Analysis of *SLC4A11*, *ZEB1*, *LOXHD1*, *COL8A2* and *TCF4* gene sequences in a multi-generational family with late-onset Fuchs corneal dystrophy

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Abstract. The aim of the present study was to determine the genetic basis of a multi-generational family with late-onset (LO) Fuchs corneal dystrophy (FCD). Five FCD causal genes [solute carrier family 4, sodium borate transporter, member 11 (*SLC4A11*), zinc finger E-box binding homeobox 1 (*ZEB1*), lipoxygenase homology domains 1 (*LOXHD1*), collagen, type VIII, alpha 2 (*COL8A2*) and transcription factor 4 (*TCF4*)], previously reported to be implicated in the pathogenesis of FCD, were screened. A total of 27 variants [including 22 known single nucleotide polymorphisms (SNPs) from the Single Nucleotide Polymorphism Database (dbSNP) and 5 variants absent from dbSNP] were detected in this FCD pedigree across the *SLC4A11*, *ZEB1*, *LOXHD1* and *COL8A2* genes as follows: i) 22 known SNPs from dbSNP, including 3 coding (p.R161R, p.S213S and p.T833T) and 11 non-coding variants of *SLC4A11*, 2 intronic SNPs of *ZEB1* from dbSNP (rs220057 and rs220060), 1 intronic SNP of *LOXHD1* from dbSNP (rs16939650), and 5 SNPs of *COL8A2* from dbSNP (p.A35A, p.R155Q, p.L335L, p.G495G and p.T502M); and ii) 5 variants that have not been previously reported in FCD patients and that are absent from dbSNP were identified across the *ZEB1* and *LOXHD1* genes; these included 3 continuous indels located at the junction of the 5'-UTR and the adjacent exon 1 of *ZEB1* [Indel 1 (c.-86_-53delins gggaggggtggaggcggaggggtGGGGGGGAAGG); Indel 2 (c.-52_-46delinsGGGAGGG); and Indel 3 (c.-45_-42delinsAGGG)], and 2 intronic variants of *LOXHD1* (c.5332-126C>T and c.1809+155G>A). Apart from one intronic SNP of *SLC4A11* from dbSNP (rs372201212), the pathologic consequence of which is uncertain, and 2 intron variants of *LOXHD1* (c.5332-126C>T and c.1809+155G>A); the variants likely represent examples of *de novo* mutations. Neither of the other 24 variants provided strong evidence of pathogenesis in this FCD pedigree. An analysis of 7 SNPs in *TCF4* from dbSNP, which have been associated with LO FCD in different populations, revealed that these 7 SNPs were not associated with FCD in this specific pedigree. A genome-wide linkage scan to search for linkage to one of the previously described FCD loci or to identify a novel locus for FCD will need to be performed in this FCD pedigree. Our observation, nevertheless, expands the knowledge of the genetic status of patients with FCD.

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Introduction

Fuchs corneal dystrophy (FCD; MIM 136800), first described by Ernst Fuchs in 1910 (1), is characterized by bilateral primary corneal guttae and a reduced endothelial cell density that can result in corneal edema, discomfort and blurred vision (2). The onset of FCD generally occurs in the 4th decade of life onwards, and FCD progresses at a slow rate over the next 2 to3 decades, causing severe impairment of endothelial cell function (3,4), ultimately leading to severely impaired vision or blindness (5,6). Currently, effective methods of restoring vision in advanced cases are corneal transplantation in the form of penetrating keratoplasty (PK) (7), Descemet's stripping endothelial keratoplasty (DSEK) (8) and Descemet's membrane endothelial keratoplasty (DMEK) (9).

FCD is genetically heterogeneous. To date, 4 loci, FCD1, FCD2, FCD3 and FCD4, on chromosomes 13, 18, 5 and 9, respectively, along with numerous linkage peaks and susceptibility loci, have been localized through linkage

analysis. In addition, 4 causal FCD genes, namely collagen, type VIII, alpha 2 (*COL8A2*) (MIM 12052) (4), solute carrier family 4, sodium borate transporter, member 11 (*SLC4A11*) (MIM 610206) (10,11), zinc finger E-box binding homeobox 1 (*ZEB1*) (MIM 189909) (12) and lipoxygenase homology domains 1 (*LOXHD1*) (MIM 613072) (13) have been identified, representing a small proportion of the total genetic load. Furthermore, a single nucleotide polymorphism (SNP) on chromosome 18q21, rs613872, in an intron of the transcription factor 4 (*TCF4*, MIM 602272) gene, which encodes a member of the E-protein family (E2-2), has been identified to be significantly associated with FCD; the association increased the probability of having FCD by a factor of 30 in individuals with 2 copies of the disease variants (homozygotes) and discriminated between case subjects and control subjects with approximately 76% accuracy (14). Another study that genotyped 18 SNPs within *TCF4* in Singaporean Chinese revealed that the minor allele of rs613872 was not present in the genotyped cohort; 2 SNPs (rs17089925 and rs17089887) located upstream and in intron 3 of *TCF4*, respectively, were significantly associated with FCD; another 3 SNPs (rs1348047, rs1452787 and rs2123392) also exhibited a marginal association with FCD (15). Another TGC trinucleotide repeat expansion (rs193922902) within intron 3 of *TCF4* has also been recently identified to be strongly associated with FCD, and a repeat length of >50 was determined to play a pathogenic role in the majority of FCD cases and is considered to be a predictor of disease risk (16).

As the susceptibility of genes to mutations can vary in different ethnicities and in view of the limited information on the genetics of FCD in southwestern China, we undertook this study. We screened for mutations in 4 causal FCD genes (*SLC4A11*, *ZEB1*, *LOXHD1* and *COL8A2*) and genotyped 7 SNPs within the *TCF4* gene to determine whether these known causal genes are responsible for causing FCD in this specific multi-generational late-onset (LO) FCD Chinese pedigree.

Subjects and methods

Case presentation. The study protocol was approved by the Ethics Committee of the First People's Hospital of Yunnan Province and was in compliance with the Declaration of Helsinki. Written informed consent was obtained from all study participants or their guardians. Family members of this LO FCD Chinese family were recruited through a proband with FCD (a 46-year-old woman) who presented to the Department of Ophthalmology, the First People's Hospital of Yunnan Province; extended pedigrees were subsequently developed through interviews. The age of the affected pedigree members in generation II and III ranged from 36 to 67 years.

A total of 191 individuals of Chinese ethnicity, consisting of 104 females and 87 males who ranged in age from 58 to 89 years with an average age of 68.6 (SD 6.9 years), who had a normal cornea upon ophthalmic examination and no history of any ocular disease in their family were recruited as healthy controls.

Clinical evaluation. All family members underwent a complete ophthalmic examination, including fundoscopy, a slit-lamp examination and specular microscopy, to document corneal guttae on December 2009. The diagnosis of FCD and the severity grading were based on a modified Krachmer scale, with grade 0 indicating an absence of guttae, grade 1 representing 12 or more central guttae, grade 5 denoting confluent central guttae with corneal edema, and grade 6 corresponding to disease severe enough to require keratoplasty (3). A detailed history was recorded for all subjects, including any family history and the duration of symptom onset.

