

Screening of the *LTBP2* gene in 214 Chinese sporadic *CYP1B1*-negative patients with primary congenital glaucoma

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Purpose: To identify deleterious mutations in the latent transforming growth factor- β -binding protein 2 (*LTBP2*) gene in sporadic patients with primary congenital glaucoma (PCG) from a Han Chinese population, which had been excluded for mutations in the *CYP1B1* gene.

Methods: In this retrospective case-control study, 36 coding exons and adjacent exon-intron boundaries of *LTBP2* were amplified with PCR and screened for mutations with Sanger sequencing in DNA samples of 214 sporadic patients with PCG. Sequence variants identified in the patients with PCG were subsequently screened in 100 unaffected control subjects and the unaffected parents of the patients with PCG who had sequence changes in *LTBP2*.

Results: Eight heterozygous single nucleotide polymorphisms (SNPs) in coding regions of *LTBP2* were identified in the patients with PCG. Four of these SNPs were missense changes that resulted in the replacement of amino acids (rs2304707, rs116914994, rs45468895, and rs763035721), two of which (rs2304707 and rs116914994) were also present in the control subjects. No significant differences in the frequencies of the missense SNPs were found between the patients with PCG and the controls. The two missense SNPs, rs45468895 and rs763035721, which were each found in one patient also existed in their unaffected parents, suggesting that these two SNPs were not segregated in these families and are unlikely to be a disease-causative variant. In addition, four synonymous SNPs were detected in the patients with PCG (rs61738025, rs862031, rs199805158, and rs12586758).

Conclusions: The results showed that no deleterious mutations were found in coding regions of *LTBP2* in patients with PCG, suggesting that it is not a causal gene for PCG in the Han Chinese population.

Primary congenital glaucoma (PCG) is a rare but severe form of glaucoma that can lead to blindness from the infantile period. The characteristic findings of PCG include the classic triad of presenting symptoms in the newborn: epiphora, photophobia, and blepharospasm [1]. Elevated intraocular pressure (IOP) causes optic atrophy in the late stage. The incidence of PCG varies from country to country depending on ethnicity. The highest incidence (1 in 1,250) occurs in the Slovakian Romani population with higher rates of consanguinity, while lower incidence (1 in 18,500 to 1 in 30,000) is observed in Western populations [2,3]. The prevalence in

the Han Chinese population is approximately 1 in 5,000 to 1 in 25,000 (1 in 10,000 on average) [4]. Several genetic loci have been linked to PCG in family linkage studies, including *GLC3A* (Gene ID: 1545, OMIM #: 601771; 2p22-p21) [5], *GLC3B* (Gene ID: 2728, OMIM #: 600975; 1p36.2-p36.1) [6], *GLC3C* (Gene ID: 399565, OMIM #: 613085; 14q24.2-q24.3) [7], and *GLC3D* (Gene ID: 4053, OMIM #: 602091; 14q24) [8,9]. Two genes have been identified from these loci: *CYP1B1*, coding for cytochrome P450, family 1, subfamily B, polypeptide 1 at *GLC3A* [10], and *LTBP2*, coding for latent transforming growth factor- β -binding protein 2 at *GLC3D* [8,9].

Previous studies showed that mutations in *CYP1B1* are the predominant cause of PCG [11,12]. Populations with a higher mutation frequency of *CYP1B1* in patients with PCG often possess a higher incidence of PCG. For instance, Arab and Romani populations have the highest incidence of PCG, corresponding to the highest mutation frequency of *CYP1B1* in patients with PCG (90% to 100%) [13]. However, in our previous study, only 17.2% of patients with PCG had *CYP1B1* mutations in a Han Chinese population [14], corresponding

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to the lower incidence of PCG in the Han Chinese population [4]. The low frequencies of *CYP1B1* mutations in patients with PCG from some populations [14,15] indicate that PCG is genetically heterogeneous, and other genes may contribute to the disease.

