Three Novel *CYP1B1* Mutations (p.L480P, p.S476P, p.R175P) in Primary Congenital Glaucoma Cases Residing in Eastern Iran

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Abstract

Background purpose: Primary Congenital Glaucoma (PCG) is typically an autosomal recessive trait and is more prevalent in community with consanguineous marriage. The aim of current study was to screen 27 familial cases of PCG for *CYP1B1*, to identify and determine common mutations, and to understand its penetrance and prevalence in the Eastern provinces of Iran.

Methods: Detailed family histories up to three generations were taken, and pedigree charts were constructed. Genomic DNA was extracted from peripheral leukocytes. Primers were designed for the two coding exons of the *CYP1B1* gene and the amplified products were sequenced. PolyPhen and SIFT were used to predict the functional impact of novel mutations identified in this study.

Results: Seventeen of 27 subjects (62.96%) had mutations in the *CYP1B1* gene. In this study, 10 specific mutations associated with disease phenotypes were found. Six missense (p. R368H, p.E229K, p.R390C, p.V364M, p.F445I, p.G61E) and one deletion mutation (c.1504_1504delA) were previously reported and 3 missense mutations (p.L480p, p.S476P and p.R175P) were novel. The most common mutation was G61E, which was identified in 8 of 17 cases (47.05%). We also notified that one of the patients was homozygous for the mutation E229K, and also R390C (tetra-allelic).

Conclusion: Mutations in *CYP1B1* was a major finding in our PCG patients. Identifying mutations in subjects at risk of developing glaucoma, particularly among relatives of PCG patients, is of clinical relevance. These findings may help in reducing the disease frequency in familial cases through proper counseling. Such studies will be of benefit in the identification of pathogenic mutations in different populations and will enable us to develop simple and rapid diagnostic tests for analyzing such cases.

Keywords: Primary congenital glaucoma; Novel mutation; *CYP1B1* gene

Introduction

Glaucoma is a group of disorders characterized by distinct optic nerve head damage and accompanying irreversible visual field defects. Progression is the natural course of the disease and absolute blindness is usually the fate of untreated cases. Glaucoma affects 2% of European and 10% of African populations and is a leading cause of blindness worldwide [1]. Usually, glaucoma is age-dependent and slowly progressive. However, it could start at any age. In its early stage, disease progression can be halted by proper medications and/or surgery; this highlights the importance of early diagnosis and management.

Primary Congenital Glaucoma (PCG) typically has an onset before age 3 and is caused by developmental anomaly in the trabecular outflow tract; hence, the patients have characteristically very high IOP [2,3]. PCG is typically an autosomal recessive trait and is more prevalent in community with consanguineous marriage [4-6]. The reported prevalence of PCG varies between 1/20,000 children in Western countries and 1/2,500 in Saudi Arabia, 1/1,250 in Slovakian Rom, and 1/3,300 in Andhra Pradesh, India [7-9]. Four loci, namely GLC3A (2p22-p21) [10], GLC3B (1p36.2-p36.1) [11], GLC3C (14q24.3) [12], and GLC3D (14q24) [5], and two genes, *CYP1B1*, coding for cytochrome P450, family 1, subfamily B, polypeptide 1 at GLC3A [13], and more recently, *LTBP2*, coding for latent transforming growth factor-beta-binding protein 2 at GLC3D [14,15] have been identified for PCG through linkage studies. *CYP1B1* have two coding exons and encodes the cytochrome P450 superfamily, subfamily B, polypeptide 1, a 543 amino acids long protein. *CYP1B1* is expressed both in the posterior segment of the eye and the trabecular meshwork [16]. Till now, at least 150 mutations associated with PCG have been found in *CYP1B1* [17].

CYP1B1 mutations have incomplete penetrance and variable expression in different ethnic groups [18,19]. *CYP1B1* mutations have been found in almost all of PCG cases in Saudi Arabia [8] and Slovakian Rom [7], and approximately 50% of Brazilian cases [20], 30% of Indonesian cases [21] and 20% of Japanese cases [22].

To investigate haplotypes associated with the mutant chromosomes in large set of PCG cases in Slovalian Rom [7], Saudi Arabia [8], Brazil [20] and United States [23], five intragenic SNPs in *CYP1B1*, rs10012 (R48G; exon 2), rs1056827 (A119S; exon 2), rs1056836 (V432L; exon 3), rs1056837 (D449D; exon 3), and rs1800440 (N453S; exon 3) were used.

