Molecular Genetic Analysis of Primary and Secondary Glaucomas in the Indian Population

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

To

THE DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
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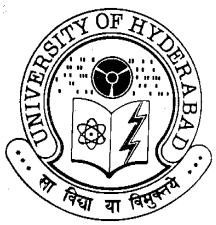

By

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Dedicated to Amma Nanna
Pedamma Pednanna
And loving Chinnu

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DECLARATION

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

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CERTIFICATE

This is to certify that this thesis entitled, "**Molecular Genetic Analysis of Primary and Secondary Glaucomas in the Indian Population**", submitted by **Ms. Sreelatha Komatireddy** for the degree of **Doctor of Philosophy** to the University of Hyderabad is based on the work carried out by her at the L. V. Prasad Eye Institute, Hyderabad, under our supervision. This work has not been submitted for any diploma or degree of any other University or Institution.

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- Sri

CONTENTS

ABBREVIATIONS

μg:	Microgram
μl:	Microlitre
μM :	Micromolar
A:	Adenine
ARA:	Axenfeld-Rieger anomaly
ARS:	Axenfeld-Rieger syndrome
bp:	Basepair
C:	Cytosine
cDNA:	Complementary DNA
DMSO:	Dimethylsulphoxide
dNTPs:	deoxy nucleotide triphosphates
dup:	Duplication
G:	Guanine
Ins:	Insertion
IOP:	Intraocular pressure
JOAG:	Juvenile open angle glauocma
kDa:	Kilo dalton
OR:	Odds ratio
PAGE:	Polyacrylamide gel electrophoresis
PCG:	Primary congenital glaucoma
PCR:	Polymerase chain reaction
POAG:	Primary open angle glaucoma

RFLP:	Restriction fragment length polymorphism
rpm:	Revolutions per minute
SNP:	Single nucleotide polymorphism
T:	Thymine
TM:	Trabecular meshwork
EDTA:	Ethylenediaminetetraaceticacid
TAE:	Tris acetate EDTA
SDS:	Sodium dodecylsulphate
Tris:	Tris(hydroxymethyl)aminomethane
PBS:	Phosphate buffered saline

Chapter 1
Introduction

INTRODUCTION

Glaucoma is a leading cause of irreversible blindness throughout the world. It affects 67 million people worldwide (Quigley *et al.,* 1996). The World Health Organization (WHO) report published in 2004 shows that it is the second leading cause of blindness in the world followed by cataract (Resnikoff *et al.,* 2004). Globally glaucoma accounts for 12.3% of total blindness and it affects 12 million people in India (Thylefors *et al.,* 1995), which represents 12.8% of the blindness burden in the country.

Glaucoma is asymptomatic and is associated with a progressive and ultimately irreversible loss of vision. Further loss of vision can be prevented, but vision loss once occurred cannot be regained. It is an optic nerve disorder characterized by glaucomatous cupping and optic nerve atrophy (Thylefors *et al.,* 1994), caused by obstruction in the aqueous humor outflow pathway that leads to raised intraocular pressure (IOP). Based on events that lead to raised IOP, glaucomas are classified into two categories, primary and secondary (Shields, 1998). Primary glaucomas are those in which the initial events lead to raise in IOP with no contribution from ocular and systemic disorders. However in secondary glaucomas, the associated ocular or systemic abnormalities are responsible for alteration in aqueous humor dynamics and raised IOP.

Primary glaucomas are those with no apparent contribution from other ocular or systemic disorders. Various forms of primary glaucomas include open angle, angle closure and congenital glaucomas. Open

angle glaucomas are those in which anterior chamber angle structures are visible by gonioscopy. Angle closure glaucomas are characterized by the apposition of peripheral iris against trabecular meshwork. Developmental abnormality of the anterior chamber angle leads to obstruction of aqueous outflow in congenital glaucomas (Shields, 1998).

Primary Open Angle Glaucoma (POAG), which is a major form of primary glaucoma, is characterized by elevated IOP (>21 mm Hg), open anterior chamber angles on gonioscopy and typical glaucomatous field defects. It is a clinically and genetically heterogeneous disorder. The molecular genetic mechanisms underlying POAG are not well characterized. So far 11 loci (*GLC1A* at *1q24.3-q25.2* (Sheffield *et al.,* 1993), *GLC1B* at *2cen-q13* (Stoilova *et al.,* 1996), *GLC1C* at *3q21-q24* (Wirtz *et al.,* 1997), *GLC1D* at *8q23* (Trifan *et al.,* 1998), *GLC1E* at *10p15-p14* (Sarfarazi *et al.,* 1998), *GLC1F* at *7q35-q36* (Wirtz *et al.,* 1999), *GLC1G* at *5q22.1* (Samples *et al.,* 2004), *GLC1H* at *2p16.3-p15* (www.gene.ucl.ac.uk/nomencleature), *GLC1I* at *15q11-q13* (Allingham *et al.,* 2005), *GLC1J* at *9q22* (Wiggs *et al.,* 2004), and *GLC1K* at *20p12* (Wiggs *et al.,* 2004) have been mapped by linkage. Of these, genes at *GLC1A* (*MYOC)*, *GLC1E* (*OPTN*) and *GLC1G* (*WDR36*) have been cloned and studied. (Stone *et al.,* 1997, Rezaie *et al.,* 2002, Monemi *et al.,* 2005). The mutational spectrum of *MYOC* and *OPTN* genes is well studied around the world.

Myocilin (*MYOC*) was the first gene identified as a candidate gene for POAG. It has 3 exons, of which the first exon carries a

myocin-like domain and the third exon the olfactomedin-like domain (Kubota *et al.,* 1997). It codes for a protein of yet to be characterized function, consisting of 504 amino acids. Mutations in the *MYOC* gene accounts for 3-5% of open angle glaucomas globally (Fingert *et al.,* 1999, Alward *et al.,* 2002). *MYOC* mutations were observed in individuals affected with open angle glaucoma, normal tension glaucoma, hypertension glauoma and secondary glaucomas (Alward *et al.,* 2002). The mutation Q368X was found to be prevalent in various populations (Fingert *et al.,* 1999, Alward *et al.,* 1998, Alward *et al.,* 2002). The mutation P370L was found to cosegregate with an early onset form of POAG with high IOP and not responding to medical treatment (Taniguchi *et al.,* 1999, Mukhopadhyay *et al.,* 2003). Individuals carrying the mutations Y437H or I477N manifested the disease four decades earlier than those carrying Q368X (Alward *et al.,* 1998). Some groups in India have studied the mutation spectrum in the South (Sripriya *et al.,* 2004, Kanagavalli *et al.,* 2003), North (Sripriya *et al.,* 2004) as well as East Indian (Mukhopadhyay *et al.,* 2002) populations and Q48H was reported to be the most prevalent mutation (Chakrabarti *et al.,* 2005). The mechanism of how mutant MYOC causes glaucoma is not known.

Optineurin (*OPTN*) is the second candidate gene identified in POAG, particularly for normal tension glaucoma (NTG) (Rezaie *et al.,* 2002). It has 16 exons, the first three being non-coding; translation starts in fourth exon. In the initial study, it was suggested to account for 16.7% of families with NTG. But the same scenario was not found in

other studies. In the initial study, the mutation E50K located in the putative bZIP motif, was observed in 7 families (Rezaie *et al.,* 2002). Later this mutation was observed in one patient with positive family history of NTG in a larger study comprising of 1048 patients (Alward *et al.,* 2003). Individuals with this mutation manifested the phenotype at an earlier age and had advanced optic disc cupping and progressing visual field loss when compared to those individuals without E50K mutation (Aung *et al.,* 2005). The variation R545Q that was initially reported as mutation, was later observed in normal controls in the Chinese (Leung *et al.,* 2003) and Japanese populations (Toda *et al.,* 2004, Funayama *et al.,* 2004). The M98K mutation, which was reported as associated risk factor (Rezaie *et al.,* 2002), was not associated with disease phenotype in other studies (Mukhopadhyay *et al.,* 2005, Alward *et al.,* 2003, Wiggs *et al.,* 2003). So far, there are two reports from India, which did not find any mutation in *OPTN* in this population (Mukhopadhyay *et al.,* 2005, Sripriya *et al.,* 2006). The R545Q variation showed association with the disease phenotype in one study (Mukhopadhyay *et al.,* 2005) and was not observed in other study (Sripriya *et al.,* 2006). The M98K variation did not show any association in a study by Mukhopadhyay *et al.,* (2005) while Sripriya *et al.,* (2006) shown M98K as a risk factor for HTG and NTG in southern Indian population especially representing Tamilanadu.

The gene WD40-repeat 36 (*WDR36*) was recently identified as a candidate for POAG (Monemi *et al.,* 2005). A single mutation, D658G, was observed in a family that was initially mapped to the *GLC1G* locus.

Further screening of 136 POAG individuals revealed mutations in 17 individuals, 11 with high-pressure glaucoma and 6 with low-pressure glaucoma. Recent report by Pang *et al.* (2006) showed no mutations in *WDR36* gene in a family that was mapped to *GLC1G* locus. Its involvement in other populations has not been reported.

The gene Cytochrome P450 1B1 (*CYP1B1*) at the *GLC3A* locus (Sarfarazi *et al.,* 1995) has been identified as a candidate gene for primary congenital glaucoma (PCG) (Stoilov *et al.,* 1997). It has 3 exons, of which parts of the second exon and third exons code for a protein of 543 amino acids. Cytochrome P450s are mono-oxygenases involved in the metabolism of steroids and xenobiotics. The mutation frequency of *CYP1B1* in PCG ranges from 20% in the Japanese population (Mashima *et al.,* 2001) to almost 100% in the Slovakian Gypsies (Plasilova *et al.,* 1999). It accounts for 37.5% of Indian PCG cases (Reddy *et al.,* 2004). It is also implicated, as part of di-genic inheritance along with *MYOC* in early onset open angle glaucoma (Vincent *et al.,* 2002), associated with early manifestation of the phenotype in individuals having mutations in both *MYOC* and *CYP1B1* genes. It was hypothesized that *CYP1B1* may be a modifier of *MYOC* gene. The *CYP1B1* mutations were noted in 4.6% of early onset open angle glaucoma cases of French (Melki *et al.,* 2004) and 10.9% of POAG cases of Spanish population (Lopez-Garrido *et al.,* 2006). In Indian POAG population the *CYP1B1* gene accounts for 4.5% of the cases (Acharya *et al.,* 2006).

Single nucleotide polymorphisms (SNPs) in three candidate genes, namely *p53*, *ApoE* and *TNF*α have been observed to be associated with open angle glaucoma in various populations. In the *p53* gene, codon 72 arginine to proline polymorphism and intron-3 16 bp duplication were associated with open angle glaucoma in Taiwanese (Lin *et al.,* 2002) and British populations (Ressiniotis *et al.,* 2004). However, no association was observed in the Indian (Acharya *et al.,* 2002) and Australian populations (Dimasi *et al.,* 2005). In the case of *ApoE* polymorphism, the distribution of $\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$ alleles was significantly different between patients and controls in Tasmanian (Vickers *et al.,* 2002) and Japanese populations (Mabuchi *et al.,* 2005), while there was no difference seen in the UK population (Lake *et al.,* 2004). In *TNF*α, the -308G>A polymorphism was found to be associated with POAG in the Chinese population (Lin *et al.,* 2003). No association was observed with *TNF*α polymorphisms –238G>A and –308G>A in Caucasian POAG patients (Mossbock *et al.,* 2005).

Secondary glaucomas are characterized by associated ocular or systemic abnormalities that appear to be responsible for the alteration in the aqueous humor dynamics (Shields, 1998). Three loci have been mapped in the Axenfeld-Rieger syndrome, one of the forms of secondary glaucomas. Of these, *PITX2* (*4q25*) (Semina *et al.,* 1996) and *FOXC1* (*6p25*) (Nishimura *et al.,* 1998) have been cloned and characterized, while another locus at 13q14 (Phillips *et al.,* 1996) is yet to be characterized. Another gene on chromosome 11p13, namely *PAX6,* has been implicated in the Rieger syndrome (Riise *et al.,* 2001).

The Forkhead box transcription factor C1 (*FOXC1*) consists of a single exon and codes for a protein of 553 amino acids. This family of transcription factors is distinguished by a highly conserved 110 residues DNA binding domain, known as fork head domain. Mutations in *FOXC1* result in various clinical phenotypes and the spectrum of mutation includes deletions, duplications (Lehmann *et al.,* 2002), missense (Nishimura *et al.,* 1998, Mears *et al.,* 1998) and nonsense mutations (Komatireddy *et al.,* 2003). There are two reports (Panicker *et al.,* 2002, Komatireddy *et al.,* 2003) from India describing the *FOXC1* mutations in the Axenfeld-Rieger anomaly phenotype. Three mutations were described in nine patients suggesting its genetic heterogeneity. Two non-sense and one missense mutation were observed resulting in variable outcomes.

Pituitary Homoeobox Transcription Factor – 2 (*PITX2*) is a member of the bicoid-like homeobox transcription factor family. The homeobox proteins contain a 60 amino acid homeodomain that binds DNA. The gene consists of 4 exons with the translation initiation codon starting in the second exon (Semina *et al.,* 1996). Mutations in *PITX2* have been found in various pathologic phenotypes including Axenfeld-Rieger malformations, Iridogonial Dysgenesis (IGD), Iris Hypoplasia (IH) and Peter's anomaly (Lines *et al.,* 2002). There are no reports on the involvement of *PITX2* in anterior chamber anomalies from India.

While molecular genetic aspects of primary and secondary glaucomas are well characterized in other populations, there are fewer reports from India. In order to understand the underlying molecular

genetic defects in candidate genes causing primary and secondary glaucomas in our country, the present study was designed with the following objectives:

1) To screen *MYOC*, *CYP1B1* and *OPTN* in primary glaucomas (POAG);
2) To screen single nucleotide polymorphisms (SNPs) in *p53*, *ApoE* and *TNF*α in POAG to understand their association with the disease phenotype;
3) To understand the molecular genetic defects underlying Axenfeld-Rieger Syndrome by screening *FOXC1* and *PITX2,* and
4) To undertake a genotype-phenotype correlation in order to determine the association of genetic factors with the clinical phenotype.

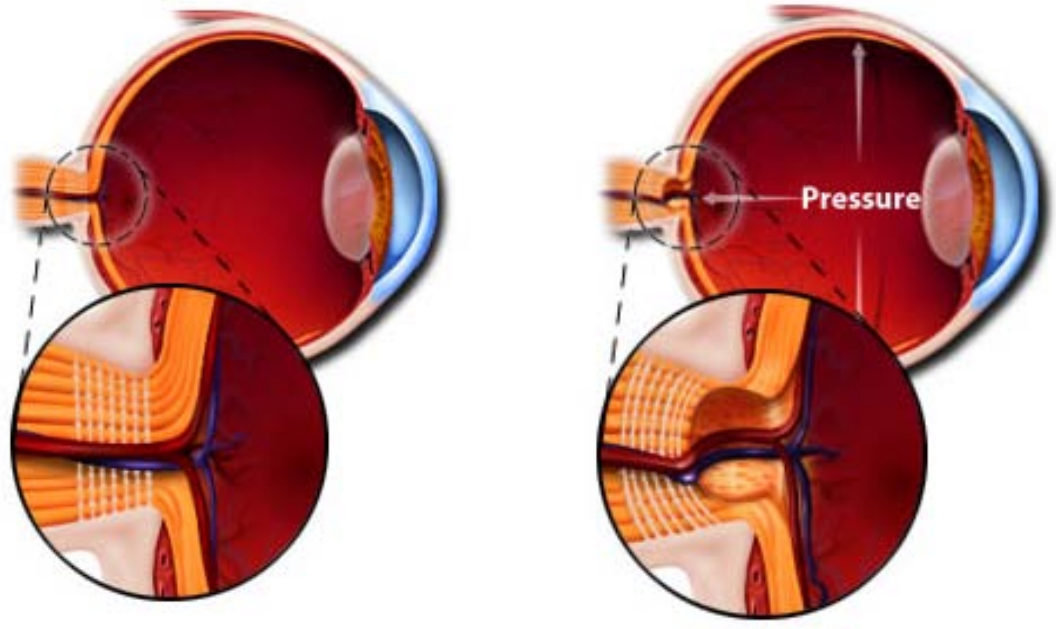
Chapter 2
Review of Literature

REVIEW OF LITERATURE

2.1 GLAUCOMA

Glaucoma is an optic neuropathy characterized by progressive loss of vision and associated with damage to the optic nerve. Selective death of retinal ganglion cells lead to the gradual enlargement of the optic cup (Figure 1) which is a small depression in the head of the optic nerve and loss of vision beginning at the periphery. Once a sufficient number of nerve cells are destroyed, "blind spots", or scotomas, begin to form in the field of vision. These scotomas usually develop in the peripheral field initially leading to peripheral field loss, gradually leading to central vision loss (Figure 2). Vision loss is an irreversible process because the degenerated nerve cells cannot be restored at present (Flammer, 2003).

Glaucoma represents a biochemical pathway resulting from different ocular conditions that are associated with elevated intraocular pressure (IOP). IOP is a function of the rate at which aqueous humor enters the eye (inflow) and the rate at which it leaves the eye (outflow). Inflow is related to the rate of aqueous humor production, while outflow depends on the resistance to the flow of aqueous from the eye and the pressure in the episcleral veins. Raise in IOP resulting from obstruction of aqueous humor outflow pathway contributes to the damage of optic nerve. An elevated IOP is considered to be a major risk factor rather than symptom for glaucoma (Shields, 1998).



A. Normal eye **B. Glaucoma eye**

Figure 1. Enlargement of optic cup in glaucoma
(http://www.merckfrosst.ca/e/health/glaucoma/glaucoma/what_is/home.html)

Figure 2. Loss of peripheral vision in glaucoma
(http://www.eyecareamerica.org/eyecare/eca/upload/glaucoma-image.jpg)

As the study of glaucoma primarily deals with the elevated IOP and its consequences, it is useful to have an overview of the physiologic factors that control IOP and the dynamics of aqueous humor outflow.

2.2 AQUEOUS HUMOR

Aqueous humor is secreted by ciliary processes and appears to be derived from the plasma within the capillary network of the ciliary processes. Aqueous humor traverses through the ciliary processes by one of the following three mechanisms (Shields 1998):

1. ***Diffusion:*** The passive movement of lipid soluble substances through lipid proteins of cell membranes in response to a concentration gradient.
2. ***Ultrafiltration:*** The passive movement of water and water soluble substances across the cell membrane in response to a non-enzymatic process regulated by both differential hydrostatic pressure in the blood and the osmotic pressure of the ciliary body.
3. ***Secretion:*** The active energy-dependent movement of solutes of greater size across the cell membrane.

Rate of aqueous humor production

The rate of aqueous humor production is measured in micro liter/min. The normal rate of production in humans is 1.82 to 4.3 μl/min.

Aqueous humor function

It serves in the metabolic function for avascular tissues like cornea, lens and trabecular meshwork. Substrates like glucose, oxygen and amino acids are supplied to these tissues and metabolic wastes like lactic acid, pyruvic acid and carbon di-oxide are carried away (Shields, 1998). The constant flow of aqueous humor helps in maintaining a stable IOP.

Aqueous humor composition

Aqueous of both anterior and posterior chambers is slightly hypertonic compared to plasma. It is acidic in nature with a pH of 7.2 in the anterior chamber. The two most striking characteristics of aqueous humor are marked excessive ascorbate (15 times greater than that of arterial plasma) and marked deficit of protein (0.02% in aqueous as compared to 7% in plasma). It also found to have amino acids and sodium hyaluronate (Shields, 1998).

2.3 AQUEOUS HUMOR OUTFLOW

The primary aqueous humor enters the posterior chamber, where its composition may be altered either by re-absorption by the iris or ciliary body or by the addition of metabolites from surrounding tissues such as lens. This secondary aqueous humor enters the anterior chamber through the pupil, from where it leaves the eye by two pathways (Figure 3).

a. ***Conventional or canalicular system:*** This accounts for 85-95% of aqueous outflow. The outflow pathway is through the trabecular meshwork into schlemm"s canal and then into the intrascleral and episcleral venous plexus.

b. ***Unconventional or extracanalicular system:*** This accounts for the remaining 5-15% of the aqueous outflow. The aqueous humor flows along the interstitial spaces of the ciliary muscles and choroids or the supra choroidal space through the sclera into the connective tissue of the orbit from there it probably drains via veins into the general circulation.

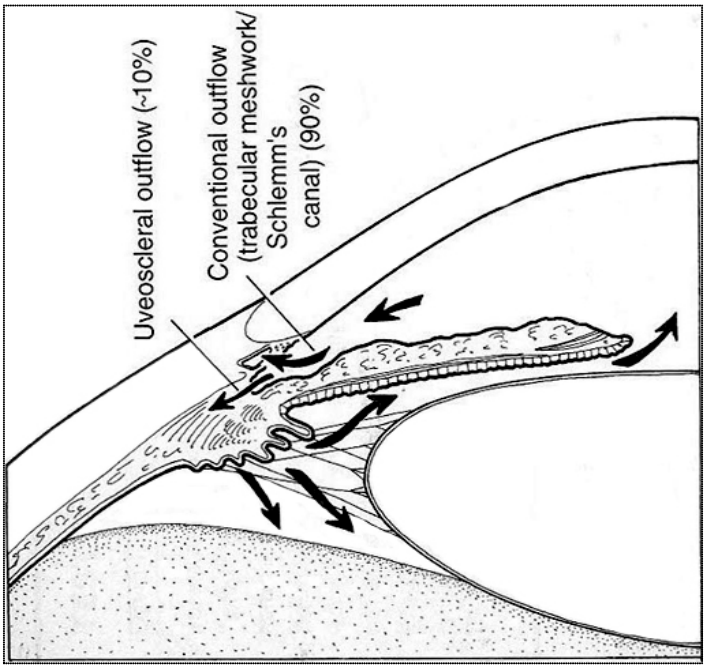


Figure 3.Schematic representation of aqueous humor outflow
(Epstein DL. *Glaucoma.* 4th ed. Baltimore, MD: Williams & Wilkins; 1997:p.19)

2.4 TRABECULAR MESHWORK (TM)

TM is the major site of resistance to the aqueous outflow. Higher primates and humans have developed a true trabecular meshwork. Lower monkeys and other mammals have a reticular meshwork, which covers a broader area of the sclera, while the Schlemm's canal is absent (Ritch *et al*., 1996).

Non-Filtering trabecular meshwork:

The trabecular meshwork can be divided into two parts (Figure 4). The most anterior portion lies adjacent to that of limbus, just posterior to Schwalbe's line. It has no contact with the Schlemm's canal and therefore called as non-filtering part of the trabecular meshwork. It consists of 3-5 trabecular beams covered by small trabecular cells (Ritch *et al*., 1996).

Filtering trabecular meshwork:

This covers the inner wall of Schlemm's canal and consists of three morphologically distinct regions (Ritch *et al*., 1996).

a) The cribriform layer (juxta canalicular tissue): This comprises the outermost part of the trabecular meshwork, adjacent to the inner wall of the endothelium of the Schlemm's canal. It contains a network of fine fibrils, elastic-like fibers and a number of elongated, fibroblast-like cells arranged in layers. These cells are embedded within the extracellular material, which appears as "empty spaces" in the electron micrograph and represent the preferential aqueous pathway.

b) Corneo-scleral meshwork: This extends from the scleral spur towards the cornea, filling the scleral sulcus. It comprises the main portion of the trabecular meshwork and consists of sheets of trabeculae that are perforated by elliptical openings. These holes become progressively smaller as the trabecular sheets approach schlemm's canal, with a diameter range of 5-50 μm.

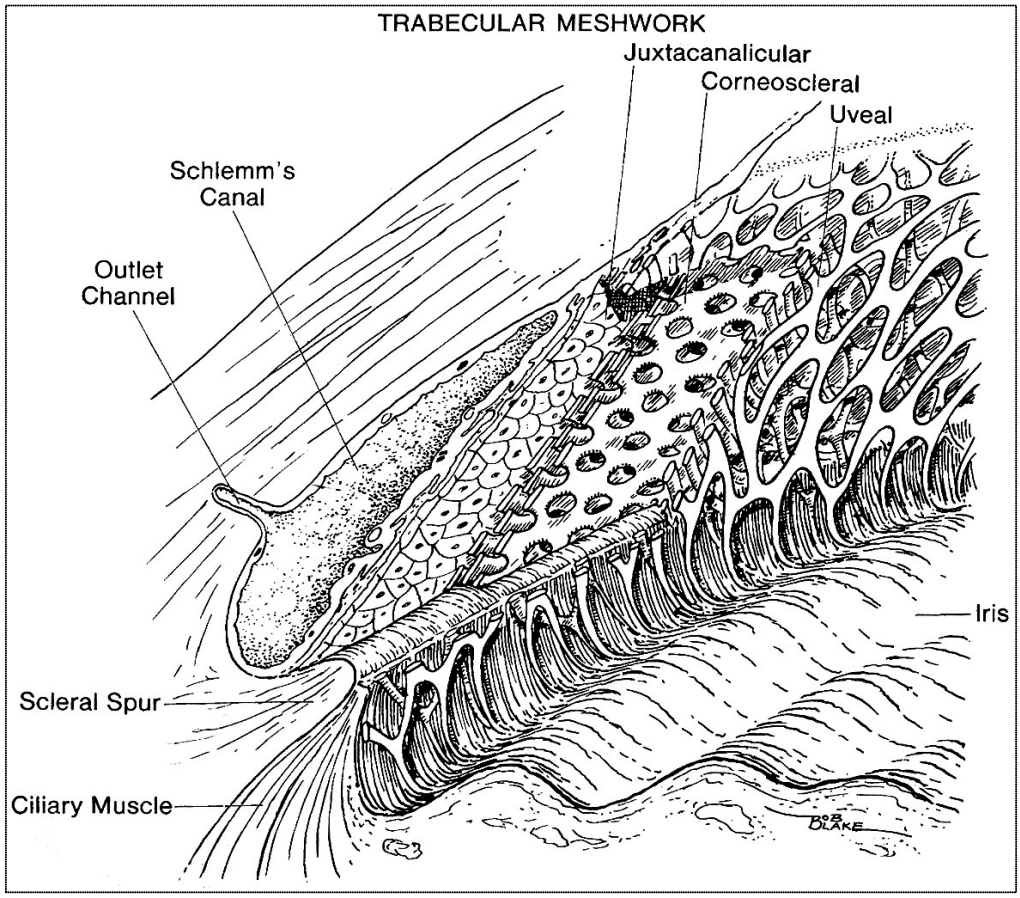


Figure 4. Three layers of the trabecular meshwork

(Shields MB. *Textbook of glaucoma*. 4th ed. Baltimore, MD: Williams & Wilkins; 1998:p.16)

c) Uveal meshwork: This continues posteriorly with the ciliary body and iris; anteriorly it tapers and is anchored at the inner layers of the corneal stroma or corneo-scleral meshwork. It consists of irregularly arranged strands or sheets and are connected with each other, forming a broad network with oval or round openings 20 μm in diameter.

Proteins in TM

The gene expression profile was characterized by expressed sequence tag (EST) analysis, as part of NEIBank project of National Eye Institute (Tomarev *et al*., 2003). Among the candidate genes for ocular disorders, *MYOC*, the candidate gene for POAG was represented by 28 clones and is the third most abundant gene in the TM. The *PITX2*, candidate gene for Rieger syndromes and anterior chamber angle defects, was also highly expressed and represented by 6 clones. Among the other genes, those for matrix protein Gla (*GLA*), vimentin (*VIM*) and elongation factor 1α were also present abundantly.

Table 1. Candidate genes for POAG and their level of expression in TM (from Tomarev *et al.,* 2003)

S.No	Gene	Number of clones
1	*MYOC*	27
2	*PITX2*	06
3	*CYPB1*	02
4	*OPTN*	01
5	*FOXC1*	00
6	*p53*	00
7	*APOE*	00
8	*TNF*α	00

2.5 CLASSIFICATION OF GLAUCOMAS

Glaucomas can be classified into various forms based on many systems, mainly based on 1) etiology underlying the disorder that leads to an alteration in aqueous humor dynamics and 2) mechanism due to specific alteration in the anterior chamber angle leading to a rise in IOP (Shields, 1998).

1) Based on etiology: They are mainly classified into primary, secondary glaucomas

a) Primary Glaucomas: The glaucomas in which the initial events lead to outflow obstruction and IOP elevation are called primary glaucomas, with no apparent contribution from other ocular or systemic disorders (Shields, 1998). These are bilateral and have a genetic basis. These

include primary open angle glaucomas (POAG), primary angle closure glaucomas (PACG) and primary congenital glaucomas (PCG).

b) Secondary glaucomas: These glaucomas are characterized by associated ocular or systemic abnormalities that appear to be responsible for the alteration in the aqueous humor dynamics. These may be bilateral or unilateral and acquired (e.g. Glaucoma secondary to cataract surgery) or inherited (e.g. Axenfeld-Rieger syndrome).

Such classification based on etiology is purely arbitrary and inadequate, as developmental glaucomas do not fit into either category. The concept of primary and secondary glaucomas is a reflection of our incomplete understanding regarding the pathophysiologic events that ultimately lead to glaucomatous optic atrophy and visual field loss (Shields, 1998).

2) Based on mechanism: This concept was introduced by Barkan in 1938. According to this classification (Shields, 1998), glaucomas are divided into:

a) Open angle glaucoma: The open angle glaucomas are those in which the anterior chamber angle structures, i.e., TM, scleral spur and ciliary body band are visible by gonioscopy (Shields, 1998). As shown in Figure 5A, aqueous humor gets accumulated in the anterior chamber due to a block in the trabecular meshwork. This results in the widening of the irido corneal angle.

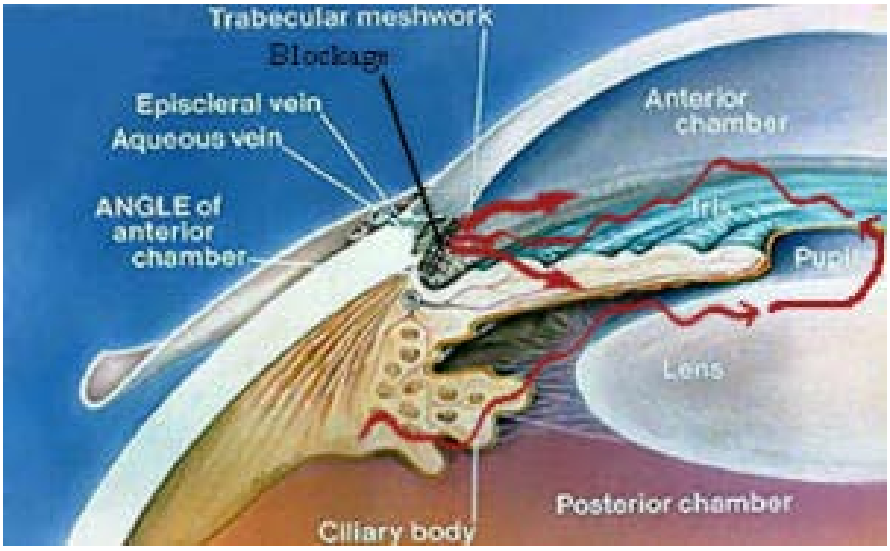
b) Angle closure glaucoma: In this form of glaucoma, the anterior chamber is shallow and aqueous outflow is blocked by the root of the

iris, which lies in apposition to the TM. As shown in Figure 5B, aqueous humor gets accumulated below the iris, pushing the iris towards the anterior chamber. This reduces the irido corneal angle.

c) Developmental anomalies of the anterior chamber angle: These glaucomas typically represent incomplete development of structures in the conventional aqueous outflow pathway (Shields, 1998). The developmental defects include incomplete development of trabecular meshwork and/or Schlemm's canal (e.g., Peters' anomaly) and iridocorneal adhesions (e.g., Axenfeld-Rieger syndrome).

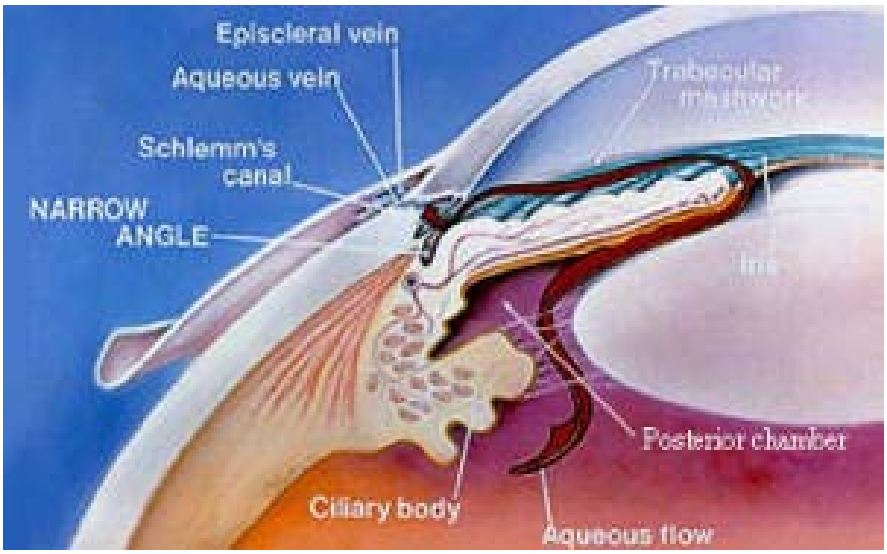
The disadvantage of this classification is that it ignores the pressure independent causative factors. Moreover many glaucomas have more than one mechanism of outflow obstruction in course of disease.

Figure 5. Aqueous humor flow in open angle and angle closure glaucomas



A. In open angle glaucoma

(http://www.pfofflaserandeye.com/The%20Informed%20Patient/Patient%20Ed%20Common%20Eye%20Disorders/Glaucoma/Glaucoma%20BG.htm)



B. In angle closure glaucoma

(http://www.opt.pacificu.edu/ce/catalog/15166-GL/Figure%208%20bot.jpg)

2.6 PRIMARY OPEN ANGLE GLAUCOMA (POAG)

"Primary" open angle glaucoma is so called because it occurs spontaneously without evident antecedent or related disease and with no known basis other than genetic or hereditary predisposition (Epstein, 1997) based on family history and race. Alternative terms that appear in literature are chronic open angle glaucoma, chronic simple glaucoma or idiopathic open angle glaucoma. It is likely that open angle glaucoma represents a spectrum of disorders in which several causative factors have varying degrees of influence.

