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Identification of *de Novo* and Novel Mutations in *LTBP2* in Pakistani Families with Inherited Primary Congenital Glaucoma

Rani Saira Saleem[1]*, Muhammad Imran Khan[2], Saba Irshad[1]*, Sorath Noorani Siddiqui[3] and Shazia Micheal[4]

[1]*School of Biochemistry and Biotechnology, University of the Punjab, 54000 Lahore, Pakistan.*
[2]*Department of Human Genetics, Radboud University Medical Centre, 6525 GA Nijmegen, The Netherlands*
[3]*Department of Pediatric Ophthalmology and Strabismus, Al-Shifa Eye Trust Hospital Jhelum Road, Rawalpindi 46000, Pakistan.*
[4]*Department of Clinical Genetics, Academic Medical Centre, Meibergdreef 9, 1105 Amsterdam, The Netherlands.*

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RSS, SM and SI conceived and designed the experiments. RSS and SM performed the experiments. RSS, SI and SM performed results and bioinformatics analysis. SNS and MIK recruited patients, collected clinical data and samples. SNS, MIK, SM and SI contributed reagents, materials, analysis tools. RSS, SI and SM wrote the manuscript. All authors have read and approved the final manuscript.

Key words
Primary congenital glaucoma, LTBP2, Homozygosity, Mutations, Microarray analysis

ABSTRACT

Primary congenital glaucoma (PCG) is the topmost reason for childhood blindness due to optic nerve impairment, enlarged globe, and loss of visual field. PCG is usually identified during the first year of life. The purpose of the recent research was to assess the involvement of the Latent transforming growth factor 2 (*LTBP2*) gene in PCG families of Pakistani origin and to find novel mutations. To extract genomic DNA from the whole blood of n=20 families were performed followed by the genotyping of the affected and unaffected persons of the families by using whole genome single nucleotide polymorphism microarray (SNP). Homozygosity mapping analysis was performed for the selected members of these families. *LTBP2* gene was screened using Sanger Sequencing in n=20 consanguineous Pakistani families diagnosed with PCG by standard ophthalmological examination. Novel homozygous mutations were identified in three families with PCG in the *LTBP2* gene. We found a new de *novo* frameshift mutation c.1762_1763del; p. (Leu588Valfs*14*)*. In a second family, we identified a splice site mutation c.2531-2A>C, and in the third family, a splice donor site c.1686G>A; p. (Gln562Gln) mutation. In this study, we report the involvement of novel *de novo* frameshift mutation and two genetic variants that affect splicing in PCG families from Pakistan. The current study will help us to extend our understanding of the part of *LTBP2* in PCG.

INTRODUCTION

Primary congenital glaucoma (PCG; OMIM 231300) is one of the types of glaucoma leading to blindness among 18% of cases in the starting years of life (DeLuise and Anderson, 1983). The disease pathology is characterized by obstruction of the aqueous humor drainage pathways owing to congenital progressive imperfections in the anterior segment of the eye (Maumenee, 1958). PCG is also commonly known as congenital buphthalmos characterized by delocalization of the sclera, damage to the optic nerve, and related structures, associated with elevated levels of intraocular pressure (IOP) (Kwitko, 1973; Shaffer, 1969). Thus, it is proposed that obstetricians should pay attention during neonatal eye development to keep a record of the corneal diameter during the growth to help with the early identification and treatment of PCG. Indeed, a strong indicator of PCG is the distensibility in the neonatal eyeball due to the excessive accumulation of elastic fibers in the sclera, optical nerve cupping, or any rise in the corneal diameter >12mm in the earlier stages of life (Hoskins, 1989; Sarfarazi *et al*., 1995). For this reason, a conventional triad of symptoms is also suggested for an early identification of PCG that includes tearing of eyes, oversensitive eyes to the light, and related inflammation issues (Hoskins, 1989).

