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Investigation of rs121918356 and rs121918355 *LTBP2* Mutations and LTBP2 Serum Levels in Primary Congenital Glaucoma in a Sample of Iraqi Children

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Abstract

Primary congenital glaucoma (PCG) is a severe type of glaucoma which occurs early in life and is a leading cause of blindness in early childhood. Latent Transforming beta Binding Protein 2 (*LTBP2*) gene is reported to be a PCG-related gene. This study is designed to investigate the involvement of *LTBP2* mutations (rs121918356 and rs121918355) in the PCG incidence in a sample of Iraqi children, and determine the *LTBP2* protein both in the patients and the control groups. Venous blood was collected from one-hundred child patients diagnosed with PCG and one-hundred healthy children. Genomic DNA was extracted, and two DNA fragments concerning the two mutations were amplified. The mutations were followed up using the restriction fragment length polymorphism technique and direct sequencing. Serum LTBP2 protein was measured using ELISA technique. No mutation was detected in any of the examined samples. A highly significant (p=0.0001) elevated serum LTBP2 protein was observed in the patients' group compared to the control group. In conclusion, *LTBP2* mutations (rs121918356 and rs121918355) are not related to the PCG in the Iraqi population. The PCG patients have higher LTBP2 protein levels compared to the healthy controls.

Keywords: Primary congenital glaucoma (PCG), LTBP2 gene, LTBP2 protein, Iraqi children, Q111X mutation, R299X mutation.

1. Introduction

Glaucoma involves a group of ocular disorders including optic nerve disintegration (Fan *et al*., 2006) and when left untreated, irreversible and permanent loss of vision can occur (Ray *et al*., 2003; Zhou *et al*., 2017). The optic nerve disintegration includes damage of retinal ganglion cells. Destruction of the retinal ganglion cells results in loss of visual field (Sarfarazi, 1997). Affecting almost sixty-five million people, glaucoma is considered as the second major reason of blindness in the world (Quigley, 1996). It is classified into three major types according to etiology, age of onset, and the anatomy of the anterior chamber (Ray *et al*., 2003). These are: primary congenital glaucoma (PCG), closed angle glaucoma, and primary open-angle glaucoma (Mohanty *et al*., 2013; Faiq *et al*., 2013). PCG is a severe type of glaucoma which occurs early in life (seen up to 3 years) (Shohdy *et al*., 2017) and is a leading reason for blindness in early childhood. PCG is featured by a developing anomaly of the trabecular meshwork (TM) which lies in the anterior chamber angle of the eye causing an elevated intraocular pressure (IOP), which leads to optic nerve damage and irreversible vision loss (Achary *et al*.,2006; Sarfarazi and Stoilov, 2000). PCG is inherited in an autosomal recessive pattern, and it is more prevalent in communities with a high ratio of consanguinity. PCG pervasiveness varies from 1:10,000 for Western communities, 1:3,300 for Southern India, 1:2,500 for Saudi Arabia, and 1:1,250 for the Slovakia Gypsy population (Ali *et al*., 2009; Khan *et al*., 2011). It is apparent that PCG is essentially a heterogenetic disorder (with a multitude of gene defects) (Shohdy *et al*., 2017). To date, four loci have been categorized under the class of GLC3 (GLC3 refers to the nomenclature of the Human Genome Organization (HGO) for congenital glaucoma), and they are: GLC3A, GLC3B, GLC3C, and GLC3D. The letters A, B and C that follow the numbers refer to the chronology of the gene mapping (Cascella *et al*., 2015). The most reported PCG- related loci are GLC3A and GLC3D; GLC3A is located on 2p21 chromosomal region and is related to the *CYP1B1* gene (El Akil *et al*., 2014; Badeeb *et al*., 2014). *CYP1B1* gene mutations are associated with 33 % of the sporadic and 80 % of the familial PCG cases (Cascella *et al*., 2015; Plasilova *et al*., 1999). Regarding GLC3D (14q24) locus, several autosomal recessive null mutations were found in this position which belongs to the *LTBP2* gene. This gene encodes for Latent Transforming beta Binding Protein 2. Null mutations related to PCG were found in consanguineous families of European Gypsy population, Pakistani families and Iranian families (Narooie-Nejad *et*

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al., 2009; Azmanov *et al*., 2011) At the same time, *LTBP2* mutations that are not related to PCG were found in other populations (Mohanty *et al.*, 2013; Safari *et al*., 2015; Lima *et al*., 2013).

