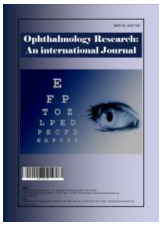

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Cytochrome P450 Family 1 B1 Gene Mutations in Primary Congenital Glaucoma Affected Egyptian Patients

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Authors' contributions

This work was carried out in collaboration among all authors. Author AFE managed the data collection, patient clinical and surgical care, obtaining blood samples, conceptualization, literature review, drafting, editing and proofing. All authors participated in experimental sections, data analysis and acquisition, manuscript preparation, editing, reviewing and guarantor. All authors managed the literature research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Primary congenital glaucoma (PCG) is a leading cause of childhood blindness. The cytochrome P450 family 1, subfamily B, polypeptide 1 (*CYP1B1*) is the most mutated gene that is associated with PCG. Very few studies have examined the promoter region and exon1 of the *CYP1B1* gene. This work was planned to contribute to the description of the possible causative mutations of *CYP1B1* gene that are related to PCG affected Egyptian patients.
Patients and Methods: Patients diagnosed as glaucomatous based on their symptoms and detailed ophthalmological examinations at the time of presentation underwent an intraocular pressure lowering surgical procedure. Investigations were further proceeded on the molecular level. Sequencing-based mutation screen for the promoter region, exon1 and the coding region of exon3

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of *CYP1B1* gene have been performed in two related consanguineous PCG affected families and four other sporadic Egyptian patients using the polymerase chain reaction (PCR) assay; where PCR products were sequenced, and further analyzed.

Results: Sequencing analysis revealed three novel mutations in PCG affected patients one in the promoter region (g.G2872A) and two in exon1 (g.C3268T and g.C3332T). Two additional mutations in exon3 (p.L432V and p.N453S) reported for the first time in PCG affected Egyptian patients. Clinical and genetic data of the two consanguineous families revealed that although the four parents have the same variations as their sons, they are ophthalmologically free.

Conclusion: Regular ophthalmic examinations of siblings and parents of these affected patients should take place for early detection of any form of glaucoma to allow prompt diagnosis and early treatment when needed.

Clinical examination and molecular genetic data could contribute to early diagnosis and prevention of the visual impairment caused by PCG. This study provides groundwork for expanded genetic investigations in Egypt paving the way for genetic counseling to help affected families make informed medical and personal decisions.

Keywords: Cytochrome P450 1B1; CYP1B1; nucleotide mutations; primary congenital glaucoma; PCG; Egypt.

ABBREVIATION

SNP: Single Nucleotide Polymorphism;

1. INTRODUCTION

Primary congenital glaucoma (PCG) is a leading cause of childhood blindness, and accounts for 4.2%-5.0% of blindness in the pediatric population [1]. The incidence of PCG occurrence varies from 1: 1250 to 1: 20,000 among different populations [2,3,4], and is reported to be higher in communities where consanguinity is common [5,6].

Primary congenital glaucoma manifests in early infancy and is characterized by an elevated IOP, large cornea, and Haab'sstriae. Variable degrees of corneal haze, photophobia, epiphora, and blepharospasm are also seen [7]. The main pathology in PCG is trabeculodysgenesis which is responsible for impairment of the aqueous humor outflow, with subsequent elevation of the intraocular pressure (IOP) and optic nerve damage [8].

The cytochrome P450 family 1, subfamily B, polypeptide 1 (*CYP1B1*) gene is identified as one of the genes causing primary congenital glaucoma [9]. It has been suggested that *CYP1B1* is involved in the development of the anterior chamber angle [10,11]. Alterations in *CYP1B1* metabolism may directly lead to an abnormal elevation of the intraocular pressure (IOP) of the eye, or indirectly affect the aqueous outflow by disrupting the proper development of the trabecular meshwork in glaucoma patients [12].

Many distinctive mutations in *CYP1B1* gene have been described in different ethnic groups [13,14,15], and most reports pointed out to the mutations in exons two and three [5,12]. Seven novel mutations have been reported in Egyptian PCG patients [16] six of these mutations were in exon2; and one in exon3. The seventh change was a homozygous mutation at g.8076-8079 del CATTinsAAAG in exon3. Very few studies have examined the promoter region of the *CYP1B1* gene as well as exon1 [17,18].

