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Pathogenic mutations of *TGFBI* and *CHST6* genes in Chinese patients with Avellino, lattice, and macular corneal dystrophies

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Abstract: Objective: To investigate gene mutations associated with three different types of corneal dystrophies (CDs), and to establish a phenotype-genotype correlation. Methods: Two patients with Avellino corneal dystrophy (ACD), four patients with lattice corneal dystrophy type I (LCD I) from one family, and three patients with macular corneal dystrophy type I (MCD I) were subjected to both clinical and genetic examinations. Slit lamp examination was performed for all the subjects to assess their corneal phenotypes. Genomic DNA was extracted from peripheral blood leukocytes. The coding regions of the human transforming growth factor β-induced (*TGFBI*) gene and carbohydrate sulfotransferase 6 (*CHST6*) gene were amplified by polymerase chain reaction (PCR) and subjected to direct sequencing. DNA samples from 50 healthy volunteers were used as controls. Results: Clinical examination showed three different phenotypes of CDs. Genetic examination identified that two ACD subjects were associated with homozygous R124H mutation of *TGFBI*, and four LCD I subjects were all associated with R124C heterozygous mutation. One MCD I subject was associated with a novel S51X homozygous mutation in *CHST6*, while the other two MCD I subjects harbored a previously reported W232X homozygous mutation. Conclusions: Our study highlights the prevalence of codon 124 mutations in the *TGFBI* gene among the Chinese ACD and LCD I patients. Moreover, we found a novel mutation among MCD I patients.

Key words: Transforming growth factor-β-induced (*TGFBI*) gene, Carbohydrate sulfotransferase 6 (*CHST6*) gene, Corneal dystrophy, Mutation

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1 Introduction

Corneal dystrophies (CDs) are traditionally characterized by their different clinical, histological, and genetic features. These dystrophies are a series of bilateral corneal diseases such that the patients often suffer a progressive increase in the opacity of the cornea. These diseases can lead to a severe visual impairment. Penetrating keratoplasty (PKP) and lamellar keratoplasty are the only treatments for CDs (Yao *et al*., 2006).

Munier *et al*. (1997) first confirmed that four autosomal dominant CDs, including Avellino corneal dystrophy (ACD), lattice corneal dystrophy type I (LCD I), granular corneal dystrophy Groenouw type I (GCDG I), and Reis-Bucklers corneal dystrophy (RBCD), are caused by the mutation of human transforming growth factor β-induced (*TGFBI*) gene on chromosome 5q31 in Caucasian subjects. Since then, new mutations in the same gene were discovered to cause these CDs (Yu *et al.*, 2006; 2008). The *TGFBI* gene is composed of 17 exons, encoding a 68-kDa secretory protein known as kerato-epithelin. Kerato-epithelin is expressed in corneal epithelium and other tissues, but the function is still unclear

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(Skonier *et al.*, 1992; Korvatska *et al.*, 1999). ACD is described as a combined CD that shows granular and linear stromal deposits in cornea. LCD I, another common autosomal dominant dystrophy, is characterized by the accumulation of fine, lattice-like deposits within the central region of cornea. ACD and LCD cause progressive accumulation of amyloid deposits in corneas, which typically develops in childhood. In the absence of clinical intervention, these patients could lose their visual function.

Another CD is macular corneal dystrophy type I (MCD I), which is less common but more severe than the above described two stromal dystrophies. It is an autosomal recessive disease in which the genetic mutations are identified in the carbohydrate sulfotransferase 6 (*CHST6*) gene, which was first identified by Akama *et al.* (2000). *CHST6* gene is located on chromosome 16 (Vance *et al.*, 1996). This gene encodes the enzyme *N*-acetylglucosamine-6-sulfotransferase (GlcNAc6ST), whose mutation causes a disorder of the corneal keratin sulfate (KS) metabolism, resulting in deposition of an unsulfated KS (Akama *et al*., 2000). The disorder is observed mostly in the offspring of consanguineous marriages. It is characterized by the presence of grayish and diffuse glycosaminoglycan deposits in keratocytes and stromal lamellae, causing severe visual impairment (Klintworth, 1980). Three biochemical subtypes have been identified based on immunology. MCD I is the most common variant of MCD, in which KS is completely absent from cornea, sera, and cartilage (Jonasson *et al.*, 1996; Hassell and Klintworth, 1997; Klintworth *et al.*, 1997). MCD is the most common stromal dystrophy in Iceland, due to its small gene pool (Jonasson *et al.*, 1996). It is also prevalent in Saudi Arabia and southern India, due to a relatively high rate of consanguinity (Klintworth *et al.*, 1997; Warren *et al.*, 2003). Patients with these dystrophies develop severely reduced vision and require keratoplasty in the end.

