DNA SEQUENCING OF CYP1B1 GENE IN FAMILIES WITH INHERITED GLAUCOMA

By

Bushra Gul

Department of Biochemistry,
Faculty of Biological Sciences,
Quaid-i-Azam University
Islamabad, Pakistan
2016

DNA SEQUENCING OF CYP1B1 GENE IN FAMILIES WITH INHERITED GLAUCOMA

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Philosophy

In

Biochemistry/Molecular Biology

By

Bushra Gul

Department of Biochemistry,
Faculty of Biological Sciences,
Quaid-i-Azam University
Islamabad, Pakistan
2016

ALLAH IS THE LIGHT OF THE HEAVENS AND THE EARTH. THE SIMILITUDE OF HIS LIGHT IS AS A NICHE WHERE IN IS A LAMP. THE LAMP IS IN A GLASS. THE GLASS IS AS IT WERE A SHINING STAR. (THIS LAMP IS) KINDLED FROM A BLESSED TREE, AN OLIVE NEITHER OF THE EAST NOR OF THE WEST, WHOSE OIL WOULD ALMOST GLOW FORTH (OF ITSELF) THOUGH NO FIRE TOUCHED IT. LIGHT UPON LIGHT, ALLAH GUIDETH UNTO HIS LIGHT WHOM HE WILL. AND ALLAH SPEAKETH TO MANKIND IN ALLEGORIES, FOR ALLAH IS KNOWER OF ALL THINGS. (SURAH 24, AYAH 35)

ACKNOWLEDGEMENTS

Praise is to Allah, the Cherisher and Sustainer of the worlds, who showed His countless blessings upon us. **Almighty Allah** enabled me to compile my humble endeavors in the shape of this thesis. All respect goes to the **Holy Prophet Hazrat Muhammad (SAW)** whose teachings strengthened our faith in Allah, who is the source of knowledge and wisdom for entire humanity, who clarified that pursuit of knowledge is a divine commandment.

I would like to extend my sincere gratitude to all those people who made this thesis possible. I am also heartily thankful to my dignified and respectable supervisor, **Dr. Muhammad Ansar**, whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop an understanding of the subject. His expertise, valuable suggestions and patience, added considerably to my experience and enabled me to complete this research project.

I owe my deepest gratitude to my lab fellows especially **Ehsan Ullah, Memoona rasheed, sundus sajid, Fatima Hashmi, Arif Mahmood, Naseeb-ullah Kakar, Nermeen Ashraf, Erica Silas** and all my juniors for their moral support and encouragement. The time spent in the company of these people has become a memorable part of my life.

I am most indebted to my sweet parents **Rao Gul Muhammad Babar** and **Roshan Ara, my** siblings **Rao Muhammad Zeeshan Gul, Irum Fatima, Anum Gul, Rao Muhammad Faizan Gul** and my friends **Fatima Liaquat, Tooba Sajid** and **Syeda Fareeha** for their endless prayers, matchless love, and care. Words become meaningless when I have to say thanks to my parents and family; their prayers gave me strength and hope to accomplish this task and to pursue my goals.

Lastly, I offer my regards and blessings to all who supported me in any respect during the completion of this thesis.

Bushra Gul

CERTIFICATE

This thesis, submitted by Ms. Bushra Gul to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor: _____

Dr. Muhammad Ansar
Associate professor

External Examiner: _____

Chairman: _____

Dr. Muhammad Ansar
Associate professor

Dated: _____

TABLE OF CONTENTS

S. No.		Page No
	List Of Abbreviations	**I**
	List Of Tables	**Iv**
	List Of Figures	**V**
	ABSTRACT	**Vii**
1.	**INTRODUCTION**	
1.1	Glaucoma	**1**
1.2	Classification of glaucoma	**1**
1.2.1	Open Angle Glaucoma	**2**
1.2.2	Closed Angle Glaucoma/Angle Closure Glaucoma	**3**
1.2.3	Developmental/ Congenital Glaucoma (DG)	**3**
1.3	Association of glaucoma with different syndromes	**3**
1.3.1	Anterior Segment Dysgenesis	**3**
1.3.1.1	Peters' Anomaly	**3**
1.3.1.2	Rieger's Anomaly	**4**
1.3.1.3	Exfoliative Glaucoma (XFG)	**4**
1.3.1.4	Iris Hypoplasia	**4**
1.3.1.5	Aniridia	**4**
1.3.1.6	Iridogoniodysgenesis	**4**
1.3.1.7	Coloboma	**5**
1.4	Structure of Eye	**5**
1.4.1	Aqueous Outflow Pathways	**6**
1.4.2	Trabecular Meshwork	**7**
1.4.3	Retina And Retinal Ganglion Cells	**7**
1.5	Pathophysiology of Glaucoma	**8**
1.6	Glaucoma Diagnosis	**9**
1.6.1	Assessment of Optic Nerve Health	**9**
1.6.1.1	Visual Acuity Test	**9**
1.6.1.2	Tonometry	**11**
1.6.1.3	Gonioscopy	**11**

1.6.1.4	Perimetry	**11**
1.7	Genetics of Glaucoma	**11**
1.7.1	Primary Open Angle Glaucoma (POAG)	**12**
1.7.2	Primary Congenital Glaucoma (PCG)	**12**
1.8	Pathophysiology of PCG	**13**
1.9	Genetics of PCG	**14**
1.9.1	*LTBP2*	**14**
1.9.2	*MYOC* (Myocilin)	**14**
1.9.3	*CYP1B1* (Cytochrome P4501b1)	**15**
1.10	CYP1B1 Mutations in PCG	**16**
1.11	Aims and Objectives	**18**
2.	**MATERIALS AND METHODS**	
2.1	Ascertainment of Families	**26**
2.2	Family Pedigrees	**26**
2.3	Sample Collection	**26**
2.4	Extraction of Genomic DNA	**26**
2.4.1	Phenol-Chloroform Method	**26**
2.4.2	Composition of solutions used for genomic DNA extraction	**27**
2.4.3	Agarose Gel Electrophoresis	**27**
2.5	Sequencing of CYP1B1	**28**
2.5.1	Exon Amplification	**28**
2.5.2	PCR Product Purification	**29**
2.5.3	DNA Sequencing	**30**
2.5.3.1	Purifying sequencing extension products by isopropanol precipitation	**30**
2.5.4	Sequence Data Analysis	**30**
2.6	Segregation Analysis via Restriction Fragment Length Polymorphisms (RFLP)	**31**
2.6.1	NEB Cutter	**31**
2.6.2	Restriction digestion analysis	**31**
3	**RESULTS**	

3.1	Description of the families studied	**34**
3.1.1	Family A	**34**
3.1.2	Family B	**34**
3.1.3	Family C	**34**
3.1.4	Family D	**35**
3.1.5	Family E	**35**
3.1.6	Family F	**35**
3.1.7	Family G	**35**
3.2	Mutation Analysis	**36**
3.2.1	Mutation analysis of Family A	**36**
3.2.2	Mutation analysis of Family B	**36**
3.2.3	Mutation analysis of Family C	**36**
3.2.3.1	Confirmation of prevalence in normal controls Via RFLP	**37**
3.2.4	Mutation analysis of Family D	**37**
3.2.4.1	Segregation Analysis via RFLP	**37**
3.2.5	Mutation analysis of Family E	**38**
3.2.6	Mutation analysis of Family F	**38**
3.2.6.1	Segregation Analysis via RFLP	**38**
3.2.7	Mutation analysis of Family G	**38**
4	**DISCUSSION**	**50**
5	**REFERENCES**	**54**

LIST OF ABBREVIATIONS

□	Male
○	Female
■	Affected male
●	Affected female
°C	Degrees Celsius
aa	Amino Acid
ABI	Applied Biosystem
APS	Ammonium persulphate
ASD	Anterior segment dysgenesis
BLAT	Blast like alignment tool
bp	Base Pair
CYP1B1	Cytochrome P4501B1
dH2O	Distilled water
DNA	Deoxy Ribonucleic Acid
dNTPs	Deoxynucleotides Triphosphates
EDTA	Ethylene Diamine tetraacetic Acid
EtBr	Ethidium bromide
FOXC1	Forkhead box C1
g	Gram
HCl	Hydrochloric acid
IOP	Intraocular pressure
JOAG	Juvenile-onset POAG
M	Molar
MAR	Minimum angle of resolution
MgCl2	Magnesium chloride
Min	Minute
ml	Milliliter
Mm	Millimetre
mM	Millimolar

mm Hg	Millimetre of Mercury
MYOC	Myocilin
NaCl	Sodium chloride
ng	Nano gram
NTG	Normal tension glaucoma
OAG	Open angle glaucoma
OCT	Optical Coherence Tomography
OPTN	Optineurin
PACG	Primary angle closure glaucoma
PAX6	Paired Box Gene 6
PCG	Primary congenital glaucoma
PCR	Polymerase Chain Reaction
PITX2	Paired like Homeodomain Transcription Factor 2
POAG	Primary open-angle glaucoma
RFLP's	Restriction fragment length polymorphism's
RGC's	Retinal ganglion cells
RNA	Ribonucleic acid
RNFL	Retinal nerve fiber layer
rpm	Revolution per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium Dodecyl sulphate
sec	Second
SIFT	Scale-invariant feature transform
Taq	Thermus aquaticus
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TIGR	Inducible trabecular meshwork glucocorticoid response
Tris	[Tris-hydroxymethyl] ainomethane
UCSC	University of California Santa Cruz
WDR36	WD repeat- domain 36
XFG	Exfoliative Glaucoma

µg	Microgram
Ml	Micro Litre

LIST OF TABLES

Table No.	Title	Page No.
1.1	Increase in Corneal Diameter and Axial length with age	**19**
1.2	CYP1B1 Reported Mutations in Pakistan	**20**
1.3	List of mutations reported worldwide	**21-25**
2.1	Primer sequences used for amplification of *CYP1B1* exon 2 and 3	**32**
2.2	Primers for amplification of exons for RFLP	**33**
2.3	Conditions for RFLP	**33**
3.1	Results of RFLP of Family C	**49**
3.2	List of Variants and Mutations in Exon 2 and 3 of CYP1B1	**50-51**

LIST OF FIGURES

Figure No.	Title	Page No.
1.1	Snellen chart	**10**
3.1	Family A-Five generation pedigree with 3 affected males (V-5, V-6 andV-7) and 2 affected females (V-3 and V-4).	**40**
3.2	Family B: Five generation pedigree with 4 affected males (IV-2, IV-7, IV-11 and V1).	**40**
3.3	Family C: Five generation pedigree with 4 affected including 1 female (V-2) and 3 males (IV-2, IV-3 and IV-4).	**41**
3.4	Family D: The four generation pedigree with two affected females (III-3 and III-4).	**41**
3.5	Family E: Five generations pedigree with two affected male members (V-2 and V-5).	**42**
3.6	The five generation pedigree with 8 affected individuals.	**42**
3.7	Family G: Four generation pedigree with two affected individuals.	**43**
3.8	Sequence Chromatogram of Exon 2 of Family A, C, D. Arrow indicates the site of SNP (c.355G>T) P.Ala119Ser.	**43**
3.9	Sequence Chromatogram of Exon 3 of Family A, B, C, D and E. Arrow indicates the site of Variant (c.1347T>C).	**44**
3.10	Sequence Chromatogram of Exon 2 of Family B. Arrow indicates the site of Heterozygous Variant (c.515G>A) p.Ser172Asn.	**44**
3.11	Sequence Chromatogram of Exon 2 of Family B. Arrow indicates the site of mutation p.Pro118Leu (c.353C>T).	**45**
3.12	Sequence Chromatogram of Exon 2 of Family C, D. Arrow indicates the SNP (c.142C>G) p.Arg48Gly.	**45**

3.13	Sequence Chromatogram of Exon 2 of Family C with reverse primer. Arrow indicates the site of SNP p.Glu229Lys (c.685G>A).	**46**
3.14	Sequence Chromatogram of Exon 3 of Family D. Arrow indicates the site of heterozygous SNP (c.1059G>A).	**46**
3.15	Sequence Chromatogram of Exon 3 of Family D. Arrow indicates the site of SNP (c.1358A>G) p.Asn453Ser.	**47**
3.16	Sequence Chromatogram of Exon 3 of Family F. Arrow indicates the site of mutation p.Arg390His (c.1169G>A).	**47**
3.17	Sequence Chromatogram of Exon 3 of Family D with reverse primer. Arrow indicates the site of Novel Variant p.Leu225Ile (c.673C>A).	**48**
3.18	Results of RFLP of Family F	**48**

ABSTRACT

Primary congenital glaucoma (PCG) is a rare autosomal recessive disorder which presents during early infancy. The signs and symptoms of PCG include reduced visual acuity, enlargement of globe, blepharospasm, edema, corneal opacification and photophobia. Two genes, *CYP1B1* and *LTBP2* along with two chromosomal loci have been mapped for PCG so far. *CYP1B1* (cytochrome P4501B1) located at GLC3A locus is considered as most commonly mutated gene in PCG. Frequency of *CYP1B1* mutations which cause PCG differs among different populations ranging from less than 10% to 100%. There is great diversity of mutation spectrum of *CYP1B1* gene studied in the pathogenesis of PCG worldwide.

In this study affected and healthy individuals of 7 Pakistani families, affected with congenital or early onset glaucoma, were collected for *CYP1B1* mutation analysis. Sanger sequencing of coding exons and splice sites of *CYP1B1* gene identify potentially pathogenic variants in three families. The identified variants include a novel homozygous c.353C>T (p.Pro118Leu) mutation and a known mutation c.1169G>A(p.Arg390His) in family B and F respectively. Both the mutations were predicted as pathogenic and disease causing by majority of the bioinformatics tools. A known mutation (p.Glu229Lys) was also identified in family C but further testing in Pakistani control individuals indicated high frequency of variant allele. This finding in family C casts doubt on the pathogenic nature of p.Glu229Lys variant and probably indicates a Pakistani population specific polymorphism.

Additionally we also found other novel variants including c.673C>A (p.Leu225Ile), c.355G>T (p.Ala119Ser), c.1347T>C, c.515G>A (p.Ser172Asn), c.142C>G (p.Arg48Gly), c.1358A>G (p.Asn453Ser) and c.1059G>Ain*CYP1B1*. But further analyses failed to indicate the pathogenic nature of these variants and majority of these were also present in public databases. The identification of *CYP1B1* mutations in 28% families (2 out of 7) in this study indicates that mutations in this gene are the major cause of PCG in Pakistan as well. Additional work is required to identify the underlying mutations in the remaining five families.

INTRODUCTION

1.1. Glaucoma

Glaucoma is a group of neurodegenerative disorders and it is considered to be second important cause of vision loss after cataract, it accounts for about 13.5% cases of blindness globally(D. M. Kumar, Simpkins, & Agarwal, 2008). Glaucoma "Silent thief of sight" is a complex disease influenced by a combination of genetic and environmental risk factors(Cook & Foster, 2012).

There are more than 60 million glaucoma patients worldwide and about 8.4 million people experience blindness due to glaucoma, and these numbers are expected to increase up to 80 million and 11.2 million respectively in 2020(Schwartz & Yoles, 2000). Glaucoma is considered as fourth most common cause for reported blindness in Pakistan(Cook & Foster, 2012).Glaucoma is associated with raised intraocular pressure (IOP) (high-tension glaucoma) but can also occur if intraocular pressure is low or normal (low-tension glaucoma)(Chakrabarti *et al*., 2006; Li *et al*., 2007).

IOP elevation might be caused by reduced drainage of aqueous humour, but association of IOP elevation with molecular pathways behind glaucoma pathogenesis is not fully known(Vasiliou & Gonzalez, 2008).Increased intraocular pressure (IOP) of the eye results in mechanical stress to the posterior structures of the eye, noticeably to the lamina cribrosa and other adjacent tissues(Quigley, Addicks, Green, & Maumenee, 1981). It can occur in both sporadic and familial patterns (Allingham *et al.,* 2012). As compared to Caucasians, glaucoma is 6-8 times more widespread, it is less than 10% of all clinical subtypes of disease in Asian populations (Chakrabarti *et al.,* 2006).

1.2. Classification of Glaucoma

Glaucoma involves apoptotic death of the retinal ganglion cells (RGC's) and their axons, tissue remodelling of optic nerve head and the retina ultimately causing optic nerve degeneration and irreversible visual field damage (Chauhan *et al.,* 2008). Glaucoma is heritably heterogeneous disorder comprising a number of different forms each with varied causes and severities(Guo *et al.,* 2007; Michels-Rautenstrauss *et al.,* 2002). Glaucoma can be subdivided on the basis of anatomy of anterior chamber i.e.

open angle and closed angle (angle closure), etiology i.e. primary and secondary and age of onset i.e. congenital, juvenile and adult (Michels-Rautenstrauss *et al.,* 2002).

Glaucoma is considered to be primary when it has no etiology and is said to be secondary when it occurs as a result of previous disease, injury, drugs, cataract or diabetes (Michels-Rautenstrauss *et al.,* 2002). Both primary and secondary glaucoma can be open angle or closed angle. Glaucoma is classified into three major types' i.e. Primary open-angle glaucoma (POAG), Primary congenital glaucoma (PCG), and Primary angle closure glaucoma (PACG)(Michels-Rautenstrauss *et al.,* 2002) .Genetically heterogeneous group of developmental disorders known as anterior segment dysgenesis (ASD) have been reported to be associated with increased IOP and glaucoma (Gould & John, 2002).

Primary congenital glaucoma and Primary open angle glaucoma have strong genetic constituent whereas primary angle closure glaucoma (PACG) is comparatively infrequent (Vasiliou & Gonzalez, 2008).In contrast to European or African populations, primary angle closure (PACG) is the leading cause of bilateral blindness in East Asian populations (Shakya & Gupta, 2006). POAG is considered as most common subtype of glaucoma accounting for more than 50% of cases of disease (C. P. Pang *et al.,* 2006).