This work relates to the possible presence of mutations in promoter-exon1 region and the coding region of exon3 of *CYP1B1* gene; using the polymerase chain reaction (PCR) assay, where PCR products were sequenced and further analyzed.

2. MATERIALS AND METHODS

This study has followed the tenets of the Declaration of Helsinki (JAMA 1997; 277:925-926) and was approved by the Medical Research Ethics Committee at the National Research Centre, Cairo, Egypt (Ethical Approval Certificate No. 18075). Written consents have been obtained from parents of six patients for surgical procedures and for blood samples. Two patients were members of two related consanguineous families, Blood samples were also taken from parents and /or siblings for genetic studies.

Patients were undergoing an intraocular pressure (IOP) lowering surgical procedure (including goniotomy, trabeculotomy, subscleraltrabeculectomy with adjunctive

mitomycin C, Ahmed glaucoma valve implantation or diode cyclo-photo-coagulation). All patients had an established diagnosis of PCG based on their symptoms and a detailed ophthalmological examination at the time of presentation to our ophthalmic clinic at Aboulreesh Children's Hospital, Cairo University. Presenting symptoms included photophobia, large globe size, and clouding of the cornea. The diagnosis of PCG was based on clinical evidence of raised IOP (>18 mmHg measured by hand held Perkins applanation tonometer (PAT) or a difference of ≥ 5 mmHg between both eyes), a large corneal diameter (>12 mm, measured intraoperatively by caliper) with or without corneal opacity, and optic nerve cupping (seen after pupil dilatation on indirect ophthalmoscopy with +20D lens, when possible, or as evidenced by ultrasonography B-scans). At our institution, IOP is assessed in-office without sedation if the child is old enough to sit at the slit lamp (for anterior segment assessment and Goldmannapplanation tonometry) or cooperate during measurement using PAT, after applying topical Benoxinate® anesthetic eye drops. In office-sedation with chloralhydrate is used in children >6 months and ≤3 years of age. If a child is too young or uncooperative, the IOP is measured in the operating theatre under inhalational anesthesia, with sevoflurane, using a PAT; and the anterior segment is assessed using the operating microscope. Examination conditions for gonioscopy are similar to IOP measurement, when the cornea is clear enough to allow for angle evaluation, using in-office Volk® 4-mirror gonioscopy lens or the Swan Jacob® gonio-prism lens intra-operatively. Suspicious cases are followed up for any signs of progression in ocular biometrics.

In this study we excluded patients with secondary or acquired childhood glaucoma. The age of onset, family history, consanguinity and sibling affection were also recorded. All patients had cycloplegic refractions, and errors of refraction were corrected when possible.

The six patients comprised of two related patients and 4 random ones. The two related patients (P1 and P2) are cousins and belong to two related consanguineous families, where the fathers are two brothers and the mothers are two sisters. Fathers and mothers were also first cousins. Patient 2 has a baby brother (3 years) who is not glaucomatous (followed up every 6 months). All four parents are ophthalmologically free, and give no family history of glaucoma. Clinical data at presentation were tabulated and analyzed using Microsoft Excel.

The present study proceeded with further investigations on the molecular level for the members of the two related families as well as four other random PCG patients including one with concurrent hydrocephalus. This approach included the investigation of the presence of mutations in three different regions of the *CYP1B1* gene (promoter, exon1 and exon3) in affected PCG subjects and members of the consanguineous family. Nucleotide sequences for *CYP1B1* promoterexon1 region and the coding region of exon3 amplimers were analyzed and aligned with the corresponding segments of the *CYP1B1* gene (acc.No. U56438.1).

2.1 Sample Collection and DNA Extraction

DNA was extracted from blood lymphocytes using (Wizard® Genomic DNA Purification Kit, Promega, USA); in accordance with the manufacturer's procedures.