POAG is defined by the following criteria (Shields, 1998):

1) IOP consistently above >21 mm Hg
2) Open anterior chamber angles with no apparent ocular abnormality that might account for elevated IOP
3) Typical glaucomatous visual field defects

Significance of IOP: The commonly used IOP level of 21 mm Hg is based on the concept that two standard deviations above the mean with in a Gaussian distribution (in Caucasian population) represents the upper limit of normal for that biological parameter (Shields *et al.,* 2005). But there are subjects with IOP>21 mm Hg and without any optic nerve or visual field defects, a condition known as ocular hyper tension. On the other hand some patients suffer from progressive glaucomatous damage despite having low pressures (<21 mm Hg) along with typical disc and field changes, known as normal / low tension glaucoma (NTG/LTG) (Shields, 1998). Though these might question the role of

IOP in the mechanism of POAG, there is a lot of evidence that support the hypothesis of IOP being a causative factor (Sommer *et al.,* 1991).

Risk factors:

Several other variables have been associated with open angle glaucoma such as:

i) Age: The prevalence of POAG increases with an increasing age. In the Beaver Dam Eye study (Klein *et al*., 1992), the prevalence increased from 0.9% in people with 43 to 54 years of age to 4.7% in those >75 years of age. Similarly in the Barbados Eye Study (Leske *et al*., 1994), a steep increase in prevalence of glaucoma was noted from 14.8% at ages 70 to 79 to 23.2% at ages 80 to 86 years.

ii) Race: Studies have shown that POAG is more prevalent in blacks compared to whites (Shields 1998). In a population based study comprising of 5308 individuals, the prevalence of glaucoma for age groups >40-80 years were 1.23% -11.26% among blacks and 0.92% - 2.16% among whites (Tielsch *et al*., 1991). The Barbados Eye Study showed that 1 in 11 Afro-Caribbeans over the age of 50 years and 1 in 6 over the age of 70 years had POAG (Leske *et al*., 1994). Studies suggested that Black Americans are more likely to become blind as a result of POAG than White Americans (Racette *et al*., 2003). Another study (Munoz *et al*., 2000) showed that black Americans are 16 times more likely than white Americans to develop POAG-associated visual impairment in terms of best-corrected visual acuity.

iii) Family history: Relatives of open angle glaucoma appear to be at a higher risk of developing glaucoma. The Barbados Family Study of open angle glaucoma showed that 39% of the recruited probands had one or more family members affected with POAG (Leske *et al*., 2001). In the Baltimore eye survey, 16.1% of the cases reported a positive family history of glaucoma among first-degree relatives compared to 7.2% controls (Tielsch *et al*., 1994). Age adjusted association of POAG with family history of glaucoma was higher in siblings (OR = 3.69), than in parents (OR = 2.17) or children (OR = 1.12) (Tielsch *et al*., 1994).

iv) Intraocular pressure: IOP is an important risk factor for development of POAG and most studies agree that it is the single most important prognostic risk factor. The degree of risk for developing glaucomatous damage is related to the level of IOP (Shields, 1998). The probability of glaucoma was found to be zero at IOP <18 mm Hg, while it reached to 0.5 at 27-28 mm Hg and 1 at 35 mm Hg (Davanger *et al*., 1991).

Apart from these, diabetes, hypertension, gender was also found to be associated with POAG, but their role as risk factors are yet to be established (Shields, 1998).

2.7 GENETICS OF POAG

Open angle glaucoma is a genetically heterogeneous disorder. So far 11 loci (Table 2) have been mapped to glaucoma by linkage. Of these, 3 genes at *GLC1A (MYOC)*, *GLC1E (OPTN)* and *GLC1G (WDR36)* have been characterized. The mutation spectrum of these genes have been determined in different populations worldwide. The *CYP1B1* gene, which is candidate gene for primary congenital glaucoma (PCG), was also implicated in early onset open angle glaucoma.

Table 2. List of candidate loci/genes identified for open angle glaucoma

S.No	Locus	Region	Gene	Reference
1	*GLC1A*	*1q24.3-q25.2*	*MYOC*	Stone *et al.*, 1997
2	*GLC1B*	*2cen-q13*	-	Stoilova *et al.*, 1996
3	*GLC1C*	*3q21-q24*	-	Wirtz *et al.*,1997
4	*GLC1D*	*8q23*	-	Trifan *et al.*, 1998
5	*GLC1E*	*10p15-p14*	*OPTN*	Rezaie *et al.*, 2002
6	*GLC1F*	*7q35-q36*	-	Wirtz *et al.*,1999
7	*GLC1G*	*5q22.*	*WDR36*	Monemi *et al.*, 2005
8	*GLC1H*	*2p16.3-p15*	-	www.gene.ucl.ac.uk/nomenclature
9	*GLC1I*	*15q11-q1*	-	Allingham *et al.*, 2005
10	*GLC1J*	*9q22*	-	Wiggs *et al.*, 2004
11	*GLC1K*	*20p12*	-	Wiggs *et al.*, 2004
12	*GLC3A*	*2p21*	*CYP1B1*	Stoilov *et al.*, 1997

A review of these candidate genes is given below:

2.7.1 MYOCILIN

The gene *MYOC* was initially linked to the *GLC1A* locus on 1q21-31 in a multi-generation, autosomal dominant juvenile form of open angle glaucoma (Sheffield *et al.*, 1993). Stone *et al*. (1997) identified mutations in *MYOC* gene in juvenile and adult onset glaucomas (JOAG/POAG) in variable frequencies. In an independent study, myocilin was described as myocin like acidic protein, expressing predominantly in photoreceptors of retina, hence the name myocilin (Kubota *et al*., 1997). Later Nguyen *et al*. (1998) identified a protein, which is overexpressed in TM cells when treated with Dexamethasone, and named it as Trabecular meshwork Inducible Glucocorticoid Response protein (*TIGR*) and is now referred to as myocilin.

MYOC gene structure

The genomic structure of *MYOC* consists of 3 exons and a 5kb promoter. Exon 1 resembles myocin like domain and exon 3 resembles olfactomedin like domain. The promoter region includes (a) the putative sequences for consensus TATA and CAAT box sequences (b) multiple hormone and cell signaling response elements including 7 glucocorticoid response elements (GREs) and 3 estrogen response elements (EREs) (c) elements that could correlate to oxidative damage, DNA damage and heat shock responses including one NF-κB and four shear stress response elements (SSRE) and two heat shock protein response elements (HSPRE). The promoter also contains a proximal 13 GT repeats and a distal 6 CA repeat unit

(Nguyen *et al*., 1998). The cDNA codes for a glycoprotein of 504 amino acids.

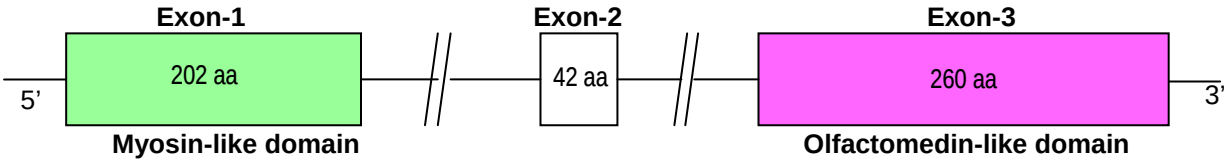


Figure 6. Schematic representation of *MYOC* gene (Nguyen *et al*., 1998).

Myocilin protein structure and properties

Myocilin is a glycoprotein that exists in glycosilated and non-glycosilated forms of molecular weights of 66 and 55 kDa, respectively. It is overexpressed by steroids like dexamethasone in a dose dependent manner (Nguyen *et al*., 1998). As shown in Figure 6, it has two major domains; an N- terminal myosin like domain having homology to the non-muscle myosin of *Dictyostelium discoideum* and the C- terminal olfactomedin-like domain resembling olfactomedin of the bullfrog (Kubota *et al*., 1997). Myocin is mainly an alpha helical protein and olfactomedin consists mainly beta sheets and turns. The secondary structure of myocilin showed N- terminal alpha helix and C-terminal beta sheet, leading to a hypothesis that myocilin might have evolved by fusion of two different proteins with the inclusion of second exon in higher organisms (Mukhopadhyay *et al*., 2002). The Myocin-like domain consists of a leucine zipper of 7 leucine residues, which might be responsible for interaction of myocilin with other leucine zippers present in other proteins (Kubota *et al*., 1997). It also contains a signal sequence of 32 amino acids at the N-terminal, generally found

in extracellularly secreted proteins. The olfactomedin-like domain is highly conserved among various species. More than 60 mutations found in JOAG/POAG are located in this domain, suggesting its role in the disease pathogenesis.

Myocilin Interactions with other proteins

Myocilin is a 55 kDa protein that is localized both intracellularly and extracellularly in the TM (Ueda *et al*., 2000). Intracellularly it was found to be associated with mitochondria, vesicles, intermediate filaments and actin stress fibers, and extracellularly in the extracellular matrices in the juxtacanalicular region. It is a secretary protein, found in the aqueous humor of human, bovine and porcine samples (Rao *et al*., 2000). Yeast two-hybrid studies and *in vitro* binding assays showed that MYOC can interact with itself (Fautsch *et al*., 2001). These myocilin-myocilin interactions occur through the leucine zipper motif involving the amino acid sequence 117-166. Extracellular localization of myocilin in the juxtacanalicular tissue was also well characterized by immunoelectron microscopic studies (Ueda *et al*., 2002). Myocilin was found to co-distribute extensively with microfibrillar-associated elements including fibrillin-1, MAGP-1, and fibronectin and moderately with decorin and type-IV collagen in juxtacanalicular tissue (Ueda *et al*., 2002).

Mechanism of pathogenesis

The exact mechanism by which mutant myocilin causes glaucoma is not known. It is not required for normal development, fertility, or viability in mice. The lack of phenotype in both +/- and -/- mice suggests that haploinsufficiency is not the mechanism by which it causes glaucoma and these mutations are likely to act by gain of function (Kim *et al*., 2001). The clustering of mutations in olfactomedin domain and the notable paucity of glaucoma associated nonsense mutations also argues against a haploinsuficiency model (Liu *et al*., 2004). A 29 year old patient who demonstrated a complex deletion of the maternal copy of chromosome 1 did not manifest the glaucoma phenotype (Wiggs *et al*., 2001). On ocular examination, IOPs were found to be 10 and 12 mm Hg in right and left eye respectively with normal macula and optic disc. A 77 year old Chinese patient harboring the R46X homozygous mutation did not manifest any symptoms of glaucoma (Lam *et al*., 2000). However a Korean patient who was homozygous for the same mutation was diagnosed of POAG at 15 years and her parents who are heterozygous for R46X did not manifest the phenotype (Yoon *et al*., 1999). All these studies suggest that haploinsufficiency is not the mechanism by which *MYOC* mutations cause glaucoma.

Mutant forms of myocilin are not secreted from the cells when expressed in HTM cell line and impaired the secretion of wild type protein when co-expressed with mutant form (Jacobson *et al*., 2001). It is likely that these mutant myocilins might associate with wild type proteins to form oligomers and multimers. Moreover these mutant

proteins are insoluble in detergents like Triton-X100 compared to the wild type, when expressed in HEK cells (Zhou *et al*., 1999). These biochemical studies provide evidence that *MYOC* mutations might be acting either by dominant negative effect or with the gain of function mechanism.

A translational pausing region was identified in the olfactomedin region of E323K mutant protein, which prolongs the time that myocilin, is processed in ER (Zimmerman *et al*., 1999). The non-secretion and translational pause of mutant myocilin suggest a mechanism in which mutant proteins overwhelm the ER associated degradation pathway leading to aggregate formation in ER and cellular dysfunction or death (Tamm *et al*., 2002), while other evidence indicates that mutant myocilin might trigger ER stress response (Joe *et al*., 2003).

In a *GLC1A* linked French–Canadian family, 83 members who were heterozygotes for the K423E mutation manifested the disease phenotype but not the homozygotes (Morissette *et al*., 1998). They authors proposed “homoallelic complementation” as a disease mechanism in which mutated K423E has a dominant negative effect and, forms defective oligomers with native myocilin and functional homodimers within itself. Contrary to this, Sarfarazi *et al*. (2000) reported a Turkish family with G326R mutation where homozygotes showed a severe phenotype than heterozygotes. Therefore the exact mechanism by which *MYOC* mutations cause glaucoma still remains debatable.

Mutation spectrum

Mutations in *MYOC* were first identified by Stone *et al*. (1997) in a JOAG family to which *GLC1A* was mapped. A Tyrosine to Histidine substitution at codon 437 was found to segregate in the family having 22 affected individuals. They observed mutations in juvenile and adult onset POAG patients. Mutations were noted in 4.4% of the cases with a family history of glaucoma and 2.9% of unrelated adult onset POAG patients. Following this, several mutations in *MYOC* were reported from different parts of the world. In a larger study comprising 1703 individuals (consisting of five different populations from Iowa, African Americans from New York, Japan, Canada and Australia), mutations were found in all the populations with a frequency of 2 - 4% (Fingert *et al*., 1999). The Q368X was the most common mutation with a frequency of 1.6% in all populations except Japanese. In another larger study (Alward *et al*., 1998) comprising of 716 patients, mutations were observed in 4.6% of the cases. The Q368X mutation was the predominant mutation and found in 2% of the cases and 0.16% of the controls. It was observed that individuals with Y437H and I477N mutations manifested the disease four decades earlier than that of individuals with Q368X mutation. Alward *et al*. (2002) screened 779 individuals affected with POAG, JOAG, normal tension glaucoma, ocular hypertension and secondary conditions like pigmentary glaucoma and cortico-steroid induced glaucoma. Disease causing variations were observed in 3% of cases among the different phenotypes and the Q368X mutation was observed in 2.9% of the

cases. In Chinese population mutations were observed in 1.5% of the POAG cases (Pang *et al*., 2002). The Arg46Stop mutation was observed in patients and controls with equal frequency and one of the controls was homozygous for the same mutation with out showing symptoms of glaucoma. In Japanese population mutations were found in 4% (Suzuki *et al*., 1997) and 2.9% (Kubota *et al*., 2000) of POAG patients. The Pro370Leu mutation, which was observed here for the first time, was found to associate with juvenile onset glaucoma, high IOP and poor response to medical treatment (Taniguchi *et al*., 1999). So far, 73 disease-causing mutations have been reported in *MYOC* gene, of which 63 were from the olfactomedin-like domain (Gong *et al*., 2004) suggesting that this is a functionally important domain (Adam *et al*., 1997).

In India, mutations in *MYOC* have accounted for 2-7% of the POAG cases (Mukhopadhyay *et al*., 2002, Kanagavalli *et al*., 2003, Sripriya *et al*., 2004). A study consisting of 56 POAG patients from Eastern India revealed mutations in 7% of the cases (Mukhopadhyay *et al*., 2002). The Q48H mutation was observed in 5.3% of the cases and was the prevalent one (Chakrabarti *et al*., 2005). It has not been reported from any other population so far, suggesting that this could be unique to India. Another novel mutation P274R was observed in a four-generation autosomal dominant family and found to segregate with the disease phenotype (Markandaya *et al.,* 2004). The previously reported mutations G367R, T377M, were also observed in India (Kanagavalli *et al*., 2003).

2.7.2 CYTOCHROME P4501B1

The gene *CYP1B1* is a recently identified member of the *CYP1* gene family (Stoilov *et al*., 2001). Cytochrome P450s have an average mass of 50 kDa along with an iron protoporphyrin IX prosthetic group liganded to a cysteine thiolate (Stoilov *et al*., 2001). They are monooxygenases and accept two reducing equivalents and use them to reduce the molecular oxygen to water molecule and oxidize the substrate molecule. The members of CYP1 family are also known as xenobiotic metabolizing enzymes and oxidize a large number of lipophilic drugs. Most of these are membrane bound and anchored to the endoplasmic reticular membrane or inner mitochondrial membrane by a transmembrane amino terminus. The structural domains (Stoilov *et al*., 2001) of the these proteins are:

1. The hydrophobic N- terminal of the protein contains a membrane insertion sequence and a stop-transfer sequence that functions as an anchor to the membrane.

2. A proline-rich hinge region, which imparts flexibility between the transmembrane region and the catalytic part of the cytosolic region.

3. The C- terminal of the protein consists of conserved core structures that include a number of α helices, β sheets and a meander region.

Gene structure

CYP1B1 was cloned from dioxin treated human keratinocyte cell line (Sutter *et al*., 1994). It showed ~40% homology with both *CYP1A1* and *CYP1A2.* It differs in its chromosomal location (chromosome 2p21-22

and 15q22-24 for A1 and 15q22-qter for A2 respectively) and in number of exons (3 versus 7) from *CYP1A1* and *CYP1A2* (Murray *et al*., 2001). It consists of three exons and two introns (Figure 7). Its open reading frame starts in the second exon and consists of 1629 bp and codes for a protein of 543 amino acids. The *CYP1B1* gene is transcriptionally activated by polycyclic aromatic hydrocarbons (Ah), which act via Ah receptor complex (Murray *et al*., 2001). It is inducible by 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD or dioxin) (Sutter *et al*., 1994). Dioxin-responsive elements and basal regulatory sequences have been identified in the 5' regulatory region (Wo *et al*., 1997). *CYP1B1* lacks the consensus TATA box in the promoter region and contains nine TCDD- responsive enhancer core-binding motifs (Tang *et al*., 1996).

CYP1B1 expression has been found in various ocular tissues like pigmentary ciliary epithelial cells, iris, trabecular meshwork, and retinal neuroepithelium and in the tissues surrounding the optic nerve (Bejjani *et al*., 2002). Low constitutive levels of mRNA was detected in non-ocular tissues including heart, brain, placenta, pancreas, lung, liver, skeletal muscle and kidneys demonstrating higher levels of expression (Stoilov *et al*., 1998).

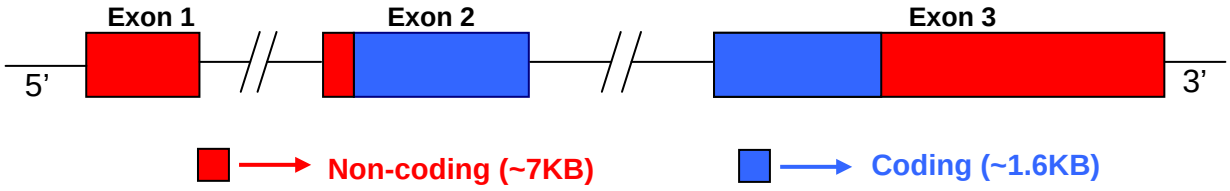


Figure 7. Schematic representation of *CYP1B1* gene (Stoilov *et al*., 1998)

It was shown that CYP1B1 is capable of metabolizing a variety of putative human carcinogens (Murray *et al*., 2001). It was also found to be overexpressed in a wide range of human cancers including cancers of the breast, colon, lung, esophagus, skin, lymph node, brain, and testis. There was no detectable immunostaining for CYP1B1 in normal tissues (Murray *et al*., 1997). Thus it appears to have potentially important roles in tumor development and progression as a target for anticancer drugs and as a tumor biomarker.

Genetics of *CYP1B1* in primary congenital glaucoma (PCG)

PCG is an autosomal recessive disorder, resulting from the maldevelopment of trabecular meshwork and anterior chamber angle leading to the obstruction of aqueous humor outflow and raised IOP. PCG is genetically a heterogeneous disorder and so far three chromosomal loci on 2p21 (*GLC3A*) (Sarfarazi *et al*., 1995), 1p36 (*GLC3B*) (Akarsu *et al*., 1996) and 14q24.3 (*GLC3C*) (Stoilov *et al*, 2002) have been mapped. Stoilov *et al*. (1997) identified *CYP1B1* as a candidate, which mapped to *GLC3A* locus gene in three Turkish PCG families. Following this many pathogenic mutations were identified in *CYP1B1* in various populations and the mutation frequency ranged from ~20% in Japanese (Mashima *et al*., 2000), ~33.3% in Indonesians (Sitorus *et al*., 2003), ~37.5% in Indians (Reddy *et al*., 2004), ~50% among the Brazilians (Stoilov *et al*., 2002) to almost 100% among the Saudi Arabians (Bejjani *et al*., 2000) and Slovakian Gypsies (Plasilova *et al*., 1999). Among various mutations G61E is the predominant

mutation in the Saudi Arabian population (Bejjani *et al*., 2000) and R368H in the Indian population (Reddy *et al*., 2003). Haplotypes analysis based on with five *CYP1B1* SNPs (R48G, A119S, V432L, and D449D), showed that the 'C-G-G-T-A' is the most common haplotype associated with different *CYP1B1* mutations (Chakrabarti *et al*., 2006).

The mechanism by which *CYP1B1* mutations lead to the PCG is not known, as the substrate for CYP1B1 in eye is not yet identified. Two possible hypotheses have been proposed to explain the involvement of *CYP1B1* in PCG (Stoilov *et al*., 2001).

1. The spatial and temporal expression of genes controlling the anterior chamber angle development may be altered by the absence of a regulatory molecule (such as steroid) produced by CYP1B1.
2. The developmental arrest might be a sign of toxic effect of a metabolite that is normally eliminated by CYP1B1.

Recently *CYP1B1* has been implicated in a di-genic mechanism in JOAG, in association with *MYOC* (Vincent *et al*., 2002). In this study, individuals harboring mutations both in *MYOC* and *CYP1B1* gene manifested JOAG phenotype and individuals with only *MYOC* mutations showed late-onset POAG phenotype suggesting that *CYP1B1* might be a modifier of *MYOC* expression and these two genes might be acting through a common biochemical pathway. Individuals carrying both *MYOC* and *CYP1B1* mutations had mean age of onset of 27 years and individuals only with *MYOC* mutations had mean age of onset of 51 years. *CYP1B1* mutations were also identified

in early onset open angle glaucoma patients in French population with 4.6% mutation frequency (Melki *et al*., 2004). The median age of diagnosis of these patients was 40 years (range from 13 - 52 years), which was significantly lesser than *CYP1B1* non-carriers. Recently *CYP1B1* mutations were also reported in Spanish POAG population with 10.9% frequency and in HTG cases with 8.1% frequency (Lopez-Garrido *et al.,* 2006). Involvement of *CYP1B1* was also reported in Indian POAG population (Acharya *et al*., 2006), where it accounted for 4.5% of the cases similar to that of French population. Six mutations were identified, of which three were reported in PCG patients. All the mutations were heterozygous except for R523T, which was homozygous.

2.7.3 OPTINEURIN

The *OPTN* gene (OMIM # 602432) was localized to *GLC1E* locus on 10p14-15 (Sarfarazi *et al*., 1998). Previously it was reported as 14.7K-interacting protein (FIP-2) (Li *et al*., 1998) and nemo related protein (NRP) (Schwamborn *et al*., 2000) and its product was found to interact with adenovirus E3-14.7K (Li *et al*., 1998), Huntingtin (Faber *et al*., 1998), transcription factor IIIA (Moreland *et al*., 2000), and RAB8 (Hattula *et al*., 2000). Rezaie *et al*., (2002) identified *OPTN* as a candidate gene for adult onset, normal tension glaucoma and designated this protein as “Optic Neuropathy Inducing protein”.

The *OPTN* gene contains 3 non-coding exons in the 5’ untranslated region and 13 coding exons (Figure 8). It codes for a 66-kDa protein consisting of 577 amino acids. Alternative splicing at 5’ untranslated region generates 3 different isoforms, having the same reading frame (Rezaie *et al*., 2002).

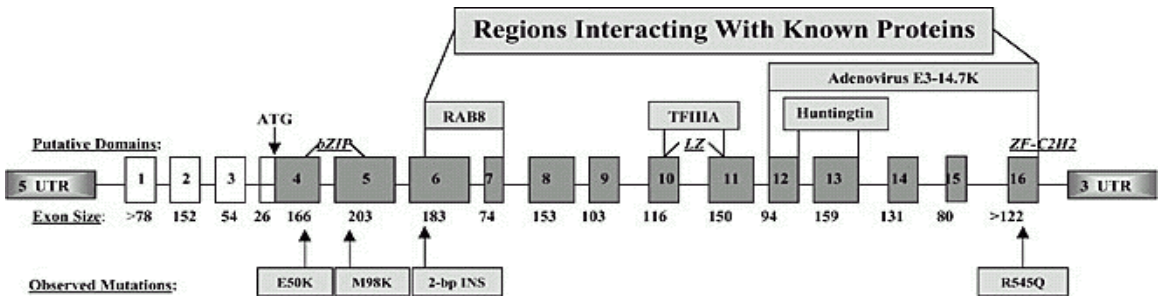


Figure 8. Schematic representation of *OPTN* gene (Rezaie *et al*., 2002)

The OPTN protein contains numerous predicted transcription factor domains, including two bZIP motifs, several leucine-zipper domains, coiled coil motifs and a C-terminal C2H2 type zinc finger domain (Li *et al*., 1998). This protein was found to be over expressed by TNFα in a dose dependent manner and co-localized with the Ad E3-14.7K in the cytoplasm near the nuclear membrane (Li *et al*., 1998).

It is expressed in ocular and non-ocular tissues like heart, brain, placenta, liver, skeletal muscle, kidney and pancreas (Li *et al*., 1998). RT-PCR analysis of *OPTN* showed its expression in ocular and non ocular tissues including trabecular meshwork, non-pigmented ciliary epithelium, retina, brain, adrenal cortex, liver, fetus, lymphoblast and fibroblast (Rezaie *et al*., 2002).

The optineurin protein blocked the protective ability of E3-14.7K on TNFα mediated cell killing suggesting that it might be a component of the TNFα signaling pathway that can shift the equilibrium towards induction of apoptosis (Li *et al*. 1998). It was hypothesized that wild-type OPTN might play a neuroprotective role in the eye and optic nerve but when defective it causes optic neuropathy and visual loss (Rezaie *et al*., 2002).

Mutation spectrum

Three mutations (E50K, R545Q, and c.691-692ins AG) in *OPTN* were identified in a large study consisting of 54 patients and accounted for 16.7% of families with hereditary POAG, most of them having NTG (Rezaie *et al*., 2002). The polymorphic variant M98K was more

prevalent in patients than controls and was suggested to be a risk factor. The E50K mutation was located within a putative bZIP motif and was associated with severe phenotype along with early age at onset, advanced optic disc cupping, smaller neuroretinal rim area and progressing visual field loss (Aung *et al*., 2005). This mutation was not observed in any other population except in one NTG proband with family history (Alward *et al*., 2003). *OPTN* mutations were also found to be associated with high-pressure JOAG in the Greater Toronto population and it was proposed that sequence variations in *OPTN* might influence the expression of *MYOC* (Willoughby *et al*., 2004). Since then many reports has been published from various populations contradicting the pathogenecity of R545Q and M98K variations. In populations including China (Leung, 2003), Japan (Toda *et al*., 2004, Funayama *et al*., 2004) and Canada (Willoughby *et al*., 2004), the R545Q variation was observed in controls with equal frequencies as that of patients. Similarly the M98K variation that was initially observed to be a risk factor was later on found in a number of controls. In a large study consisting of 1,048 patients, there was a marked difference in Japanese NTG patients and controls, but on a relatively lesser sample size (Alward *et al*., 2003). Another study in Japan revealed M98K to be associated with POAG as well as NTG (Fuse *et al*., 2004), while an independent study from the same population (Funayama *et al*., 2004) observed high frequency of M98K in NTG patients compared to POAG and controls. This was however not observed in another study (Toda *et al*., 2004).

There are two reports on the involvement of *OPTN* in POAG from India (Mukhopadhyay *et al*., 2005, Sripriya et al., 2006). No pathogenic mutations were observed. The R545Q variation was observed in 3% of the patients from eastern Indian region and not observed in controls (Mukhopadhyay *et al*., 2005) while it was not observed in HTG and POAG patients from South India (Sripriya et al., 2006). The M98K variation was not associated with disease phenotype in one study (Mukhopadhyay *et al*., 2005) while in another study it showed association with both HTG and POAG cases (Sripriya et al., 2006). This supports the fact that *OPTN* has limited involvement. The IVS+24G>A polymorphism present in HTG group showed association with increased IOP at diagnosis and also created a binding site for transcription factors NF-1 and CPE which were absent in wild type (Sripriya et al., 2006).

2.8 SINGLE NUCLEOTIDE POLYMORPHISM SCREENING IN POAG

POAG is a complex disorder and multi-factorial in nature. Apart from the mutations in the candidate genes, SNPs in 16 other candidate genes were found to be associated to POAG (Fan *et al*., 2006). Of these, SNPs in the *p53*, *ApoE* and *TNF*α genes were evaluated with glaucoma in the present study.

2.8.1 *P53* POLYMORPHISM

The *p53* gene codes for a transcription factor which regulates the expression of wide variety of genes involved in cell cycle regulation and apoptosis in response to genotoxic or cellular stress. The *p53* gene is located on chromosome 17p13.1 and has 11 exons spanning 20 kb. It is a 53 kDa protein with 393 amino acids and is organized into 5 structural and functional domains: transcriptional activation domain, DNA binding domain, proline rich region, oligomerization domain and regulatory domain.

Under normal conditions, p53 protein levels are low in cells and get accumulated following damage to the cell. p53 suppresses cell growth in response to DNA damage by two mechanisms: cell-cycle arrest (for DNA to repair) and apoptosis (if damage is not repaired) (Janus *et al*., 1999).

Mechanisms of p53 mediated cell cycle arrest

1. DNA damage arrests the cell cycle at two checkpoints: the G1 / S phase before DNA replication and the G2 / M phase before

chromosome segregation. The p53 arrests the cell cycle at G1 / S phase by *p16-cyclin D1-cdk4-Rb* pathway (Levine *et al*., 1997).

2. Upon DNA damage the p53 protein gets accumulated which in turn induces the expression of *p21* gene.
3. p21 inactivates Cyclin Dependent Kinases (CDK) thus leading the cell into G1 arrest.
4. Formation of these complexes leads to the accumulation of hypo-phosphorylated retinoblastoma protein, causing the release of the E2F transcription factor, which is necessary for DNA synthesis.

Mechanisms of p53-mediated apoptosis

1) The loss of integrity of the mitochondrial membrane during apoptosis is followed by release of cytochrome C into the cytosol, which in turn leads to activation of caspase cleavage. Antiapoptotic genes *Bcl-2* and proapoptotic genes *Bax* regulate release of cytochrome C. *Bax* has been shown to be upregulated in response to DNA damage and increased p53 levels (Miyashita *et al*., 1995). *Bcl-2* has been shown to be transcriptionally repressed by p53.
2) Reactive oxygen species (ROS) are powerful activators of mitochondrial damage and apoptosis. A number of genes that increase production of ROS and therefore oxidative stress, have

been found to be induced by p53 or p53-induced genes (PIGs). (Lotem *et al*., 1996, Johnson *et al*., 1996).

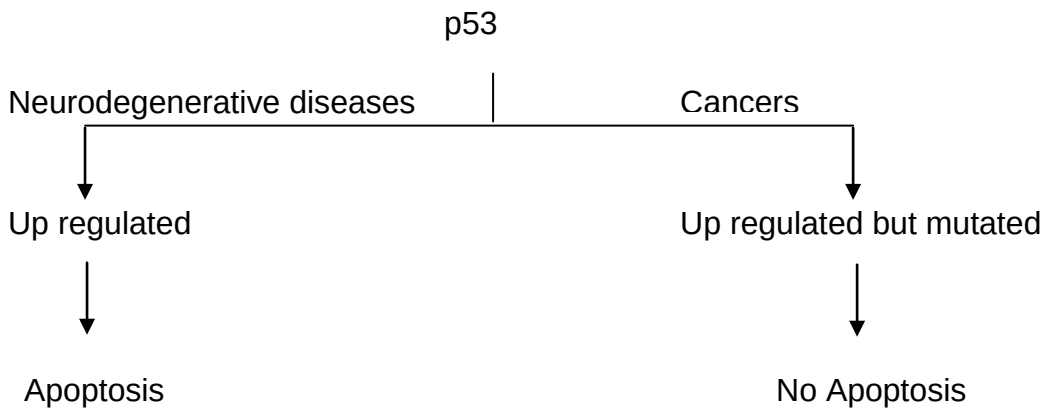
3) p53 may upregulate Fas, death receptor and induce Fas mediated death (Bennett *et al*., 1998). Fas when activated by FasL, its ligand, trimerizes resulting in caspase activation. Overexpression of Fas can cause apoptosis and is thought to be p53 dependent, although the mechanism is not yet clear (Owen-shaub *et al*., 1995).

Role of p53 in POAG

POAG is a complex neurodegenerative disorder resulting in an irreversible loss of vision due to ganglion cell death (Flammer, 2003). The exact mechanism underlying the loss of RGCs in POAG is not known. Nickells *et al*. (1999) suggested that RGC death by apoptosis is an important mechanism behind glaucoma. During apoptosis, cellular distress signals activate p53, which in turn regulates the subsequent apoptotic events by regulating the expression of proapoptotic and antiapoptotic genes.

Mutations in *p53* gene have been detected in about 50% of human malignancies (Hollstein *et al*., 1994). The mutated p53 fails to initiate the apoptotic process resulting in the uncontrolled growth of damaged cells (Sekido *et al*., 2003). However, in neurodegenerative diseases, the *p53* gene is up regulated in response to cellular stress and promotes cell death by apoptosis. Hence there is a possibility that genetic variants of p53 might influence the rate of neuronal cell loss in

neurodegenerative diseases including POAG (Ressiniotis *et al*., 2004). Therefore it is important to study the association of p53 polymorphisms and POAG.



The codon 72 polymorphism in *p53* resulting in the substitution of arginine with proline was associated with cancers of lung (Wang *et al*., 1999), breast (Sjalander *et al*., 1996) and cervix (Zehbe *et al*., 2001). Hence Lin *et al*. (2002) suspected a possible role of this polymorphism in POAG. Their study on 58 POAG patients and 59 controls revealed that the proline allele was significantly associated with POAG (P=0.00782). In another study, Ressiniotis *et al*. (2004) reported an association (P<0.0001) between arginine allele with patients in a cohort of 140 POAG patients and 73 controls from British population, while Dimasi *et al*. (2005) found no association with codon 72 and intron duplication in a cohort of normal tension and high-tension glaucomas. In Indian population, Acharya *et al*. (2002) observed that the codon 72 polymorphism and 16 bp duplication in 3rd intron was not associated with POAG.