The *GLC3C* locus spans a region of around 5.77 Mb that contains more than 40 genes, and it has been confirmed and further mapped within a genetic distance of 0.006 cM [16]. *LTBP2* is around 1.3 Mb proximal to the *GLC3C* locus [8,9]. Ocular expression studies have determined the presence of *LTBP2* in the trabecular meshwork and ciliary processes [17]. Recently, *LTBP2* has been verified as essential for the development of ciliary zonule microfibrils [18]. Several studies have shown that *LTBP2* mutations are associated with secondary glaucoma [19-22]. Null mutations have been found in patients with PCG from Pakistani, Iranian, and Slovakian Romani consanguineous families [8,9,23]. However, the exact role of *LTBP2* in the development of PCG remains unclear. Several studies did not discover any deleterious mutations in *LTBP2* in sporadic PCG North Indian, British, and American cases [24-26]. Intriguingly, a possible role of *LTBP2* has been suggested in the etiology of primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG), and secondary glaucoma, such as megalocornea, spherophakia, ectopia lentis, Weill-Marchesani syndrome, and pseudoexfoliation syndrome [19-22,27,28]. In the present study, we screened for mutations in the *LTBP2* gene in a large sample of Chinese patients with PCG, aiming to evaluate the role of *LTBP2* in Chinese patients with PCG.

METHODS

Study subjects: A total of 214 unrelated patients diagnosed with PCG but without mutations in the *CYP1B1* gene were included in this study [14,29]. Briefly, we amplified the coding regions (exons 2 and 3) and the promoter region of *CYP1B1* with PCR and screened for *CYP1B1* mutations with direct sequencing with forward and reverse primers [14,29]. PCR was performed in a 25 μ l mixture containing genomic DNA (50 ng), forward and reverse primers (0.5 μ mol/l), magnesium chloride (1.5 mmol/l), deoxyribonucleotide triphosphate (dNTP; 0.2 μ mol/ml) and Taq polymerase (1 U; Takara Bio Inc, Shiga, Japan), as per the following procedure: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at a primer-specific temperature for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. We excluded patients who carried *CYP1B1* mutations from the present study. Among these sporadic cases, 152 trios that consisted of one affected proband and two unaffected parents had been used in our

previous study of *GLC3C* with the transmission disequilibrium test (TDT) [16]. As the other 62 sporadic cases had only one parent available for genotyping, they were excluded from TDT in our previous study [16]. The diagnosis of PCG was based on the following criteria [29]: 1) Age of diagnosis was less than 3 years old, 2) the cornea was enlarged so that the parameter was bigger than 11 mm or edema with or without Haab striae, 3) IOP was greater than 21 mmHg, and 4) the cup to disk ratio was enlarged or dissymmetric between two eyes. Patients with other ocular or systemic anomalies, such as aniridia, anterior segment dysgenesis, congenital hereditary endothelial dystrophy, neurofibromatosis, and Sturge-Weber syndrome, were excluded.

All patients underwent comprehensive examinations of the anterior segments with slit-lamp and surgical microscope, the structure of anterior chamber angles with gonioscope, and the ocular fundus with a direct ophthalmoscope. IOP was measured with Tono- PEN[®] (Reichert, Depew, NY) before or during surgery under general anesthesia. Corneal opacity was scored based on the microscope examination, and a higher corneal opacity score indicated a more opaque cornea [29]. The more severe eye was recorded and analyzed when cases were bilateral present.

One hundred ethnically matched unrelated individuals without any ocular or systemic disorders were enrolled as controls. The unaffected parents of the patients with PCG who had sequence changes in *LTBP2* were also included in the study. All the controls and healthy parents were older than 18 years and had normal IOP, open angles on gonioscopy, and normal optic nerves on ocular fundus examination. Peripheral blood samples were collected from patients and controls by venipuncture in EDTA vacutainers and stored in -20 °C until further use.

All study subjects were Han Chinese who live in Mainland China. This study adhered to the ARVO statement on human subjects. This study was approved by the Ethics Committee of the Eye and ENT hospital, Fudan University. Informed written consent was obtained from all study subjects after explanation of the nature and possible consequences of the study, in accordance with the tenets of the Declaration of Helsinki.

Screening for *LTBP2* mutations with Sanger sequencing: DNA extraction and Sanger sequencing were performed for all 214 patients with PCG using standard methods [30]. The primers were designed to include the intron-exon boundaries of *LTBP2* using the software Primer Premier 5.0 (Table 1). Standard PCR conditions were run to amplify all exons and exon-intron boundaries of *LTBP2*. PCR was performed in a 25 μ l mixture containing genomic DNA (50 ng), forward