2.2 Primers Design

Based on the *CYP1B1* gene (genBank acc. no.U56438.1), two sets of primer pairs were used in this investigation. The first pair of primers (F:5'AGCGGCCGGGGCAGGTTGTACC3'; R: 5'ATCCATCTGAAGAGGTCGCC3') was designed to amplify a 708 bp segment of DNA representing the promoter region from g.2753 to g.3069 and exon1 (non coding) from g.3070 to g.3461. The second primer pairs (F:5'AATGTGCTTTCTAGATGAAATAAGAA3'; R: 5'CAGCACAAAAGAGGAACTGGA3') amplify a 751 bp segment that cover 69 bp of intron2, coding region of exon3 (588 bp) and 92 bp from the non-coding region of exon3 [19].

2.3 PCR Reactions and Amplification Conditions

PCR reactions were performed in a total volume of 25 µl using 1X NH_4 buffer, 1.5 mM $MgCl_2$, and 10%dimethyl sulfoxide, 0.2 mMdeoxyribonucleoside triphosphate, 2.5 U of ThermoScientific^{TM}DreamTaq DNA Polymerase, 0.5 µM of each primer, and 200–500 ng of human genomic DNA.

PCR reactions were conducted in a Bio-Rad MJ Research PTC-100 thermal cycler. Thermal cycling consisted of denaturation at 95ºC for 4 min, followed by 35 cycles of denaturation at

95ºC for 60s, annealing for 60s and extension at 72ºC for 1 min. Annealing temperatures were 60ºC and 54.6ºC for the first and second primers, respectively.

2.4 Sequencing and Identification of the Genetic Variation Sites

PCR amplicons of the targeted fragments were purified using total fragment DNA purification kit (MEGAquick-spin™, iNtRON Biotechnology, Inc). Sequences were determined in Macrogen company using ABI3730 automated DNA sequencer (Applied Biosystems). The sequences were searched for nucleotide variations by using the BLAST http://www.ncbi.nlm.nih.gov/blast. Polymorphic sites were determined by visual examination of sequence's charts.

3. RESULTS

Six patients (11 eyes) were included in our analysis. The average age at presentation was 4±4.85 months. The mean intraocular pressure (IOP) for all eyes was 26.5±8.1 mmHg at presentation and 12.58±3.99 mmHg at the last visit (mean follow up period = 54.2 months-range= 11-144 months). The average number of operations for each eye was 2.25±1.48. Table 1 shows the clinical data at presentation and last follow up visit for all 6 patients. Data are expressed as mean ± standard deviation.

In the present work *CYP1B1* gene Promoter–exon1 region and sequence flanking exon3 were investigated for the presence of genetic variations in P1 and P2, their parents and sibling as well as four random patients (P3, P4, P5 and P6) and were further submitted to the Genbank data base (MG181951.1 and MG014360.1, respectively).

Sequence analysis revealed the presence of six polymorphic sites. Three in the coding region of exon3 C>G, C>T and A>G at nucleotide positions (nt) 303, 356 and 367 (MG014360.1) that correspond to g.8131 (c.1294), g.8184 (c.1347) andg.8195 (c.1358) positions, respectively in the reference *CYP1B1* gene acc. no. U56438.1. Mutations at c.1294 and c.1358 were nonsynonymous, they are translated to p.L432V and p.N453S, respectively. Two polymorphic sites located in exon1 at nt.503 and nt.566 (MG181951.1) that correspond to g.3269 and g.3332 positions, and one polymorphic site was in the promoter region, at nt.106 (MG181951.1) which corresponds to g.2872 in GenBank entry 56438.1.

The first Patient (P1) presented at the age of seven months with large corneas, however his IOP was 8 and 10 mmHg in the right and left eyes respectively. A final diagnosis was reached at 13 months and he received bilateral goniotomy then. His glaucoma showed a refractory course and there was advanced bilateral optic nerve cupping denoting advanced disease. He developed cataract in the left eye and had cataract surgery and intraocular lens (IOL) implantation at the age of 3 and a half years. A year later he required bilateral Ahmed Glaucoma Valve (AGV) implantation for IOP control (after failure of filtering procedures).