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This study was aimed to screen 27 familial cases of PCG for *CYP1B1*, to identify and determine common mutations, and to understand its penetrance and prevalence in the Eastern provinces of Iran.

Materials and Methods

Clinical evaluation and patient selection

A cross-sectional study was designed and retrospective evaluation of patients' records was done. The study adhered to the tenets of declaration of Helsinki. All included cases or their legal guardians provided written informed consent to be included in the study. Twenty-seven PCG families were enrolled in this study and pedigrees were drawn to determine inheritance pattern. To confirm PCG diagnosis, systemic evaluation was performed in pediatric service as a part of routine pediatric care and ocular examinations were performed both in clinics during preoperative evaluation and during Examination Under Anesthesia (EUA) prior to proceeding to surgery. PCG diagnosis was based on an increased corneal diameter (more than 10 mm in premature neonates, more than 11 mm in term neonates and more than 12 mm after 1st birthday), high intraocular pressure (more than 18 mmHg) and the presence of glaucomatous optic nerve cupping. All included cases were diagnosed before 3 year-old and had at least one ocular examination before their 3rd birthday. Neglected PCG cases were not included in this study to ascertain the homogeneity of the study group. The presence of Haab's striae was a confirmatory finding, but was not essential for diagnosis. None of the included cases had other major ocular anomalies contributable to a secondary glaucoma. Moreover, none of the included cases was a syndromic case. All cases were evaluated by a single glaucoma specialist (RD) and a diagnosis of PCG was confirmed by him. Detailed family histories up to three generations were taken, and pedigree charts were constructed by a clinical geneticist (FA). The history of ocular or other hereditary disorders was recorded. Fifty ethnically matched normal individuals without any ocular disorders were enrolled as controls.

Mutation screening and sequence analysis

Genomic DNA was extracted from peripheral leukocytes with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored at -20°C. Primers were designed for two coding exons of the *CYP1B1* gene using Primer3 web tool (Table 1). PCR amplification was accomplished in a thermal cycler T-Personal (Biometra, Germany) in a final volume of 50 μl. The PCR mix contained 300 ng of genomic DNA, 2 mM MgCl$_2$, 200 μM each dNTP, 5% of DMSO, Promega 10X Reaction Buffer diluted to a 1X, 2 units of Taq DNA Polymerase (Cat.# M1861) and 1 μM of each primer in 50 μl.

The cycling conditions included an initial denaturation (95°C/10 min), followed by 33 cycles of denaturation at 95°C for 0.5 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. PCR products were visualized on 1% agarose gel and purified. The amplified products were sequenced in both forward and reverse directions with the same primers as used in the PCRs, using the ABI Big Dye Terminator chemistry and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA) and were analyzed by using Chromas v 2.4.

Primer sequence(5-3)	PCR product (bp)
2F- AGACCACGCTCCTGCTACTC	930
2R- CGCCGAAGATGTCAGTGATAGTG	
3F- TCGAGTGCAGGCAGAATTGGAT	717
3R- CACCTCACCTGATGGACAGTTGAT	

Table 1: PCR primers used for *CYP1B1* amplification.

Computational assessment of missense mutations

In this study, two homology-based programs PolyPhen (polymorphism phenotyping; Division of Genetics, Department of Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, MA) [24] and SIFT (sorting intolerant from tolerant; the J. Craig Venter Institute Rockville, MD and La Jolla, CA) [25] were used to predict the functional impact of identified novel mutations.

Results

Twenty seven families with one or more PCG cases were included in this study. All of the included cases had regular examination and paper charts in Khatam Eye Hospital. This hospital is the major tertiary referral eye hospital in Northeastern part of Iran and serves the adjacent 7 provinces out of a total 31 provinces of the country. Among included families, 19 had consanguineous marriage. Pedigree analysis demonstrated an autosomal recessive pattern of inheritance.

In direct sequencing of *CYP1B1* coding exons, 17 families (62.96%) have had mutations in this locus and 10 specific mutations related to patient phenotype were found. This article describes these seventeen families. Among the ten variants, six missense mutations (p.R368H, p.E229K, p.R390C, p.V364M, p.F445I, p.G61E) and a 1 bp frameshift deletion mutation (c.1504_1504delA) had previously been reported (Table 2); while three missense mutations (p.L480P, p.R175P, p.S476P) were novel (Table 3). The clinical features of all included subjects with *CYP1B1* mutations have been summarized in Supplementary file 1. We performed Next Generation Sequencing (NGS) in one of the ten families without a *CYP1B1* mutation and the affected case had a homozygote mutation in *LTBP2*. This homozygous mutation was c.895C>T p.Arg299* on gene *LTBP2*. The gene *LTBP2* is Glaucoma, primary type 3D, late infantile, variant.

