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Original article

Mutational analysis of *CYP1B1* (rs56010818) variant in primary open angle glaucoma (POAG) affected patients of Pakistan

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ABSTRACT

Background: Primary open angle glaucoma (POAG) occurs due to the discrepancies in the angle of anterior chamber characterized by the alterations in intraocular pressure, optic nerves head changes and central loss of visual field. In molecular research, *CYP1B1* mutations modulates an integral role in association with glaucoma. Current study was undertaken to reveal the homozygous and heterozygous patterns of *CYP1B1* c.1169 G > A variant (rs56010818) in POAG patients of Pakistan.
Methods: After consent, total n = 88 POAG patients undergone through standard ophthalmological investigations before their recruitment in this study. The blood samples were utilized for DNA isolation. The genotyping of *CYP1B1* c.1169 G > A variant was carried out by Sanger sequencing. The mutational patterns and its association with clinical variables were demonstrated by statistical and bioinformatic tools.
Results: It was evident that the frequencies of heterozygous G/A and homozygous mutants A/A genotypes were higher in males (36.5%, 7.7%) than females (30.6%, 2.8%) of POAG population. Furthermore, the juvenile patients exhibit high manifestation of carrier genotype (66.6%) in comparison to adult patients (31.7%). The results also indicated the significant relationship of intraocular pressure with homozygous mutant A/A genotype of *CYP1B1* variant in POAG patients ($p < 0.05$).
Conclusions: Our study provided the mutational data of *CYP1B1* R390H variant and the patterns of homozygosity and heterozygosity along with clinical associations. Overall, this study revealed the genetic predisposition of *CYP1B1* c.1169 G > A variant in the patients of POAG in Pakistan. The findings could be helpful for genetic screening and in-depth understanding of underlying causes in the pathogenesis of POAG.

1. Introduction

Glaucoma is pronounced by progressive disintegration of ganglion cells in retina, that triggers the dissociation of optic nerve associated with elevated intraocular pressure lead to cause pathogenesis (Wiggs et al., 1996). The pathway which carries the fluid aqueous humor in the anatomy of eye, chiefly contributes to the regulation and alteration of intraocular pressure, localized at the junction of iris and cornea also referred as an angle (Ramos et al., 2011). However, the appearance of the anterior chamber angle modulates the discrepancies in the clinical demonstration of glaucoma categories. Particularly, it has been recognized into two major forms; open and closed angles (Babizhayev and Yegorov, 2011). Closed angle glaucoma is extremely infrequent usually appeared due to structural anomalies. On the other hand, primary open angle glaucoma (POAG) is regarded as frequently reported form, developed due to various physiological, biochemical and genetic anomalies (Weinreb et al., 2014).

POAG mainly considered as an optic neuropathy that drives irregularities in visual field (Melki et al., 2004). According to recent data, approximately 6.9 million individuals got affected by

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glaucoma induced blindness internationally (Bourne et al., 2017). The disease is typically accompanied by high intraocular pressure, epiphora, edema in cornea, blepharospasm, buphthalmia, and photophobia (Kumar et al., 2007). The manifestation of primary open angle glaucoma occurred in two distinct forms. The juvenile form is relatively rare, that diagnose POAG in age<40 years. While, adult form is frequently reported after the age of 40 years (Wiggs et al., 1996; Shimizu et al., 2000). >20 loci have been identified till date in association with juvenile form of POAG, which exhibit autosomal dominant inheritance (Allingham et al., 2009). Though, adult form of POAG demonstrated complex pattern of inheritance (Waryah et al., 2013). The predisposition of POAG has been associated with several genetic factors but their exact function in the pathogenesis mechanism is quite complex (Wolfs et al., 1998; Hulsman et al., 2002).