The mode of inheritance for PCG is autosomal

* Corresponding author: saba.sbb@pu.edu.pk, rani.phd.ibb@pu.edu.pk
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recessive with variable penetrance indicating that PCG is more prevalent in the populations with consanguinity. Genetic investigation of the families with PCG revealed four genetic loci linked to the disease; GLC3A at 2p22-p21 (Sarfarazi *et al*., 1995), GLC3B at 1p36.2-p36.1 (Akarsu *et al*., 1996), GLC3C at 14q24.3 (Stoilov and Sarfarazi, 2002) and GLC3D at 14q24 (Firasat *et al*., 2008). To date, disease-causing mutations have been known in three genes within these loci. Cytochrome P450 1B1 (*CYP1B1*) is the most common gene identified for its association with PCG and is encompassed within the *GLC3A* locus (Stoilov *et al*., 1997). *LTBP2* is another important disease-causing gene localized within the *GLC3D* locus (Ali *et al*., 2009). Recently, mutations have also been known in the third *PCG* gene, Tunica internal endothelial cell kinase **(***TEK*) (Souma *et al*., 2016).

The most frequent cause of PCG in consanguineous families are disease producing changes in the *CYP1B1* gene. For example, 80%-100% of Saudi Arabian PCG families were reported to harbor *CYP1B1* mutations (Abu-Amero, 2011). So far, about more than 150 different mutations including synonymous, non-synonymous, coding, non-coding, and frameshifts have been listed in *CYP1B1* for causing PCG in families with different origins. However, only 37-40% of Pakistani PCG families had mutations in the *CYP1B1* gene, which further suggests genetic heterogeneity of the disease (Micheal *et al*., 2014). Recently three novel alterations have been stated in Iranian families with PCG linked to the *CYP1B1* gene (Emamalizadeh, 2021). In addition to *CYP1B1*, a few mutations in the *LTBP2* gene have been reported for Pakistani families and other populations linked with PCG. Since the expression of *LTBP2* in trabecular meshwork cell lines is 12.7 times that of *CYP1B1,* it is tempting to speculate on the importance of *LTBP2* in the pathogenesis of PCG (Wang *et al*., 2001).

The purpose of the current study was to screen the probands of twenty Pakistani PCG families for mutations in the *LTBP2* gene. All these families had been previously excluded for variants in the *CYP1B1* gene.

MATERIALS AND METHODS

Subjects

The probands were engaged by the Ophthalmology Department of Al-Shifa Eye Trust Hospital, Rawalpindi, Pakistan. The ocular investigation was executed for both affected and unaffected persons of families.

Extraction of DNA and PCR amplification of LTBP2

Extraction of DNA was done from peripheral leukocytes by using the QIAGEN DNA Blood Midi Kit (QIAGEN, Germantown, Maryland, USA) of twenty PCG families. Genotyping will be followed by whole genome SNP microarray (Illumina GSA Beadchip) analysis by using DNA samples of selected affected and unaffected individuals of these 20 families. An online mapping tool Homozygosity Mapper was deployed to evaluate the information and find the respective regions. *LTBP2* gene was screened for the presence of mutations. Sanger sequencing of all 36 exons inclusive of exon borders of the gene was performed by using an automated ABI 3730 Sequencer (Applied Biosystems, Inc.) according to the provided protocol. CodonCode Aligner (version 6.1) was deployed for performing sequence alignment with the reference sequence to identify the novel variants. The primer sequences, reactions, and PCR conditions were modified from the literature.

In silico studies

In the current study, an in-silico investigation was completed for the variants found by using ALAMUT® VISUAL software to determine the functional impact. The prediction tools included to examine the pathogenicity of missense variations were SIFT, Mutation Taster, and PolyPhen-2. In the case of splice site variants, GeneSplicer, MaxEntScan, HSF, NNSPLICE, and ESE were considered for splicing site calculations. The links used to access these analytical tools were;

Human Splicing Finder (HSF) http://www.umd.be/HSF3/technicaltips.html

Maximum entropy (MaxENt) score: http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html

Sorting Intolerant from Tolerant (SIFT): http://sift.bii.a-star.edu.sg/

Polymorphism Phenotyping v2 (PolyPhen-2): http://genetics.bwh.harvard.edu/pph2/

NNSPLICE: https://omictools.com/nnsplice-tool

Exon Splicing Enhancer (ESE): http://rulai.cshl.edu/tools/ESE2/index.html

GeneSplicer: https://ccb.jhu.edu/software/genesplicer/

RESULTS

*Identification of De-Novo c.1762_1763del; p.(Leu588Valfs*14) mutation*

In family 1, proband II.I was a 3-months-old boy when presented in the patient outdoor clinic. He was diagnosed with PCG. Both his parents were unaffected upon clinical examination. Both parents did not suffer from elevated IOP or glaucoma but, soon after birth, the child was reported with raised IOP > 22 mmHg. Along with this, the corneal diameter of the child was recorded to be larger than 13mm. The reported cup-to-disc ratio was >0.7.

