·Basic Research·

TGFBI and *CHST6* gene analysis in Chinese stromal corneal dystrophies

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Abstract

• AIM: To investigate whether mutations in *TGFBI* gene or *CHST6* gene correlated with stromal corneal dystrophies (CD) in 8 Chinese probands.

• METHODS: Eight unrelated patients with stromal corneal dystrophies were recruited in this study; all affected members were assessed by completely ophthalmologic examinations. Genomic DNA was extracted from peripheral leukocytes, 17 exons of *TGFBI* gene and the exon of *CHST6* gene were amplified by polymerase chain reaction (PCR), sequenced directly and compared with the reference database.

• RESULTS: Three heterozygous mutations in *TGFBI* gene were identified in six patients: c. 370C>T (p.Arg124Cys) was found in exon 4 of *TGFBI* gene in three members, c. 371G>A (p.Arg124His) was found in one patient; c. 1663C>T (p. Arg555Trp) was found in exon 12 in other two members. In addition, four polymorphisms with the nucleotide changes rs1442, rs1054124, rs4669, and rs35151677 were found in *TGFBI* gene. Mutations were not identified in the rest of 2 affected individuals in *TGFBI* gene or *CHST6* gene.

• CONCLUSION: Within these patients, R124C, R124H and R555W mutations were co-segregated with the disease phenotypes and were specific mutations for lattice corneal dystrophy type I (LCD I), Avellino corneal dystrophy (ACD, GCD Ⅱ), granular corneal dystrophy type I (GCD I), respectively. Our study highlights the prevalence of codon 124 and codon 555 mutations in the *TGFBI* gene among the Chinese stromal corneal dystrophies patients.

• KEYWORDS: corneal dystrophies; Mutation screening; TGFBI gene; TGFBI protein; carbohydrate; sulfotransferse *CHST6*

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INTRODUCTION

Corneal dystrophies (CDs) define a heterogeneous group of inherited, bilateral, primary alterations of the cornea that are not associated with prior inflammation or systemic diseases. Based on the affected anatomical layer, corneal dystrophy can be classified as epithelial and subepithelial or Bowman layer and the superficial corneal stroma (anterior corneal dystrophies), stroma layer (stromal corneal dystrophies), Descemet membrane and the endothelium (posterior corneal dystrophies). *TGFBI* (BIGH3, OMIM: 601692) and *CHST6* (OMIM: 605294) are more likely two candidate virulence genes for stromal corneal dystrophies[1].

Human *TGFBI* gene locates in chromosome 5q31 [2] and encodes a protein named TGFBIp or keratoepithelin (KE), which is constitutively expressed in numerous tissues of human. However, mutations in the *TGFBI* gene so far reported are correlating with corneal epithelial and stromal diseases exclusively[3].

Furthermore, point mutations of *TGFBI* bring about five phenotype-specific corneal dystrophies: p.R555W in granular CD type I (GCD I,OMIM: 121900), p. R124C in Lattice CD type I (LCD I, OMIM: 122200), p. R124H in Avellino CD, (ACD,GCD Ⅱ, OMIM: 607541), p.R555Q in Thiel-Behnke CD (CDTB, OMIM: 602082), p.R124L in Reis-Bucklers CD (CDRB,OMIM:608470)[4]. Particularly noteworthy is the fact that specific mutations in *TGFBI* consistently cause specific types of corneal deposits. The nature of corneal deposits associated with TGFBI mutations is of amyloid (R124C), non-amyloid (R555W, R555Q and R124L) or mixture of both (R124H) [5]. A progressive alteration of the cornea resulting in loss of transparency and finally blind. KE is found not only in primary amyloid deposits of hereditary cornea dystrophies, but also in secondary amyloidosis of the cornea of diverse ethiologies [6]. These mutations were reported independently in various ethnic populations around world including Chinese [7-12]. Among them, mutations at two positions (R124 and R555) are responsible for approximately 50% of all *TGFBI* related corneal dystrophies that are diagnosed [13], indicating the amino acids R124 and R555 represent hot spots for mutations in the *TGFBI* gene-linked corneal dystrophies[14].