The *CYP1B1* gene encoded protein is categorized as the member of cytochrome P450 family falls into I subfamily. The gene is positioned on chromosome 2p22-21 into the genetic map. It comprised of total three exons and two introns in which translated region originated from 5′ site of second exon (http://ncbi.nlm.nih.gov/dbSNP). The elevated levels of *CYP1B1* mRNA also reported in various ocular regions including ciliary body, cornea, iris, retina and pigment epithelial membrane (Stoilov et al., 1998). The translated protein fundamentally functions as monooxygenase incorporated into the membrane. Previously, it is suggested that this cytochrome protein showed involvement in the formation of iridocorneal angle (Libby et al., 2003). Thereby, lead to stimulate variations in activity of *CYP1B1* which caused impairment in morphogenesis process that produced outflow angle which ultimately enhanced IOP and develops glaucoma. There are several mutations identified in familial based studies in the patients of primary congenital glaucoma and open angle glaucoma reported so far, but their association varies sporadically among various populations and ethnicities (Belmouden et al., 2002; Colomb et al., 2003; Lopez-Garrido et al., 2006; Chen et al., 2016).

Interestingly, clinical reports revealed that mutations of *CYP1B1* gene such as in the N-terminus hinge depicted more complex phenotypes of glaucoma (Li et al., 2011). Numerous variants (>150) were recognized in *CYP1B1* gene in the patients of congenital glaucoma throughout the world (Song et al., 2019). The genetic variant of *CYP1B1* c.1169 G > A was identified in the nucleotide sequence of third exon responsible for the substitution of arginine (R) at 390 position with histidine (H) amino acid, reported in only 20 % cases of congenital glaucoma in a previous Pakistani study (Khan et al., 2019). However, p. R390H was significantly observed as a most abundant mutation (50%) in five Pakistani families of congenital primary glaucoma (Sheikh et al., 2014).

To date, no prospective study has identified the gender-based distribution pattern of homozygosity and heterozygosity of *CYP1B1* c.1169 G > A variant in the pathogenicity of primary open angle glaucoma. Collectively, considering its significant role in PCG from above data, *CYP1B1* c.1169 G > A variant might play an essential role in the predisposition of primary open angle glaucoma. Herein, the present study aimed to evaluate the pattern of homozygosity and heterozygosity of *CYP1B1* c.1169 G > A (rs56010818) variant in unrelated Pakistani individuals affected with primary open angle glaucoma.

2. Material and Methods

2.1. Patient recruitment

It is a descriptive study carried out from October 2017 to December 2020 by Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro and Department of Molecular Biology, Liaquat University of Medical and Health Sciences, Jamshoro Pakistan. The study was approved by the ethical research committee and board of research and graduate studies, University of Sindh, Jamshoro Pakistan by following the guidelines of Helsinki Declaration from the respective institutions. A total of n = 88 blood samples as per WHO 7.1 calculator were collected in EDTA vacutainers after getting the informed consent from diagnosed patients of primary open angle glaucoma from SIOVS, Hyderabad, Ophthalmic unit LUMHS and LRBT hospitals of Sindh Province. Complete medical history and basal parameters were recorded from all the participating individuals which includes; age, gender, visual acuity, intraocular pressure (IOP), and cup-to-disc (C/D) ratio. The confirmation of diagnosis was performed by perimetry, gonioscopy and slit lamp bio microscopy. The anterior chamber angles were measured by using Goldmann goniolens. The field of vision was assessed by using Humphrey perimetry. While, the fundus examination was undertaken using 78 plus diopter lens with slit-lamp bio microscopy.

2.2. Genotyping of CYP1B1 (rs56010818) variant

The EDTA vacutainers filled with whole blood of study participants (n = 88) were utilized for the isolation of DNA using standard inorganic protocol (Grimberg et al., 1989). The sequencing primers for *CYP1B1* gene were designed by using the online web software Primer3 for the genotyping of c.1169 G > A variant (Table 1). The PCR reaction was done using the optimized composition of 1.5 pM primers, 0.3 Units of Taq polymerase, 0.125 mM of dNTPs and 1.5 mM of $MgCl_2$ in Applied Biosystems 2720 thermal cycler was used. The following conditions were used in optimized program operated by thermal cycler; 5 min initiation denaturation at 95 °C, followed by 35 cycles, each cycle processed through 20 secs denaturation at 95 °C, 40 sec annealing at 58 °C, 50 sec primer extension at 72 °C, and then 10 min final extension at 72 °C. After the amplification, the products were resolved on 2% agarose gel by gel electrophoresis. While, the purification of PCR product was done by improvised standard ethanol precipitation method (Fregel et al., 2010). Finally, the DNA sequencing was carried out using BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) following Sanger's dideoxy chain termination method in accordance with literature (Sanger et al., 1977; Waryah et al., 2013). The ABI 3130 Genetic Analyzer was used to accomplished the reaction of sequencing, whereas the output file was analyzed and electropherograms were generated by Chromas software v 1.45.

