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Mutational Analysis of *CYP1B1* gene in families with Primary Congenital glaucoma

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ABSTRACT

Primary congenital glaucoma (PCG) is a rare form of autosomal recessive disorder which shows its symptoms during early infantile period. These symptoms may include enlargement of globe, reduced visual acuity, blepharospasm, edema, photophobia and corneal opacification. *CYP1B1* (cytochrome P4501B1) gene sited at locus GLC3A is considered as most common mutated gene in PCG patients. Frequency of disease causing *CYP1B1* mutations differs among different populations ranging from less than 10% to 100%. There is a great diversity of mutations of *CYP1B1* gene studied in the PCG pathogenesis worldwide. In this study, blood samples of both diseased and healthy individuals of 6 Pakistani families affected with PCG, were collected mutation analysis of *CYP1B1*. Sanger sequencing of exons of *CYP1B1* gene identify potentially pathogenic variants in these families. The identified variants were solved with one novel mutation (c.1551_1551delA), chr2:38297946_38297946delT, one known (c.1347T>C), chr2:38298150A>G, rs1056837 and homozygous nonsynonymous SNP (c.406C>A) in were found in two of the families respectively. Another known polymorphism (c.1294G>C) chr2:38298203C>G was observed in one of our patient, resulting in protein change V432L, signifying its role in disease pathogenesis. Additionally two more novel polymorphisms are found in two of our families i.e. cDNA.2218A>C, chr2:38297681T>G with no effect at protein level, and cDNA.2215G>T, chr2:38297684C>A, 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 33% families (2 out of 6) 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 four families.

Keywords: Glaucoma, Interocular pressure, ophthalmology, primary congenital glaucoma

INTRODUCTION

Glaucoma is a category of neurodegenerative disorders that is the second most common vision loss cause after cataracts. About 13.5 percent of all blindness cases worldwide are due to glaucoma (D.M. Kumar *et al*., 2008). Glaucoma also known as "Silent thief of sight" is a multifaceted disease affected by both genetic and environmental factors (Cook & Foster, 2012). It is characterized by the changes to optical nerve head and retinal nerve fiber layer.

Primary congenital glaucoma is an inherited ocular abnormality with signs such as eye rubbing, photophobia, and irritability that usually appear before the age of six. (Stoilov, 1997; Monemi *et al*., 2005).

Pathway of aqueous outflow

The limbus is the region between the cornea and the sclera. It has functions such as corneal wound healing and peripheral cornea nourishment; it also regulates IOP and has aqueous outflow pathways. Internal scleral sulcus and shallow external scleral sulcus produced by variation in radius of curvature of limbus, internal scleral sulcus contains trabecular meshwork and schlemm canal. This outflow pathway is associated with 70-90% flow of the fluid aqueous humour (Manivannan *et al*.,2001). Rest of 10-30% of aqueous humour is flows by spaces between ciliary muscle fibers and loose connective tissues of suprachoroidal space, these are nonconventional outflow pathways (Neri *et al*., 2004).

Major sites of damage in Glaucoma patients

The most common site of damage in glaucoma is the optic nerve, and it is not always linked with i intraocular pressure (IOP) increase (Shields *et al*., 1996). Visual information is transmitted from the retina to the brain through the optic nerve (cranial nerve II). RGCs have 1.2 million nerve fibers in the head of the optic nerve. The optic nerve is referred to as the optic disc because it appears circular at the point of exit. The nourishment of the retinal layers is supported by blood vessels. The optic disc tends to be a 3 mm2 oval white region with a physiologic cup in the middle caused by transmission of the central retinal vein and artery. A hole in the center of the neuroretinal rim is defined as a normal cup size, which is about 1/3 the size of the disk (Elolia and Stokes 1998; Alexander, 1991).