and reverse primers (0.5 $\mu\text{mol/l}$), magnesium chloride (1.5 mmol/l), deoxyribonucleotide triphosphate (dNTP; 0.2 $\mu\text{mol/ml}$) and Taq polymerase (1 U; Takara Bio Inc, Shiga, Japan), as per the following procedure: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at a primer-specific temperature for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. All PCR products were analyzed on 2% agarose gel and stained with ethidium-bromide (EB, 10 mg/ml). Then the right size products were purified with spin-columns technology (QIAQuick PCR Purification Kit; Qiagen, Valencia, CA). Using ABI BigDye chemistry (Applied Biosystems Inc., Foster City, CA), Sanger sequencing was performed on the PCR amplicons and was processed through an automated ABI 3730 XL Sequencer (Applied Biosystems). The sequences were scrutinized for variations alongside reference sequences from the University of California, Santa Cruz [Genome Browser website](#) using Sequencher 5.0 software (Gene Codes, Ann Arbor, MI). PCR and Sanger sequencing were subsequently performed on 100 unaffected control samples with identified sequence variants. The unaffected parents of the patients with PCG who had sequence changes in *LTBP2* were also screened for the specific SNPs using Sanger sequencing. The PCR products were first sequenced with forward primers. Further sequencing with reverse primers was performed to confirm the identified variants.

Statistical analysis: Fisher's exact test was used to compare the allele frequencies of the *LTBP2* variants between patients with PCG and controls. A Bonferroni corrected p value of 0.0125 was considered statistically significant.

RESULTS

Clinical features of the patients: Some clinical features of the patients with PCG in this study are shown in Figure 1. At the preoperative examination, the median IOP was 36 mmHg, with an average corneal diameter of 13.5 mm. The median age of diagnosis was 4.5 months, ranging from birth to 3 years old. The median corneal opacity score was 2, indicating mild corneal haze with visible iris. Figure 2 shows the classic clinical features seen in the left eye of a patient with PCG.

Screening for *LTBP2* mutations: Eight single nucleotide polymorphisms (SNPs) were identified in the coding regions of the *LTBP2* gene in the patients with PCG. All these variants were inherited in a heterozygous manner. Four missense SNPs resulted in the replacement of amino acids ([rs2304707](#), [rs116914994](#), [rs45468895](#), and [rs763035721](#), Table 2). Two of the SNPs, [rs2304707](#) and [rs116914994](#), which changed amino acids p.Pro319Gln and p.Arg1429Gln, respectively, were also present in the control subjects. No significant differences in

the frequencies of these missense SNPs were found between the patients with PCG and the controls. The other two missense SNPs, [rs45468895](#) and [rs763035721](#), which changed amino acids p.Ala1204Val and p.Glu1794Lys, respectively, were each found in one patient with PCG. Their unaffected parents also had the same sequence changes, suggesting that these two SNPs were not segregated in these families and, thus, were unlikely to be a disease-causative variant. In addition, four synonymous SNPs were detected in the patients with PCG ([rs61738025](#), [rs862031](#), [rs199805158](#), and [rs12586758](#)).

DISCUSSION

Null mutations in *LTBP2* were initially found to cause PCG in four consanguineous families from Pakistan and in patients of Romani ethnicity [8]. Subsequently, mutations in *LTBP2* were identified in patients with PCG from several other populations, such as Iranian and Slovakian Romani families (Table 3) [9,23]. However, several other studies did not discover deleterious mutations in *LTBP2* in sporadic North Indian, British, and American patients with PCG [24-26]. In the present study, we screened 214 sporadic patients with PCG from the Han Chinese population for mutations in the *LTBP2* gene. We did not find disease-causative mutations in *LTBP2* in the patients with PCG, which is consistent with previous studies [24-26]. None of the previously reported pathogenic mutations was detected in our study (Table 3) [8,9,23]. In addition, the SNP [rs2304707](#) identified in this study was also reported as a polymorphism in consanguineous Iranian families with PCG and a Turkish *GLC3C*-linked family with PCG [9,25]. Another SNP [rs862031](#) has been reported in patients in the United States [26]. None of the other SNPs identified in the present study was reported in patients with PCG in other populations [8,9,23].