1.2.1 Open Angle Glaucoma

Open angle glaucoma (OAG) tends to develop slowly over time and is painless mostly with no symptoms till significant progression of disease(J. L. Wiggs, Damji, Haines, Pericak-Vance, & Allingham, 1996). It is most common form of glaucoma and its occurrence vary considerably across countries. Open angle glaucoma is due to specific morphology of the drainage canals between iris and cornea, preventing outflow of liquid that circulate within the eye and hence elevate IOP (Sarfarazi, 1997).

There are two variants of OAG i.e. primary OAG and normal tension OAG. Primary OAG relates with late onset and Normal tension OAG has normal IOP risk factors of normal tension OAG include diabetes and high blood pressure (Paton and Craig 1976). Juvenile-onset primary open-angle glaucoma has an unknown pathophysiology like adult primary open-angle glaucoma(Anderson, 1983). POAG is shown to be more

prevalent with severe disease progression among people of African-Caribbean as compared to European descent, Hispanics, and Asians (He *et al.,* 2006).

1.2.2 Closed Angle Glaucoma/Angle Closure Glaucoma

In closed angle glaucoma there is congenital configuration of iris, right side of which blocks outflow of liquid. Angle-closure glaucoma is common in persons with nanophthalmos (reduced axial length and thickening of the sclera). It accounts for less than 10% of glaucoma cases and develop with symptoms like severe pain accompanied by rapid progression of optic nerve damage (Michels-Rautenstrauss *et al.,* 2002).

1.2.3 Developmental/ Congenital Glaucoma (DG)

Developmental glaucoma involves a variety of ocular and non-ocular abnormalities, which mainly affect the cornea, iris and anterior chamber angle(Liu & Allingham, 2011). It includes rare types of glaucoma that commonly affects children due to incomplete development of drainage canal during prenatal period (Sarfarazi *et al.,* 1995). They are usually diagnosed at 1st year of life. First and most important variant of Developmental glaucoma is Primary congenital glaucoma, most prevalent form of glaucoma (AlFadhli, Behbehani, Elshafey, Abdelmoaty, & Al-Awadi, 2006). Other types include infantile glaucoma and glaucoma associated with hereditary of familial diseases (Sarfarazi *et al.,* 1995).

1.3. Association of Glaucoma with Different Syndromes

1.3.1 Anterior Segment Dysgenesis

Anterior segment dysgenesis is known to be linked with raised IOP and glaucoma(Gould & John, 2002). Anterior segment anomalies as a whole are associated with approximate 50% risk of glaucoma (Sowden, 2007). These include Peters' anomaly, Rieger's anomaly, iris hypoplasia, Coloboma, aniridia and iridogoniodysgenesis(Vasiliou & Gonzalez, 2008).

1.3.1.1 Peters' Anomaly

It is considered as rare form of anterior segment dysgenesis. It is referred as incomplete separation of cornea from iris and lens. *CYP1B1* are usually linked with primary congenital glaucoma but in few reports its mutations in patients have been

associated with Peter's anomaly, in association with glaucoma(Chavarria-Soley *et al.,* 2006).

1.3.1.2 Rieger's Anomaly

Rieger's anomaly is result of malformations of anterior segment of eye and occurs normally in association with that of secondary glaucoma. Main symptoms of Rieger's syndrome include abnormalities like underdeveloped iris, small cornea (micro cornea) with an opaque ring around outer edge of cornea, pupil displacement. It is inherited as autosomal dominant trait(Gould & John, 2002).

1.3.1.3 Exfoliative Glaucoma (XFG)

In some patients glaucoma develops with a syndrome known as Exfoliative syndrome. XFG is also known as pseudo exfoliation(Ritch, 2008). It occurs due to abnormal accumulation of proteins in drainage canals and other related structures of eye. It is the most common recognizable form of open-angle glaucoma with faster progression as compared to OAG(Ritch & Schlötzer-Schrehardt, 2001). XFG with aggressive clinical course is considered to be a greater risk of vision loss and blindness than POAG (Ritch, 2008).

1.3.1.4 Iris Hypoplasia

Iris hypoplasia is a condition that usually occurs due to inflammation in the eye or due to inherited conditions that prevent from developing. It affects the ability of iris to block light. This condition is inherited as autosomal dominant character (Weatherill & Hart, 1969).

1.3.1.5 Aniridia

Aniridia is absence of iris in the eyes. It can be congenital or due to an injury. Vision is severely affected and disorder is commonly associated with number of ocular complications like buphthalmos, glaucoma and cataract(L. B. Nelson, Spaeth, Nowinski, Margo, & Jackson, 1984).

1.3.1.6 Iridogoniodysgenesis

It is characterized by malfunction of anterior chamber of eye and irido-corneal angle. Iridogoniodysgenesis is due to terminal induction or abnormal migration of neural

crest cells which leads to formation of most of anterior segment structures of eye i.e. corneal endothelium, stroma and iris stroma(François, 1981).

1.3.1.7 Coloboma

Coloboma (Greek koloboma) is referred as a hole in eye structures like retina, choroid and iris. Hole appears during prenatal development and it fails to close completely before child birth. Depending upon size and location of gap Coloboma effect on vision could be mild or severe. If large part of optic nerve or retina is missing it can cause problems like glaucoma (Cunliffe *et al.,* 1998).

POAG is further classified into adult and juvenile onset primary open angle glaucoma. Juvenile-onset POAG (JOAG) inherits as Mendelian-dominant character while adult-onset POAG shows complex inheritance (C. P. Pang *et al.,* 2006; Wang *et al.,* 2006). POAG is a common disease type of glaucoma diagnosed at later ages(C. P. Pang *et al.,* 2006).

Primary congenital glaucoma accounts for about 0.01 to 0.04 percent of blindness globally(Akarsu *et al.,* 1996). PCG is most prevalent form of glaucoma among infants. Above 80% cases have been observed with in first year of child's life affecting more males (65%) as compared to females (35%), in 60-8-% cases both eyes are affected (Vasiliou & Gonzalez, 2008).

1.4. Structure of Eye

Human eye is specialized organ for photoreception, the mechanism by which the light rays coming from object are congregated and focused on a group of photoreceptors i.e. the rods and the cones.Action potentials generated by nerves in various photoreceptors are then transmitted to the optic nerve brain, where the information is administered and results in vision. Retina and optic nerve of the eye are involved in this basic physiological process. Other parts of the eye are required for the transmission of light and focusing on retina and for supporting and nourishing tissues of eye.

Ciliary body the forward extension of choroid and is about 5- 6 mm wide sphere of tissues divided in two regions, an anterior pars plicata and posterior pars plana.

Ciliary body produces aqueous humour in posterior chamber of eye, at a rate of 2.0 - 3.0 µl/min which enters at the posterior chamber through pupil leaving the eye

through the porous trabecular meshwork and then pours into Schlemm's canal, which then drains into blood (Neri, Azuara-Blanco, & Forrester, 2004).

Aqueous humour is slightly alkaline, optically clear liquid occupying anterior and posterior chamber of eye (Neri *et al.,* 2004). Nutrients and oxygen is provided by aqueous humour to the tissue of the eye lacking direct blood supply. It also provides intraocular pressure (IOP), internal pressure that keeps the eye ball in proper form. Range of normal inter ocular pressure is 10-21 mm Hg(Pensiero, Da Pozzo, Perissutti, Cavallini, & Guerra, 1992). From blood, aqueous humour is derived with in capillary network via three mechanisms i.e. Diffusion, Ultra filtration and active It is decreased by inhibitor of active metabolism, hypoxia and hypothermia (Gupta, 2005).

Mesodermal lineage and neural crest are the sites from which structures are derived that comprise drainage angle. Formation of trabecular meshwork occurs at 12 to 22 weeks of gestation. Mesenchymal cells form wedge shaped structure that is between the stroma and corneal endothelium(McMenamin, 1991). Schlemm canal formed from venous plexus (located anterior to the trabecular anlage), becomes visible at 16th week of gestation and turn out to be along with inter-canal links after 36 weeks(Ramírez *et al.,* 2005). The developmental event continues even after birth and look like an adult structure till 8th year of life (Ramírez *et al.,* 2005).

The developmental arrest of tissues of neural crest at third trimester can result in immature angle appearance of PCG .The severity of disease depending upon the stage at which developmental arrest occur in the patient. For example obstruction in outflow results in the condensed trabecular beams that prevent normal posterior migration of iris and ciliary body(Anderson, 1981).

1.4.1 Aqueous Outflow Pathways

Border zone between cornea and sclera is called limbus. It performs many functions like corneal wound healing, nourishment of peripheral cornea and hypersensitivity responses. It also controls IOP and contains aqueous humour outflow pathways. Alteration in radius of curvature between cornea and sclera produces internal scleral sulcus and shallow external scleral sulcus. Trabecular meshwork and canal of schlemm are contained in internal scleral sulcus. Conventional outflow pathway is involved in 70-90% of outflow of aqueous humour (Forrester *et al.,* 2001). 10-30% of aqueous humour is drained by non-conventional outflow pathways that involve

spaces between loose connective tissues of suprachoroidal space and ciliary muscle fibres(Neri *et al.,* 2004).

1.4.2 Trabecular Meshwork

Trabecular Meshwork is a Sponge like connective tissue lying in the chamber and is lined by trabeculocytes which are responsible for outflow of aqueous humour form anterior chamber of eye. There are three anatomical parts of trabecular meshwork with different ultra-structures i.e. (Inner uveal meshwork, corneo-scleral meshwork and outermost cribriform meshwork) (Neri *et al.,* 2004).

1.4.3 Retina and Retinal Ganglion Cells

Innermost sensory layer of eye contains sensory neurons, which are responsible for the transmission of neural impulse from external environment to brain for decoding and analysis hence responsible for image formation. Retina consists of mainly five types of neural cells(Barker, 2002).

Photoreceptors: Photoreceptors are responsible for absorbing quanta of light and transforming them into chemical signals (glutamic acid).

Bipolar cells: Bipolar cells connect photoreceptors to the ganglion cells. Bipolar cells signal light either OFF or light ON further down to the ganglion cells.

Ganglion cells: Ganglion cells collect the information that is generated in the retina and transmit it to brain.

Horizontal cells: Horizontal cells are interneurons linking cones and bipolar cells. They aid in signal processing.

Amacrine cells: Amacrine cells are also interneurons and they link bipolar cells and retinal ganglion cells.

Retinal ganglion cells (RGC's) are located near inner surface of the retina. It receives visual information from the photoreceptors by mean of two intermediate neuron types (amacrine cells and bipolar cells). There are 1.2 to 1.5 million RGC's in human retina and around 105 million photoreceptors per retina. Normally each retinal 13 ganglion cells receive inputs from 100 photoreceptors (http://en.wikipedia.org/wiki/Retina). In centre of retina (fovea) single photoreceptor connects with five ganglion cells. In

extreme ends of the retina a single ganglion cell receives information from thousands of photoreceptors. RGC's collectively transmit visual information from retina to numerous sections in the brain. These vary greatly in their size, connections, and responses to the visual stimulation but they all share defining property of having long axon extending into brain. These axons form optic tract, optic nerve, and optic chiasm.

Optic nerve is the major site of damage in glaucoma and is not always associated with elevated intraocular pressure (IOP) (Shields, Varley, Broks, & Simpson, 1996). Optic nerve (cranial nerve II) is involved in transmission of visual information from retina to brain. Optic nerve head contains 1.2 million nerve fibres of retinal ganglion.

Retinal ganglion cells leave the eye collectively. Optic nerve appears round at point of leaving and hence it is referred as optic disc. Blood vessels provide nourishment to retinal layers. Optic disc appears to be oval white area of 3 mm^2 having physiologic cup at centre, resulting from transmission of central retinal vein and artery. Normal cup is about 1/3 that of the disc and is described as a hole in the centre of neuro-retinal rim (Alexander, 1991; Elolia and Stokes, 1998). Increased optic disc cupping is indicative of pathologic alteration of glaucoma(Klein, Klein, Lee, & Hoyer, 2006).

1.5 Pathophysiology of Glaucoma

Degeneration of retinal ganglion cells decrease in width of neuro-retinal rim along with the enlargement of cup. The pathological process in glaucoma patients affects axon, RGC's and dendrites, which eventually damage glial cells these events results in the loss of neurons in the visual cortex and also in the lateral geniculate nucleus (Weinreb & Khaw, 2004). The principle symptom of glaucoma is elevated IOP, which is caused due to the decreased outflow of aqueous humour via meshwork outflow pathways.

Molecular mechanism for normal and impaired outflow of aqueous humour is not clear (Lütjen-Drecoll, B'Ann, Tian, & Kaufman, 2001). Raised IOP compresses optic nerve at lamina cribosa, blocks axoplasmic flow interfering neurotrophin backward transport to RGS's (Guo *et al.,* 2005; A. Kumar *et al.,* 2007). Elevated IOP also

affects blood supply leading to apoptotic death of RGC's (López-Garrido *et al.,* 2006).

There is no well-known relationship between elevated IOP and death of RGC's (J. Wiggs *et al.,* 2004). However deficiency of neuronal growth factors, increased retinal glutamate, oxidative stress and nerve damage can play role in the initiation and advancement of glaucoma (Libby *et al.,* 2005; Mozaffarieh, Grieshaber, & Flammer, 2008; Weinreb & Khaw, 2004). This is also supported by the fact that Reduction of IOP decreases the progression of glaucoma in patients with or without elevated IOP (Caprioli & Coleman, 2008).

1.6. Glaucoma Diagnosis

Glaucoma diagnosis is usually based on defects in visual field and damage of optic disc (Martus *et al.,* 1998). Family having history of glaucoma and elevated inter-ocular pressure are given attention (I.-H. Pang & Clark, 2002). As a result of raised IOP glaucomic patients often suffer from photophobia, buphthalmos, tearing and corneal opacification (Burgoyne, 2011). Optic nerve damage is also associated with elevated IOP (Girgis & Frantz, 2007). Identification of glaucoma should include a comprehensive eye examination that covers visual acuity (tests resolution of eye), family history, biomicroscopy, gonioscopy, perimetry, tonometry, nerve fibre layer assessment, optic nerve evaluation and visual field testing (Sommer *et al.,* 1991).

1.6.1. Assessment of Optic Nerve Health

Optic nerve health can be checked by measuring cup to disc ratio i.e. the ratio of the diameter of optic cup to total diameter of optic disc expressed in decimals. A diameter 0.3 is considered as normal (Elolia & Stokes, 1998). Retinal nerve fiber layer (RNFL) evaluation is also an important factor for diagnosis of glaucoma and this can be done by Optical Coherence Tomography (OCT) that is non-invasive and non-contact method to calculate thickness of RNFL via high resolution images. It can detect diffused retinal nerve fiber layer that occurs in glaucoma (Bagga, Feuer, & Greenfield, 2006; Sihota, Sony, Gupta, Dada, & Singh, 2006).

1.6.1.1 Visual Acuity Test:

This is an important test for valuation of visual function. The resolving power of eye to differentiate object as separate is known as Visual Acuity. Distance is an important factor because resolution for close objects is better than for far away. It is typically measured by the Snellen chart. Snellen chart is a white chart with black letters with larger letters at the top and smaller and more numerous at the bottom of the list (Figure 1.1)

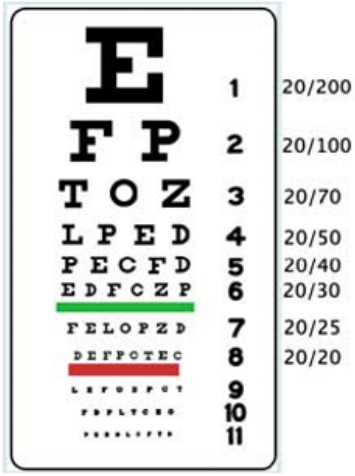

Figure 1.1: Snellen chart.

Visual acuity based on the concept of minimum angle of resolution (MAR) which is the angle subtended by the thickness of a letter with width and height of letter. One eye is tested covering other eye. The visual acuity is noted in terms of snellen fraction in which numerator is testing distance i.e. 20 feet or 6 meters and the denominator is distance at which a normal observer canread the letter. Visual acuity of 20/20 (6/6) refers to MAR of one min of arc thickness and is considered standard for normal vision.

1.6.1.2 Tonometry:

Increased intraocular pressure (IOP) is reflected as main risk factor for glaucoma(Klein *et al.,* 2006). Indirect measurement of IOP by determining response of eye to the external pressure is known as tonometry. Range of IOP i.e. 10 to 21.5 mm Hg is considered to be normal. IOP is usually measured by two methods i.e. a) Goldmann applanation method and b) non-contact method. For both of these two methods patient is asked to sit on examination chair, chin is positioned in special cradle to stop sudden movement or jerking of the head.

1.6.1.3 Gonioscopy:

It is essential in identifying the type and cause of glaucoma. Gonioscopy is done to examine that anterior chamber angle is open or close. Gonioscopy is significant to assess symptoms of goniodysgenesis and pigment, psedoexfoliation, secondary glaucoma in the open angles. Slit lamp bio-microscope with the Goldmann lens is usually used by ophthalmologists for indirect gonioscopy.

1.6.1.4 Perimetry:

Test is done while keeping gaze of subject fixed whereas light flash is given at several places in the visual field. Computer records spot of each flash and if patient pressed the button when light flashed in that particular spot. Perimetry is very useful to find boundaries of visual field and for finding progression of glaucoma.