DNA sample preparation. After obtaining written informed consent, peripheral blood samples were collected from the proband and her family members. Genomic DNA was extracted from the leukocytes of the peripheral blood using a cell/tissue genomic DNA extraction kit (BioTeke Corp., Beijing, China) according to the manufacturer's instructions. In addition, genomic DNA from 191 healthy individuals was extracted and used as the control DNA.

Sequencing analysis of SLC4A11, ZEB1, LOXHD1, COL8A2, and TCF4. A total of 69 sets of primers (Table I) were designed to completely incorporate the exon and intron boundaries of the *SLC4A11* (NM_032034.3), *ZEB1* (NM_030751.5), *LOXHD1* (NM_144612.6) and *COL8A2* (NM_005202.3) genes, and all primers were designed such that they would be positioned on intronic segments at least 80 nucleotides on either side of the intron-exon boundary, to ensure complete reading of the exons. Primers were also designed to amplify the fragment of the *TCF4* gene containing 7 SNPs. Polymerase chain reaction (PCR) was carried out with 25 ng of genomic DNA as a template in a mixture of PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 0.75 units of rTaq DNA polymerase (Takara Bio, Dalian, China). After an initial denaturation step at 95˚C for 5 min, 35 PCR cycles were performed as follows: 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min, followed by a final extension at 72˚C for 5 min. The PCR-amplified products were purified and sequenced on an ABI 3130 Genetic Analyzer using the BigDye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. Sequence assembly and analysis were performed using the DNASTAR Lasergene.v7.1 program (DNAstar Inc., Madison, WI, USA).

Strand-specific sequencing. To confirm the indels we observed as mixed sequences, the 2 alleles were cloned so they could be sequenced separately. Fragments containing insertion or deletion alleles were amplified as described above and subcloned within the pZeroBack/blunt vector by using ZeroBack Fast Ligation kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the instructions provided by the manufacturer. Plasmid DNA was isolated using the MiniPrep kit (Qiagen China Co., Ltd., Shanghai, China), followed by bidirectional sequencing according to the method described above, with a pZeroBack/blunt forward primer, 5'-CGACTCACTATA GGGAGAGCGGC-3' and reverse, 5'-AAGAACATCGA TTTTCCATGG CAG-3'.

Short tandem repeat (STR) assay. For TGC trinucleotide repeat expansion analysis of the *TCF4* gene, a 5'-FAM-conjugated forward primer corresponding to a location upstream of the STR

Table I. Primer sequences used to amplify exons of the *SLC4A11*, *ZEB1*, *LOXHD1* and *COL8A2* genes, and 7 fragments containing 7 SNPs within the *TCF4* gene.

Gene	Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Coding region size (bp)
SLC4A11	1	GCCCGGTCCCTTCCTCTC	GCCAAAAGCATTCCAGCACTAG	553	136
	2, 3	CGGCTAGGGAATGCTGGAGA	GGAGCAGCGGGAGGATTCT	631	153, 50
	4, 5	CCCGCTGCTCCCCTCTTC	GCAGTGCTCCAGCCCTCTTC	720	232, 82
	6	GGCGGCCCAACCAACTT	CCGCGTGTTTGAATAGGGATAG	556	124
	7, 8, 9A	GGGGAGAGCACCTTCACCTG	CCCCGTCTGTGTTCTCGTCA	688	219, 94, 126
	9B, 10, 11	TCCCCAGCAAACCCTCTCTC	TGGGGCAGCAATATGGTGG	687	114, 133
	12	TGCGCTTTATGCCTTTTTCAA	CACGGGCACACACTCAGCTT	493	74
	12, 14, 15A	CCCCCTGGAGCCCTTTCT	GGCGGCCACCAAGTTCTG	755	253, 107, 169
	15B, 16, 17A	TCCGGGAAATCGAGAGTGAGT	AGCGCGATGTAGAGGAAGAGG	791	174, 196
	17B, 18	GCCGTGGACCCTGAGGAGT	CCCGCCCATTCTCCACAC	617	170
	19	TGGGCTGGGATGGGTGTC	GGCAGTAGCAGGGACACAGGT	537	70
ZEB1	1	CCGCCCGGTCCCTAGCAACAAG	CGGAGGGGGGCAGAGAGCACTACTT	413	58
	2	TTGCTGTTAAAATCCTGGCTCTG	TCCTTCCACTCAGCCATACTTTG	1,115	20
	3	TCCTTTTCAGATTTCGGGAAGTT	TGATTCTCGTTTGCTGTGACATG	789	60
	4	GGGGCTGTCTATTGTCCAACTTT	AAGGCAGATTCAGGAAAACCACT	994	162
	5	AGCCCGTATTTGAACCCTGACT	TTCCATTGAGGGCTGAGTTGTC	512	203
	6	CAAAACAACCATCAGGCTCACA	TTCACTCCCTCTCATTGCCTCTA	674	106
	7A	CAGTTCTGTCACAAGCATGCATG	TGGCTAGGCTGCTCAAGACTGT	791	1,811[a]
	7B	CCCATTACAGGCAACCAGTTCT	TGGGGTTCATTTGCATTTGC	876	
	7C	TGAAAAGATGCAAGCTGGACAG	GGCTGGATCACTTTCAAGGGT	702	
	7D	GGCCATTGCTGACCAGAACA	GGTTCACAGCCACACTTCCTCAT	743	
	8	TTCGGTGTCCTTGCTTTCTTTC	GCCGAGATTGAGATTGCGTG	640	181
	9	AACCCTCCCCTTTCTACAACATG	GGCACACCCGGATTTATTTTG	900	593
LOXHD1	1	AGAAGGCAGAGGGAACA	ATGGGATAATCAGTGAGGAA	439	130
	2	GTTGTGCTGGAAAGATTAC	CTGGTCCCTGGTGAGA	844	115
	3	TTCCTCCTCCATCCAC	CAATCCCTCACTTTCATC	508	81
	4	ACCGAGGTTCAGGAGA	GCAAGACAGGCACGA	405	185
	5	GGGGTAAGTGTAGATGGTG	TTCTTGCTTTCCCTGTG	650	99
	6	AAGGAAGTCTGTAGGCTGAA	CTGGCTTAGGTAGAAGAGTGG	657	149
	7	AAGGTAATCGCCAGTCA	TTCAGGAGCAGGAGGA	473	124
	8	ATTCTTAGCCAACCCG	GGATAATCATAACCACCAA	822	251
	9, 10	TGGGTGATACCTACTTTG	ATCCCTTCCTCCTTTC	1,116	136, 161
	11, 12	GTTTATTGCTTGGAGGAT	ACTTGGAGATGGCTTTT	1,127	87, 136
	13	GGAAGGTCAGCCCAGAT	TCCCAGGAGTCCAACAG	546	155
	14	GAGCAGGATGTTGTGG	AGTAGGGCTGGGTCTT	913	161
	15	TCCAATCTCAGCCAAAC	GCACAGGCAGGAACTCT	452	77
	16	ATTTACACGCTTTCCTG	TCTTAGTCCTCCCTTCTC	857	197
	17	CCCCTCTTGTTTCTCAC	CATTGGGTCCTCAGTTT	677	193
	18	GCTGGTATGTGACTCCTC	GATTTGCCTGGTATGG	737	161
	19, 20	CTGGCTCTTTGTTGGG	TGTTTGTTCCTGTGGGT	1,423	463, 155
	21	TCCAGCAAACCTATCTTC	GTCTTCTTCCAGGACTACC	513	134
	22	CAGGCAAATGACTAATGG	GAGGGAAGGAAGATGGA	428	164
	23	GGGCTCACAGATACAGG	GACCCAACACTAAACACC	640	105
	24, 25	AACTCACCCATGTAACCA	CAGGGATGAAAGACCAA	1,441	129, 165
	26	GGGATACGGAGAAGAGTG	AGGAGGAGCAGGGTGAG	897	182
	27, 28	GGCAAGACAGGAGCAT	GGACGAGGATAAACCAG	1,984	117, 163
	29	TGGCAGGTAGATTTAGTGA	AGGGCAAGGGCAGA	661	155
	30, 31	GAGTGGGTTGAGTGGG	ATCGGTGATGGTGGG	608	210, 136
	32	GAAACCTACCCAACAATG	GTGGCTCACCCAAAGT	1,003	209