2.8.2 *APOE* POLYMORPHISM

The protein components of lipoproteins are known as apolipoproteins (APOEs). In humans, 9 different kinds of lipoproteins are distributed in significant amounts in the plasma. ApoE is a 229 amino acid long arginine-rich lipoprotein and its gene *ApoE* is located on chromosome 19q13.2. It is a monomeric protein consisting of two independently folded domains. The N- terminal domain binds strongly to the low-density lipoprotein (LDL) receptors and C- terminal binds to the lipoprotein surface (Voet *et al*., 1995). It is an essential component of circulating lipoproteins and acts as a ligand for low-density lipoprotein receptors. It has three major isoforms: apoE2, apoE3 and apoE4. These three forms are coded by three different alleles ε2, ε3, ε4 and were identified by isoelectric focusing. The ε2, ε3, ε4 isoforms differ in amino acid sequence at two positions, residue 112 (site A) and residue 158 (site B). At sites A/B, ε2, ε3 and ε4 alleles will contain cysteine/cysteine, cysteine/arginine, and arginine/arginine residues, respectively (Weisgraber *et al*., 1981, Rall *et al*., 1982. ε3 is the most frequent allele (representing 74 to 86% of all alleles) in European and American Caucasian populations (Souza *et al*., 2003).

Association with diseases

Although the mechanism by which *ApoE* participates in different pathogenic processes remains unclear, it seems to play an important role in neuronal degeneration and beyond this appears to be involved in stress-induced injury that could affect many tissues and organs

(Copin *et al*., 2002). The ε4 allele was associated with late onset Alzheimer's disease (AD) in Brazilians (Souza *et al*., 2003) and the ε3/ε4 and ε4/εE4 genotypes were found to result in earlier age of onset compared to the patients with ε3/ε3 allele in Parkinson disease (Zareparsi *et al*., 2002). But the same ε4 allele was identified as protective allele and observed to reduce the risk of age related macular degeneration (AMD) in a large cohort of AMD patients (Zareparsi *et al*., 2004).

Role in Glaucoma

Copin *et al*. (2002) reported that two *APOE* promoter polymorphisms which were previously associated with Alzheimer disease modify the POAG phenotype. They observed that *APOE* –219 polymorphism was associated with increased optic nerve damage and –419 along with the *MYOC* (-1000) promoter resulted in increased IOP and poor response to IOP lowering treatment. These findings could explain the linkage to chromosome 19q and an increased frequency of glaucoma in patients with AD. However this association was not observed in NTG (Lake *et al*., 2004) and in POAG patients (Ressiniotis *et al*., 2004).

Based on similarities in cellular events leading to degeneration in Alzheimer's disease and glaucoma, Vickers *et al*. (2002) looked for an association of APOE isoforms to glaucoma susceptibility. They studied 70 NTG and 72 HTG cases from Tasmania and found the ε4 allele to be associated with elevated risk for glaucomatous changes in NTG and HTG. In contrast, no association was found between ε4 allele

and POAG from Northeast England (Ressiniotis *et al*., 2004). In the Japanese population, the ε3 allele was observed with higher frequency in POAG patients compared to controls. But the role of ε3 allele as a risk factor is debatable as it is a common allele in normal population in Japan (Mabuchi *et al*., 2005).

2.8.3 TUMOR NECROSIS FACTOR ALPHA (*TNFα*)

TNFα is a pleiotropic pro-inflammatory cytokine produced mainly by activated macrophages and in smaller amounts by several other types of cell (Ding *et al*., 2004). The gene *TNFα* has a length of approximately 5 kb and contains five exons. It maps to the human chromosome 7p14-21. TNFα is a protein of 185 amino acids glycosylated at positions 73 and 172. It is synthesized as a precursor protein of 212 amino acids. The cytokine possesses both growth stimulating properties and growth inhibitory processes. For instance, TNFα induces neutrophil proliferation during inflammation, but it also induces neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray, *et al*., 1997).

TNFα has two receptors, p55 and p75. The p55 receptor is a 55 kDa protein termed as TNF-R1. The p75 receptor is a 75 kDa protein termed as TNF-R2. All the TNF functions are mediated by these two receptors and the apoptotic effects are only mediated by TNF-R1. In contrast, TNF-R2 may serve to potentiate the effects of TNF-R1 in promoting cell death or promoting inflammation (Wang *et al*., 2003).

Activation of caspase-8, a proximal effector protein, is known to be an important step in the TNF receptor family cell death pathway (Hsu *et al*., 1995). Therefore the observation of retinal caspase-8 activation in vitro and in vivo led to the idea that TNFα mediated cell death may be involved in glaucomatous neurodegeneration (Tezel *et al*., 2001). Supporting this hypothesis, histopathological studies revealed an increased immunostaining for TNFα and its receptors in the glaucomatous optic nerve head compared to age matched control eyes (Yuan *et al*., 2000). The levels of TNFα and TNFα receptors were also found to be increased in the retina of glaucomatous eyes when compared to normal eyes (Tezel *et al*., 2001). The localization of TNFα was prominent in glial cells, TNFα receptor-1 was mainly localized to RGCs. This provides evidence that RGCs are sensitive targets for the cytotoxic effects of TNFα that is produced by glial cells in glaucomatous retina. *In vitro* studies using primary co-cultures of RGCs and glial cells provided direct evidence that elevated pressures and ischemia, which are the prominent stress factors in glaucoma patients, can initiate apoptosis cell death cascade in retinal ganglion cells (Tezel *et al*., 2000). All these observations provided additional evidence that *TNFα* may have a role in neurodegeneration in glaucoma.

Herrmann *et al*. (1998) screened the entire coding region *TNFα* and 1053 bp upstream of the transcription start site, by PCR-SSCP and by sequencing and observed four SNPs at -857, -851, -308 and –238 in the upstream region and one in the non-translating region. The –308G>A polymorphism was associated with various diseases like

cerebral infarction (Um *et al*., 2003) and diabetes mellitus (Furuta *et al*., 2002).

Role in glaucoma

In glaucoma, association of *TNF*α –308G>A polymorphism was found to be associated with POAG in Chinese population (Lin *et al.,* 2003). In Japanese patients, *TNF*α/□/□polymorphisms in combination with *OPTN* variations resulted in a severe phenotype (Funayama *et al.,* 2004). Individuals with *TNF*α/-857T and *OPTN*/412A alleles had significantly worse visual fields than those without *OPTN*/412A. Patients with *TNF*α/ -863A and Lys98 alleles showed worse visual field scores than those without *OPTN*/Lys98. A recent report (Mossbock *et al.,* 2005) showed no association between POAG and *TNF*α polymorphisms –238G>A and -308 G>A in Caucasian populations. There are no reports so far from India on association between *TNF*α polymorphisms and POAG.

2.9 SECONDARY GLAUCOMAS

Secondary glaucomas are characterized by associated ocular or systemic abnormalities that appear to be responsible for the alteration in the aqueous humor dynamics. These glaucomas are classified either based on the underlying etiologic factor that ultimately leads to elevated IOP or through mechanism that leads to elevation of IOP (Ritch *et al*., 1989). Classification by etiology is limited by the fact that the primary factor is not completely understood in many forms of secondary glaucoma. Based on their mechanism, these glaucomas are classified into two types: Secondary open angle glaucomas and secondary angle closure glaucomas (Ritch *et al*., 1989).

1. **Secondary open angle glaucomas** are those in which anterior chamber angle structures are visible by gonioscopy. These are again divided into pre-trabecular, trabecular and post-trabecular depending on site where aqueous humor flow is getting obstructed (Ritch *et al*., 1989).
2. **Secondary angle closure glaucomas** include conditions in which the peripheral iris is in apposition to the trabecular meshwork or peripheral cornea. These are again divided into anterior, posterior or congenital depending on whether peripheral iris is pushed (posterior), pulled (anterior) or as a result of congenital anomaly.

Anterior Segment Dysgenesis (ASD)

ASD include various kinds of anomalies such as Axenfled Rieger group anomalies, which are characterized by malformation of eyes, teeth and umbilicus. This group of anomalies includes different kinds of phenotypes such as Rieger syndrome, Axenfeld anomaly and Rieger anomaly (Shields, 1998). It was observed that most of the ocular and facial structures involved in these developmental disorders have an origin in the neural crest.

In 1920, Axenfeld described a patient with a white line on the posterior aspect of the cornea near the limbus and tissue strands extending from the peripheral iris to this prominent line and termed these clinical features as “posterior Embryotoxon of the cornea”. In 1934, Rieger reported cases with similar anterior segment anomalies but with additional changes in the iris, including corectopia, atrophy and hole formation ad coined the term “mesodermal dysgenesis of the cornea and iris”. Some of these patients were also having systemic developmental defects, especially of the teeth and facial bones.

Traditionally, all these clinical features are classified into the following three categories (Shields, 1998):

Table 3. Clinical features of Axenfeld’s clinical phenotypes

S.No.	Phenotype	Clinical features
1	Axenfeld anomaly	Defects limited to peripheral anterior segment
2	Rieger anomaly	Peripheral abnormalities with additional changes in the iris
3	Rieger syndrome	Ocular anomalies plus extra ocular developmental defects

2.10 AXENFELD RIEGER SYNDROME

An alternative term Axenfeld Rieger Syndrome (AR syndrome) was proposed for all the clinical variations within this spectrum of developmental disorders because of their overlapping phenotpes (Alward *et al*., 2000).

Clinical features

The AR syndrome manifests generally during infancy or early childhood but may also manifests in adulthood. All patients with AR syndrome share the same clinical features, irrespective of their ocular manifestations. The commonly involved structures are peripheral cornea, iris and anterior chamber angle. The characteristic abnormality of the cornea is a prominent anteriorly displaced Schwalbe's line. A prominent schwalbe's line with no other evidence of the AR syndrome is referred as posterior embryotoxon and is reported to occur in 8 to 15% of general population. Tissue strands bridge the anterior chamber angle from the peripheral iris to the prominent ridge. Beyond the tissue strands, anterior chamber angle is open and TM is visible but the scleral spur inserts into the posterior portion of the meshwork. In AR syndrome defects in iris will range from mild stromal thinning to marked atrophy with hole formation, corectopia and ectropion uveae. Around 50% of the ARS patients progress to glaucoma. Generally glaucoma manifests during childhood or young adulthood, sometimes even during infancy (Shields, 1998).

The systemic abnormalities associated with AR syndrome are redundant periumbilical skin, developmental defects of the teeth

(Figure 9) and facial bones. The dental abnormalities include a reduction in crown size (microdontia), a decreased but evenly spaced number of teeth (hypodontia) and absence of teeth (anodontia). Facial anomalies include maxillary hypoplasia with flattening of mid face and a receding upper lip and prominent lower lip, especially in association with dental hypoplasia (Shields, 1998).

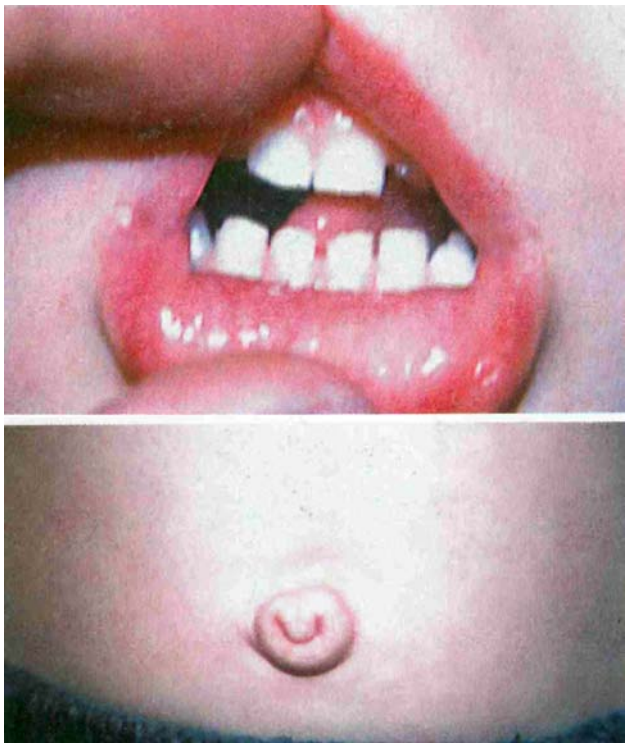


Figure 9. Dental abnormalities (top) and redundant periumbilical skin (bottom) in AR patients (Kanski JJ. *Clinical Ophthalmology: A systematic approach*. 5th ed. Butterworth-Heinemann; 2003: p.250)

Mechanism

Based on clinical and histopathologic observations, a developmental arrest of certain anterior segment structures derived from neural crest cells has been postulated as the mechanism of the AR syndrome (Shields, 1998). During embryogenesis, neural crest

cells differentiate to form a large portion of the anterior segment of the eye. The neural crest cells and mesoderm migrate into the developing eye in three waves after the basement membrane of the surface ectoderm and the lens vesicle separate. The clinical manifestations of ARS are thought to arise when there is a faulty migration of the third wave of neural crest cell migration (Churchill *et al*., 1996, Figure 10).

This leads to the abnormal retention of the primordial endothelial layer on portions of the iris and anterior chamber angle and alterations in the aqueous humor outflow structures. The developmental arrest also accounts for high insertion of anterior uvea into the posterior TM and results in the incomplete maturation of the TM and schelmm's canal.

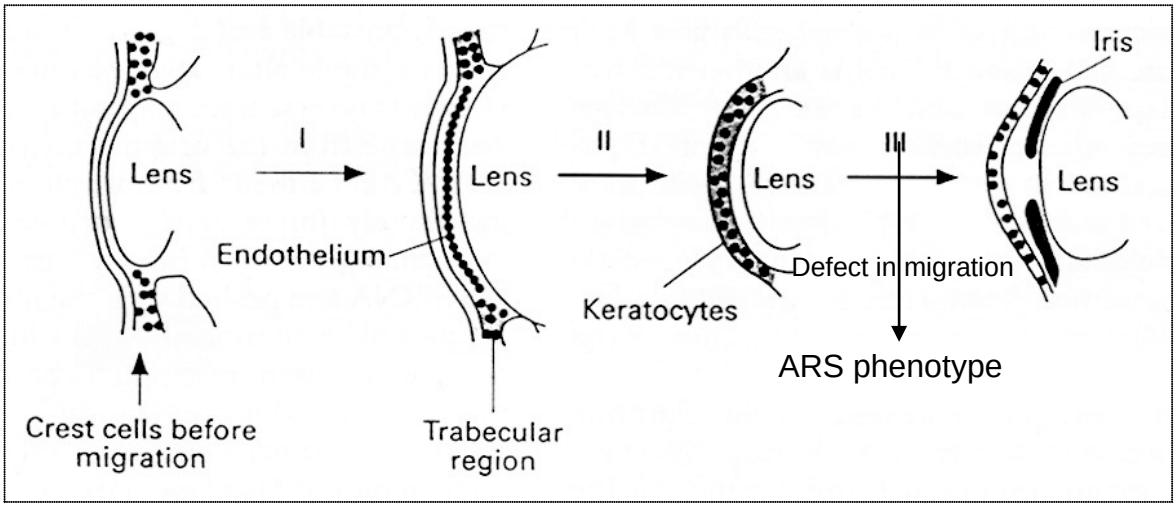


Figure 10. Schematic diagram of neural crest cell migration

(Churchill *et al*., 1996)

2.11 GENETICS OF AXENFELD RIEGER SYNDROME

ARS has been linked to four loci on chromosomes 4q25 (*PITX2*), 6p25 (*FOXC1*), 13q14 (gene not yet identified) and chromosome 11 (*PAX6*). The *PITX2* gene was identified by positional cloning and 6 different mutations were identified in 10 ARS families (Semina *et al*., 1996). The *FOXC1* gene was identified in two PCG patients having other anomalies by cloning the balanced translocation break points between 6p25 and 13q22 (Nishimura *et al*., 1998).

2.11.1 Forkhead box transcription factor C1 (*FOXC1*)

FOXC1 (OMIM # 601090) is a member of winged-helix/forkhead family of transcription factors. This family of transcription factors is distinguished by a highly conserved 110 amino acids DNA binding domain, known as fork head domain (FHD). This FHD was first identified as a region of homology between the *Drosophila melanogaster* protein fork head (Weigel *et al*., 1989) and rat hepatocyte nuclear factor 3 protein (Lai *et al*., 1991). Forkhead domains are evolutionary conserved and exist in a wide range of species from yeast to humans (Kaufmann *et al*., 1996). This DNA binding motif is a variant of helix–turn-helix motif and consist of three α helices and two large loops that form wing structure, hence they named it as the winged helix (Clark *et al*., 1993). It was first cloned from a craniofacial cDNA library along with six other human forkhead orthologues (Pierrou *et al*., 1994). Later it was mapped to a subtelomeric region at 6p25 by fluorescence in situ hybridization and

somatic cell hybrid analysis (Larsson *et al*., 1995). Members of this family of transcription factors play key roles in development including morphogenesis and cell fate specification (Kaufmann *et al*., 1996).

FOXC1 gene has a single exon without introns and codes for a protein of 543 amino acids. Recent studies using *FOXC1* deletion constructs identified functionally important domains, which are involved in nuclear localization and transcriptional regulation (Berry *et al*., 2002). Two regions in Forkhead domain are required for correct localization of FOXC1 into the nucleus. The first region spanning from residues valine at 77 to asparagine at 93 is necessary for proper nuclear localization of FOXC1, serving as nuclear localization signal (NLS) accessory domain. The second region contains a stretch of basic amino acids from 169 to 176 at the C–terminal end of the FHD and is necessary for FOXC1 nuclear localization, representing a bonafied NLS.

FOXC1 contains N- and C- terminal transcriptional activation domains form positions 1 (methionine) to 51 (alanine), and 466 (histidine) to 553 (phenylalanine), respectively (Figure 11). Both regions are required for full activation of FOXC1 responsive reporter gene and each region is individually capable of activating transcription.

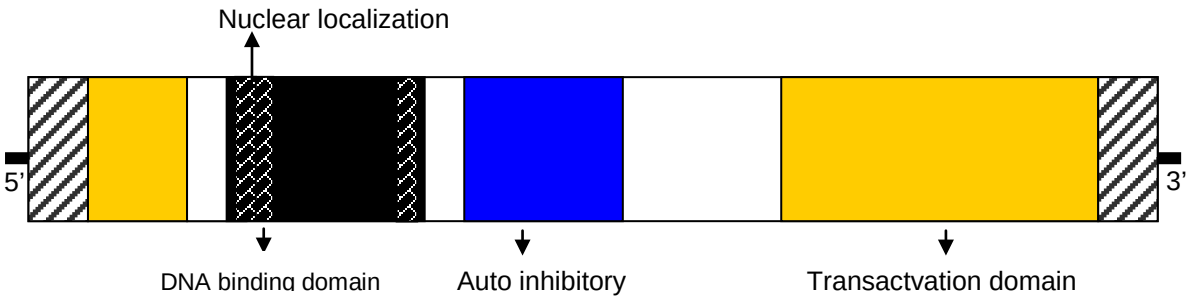


Figure 11. Schematic representation of *FOXC1* gene (Lines *et al*., 2002)

The mouse gene *Mf1*, which encodes a forkhead/winged helix transcription factor, is the mouse homolog of *FKHL7*. Murine models of *FOXC1* null mutations show the spectrum of ocular and systemic defects seen in humans, revealing a broad role for FOXC1 throughout development. *FOXC1*$^{-/-}$ homozygous null mice die at birth with hydrocephalus and several skeletal anomalies, including absence of anterior chamber and open or absent eyelids (Kume *et al*., 1998; Hong *et al*., 1999; Kidson *et al*., 1999). FOXC1$^{+/-}$ heterozygous mice showed anterior segment defects similar to those found in human patients with *FOXC1* mutations, including iris hypoplasia, a displaced Schwalbe line and iridocorneal angle dysgenesis (Smith *et al*., 2000). Libby *et al*. (2003) demonstrated that Tyr activity modifies the phenotype in *FOXC1* +/- mice and also in mice deficient in *CYP1B1*, which have ocular drainage structure abnormalities resembling those reported in human primary congenital glaucoma patients.

FOXC1 was identified as a candidate gene in PCG patients having chromosomal anomalies involving 6p25, by cloning

chromosomal breakpoints with balanced translocation between 6p25 and 13q22 (Nishimura *et al*., 1998). They screened 19 probands, 13 individuals with anterior chamber defects and 6 with PCG. Four mutations were found in individuals mainly with Rieger anomaly. In a study comprising of 70 probands with anterior chamber defects, 9 mutations were observed resulting in various phenotypes, major being Reiger anomaly and Axenfeld anomaly (Nishimura *et al*., 2001). Mears *et al*. (1998) studied 21 patients including 5 familial cases and observed 3 mutations in individuals with Axenfeld Rieger anomaly (ARA) phenotype. Interstitial deletions and duplications were observed in individuals with Axenfeld Rieger syndrome and Iris hypoplasia patient (Lehmann *et al*., 2002). There are only two reports on ARA from India. Two novel nonsense mutations and a missense mutation (M161K) were observed in two autosomal dominant ARA families (Panicker *et al*., 2002, Komatireddy *et al*., 2003).

The effect of these mutations on structure and function of FOXC1 was studied by bioinformatic and biochemical analysis (Saleem *et al*., 2001, Saleem *et al*., 2003). Biochemical analysis indicated that all mutant proteins localize to the cell nucleus except P79L, P79T, I91S and I91T mutations, which involves mutation in putative nuclear localization signal. DNA binding experiments revealed that mutations decreased DNA binding capacity except for F112S and I126M, but these mutations reduced the *trans*-activation activity of FOXC1, along with other missense mutations. Biochemical analysis of missense mutations (M161K, G165R and R169P) in wing 2 demonstrated that

the M161K and R169P mutations disrupted the DNA binding activity of FOXC1 consistent with the hypothesis that wing 2 is necessary for DNA binding activity. But all these mutations exhibited reduced levels of transactivation activity (Murphy *et al*., 2004). This data provides strong evidence that explains the haploinsufficiency as a mechanism by which it causes AR malformations. The chromosomal duplications of 6p25 regions also resulted in anterior segment anomalies suggesting the gene dosage as a mechanism for *FOXC1* mutations (Nishimura *et al*., 2001).

2.11.2 Pituitary Homoeobox Transcription Factor – 2 (*PITX2*)

PITX2 (OMIM # 601542) is a member of the bicoid-like homeobox transcription factor family. The homeobox gene family members play fundamental roles in the genetic control of development including pattern formation and cell fate determination. The homeobox proteins contain a 60 amino acid homeodomain that binds DNA (Figure 12).

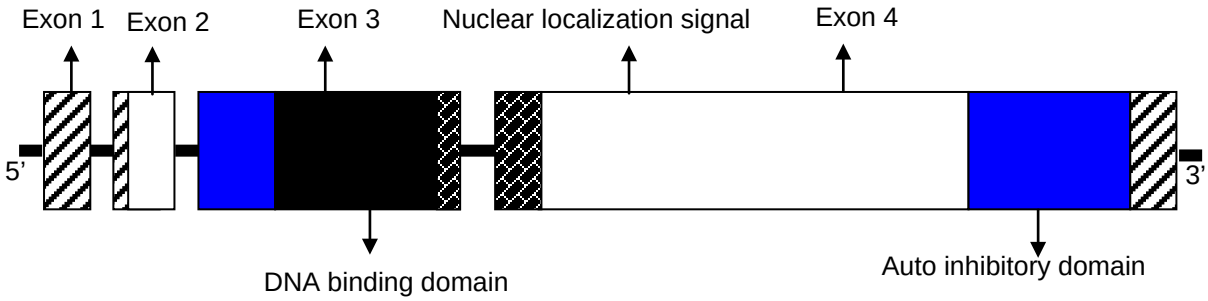


Figure 12. Schematic representation of *PITX2* gene (Lines *et al*., 2002)

PITX2 was identified as candidate gene for Rieger syndrome on 4q25, and cloned and characterized by Semina *et al*. (1996). It consists of 4 exons with translation initiation codon starting in the second exon. Its open reading frame codes for a protein of 271 amino acids. It contains a lysine residue at position 50 in the third helix of homeodomain, characterisic of the bicoid related protein. This lysine residue selectively recognizes the 3'-CC dinucleotide adjacent to the TAAT core. PITX2 binds to the DNA sequence 5'-TAATCC-3' (Amendt *et al*., 1998). In mice during embryogenesis, PITX2 was found to express in mesenchyme around the eye, dental lamina, the vitelline and umbilical vessels, limb bud and dorsal mesentry (Semina *et al*., 1996).

Mutations in *PITX2* have been found in various phenotypes including AR malformations, Iridogonio Dysgenesis (IGD), Iris Hypoplasia (IH) and Peter's anomaly (Lines *et al*., 2002). In a study comprising of 76 patients with a spectrum of phenotypes, 8 mutations were observed in individuals with Rieger anomaly, Axenfeld anomaly, Peters' anomaly and Aniridia (Perveen *et al*., 2000). Phillips *et al*. (2002) identified four novel mutations in Axenfeld Rieger syndrome patients. There are no reports on the involvement of *PITX2* in anterior chamber defect patients from Indian population.

Biochemical characterization including DNA binding and transactivation studies of the mutants causing IH, IGD and ARS showed residual activity for IH and IGD mutant proteins, whereas ARS mutant proteins showed no activity (Kozlowski *et al*., 2000). These

results were consistent with the hypothesis that mutant PITX2 proteins that retained residual or partial function result in the milder anterior segment abnormalities. Functional studies on V45L mutation in ARS showed decreased DNA binding activity but increased transactivation activity, suggesting that increased and reduced PITX2 activities is deleterious during development (Priston *et al*., 2001). Recently the link between the FOXC1 and PITX2 was identified (Berry *et al*., 2006) and it was shown that these two transcription factors interact physically by functional domains on both the proteins. It was also shown that PITX2A acts as a negative regulator of FOXC1 transactivity (Berry *et al*., 2006).

Chapter 3
Materials and Methods

MATERIALS AND METHODS

3.1 Enrollment of primary and secondary glaucoma cases and controls

The study protocol was approved by the Institutional Review Board and adhered to the guidelines of the Declaration of Helsinki. With prior informed consent, blood samples were collected from patients, who presented at the L V Prasad Eye Institute from different states of India between June 2002 and July 2005 (Table 4). Majority of the patients were from Andhra Pradesh. All the patients and their family members underwent complete ophthalmic examination that included gonioscopy, slit lamp bio-microscopy and Humphrey visual fields. Intra ocular pressure (IOP) was measured by applanation tonometry. Pedigrees were drawn for each family based on information from the probands and their relatives. The entire clinical data were documented in the form of medical records that included the age at onset, diagnosis, initial and final IOPs, initial and final cup to disc (CD) ratios, visual acuities and visual field defects.

3.1.1 Clinical examinations

All the clinical examinations were done by qualified ophthalmologist colleagues at the institute to whom we are deeply grateful.

All the patients were clinically evaluated by following methods.

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

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

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

d) Ophthalmoscopy (Direct and indirect, Heine, Germany)

A complete description of the methods used for clinical diagnosis are provided in Appendix-I.

Table 4. Geographical distribution of OAG and ARS cases

S.No.	State	Primary glaucomas (n=109)	Secondary glaucomas (n=25)
1	Andhra Pradesh	73 (66 %)	14 (56 %)
2	West Bengal	20 (18 %)	01 (04 %)
3	Maharashtra	06 (06 %)	-
4	Karnataka	04 (04%)	02 (08 %)
5	Chattisgarh	01 (01 %)	-
6	Jharkand	01 (01 %)	-
7	Kerala	01 (01 %)	-
8	Madhya Pradesh	01 (01 %)	02 (08 %)
9	Orissa	01 (01 %)	04 (16 %)
10	Rajasthan	01 (01 %)	-
11	Goa	-	01 (04 %)
12	Gujarat	-	01 (04 %)

3.1.2 Inclusion and Exclusion criteria

3.1.2.1 Inclusion and Exclusion Criteria for OAG patients

Patients with the following characteristics were included in the study:

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

Patients with IOP<21 mm Hg with open angles on gonioscopy and glaucomatous visual field defects were also included.

Exclusion criteria for OAG patients

Patients with the following characteristics were excluded from the study:

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

One hundred and nine OAG cases were enrolled for the study, which had a male to female ratio of 6.3:1. These patients were divided into 2 subgroups, adult-onset and juvenile onset OAG. Patients with onset at 30 years or more were classified as adult onset OAG and those below 30 years were enrolled as juvenile onset OAG.

3.1.2.2 Inclusion criteria for Axenfeld-Rieger Syndrome (ARS) cases

Patients with the following ocular and non-ocular findings were included in the study:

Ocular findings:

1. Posterior embryotoxon (a prominent, anteriorly displaced Schwalbe's line)
2. Iris strands adherent to Schwalbe's line, iris hypoplasia, focal iris atrophy with hole formation, corectopia, and ectropion uveae

Non-ocular findings:

1. Developmental defects of the teeth and facial bones
2. Pituitary anomalies
3. Cardiac disease
4. Oculocutaneous albinism, and redundant periumbilical skin.

Twenty-five ARS cases were collected with a male to female ratio of 2:3.

3.1.2.3 Inclusion criteria for controls

1. Older than 40 years of age
2. No family history of glaucoma and any other ocular disease
3. Normal optic disc (<0.4:1)
4. IOP < 18 mm Hg
5. Individuals without any systemic diseases

One hundred normal subjects who satisfied the above criteria were enrolled for the study. All the cases were independently diagnosed by two clinicians based on the inclusion criteria. Cases that had a disagreement among observers were excluded.

3.1.3 Clinical analysis

Clinical analysis was performed to compare the phenotypes of patients with and without mutations. The clinical parameters like IOP, CD ratio, visual acuity and visual fields were considered.

a) Intraocular Pressure

IOP readings were measured in both the eyes at the time of presentation and during subsequent follow-ups. The tonometer readings prior to pupil dilatation were considered.

b) Cup to Disc Ratio:

The enlargement of the cup with respect to optic disc was measured and the cup to disc (CD) ratio was estimated. The CD ratios were recorded for both the eyes at presentation and subsequent follow-ups.

c) Visual fields

Visual fields were evaluated through Humphrey visual filed analyzer to diagnose the disease at the time of presentation and monitor further progression. Visual fields were analyzed and categorized into mild (1), moderate (2) and severe (3) as per Anderson's criteria (Anderson *et al.,* 1987a).

d) Visual acuity

The best-corrected visual acuity measured through ETDRS chart was recorded at the time of presentation and subsequent visits.

3.2 Molecular analysis

Molecular analysis included the isolation of DNA from blood samples and its quantification, amplification of candidate genes by PCR and mutation screening by SSCP and sequencing.

3.2.1 Genomic DNA extraction

Blood samples from patients and controls were collected in heparinised vacuettes (Vacuette, Greiner bio-one GmbH, Austria) and stored at – 20º C until the DNA was extracted. The DNA was isolated by phenol-chloroform extraction method with slight modifications (Sambrook *et al.,* 1989). Further details of this method are provided in Appendix-II.

3.2.2 Estimation of DNA quantity

The concentration and purity of DNA was estimated by UV spectrophotometry (UV-1601, Shimadzu). DNA was diluted 100 times using autoclaved MilliQ water and absorbance values at 260 nm and 280 nm were recorded. The OD at 260 nm was used to calculate the concentration of DNA. The ratio of ODs at 260 nm and 280 nm was used to estimate the purity of DNA. Normally a ratio of 1.8 indicates good quality DNA, while that below 1.8 indicates protein or phenol contamination and above 1.8 indicates RNA contamination. The

concentration of DNA was calculated using the following formula (Sambrook *et al.,* 1989).

Concentration of DNA = OD at 260 nm X 50 X 100 (dilution factor)

(1 OD at 260 nm = ~ 50 ng/μl for the double stranded DNA)

3.2.3 Mutation screening

The candidate genes were screened for mutations by Single Strand Conformation Polymorphism (SSCP) followed by direct sequencing. The coding region of all the candidate genes were amplified by polymerase chain reaction (PCR) and screened for variations by different methods as detailed in Table 5.

Table 5. Different methods used in candidate gene screening

S.No.	Disease	Gene	Region	Screening method
1	Open Angle Glaucoma	*MYOC*	Coding and promoter	SSCP and Sequencing
2		*CYP1B1*	Coding	Direct sequencing
3		*OPTN*	Coding	SSCP followed by sequencing
4		*P53*	Codon-72 and intron-3 polymorphisms	Restriction digestion
5		*ApoE*	ε2, ε3 and ε4 alleles	Restriction digestion
6		*TNF*	Promoter -308G>A	Restriction digestion
7	Axenfeld Rieger Syndrome	*FOXC1*	Coding	Direct sequencing
8		*PITX2*	Coding	Direct sequencing

3.2.3.1 Polymerase chain reaction:

Polymerase chain reaction (PCR) was used to amplify the coding regions of all the candidate genes. The primer sequences of the candidate genes along with their amplifying conditions (annealing temperatures, $MgCl_2$ concentrations) are given in Table 6. All PCR reactions were done in a PTC-200 thermal cycler (MJ Research, Watertown, MA). A 25 μl PCR reaction was set up with the following reagents (Table 7).