LTBP2 is a large gene with thirty-six exons which codes for 1821 amino acids of latent transforming growth factor (TGF)-β binding protein 2 (Safari *et al*., 2015). *LTBP2* mutations were found to be linked to severe PCG cases (Azmanov *et al*., 2011). Ali *et al*. selected *LTBP2* as a disease-related gene because of its elevated expression in the anterior chamber of the eye. Homozygous nonsense mutations (c.895 C/T; p.R299X; rs121918356 and c.331 C/T; p.Q111X; rs121918355) were identified in some consanguineous Pakistani and Slovak Gypsy families (Ali *et al*., 2009; Azmanov *et al*., 2011). These two mutations are considered as the most reported pathogenic mutations related to PCG. When occurring, these two mutations cause stop codons, thus producing truncated non-functional LTBP2 protein (Ali *et al*., 2009).

This study is the first genetic study which investigates the role of *LTBP2* mutations (rs121918356 and rs121918355) in the incidence of primary congenital glaucoma in a sample of Iraqi patients and compares the serum LTBP2 protein levels among patients and controls.

2. Materials and Methods

2.1. Study Subjects

This study adheres to the tenets of the Declaration of Helsinki and is approved by the Ethics Committee of the Department of Chemistry, College of Science at Mustansiriyah University in Baghdad, Iraq, it is also approved by the Iraqi Ministry of Health as well. All the samples were provided after obtaining informed consents of the children's parents prior to children's inclusion in the study.

One hundred unrelated PCG Iraqi child patients (58 males and 42 females) were included in this study and one-hundred unrelated healthy children (60 males and 40 females) from the same ethnicity without any systemic or ocular disease served as controls; the baseline characteristics of the PCG patients are illustrated in table 2. Five mL of venous blood was obtained from all the enrolled children by using plastic disposable syringes and were divided into two parts. The first part (2 mL) was put in EDTA tubes, and was stored at −20 °C until further use in the genetic analysis, while the second part (3 mL) was put into gel tubes and was left for fifteen minutes at a room temperature 25 °C, then the blood was centrifuged at 2000 ×g for ten minutes to collect sera. Aliquots were placed in Eppendorf tubes and stored at -40 ºC until use for the LTBP2 protein evaluation. The patients were recruited from Ibn Al-Haitham Teaching Eye Hospital in Baghdad, Iraq, while the healthy children were volunteers. All the patients were diagnosed by a glaucoma specialist (Ali N.M.Al-Sharifi, C.A.B.Opth). The standards adopted to diagnose PCG are as follows (Chen *et al*., 2016): Age of onset runs between one month and three years with a mean of twenty-four months, the elevated IOP (more than 21 mmHg), enlarged cornea so the parameter was bigger than 11 mm and increased cup to disk ratio. The exclusion criteria according to Chen and co-workers (Chen *et al*., 2016) included other ocular anomalies, such as the anterior segment dysgenesis, aniridia, neurofibromatosis, Sturge–Weber syndrome and congenital hereditary endothelial dystrophy.

