V), a silent change (P151P), two 3' UTR changes and one reported polymorphism (rs11669977).Only the A88V and rs11669977 variations were observed in PACG (Table 4.4). The chromatograms of the observed variations are provided in Figure 4.4.

Table 4.4. Distribution of heterozygous variations in *NTF4* observed in primary glaucomas and controls in the Indian cohort

Nucleotide Change	Location in the gene	Amino acid Change	Variations observed		
			POAG (n, %)	PACG	Controls
c.263C>T	Exon 2	A88V	3/140 (2.14%)	3/105 (2.85%)	14/285 (4.91%)
c.453G>A	Exon 2	P151P	1/141 (0.71%)	0/111	0/285
c.790T>G	3'UTR	-	1/141 (0.71%)	0/111	1/227 (0.44%)
c.811G>A	3'UTR	-	1/141 (0.71%)	0/111	0/285

NCBI Reference Sequence: NC_000019.9

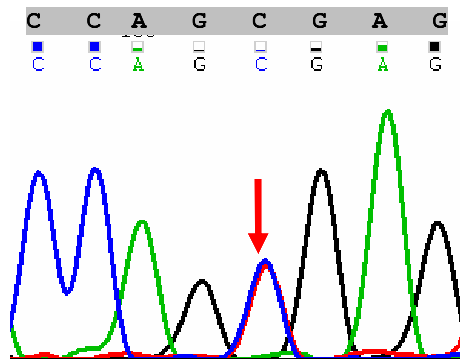


Figure A Electropherograms showing heterozygous missense variation (c..263 C>T (A88V) in *NTF4*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.

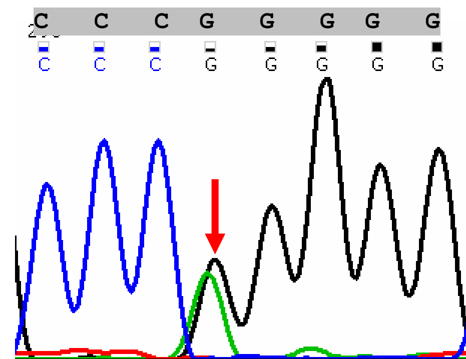


Figure B Electropherograms showing heterozygous variation (c. 453G>A (P151P) in *NTF4*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.

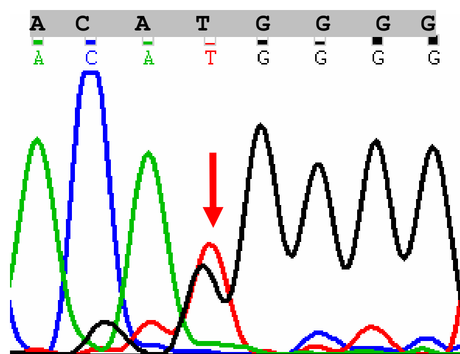


Figure C. Electropherograms showing heterozygous variation c.790T>G (3'UTR) in *NTF4*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.

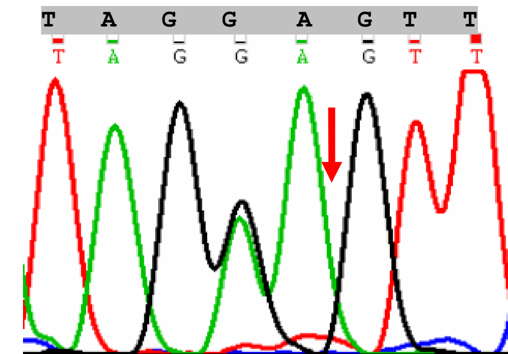


Figure D. Electropherograms showing heterozygous variation c.811G>A (3'UTR) in *NTF4*. The sequence above the electropherogram in the shaded portion represent the wild type sequence. The nucleotide change is indicated by an arrow.

Figure 4.4. Electropherograms representing the *NTF4* variations

Interestingly, the most predominant mutation (A88V) in the European population, was more prevalent in the normal subjects (4.91%) compared to POAG (2.14%; P_{exact} = 0.200) and PACG (2.85%; P_{exact} = 0.577) in the present study (Table 4.4) (Pasutto *et al.*, 2009). The other variations P151P and the 3'UTR changes were observed in two unrelated patients. Analysis of the clinical features at presentation with respect to IOP, cup to disc ratio and visual field defects in POAG and PACG cases and controls harboring the A88V variation showed no significant difference between POAG and PACG cases and controls (Table 4.5). Based on these observations, the A88V is likely to be non pathogenic and appears to be a polymorphism rather than a mutation.

Table 4.5. Clinical features of patients with A88V variation at presentation in Indian cohort

Subject ID	Phenotype	Age at Onset/ Examination	Gender	IOP at Presentation		CDR		VFD	
				OD	OS	OD	OS	OD	OS
PO038	POAG	37	M	30	22	0.7	0.5	Severe	Moderate
PO083	POAG	40	M	34	40	0.9	0.9	Severe	Severe
PO113	POAG	62	F	28	22	0.9	0.9	Severe	Severe
PA083	PACG	50	F	40	24	0.9	0.5	Severe	Severe
PA139	PACG	65	M	22	24	0.8	0.7	Severe	Severe
PA143	PACG	52	M	22	44	0.6	0.9	Early	severe
CO025	Cataract	66	F	17	12	0.3	0.3	-	-
CO030	Cataract	70	F	14	16	0.3	0.3	-	-
CO045	Cataract	68	M	14	14	0.3	0.3	-	-
CO124	Cataract	65	F	16	16	0.2	0.2	-	-
AO042	Cataract	68	M	14	14	0.3	0.3	-	-
AO049	Cataract	65	F	12	12	0.3	0.3	-	-
AO059	Cataract	67	F	12	14	0.2	0.1	-	-
AO102	Cataract	65	F	15	15	0.2	0.2	-	-
AC002	Cataract	68	M	12	13	0.3	0.3	-	-
AC006	Cataract	66	M	16	16	0.3	0.3	-	-
AC011	Cataract	65	F	16	12	0.3	0.3	-	-
AC035	Cataract	65	M	17	16	0.3	0.2	-	-
AC036	Cataract	69	F	17	15	0.3	0.1	-	-
AC051	Cataract	65	M	10	12	0.2	0.2	-	-

4.2.3. Analysis of *mtND1* in glaucoma

The *mtND1* (OMIM 516000, located on mtDNA between 3307 and 4262 bps) was sequenced in cases of POAG (n=122) and controls (102). A total of 47 nucleotide changes were observed of which, 11 (23.4%) were nonsynonymous and 36 (76.5%) were synonymous changes. These 11 non synonymous changes were observed in 21 POAG patients (17.2 %) and 10 controls (9.8%) (Table 4.6). Of the 11 nonsynonymous changes 6 were exclusively present in 8 POAG patients (6.5%) and remaining 5 were detected in 13 POAG (10.6%) and 10 control subjects (9.8%). The T67A variation was observed in 3 POAG patients and absent in 102 normal controls, which was not conserved across the species and predicted to be non pathogenic. Most of the non-synonymous variations were novel and have not been reported earlier in POAG and PACG. However, the variants A4T, T263A and Y304H were previously reported in PCG (Abu-Amero *et al*., 2006, Abu-Amero *et al*., 2007, Tanwar *et al*., 2010) and also in other diseases like Parkinson's, LHON and diabetes (Nakagawa *et al*., 1995, Brown *et al*., 1995, Kumar *et al*., 2010) and were located in the inner mitochondrial space (Fig 4.5). Of the 8 nonsynonymous variations, M31V was pathogenic based on Polyphen prediction and was highly conserved across different species (Fig 4.6). The patient with the M31V was diagnosed as POAG at the age of 45 years with elevated IOP of 30 and 25 mmHg in both eyes and along with CDR of 0.9:1 and severe visual field loss. The clinical features of cases and controls containing all the nonsynonymous variations were provided in Tables 4.7 and 4.8. There were no significant differences in the clinical profile between the POAG and controls harboring these variations. Of the 36 synonymous variations 24 were present exclusively in POAG cases (Table 4.9).

Table 4.6. Non synonymous *mtND1* Sequence Changes in POAG and controls

S.No	Nucleotide change	Amino acid change	POAG	Controls	Polyphen prediction	Summary	Reported
1	m.3316 G>A	A4T	3	1	Benign	non pathogenic	Non-insulin-dependent diabetes mellitus, LHON, PCG (Nakagawa *et al*., 1995, Matsumoto *et al*., 1999, Tanwar *et al*., 2010)
2	m.3397 A>G	M31V	1	0	probably damaging	Pathogenic	AD/PD (Brown *et al*., 1996)
3	m.3505 A>G	T67A	3	0	Benign	non pathogenic	PD (Grazina *et al*., 2005)
4	m.3511 A>T	T73S	1	0	Benign	non pathogenic	Leigh-like syndrome (Blok *et al*., 2007)
5	m.4025C>T	T240M	1	2	Benign	non pathogenic	LHON (Zou *et al*., 2010)
6	m.4070 A>T	Y255F	1	0	Benign	non pathogenic	Novel
7	m.4093 A>G	T263A	1	2	Benign	non pathogenic	PCG, LHON (Tanwar *et al*., 2010, Kumar *et al*., 2010)
8	m.4129 A>G	T275A	1	0	Benign	non pathogenic	Novel
9	m.4132 G>A	A276T	1	0	Benign	non pathogenic	Non-arteritic anterior ischemic optic neuropathy (Fingert *et al*., 2007)

10	m.4216 T>C	Y304H	7	4	Benign	non pathogenic	LHON/Insulin Resistance, PCG, Acute lymphoblastic leukaemia (Johns *et al*., 1991, Crispim *et al*., 2006, Tanwar *et al*., 2010., Back *et al*., 2011)
11	m.4232T>C	I309T	1	1	Benign	non pathogenic	Ischemic optic neuropathy (Fingert *et al*., 2007)

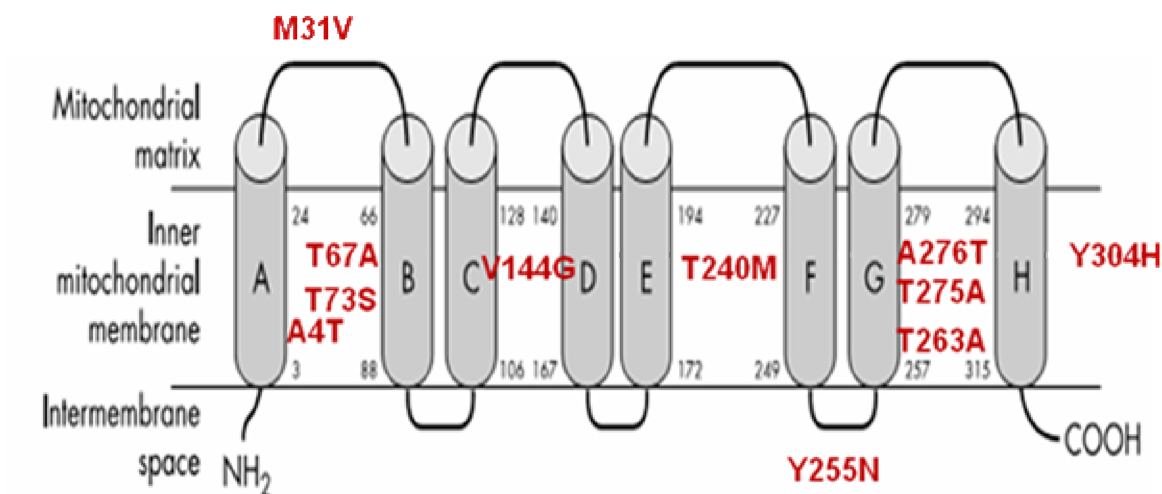


Figure 4.5. Schematic representation of *mtND1* and location of observed variations

M31V

Figure 4.6. Conservation of M31V across different species

Table 4.7. Clinical features of patients carrying *mtND1* nonsynonymous variations

				IOP		CDR		VFD	
Subject Code	**Nucleotide change**	**Amino acid change**	**Age at Onset/ Examination**	**OD**	**OS**	**OD**	**OS**	**OD**	**OS**
P127	m.3316 G>A	A4T	42	28	22	0.7	0.9	moderate	Severe
P123	m.3316 G>A	A4T	37	25	16	0.9	0.8	Severe	Severe
P58	m.3316 G>A	A4T	58	16	6	0.9	0.9	Severe	Severe
P22	m.3397 A>G	M31V	45	30	25	0.9	0.9	Severe	Mild
P122	m.3505 A>G	T67A	50	21	19	0.9	0.8	Severe	Severe
P128	m.3505 A>G	T67A	41	19	25	Inf nocth	0.9	Mild	Severe
P64	m.3505 A>G	T67A	45	24	19	0.6	0.4	Severe	Severe
P1	m.3523 A>T	T73S	12	42	30	0.9	0.4	Mild	Mild
P158	m.4025 C>T	T240M	50	19	17	0.8	0.8	Mild	Mild
P136	m.4070 A>T	Y255F	26	26	24	0.3	0.3	Severe	Severe
P22	m.4093 A>G	T263A	50	21	19	0.9	0.8	Severe	Severe
P51	m.4129 A>G	T275A	77	12	12	0.9	0.9	Severe	Severe
P26	m.4132 G>A	A276T	35	22	22	0.9	0.8	Severe	Mild
P18	m.4216 T>C	Y304H	10	28	28	0.2	0.4	Mild	Mild
P31	m.4216 T>C	Y304H	63	19	14	0.9	0.7	Severe	Severe
P38	m.4216 T>C	Y304H	36	30	22	0.4	0.5	Mild	Mild
P89	m.4216 T>C	Y304H	53	14	14	0.5	0.5	Mild	Mild
P108	m.4216 T>C	Y304H	42	32	33	0.9	0.9	Severe	Severe
P145	m.4216 T>C	Y304H	37	21	15	0.9	0.9	Severe	Severe
P165	m.4216 T>C	Y304H	45	24	22	0.9	0.9	moderate	Severe
P27	m.4232 T>C	I309T	49	26	28	0.9	0.9	Severe	Severe

Table 4.8. Clinical features of controls containing *mtND1* variations

Subject Code	Nucleotide change	Amino acid change	Age at Onset/ Examination	IOP		CDR	
				OD	OS	OD	OS
C97	m.3316G>A	A4T	62	10	12	0.3	0.3
C45	m.4025C>T	T240M					
C132	m.4025C>T	T240M	58	16	16	0.3	0.3
C77	m.4093A>G	T263A	65	18	16	0.2	0.2
C82	m.4093A>G	T263A	62	16	16	0.3	0.3
C23	m.4216T>C	Y304H	56	10	11	0.4	0.3
C26	m.4216T>C	Y304H	60	14	14	0.3	0.4
C69	m.4216T>C	Y304H	64	16	16	0.3	0.3
C98	m.4216T>C	Y304H	60	12	14	0.3	0.3
C67	m.4232T>C	I309T					