Patient1 (P1) was heterozygous at three sites in exon3; (c.1294C/G:p.L432V), (c.1347C/T:p.D449D) and (c.1358A/G:p.N453S). His parents were also heterozygous at the three sites as their son. P1 and his mother also exhibited a C/T heterozygous site in exon1 at nt g.3332. Patient 2 (P2) (a cousin of P1) on the other hand, presented within the first month of life with bilateral raised IOP, large corneas (with left dense central opacity) and evidence of bilateral cupping of the optic nerve head. P2 had more advanced disease in the left eye from the start, which required AGV implantation for IOP control at the age of 13 months. The right eye, however, achieved a qualified success after subscleraltrabeculectomy with adjunctive mitomycin C. Sequencing analysis for the investigated segment, in P2 revealed only one homozygous mutation in exon3 at c.1358A>G:p.453S. Both his father and brother had the same homozygous mutation (c.1358A>G:p.453S), while his mother was heterozygous at this site. No mutation was found in the promoter-exon1 region in P2 or members of his family.

Patient 3 is non consanguineous and has three older siblings who are not glaucomatous. Glaucomatous changes in his left eye are more advanced and resistant to treatment than the right eye. He has three heterozygous sites; one in exon3 at c.1358A/G:p.N453S, a second one in the promoter region at nt. g.G2872A and the third one is in exon1 at nt. g.C3269T.

Patient P4 had left AVG implantation and bilateral cataract surgery and intraocular lens (IOL) implantation. Her best corrected visual acuity (BCVA) at last follow up was 6/24 (OD) and 6/36 (OS). Patient P5 has 4 siblings, one of them is known glaucomatous. P5 received one angle surgery in each eye (180˚infero-nasal

trabeculotomy), and her IOP at last follow up (2 years) was 10 and 12 in the right and left eyes respectively without medications. Both P4 and P5 had two distinct mutations in exon3. They were G and T homozygous at c.1294 and c.1347, respectively. Mutation at Position c.1294 is non-synonymous c.1294C>G:p.L453V which causes amino acid change from leucine (L) to valine (V), whereas the other mutation c.1347 C>T:p.D449D was synonymous. No mutations were detected in the promoter-exon1 region in both patients.

Patient number six (P6) is hydrocephalic and had unilateral glaucomatous changes in his right eye with raised IOP 22 mmHg at the age of six months. He underwent a unilateral surgery. His IOP at the last follow-up was 18 mmHg and 14 mmHg for his right and left eyes respectively. P6 showed no heterozygosity or mutations at any of the above mentioned sites. He has homozygous wild type alleles.

For IOP control; all patients except P5 required combination eye drops of beta-blocker/carbonic anhydrase inhibitor (administered twice daily) to reach a qualified success. P5 attained complete success after angle surgery without the need for such medications. Table 2 represents the collective genotype/phenotype of patients positive for the *CYP1B1* mutations in promoter-exon1 region and exon3.

4. DISCUSSION

In the present work, we investigated, for the first time in Egypt, the presence of mutations in exon1 and the promoter region of *CYP1B1* gene. Relatively limited studies have examined the promoter region and exon1 of this gene [17,18]. In addition, the coding region of exon3 was also investigated in members belonging to two PCG affected consanguineous families as well as four other sporadic PCG affected Egyptian patients. Sequencing of the *CYP1B1* promoter and the non coding exon1 in our patients revealed three novel variations (g.G2872A, g.C3269T and g.C3332T).

Three mutations were identified in exon3: p.L432V, p.D449D, and p.N453S. Although the first and third mutations (p.L432V and p.N453S) have been reported in other communities [12,14,20]; yet this is the first time to be reported in PCG affected Egyptian patients. The previously reported mutations in exon3 in PCG affected Egyptian population were P.D449D and H413_I414 delins QK P [16], N498D [21] and R368H [22]. The later three mutations were not detected in our studied patients.

In order to predict whether an amino acid substitution due to the presence of the heterozygosity would affect protein function and hence, potentially alter phenotype, sorting intolerant from tolerant (SIFT) amino acids prediction analysis [23,24,25] was applied.