2.2. Genotyping

Genomic DNA was extracted from whole blood using a genomic DNA extraction kit according to the manufacturer's instructions (Geneaid, Taiwan). The purity and concentration of the genomic DNA were assessed by a nanodrop (BioDrop μLITE, BioDrop co., UK), while the DNA integrity was confirmed using 0.8 % (w/v) agarose gel electrophoresis which is pre-stained with ethidium bromide (0.5 μg/mL) in Tris-Borate-Ethylene-diamine tetra-acetic acid (TBE) buffer. Two DNA fragments (850 and 247 bp), lying on exon 1 and exon 4 of the *LTBP2* gene respectively were amplified by polymerase chain reaction (PCR) using thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Korea). PCR was worked in a total volume of 25 μL mixture containing 3μL;100-150ng of genomic DNA, 2μL; 20 p mol/μL of each primer (forward and reverse primers),13 μL of free DNAase distilled water and 5 μL AccuPower PCR premix (Bioneer, Korea), depending on the following protocol: initial denaturation at 95 °C for five minutes, thirty cycles of amplification (denaturation at 94 °C for forty-five seconds, annealing at primer specific annealing temperature for forty-five, seconds extension at 72 °C for one minute and final extension at 72 °C for five minutes). All the lyophilized primers were purchased from Bioneer (Bioneer, Korea) and they are listed in Table 1. The PCR products integrity was checked using 1.5 % (w/v) agarose gel electrophoresis (Figure 1A and B).

Restriction fragment length polymorphism- Polymerase chain reaction (RFLP-PCR) technique was used to identify the mutations in exon 1 and exon 4 (c.331 C/T; p.Q111X; rs121918356 and c.895 C/T; p.R299X; rs121918355) of *LTBP2* gene respectively. An 850 bp fragment encompassing c.331 C>T mutation position on exon 1 of *LTBP2* gene was amplified using specific primer sequences (Table 1).The detection of the mutation was done using AlwNI endonuclease (Biolabs, United Kingdom). In case of c.895C/T mutation on exon 4, a 247 bp fragment containing the whole exon was amplified using specific primers sequences (Table 1), AlwNI endonuclease enzyme was used to detect the mutation. The products of digestion (restricted fragments) were visualized using 3 % (w/v) agarose gel electrophoresis (Figure 2A and Figure 2B). Sanger sequencing was performed for all samples concerning the two exons as further screening using the standard methods (Figure 3A and B).

Table 1. PCR primers, restriction enzymes and digestion conditions used to genotype *LTBP2* gene.

Exon	Primers	Ta (^{0}C)	Product Size (bp)	Restriction enzyme	Digestion conditions	Sizes of restricted fragments
1	F-GGGCCTGGTGTGGATAAAAG R- TTCCCTCTCCCATGCTCAC	65	850	A1wNI	10 µl of PCR product, 0.5 (5 unit) µl of A1wNI enzyme, 5 µl of buffer and 4.5 µl of DDW in a total volume of 20 µl were incubated for 2 hrs. at 37 ^{0}C.	Two pieces of 527 bp and 323bp
4	F-GCGGTTGTCTCCACAGGA R- AGGCCCTGCTCTTCTAGGAC	64	247	A1wNI	10 µl of PCR product, 0.5 (5 unit) µl of A1wNI enzyme, 5 µl of buffer and 4.5 µl of DDW in a total volume of 20 µl were incubated for 2 hrs. at 37 ^{0}C.	One intact fragment of 247 bp.

Ta: annealing temperature. DDW: deionized sterile DNAase and RNAase free distilled water.

2.3. LTBP2 Protein Measurement

The level of LTBP2 protein was measured in the sera for all patients and control groups using the enzyme-linked immune sorbent assay (ELISA) kit according to the manufacturer's instructions (Cat # MBS760698, Mybiosource/ US).

2.4. Statistical Analysis

SPSS (statistical package for social sciences) version 19 was used for the statistical analysis of serum LTBP2 protein in both the patients and control groups. T- Test was used for the statistical comparison between means and $p<0.01$ was considered statistically significant.

3. Results

Two fragments for exon 1 and exon 4 of the *LTBP2* gene were amplified and *LTBP2* mutations (c.331 C/T; p.Q111X; rs121918356 and c.895 C/T; p.R299X; rs121918355) were tracked using RFLP-PCR technique. In case of c.331 C/T mutation, the product of the enzyme digestion for the wild-type is two pieces of the size 527 bp and 323bp (Figure 2A). Mutation is supposed to change the recognition site for AlwNI endonuclease and give an intact 850 bp fragment; all the DNA samples included in this study showed the wild type pattern. In case of c.895 C/T mutation, according to Ali (Ali *et al*, 2009) an AlwNI recognition site is formed in case the mutation is present, while in the wild type, the 247bp fragment would remain intact. All the DNA samples included in this study showed the wild type pattern (Figure 2B). Sequencing using Sanger protocol was done as further screening for all the samples concerning both the DNA fragments, and to discover if there were other nucleotide variations in these loci (Figure 3A and B). No variation was observed in any of the examined samples. Highly-significant elevation ($p=0.0001$) of the serum LTBP2 protein was noticed in the patients groups compared to the control groups (Table 3).