In the DNA of the proband (II-1), we found a

homozygous *de novo* mutation c.1762_1763del by Sanger sequencing in the *LTBP2* gene. The absence of this somatic, non-hereditary variant in both parents suggests this particular variant is a novel cause of PCG. This 2bp deletion in Exon 8 (Fig. 1B) causes a frameshift at codon Leu588 and, subsequently, a stop codon 13 positions downstream. This may result in the non-sense-mediated decay of the mRNA.

LTBP2 canonical splice site mutation (c.2531-2A>C)

In family 2, two affected individuals were diagnosed with bilateral PCG. Male Proband IV: 2 and his affected sister (IV: 3) were, respectively, 6 and 3 months old at the time of diagnosis. All the affected (IV:2, IV:3) and unaffected (III:1, III:2, IV:1, IV:4) members of the family were checked for the presence of any other related abnormalities. After the Whole genome analysis, the homozygosity mapper yielded two relevant regions: one

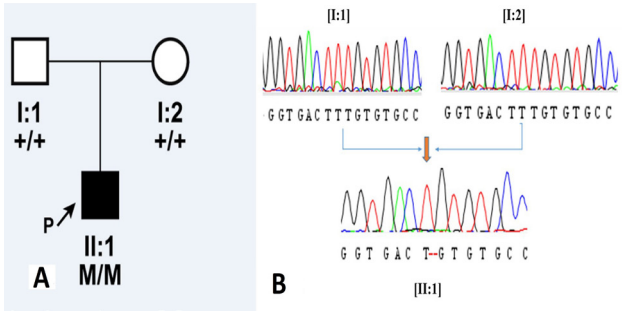

Fig. 1. Family I: *LTBP2 de Novo* Mutation c.1762_1763del; p. (Leu588Valfs*14).
(A) A two-generation family pedigree. PB (II: 1) is indicated with an arrowhead. Affected members are indicated with black filled box. (B) sanger sequence analysis of the *de novo* mutation showing the chromatograms of unaffected father, Mother and the affected son.