In the present work, the substitution at pos 432 from Leucine to Valine present in patients P1, P4 and P5 was predicted to be tolerated which was previously reported to be benign to protein function [10]. However Bhattacharjee and his colleagues [26] reported that *CYP1B1* protein with valine at position 432, which is present in patients P4 and P5, generates more free radicals and causes oxidative damage to retinal pigment epithelial (RPE) cells. Two mutations (p.R117W and p.G329V) reported in Turkish PCG families [27] showed considerably reduced CYP1B1 enzyme activity. On the other hand, SIFT analysis showed that substitution present in patient P2 at pos 453 from Asparagine (N) to Serine (S), has a score of 0.00 which may affect the protein function. This low prediction confidence may be attributed to the limited number of investigated sequences. Therefore analysis of a larger number of patient samples are required to confirm SIFT analysis results for this site. The third substitution reported in this study in exon3, p.D449D was predicted to be tolerated with a SIFT score of 1.00. This substitution has been previously reported to be benign to protein function [15].

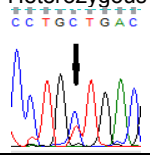
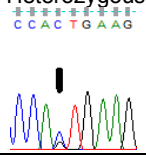
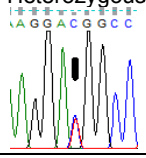
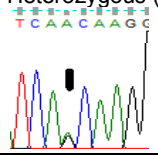
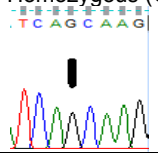
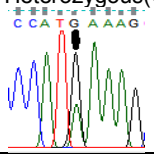
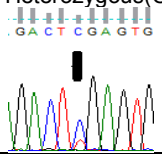
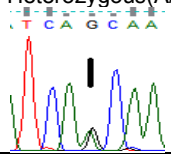
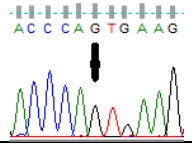
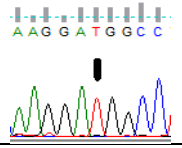
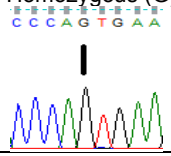
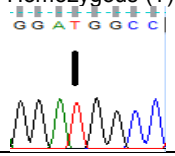
In the present study, although P1 and P2 have variable clinical parameters (Table 1) both patients had advanced disease in both eyes which required the implantation of Ahmed glaucoma valves (AGV) and several diode cyclophotocoagulation procedures. It is noteworthy that P1 is heterozygous at four of the identified sites and P2 has only a single homozygous mutation (at c.1358G; p. 453S); P1 has bilateral clear corneas and a higher cup to disk (CD) ratio (0.8) in both eyes. It has been reported that patients with compound heterozygous *CYP1B1* alterations may exhibit less disease severity than those with homozygous alterations [28], since in case of patients with heterozygous alterations; the normal allele is able to compensate for the mutant allele [15]. However, in case of P1, despite his advanced disease as evidenced by the large optic nerve cups, he developed the full picture of PCG as late as the second year of his

Table 1. Clinical data of patients at presentation and at last follow up

Patient	Sex	Age at presentation (months)	laterality	IOP at presentation		IOP at last follow up		No. of surgeries		AXL* (mm)		Corneal diameter*(mm)		CD ratio*		Refraction* (Diopters)†		Notes
				R	L	R	L	R	L	R	L	R	L	R	L	R	L	
1	M	13	bilateral	32	32	14	7	3	5	27	27	15	15	0.8	0.8	-16	-17	Bilateral AGV, (L)Cataract
2	M	1	bilateral	24	37	15	4	2	4	21	28	15	14	0.7	0.6	-11	N/A	(L) dense corneal opacity, AGV, RD
3	M	2.5	bilateral	22	38	12	14	1	3	22	23	12	14	0.4	0.6	+0.5	-2.0	left eye is more advanced and resistant to treatment than the right eye
4	F	0.5	bilateral	21	28	14	17	3	3	25	25	13	13	0.5	0.7	-4.0	-3.0	Bilateral AGV, cataract and IOL
5	F	1	bilateral	20	32	10	12	1	1	N/A	N/A	12.5	12.5	0.4	0.7	+6.0	+4.0	Required no medical treatment
6	M	6	unilateral	22	10	18	14	1	0	28	24	12	12	0.2	no	-17	-16	Hydrocephaly