G61E was the most frequent mutation, found in eight out of 17 families (47.05%) with PCG and mutation in *CYP1B1*. In F1 family, there was one subject with a G61E homozygous mutation who was normal in clinical investigation. On the other side, in F4 family, a definite PCG case was heterozygote for G61E mutation; no other mutation in *CYP1B1* was detected in this case. We checked the gene coding in these two exons and did not find any other mutations in this patient. Also, the patient's father did not have any mutation.

Family history	Consanguinity	Exon 3	Exon 2	Hom/ Het*	Family ID
-	Yes		G61E	Hom	F1
-	Yes		G61E	Hom	F2
-	No		G61E	Hom	F3
-	Yes		G61E	Het	F4
Yes	Yes		G61E	Hom	F5
Yes	Yes		G61E	Hom	F6
-	Yes		G61E	Hom	F7
-	No		G61E	Hom	F8
-	No	R390C	E229K	Hom	F9
Yes	Yes	F445I		Hom	F12
-	Yes	R368H		Hom	F13
-	Yes	V368M		Hom	F14
-	Yes	V368M		Hom	F15
-	Yes	c.1504delA		Hom	F16

*Hom, homozygous; Het, heterozygous

Table 2: PCG patients with *CYP1B1* mutations.

Polyphen	Mutation type	Hom/ Com Het	cDNA location*	Gene location*	New Mutation	Family ID
Probably damaging	Missense	Com Het with R368H	c.1426T>C	g.10254T>C	S476P	F10
Probably damaging	Missense	Com Het with G61E	c.524G>T	g.6317G>C	R175P	F11
Probably damaging	Missense	Com Het with R368H	c.1430T>C	g.10267T>C	L480P	F17

Table 3: Analysis of novel mutation detected in PCG patients.

*Reference sequences used were NT_022184.16, NM_000104.3, NP_000095.2. (Com Het, compound heterozygous).

Homozygous R368H mutation was found in a member of a PCG family (F13). This patient had a peak IOP of 26 mmHg and a haze cornea. In F9 family, a PCG patient was homozygous for E229K and R390C mutations. The patient was 8 years old at the time of manuscript preparation with a maximum IOP of 32 mmHg; unfortunately his left eye was lost during a glaucoma shunting procedure elsewhere. His parents were clinically normal and compound heterozygote for E229K and R390C mutations.

Homozygous V364M mutation was found in two patients of families F14 and F15. Also F445I mutation was found in a PCG family (F12). In this case the first recorded IOP was 30 mmHg and the cornea was haze. Other members of this large family were heterozygote for this mutation. In F16 family, a PCG case had a 1 bp deletion mutation in c.1504delA as a homozygous mutation. This case was 11 month old at the time of manuscript writing with the first recorded IOP of 46 mmHg and a haze cornea. Nucleotide A deletion in genomic location g10332 and coded nucleotides c.1504 (c.1504_1504 delA) cause a frameshift in exon 3 of *CYP1B1* gene. This deletion mutation changes the methionine codon in location 503 to a termination codon and produces a truncated protein in which the aminoacids of carboxyl end of *CYP1B1* polypeptide are omitted.

Identification of three novel mutations

CYP1B1 sequencing in a family with a PCG case (F11) detected compound heterozygote mutations for known G61E and novel R175P missense mutation (Figure 1). This patient was 5 years old at the time of manuscript preparation and the first recorded IOP was 25 mmHg. This p.Arg175Pro mutation in g.6317G>C genomic location and c.524 (c.524 G>C) coded nucleotide, substitutes a guanine base with a cytosine base and changes an arginine amino acid to a proline. PolyPhen bioinformatics software predicted this as a pathogenic mutation. Notably, we did not find this mutation in any of the control chromosomes.