Table 6. List of primers used in candidate gene screening

Gene	Exon	Primers used for PCR	Amplicon size (bp)	$Mgcl_2$ (mM)	Ann. temp (^0C)	DMSO
MYOC	1	1F: 5'GGCTGGCTCCCCAGTATATA 3' 1R: 5' ACAGCTGGCATCTCAGGC 3'	180	1.5	58	-
	1	2F: 5' ACGTTGCTCCAGCTTTGG 3' 2R: 5' GATGACTGACATGGCCTGG 3'	196	1.5	58	-
	1	3F: 5'AGTGGCCGATGCCAGTATAC 3' 3R: 5'CTGGTCCAAGGTCAATTGGT 3'	190	1.5	58	-
	1	4F: 5'AGGCCATGTCAGTCATCCAT 3' 4R: 5'TCTCTGGTTTGGGTTTCCAG 3'	214	1.5	58	-
	1	5F: 5' TGACCTTGGACCAGGCTG 3' 5R: 5'CCTGGCCAGATTCTCATTTT 3'	200	1.5	58	-
	1	6F: 5'TGGAGGAAGAGAAGAAGCGA3' 6R: 5'CTGCTGAACTCAGAGTCCCC3'	185	1.5	58	-
	2	7F: 5'AACATAGTCAATCCTTGGGCC3' 7R: 5'TAAAGACCATGTGGGCACAA3'	223	1.5	58	-
	3	8F: 5'TTATGGATTAAGTGGTGCTTCG3' 8R: 5' ATTCTCCACGTGGTCTCCTG 3'	177	1.5	58	-
	3	9F: 5' AAGCCCACCTACCCCTACAC 3' 9R: 5'AATAGAGGCTCCCCGAGTACA 3'	184	1.5	58	-
	3	10F: 5' ATACTGCCTAGGCCACTGGA 3' 10R: 5' CAATGTCCGTGTAGCCACC 3'	192	1.5	58	-
	3	11F: 5'TGGCTACCACGGACAGTTC 3' 11R: 5'CATTGGCGACTGACTGCTTA 3'	197	1.5	58	-
	3	12F: 5' GAACTCGAACAAACCTGGGA 3' 12R: 5'CATGCTGCTGTACTTATAGCGG3'	195	1.5	58	-
	3	13F: 5' AGCAAGACCCTGACCATCC 3' 13R: 5' AGCATCTCCTTCTGCCATTG 3'	179	1.5	58	-
CYP1B1	2	P1F: 5'TCTCCAGAGAGTCAGCTCCG 3' P1R: 5' GGGTCGTCGTGGCTGTAG 3'	786	2.5	56	10X
	2	P2F: 5' GATGCGCAACTTCTTCACG 3' P2R: 5' CTACTCCGCCTTTTTCAGA 3'	648	2.5	56	10X
	3	P3F: 5' GCTCACTTGCTTTTCTCTCT 3' P3R: 5' AAATTTCAGCTTGCCTCTTG 3'	653	2.5	60	-

OPTN	4	4AF: 5-GGGGGACAGCTCTATTTTCA 3’ 4AR: 5’ CTGCTCACCTTTCAGCTGGT 3’	224	1.5	55	-
	4	4BF: 5’ AAACCTGGACACGTTTACCC 3’ 4BR: 5’TAGTGCAAAGGGATGGCATT 3’	146	1.5	55	-
	5	5AF: 5’ TCCACTTTCCTGGTGTGTGA 3’ 5AR: 5’ TTTTCCAAGCTCTTCCTTCAA 3’	218	1.5	55	-
	5	5BF: 5’ CAGAAGGAAGAACGCCAGTT 3’ 5BR: 5’ CATCACAATGGATCGGTCTG 3’	160	1.5	55	-
	6	6AF: 5’ ATGGTGCCCAGCCTTAGTTT 3’ 6AR: 5’ CGCTGGAGTTCAGCTTGAG 3’	201	1.5	55	-
	6	6BF: 5’ CCAGGTGGTGAGGCTACAAG 3’ 6BR: 5’ CAATCCTTGGCTTGTGTTGA 3’	219	1.5	55	-
	7	7F: 5’ TGGGTTGCATGTCACAAAAA 3’ 7R: 5’ GACAGCCCGAGTCTTCCTTC 3’	206	1.5	55	-
	8	8AF: 5’TTGGAATTTTTCTGATGAAAACC3’ 8AR: 5’ CTGATTCCCTTCCCTTAGGC 3’	154	1.5	55	-
	8	8BF: 5’ CAGATGGGGCCAAGAATTAC 3’ 8BR: 5’CTTTAAATGGGTGAACTGTATGG3’	214	1.5	55	-
	9	9F: 5’CCCAATTGTAAACAATGTTCTTTTT3’ 9R: 5’ GTGTGTGGGTGTGGTAGTGG 3’	244	1.5	55	-
	10	10F: 5’ TGGTTCAGCCTGTTTTCTCC 3’ 10R: 5’TCAAAGGAGGATAAAATTGCTCTC3’	235	1.5	55	-
	11	11F: 5’ TTAAAAGCCACTGCGACGTA 3’ 11R: 5’ GCTGCCCTTCTGACTCAACA 3’	239	1.5	55	-
	12	12F: 5’ ATTTTCCCCAGGATTCCATT 3’ 12R: 5’AACGTTCAACAGTTTCTGTTCATT3’	194	1.5	55	-
	13	13F:5’ACTAAAACAGGCAGAATTATTTCAA3’ 13R: AGCTGGGGTTTTGGAAGGT 3’	242	1.5	55	-
	14	14F: 5’CGCATAAACACTGTAAGAATCTGC3’ 14R: 5’ GATGTGAGCTCTGGGTCCTC 3’	236	1.5	55	-
	15	15F: 5’ TGTCATGTTTCGGGGTTGTA 3’ 15R: 5’TGAAGTGGAATTTTTCTTCAAGC3’	213	1.5	55	-
	16	16F: 5’ CGCCATCTGTTCTTCAAGTG 3’ 16R: 5’ ACCAACAGTTTTGGGGAGGT 3’	232	1.5	55	-
FOXC1	1	A1F: 5’ CCCGGACTCGGACTCGGC 3’ A1R: 5’ AAGCGGTCCATGATGAACTGG 3’	428	1 – 1.2	62	10X
	1	A2F: 5’ ATCAAGACCGAGAACGGTACG 3’ A2R: 5’ CTGAAGCCCTGGCTATGGT 3’	710	1 – 1.2	58	10X

	1	A3F: 5' ATCAAGACCGAGAACGGTACG 3' A3R: 5' GTGACCGGAGGCAGAGAGTA 3'	634	1	58	10X
	1	A4AF: 5' GAGCTCCCTCTACAGCTCCC 3' A4AR: 5' GTGACCGGAGGCAGAGAGTA 3'	240	1	55	10X
	1	A4BF: 5' CAAGCCATGAGCCTGTACG 3' A4BR: 5' GGGTTCGATTTAGTTCGGCT 3'	502	1	55	10X
PITX2	2	Ex2F: 5' AAAACACGCCTGAAGCCT 3' Ex2R: 5' CTGGCGATTTGGTTCTGATT 3'	331	1	58	-
	3	X1F: 5' GATAAAAGCCAGCAGGGGAA 3' X1R: 5' GAGGGAACTGTAATCTCGCA 3'	376	1.5	56.5	-
	4	X2F: 5' GTAATCTGCACTGTGGCATC 3' X2R: 5' CCAGTTGTGTAGGAATAGCC 3'	197	1.5	56.5	-
	4	X3F: 5' AGTTCAATGGGCTCATGCAG 3' X3R: 5' CATCCGGCAAGGTCCTAGGAT 3'	502	1.5	58	-
p53	*p53*	F: 5' CCTGAAAACAACGTTCTGGTAA 3' R: 5' GCATTGAAGTCTCATGGAAG 3'	432/448	1.5	56.5	-
TNF	*TNF*	F: 5' AGGCAATAGGTTTTGAGGGCCAT 3' R: 5' ACACTCCCCATCCTCCCGGCT 3'	107	1.5	62	-
ApoE	*ApoE*	F: 5' ACTGACCCCGGTGGCGGAGGAGAC GCG**T**GC 3'	318	1.0	62	10X
	ApoE	R: 5' TGTTCCACCAGGGGCCCCAGGCGC TCGCGG 3'				

10X DMSO (Sigma Chemical Co. St.Louis, USA) was used in PCR reactions when required (mentioned in Table 6).

Table 7. Reagents and their concentrations used in 25 μl PCR reaction

S.No.	Reagents	Concentration	Total volume for one reaction (μl)
1	Taq buffer	1X	1.0
2	dNTP	200 μM	2.5
3	F primer	10 pm	2.0
4	R primer	10 pm	2.0
5	Taq polymerase	1U	0.2
6	Genomic DNA	50 – 100 ng	1 - 2
7	MilliQ water		15 - 16

Amplification conditions

1. Initial denaturation 94^{o} C - 3 minutes
2. Denaturation 94^{o} C - 30 seconds
3. Annealing X ^{o} C (Table 6) - 30 seconds
4. Extension 72^{o} C - 30 to 60 seconds

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

Confirmation of PCR amplification

The amplified products were checked on a 1.5% agarose gel containing ethidium bromide (USB, Amersham Biosciences, New Jersey, USA). Samples were mixed with 6X loading dye containing bromophenol blue (USB, Amersham Biosciences, New Jersey, USA) and xylene cyanol (USB, Amersham Biosciences, New Jersey, USA) and loaded in the gel along with 100 bp ladder (MBI fermentas, Lithuania). The gel was run using 1X Tris-Acetate-EDTA (TAE) buffer at 100 mA for 45 minutes and observed under Gel Documentation system (Uvitec Ltd., Cambridge, UK) and photographed.

3.2.3.2 Single Strand Conformation Polymorphism (SSCP)

SSCP has the ability to detect nucleotide changes in the DNA, depending on differences in the mobility of their single strands due to mismatches. It is capable of identifying variations present in a single strand of DNA typically between 150 to 300 bp in length. Under non-denaturing conditions, the single stranded DNA will adopt a conformation that is unique and dependent on its sequence composition. The change will be detected as mobility shift when the samples are subjected to electrophoresis. Sensitivity of SSCP depends on the fragment length of the amplicon and is usually between 70% to 97% for the fragments of 200 bp to 155 bp (Sheffield *et al.,* 1993).

SSCP protocol

Sample preparation

1. Two μl of PCR product was mixed with 4 μl of 95% Formamide dye containing bromophenol blue and xylene cyanol.
2. Samples were denatured at 95° C for 5 minutes and snap chilled immediately in salt mixed ice to prevent re-annealing of denatured strands.
3. Samples were loaded into the gels along with undenatured samples.

Gel preparation

1. Samples were run in 8% non-denaturing polyacrylamide gel using Hoefer SE600 (Amersham Biosciences, New Jersey, USA) gel apparatus.
2. Gels were prepared using 18X16 cm glass plates with 1.5 mm spacers.
3. The gel constituents were: 8% acrylamide (19.5:0.5 acrylamide to bis acrylamide), 0.5X TBE, 0.03% APS (Sigma Chemical Co. St.Louis, USA), TEMED (USB, Amersham Biosciences, New Jersey, USA). Sometimes the gels were run with 5% glycerol (Qualigens Fine Chemicals, Mumbai, India).
4. The reagents were mixed properly and poured between the plates for polymerization.

5. On polymerization, the wells were washed with MilliQ water to remove the acrylamide pieces.

Electrophoresis

Electrophoresis was performed at two different temperatures. Gels were run either at 75 mA at room temperature or 110 mA at 4° C overnight. The gels were also pre run for 30 minutes at 80 V to normalize the conditions.

Staining and detection of variants

1. The gels were removed and kept in fixative containing 10% ethanol and 0.5% glacial acetic acid (Qualigens Fine Chemicals, Mumbai, India) for 45 minutes on shaker (Amersham Biosciences, New Jersey, USA).
2. The gels were washed twice for 5 minutes with MilliQ water and stained with 2% silver nitrate solution (Qualigens Fine Chemicals, Mumbai, India) for 15 minutes.
3. The gels were then washed twice for 5 minutes with MilliQ water and transferred to the developer solution containing NaOH (Qualigens Fine Chemicals, Mumbai, India) and formaldehyde (Qualigens Fine Chemicals, Mumbai, India).
4. The gels were developed on a shaker till the bands became visible.
5. Samples with altered mobility were observed under a gel documentation system (Uvitec Ltd., Cambridge, UK).

6. Samples with mobility shift were further characterized by sequencing.

3.2.3.3 Bi-directional sequencing

Bi-directional cycle sequencing was performed on an automated DNA sequencer ABI 310 (Applied Biosystems, Foster city, CA) using BigDye terminator chemistry (Version 3.1).

Purification of PCR products

Prior to sequencing, amplicons were purified by Millicon (Millipore corporation, Billerica, MA) columns to remove the unutilized primers, dNTPs and salts by the following methods:

1. Amplicons were diluted to a final volume of 500 μl using autoclaved MilliQ water.
2. The sample was loaded in the column and spun at 5000 rpm for 15 minutes.
3. The column was transferred to a fresh 1.5 ml eppendorf tube in an inverted position.
4. The product was eluted by adding 15 μl of autoclaved MilliQ and spinning at 5000 rpm for 2 minutes.
5. The amplicons were rechecked on 1.5% agarose gel after purification.

Sequencing PCR

Sequencing PCR was performed to generate the amplicons that are terminated by the incorporation of fluorescently labelled dideoxynucleotides ddNTPs. It contains template, primer, polymerase and deoxynucleotides (dNTPs) along with fluorescently labeled ddNTPs. The dNTPs and ddNTPs are present at concentrations that a ddNTP will be incorporated instead of a dNTP at each nucleotide position in the synthesizing fragments. The dNTP differs from ddNTP in having a hydrogen atom instead of a hydroxyl group at its 3' end and cannot participate in further extension. Therefore, when a ddNTP is incorporated, further chain elongation is blocked and this results in a population of truncated products of varying lengths. The list of reagents used is given in Table 8.

Table 8. List of reagents used for sequencing PCR

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

Conditions for sequencing PCR

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

Precipitation:

1. The PCR product was diluted to a final volume of 100 μl as per ABI manufacturer's protocol (Applied Biosystems, Foster city, CA) using autoclaved MilliQ water.
2. To the sample 3 μl of 3M sodium acetate (pH 4.6) and 250 μl of ethanol was added.
3. Samples were spun at 12000 rpm for 20 minutes at room temperature.
4. Supernatant was discarded and 250 μl of 70% ethanol was added.
5. After mixing gently, samples were spun at 12000 rpm for 10 min.
6. The supernatant was discarded and washed again with 70% ethanol
7. Samples were dried and dissolved in 15 μl of Hidi formamide (Applied Biosystems, Foster city, CA).

Electrophoresis

The ABI-310 genetic analyzer (Applied Biosystems, Foster city, CA) was used to sequence the DNA fragment.

1. A POP-6 (Applied Biosystems, Foster city, CA) polymer was brought to room temperature and loaded the injection syringe without air bubbles.
2. Genetic analyzer buffer (10X) was diluted to 1X and used.
3. Matrix files were created for all the four bases by running the matrix standards, to correct the spectral overlap
4. Samples were denatured at 95° C were 5 minutes followed by snap chilling.
5. Samples were loaded into auto sampler, which brings each sample into contact with the cathode electrode and one end of glass capillary filled with polymer.
6. Sample sheet was prepared according to the order of the samples loaded in auto sampler.
7. Injection list was prepared giving information about order for running samples, number of injections and running conditions like run time, injection time and injection voltage.
8. Reactions were run for 60 to 120 minutes depending on the size of the amplicon.

Analysis:

1. The raw data was obtained through the Data Collection software (Version 3.0).
2. The Sequencing software (Version 3.7) analysis software was opened and the start and end points were set.
3. The auto analysis function was used to analyze and the results were obtained in the form of electropherograms.
4. Sequences were compared with the reference GENBANK sequences (www. ncbi.nlm.nih.gov) for each of the candidate genes.

3.2.3.4 Multiple Sequence alignment

Multiple sequence alignment was done to compare the protein sequences of different species for a candidate gene to check for conservation of the mutated residues.

1. The protein sequences of the different species were obtained from NCBI database (www.ncbi.nlm.nih.gov/protein).
2. The protein sequences from all the available species were converted to FASTA format.
3. Multiple sequence alignment was performed using CLUSTALW software, available at the European Bio-informatics Institute (EBI) (www.ebi.ac.uk/clustalw).

4. Alignment was performed using score type as absolute and rest of the parameters like gap openings, gap extension, matrix, word size, window length were set as default.
5. Each amino acid was denoted by its their specific color.

3.2.4 PCR based Restriction Digestion

PCR based restriction digestion was used to confirm nucleotide variations in normal controls and ascertain segregation in other family members. Nucleotide changes that resulted in either creation or abolition of the restriction site for a particular restriction enzyme were used for screening the variation. Further details of these enzymes are provided in Table 9.

1. NEBcutter software was used to select the appropriate restriction enzyme, (http://tools.neb.com/NEBcutter2/index.php).
2. 5 μl of PCR product was mixed with 1X restriction buffer and 2 to 4 units of the restriction enzyme.
3. All the reactions were incubated overnight at appropriate temperatures.
4. Following digestion, fragments were separated on an 8% non-denaturing polyacrylamide gel and bands were visualized under UV light after staining with ethidium bromide.

Table 9. List of restriction enzymes used in PCR-RFLP

S.No	Gene	Variation	Restriction Enzyme	Temp. (°C)	Obtained from
1	*MYOC*	Q48H	Acc I (-)	37	NEB
2		S231R	Alw 26I (+)	37	MBI
2		P370L	Cai I (+)	37	MBI
3		-83G>A	Eco 88I (+)	37	MBI
4		R76K	Alw 26I (+)	37	MBI
5		-1000.mt	Cai I (-)	37	MBI
6	*CYP1B1*	G61E	Taq I (+)	65	MBI
7		Y81N	Hae II (-)	37	NEB
8		Q144R	Msp A1I (-)	37	NEB
9		E229K	Eam 11041 (-)	37	MBI
10		P193L	Eco 8II (+)	37	MBI
11		R368H	Taa I (-)	65	MBI
12		W434R	Bst XI (-)	55	MBI
13		P437L	Hpa II (-)	37	MBI
14		F445C	Hinf I (-)	37	MBI
15	*OPTN*	T34T	Tai I (-)	65	MBI
16		M98K	Eco 147I (+)	37	MBI
17		R545Q	Aci I (-)	37	NEB
18		IVS15+10G>A	Nla III (+)	37	NEB

+ indicates creation of site; - indicates abolition of site; MBI indicates MBI fermaentas; NEB - New England Biolabs.

3.2.4.1 Restriction digestion for SNP screening

a) *p53* screening

PCR based restriction digestion was used to determine the association between *p53* codon-72 polymorphism and 16bp intron duplication with POAG. A single nucleotide change in exon 4 from G>C (CGC $\rightarrow$ CCC) replacing the arginine amino acid with proline, and 16 bp duplication in 3rd intron were screened. The Arg72Pro substitution abolished the restriction site for *Bsh1236I* enzyme.

Methods

1. PCR was performed in a 25 μl reaction volume using pre-designed primers (Acharya *et al.,* 2002), 1X buffer, 0.2 mM dNTPs, 10 pM forward and reverse primer, 1.5 mM $MgCl_2$ and 1 unit *Taq* polymerase.
2. The size of the amplicon consisted of 432bp in the absence of 3rd intron 16 bp duplication or 448 bp in the presence of 16 bp duplication.

Restriction digestion

5 μl of PCR product was digested with 2 units of *Bsh*1236I restriction enzyme by incubating at 37°C for 3 hours. Fragments comprising of an undigested product along with digested product were resolved on a 8% polyacrylamide gel. Gels were stained with ethidium bromide to visualize the bands and photographed under a UV-gel documentation system.

Haplotyping

Haplotyping was done by comparing the fragments of the digested and undigested products. PCR product with 16 bp duplication, harboring arginine at codon 72 should have the restriction site for *Bsh*1236I and provide fragments of 202 bp and 246 bp while the one harboring proline at codon 72 without restriction site will have a fragment of 448 bp. PCR product without 16 bp duplication, harboring arginine at codon 72 will cleave into 202 bp and 230 bp fragments, similarly one with proline at codon 72 will have a 432 bp fragment. Thus the digested fragments and corresponding haplotypes would be- Del-Pro: 432bp, Del-Arg: 202 bp and 230 bp, Ins-Pro: 448 bp, Ins-Arg: 202 bp and 246 bp (where Del indicates the absence of 16 bp duplication in 3rd intron and Ins indicates the presence of 16 bp duplication in 3rd intron).

b) *ApoE* polymorphism

ApoE isoforms were genotyped by PCR-RFLP technique as given by QIAGEN Corp. (Bothell, WA).

Methods

1. PCR was performed in a 25 μl reaction volume using 1X buffer, 0.2 mM dNTPs, and 10 pm forward and reverse primer, 1.0 mM $MgCl_2$ and 1unit *Taq* polymerase.

The primer details were given in Table 6. Forward primer differs from genomic sequence at one position (indicated in bold) to create an additional restriction site for *Afl*III in the amplicon.

Restriction digestion

Two different digestions were performed for each amplicon.

1. a) 5 μl of PCR product was digested with 4 units of *Afl*III restriction enzyme by incubating at 37 °C for overnight.
2. b) 5 μl of PCR product was digested with 2 units of *Hae*II restriction enzyme by incubating at 37 °C for overnight.
3. Fragments were resolved on a 8% polyacrylamide gel by loading two digestions in two consecutive wells.
4. Gels were stained with ethidium bromide to visualize the bands and photographed under UV-gel documentation system.

Haplotyping

Alleles were scored depending on fragment sizes generated from digestions with both *Afl*III and *Hae*II enzymes. An ε3 allele would give fragments of sizes 231 bp and 232 bp on digestion with *Afl*III and *Hae*II respectively. An ε2 allele lacks the site for *Hae*II variable site and result in a 267 bp fragment. The variable site for *Afl*III is absent in an ε4 allele and result in a fragment of 295 bp.

c) *TNFα (-308G>A)* polymorphism

The -308G>A polymorphism of *TNFα* was screened by PCR-RFLP by digestion with *Nco*I enzyme. Genomic DNA was amplified by PCR and 5 μl of PCR product was digested with 4 units of *Nco*I enzyme. Fragments were resolved on a 8% polyacrylamide gel and samples were genotyped based on fragment sizes. In the presence of wild type G allele, amplicons would be digested to yield fragments of 97 bp and 20 bp. In the presence of mutant A allele, amplicon will not be digested and generate a fragment of 107 bp. In the heterozygous condition (GA), all the three fragments will be present.

3.3 Statistical analysis

Allele and gene frequencies were calculated by gene counting method. The $\chi 2$ test was used to compare the allele and genotype frequency distributions between patients and controls. A p value <0.05 was considered statistically significant. Odds ratios and 95% confidence intervals were calculated to determine the likelihood of developing the disease by individuals having the mutant alleles.

Chapter 4
Results

RESULTS

4.1 Screening in primary glaucomas

4.1.1 Mutation screening in candidate genes

Open angle glaucoma (OAG) is clinically and genetically a heterogeneous disorder. In order to understand the mutation spectrum underlying OAG in Indian population, we screened candidate genes like *MYOC*, *CYP1B1* and *OPTN* in our patient cohort. Several novel and known mutations were observed in *MYOC* and *CYP1B1*, while the *OPTN* did not show any involvement in the disease pathogenesis.

4.1.1.1 Mutational Analysis of *MYOC*

Direct sequencing of the coding region of *MYOC* in 109 OAG cases revealed 4 mutations in 5 cases (4.6%). Of these, three mutations were observed in third exon (P370L, D395N and Y479H) and one in the first (Q48H) exon. The D395N and Y479H mutations were novel. All the mutations were observed in heterozygous state. A novel variation (S231R) was also observed in the second exon. Five polymorphisms in the promoter (-1000C>G, -83G>A), exon 1 (R76K, G122G) and exon 3 (Y347Y) were also observed.

Figure 13 and 14 shows the corresponding electropherograms and the multiple sequence alignment of *MYOC* across different species, respectively. Table 10 provides an overview of *MYOC* mutations observed in OAG that are detailed below:

Gln48His: c.144 CAG > CAT

A heterozygous change from Guanine to Thymine at cDNA position c.144 (Fig. 13A) resulted in the replacement of glutamine by histidine at codon 48 (Gln48His) in a sporadic case (P-026). It co-segregated with P437L mutation of *CYP1B1* (Figure 15) suggesting a possible digenic inheritance. This change resulted in the loss of restriction site for *Acc*I enzyme. The patient manifested the disease at the age of 35 years with an IOP of 22 mm Hg along with total cupping of the optic disc and a visual acuity of 20/20 in both eyes (Table 11). Visual field defects were graded as severe and mild in right and left eyes, respectively. Glutamine at this site is highly conserved across all the species (Figure 14). However it was also observed in 7 normal controls without any glaucoma symptoms at presentation. These individuals could be at risk for developing glaucoma at a later stage. The clinical features of these subjects are given in Table 12. So far, there is no functional study to support the pathogenic nature of this mutation.

Pro370Leu: c.1109 CCG > CTG

A heterozygous Cytosine to Thymine change at cDNA position c.1109 (Fig. 13B) resulted in a change from proline to leucine at codon 370 (Pro370Leu) in two autosomal dominant families (P-018 and P-025). proline at this codon is highly conserved across all species (Figure 14). This mutation created a restriction site for *Cai* I enzyme and was found to segregate with the disease in both the families (Figure 16) and was

not observed in the controls. Both the patients were diagnosed with JOAG at ages of 7 and 11 years. Despite raised IOPs of 28 and 35 mm Hg at presentation, their IOPs have reduced to 14 and 16 mm Hg after surgery (Table 11).

Asp395Asn: c.1183 GAT > AAT

Another heterozygous change from Guanine to Adenine at cDNA position c.1183 (Fig. 13C) resulted in the replacement of aspartic acid by asparagine at codon 395 (Asp395Asn) in a sporadic case (P-020). This is a novel variant but the residue is not conserved across species (Figure 14). The Aspartic acid residue is present only in humans and *Macaca sp.* Therefore this change might have a milder effect on protein function. The patient was diagnosed with POAG at the age of 56 years, with an IOP of 17 mm Hg in both eyes and total cupping of the optic disc. After surgery, IOPs are maintained at 12 and 10 mm Hg in right and left eyes, respectively, with a visual acuity of 20/30 in both eyes and the field defects were severe in both eyes.

Tyr479His: c.1457 TAC > CAC

Another novel heterozygous substitution of Thymine with Cytosine at cDNA position c.1457 (Fig.13D) resulted in the replacement of amino acid tyrosine with histidine at codon 479 (Tyr479His) in a sporadic POAG case (P-083). This change was also novel and not observed in the normal controls. The tyrosine residue is highly conserved across most species (Figure 14). The patient was diagnosed of JOAG at the

age of 30 years with an initial IOP of 34 and 40 mm of Hg in the right and left eyes, respectively, along with a total cupping of the optic disc. Presently the IOPs are under control with visual acuity of 20/20 in both eyes after surgery.

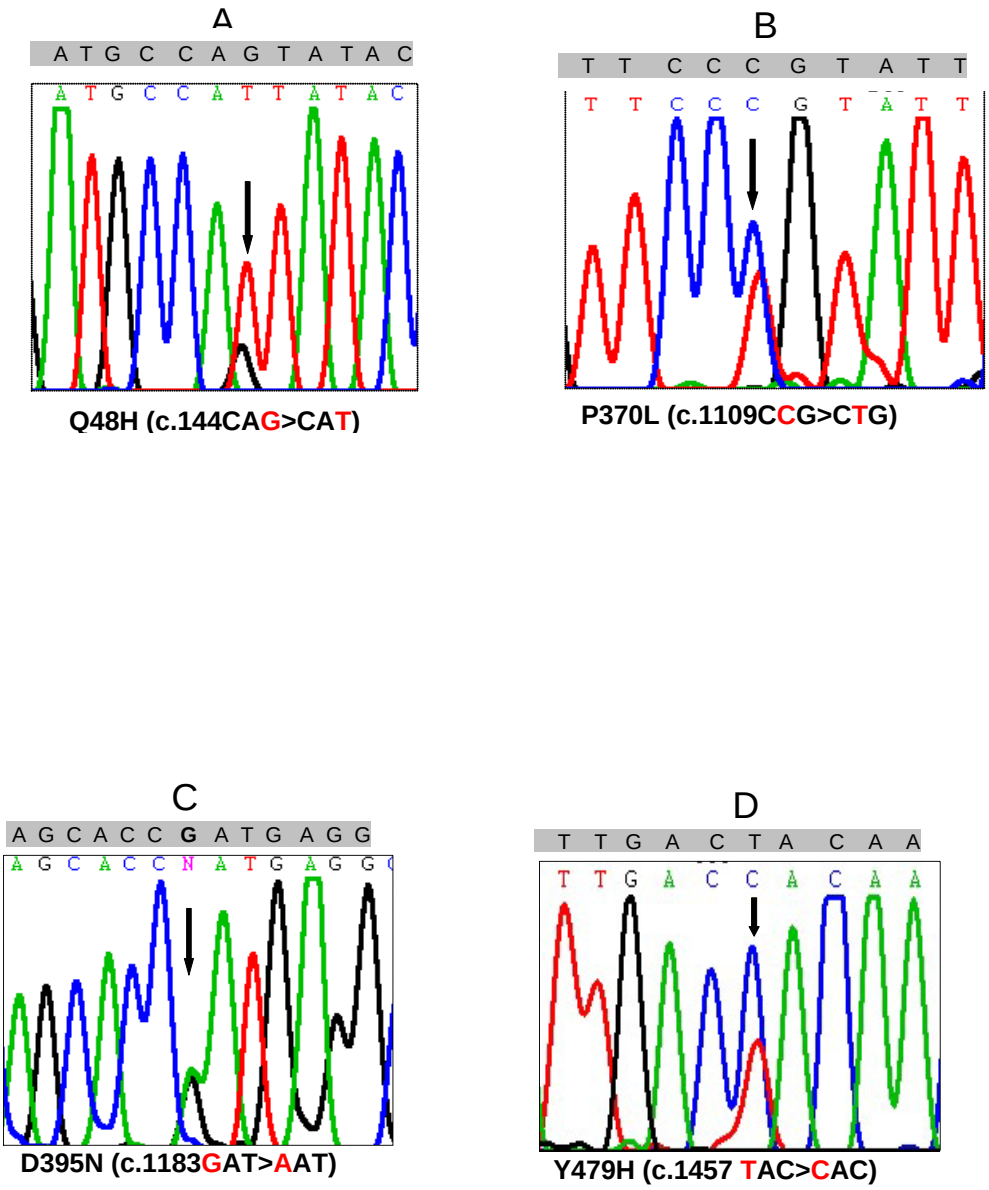


Figure 13. Electropherograms representing the *MYOC* mutations. The shaded sequence represents the wild type sequence and arrow shows the point of mutation.
(A) A heterozygous substitution at c.144G>T resulting in the Gln48His mutation.
(B) A Heterozygous transition of C>T at c.1109 resulting in the Pro370Leu mutation.
(C) A heterozygous substitution of G>A at c.1183 resulting in Asp395Asn mutation
(D) A heterozygous substitution at c.1457 T>C resulting in the Tyr479His mutation.

Figure 14. Multiple sequence alignment of *MYOC* across various species

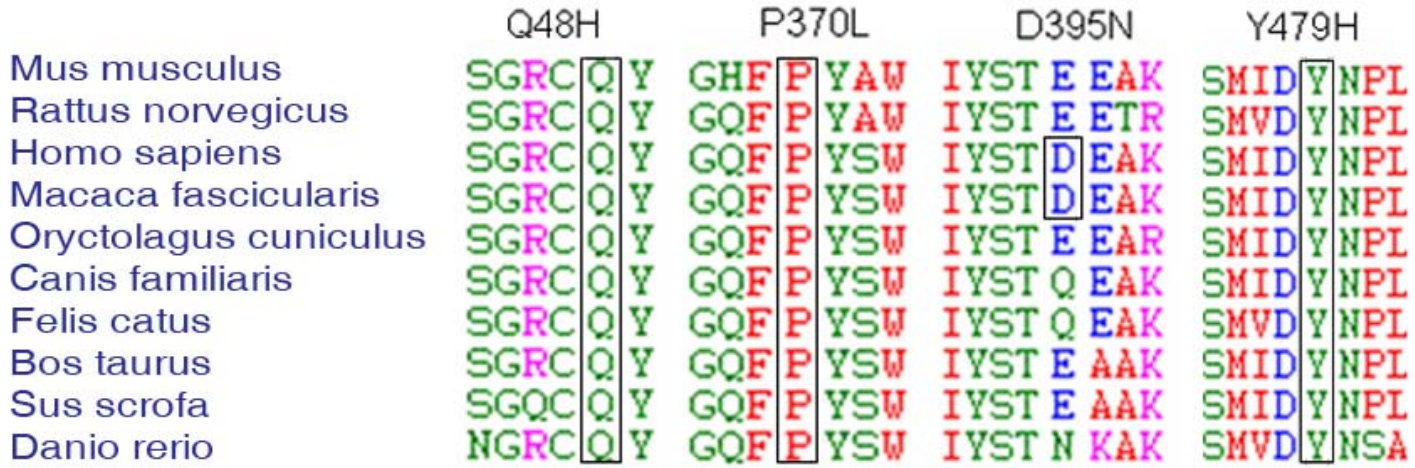


Figure 14: Multiple sequence alignment of the Myocilin protein across ten different species. The conservation of the wild type residue is indicated by boxes.

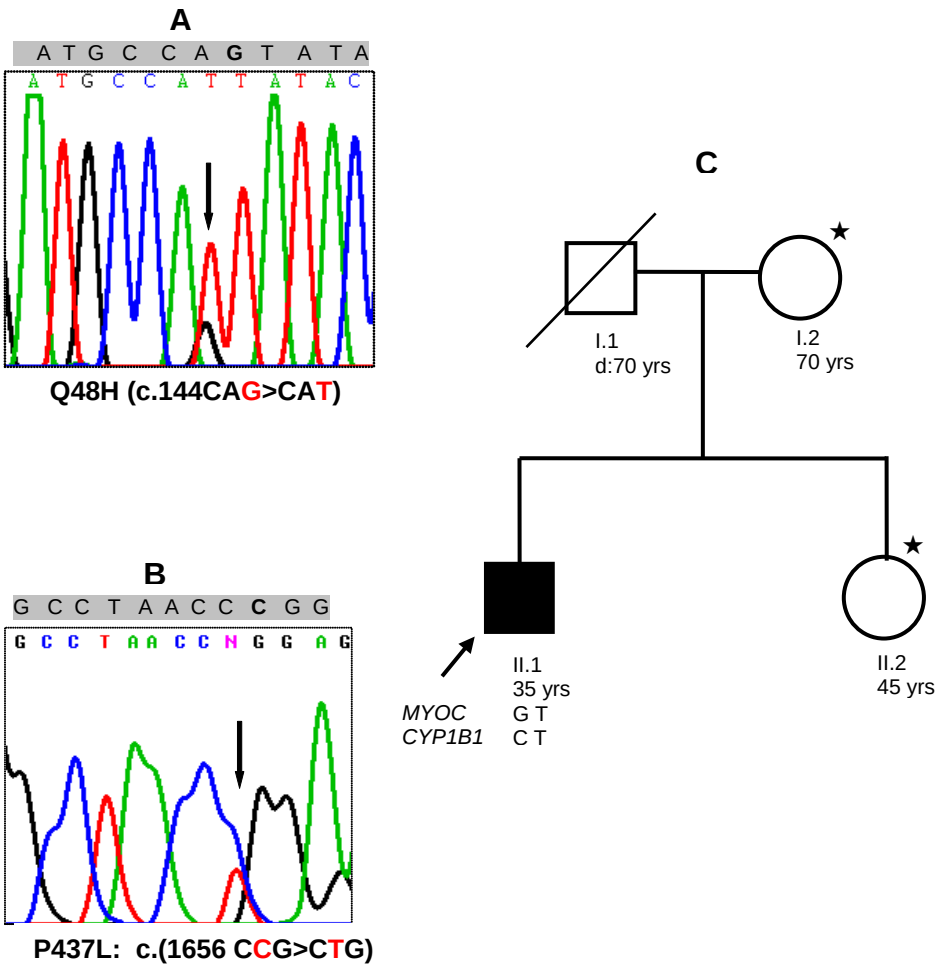
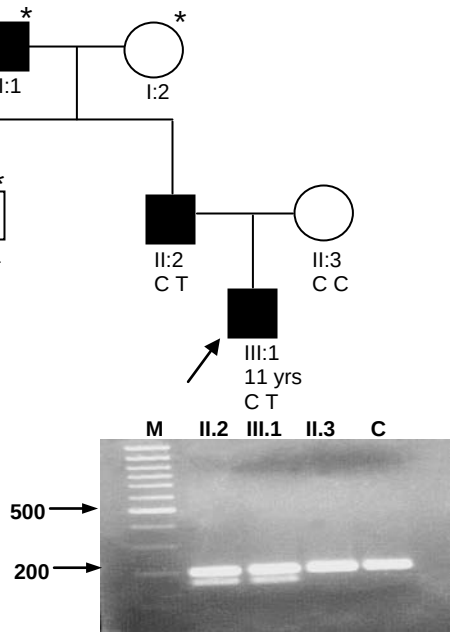


Figure 15. Possible digenic inheritance of *MYOC* and *CYP1B1* in P-026 family. The shaded sequence represents the wild type sequence and arrow shows the point of mutation. DNA was not available from individuals marked with asterisk. (A) The electropherograms showing the heterozygous substitution at c.144G>T resulting in the Gln48His mutation. (B) The electropherograms showing the heterozygous substitution at c.1656 C>T resulting in the Pro437Leu mutation. (C) Pedigree of P-026 family with the proband showing both the *CYP1B1* and *MYOC* mutations. Darkened symbols indicate the affected person and clear symbols indicate normal individuals. The proband is indicated by an arrow. The genotypes are indicated below the symbols.