M=male, F-female, R=right, L=left, AXL= axial length, IOP=intraocular pressure, CD= cup to disc ratio, AGV= Ahmed glaucoma valve, RD= retinal detachment, IOL= Intraocular lens.
** At the last follow up visit. † In spherical equivalent. N/A= not applicable*

Table 2. Illustrated collective genotype/phenotype status of patients positive for the *CYP1B1* mutations in promoter-exon1 region and exon3

Patients / Phenotype*	Genotype‡					
	Promoter	Exon one		Exon three		
	g.2872 G>A	g.3269 C>T	g.3332 C>T	c.1294 C>G	c.1347 C>T	c.1358 A>G
P1 Sever status	Normal (G)	Normal (C)	Heterozygous (C/T) 	Heterozygous (C/G) 	Heterozygous (C/T) 	Heterozygous (A/G) 
P2 Sever status	Normal (G)	Normal (C)	Normal (C)	Normal (C)	Normal (C)	Homozygous (G) 
P3 Moderate to sever status	Heterozygous(G/A) 	Heterozygous(C/T) 	Normal (C)	Normal (C)	Normal (C)	Heterozygous(A/G) 
P4 Moderate to sever status	Normal (G)	Normal (C)	Normal (C)	Homozygous(G) 	Homozygous (T) 	Normal (A)
P5 Mild to moderate	Normal (G)	Normal (C)	Normal (C)	Homozygous (G) 	Homozygous (T) 	Normal (A)

*All changes were assigned a nucleotide number according to the GenBank entry U56438.1; * Patient's phenotype (status) based on C/D ratio and corneal clarity at presentation;*
‡Electropherograms of DNA sequences representing genotypic variations in promoter-exon1 and exon3 regions of CYP1B1 gene in PCG affected patient in the present study

life and at the last follow up at the age of six years (Table 1), his best corrected visual acuity (BCVA) was 6/36 (OD) and 3/60 (OS). It has been stated that different combinations of *CYP1B1* mutations may produce various degrees of structural alterations in the trabecular meshwork and Schlemm's canal that closely correlate with the severity of the disease [29]. It is noteworthy to mention that P1 exhibited alterations at position 432 which was previously reported to cause damage to the RPE cells; this may explain, in part, the advanced changes seen in his posterior segment (namely a CD ratio= 0.8) despite bilateral clear corneas [26]. P2 on the other hand, presented at the age of one month (earlier than P1) with advanced diseases. He has a homozygous mutation at p.453S in exon3, while no mutations were present in the promoter-exon1 region. Previous studies showed that, patients with homozygous *CYP1B1* mutations have more severe phenotypical presentation compared to patients with heterozygous mutations for this gene [15]. P2 had a full picture of raised IOP, large corneas and optic nerve head cupping which was more advanced on the left side as evidenced by the dense corneal opacity. P2 also had a measured C/D ratio equal to 0.7 and 0.6 for right and left eyes respectively (Table 1). Decreased cupping of the optic disk with a milder alteration of the visual field is known to be associated with the p.453S serine allele [17].

P2 shared the same homozygous mutation p. 453S with both his father and brother; who are both non-affected. While his mother, who is also non-affected, has a heterozygous change at the same locus (p.N453S).The disease phenotype was not represented in any of the family members of this patient (P2). These observations suggest that the reported mutations have low phynotypic penetrance association with primary congenital glaucoma. Lack of the disease phenotype [30] was also reported, in some cases of non-affected individuals having the same mutations in *CYP1B1* gene as affected ones.

Many authors reported variable rates of penetrance among PCG affected families ranging from incomplete penetrance [31] to high penetrance rate (9). Other authors suggested the presence of a dominant modifier locus that is not linked genetically to *CYP1B1* (14), or that the affected *CYP1B1* genotypes may exhibit variable expressivity rather than nonpenetrance [32]. Moreover, López-Garrido and colleagues [33], suggested a multi-gene involvement in PCG inheritance patterns. This in its turn suggests that additional genetic studies besides *CYP1B1* should be investigated and that regular ophthalmic examinations of siblings and parents of these affected patients should take place for early detection of any other form of glaucoma to allow prompt diagnosis and early treatment if needed.