In this study, we reported on two subjects with ACD, four subjects from the same family with LCD I, and three subjects with MCD I. All showed clinical symptoms with progressive opacities of the cornea, leading to severe visual loss. Six of them accepted PKP or deep lamellar keratoplasty (DLKP) in our hospital.

2 Materials and methods

2.1 Clinical data

All subjects were clinically assessed by slit lamp and photographed before peripheral leukocytes were collected. Fifty healthy volunteers were used as control. The study was approved by the Institutional Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University, China. Informed consent was obtained from each subject. All subjects were free of systemic disease.

2.2 DNA extraction

After each subject provided informed consent, DNA was extracted from the peripheral blood leukocytes by standard methods (Grimberg *et al.*, 1989; Yu *et al.*, 2008).

2.3 Polymerase chain reaction amplification of *TGFBI* gene

All the 17 exons from the entire coding region of *TGFBI* gene were amplified by polymerase chain reaction (PCR). The primers we used were designed according to Klintworth *et al*. (2004).

Genomic DNA (100 ng) was suspended in a 50-μl PCR mixture, consisting of 0.2 mmol/L deoxyribonucleoside triphosphate (dNTP) mixture, 1.5 mmol/L $MgCl_2$, 1 μmol/L of each primer, and 2 U of Platinum Taq DNA polymerase (Sangon, Shanghai, China). Amplification was performed using a PTC-100 PCR System (MJ Research, Waltham, USA)) started with denaturing at 97 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56–61 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 7 min.

2.4 Polymerase chain reaction amplification of *CHST6* gene

Four pairs of primers were designed to overlap the whole coding region of *CHST6* (Table 1). The procedure was similar to that of *TGFBI* gene. Genomic DNA (100 ng) was suspended in a 50-μl PCR mixture, consisting of 0.2 mmol/L dNTPs mixture, 1.5 mmol/L $MgCl_2$, 1 μmol/L of each primer, 2 U of Platinum Taq DNA polymerase. Amplification was performed using a PTC-100 PCR System started with 5 min at 97 °C for denature, followed by

35 cycles of 94 °C for 45 s, 54–61 °C for 45 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min.

Table 1 PCR primers of *CHST6* gene

Primer	Length (bp)	Sequence
H	498	F: 5′-GCCCCTAACCGCTGCGCTCTC-3′ R: 5′-GGCTTGCACACGGCCTCGCT-3′
M1	372	F: 5′-GACATGGACGTGTTTGATGC-3′ R: 5′-GCACGATGCCGTTGTCAC-3′
M2	393	F: 5′-GCTCAACCTACGCATCGTG-3′ R: 5′-ATCCGTGGGTGATGTTATGG-3′
L	407	F: 5′-GAGCCGCTGGCAGAAATC-3′ R: 5′-TGCACCATGCACTCTCCTC-3′

2.5 DNA sequencing

Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), following the manufacturer's procedure. DNA was directly sequenced (Mega BACE 1000, Amersham Pharmacia Biotech Inc., Piscataway, USA) in both directions.

3 Results

3.1 Clinical findings

Case 1 is a 38-year-old female subject who was referred to us for progressive loss of vision in both eyes, first developing when she was six years old. Slit lamp examination revealed the presence of gray-white granular and partially confluent corneal opacities in the superficial corneal stroma. The deep corneal stroma could not be observed clearly due to the severe superficial opacities. However, we could still observe fine, linear patterns of opacities in stroma by slit lamp examination (Fig. 1a). This patient was diagnosed as ACD, underwent a left full-bed (FB)-DLKP at 32 years of age, and had a right FB-DLKP one year later. Fourteen months later, whitish recurrent deposits were observed along the suture tracts and the incision line in her right eye.

Case 2 was a 60-year-old male subject who was referred to us because of his corneal opacities in both eyes, present for over 30 years. This subject had poor vision since his early childhood. He was diagnosed with ACD. Slit lamp examination showed similar characteristics to those of Case 1, such as the granular and partially confluent lesions in the superficial corneal stroma. He underwent a left PKP at the age of 52 years. No recurrence was observed eight years after the surgery.