Table 1 Primers and annealing temperatures for PCR of *TGFBI* gene and *CHST6* gene

Exon	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp (℃)
TGFBI-E1-F	GCGCTCTCACTTCCCTGGAG	252[18]	62
TGFBI-E1-R	GACTACCTGACCTTCCGCAG		
TGFBI-E2-F	AGGCAAACACGATGGGAGTCA	279[19]	62
TGFBI-E2-R	TAGCACGCAGGTCCCAGACA		
TGFBI-E3-F	ACCTGTGAGGAACAGTGAAG	200[2]	64
TGFBI-E3-R	GCCTTTTATGTGGGTACTCC		
TGFBI-E4-F	TTCCCACATGCCTCCTCGTC	390	58
TGFBI-E4-R	AGTTCCACAGCCTTTTCTAA		
TGFBI-E5-F	TCACGAGGGCTGAGAACA	351	60
TGFBI-E5-R	AGGAGAAGCAGGCACAAA		
TGFBI-E6-F	CATTTTGCTACTGTGTTTGAA	306	57
TGFBI-E6-R	AGCCATCCTCTACCTCTCC		
TGFBI-E7-F	GTGAGCTTGGGTTTGGCTTC	347[19]	56
TGFBI-E7-R	ACCTCATGGCAGGTGGTATG		
TGFBI-E8-F	TGAGGTTATCGTGGAGTG	399[19]	55
TGFBI-E8-R	CACATCAGTCTGGTCACA		
TGFBI-E9-F	GTGTTTAGAGGGGTTGTTGACT	372	62
TGFBI-E9-R	TCTCTGCACTGCCTAATGAA		
TGFBI-E10-F	TAGAAGATACCAGATGTTAAGG	384[19]	54
TGFBI-E10-R	TGTCAGCAACCAGTTCTCAT		
TGFBI-E11-F	CTTAATAACCCATCCCAGTGT	332	58
TGFBI-E11-R	TGAATCCCCAAGGTAGAAGA		
TGFBI-E12-F	GTGGCCTGGACTCTACTATC	384	64
TGFBI-E12-R	CCCTGGTTGGCCTCATCCTT		
TGFBI-E13-F	CATTAGACAGATTGTGGGTCA	378[19]	60
TGFBI-E13-R	GGGCTGCAACTTGAAGGTT		
TGFBI-E14-F	CAGCCTGGGCGACAAGAT	396	64
TGFBI-E14-R	GTGCATTCAAAACCAACAG		
TGFBI-E15-F	CCCTCAGTCACGGTTGTT	312[19]	56
TGFBI-E15-R	GGAGTTGCCTTGGTTCTT		
TGFBI-E16-F	CTTGCACAACTTATGTCTGC	279[19]	56
TGFBI-E16-R	TGCACCATGATGTTCTTATC		
TGFBI-E17-F	CAGGAGAGCATGGCAGAAGGA	178	58
TGFBI-E17-R	AGAGAAATTGGCGGAGAG		

Carbohydrate sulfotransferase 6 gene (*CHST6*), encodes the enzyme N-acetylglucosamine-6- sulfotransferase (GlcNAc6ST). *CHST6* gene mutation causes the corneal keratin sulfate (KS) metabolism, results the deposition of an unsulfated KS [15] and macular corneal dystrophy (MCD, OMIM: 217800). It is characterized by the presence of grayish and diffuse glycosaminoglycan deposits in keratocytes and stromal lamellae, causing severe visual impairment [16].

In this study, corneal lesions of 8 subjects were located in different depth of stromal layer. So we selected *TGFBI* and *CHST6* genes for research. Of the eight cases, mutations were identified in 6 patients in TGFBI gene, which corresponded to three forms of corneal dystrophies:p. R124C in LCD I,p. R124H in GCDII, and p.R555W in GCD I.

MATERIALS AND METHEDS

Subjects Eight sporadic probands with corneal dystrophies from 8 Chinese families were recruited in this study. All the patients were performed with complete ophthalmologic examination by the experienced ophthalmologists (Q.Z.). They were initial treatment, but none underwent surgery. 3 patients were diagnosed as lattice CD type I (LCDI) which was characterized by refractile lattice lines that were associated with white dots and faint haze in the anterior stroma. Twe patients presented with granular CD type I (GCDI) which was characterized by discrete deposits of gray-white, rounded, crumb-shaped opacities in the anterior central stroma. One presented with Avellino CD (GCDII), combined features of both granular, stellate opacities and lattice lesions. Two exhibited 'atypical phenotypes' CD with irregular gray-white haze in the subepithelium and superficial stroma of central corneal. Informed consent conforming to the tenets of the Declaration of Helsinki was obtained from each of participants. This study was approved by a local institutional medical ethics committee.