In addition to PCG, *LTBP2* has been related to the development of secondary glaucoma, such as megalocornea, spherophakia, ectopia lentis, Weill-Marchesani syndrome, and pseudoexfoliation syndrome [19,21,22,27]. This can be well explained by recent findings in *Ltbp2*^{-/-} mice [18]. The mice survived to adulthood but developed lens luxation caused by compromised ciliary zonule formation. The suppression of *LTBP2* expression in cultured human ciliary epithelial cells by siRNA disrupted the formation of the microfibril meshwork by the cells [18]. Supplementation of recombinant *LTBP2* in culture medium not only rescued the microfibril meshwork formation in *LTBP2*-suppressed ciliary epithelial cells but also restored unfragmented and bundled ciliary zonules in *Ltbp2*^{-/-} mouse eyes under organ culture [18]. The *LTBP* proteins are important components

TABLE 1. PCR PRIMERS USED FOR AMPLIFICATION OF *LTBP2* GENE.

Primer name	Forward Primer	Reverse Primer	Product size (bp)
Exon1	GGGAGAGATGAAAGCAGGCAAGG	AGGTGGAGGCAGCCAGCAGA	350
Exon2	CGTTCAAGTCATCACACTCATTACA	AAGGGCGAGGGTCCCAGCATT	666
Exon3	TGTGATAGTTTATGACGGAGTTGTTAGGG	GCATTGAGGCAGAGCGGGAG	456
Exon3-5	CTCTGGCTTGGTGTGTCTTTCCC	CAATGCCCCACCCTTTCTCTG	1110
Exon5-6	GTGGATGTCGTGGTCAGGGAGGT	GCTAGTCTTGAGAAGGAAATGATGGAG	982
Exon7	TGGGACAGAAAAGGTGGAGGC	GTGACACTGAAAGAAGGGGAGAGA	289
Exon8	CGTCCAGGCTCCTCCACAG	TCTTGAGATGATGATTAGGCCACTT	559
Exon9	GGACACGCTCTCTGTAAACCTCTGA	TGCCACTCCTCTCCTTTTTTCTCT	100
Exon10-11	CCCCTGGGCGAGGAGTTGAA	GGCAGTGGAGGAAAAGGAGTG	1027
Exon12	CATCTGTGGAAAGTGCTCTGCTC	TCCCTGACTCTCCTATGGGCTACT	329
Exon13-14	CCTCACATCACTCCTCGCTCCC	GCTGCCATCGGCTGTCTTCC	578
Exon15	AGAGGGGGCATCCCATTGTATC	AGGAGGAAAAGCCAGTCCCAGAG	354
Exon15-16	GGGGGCATCCCATTGTATCCT	CCTATTCACACTGACCCAACACATCC	789
Exon17	CATTCACCCAGTACCCGTTCTCAC	GCCCACCGTCTTGGACTTCTCTC	488
Exon18	ACAACAGCCTCCAAGCCACCTCC	GTCCATCCCTCTCCCCAACCAA	344
Exon19	GCAACAGGGCGGAGGTTACAG	GCAGACCCGAAGGGGATATAGTG	788
Exon20	ATTCTGCGGCACGGTGTTTG	ACTGGGCTGACTTTATGGCTTCC	409
Exon21	ACTGCTTGGACCTTCTGCTTCTC	GGGGGATGCCCTACTATGACCT	465
Exon22	TCCCCCTTCTGGGCTTGG	GGCTTACCCTTCGTTCTTGG	445
Exon23	TCTGATTATGTGCTAAGTGGTGC	GCTTCTGTTACTGTGTTGAGATGCT	654
Exon24	TCCGTGAGGTGGTTTGAGGC	GGGCAGAGGCAGGTAAGAAGAGT	645
Exon25	ATGGAAGAGGAAGTGGGTGGGG	ATGGGGCAGCGAGTGTGAGAA	710
Exon26	AAGCCAACAGCCAGAGGACAAAC	TGGGGAGTCACGAGTCTCATCAAG	657
Exon27	TCCAGGTTGAATAGCCGACAC	AAACTGAGGCACAGGGAGAGG	422
Exon28	GGCTGACTTGATTTCCACCCTA	TTTGTCTTTCTGTGCCTGTCTTATTC	697
Exon29	GCTGGAACAGGAGGTAAGGAGATAA	TGGCAGAATGTGGGGAGTGAG	626
Exon30	AACAAAGAGGACAGAGAGGAGGAGAAG	TCAGAGGGTTGGAAATGAGGGTG	443
Exon31	TCCACCCAAGTGGCACTCCTC	CCAAGTCTGGCTTCCGCATCT	783
Exon32	AGAGAAACAGGCGGCAGAACC	AAAATACCAACACTCAGCCATAGGA	897
Exon33	ATGATAGATTGGGCAGAAATAGAGC	GTAGGGAGTAGGTACAGGACTTGTT	846
Exon34	ATGCCAGGGAAAGCCAAGGA	TGTGGGACGTGGTGGTGTGAG	680
Exon35	TGGGAGTAGGGTGGGGAATGG	AGAAGGTGGGACTGTCCGAGGG	225
Exon36	GCCTGATGTCACGGTGTCTTCC	GCTCCCTACAGCACTCTCCTTGG	281