A) Segregation of the P370L mutation in P-018 family



B) Segregation of the P370L mutation in P-025 family

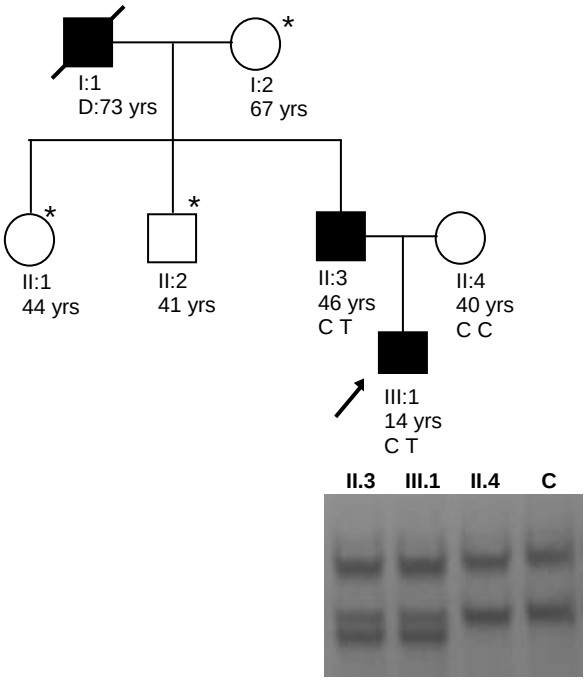


Figure 16. Segregation of P370L (c.1109C>T) in two autosomal dominant families, P-018 (A) and P-025 (B). Darkened symbols indicate the affected person and clear symbols indicate the normal individuals. The proband is indicated by an arrow. The genotypes are indicated below the symbols. DNA was not available from individuals marked with asterisk (*). The corresponding gel images denotes the segregation of the P370L mutation through PCR – based Cai I digestion in P-018 (A) and PCR based SSCP in P-025 (B) families.

Table 10. List of *MYOC* mutations observed in patients with open angle glaucoma

S.No.	c. DNA position *	Exon	Codon change	Amino acid change	Mutation	Frequency N (%)	Population	Reference
1	c.0153G>T	1	CA**G** > CA**T**	Gln>His	Q48H	1 (0.9 %)	India	Mukhopadhyay *et al.,* 2002 Sripriya *et al.,* 2004 Chakrabarti *et al.,* 2005
2	c.1109C>T	3	C**C**G > C**T**G	Pro>Leu	P370L	2 (1.8 %)	French Japanese North America USA Brazil India	Adam *et al.,* 1997 Suzuki *et al.,* 1997 Wiggs *et al.,* 1998 Shimizu *et al.,* 2000 Vasconcellos *et al.,* 2000 Mukhopadhyay *et al.,* 2002
3	c.1183G>A	3	**G**AT > **A**AT	Asp>Asn	D395N	1 (0.9 %)	Novel	
4	c.1457T>C	3	**T**AC > **C**AC	Tyr>His	Y479H	1 (0.9 %)	Novel	

* GenBank ID # NM_000261

Table 11. Clinical features of patients with different *MYOC* mutations

Case No	Mutation	Age at onset	IOP at		CD ratios OD;OS	Visual acuity at		Field Defects	Treatment
			Presentation OD;OS	Treatment OD;OS		Presentation OD;OS	Treatment OD;OS		
P-26	Q48H	35	22; 22	14; 12	0.9;0.8	20/20; 20/20	20/20; 20/20	S; Mi	Surgery OD
P-18	P370L	11	28; 28	14; 14	0.3;0.3	20/15; 20/15	20/25; 20/25	Mi; Mi	Surgery OU
P-25	P370L	7	35; 35	16; 14	0.6;0.6	20/30; 20/20	20/20; 20/20	Mi; Mi	Surgery OU
P-20	D395N	56	17; 17	12; 10	0.9;0.9	20/25; 20/20	20/30; 20/30	S; S	Surgery OU
P-83	Y479H	30	34; 40	10; 12	0.9;0.9	20/20; 20/20	20/20; 20/20	S; S	Surgery OU

Clinical features of the patients with *MYOC* mutations indicating the mutation, age of onset, IOP and visual acuity at presentation and treatment along with CD ratios, field defects and treatment. OU = Both eyes; OD = Right eye; OS = Left eye; S = Severe; Mi = Mild; Mo = Moderate; N = Normal; PLPR = Perception of light with accurate projection of rays; CF = Counting fingers;

Table 12. Clinical features of the control subjects with Q48H mutation

S.No	Age at presentation	IOP at		CD ratios OD; OS	Visual acuity at	
		Presentation OD;OS	Treatment OD;OS		Presentation OD;OS	Treatment OD;OS
1	53	16; 17	16; 16	0.3; 0.4	20/20; 20/200	20/25; 20/25
2	50	14; 14	10; 12	0.3; 0.3	PLPR; 6/9	6/12; 6/6
3	75	10; 10	NA; 14	0.3; no view **	6/36; PLPR	NA; 20/200
4	65	16; 14	16; 16	0.3; 0.4	PLPR; CF 30cm	20/30; NA
5	50	10; 10	14; 16	0.3; 0.3	20/30; 6/36	20/160; 20/30
6	63	14; 14	12; 11	0.3; 0.3	20/50; 20/40	20/20; 20/20
7	60	10; 11	5; NA	No view ** OU	PLPR; CF 1m	6/36; NA

OU = Both eyes; OD = Right eye; OS = Left eye; PLPR = Perception of light with accurate projection of rays; CF = Counting fingers; NA = Not available ** Due to presence of cataract

4.1.1.1.1 *MYOC* Polymorphisms

Five previously reported SNPs, in the promoter (–1000C>G, -83G>A) and coding regions (R76K, G122G, Y347Y) were noted. These polymorphisms showed no association to the disease phenotype. The distribution of –83 G>A and R76K allele frequencies in patients and controls were not statistically significant (P = 0.304). Details of these SNPs in patients and controls are provided in Tables 13 and 14.

A variation S231R was observed in a sporadic POAG case. This was not reported previously and was also observed in an unaffected individual.

MYOC.mt1 polymorphism

A change from Cytosine to Guanine in the promoter at 1000 bp upstream of *MYOC* resulted in the abolition of restriction site of *Cai*1 enzyme. The mutant allele 'G' was found to be associated with the disease severity in terms of IOP and visual fields in the French (Colomb *et al.*, 2001) and US populations (Polansky *et al.*, 2003). On the other hand no association was reported in Turkish population (Ozgul *et al.*, 2005). The present study also did not reveal any association of the *MYOC*.mt1 SNP in OAG (Tables 15 and 16). The severity of phenotype of individuals were similar for all the genotypes.

Table 13. Allele frequencies of –83G>A AND R76K polymorphisms

Subjects	-83 G>A		R76K (G>A)	
	G allele	A allele	G allele	A allele
Patients	67	33	67	33
Controls	60	40	60	40
Significance	χ^2 = 1.06, P = 0.304		χ^2 = 1.06, P = 0.304	

Table 14. Genotype distribution of –83 G>A and R76K polymorphisms

Subjects	–83 G>A			R76K (G>A)		
	GG (%)	GA (%)	AA (%)	GG (%)	GA (%)	AA (%)
Patients (N = 109)	49 (44.9)	45 (41.2)	15 (13.7)	49 (44.9)	45 (41.2)	15 (13.7)
Controls (N = 105)	38 (36.2)	43 (41.0)	24 (22.9)	38 (36.2)	43 (41.0)	24 (22.9)
Significance	χ^2 = 3.43, P = 0.179			χ^2 = 3.43, P = 0.179		

Table 15. Allele frequencies of *MYOC*.mt1 polymorphism

Subjects	C allele	G allele
Patients	87	13
Controls	90	10
Significance	$\chi^2 = 0.44$, P = 0.506	

Table 16. Genotype frequencies of *MYOC*.mt1 polymorphism

Subjects	CC (%)	CG (%)	GG (%)
Patients (N = 109)	82 (75.2)	25 (22.9)	2 (1.8)
Controls (N = 101)	82 (81.2)	18 (17.8)	1 (0.9)
Significance	$\chi^2 = 1.17$, P = 0.557		

4.1.1.2 Mutation analysis of *CYP1B1*

Direct sequencing of the coding region of *CYP1B1* in 109 POAG cases revealed 10 pathogenic mutations in 22 (20.18%) cases. Of these, four mutations (Q144R, W434R, c.1657del5bp and F445C) were novel and the rest were reported as pathogenic mutations in PCG in different populations. Five mutations were noted in exon 2 and the rest in exon 3. All the mutations were observed either in heterozygous or in compound heterozygous states except c.1657del5bp.

An outline of the mutations and the corresponding electropherograms are provided in Table17 and Figure 17. Multiple sequence alignment of CYP1B1 across different CYP1 families and species indicating the conservation of the mutated residues are shown in Figure 18. A brief description of mutations is presented below.

Gly61Glu: c.528 GGA>GAA

A heterozygous change from Guanine to Adenine at cDNA position c.528 (Fig. 17A) resulting in the change of glycine to glutamic acid at codon 61 (Gly61Glu) was observed in a sporadic case (P-008). This variation resulted in the gain of *Taq* I restriction site and was not observed in the normal controls. The residue at this site is highly conserved across different CYP1 family members. It occurred in the proline rich region that connects the membrane bound region to cytosolic C-terminal domain of the protein. This hinge region, characteristic of microsomal cytochrome P450s, permits flexibility

between the membrane spanning domain and cytoplasmic portion of the molecule (Yamazaki et al., 1993). The Gly61 is a functional part of the hinge region and its substitution might interfere with proper folding and heme-binding properties of the cytochrome P450 molecules (Stoilov *et al.*, 1998).

This mutation co-segregated with another mutation R368H of *CYP1B1*. Unfortunately the parent's DNA samples were not available to analyze the segregation of this mutation. The proband manifested the JOAG phenotype at the age of 9 years with an IOP of 38 and 40 mm Hg in right and left eye, respectively along with a CD ratio of 0.9 in both eyes. After surgery the IOPs have reduced to 15 and 16 mm Hg in the right and left eye, respectively. However the vision in the right eye deteriorated to counting fingers at 50 cm and remained at 20/50 in the left eye.

Tyr81Asn: c.587 TAC>AAC

A heterozygous substitution from Thymine to Adenine at cDNA position c.587 (Fig. 17B) resulted in the change of tyrosine to asparagine at codon 81 (Tyr81Asn) in an autosomal dominant family (P-105). Unfortunately, the DNA samples of other affected members in this family were not available to analyze the segregation of this mutation. This residue is also highly conserved among CYP1A and CYP1B families across species (Figure 18) and resulted in the loss of *Hae* II restriction site. This mutation was also reported to be pathogenic in early onset open angle glaucoma patients from French population

(Melki et al., 2004). The patient with this mutation was diagnosed as JOAG at the age of 30 years and presented with an IOP of 15 mm Hg in both eyes and total cupping of the optic disc. After surgery, the patient had a visual acuity of 20/50 and 20/20 in right and left eye, respectively.

Gln144Arg: c.777 CAG>CGG

A novel heterozygous substitution from Adenine to Guanine at cDNA position c.777 (Fig. 17C) resulted in the replacement of glutamine to arginine at codon 144 (Gln144Arg) in three POAG cases (P-001, P-033 & P-086). It co-segregated with disease phenotype in P-001 family, while the remaining cases were sporadic. This mutation was not observed in the controls.

In P-001 family (Figure 19), two members (III:2 and IV:2) who are phenotypically normal also harbored this mutation. These two individuals might be at risk of developing the disease in future, as POAG is a late onset disorder or it might be due to the incomplete penetrance of *CYP1B1*, which is well documented in PCG. Multiple sequence alignment showed that glutamine is present only in human and mouse CYP1B1, while arginine is present in rat CYP1B1 and CYP1A1 and CYP1A2 members of other species. This variation resulted in the loss of *MSP*A1I restriction site.

This mutation resulted in both JOAG and POAG phenotypes with age of onset ranging from 12 years to 65 years with presenting IOP

ranging from 12 to 44 mm Hg along with a CD ratio of 0.6 to 0.9. All the three patients underwent surgery with variable outcomes (Table 18).

Pro193Leu: c.923 C<u>C</u>G>C<u>T</u>G

A heterozygous substitution of Cytosine with Thymine at cDNA position c.923 (Fig. 17D) position resulted in the replacement of proline with leucine at codon 193 (Pro193Leu) in a sporadic case (P-173). It co-segregated with another missense mutation E229K of *CYP1B1* gene (Figure 20). She inherited P193L mutation from her mother and E229K from her father. She manifested the phenotype at 7 years; IOPs of 24 and 22 mm Hg in the right and left eyes, respectively, and total cupping of the optic disc. Her IOPs are under control with medication along with a visual acuity of 20/20 in both eyes.

Glu229Lys: c.1031 <u>G</u>AA>A<u>A</u>A

A heterozygous change from Guanine to Adenine at cDNA position c.1031 (Fig. 17E), due to the replacement of glutamic acid with lysine at codon 229 (Glu229Lys) was observed in 6 POAG (P-012, P-013, P-039, P-081, P-104, P-173) cases. It lay in the C-terminal region of the F-helix. This mutation resulted in loss of restriction site for the *Eam*1104I enzyme.

In a study on French POAG patients, the proband with this mutation manifested the disease at the age of 35 years with IOP of 25 and 21 mm Hg in right and left eye, respectively (Melki et al., 2004). In the present study, this mutation was present in patients with juvenile

and adult onset OAG with variable degrees of severity. Age of onset varied between 32 to 64 years with initial IOPs ranging from 12 to 24 mm Hg. After treatment, IOPs were under control in four patients while it raised to 46 mm Hg in the right eye of one patient. Visual acuity in three patients ranged between 20/20 to 20/30, while it was worsened to 20/400 and PLPR in the right eyes of two patients (Table 18).

Arg368His: c.1449 CGT>CAT

A heterozygous substitution of Guanine with Adenine at cDNA position c.1449 (Fig. 17F) resulted in replacement of arginine with histidine at codon 368 (Arg368His) in 8 OAG cases. Of these, four were sporadic, three were autosomal dominant, and one was as autosomal recessive family. This variation was found in heterozygous state in 2 control subjects. It was located in the meander region of the protein, connecting the J and K helices and resulted in the loss of *Taa*I restriction site. This was the most predominant mutation observed in 8 Indian OAG cases (7.3%). It was also observed in early onset open angle glaucoma patients in French population (Melki *et al.*, 2004). This mutation was associated with both JOAG and POAG and resulted in varying degrees of severity. Patients with this mutation presented with IOPs ranging from 10 to 42 mm Hg along with CD ratios from 0.3 to 0.9. After treatment, IOPs were under control in six patients, while it raised in one patient 45 mm Hg in the left eye. Visual acuities ranged from 20/20 to 20/30 in four patients and worsened to PLPR in three patients in one of their eyes (Table 18).

Trp434Arg: c.1646 TGG>CGG

In a sporadic case (P-116), a novel heterozygous change from Thymine to Cytosine at cDNA position c.1646 (Fig. 17G) resulted in the replacement of tryptophan to arginine at codon 434 (Trp434Arg). This residue was highly conserved across CYP1 family members in different species (Figure 18) and was not seen in the controls. This substitution resulted in the abolition of restriction site for *Bst*XI enzyme. This change resulted in the change of aromatic to aliphatic amino acid, and might be changing its hydrophobic interactions. The patient manifested the disease at an age of 67 years with IOPs of 14 mm Hg in both eyes and a vision of 20/40 and 20/25 in right and left eyes, respectively. Currently the patient is under medication with no further deterioration of his condition.

Pro437Leu: c.1656 CCG>CTG

A heterozygous substitution of Cytosine with Thymine at cDNA position c.1656 (Fig. 17H), replacing the amino acid proline with leucine at codon 437 (Pro437Leu) was observed in a sporadic case (P-026). This residue is highly conserved in the CYP1A1, CYP1A2 and CYP1B1 proteins across species (Figure 18). This mutation occurred between the K and L helices of the protein. It was absent in the controls and co segregated with another mutation Q48H of *MYOC* in P-026 family suggesting a digenic inheritance (Figure 15). The proband harbored two heterozygous mutant alleles of *MYOC* and *CYP1B1*, but as the parents'

DNA samples were not available for genotyping, the segregation of these alleles could not be determined (Figure 15).

c.1657delGGAGA

A homozygous deletion of 5bp at cDNA position c.1657 (Fig. 17J) was observed in a sporadic case (P-177). This mutation was not reported previously and not observed in the controls. The proband manifested the disease at the age of 15 years with initial IOPs of 30 mm Hg along with a CD ratio of 0.9 in both eyes. On surgery IOP in the right eye was16 mm Hg along with a visual acuity of 20/1200. The other eye had a vision of PLPR at the time of presentation, hence no further treatment was given (Table 18).

Phe445Cys: c.1680 TTC>TGC

A heterozygous change from Thymine to Guanine substitution at cDNA position c.1680 (Fig. 17I) resulted in the replacement of phenylalanine with cysteine at codon 445 (Phe445Cys) in a sporadic case (P-016). This residue is highly conserved across CYP1A1, CYP1A2 and CYP1B1 families among most species (Figure 18). This mutation has not been reported previously from other populations either in PCG or POAG and was not observed in the controls. This substitution resulted in the abolition of restriction site for *Hinf* I. The patient was diagnosed of POAG at the age of 44 years and presented with high IOPs (28 and 26 mm Hg in the right and left eye, respectively), along with total cupping of

the optic disc and a visual acuity of 20/400 in right eye and 20/100 in left eye. The patient was also myopic with a refraction of –9.5D and –7D in right and left eye, respectively. On surgery, his IOP is under control and the vision improved to 20/50 in right eye.

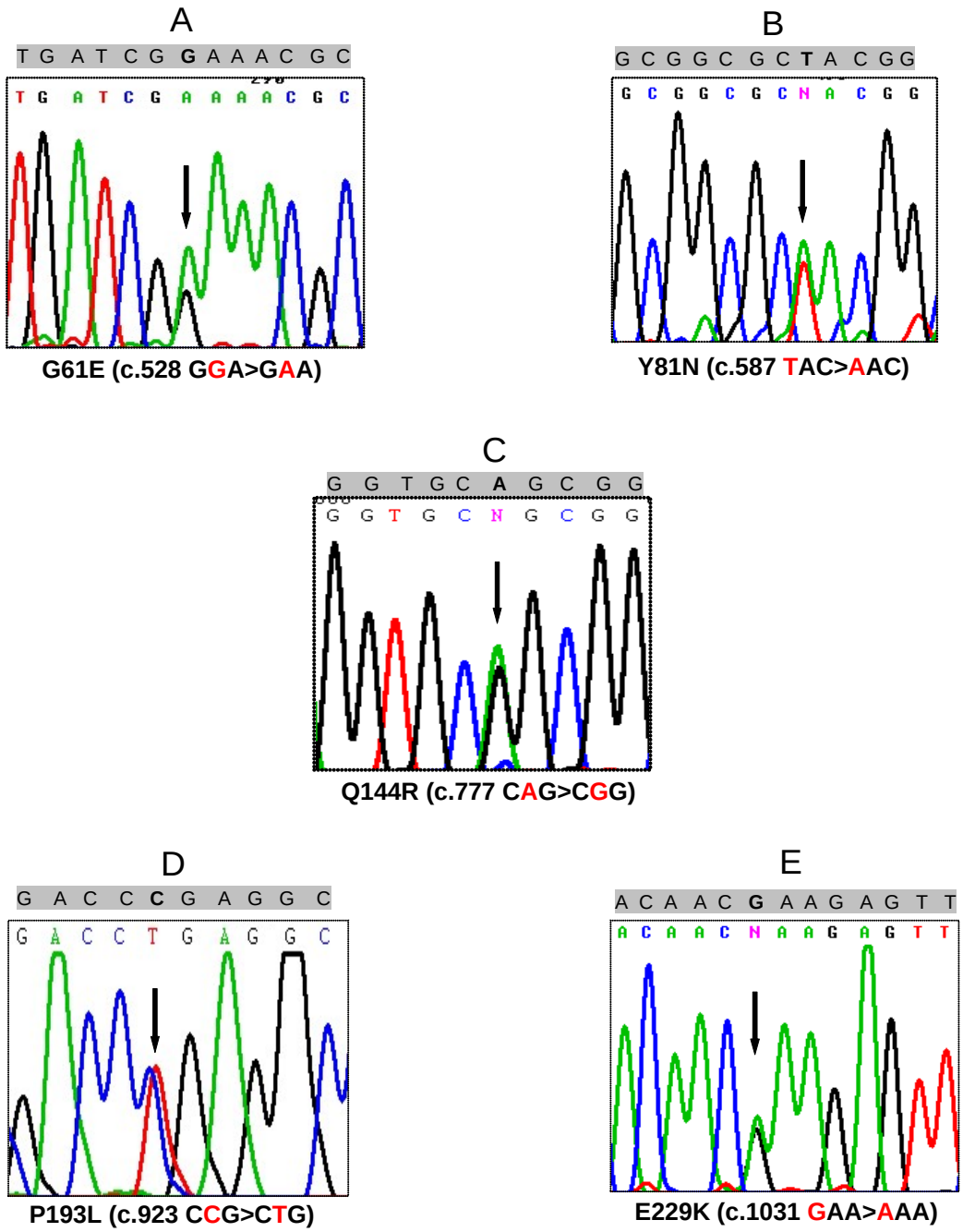
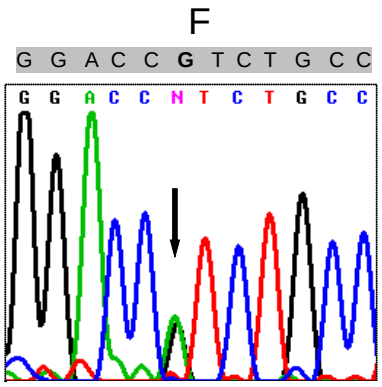
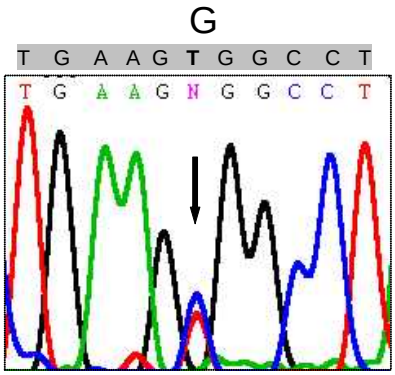


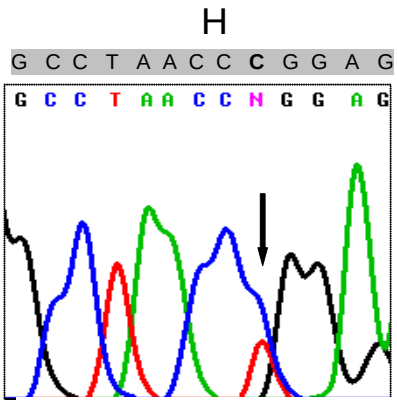
Figure 17. Electropherograms representing the *CYP1B1* mutations. The sequence above the electropherograms in shaded portion represents the wild type sequence and arrows shows point of mutation.
(A) A heterozygous substitution at c.528G>A resulting in the Gly61Glu mutation.
(B) A heterozygous sbstitution at c.587T>A resulting in the Tyr81Asn mutation.
(C) A heterozygous substitution of A>G at c.777 resulting in the Gln144Arg mutation.
(D) A heterozygous substitution of C>T at c.923 resulting in the Pro193Leu mutation.
(E) A heterozygous substitution of G>A at c.1031 resulting in the Glu229Lys mutation.



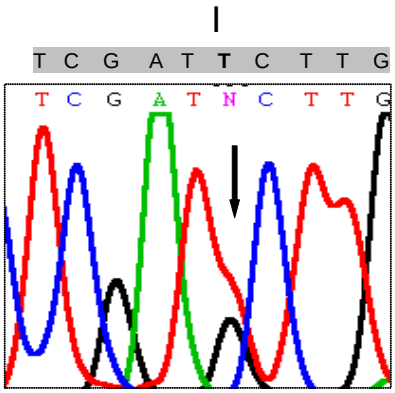
R368H (c.1449 CGT>CAT)



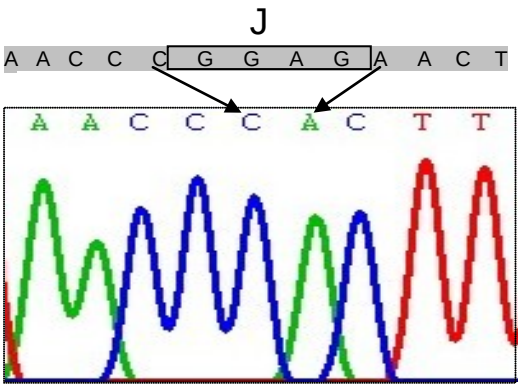
W434R (c.1646 TGG>CGG)



P437L (c.1656 CCG>CTG)



F445C (c.1680 TTC>TGC)



C.1657delGGAGA

Figure 17 (continued).
(F) A heterozygous substitution at c.1449G>A resulting in the Arg368His mutation.
(G) A heterozygous substitution at c.1646T>C resulting in the Trp434Arg mutation.
(H) A heterozygous substitution at c.1656C>T resulting in the Pro437Leu mutation.
(I) A heterozygous substitution at c.1680T>G resulting in the Phe445Cys mutation.
(J) A homozygous deletion of 5bp at c.1657.

Figure 18. Multiple sequence alignment of CYP1 protein across various species

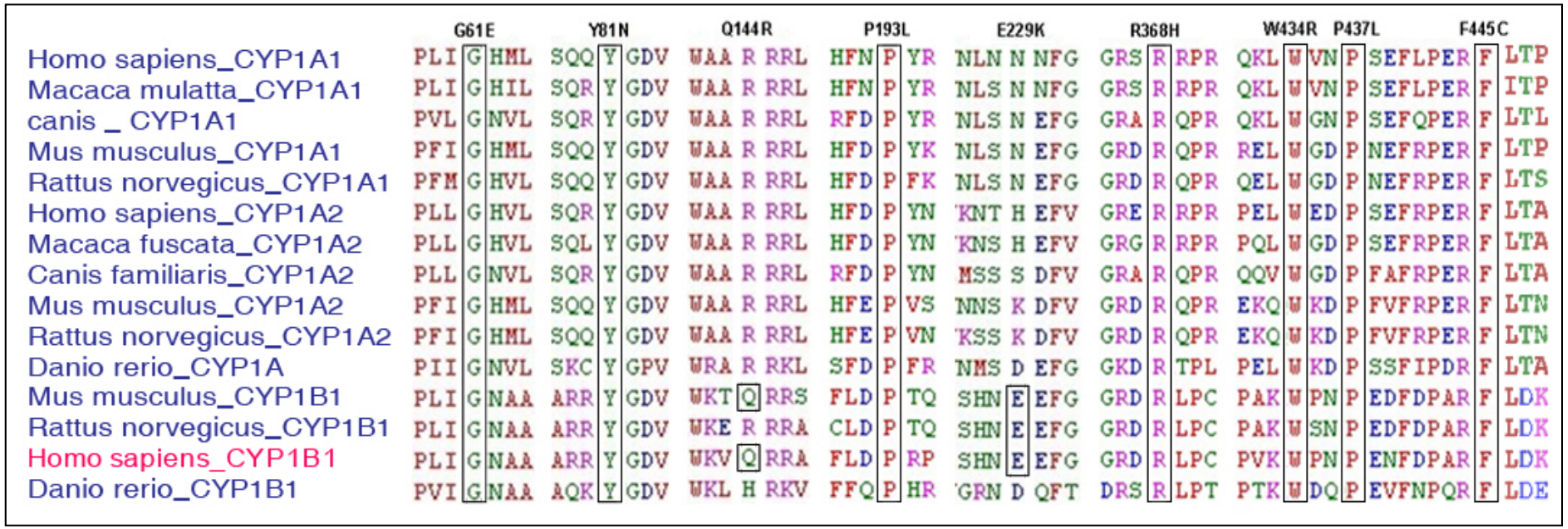


Figure 18. Multiple sequence alignment of Cytochrome P450 families across different species. The conservation of wild type residue is indicated by boxes.

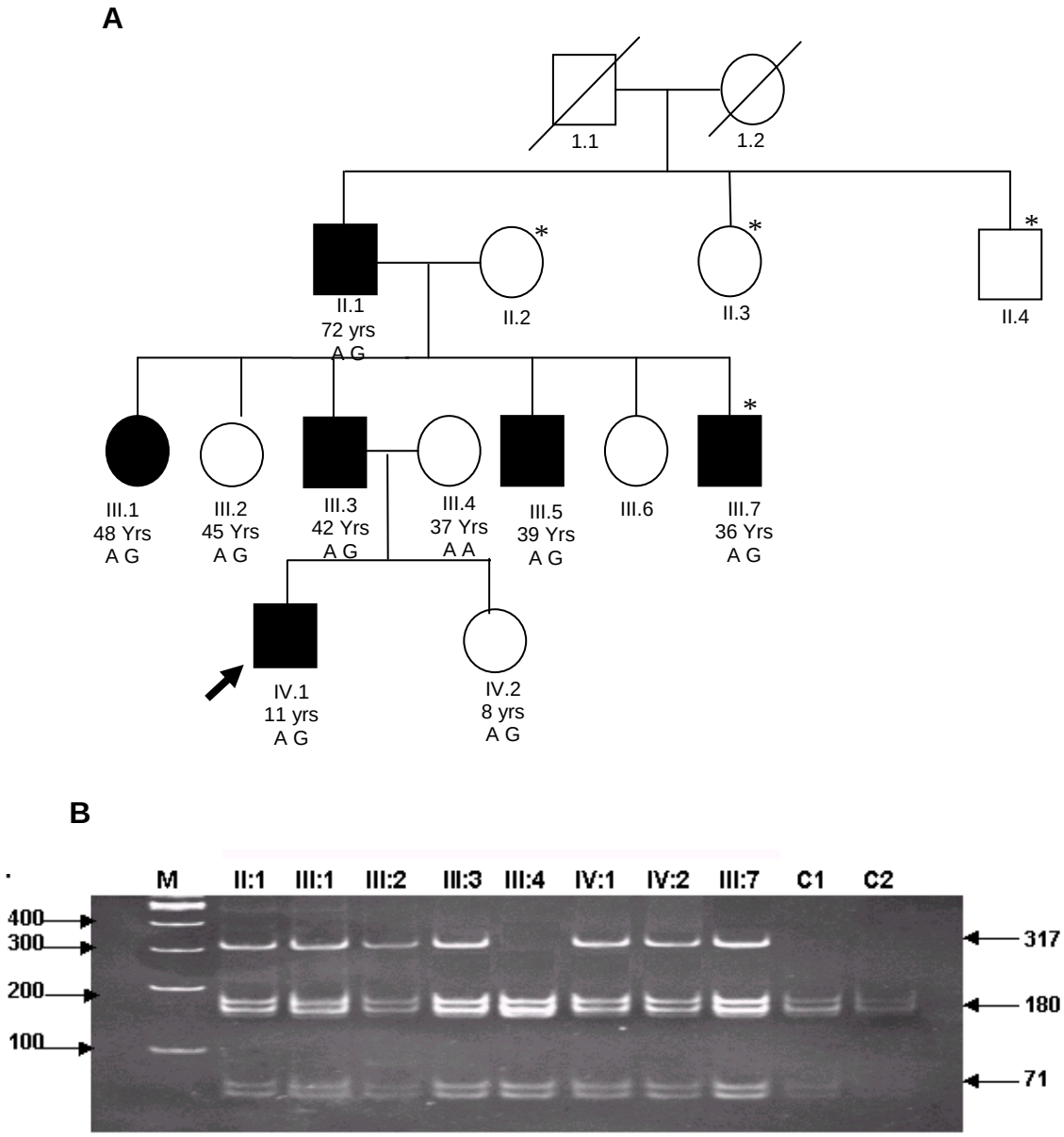
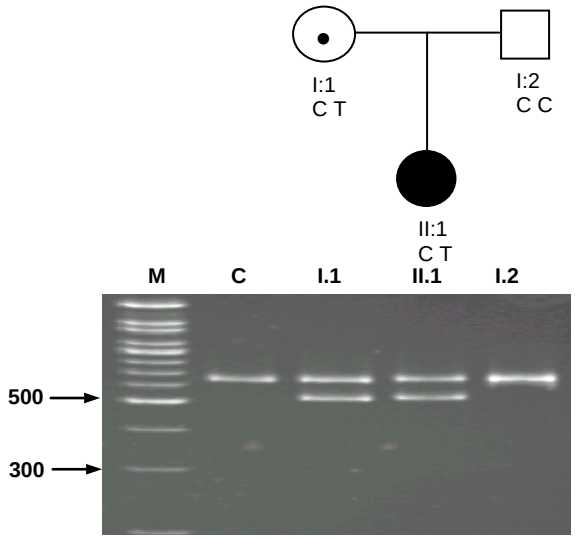


Figure 19. Segregation of Q144R in a multi generation family. A) Pedigree of P-001 family showing the segregation of Q144R mutation. DNA was not available from individuals marked with asterisk (*). The filled symbols indicate the affected individuals and clear symbols indicate the normal individuals. The proband is indicated by an arrow. The genotypes are indicated below the symbols. However, two individuals (III.2 and IV.2) heterozygous for Q144R did not manifest the disease phenotype. B) The 8% polyacrylamide gel showing the *MSP*A1I digestion pattern for Q144R mutation. M is the marker, C1 and C2 are the normal controls.