The optic nerve or cranial nerve II plays its role in conduction of received visual information from the retina towards the brain. Head of optic nerve is the segment of retina from where the optic nerve originates and contains 1.2 million nerve fibers or axons of RGCs exiting the eye collectively. Upon leaving the optic nerve seems to be a round shaped site called as optic disc. The optic disc seems to be an oval shaped white zone of 3 mm^2 diameter. As a result of transmission of the central retinal artery and vein a small concave cut or pit appears which is called as the physiologic cup and is located in the center. These blood vessels help in vascularization of the retinal layers. The cup is basically a space in the central part of the neuro-retinal rim which is the situated between the cup and the periphery of the disc (Alexander *et al*., 1991; Elolia and Stokes 1998). Glaucoma's pathology can be easily and strongly determined by observing the gradual increase in optic disc cupping (Klein *et al*., 2006).

The optic nerve comprises of axons of RGCs that are stretched towards brain and incoming blood vessels have an opening passage into the retina.

Any severe kind of damage to optic nerve can result in blindness which can't be repaired because regeneration is not a luxury that nerve fibers can afford.

Classification

Classification of glaucoma is based on relating to the cause (primary VS secondary), and stage of onset of glaucoma (juvenile VS adult), composition of the frontal compartment (open angle VS closed angle) (Stoilov, 1997).

Glaucoma can be said to as primary glaucoma once it happens with no recognized etiology or categorized as secondary if earlier damage or illness is contributing. Extensively, it can be classified into 3

major types: Primary open angle Glaucoma (POAG), Primary angle closure Glaucoma (PACG), Primary congenital Glaucoma (PCG).

Genetics of Primary Congenital Glaucoma

PCG is the ultimate persistent babyhood glaucoma, can lead to the loss of vision during early childhood (Ghate and Wang, 2015) PCG is established as an autosomal recessive trait from the molecular genetic studies conducted in the last few years. Four chromosomal loci on GLC3A bearing the cytochrome P4501B1 (*CYP1B1*) gene have been concerned in Primary congenital glaucoma (Stoilov *et al*., 1997).

Diagnosis and Medications

Glaucoma can be diagnosed using techniques like Tonometry, Perimetry, Ophthalmoscopy, Gonioscopy & Pachymetry (Anderson *et al*., 1993).

If detected in early stages, can be treated using medications and surgery (Mendell, 1993). Medications comprise controlling the flow of aqueous outflow through the eyes. It is alternative approach adopted (Woodward and Gil, 2004; Schwartz and Budenz, 2004).

Table1. Increase in corneal thickness 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
01 year	11-12	20-21
06 years	12	23
Adult	12	22-24

FOXC1, PAX6, CYP1B1, MYOC, PITX2, and *LTBP2* all have mutations that are implicated in the pathogenesis of juvenile and congenital glaucoma (Fan *et al* 2010). In the Human Gene Mutation Record, there are over 100 CYP1B1 mutations. Missense, frame shift, small deletion/insertion, premature stop codon, and a large deletion have all been identified as disease-causing mutations in *CYP1B1* (Tham *et al*., 2014; Vasiliou & Gonzalez, 2008) The presence of cytochrome P-450 1B1 may be responsible for the metabolism of ocular development-related compounds (D Choudhary *et al*., 2009). A global study of *CYPB1* gene mutations in patients from various populations showed a broad range of *CYP1B1* mutation spectrum in disease pathogenesis, ranging from 20% in Caucasians to 100% in Saudi Arabians and Slovakian Roms (Rao *et al*., 2011)

Table 2. *CYP1B1* Reported Mutations in Pakistan (Adapted from Michael *et al*., 2015)

Nucleotide change	Aminoacid 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

METHODOLOGY ADOPTED FOR THIS STUDY

Blood samples of 6 Pakistani families with Primary congenital Glaucoma were collected. Blood was shifted to EDTA containing vacutainer tubes for processing for DNA extraction, and placed at 4°C temperature in laboratory. For the extraction of genomic DNA phenol chloroform method (organic technique) were put to use. After the isolation of DNA, the exons of *CYP1B1* were amplified using Polymerase Chain reaction (PCR). Sanger dideoxy sequencing were carried out on purified PCR products. Analysis of sequenced data using Sequencher software version 5.4.6 (http://www.mbio.ncsu.edu/sequencher.html) identified potentially pathogenic variants.