of the extracellular matrix (ECM) that interact with fibrillin microfibrils and have several different roles in microfibril biology. There are four LTBP isoforms in the human genome (LTBP-1, -2, -3, and -4). LTBP-2 is expressed abundantly in elastic tissues and unique in the family as it is the only one that does not bind to latent transforming growth factor-beta (TGF- β) [31]. Several reports indicate that LTBP-2 has a role in the elastic fiber assembly process, especially fibrillin-1 and -2 [18,32]. Whether increased IOP is a primary symptom or

a secondary effect of another abnormality caused by *LTBP2* mutations in childhood glaucoma remains controversial.

In summary, we screened for mutations in the *LTBP2* gene in a large sample of Chinese patients with PCG. We did not detect deleterious mutations in *LTBP2*, suggesting that this gene is not a causal gene for PCG in the Han Chinese population.

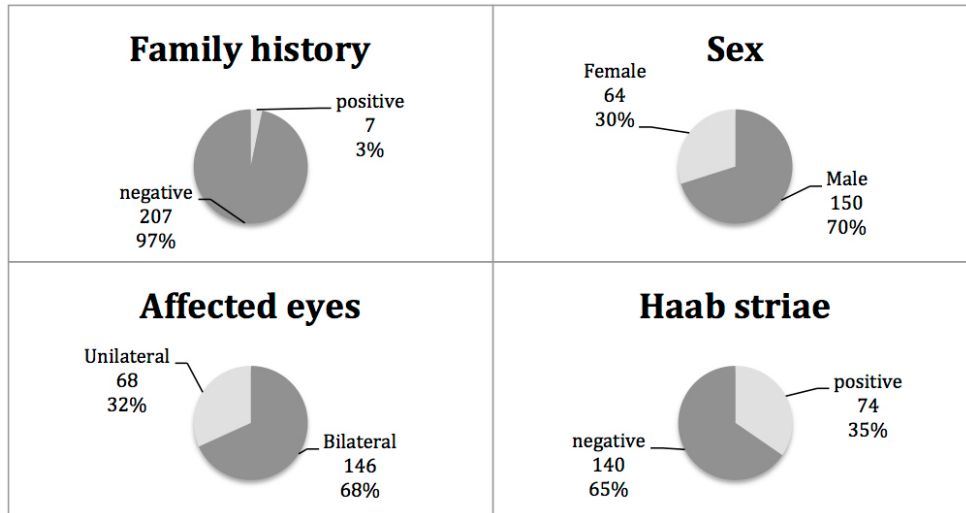


Figure 1. Clinical features of Chinese patients with primary congenital glaucoma.

TABLE 2. *LTBP2* POLYMORPHISMS IDENTIFIED FROM PATIENTS WITH PRIMARY CONGENITAL GLAUCOMA.

SNP ID	Exon	Sequence change	Amino acid change	Minor allele frequency (%)		P value
				Affected	Unaffected	
rs2304707	4	c.956C>A	p. Pro319Gln	35/370 (9.5)	7/90 (7.8)	0.62
rs45468895	24	c.3611C>T	p. Ala1204Val	1/428 (0.2)	0/200 (0.0)	0.49
rs116914994	29	c.4286G>A	p. Arg1429Gln	22/428 (5.1)	16/200 (8.0)	0.16
rs763035721	36	c.5380G>A	p. Glu1794Lys	1/428 (0.2)	0/200 (0.0)	0.49

Fisher's exact test was used to compare the allele frequencies of the *LTBP2* variants between PCG patients and controls. A Bonferroni corrected p value of 0.0125 was considered significant.