Table 4.9. Synonymous *mtND1* Sequence Changes in Patients with POAG and controls

S.No	Nucleotide change	Amino acid	POAG	Controls
1	m.3357 G>A	M17M	1	0
2	m.3384 A>G	K26K	4	0
3	m.3396 T>C	Y30Y	1	0
4	m.3438 G>A	G44G	2	0
5	m.3465 A>G	M53M	1	0
6	m.3480 A>G	K58K	1	0
7	m.3483 G>A	E59E	3	1
8	m.3486 C>T	P60P	2	5
9	m.3531 G>A	P75P	1	1
10	m.3534 C>T	T76T	1	0
11	m.3537 A>G	L77L	2	5
12	m.3552 T>C	A82A	1	0
13	m.3591 G>A	L95L	1	2
14	m.3630 C>T	T108T	2	1
15	m.3637 C>T	L111L	1	0
16	m.3672 A>G	A122A	1	0
17	m.3705 G>A	L133L	1	0
18	m.3714 A>G	V136V	1	0
19	m.3720 A>G	Q138Q	2	0
20	m.3729 A>C	S141S	2	0
21	m.3741 C>T	T145T	1	3

22	m.3744 A>G	L146L	1	1
23	m.3780 C>T	G158G	1	0
24	m.3834 G>A	L176L	2	1
25	m.3915 G>A	G203G	2	3
26	m.3921 C>T	S205S	5	6
27	m.3954 C>T	A216A	1	0
28	m.3970 C>T	L222L	1	0
29	m.4020 C>T	T238T	2	0
30	m.4023 T>C	T239T	1	0
31	m.4053 A>T	A249A	1	0
32	m.4101 T>C	L265L	2	0
33	m.4113 G>A	L269L	1	1
34	m.4191 A>G	P295P	1	0
35	m.4242 C>T	S312S	1	0
36	m.4254 T>C	P316P	1	0

4.2.4. Analysis of *BAX* gene in POAG patients

Resequencing of *BAX (*OMIM 600040, 19q13.3*)* did not reveal any disease associated mutations in POAG. Seven variations were observed that included a nonsynonymous variation (G39W), two silent variations (D98D and V111V) and four intronic known polymorphisms (Table 4.10). A novel heterozygous change at g.856G>T resulted in replacement of Glycine with Tryptophan at codon 39 was observed in one POAG patient. The presence of glycine at this position is necessary for proteins function, however, the substitution at position 39 from G to W is predicted to affect protein function with a SIFT score of 0.02. Multiple sequence analysis (ClustalW) showed that the Glycine residue at the 39th position was not conserved across species (Figure 4.8). The clinical features of patients with coding variations in *BAX* is provided in Table 4.11. The patient with G39W had a late onset of POAG with optic disc cupping (0.9:1 in both eyes) along with severe visual field defects. Patients with the D98D variation had CDR 0.9:1 in both eyes with severe visual field defects and with V111V had CDR of 0.7:1 in both eyes with mild visual filed defects. The intronic variations rs1805419

(p=0.07), rs4645886 (p=0.36), rs11358529 (p=0.56) and rs4645900 (p=0.73) were not significantly associated with POAG (Table 4.12).

Table 4.10. Variations observed in the coding region of *BAX* gene

Nucleotide change	Location	Amino acid change	POAG (n, %)	Controls
g.856G>T	3rd exon	G39W	1 (0.7)	0
g.1399 C>T	4th exon	D98D	1(0.7)	0
g.1438C>T	4th exon	V111V	1 (0.7)	0

Table 4.11. Clinical features of patients with variations in coding region of *BAX* at presentation

Variation	Patient ID	Age at onset	IOP OD ; OS	CDR OD ; OS	VFD OD ; OS
G39W	P145	37	21;15	0.9;0.9	Severe, Severe
D98D	P105	30	21;15	0.9;0.9	Severe, Severe
V111V	P67	21	40;31	0.7;0.7	Mild, Mild

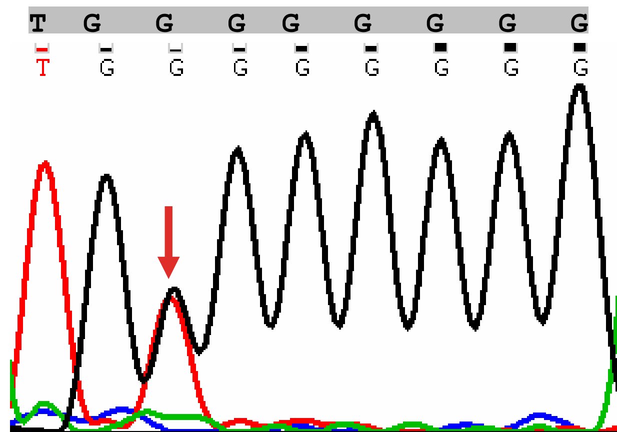


Figure 4.7. Electropherograms showing heterozygous variation g.856G>T (G39W) in *BAX*. The sequence above the electropherogram in the shaded portion represent wild type sequence. The nucleotide change is indicated by an arrow.

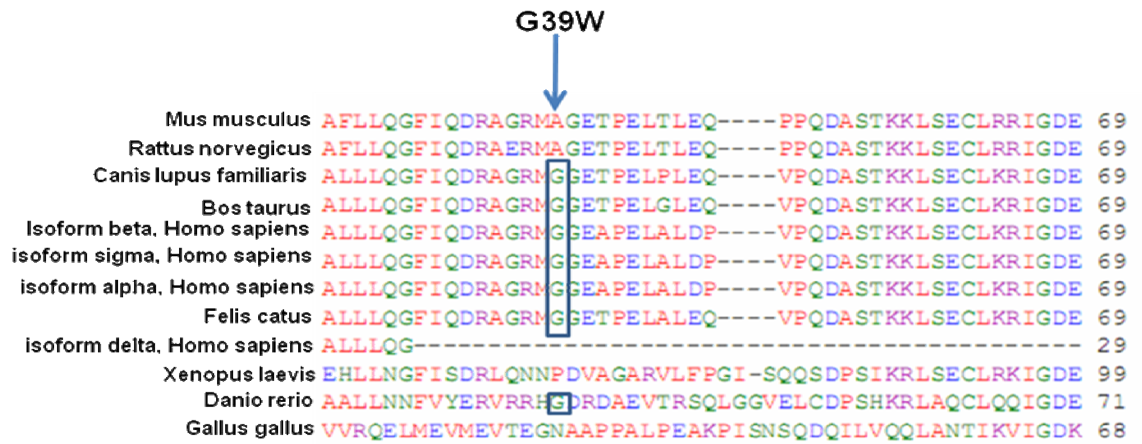


Figure 4.8. Multiple sequence alignment for G39W variation

Table 4.12. Reported polymorphism in the *BAX* gene

Nucleotide Change	Location	Amino acid Change	Allele frequency		OR (95%CI)	p Value
			POAG (n=141)	Controls (n=101)		
rs1805419	3rd intron	-	0.449	0.341	1.60 (0.94-2.73)	0.07
rs4645886	4th intron	-	0.010	0.020	0.34(0.03-3.82)	0.36
rs11358529	4th intron	-	0.264	0.299	0.84(0.47-1.49)	0.56
rs4645900	6th intron	-	0.018	0.020	0.71(0.098-5.13)	0.73

4.3. Analysis of gene variants (single nucleotide polymorphisms, SNPs) in POAG, PACG cases and controls

In order to replicate the findings of GWAS, we further validated the previously observed associations of *LOXL1* and variants proximity to *ZP4, PLXDC2, DKFZp762A217, CAV1* and *CAV2* in IOP-related primary glaucomas (POAG and PACG) and also secondary glaucomas (PG and PDS).

4.3.1. Analysis of *LOXL1* SNPs in POAG and PACG

The *LOXL1* variants were screened in a cohort of 313 subjects, which included 112 POAG, 96 PACG cases, and 105 ethnically and age matched normal controls. All the variants conformed to Hardy-Weinberg equilibrium (>0.05) in the normal controls. Their allele frequencies are provided in Table 4.13. There was no significant difference in the allele frequencies for all the three variants between POAG, PACG and controls. The T allele of rs2165241 that was significantly associated with POAG of Iceland population (OR=1.36, 95% CI, 1.01–1.83; p= 0.04) was not associated either with POAG (OR=0.95, 95%CI, 0.54–1.67; p= 0.426) or PACG (OR=0.82, 95%CI, 0.452-1.51; p=0.262). The G allele of the coding variants rs1048661 (OR=0.70, 95%CI, 0.40–1.24; p= 0.112) and rs3825942 (OR=1.53, 95%CI, 0.78–2.98; p=0.105) were not associated with POAG and PACG (rs1048661; OR= 0.88, 95% CI, 0.49–1.59; p=0.332

and rs3825942; OR=0.94, 95%CI, 0.49–1.79; p=0.456) in the present study. The genotype frequency of these alleles also did not associate either with POAG or PACG (Table 4.14).

Strong LD was observed between rs1048661 and rs3825942 (D'1), between rs3825942 and rs2165241 (D'0.93). Four different haplotypes were generated with these three variants among POAG, PACG and normal subjects. None of the haplotypes were significantly associated either with POAG or PACG (Table 4.15). The frequency of the risk haplotype 'G-G' (generated with rs1048661 and rs3825942) was similar in cases and controls compared to the previous studies. The frequency of the 'T-G' haplotype, was slightly higher in cases compares to the controls but was not statistically significant (Table 4.15).

Table 4.13. Distribution of Allele Frequencies and Their Odds Ratios for the *LOXL1* SNPs across POAG and PACG Cases in the Present Cohort

			Allele frequency			POAG vs. Controls		PACG vs. Controls	
SNP	Genomic position	cDNA position	POAG	PACG	Controls	OR (95%CI)	P	OR (95%CI)	p
rs1048661	g.5758G>T	c.422 G>T (R141L)	0.616	0.667	0.7	0.70 (0.40–1.24)	0.112	0.88(0.481-1.59)	0.332
rs3825942	g.5794G>A	c.458G>A (G153D)	0.83	0.757	0.752	1.53 (0.78–2.98)	0.105	0.94 (0.49–1.79)	0.456
rs2165241	g.8414T>C	IVS1	0.321	0.296	0.328	0.95 (0.54–1.67)	0.426	0.82(0.452-1.51)	0.262

Table 4.14. Distribution of Genotype Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs in POAG and PACG

			Genotype frequency			POAG vs. Controls		PACG vs. Controls	
SNP	Genomic position	Genotype	POAG	PACG	Controls	OR (95% CI)	p value	OR (95% CI)	p value
rs1048661	g.5758G>T	GG	0.37	0.4	0.49	0.56 (0.23–1.40)	0.106	1.09 (0.36–3.27)	0.439
		GT	0.5	0.52	0.41	0.93 (0.38–2.30)	0.437	1.70 (0.57–5.10)	0.168
		TT	0.13	0.08	0.1				
rs3825942	g.5794G>A	GG	0.71	0.61	0.6	2.11(0.73-6.13)	0.080	1.04(0.395-2.73)	0.496
		GA	0.23	0.30	0.30	1.35(0.434-4.21)	0.300	0.97(0.345-2.73)	0.478
		AA	0.06	0.09	0.1				
rs2165241	g.8414T>C	CC	0.47	0.49	0.44	1.25(0.491-3.20)	0.318	0.83 (0.28–2.43)	0.368
		CT	0.41	0.42	0.48	0.8(0.455-1.40)	0.216	0.79 (0.44–1.43)	0.193
		TT	0.12	0.09	0.08				

Table 4.15. Estimated *LOXL1* Haplotype Frequencies across POAG and PACG Cohorts

Haplotypes	%POAG	%Controls	P	PACG	%Controls	p
T-G-C	37.9	29.8	0.079	30.8	29.5	0.781
G-G-T	32.1	31.9	0.963	25.7	31.8	0.183
G-A-C	16.4	22.3	0.123	23	22.4	0.886
G-G-C	13	14.3	0.707	16.8	14.2	0.475

4.3.2. Analysis of *LOXL1* SNPs in PDS and PG

There was no significant difference in allele frequencies for these three variants in PG, PDS and controls (Table 4.16). The allele frequencies of the risk variants rs1048661 (G) and rs3825942 (G) in PG, PDS and controls were similar to POAG, PACG of Indian population. The frequency of rs2165241 (T) was higher in PG and PDS (52 and 50%) compared to the POAG and PACG in Indian population (32.1% and 29.6%). But, none of these were significantly associated either with PDS or PG (Table 4.16). The genotype frequencies of these alleles also did not exhibit any significant difference either with PDS or PG (Table 4.17).