2.3. Data analysis

The distribution of study variables such as age, gender, IOP, and C/D ratio was performed by SPSS software v 16.0. The frequencies of heterozygosity and homozygosity of *CYP1B1* c.1169 G > A variant were estimated and differences were observed among genders and

Table 1
Sequencing primers used for *CYP1B1* gene amplification.

Primer ID	Primer Sequence
CYP-E2a-F	5'-AGCCTATTTAAGAAAAAGTGGAAT TA-3'
CYP-E2a-R	5'-GAATCCAGCTGGATCAAAGTT-3'
CYP-E2b-F	5'-CTACCACATTCCCAAGGACAC T-3'
CYP-E2b-R	5'-AGAAGCAGCACAAAAGAGGAA CT-3'
CYP-E1a-F	5'-CCTTCTCTTCTCCAAGGGAGAGT-3'
CYP-E1a-R	5'-CTCGCCATTCAGCACCACTAT-3'
CYP-E1b-F	5'-TACGGCGACGTTTTCCAG AT-3'
CYP-E1b-R	5'-CTCTTCGTTGCTGAGCA-3'
CYP-E1c-F	5'-ACGTCATGAGTGCCGTGTGT-3'
CYP-E1c-R	5'-GTCTCTACTCCGCCTTTTTCAGAC-3'

age groups. The bar graph used for the interpretation of visual acuity in normal and corrected ranges produced by Microsoft Office v 2016. The relationship of *CYP1B1* c.1169 G > A variant genotype with study variables was evaluated by regression statistics in SPSS® software v16. In bioinformatic analysis, PolyPhen2 online tool was utilized for the prediction of potentially hazardous nature of the studied *CYP1B1* variant on the functionality of its protein (Adzhubei et al., 2010). The online tool Clustal Omega was used for multiple sequence alignment of *CYP1B*1 c.1169 G > A variant and to observe its effect on R390H amino acid substitution among various species (Sievers et al., 2011).

3. Results

Total n = 88 patients of primary open angle glaucoma (POAG) were divided into n = 52 male and n = 36 female subjects. The mean, standard deviation and range of age categorized in both genders were described in Table 2.

The comparison of study variables between male and female POAG patients was demonstrated in Table 2. No statistical difference was observed in age, intraocular pressure (IOP), and C/D ratio of right (OD) and left (OS) eyes among the genders of the patients ($p > 0.05$). The frequencies distribution of *CYP1B1* c.1169 G > A variant genotypes and alleles was presented in Table 3. The heterozygous G/A and mutant A/A genotype frequencies were elevated in males (36.5%, 7.7%) in comparison to females (30.6%, 2.8%) respectively. In males, the frequency of G allele was 74% and A allele was 26%. Whereas, in females, the G allelic frequency was 82% and A allelic frequency was 18%. The juvenile form (<40 yrs.) of POAG contains higher frequency of heterozygous G/A genotype (66.6%) and similar frequency of wild type and mutant genotypes (16.7%). While, the adult form (>40 yrs.) of POAG encompasses higher manifestation of heterozygosity G/A (31.7%) as compared to the homozygosity A/A (4.9%) of mutant genotype. Although, the distribution of G (50%) and A (50%) allele was equivalent in juvenile patients, whereas G allele frequency (79%) was evident to be higher than A allele (21%) frequency in adult POAG patients.

The relationship of study variables with genotypes of *CYP1B1* c.1169 G > A variant was analyzed by regression statistics described in Table 4. It was observed that IOP of left eye (OS) was statistically higher in females as compared to males among the individuals carrying homozygous mutant A/A genotype ($p < 0.05$). Hence, it was anticipated that higher intraocular pressure was significantly associated with mutant homozygosity in female POAG patients. Moreover, no statistical difference was observed in other study variables among three genotypes of *CYP1B1* variant.