Table I. Continued.

Gene	Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Coding region size (bp)
	33	GCCTGGACTTAGGTTGG	GAAGAAATGTTATGGGTAGA	657	128
	34	GCAGCATTACCTTCTATTT	AAGCCAGAGGAACCAG	786	118
	35	GCAAATGTCAGCGTTCT	GTTGGAGTGGTAAGGGA	601	175
	36	AACTTCCCTGCTTCCT	AACACTGGCTCTTTCATAC	743	186
	37	CTCTGAGACCACCTAACC	ACAAGCCTCTTCCAATC	879	171
	38	CTACCTACAACGCCTCAA	TGGGCATCCGAACAG	620	133
	39	AAATGCTTACCTGCTTCA	TCTGGTCCCTACTCTGC	646	159
	40	CCAAGTAGCAGGAGGG	TTGCCACTGGGTTTATT	1,449	699
COL8A2	1	CCCCGCGACTTTGAAAATTG	GGGCGCCTGAGGATCTGAT	470	193
	2A	CCCATTCTTCCTCTCCCGTGTA	TTGCCTAAGCCAGCTGGACC	630	1,919[a]
	2B	GGCCTCAAGGGGGATAATGG	TTTCCCAGCCAGGCCACTAG	606	
	2C	GGGCTTCCTGGCAGACGTG	CGGTGTGGCATGGGCAGA	627	
	2D	TGGGGCCTTCGATGAGACTG	GCCGCCTCTGTTCAGCTTTT	624	
TCF4	Including rs613872	CCCAGGCACTCCCCATTTACT	GGACGTTGAACAGCTTGACAGG	579	
	Including rs17089925	TTCCTGCTTCTGACCC	AGTGACCTGCTTGCTC	924	
	Including rs17089887	GCATAGAAGGCAAGA	GTAAGGAAGAGGCAAT	972	
	Including rs1348047	GGGAATCATAAGCACG	GGCGAAAGGTAGCG	576	
	Including rs1452787	GAATGGGAATCAAATAG	GCAAACTGTGGGAGG	760	
	Including rs2123392	AAGAATGTCAGGGAAAG	CAGAATCACTGCGAAA	960	
	Including rs193922902 (TGC repeat)	CAGATGAGTTTGGTGTAAGATG	ACAAGCAGAAAGGGGGCTGCAA	230+(TGC)12-100	
	5-FAM-TCF-Fuchs, including rs193922902 (TGC repeat)	FAM-CAGATGAGTTTGGTGTAAGATG	ACAAGCAGAAAGGGGGCTGCAA		

[a]Exons were amplified as multiple fragments because of their large size. SNPs, single nucleotide polymorphisms.

region was used in PCR as previously described (16) (Table I). Following PCR, 2 μl of DNA were mixed with 12 μl of diluted Map Marker 1000 (BioVentures, Inc., Murfreesboro, TN, USA). The gene scan was carried out using an ABI 3130 Genetic Analyzer (Applied Biosystems Life Technologies).

Statistical analysis. Statistical analysis was performed using the SPSS 16 software package. A χ^2 test and Fisher's exact test were performed to compare the minor allele frequency (MAF) between data from the 1000 Genomes database and the Chinese healthy controls tested in the present study.

Results

Findings on ocular examination. Microscopic investigation of the proband II-9, a 46-year-old woman, revealed the pleomorphism of corneal endothelial cells and the presence of corneal guttae in both eyes of the proband at her first presentation to our hospital on December 2009 (Fig. 1). A 5-generation Chinese pedigree with 8 affected individuals was subsequently assembled through interviews with the initial proband (Fig. 2, arrow). FCD was diagnosed using slit-lamp biomicroscopy and assigned severity grades as described in the Subjects and methods (Fig. 2). The presence of an age-severity profile in this family was found to be generally consistent with that of LO FCD, which typically progresses from onset to end-stage disease over a period of approximately 2 decades (17,18). Those affected in generation II, whose aged ranged from 56 to 67 years, all exhibited advanced advanced FCD (II-1, II-3, and II-5 all had grade 6 FCD; II-7 had grade 5 FCD), whereas in generations II and III, the affected individuals ranged in age from 36 to 46 years and typically had grades 3 and 4 disease (II-9 had grade 4 FCD; III-7, III-9, and III-19 all had grade 3 FCD) (Fig. 2).

Genetic analysis

Analysis of SLC4A11 gene. A total of 14 known variants (3 coding and 11 non-coding variants) from the Single Nucleotide Polymorphism Database (dbSNP) were detected in our analysis of the *SLC4A11* gene (Table II). The 3 coding variants were synonymous variants that have been previously reported in Asian FCD cases and controls, namely, p.R161R (rs3827075, MAF: G=0.4798/2402), p.S213S (rs3803956, MAF: T=0.1663/833) (19,20), and p.T833T (rs58757394, MAF: T=0.0901/450) (20) (Fig. 3A). All 3 variants were detected in both the affected members of this FCD pedigree (p.R161R, 5/16;