There was a strong LD between the coding variants (rs1048661 and rs3825942) (D'=0.89, 95%CI, 0.57-0.97) and between the rs3825942 and rs2165241 (D'1.0, 95%CI, 0.81-1.00). None of the haplotypes were significantly associated with either PG or PDS in the present study (Table 4.18). These observations indicate that *LOXL1* SNPs may not play a major role in secondary glaucomas like PG and PGS

Table 4.16. Distribution of Allele Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs across PG and PDS Cases in the Present Cohort

			Allele frequency				PG Vs Controls		PDS Vs Controls		PG+PDS Vs Controls	
SNP	**Genomic position**	**cDNA position**	**PG**	**PDS**	**PG+PDS**	**Normal**	**OR (95% CI)**	**p value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**p value**
rs1048661	g.5758G>T	c.422 G>T (R141L)	0.68	0.667	0.674	0.732	0.81 (0.337-1.97)	0.439	0.731 (0.279-1.91)	0.389	0.81 (0.42-1.56)	0.309
rs3825942	g.5794G>A	c.458G>A (G153D)	0.87	0.834	0.852	0.851	1.17 (0.355-3.89)	0.368	0.81 (0.241-2.75)	0.844	1.17 (0.52-2.65)	0.461
rs2165241	g.8414T>C	IVS1	0.52	0.5	0.514	0.509	1.06 (0.474-2.36)	0.417	0.964 (0.396-2.34)	0.91	1.2 (0.66-2.20)	0.432

Table 4.17. Distribution of Genotype Frequencies and Their Odds Ratios for the Three *LOXL1* SNPs in PG and PDS

			Genotype frequency			PG vs. Controls		PDS vs. Controls	
SNP	**Genomic position**	**Genotype**	**PG**	**PDS**	**Normal**	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
rs1048661	g.5758G>T	GG	0.48	0.43	0.52	0.41(0.08-1.93)	0.25	0.44(0.08-2.52)	0.35
		GT	0.4	0.47	0.43	0.42(0.89-2.02)	0.27	0.58(0.10-3.29)	0.54
		TT	0.12	0.1	0.05				
rs3825942	g.5794G>A	GG	0.78	0.7	0.72	0.39(0.03-4.49)	0.44	0.51(0.03-8.60)	0.64
		GA	0.17	0.27	0.263	0.23(0.017-3.02)	0.24	0.53(0.03-9.70)	0.67
		AA	0.05	0.03	0.017				
rs2165241	g.8414T>C	TT	0.33	0.17	0.25	1.08(0.368-3.18)	0.88	0.92(0.21-3.96)	0.92
		TC	0.38	0.66	0.51	0.61(0.228-1.67)	0.34	1.85(0.57-6.04)	0.3
		CC	0.29	0.17	0.24				

Table 4.18. Estimated *LOXL1* haplotype frequencies of PG/PDS patients and controls

Haplotype	Phenotype	%cases	%controls	p value
GGT	PG+PDS	46.6	45.8	0.881
	PG	48	45.8	0.729
	PDS	44.9	45.9	0.883
TGC	PG+PDS	27.9	24.8	0.511
	PG	27.1	24.8	0.683
	PDS	29.1	24.9	0.495
GAC	PG+PDS	13.8	16.8	0.451
	PG+PDS	12.4	16.7	0.364
	PDS	15.5	16.6	0.831
GGC	PG+PDS	7.7	10	0.464
	PG+PDS	7.4	10	0.483
	PDS	8.1	9.9	0.662

4.3.3. Analysis of three susceptible loci in POAG and PACG

The involvement of three susceptible loci in the Japanese population were validated in a cohort of 470 subjects, which included cases of POAG (n=140), PACG (n=111) and ethnically matched normal controls (n=219). Allele frequencies of these variants and their associations are provided in Table 4.19. From the table, it is evident that allele frequencies of these six variants were not significantly different between POAG, PACG and controls. Similarly genotype frequencies were calculated but neither of the genotype were associated with POAG or PACG (Table 4.20). The four SNPs in chromosome 1 (rs547984, rs540782, rs693421 and rs2499601) were in strong linkage disequilibrium between them (D'= 0.90–1.0) and haplotypes generated with these did not indicate any significant differences between the cases and controls (Table 4.21).

Table 4.19. Distribution of Allele Frequencies and Their Odds Ratios for the six SNPs across POAG and PACG Cases in the Present Cohort

						POAG vs. Controls		PACG vs. Controls	
SNP	**Chr**	**Gene (Nearest gene)**	**POAG**	**PACG**	**Controls**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
rs547984	1	*ZP4*	0.417	0.441	0.363	1.29(0.84-2.00)	0.437	1.38(0.87-2.20)	0.271
rs540782	1	*ZP4*	0.409	0.436	0.356	1.24(0.80-1.92)	0.443	1.41(0.88-2.24)	0.255
rs693421	1	*ZP4*	0.405	0.444	0.357	1.23(0.79-1.91)	0.616	1.46(0.91-2.34)	0.190
rs2499601	1	*ZP4*	0.482	0.505	0.413	1.31(0.86-2.02)	0.286	1.45(0.91-2.30)	0.168
rs7081455	10	*PLXDC2*	0.424	0.390	0.407	1.06(0.69-1.64)	0.629	0.92(0.5701.47)	0.676
rs7961953	12	*DKFZp762A217 (TMTC2)*	0.814	0.830	0.778	1.25(0.72-2.14)	0.251	1.32(.73-2.38)	0.124

Table 4.20. Distribution of Genotype Frequencies and Their Odds Ratios for the 6 SNPs in POAG and PACG

		Genotype frequency			POAG Vs Controls		PACG Vs Controls	
SNP	**Genotype**	**POAG**	**PACG**	**Controls**	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
rs547984	AA	0.181	0.218	0.124	1.67(0.88-3.18)	0.11	2.08 (1.07-4.05)	0.03
	AC	0.471	0.446	0.478	1.12(0.70-1.79)	0.61	1.10 (0.66-1.83)	0.7
	CC	0.348	0.336	0.398				
rs540782	CC	0.167	0.218	0.115	1.69(0.87-3.28)	0.12	2.23(1.14-4.37)	0.02
	CG	0.485	0.437	0.478	1.18(0.74-1.88)	0.46	1.07(0.64-1.78)	0.78
	GG	0.348	0.345	0.407				
rs693421	GG	0.345	0.333	0.406				
	TG	0.497	0.445	0.481	1.21(0.75-1.93)	0.65	1.12(0.66-1.88)	0.66
	TT	0.158	0.222	0.113	1.64(0.83-3.23)	0.15	2.38(1.20-4.74)	0.01
rs2499601	CC	0.232	0.324	0.179	1.64(0.89-3.01)	0.11	1.95(1.06-3.59)	0.03
	CT	0.5	0.361	0.48	1.32(0.80-2.17)	0.27	0.81(0.47-1.40)	0.46
	TT	0.268	0.315	0.341				
rs7081455	GG	0.158	0.167	0.158	1.11(0.57-2.14)	0.75	0.93(0.47-1.85)	0.84
	GT	0.532	0.444	0.498	1.19(0.73-1.92)	0.51	0.79(0.47-1.31)	0.36
	TT	0.31	0.389	0.344				
rs7961953	GG	0.657	0.697	0.6	1.36(0.39-4.66)	0.62	1.15(0.33-3.95)	0.82
	GA	0.314	0.266	0.364	1.07(0.30-3.77)	0.91	0.72(0.20-2.58)	0.62
	AA	0.029	0.037	0.036				

Table 4.21. Haplotype frequencies in POAG and PACG cases and normal controls in an Indian population

Haplotypes	Frequency in POAG cases	Frequency in controls	p value	Frequency in PACG cases	Frequency in controls	p value
C-G-G-T	0.485	0.564	0.143	0.466	0.564	0.13
A-C-T-C	0.383	0.332	0.523	0.403	0.332	0.354
C-G-G-C	0.088	0.058	0.376	0.051	0.058	0.999
C-G-TC	0.007	0.012	0.999	0.015	0.012	0.999

4.3.4. Analysis of *VAV2* and *VAV3* SNPs in POAG and PACG

To replicate the involvement of *VAV2* and *VAV3,* we screened these variants in a cohort of 352 patients which include 141 POAG subjects, 111 PACG and 100 normal subjects. The allele frequency of these variants are provided in Table 4.22. The allele frequency of the risk variant rs2156323 was not statistically different between POAG (OR= 1.34, 95%CI, 0.62-2.85, p=0.533), PACG (OR= 1.08, 95%CI, 0.49-2.34, p= 0.223) compared to the controls. Similarly, the allele frequency of rs2801219 was not associated with POAG (OR 1.30, 95%CI, 0.75-2.25, p=0.133) and PACG (OR= 1.46, 95%CI, 0.82-2.59, p= 0.394). The genotype frequency for rs2156323 and rs2801219 are given in Table 4.23. The OR for the risk allele of rs2156323 (OR_{Het} = 0.84, 95%CI, 0.45–1.56 and OR_{Hom} = 0.48, 95%CI, 0.08–2.94) was lower than the Japanese population (OR_{Het}=5.65, 95%CI, 1.99–16.0) and was not statistically significant (p=0.38). Similarly OR for the risk variant of rs2801219 (OR_{Het}=1.18, 95%CI, 0.68 –2.05 and OR_{Hom} =1.54, 95%CI, 0.56 – 4.26) was lower than the Japanese cohort (OR_{Het} =2.03, 95%CI, 1.01–4.09) and was not significantly associated with POAG (p=0.47).

Although there was a tight LD between the three SNPs on *VAV2* and six SNPs of *VAV3* (both D'=1.0), haplotypes generated with these SNPs (frequency >5%) were not associated with POAG or PACG (Table 4.24). Overall these observations indicated that *VAV2* and *VAV3* were not the major candidates for both POAG and PACG in Indian population.

Table 4.22. Distribution of Allele Frequencies and Their Odds Ratios for the *VAV2* and *VAV3* SNPs across POAG and PACG Cases in the Present Cohort

						POAG vs Controls		PACG vs Controls	
Gene	SNP	Risk allele	POAG	PACG	Controls	OR (95%CI)	p value	OR (95%CI)	p value
VAV2	Rs2156323	G	0.885	0.861	0.851	1.34(0.62-2.85)	0.533	1.08(0.49-2.34)	0.233
	Rs3819500	C	0.869	0.86	0.847	1.15(0.55-2.41)	0.71	1.06(0.49-2.31)	0.86
	Rs2073930	G	0.891	0.882	0.87	1.21(0.55-2.68)	0.63	1.06(0.46-2.43)	0.87
VAV3	Rs6689477	G	0.04	0.033	0.03	1.46(0.35-6.02)	0.59	1.25(0.27-5.75)	0.77
	Rs6689476	G	0.04	0.033	0.03	1.46(0.35-6.02)	0.59	1.25(0.27-5.75)	0.77
	Rs6697852	G	0.04	0.033	0.03	1.46(0.35-6.02)	0.59	1.25(0.27-5.75)	0.77
	Rs6686831	C	0.04	0.033	0.03	1.46(0.35-6.02)	0.59	1.25(0.27-5.75)	0.77
	Rs2801219	G	0.37	0.4	0.31	1.30(0.75-2.25)	0.133	1.46(0.82-2.59)	0.394
	rs59614404	A	0.04	0.033	0.03	1.46(0.35-6.02)	0.59	1.25(0.27-5.75)	0.77

Table 4.23. Distribution of Genotype Frequencies and Their Odds Ratios for the *VAV2* and *VAV3* SNPs across POAG and PACG Cases in the Present Cohort

				POAG Vs Controls		PACG Vs Controls	
rs2156323	**POAG**	**Control**	**PACG**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**
CC	0.784	0.732	0.769				
CT	0.201	0.238	0.185	0.84 (0.45–1.56)	0.38	0.74(0.37-1.45)	0.38
TT	0.015	0.03	0.046	0.48 (0.08 –2.94)	0.46	1.48(0.34-6.43)	0.59
rs2801219							
GG	0.122	0.07	0.17	1.54 (0.56 – 4.26)	0.14	2.96 (1.12-7.84)	0.02
GT	0.497	0.48	0.462	1.18(0.68 –2.05)	0.47	1.17(0.65-2.11)	0.58
TT	0.381	0.45	0.368				

Table 4.24. Distribution of haplotype frequencies at the *VAV2* and *VAV3* loci in POAG, PACG and normal controls in the Indian cohort

Gene	Haplotypes	Freq. in POAG	Freq. in controls	p values (POAG vs controls)	Freq in PACG	Freq. in controls	p values (PACG vs controls)
VAV2	C-C-G	0.866	0.836	0.365	0.854	0.836	0.643
	T-A-T	0.155	0.134	0.533	0.091	0.134	0.206
VAV3	A-A-C-T-T-C	0.651	0.687	0.416	0.605	0.687	0.098
	A-A-C-T-G-C	0.311	0.298	0.771	0.367	0.298	0.155

4.3.5. Analysis of *CAV1* and *CAV2* polymorphisms in Indian POAG and PACG patients

We analyzed seven variants located around *CAV1* and *CAV2* (rs4236601, rs4730742, rs8940, rs1052990, rs10227696, rs10258482 and rs926198) in a cohort of 577 subjects that included cases of POAG (n=196), PACG (n=111) and ethnically matched normal controls (n=270). None of the variants were associated either with POAG or PACG in the present cohort (Table 4.25). The risk allele of rs4236601 was not associated either with POAG (OR=1.10, 95%CI, 0.71-1.69, p= 0.52) or PACG (OR= 0.87, 95%CI, 0.46-1.66, p= 0.69). The profile of the remaining six variants were also similar in POAG and PACG (Table 4.25). Genotype frequencies of these alleles also did not exhibit any significant association in POAG and PACG (Table 4.26)

Table 4.25. Distribution of Allele Frequencies and Their Odds Ratios for the *CAV1*, *CAV2* SNPs across POAG and PACG Cases in the Present Cohort

SNP		Allele Frequency			POAG Vs controls		PACG Vs Controls	
	Risk allele	POAG	Controls	PACG	OR (95%CI)	P	OR (95%CI)	P
rs4730742	G	0.182	0.158	0.181	1.18(0.65-2.13)	0.57	1.13(0.59-2.16)	0.69
Rs8940	G	0.169	0.153	0.163	1.14(0.62-2.09)	0.65	1.10(0.57-2.12)	0.77
rs1052990	G	0.274	0.251	0.245	1.12(0.67-1.86)	0.65	0.96(0.55-1.69)	0.91
rs10227696	G	0.181	0.154	0.179	1.21(0.66-2.20)	0.53	1.22(0.64-2.31)	0.53
rs4236601	A	0.187	0.166	0.144	1.10(0.71-1.69)	0.52	0.87(0.46-1.66)	0.69
rs10258482	A	0.088	0.073	0.132	0.64(0.31-1.34)	0.24	0.54(0.23-1.25)	0.15
Rs926198	C	0.346	0.301	0.381	1.22(0.76-1.97)	0.39	1.40(0.84-2.34)	0.19

Table 4.26. Distribution of Genotype Frequencies and Their Odds Ratios for the rs1052990 and rs4236601 across POAG and PACG Cases in the Present Cohort

					POAG vs Controls		PACG vs Controls	
	Genotype	POAG	PACG	Controls	OR (95%CI)	p	OR (95%CI)	P
rs105990	GG	0.059	0.069	0.06	1.07(0.41-2.80)	0.88	1.1(0.40-2.99)	0.85
	TG	0.43	0.353	0.383	1.22(0.77-1.94)	0.39	0.88(0.53-1.48)	0.65
	TT	0.511	0.578	0.557				
rs4236601	AA	0.021	0.03	0.028	0.79(0.22-2.77)	0.72	0.97(0.24-3.88)	0.97
	GA	0.332	0.228	0.276	1.28(0.85-1.94)	0.23	0.77(0.44-1.33)	0.35
	GG	0.647	0.742	0.696				

LD analysis of *CAV1* and *CAV2* variants

There was a tight LD (Figure 4.9) across all the seven variants (D'=1.0), but haplotypes generated with these SNPs (frequency >5%) did not exhibit association to either POAG or PACG.