A) Segregation of the P193L mutation in P-173 family



B) Segregation of the E229K mutation in P-173 family

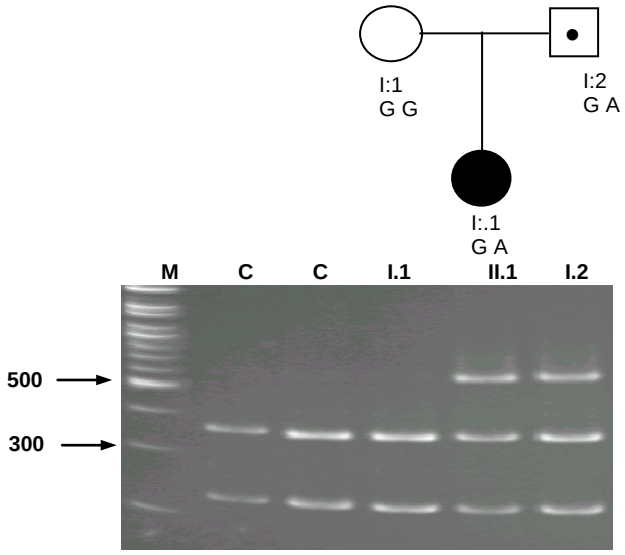


Figure 20. Co-segregation of P193L and E229K mutations in P-173 family. The filled symbols indicate the affected individuals and clear symbols indicate the normal individuals. Symbols with dot indicate carriers. A) Segregation of P193L (c.950 C>T) mutation indicated by PCR – based *Eco* 811 digestion. The proband is heterozygous like her mother. Father is normal. B) Segregation of E229K (c.1031 G>A) mutation indicated by PCR – based *Eam* 11041 digestion. The proband is heterozygous like her father and mother is normal. 'M' indicates DNA ladder and 'C' indicates normal control in both gels.

Table 17. List of *CYP1B1* mutations observed in patients with open angle glaucoma

S.No.	c.DNA position*	Exon	Codon change	Amino acid change	Mutation	Frequency (%)	Other POAG populations	Reference
1	c.528 G>A	2	GGA>GAA	Gly>Glu	G61E	1 (0.9 %)	Spanish	Lopez-Garrido *et al.,* 2006
2	c.587 T>A	2	TAC>AAC	Tyr>Asn	Y81N	1 (0.9 %)	French Spanish	Melki *et al.,* 2004, Lopez-Garrido *et al.,* 2006
3	c.777A>G	2	CAG>CGG	Gln>Arg	Q144R	3 (2.7 %)	Novel	
4	c.950 C>T	2	CCG>CTG	Pro>Leu	P193L	1 (0.9 %)	-	
5	c.1031G>A	2	GAA>AAA	Glu>Lys	E229K	6 (5.5 %)	India French Spanish	Melki *et al.,* 2004 Acharya *et al.,* 2006 Lopez-Garrido *et al.,* 2006
6	c.1449G>A	3	CGT>CAT	Arg>His	R368H	8 (7.3 %)	India	Acharya *et al.,* 2006
7	c.1646 T>C	3	TGG>CGG	Trp>Arg	W434R	1 (0.9 %)	Novel	
8	c.1656 C>T	3	CCG>CTG	Pro>Leu	P437L	1 (0.9 %)	-	
9	c.1657 del 5bp	3	-	-	del 5bp	1 (0.9 %)	Novel	
10	c.1680 T>G	3	TTC>TGC	Phe>Cys	F445C	1 (0.9 %)	Novel	

*GenBank ID # NM_000104

Table 18. Clinical features of patients with different *CYP1B1* mutations

Case No	Mutation	Age at onset	IOP at Presentation OD;OS	IOP at Treatment OD;OS	CD ratio	Visual acuity at Presentation OD;OS	Visual acuity at Treatment OD;OS	Field Defects	Treatment
P-008	G61E / R368H	9	38; 40	15; 16	0.9;0.9	20/70; 20/40	Cf50cm;20/50	S; S	Surgery OU
P-105	Y81N	30	15; 15	16; 16	0.9;0.9	20/30; 20/20	20/50; 20/20	S; S	Surgery OU
P-001	Q144R	12	42; 30	13; 13	0.6;0.6	20/25; 20/20	20/20; 20/20	Mi; Mi	Surgery OU
P-033	Q144R	65	39; 44	14; 38	0.9;0.6	20/125;plpr-	20/25; plpr	S; S	Surgery OS
P-086	Q144R	50	12; 12	04; 06	0.9;0.6	20/20; 20/20	20/40; 20/20	S; Mi	Surgery OU
P-173	P193L / E229K	7	24; 22	20; 21	0.8;0.8	20/30; 20/30	20/20; 20/20	Mi; Mi	Medication
P-012	E229K	40	16; 19	16; 17	0.9;0.9	20/20; 20/20	20/20; 20/30	S; S	Surgery OU
P-013	E229K	53	10; 18	08; 12	0.9;0.9	20/100; 20/20	20/400; 20/25	S; Mo	Medication
P-039	E229K	64	18; 18	12; 12	0.6;0.8	20/20; 20/20	20/20; 20/30	Mi; Mo	Medication
P-081	E229K	32	24; 14	46; 12	0.9;0.9	plpr-; 20/30	plpr-; 20/25	S; S	Surgery OS
P-104	E229K	39	14; 12	10; 11	0.9;0.9	20/25; 20/20	20/20; 20/20	S; S	Surgery OU
P-024	R368H	57	20; 16	10; 06	0.9;0.9	plpr; 20/30	plpr; 20/30	NA; Mi	Surgery OU

P-027	R368H	49	26; 28	13; 13	0.9;0.9	Plpr-; 20/30	plpr ina; 20/30	S; S	Surgery
P-035	R368H	35	20; 22	10; 45	0.9;0.9	20/30; 20/60	20/160;plpr	S; S	Surgery OU
P-084	R368H	63	15; 17	14; 10	0.9;0.9	20/25; 20/20	20/25; 20/30	Mi; S	Surgery OS
P-112	R368H	20	42; 30	10; 10	0.9;0.3	20/25; 20/20	20/20; 20/20	S; Mi	Surgery OD
P-114	R368H	80	18; 16	14; 14	0.9;0.8	20/30; 20/30	20/30; 20/30	S; Mi	Medication
P-078	R368H	39	18; 10	18; 14	0.4;0.3	20/20; 20/20	20/20; 20/20	N; N	Medication
P-116	W434R	67	14; 14	10; 12	0.4;0.8	20/40; 20/25	20/40; 20/30	Mi; Mo	Medication
P-026	P437L	35	22; 22	14; 12	0.9;0.8	20/20; 20/20	20/20; 20/20	S; Mi	Surgery OD
P-177	Del 5BP	15	30; 30	16; 31	0.9;0.9	CF1m; pl+pr-	20/1200;plpr	S; S	Surgery OD
P-016	F445C	44	28; 26	14; 14	0.9,0.9	20/400;20/100	20/50; 20/100	S; S	Surgery OD

Clinical features of probands with *CYP1B1* mutations indicating their mutation, age of onset, IOP and visual acuities at presentation and treatment along with the CD ratios, field defects and treatment. OU = Both eyes; OD = Right eye; OS = Left eye; S = Severe; Mi = Mild; Mo = Moderate; N = Normal; plpr = Perception of light with accurate projection of rays; pr- = inaccurate projection of rays; CF = Counting fingers; NA = Not available

4.1.1.2.1 Single Nucleotide Polymorphisms in *CYP1B1*

In addition to the mutations, five intragenic SNPs were observed in exon II (R48G and A119S) and exon III (V432L, D449D and N453S). The SNPs R48G and A119S in exon II, V432L and D449D in exon III were found to be in linkage disequilibrium. The details of these SNPs are given in Table 19.

Table 19. List of *CYP1B1* polymorphisms observed in OAG

S.No	dbSNP ID	Location	Codon Change	Amino acid change
1	rs10012	Exon II	CGG>GGG	R48G
2	rs1056827	Exon II	GCC>TCC	A119S
3	rs1056836	Exon III	GTG>CTG	V432L
4	rs1056837	Exon III	GAT>GAC	D449D
5	rs1800440	Exon III	AAC>AGC	N453S

There was no significant difference in the frequency distribution of these alleles between patients and controls (Table 20). However a formal haplotype analysis was not done.

Table 20. Genotype frequencies distribution of *CYP1B1* SNPs in patients and controls

S.No	Polymorphism		Patients N (%)	Controls N (%)
1	R48G	CC	37 (34.2)	32 (32.0)
		CG	54 (50.0)	42 (42.0)
		GG	17 (15.7)	26 (26.0)
2	A119S	GG	37 (34.2)	32 (32.0)
		GT	54 (50.0)	42 (42.0)
		TT	17 (15.7)	26 (26.0)
3	V432L	GG	06 (5.5)	04 (4.0)
		GC	29 (26.8)	33 (33.0)
		CC	73 (67.5)	63 (63.0)
4	D449D	TT	06 (5.5)	04 (4.0)
		TC	29 (26.8)	33 (33.0)
		CC	73 (67.5)	63 (63.0)
6	N453S	AA	75 (69.4)	74 (74.0)
		AG	27 (50.0)	22 (22.0)
		GG	06 (5.5)	4 (4.0)

4.1.1.3 Mutational analysis of *OPTN*

Screening of *OPTN* by SSCP in 103 unrelated POAG patients did not reveal any pathogenic mutations. Six different nucleotide variations were observed, of which the Thr34Thr, Met98Lys and Arg149Arg were in the coding region and the remaining were intronic (Table 21). None of these SNPs exhibited significant association with the disease phenotype. A brief description of the variations is given below.

Met 98 Lys: c.619 A<u>T</u>G>A<u>A</u>G

A heterozygous change of Thymine to Adenine at cDNA position c.619 resulted in the replacement of amino acid from Methionine to Lysine at codon 98 (Met98Lys). This change resulted in the gain of *Stu*I restriction site. The allele frequency distribution among patients and controls was not statistically significant (p = 0.299) (Table 21). This variant was initially identified as a risk factor and was observed in higher frequencies in patients than controls (Rezaie *et al.,* 2002).

Silent changes:

Two silent changes were observed, one in exon 4 (Thr34Thr) and other in exon 6 (Arg149Arg). The Arg149Arg variation was reported here for the first time where as Thr34Thr was noted previously (Rezaie *et al.,* 2002, Alward *et al.,* 2003, Willoughby *et al.,* 2004). This silent change at cDNA position c.428 G>A at codon 34 abolished the restriction site for *Tai*I enzyme. There was no significant difference in the frequency distribution of these alleles among patients and controls. This variation

was observed in almost all the studies across different populations (Table 21).

Intronic variations

Three intronic variations were observed, two of them in the upstream region of exon 7, at IVS6 –5C>T, and IVS6 –10G>A, and the third one downstream of exon 15 at IVS15 +10G>A. None of these variations were predicted to create cryptic splice sites and hence their role in causing the disease could be ruled out. The frequency distribution of these alleles was similar in patients and controls (Table 21).

Table 21. Distribution frequencies of *OPTN* SNPs between patients and controls

S.No	Variation		Patients N (%)	Controls N (%)	Population	Reference
1	T34T	GG	72 (69.9)	49 (49)	USA USA Canada	Rezaie *et al.,* 2002 Alward *et al.,* 2003 Willoughby *et al.,* 2004
		GA	29 (28.1)	45 (45)		
		AA	2 (1.9)	6 (06)		
2	M98K	TT	91 (88.3)	95 (95)	USA USA Canada USA Japan	Rezaie *et al.,* 2002 Alward *et al.,* 2003 Willoughby *et al.,* 2004 Wiggs *et al.,*, 2004 Fuse *et al.,* 2004
		TA	12 (11.7)	5 (05)		
3	R149R	GG	102 (99)	100 (100)	Novel	
		GA	1 (01)	00 (00)		
4	IVS6 -5T>C	TT	66 (64)	33 (64.7)	USA Canada Japan	Alward *et al.,* 2003 Willoughby *et al.,* 2004 Fuse *et al.,* 2004
		TC	31 (30)	12 (23.5)		
		CC	6 (5.8)	6 (11.7)		
5	IVS6 –10G>A	GG	102 (99)	51 (100)	USA	Alward *et al.,* 2003
		GA	1 (01)	0 (00)		
6	IVS15 +10G>A	GG	102 (99)	100 (100)	USA	Alward *et al.,* 2003
		GA	1 (01)	00 (00)		

4.1.2 SNP screening in Primary glaucomas

Four SNPs in three candidate genes *p53*, *ApoE* and *TNFα* were screened and their allele and genotype frequencies were compared between cases and controls to determine their association with the disease phenotype. Genotypes were also compared with *MYOC* and *CYP1B1* mutations. Odds ratios were calculated to assess the risk of individuals with a particular genotype in developing the disease.

4.1.2.1 p53 polymorphism

The allele and genotypes frequencies in patients and controls are shown in Tables 22 and 23. Figure 21 shows the observed haplotypes based on the genotypes. No significant differences were observed in the distribution of genotypes of *p53* codon-72 polymorphism (P = 0.189) and intron-3 polymorphism (P = 0.151) between patients and controls. The association of the *p53* genotypes were assessed with respect to IOP, CD ratio and mutation background. The parameters were above and below 21 mm Hg for IOP and for CD ratio above and below 0.7. Patients with and without *MYOC* and *CYP1B1* mutations were also considered for association to *p53* polymorphism. However no associations were noted with these parameters (Tables 24 and 25). Odds ratios for these genotypes were not significant (Table 24 and 25).

Figure 21. *p53* haplotypes after digestion with *Bsh*1236I

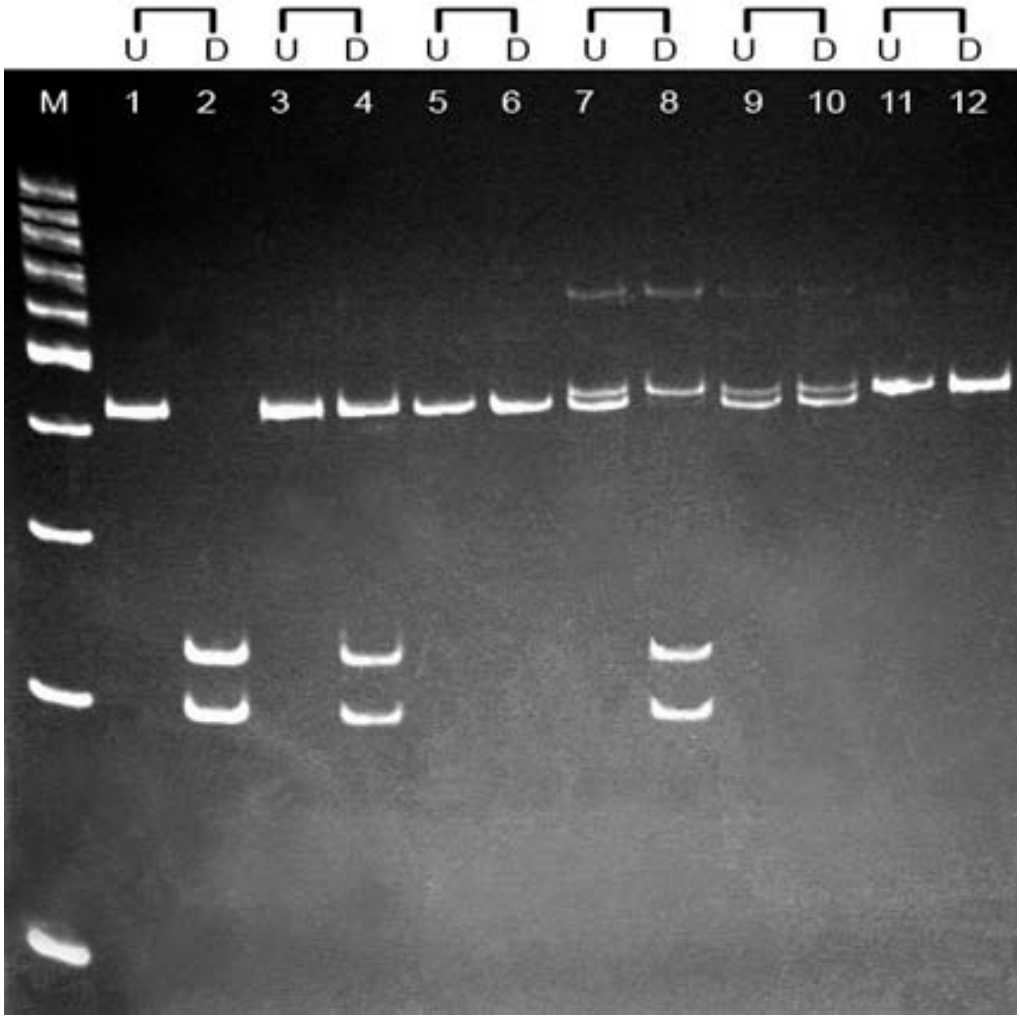


Figure 21. The 8% polyacrylamide gel showing observed *p53* haplotypes. Lane 1 & Lane 2: Undigested and digested products of del/Arg, del/Arg. Lane 3 & Lane 4: Undigested and digested products of del/Arg, del/Pro. Lane 5 & Lane 6: Undigested and digested products of del/Pro, del/Pro. Lane 7 & Lane 8: Undigested and digested products of del/Arg, Ins/Pro. Lane 9 & Lane 10: Undigested and digested products of del/Pro, Ins/Pro. Lane 11 & Lane 12: Undigested and digested products of Ins/Pro, Ins/Pro. U - Undigested, D - Digested, M – Marker.

Table 22. Allele frequencies of *p53* codon-72 and intron-3 polymorphisms

Subjects	Codon-72 polymorphism		Intron-3 polymorphism	
	Arg	Pro	Del	Ins
Patients	43	57	77	23
Controls	54	46	70	30
Significance	χ^2 = 2.422, P = 0.12		χ^2 = 1.258, P = 0.262	

Table 23. Genotype distribution of *p53* codon-72 (A) and intron-3 (B) polymorphisms

(A) Codon-72 polymorphism			
Geno Types	**Patients** (N = 107)	**Controls** (N = 106)	**Odds Ratio**
Arg/Arg (%)	20 (18.7)	31 (29.2)	1
Arg/Pro (%)	55 (51.4)	49 (46.2)	1.74 (0.8-3.6)
Pro/Pro (%)	32 (30.0)	26 (24.5)	1.9 (0.8-4.4)
Signifcance	χ^2 = 3.334, P = 0.189		

(B) Intron-3 polymorphism			
Geno types	**Patients** (N = 107)	**Controls** (N = 106)	**Odds Ratio**
Del/Del (%)	63 (58.8)	73 (68.9)	1
Del/Ins (%)	42 (39.2)	29 (27.3)	1.69 (0.9-3.1)
Ins/Ins (%)	2 (1.8)	4 (3.8)	0.58 (0.1-3.3)
Signifcance	χ^2 = 378, P = 0.151		

Table 24. Association between Arg/Pro genotype and phenotypic traits

Patients	IOP <21	IOP ≥ 21	CD ratio ≤ 0.7	CD ratio > 0.7	*Cases with mutations	Cases without mutations
Arg-Arg	7	13	3	17	7	13
Arg-Pro	21	34	10	42	14	41
Pro-Pro	14	18	2	30	8	24
Significance	$\chi 2 = 0.449$, P=0.799		$\chi 2 = 2.7$, P=0.258		$\chi 2 = 0.778$, P=0.678	

* Cases with *MYOC* or *CYP1B1* mutations

Table 25. Association between Indel polymorphism and phenotypic traits

Patients	IOP <21	IOP ≥ 21	CD ratio ≤ 0.7	CD ratio > 0.7	*Cases with mutations	Cases without mutations
Del-del	23	40	12	50	18	45
Del-Ins	19	23	3	37	10	32
Ins-Ins	0	2	0	2	1	1
Significance	$\chi 2 = 2.122$, P=0.346		$\chi 2 = 3.1$, P=0.211		$\chi 2 = 0.83$, P=0.66	

* Cases with *MYOC* or *CYP1B1* mutations

4.1.2.2 *ApoE* polymorphism

The allele and genotypes frequencies in patients and controls are shown in Tables 26 and 27. Figure 22 shows the gel picture with the observed genotypes and the corresponding fragments. No significant differences were observed in the distribution of *ApoE* genotype (p = 0.35) and allele (p = 0.572) frequencies between patients and controls. There was no association with IOP, CD ratio or the mutation spectrum (as categorized in section 4.1.2.1) with any of these genotypes (Table 28).

Figure 22. Gel picture showing *ApoE* haplotypes

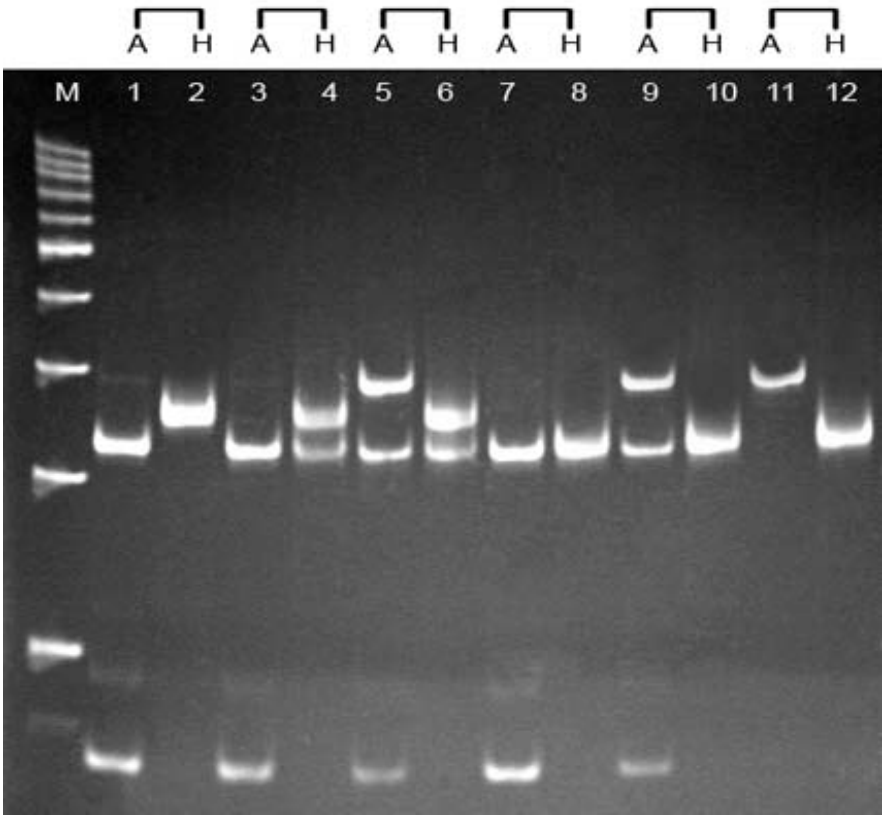


Figure 22. The 8% polyacrylamide gel showing observed *APOE* haplotypes. A: *Afl* III digestion, H: *Hae* II digestion, M: Marker. Lane 1 & Lane 2: *Afl* III and *Hae* II digested products of ε2/ ε2. Lane 3 & Lane 4: *Afl* III and *Hae* II digested products of ε2/ ε3. Lane 5 & Lane 6: *Afl* III and *Hae* II digested products of ε2/ ε4. Lane 7 & Lane 8: *Afl* III and *Hae* II digested products of ε3/ ε3. Lane 9 & Lane 10: *Afl* III and *Hae* II digested products of ε3/ ε4. Lane 11 & Lane 12: *Afl* III and *Hae* II digested products of ε4/ ε4

Table 26. Allele frequencies of *ApoE* polymorphism

Subjects	ε 2	ε 3	ε 4
Patients	3	89	8
Controls	5	84	11
Significance	χ2=1.118, P = 0.572		

Table 27. Genotype distribution of *ApoE* polymorphism

Subjects	**Patients** (N = 108)	**Controls** (N = 104)	**Odds ratio**
ε3 / ε3 (%)	86 (79.6)	74 (71.2)	1
ε2 / ε3 (%)	06 (5.5)	09 (8.7)	0.57 (0.2-1.7)
ε3 / ε4 (%)	16 (14.8)	21 (20.2)	0.655 (0.3-1.3)
Significance	χ^2 = 2.1, P = 0.35		

Table 28. Associations between *ApoE* genotypes and phenotypic traits

Patients	**IOP <21**	**IOP ≥ 21**	**CD ratio ≤ 0.7**	**CD ratio > 0.7**	**Cases with mutations**	**Cases without mutations**
ε2 / ε3	2	4	0	6	2	4
ε3 / ε3	37	49	10	74	22	64
ε3 / ε4	3	13	5	10	6	10
χ2 test	χ2 = 3.42, P=0.19		χ2 = 5.83, P<0.054		χ2 = 1.05, P=0.591	

4.1.2.3 *TNFα (-308G>A)* polymorphism

The allele and genotype frequencies of *TNFα* -308G>A are given in Tables 29 and 30, respectively. No significant differences were observed in the distribution of allele and genotype frequencies among patients and controls.

Table 29. Allele frequencies of *TNFα* polymorphism

Subjects	G allele	A allele
Patients	94	6
Controls	94	6

Table 30. Genotype distribution of *TNFα* polymorphism

Subjects	GG (%)	GA (%)
Patients (N = 107)	94 (87.8)	13 (12.1)
Controls (N = 101)	89 (88.1)	12(11.8)
Significance	χ^2 = 0.0035, P = 0.953	

4.2 Mutation screening in secondary glaucomas

4.2.1 Mutation screening in candidate genes

In order to determine the molecular mechanism underlying Indian ARS cases, we screened *FOXC1* and *PITX2* by direct sequencing.

4.2.1.1 Mutational analysis of *FOXC1*

Direct sequencing of *FOXC1* coding region in 25 ARS cases revealed six different mutations in 7 unrelated cases. This included, three nonsense (Q2X, Q92X and Q123X), two missense (M161K and R170Q) and one frameshift (c.847 ins 4bp) mutation. Table 31 shows the list of mutations observed in *FOXC1* in ARS patients. Figure 23 shows the electropherograms representing the *FOXC1* mutations and Figure 24 shows the multiple sequence alignment of FOXC1 across various species. A brief description of the mutations is given below.

Gln2Stop: c.4 C*AG >*T*AG

A heterozygous substitution of Cytosine with Thymine at cDNA position c.4 (Fig. 23A) position resulted in the truncation of protein at the second amino acid in a sporadic case (ARA-10). To the best of our knowledge this mutation was not reported previously and resulted in a null allele. The wild type residue is highly conserved across FOXC1 families in different species but absent in other FOX proteins of humans (Figure

24). The proband presented at the age of 11 years with normal IOPs of 12 mm Hg in both eyes and a CD ratio of 0.5 and 0.6. There was no evidence of secondary glaucoma. He showed good prognosis on medication with a final visual acuity of 20/20 both eyes.

Gln92Stop: c.274 CAG>TAG

A heterozygous change from Cytosine to Thymine at cDNA position c.274 (Fig. 23B) position resulted in a nonsense mutation at codon 92 was observed in a sporadic case (ARS-26). This mutation truncated the protein after 92 amino acids. This residue is highly conserved across different species. To the best of our knowledge, this mutation has not been reported previously. The proband manifested the disease phenotype at birth. After surgery, the patient has IOPs of 11 mm Hg along with a CD ratio of 0.2 in both eyes.

Gln123Stop: c.367 CAG >TAG

Another heterozygous change from Cytosine to Thymine at cDNA position c.367 position (Fig. 23C) resulted in a nonsense mutation at codon 123 in an autosomal dominant family (ARS-7). This mutation truncated the protein in helix 3 region of the forkhead domain. This mutation was reported previously in a murine homolog of *FOXC1* (*Mf1)*, in a mouse with congenital hydrocephalus (Hong *et al.,* 1999). This is perhaps the first report of this mutation in humans and segregated with the disease phenotype. The unaffected grandmother in this family also harbored the same mutation but did not manifest the phenotype (Figure

25). This residue is highly conserved across all the FOXC1 proteins (Figure 24). The proband presented at eighteenth day after birth with IOPs of 28 and 30 mm Hg in the right and left eye, respectively, along with CD ratios of 0.5 and 0.7 in the right and left eye, respectively. The proband showed relatively fair prognosis compared to his mother and uncle. Both of them were diagnosed at the age of 10 and 8 years, respectively, and had developed pthisis bulbi in one of their eyes at the time of presentation. Despite surgical intervention, their IOPs were high (30 and 36 mm Hg for mother and maternal uncle respectively), with total cupping. The mother was having no vision in her right eye and 20/200 in left eye, while the uncle had a vision of light perception and no light perception in right and left eye respectively. The unaffected grandmother with the same mutation was having normal vision and did not show any symptoms of ARS or secondary glaucoma.

Met161Lys: c.482 A<u>T</u>G > A<u>A</u>G

A heterozygous change from Thymine to Adenine at cDNA position c.482 position (Fig. 23D) resulted in the replacement of methionine with lysine at codon 161 in two autosomal dominant families (ARS-3 and ARS-8). This mutation was not observed in the normal controls and found to segregate with the disease phenotype in both the families. The substitution abolished the restriction site for *Nla*III enzyme. This residue is highly conserved across all species (Figure 24). In ARS-3 family, the proband and his brother showed better prognosis, perhaps due to early intervention when compared to their father who was

intervened at the age of 24 years (Table 32). In another family (ARS-8) harboring the same mutation, proband showed poor prognosis with a vision of 20/260 and fixes and follows light in right and left eye, respectively, probably due to secondary glaucoma at the time of presentation when compared to ARA-3 proband (Table 32).

Arg170Gln: c.509 CGG>CAG

A heterozygous substitution of Guanine with Adenine at cDNA position c.509 position (Fig. 23E) resulted in the replacement of arginine by glutamine in a sporadic case (ARS-28). To the best of our knowledge it is a novel mutation and was not observed in normal controls. This mutant residue lies in the nuclear localization signal and might interfere with the localization of the protein. But so far none of the observed mutations appeared to affect the localization of the protein (Lines *et al.,* 2002). The patient presented at the age of 17 years with raised IOPs of 40 and 32 mm Hg in the right and left eye, respectively, along with a total cupping of the optic nerve. After surgery, the patient's IOPs are 14 and 19 mm Hg along with a visual acuity of 20/25 and 20/50 in right and left eye, respectively.

c.847insCCGC

A heterozygous insertion of 4 bp (CCGC) at cDNA position c.847 (Fig. 23F) resulted in a frameshift in a sporadic case (ARA-19). This mutation has not been reported previously and abolished the restriction site for *Mbi*I enzyme. The patient presented at the age of 16 years with

initial IOPs of 20 and 50 mm Hg in the right and left eyes, respectively. Currently IOPs are maintained at 16 and 26 mm Hg along with a vision of 20/40 and no light perception in right and left eyes, respectively.

FOXC1 Polymorphisms

An insertion deletion (Indel) of GGC nucleotide was observed after codon 375, in a stretch of 6 nucleotides of (GGC) and after codon 447, in a stretch of 7 nucleotides of (GGC). This variation has been reported previously in both patients and controls (Mears *et al.,* 1998).

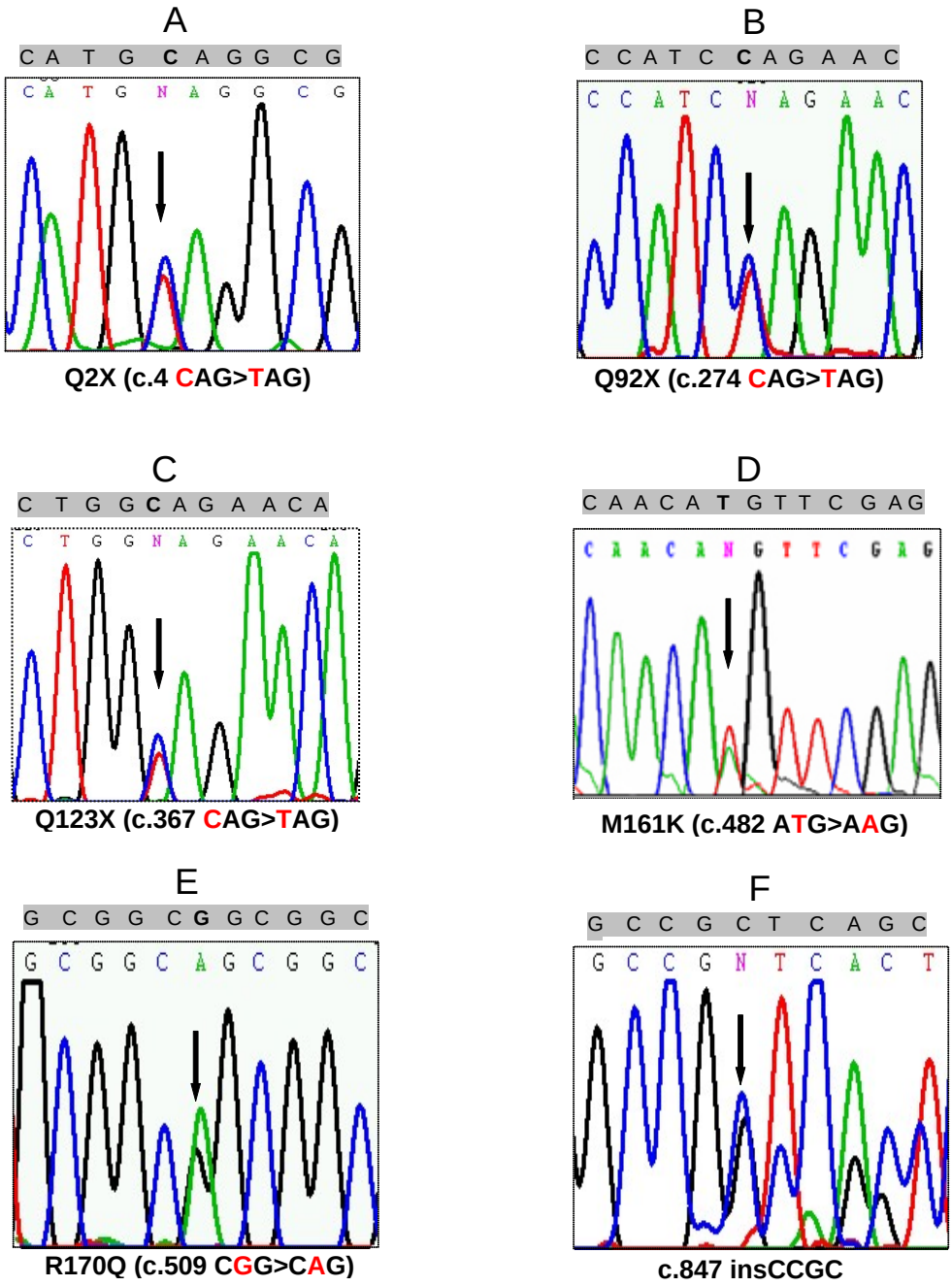


Figure 23. Electropherograms representing different *FOXC1* mutations. The shaded sequence represents the wild type sequence and arrow shows the point of mutation. A). A heterozygous substitution at c.4 C>T resulting in the protein termination at second amino acid. B). A heterozygous substitution at c.274 C>T resulting in the protein termination at codon 92. C). A heterozygous substitution from C>T at position c.367 resulting in the protein termination at codon 123. D). A heterozygous substitution at c.482 T>A resulting in the replacement of Met with Lys at codon 161. E). A heterozygous substitution at c.509 G>A resulting in the replacement of Arg with Gln at codon 170. F). A heterozygous insertion of 4bp at position c.847 resulting in frameshift mutation.