The range of visual acuity of POAG patients in right (OD) and left (OS) eyes was illustrated in Fig. 1. The visual acuity of 6/60 was observed in highest number of individuals in both OD (15) and OS (16) eyes. Visual acuities of 6/24 and 6/18 were more abundant in OS eyes, whereas 6/36 and 6/12 were more frequent in OD eyes. The corrected range of visual acuity in the patients of POAG was evident in Fig. 2. The visual acuity of 6/60 was found to be most frequently observed in left eyes (OS), whereas, 6/6 was more prevalent in right eye (OD) of the patients. In addition, visual acuities of 6/24, 6/18, 6/12 and 6/9 were also detected adequately in both eyes of POAG patients.

The electropherogram of *CYP1B1* c.1169 G > A variant in POAG patients generated by DNA sequencing was represented in Fig. 3.

Table 4
The association of study variables with genotypes of *CYP1B* variant in POAG patients.

Parameters	Genotypes of *CYP1B1* variant		
	G/G	G/A	A/A
Age (years)			
Male	55.59 ± 10.91	47.89 ± 16.35	42.50 ± 16.58
Female	55.57 ± 11.03	56.55 ± 15.97p = 0.170	45.00 ± 0.00
	p = 0.993		p = 0.901
IOP (OS) mmHg			
Male	26.50 ± 9.14	22.63 ± 8.42	20.30 ± 1.83
Female	22.00 ± 0.00	24.18 ± 8.26	24.85 ± 3.02
	p = 0.690	p = 0.629	p = 0.031*
IOP (OD) mmHg			
Male	18.83 ± 5.278	19.66 ± 7.60	13.25 ± 5.85
Female	20.53 ± 7.315	18.91 ± 6.02	18.00 ± 0.00
	p = 0.334	p = 0.782	p = 0.520
C/D (OS) Ratio			
Male	0.65 ± 0.19	0.71 ± 0.186	0.77 ± 0.05
Female	0.72 ± 0.16	0.66 ± 0.27	0.70 ± 0.00
	p = 0.161	p = 0.542	p = 0.272
C/D (OD) Ratio			
Male	0.66 ± 0.18	0.65 ± 0.20	0.62 ± 0.17
Female	0.72 ± 0.16	0.69 ± 0.14	0.50 ± 0.00
	p = 0.185	p = 0.648	p = 0.559

* $p < 0.05$.

Table 2
The study variables distribution between males and females including age distribution of POAG patients.

Parameter	Primary Open Angle Glaucoma Patients (n = 88)			P Value
	Males	Females	Total	
Number (%)	52 (59.1%)	36 (40.9%)	88 (100%)	
Range (yrs.)	16–80	26–80	16–80	
Age (Mean ± S.D)	52.62 ± 12.33	55.22 ± 12.54	53.68 ± 12.41	0.335
IOP (OS) mmHg	21.63 ± 7.20	24.56 ± 8.56	23 ± 7.88	0.086
IOP (OD) mmHg	18.51 ± 6.40	19.96 ± 6.79	19 ± 6.53	0.312
C/D (OS) Ratio	0.68 ± 0.18	0.70 ± 0.19	0.69 ± 0.19	0.646
C/D (OD) Ratio	0.65 ± 0.18	0.71 ± 0.16	0.67 ± 0.18	0.187

Table 3
The genotypic and allelic distribution of *CYP1B1* c.1169 G > A variant in POAG patients.

Genotype & Alleles	Total % (n = 88)	Males % (n = 52)	Females % (n = 36)	Juvenile Patients % (n = 6)	Adult Patients % (n = 82)
G/G	60.2% (53)	55.8% (29)	66.6% (24)	16.7% (1)	63.4% (52)
G/A	34.1% (30)	36.5% (19)	30.6% (11)	66.6% (4)	31.7% (26)
A/A	5.7% (5)	7.7% (4)	2.8% (1)	16.7% (1)	4.9% (4)
G allele	77% (136)	74% (77)	82% (59)	50% (6)	79% (130)
A allele	23% (40)	26% (27)	18% (13)	50% (6)	21% (34)
Juvenile Patients: Age < 40 yrs., Adult Patients: Age > 40 yrs.					