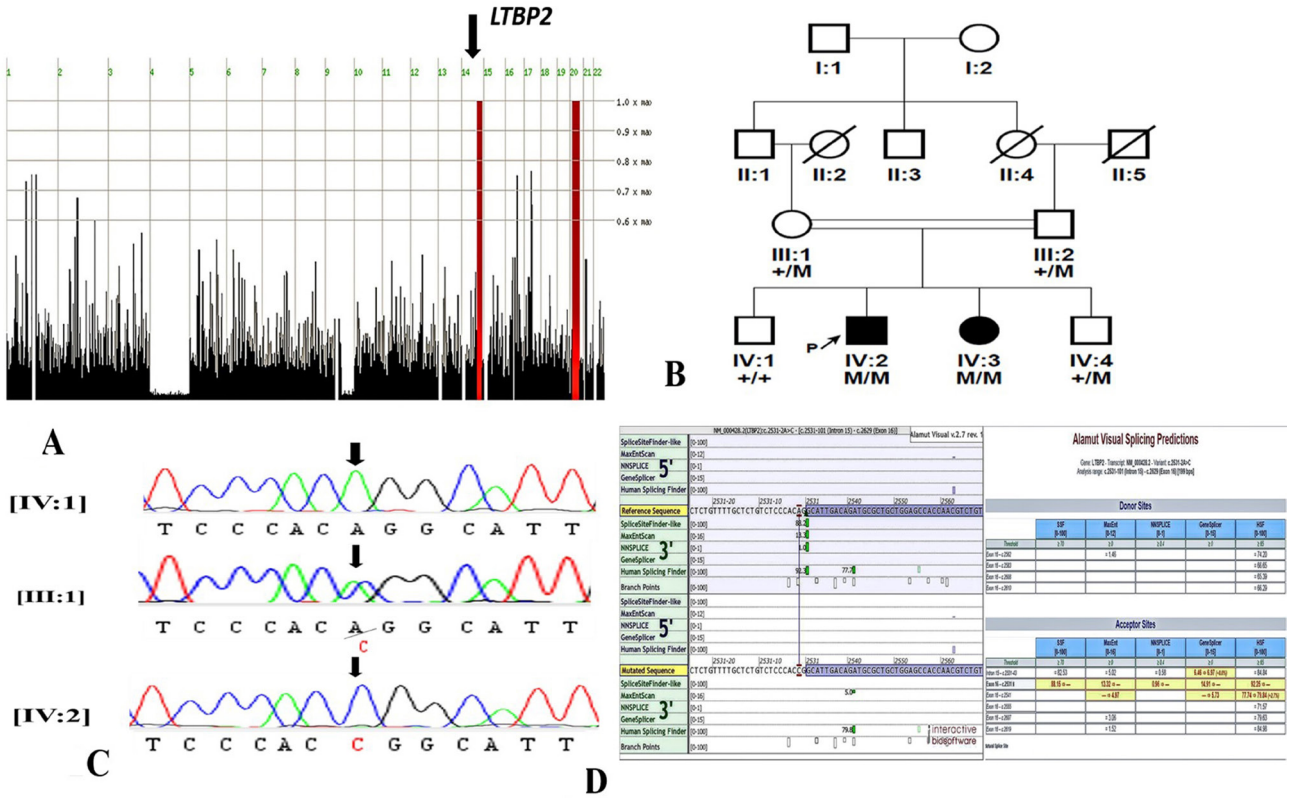

Fig. 2. Family II: *LTBP2* canonical splice site mutation (c.2531-2A>C).
(**A**) Homozygosity mapping in two affected and two unaffected individuals of family II. Analysis was done by using online web tool homozygosity mapper (http://www.homozygositymapper.org/). The red line indicates the homozygous regions in the SNP array analysis, one of them corresponding to 22Mb region at chromosome 14 for *LTBP2*. (**B**) Pedigree of consanguineous family II showing segregation of a novel mutation c.2531-2A>C. Proband is indicated by a black arrowhead. (**C**) Chromatogram showing Sanger sequencing results for the canonical splice site mutation c.2531-2A>C indicated with respect to homozygous normal (IV:1), heterozygous carrier (III:1) and homozygous mutant (IV:2). (**D**) In-Silico predictions for mutation (c.2531-2A>C) by ALAMUT Visual. The score for natural 3' splice site was decreased in the mutant.

confined to chromosome 14 and the other referred to chromosome 20; as revealed in Figure 2A. *LTBP2* gene spans at the 22Mb localized homozygous region of chromosome 14. Sanger sequencing of the entire coding sequence and intronic boundaries identified a novel homozygous acceptor splice site mutation c.2531-2A>C neighboring exon 16. The mutation was found cosegregating with the disease in the family. In the affected patients, the mutation is existing in homozygous form, as shown in Figure 2C. The prediction analysis of human splicing finder (HSF) and other tools, such as MaxEntScore and NNSPLICE, and ExoSKIP, all predicted a pathogenic modification of the wild acceptor site. Most likely, the consequence of this change is of the skipping of *LTBP2* exon 16, as indicated by ExoSKIP in Figure 2D. This change has not been stated in any of the databases including genomAD.

LTBP2, synonymous splice site c.1686G>A; p. (Gln562Gln) mutation

In family 3 (Fig. 3B), proband IV: 7 was identified with PCG when he was just 9 months old. In addition to this, members IV: 2, IV: 5, and IV: 7 were also diagnosed with bilateral PCG with corneal diameters >13mm and IOP > 23 mmHg. No secondary ocular anomalies were observed in these individuals. Whole genome homozygosity mapping analysis revealed multiple homozygous regions. Among them was a 14Mb region on chromosome 14 encompassing for *LTBP2* gene, found to be homozygous (Fig. 3A). Sanger Sequencing of the *LTBP2* gene in the affected members of the family caused the recognition of a c.1686G>A; p. (Gln562Gln) mutation at the splice donor site. The genetic variant was found to be cosegregated with the disease in the family (Fig. 3C). The consensus nucleotide positions flanking this canonical splice donor