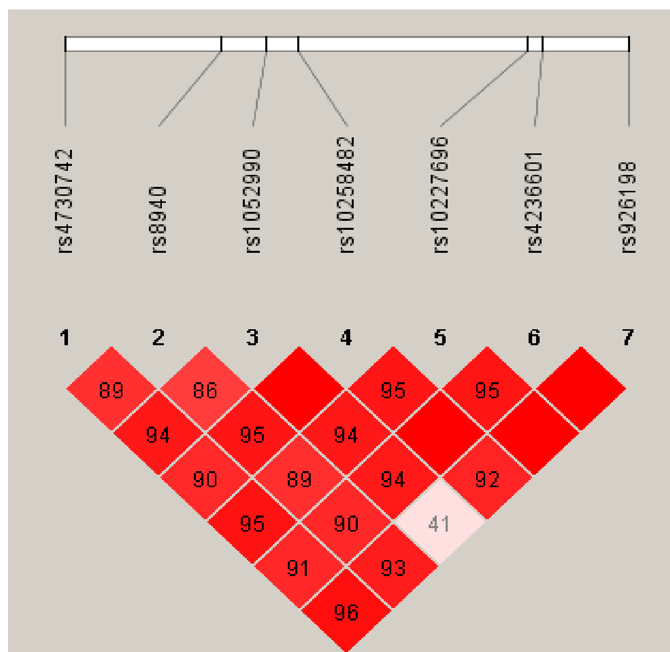


Figure 4.9. LD plot for all the 7 SNPs

Meta-analysis of rs4236601

A meta analysis was done after extraction of the genotype data for rs4236601 from 11 cohorts (including the present one), which comprised a total of 4429 cases and 38,015 controls. There was moderate evidence of association between the rs4236601 and POAG (OR=1.22) and the pooled estimate of the odds ratios for the risk allele had very narrow confidence interval (95%CI, 1.11-1.34). Majority of the cohorts including the present one clustered around the pooled estimate with overlapping confidence intervals. However, there was a marked degree of heterogeneity as the Asian cohorts from Hong Kong and Shantou clustered further away from the pooled estimate with wide

confidence intervals. Additionally, the lower limit of the confidence interval was less than 1 in cohorts from Iowa, Sweden, UK (Leicester and Southampton), Australia (BMES and GIST) and India (Figures 4.10 and 4.11).

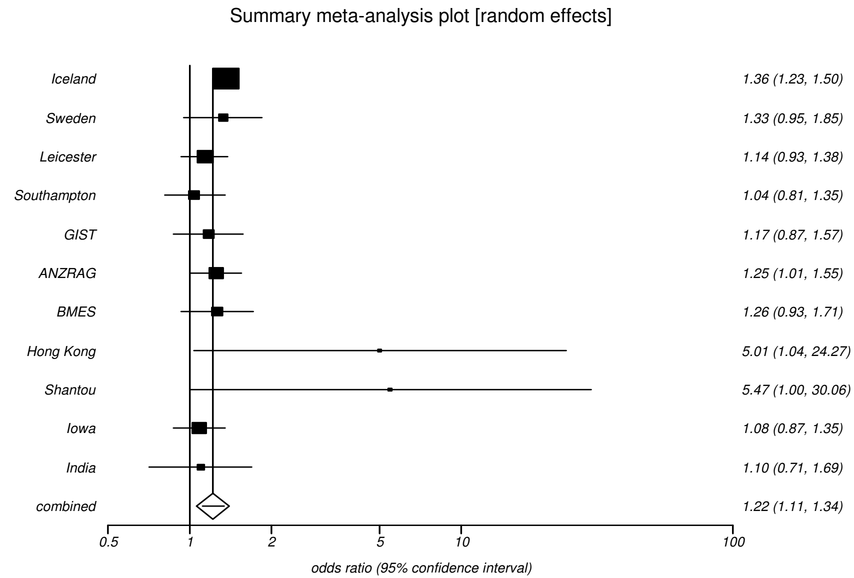


Figure 4.10. Summary of meta- analysis plot (random effects)

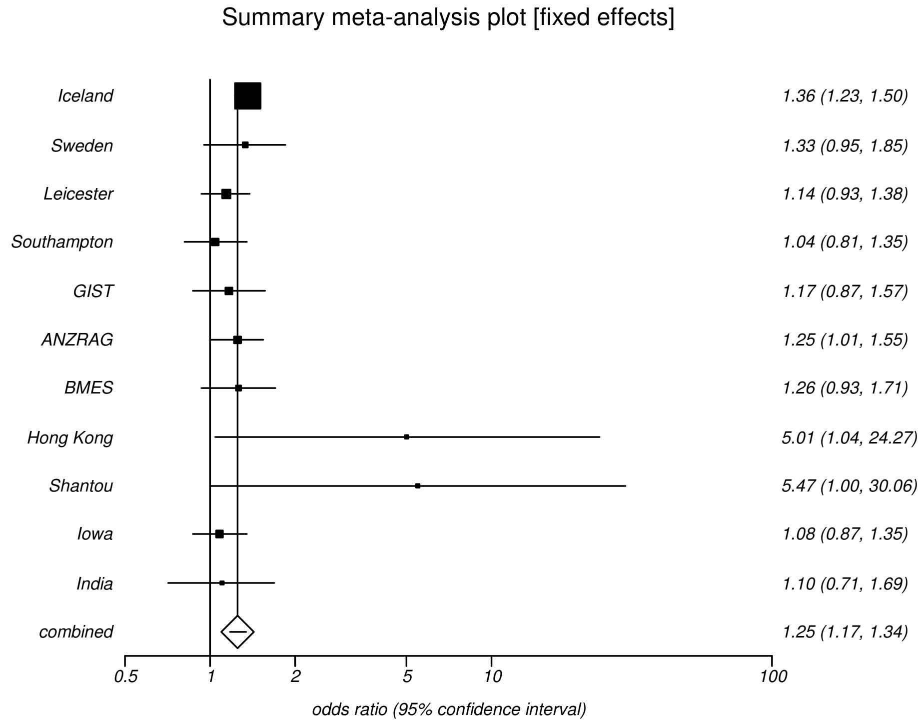


Figure 4.11. Summary of meta- analysis plot (fixed effects)

Stratification of studies based on ethnicity indicated that this variant was very rare in the Asian populations from China and India and the significance of the effect observed was based largely on the higher frequency of this variant within individuals of European descent, particularly from Iceland.

Thus the present study indicates a lack of involvement of variants on chromosome 7q31 in our cohort suggesting that these variants may not have a strong effect in primary glaucoma cases of Indian origin.

4.3.6. Analysis of *CAV1* and *CAV2* SNPs in PG and PDS

The two risk variants, rs1052990 and rs4236601 were screened in a cohort of 136 subjects which include 34 PDS, 44 PG and 58 normal subjects. The allele frequencies of both these variants are provided in Table 4.27. From the table it is evident that the allele frequencies of both the SNPs were not statistically significant either with PDS or PG. The risk variant of rs4236601 was not significantly associated either with PG (OR=

2.15, 95%CI, 0.88-5.96; p= 0.09) or PDS (OR=1.43, 95%CI, 0.52-3.91; p= 0.48). Similarly the other risk variant of rs1052990 was also not associated either with PG (OR=2.15, 95%CI, 0.88-5.22; p=0.09) or PDS (OR=1.69, 95%CI, 0.64-4.41; p value=0.28). The genotype frequencies of these variants were not associated with PG and PDS (Table 4.28).

Table 4.27. Allele frequency and association of rs1052990 and rs4236601 with PG and PDS

						PG Vs Control		PDS Vs Controls		PG+PDS Vs Controls	
SNP	**Risk allele**	**PG**	**PDS**	**PG+PDS**	**Normal**	**OR(95%CI)**	**p**	**OR(95%CI)**	**P**	**OR(95%CI)**	**p**
rs1052990	T	0.388	0.344	0.368	0.245	2.15 (0.88-5.22)	0.09	1.69 (0.64-4.41)	0.28	1.82(0.83-4.1)	0.13
rs4236601	A	0.381	0.306	0.349	0.231	2.15 (0.88-5.26)	0.09	1.43 (0.52-3.91)	0.48	1.83(0.81-4.06)	0.14

Table 4.28. Distribution of Genotype Frequencies and Their Odds Ratios for the rs1052990 and rs4236601 across POAG and PACG Cases in the Present Cohort

		Genotype frequency			PG vs Controls		PDS vs Controls	
SNP	**Genotype**	**PG**	**PDS**	**Controls**	**OR(95%CI)**	**P**	**OR(95%CI)**	**p**
rs1052990(T>G)	GG	0.175	0.124	0.073	3.5(0.89-13.7)	0.06	2.28(0.49-10.46)	0.28
	TG	0.425	0.438	0.345	1.78(0.73-4.34)	0.2	1.68(0.66-4.28)	0.27
	TT	0.4	0.438	0.582				
rs4236601(A>G)	AA	0.143	0.129	0.074	3.09(0.76-12.52)	0.1	2.06(0.45-9.32)	0.34
	GA	0.476	0.355	0.315	2.42(1-5.84)	0.05	1.33(0.51-3.50)	0.56
	GG	0.381	0.516	0.611				

4.3.7. Analysis of complement factor H (*CFH*) variant (Y402H; rs1061170, T > C)

Based on potential role of *CFH* in complement activation, we analyzed the rs1061170 variant in a cohort of 437 samples, which included 124 POAG, 94 PACG and 219 normal individuals. There was no significant difference between the cases and controls with respect to the allele (Table 4.29) and genotype frequencies (Table 4.30).

Table 4.29. Allele frequency and its association of rs1061170 between cases and controls

				POAG vs Controls		**PACG vs Controls**	
rs1061170	**POAG**	**PACG**	**Controls**	**OR (95%CI)**	**p value**	**OR (95%CI)**	**p value**
C	0.274	0.165	0.272	1.04 (0.63-1.71)	0.87	0.54 (0.29-1.00)	0.05

Table 4.30. Genotype frequency and its association of rs1061170 between cases and controls

				POAG vs Controls		PACG vs Controls	
rs1061170	**POAG**	**PACG**	**Controls**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**
TT	0.524	0.67	0.498	--	--	--	
TC	0.403	0.33	0.461	0.82 (0.48-1.40)	0.47	0.53 (0.319-0.882)	0.01
CC	0.073	0	0.041	2.01 (0.70-5.76)	0.18	--	--

4.3.8. Analysis of *FBLN5* SNPs in POAG

The two risk variants, which are located in *FBLN5* were shown to be significantly ssociated with POAG. After multiple corrections the SNPs rs2244158 ($p=5x10^{-3}$) and rs2160079 ($p=5x10^{-3}$) were shown to be significantly associated with POAG. The haplotypes generated with these risk variants statistically significant with POAG ($p= 2x10^{-4}$). The association of *FBLN5* variants was provided in table 4.31 and 4.32.

Table 4.31. Allele frequency and its association of *FBLN5* with POAG

S. No.	SNPs	Associated allele	Freq. Cases	Freq. Controls	Chi square	p value	p value (after correction)
1	rs929608	A	0.524	0.454	2.833	0.0923	
2	rs10484030	G	0.736	0.689	1.618	0.2033	
3	rs2244158	G	0.78	0.643	13.1	$3x10^{-4}$	0.005
4	rs2017488	T	0.886	0.88	0.048	0.8263	
5	rs741198	G	0.85	0.754	8.333	0.0039	0.0594
6	rs1861085	G	0.736	0.617	9.399	0.0022	0.0348
7	rs726063	A	0.047	0.031	1.005	0.3161	
8	rs2160079	C	0.783	0.649	12.885	$3x10^{-4}$	0.0054
9	rs2160080	A	0.906	0.903	0.012	0.913	
10	rs12589592	A	0.256	0.2	2.651	0.1035	
11	rs2284340	A	0.291	0.223	3.665	0.0556	
12	rs2246416	A	0.65	0.571	3.762	0.0524	
13	rs2268002	C	0.701	0.606	5.815	0.0159	0.2537
14	rs2474028	A	0.382	0.309	3.529	0.0603	
15	rs3783937	T	0.323	0.243	4.706	0.0301	
16	rs12432450	A	0.35	0.349	0.002	0.963	
17	rs3814835	T	0.913	0.909	0.042	0.8378	
18	rs2430378	T	0.059	0.051	0.166	0.6839	

Table 4.32. Distribution of haplotype frequencies of *FBLN5* SNPs in POAG and normal controls in the Indian cohort

Haplotype Associations	Overall Freq.	%Cases	%Controls	chi square	p value	p value (after correction)
Block 1						
G-C	69.9	78	64	13.611	$2x10^{-4}$	0.0009
A-A	29.3	21.7	34.9	12.385	$4x10^{-4}$	0.0019
Block 2						
G-G-G	39.3	34.8	42.6	3.716	0.0539	
G-G-A	34.7	34.9	34.5	0.006	0.9383	
A-A-A	21.5	24.4	19.4	2.165	0.1412	
G-A-A	3.7	4.8	2.9	1.448	0.2288	
Block 3						
G-C	65.2	61	68.2	3.429	0.0641	
A-T	26.8	31.4	23.4	4.876	0.0272	
A-C	7.2	6.8	7.5	0.117	0.7328	

4.4. Functional analysis of *CFH* and *FBLN5*

The results of the association were confirmed by functional analysis. The expression of *CFH* was analyzed in serum samples from cases and controls carrying the Y402H risk genotype. The *FBLN5* was analyzed in aqueous humor samples from cases and controls by using immunoblotting. Further, the expression of *FBLN5* in the eye was demonstrated by immunohistochemistry.

4.4.1. Functional analysis of *CFH*

To determine whether the *CFH* (Y402H) polymorphism influenced function, the serum samples from 3 POAG patients and 3 controls homozygous for Y402H (CC) genotype were analyzed by immunoblotting using mouse monoclonal Abs directed against CFH. The Y402H SNP lies in the short consensus repeat 7 (SCR7) of *CFH* that has a binding site for C reactive protein (*CRP*) and is known to possibly affect the *CFH-CRP*

interactions, thus altering the levels of CRP (Giannakis *et al*., 2003). Similarly the expression of *CRP* was also studied in the serum of POAG cases and controls with the risk genotype (CC) by using immunoblotting and ELISA.

There was a similar expression of the CFH protein in the sera of patients and controls (Figure 4.12). There was no difference in the expression of *CRP* in patients and controls carrying *CFH* risk genotype (Figure 4.13). A similar expression of CFH was observed in POAG and controls carrying the risk genotype as evident from ELISA (Figure 4.14).

