J.Natn.Sci.Foundation Sri Lanka 2021 50 (3): 589-594
DOI: http://dx.doi.org/10.4038/jnsfsr.v50i3.10580

RESEARCH ARTICLE

Ophthalmic Genetics

High prevalence of glaucoma-associated CYP1B1 mutation (p.G61E) in primary congenital and open angle glaucoma patients in Pakistan

K Yousaf, R Bashir*, K Balqees, S Naz, N Munir and F Aslam
Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.

Submitted: 17 June 2021; Revised: 22 December 2021; Accepted: 25 February 2022

Abstract: The purpose of this research study was to investigate the prevalence of the p.G61E variant of the *CYP1B1* gene among primary congenital and open angle glaucoma (PCG and POAG) patients in the province of Punjab, Pakistan. A total of 112 POAG and 50 PCG patients were enrolled in this study. Detailed clinical examination was carried out on all patients. Screening of G61E was done by direct Sanger sequencing. Different *in silico* tools, *e.g.*, Clustal Omega, PSIPRED, and Franklin tools were used to check the conservation, secondary structure, and pathogenicity, respectively, of this variant. Sanger sequencing of the whole *CYP1B1* gene revealed a homozygous missense transition, c.182G>A, p.G61E in 25/50 (50%) of PCG and in 42/112 (37.5%) of POAG cases, which co-segregated with the disease phenotype. This study revealed that p.G61E is the relatively major contributor of PCG. However, 42 POAG patients harbouring the G61E mutation showed moderate to severe phenotype, suggesting the genetic heterogeneity of this variant in Pakistani population.

Keywords: Juvenile open angle glaucoma, intra ocular pressure, latent transforming growth factor-beta protein 2, primary congenital glaucoma.

INTRODUCTION

Glaucoma is a heterogeneous group of visual neuropathies which leads to optic nerve damage and permanent loss of vision if left untreated (Allingham *et al*., 2009). Primary congenital glaucoma (PCG) is a more severe form of the disease categorized by atypical development of trabecular meshwork and elevated intraocular pressure (IOP) at the time of birth or within the first three years of life, which manifest itself with photophobia, excessive tearing, and buphthalmos (Liu & Allingham, 2017). PCG mostly shows autosomal recessive form of inheritance (Firasat *et al*., 2008). In the Western region, the incidence of PCG is 1 in 10,000–20,000 live births while in the Middle Eastern region the incidence is 1 in 2500 to 8200 (Lewis *et al*., 2017). Primary open angle glaucoma (POAG) is a common cause of irreparable blindness in the world. It is an optic neuropathy characterized by open anterior chamber, complete cupping of nerve head, loss of visual field and elevated IOP (Liu & Allingham, 2017). On the basis of age of onset, POAG is subcategorized into adult-onset open angle glaucoma (AOAG) and juvenile-onset open angle glaucoma (JOAG). JOAG as an idiopathic glaucoma occuring in children above three years of age and it is associated with myopia and elevated intraocular pressure with fluctuation (Kwun *et al*., 2016). The age of onset of JOAG is 3-40 years and shows autosomal dominant inheritance; however a few cases of autosomal recessive inheritance from Saudi Arabia and India have been reported (Khan *et al*., 2011). Adult-onset POAG manifest itself after the age of 40 years and shows a complex pattern of inheritance because multiple genes and environmental factors are involved (Rauf *et al*., 2016). The estimated prevalence of JOAG ranges from 0.38 to 2 in 100,000 in individuals between 4 and 20 years of age (Taqi *et al*., 2011). JOAG comprises 4% of childhood glaucoma (Kwun *et al*., 2016). POAG is the

* Corresponding author (rashidasbs@yahoo.com; https://orcid.org/0000-0003-1270-3742)

most common form of glaucoma worldwide accounting for 90% of all cases reported (Qureshi *et al.*, 2006). POAG is also the common types of glaucoma in Pakistan (Taqi *et al*., 2011).