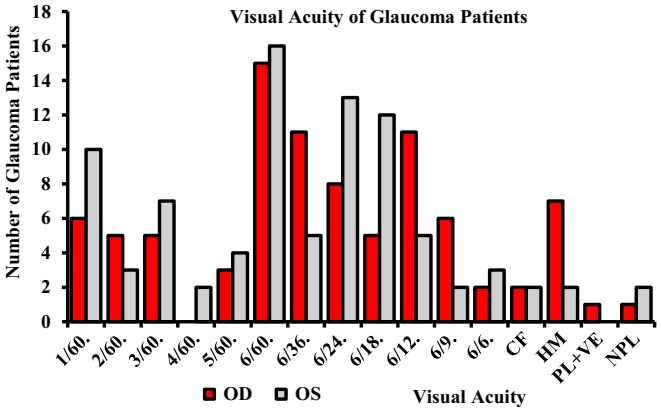

Fig. 1. The range of visual acuity observed in POAG patients in left (OS) and right (OD) eyes. CF: Counting finger, HM: Hand Movement, PL + VE: Perception of light positive, NPL: No perception to light in studied group.

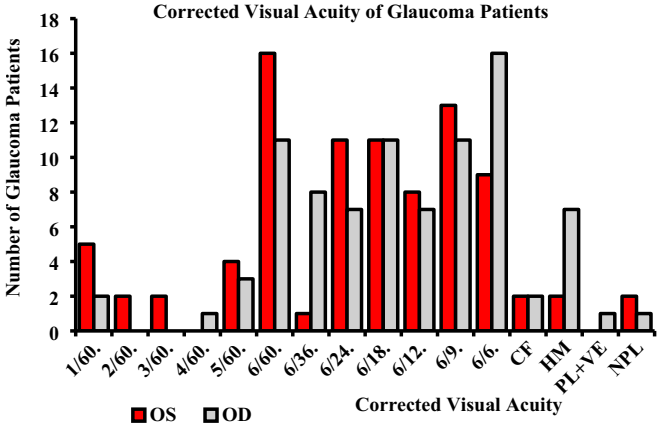

Fig. 2. The range of corrected visual acuity detected in POAG patients in left (OS) and right (OD) eyes. CF: Counting finger, HM: Hand Movement, PL + VE: Perception of light positive, NPL: No perception to light in studied group.

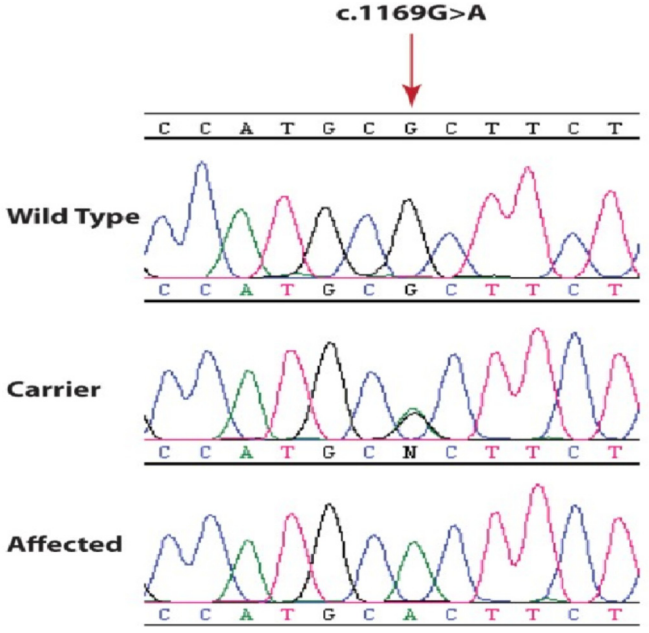

Fig. 3. Electropherogram of wild type, carrier and mutant genotypes of *CYP1B1* 1169G > A variant in primary open angle glaucoma patients.