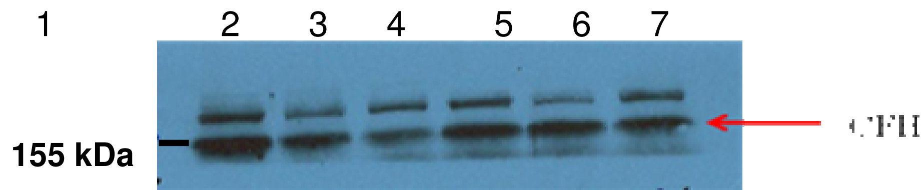


Figure 4.12. Immuno blotting analysis of CFH in human POAG and control serum samples
1st well marker, 2nd well C1, 3r well C2, 4th well C3, 5th well P1, 6th well P2 and 7th well P3

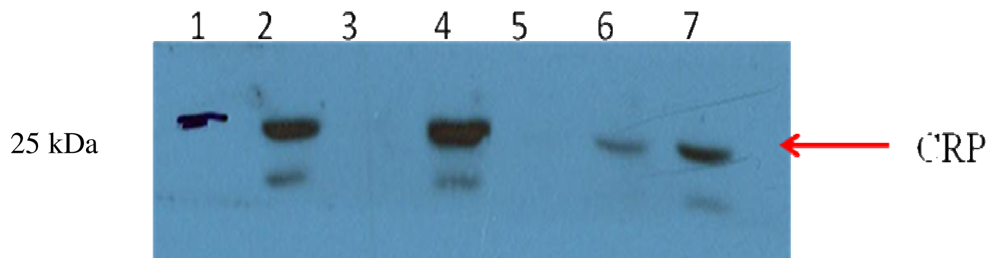


Figure 4.13. Immuno blotting analysis of CRP in human POAG and control serum samples
1st well marker, 2nd well C1, 3r well C2, 4th well C3, 5th well P1, 6th well P2 and 7th well P3

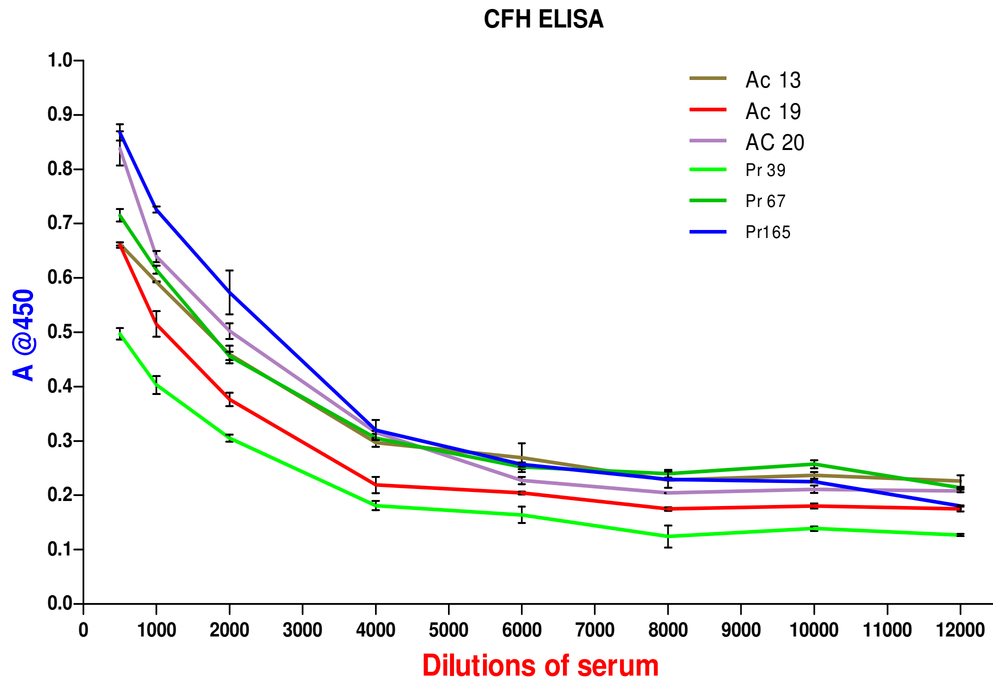


Figure 4.14. Analysis of CFH in different dilutions of serum samples by ELISA in patients and controls carrying the risk genotype. (Serum dilutions 1:500, 1:1000, 1:2000, 1:4000, 1:6000, 1:8000, 1:10,000 and 1:12000 with 1X PBS)

4.4.2. Analysis of *FBLN5* in aqueous humor by immunoblotting

Since variants in the *FBLN5* were significantly associated with POAG, we compared the expression of *FBLN5* in the aqueous humor of POAG and normal cataract subjects. Western blot analyses were performed on equal volumes (20 µL) of aqueous humor from three controls and three POAG on the same gel. We detected the expression of FBLN5 in aqueous humor of controls as well as in POAG samples. We observed two bands at 64kDa and at 54kDa. Fibulin 5 is a glycoprotein present as glycosylated and non glycosylated forms. The non glycosylated form observed at 54 kDa had a similar expression in both cases and controls. The glycosylated form at 64 kDa was relatively more intense in POAG than normal individuals (Figure 4.15).

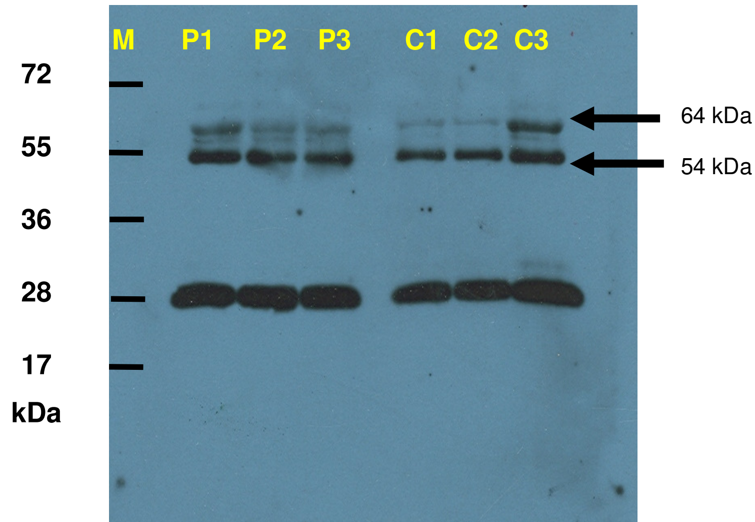


Figure 4.15. Western blot analysis for FBLN5 in aqueous humor of POAG and normal subjects. 1st well Marker, 2nd well no sample, 3rd well P1, 4th well P2, 5th well P3, 6th well no sample, 7th well C1, 8th well C2, 9th well C3. The marker (SM≠1811) (corresponds to 72, 55, 36, 28, 17 kDa). Upper band (64 kDa) corresponds to glycosylated form and other one (54 kDa) unglycosylated form

4.4.3. Immunohistochemical localization of FBLN5 in the eye

IHC was performed to examine the expression of *FBLN5* in human eye and the expression was demonstrated in cornea, TM and retina. In the normal eye we found the strong expression of *FBLN5* in the corneal epithelium and no positive reaction was observed in the stroma and endothelium (Figures 4.16 a, b).

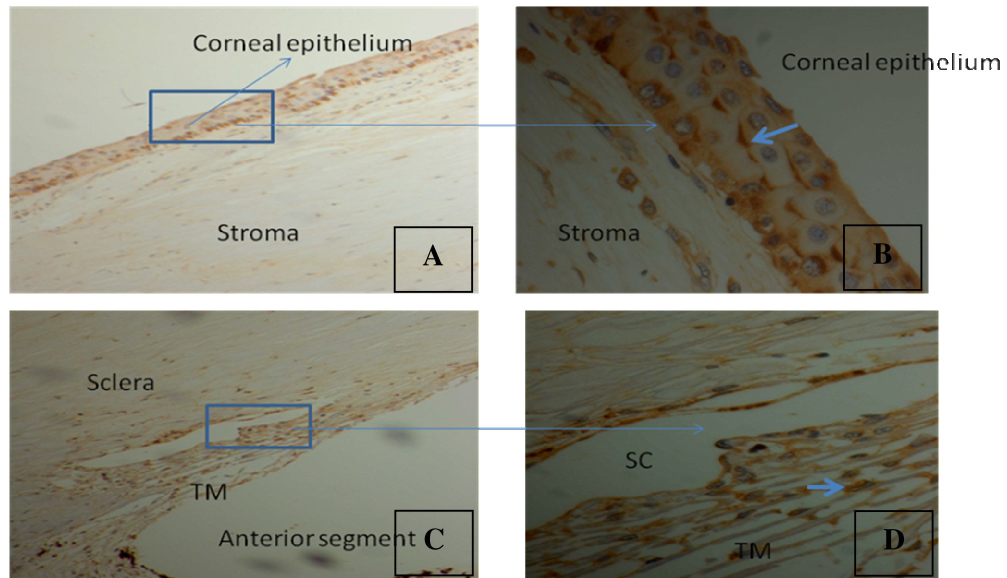


Figure 4.16. Immuno histochemical analysis of *FBL5* in human corneal epithelium (A, B) and TM region adjoining Schelm's canal (C, D). Arrows indicate the site of expression.

In the anterior segment we observed moderate to strong expression for FBLN5 in the TM. We also observed the expression of FBLN5 in endothelial cells of schlems canal (Figures 4.16 c and d). In the retina, we observed a weak expression of FBLN5 in RGC and retinal pigment epithelial cells (Figure 4.17).

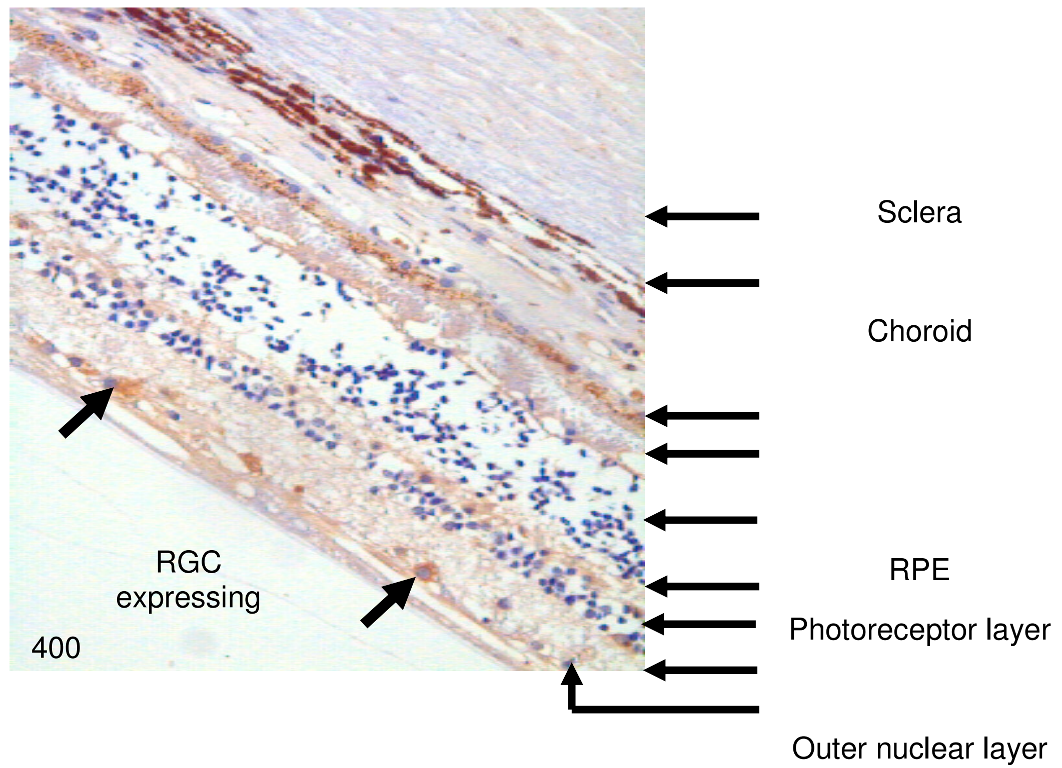


Figure 4.17. Immunohistochemical analysis of *FBLN5* in human retina (arrows indicate the site of expression)

CHAPTER 5: DISCUSSION

POAG is a complex disease attributed to multiple genes with varying magnitude of effects. Several chromosomal loci that have been mapped by linkage analysis have resulted in the identification of a few genes that do not contribute to a large proportion of POAG cases. The genes identified by case control based association studies are still in their infancy and await to be validated across multiple populations and their involvement in POAG is yet unclear (Rao *et al.,* 2011; Fan *et al.,* 2010). Expression studies and animal models have suggested several genes, which are supposed to be candidates for the regulation of IOP and RGC death (Borras *et al.,* 2003; Wang *et al.,* 2010). Although GWAS have revealed several gene variants to be associated with POAG in different populations (Thorleifsson *et al.,* 2007, Nakano *et al.,* 2009; Thorleifsson *et al.,* 2010, Burdon *et al.,* 2011), their involvement would be biologically more meaningful if they are replicated across different populations, as it helps to understand their potential role in the disease pathogenesis. Understanding the genetic basis of POAG would be helpful both in the diagnosis and management of this disease. In this context we attempted to understand the role of candidate genes like *CTGF, BAX, mtND1* and *NTF4* and the recently identified genetic variants in order to validate their involvement in IOP related primary glaucomas (POAG and PACG) in an Indian population. We also evaluated the role of an ECM related gene in POAG that was further confirmed by functional analysis.

Earlier studies have demonstrated that regulation of *CTGF* expression in tissues involved in the outflow pathway may play a crucial role by modulating the ECM production in the TM, thereby regulating IOP (Chudgar *et al.,* 2006). Recently study by

Junglas *et al*., 2012, has shown that over expression of *CTGF* in mice eyes lead to increased IOP and ONH degeneration. To understand the role of *CTGF*, we screened this gene in a cohort of IOP related primary glaucomas (POAG and PACG). We observed three novel variants in coding region (R22P, P36S, P102S), along with an intronic variation (g.325G>A) and two polymorphisms in the promoter region (g.-444G>C, g.-251delA). Except for the R22P and P102S, all other variations were observed in equal frequencies amongst the POAG cases and normal controls (Table 4.2). Genotype-phenotype correlation indicated a severity of the phenotype in probands harboring the R22P and P102S variations, who presented with juvenile onset POAG along with absolute cupping of the optic discs (> 0.8:1 in both eyes). *CTGF* is involved in multiple physiological pathways and is a highly expressed gene in the TM and regulates the synthesis of several ECM molecules (Blom *et al.,* 2002; Tomarev *et al.,* 2003). Elevated levels of *CTGF* have been observed in various fibrotic disorders and also in aqueous humor of POAG patients, wherein, it affects the ECM metabolism by directly promoting collagen and fibronectin synthesis (Iman *et al.,* 2008, Junglas *et al.,* 2009).