1.7. Genetics of Glaucoma

Molecular basis of glaucoma has always been very difficult to identify because of little knowledge underlying association of reduced IOP drainage and function of RGC's (Guo *et al.,* 2005). Pathophysiology of glaucoma can be understood by

identifying the genes and their products that could be the cause of glaucoma. Glaucoma is a heterogenetic disorder as it involves polygenic inheritance, heterogeneity of locus, incomplete penetrance and phenocopies (Gong, Kosoko-Lasaki, Haynatzki, & Wilson, 2004; Hogewind *et al.,* 2007). Various loci have been mapped and traced up to gene level for different forms of glaucoma. Furthermore genetic studies also showed the role of, many other regions of chromosomes for pseudo exfoliation glaucoma, JOAG, angle closure glaucoma, POAG, and primary congenital glaucoma.

1.7.1. Primary Open Angle Glaucoma (POAG)

POAG exhibits complex inheritance along with sporadic appearance in most cases (Sarfarazi, 1997). Depending on age of onset we can divide POAG into two sub-types i.e. Juvenile-onset POAG (JOAG) appearing earlier in lives like before 40 years and is normally inherited as autosomal dominant trait, and adult-onset POAG.

JOAG is severe form associated with elevated IOP, optic disc damage and visual field loss and requires surgical therapy (Wang *et al.,* 2006). Adult-onset POAG involves complex pattern of inheritance and is usually diagnosed after 40 years of age (J. L. Wiggs *et al.,* 1996)with comparatively different phenotype like moderate IOP elevation, having mild presentation and progressive development.

Adult-onset POAG has complicated prognosis because of lack of symptoms like pain, and hence cannot be diagnosed earlier (Sarfarazi, 1997). In Normal tension glaucoma (NTG) a subset of adult onset POAG affected individuals suffer from vision loss because of optic nerve damage at normal IOP level (Anderson, 2003). Several genes have been studied whose mutations play role in the development and prognosis of POAG, including optineurin (OPTN), Myocilin (MYOC), and WD repeat- domain 36 (WDR36) which are located at GLC1A, GLC1E, and GLC1G loci respectively. Mutations in these gene accounts for only a very few fraction of POAG indicating contribution of other gene and loci in etiology of disorder(Rezaie *et al.,* 2002; Stone *et al.,* 1997) .

1.7.2 Primary Congenital Glaucoma (PCG)

Its symptoms are mostly notable before 6 years of age such as eye rubbing, irritability and photophobia (Monemi *et al.,* 2005; Sarfarazi, 1997). Corneal clouding may also be noticed with increased diameter of cornea and breaks in Descemet membrane or

enlarged axial length as mentioned in thetable 1.1. In premature infants and new-borns slight corneal can occur, but if cornea thins from 960mm at birth to 520 nm at 6th month, then it can result in pathogenesis (Saw *et al.,* 2004).

Primary congenital glaucoma is a heterogeneous group of inherited ocular anomaly of anterior chamber angle and trabecular meshwork leading to obstruction of aqueous drainage, optic nerve damage and increased IOP which results in early childhood blindness (Anderson, 1981). PCG signs and symptoms include enlargement of globe, blepharospasm, edema, opacification and photophobia and it establishes in newborn or infantile period (Sarfarazi *et al.,* 1995). Prevalence of PCG is very high in inbred populations where cousin marriages are customary (AlFadhli *et al.,* 2006). Its inheritance pattern is mainly autosomal recessive but also there are very few cases of pseudo-dominance in some of families(Papadopoulos, Cable, Rahi, & Khaw, 2007; A. Reddy *et al.,* 2004). Additionally evidences have been found for its reduced penetrance with varying expressions of phenotypes (Bejjani *et al.,* 2000). In 70% cases glaucoma is bilateral and in others it is asymmetric. Sporadic cases are more in males while familial cases are equally prevalent in both males and females (Suri *et al.,* 2009; Walton, Nagao, Yeung, & Kane, 2013).

1.8 Pathophysiology of PCG

There are 2 main theories about pathophysiology of PCG. The first commonly known theory states that PCG is caused by the "thickened beams" in trabecular meshwork which leads to shrill trabecular spaces and condensed aqueous outflow(Anderson, 1981). The trabecular meshwork is not fully differentiated at birth in PCG patients because of the immature trabecular meshwork. There is also developmental arrest of ciliary muscle, iris, and the anterior chamber angle. Therefore, at birth the iris, ciliary muscle, and the anterior chamber angle end up into an anterior location overlapping the trabecular meshwork,and reducing aqueous drainage and stopping further differentiation of trabecular meshwork (Anderson, 1981)

Second theory is based on Barkan's membrane, which is an imperforate membrane that covers the anterior chamber angle thus reduce aqueous drainage. Reduced outflow leads to raised IOP resulting in congenital glaucoma. In recent years, this theory has largely been disproven largely because there is no histologic proof of Barkan membrane (Rummelt & Naumann, 1997).

PCG consists of 3 subclasses that are classified by age of onset including, a: Primary congenital glaucoma (40%) with age of onset from birth to age of 2 months, b; Primary infantile glaucoma (55%) with age of onset from age 2 months to 2-3 years, c; Late-onset primary infantile glaucoma occur at 3 years of age(Beck, 2001).

1.9. Genetics of PCG

Mutations of 6 genes are primarily involved in pathogenesis of juvenile and congenital glaucoma these include *FOXC1, PAX6, CYP1B1, PITX2, MYOC*, and *LTBP2* (Fan *et al* 2010). Some unilateral cases of PCG have also been identified suggesting possibly different etiology as not linked with mutations of *CYP1B1*(Lim *et al.,* 2013). It has also been found that *MYOC* gene which was supposed to be involved in pathogenesis of POAG can also play role in development of PCG pathogenesis (Lim *et al.,* 2013). Autosomal recessive PCG has also been mapped for four chromosomal loci including: GLC3A (2p21), GLC3B (1p36), GLC3C (14q24.3) and 14q24.2-q24.3 (Firasat, Riazuddin, Hejtmancik, & Riazuddin, 2008; Lim *et al.,* 2013)*CYP1B1* gene (OMIM 601771) cytochrome P4501B1, located at GLC3A locus has been reported to be most mutated gene in PCG (Firasat *et al.,* 2008; Stoilov, Akarsu, & Sarfarazi, 1997). While other causes like lens dislocation, called as secondary may also contribute in pathogenesis (Gatzioufas *et al.,* 2013). Mitochondrial dysfunction is also related with PCG (M. Kumar *et al.,* 2013).

1.9.1*LTBP2*

A gene known as Latent transforming growth factor beta-binding protein 2(*LTBP2*) is located at GLC3C and has been found to be associated with PCG(Ali *et al.,* 2009; Lim *et al.,* 2013; Sarfarazi, Stoilov, & Schenkman, 2003).Though it was reported originally as a cause of PCG in Pakistani and Iranian families, but further studies ruled out its evolvement in Turkish, Indian and American populations(Ali *et al.,* 2009; Lim *et al.,* 2013; Sharafieh, Child, Khaw, Fleck, & Sarfarazi, 2013) .

Among ethnically diverse population *LTBP2* seems to be a rare cause of congenital glaucoma, although there are reports suggesting that null mutations in *LTBP2* is a cause of PCG (Lim *et al.,* 2013).

1.9.2 *MYOC* (Myocilin)

MYOC was the first glaucoma-causing gene identified. MYOC is located at chromosome 1q24.3– q25.2(Zhuo, Wang, Wei, Huang, & Ge, 2006), and encodes a bipartite protein Myocilin. This gene consists of 3 exons (encoding 504 amino acid) and a 5kb promotor region, containing olfactomedin homology COOH-terminal domain, myosin-like NH2-terminal domain and TIGR (inducible trabecular meshwork glucocorticoid response) (Tamm, 2009). Most of the mutations have been found in olfactomedin-like domain (López-Garrido *et al.,* 2013). Mutations of MYOC gene have been studied extensively in juvenile-onset and congenital glaucoma cases globally and are proved to be involved in pathogenicity of disorder (V. Kumar, Abbas, Fausto, & Aster, 2014).

Mutated variants of *MYOC* alter ciliary body architecture and trabecular meshwork hindering aqueous humour outflow and hence raising IOP (Takahashi *et al.,* 2000). Disease causing sequence variants of *MYOC*, have been reported in the families with primary congenital glaucoma (Chakrabarti *et al.,* 2005). Mutations of *MYOC* are also related in cohort of non-consanguineous primary congenital glaucoma families (Abu-Amero *et al.,* 2011).

1.9.3 *CYP1B1* (Cytochrome P4501b1)

GLC3A locus was mapped on short arm of the chromosome number 2 and contains *CYP1B1* which is known to be involved in PCG(Sarfarazi *et al.,* 1995; Stoilov *et al.,* 1997).

CYP1B1 is a gene consisting of 3 exons which encodes a protein of 543 amino acids. Crystal structure of protein encoded by *CYP1B1* is not known but it can be anticipated on basis of conserved structural elements of all cytochrome P450 molecules (Vasiliou and Gonzalez *et al.,* 2008). All Cytochrome P450 shares highly conserved carboxyl terminal core, segment including four helix bundles, and heme-binding region and the meander regions (Acharya *et al.,* 2006; Sutter *et al.,* 1994).

CYP1B1 is a member of CYP450 super family of hemoproteins which contains 58 genes in humans and 102 in mice (Lim *et al.,* 2013).

CYP1B1 also metabolizes endogenous substrates like steroids, melatonin and retinoic acid (Vasiliou and Gonzalez, 2008). It is expressed in fetal and adult human tissues like kidney, brain, breast, prostate, uterus, ovary, eye, lymph nodes and cervix(Bejjani, Xu, Armstrong, Lupski, & Reneker, 2002). Relatively high level of

CYP1B1 mRNA has been observed in human eye tissue like ciliary body and iris as compared to retina, cornea and retinal pigment epithelium (Stoilov *et al.,* 1998).

CYP1B1 mRNA and CYP1B1 transcripts are identified in adult trabecular meshwork cDNA library, by semi-quantitative RT-PCR (Stoilov *et al.,* 1998; Tomarev *et al.,* 2003). In eye of an adult mouse (C57BL/6), *CYP1B1* protein was identified in inner ciliary epithelial cells, corneal epithelium, retinal ganglion cells, and inner nuclear layers and in lens epithelium with trace expression(D Choudhary, Jansson, Sarfarazi, & Schenkman, 2006). Studies have shown that during development of chick embryo *CYP1B1* is expressed in ectodermal, endodermal and mesodermal derivatives containing retina and anterior segment of eye (Dawczynski, Koenigsdoerffer, Augsten, & Strobel, 2007).

CYP1B1 has differential distribution as its level is high in fetal than in adults suggesting that it plays role in ocular development and function. Ciliary body is considered as prime source of aqueous humour generation so as a result of mutated *CYP1B1,* metabolism of important endogenous substrate will be affected leading to developmental abnormalities resulting in eye pathophysiology (Vasiliou & Gonzalez, 2008). *CYP1B1* is a modifier gene and causative agent for POAG and is also related to anterior segment disgenesis, peter's anomaly, and peter's anomaly (Vasiliou & Gonzalez, 2008).*CYP1B1* deficient mice develop irido-corneal abnormalities along with increase in endothelial cells in trabecular meshwork and decreased collagen(Johnson & Tomarev, 2016). Similarly A knockout Cyp1b1-/- mice model for *CYP1B1* develop ocular drainage abnormalities, like small or complete absent Schlemm's canal, Hypo plastic trabecular meshwork, and Focal angle abnormalities similar to that of phenotypes in human PCG patients (Libby *et al.,* 2003).

Mutations of *CYP1B1* are variably expressive and penetrating (Suri *et al.,* 2009). *CYP1B1* mutations are associated with less severe PCG but they are more likely to be associated with childhood PCG(Khan, 2011). There are very few evidences showing that severity of disease and severity of angle dysgenesis are directly correlated with mutations of *CYP1B1*(Hollander *et al.,* 2006).

1.10 CYP1B1 Mutations in PCG

More than 100 *CYP1B1* mutations are reported in Human Gene Mutation Database. Disease causing mutations have been identified in *CYP1B1* including frame shift,

missense, small deletion/insertion, premature stop codon and large deletion(Tham *et al.,* 2014; Vasiliou & Gonzalez, 2008). *CYP1B1* gene product (cytochrome P-450 1B1) digests many complex molecules like 17-β-estradiol and polycyclic aromatic hydrocarbons(Tokizane *et al.,* 2005; Tsuchiya, Nakajima, & Yokoi, 2005). P-450 1B1 activity might be responsible for metabolism of a compound essential for ocular development (Dharamainder Choudhary, Jansson, Sarfarazi, & Schenkman, 2008; D Choudhary, Jansson, & Schenkman, 2009).

Mutations that result in premature truncation of gene i.e. deletion, insertion, frmaeshifts and nonsense mutations, results in earlier onset and more severity of disease. Disease caused by nonsense mutations are less severe(Bejjani *et al.,* 1998; Bejjani *et al.,* 2000; López-Garrido *et al.,* 2013).

Broad analysis of *CYPB1* gene among patients of different populations worldwide revealed great diversity with respect to *CYP1B1* mutation spectrum in disease pathogenesis ranging from 20% in Caucasians,100% in Saudi Arabians and Slovakian Roms (Rao *et al.,* 2011). PCG being recessive disorder carry homozygous *CYP1B1* mutations in patients (Stoilov *et al.,* 1998).Variants which proved to be pathogenic for glaucoma include p.Glu229Lys and p.Gly61Glu on exon 2, with later typically homozygous and former heterozygous (Badeeb, Micheal, Koenekoop, den Hollander, & Hedrawi, 2014).

Glutamine 229 is found to be located at periphery of the active site at the carboxyl terminal and it forms charged pair through Arg233(Badeeb *et al.,* 2014). When Glu229 is replaced with lysine residue it leads to the charge repulsion which affects structure of the substrate binding region and may also influence metabolite formation. Variant p.Glu229Lys destabilizes holoenzyme resulting in 25% decline in enzyme activity than wild type. Mutant protein results in 75% decrease in *CYP1B1* activity to break down metabolites produced by metabolism of Arachidonic acid (Dharamainder Choudhary *et al.,* 2008). The Arg469Trp and p.Gly61Glu variants are primarily associated with more severe forms(Badeeb *et al.,* 2014). The p.Arg390His mutation is previously found in homozygous condition in PCG patients (Tanwar *et al.,* 2009). Till now more than 200 *CYP1B1* mutations are known. Most commonly reported mutations of *CYP1B1* from Pakistan and worldwide are given in tables 1.2 and 1.3.

1.11. Aims and Objectives

Various CYP1B1 mutations are known among diverse ethnic groups including Pakistani families, some of these mutations are population specific.

1. To investigate the CYP1B1 mutations in 7 consanguineous families with PCG.
2. Identification of disease causing mutations and pathogenicity analysis of the identified mutations.

Table 1.1 Increase in Corneal Diameter and Axial length with age (Adapted from basic and clinical science course. American Academy of Ophthalmology 2010-2011)

Age	**Corneal diameter**	**Axial length**
At birth	9.5-10.5	16-18
1 Year	11-12	20-21
6 Year	12	23
Adult	12	22-24

Table 1.2 *CYP1B1* Reported Mutations in Pakistan (Adapted from Michael *et al.,* 2015)

Nucleotide change	Amino acid change	Previous reports
c.182G>A	p.Gly61Glu	PCG, POAG
c.685G>A	p.Glu229Lys	PCG, POAG
c.701C>T	p.Thr234Met	POAG
c.725A>C	p.Asp242Ala	PCG, POAG
c.859G>C	p.Ala287Pro	POAG
c.862G>C	p.Ala288Pro	PCG, POAG
c.868dup	p.Arg290Pro	PCG, POAG
c.947A>T	p.Asp316Val	POAG
c.1063C>T	p.Arg355	PCG
c.1084C>T	p.Gln362	POAG
c.1103G>A	p.Arg368His	PCG, POAG
c.1169G>A	p.Arg390His	PCG, POAG
c.4335T>G	P.Leu177Arg	PCG
c.8297T>C	P .Leu487Pro	PCG