Methods

DNA extraction and polymerase chain reaction Peripheral blood samples (5mL) were collected from all participators and genomic DNA was extracted with phenol chloroform isoamyl alcohol method [17]. TGFBI (NCBI Reference Sequence: NC_000005.9) gene was amplified in these 8 patients and CHST6 (NCBI Reference Sequence: NC_000016.9) gene was amplified in the 2 'atypical phenotypes' patients, using polymerphrase chain reaction. The primers and conditions for PCR are listed in Table 1 and Table 2. All primers were synthesized by Shanghai

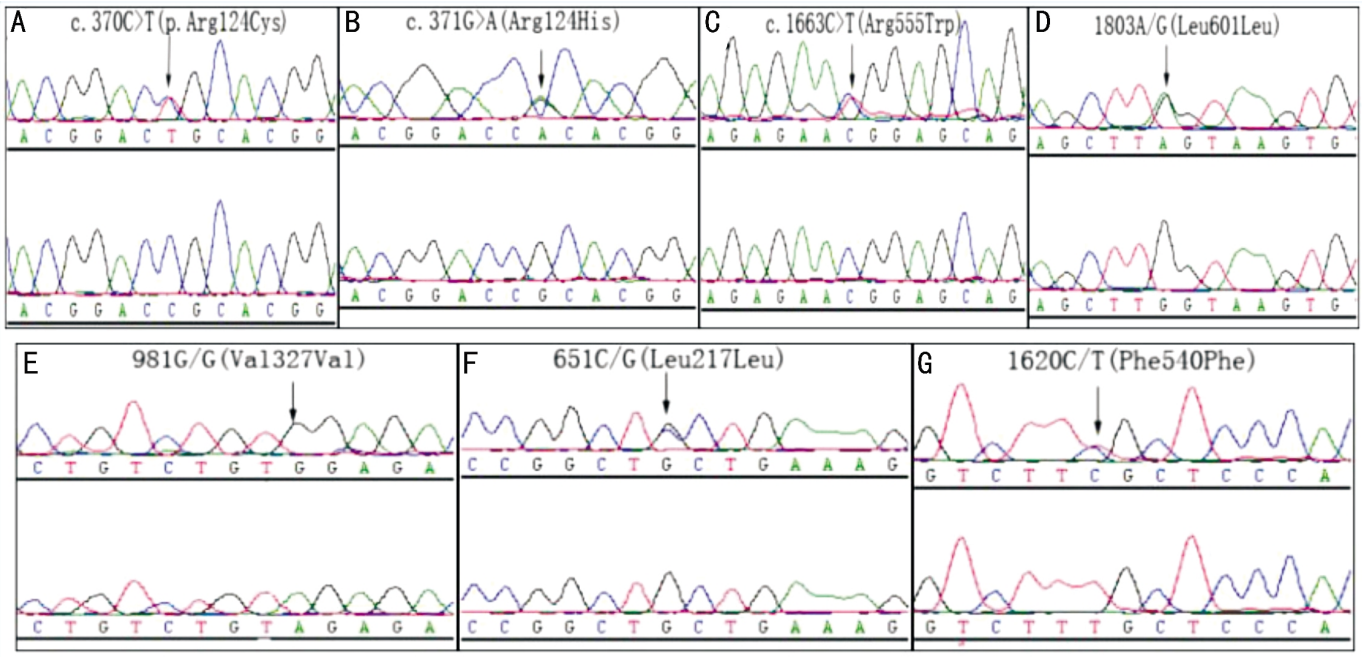

Figure 1 The experimental maps of three loci mutations and four SNPs A: The heterozygous c.370C>T (p.R124C) mutation in exon 4 in one of three CDLI families; B: The heterozygous c.371G>A (p.R124H) mutation in exon 4 in the GCDII family; C: The heterozygous c. 1663C>T (p.R555W) mutation in exon 12 in two GCDI families; D: A heterozygous 1803A/G (L601L)SNP in exon 13; E**:** A homozygous 981G/G (V327V) SNP in exon 8; F: A heterozygous 651C/G(L217L) SNP in exon 6; G:A heterozygous 1620C/T(F540F)SNP in exon 12. Blank arrows highlight the position of nucleotide substitutions. Control electropherograms are shown for comparison purposes (A-G, bottom panel).