Although *CTGF* was shown to play a major role in the regulation IOP *in vitro* (Chudgar *et al.,* 2006) and *in vivo* (Junglas *et al*., 2012) , the present study indicated that *CTGF* variations were unlikely to be involved as a major candidate as it exhibited a limited involvement in POAG. To the best of our knowledge, this is the first study to observe the role of *CTGF* in POAG and PACG.

Recently, in the European population, it was demonstrated that heterozygous mutations in *NTF4* on 19q13.3 were involved in POAG (Pasutto *et al.,* 2009). In an effort to replicate these findings we screened entire coding regions of *NTF4* in a cohort

of POAG and PACG. Although 5 variations were observed that included a nonsynonymous change (A88V), a silent variation (P151P), two 3’ UTR variations and a known polymorphism (rs11669977) in case of POAG and A88V, rs11669977 in case of PACG, none of these were associated either with POAG or PACG (Rao *et al.,* 2010). Our results were similar to another study on a Caucasian POAG cohort from the southeastern United States that exhibited nine nonsynonymous variations in *NTF4,* including two common variants observed in the European cohort (A88V and R206W), with none of them being associated to POAG (Liu *et al.,* 2010). Similarly, another study in a Chinese population did not observe these reported mutations in their cohort (Vithana *et al.,* 2010). Interestingly, the more predominant mutation A88V in the European population, was seen to be more common among the normal controls (4.91%) compared to POAG (2.14%; *P* exact = 0.200) and PACG (2.85%; *P* exact = 0.577) in the present study (Table 5.1). In the European population the A88V was observed in five POAG patients, of whom four had NTG, while in the Indian scenario, we observed A88V both in POAG and PACG cases with raised IOP as well as in the normal controls with no signs or symptoms of glaucoma at presentation.

Table 5.1. Distribution of A88V variation in different populations

Population	POAG	Normal individuals	P value	Reference
European	5/892 (0.56%, 95% CI, 0.24–1.30)	0/895 0 (95% CI, 0–0.43)	--	Pasutto *et al.,* 2009
American	1/443 (0.22%, 95% CI, 0.04–1.27)	5/533 (0.94%, 95% CI, 0.40–2.18)	0.097	Liu *et al.,* 2010
Chinese	0/174	0/91	--	Vithana *et al.,* 2010
Indian	3/140 (2.14%, 95% CI, 0.73–6.11)	14/285 (4.91%, 95% CI, 2.95–8.07)	0.200	Present study

Our present data indicated that non-synonymous coding changes in *NTF4* were not associated either with POAG or PACG. The differences in our findings compared to the European population could be attributed to different genetic profiles between Indian and European populations as A88V was observed more in our normal population (4.91%) compared to the Europeans (0%) (Table 5.1). This could also be due to the clinical heterogeneity between the Indian and European cohorts as most of the mutations in European population were observed in NTG but none of our cases had NTG. However, these differences could not be due to the screening techniques since our primers and sequencing methods were identical to the European study. Although A88V alters the neurotrophin signaling by reducing the binding of *NTF4* to TrkB receptor, the genetic data in the present study suggested that this variant might not play a major role in *in vivo*.

Wide spectrums of human diseases are associated with variations in the mitochondrial DNA (mtDNA) (Zeviani *et al.,* 1991). Recent studies have shown that mitochondrial abnormalities are associated with elevated IOP and visual field loss (Izzotti *et al.,* 2011). As nuclear genetic abnormalities were shown to be associated with only a small fraction of POAG patients, we investigated the possible link between *mtND1* and POAG. Screening of *mtND1* revealed a total of 51 nucleotide changes of which 13 (25.4%) were nonsynonymous and 38 (74.6%) were synonymous changes in POAG and normal controls. Of the 13 nonsynonymous changes, eight were exclusively present in 8.1% of POAG patients and the remaining five were detected both in POAG (9.8%) and normal subjects (8.8%). Except the M31V, all other variants were non pathogenic. The M31V was previously reported in Alzheimer's and Parkinson's disease (Shoffner *et al.,* 1993). Interestingly, the previous studies did not observe these

variations in either POAG or PACG (Abu-Amero *et al.,* 2006; Abu-Amero *et al.,* 2007) except for the A4T and Y304H that was previously reported in PCG (Tanwar *et al.,* 2010). Neither the LHON mutations were observed in the present study. It has been demonstrated through *in vitro* experiments that mutations in *mtND1* functionally interacts with *BAX* and induces cell death (Perier *et al.,* 2005). But, we did not find any pathogenic mutations in the present study. Thus the role of *mtND1* and other mitochondrial genes still remain to be explored in POAG.

As *BAX* is a major candidate gene for RGC death and IOP regulation, we investigated the role of *BAX* in POAG. A single missense (G39W) and two synonymous variations (D98D, V111V) were observed, but none of them were associated with POAG. We also observed four intronic variations but none of these were statistically associated with POAG. Animal models have demonstrated that RGC death is mediated by *Bax* dependent apoptotic pathway. *Bax* null ($Bax^{-/-}$) mice and heterozygous ($Bax^{+/-}$) mice exhibited reduced IOP and RGC death indicating that *BAX* could be candidate for RGC death (Libby *et al.,* 2005). Although *BAX* is a candidate gene for IOP regulation and RGC death in mice model, the results of the present study indicated that *BAX* was not a major candidate for POAG in humans. To the best of our knowledge, this is also the first study to observe variations in the *BAX* gene in POAG.

The association of *LOXL1* has been established in multiple populations with XFS/XFG. Although POAG and XFG share similar clinical features with glaucomatous optic neuropathy, the XFG associated intronic variant rs2165241 was weakly associated with POAG in the Icelandic population (Thorleifsson *et al.,* 2007). The intronic variant rs2165241, which was associated with POAG (P=0.04) in Icelandic population was not

associated either with POAG (OR=0.95, 95%CI, 0.54-1.67, p=0.426) or PACG (OR=0.82, 95%CI 0.45-1.50, p=0.262). We found that all the three XFS/XFG associated variants were not involved with either POAG or PACG in the present study (Chakrabarti *et al.,* 2008).

However, the risk allele frequencies of rs2165241 (0.321), rs1048661 (0.616) and rs3825942 (0.830) were similar to POAG in populations of Iceland and Sweden. Neither the genotype frequencies for these variants were significantly associated with either POAG or PACG. This lack of association was further confirmed by haplotype analysis, wherein, the risk haplotype (G-G) generated with rs1048661 and rs3825942 in XFG in previous studies had a similar frequency in POAG, PACG and control subjects. In contrast to the previous studies, frequency of the risk haplotype (T-G) was more in POAG and PACG than normal controls, but none of them were statistically significant. This was perhaps the first study other than Iceland population to screen *LOXL1* variants in POAG and PACG. Later, these variants were screened in Caucasian, Japanese, Saudi Arabian, and Chinese populations with POAG, which further confirmed the lack of *LOXL1* association with POAG (Table 5.2 and 5.3). Recently a meta-analysis was performed to understand the involvement of these variants in POAG across multiple populations (Chen *et al.,* 2010). No significant association was observed for these variants with POAG. The pooled OR was 0.93, (95%CI, 0.84-1.03, p= 0.16) for the ‘G’ allele of rs1048661, 1.06, (95%CI, 0.94-1.19, p= 0.34) for the ‘G’ allele of rs3825942 and 0.95 (95%CI 0.82-1.10, p= 0.50) for the ‘T’ allele of rs2165241. The present data in conjunction with other studies indicated that *LOXL1* variants were not associated with primary glaucomas. This lack of association indicated that these are more complex disorders and *LOXL1* could be involved in XFG but did not have a direct role in IOP

associated primary glaucomas.

Table 5.2. Distribution of Allele Frequencies and Their odds Ratios for the *LOXL1* SNPs across POAG Cases of different populations

Population	Phenotype	SNP	Allele frequency	OR (95%CI)	p Value	Reference
Iceland	POAG	rs1048661	0.711	1.32 (0.96–1.82)	0.085	Thorleifsson *et al.,* 2007
	POAG	rs3825942	0.872	1.25 (0.81–1.91)	0.32	
	POAG	rs2165241	0.550	1.36 (1.01–1.83)	0.04	
Sweden	POAG	rs1048661	0.638	0.82 (0.61–1.10)	0.19	
	POAG	rs3825942	0.863	0.87 (0.57–1.31)	0.49	
	POAG	rs2165241	0.488	0.83 (0.63–1.09)	0.18	
India	POAG	rs1048661	0.616	0.70 (0.40–1.24)	0.112	Chakrabarti *et al*., 2008
	POAG	rs3825942	0.83	1.53 (0.78–2.98)	0.105	
	POAG	rs2165241	0.321	0.95	0.426	

				(0.54–1.67)		
Caucasian	POAG	rs1048661	0.724	1.02 (0.70, 1.51)	0.92	Fan *et al.*, 2008
	POAG	rs3825942	0.771	0.86 (0.57, 1.30)	0.54	
	POAG	rs2165241	0.412	0.83 (0.59, 1.18)	0.33	
China	POAG	rs1048661	0.42	NA	0.084	Gong *et al.*, 2008
	POAG	rs3825942	0.894	NA	0.35	
	POAG	rs2165241	0.084	NA	0.3	
Caucasian	POAG	rs3825942	0.829	NA	0.583	Liu *et al.*, 2008
	POAG	rs2165241	0.424	NA	0.056	
African American	POAG	rs3825942	0.617	NA	0.591	
	POAG	rs2165241	0.237	NA	0.408	
Ghana	POAG	rs3825942	0.622	NA	0.217	
	POAG	rs2165241	0.226	NA	0.472	
Japan	POAG	rs1048661	0.472	NA	0.57	Mabuchi *et al.*, 2008
	POAG	rs3825942	0.85	NA	0.92	
Saudi Arabia	POAG	rs1048661	0.75	NA	0.866	Abu-Amero *et al.*, 2011
	POAG	rs3825942	0.844	NA	0.477	
	POAG	rs2165241	0.464	NA	0.176	

Table 5.3. Distribution of Estimated Haplotype Frequencies and Their Odds Ratios for the Two SNPs (rs1048661 and rs3825942) across POAG and PACG in the Present Cohort and Other Populations

		G-G Haplotype				T-G Haplotype			
Populations [n (Cases, Controls)]	Phenotype	% Cases	% Controls	OR (95% CI)*	P	% Cases	% Controls	OR (95% CI)*	p
Sweden [399, 198]	XFG	83.3	56.1	35.72†	2.2×10^{-16}	16.2	31.8	12.36†	1.6×10^{-6}
Iceland [195, 14474]	XFG	81.4	49.8	18.94†	3.3×10^{-12}	17.3	34.9	5.74†	0.0027
Iowa (USA) [72, 75	XFS	80.6	48	14.50†	2.7×10^{-5}	18.1	40	3.90†	0.12
Australia [86, 2422]	XFS	74	51	2.71 (1.91–3.92)	3.8×10^{-9}	22	34	0.54 (0.36–0.78)	7.8×10^{-4}
India [112, 105] Present study	POAG	45.2	46.1	1.45 (0.70–2.98)	0.158	37.8	29.9	1.88 (0.87–4.03)	0.052
India [96, 105] Present study	PACG	42.1	46	0.87 0.43–1.75)	0.346	33.4	30	1.07 (0.51–2.27)	0.424

The odds ratios were calculated with respect to G-A haplotype

†95%CI were not reported in the studies

Since, *LOXL1* variants were not associated with primary glaucomas, we further studied their involvement in PG/PDS in a Caucasian cohort to establish the specificity of their association. Similar to primary glaucomas, it was observed that the XFG associated *LOXL1* variants were not associated with PG/PDS (Rao *et al.,* 2008). This association was not significant either with the combined phenotype (PG+PDS) or after categorizing the data in to PG and PDS. The allele frequency for rs1048661 (0.674) and rs3825942 (0.852) was similar with primary glaucomas and the allele frequency of rs2165241 (0.514) was higher in PG/PDS than primary glaucomas (0.321 for POAG and 0.296 for PACG). There was no statistical difference for genotype frequencies for these three variants. Haplotype analysis also did not reveal any association with PG or PDS. The frequency of the risk haplotype (G-G) was higher in controls than PG/PDS but was not statistically significant (OR= 1.08, 95%CI, 0.59-1.97, p =0.643). Similar to the primary glaucomas, the T-G risk haplotype was observed more in cases (32.3%) than in controls (26.9%) and this was not statistically significant (OR= 1.35, 95%CI, 0.70-2.60, p= 0.266). Even though XFG and PG are classified under the category of secondary glaucomas, the present data indicated these two disorders were genetically different. Similar findings have been observed in European population and the allele frequencies of risk variants were similar to the present study but none of these were statistically significant (Wolf *et al.,* 2010).

This is perhaps the first study to screen *LOXL1* variants in PG, which indicated that *LOXL1* was specific to XFS/XFG and did not exhibit any involvement either with primary glaucomas (POAG and PACG) or secondary glaucomas (PG and PDS).

To identify specific associations of POAG susceptible loci with high pressure glaucomas, we tried to replicate six variants previously implicated in the Japanese

population, in our POAG and PACG cohorts. There was no significant association to any of these six variants with POAG and PACG in our study (Rao *et al.,* 2009). However, the risk allele frequencies of these variants were similar to POAG from Japanese population (Nakano *et al.,* 2009). Neither the genotype frequencies for these variants were significantly associated with POAG or PACG. This lack of association was further confirmed by haplotype analysis generated for four variations on chromosome 1 (rs547984, rs540782, rs693421 and rs2499601) and the frequencies of these haplotypes were similar in cases and normal individuals and these are not statistically significant. The discrepant of these findings between these two studies could be due to the stringency of our enrolment criteria that included only POAG and PACG cases with an elevated IOP unlike the Japanese cohort that had a combination of high and low pressure glaucomas. The mean age of our controls subjects (60.9 years) was much higher than the Japanese (53.6 years). Except for the rs2499601 (6.9%), the differences in the MAF of other variants was not more than 5% between cases and controls in the present study. It was also observed that the allele frequencies among normal controls in Japanese cohorts were much higher (>10%) than controls from India and it could be due to different genetic profile between Indian and Japanese population.