Table.1.3 List of mutations reported in PCG worldwide

Nucleotide change	Amino acid change	Previous reports
c.553G>A	p. Gly61Glu	(Bejjani *et al.,* 1998)
c.7957G>A	p. Asp374Asn	(Bejjani *et al.,* 1998)
c.8242C>T	p. Arg469Trp	(Bejjani *et al.,* 1998)
c.517G>C	p. Trp57Cys	(Stoilov *et al.,* 1998)
c.528G>A	p. Gly61Glu	(Stoilov *et al.,* 1998)
c.847insT	Frame shift	(Stoilov *et al.,* 1998)
c.1187 G>T	Glu281→stop	(Stoilov *et al.,* 1998)
c.1209insC	Frame shift	(Stoilov *et al.,* 1998)
Large deletion	Splicing error	(Stoilov *et al.,* 1998)
1410del13	Frame shift	(Stoilov *et al.,* 1998)
c.1439G>T	p. Gly365Trp	(Stoilov *et al.,* 1998)
c.C1482>T	p. ro379Leu	(Stoilov *et al.,* 1998)
c.G1505>A	p. Glu387rLys	(Stoilov *et al.,* 1998)
c.G1515>A	p. Arg390rHis	(Stoilov *et al.,* 1998)
c.1546dup10	Frame shift	(Stoilov *et al.,* 1998)
c.1656C>T	p. Pro437Leu	(Stoilov *et al.,* 1998)
c.G1691del	Frame-shift	(Stoilov *et al.,* 1998)
c.C1751>T	p. Arg469Trp	(Stoilov *et al.,* 1998)
c.1749dup27	Frame-shift	(Stoilov *et al.,* 1998)
c.182G>A	p. Gly61Glu	(Chitsazian *et al.,* 2007)
c.1103G>A	p. Arg368His	(Chitsazian *et al.,* 2007)

c.1169G>A	p. Arg390His	(Chitsazian *et al.,* 2007)
c.688C>A	p. Ala106Asp	(Campos-Mollo *et al.,* 2009)
c.888G>T	p. Glu173X	(Campos-Mollo *et al.,* 2009)
c.1154C>A	p. Phe261Leu	(Campos-Mollo *et al.,* 2009)
c.1155G>T	p. Glu262X	(Campos-Mollo *et al.,* 2009)
c.1394G>A	p. Trp341X	(Campos-Mollo *et al.,* 2009)
c.1907_1912del ACCCAA	p. Pro513_Lys514del	(Campos-Mollo *et al.,* 2009)
c.526C>T	p. Pro52Leu	(Campos-Mollo *et al.,* 2009)
c.612T>A	p. Tyr81Asn	Melki*et al.,* 2004
c.1539C>A	p. Arg390Ser	(A. B. Reddy, Panicker, Mandal, Hasnain, & Balasubramanian, 2003)
c.1569C>T	p. Pro400Ser	(Dimasi *et al.,* 2007)
c.1681C>T	p. Pro437Leu	(Chakrabarti *et al.,* 2005)
c.1776C>T	p. Arg469Trp	(Stoilov *et al.,* 1998)
c.4124C>G	p. leu107Val	(Chen *et al.,* 2008)
c.4157C>A	p. Pro118Thr	(Chen *et al.,* 2008)
c.352C>T	p. Pro118Ser	(Chen *et al.,* 2008)
c.349C>T	p. Arg117Trp	(Bagiyeva, Marfany, Gonzalez-Angulo, & Gonzalez-Duarte, 2007)

c.350G>C	p. Arg117pro	(Hollander *et al.,* 2006)
c.343G>C	p. Ala115Pro	(A. Reddy *et al.,* 2004)
c.319C>G	p. Leu107Val	(Chen *et al.,* 2008)
c.284T>C	p. Val95Ala	(Chen *et al.,* 2008)
c.241T>A	p.Tyr81Asn	(Melki, Colomb, Lefort, Brezin, & Garchon, 2004)
c.1033C>T	p. Leu345phe	(A. L. Vincent *et al.,* 2002)
c.988G>T	p. Ala330Ser	(López-Garrido *et al.,* 2006)
c.896G>T	p. Gly329Val	(Bagiyeva *et al.,* 2007)
c.986G>A	p. Gly329Asp	(Dimasi *et al.,* 2007)
c.958G>T	p. Val320Leu	(Mashima *et al.,* 2001)
c.956A>G	p. Asn319Ser	(Chen *et al.,* 2008)
c.875T>A	p. Met292Lys	(A. Kumar *et al.,* 2007)
c.872A>G	p. Asp291Gly	(Chitsazian *et al.,* 2007)
c.859G>A	p. Ala287Thr	(Chen *et al.,* 2008)
c.845G>A	p. Ser282Asp	(Weisschuh, Wolf, Wissinger, & Gramer, 2009)
c.835C>G	p. His279Asp	(Tanwar *et al.,* 2009)
c.783C>A	p. Phe261Leu	(Campos-Mollo *et al.,* 2009)

c.756C>A	p. Asn252Lys	(Samant, Chauhan, Lathrop, & Nischal, 2016)
c.715A>C	p. Ser239Arg	(A. Reddy *et al.,* 2004)
c.694G>C	p. Gly232Arg	(Colomb, Kaplan, & Garchon, 2003)
c.608A>G	p. Asn203Ser	(Chavarria-Soley *et al.,* 2006)
c.605C>A	p. Ala202Asp	(Chitsazian *et al.,* 2007)
c.592G>A	p.Val198Ile	(Mashima *et al.,* 2001)
c.578C>T	p. Pro193Leu	(Mashima *et al.,* 2001)
c.565G>C	p. Ala189pro	(López-Garrido *et al.,* 2006)

2. Materials and Methods

2.1 Ascertainment of Families

Seven Pakistani families (Family A- G) affected with recessive glaucoma were included in this study. Families with at least two affected members were visited in remote areas of Pakistan. During these visits detailed family history was collected for each family to document the clinical profile, photograph were taken to document visible ophthalmic feature.

2.2 Family Pedigrees

Genetic relationship between different individuals of families was clarified by taking interview of normal elders or guardians. Pedigrees were drawn using Haplopainter software (Thiele & Nürnberg, 2005). Males are represented by squares in the pedigree whereas females are represented by circle. Normal individuals are shown by hollow symbols while glaucoma patients are represented by filled symbols. Diagonal line on circle or square represents deceased members. Cousin marriage between partners is shown by double line. Different generations are shown by roman numerals while individuals with in single generation are denoted by Arabic numbers.

2.3 Sample Collection

With written consent peripheral blood samples of glaucoma patients and normal individuals of each family were collected via 10 ml sterile syringes. Blood was then transferred to EDTA vacutainer tubes and kept at 4°C in laboratory, before further processing for DNA extraction.

2.4 Extraction of Genomic DNA

2.4.1 Phenol-Chloroform Method

For genomic DNA extraction, Phenol-chloroform isolation technique (organic method) was used (Sambrook, Fritsch, & Maniatis, 1989). In this method 750 µL of blood from each of the individual was added in 1.5 ml tube. Tube was then inverted 4-5 times and then incubated at room temperature for about 10-15 minutes. Afterward tubes were centrifuged in micro centrifuge for 2 minutes at 13,000 rpm (revolution

per minute) (HittichMikro 120, Germany). Supernatant was discarded and the pellet was dissolved in solution A (400 µL) and again centrifuged for 2 minutes at 13,000 rpm. Again supernatant was discarded and to the pellet solution B (400 µL), 10% SDS (25 µL) and proteinase K, 5 µL were added. Pellet was re-dissolved and tubes were incubated overnight at 37°C in incubator B28 (Binder, Germany).

After that equi-volume mixture of freshly prepared solution C and D (500 µL) were added and tubes were inverted several times to thoroughly mix the solution. Then 10 minutes centrifugation was done at 13,000 rpm. To separate three phases (upper, middle and lower), we transferred upper aqueous phase in new eppendorf tube (1.5 mL) and added solution D (500 µL) followed by centrifugation for 10 minutes at 13,000 rpm. Centrifugation again resulted in the separation of aqueous and organic phase's separation. While the earlier was collected in a new eppendorf. For precipitation of genomic DNA we added sodium acetate (60 µL 3 M, pH 4.5-6) and chilled isopropanol (500 µL), was added and tubes centrifuged for 10 minutes at 13,000 rpm. On centrifugation genomic DNA goes into the pellet, supernatant was cast-off and 70% ethanol (200 µL) was then added to the pellet and centrifuged for 7 min at 13,000 rpm. Supernatant was cast-off and the pellet was kept in incubator at 37°C to dry it. After that to the DNA suitable volume of TE (Tris-EDTA) (80-150 µL) was added and incubated at 37°C overnight.

2.4.2 Composition of the Solutions Used For Genomic DNA Extraction

Solution A is composed of sucrose (0.32 M), Tris (10 mM, pH 7.5), $MgCl_2$ (5 mM) and Trition X-100 (1% (v/v)). Solution B contains NaCl (400 mM), Tris (10 mM, pH 7.5) and EDTA (2 mM, pH 8). Solution C is actually phenol (pH 8). Solution D is composed of Iso-amyl alcohol (i.e. 1 volume) and Chloroform (24 volumes). T.E buffer contains EDTA (0.1 mM) and Tris (10 mM, pH 8).

2.4.3 Agarose Gel Electrophoresis

For qualitative/quantitative analysis agarose gel electrophoresis was used. For preparation of 1% agarose gel we added 0.4 g of powdered agarose in 40 mL of 1X TBE buffer in conical flask and mixed it. To prepare 1X TBE, 36 mL of the distilled water and 4 mL of 10X TBE were mixed together (10X TBE contains 0.89 M Tris Borate with pH 8.3 and 0.032 M EDTA). Mixture was then heated for 1 minute in

microwave oven and then it was allowed to cool for few seconds and for staining purpose 8 µL Ethidium bromide (EtBr) dye was added in gel mixture. Mixture was poured in the casting apparatus and kept then for 20 to 30 minutes, at room temperature for polymerization. After that 5 µL of DNA sample along with 3 µL of loading dye (bromophenol blue0.25% and 40% sucrose) was loaded in the gel. This gel was kept in the gel tank (Biometra, Germany) for electrophoresis. Gel tank was loaded with TBE running buffer (1X) and voltage was applied (100 volts) for 25 minutes. Gel was then visualized via gel documentation system (SYNGENE, UK) and results were recorded.

2.5 Sequencing of CYP1B1

Various genes have been found to be associated with glaucoma, but few are more common among Asian populations (Stoilov et al. 1997). A detailed literature survey was conducted to link the prevalent genes and *CYP1B1* gene was further selected for sequencing in seven families. *CYP1B1* gene is located on chromosome 2 and it has 3 exons. CYP1B1 (ENST00000260630) sequence was obtained from the ensemble genome browser (http://asia.ensembl.org/index.html). Primers were designed using primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), for two exons through flanking regions of the exons including intron-exon junctions. Blast like alignment tool (BLAT) (http://genome.ucsc.edu/cgi-bin/hgBlat) was used to determine specificity of each primer and primers which gave single hit were selected. Insilico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) was also done in order to confirm primer product size, melting temperature. List of primers is given in Table 2.1.

2.5.1 Exon Amplification

PCR amplification was carried out in the PCR tubes, 200 µL (Axygen, USA). PCR reaction mix comprises following components;

- sample DNA (1 µL) (40 ng)
- PCR buffer (2.5 µL) (750 mM Tris-HCl, pH 8.8, Ammonium sulphate 200 mM, Tween-20 0.1%)
- $MgCl_2$(1.5 µL) (25 mM)
- dNTPs (0.5 µL) (10 mM, Fermentas, UK)
- Forward and reverse primer (0.5 µL each) (0.1 µM)

- Taq DNA Polymerase (0.5 µL) (5 U/ µL, Fermentas, UK)
- PCR water (18 µL)

PCR reaction mixture for amplification of Exon (1-3) was prepared by adding sample DNA (1 µL), forward and reverse primers (0.5 µL), $MgCl_2$ (1.5 µL), PCR buffer (2.5 µL), dNTPs (0.5 µL), Taq DNA polymerase (0.5 µL), and PCR water (18 µL). 11 µL of PCR water was added to adjust final volume to 25 µL. After preparation of PCR reaction mixture, vortexing and centrifugation was done and then PCR tubes were placed in T1 Thermocycler (Biometra, Germany) for amplification. Following were thermo-cycling conditions for PCR

- DNA Template denaturation for 10 min at 95°C
- Amplification cycles , (39 cycles)

i. Denaturation for 40 sec at 95°C
ii. Primer annealing for 60 sec at 56 and 60°C
iii. Primer extension for 60 sec at 72°C

- Final extension for 10 min at 72°C
- Holding at 4°C

Amplified product was confirmed with 2% agarose gel (2% agarose gel, In 40 mL of 1X TBE buffer). After polymerization gel was loaded with PCR product (5 µL) mixed with loading dye (3 µL).Voltage was applied (100 volts) for 25 minutes and results were recorded via gel documentation system (SYNGENE, UK). The size specific PCR products for each exon were further purified before Sanger sequencing.

2.5.2 PCR Product Purification

ExoSAP-IT (AffymetrixUK) reagent was used to purify PCR product in order to remove unused primers and the nucleotides. 2 µL of ExoSAP reagent was added to 5 µL of PCR product. Mixture was then incubated at 37°C for 15 minutes in order to degrade remaining primers and the nucleotides followed by incubation at 80°C for 15 minutes in order to inactivate the ExoSAP-IT reagent.

2.5.3DNA Sequencing

Standard DNA sequencing protocol for ABI Big Dye Terminator Cycle Sequencing Kit was followed to determine any pathogenic variants (https://www.thermofisher.com/order/catalog/product/4337455).

0.2 ml microamp tubes were taken separately for each template and following reagents were added, 4.0 µL Terminator Ready Reaction Mix, template (dsDNA) up to 4 µL (30-90 ng) (dsDNA template at a concentration of 2 ng/ µL per 100 bp length required for every reaction), primer 1.0 µL, ddH_2O to make final volume equal to 10 µL and then it was mixed and spin briefly.Tubes were placed in thermal cycler and it was programmed to temperature cycling protocol i.e. 25 cycles of [96 C for 10 sec, 50 C for 5-10 sec, 60 C for 4 min], then ramp to 4 C.

2.5.3.1 Purifying sequencing extension products by isopropanol precipitation

Tubes were then spun and transferred into 1.5 ml micro-centrifuge tubes by pipetting the entire sequencing reactions.

After that 75% isopropanol (40 µL) and 100% isopropanol (30 µL) were added and mixed by vortexing briefly. After vortexing it was left at room temperature for >15 min (and < 24 hrs) in order to precipitate products. Then tubes were centrifuged at maximum speed for 20 minutes. Supernatant was carefully discarded so that DNA pellet is not disturbed. After that we added 75% isopropanol (125 to 250 µL) to the tubes and vortex and then again centrifuged at maximum speed for 5 minutes and discarded the supernatant. Samples were dried in vacuum centrifuge for 10 - 15 minutes, and stored at -20 C until ready for electrophoresis.Sample pellet was re-dissolved in 3 µL loading buffer i.e. Hi-Di Formamide, pH 8.0 with blue dextran i.e. 50 mg/ml. Vortexed and centrifuged and then heated for 2 minutes at 95 C and then directly placed on ice. Purified product was sequenced on ABI 310 sequencer.

2.5.4 Sequence Data Analysis

Variations in sequence data were analyzed using BioEdit software version 7.1.3.0 (http://www.mbio.ncsu.edu/bioedit.html).Sequence of *CY1B1* gene (ENST00000260630) was obtained from Ensembl database and was used as template

to align the obtained sequence of each exon by using BioEdit. For alignment of sequence data with reference data "ClustalW Multiple Alignment" option was used.

The observed variants were analyzed by using MutationTaster(http://www.mutationtaster.org/), Mutalyser (https://mutalyzer.nl/), Polyphen-2.0 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php), SIFT (http://sift.jcvi.org/) and Mutation Assessor (http://mutationassessor.org/r3/) to predict their pathogenicity. Public databases including ExAC (http://exac.broadinstitute.org/), 1000 Genomes Browser (http://browser.1000genomes.org/index.html) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) were consulted to determine if the variants were rare.

2.6 Segregation Analysis via Restriction Fragment Length Polymorphisms (RFLP)

RFLP was performed to evaluate transmission of disease within pedigrees. Exons 2 and 3 were amplified with new sets of primers so that each primer product has single restriction sites for the enzymes which have to be selected. Primers which were used to amplify exons for RFLP are given in Table 2.2. First step was to select an enzyme which has only 1 restriction site at the site of mutation. For selection of restriction enzyme a web tool NEB CUTTER was used.

2.6.1 NEB Cutter

Sequence of respective Primer product was pasted in NEB Cutter and submitted to analyze sites for different restriction enzymes. Then by selecting custom digest those enzymes were selected which had only 1 restriction site for given primer product. After that product was restricted by that restriction enzyme and its gel was analyzed in order to get fragments sizes. Restriction sites are lost for all the four enzymes, so wild type should have 2 bands, heterozygous individuals should have 3 particular variant and mutants should have only 1 uncut band.

2.6.2 Restriction digestion analysis

1µL of restriction enzyme was added to 5 µL of PCR product, 7µL of ultra-pure water and 2µL of buffer. Followed by 16-17 hours digestion in case of low digest and 5

minutes digestion in case of fast digest. Green buffer was also added in case of fast digest. Restricted product was then visualized on agarose gel. Nae1 is used for restriction of Family B, Ear1 is used for restriction of Family C, BIPI is used for restriction of Family D andHha1 is used for the restriction of Family G. Ear1 in Family C is used for 20 control samples to find out prevalence of polymorphism in normal control population. List of the enzymes used for digestion is given in table 2.3 along with respective families.

Table 2.1: Primer sequences used for amplification of *CYP1B1* exon 2 and exon 3.

Exon No.3	PRIMER (5′→3′)		Product Size	Annealing
	Forward	Reverse		
EX3 AF/ EX3 BR	AGTGAGAAATTAGGAA GCTGTTTT	TGAGAAGCAGCACAA AAGAGG	862	60ºc

Exon No.2	PRIMER (5′→3′)		Product Size	Annealing
	Forward	Reverse		
EX2AF/ EX2BR	TCTTCTCCAAGGGA GAGTGG	TCGCAGTGCCTCAA GAACTT	1024	60ºc
EX2CF/ CYP1B1_E X2CR	CATGAGTGCCGTGT GTTTCG	GATCTTGGTTTTGAG GGGTGG	569	59ºc

Table 2.2 Primers for amplification of exons for RFLP

Exon No.2	PRIMER (5′→3′)		Product Size (base	Annealing
	Forward	Reverse		
CYP1B1EX2P1-F/R	AGAGTCAGCTCCGACCTCTC	GAACTCTTCGTTGTGGCTGAG	816	58 ºc
CYP1B1_rest_F/R	ACGTCATGAGTGCCGTGTGT	CGGTACGTTCTCCAAATCCA	356	60 ºc

Exon No.3	PRIMER (5′→3′)		Product Size	Annealing
	Forward	Reverse		
CYP1B1EX3-F/R	AGTGAGAAATTAGGAAGCTGTTTT	GCTAATTGAGAAGCAGCACAAA	868	59ºc

Table 2.3: Conditions for Restriction Fragment Length Polymorphism

Enzyme Name	Family	PCR Product	Restriction Buffer	Ultra-Pure Water	Quantity of restriction Enzyme	Restriction Time
Nae1	Family B	5 µL	2 µL	7µL	1µL	16 hours
Ear1	Family C	5 µL	2 µL	7µL	1µL	5 min
BIPI	Family D	5 µL	2 µL	7µL	1µL	16 hours
Hha1	Family G	5 µL	2 µL	7µL	1µL	16 hours

RESULTS

3.1 Description of the families studied

Affected and healthy individuals of 7 consanguineous Pakistani families affected with congenital glaucoma were involved in this study. The signs and symptoms of PCG include reduced visual acuity, enlargement of globe, blepharospasm, edema, corneal opacification and photophobia. Affected member of each family were diagnosed by local ophthalmologist and clinical phenotypes were recorded which segregates in the family.