Table 2. Characteristics of child patients enrolled in the study.

Group description		Total patients=100
Sex	Male	58
	Female	42
Consanguinity	Yes	74
	No	26
Family history of glaucoma	Yes	8
	No	92

Table 3. Statistical analysis of Serum LTBP2 protein distributed among total patients and total control, male Patients and male control and female patients and female control.

Group	LTBP2 Protein ng/ml Mean ±SE	95 % C.I. for Mean L.b.	U.b.	*P*-Value
Total patients	1.955±0.137	0.506	1.064	
Total controls	1.169± 0.323	0.505	1.065	.0001**
Male patients	1.952± 0.172	0.449	1.143	
Male controls	1.156± 0.042	0.441	1.151	.0001**
Female Patients	1.957± 0.226	0.297	1.243	
Female controls	1.187± 0.049	0.302	1.238	.0001**

SE: Standard error, 95% C.I.: 95% Confidence interval, L.b.: Lower Bound, U.b.: Upper Bound. ** Significant at $p<0.01$

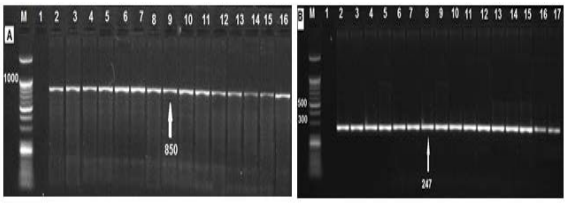

Figure 1. Electropherograms representing. A: Agarose gel electrophoresis photographs of an 850 bp PCR product of *LTBP2* gene on 1.5 % agarose gel. M: 25bp DNA marker. Lane 1 shows negative control and Lanes 2-16 show the PCR products. B: Agarose gel electrophoresis photographs of a 247 bp PCR product of *LTBP2* gene on 1.5% agarose gel. M: 25bp DNA marker. Lane 1 shows negative control and Lanes 2-17 show the PCR products.

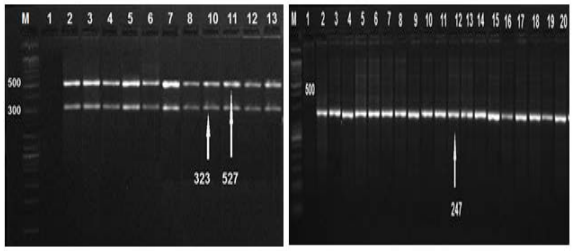

Figure 2. PCR-RFLP genotyping. A: PCR-RFLP genotyping of Q111X mutation using A1wNI endonuclease on 3 % agarose gel. M: 25bp DNA marker. Lane 1 shows negative control and lanes 2-13 represent wild type pattern of digestion producing two bands at 527bp and 323 bp. B: PCR-RFLP genotyping of R299X mutation using A1wNI endonuclease on 3 % agarose gel. M: 25bp DNA marker. Lane 1 shows negative control and lanes 2-20 represent wild type pattern producing intact 247 bp fragments.

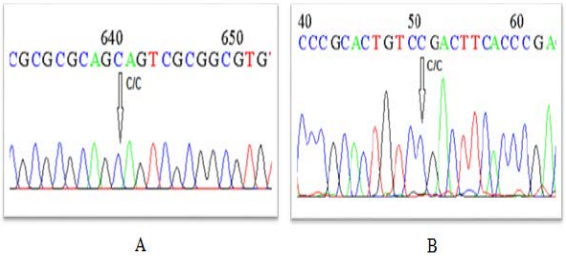

Figure 3. Sanger sequencing of PCR product. A: Regarding Q111X mutation, direct sequencing shows homozygous wild type pattern C/C. B: Regarding R299X mutation, direct sequencing shows homozygous wild type pattern C/C.