The lack of association could not be due to population stratification, because the cases and controls were collected from the same ethnic back ground (all the subjects are from south India). These differences could not be due to experimental errors because the screening was done by resequencing. This lack of association was further supported by a recent study on a population from Iceland (Table 5.4, Thorleifsson *et al.,* 2010). Overall these observations indicated that the associations of these variants were not an universal phenomenon.

Table 5.4. The distribution of three chromosomal loci implicated in the Japanese population in the Indian POAG and PACG cohort

			Japanese population		Indian population		Icelandic population	
dbSNP ID	**Chr**	**Nearest gene**	**OR (95%CI)**	**p value**	**OR(95%CI)**	**p value**	**OR†**	**p value**
rs547984	1	*ZP4*	1.34 (1.16–1.54)	0.00006	1.29 (0.84-2.00)	0.437	1.06	0.23
rs540782	1	*ZP4*	1.34 (1.16–1.54)	0.00006	1.24 (0.80-1.92)	0.443	1.06	0.19
rs693421	1	*ZP4*	1.35 (1.17–1.56)	0.00004	1.23 (0.79-1.91)	0.616	1.06	0.19
rs2499601	1	*ZP4*	1.33 (1.15–1.53)	0.00009	1.31 (0.86-2.02)	0.286	1.02	0.68
rs7081455	10	*PLXDC2*	1.49 (1.25–1.77)	0.00001	1.06 (0.69-1.64)	0.629	1.06	0.18
rs7961953	12	*TMTC2*	1.37 (1.18–1.61)	0.00007	1.25 (0.72-2.14)	0.251	1	0.95

†95% CIs were not reported in this study

The haplotypes generated with four variants on chromosome 1 did not reveal any association with either POAG or PACG. The frequencies of haplotypes were similar in cases and controls and were not statistically significant.

Continuing with our replication studies, we examined the role of variations in *VAV2* and *VAV3* in POAG and PACG cases from Indian population. The risk allele rs2156323 (*VAV2*) was not associated with either POAG (OR=1.34, 95% CI, 0.62-2.85; p=0.533) or PACG (OR=1.08, 95%CI, 0.49-2.34; p=0.223) in the present study (Rao *et al.,* 2010). Similarly, the risk variant in *VAV3* (rs2801219) was also not associated with either POAG (OR=1.30, 95%CI, 0.75-2.25; p=0.133) or PACG (OR=1.46, 95% CI, 0.82-2.59; p=0.394). The relative risks for the heterozygote genotypes of rs2156323 (OR_{Het} = 0.84, 95% CI, 0.45–1.56) and rs2801219 (OR_{Het}=1.18, 95% CI, 0.68 –2.05) were lower in the Indian cohort compared to the Japanese POAG cases where the

heterozygous genotypes for rs2156323 (OR $_{Het}$, 5.65 (95%CI 1.99-16), p=4.38X10^{-4}) and rs2801219 (OR $_{Het,}$ 2.03 (1.01-4.09), p=5.42X10^{-4}) (Fujikawa *et al.,* 2010). The haplotype analysis also did not reveal any association with either POAG or PACG. The frequencies of haplotypes were similar in cases and controls and were not statistically significant. The lack of association of these variants in our cohort could be due to clinical heterogeneity (as present had raised IOP) or due to different genetic profile between these populations.

However, the present study was performed by screening POAG associated alleles within a specific region, which may not completely rule out the role of *VAV2* and *VAV3* in POAG. *VAV2* and *VAV3* deficient mice exhibited buphthalmos, along with iridocorneal changes, elevated IOP and ONH cupping and these features are similar to the developmental glaucomas indicating that these genes could be a candidates for PCG (Fujikawa *et al.,* 2010).

A recent study by Thorleifsson *et al.,* 2010 identified several variants located on 7q31 of which, the rs4236601 (OR=1.364, p=5.0×10^{-10}) and rs1052990 (OR=1.319, p=1.1X10^{-9}) were significantly associated with POAG. These variants located near *CAV1* and *CAV2* were thought to be involved in POAG by interacting with *TGFβ* and *eNOS* signaling (Garcia-Cardena *et al.,* 1997; Razani *et al.,* 2001). However, data from the present study indicated that the seven SNPs which were associated with POAG in the Iceland population were not associated with either POAG or PACG. There was no statistically significant difference in the allele frequencies between POAG or PACG and the normal controls. The allele frequency for rs4236601 was 18.7% in POAG and 16.6% in the normal controls and this was lower as compared to Iceland, European and Australian population, while it was much higher than the Chinese population but was

not statistically significant (OR= 1.10, 95%CI, 0.71-1.69; p=0.52) in comparison with previous study (OR=1.364, p=5.0×10^{-10} [Table 5.5]) . Neither the genotype frequencies for these variants were significantly associated with either POAG or PACG. This lack of association was further confirmed by haplotype analysis, where in the haplotypes generated with risk variants had a similar frequencies in POAG, PACG and normal controls. Recently, screening of these variants in Caucasian populations revealed that rs1052990 was marginally associated with NTG (OR= 1.27, p= 0.0392), but not the rs4236601 variant (Wiggs *et al.,* 2011 [Table 5.5]).

Table 5.5. Distribution of allele frequencies of rs4236601 with POAG in different populations

Population	Allele frequency	OR (95%CI)	p Value	References
Iceland	0.287	1.36 (1.23–1.50)	5.0×10^{-10}	Thorleifsson *et al.,* 2010
Sweden	0.258	1.33 (0.95–1.85)	0.092	
Leicester, UK	0.293	1.14 (1.93–1.38)	0.2	
Southampton, UK	0.29	1.04 (0.81–1.35)	0.75	
Australia	0.3	1.23 (1.06–1.43)	0.0063	
China	0.018	3.33 (1.56–7.08)	0.003	
USA	0.285	NA	0.5	Kuehn *et al.,* 2011
USA	NA	1.14	0.2639	Wiggs *et al.,* 2011
Australians of European descent	0.290	1.07 (NA)	0.17	Burdon *et al.,* 2011
India	0.187	1.10(0.71-1.69)	0.52	Present study

NA: Not available

Since the association of rs4236601 varied between different ethnic groups we performed meta-analysis on these 11 cohorts comprising 4429 cases and 38,015

controls. There was moderate evidence for association between rs4236601 and POAG and the pooled estimate of the odds ratios (OR=1.22) for the risk allele had a very narrow confidence interval (95%CI, 1.11-1.34). Majority of the cohorts including the present one clustered around the pooled estimate with overlapping confidence intervals.

However, there was a marked degree of heterogeneity as the Asian cohorts from Hong Kong and Shantou clustered further away from the pooled estimate with wide confidence intervals. Additionally, the lower limit of the confidence interval was less than 1 in cohorts from Iowa, Sweden, UK (Leicester and Southampton), Australia (BMES and GIST) and India (Figure 4.10 and 4.11).

Stratification of studies on the basis of ethnicity indicated that this variant is very rare in Asian populations from China and India and the significance of the effect observed is based largely on the high frequency of this variant within individuals of European descent, particularly from Iceland. Thus the present study indicated a lack of involvement of SNPs on chromosome 7q31 in our cohort suggesting that these variants may not have a strong effect in primary glaucoma cases of Indian origin.

These variants were also analyzed in secondary glaucomas like PG and PDS. An earlier study by Andersen *et al.,* 1997 had demonstrated that the gene responsible for PG could be located on 7q35-q36. As *CAV1* and *CAV2* were localized on 7q31.2 and were strongly associated with POAG and as there are similarities in the clinical features between PG and POAG, we hypothesized that there may be common genetic factors in these two phenotypes. Hence, we tried to investigate the involvement of *CAV1* and *CAV2* variants in PG and PDS. Similar to the primary glaucomas, *CAV1* and *CAV2* variants were not associated with PG/PDS. The association was neither significant with the combined phenotype (PG+PDS) nor after categorizing the data into PG and PDS.

The allele frequency of rs4236601 was higher in PG (0.388) and PDS (0.344) compared to POAG from different population (Thorleifsson *et al.,* 2010). But there was no significant association either with PG (OR= 2.15, 95%CI 0.88-5.22; p=0.09) or PDS (OR= 1.69, 95%CI 0.64-4.41; p=0.28). Similarly the rs1052990 was higher in PG and PDS but was not statistically significant. Neither the genotypes nor haplotypes were associated with PG or PDS indicating that POAG and these two disorders have different genetic etiologies.

Increasing evidences have suggested that abnormal activation of the complement system may be involved in POAG (Ren *et al.,* 2010). Abnormal activation of complement system is well established in AMD and the coding polymorphism (rs1061170, T > C) in exon 9 of *CFH,* which results in the substitution of Tyrosine by Histidine at 402 amino acid position (Y402H) was shown to increased the risk of AMD in different ethnic groups (Klein *et al.,* 2005 and Edwards *et al.,* 2005). Recent studies have shown the activation of the complement system and down regulation of *CFH* in POAG patients (Khalyfa *et al.,* 2007; Tezel *et al.,* 2010). However, the present study showed that the Y402H variant was not associated with POAG in the Indian population. The risk genotype and allele frequencies were similar in both POAG and normal controls. Functionally we also showed that the expression of *CFH* was similar in POAG and normal individuals harboring risk genotype (CC). Since, the Y402H in *CFH* alters its interaction with CRP (Johnson *et al.,* 2006) we also analyzed the expression of *CRP* levels in patients and controls carrying *CFH* risk genotype. However, we did not find any difference in the levels of *CRP* in cases and controls. Overall, the present study showed that the risk variant rs1061170 in *CFH* was not involved in POAG.

Since upregulation of several ECM molecules have been observed in the TM of POAG patients, we also analyzed genetic variations in ECM-related genes and observed that variants in fibulin 5 (*FBLN5)* were strongly associated with POAG (rs2244158 and rs2160079). *FBLN5* is a calcium-binding glycoprotein and it is critical for assembly of elastic fibers. Mutations in fibulin 5 have been shown to cause cutis laxa and have been associated with AMD (Loeys *et al.,* 2002; Stone *et al.,* 2004). Functional analysis showed that *FBLN5* was expressed in TM, retina and cornea indicating that this gene may have a pathogenic role in POAG.

Several recent findings indicated that fibulins are involved in human disease (Argraves *et al.,* 2003). The exact mechanism by which fibulin-5 mutations are responsible for human diseases was not understood. Knockout experiments have demonstrated that *FBLN5* is involved in elastic fibre assembly (Nakamura *et al.,* 2002). Mice deficient in the expression of fibulin 5, are viable but showed the symptoms of defective elastic fibre formation, including a tortuous aorta, severe emphysema and loose skin. Elastin is a major component of the ECM of the lamina cribrosa in the ONH in humans and disruption of elastin formation in ONH could lead to greater susceptibility of neurons to elevated IOP (Hernandez *et al.,* 2000). *FBLN5* also bind to superoxide dismutase and variations in *FBLN5* may inhibit these interactions leading to increased oxidative damage (Nguyen *et al.,* 2004). Oxidative stress is one of the mechanisms for IOP elevation and RGC death in POAG and this further supports the role of *FBLN5* in POAG (Izzotti *et al.,* 2011). We have demonstrated the expression of *FBLN5* in RGC and TM indicating that this protein could have important role in regulation of IOP and RGC death. Recent reports have shown that abnormal accumulation of ECM in TM could be an important mechanism for IOP elevation (Tamm *et al.,* 2007). *FBLN5* is involved in proper elastin formation and variations

could lead to aggregation of this elastin in TM that may block its channels thereby contributing to elevated IOP. Recently, it has been shown that overexpression of FBLN5 in RPE cells inhibited cell proliferation and migration and downregulated the expressions of VEGF, CXCR4, and TGFB1 in cocultured choroidal endothelial cells (Li *et al*., 2012). This data however needs further replication in other populations.

The data obtained from the current study provided information on the role of different candidate genes (*CTGF, NTF4, mtND1, BAX* and *FBLN5*) and the status of possible involvements of the recently identified genetic variations in IOP related glaucomas in Indian population. The results indicated lack of associations with the previously identified genetic variations indicating the genetic heterogeneity of POAG. Further, we were unable to find any significant associations of candidate genes indicating the underlying complex molecular mechanisms involved with IOP regulation in POAG. The gene variants identified through GWAS could not be replicated in our POAG cohort indicating the clinical and genetic heterogeneity of the disease. However, we found a significant association of variants in an ECM related gene (*FBLN5*) whose potential role was further understood by functional analysis. The findings in this present study demonstrate the involvement of the previously identified genes and gene variants in the Indian scenario. It was observed that majority of these genes are either not genetically involved with POAG and PACG or may have a moderate effect that could not be captured with our existing sample size. Nevertheless, these results provide a better understanding of the different pathways, which are involved in IOP associated glaucomas and may further help in designing reliable molecular diagnostics for predictive testing in future.

CHAPTER 6: CONCLUSIONS OF THE PRESENT STUDY

The present study indicated that *CTGF* variations were unlikely to be involved as a major candidate as it exhibited a limited involvement in POAG. Our present data indicated that non-synonymous coding changes in *NTF4* were not associated either with POAG or PACG. Although *MTND1* and *BAX* are candidate genes for IOP regulation and RGC death in mice model, the results of the present study indicated that *MTND1* and *BAX* were not a major candidate for POAG in humans. We found that all the three XFS/XFG associated *LOXL1* variants were not involve with either POAG or PACG and PG and PDS in the present study. There was no significant association to any of the six GWAS variants in Japanese with POAG and PACG in our study. The risk allele rs2156323 (*VAV2*) and *VAV3* (rs2801219) were not associated with either POAG or PACG. Data from the present study indicated that the seven SNPs located near to *CAV1* and *CAV2* which were associated with POAG in the Iceland population were not associated with either POAG or PACG. The present study showed that the CFH (Y402H) variant was not associated with POAG in the Indian population. We observed that variants in fibulin 5 (*FBLN5)* were strongly associated with POAG (rs2244158 and rs2160079). Functional analysis showed that *FBLN5* was expressed in aqueous humor, TM, retina and cornea indicating that this gene may have a pathogenic role in POAG. These results provide a better understanding of the different pathways, which are involved in IOP associated glaucomas and may further help in designing reliable molecular diagnostics for predictive testing in the Indian scenario.