The CYP1B1 (OMIM 601771) gene is a major contributor in glaucoma pathogenesis (Achary *et al*., 2006). CYP1B encodes a member of the Cytochrome p450 superfamily, subfamily I. The *CYP1B1* gene is localized in chromosome number 2 and consists of three exons out of which two are coding (López-Garrido *et al*., 2015). It has been reported that the CYP1B1 protein plays role in the development of trabecular meshwork, iridocorneal angle, and in the formation of the ciliary (Rauf *et al*., 2016). Pathogenic and potentially pathogenic mutations in *CYP1B1* have been reported in JOAG, POAG, and PCG patients from various populations around the globe (Micheal *et al*., 2014). Mutation p.G61E has been reported previously in POAG and PCG patients from different ethnicities (Qashqai *et al*., 2018). Recent investigations on the functional role of the homozygous p.G61E *CYP1B1* mutation in a glaucoma family have shown that this mutation disturbs the microfibrils of the extracellular matrix (ECM), making its presence less abundant, and increases more fragmented protein in affected individuals. This study has suggested that disruption in development of the ECM-trabecular meshwork leads to increased resistance to aqueous outflow, causing elevated IOP, and eventually causes development of glaucoma (Banerjee *et al*., 2016). The G61E mutation has been reported to be the commonest mutation in Lebanon, Iran, Egypt, and other Middle Eastern countries (Qashqai *et al*., 2018). This variant is a founder mutation in Middle Eastern populations (Saudi Arabia, Iran, Morocco, Kuwait, Israel, Oman, and Egypt) (Campos-Mollo *et al*., 2009; Li *et al*., 2011). In Saudi POAG patients the frequency of this variant accounted for 64.3% (Qashqai *et al*., 2018). Heterozygosity for the mutation p.G61E has already been reported as a cause of adult-onset POAG in two Pakistani sporadic cases (Micheal *et al*., 2014). In heterozygous form, p.G61E has been reported with mild phenotype in PCG and POAG patients of different ethnicities (Khan *et al*., 2011; López-Garrido *et al*., 2015; Wiggs & Pasquale, 2017). Previously, our study demonstrated that the p.G61E variant was segregated in a consanguineous Pakistani family with co-existence of JOAG and PCG in two successive generations (Bashir *et al*., 2015). Only one study showed heterozygous p.G61E mutation in sporadic POAG patients from Pakistan (Micheal *et al*., 2014). Mutation Taster and PolyPhen predicted this variant as disease causing, and the Franklin tool qualified this variant to be deleterious.

POAG and PCG are the most prevalent types of glaucoma in Pakistan (Iqbal *et al*., 2011). However, few studies are available on the contribution of the p.G61E mutation in the Pakistani population. In the current study, the aim was to investigate the frequency of the p.G61E variant in PCG and POAG cases.

MATERIALS AND METHODS

Selection of study subjects and clinical examination

The study followed the guidelines of the Declaration of Helsinki. This study was approved by the Ethical Review Committee of the Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan with reference number 2019-07. Informed consent was obtained from all participants or from the parents in case of infants.

Patients identified as having POAG and PCG were recruited from 2017-2020 (a 3-year period) from the Mughal Eye Hospital Lahore and the eye ward of the Children Hospital and Institute of Child Health (CHICH) Punjab. CHICH is the major ophthalmology hospital recommended for congenital cases in Punjab, and patients travel from all parts of Pakistan to this hospital. 112 unrelated Pakistani POAG and 50 PCG patients from consanguineous marriage were enrolled. 196 unrelated ethnically matched controls were also recruited.

For this study, the following inclusion criteria were used for POAG and PCG cases: (1) Age at the time of diagnosis, (2) Intraocular pressure > 21 mm Hg in adults and >12mm Hg in at least one eye in case of PCG, (3) Anterior chamber angle that was open to scleral spur or ciliary body, (4) Evidence of optic nerve damage and loss of visual field consistent with glaucoma in at least one eye, and (5) cup-to-disc (C/D) ratio > 0.3 of optic nerve. Megalocornea with corneal diameter >12 mm and rupture in Descemet's membrane have been considered. Inclusion criteria for POAG: ethically matched controls (50 males, 50 females) and age ranges from 40 to 67 years were selected after ophthalmic screening, and individuals with normal range of IOP (not having anti-glaucoma medication), open angles after gonioscopy examination and healthy optic disk with no history of eye disease(s) or any ophthalmic surgeries.

For PCG, ethnically matched controls (56 males, 40 females), age ranges from 2 to 15 years with normal IOP range, normal cup/disc ratio, normal corneal diameter and intact Descemet's membrane were included.