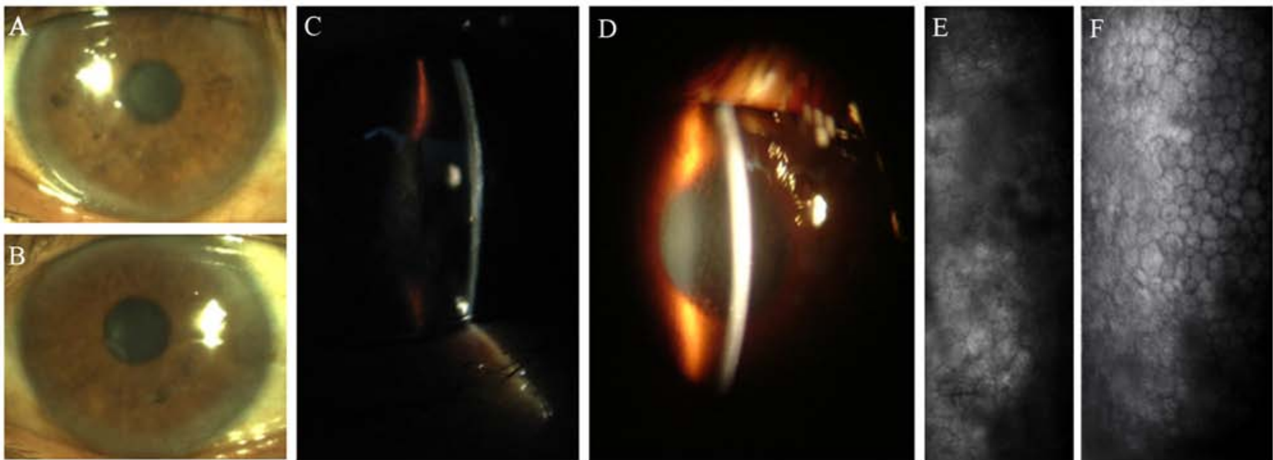

Figure 1. Clinical features of proband with late-onset (LO) Fuchs corneal dystrophy (FCD). (A and B) A clinical image shows severe corneal guttae caused by FCD, with an associated loss of corneal clarity in both (A) right and (B) left eyes. (C and D) Slit-lamp image of the corneal guttae were observed in both the (C) right and (D) left eyes. (E and F) Specular microscopy of the both the (E) right and (F) left eyes revealed pleomorphism of the corneal endothelial cell and corneal guttae (proband was 46 years of age when the microscopic investigation performed in December 2009).

Figure 2. Pedigree of a Chinese family with late-onset (LO) Fuchs corneal dystrophy (FCD) with genotypes of 11 variantions identified across the *SLC4A11*, *LOXHD1*, *ZEB1* and *COL8A2* genes. Squares, males; circles, females; diagonal lines, deceased; filled symbols, affected individuals; unfilled symbols, unaffected individuals or not known to be affected; arrowhead, the proband. The double cross symbol indicates individuals in whom DNA collection and genetic analysis were performed, age is presented in years (y) and a severity grade is indicated for affected and unaffected individuals examined in detail in December 2009. Genotypes of 11 variantions identified across the *SLC4A11*, *LOXHD1*, *ZEB1* and *COL8A2* genes are shown below the individual symbols in the following order: i) *SLC4A11*: c.777+10T>C (rs372201212); ii) *LOXHD1*: c.5332-126C>T and c.1809+155G>A; iii) *ZEB1*: Indel1, Indel2 and Indel3; and iv) COL8A2: p.A35A, p.R155Q, p.L335L, p.G495G and p.T502M. Genotypes of 3 intronic variations (*SLC4A11*: c.777+10T>C; and *LOXHD1*: c.5332-126C>T and c.1809+155G>A) and 3 synonymous variations of *COL8A2* (p.A35A, p.L335L and p.G495G) are shown in alleles. Three indels of *ZEB1* (Indel 1, Indel 2 and Indel 3) are showed as: D, deletion; I, insertion. Two missense variations of *COL8A2* (p.R155Q and p.T502M) are shown as: +, wild-type; M, missense mutation.

Table II. Sequence variants identified across the *SLC4A11*, *ZEB1*, *LOXHD1* and *COL8A2* genes, and genotypes of 7 SNPs within the *TCF4* gene in 8 cases of this FCD pedigree, 14 unrelated spouses married into the family, and ethnically matched healthy controls (n≥100).

Gene	Chr position	rs ID	mRNA	Amino acid change	Functional consequence	MAF			
						1000 Genomes	8 Cases	14 Unrelated spouses	Healthy controls
SLC4A11			NM_032034.3	NP_114423.1			n=16	n=28	n≥100
	20:3237709	rs3827076	c.-30G>C		nc	G=0.4503/2255	5/16 (0.3125)	6/28 (0.2143)	
	20:3235025	rs3803958	c.137-131A>G		nc	C=0.0038/19	1/16 (0.0625)	3/28 (0.1071)	
	20:3234400	rs6139040	c.340-86G>C		nc	G=0.1697/849	1/16 (0.0625)	5/28 (0.1786)	
	20:3234173	rs3827075	c.481A>C	p.R161R	syn	G=0.4798/2402	5/16 (0.3125)	13/28 (0.4643)	108/280 (0.3857)[a]
	20:3233935	rs3803956	c.639G>A	p.S213S	syn	T=0.1663/833	2/16 (0.1250)	5/28 (0.1786)	45/274 (0.1642)
	20:3233504	rs372201212	c.777+10T> C		nc	G=0.0014/7	4/16 (0.2500)	0/28 (0.0000)	0/382 (0.0000)
	20:3233480	rs3803955	c.777+34G>A		nc	T=0.2498/1250	9/16 (0.5625)	11/28 (0.3929)	
	20:3233374	rs2144771	c.777+140C>A		nc	G=0.4477/2242	0/16 (0.0000)	16/28 (0.5714)	
	20:3231077	rs3803954	c.1091-19T>C		nc	G=0.0669/335	3/16 (0.1875)	9/28 (0.3214)	
	20:3231073	rs3803953	c.1091-15A>C		nc	G=0.4006/2005	2/16 (0.1250)	6/28 (0.2143)	
	20:3230418	rs3810561	c.1463+97T>G		nc	A=0.2117/1059	5/16 (0.3125)	12/28 (0.4286)	
	20:3228711	rs10048856	c.2241-4G>A		nc	T=0.1160/580	8/16 (0.5000)	6/28 (0.2143)	
	20:3228437	rs41281858	c.2437-9C>T		nc	A=0.1591/796	5/16 (0.3125)	4/28 (0.1429)	
	20:3228366	rs58757394	c.2499G>A	p.T833T	syn	T=0.0901/450	5/16 (0.3125)	6/28 (0.2143)	18/264 (0.0682)
ZEB1			NM_030751.5						
	10:31319149	NA	c.-86_-53delinsgggaggggt ggaggcggaggggtGGGGG GGAAGG		utr 5 prime, ex	NA	Del=16/16 (1.0000)	28/28 (1.0000)	368/370 (0.9946)
	10:31319183	NA	c.-52_-46delinsGGGAGGG		ex	NA	Del=9/16 (0.5625)	14/28 (0.5000)	183/370 (0.4946)
	10:31319190	NA	c.-45_-42delinsAGGG		ex	NA	Del=5/16 (0.3125)	12/28 (0.4286)	162/370 (0.4378)
	10:31502731	rs220057	c.481+222C>T		in	C=0.2524/1264	0/16 (0.0000)	2/28 (0.0714)	
	10:31504588	rs220060	c.685-15G>A		in	G=0.0787/394	0/16 (0.0000)	1/28 (0.0357)	
LOXHD1			NM_144612.6						
	18:46507838	NA	c.5332-126C>T		in	NA	T=1/16 (0.0625)	0/28 (0.0000)	0/382 (0.0000)
	18:46579407	rs16939650	c.1809+223G>A		in	T=0.2584/1294	7/16 (0.4375)	9/28 (0.3214)	
	18:46579475	NA	c.1809+155G>A		in	NA	A=1/16 (0.0625)	0/28 (0.0000)	0/382 (0.0000)
COL8A2			NM_005202.3	NP_005193.1					
	1:36100138	rs57985157	c.105G>A	p.A35A	in, syn	T=0.0966/484	5/16 (0.3125)	12/28 (0.4286)	128/364 (0.3516)[a]
	1:36099217	rs75864656	c.464G>A	p.R155Q	mis	T=0.0377/188	3/16 (0.1875)	3/28 (0.1071)	23/364 (0.0632)
	1:36098676	rs79833067	c.1005C>G	p.L335L	syn	C=0.0413/207	0/16 (0.0000)	6/28 (0.2143)	88/364 (0.2418)[a]
	1:36098196	rs35495320	c.1485G>A	p.G495G	syn	T=0.1815/909	12/16 (0.7500)	19/28 (0.6786)	202/364 (0.5549)[a]
	1:36098176	rs117860804	c.1505C>T	p.T502M	mis	A=0.0587/294	7/16 (0.4375)	6/28 (0.2143)	79/364 (0.2170)[a]