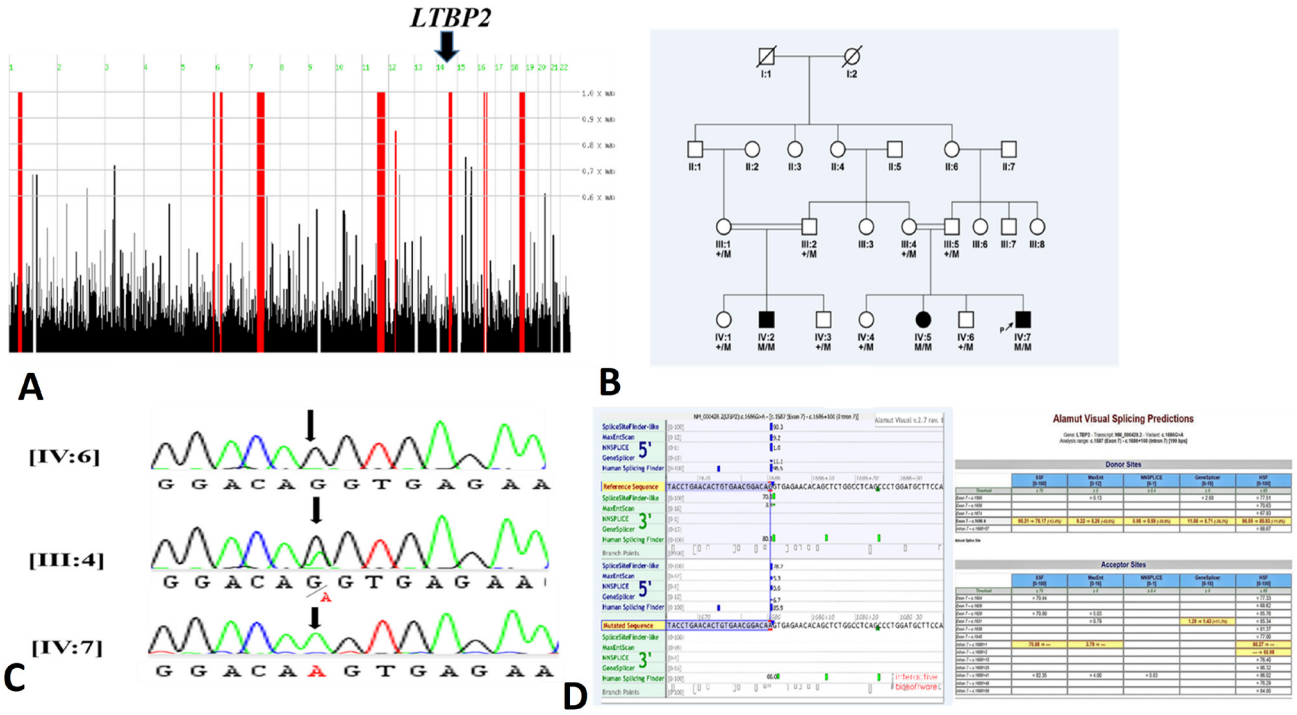

Fig. 3. Family III: *LTBP2*, synonymous splice site c.1686G>A; p. (Gln562Gln) mutation.
(**A**) Homozygosity mapping in three affected and three unaffected individuals of family III. Analysis was done by using online web tool Homozygosity mapper (http://www.homozygositymapper.org/). The red line indicates the homozygous regions in the SNP array analysis, one of them corresponding to 14Mb region at chromosome 14 for *LTBP2*. (**B**) Pedigree of consanguineous family III showing segregation of a novel c.1686G>A mutation. Proband indicated by a black arrowhead. (**C**) Chromatogram showing Sanger sequencing results for the splice donor site mutation c.1686G>A indicated with respect to homozygous normal (IV: 6), heterozygous carrier (III: 4) and homozygous mutant (IV: 7). (**D**) In-Silico predictions for mutation c.1686G>A; p. (Gln562Gln) by ALAMUT Visual. The score for natural 5' splice site was decreased in the mutant. Respective HSF and SSF scores have also been listed in the table.

site are highly conserved indicating its highly pathogenic potential (Ma *et al*., 2015). The pathogenicity of the synonymous change c.1686G>A is further supported *in silico* by the computer program scores, SSF, MaxEnt, and HSF (Fig. 3D). These scores indicate that as a result of the G>A change at position c.1686 the splice donor site will not be recognized by the splicing machinery anymore, and most likely will lead to intron retention. As introns frequently have stop codons, so this synonymous modification in the open reading frame will likely be introducing a premature stop codon.