4. Discussion

Null mutations (c.331 C/T; p.Q111X; rs121918356 and c.895 C/T; p.R299X; rs121918355) of the *LTBP2* gene were primarily found to cause PCG in some individuals from Pakistani and Romani populations (Ali *et al*., 2009; Azmanov *et al*., 2011). PCG patients from several other populations, such as Slovakian Romani and Iranian populations were found to have mutations in *LTBP2* (Narooie-Nejad *et al*., 2009; Azmanov *et al*., 2011). However, other studies on British, American, and North Indian PCG patients did not discover deleterious mutations in *LTBP2* (Mohanty *et al*., 2013; Lima *et al*., 2013). In this study, DNA of one-hundred sporadic patients with PCG and one-hundred healthy Iraqi children (control) were screened for mutations in exon 1 and exon 4 of *LTBP2* gene. Mutations were not found in the population of this study, and this finding is consistent with the results of a previous study on a Saudi population (Abu-Amero *et al*., 2011) where no mutation was found in the LTBP2 gene in the Saudi PCG patients as illustrated in Table 4.

This study shows a little prevalence of PCG in males over females (Table 2). PCG is reported to be more prevalent in male subjects than females; males account for approximately 65 % of PCG cases (El Akil *et al*., 2014). The cases that have a family history in this study represent eight (8%) of the total cases (Table 2). It was reported that most of the PCG cases are sporadic, nonhereditary, and not familial (Sarfarazi, 1997). From 10 % to 12 % have a family tendency with variable penetrance (40-100 %) (Sarfarazi, 1997). Consanguinity is a factor in seventy-four (74%) of the cases in this study (Table 2). Regarding the autosomal recessive pattern of PCG inheritance, consanguinity is considered a risk factor for PCG in some populations such as Saudi, Iranian, Pakistani, and Slovak Gypsy populations, (Narooie-Nejad *et al*., 2009; Azmanov *et al*., 2011; Abu-Amero *et al*.2011). It can be said then that consanguinity plays an important role in the PCG incidence in the Iraqi population as a community with high consanguinity.

LTBP2 protein is the biggest member in the LTBPs family. It has a structure domain analogous to that of other LTBPs, particularly the form of LTBP1, but it also involves distinctive regions, which makes it related to fibrillin-1 (Hirani, 2007). LTBP2 co-localizes with fibrillin, and its aggregation largely associates with the fibers of fibrillin-1 (Robertson *et al*., 2015). Fibers are largely expressed in the eye especially in the TM. The interconnected circumferential network of the elastic fiber is much similar to the elastic fiber structure in blood vessels, and likely provides basic elastic properties to the aqueous humour (AH) outflow system, which is constantly accompanied with pulsatile force. Expression of fibrillin-1 in the TM confirms a distribution of microfibrils overlapped with TM elastic fibers (Kuchtey and Kuchtey, 2014). The flow of aqueous homour is concomitant with the ocular pulse. Microfibril defects could alter elasticity of the TM, collector channels, and episcleral veins, which could obstruct the pumping action of the TM and the pulsatile outflow of AH causing accumulation of the AH and increasing of the IOP in glaucoma. LTBP2 is the microfibril-associated protein, therefore, *LTBP2* mutations had been shown to cause PCG (Kuchtey, 2011; Johnstone, 2004). Microfibril defects could be involved in glaucoma through changing the biomechanical properties of the tissue and through affecting the signaling of transforming growth factor beta (TGFβ) (Kuchtey and Kuchtey, 2014). Microfibrils are the main reservoir of latent TGFβ and are essential for TGFβ signaling and localization. Abnormal microfibrils could be a reason for TGFβ elevation in the AH of glaucomatous eyes (Kuchtey and Kuchtey, 2014). LTBP2 co-localizes with fibrillin-1, and it is involved in the activation and secretion of TGFβ. In addition, some studies show that the assembly and secretion of TGFβ require the co-expression of LTBP (Miyazono *et al*., 1991; Rahmi and Hesketh, 2000). Other studies reported that the concentration of TGFβ2 in the AH of pseudoexfoliation syndrome patients was high compared to the controls, and that the LTBP2 protein is co-expressed with TGFβ2 in the eye; thus, it was found to be elevated as well in the pseudoexfoliation syndrome patients (Schlotzer-Schrehardt *et al*., 2001). This fact is consistent with the results obtained from the present study in which LTBP2 protein levels in patients' sera are significantly higher (p=0.0001) than those of the healthy control (Table 3). The increased expression of *LTBP2* is required for the increased secretion of TGFβ in PCG patients and that explains the cause (other than *LTBP2* mutations) underling the elevation of LTBP2 protein level in the PCG patients participating in this study. These findings indicate that the high LTBP2 protein levels can be detected in the PCG patients' sera as well as in the patients' AH. On the other hand, there is no observed differentiation in LTBP2 protein levels among the male and female patients, which indicates that the sex is not a controlling factor concerning the LTBP2 protein level. Limitation of this study include: the small size of the sample, only few mutations in LTBP2 gene were examined. The observed elevation in LTBP2 might not reflect the situation in the eyes.