Moreover, we found two other novel mutations in two families with PCG cases. In F17 family, a PCG case was compound heterozygote for a novel missense L480P mutation and R368H mutation (Figure 1). The
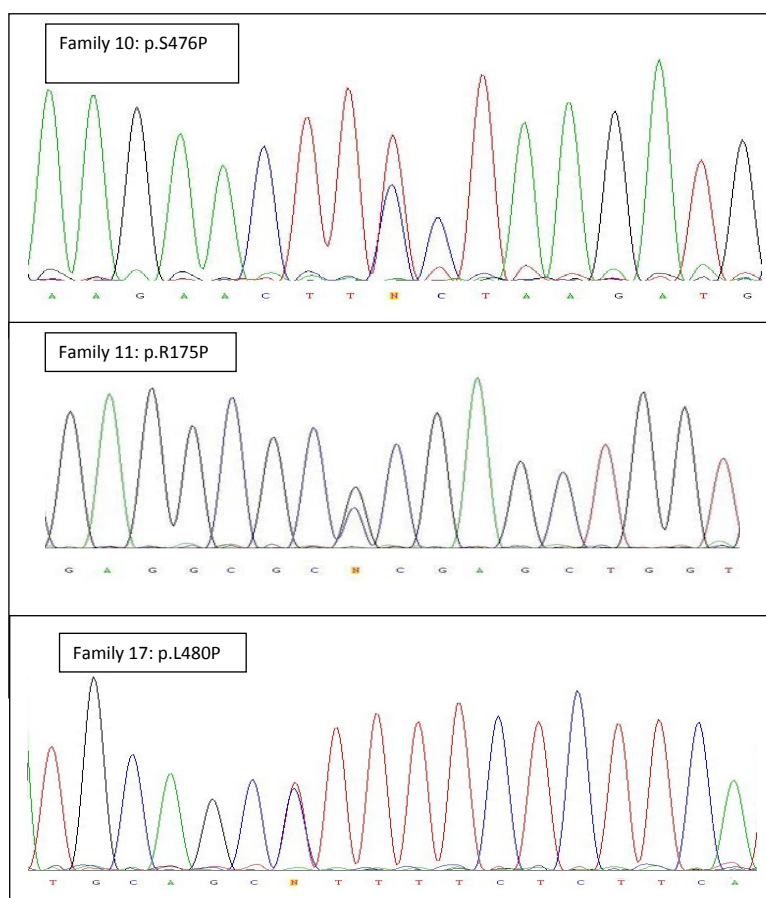

Figure 1: Novel mutations found in three families (A, B, C) with primary congenital cases and non-consanguineous marriage.

newly found p.Leu480Pro mutation is a missense mutation in genomic position g.10267T>C and coding nucleotide number c.1439 (C.1439 T>C), which results in substitution of a thymine base with a cytosine base. This substitution changes the amino acid codon from CTT to CCT and hence, leucine amino acid is changed to proline. PolyPhen bioinformatics software predicted this change as a pathogenic one and we could not find this mutation in any of the control chromosomes.

A PCG case in F10 family was compound heterozygote for known R368H and novel S476P mutations. This family had an Afghani descent and was permanent legal residents in Iran for 2 generations (Figure 1). In the new missense p.Ser476Pro mutation, a thymine base is substituted by cytosine in g.10254T>C genomic position and coding nucleotide number c .1426 (c.1426T>C). This substitution results in changing codon TCT to CCT and serine amino acid to proline. PolyPhen bioinformatics software predicted this change as a pathogenic one and we did not find similar mutation in any of the control chromosomes.

Six previously known SNPs, used to produce *CYP1B1* mutant haplotypes, were also detected in our study. In these patients, three different haplotypes were detected. Eight families carrying G61E mutation and one family carrying homozygote R368H mutation demonstrated similar haplotype (C-G-G-T-A). Two families carrying V364M mutations had similar haplotype (C-G-C-C-A), too. Five families (F10, F11, F12, F16, and F17) had also similar C-G-C-C-G haplotype.

None of the newly found mutations (L480P, S476P, and R175P) was previously reported in the Exome Aggregation Consortium (ExAC) database. All members of these three families were investigated using both clinical and molecular techniques and a trans condition was confirmed. According to data available Iran Variation database (available at http://genet.ir/variome/genes; accessed on March 1st, 2017), frequency of homozygous variants for these mutations was zero in the Iranian population.