DISCUSSION

The *LTBP2* gene lies near in to the GLC3C locus that was reported for the first time in a large Turkish PCG family (Firasat *et al*., 2008; Stoilov and Sarfarazi, 2002). In the recent study, three novel mutations in *LTBP2* are reported which includes a canonical splice site mutation c.2531-2A>C, a *de novo* mutation c.1762_1763del; p. (Leu588Valfs*14), and a synonymous missense c.1686G>A; p. (Gln562Gln) in 3 out of 20 Pakistani PCG families. Previously, more than 28 different alterations in the *LTBP2* gene have been implicated in various forms of glaucoma, especially PCG (Fig. 4). The previously reported mutations for PCG in Pakistani families include a c.412delG; p. (Ala138Prfs*278) mutation in exon 1; a nonsense variant in exon 4, c.895C>T; p. (Arg299X); a c.1243-1256del; p. (Glu415Argfs*596) homozygous removal of 14 bp in exon 6; and c.331C>T; p. (Gln111X) in exon 1 (Ali *et al*., 2009). One of these mutations (c.895C>T; p. (Arg299X); exon 4) has also been found in a European gypsy family supporting the idea that this mutation may have a common origin. Interestingly, another recent study described null variants in *LTBP2* in 2 Pakistani families characterized by secondary glaucoma along with ectopia lentis, myopia, microspherophakia, megalocornea and Merfanoid structures in elder children. These null mutations included novel homozygous mutations consisting of exons 4 and 9 (Désir *et al*., 2010). Whole genome homozygosity mapping for two additional Iranian PCG families also revealed two disease-segregating mutations in *LTBP2*, p.(Ser472fsX3) and p.(Tyr1793fsX55) resulting in a nonfunctional protein. These mutations were suggested to affect significantly the protein assembly and its role thereby interfering with both fibrillin 5 and fibrillin 1 interaction which is critical for the role of *LTBP2* (Narooie-Nejad *et al*., 2009). WES in the two PCG families with Pakistani origin led to the identification of two additional new missense p. (Arg1645Glu) and a new variant p. (Asp1345Glyfs*6) resulting in a frameshift in *LTBP2* found segregating with the diseased phenotype (Micheal *et al*., 2016).

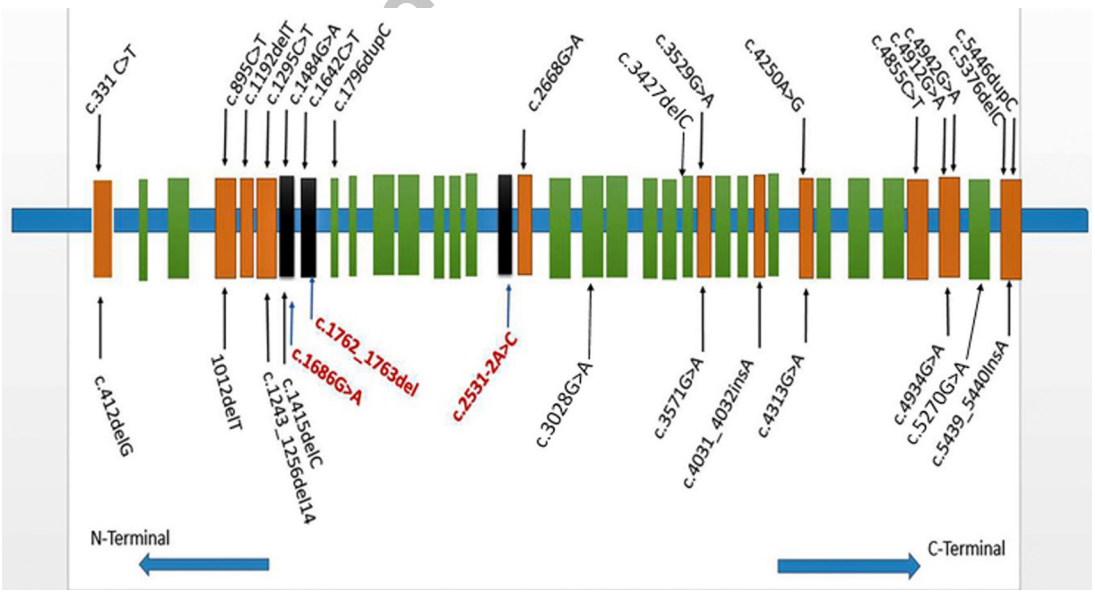

Fig. 4. Structural representation of *LTBP2* exons showing the N and C-Terminal of the protein. The exons with previously reported mutations have been shown in rust red color. Current study mutations and exons have been marked with red and black color, respectively.