RESULTS

Mutation analysis of affected member of a family revealed homozygous nonsynonymous SNP (c.406C>A) in exon 2, showing no pathogenic sequence change as shown in Fig 1 and already known homozygous polymorphism (c.1347T>C) in exon 3, showing no pathogenic sequence change Fig 2. Another novel disease causing frameshift mutation (c.1551_1551delA) was observed in exon 3 of the same affected individual and individual of second family (Fig 3), predicting loss of stop codon and prolonged protein structure.

Mutational analysis of affected member of another family revealed a novel polymorphism (cDNA.2218A>C) in exon 3 with splice site change after stop codon at amino acid 607 (Fig 4). Another novel polymorphism (cDNA.2215G>T) with no alteration in the protein structure Fig 5. A already reported homozygous polymorphism (c.1294G>C) was observed in the variant with amino acid changes Val432Leu and no alteration in the protein sequence Fig 6.

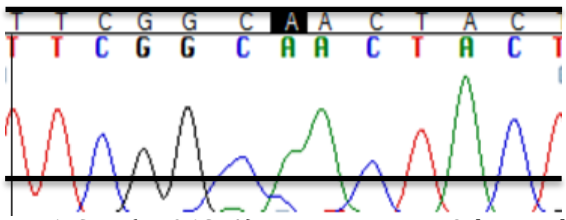

Fig 1. SNP (c.406C>A)

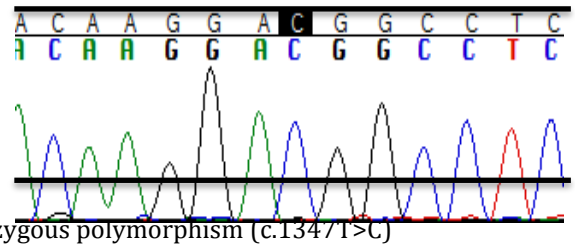

Fig2. known homozygous polymorphism (c.1347T>C)

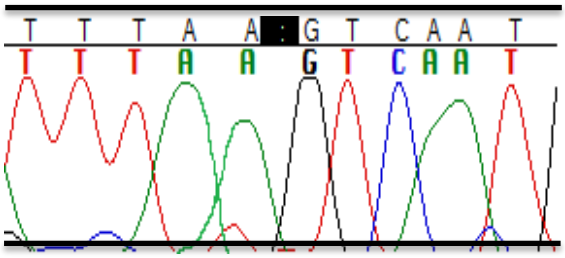

Fig 3. Frameshift mutation (c.1551_1551delA)

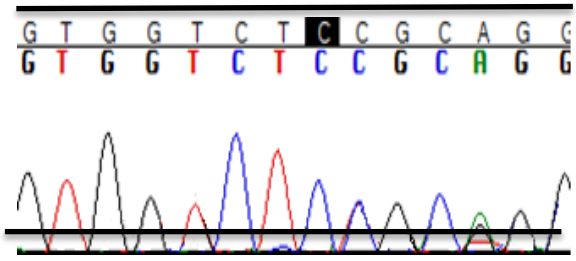

Fig 4. Novel polymorphism(cDNA.2218A>C)

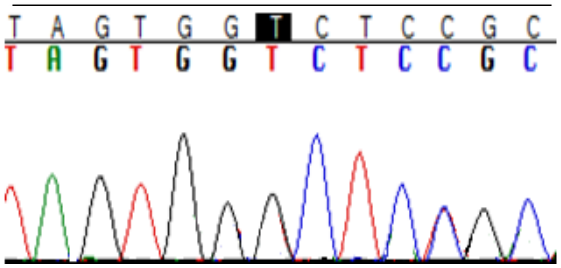

Fig 5. Novel polymorphism (cDNA.2215G>T)