In the present work, three novel mutations were also identified in the promoter–exon1 region. Patient P3 has two heterozygous changes one in the promoter (g.G2872A) and the other in exon1 (g.C3269T) in addition to the heterozygous change p.N453S in exon3. It has been suggested that variation in *CYP1B1* promoter, may be responsible for the disease [34]. Chakrabarti and colleagues [34] reported a strong evidence of a functionally characterized association of rs2567206 (T2805C) SNP in PCG affected patients. Glaucomatous changes in the left eye of P3 are more advanced and resistant to treatment than the right eye. Despite the presence of a number of PCG-associated mutations in *CYP1B1* gene yet, phenotypic variability has been documented in PCG affected patientshaving these mutations [35,36]. Direct correlation of the mutant genotype with the disease phenotype [37] needs more confirmation to be established.

Two of the sporadic patients P4 and P5 had the same genotypic pattern in all investigated sites. Both had homozygous mutations C>G at c.1294 (p.432V) and C>T at c.1347 (p.449D). However they differed in the severity of the disease, P4 presented as early as six months, she required three surgeries in each eye to reach a controlled IOP equal to 14 mmHg and 17 mmHg for right and left eyes respectively whereas P5 required only one surgery in each eye to reach a satisfactory IOP at last follow-up and did not require medical treatment. In previous studies, patients having the same mutations in *CYP1B1* gene were noticed to show different degrees of disease severity, age of onset, or even absence of the disease phenotype [30].

P6, the hydrocephalic patient, is a unilateral PCG patient with small cupping at the optic nerve head has no detected mutations. The association of PCG and hydrocephalus has been previously reported [38,39]. The milder phenotypic glaucoma presentation of P6 could also be attributed to the lack of either homozygous or heterozygous mutations in the coding region of CYP1B1 [15]. It has been suggested that patients lacking mutations in CYP1B1 may have

other genetic loci involved in the pathogenesis of PCG [5].

Because of the scarcity of studies in understanding the genetic basis of cases without *CYP1B1* mutations, some efforts were made to study the molecular involvement of other genes. Chakrabarti [40] and his colleagues identified two novel candidate genes in PCG; the kelch like family member 26 (*KLHL26*) and teashirt zinc finger homeobox 2 (*TSHZ2*). These have been earlier implicated in regulating aqueous humor outflow and embryonic development respectively.

A major limitation to our study is the small sample size and the absence of a control group. Certainly a larger scale study involving all patients attending our glaucoma service, at Aboulreesh Children's Hospital, in Cairo University, would provide us with a complete understanding of how prevalent these novel mutations are amongst our PCG patients; it could help us link them to the clinical presentation or even design strategies for prevention of such disabling morbidity. However, this would require a larger scale program and funding.

5. CONCLUSION

The present investigation revealed the presence of six different mutations distributed on the promoter-exon1 region and the coding region of exon3 of *CYP1B1* gene. To the best of our knowledge, three of these mutations are novel mutations reported for the first time in the present study which in its turn provides groundwork for expanded genetic investigations in Egypt.

Clinical and genetic data of the two consanguineous families, in the present study revealed that although the four parents have the same variations as their sons, they are ophthalmologically free giving no family history of glaucoma and did not develop glaucoma to the present. These observations suggest that additional genetic studies besides *CYP1B1* should be investigated and that regular ophthalmic examinations of siblings and parents of these affected patients should take place for early detection of any other form of glaucoma to allow prompt diagnosis and early treatment if needed. Clinical examination coupling with molecular genetic data could provide a basis to contribute to early diagnosis and preventing the visual impairment caused by primary congenital glaucoma.

CONSENT

All authors declare that "written consents have been obtained from parents of six patients for surgical procedures and for blood samples. Two patients were members of two related consanguineous families, Blood samples were also taken from parents and /or siblings for genetic studies".

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Medical Research Ethics Committee (Ethical Approval Certificate No. 18075) at the National Research Centre, Cairo Egypt, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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