Table II. Continued.

Gene	Chr position	rs ID	mRNA	Amino acid change	Functional consequence	MAF 1000 Genomes	MAF 8 Cases	MAF 14 Unrelated spouses	MAF Healthy controls
TCF4			NM_003199.2				n=16	n=28	n≥100
	18:55382827	rs1348047	c.369+20627C>A		in	T=0.2780/1392	10/16 (0.6250)	16/28 (0.5714)	
	18:55539976	rs1452787	c.145+45304T>C		in	G=0.2708/1356	8/16 (0.5000)	14/28 (0.5000)	
	18:55541025	rs17089887	c.145+44255A>G		in	C=0.1689/846	3/16 (0.1875)	6/28 (0.2143)	
	18:55543071	rs613872	c.145+42209C>A		in	G=0.0697/348	0/16 (0.0000)	0/28 (0.0000)	1/382 (0.0026)[a]
	18:55547634	rs2123392	c.145+37646A>G		in	C=0.3005/1504	13/16 (0.8125)	11/28 (0.3929)	
	18:55586156:55586227	rs193922902	c.72+818_73-804CTG(10_37)		STR	NA	$TGC_{(11)}$=8/16 (0.5000)	9/28 (0.3214)	
	18:55733603	rs17089925	NA		in	T=0.1569/786	1/16 (0.0625)	10/28 (0.3571)	

There were 8 cases and 14 unrelated spouses (in whom DNA collection and genetic analysis were performed); thus, there were 16 and 28 chromosomes, respectively. As we did not obtain good sequencing results for all the 191 controls, the total number of samples for each gene analysis differed, n≥100). MAF, minor allele frequency; NA, not available; in, intron variant; ex, exon variant; utr, untranslated regions variant; nc, non-coding transcript variant; syn, synonymous codon; mis, missense; STR, short tandem repeat. Lower case superscript letters indicates statistical significance ([a]P<0.01) when comparing the MAF data from the 1000 Genomes database with that from the Chinese ancestry healthy controls tested in the present study.

p.S213S, 2/16; and p.T833T, 5/16) and in 14 unaffected spouses who married into this family (the 14 spouses in whom DNA collection and genetic analysis were performed; p.R161R, 13/28; p.S213S, 5/28; and p.T833T, 6/28), as well as in unrelated, ethnically matched, healthy control subjects (n≥100) (Table II).

Among the 11 non-coding variants identified in this FCD pedigree, 9 variants (rs3827076, rs3803958, rs6139040, rs3803955, rs3803954, rs3803953, rs3810561, rs10048856 and rs41281858) were detected in both the affected members of this FCD pedigree and in 14 unaffected individuals who married into this family. As for rs2144771, it was absent in the 8 affected members of this FCD pedigree (0/16), whereas it was detected in the 14 unaffected individuals who married into this family (16/28) (Table II). For an intronic variant (rs372201212, MAF: G=0.0014/7), its minor allele (G) was detected in 4 of the 8 affected members of this FCD pedigree, II-1, II-5, III-9 and III-19 (4/16), and in 3 of 20 healthy descendants in this family (in whom DNA collection and genetic analysis were performed), III-3, IV-4 and IV-16 (3/40). This variant was absent in the other 4 affected members of this FCD pedigree (II-3, II-7, II-9 and III-7). As this variant was not identified in the 14 unaffected individuals who married into this family (0/28) (Table II) (Fig. 2), this variant was further tested in unrelated ethnically matched controls (n≥100), and the results revealed that it was also absent in the 191 healthy samples we tested (0/382) (Table II).

Analysis of ZEB1 gene. Bidirectional sequencing of the PCR product encompassing the 5'-UTR region and exon 1 of the *ZEB1* genomic DNA (GenBank reference ID: NC_000010.11) of the proband (II-9) of this pedigree revealed a homozygous 34 bp deletion involving 23 bp of the 5'-UTR region and the adjacent 11 bp at the 5' end of exon 1 (GenBank reference ID: NM_030751.5): c.-86_-53delins gggaggggtggaggcgg aggggtGGGGGGGAAGG (exon and 5'-UTR sequences are depicted by capital and lower case letters, respectively), as well as a heterozygous 7 bp indel in exon 1: c.-52_-46delins GGGAGGG. Follow-up screening of the other family members of this specific FCD pedigree revealed that there was another 4 bp indel: c.-45_-42delinsAGGG (Fig. 4A). These 3 indels were named Indel 1, Indel 2 and Indel 3; however, these 3 indels were present in both the affected members of this FCD pedigree (Indel 1: Del, 16/16; Indel 2: Del, 9/16; and Indel 3: Del, 5/16) and in the 14 unaffected individuals who married into this family (Indel 1: Del, 28/28; Indel 2: Del, 14/28; Indel 3: Del, 12/28) (Table II) (Fig. 2), as well as in the unrelated, ethnically matched, healthy control subjects (Indel 1: Del, 368/370; Indel 2: Del, 183/370; Indel 3: Del, 162/370) (Table II).