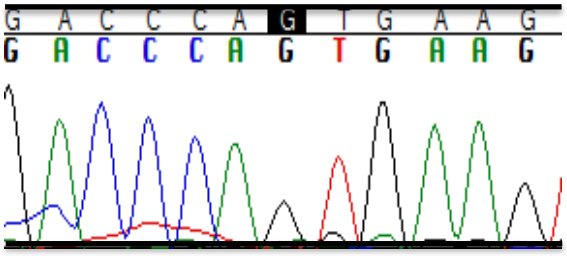

Fig 6. Reported homozygous polymorphism (c.1294G>C)

Table 3: Mutations found in PCG patients

Family	**Exon**	**Hg19 coordinate**	**HGVSc**	**HGVSp**	**Rs-ID**	**Mutation Taster**	**ExAc**
H3BF003	3	chr2:38298150A>G	c.1347T>C	N/A	rs1056837	Reported polymorphism	0.622
HA3CF004	3	chr2:38297946_38297946delT	c.1551_1551delA	Stop codon lost	N/A	**Novel Mutation**	N/A
A3BF005	3	chr2:38298203C>G	c.1294G>C	V432L	rs1056836	Reported polymorphism	**25066**
A3CF006	3	chr2:38297681T>G	cDNA.2218A>C	N/A	N/A	**Novel polymorphism**	N/A
A3CF007	3	chr2:38297684C>A	cDNA.2215G>T	N/A	N/A	**Novel polymorphism**	N/A
	2	chr2:38302126G>T	c.406C>A	N/A	rs538072907	Reported polymorphism	0.5

DISCUSSION AND CONCLUSION

Glaucoma is a neurodegenerative eye disease that may lead to damage to the optic nerve and consequent vision loss. It is the leading cause of irreversible blindness globally (Quigley *et al.*, 2018). According to a recent report there will be ~79.6 million people with glaucoma by 2020 (Thakur *et al.*, 2017). Research on glaucoma inheritance has been improved by advancement of genetic approaches that are involved in mutations that account for glaucoma. Many genes that cause glaucoma remain unidentified, but we continue to look for fundamental insights into the condition. Primary congenital glaucoma (PCG) is a common autosomal recessive trait that affects people who marry their cousins. (Al-Hazmi *et al.*, 2005). *CYP1B1* belongs to the CYP450 superfamily. It is the most mutated gene in PCG, with 58 functional genes in the human genome (Grinvald *et al.*, 2004). *CYP1B1* was previously mapped to the 2p21-22 region and consists of three

exons, one of which is noncoding and the other two are coding. (Stoilov et al., 1998). *CYP1B1* was related to the 2p21 region and was considered a candidate gene for PCG (GLC3A) (Sarfarazi *et al.,* 1995). Mutations in CYP1B1 reduce its activity by affecting functionally essential and highly conserved regions. (Acharya *et al*., 2006; Chavarria-Soley *et al*., 2008; Kumar *et al*., 2007).

In this study, *CYP1B1* gene of 6 PCG families was sequenced out of which 2 families were found with one novel mutation (c.1551_1551delA), chr2:38297946_38297946delT, and one known polymorphism(c.1347T>C), chr2:38298150A>G, rs1056837 and homozygous nonsynonymous SNP (c.406C>A) were found. The Novel deletion mutation found in two of our patients results in loss of stop codon, the resulted malfunctioned protein must be playing role in disease pathogenesis.

Another known polymorphism (c.1294G>C) chr2:38298203C>G was observed in one of our patient, resulting in protein change V432L, signifying its role in disease pathogenesis. Two more novel polymorphisms are found in two of our families i.e. cDNA.2218A>C, chr2:38297681T>G with no effect at protein level, and cDNA.2215G>T, chr2:38297684C>A.

Because of such pathogenic variants in candidate genes of Primary congenital Glaucoma, early detection, diagnosis and treatment is necessary before the sufficient irreversible damage to optic nerve takes place. The hereditary and genetic characteristics of disease demand genetic counseling and awareness of ophthalmologists to detect such type of genetic disorders at early stages.

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