3.1.1 Family A

Family A shows optic atrophy and glaucoma. Pedigree design that is presented in figure 3.1 indicates five generations with 3 affected males (V-5, V-6 andV-7) and 2 affected females (V-3 and V-4). From the pedigree it is clear that PCG is transmitted in autosomal recessive pattern as affected status is independent of the sex of a person. Parents (IV-1 and IV-2) were phenotypically normal but they produced five affected children. All affected members were blind since early childhood. Affected members cannot fully open there eyes suggesting light sensitivity and photophobia.

3.1.2 Family B

Family Bdemonstrates autosomal recessive glaucoma since the age of six years. The five generation pedigree (Figure 3.2) contains 30 members 4 of which are affected. All the 4 affected members are male (IV-2, IV-7, IV-11 and V1). All the parents of affected members are showing normal phenotype, suggesting autosomal recessive mode of inheritance. Pedigree analysis is also suggesting that consanguineous loops might account for affected members to have homozygous abnormal alleles.

3.1.3 Family C

Affected individuals of family C haveglaucoma with cataract. Five generation pedigree in figure 3.3 shows 17 members 4 of which are affected including 1 female (V-2) and 3 males (IV-2, IV-3 and IV-4). One of the parent of affected female (V-2) was also phenotypically abnormal. Consanguineous loop may account for affected female being homozygous for an abnormal allele. Affected members of family C were diagnosed with blindness along with hazy cornea.

3.1.4 Family D

Family D is found with glaucoma (Figure 3.4). The four generation pedigree consists of 18 members including two females (III-3 and III-4). One of the members (III-1) of the family is deceased. Affected member of family D had autosomal recessive glaucoma since early childhood which resulted in complete blindness at the later stages.

3.1.5 Family E

Family E comprise of five generations (Figure 3.5). Two male members (V-2 and V-5) of fifth generation present symptoms of glaucoma. Parents of affected members are phenotypically normal showing autosomal recessive pattern of inheritance of PCG.

3.1.6 Family F

Family F was identified with vision loss at earlier stages of life and complete vision loss at later stages (Figure 3.6). The five generation pedigree consists of 30 members 8 of which segregate PCG. Out of 8 three members are male (V-1, V-8 and V-9) while rest are female (IV-1, IV-3, V-3, V-6 and V-7). None of the parents of affected members showed any phenotypic abnormality. Family F had initially a little bit vision but lost completely at later stages. Their eyes were covered by deposits and had no vision. The PCG patients from this family also underwent surgery to remove deposits but these procedures failed to improve the vision. Further evaluations also showed the raised inter-ocular pressure in affected individuals of this family.

3.1.7 Family G

Family G was identified with blindness at earlier stages of life (Figure 3.7). The pedigree depicts four generations consisting of 12 members out of which 1 male (IV-3) and 1 female (IV-1) are affected. Both affected male and female are siblings with phenotypically normal parents showing autosomal recessive inheritance pattern. The patients from family G had glaucoma with bulging cornea, increased intraocular pressure and complete vision loss.

3.2 Mutation Analysis

The gene *CYP1B1*, cytogenetic location 2p22.2, was reported as acontributing factor for PCG (Stoilov et al. 1997). Therefore *CYP1B1*gene was sequenced by using gene specific primers in one affected individual from each family and analysis of sequenced data was carried out using BioEdit (Version 7.0.9.0).

3.2.1 Mutation analysis of Family A

Mutation analysis of affected member (V-4) of family A revealed heterozygous non-synonymous SNP (c.355G>T) in exon 2, predicting amino acid change P.Ala119Ser, showing no pathogenic sequence change (Figure 3.8). Another homozygous synonymous SNP (c.1347T>C) was observed in exon 3 of the same affected (V-4) individual. It also had no role in pathology of disease (Figure 3.9).

3.2.2 Mutation analysis of Family B

During sequence analysis of Family B (individual IV-11), we found a heterozygous non-synonymous SNP (c.515G>A) coding for amino acid change p.Ser172Asn (Figure 3.10). It was shown to be non-pathogenic variant by Polyphen-2, Provean, Mutation assessor and SIFT tools.In this family a novel disease causing missense mutation (c.353C>T) was also found in exon 2 predicting amino acid change p.Pro118Leu (Figure 3.11). The mutation was shown to be damaging by polyphen-2 and deleterious by Provean(Table 3.2).

3.2.3 Mutation analysis of Family C

In family C affected member IV-2 showed two homozygous non-synonymous variants (c.355G>T) (Figure 3.8) and (c.142C>G) (Figure 3.12) in exon 2. These changes results in amino acid substitutions p.Ala119ser and p.Arg48Gly, respectively but none of these variants was found pathogenic by bioinformatics tools.

We also found already known pathogenic mutation p.Glu229Lys corresponding to (c.685G>A) in exon 2 (Figure 3.13). The mutation was shown to be damaging by polyphen-2 result with score of 0.656, deleterious by provean with score of -8.405, Various restriction sites have been deleted including site for EarI, its impact is low according to mutation assessor. It is damaging according to SIFT. Presence of 41 homozygotes of p.Glu229Lys in normal population provides evidence of it being least likely a disease causing mutation.

3.2.3.1 Confirmation of prevalence in normal controls Via RFLP

In order to confirm prevalence ofp.Glu229Lys mutationin normal population we analysed 20 normal individuals with EarI restriction enzyme. The EarIrestriction site was deleted as a result of mutation. Wild type should have 2 bands, carriers should have 3 bands (i.e. 1 cut and 1 uncut) while mutants should have only 1 (uncut) band. Frequency of mutant allele among normal control population was 55% proving itsnon-pathogenic nature. The restriction analysis results are shown in table 3.1.

3.2.4 Mutation analysis of Family D

Mutation analysis of affected member of family D (III-4) revealed two homozygous non-synonymous variants (c.142C>G) resulting in amino acid change p.Arg48Gly (Fig 3.12) and (c.355G>T) depicting P.Ala119Ser (Figure 3.8). Both of these variants were benign according to polyphen-2 result, Neutral according to provean, their impact is low according to mutation assessor and are Tolerated according to SIFT.

We also found three SNPs in exon 3 i.e. a disease causing SNP (c.1059G>A) with no change on amino acid level (Figure 3.14), a homozygous synonymous SNP (c.1347T>C) having no impact on amino acid level (Figure 3.9), and a heterozygous non-synonymous SNP (c.1358A>G) corresponding to amino acid change p.Asn453Ser (Figure 3.15).

Mutation analysis of affected member (III-4)of family D also revealed a disease causing mutation p.Leu225Ile (c.673C>A) in exon 2 (Figure 3.17). This mutation was benign according to polyphen-2, Neutral according to provean, low impact according to mutation assessor and tolerated according to SIFT. It was shown to be a mutation according to mutation taster so we confirmed its segregation.

3.2.4.1 Segregation Analysis via RFLP

In order to check the segregation of (c.673C>Ain the entire family, restriction enzyme BlpIwas used. Segregation analysis in rest of individuals (affected and normal) of this family negates its segregation in the family.

3.2.5 Mutation analysis of Family E

Mutation analysis in affected member (V-5) revealed heterozygous non-synonymous SNP (c.515G>A) that results in amino acid change p.Ser172Asn in exon 2 (Figure 3.10). This variant was benign according to polyphen-2, Neutral according to provean,low impact according to mutation assessor and tolerated according to SIFT.

Two variants were also obtained in exon 3. One of which was homozygous synonymous (c.1347T>C) showing no amino acid change (Figure 3.9). Other variant was shown to be a disease causing variant (c.1059G>A) according to mutation taster (Figure 3.14). But it was proved to be a non-pathogenic variant by to polyphen-2 result, provean, mutation assessor and SIFT.

3.2.6 Mutation analysis of Family F

During sequence analysis of Family F, member (V-6) we found Heterozygous non-synonymous variant (c.515G>A) for amino acid change p.Ser172Asn in exon 2 was observed (Fig 3.10). This was the same variant which was observed in exon 2 of family E. It showed no pathogenic sequence change.

In exon 3 we found two Homozygous synonymous non-pathogenic variants (c.1347T>C) (Fig 3.9) and a variant (c.1059G>A) (Fig 3.14), and a known disease causing mutation (c.1169G>A) corresponding to amino acid change p.Arg390His.

The mutation was shown to be damaging by polyphen-2 result with score of 1, deleterious by provean with score of -4.85, Various restriction sites have been deleted including site for HhaI, its impact is very high according to mutation assessor with score of 4.295. It is damaging with ‘0’ score according to SIFT.

3.2.6.1 Segregation Analysis via RFLP

In order to confirm segregation of mutation p.Arg390His in other Family members we restricted other healthy and affected members of the family with restriction enzyme HhaI. Restriction site for HhaI is deleted as a result of mutation so wild type should have 2 bands, carriers should have 3 bands (i.e. 1 cut and 1 uncut) while mutants should have only 1 (uncut) band. RFLP results confirmed it to be a mutation that is segregating in the family is shown in figure 3.18.

3.2.7 Mutation analysis of Family G

Sequence analysis of family G did not show any mutation or SNP in either of exon 2 or exon 3.

Sanger sequencing in the probands revealed mutations in four families including one novel mutation c.353C>T predictingp.Pro118Leu. Three mutations c.685G>A predicting p.Glu229Lys, c.673C>A predicting p.Leu225Ile and c.1169G>A predicting p.(Arg390His) were found in three different families which have been previously reported as disease causing mutations in scientific literature. Pathogenicity analysis by bioinformatics web tools predicted all mutations to be disease causing. The frequencies of p.(Glu229Lys) and p.(Arg390His) was 0.01423 (41 homozygotes) and 0.0001522 respectively while p.(Leu225Ile) was absent in ExAC. List of Variants found in exon 2 and exon 3, and list of mutations are given in in table 3.2.

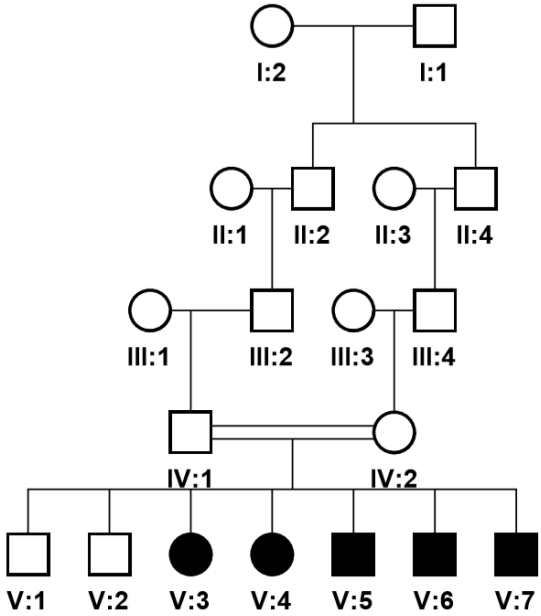

Figure 3.1 Family A: Five generation pedigree with 3 affected males (V-5, V-6 andV-7) and 2 affected females (V-3 and V-4).

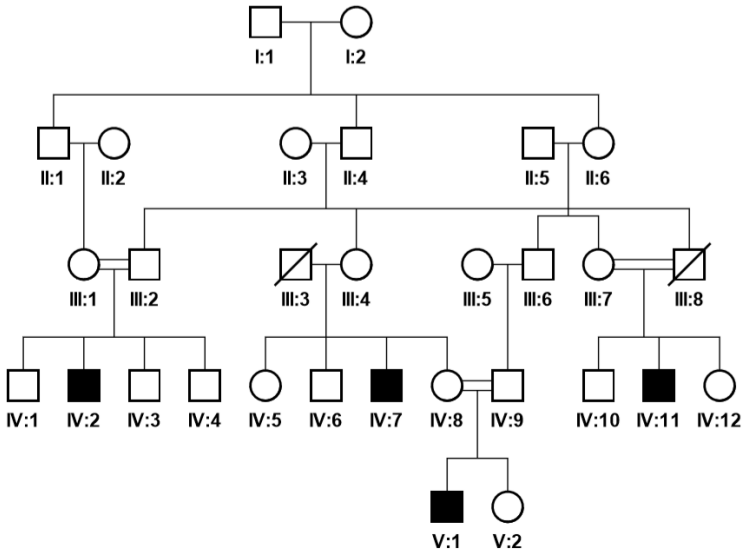

Figure 3.2 Family B: Five generation pedigree with 4 affected males (IV-2, IV-7, IV-11 and V1).

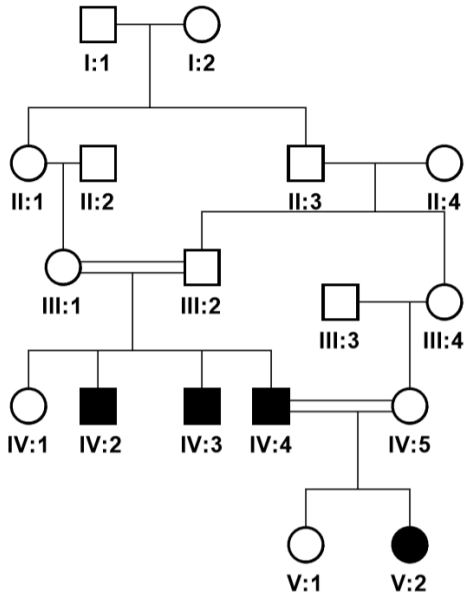

Figure 3.3 Family C: Five generation pedigree with 4 affected including 1 female (V-2) and 3 males (IV-2, IV-3 and IV-4).

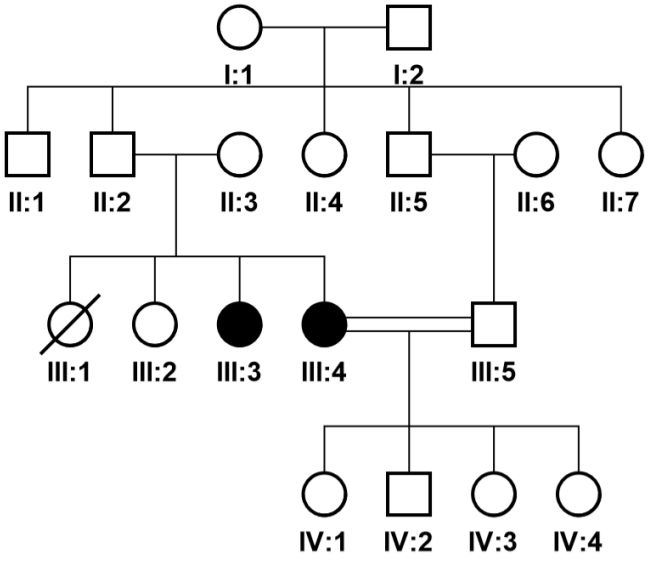

Figure 3.4 Family D: The four generation pedigree with two affected females (III-3 and III-4).

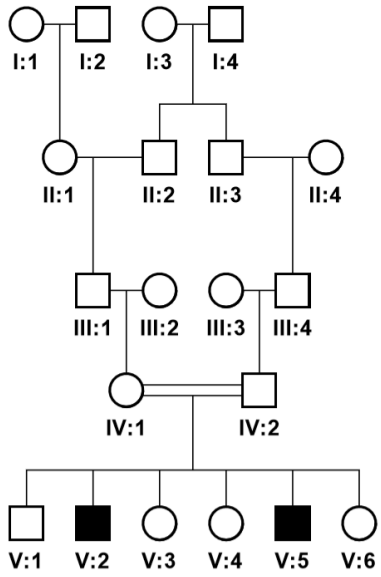

Figure 3.5 Family E: Five generations pedigree with two affected male members (V-2 and V-5).

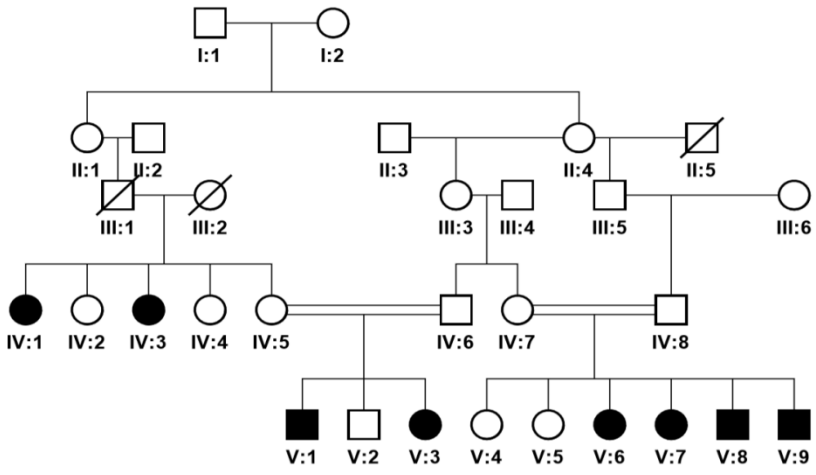

Figure 3.6 Family F: The five generation pedigree with 8 affected individuals.

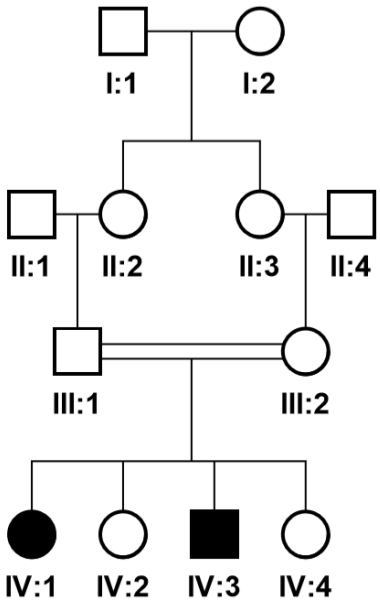

Figure 3.7 Family G: Four generation pedigree with two affected individuals.