Screening of p.G61E mutation

Primers were designed for coding exon 2 of *CYP1B1* with Primer3 including 50–80 bp of intronic region from the upstream and the downstream region of coding exon with Primer3 software (*http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi*). Polymerase chain reaction (PCR) amplification was performed from genomic DNA. Each reaction was performed in a final volume of 50 µL under standard conditions with 50 ng DNA in 2.5 µL of Taq buffer, 2 mM $MgCl_2$ (1 µL), 0.2 mM DNTPs (1.5 µL), 0.24 µL forward primer, 0.24 µL reverse primer, 25 ng genomic DNA and then PCR water was added to raise its volume to 50 µL. Denaturation of PCR products was performed at 95 °C for 30 s and annealed at 55 °C for 30 s. The initial extension was performed at 75 °C for 1 min and final extension was performed at 75 °C for 5 min. PCR products were then purified by gene cleaning column (Gel Extraction Kit Thermo Scientific GeneJet USA). After purification, samples were sequenced by direct sequencing with Big Dye Terminator® (V 3.1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

In silico analysis

ClustalW alignments were performed to determine evolutionary conservation, Figure 1C. To predict secondary structure, elements from amino acid sequence PSIPRED v.3.3 were used, Figure 1D. Franklin tool classified the variant as potentially pathogenic (*https://franklin.genoox.com/clinical-db/variant/snp/chr2-38302350-C-T?app=assessment-tools*).

RESULTS AND DISCUSSION

Table 1 shows the clinical characteristics of all PCG and POAG patients clinically examined. In 50 PCG and 112 POAG patients, the mean age in POAG was 52.8 ± 11.1 years with range 40-70 years. In PCG cases the mean age was 12.2 ± 9.7 years and range was 1 month to 9 years. The mean age of IOP in PCG cases was 19.5 ± 6 years whereas in POAG the mean age of IOP was 24.3 ± 7.8 years (Table 1). Clinical features of POAG patients showed watery eyes with enlarged globe and hazy cornea. They felt pain, headache, itching, and blurred vision. Some of them had maintained normal IOP pressure by regular treatment and prescribed medications. Clinical features of PCG patients showed hazy cornea, photophobia, complete opacification of corneas, and excessive tearing with enlarged globe. Most of the PCG patient underwent trabeculectomy in both or one eye to maintain normal IOP pressure along with the use of prescribed medications. IOP of PCG and POAG was positively associated with glaucoma development (Figure 1 and Figure 2).

Sequencing results of CYP1B1 showed a missense mutation c.182G>A (p.G61E) in exon 2 which was homozygous in 25/50 (50%) PCG cases and in 42/112 (37.5%) POAG cases; the mutation co-segregated with the disease phenotype in 50 PCG and 42 POAG patients (Figure 2A, 2B). The variant was absent in 196 ethnically matched control chromosomes, further confirming that this mutation is pathogenic in the Pakistani population. MutationTaster and PolyPhen predicted this variant as disease causing. Franklin tool qualified this variant to be deleterious. PSIPHRED sequence plot showed that residue G61 lies in the Protein Helix region. Genetic variants in *CYP1B1* gene are major contributing factors in the pathogenesis of PCG and POAG in

Table 1: Clinical parameters of PCG and POAG patients and control groups

Parameters	Status	PCG	POAG	Control POAG	Control PCG
Gender	Male	30	66	50	56
	Female	20	46	50	40
Age	Mean ± SD	12.18 ± 9.7	52.8 ± 11.1	65.9 ± 11.6	13.2 ± -6.4
Unit: (mth,yrs)	Range	1 mth–14 yrs	40 – 70 yrs	40 yrs – 67 yrs	2–15 yrs
Max. IOP (Unit: mmHg)	Mean ± SD	19.46 ± 5.96	24.32 ± 7.8	15.0 ± 2.7 8–21	16.5 ± 3.7
CD ratio	Range	0.3–0.9	0.4 – 0.9	0.2–0.3	0.2–0.3

C/D: cup to disk ratio, mth: month yrs: years

various populations of the world (Qashqai *et al*., 2018; Youngblood *et al.*, 2019; Ling *et al*., 2020). The variant p.G61E has been found with varying frequency in different populations; however, data on genetic spectrum of this variant in Pakistani population is very limited. To explore the clinical spectrum of this variant, the present study was performed to determine the frequency and to make genotype-phenotype comparison of the p.G61E mutation associated with PCG and POAG in the Pakistani population. This genetic study was done to demonstrate the role of the p.G61E variant of *CYP1B1* gene in Pakistani PCG and POAG patients. This cohort included 162 patients (n = 50 PCG, n = 112 POAG) as well as 196 control.