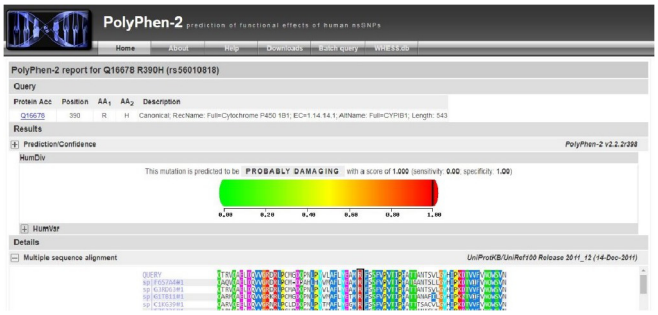

Fig. 4. Screenshot of Polyphen 2 online tool for analysis of CYP1B1 R390H substitution (http : //genetics.bwh.harvard.edu/pph2/).

The wild type, carrier and mutant genotypes showed the presence of both A and G alleles of this variant in nucleotide sequence of *CYP1B1* gene in POAG patients revealed by Chromas v 1.45 software. Hence, the results validated the existence of both homozygosity and heterozygosity of *CYP1B1* c.1169 G > A variant in the population of primary open angle glaucoma in Pakistan.

In silico analysis was carried out to investigate the hazardous impact of *CYP1B1* c.1169 G > A variant on the functionality of its protein structure due to the substitution of R to H at 390 amino acid position. The PolyPhen 2 online web tool was used to perform the analysis (Adzhubei et al., 2010). The score observed for R390H variant was equal to 1.00 which suggested its higher probability of stimulating benign effect on the functioning of CYP1B1 protein. The screenshot of the analysis was given in Fig. 4. The multiple sequence alignment was also performed for CYP1B1 human protein sequence with other related species using Clustal Omega online tool (Sievers et al., 2011) displayed in Fig. 5. It disclosed the conservation of arginine amino acid at 390 position, emphasized its significance in CYP1B1 protein sequence of Human, pig, bovine, rat, horse, goat, cat, and rabbit species.

4. Discussion

The *CYP1B1* c.1169 G > A variant has been investigated previously in different types of glaucoma among various populations which revealed great diversity (Su et al., 2012; Huang et al., 2018). Some of them were focused on familial based consanguinity for congenital glaucoma (Khan et al., 2019), while some based on case-control comparisons in juvenile and adult forms of primary open angle glaucoma (Suri et al., 2008). Our study revealed the genetic distribution of homozygosity and heterozygosity of *CYP1B1* c.1169 G > A variant among male and females of both juvenile and adult cases of POAG in Jamshoro district of Sindh, Pakistan.

The findings of the current study suggested that the frequencies of heterozygous G/A and homozygous mutant A/A genotypes were elevated in males in comparison to females. Moreover, the frequency of mutant A allele was greater in males (26%) and lower in females (18%). In agreement with our findings, former study reported that the frequency of heterozygous genotype of *CYP1B1* c.1169G > A variant was higher in males 21(60%) compared to the frequency in females 14(40%) in primary congenital glaucoma (PCG) patients (Khan et al., 2019). Another supported study conducted by Vasiliou and Gonzalez (2008), revealed the higher frequency of male subjects (65%) in comparison to female subjects (35%) in primary glaucoma patients. However, the frequency of *CYP1B1* c.1169 G > A variant was reported to be fluctuated from 10 to 100% in distinctive populations of primary glaucoma patients (Bejjani et al., 2000; Sitorus et al., 2003; Reddy et al., 2004). Moreover, the outcomes of the current study are comparable with the prevalence of *CYP1B1* c.1169G > A variant in Europe (22.2%), China

Fig. 5. Multiple sequence alignment of CYP1B1 R390H variant in protein sequence of eight species by Clustal Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

(17.2%) and in Indonesia (33.3%) (Sitorus et al., 2003; Chen et al., 2008; Tanwar et al., 2009). However, the higher prevalence of *CYP1B1* variants was reported from Iranian (70%) and Indian (44%) populations so far (Chitsazian et al., 2007). A previous report evident that the carrier genotype of *CYP1B1* c.1169G > A variant was observed in approximately 20% of total PCG cases (Khan et al., 2019). Thus, the findings indicated that the heterozygosity and homozygosity of *CYP1B1* c.1169G > A variant might be associated with the pathogenesis of POAG in Pakistani population.