In yet another family of south Indian origin, diagnosed with microspherophakia (a rare eye disorder characterized by spherical lens), in exon 36 a variant c.5446dupC has been stated. Thus, an elongation of 21 residues was resulted due to this variant in LTBP2 protein which replaced the preceding 6 with an additional 27 residues (Kumar *et al*., 2010). Finally, three novel changes have also been described segregating in three consanguineous families with Saudi Arabian origin, one in exon 4; p. (Ser338PfsX4), in exon 33, p. (Gln1619X) and exon 29, p. (Cys1438Tyr). The medical aspects of the disease carrying individuals in these families consisted of an ocular syndrome of congenital megalocornea and associated secondary glaucoma (Khan *et al*., 2011). Recently, another study reported three new pathogenic *LTBP2* variants p. (Gln1143Argfs*35), p. (Cys1757Tyr), and p. (Asp1010Asn) in Pakistani PCG families (Rauf *et al*., 2020). *LTBP2* changes have also been associated with Weill Marchesani-like syndrome, primary open-angle glaucoma, pseudo exfoliative glaucoma, and microspherophakia in addition to PCG (Alías *et al*., 2018; Jelodari-Mamaghani *et al*., 2013; Javadi *et al*., 2012). The *LTBP2* gene has also been investigated in northern India, the United States, the United Kingdom, and Chinese PCG cohorts but, in these countries, no mutations were found associated with PCG or related disease (Wang *et al*., 2018; Lim *et al*., 2013). A recent study regarding phenotype-genotype association for childhood glaucoma revealed a strong association between Weill Marchesani-like syndrome, childhood glaucoma, and *LTBP2* mutations by presenting a ratio of about 12.5% among all the cases studied for childhood glaucoma (Stingl *et al*., 2022).

The LTBP2 protein relates to the transforming growth factor superfamily that performs a significant part in the differentiation of organs and tissues. The gene codes for 1821 amino acids long protein molecule containing (Ali *et al*., 2009). LTBP2 is known as an extracellular matrix microfibril protein. The actual mechanism of *LTBP2* mutations causing PCG is still not clear. Interestingly, however, its high expression has been profiled in Trabecular Meshwork (TM), in the frontal chamber of the eye, and in the ciliary zonule microfibrils. At the cellular level, various causative mutations have been reported for PCG along with its role in ECM maintenance (Lim *et al*., 2013; Horiguchi *et al*., 2012). The protein is involved in the regulation of TGFβ1 signaling. It is important to regulate the downstream signaling and initiation of transcription factors by phosphorylation and activation of TGFBR1, upon the binding of TGFβ1 to TGFBR2 (Kielty *et al*., 2005). While interacting, the C-terminal of LTBP2 come in close association with the N-terminus of fibrillin 1, a critical component of the microfibrils (Verstraeten *et al*., 2016). The participation of LTBP2 in the development of elastic fibers during elastic tissues development has also been shown (Hirani *et al*., 2007). It is hypothesized that due to the presence of mutations in *LTBP2*, there results in changes in the structure of the protein that cease it to bind with FBN1 (Hirani *et al*., 2007). Furthermore, lens dislocation has also been reported in *LTBP2* null mice, this may be the result of the loss of arrangement of ciliary zonules, consisting of microfibrils (Inoue *et al*., 2014). Thus, the loss of ciliary organization and associated structures and tissues may result in high IOP and apoptosis of RGCs thus contributing towards glaucoma.

CONCLUSIONS

In conclusion, we report here a *de novo* pathogenic variant c.1762_1763del; p. (Leu588Valfs*14) in a family for PCG. We also report two novel splice site mutations c.2531-2A>C and c.1686G>A identified in *LTBP2* for two consanguineous families of Pakistan. This study along with the previous studies on the roles of *LTBP2* predicts that there is a lot more to explore for elucidating the exact mechanisms involved in the pathogenesis of PCG.

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Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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IRB approval

The consent for the research was obtained from the Institutional Review Board of the Al-Shifa Eye Trust Hospital (Rawalpindi, Pakistan).

Ethical statement

Ethical Committee of the University of Punjab Lahore, approved the study following the tenets of the Declaration of Helsinki. For inclusion in research, written

well-versed consensus was gained either from the members and/or their parents, as a pre-requisite for participation in the study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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