Table 2 Summary of the annealing temperatures and PCR products length of *CHST6* gene, which were divided into 3 overlapping amplicons

Exon	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp (℃)
CHST6PF1	AGTGGGGTACGGGATGGG	559	62
CHST6PR1	GCCAGGGTGAAGGACTGC		
CHST6PF2	GCAACCTGTCCGACCTCT	531	62
CHST6PR2	CAGTGAAGGCGTAGAGCG		
CHST6PF3	AAGCCGCCACCCTTTCTG	500	60
CHST6PR3	GGGACCTGCTTCTCCGTGC		

Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China)

Primer 1 was quoted from Afshari *et al* [18]. Primer 3 was quoted from Munier *et al*[2]. Primer 2, 7,8,10,13,15,16 were cited from Ma *et al* [19].The rests were designed by DNA Star primerSlect Programme.(DNA Star Inc., Madison, WI).

Mutation screening The 25μL PCR reaction mixture included 10×PCR buffer (Mg^{2+} plus) (2×GC buffer I was used in exon 1), 80ng DNA, 5.0mmol/L of each of dNTPs, 2.0U rtaq DNA polymerase, and 5.0μmol/L of each of the forward and reverse primers. All reagents used in this procedure were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Thermocycling included an initial denaturation step at 94℃ for 5 minutes , followed by 36 cycles of denaturation at 94℃ for 30 seconds, annealing at 53-64℃ for 30 seconds, and extension at 72℃ for 30-40 seconds, a final extension at 72℃ for 5 minutes.

The PCR products were purified and terminator cycle sequencing was carried out using the Big Dye v3.1 kit and unidirectional sequencing reactions were analyzed in ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing results from patients as well as the consensus sequences from the NCBI Human Genome Database were imported into the SeqManII program of the Lasergene package (DNAStar Inc., Madison, WI) and aligned to identify variations.

RESULTS

Sequencing Analysis We identified a heterozygous C to T transition at position c.370 (CGC to TGC) in exon 4 of *TGFBI* in three patients, which caused the substitution of Arg for Cys at codon 124, can be related to LCDI(Figure 1). We detected a heterozygous G to A transition at nucleotide c.371 (CGC to CAC), located in exon 4 in one GCDII patient. This nucleotide substitution resulted in the missense

Table 3 Details of *TGFBI* mutations including amino acid change and cSNP in each patient

Patients	Dystrophy	TGFBI mutations	
		Amino Acid Change	cSNP
1	GCDI	c.1663C>T, R555W	651C/G,L217L 981G/G, V327V 1620C/T,F540F
2	GCDII	c.371G>A ,R124H	—
3	GCDI	c.1663C>T, R555W	981G/G,V327V 1620C/T,F540F 1803A/G,L601L
4	LCDI	c.370C> T ,R124C	651C/G, L217L
5	LCDI	c.370C> T ,R124C	651C/G, L217L
6	Atypical CD	—	651C/G,L217L 981G/A,V327 V 1620C/T,F540F
7	LCDI	c.370C>T ,R124C	651C/G,L217L 981G/A,V327 V 1620C/T,F540F
8	Atypical CD	—	651C/G,L217L 981G/A, V327V 1620C/T,F540F 1803A/G,L601L,

mutation Arg124His (Figure 1). We found a heterozygous C to T transition at nucleotide c.1663 (CGG to TGG), located in exon 12 in other two GCDI patients (Figure 1). This mutation caused an Arginine to Tryptophan substitution on protein level at codon 555. In addition, we found four inconsequential heterozygous single nucleotide polymorphisms in *TGFBI* gene,with the nucleotide changes 651C/G (L217L, rs1442) in exon 6,981 G/A (V327V, rs1054124) in exon 8, 1620 C/T (F540F , rs4669)in exon 12, 1803A/G (L601L, rs35151677) in exon 13. To the best of the authors' knowledge, L601L was rarely reported in research. Mutations were not identified in the rest two atypical individuals either in *TGFBI*or *CHST6*gene. Detail mutations in *TGFBI*gene were listed in Table 3 and Figure 1.

DISCUSSION

In this study, 3 point mutations in *TGFBI* gene were identified in the 8 individuals with corneal dystrophies. That were, Arg124Cys mutation in three patients with LCDI, Arg555Trp mutation in two patients with GCDI and Arg124His mutation in one patient with GCDII. Remaining two atypical CDs may be caused by some other genes excluding from *TGFBI* or *CHST6* gene. It confirmed a strong correlation between *TGFBI* gene specific mutations and stromal corneal dystrophies in Chinese.