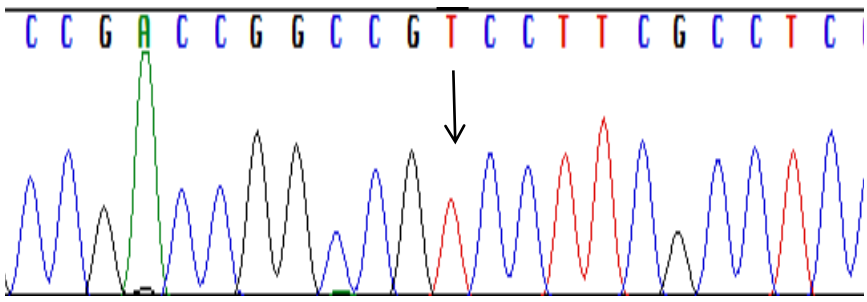

Figure 3.8 Sequence Chromatogram of Exon 2 of Family A, C, D. Arrow indicates the site of SNP (c.355G>T) P.Ala119Ser.

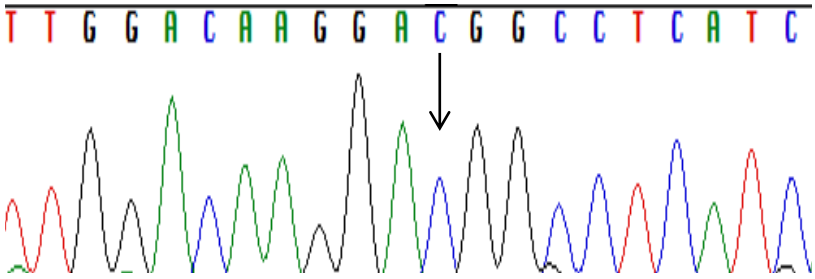

Figure 3.9 Sequence Chromatogram of Exon 3 of Family A, B, C, D and E. Arrow indicates the site of Variant (c.1347T>C).

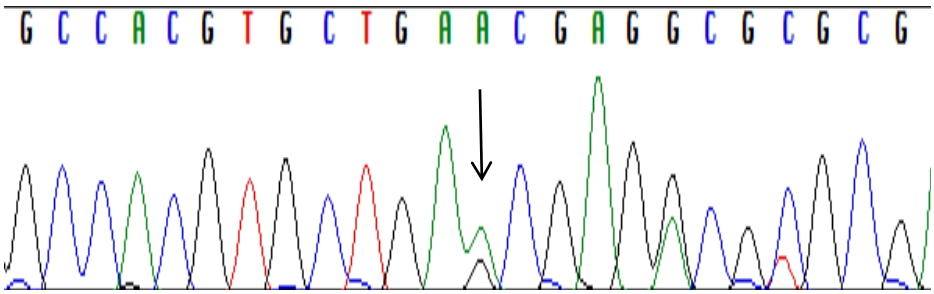

Figure 3.10 Sequence Chromatogram of Exon 2 of Family B. Arrow indicates the site of Heterozygous Variant (c.515G>A) p.Ser172Asn**.**

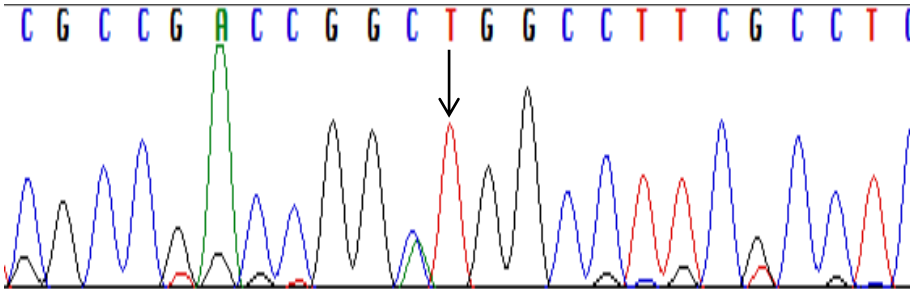

Figure 3.11 Sequence Chromatogram of Exon 2 of Family B. Arrow indicates the site of mutation p.Pro118Leu (c.353C>T).

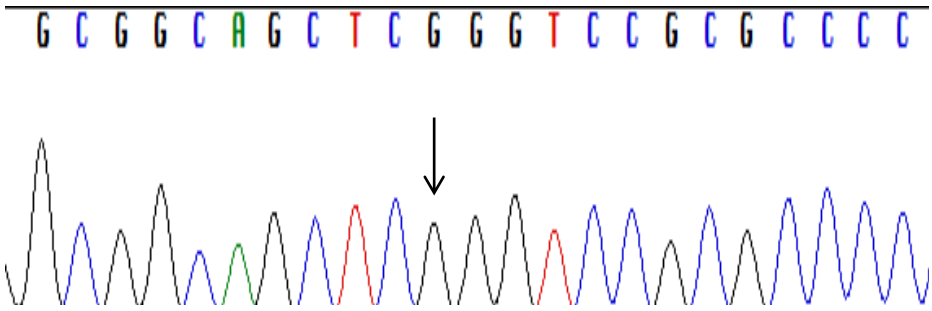

Figure 3.12 Sequence Chromatogram of Exon 2 of Family C, D. Arrow indicates the site of SNP (c.142C>G) p.Arg48Gly.

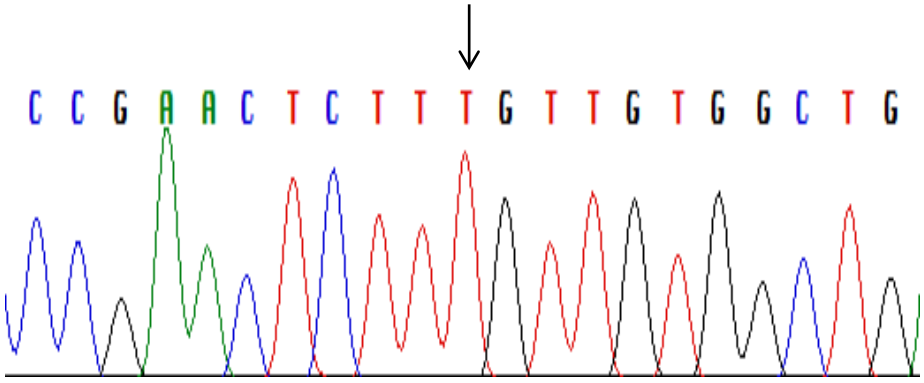

Figure 3.13 Sequence Chromatogram of Exon 2 of Family C with reverse primer. Arrow indicates the site of SNP p.Glu229Lys (c.685G>A).

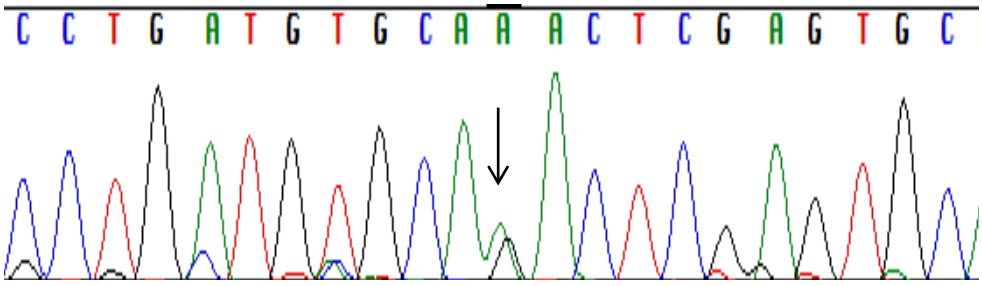

Figure 3.14 Sequence Chromatogram of Exon 3 of Family D. Arrow indicates the site of heterozygous SNP (c.1059G>A).

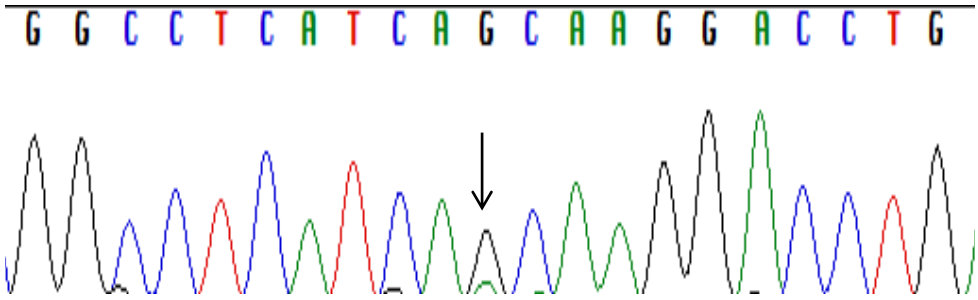

Figure 3.15 Sequence Chromatogram of Exon 3 of Family D. Arrow indicates the site of SNP (c.1358A>G) p.Asn453Ser.

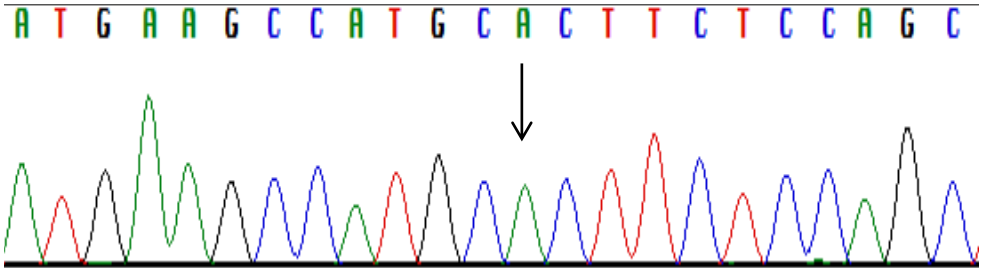

Figure 3.16 Sequence Chromatogram of Exon 3 of Family F. Arrow indicates the site of mutation p.Arg390His (c.1169G>A).

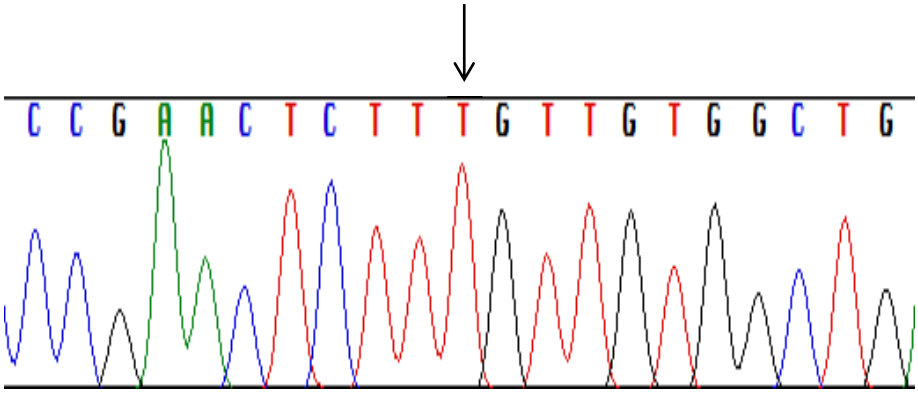

Figure 3.17 Sequence Chromatogram of Exon 3 of Family D with reverse primer. Arrow indicates the site of Novel Variant p.Leu225Ile(c.673C>A).

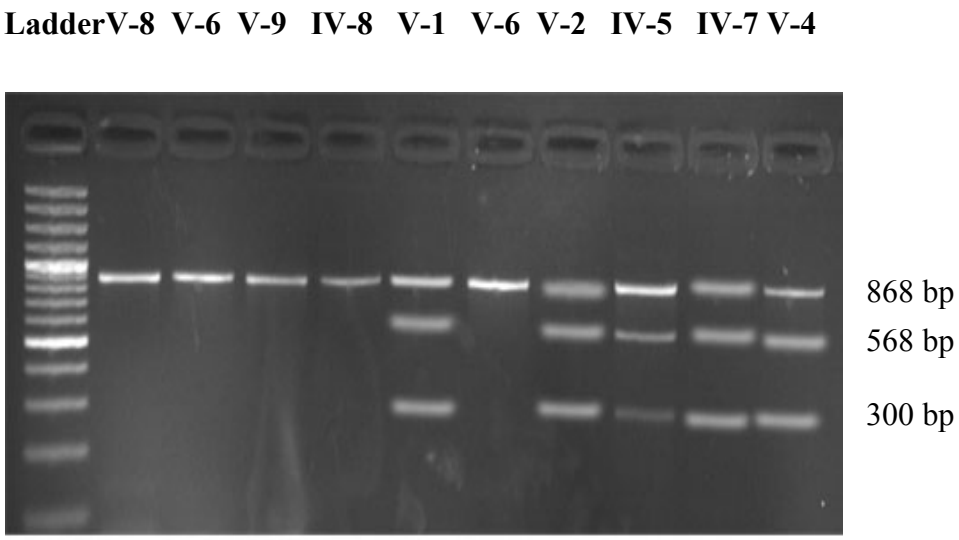

Fig 3.18 Results of RFLP of Family F

Table 3.1 Results of RFLP of Family C

Total number of Alleles	**Mutant Alleles**	**Wild type Alleles**	**Frequency of mutant Alleles**
40	22	18	55

Table3.2: List of Variants and Mutations in Exon 2 and 3 of *CYP1B1*.

Family	Exon	Hg19 coordinate	HGVSc	HGVSp	RS_ID	Mutation Taster	ExAC	PolyPhen-2	PROVEAN	Mutation assessor	SIFT
A,C,D	**2**	chr2:38302177C>A	c.355G>T	P.Ala119Ser	rs1056827	P	0.412	B	N	N	D
A,B,C,D,E,F	**3**	chr2:38298150A>G	c.1347T>C	no AA changes	rs1056837	P	0.622	NA	NA	NA	NA
B	**2**	chr2:38302179G>A	c.353C>T	**p.Pro118Leu**	rs73625096	DC	NA	PD	De	H	D
B,E,F	**2**	chr2:38302017C>T	c.515G>A	p.Ser172Asn	Not Found	P	NA	B	N	N	D
C	**2**	chr2:38301847C>T	c.685G>A	**p.Glu229Lys**	rs57865060	P	0.01423	D	NA	L	NA
C,D	**2**	chr2:38302390G>C	c.142C>G	p.Arg48Gly	rs10012	P	0.3777	B	N	N	T
D	**2**	chr2:38301859G>T	c.673C>A	**p.Leu225Ile**	Not Found	DC	NA	B	N	L	T
D,E,F	**3**	chr2:38298438C>T	c.1059G>A	**no AA changes**	Not Found	P	NA	NA	NA	NA	NA
E,F	**3**	chr2:38298139T>C	c.1358A>G	p.Asn453Ser	rs1800440	P	0.1547	PD	De	L	D
F	**3**	chr2:38298438C>T	c.1059G>A	no AA changes	Not Found	P	NA	NA	NA	NA	NA
F	**3**	chr2:38298328C>T	c.1169G>A	**p.Arg390His**	rs56010818	D	0.0001522	PD	De	H	D

HGVSc = Human Genome Variation Society cDNA Change; HGVSp = Human Genome Variant Society Protein Change; P = Polymorphism; B = Benign; N = Neutral; D = Damaging; NA = Not Applicable; DC = Disease Causing; PD = Probably Damaging; De = Deleterious; H = High; L = Low

DISCUSSION

Glaucoma is very vexing optic neuropathy having great heterogeneity which causes optical complications and if left untreated, can lead to irreversible vision loss(Shields *et al.,* 1996) .For prenatal and pre-symptomatic diagnosis of individual with risk of glaucoma, genetic screening is of great use. It is useful to find out basic underlying genes, biochemical and molecular pathways and proteins involved in this group of disorder, which is not very easy because of lack of accurate clinical diagnosis, primary vs. secondary disease and genetic heterogeneity (Allingham *et al.,* 2005).

Many glaucoma causing genes are still unidentified but still we try to find fundamental insights of this disease. Primary congenital glaucoma (PCG) is inherited commonly as autosomal recessive characteristic and is predominant in populations where cousin marriages are more common (Al-Hazmi *et al.,* 2005; Plášilová *et al.,* 1998; Zenteno *et al.,* 2008).

CYP1B1 is member of CYP450 super family. It contains 58 functional genes in human genome *and* is the most mutated gene in PCG(D. R. Nelson *et al.,* 2004).*CYP1B1* previous had been mapped to the 2p21-22 region, consists of three exons, of these 3 exons one exon is noncoding and two are coding(Stoilov *et al.,* 1998; Tang *et al.,* 1999). *CYP1B1* was considered as a candidate gene for PCG (GLC3A), and had been linked to the 2p21 region(Sarfarazi *et al.,* 1995). Mutations in *CYP1B1* affect functionally important and highly conserved regions, reducing its activity (Acharya *et al.,* 2006; Bagiyeva *et al.,* 2007; Chavarria-Soley *et al.,* 2008).

Frequency of *CYP1B1* mutations which cause PCG differs among different populations ranging from less than 10% to 100%(Zenteno *et al.,* 2008). There is great diversity of mutation continuum of *CYP1B1* gene studied in pathogenesis of PCG worldwide. PCG is recessive disorder and mostly homozygous mutations of *CYP1B1* are reported among patients(Stoilov *et al.,* 1998).

In current study *CYP1B1* gene of 7 PCG families was sequenced of which 2 families were solved with one homozygous novel mutation and one known homozygous missense mutations i.e. c.353C>T predicting p.Pro118Leu,c.1169G>A predicting p.Arg390His(Stoilov *et al.,* 1998) respectively. We negated c.685G>A predicting p.Glu229Lys to be a mutation and proved it to be a polymorphism as its frequency

was very high in normal control population. Although it has been published many time as a mutation in PCG and POAG patients (Michels-Rautenstrauss *et al.,* 2001).