To further characterize the indels observed, 8 cases (II-1, II-3, II-5, II-7, II-9, III-7, III-9 and III-19), 3 healthy individuals who married into the family (II-4, II-6 and II-8), 2 healthy descendants of the family (III-11 and III-23) and 7 healthy control subjects (H4, H22, H31, H67, C8, C30 and C52) were enrolled in the validation set. PCR products of the segment encompassing the 5'-UTR region and exon 1 of the *ZEB1* gene were then subcloned into a pZeroBack/blunt vector. Plasmids were extracted from 10-20 positive colonies in each sample and sequenced bidirectionally by ABI 3130, according to the method described above. Subsequent sequence analysis demon-

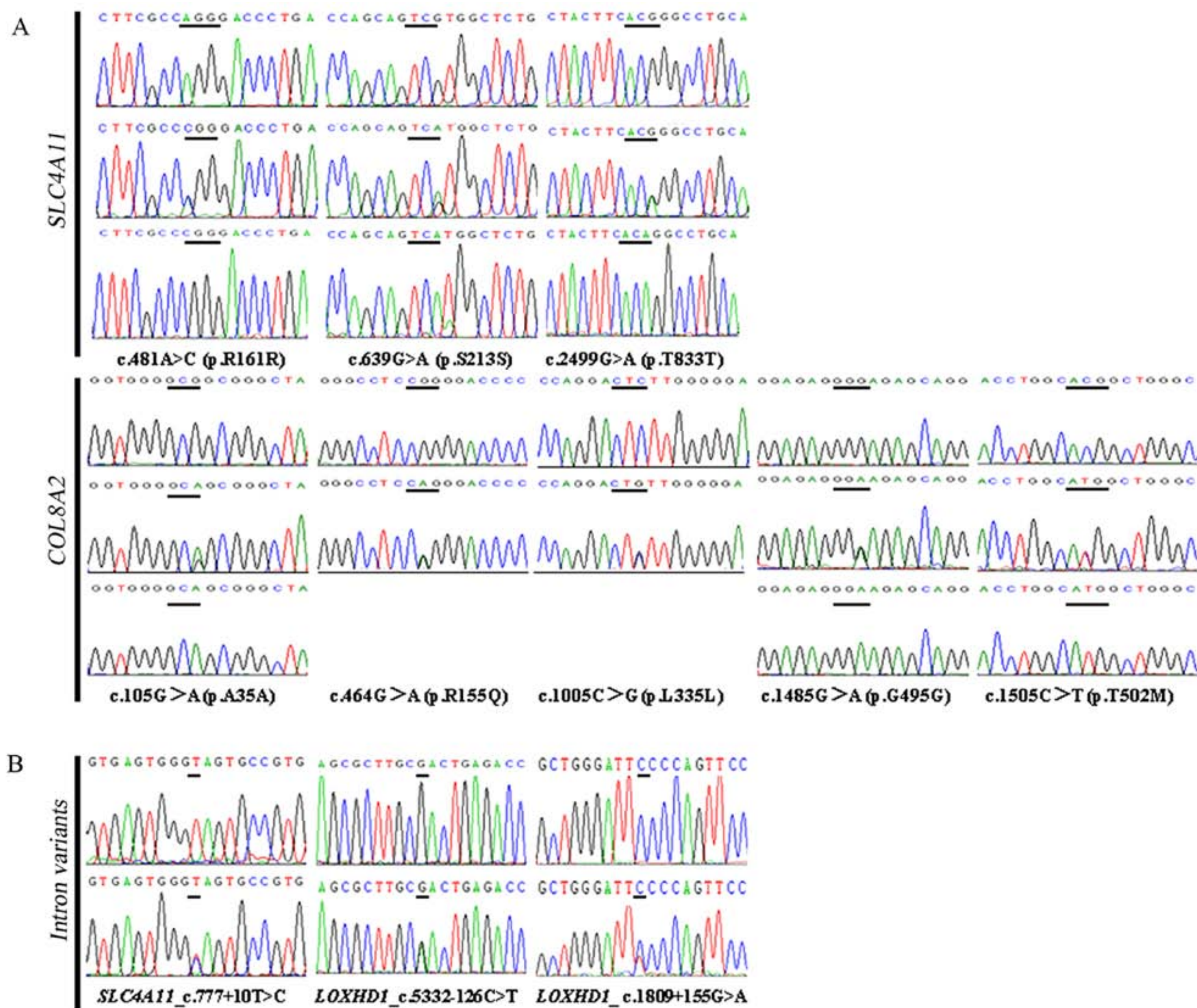

Figure 3. Sequence electropherograms of 8 coding variants detected across the *SLC4A11* and *COL8A2* genes and 3 intronic variants detected in *SLC4A11* and *LOXHD1* genes. (A) Eight coding variants detected in *SLC4A11* and *COL8A2* genes. *SLC4A11*: c.481A>C (p.R161R), c.639G>A (p.S213S) and c.2499G>A (p.T833T); and *COL8A2*: c.105G>A (p.A35A), c.464G>A (p.R155Q), c.1005C>G (p.L335L), c.1485G>A (p.G495G) and c.1505C>T (p.T502M). Wide-type sequences are shown in the top panel for comparison. The underline marks the variated codon. (B) Three intron variants detected in *SLC4A11* and *LOXHD1* genes. *SLC4A11*: c.777+10T>C (rs372201212); and *LOXHD1*: c.5332-126C>T and c.1809+155G>A (both absent from dbSNP). The underline marks the variant nucleotide.

strated that 4 haplotypes (ordered as Indel 1/Indel 2/Indel 3), I/I/I, D/I/I, D/D/I and D/D/D, were detected in the present study (Fig. 4B), and these observations were consistent with our bidirectional sequencing results. A schematic illustration of the *ZEB1* genomic DNA and the position of the 3 continuous indels (Indel 1, Indel 2 and Indel 3) relative to exon 1 is shown in Fig. 4C. Although these 3 indels have not been previously reported in patients with FCD and are absent from dbSNP, they were detected in both the cases and healthy controls (n≥100) (Table II) (Fig. 2), leading to the conclusion that these 3 indels have no pathogenic correlation with FCD.

Another 2 known dbSNP intron variants were detected in our analysis of the *ZEB1* gene (rs220057, MAF: C=0.2524/1264 and rs220060, MAF: G=0.0787/394), both of them were detected in 14 healthy spouses who married into the family (rs220057, 2/28; rs220060, 1/28) (Table II).

Analysis of LOXHD1 gene. Only one known dbSNP intron variant was detected in our analysis of the *LOXHD1* gene (rs16939650, MAF: T=0.2584/1294), and it was detected in both the cases in this FCD pedigree (7/16) and in the 14 healthy spouses who married into the family (9/28) (Table II). Another 2 intron variants were identified in the cases in this FCD pedigree that have not been previously reported in patients with FCD, namely, c.5332-126C>T and c.1809+155G>A (GenBank reference ID: NM_144612.6) (Table II) (Fig. 2). Both of these variants were absent from dbSNP and were not identified in the 14 unaffected spouses who married into this family (0/28) or in the 20 healthy descendants of this family (0/40) (Table II and Fig. 2). Therefore, these 2 variants were further tested in unrelated, ethnically matched controls (n≥100), and the results revealed that both of the variants were also absent from the 191 healthy samples we tested (0/382) (Table II). Heterozygous alterations in each variant were only identified in a single case each in this FCD pedigree (c.5332-126C>T was found in II-3, and c.1809+155G>A was found in II-5; Fig. 2) and are likely examples of *de novo* mutations, the pathological consequences of which are uncertain.