Case 3 was a 40-year-old female subject who was diagnosed as LCD I at our ophthalmology clinic center. She is the proband of Family III (Fig. 1c). Her elder brother (Case 4), elder sister (Case 5), and nephew (Case 6), all of whom suffered from the same disorder, were included in this study, as described later. The cornea of Case 3 showed opacities composed of radially arranged branching refractile lines and diffuse haze-like opacities in corneal stroma (Fig. 1b). Ocular pain and recurrent corneal erosions were mentioned in the medical history of all the subjects of Family III. Case 3 underwent a PKP on her left eye at 32 years of age. The graft failed during the first three years after PKP.

Fig. 1d shows the diseased cornea of Case 7 with MCD I, with multiple, irregular, gray-white opacities, involving both center and peripheral stromata. The superficial opacities were diffuse. This female subject underwent a PKP on her left eye at 33 years of age. No recurrence was observed six years after the surgery.

Cases 8 and 9 were referred to us with corneal opacities of both eyes for over 20 years. Slit lamp examination revealed superficial and moderate diffuse, cloudy stromal opacities. Case 8 (female, 42-year-old) underwent FB-DLKP on her both eyes at 35 years of age. Case 9 (male, 40-year-old) underwent a right PKP at 33 years of age and a left FB-DLKP two years later. No recurrence was observed until now.

The clinical and molecular features of these subjects are summarized in Table 2. None of the subjects was aware of consanguineous marriage.

3.2 Molecular findings

DNA sequencing from two ACD subjects, Cases 1 (Fig. 2a) and 2 (Fig. 2b), revealed a homozygous CGC to CAC change in exon 4 of *TGFBI* gene, resulting in an arginine to histidine substitution at codon 124 (R124H). DNA sequencing from Case 3 (Fig. 2c) detected a heterozygous C to T change at the first nucleotide position of codon 124 in exon 4 of *TGFBI* gene, resulting in an arginine to cysteine (R124C) mutation. The same mutation was also detected in

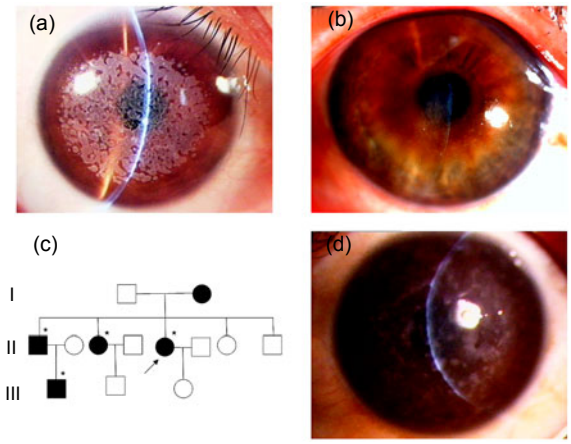

Fig. 1 Phenotypes of ACD (a), LCD I (b), and MCD I (d)
(a) Clinical photograph from Case 1 shows that dense subepithelial and anterior stromal lesions are approaching confluence, involving both center and peripheral stromata; (b) The corneal image from the proband of Family III (Case 3) shows small opaque spots and refractile lattice of deposition in the stroma; (c) Pedigree of the Family III (circles indicate females; squares indicate males; solid symbols indicate affected members; open symbols indicate unaffected members; arrows indicate the proband; asterisks indicate members who were examined clinically and genetically); (d) The cornea of Case 7 showed typical diffuse, cloudy opacities in the superficial stroma