Our study also revealed that the juvenile patients of POAG exhibit higher frequency of heterozygosity (G/A). In contrast, the adult patients of POAG showed lowered frequency of heterozygous and homozygous mutant genotypes of *CYP1B1* c1169 G > A variant. Earlier studies from Spain, France and Canada demonstrated the heterozygosity of *CYP1B1* variants in the diagnosed patients of POAG (Vincent et al., 2002; Melki et al., 2004; Lopez-Garrido et al., 2006). Contrastingly, another study defined higher prevalence of homozygosity for R390H variant recognized in POAG patients. While, the onset age of POAG patients were mostly nearby 40 years and PCG patients was around 2 years. This indicated that POAG was more likely to be associated with homozygosity of *CYP1B1* variants (Micheal et al., 2015). Our findings also elaborated the significant association of IOP variable with homozygous A/A mutant genotype of *CYP1B1* variant in females of POAG patients. Correspondingly, the homozygous A/A genotype of *CYP1B1* R390H variant was observed to be frequently associated with primary congenital glaucoma patients in various populations of the world (Suri et al., 2009; Bashir et al., 2018). Besides that, heterozygous G/A genotype was majorly reported in adult form of POAG patients in France (Melki et al., 2004) and homozygous G/G genotype in JOAG patients in Tehran (Suri et al., 2009). Scientists suggested that gene dosage is probably a rare mechanism in modulation of late expression in the cases of POAG, as their familial pedigree did not demonstrate any evidence of glaucoma. The likely explanation might be the participation of modifier locus, revelation to extrinsic factors, and possible variabilities in dopamine pathway (Micheal et al., 2015).

Current study also revealed the high value to Poly Phen 2 score, and thus validated its benign impact on the adequate regulation and functioning of CYP1B1 protein. The R390H alteration influence the conserved motif of arginine amino acid into the loop of helical turn of *CYP1B1* protein K helix (Khan et al., 2019). This mutation was also projected to be involved in development of salt bridges, which becomes disoriented by the incorporation of histidine residue at 390 position (Monemi et al., 2005). Interestingly, a former study observed that eight out of eleven POAG cases carries two mutated alleles of *CYP1B1* genetic variants (Bayat et al., 2008). Recently, Emamalizadeh et al. (2021) identified three novel *CYP1B1* mutations in Iranian families affected with primary congenital glaucoma. But, the occurrence of this discordance might be the outcome of synergistic effect of variations in *CYP1B1* variants both in adult and juvenile form of POAG, and the variance in proportion of samples. Though, the US National Library of Medicine (https://medlineplus.gov/genetics/), denoted that the juvenile form (JOAG) influenced approximately one out of fifty thousand individuals and adult form (POAG) affects nearly one percent of aged 40 years or above individuals (Suri et al., 2008).

The contradictory trends detected in *CYP1B1* genetic variants might be due to the dissimilar impact of its alleles in different populations and ethnicities, probably due to the presence of linkage disequilibrium with nearby and distinct variants of *CYP1B1* gene. Though, only a limited proportion of research have disclosed the mutational spectrum of *CYP1B1* R390H variant in adult patients of POAG disorder (Patel et al., 2012); (Micheal et al., 2015; Gong et al., 2015). Further studies are required for comprehensive analysis of *CYP1B1* whole exome variants, haplotypic associations, linkage disequilibrium plots and their single and synergistic roles in the progression towards the pathogenesis of POAG.

5. Conclusion

The outcomes concluded that *CYP1B1* c.1169 G > A (rs56010818) is a predominant variant which could be considered as a probable cause of primary open angle glaucoma (POAG). The heterozygosity of *CYP1B1* variant was more abundant in males and mutant homozygosity was observed to be more prevalent in females. The data will provide a reference line for the correlation analysis of genotypes with distinctive phenotypes of POAG patients in future. The characterization of homozygosity and heterozygosity in *CYP1B1* c.1169 G > A variant will be helpful in comprehension of the genetic underlying causes of origin for the pathogenicity of POAG.

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None.

7. Authors' contributions

All authors contributed in data analysis, drafting or revising the article, gave final approval of the version to be submitted for publication and agree to be accountable for all aspects of work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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