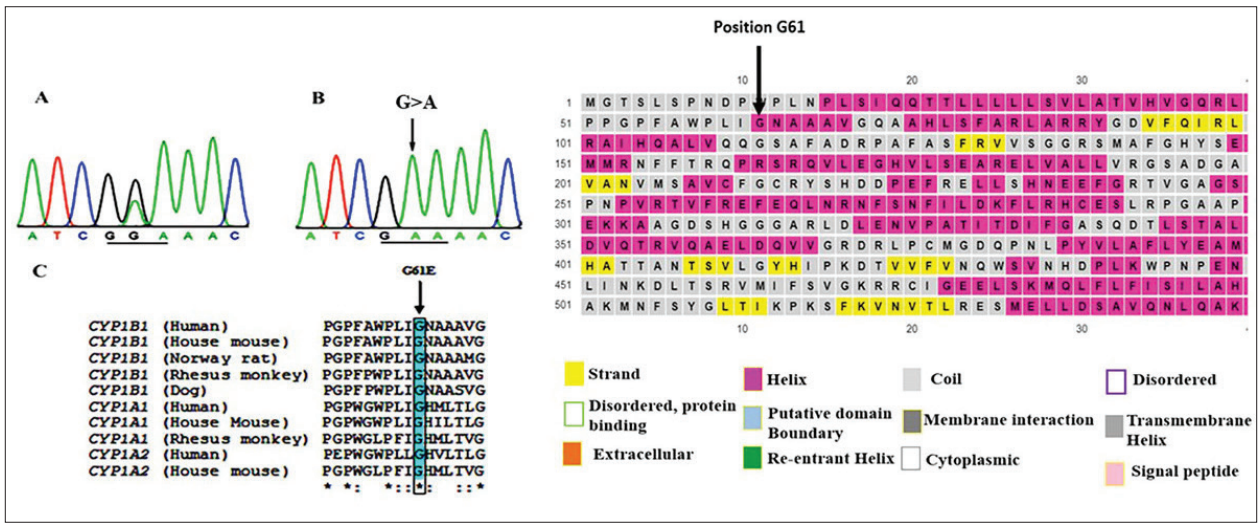

Figure 1: Electrogram of G61E mutation sequence. (A) normal sequence in control sample; (B) G>A transition in patient's DNA; (C) ClustalW sequence alignment indicates conservation of p. G61E mutation in *CYP1B1, CYP1A1* and *CYP1A2* among different vertebrate species; (D) PSIPhred sequence plot indicating that residue G61 lies in Protein Helix.

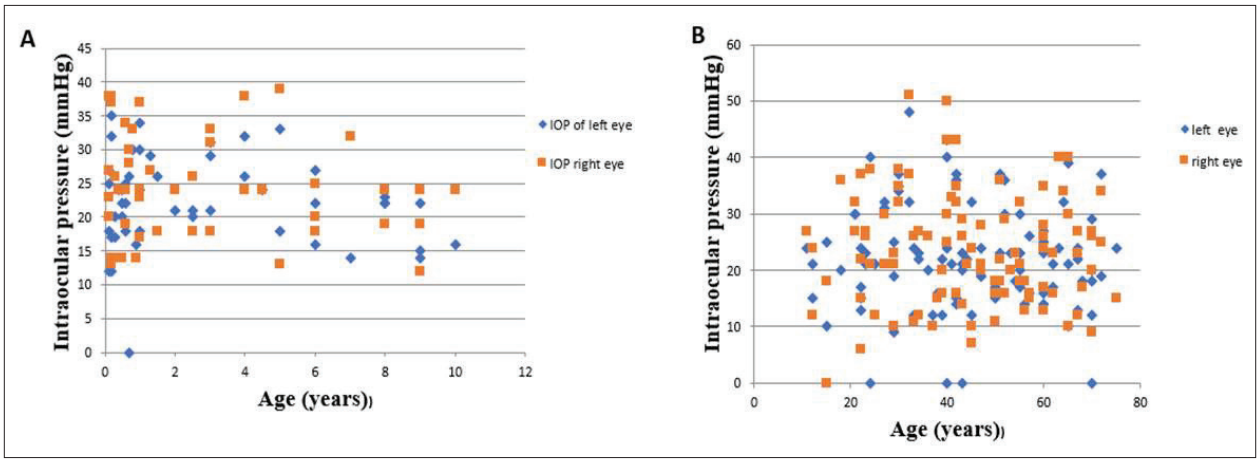

Figure 2: Graph showing IOP among both groups, *i.e.*, PCG patients (A) and POAG (B) patients.