Acknowledge the structure and function of TGFBIp will help us revealing the disease mechanism.

Humans TGFBIp in corneas from individuals ranging from 6 months to 86 years of age, TGFBIp is more abundant in mature corneas than in the developing cornea and the processing of TGFBIp changes during postnatal development of the cornea [20]. In mouse embryon, at 18.5 dpc, the expression of TGFBIp in the epithelium appears more than the developing stroma cornea. Nolabeling is observed in the retina[21]. The origination of TGFBIp was not imported into the cornea from plasma but from local synthesis suggested corneal deposits with inherited corneal diseases caused by mutations in the TGFBI gene[22].

The human TGFBIp is composed of 683-amino acid protein is highly conserved between species and contains an N-terminal secretory signal peptide, four 140-amino acid tandemly internally domains homologous to one another, known as fasciclin-1, denoted "FAS1" domains, and an arg-gly-asp (RGD) motif at the C terminus [23]. The FAS1 domain represents an ancient cell adhesion domain homologous to fasciclin I protein in Drosophila [24]. In humans, there are four proteins containing FAS1 domains, specifically, two secretory proteins, TGFBIp and periostin, and two membrane proteins, stabilin-1 and stabilin-2 [21]. TGFBIp as a secreted protein and plays a "linker" role in the interaction between extracellular matrixc (ECM) and integrins [25]. The FAS1 domain mediates cell adhesion and migration via interactions with integrins a3β1 [26] and avβ5 [27]. Missense mutations in the FAS1 domains of the human TGFBIp cause corneal dystrophies. Most of *TGFBI* mutations so far reported are located in the FAS1 domain with two mutational hot spots in R124 and R555. Arg-124 is located in the turn between helicesα1 andα2 of FAS1 domain 1,whereas Arg-555 is located in the helicesα3-α4 of FAS1 domain 4,whichis the most variable region of the FAS1 fold [28]. Amino acid changes in FAS1-1 did not affect the stability of the intact TGFBIp, mutations in the FAS1-4, in contrast to the FAS1-1, appeared to affect the stability of

TGFBIp, the similar behavior of intact TGFBIp and the isolated FAS1- 4, suggests that the stability of FAS1-4 may be the determiner of CD's mechanism [29].

Moreover, the common mutations of *TGFBI* at positions 124 and 555 are likely to affect protein-protein interactions directly and reduced TGFBIp stability or solubility as the cause of amyloid deposition, do not apparently affect its binding to type I collagen, fibronectin and laminin or to forming fibrillar structures [27], whereas the rare mutations that affected FAS1 core residues are likely to cause misfolding and secretion of the protein within the cell [30]. While, the deposits of mutant TGFBIp are probably present only in the cornea of affected patients, suggesting that mutant forms of TGFBI may require other cornea-specific factors to trigger the deposition of abnormal TGFBI protein [31].

The general notion is that *TGFBI* mutations may impair protein secretion, folding, degradation or interaction with other macromolecular components thus promoting the accumulation of corneal deposits.

Clinically, disease diagnoses are mostly based on the silt-lamp biomicroscope examination and the ophthalmologists' experience. The *TGFBI* gene-linked corneal dystrophies mainly include the lattice and granular types, as well as a lot of subtypes. It is difficult to make the accurate diagnosis and classification just relying on the clinical features. But the corneal tissue for histopathology was difficult to obtain in the initial treatment of patients, repeated grafts and bullous keratopathy with deficient corneal endothelial cells was not apparent in the originally grafted tissue. In our researches, followed by clinical examination, genetic screen was the efficient method to confirm the diagnosis, we establish the specific correlations between phenotype-genotype in *TGFBI* mutations: Arg124Cys and LCDI, Arg124His and GCDII, Arg555Trp and GCDI. Unraveling the genetic defects of corneal dystrophies will aid efforts directed at treatment.

Overall, our results about *TGFBI* and *CHST6* gene analysis indicating that R124 and R555 codons were also the hot point mutations in Chinese as other populations in *TGFBI* gene. Genetic analysis will serve as the accurate and rapid adjacent tool to diagnose distinct CD populations. Patients will benefit from genetic counseling.

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