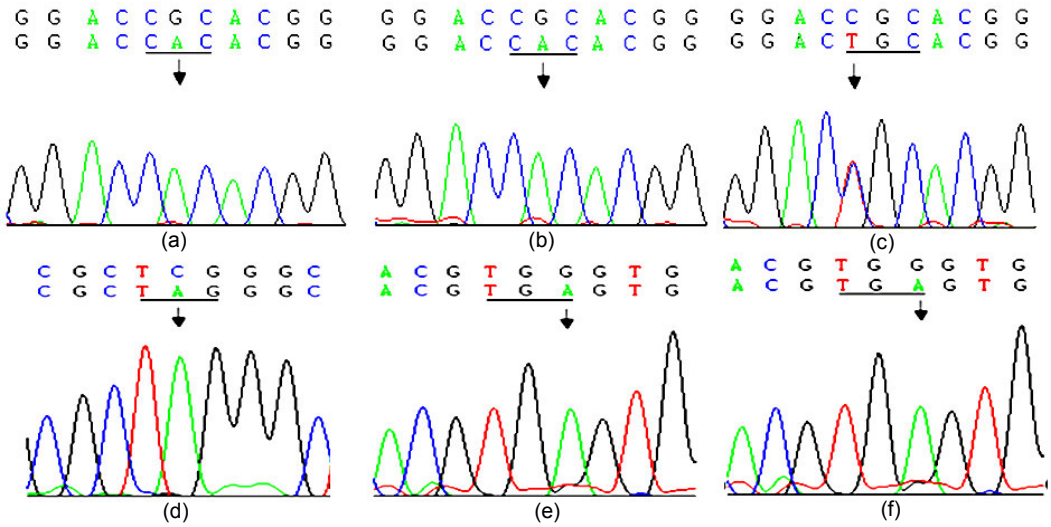

Fig. 2 Polymerase chain reaction sequencing analysis of *TGFBI* and *CHST6* genes
Top line: normal sequence; bottom line: mutated sequence. (a) Arrow indicates a G to A mutation at the second nucleotide position of codon 124 of *TGFBI* gene in Case 1; (b) Arrow indicates the same mutation at the second nucleotide position of codon 124 in Case 2 as in Case 1; (c) Direct sequencing analysis of exon 4 of the *TGFBI* gene in Case 3, revealed a C to T mutation at codon 124, and arrow indicates T and C co-existing at the first nucleotide position of codon 124; (d) Arrow indicates a C to A mutation at the second nucleotide position of codon 51 of *CHST6* gene in Case 7; (e) Arrow indicates a G to A mutation at the third nucleotide position of codon 232 of *CHST6* gene in Case 8; (f) Arrow indicates a G to A mutation at the second nucleotide position of codon 232 of *CHST6* gene in Case 9. No mutations were detected in healthy controls

Table 2 *TGFBI* and *CHST6* gene mutations in subjects and their clinical characteristics

Case	Sex	Family	Age of onset (year)	VA before surgery		Mutation gene	Amino acid change	Dystrophy	Previous surgery
				OD	OS				
1	F	I	6	20/400	20/800	*TGFBI*	R124H (homo)	ACD	OU FB-DLKP
2	M	II	~12	20/1600	20/100	*TGFBI*	R124H (homo)	ACD	OS PKP
3	F	III	27	20/80	20/1600	*TGFBI*	R124C (heter)	LCD I	OS PKP
4	M	III	26	20/40	20/40	*TGFBI*	R124C (heter)	LCD I	NA
5	F	III	22	CF/20 cm	20/200	*TGFBI*	R124C (heter)	LCD I	NA
6	M	III	11	20/80	20/600	*TGFBI*	R124C (heter)	LCD I	NA
7	F	IV	17	20/250	20/300	*CHST6*	S51X (homo)	MCD I	OS PKP
8	M	V	~13	20/400	20/400	*CHST6*	W232X (homo)	MCD I	OU FB-DLKP
9	M	VI	12	20/250	20/200	*CHST6*	W232X (homo)	MCD I	OD PKP, OS FB-DLKP

F: female; M: male; VA: visual acuity; OD: right eye; OS: left eye; CF: count fingers; homo: homozygous; heter: heterozygous; ACD: Avellino corneal dystrophy; LCD I: lattice corneal dystrophy type I; MCD I: macular corneal dystrophy type I; OU: both eyes; NA: not available; FB-DLKP: full-bed deep lamellar keratoplasty; PKP: penetrating keratoplasty

Cases 4–6 from the same family with Case 3. Among the MCD I patients, one novel mutation was identified within the coding region of *CHST6* gene in Case 7. A homozygous C to A change was identified at the second nucleotide position of codon 51, changed the amino acid from serine to a stop codon (S51X) (Fig. 2d). In Cases 8 (Fig. 2e) and 9 (Fig. 2f), a homozygous G1388A mutation was revealed at the third nucleotide position of codon 232, changing the amino acid from tryptophan to a stop codon (W232X). None of these changes was detected in the control population in this study. The molecular genetic findings confirmed our clinical diagnoses.