Figure 24. Multiple sequence alignment of FOXC1 in various species

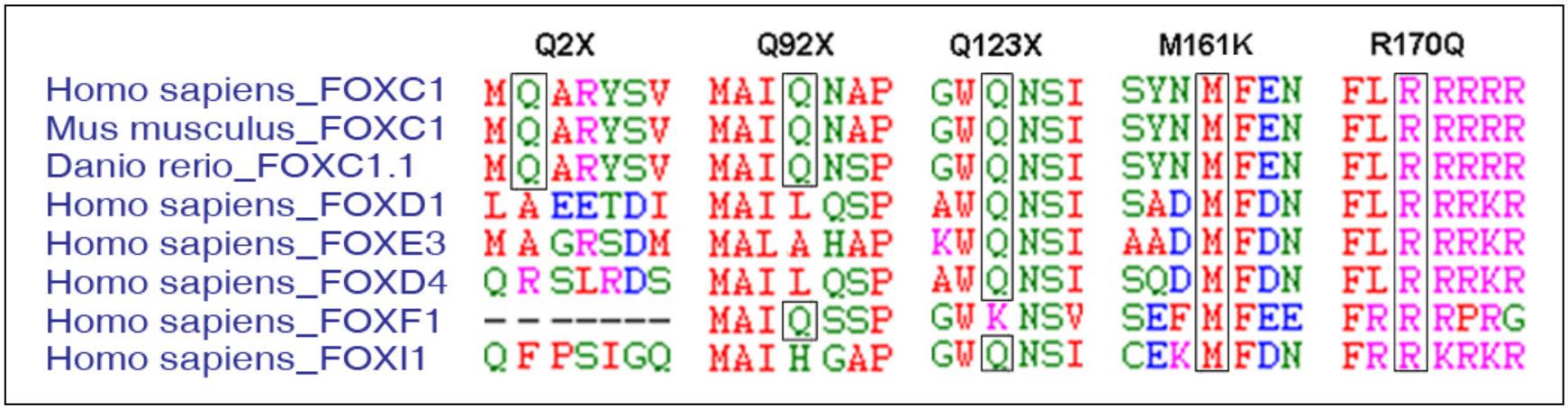


Figure 24. Multiple sequence alignment of FOX family of proteins across different species. The wild type residues are indicated in a box.

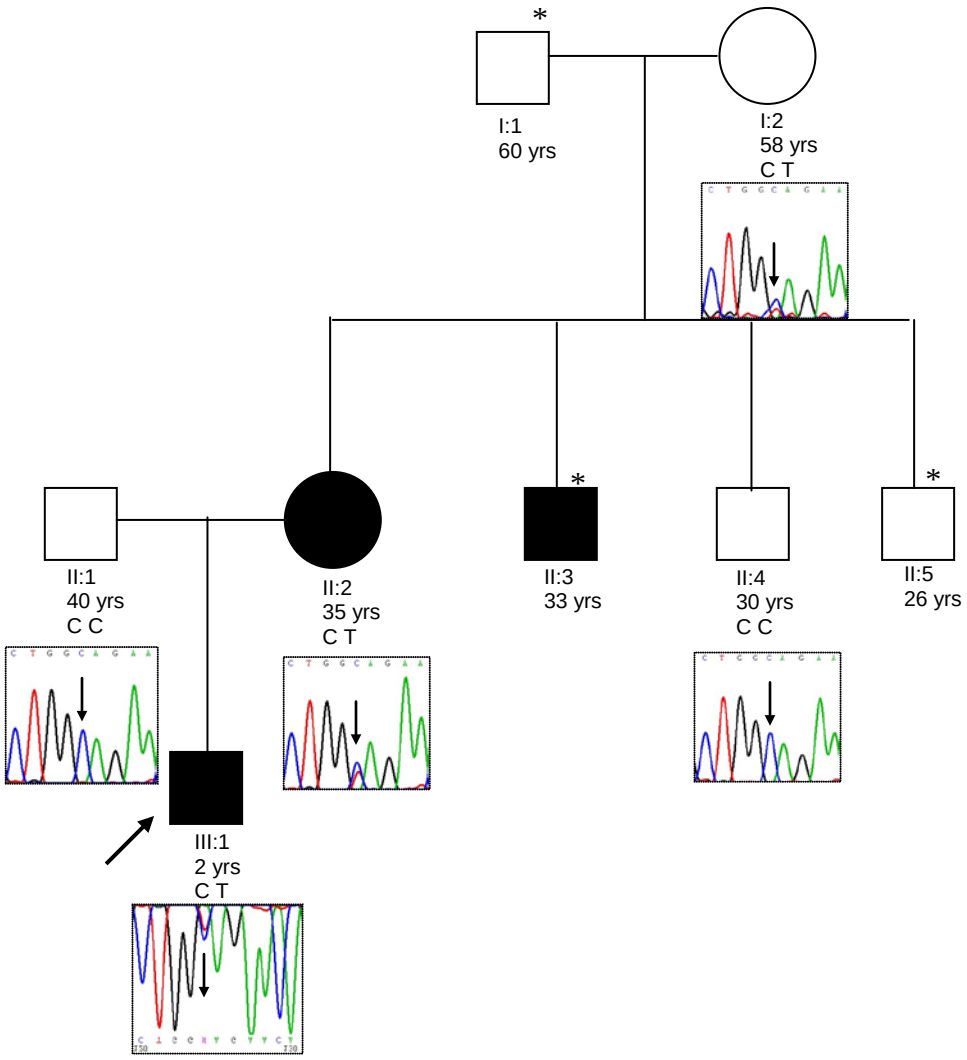


Figure 25. Segregation of the Q123X (c.367 C>T) mutation in ARS-07 family. Darkened symbols indicate the affected persons. Arrow indicates the proband. The genotypes are indicated below the symbols. The corresponding electropherograms indicate the Q123X mutation in individuals I:2, II:2 and III:1. The grandmother of the proband was asymptomatic but carried the heterozygous null allele. The DNA was not available from subjects marked with * (asterisk).

Table 31. List of *FOXC1* mutations observed in patients with ARS

S.No	c. DNA position*	Exon	Codon change	Amino acid change	*Mutation*	Frequency (%)	Other populations
1	c.4 C>T	1	CAG >TAG	Gln > Stop	Q2X	1 (4)	Novel
2	c.274 C>T	1	CAG > TAG	Gln > Stop	Q92X	1 (4)	Novel
3	c.367 C>T	1	CAG >TAG	Gln > Stop	Q123X	1 (4)	Novel
4	c.482 T>A	1	ATG > AAG	Met > Lys	M161K	2 (8)	India (Panicker *et al.,* 2002)
5	c.509 G>A	1	CGG>CAG	Arg > Gln	R170Q	1 (4)	Novel
6	c.847 ins 4bp	1	-	Frameshift	Ins 4bp	1 (4)	Novel

*****Gen bank ID # NM_001453

Table 32. Clinical features of individuals with different *FOXC1* mutations

Case ID	Mutation	Age		Corneal Diameter		Initial IOP		Final IOP		CD Ratio	Final VA	
		Onset	Diagnosis	OD	OS	OD	OS	OD	OS	OD OS	OD	OS
ARS-10	Q2X	Birth	11yrs	NA	NA	12	12	16	12	0.5; 0.6	20/20	20/20
ARS-26	Q92X	Birth	1 month	13	13	dgn	dgn	11	11	0.2; 0.2	NA	NA
ARS-7	Q123X	Birth	18 days	12	12.5	28	30	14	12	0.5; 0.7	6/30	6/38
ARS-7.M	Q123X	Birth	10yrs	NA	12	NA	NA	PB	30	NA; 0.9	NLP	20/200
ARS-7.U	Q123X	Birth	8yrs	13	NA	NA	NA	36	PB	NA; NA	LP	NLP
ARS-3A	M161K	Birth	2.5 months	12	12	30	28	12	14	0.4; 0.3	20/80	20/80
ARS-3B	M161K	Birth	2 weeks	11	11.5	24	26	11	12	0.2; 0.3	20/80	20/80
ARS-3C	M161K	Birth	24 yrs	NA	NA	42	35	14	14	NA; 0.9	NLP	CF 1m

M161K	ARS-8A	Birth	3 days	13	12	32	36	12n	13	0.4; NA	20/260	FF
M161K	ARS-8C	Birth	28 yrs	12.5	NA	21	NA	18	PB	0.7; NA	20/30	NLP
M161K	ARS-8G	Birth	54 yrs	NA	12.5	NA	16	PB	16	NA; 0.3	NLP	20/30
R170Q	ARS-28	12yrs	17yrs	Rai-sed	Rai-sed	40	32	14	19	0.9; 09.	20/25	20/50
Ins 4bp	ARS-19	6yrs	16yrs	NA	NA	20	50	16	26	0.4; 0.6	20/40	NLP

Clinical features of the patients with different *FOXC1* mutations indicating their mutations, age at onset and diagnosis, corneal diameter, CD ratio, initial IOP and final visual acuity. OU = Both eyes; OS = Left eye; OD = Right eye; NA = Not available; NLP = No light perception; dgn = Digitally normal; CF 1m = Counting fingers 1 meter; FF = Fixes and follows light.

4.2.1.2 Mutational analysis of *PITX2*

Direct sequencing of *PITX2* did not reveal any pathogenic mutations. Two intronic changes and a synonymous change were observed (Table 33).

Intronic changes

An insertion of GTT due to a deletion of C in second intron was observed in sporadic case (ARS-17). This indel was observed in a control subject also. The proband manifested the disease at the age of 22 years with IOPs of 18 and 16 mm Hg in the right and left eyes, respectively, along with CD ratios of 0.6 in both eyes. After surgery, the proband's IOP is under control and he has a visual acuity of 20/60 in both the eyes.

Another change from Cytosine to Thymine (C>T) was observed at 40 bp upstream to ATG. It was in heterozygous (CT) in 3 patients and homozygous (TT) in 3 other patients. This variation was not checked in the controls.

Silent changes

A change from Guanine to Adenine (G>A) at cDNA position c.660 resulted a synonymous transition at codon position 220 (F220F). This silent variation was in homozygous (AA) in one patient and heterozygous (GA) in another patient. This variation was also not checked in the controls.

Table 33. List of *PITX2* variations observed in patients with ARS

S.No	Exon/ Intron	Position	Codon Change	Amino acid change
1	Intron 2	IVS2 +6	Del of C Ins of GTT	-
2	Upstream of ATG	–40C>T	-	-
3	Exon 4	c.660 G>A	CCG>CCA	Phe 220 Phe

Gen Bank ID# NM_153427

Chapter 5
Discussion

DISCUSSION

Glaucoma comprises a group of clinically and genetically heterogeneous disorders of the eye leading to irreversible loss of vision if left untreated (Flammer, 2003). Both genetic and environmental factors are implicated in the pathophysiology. POAG is a major form of primary glaucomas in different populations worldwide. The juvenile form of POAG (JOAG) is inherited as an autosomal dominant trait, whereas the inheritance of adult onset POAG is varied (Fan *et al*., 2006). The molecular genetics of POAG has been studied by linkage analysis and whole genome scans but the underlying molecular mechanism is yet unknown. So far eleven chromosomal loci have been mapped in POAG, of which genes at *GLC1A* (*MYOC*), *GLC1E* (*OPTN*), and *GLC1G* (*WDR36*) have been characterized (Stone *et al.,* 1997, Rezaie *et al.,* 2002, Monemi *et al.,* 2005). Myocilin is an olfactomedin-like protein expressed in the trabecular meshwork and is hypothesized to cause the disease by forming aggregates with mutant myocilin protein, which are not secreted outside from the cells (Jacobson *et al*., 2001). The gene *OPTN* on the other hand codes for a protein hypothesized to play a neuroprotective role in the optic nerve (Rezaie *et al*., 2002). The gene *WDR36* codes for a protein with multiple G-beta WD40 repeats that was recently identified as one of the gene involved in T-cell activation and highly coregulated with interlukin-2 (*IL2*) (Monemi *et al*., 2005). While there is a wide spectrum of mutations in POAG across different populations, their precise role leading to the disease pathogenesis is poorly understood. The *CYP1B1* gene was

initially identified as a candidate gene for primary congenital glaucoma (PCG). However its involvement in JOAG (Vincent *et al*., 2002) through a digenic mechanism in combination with *MYOC* has eventually made it a strong candidate for POAG (Melki *et al*., 2004, Acharya *et al*., 2006, Lopez-Garrido *et al*., 2006). Apart from mutations in these genes, other candidate genes namely *p53*, *APOE* and *TNF*α, (Fan *et al*., 2006) *IL2* (Wang *et al*., 2001) etc., have also been implicated in POAG. Specific SNPs in these genes have been associated with certain phenotypic traits such as raised IOP and worsening of visual fields in POAG. While most of these results could not be replicated across different populations, nevertheless, they have provided significant insights in POAG pathogenesis.

India is a country with diverse ethnic groups. POAG is the major cause of blindness in India affecting 12 million people (Thylefors *et al*., 1995) resulting in 1.5 million blind people in the country (Balasubramanian D, 2002). POAG is a late onset disorder resulting in irreversible vision loss with no detectable symptoms. The progression of vision loss due to the death of retinal ganglion cells can be controlled, if detected earlier. Hence there is a need to identify individuals at risk and screen for the underlying molecular defect. There are a few reports describing the involvement of *MYOC, CYP1B1* and *OPTN* in POAG among Indian patients. Based on the involvement of these genes along with their mutation spectrum globally, it is intriguing to understand their role in diverse Indian population affected with POAG. Hence the present study was undertaken to screen

MYOC, *OPTN* and *CYP1B1*, in an uncharacterized patient cohort from India to generate genetic data that could be used for a better management of the disease. We hypothesized that molecular defects in multiple genes could lead to POAG. We also assessed the role of intragenic polymorphisms in *p53*, *APOE* and *TNF*α genes that exhibited some association with POAG in other populations.

In the present study, *MYOC* mutations accounted for 4.6% open angle glaucoma cases. The observed frequency was comparable to other reports from Eastern India [7 % - (Mukhopadhyay *et al*., 2002)], Southern India [~ 2% - (Kanagavalli *et al*., 2003)], Southern and Northern India [2 % - (Sripriya *et al*., 2004)], and globally (Gong *et al*., 2004) the mutation frequency was found to be 4% in the Caucasians, 3% in Africans and 4.4% in Asians. Of the 73 mutations reported so far (Gong *et al*., 2004), only 10 mutations were observed in the first exon, one in the second and the remaining in the third exon. As majority of the variants are clustered in this exon, it is also a hot spot for pathogenic mutations (Gong *et al*., 2004). This also suggests the importance of olfactomedin-like domain in protein function (Adam *et al*., 1997). The distribution of *MYOC* mutations indicates a wide geographical spread of these variants over time. Certain mutations are common across geographically and ethnically diverse populations indicating possible founder effects (Baird *et al*., 2003).

In the Indian scenario, the Q48H mutation in the first exon was specific to different populations. This mutation was reported earlier in

two other studies from Eastern (Mukhopadhyay *et al*., 2002), Southern and Northern (Sripriya *et al*., 2004) India. It was also observed in 7 asymptomatic individuals in the current study but not in two other studies. As POAG is a late onset disorder, these individuals are at the risk of developing the disease in later part of their of life.

As the Q48H mutation is unique to different populations in India, we explored the nature of phenotype across patients harboring this mutation (Table 34). The Q48H mutation manifested both with JOAG and POAG phenotypes (Table 34) with ages at onset ranging from 17 – 70 years along with presenting IOPs of 21 - 38 mm Hg. Their cup to disc ratios ranged from 0.4 to 0.9. Four of these 6 patients underwent trabeculectomy. The Q48H phenotype exhibited a wide variation across different populations (Table 34). While a marked severity was noted with respect to visual field defects in individuals with POAG than JOAG, there was no other significant difference in the clinical presentation in patients with Q48H. A founder effect could not be determined due to lack of haplotype data from the other studies (Mukhopadhyay *et al*., 2002, Sripriya *et al*., 2004).

The Q48H mutation was also observed in association with another *CYP1B1* mutation (P437L) in a sporadic case suggesting a possible digenic inheritance. The involvement of *MYOC* and *CYP1B1* in digenic inheritance was reported previously in JOAG (Vincent *et al*., 2002) and PCG (Kaur *et al*., 2005). In the former study, JOAG patients with mutations in *MYOC* and *CYP1B1* had an onset at 27 years while

Table 34. Comparison of phenotypes of JOAG/POAG patients with Q48H mutation in Indian populations

Region	Pheno type	Age at presentation	IOP at presentation	CD ratio OD; OS	Visual fields	Surgery	Reference
North India	JOAG	17	33; 26	0.6; 0.4	Generalized reduction of sensitivity	Trabeculectomy OU	Sripriya *et al*., 2004
South India	POAG	65	21; 23	0.6; no view	Consistently unreliable	Medication	
Eastern India	POAG	70	24; 36	0.9; 0.5	Diffuse depression with a scotoma OS	Trabeculectomy OS	Mukhopadhyay *et al*., 2002
	JOAG	20	20; 14	0.9; 0.9	Glaucomatous changes	Trabeculectomy	
	JOAG	32	28; 38	0.5; 0.7	Glaucomatous changes	Medication	
Andhra Pradesh	POAG	35	22; 22	0.9; 0.8	Severe; Mild	Trabeculectomy OD	Present study

OU = Both eyes; OD = Right eye; OS = Left eye.

those with only *MYOC* mutations had POAG with mean age at onset of 51 years. In the latter case, the proband with both *MYOC* and *CYP1B1* mutations had PCG while his parents were asymptomatic and carriers for either of these mutations. Apart from glaucomas, digenic inheritance was also reported in other ocular diseases like Retinitis Pigmentosa involving *RDS* and *ROM1* genes (Kajiwara *et al*., 1994). In the present study, the DNA samples from other family members were not available to determine the digenic nature of these mutations.

As the Q48H mutation has been observed in various forms of glaucomas in the Indian population, this might be useful to develop screening methods for identifying asymptomatic individuals who may be at risk of developing the disease. An initial screening of 200 POAG and 200 PCG cases from different parts of India along with 300 ethnically matched normal controls indicated that the Q48H mutation was equally distributed among POAG and PCG phenotypes (Chakrabarti *et al.,* 2005). This calls for further investigation in other populations in India and could represent a major diagnostic marker across a spectrum of glaucoma phenotypes, similar to the Q368X mutation in different Caucasian populations (Fingert *et al*., 1999, Alward *et al*., 2002, Alward *et al*., 1998).

Although a functional analysis of this mutation was not done, in silico secondary structure prediction by the Chou-Fasman method showed that the substitution of glutamine by histidine resulted in the removal of extended sheets in the glycosaminoglycan initiation site

along with a difference in the core-surface ratio when compared to the wild type protein (Sripriya *et al*., 2004).

The P370L mutation was observed to segregate in two autosomal dominant JOAG families (Figure 16). This mutation was previously reported in the French (Adam *et al*., 1997), Japanese (Suzuki *et al*., 1997), English (Wiggs *et al*., 1998), North American (Shimizu *et al*., 2000), Brazilian (Vasconcellos *et al*., 2000) and Indian (Mukhopadhyay *et al*., 2002) JOAG patients. This mutation was associated with autosomal dominant families resulting in the disease manifestation at a younger age (Table 35), along with raised IOP that was largely uncontrolled even on medical treatment (Taniguchi *et al*., 1999, Mukhopadhyay *et al*., 2003). The other family members with this mutation also manifested the disease at ages ranging from 5 – 27 years along with IOPs ranging from 25 – 66 mm Hg (Shimizu *et al*., 2000). All the cases reported so far exhibited a severe phenotype in terms of earlier age at onset and high IOPs, which were not controlled on medication. Rozsa *et al*. (1998) predicted that proline to leucine substitution at codon 370 might bring more than one change apart from failure of CK2 motif phosphorylation.

Similar to the Q48H mutation, the origin of the P370L mutation could not be determined as haplotype data from other studies were not available.

Table 35. Comparison of phenotypes of JOAG patients harboring the P370L mutation in different populations

Population	Age at diagnosis	IOP at diagnosis	Surgery/remarks	Reference
Japan	15	28	Underwent surgery as IOP is not controlled on medication	Suzuki *et al.,* 1997, Taniguchi *et al.,* 1999
French	10	NA	NA	Adam *et al.,* 1997
French	11			Adam *et al.,* 1997
British/English	06	38	NA	Wiggs *et al.,* 1998
USA	16	44	NA	Shimizu *et al.,* 2000
Greek	10	20 to 30	NA	Vincent *et al.,* 2002
India	18	24; 32	NA	Mukhopadhyay *et al.,* 2002
India	11	28	Trab and Trab	Present study
India	07	35	Trab and Trab	Present study

NA = Not available, Trab and Trab = Combined Trabeculectomy and Trabeculotomy.

The S231R mutation was a novel variation observed in the second exon. It has also been seen in a normal control. Two polymorphisms (D208E and L215P) in this exon were previously reported in the Chinese population (Pang *et al*., 2002). But the phenotypic details of those patients are not available. The presence of this exon only in higher organisms and the absence of proteins with complete homology to myocilin have led to the hypothesis that *MYOC* evolved by the fusion of myocin- related and olfactomedin- related proteins (Mukhopadhyay *et al.*, 2002). The functional importance of the second exon and the role of the S231R variation in the disease pathogenesis need to be established.

Among other *MYOC* mutations, Y479H was seen to result in a severe phenotype in a JOAG case with an early age at onset and raised IOPs at presentation, compared to the D395N mutation (Table 11). The phenotype of the patient with the Y479H mutation was similar to the patient with a P370L mutation in terms of high IOP and response to medical treatment. But the phenotype of the patient with the D395N mutation did not resemble the phenotype of other known mutations.

Apart from the potential disease causing mutations, several SNPs in the promoter [*MYOC*.mt1 (–1000C>G), –83G>A] and coding regions (R76K and Y347Y) were observed. The –83 G>A and R76K variations were found together and were not associated with the disease phenotype (Table 13 and 14), as was noted in other studies (Mukhopadhyay *et al*., 2002, Pang *et al*., 2002).

The *MYOC*.mt1 polymorphism that was earlier shown to be associated with glaucoma progression showed no association in the present study. There was no difference in the distribution of allele and genotype frequencies among patients and controls (Table 15 and 16). While there was no association with the disease phenotype in the Turkish population (Ozgul RK *et al*., 2005), using the Cox proportional hazards model, Polansky *et al*. (2003) showed that *MYOC*.mt1 (+) variant accelerates the worsening of both optic disc and visual field defects. Although the allele frequencies were not different between French patients and controls, *MYOC*.mt1 carriers exhibited poor IOP control on medication and greater degree of visual field loss in their cohort (Colomb *et al*., 2001). The lack of association in the present study could be attributed to ethnic variation or low power due to a relatively smaller sample size (n=109) compared to previous studies.

The involvement of *CYP1B1* in open angle glaucoma was first reported along with *MYOC* as a digenic mechanism in JOAG (Vincent *et al*., 2002). Individuals with both *MYOC* and *CYP1B1* mutations showed juvenile age of onset while the individuals only with *MYOC* mutation exhibited late age of onset. Based on these evidence, it was hypothesized to be a potential modifier of *MYOC*, and that these two genes might be acting through a common biochemical pathway (Vincent *et al*., 2002). Later studies showed *CYP1B1* to be associated with 4.5% of French JOAG (Melki *et al*., 2004), 4.6% of Indian POAG (Acharya *et al*., 2006) and 10.9% of Spanish POAG (Lopez-Garrido *et al*., 2006) populations. In the present study, the mutation frequency of

*CYP1B*1 was 20.2% (22/109), that was relatively higher compared to previous studies.

Screening of *CYP1B1* in 109 open angle glaucomas cases by direct sequencing revealed 10 different mutations in 22 patients, of which four (Q144R, W434R, F445C, c.1657del5GGAGA) were novel and were not observed even in PCG. Majority of the mutations (86%) were observed in the heterozygous state in 19/22 of the patients; two patients were compound heterozygous and one was homozygous for *CYP1B1* mutation. Genotype – phenotype correlations showed variable phenotypes for different mutations.

The R368H mutation was the most prevalent, observed in 7.3% (8/109) of the cases. It was also observed in POAG population from India (Acharya *et al*., 2006) but not in French (Melki *et al*., 2004) and Spanish (Lopez-Garrido *et al*., 2006) populations. This was also reported to be a pathogenic mutation in PCG in India (Reddy *et al.,* 2004, Panicker *et al.*, 2002), Brazil (Stoilov *et al.*, 2002), and Saudi Arabia (Bejjani *et al.*, 2000) and was also the most predominant mutation among Indian PCG patients (Reddy *et al*., 2003, Reddy *et al*., 2004). This suggests a wide prevalence of this mutation across multiple glaucoma phenotypes. Similar to the *MYOC* Q48H mutation, this calls for a PCR based diagnostic test for predictive testing.

The age of onset in these patients ranged from 20 to 80 years with IOPs of 10 - 42 mm Hg (Table 18). The proband (P-008) who was compound heterozygous for both G61E and R368H mutations

exhibited a relatively severe phenotype compared to other patients with only R368H mutation and manifested the disease at 9 years. His initial IOPs were 38 and 40 mm Hg in right and left eyes, respectively, and showed severe field defects. His IOPs reduced to 15 and 16 mm Hg in right and left eyes, respectively, upon surgery. The severity could be attributed to the cumulative effect of both the mutations. The DNA samples from the parents were not available to analyze the segregation of the mutation. In another report from India (Acharya *et al*., 2006), this mutation resulted in POAG with an onset at 37 years and IOPs of 16 and 22 mm Hg. We could not decipher the origin of R368H in POAG in India as the haplotype data was not available from the other study (Acharya *et al*., 2006).

The E229K mutation was the second most predominant mutation in the present study and accounted for 5.5% (6/109) of the cases. It was observed in all POAG populations screened so far suggesting that this could be a predominant *CYP1B1* mutation in POAG. The frequency of this mutation was 0.4% (1/236) in French (Melki *et al*., 2004), 0.5% (1/200) in Indian (Acharya *et al*., 2006) and 2.4% (2/82) in Spanish (Lopez-Garrido *et al*., 2006) populations with mixed glaucomas. A comparison of phenotypes of these individuals is given in Table 36.

Table 36. Comparison of phenotypes of POAG individuals with E229K mutation across different populations

Population	Genotype		Age at diagnosis	IOP at Presentation OD; OS	CD ratio (OD; OD)	Visual fields	Reference
	E229K (c.1031G>A)	**P193L (c.950 C>T)**					
French	G,A	C,C	35	25; 21	NA	NA	Melki *et al.,* 2004
Spanish	G,A	C,C	66	18; 18	0.5; 0.4	E; N	Lopez-Garrido *et al.,* 2006
	G,A	C,C	48	20; 21	0.7; 0.7	E; E	
Indian	G,A	C,C	17	24; 32	0.7; 0.8	Glaucomatous changes	Acharya *et al.,* 2006
	G,A	**C,T**	7	24; 22	0.8;0.8	Mi; Mi	Present study
	G,A	C,C	40	16; 19	0.9;0.9	S; S	
	G,A	C,C	53	10; 18	0.9;0.9	S; Mo	
	G,A	C,C	64	18; 18	0.6;0.8	Mi; Mo	
	G,A	C,C	32	24; 14	0.9;0.9	S; S	
	G,A	C,C	39	14; 12	0.9;0.9	S; S	

E= Early, N = Normal; Mi = Mild; S = Severe (Based on Anderson's criteria); NA = Not available

In the present study, the proband (P-173) was compound heterozygous for the E229K and the P193L mutations. She inherited the E229K mutation from her father and the P193L mutation from her mother (Figure 20) and manifested the disease at 7 years of age, probably due to the cumulative effect of both the mutations. The clinical phenotype of the parents was not diagnosed. This was also reported to be pathogenic in PCG patients from Lebanon (Michels-Rautenstrauss *et al.*, 2001), India (Panicker *et al.*, 2002), and France (Colomb *et al.*, 2003).

The G61E mutation was observed along with the R368H mutation of *CYP1B1* in a sporadic case. It was observed earlier in a Spanish POAG patient who manifested the disease at the age of 65 years with early visual field defects; IOPs were not mentioned. In the present study, the proband (P-008) was compound heterozygous for G61E and R368H mutations and manifested the disease at an early age (9 years) with IOPs of 38 and 40 mm Hg in right and left eyes, respectively. He had severe field defects in both eyes, which could be attributed to the presence of two different mutations. The DNA samples of the parents were not available to determine the segregation of these mutations. Based on circumstantial evidence, the severity was apparently more in the Indian patient, compared to the Spanish. It was also noted that this mutation resulted in severe phenotype in Indian PCG patients (Panicker *et al*., 2004). By homology modeling Stoilov *et al*. (1998) suggested that it is the functional part of the hinge region and mutations in this hinge region were shown to

interfere with proper folding and heme binding properties of cytochrome P450s. Therefore this mutation might affect the protein function significantly resulting in severe phenotype. The G61E mutation was found to be the prevalent mutation in the Saudi Arabian PCG population (Bejjani *et al*., 2000). It was also reported to be pathogenic in PCG in populations of Turkey (Stoilov *et al.*, 1998), Ecuador (Curry *et al.*, 2004) and Morocco (Belmouden *et al.*, 2002).

The Y81N mutation was the predominant *CYP1B1* mutation in POAG population reported in literature. It was observed in 3.6% (3/82) of the Spanish (Lopez-Garrido *et al*., 2006) and 0.85% (2/236) of the French population (Melki *et al*., 2004). The comparison of phenotypes of these individuals is given in Table 37. The proband in the present study showed relatively severe phenotype with age of onset at 30 years and severe field defects in both eyes. This mutation was also observed in two POAG suspects with CD ratios of 0.5 and 0.6 without visual field defects in Spanish population (Lopez-Garrido *et al*., 2006).

Table 37. Comparison of phenotypes of patients with Y81N mutation across different populations

Population	Phenotype	Age at diagnosis	IOP (OD; OS)	CD ratio (OD; OS)	Visual fields	Reference
French	POAG	52	NA	NA	NA	Melki *et al*., 2004
	POAG	37	28; 28	NA	NA	
Spanish	POAG	77	22; 22	0.7; 0.7	M; M	Lopez-Garrido *et al*., 2006
	POAG	49	17; 17	ND; ND	E; E	
	POAG	58	22; 20	0.5; 0.3	E; M	
Indian	JOAG	30	15; 15	0.9; 0.9	S; S	Present study

E = Early; M = Moderate; S = Severe (Based in Anderson's criteria); ND = Not determined; OD = Right eye; OS = Left eye; NA = Not available.

The Q144R mutation was observed in three POAG cases, of which one was familial. In a four-generation autosomal dominant family (P-001) it segregated with the disease phenotype (Figure 19). But two asymptomatic individuals (III:2 and IV:2) also harbored this mutation. These two individuals might perhaps manifest the disease in future, as POAG is a late onset disorder. In this family, the mean age of POAG patients harboring the mutation was 42 ± 4.74 years (range 36 to 48 years), therefore the asymptomatic individual (III:2) of 45 years of age may still manifest the disease in the future. Similarly another asymptomatic individual (IV:2) of 8 years may probably develop the disease in the future, as her brother (IV:1) with same mutation manifested the disease at the age of 11 years. It could also be due to the incomplete penetrance of *CYP1B1*, which is well documented in PCG (Bejjani *et al.*, 2000). In a study consisting of 52 Saudi Arabian families (Bejjani *et al.*, 2000), 40 members from 22 different families carried the *CYP1B1* mutations and haplotypes identical to their affected siblings but did not manifest the phenotype suggesting incomplete penetrance. Hence there could be a possibility of incomplete penetrance in the asymptomatic individuals in this study.

The glutamine residue is not conserved across CYP1 families in different species and is present only in the human and mouse CYP1B1 proteins. It is replaced by arginine in rat CYP1B1 and CYP1A1 and

CYP1A2 proteins of *Homosapians*, *Macaca*, *Canus*, *Mus* and *Rattus* species. A change from Glutamine to Proline at this codon was reported in PCG (Reddy *et al*., 2004) and Glutamine to Histidine in POAG (Lopez-Garrido *et al*., 2006). The proband with Gln144His manifested the disease at the age of 58 years, with an IOP of 18 and 16 mm Hg in right and left eyes, respectively (Table 38) (Lopez-Garrido *et al*., 2006). In the present study, the age at onset of the probands with Gln144Arg mutation was 12 years in familial case and 50 and 65 years in sporadic cases with IOPs ranging from 12 to 42 mm Hg.

Table 38. Comparison of phenotypes in patients with mutation at codon 144 in different populations

Population	Phenotype	Mutation	Age at Diagnosis	IOP at presentation OD; OS	Visual fields	Reference
Spanish	POAG	Q144H	58	18; 16	ND; M	Lopez-Garrido *et al*., 2006
Indian	JOAG	Q144R	12	42; 30	Mi; Mi	Present study
Indian	POAG	Q144R	65	39; 44	S; S	Present study
Indian	POAG	Q144R	50	12; 12	S; Mi	Present study

Mi = Mild; M = Moderate; S = Severe (Based on Anderson's criteria); ND = Not determined; OD = Right eye; OS = Left eye.

The deletion of 5bp (GGAGA) was the only mutation observed in homozygous condition resulting in a severe phenotype. The patient (P-177) manifested the disease at an early age with high IOPs when compared to phenotype of other novel mutations. He had severe visual field defects (Table 18) with a cup to disc ratio of 0.9 and underwent surgery in the right eye. On surgery, IOP was under control. Another novel mutation F445C also resulted in severe phenotype in terms of early onset, high initial IOPs and severe field defects in both eyes compared to other novel mutation W434R which resulted in less severe phenotype. As the reported mutations in *CYP1B1* resulted in phenotypes with varying degrees of severity in terms of age at onset, IOP and field defects, the phenotypes of novel mutations could not be compared with that of reported ones. However, it was observed that the phenotype of the proband with F445C mutation was similar to the proband (P-027) with R368H mutation in terms of presentation and response to surgical treatment (Table 18).