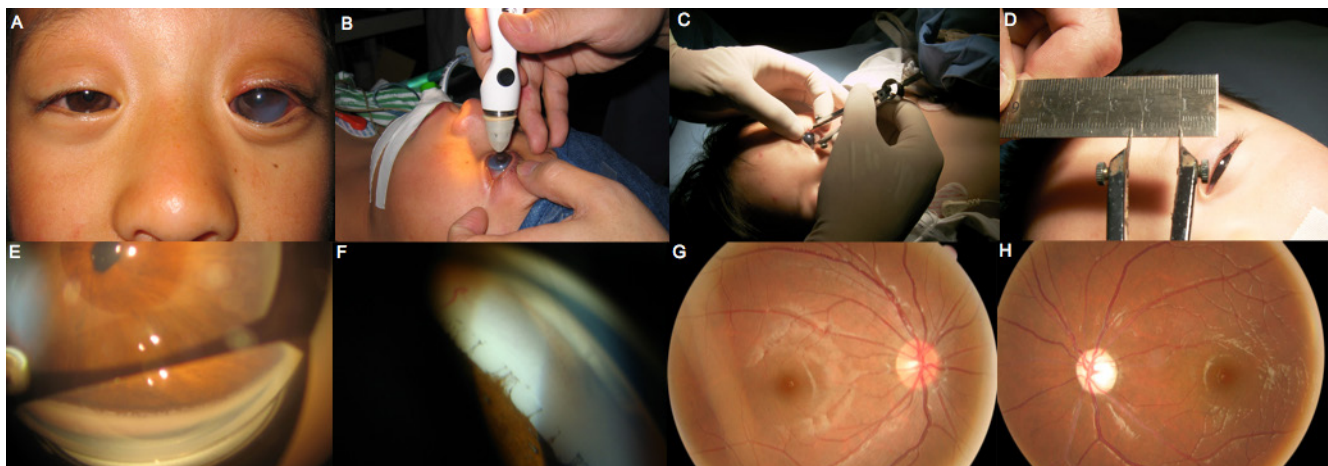


Figure 2. Classic features of Chinese patients with primary congenital glaucoma. **A:** The left eye is obviously bigger than the right eye and mild corneal haze with visible iris. **B:** Intraocular pressure (IOP) was measured with Tono-PEN®. **C, D:** The horizontal diameter of the cornea is about 13 mm measured with bisecting compasses. **E, F:** The structure of the anterior chamber angle on gonioscopy; absence of angle recess; peripheral iris hypoplasia; tenting of the peripheral iris pigment epithelium. **G, H:** The fundi photos show a dissymmetric cup disc ratio.

TABLE 3. *LTBP2* MUTATIONS/ POLYMORPHISMS PREVIOUSLY REPORTED IN PATIENTS WITH PRIMARY CONGENITAL GLAUCOMA FROM OTHER POPULATIONS.

Exon	Sequence change	Amino acid change	SNP ID	Population	References
1	c.331C>T	p.Gln111X p. Pro319Gln	rs121918356 *	Pakistan/ Gypsy *	8 *
1	c.412delG	p.Ala138ProfsX278	N.A.	Pakistan/ Gypsy	8, 23
4	c.895C>T	p.Arg299X	rs121918355	Pakistan, Roma / Gypsy	8
4	C>A	p.Pro319Gln	rs2304707	American	26
6	c.1243-1256 del	p.Glu415ArgfsX596	N.A.	Pakistan/ Gypsy	8
6	c.1287G>A	p.Leu429Leu	rs61738025	Iranian	9, 26
Intron 6-7	N.A.	g.75070493	rs3742793	North Indian	24
7	c.1415delC	p.Ser472fsX3	N.A.	Iranian	9
7	G>T	p.Ser518Ile	N.A.	American	26
7	C>T	p.Arg548X	N.A.	American	26
7	C>T	p.Asn560Asn	N.A.	American	26
14	T>C	p.Thr802Thr	rs699374	American	26
15	C>T	p.Thr834Thr	rs862031	American	26
17	C>T	p.Arg905Arg	rs7145480	American	26
19	c.2966C>G	p.Pro989Arg	rs76172717	Iranian	9
20	C>T	p.Ser1031Ser	rs45473602	American	26
22	G>A	p.Thr1111Thr	rs61729544	American	26
26	C>T	p.Arg1284Arg	rs61736977	American	26
26	G>A	p.Prol297Pro	rs61738013	American	26
29	G>A	p.Ala1421Ala	rs61738017	American	26
33	c.4808G>A	p.Arg1603His	rs75200417	Iranian	9
36	c.5376delC	p.Tyr1793fsX55	N.A.	Iranian	9

N.A., not available.

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