CHAPTER 7: SPECIFIC CONTRIBUTIONS OF THE STUDY

The present study was conducted to identify genes which are involved in POAG, which would further helps in better understanding of the disease pathogenesis and help in better management. We have screened candidate genes and validated the role of recently identified variations in POAG and PACG in Indian population. The results of the present study suggested the lack of involvement of novel candidate genes *CTGF* and *BAX* in POAG in Indian population. *In vitro* studies have shown that *CTGF* is a major regulator of ECM synthesis in TM and thereby it regulates IOP. However, the present data demonstrated that *CTGF* could not play role in POAG and this data suggesting that *in vitro* system may not correlates with what occurs in *in vivo* system. Animal model has shown that *BAX* deficiency protected RGC and limits the IOP elevation, suggesting *BAX* could be a candidate gene for glaucoma. However, we did not find any pathogenic mutation in *BAX* in the present study indicating there may be differences in drainage structures of eye among animals and humans. The lack of association of *NTF4* with POAG in Indian population suggesting the clinical heterogeneity of the cases between Indian and European population and it could be due to different genetic profile. Another important observation in present study is that *NTF4* variants are present more in control subjects suggesting that these variations do not have a major functional role *in vivo*. The lack of association of recently identified genetic variants in Indian population suggesting that clinical and genetic heterogeneity of POAG. We observed that variants in a novel gene fibulin 5 (*FBLN5)* were strongly associated with POAG. Functional analysis showed that *FBLN5* was expressed in aqueous humor, TM, retina and cornea indicating that this gene may have a pathogenic

role in POAG. These results provide a better understanding of the different pathways, which are involved in IOP associated glaucomas and may further help in designing reliable molecular diagnostics for predictive testing in the Indian scenario. However, similar other studies are warranted to substantiate the findings of the present study.

CHAPTER 8: LIMITATIONS OF THE STUDY

There are different pathways that have been identified in the pathogenesis of POAG of which, ECM and apoptosis play an important role in elevation of IOP and RGC death. ECM and apoptosis are regulated by a wide array of genes. In the present study the candidate gene screening was focused on the specific genes involved in different pathways. Hence, analysis of the entire pathway will give more insight into the disease pathogenesis rather than screening specific genes. We did not find the association of recently identified variants in other populations. However, the effect sizes of these variants were low to draw specific conclusions on these genetic associations. We could not analyze *FBLN5* in patients TM due to the unavailability of surgical samples. Screening of entire gene rather than specific variants in *VAV2* and *VAV3* would have indicated its role in POAG and PACG.

CHAPTER 9: FUTURE SCOPE OF THE STUDY

- Screening of the entire *FBLN5* gene will give more information on its role in POAG. Functional studies on *FBLN5* will provide further insights on the biological pathways involved in the disease pathogenesis.
- Screening of entire mitochondrial DNA can provide better insight on the role of mitochondria in POAG.
- As POAG is associated with different pathways, screening of variants based on pathways will lead to the identification of novel disease-susceptibility genes.
- Whole genome sequencing would be an ideal choice for understanding the unknown genes involved in glaucoma pathogenesis.

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APPENDICIES

1. Reagents used for agarose gel electrophoresis

50X TAE [(Tris- acetate EDTA (ethylenediaminetetraacetic acid)]

242 g of Tris-base and 100 ml of 0.5M EDTA were added to 800 ml of autoclaved deionized water and allowed to dissolve on a magnetic stirrer. To this, 57.1 ml of glacial acetic acid was added and the volume was made up to 1 liter with double distilled water. The contents were mixed until a clear solution was obtained and stored at room temperature.

EtBr (Ethidium Bromide)

1g of Ethidium Bromide was added in 100 ml of autoclaved deionized water and stirred with a magnetic stirrer to give a concentration of 10 mg/ml. The solution was stored in a dark bottle, wrapped with aluminum foil and stored at room temperature.

6X loading dye

To make 100 ml of the dye, 40 g of sucrose, 0.025 g of Xylene cyanol and 0.025 g of Bromophenol blue were added to obtain a final volume of 100 ml with autoclaved deionized water.

2. Reagents used for PAGE

30% acrylamide (acrylamide:bisacrylamide, 29:1)

29 g of acrylamide and 1g of N,N'-Methylenebisacrylamide was added to autoclaved deionized water to make a stock solution of 100 ml. The solution was made homogenous by mixing on a magnetic stirrer.

10X TBE (Tris-Borate EDTA)

54 g of Tris-base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8) were added to 900 ml of autoclaved deionized water and allowed to dissolve on a magnetic stirrer to give a final volume of 1 liter.

0.5 M EDTA

186.1 g of disodium EDTA.2H2O was added to 800 ml of autoclaved deionized water and stirred on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets (approximately 20 g of pellets). The solution was sterilized by autoclaving.

10% APS (Ammonium per sulphate)

0.1 g of APS was added to 1 ml of autoclaved deionized water in a 1.5 ml eppendorrf tube and mixed thoroughly by inverting the tube. The tube was wrapped with aluminium foil at stored at 4˚C.

3. Preparation of reagents for DNA sequencing

Sodium Acetate (3M pH5.2)

For 100 ml of the solution, 40.83 g of sodium acetate was added to 80 ml of autoclaved deionized water in a beaker and dissolved on a magnetic stirrer. The pH of the solution was adjusted to 5.2 with glacial acetic acid and the volume was adjusted with water and autoclaved.

70% ethanol

7 ml of absolute ethanol (100%) was diluted with 3ml of autoclaved deionized water to make 70% ethanol.

4. Reagents for western blotting

1X PBS

8 grams of sodium chloride (NaCl), 0.2 grams of potassium chloride (KCl) and 1.44 grams of sodium biphosphate (Na_2HPO_4) were added to 800 milliliters (ml) of autoclaved deionized water. The pH of the solution was adjusted to 7.4 with 1M hydrochloric acid. The final volume of the solution was made to 1000 ml with autoclaved autoclaved deionized water. The final concentration of the solution was 10X. The working solution of 1X was prepared by diluting the stock solution with autoclaved deionized water.

30% acrylamide mix

29 g of acrylamide and 1g of N,N'-Methylenebisacrylamide was added to autoclaved deionized water to make a stock solution of 100 ml. The solution was made homogenous by mixing on a magnetic stirrer.

Tris-HCl (1.5M –pH 8.8)

For 200 ml of the solution, 36.3 g of Tris base was added to 150 ml of deionized water in a beaker and dissolved on a magnetic stirrer. The pH of the solution was adjusted to 8.8 with HCl and the volume was adjusted with water and autoclaved.

Tris-HCL (1M-pH 6.8)

For 100 ml of the solution, 12.21 g of Tris base was added to 50 ml of deionized water in a beaker and dissolved on a magnetic stirrer. The pH of the solution was adjusted to 6.8 with HCl and the volume was adjusted with water and autoclaved.

10% SDS

For 50 ml of the solution, 5 g of SDS was added to 40 ml of autoclaved deionized water in a beaker and dissolved by heating in a water bath. The final volume was adjusted to 50mL with autoclaved deionized water.

Sample buffer

The 2X sample buffer was prepared by adding following reagents

Reagent	Volume	Concentration (2X)
1M Tris HCL pH 6.8	2 Ml	100Mm
80% Glyceral	5 mL	20%
10% SDS	8 mL	4%
β Mercapto ethanal	0.4 mL	2%
Bromophenol Blue	0.04g	0.2%
Water	5 mL	
Final Volume	20 mL	

5X running buffer

15.1g of Tris base, 5g of SDS and 94g of Glycine were added to 800 mL of autoclaved deionized water. The pH of the solution was adjusted to 8.3 and the final volume of the solution was made to 1000 ml with autoclaved deionized water. The final concentration

of the solution was 5X. The working solution of 1X was prepared by diluting the stock solution with autoclaved deionized water.

Transfer buffer

Transfer buffer was prepared by adding 20 mL of methanol to 80 mL of 1X running buffer.

5% Blocking reagent

2.5g of nonfat milk was added to 30 mL of 1X PBS dissolved properly and made the volume to 50 mL with autoclaved deionized water.

Preparation of detection reagent

Added 0.5 mL of reagent A with 0.5 mL of reagent B in a ratio of 1:1 in a 1.5ml tube and covered with aluminum foil.

5. Reagents for IHC

3%H2O2/CH3OH solution

30mL of 6%H_2O_2 was added to the 30 mL of CH3OH and mixed properly

Preparation of Trypsin solution

5mL of autoclaved deionized water was added to the 15mL falcon tube and 10 mg of $CaCl_2$ (0.1%) and Trypsin (0.1%) (cell culture grade) was added and mixed properly. 2mL of 20mM Tris Hcl (pH 7.6) was added and made the final to 10mL with autoclaved deionized water.

Blocking solution

250mg of BSA (Bovine Serum Albumin) was added to the 8mL of autoclaved deionized water and dissolved properly and made the volume to 10 mL with autoclaved deionized water

Preparation of DAB

DAB (Diaminobenzidine) was prepared by adding 36 μL of DAB to the 964 μL of HRP substrate buffer.

LIST OF PUBLICATIONS

1. **Rao KN**, Nagireddy S, Chakrabarti S. Complex genetic mechanisms in glaucoma: an overview. Indian J Ophthalmol. 2011; 59 Suppl: S31-42.

2. Chakrabarti S, Ghanekar Y, Kaur K, Kaur I, Mandal AK, **Rao KN**, Parikh RS, Thomas R, Majumder PP. A polymorphism in the *CYP1B1* promoter is functionally associated with primary congenital glaucoma. Hum Mol Genet. 2010; 19: 4083-90.

3. **Rao KN**, Kaur I, Parikh RS, Mandal AK, Chandrasekhar G, Thomas R, Chakrabarti S. Variations in *NTF4, VAV2*, and *VAV3* genes are not involved with primary open-angle and primary angle-closure glaucomas in an Indian population. Invest Ophthalmol Vis Sci. 2010; 51:4937-41.

4. **Rao KN**, Kaur I, Chakrabarti S. Lack of association of three primary open-angle glaucoma-susceptible loci with primary glaucomas in an Indian population. Proc Natl Acad Sci U S A. 2009; 106(44):E125-6.

5. Chakrabarti S, Kaur K, **Rao KN**, Mandal AK, Kaur I, Parikh RS, Thomas R. The transcription factor gene *FOXC1* exhibits a limited role in primary congenital glaucoma. Invest Ophthalmol Vis Sci. 2009; 50: 75-83.

6. **Rao KN**, Ritch R, Dorairaj SK, Kaur I, Liebmann JM, Thomas R, Chakrabarti S. Exfoliation syndrome and exfoliation glaucoma-associated *LOXL1* variations are not involved in pigment dispersion syndrome and pigmentary glaucoma. Mol Vis. 2008; 14: 1254-62.

7. Chakrabarti S, **Rao KN**, Kaur I, Parikh RS, Mandal AK, Chandrasekhar G, Thomas R. The *LOXL1* gene variations are not associated with primary open angle and primary angle-closure glaucomas. Invest Ophthalmol Vis Sci. 2008; 49: 2343-7.

LIST OF PRESENTATIONS

1. Presented a paper at paper at ISHG- 2012 (Chandigarh, India) on Molecular Genetic Analysis of Intraocular pressure related glaucomas in Indian Population**.**

2. Presented a poster at ARVO (Fort Lauderdale, USA) on 'Evaluation of *NTF4*, *VAV2* and *V AV3* in POAG and PACG of Indian patients' held on May, 2010.

3. Presented a Paper at IERG (Hyderabad, India) on "Evaluation of *BAX* gene in primary open angle glaucoma in an Indian population" held on July, 2010.

4. Presented a poster at Asia ARVO (Hyderabad, India) on 'Evaluation of Connective Tissue Growth Factor (*CTGF*) in primary open angle glaucoma' held on Jan, 2009.

AWARDS & FELLOWSHIPS RECEIVED

1. Recipient of Council for Scientific and Industrial Research- Senior Research Fellowship (CSIR-SRF), 2008.

2. Won the best poster award at Asia ARVO held in Hyderabad, India, 2009.

3. Received Travel grant provided by DST (India) to attend the ARVO (2010), Fort Lauderdale, US A.

BRIEF BIOGRAPHY OF THE CANDIDATE

Kollu Nageswara Rao has been working at Kallam Anji Reddy Molecular Genetics Laboratory at LV Prasad Eye Institute (LVPEI) for past five years (August 2006- till date). He joined this institute after his academic training (M. Tech Medical Biotechnology) at University of Hyderabad, Hyderabad.

After her joining LVPEI, he was involved in research related to 'Molecular Genetic Analysis of IOP related glaucomas in Indian population'. He was also involved in teaching the graduate students at the Bausch and Lomb School of Optometry, LVPEI.

His Ph.D work has been published in International journals of repute. He has presented his work in national and international scientific meetings. He was selected to present his work at the Asia ARVO 2009 and ARVO 2010, USA and was given the Travel Fellowship Award for the same.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr.Subhabrata Chakrabarti (b.1972) is a associated director (Research) at the L.V.Prasad Eye Institute, Hyderabad, India, for the last 7 years. He obtained his Ph.D. in human genetics and was also a visiting scientist at the prestigious National Eye Institute at NIH, USA (2006). He has done some pioneering work in the area of molecular genetics of eye diseases in India. His main thrust lies in understanding the molecular mechanisms underlying complex ocular diseases like glaucoma, anterior segment disorders, age-related macular degeneration (AMD) and myopia.

Dr. Chakrabarti collaborates widely with his peers within and outside the country and has lectured at all the major eye institutes in the world. All his research works have been done in India and published in peer reviewed international journals. He is an editorial board member for two journals and a reviewer for 13 other international journals and various granting agencies. His research work has been funded through competitive extramural research grants from various international and national funding agencies. He has organized scientific conferences, chaired sessions, conducted workshops and presented widely at the international and national levels.

His research works have been widely acknowledged and the Association of International Glaucoma societies (AIGS) gave him the Clinician Scientist Award in Sciences (2004), Indian National Science Academy (INSA) medal for Young Scientist (2006), Platinum Jubilee Award of the National Academy of Sciences India (2007), Young Scientist Award at the Asia-Pacific Joint Glaucoma Congress (2010) and World Glaucoma Association Research Award (WGA) for 2010 in basic sciences

Dr. Chakrabarti is a Ph.D. examiner and supervisor for the University of Melbourne, Australia, BITS, Pilani and the University of Hyderabad, Hyderabad. He has supervised three Ph.D. candidates and a post-doctoral fellow. He is a member of several national and international professional bodies, notable among them being the advisory board member of the World Glaucoma Association and executive council member of the Indian Society of Human Genetics. Currently he is a chair of the scientific committee and a member of the ethics committee of the Institutional Review Board.

He is on the Advisory Board of the World Glaucoma Association, and Editorial Board Member and reviewer for various international journals and funding agencies.