Apart from above disease causing mutations, five intragenic polymorphisms were also observed, which were not associated with disease phenotype. Two SNPs in exon 2 (R48G and A119S) and two in exon 3 (V432L and D449D) were observed together in patients and controls. However, a formal haplotype analysis was not done to study the role of these polymorphisms in POAG.

Mutations in *OPTN* were initially reported in NTG cases (Rezaie *et al*., 2002), but studies have also evaluated its role in POAG (Alward *et al*., 2003, Leung *et al*., 2003, Wiggs *et al*., 2003) and JOAG (Willoughby *et al*., 2004) cases. It is one of the three genes cloned and characterized for open angle glaucomas and showed varying degrees of involvement in high and low tension glaucomas. As there are no available data on its involvement in Indian POAG cohort, we screened *OPTN* to determine its role in Indian POAG patients. Screening of *OPTN* gene in our patient cohort did not reveal any pathogenic mutations. We did not observe the R545Q and E50K mutations, which were observed mainly in NTG cases of other populations. In our cases, most of the patients were high-tension glaucomas (n=93). Six polymorphisms were observed, which were not associated to the disease phenotype.

The distribution of M98K variation was not significantly different from that of controls. It was initially reported to be a risk factor in Caucasian population (Rezaie *et al*., 2002), but was not universally replicated across other populations. In a larger study consisting of 1,048 patients, no association was found with M98K variation (Alward *et al*., 2003), while another study from Japanese population showed M98K to be associated with POAG and NTG (Fuse *et al*., 2004). A different study from same population revealed high frequency of M98K in NTG population compared to POAG, however the difference was not statistically significant (Funayama *et al*., 2004). The M98K variation was also reported to be a

modifier of IOP in POAG patients (Melki *et al*., 2003). A meta-analysis of the published literature using this variant showed a weak but highly significant association with POAG (Craig *et al*., 2006). In a haplotype analysis done with 12 intragenic SNPs, it was observed that frequency of haplotype containing minor alleles of 3 SNPS [(T34T (c.412G>A), IVS5+38T>G and IVS7+24G>A)] and major alleles of 9 SNPs [T49T (c.457C>T), M98K (c.603T>A), IVS6-5T>C, IVS6-10G>A, INS8+20G>A, IVS8-53T>C, IVS15+10G>A, IVS15-48C>A and R545Q (c.1944G>A)] was higher in NTG than in controls (Fan *et al*., 2005). Due to the inconsistent results on the involvement of *OPTN* in HTG across different populations, it may be worthwhile to conduct a study specifically on NTG patients in India, which might provide further information on the involvement of *OPTN*.

Overall, mutations in *MYOC* (4.6%) and *CYP1B1* (20.2%) accounted for 25.7% of Indian OAG cases. No mutations were observed in the *OPTN* gene. The molecular defects underlying the remaining 75% cases still remain unknown. There could be other loci/genes involved in glaucoma pathogenesis. Being a complex disease, association studies using candidate gene variants have been proposed as a powerful means to determine their role in disease pathogenesis (Fan *et al*., 2006). Therefore we have looked at the association of specific SNPs in genes namely *p53* (Pro72Arg and intron-3), *ApoE* (ε2, ε3, ε4 isoform distribution) and *TNF*α (–308G>A) with the disease phenotype. It was hypothesized

that these genes play a role in glaucoma pathogenesis and their SNPs were shown to be associated with POAG in some populations, while it was not replicated in other studies (Fan *et al*., 2006).

The *p53* gene has been hypothesized to play a role in glaucoma where it was believed that retinal ganglion cells (RGCs) undergo apoptosis mediated by *p53* gene. Lin *et al*. (2002) observed that Proline allele at codon 72 was significantly associated with POAG in Chinese patients. Association with Ins-Arg haplotype was observed in the British population (Ressiniotis *et al*., 2004), while no association was noted in an Australian population (Dimasi *et al*., 2005) (Table 39). There were no differences in the distribution of allele and genotype frequencies among patients and controls in the present study similar to a study from eastern India (Acharya *et al*., 2002). The allele frequencies observed in our study are comparable to frequencies reported from India (Acharya *et al*., 2002) and Taiwan (Lin *et al*., 2002). The reason for not finding any association in our study could be attributed to the ethnic variation or small sample size. More studies from other populations with larger sample size are warranted to determine the role these candidate genes in glaucoma pathology.

Table 39. Comparison of *p53* polymorphism across different populations

Population	Polymorphism Studied	No of cases		No of controls	Association	Reference
		POAG/HTG	NTG			
Taiwan	Codon- 72	58 POAG	0	59	Pro allele (P=0.00782)	Lin *et al.*, 2002
India	Arg 72 Pro Intron - 3	67 POAG	0	117	No	Acharya *et al.*, 2002
British	Arg 72 Pro Intron - 3	140 POAG	0	73	Ins Arg (P<0.0001)	Ressiniotis *et al.*, 2004
Australia	Arg 72 Pro Intron - 3	283 HTG	62	178	No	Dimasi *et al.*, 2005
India	Arg 72 Pro Intron - 3	107 POAG	0	106	No	Present study

For the first time, Copin *et al*., (2002) showed a link between *APOE* and *MYOC* in POAG pathogenesis and hypothesized *APOE* as a potential modifier of POAG phenotype. It was observed that a promoter polymorphism in *APOE* (-491T) in association with the *MYOC* promoter polymorphism (*MYOC*.mt1) resulted in increased IOP and limited effectiveness of IOP lowering treatments in POAG patients. Bunce *et al*. (2003) suggested that the clinical parameters in this study was subjective and small fractional differences could not be interpreted as an indication of disease severity and progression. However, it led for further studies to determine the role of *APOE* in glaucoma pathogenesis. In the Tasmanian NTG population, an association was observed with ε4 allele where they studied only 70 patients and 51 controls (Vickers *et al*., 2002). But no association of *APOE* polymorphism was observed in the UK NTG population despite screening a larger cohort of 155 patients and 349 controls (Lake *et al*., 2004). In the present study, no association was observed with the disease phenotype. Similarly, no association was observed with north England POAG patients in a study comprising of 137 patients and 75 controls (Ressiniotos *et al*., 2004). But an association with ε3 allele was observed in Japanese OAG patients in a larger cohort of 310 patients and 179 controls (Mabuchi *et al*., 2005). However, it was suggested that the ε3 allele may not be a true risk factor as it was a common allele in the population. In a recent study it was observed that e4 allele decreased the risk of NTG in Chinese population (Lam *et al*., 2006).

There has not been a clear-cut indication on the role of *APOE* in glaucoma pathogenesis. This could be attributed to either ethnic variation or less power due to smaller sample size. Therefore more studies are warranted from various populations to determine the role of *APOE* in glaucoma.

Similarly no association was observed with *TNF*α –308G>A polymorphism in the present study. The role of *TNF*α was hypothesized based on raised levels *TNF*α and its receptors in glaucomatous retinas compared to normal eyes (Yuan *et al*., 2000). An association with –308 G>A polymorphism was observed in the Chinese POAG population (Lin *et al*., 2003) but not in Caucasian populations (Mossbock *et al*., 2005). In summary, no association was observed to any of the SNPs in the candidate genes in the present study. This could be attributed to either ethnic variation or small sample size. Therefore more studies from different populations across the world, consisting large numbers might provide insight on the role of these SNPs in disease pathogenesis. Also association studies using genetic variants of large effects (with odds ratios of 2 or more) or studies based on proper meta-analyses might provide some useful information on the disease pathogenesis (Todd *et al*., 2006).

Anterior Segment Dysgenesis (ASD) comprises a group of genetically heterogeneous developmental disorders with overlapping clinical features and about half of them progress to glaucoma (Alward *et al*., 2000). Therefore understanding molecular mechanisms in these

diseases may provide some insight on the implications of these candidate genes in ASD and glaucoma pathogenesis. Some of these genes like *FOXC1*, *PITX2*, *PAX6* etc. have been characterized in ASD leading to secondary glaucoma in some populations (Lines *et al*., 2002). These results have provided valuable information on the potential role of these genes in glaucoma pathogenesis. However there were no previous reports on the involvement of these genes in ASD and glaucoma in Indian population. Hence an attempt was made to screen *FOXC1* and *PITX2* genes in AR syndrome to understand their possible role in Indian patients. This data would be valuable in understanding their potential implications in glaucoma.

The spectrum of *FOXC1* mutations varies widely in AR syndrome. It has been suggested that proper dosage of the protein is required for proper ocular development (Lines *et al*., 2002). Although there are not many large studies on AR syndrome, the mutation frequency ranges from 12.8% (Nishimura *et al*., 2001) to 14.2% (Mears *et al*., 1998) in different studies.

FOXC1 mutations accounted for 28% (7/25) of the AR cases and resulted in variable phenotypes suggesting genetic heterogeneity. All the nonsense mutations Q2X, Q92X and Q123X truncated the protein ahead of the forkhead domain and also resulted in an early onset of the disease. The phenotype of Q123X mutation was initially described in a congenital hydrocephalus mouse model (Hong *et al*., 1999). In the present study,

Q123X was observed in an autosomal dominant family and segregated with the disease phenotype (Figure 25). The proband showed better prognosis with a visual acuity of 6/30 and 6/38 in left and right eyes, respectively, compared to his mother and uncle, probably due to his early intervention (Table 32). The unaffected grandmother of 58 years also harbored the same mutation, but did not manifest the disease phenotype. This might be due to the presence of a modifier locus, which supressed the expression of the disease phenotype or she might be a somatic mosaic for the modifier locus. She might have transmitted the mutation through her germline to the subsequent generations.

Among the missense mutations, M161K was observed in two autosomal dominant families resulting in variable clinical phenotypes. In ARS-3 family, proband and sibling harboring M161K mutation showed a relatively better prognosis with 20/80 visual acuity, because of early intervention compared to his father with same mutation, who was intervened at a later age (Table 32). The proband in ARS-8 family, showed poor prognosis in spite of early intervention, probably due to development of secondary glaucoma at presentation (Table 32). Biochemical analysis (Murphy *et al*., 2004) of this mutation showed that wing 2 region is necessary for DNA binding and plays a role in gene activation. However, between the two missense mutations observed, M161K resulted in severe phenotype in terms of earlier age of

manifestation and high CD ratio when compared to the phenotype of an individual with R170Q mutation.

An indel of GGC nucleotides was observed in a stretch of 6 and 7 nucleotides after codons 375 and 447, respectively. It was previously reported to be non-disease associated polymorphism (Mears *et al*., 1998). However, the clinical phenotype of these patients was not available to make a comparison with the present study. *PITX2* did not reveal any pathogenic mutations in the patient cohort, similar to another study on ARS patients with developmental glaucoma (Cella *et al.*, 2006).

In summary, *MYOC* was involved in 4.6% of OAG cases, which was similar to the global frequency (Gong *et al*., 2004). As seen earlier, the mutation P370L was associated with familial JOAG, high IOPs and poor response to medical treatment (Mukhopadhyay *et al*., 2003). No association was found with the coding and promoter SNPs of *MYOC*. The *CYP1B1* showed involvement in 20.2% of the cases, which was relatively higher compared to those in French (Melki *et al*., 2004), Indian (Acharya *et al*., 2006) and spanish (Lopez-Garrido *et al*., 2006) populations. All the *CYP1B1* mutations resulted in varying degrees of disease severity. Homozygous and compound heterozygous mutations resulted in relatively severe phenotype compared to heterozygous mutations. The observed intragenic polymorphisms in *CYP1B1* did not show any association with the disease phenotype. *OPTN* did not show any involvement in OAG cases. Neither the SNPs in *p53*, *ApoE* and *TNF*α were associated to the

disease phenotype. More studies with large sample sizes are warranted to determine the role of these SNPs in POAG.

In secondary glaucomas, *FOXC1* was involved in 28% of the ARS cases, while *PITX2* did not reveal any pathogenic mutations. Early intervention resulted in better prognosis in patients harboring *FOXC1* mutations than those who were intervened at later stages.

The data obtained from the current study provides information on the molecular genetic defects underlying primary and secondary glaucomas in India. This data exhibits the complex nature of glaucoma in terms of clinical and genetic heterogeneity. It may be helpful in developing a reliable diagnostic method for screening in populations.

SUMMARY

Chapter 1: Introduction

It gives a brief note on glaucoma and its impact on blindness. It includes a short description on various forms of glaucoma and different genes implicated in primary and secondary glaucomas. With this background, it also lists the aims and objectives of the present study.

Chapter 2: Literature review

This chapter provides a detailed description of the aqueous humor and its outflow pathways, and trabecular meshwork, which are mainly involved in glaucoma pathogenesis. The classification of glaucomas based on etiology and mechanism are summarized. It also describes the epidemiology, risk factors in primary glaucomas and a complete review of genes implicated in POAG along with a description of SNPs that were found to be associated with POAG. A brief note on secondary glaucomas including its characteristic features and genes involved with respect to Axenfeld-Rieger Syndrome is also included.

Chapter 3: Materials and Methods

This chapter details the inclusion and exclusion criteria for OAG, ARS cases and controls along with the demographics of cases and controls. It also includes a short note on the phenotypic parameters used in genotype-phenotype correlations. The details on molecular analysis, genomic DNA extraction, polymerase chain reaction, mutation

detection methods like SSCP and bi-directional sequencing, and PCR based restriction digestion are provided along with the statistical analysis undertaken.

Chapter 4: Results

This chapter provides the results of candidate genes screening in primary (*MYOC*, *CYP1B1* and *OPTN*) and secondary (*FOXC1* and *PITX2*) glaucomas. It also describes the clinical phenotype of the individuals with mutations. The screening of SNPs (*p53*, *APOE* and *TNF*α) in POAG and the association studies are also provided.

Chapter 5: Discussion

This chapter discusses the results obtained in the present study and analyzes the data in the background of published literature.

Important findings of the present study:

- Mutations in the *MYOC* gene were observed in 4.6% of the POAG cases
- *CYP1B1* mutations were observed in 20.2% of OAG cases, which is high when compared the frequency observed in French (4.6%), Spanish (10.9%) and Eastern Indian (4.5% populations).
- *OPTN* did not show any involvement in Indian OAG patients.
- Involvement of more than one gene viz. *MYOC* and *CYP1B1* indicates genetic heterogeneity in POAG.

- No association was observed with SNPs in *p53*, *APOE* and *TNF*α in POAG patients.
- *FOXC1* screening revealed mutations in 28% of ARS cases.
- *PITX2* screening did not show any involvement in ARS.
- The data obtained from the present study may be helpful in developing a reliable diagnostic method for screening in populations.

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URLs:

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2. http://www.eyecareamerica.org/eyecare/eca/upload/glaucoma-image.jpg
3. http://www.pfofflaserandeye.com/The%20Informed%20Patient/Patient%20Ed%20Common%20Eye%20Disorders/Glaucoma/Glaucoma%20BG.htm
4. http://www.opt.pacificu.edu/ce/catalog/15166-GL /Figure %208 % 20bot.jpg
5. www.gene.ucl.ac.uk/nomencleature

Appendix

APPENDIX-1

Methods used in clinical examination

a. Applanation tonometry

Tonometry is the objective measurement of IOP, based on the force required to flatten the cornea or the degree of corneal indentation produced by a fixed force (Kanski, 2003).

Methods:

1. Topical anaesthetic and fluorescein, to stain the tear film are instilled into the conjunctiva.
2. At the slit lamp, a Goldmann prism is applied axially to the cornea surface.
3. A pattern of two semicircles will be seen, one above and one below the horizontal midline.
4. The dial on the Tonometer is rotated to align the inner margins of the semicircles
5. The reading on the dial, multiplied by 10 gives the measure of IOP.

b. Gonioscopy

Gonioscopy is used to view the anterior chamber angle structures with direct observation. It facilitates the identification of abnormal angle structures and estimation of the width of the anterior chamber angle. The angle of the anterior chamber cannot be visualized directly through an intact cornea because light emitted from angle structures undergo

total internal reflection. A goniolens eliminates this by replacing the cornea-air interface with a new interface, which has a refractive index greater than that of the cornea and tears. These goniolenses are two types: Indirect and direct (Kanski, 2003).

Indirect goniolenses provides a mirror image view of the opposite angle and can be used in conjunction with a slit lamp. *Direct goniolenses* provide a direct view of the angle and are usually used with the patient in th supine position.

c. Perimetry

Perimetry involves the evaluation of the visual fields, which may be described as an island of vision surrounded by a sea of darkness. The visual field extends approximately 50° superiorly, 60° nasally, 70° inferiorly and 90° temporally (Kanski, 2003).

The patient is made to sit facing a computerized screen and asked to press a button whenever a flash of light appears. If the flash of light falls into a scotoma, it is not seen, and this will be registered on the printout as a blind spot. Sequential visual fields in a glaucoma patient can be used to determine the progression of the disease.

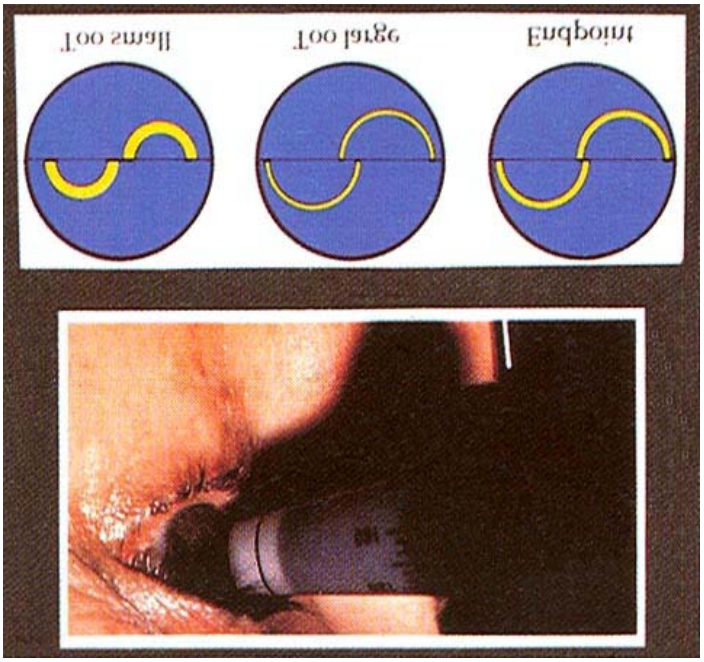


Figure I. Fluorescein-stained semicircles seen during tonometry

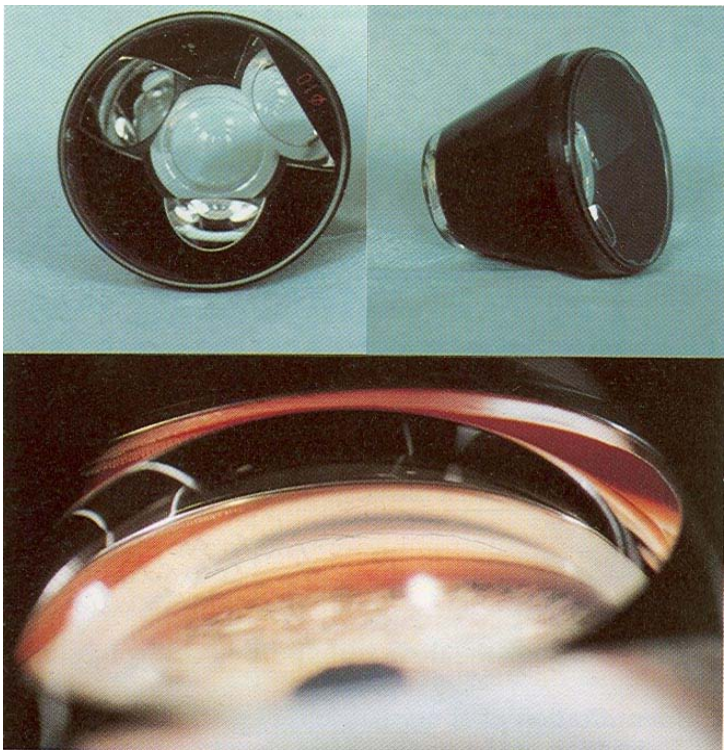
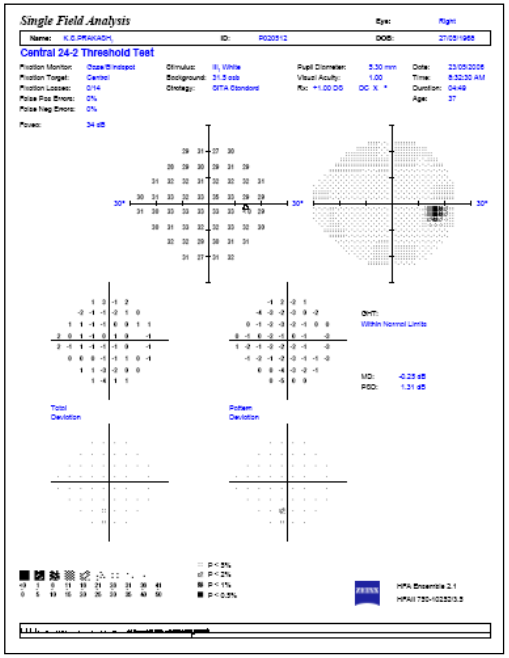


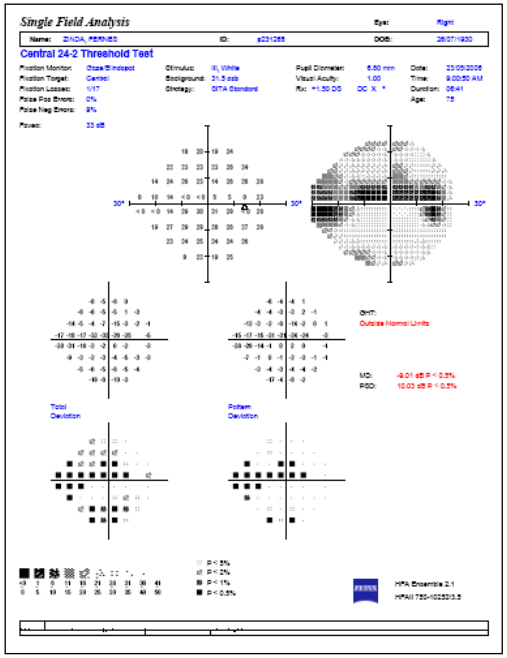
Figure II. Goldmann triple mirror lens (Top) and wide open angles on gonioscopy (down)

d. Ophthalmoscopy

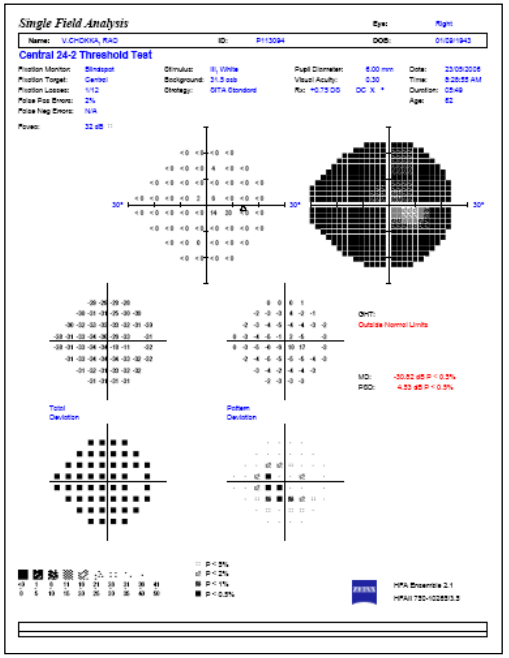
In glaucoma patients, progressive optic nerve cupping may occur without causing recordable visual field loss. Evaluation of the optic nerve head is, therefore, an important part of glaucoma evaluation. The optic nerve head can be examined by direct and indirect ophthalmoscopy, slit lamp biomicroscopy and also by photography. These methods are, however, subjective and may not be reproducible.



a) No field defect



b) Early glaucoma defect



c) Advanced glaucoma defect

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

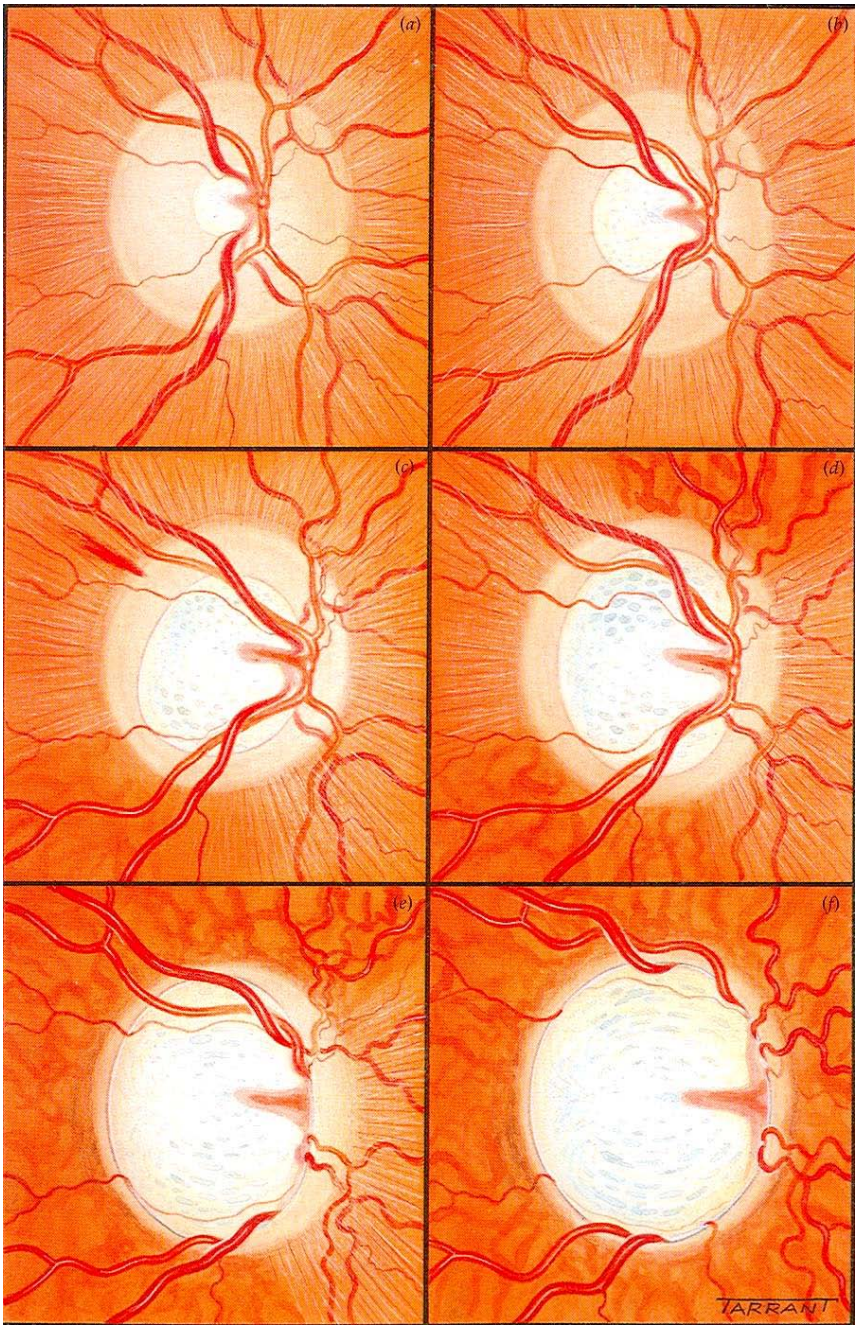


Figure IV. Progression of glaucomatous optic nerve damage. (a) Normal disc (b) Concentric enlargement with CD ratio 0.5 (c) Inferotemporal expansion of the cup and haemorrhage on the disc margin, loss of inferior retinal nerve fibre layer (d) superior expansion of the cup and superior nerve fibre loss (e) Advanced cupping (f) Total cupping

e. Optic nerve imaging

Objective computerized image analysis methods have recently been used to avoid this problem. The main computerized image analysers used at present are (Kanski, 2003):

i. Heidelberg Retinal tomograph (HRT)

It is a confocal laser-scanning microscope designed for three-dimensional imaging of the posterior segment of the eye. It enables the quantitative assessment of the topography of ocular structures. The depth of scanning ranges from 0.5 to 4.0 mm with 0.5-mm increments. The instrument performs 32 scans within this depth. The computer then forms a three-dimensional image of the disc.

ii. Optical Coherence Tomography (OCT)

It provides high-resolution cross-sectional images of the retina without contacting the eye. It works on the same principle as ultrasound but by using optical, rather than, ultrasound beam. It is not affected by the refractive status of the eye.

iii. Scanning laser polarimetry (GDX)

It is based on the assumption that the retinal nerve fibre layer has birefringent properties. The change in polarization called retardation can be quantified by determining the phase shirt between polarization of light returning from the eye with that of illuminating laser beam. The degree of retardation is linearly related to retinal nerve fibre thickness.

APPENDIX-2

Protocol for DNA isolation from blood samples (Sambrook *et al.,*1989)

1. Blood samples were thawed at room temperature and mixed with equal amount of 1X Phosphate buffer saline (PBS), to lyse the RBCs.
2. The samples were spun at 3800 rpm for 10 minutes.
3. The supernatant was removed by retaining the buffy coat at the bottom of the tube.
4. The procedure was repeated two to three times till the RBCs were completely removed and the solution had a white pellet of WBCs.
5. The pellet was resuspended in 5ml of extraction buffer containing 0.5% SDS (SISCO research laboratories PVT LTD Bombay. India) to lyse the WBCs, 10 mM Tris (Sigma chemicals Co. St.Louis, USA) (pH 8.0) and 0.1 M EDTA (Sigma chemicals Co. St.Louis, USA).
6. The proteinase K (Bangalore Genei, Bangalore, India) was added to the final concentration of 100μg/ml to digest the proteins and RNAse (Bangalore Genei, Bangalore, India) to remove RNA contamination and incubated at 37 °C overnight.
7. In between samples were vortexed in a cyclomixer (Remi Equipments, Mumbai, India) for 5 to 10 seconds at for complete dissolution of pellet.

8. Equal amount Phenol (Ambion, Texas, USA) equilibrated with 0.5 M Tris (pH 8.0) was added and mixed gently for mixing of organic and inorganic phases.
9. Samples were then spun at 4800 rpm for 10 minutes.
10. Upper aqueous layer was carefully transferred to a fresh tube with the help of Pasteur pipette.
11. Phenol and chloroform (Qualigens Fine Chemicals, Mumbai, India) were added in ratio of 1:1 to the aqueous layer for complete removal of denatured proteins.
12. Samples were spun at 4800 rpm for 10 minutes and the organic phase was removed.
13. The procedure was repeated once with equal amount of Chloroform to remove the phenol contamination.
14. Aqueous layer was separated in a fresh tube containing 1.5 ml of 10 M ammonium acetate and the DNA was precipitated by using 2.5 volumes of absolute ethanol (Qualigens Fine Chemicals, Mumbai, India).
15. The precipitated DNA was washed twice with 70% ethanol to remove the excess of salt and air-dried.
16. The pellet was dissolved in autoclaved MilliQ water and stored at –20 °C for further use.

INDEX

10X PBS: (per liter)

NaCl	40 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO4	2.4 g
Milli Q	1 lit

Adjust the pH to7.4 with 1 M HCl.

Working concentration (1X): Prepared by adding 100 ml of stock solution to 900 ml of Milli Q water.

10X TBE: (per liter)

Tris base	108 g
Boric acid	55 g
0.5M EDTA (pH 8)	40 ml

Make up the volume to 1000 ml.

Working concentration (1X): Prepared by adding 100 ml of stock solution to 900 ml of Milli Q water.

50X TAE: (per liter)

Tris base	242 g
Glacial acetic acid	57 ml
0.5M EDTA (pH 8)	100 ml

Make up the volume to 1000 ml.

Working concentration (1X): Prepared by adding 20 ml of stock solution to 980 ml of Milli Q water.

1M Tris: pH 8.0 (per liter)

Tris Base	121.1 g
Milli Q	800 ml

Adjust the pH to 8.0 by adding 42 ml of Conc. HCl Make up the volume to 1000 ml and autoclave.

0.5M EDTA: pH 8.0 (per liter)

EDTA	186.1g
MilliQ	800 ml

Adjust the pH to 8.0 by adding 20 g of NaOH. Make up the volume to 1000 ml and autoclave.

Extraction buffer: (100ml)

1M Tris	1.0 ml
0.5M EDTA	20 ml
10% SDS	5 ml

Make up the volume to 100 ml using MilliQ water.

LIST OF PUBLICATIONS

1. Arijit Mukhopadhyay, **Sreelatha Komatireddy**, Moulinath Acharya, Ashima Bhattacharjee, Anil Kumar Mandal, Sanjay K. D. Thakur, Garudadri Chandrasekhar, Arun Banerjee, Ravi Thomas, Subhabrata Chakrabarti, Kunal Ray. ***Evaluation of Optineurin as a candidate gene in Indian patients with primary open angle glaucoma***. **Mol Vis. 2005**;11:792-797. **(First two authors contributed equally).**

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

3. **Sreelatha Komatireddy**, Subhabrata Chakrabarti, Anil Kumar Mandal, Aramati Bindu Madhava Reddy, Srirangan Sampath, Shirly George Panicker, Dorairajan Balasubramanian. ***Mutation Spectrum of FOXCI and clinical genetic heterogeneity of Axenfeld-Rieger anomaly in India*****. Mol. Vis. 2003**;9:43-48.

LIST OF PRESENTATIONS

- Presented a poster titled "**Identification of *CYP1B1* mutations in Indian Primary Open Angle Glaucoma population "** in 72nd **SBCI 2002,** held at LUDHIANA.

- Presented a Paper titled " **Primary Open Angle Glaucoma in Indian Population: Involvement of *CYP1B1"*** in **IERG - 2003** held at LVPEI, HYDERABAD and was awarded the **BEST PAPER PRESENTATION**.

- Presented a poster titled " **Molecular Genetic analysis of Primary Open Angle Glaucoma" at 10th FAOBMB, 2003** held at Indian Institute of Science**,** BANGALORE.

- Presented a Paper titled " **Genetic Heterogeneity of Primary Open Angle Glaucoma: Involvement of *CYP1B1***" at **KVR scientific society,** 2004, HYDERABAD.

- Presented a poster titled **" The involvement of myocilin gene in Indian primary open angle glaucoma patients"** in **Vision Research Foundation, 2004** held by Shankara Netralaya, CHENNAI.