Discussion

PCG is a clinically and genetically heterogeneous disorder and *CYP1B1* was the first gene attributed to it. To the best of our knowledge, this is the first study to investigate *CYP1B1* mutation in 27 families with PCG in Eastern provinces of Iran. There was a wide spectrum of molecular defects in this cohort and some of the detected mutations were not described previously. *CYP1B1* mutations were detected in seventeen (62.9%) of 27 families with apparently diseased member; this is similar to the previously reported penetrance level in North and Northwestern part of Iran and higher than PCG cases in Pakistan (50%) and India (44%) [26]. The most frequent mutation in *CYP1B1* was G61E mutation which was found in 47% of cases (8 out of 17) with diseased phenotype. Noteworthy, G61E is the principle disease-associated allele in Saudi Arabian PCG cases with a *CYP1B1* mutation (approximately 75% of cases) [27]. The frequency of this mutation in Iran was previously reported to be 21.6% [28], dramatically less than the observed frequency in Eastern Iran in our study.

R368H mutation was found as compound heterozygote/homozygote mutation in 17.65% of families with diseased phenotype; significantly higher than the previously reported frequency of 11.5% in Iran. This is the most frequent mutant allele of *CYP1B1* in India (59.46%) [26]. G61E and R368H mutations along with R390H and R469W are the most frequent mutation described in Iranian PCG cases in a study conducted in 2007 [28]; the last two mutations were not detected in any of our patients. Together, G61E and R368H mutations summed up to 64.65% of mutations

in the present study. Interestingly, we had a clinically normal subject with a homozygote G61E mutation; this could be explained by variable expression or non-penetrance of *CYP1B1* mutations as previously described [18,19]. This subject was 11-year-old at the time of preparing this manuscript and may have one or more modifier genes.

The known E229K mutation was identified as a homozygous mutation in combination with R390C mutation in the family F9; this mutation was previously reported in Turkish [29], Indian [27], and Saudi Arabian [30] PCG patients and was also reported in an Omani family [31]. R390C mutation was not previously reported in Iran. Actually, this is a "tetra-allelic mutation" in this case. Recently, "tetra-allelic mutation" of concurrent homozygous F231I and P437A mutations was reported in a Tunisian PCG case [32]. Unrelated clinically normal parents of the proband had both mutations which can be explained due to *cis* positions of the mutations relative to each other on the gene.

V364M mutation, with a frequency of 11.8%, is another mutation in our cohort which was not previously described in Iranian PCG patients; this is the principle *CYP1B1* mutation in Indonesian PCG cases [21]. This mutation was first reported as a compound heterozygote mutation in Japanese cases [22].

F445I mutation was found in only one family as a homozygote mutation and is still among rare alleles in this geographic area [33]. This mutation was first described in Gypsy patients [34] and was not previously reported in Iranian patients [28]. Also, in our study c.1504delA mutation was found in a patient in F16 family as a homozygote mutation; while this was previously described as a compound heterozygote mutation with G61E in an Iranian patient [28].

Huang and colleagues recently reported that heterozygous and homozygous *CYP1B1* mutations also play a role in the development of JOAG [35].

Chitsazan et al. previously reported the genetics finding in PCG cases based on the data of 3 principle referral hospitals in the capital city, Tehran [28]. The patients in these hospitals are usually referred from all around the country. However, our patients were selected among a cohort of patients treated in the major referral hospital of Northeastern part of Iran. This part of the country has a different ethnic background with some similarities to the neighbor countries like Afghanistan, Pakistan, and Turkmenistan. Regarding the long distance to the capital city and the implemented referral system, most of these cases are treated at this hospital and are not referred to Tehran. Regarding these, it is valuable to investigate these cohorts of patients.

Six SNPs are widely used in different ethnic groups to produce *CYP1B1* mutant haplotypes. The most frequent haplotype was C-G-G-T-A. G61E mutation occurs in a similar background in Iranian and Saudi Arabia population, probably denoting a common ancestry [18]. Similarly, R368H has a similar haplotype background between Iranians and Indians [36].

As we could not detect *CYP1B1* mutations in 10 out of 27 families, further studies using next generation sequencing (NGS) are underway to investigate other genetic loci, including *LTBP2*, in this cohort of patients. Modifier genes appear to affect patient phenotypes. Because healthy people had the same mutations with people with glaucoma, this form can only be justified by modifier genes in the normal person. Moreover, some cultural and other demographic factors may affect the phenotypic presentation of the disease [37-39].

Conclusion

Three novel missense mutations (p.R175P, p.S476P, and p.L480P)

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were detected for the first time in Iranian patients with PCG phenotype and reported herein. Potentially, one could classify these mutations as pathogenic, as they were not found in any of 100 normal chromosomes. Moreover, PolyPhen software predicted these mutations as pathogenic.

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