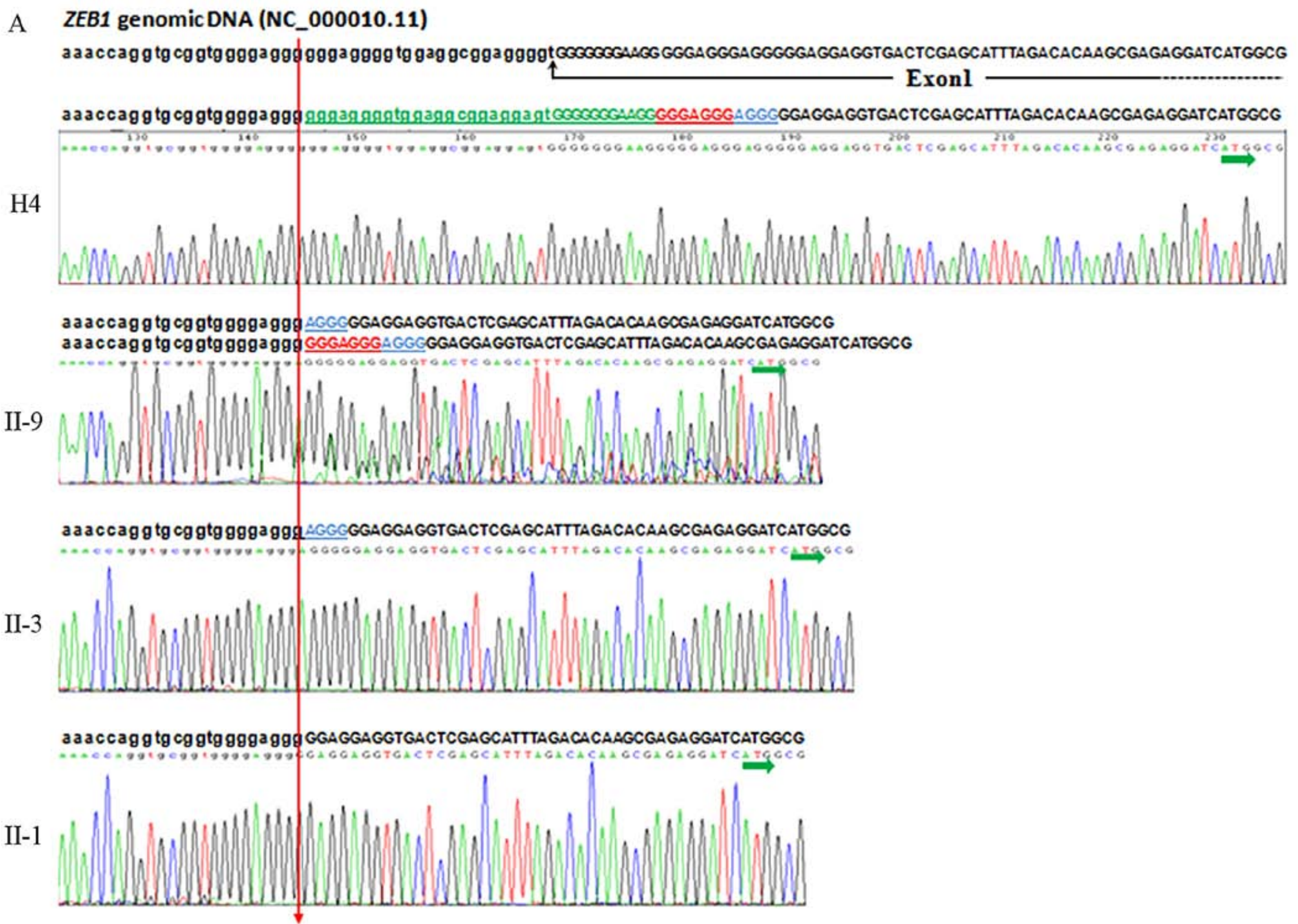

Figure 4. Sequencing analysis of the *ZEB1* gene. (A) Sequence electropherograms of PCR products encompassing the 5'-UTR region and exon 1 of the *ZEB1* genomic DNA. Sequences and sequencing chromatograms of PCR products encompassing 5'-UTR region and exon 1 of the *ZEB1* genomic DNA from H4 (healthy control), II-9 (proband), II-3 (FCD case), and II-1 (FCD case) are shown from top to bottom (homozygous is shown in only one sequence, heterozygous is shown in both sequences). *ZEB1* genomic DNA sequence (GenBank reference ID: NC_000010.11) was show above (exon and 5'-UTR seqences are depicted by upper and lower case letters, respectively) The breakpoint is indicated by the red arrow. Three indels detected in the present study are indicated in different colors: green for Indel 1, 34 bp indel containing 23 bp of the 5'-UTR region and 11 bp 5' end of exon 1 (NM_030751.5: c.-86_-53delinsgggaggggtggaggcggaggggtGGGGGGGAAGG); red for Indel 2, 7 bp (NM_030751.5:c.-52_-46delinsGGGAGGG); blue for Indel 3, 4 bp indel (NM_030751.5:c.-45_-42delinsAGGG). Transcription start site (TSS) is indicated by a horizontal green arrow underneath ATG. The numbering system used for sequence variations is based on cDNA sequence with +1 corresponding to the A of the ATG TSS (GenBank Reference ID: NM_030751.5). FCD, Fuchs corneal dystrophy.

Analysis of COL8A2 gene. Five known dbSNP variants were detected in our analysis of the *COL8A2* gene, including 3 synonymous variants, p.A35A (rs57985157, MAF: T=0.0966/484), p.L335L (rs79833067, MAF: C=0.0413/207) and p.G495G (rs35495320, MAF: T=0.1815/909), and 2 missense variants, p.R155Q (rs75864656, MAF: T=0.0377/188) and p.T502M (rs117860804, MAF: A=0.0587/294) (Table II) (Fig. 3). Four of these SNP coding variants from dbSNP (p.A35A, p.G495G, p.R155Q and p.T502M) have been reported in patients with FCD and unaffected individuals previously (21-23) and were present in both the affected members of this FCD pedigree (p.A35A, 5/16; p.G495G, 12/16; p.R155Q, 3/16; and p.T502M, 7/16) and in the 14 healthy spouses who married into the family (p.A35A, 12/28; p.G495G, 19/28; p.R155Q, 3/28; and p.T502M, 6/28) (Table II) (Fig. 2), as well as in the unrelated, ethnically matched, healthy control subjects (p.A35A, 128/364; p.G495G, 202/364; p.R155Q, 23/364; and p.T502M, 79/364) (Table II).

In addition, the synonymous variant p.L335L, which has been previously reported in 2 patients with posterior polymorphous corneal dystrophy (PPCD; MIM 122000) (18), was present in 10 unaffected family members, 6 of whom were unrelated spouses who married into this family, and none of them displayed any clinical features of FCD (Table II) (Fig. 2). Furthermore, the finding of this synonymous variant in 182 healthy control individuals (p.L335L, 88/364) (Table II), along with the absence of the p.L335L synonymous change in any of the 8 affected individuals in this Chinese FCD family (Table II) (Fig. 2) and the detection of this silent variant in 1 out of 116 healthy controls previously reported (18), leads to the conclusion that this substitution is a known polymorphism (dbSNP: rs79833067), and it has no association with FCD.