4 Discussion

In this study, we examined the *TGFBI* and *CHST6* coding regions for mutations in nine subjects with three different CDs. Sequencing analysis revealed that two ACD subjects are associated with R124H homozygous mutation, while four LCD I subjects are associated with R124C heterozygous mutation. Moreover, one novel nonsense mutation S51X and a previously reported W232X mutation were revealed in the subjects with MCD I.

In most cases, mutations in *TGFBI* gene-related CDs are heterogeneous, and R124H mutation of a *TGFBI* gene has been reported as the most common mutation in ACD worldwide (Yoshida *et al*., 2002; Huerva *et al*., 2008; Cao *et al*., 2009). Here we report two cases with an unusual severe form of ACD from China. The clinical features include more severe symptoms, earlier onset, and faster progression than other heterozygous ACD. However, the details of these types of cases have not been well studied to date. There are a few reports regarding homozygous ACD, mostly in Japan with consanguineous marriage (Fujiki *et al*., 1998; Okada *et al*., 1998; Tsujikawa *et al*., 2007). Moon *et al*. (2007) reported 18 homozygous ACD patients in Korea, none of the subjects was aware of any consanguineous marriages. Early keratoplasty is required in these homozygous ACD patients. Recurrence is earlier and more severe, requiring repeated surgery. Three cases were reported previously as homozygous R124H mutation from northern China as the offsprings of two consanguineous marriages (Yu *et al*., 2003; Cao *et al*., 2009). In our study, both of the two ACD subjects were from southern China, and neither of them was aware of consanguineous marriage.

R124C mutation is one of the most common mutations of LCD I worldwide. Our study also identified the R124C mutation among all four affected members from one family (Family III) with LCD I. All the patients exhibited the similar phenotype of LCD I. Typical progressive vision acuity loss and cornea opacity were present in their first decade of life, accompanied by ocular pain and recurrent corneal erosions. Our study supported R124C as the hot mutation spot of LCD I. It appears that the different phenotypes of ACD and LCD I are caused by particular amino acid change, as we found that a different amino acid change occurred in the same position (R124H) and resulted in ACD. LCD II is a rare form that associated with systemic amyloidosis. None of the subjects in this study had systemic disease.

MCD is the least common of the three classic stromal dystrophies. It is an autosomal recessive disorder of corneal KS metabolism that causes the deposition of unsulfated KS. *CHST6* gene mutations have been identified as the cause for MCD (Akama *et al*., 2000). A number of missense, nonsense, and frameshift mutations have been reported in the past (Klintworth *et al*., 1997; 2006; Ha *et al*., 2003b; Sultana *et al*., 2003; Warren *et al*., 2003). This type of mutation is common in consanguineous marriages. In our study, three subjects (Cases 7–9) from unrelated Chinese families with MCD I were studied. The results of the sequence analysis for Case 7 identified one novel nonsense mutation S51X. This mutation results in a premature stop condon which may cause a loss of most amino acids of the CHST6 protein. We also found other homozygous nonsense mutation W232X in both Cases 8 and 9. MCD patient with nonsense mutation is rarely reported in the literature (Ha *et al*., 2003a; 2003b). To date, only one study of a Vietnamese MCD I patient mentioned the W232X mutation (Ha *et al*., 2003a).

5 Conclusions

In conclusion, we report in this study several cases of ACD, LCD I, MCD I, and the associated genetic mutations in Chinese patients. The direct

sequencing data of the *TGFBI* and *CHST6* genes revealed the presence of a rare homozygous R124H mutation in two ACD subjects, heterozygous R124C mutation in an LCD I family, one novel S51X nonsense mutation and W232X mutation in three MCD I subjects. None of these mutations was detected in 50 healthy volunteers. The final treatment for CDs is surgical intervention, in which FB-DLKP should be preferred over PKP as the primary treatment for CDs for its markedly lower risk of endothelial dysfunction and corneal decompensation (Yao, 2008). Repeated treatment may be required for recurrence. PKP should be a treatment of last resort.

The diagnosis of CDs in clinic is still dependent on clinical appearance and histological examinations. However, the phenotype-genotype correlation of some CDs is not clear because of phenotypic variations. Therefore, analysis of genetic mutation may help to confirm the diagnosis, particularly in the early stages of the disease.

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