We found a novel variant c.673C>A predicting p.(Leu225Ile) and six other variants including c.355G>T, Predicting (P.Ala119Ser),c.1347T>C having no effect on amino acid sequence, c.515G>A predicting (p.Ser172Asn), c.142C>G predicting (p.Arg48Gly), c.1358A>G predicting (p.Asn453Ser) and c.1059G>A having no effect on amino acid sequence.

A homozygous missense mutation at amino acid position 118, where proline is replaced by leucine, is identified in affected individuals of family B (Figure 3.2). Previously, proline to threonine substitution and proline to serine substitution at same point has been reported to be leading cause of PCG phenotype in patients (Chen *et al.,* 2008; A. Vincent *et al.,* 2006). These missense mutations altered evolutionarily conserved amino acids affecting substrate recognition site of *CYP1B1*. The replacement of proline with other amino acid is destined to impair the native protein structure and therefore affect the protein function.

The homozygous missense mutation p.Arg390His was supposed to interfere with some universal properties of the cytochrome P450 molecule like heme binding motif(Stoilov *et al.,* 1998). Extraordinary conservation of this motif specifies that it is vital for the usual function of P450 molecule (Stoilov *et al.,* 1998). The segregation of mutant allele of *CYP1B1* was seen to be consistent with autosomal recessive inheritance of the disease in the families being investigated.

Glutamic acid 229 is located in the surrounding area of the substrate-binding region (SBR) and is said to be the cause of conformational alterations in *CYP1B1*(Michels-Rautenstrauss *et al.,* 2001). Homozygous p.E229K has been reported as segregating with PCG in different populations including Pakistani population (Firasat *et al.,* 2008). Glutamic acid to lysine change at position 220 has been reported to be a cause of PCG in French, German, Indian, and Iranian patients(Chitsazian *et al.,* 2007). Compound heterozygous carriers of E229Kshowed no symptoms of PCG (Michels-Rautenstrauss *et al.,* 2001). We also found same mutation in our family (Family C). It was shown to be a variation according to mutation taster, but its frequency was very high in ExAC. Presence of 41 homozygotes of p.(Glu229Lys) in normal population provide evidence of it being least likely a disease causing mutation. We checked

frequency of this mutant allele in normal control population. For this we extracted and amplified DNA of normal controls and restricted them with the enzyme which had single restriction site in the site of mutation. As a result of restriction it was observed that frequency of mutant allele was very high in normal population disproving it to be a disease causing mutation. Our study proved it to be a polymorphism, not a mutation.

Eight coding sequence SNPs were also found in the *CYP1B1* gene (Table 3.2) including five variants in exon 2, while 3 variants were in exon 3. Among these variants we had a novel polymorphism c.673C>A predicting p.(Leu225Ile) in family D which was shown to be a mutation by mutation taster but it do not segregates in the family. We found heterozygous non-synonymous variant c.355G>T P.Ala119Ser in three families (A, C and D), homozygous synonymous variantc.1347T>C in families (A, B, C, D, E and F), heterozygous non-synonymous variant c.515G>A predicting p.Ser172Asn in families (B, E and F), Homozygous non-synonymous variant c.142C>G predicting p.Arg48Gly in families (C and D), heterozygous synonymous splice variant c.1059G>A in families (D, E and F), heterozygous non-synonymous variant c.1358A>G predicting p.Asn453Ser in families E and F) and heterozygous synonymous splice variant c.1059G>A in family (F) Table 3.2.

We report the identification of a novel and 02 previously reported mutations of *CYP1B1* gene in consanguineous Pakistani families affected with PCG. The presence of 41 homozygotes of p.Glu229Lysvariant in normal population provides evidence of it being least likely a disease causing mutation. Our data specify that mutations identified in this study possibly interfere with the heme-binding ability of the CYP1B1 molecule by either deleting the heme-binding region or by affecting discrete components of hinge region and the CCS, which are determinants for appropriate heme-binding and also folding ability of the cytochrome P450 molecules.

Glaucoma is irreversible vision loss and there is no treatment of glaucoma so far. Molecular diagnostics for prognostic testing and early intervention is vital to reduce the influence of visual damage and blindness(Wadhwa & Higginbotham, 2005). For this, there is need to characterize the subtypes of glaucoma at molecular level and to identify unknown genes/ loci causative for this ocular disease among different population. Present study was conducted to understand the genetic basis of glaucoma in Pakistani families.

Precise role of *CYP1B1* gene is still not very clear, but some of the in-vivo and in-vitro studies have shown its involvement in the development based on the retinoic acid signalling and 17bestradiol formation (Vasiliou & Gonzalez, 2008). Studies also revealed angle dysgenesis in PCG patients with *CYP1B1* mutations(Hollander *et al.,* 2006).

During this study, identification of 1 novel, 2 known missense *CYP1B1* mutations and 8 SNPs in consanguineous Pakistani families affected with PCG confirms varied allelic heterogeneity of the gene*CYP1B1* in pathogenesis of congenital glaucoma. In future this study will further help to elucidate structural and functional relationship of *CYP1B1* protein and will aid in better management of glaucoma.

REFERENCES

Abu-Amero, K. K., Osman, E. A., Mousa, A., Wheeler, J., Whigham, B., Allingham, R., . . . Al-Obeidan, S. A. (2011). Screening of CYP1B1 and LTBP2 genes in Saudi families with primary congenital glaucoma: genotype-phenotype correlation.

Acharya, M., Mookherjee, S., Bhattacharjee, A., Bandyopadhyay, A. K., Daulat Thakur, S., Bhaduri, G., . . . Ray, K. (2006). Primary role of CYP1B1 in Indian juvenile-onset POAG patients. *Mol Vis, 12*(1), 399-404.

Akarsu, A. N., Turacli, M. E., Aktan, S. G., Barsoum-Homsy, M., Chevrette, L., Sayli, B. S., & Sarfarazi, M. (1996). A second locus (GLC3B) for primary congenital glaucoma (Buphthalmos) maps to the 1p36 region. *Human molecular genetics, 5*(8), 1199-1203.

Al-Hazmi, A., Awad, A., Zwaan, J., Al-Mesfer, S., Al-Jadaan, I., & Al-Mohammed, A. (2005). Correlation between surgical success rate and severity of congenital glaucoma. *British Journal of Ophthalmology, 89*(4), 449-453.

AlFadhli, S., Behbehani, A., Elshafey, A., Abdelmoaty, S., & Al-Awadi, S. (2006). Molecular and clinical evaluation of primary congenital glaucoma in Kuwait. *American journal of ophthalmology, 141*(3), 512-516.

Ali, M., McKibbin, M., Booth, A., Parry, D. A., Jain, P., Riazuddin, S. A., . . . Shires, M. (2009). Null mutations in LTBP2 cause primary congenital glaucoma. *The American Journal of Human Genetics, 84*(5), 664-671.

Allingham, R. R., Damji, K. F., Freedman, S. F., Moroi, S. E., Rhee, D. J., & Shields, M. B. (2012). *Shields textbook of glaucoma*: Lippincott Williams & Wilkins.

Allingham, R. R., Wiggs, J. L., Hauser, E. R., Larocque-Abramson, K. R., Santiago-Turla, C., Broomer, B., . . . Pericak-Vance, M. A. (2005). Early adult-onset POAG linked to 15q11-13 using ordered subset analysis. *Investigative ophthalmology & visual science, 46*(6), 2002-2005.

Anderson, D. R. (1981). The development of the trabecular meshwork and its abnormality in primary infantile glaucoma. *Transactions of the American Ophthalmological Society, 79*, 458.

Anderson, D. R. (1983). Primary infantile glaucoma (congenital glaucoma). *Survey of ophthalmology, 28*(1), 1-19.

Anderson, D. R. (2003). Collaborative normal tension glaucoma study. *Current opinion in ophthalmology, 14*(2), 86-90.

Badeeb, O. M., Micheal, S., Koenekoop, R. K., den Hollander, A. I., & Hedrawi, M. T. (2014). CYP1B1 mutations in patients with primary congenital glaucoma from Saudi Arabia. *BMC medical genetics, 15*(1), 1.

Bagga, H., Feuer, W. J., & Greenfield, D. S. (2006). Detection of psychophysical and structural injury in eyes with glaucomatous optic neuropathy and normal standard automated perimetry. *Archives of ophthalmology, 124*(2), 169-176.

Bagiyeva, S., Marfany, G., Gonzalez-Angulo, O., & Gonzalez-Duarte, R. (2007). Mutational screening of CYP1B1 in Turkish PCG families and functional analyses of newly detected mutations. *Mol Vis, 13*, 1458-1468.

Barker, E. M. (2002). *Neuroscience nursing: A spectrum of care*: Mosby Incorporated.

Beck, A. D. (2001). Diagnosis and management of pediatric glaucoma. *Ophthalmology Clinics, 14*(3), 501-512.

Bejjani, B. A., Lewis, R. A., Tomey, K. F., Anderson, K. L., Dueker, D. K., Jabak, M., . . . Lupski, J. R. (1998). Mutations in CYP1B1, the gene for cytochrome P4501B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia. *The American Journal of Human Genetics, 62*(2), 325-333.

Bejjani, B. A., Stockton, D. W., Lewis, R. A., Tomey, K. F., Dueker, D. K., Jabak, M., . . . Lupski, J. R. (2000). Multiple CYP1B1 mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest frequent de novo events and a dominant modifier locus. *Human molecular genetics, 9*(3), 367-374.

Bejjani, B. A., Xu, L., Armstrong, D., Lupski, J. R., & Reneker, L. W. (2002). Expression patterns of cytochrome P4501B1 (Cyp1b1) in FVB/N mouse eyes. *Experimental eye research, 75*(3), 249-257.

Burgoyne, C. F. (2011). A biomechanical paradigm for axonal insult within the optic nerve head in aging and glaucoma. *Experimental eye research, 93*(2), 120-132.

Campos-Mollo, E., López-Garrido, M.-P., Blanco-Marchite, C., Garcia-Feijoo, J., Peralta, J., Belmonte-Martínez, J., . . . Escribano, J. (2009). CYP1B1 mutations in Spanish patients with primary congenital glaucoma: phenotypic and functional variability.

Caprioli, J., & Coleman, A. L. (2008). Intraocular pressure fluctuation: a risk factor for visual field progression at low intraocular pressures in the Advanced Glaucoma Intervention Study. *Ophthalmology, 115*(7), 1123-1129. e1123.

Chakrabarti, S., Kaur, K., Kaur, I., Mandal, A. K., Parikh, R. S., Thomas, R., & Majumder, P. P. (2006). Globally, CYP1B1 mutations in primary congenital glaucoma are strongly structured by geographic and haplotype backgrounds. *Investigative ophthalmology & visual science, 47*(1), 43-47.

Chakrabarti, S., Kaur, K., Komatireddy, S., Acharya, M., Devi, K. R., Mukhopadhyay, A., . . . Thomas, R. (2005). Gln48His is the prevalent myocilin mutation in primary open angle and primary congenital glaucoma phenotypes in India.

Chauhan, B. C., Garway-Heath, D. F., Goñi, F. J., Rossetti, L., Bengtsson, B., Viswanathan, A. C., & Heijl, A. (2008). Practical recommendations for measuring rates of visual field change in glaucoma. *British Journal of Ophthalmology, 92*(4), 569-573.

Chavarria-Soley, G., Michels-Rautenstrauss, K., Caliebe, A., Kautza, M., Mardin, C., & Rautenstrauss, B. (2006). Novel CYP1B1 and known PAX6 mutations in anterior segment dysgenesis (ASD). *Journal of glaucoma, 15*(6), 499-504.

Chavarria-Soley, G., Sticht, H., Aklillu, E., Ingelman-Sundberg, M., Pasutto, F., Reis, A., & Rautenstrauss, B. (2008). Mutations in CYP1B1 cause primary

congenital glaucoma by reduction of either activity or abundance of the enzyme. *Human mutation, 29*(9), 1147-1153.

Chen, Y., Jiang, D., Yu, L., Katz, B., Zhang, K., Wan, B., & Sun, X. (2008). CYP1B1 and MYOC mutations in 116 Chinese patients with primary congenital glaucoma. *Archives of ophthalmology, 126*(10), 1443-1447.

Chitsazian, F., Tusi, B. K., Elahi, E., Saroei, H. A., Sanati, M. H., Yazdani, S., . . . Mehrjerdi, M. A. Z. (2007). CYP1B1 mutation profile of Iranian primary congenital glaucoma patients and associated haplotypes. *The Journal of Molecular Diagnostics, 9*(3), 382-393.

Choudhary, D., Jansson, I., Sarfarazi, M., & Schenkman, J. (2006). Physiological significance and expression of P450s in the developing eye. *Drug metabolism reviews, 38*(1-2), 337-352.

Choudhary, D., Jansson, I., Sarfarazi, M., & Schenkman, J. B. (2008). Characterization of the biochemical and structural phenotypes of four CYP1B1 mutations observed in individuals with primary congenital glaucoma. *Pharmacogenetics and genomics, 18*(8), 665-676.

Choudhary, D., Jansson, I., & Schenkman, J. (2009). CYP1B1, a developmental gene with a potential role in glaucoma therapy. *Xenobiotica, 39*(8), 606-615.

Colomb, E., Kaplan, J., & Garchon, H. J. (2003). Novel cytochrome P450 1B1 (CYP1B1) mutations in patients with primary congenital glaucoma in France. *Human mutation, 22*(6), 496-496.

Cook, C., & Foster, P. (2012). Epidemiology of glaucoma: what's new? *Canadian Journal of Ophthalmology/Journal Canadien d'Ophtalmologie, 47*(3), 223-226.

Cunliffe, H., McNoe, L., Ward, T., Devriendt, K., Brunner, H., & Eccles, M. (1998). The prevalence of PAX2 mutations in patients with isolated colobomas or colobomas associated with urogenital anomalies. *Journal of medical genetics, 35*(10), 806-812.

Dawczynski, J., Koenigsdoerffer, E., Augsten, R., & Strobel, J. (2007). Anterior segment optical coherence tomography for evaluation of changes in anterior chamber angle and depth after intraocular lens implantation in eyes with glaucoma. *European journal of ophthalmology, 17*(3), 363-367.

Dimasi, D., Hewitt, A., Straga, T., Pater, J., MacKinnon, J. R., Elder, J., . . . Craig, J. (2007). Prevalence of CYP1B1 mutations in Australian patients with primary congenital glaucoma. *Clinical genetics, 72*(3), 255-260.

Elolia, R., & Stokes, J. (1998). Monograph series on aging-related diseases: XI. Glaucoma. *Chronic Diseases and Injuries in Canada, 19*(4), 157.

Firasat, S., Riazuddin, S., Hejtmancik, J. F., & Riazuddin, S. (2008). Primary congenital glaucoma localizes to chromosome 14q24. 2-24.3 in two consanguineous Pakistani families.

François, J. (1981). Genetic predisposition to glaucoma *Current Genetic, Clinical and Morphologic Problems* (pp. 1-5): Karger Publishers.

Gatzioufas, Z., Labiris, G., Stachs, O., Hovakimyan, M., Schnaidt, A., Viestenz, A., . . . Seitz, B. (2013). Biomechanical profile of the cornea in primary congenital glaucoma. *Acta ophthalmologica, 91*(1), e29-e34.

Girgis, N. M., & Frantz, K. A. (2007). A case of primary congenital glaucoma: A diagnostic dilemma. *Optometry-Journal of the American Optometric Association, 78*(4), 167-175.

Gong, G., Kosoko-Lasaki, O., Haynatzki, G. R., & Wilson, M. R. (2004). Genetic dissection of myocilin glaucoma. *Human molecular genetics, 13*(suppl 1), R91-R102.

Gould, D. B., & John, S. W. (2002). Anterior segment dysgenesis and the developmental glaucomas are complex traits. *Human molecular genetics, 11*(10), 1185-1193.

Guo, L., Moss, S. E., Alexander, R. A., Ali, R. R., Fitzke, F. W., & Cordeiro, M. F. (2005). Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix. *Investigative ophthalmology & visual science, 46*(1), 175-182.

Guo, L., Salt, T. E., Luong, V., Wood, N., Cheung, W., Maass, A., . . . Cheetham, M. E. (2007). Targeting amyloid-β in glaucoma treatment. *Proceedings of the National Academy of Sciences, 104*(33), 13444-13449.

Gupta, D. (2005). *Glaucoma diagnosis and management*: Lippincott Williams & Wilkins.

He, M., Foster, P. J., Ge, J., Huang, W., Zheng, Y., Friedman, D. S., . . . Khaw, P. T. (2006). Prevalence and clinical characteristics of glaucoma in adult Chinese: a population-based study in Liwan District, Guangzhou. *Investigative ophthalmology & visual science, 47*(7), 2782-2788.

Hogewind, B. F., Gaplovska-Kysela, K., Theelen, T., Cremers, F. P., Yam, G., Hoyng, C. B., & Mukhopadhyay, A. (2007). Identification and functional characterization of a novel MYOC mutation in two primary open angle glaucoma families from The Netherlands.

Hollander, D. A., Sarfarazi, M., Stoilov, I., Wood, I. S., Fredrick, D. R., & Alvarado, J. A. (2006). Genotype and phenotype correlations in congenital glaucoma: CYP1B1 mutations, goniodysgenesis, and clinical characteristics. *American journal of ophthalmology, 142*(6), 993-1004. e1002.

Johnson, T. V., & Tomarev, S. I. (2016). Animal Models of Glaucoma *Animal Models of Ophthalmic Diseases* (pp. 31-50): Springer.

Khan, A. O. (2011). Conditions that can be mistaken as early childhood glaucoma. *Ophthalmic genetics, 32*(3), 129-137.