Table 4. LTBP2 polymorphisms/ mutations reported in other populations for patients with primary congenital glaucoma.

No.	Nucleotide change	Exon / Intron location	Heterozygous/ Homozygous	Type of mutation	Amino acid change	Reference SNP number	Population	Reference
1	g.75070493 C>G	Intron 5	homozygous			rs3742793	North Indian	Mohanty *et al.*, 2013
2	c.331C>T	Exon 1	homozygous	Nonsense	p.Q111X	rs121918356	Pakistani	Ali *et al*., 2009
3	c.412delG	Exon 1	homozygous	Frame shift	p.A138PfsX278		Pakistani,	Ali *et al*., 2009
							Gypsy	Azmanov *et al*., 2011
4	c.1243_1256del14	Exon 6	homozygous	Frame shift	p.E415RfsX596	N A	Pakistani,	Ali *et al*., 2009
							Gypsy	Narooie-Nejad *et al*., 2009
5	c.895C>T	Exon 4	homozygous	Nonsense	p.R299X	rs121918355	Gypsy	Azmanov *et al*., 2011
6	c.5376delC	Exon 36	homozygous	Frame shift	p.W1793fsX55	NA	Iranian	Narooie-Nejad *et al*., 2009
7	c.1415delC	Exon 7	homozygous	Frame shift	p.S472fsX3	NA	Iranian	Narooie-Nejad *et al*., 2009
8	c.1287G>A	Exon 6		Silent	p.L429L	rs61738025	Iranian	Narooie-Nejad *et al*., 2009
	c.4808G>A	Exon 33		Missense	p.R1603H	rs75200417	Iranian	Narooie-Nejad *et al*., 2009
9	c.956C>A	Exon 4	heterozygous	Missense	p. P319Q	rs2304707	Chinese,	Chen *et al*., 2016
							Iranian,	Narooie-Nejad *et al*., 200920
							American,	
							British	Sharafieh et al., 2013
10	c.3611C>T	Exon 24	heterozygous	Missense	p. A1204V	rs45468895	Chinese	Chen *et al*., 2016
11	c.4286G>A	Exon 29	heterozygous	Missense	p. R1429Q	rs116914994	Chinese	Chen *et al*., 2016
12	c.5380G>A	Exon 36	heterozygous	Missense	p. E1794K	rs763035721	Chinese	Chen *et al*., 2016
13	No detected mutation						Saudi	Abu-Amero *et al*.2011
14	No detected mutation							Current study

NA: Not Available

5. Conclusion

The null mutations (c.331 C/T; p.Q111X; rs121918356 and c.895 C/T; p.R299X; rs121918355) which are considered as pathogenic mutations in some populations are not found in the current study's groups, which means that they have no relation to the PCG disease in the selected sample of Iraqi population. The PCG patients have higher LTBP2 protein levels when compared to controls. The elevated levels of LTBP2 protein in the patients may suggest the involvement of other loci of *LTBP2* gene in the incidence of the disease; at the same time, this elevation may not have other genetic reasons.

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