The p.G61E mutation has already been reported to be a frequent cause of PCG and POAG in many different populations of the world including Arab, Middle Eastern and Mediterranean countries (Khan *et al*., 2011; Micheal *et al*., 2014; López-Garrido *et al*., 2015), whereas this variant has been reported with incomplete penetrance as well in different populations (Campos-Mollo *et al*., 2009 Lewis *et al*., 2017;). This mutation has been shown to be a 'hypomorphic' allele, having reduced enzymatic activity of the CYP1B1 protein (Campos-Mollo *et al*., 2009). In previous studies, it was reported that the homozygous p.G61E mutation was pathogenic in PCG patients of Saudi Arabia (Abu-Amero *et al.*, 2011). In a recent study from Pakistan, this variant has been identified in homozygous state in a PCG patient belonging to a consanguineous family. However, in some cases the p.G61E mutation may exhibit decreased penetrance which has been reported previously in Spanish and Saudi Arabian patients (Campos-Mollo *et al*., 2009; Youngblood *et al*, 2019). In a cohort of Saudi POAG patients, the p.G61E variant accounted for 64.3% (Abu-Amero *et al*., 2011). However, in another study of the Saudi population, the mutation was reported in heterozygous form and accounted for 4% of POAG cases and 2% of controls (Abu-Amero *et al*., 2011). Mutation p.G61E has been the most frequent mutated *CYP1B1* allele in Iranian PCG patients. It is also the most commonly detected mutation among the POAG patients (Qashqai *et al*., 2018). In adult-onset POAG patients, the variant p.G61E is mostly reported in heterozygous and compound heterozygous state, with a milder disease phenotype (López-Garrido *et al*., 2015). In homozygous state the p.G61E variant showed severe phenotype in Indian and Saudi Arabian PCG patients. In Saudi JOAG patients, this mutation is reported to result in a severe phenotype in recessive form. In a report from the Pakistani population, the heterozygous p.G61E variant was reported in sporadic cases of POAG (Micheal *et al*., 2014). Moreover, in our previous study from Pakistan we reported the homozygous p.G61E variant, which, co-segregating in two generations of JOAG and PCG patients from a consanguineous family, showed a severe phenotype with variable penetrance of this p.G61E variant (Bashir *et al*., 2015).

The residue p.G61E lies in the functional part of a very highly conserved hinge region of the *CYP1B1* protein, from which region the polypeptide chain shows a sharp turn (Panicker *et al*., 2004). It is present right after the N-terminus of a region rich in proline. The proline–proline–glycine–proline motif might make the link between the membrane binding N-terminus and the globular region of the P450 protein. The change of glycine to glutamic acid at position 61 causes a complete loss of haem binding, resulting in minimal catalytic activity of the enzyme and results in disease manifestation (Panicker *et al*., 2004). Furthermore, MutationTaster, SIFT, Franklin tool, and PolyPhen predicted this variant as disease causing.

In this study, screening for mutation p.G61E in the *CYP1B1* gene revealed that 50% of PCG cases and 37.5% of POAG cases carried this mutation in homozygous state. The frequency of the p.G61E variant in PCG cases is higher in the Pakistani population as compare to Chinese, Iranian, and Spanish populations (López-Garrido *et al*., 2010; Safari *et al.,* 2016; Abu-Amero *et al*., 2018). These variations in mutational frequency may be due to differences in geographical region, and epigenetic factors such as age, gender, race, and family history also contribute in causing PCG and POAG. IOP and CD ratio of PCG cases in p.G61E positive patients was high, resulting in opacification of cornea and need of surgical interventions. The patients affected with PCG showed severe phenotype indicating complete penetrance of this mutation in congenital cases. However, 19 patients affected with POAG showed mild phenotype whereas 23 cases showed severe phenotype, which showed the genetic heterogeneity of this variant in POAG.

CONCLUSION

We conclude that the *CYP1B1* gene is important in the aetiology of PCG as well as POAG, in the Pakistani population. This study reveals that p.G61E is more common among primary congenital glaucoma in the Pakistani cohort. However, the phenotypic variability associated with p.G61E variant in POAG patients of our population is possibly the effect of genetic modifiers or epigenetic factors that may modify the manifestation and variable penetrance. Further detailed studies applying whole-exome sequencing on the DNA of a large number of severely affected cases and those of less affected patients could reveal further genetic modifiers.

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