Klein, B. E., Klein, R., Lee, K. E., & Hoyer, C. J. (2006). Does the intraocular pressure effect on optic disc cupping differ by age? *Transactions of the American Ophthalmological Society, 104*, 143.

Kumar, A., Basavaraj, M. G., Gupta, S. K., Qamar, I., Ali, A. M., Bajaj, V., . . . Dorairaj, S. K. (2007). Role of CYP1B1, MYOC, OPTN, and OPTC genes in adult-onset primary open-angle glaucoma: predominance of CYP1B1 mutations in Indian patients. *Mol Vis, 13*, 667-676.

Kumar, D. M., Simpkins, J. W., & Agarwal, N. (2008). Estrogens and neuroprotection in retinal diseases. *Molecular vision, 14*, 1480.

Kumar, M., Tanwar, M., Faiq, M. A., Pani, J., Shamsi, M. B., Dada, T., & Dada, R. (2013). Mitochondrial DNA nucleotide changes in primary congenital glaucoma patients. *Molecular vision, 19*, 220.

Kumar, V., Abbas, A. K., Fausto, N., & Aster, J. C. (2014). *Robbins and Cotran pathologic basis of disease*: Elsevier Health Sciences.

Li, H., Leung, C. K. S., Cheung, C. Y. L., Wong, L., Pang, C. P., Weinreb, R. N., & Lam, D. S. C. (2007). Repeatability and reproducibility of anterior chamber angle measurement with anterior segment optical coherence tomography. *British Journal of Ophthalmology, 91*(11), 1490-1492.

Libby, R. T., Anderson, M. G., Pang, I.-h., Robinson, Z. H., Savinova, O. V., Cosma, I. M., . . . Clark, A. F. (2005). Inherited glaucoma in DBA/2J mice: pertinent disease features for studying the neurodegeneration. *Visual neuroscience, 22*(05), 637-648.

Libby, R. T., Smith, R. S., Savinova, O. V., Zabaleta, A., Martin, J. E., Gonzalez, F. J., & John, S. W. (2003). Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science, 299*(5612), 1578-1581.

Lim, S.-H., Tran-Viet, K.-N., Yanovitch, T. L., Freedman, S. F., Klemm, T., Call, W., . . . Nading, E. B. (2013). CYP1B1, MYOC, and LTBP2 mutations in primary congenital glaucoma patients in the United States. *American journal of ophthalmology, 155*(3), 508-517. e505.

Liu, Y., & Allingham, R. R. (2011). Molecular genetics in glaucoma. *Experimental eye research, 93*(4), 331-339.

López-Garrido, M.-P., Medina-Trillo, C., Morales-Fernandez, L., Garcia-Feijoo, J., Martínez-de-la-Casa, J.-M., García-Antón, M., & Escribano, J. (2013). Null CYP1B1 genotypes in primary congenital and nondominant juvenile glaucoma. *Ophthalmology, 120*(4), 716-723.

López-Garrido, M.-P., Sánchez-Sánchez, F., López-Martínez, F., Aroca-Aguilar, J.-D., Blanco-Marchite, C., Coca-Prados, M., & Escribano, J. (2006). Heterozygous CYP1B1 gene mutations in Spanish patients with primary open-angle glaucoma. *Mol Vis, 12*(1), 748-755.

Lütjen-Drecoll, E., B'Ann, T. G., Tian, B., & Kaufman, P. L. (2001). Outflow of aqueous humor. *Journal of glaucoma, 10*(5), S42-S44.

Martus, P., Korth, M., Horn, F., Jünemann, A., Wisse, M., & Jonas, J. B. (1998). A multivariate sensory model in glaucoma diagnosis. *Investigative ophthalmology & visual science, 39*(9), 1567-1574.

Mashima, Y., Suzuki, Y., Sergeev, Y., Ohtake, Y., Tanino, T., Kimura, I., . . . Inatani, M. (2001). Novel cytochrome P4501B1 (CYP1B1) gene mutations in Japanese patients with primary congenital glaucoma. *Investigative ophthalmology & visual science, 42*(10), 2211-2216.

McMenamin, P. G. (1991). A quantitative study of the prenatal development of the aqueous outflow system in the human eye. *Experimental eye research, 53*(4), 507-517.

Melki, R., Colomb, E., Lefort, N., Brezin, A., & Garchon, H. (2004). CYP1B1 mutations in French patients with early-onset primary open-angle glaucoma. *Journal of medical genetics, 41*(9), 647-651.

Michels-Rautenstrauss, K. G., Mardin, C. Y., Zenker, M., Jordan, N., Gusek-Schneider, G.-C., & Rautenstrauss, B. W. (2001). Primary congenital glaucoma: three case reports on novel mutations and combinations of mutations in the GLC3A (CYP1B1) gene. *Journal of glaucoma, 10*(4), 354-357.

Michels-Rautenstrauss, K., Mardin, C., Wakili, N., Jünemann, A. M., Villalobos, L., Mejia, C., . . . Naumann, G. O. (2002). Novel mutations in the MYOC/GLC1A gene in a large group of glaucoma patients. *Human mutation, 20*(6), 479-480.

Monemi, S., Spaeth, G., DaSilva, A., Popinchalk, S., Ilitchev, E., Liebmann, J., . . . Child, A. (2005). Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22. 1. *Human molecular genetics, 14*(6), 725-733.

Mozaffarieh, M., Grieshaber, M. C., & Flammer, J. (2008). Oxygen and blood flow: players in the pathogenesis of glaucoma. *Mol Vis, 14*, 224-233.

Nelson, D. R., Zeldin, D. C., Hoffman, S. M., Maltais, L. J., Wain, H. M., & Nebert, D. W. (2004). Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics and genomics, 14*(1), 1-18.

Nelson, L. B., Spaeth, G. L., Nowinski, T. S., Margo, C. E., & Jackson, L. (1984). Aniridia. A review. *Survey of ophthalmology, 28*(6), 621-642.

Neri, P., Azuara-Blanco, A., & Forrester, J. V. (2004). Incidence of glaucoma in patients with uveitis. *Journal of glaucoma, 13*(6), 461-465.

Pang, C. P., Fan, B. J., Canlas, O., Wang, D. Y., Dubois, S., Tam, P., . . . Ritch, R. (2006). A genome-wide scan maps a novel juvenile-onset primary open angle glaucoma locus to chromosome 5q. *Mol Vis, 12*, 85-92.

Pang, I.-H., & Clark, A. F. (2002). Advances in glaucoma therapeutics. *Expert opinion on emerging drugs, 7*(1), 141-163.

Papadopoulos, M., Cable, N., Rahi, J., & Khaw, P. T. (2007). The British infantile and childhood glaucoma (BIG) eye study. *Investigative ophthalmology & visual science, 48*(9), 4100-4106.

Pensiero, S., Da Pozzo, S., Perissutti, P., Cavallini, G. M., & Guerra, R. (1992). Normal intraocular pressure in children. *Journal of pediatric ophthalmology and strabismus, 29*(2), 79-84.

Plášilová, M., Feráková, E., Kádasi, L., Poláková, H., Gerinec, A., Ott, J., . . . Ferák, V. (1998). Linkage of autosomal recessive primary congenital glaucoma to the

GLC3A locus in Roms (Gypsies) from Slovakia. *Human heredity, 48*(1), 30-33.

Quigley, H. A., Addicks, E. M., Green, W. R., & Maumenee, A. (1981). Optic nerve damage in human glaucoma: II. The site of injury and susceptibility to damage. *Archives of ophthalmology, 99*(4), 635-649.

Ramírez, J. M., Ramírez, A. I., Salazar, J. J., Rojas, B., De Hoz, R., & Triviño, A. (2005). Schlemm's canal and the collector channels at different developmental stages in the human eye. *Cells Tissues Organs, 178*(3), 180-185.

Reddy, A., Kaur, K., Mandal, A. K., Panicker, S. G., Thomas, R., Hasnain, S. E., . . . Chakrabarti, S. (2004). Mutation spectrum of the CYP1B1 gene in Indian primary congenital glaucoma patients. *Mol Vis, 10*, 696-702.

Reddy, A. B., Panicker, S. G., Mandal, A. K., Hasnain, S. E., & Balasubramanian, D. (2003). Identification of R368H as a predominant CYP1B1 allele causing primary congenital glaucoma in Indian patients. *Investigative ophthalmology & visual science, 44*(10), 4200-4203.

Rezaie, T., Child, A., Hitchings, R., Brice, G., Miller, L., Coca-Prados, M., . . . Kreutzer, D. (2002). Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science, 295*(5557), 1077-1079.

Ritch, R. (2008). The management of exfoliative glaucoma. *Progress in brain research, 173*, 211-224.

Ritch, R., & Schlötzer-Schrehardt, U. (2001). Exfoliation (pseudoexfoliation) syndrome: toward a new understanding. *Acta Ophthalmologica Scandinavica, 79*(2), 213-217.

Rummelt, V., & Naumann, G. O. (1997). Cystic epithelial ingrowth after goniotomy for congenital glaucoma: a clinicopathologic report. *Journal of glaucoma, 6*(6), 353-356.

Samant, M., Chauhan, B. K., Lathrop, K. L., & Nischal, K. K. (2016). Congenital aniridia: etiology, manifestations and management. *Expert Review of Ophthalmology, 11*(2), 135-144.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning* (Vol. 2): Cold spring harbor laboratory press New York.

Sarfarazi, M. (1997). Recent advances in molecular genetics of glaucomas. *Human molecular genetics, 6*(10), 1667-1677.

Sarfarazi, M., Akarsu, N. A., Hossain, A., Turacli, E. M., Aktan, G. S., Barsoum-Homsy, M., . . . Sayli, S. B. (1995). Assignment of a locus (GLC3A) for primary congenital glaucoma (Buphthalmos) to 2p21 and evidence for genetic heterogeneity. *Genomics, 30*(2), 171-177.

Sarfarazi, M., Stoilov, I., & Schenkman, J. B. (2003). Genetics and biochemistry of primary congenital glaucoma. *Ophthalmology Clinics, 16*(4), 543-554.

Saw, S., Tong, L., Chia, K., Koh, D., Lee, Y., Katz, J., & Tan, D. (2004). The relation between birth size and the results of refractive error and biometry measurements in children. *British Journal of Ophthalmology, 88*(4), 538-542.

Schwartz, M., & Yoles, E. (2000). Neuroprotection: a new treatment modality for glaucoma? *Current opinion in ophthalmology, 11*(2), 107-111.

Shakya, S., & Gupta, H. R. (2006). Angle closure glaucoma: a cause for bilateral visual threat. *Nepal Med Coll J, 8*(3), 153-155.

Sharafieh, R., Child, A. H., Khaw, P. T., Fleck, B., & Sarfarazi, M. (2013). LTBP2 gene analysis in the GLC3C-linked family and 94 CYP1B1-negative cases with primary congenital glaucoma. *Ophthalmic genetics, 34*(1-2), 14-20.

Shields, J., Varley, R., Broks, P., & Simpson, A. (1996). Social cognition in developmental language disorders and high-level autism. *Developmental Medicine & Child Neurology, 38*(6), 487-495.

Sihota, R., Sony, P., Gupta, V., Dada, T., & Singh, R. (2006). Diagnostic capability of optical coherence tomography in evaluating the degree of glaucomatous retinal nerve fiber damage. *Investigative ophthalmology & visual science, 47*(5).

Sommer, A., Tielsch, J. M., Katz, J., Quigley, H. A., Gottsch, J. D., Javitt, J. C., . . . Ezrine, S. (1991). Racial differences in the cause-specific prevalence of blindness in east Baltimore. *New England Journal of Medicine, 325*(20), 1412-1417.

Sowden, J. (2007). Molecular and developmental mechanisms of anterior segment dysgenesis. *Eye, 21*(10), 1310-1318.

Stoilov, I., Akarsu, A. N., Alozie, I., Child, A., Barsoum-Homsy, M., Turacli, M. E., . . . Brice, G. (1998). Sequence analysis and homology modeling suggest that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. *The American Journal of Human Genetics, 62*(3), 573-584.

Stoilov, I., Akarsu, A. N., & Sarfarazi, M. (1997). Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Human molecular genetics, 6*(4), 641-647.

Stone, E. M., Fingert, J. H., Alward, W. L., Nguyen, T. D., Polansky, J. R., Sunden, S. L., . . . Nichols, B. E. (1997). Identification of a gene that causes primary open angle glaucoma. *Science, 275*(5300), 668-670.

Suri, F., Yazdani, S., Narooie-Nejhad, M., Zargar, S. J., Paylakhi, S. H., Zeinali, S., . . . Elahi, E. (2009). Variable expressivity and high penetrance of CYP1B1 mutations associated with primary congenital glaucoma. *Ophthalmology, 116*(11), 2101-2109.

Sutter, T. R., Tang, Y. M., Hayes, C. L., Wo, Y., Jabs, E. W., Li, X., . . . Greenlee, W. F. (1994). Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *Journal of Biological Chemistry, 269*(18), 13092-13099.

Takahashi, H., Noda, S., Mashima, Y., Kubota, R., Ohtake, Y., Tanino, T., . . . Shimizu, N. (2000). The myocilin (MYOC) gene expression in the human trabecular meshwork. *Current eye research, 20*(2), 81-84.

Tamm, E. R. (2009). The trabecular meshwork outflow pathways: structural and functional aspects. *Experimental eye research, 88*(4), 648-655.

Tang, Y. M., Chen, G.-F., Thompson, P. A., Lin, D.-X., Lang, N. P., & Kadlubar, F. F. (1999). Development of an antipeptide antibody that binds to the C-terminal region of human CYP1B1. *Drug metabolism and disposition, 27*(2), 274-280.

Tanwar, M., Dada, T., Sihota, R., Das, T. K., Yadav, U., & Dada, R. (2009). Mutation spectrum of CYP1B1 in North Indian congenital glaucoma patients.

Tham, Y.-C., Li, X., Wong, T. Y., Quigley, H. A., Aung, T., & Cheng, C.-Y. (2014). Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis. *Ophthalmology, 121*(11), 2081-2090.

Thiele, H., & Nürnberg, P. (2005). HaploPainter: a tool for drawing pedigrees with complex haplotypes. *Bioinformatics, 21*(8), 1730-1732.

Tokizane, T., Shiina, H., Igawa, M., Enokida, H., Urakami, S., Kawakami, T., . . . Tanaka, Y. (2005). Cytochrome P450 1B1 is overexpressed and regulated by hypomethylation in prostate cancer. *Clinical cancer research, 11*(16), 5793-5801.

Tsuchiya, Y., Nakajima, M., & Yokoi, T. (2005). Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer letters, 227*(2), 115-124.

Vasiliou, V., & Gonzalez, F. J. (2008). Role of CYP1B1 in Glaucoma*. *Annu. Rev. Pharmacol. Toxicol., 48*, 333-358.

Vincent, A., Billingsley, G., Priston, M., Glaser, T., Oliver, E., Walter, M., . . . Héon, E. (2006). Further support of the role of CYP1B1 in patients with Peters anomaly. *Mol Vis, 12*(1), 506-510.

Vincent, A. L., Billingsley, G., Buys, Y., Levin, A. V., Priston, M., Trope, G., . . . Héon, E. (2002). Digenic inheritance of early-onset glaucoma: CYP1B1, a potential modifier gene. *The American Journal of Human Genetics, 70*(2), 448-460.

Wadhwa, S. D., & Higginbotham, E. J. (2005). Ethnic differences in glaucoma: prevalence, management, and outcome. *Current opinion in ophthalmology, 16*(2), 101-106.

Walton, D. S., Nagao, K., Yeung, H. H., & Kane, S. A. (2013). Late-recognized primary congenital glaucoma. *Journal of pediatric ophthalmology and strabismus, 50*(4), 234-238.

Wang, D. Y., Fan, B. J., Chua, J. K., Tam, P. O., Leung, C. K., Lam, D. S., & Pang, C. P. (2006). A genome-wide scan maps a novel juvenile-onset primary open-angle glaucoma locus to 15q. *Investigative ophthalmology & visual science, 47*(12), 5315-5321.

Weatherill, J., & Hart, C. (1969). Familial hypoplasia of the iris stroma associated with glaucoma. *The British journal of ophthalmology, 53*(7), 433.

Weinreb, R. N., & Khaw, P. T. (2004). Primary open-angle glaucoma. *The Lancet, 363*(9422), 1711-1720.

Weisschuh, N., Wolf, C., Wissinger, B., & Gramer, E. (2009). A clinical and molecular genetic study of German patients with primary congenital glaucoma. *American journal of ophthalmology, 147*(4), 744-753.

Wiggs, J., Lynch, S., Ynagi, G., Maselli, M., Auguste, J., Del Bono, E., . . . Haines, J. (2004). A genomewide scan identifies novel early-onset primary open-angle glaucoma loci on 9q22 and 20p12. *The American Journal of Human Genetics, 74*(6), 1314-1320.

Wiggs, J. L., Damji, K. F., Haines, J. L., Pericak-Vance, M. A., & Allingham, R. R. (1996). The distinction between juvenile and adult-onset primary open-angle glaucoma. *American journal of human genetics, 58*(1), 243.

Zenteno, J. C., Hernandez-Merino, E., Mejia-Lopez, H., Matías-Florentino, M., Michel, N., Elizondo-Olascoaga, C., . . . Garcia-Ortiz, J. E. (2008). Contribution of CYP1B1 mutations and founder effect to primary congenital glaucoma in Mexico. *Journal of glaucoma, 17*(3), 189-192.

Zhuo, Y.-H., Wang, M., Wei, Y.-T., Huang, Y.-L., & Ge, J. (2006). Analysis of MYOC gene mutation in a Chinese glaucoma family with primary open-angle glaucoma and primary congenital glaucoma. *Chinese medical journal, 119*(14), 1210-1214.