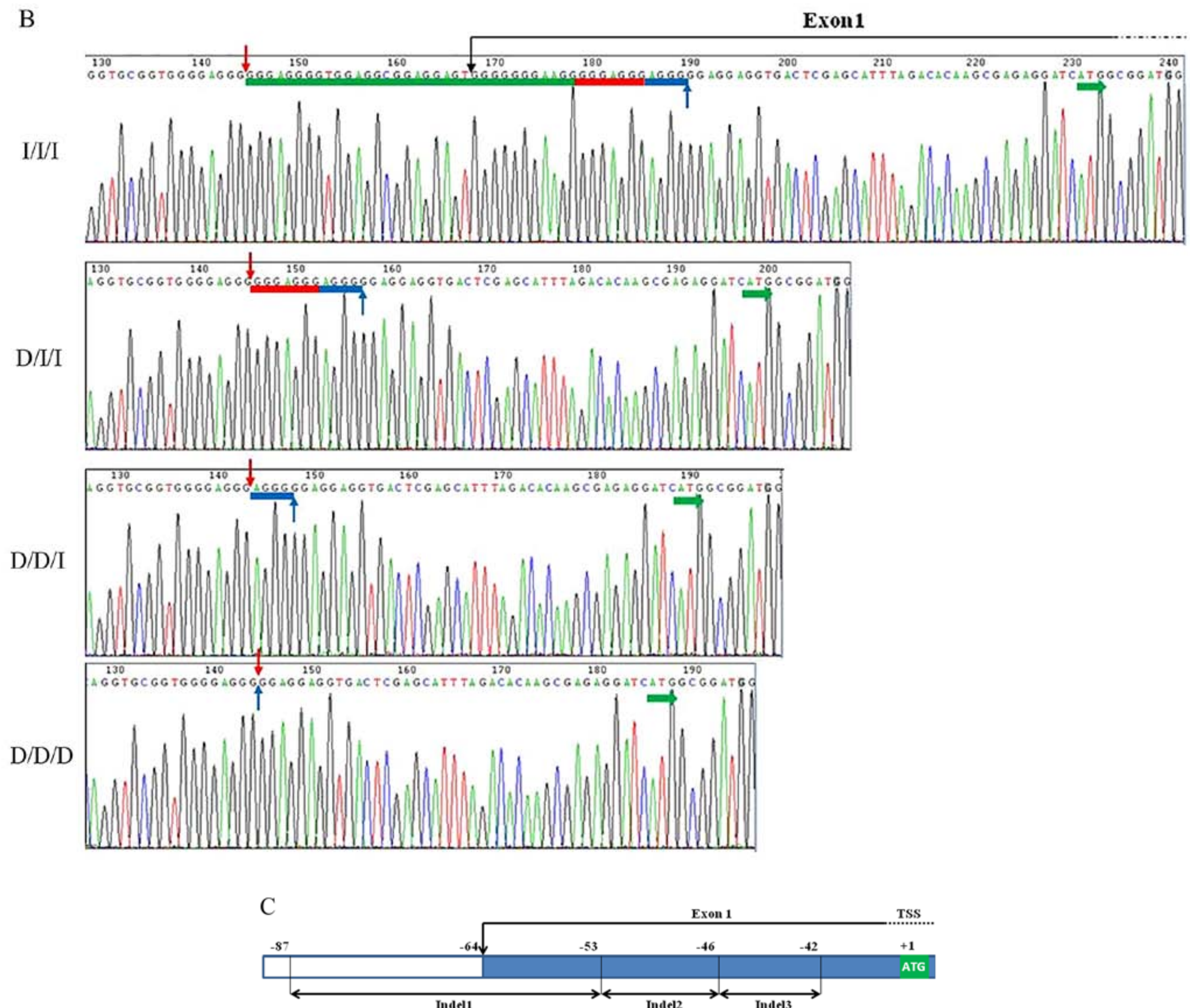

Figure 4. Continued. Sequencing analysis of *ZEB1* gene. (B) DNA sequencing results of the four haplotypes subcloned into pZeroBack/blunt vector. Three indels were underlined in different colors: green for Indel 1; red for Indel 2; and blue for Indel 3. Sequencing chromatograms of 4 haplotypes (ordered as Indel 1/Indel 2/Indel 3), I/I/I, D/I/I, D/D/I, D/D/D, are shown from top to bottom. The 5' and 3' boundaries of indel are shown by a vertical red and a blue arrow, respectively. TSS is indicated by a horizontal green arrow underneath ATG. (C) Schematic illustration of the ZEB1 genomic DNA and distribution of 3 continuous Indels (Indel 1, 2 and 3) relative to exon 1. The numbering system used for sequence variations is based on cDNA sequence with +1 corresponding to the A of the ATG TSS (GenBank Reference ID: NM_030751.5). FCD, Fuchs corneal dystrophy.

TCF4 genotype. The PCR products of the *TCF4* gene, which contains 7 previously reported SNPs significantly associated with LO FCD, were sequenced. An analysis of an intronic SNP in the *TCF4* gene, rs613872 (MAF: G=0.0697/348), the risk allele (G) that has been identified to be significantly associated with FCD among Europeans through genome-wide association studies (GWAS) (14), revealed that the risk allele (G) was not present in any subject in our FCD pedigree (0/84; 84 refers to the total number of chromosomes detected for the 42 members of the pedigree in whom DNA collection and genetic analysis were performed), and only one individual was heterozygous for the risk allele (G) out of the 191 unrelated healthy controls we tested (1/382) (Table II). This result was consistent with a previous study in which rs613872 was not present in Singaporean Chinese (15). The detection of 2 other SNPs (rs17089887, MAF: C=0.1689/846 and rs17089925, MAF: T=0.1569/786), which had been found to be significantly associated with FCD in Singaporean Chinese (15), revealed that only 3 out of 8 cases carried the heterozygous risk allele (C) of rs17089887 (3/16) and that only 1 out of 8 cases carried the heterozygous risk allele (T) of rs17089925 (1/16). Both of these risk alleles were also present in the 14 healthy individuals who married into this family (rs17089887: 6/28; rs17089925: 10/28) (Table II). The analysis of another 3 SNPs that exhibited a marginal association with FCD in Singaporean Chinese (rs1348047, MAF: T=0.2780/1392; rs1452787, MAF: G=0.2708/1356; and rs2123392, MAF: C=0.3005/1504) (15) revealed that 7 out of 8 cases carried the risk allele (T) of rs1348047 (3 homozygous and 4 heterozygous for the risk allele, 10/16), 7 out of 8 cases carried the risk allele (G) of rs1452787 (1 homozygous and 6 heterozygous for the risk allele, 8/16), and all 8 cases carried the risk allele (C) of rs2123392 (5 homozygous and 3 heterozygous for the risk allele, 13/16). While these 3 risk alleles were also present in the 14 healthy individuals who

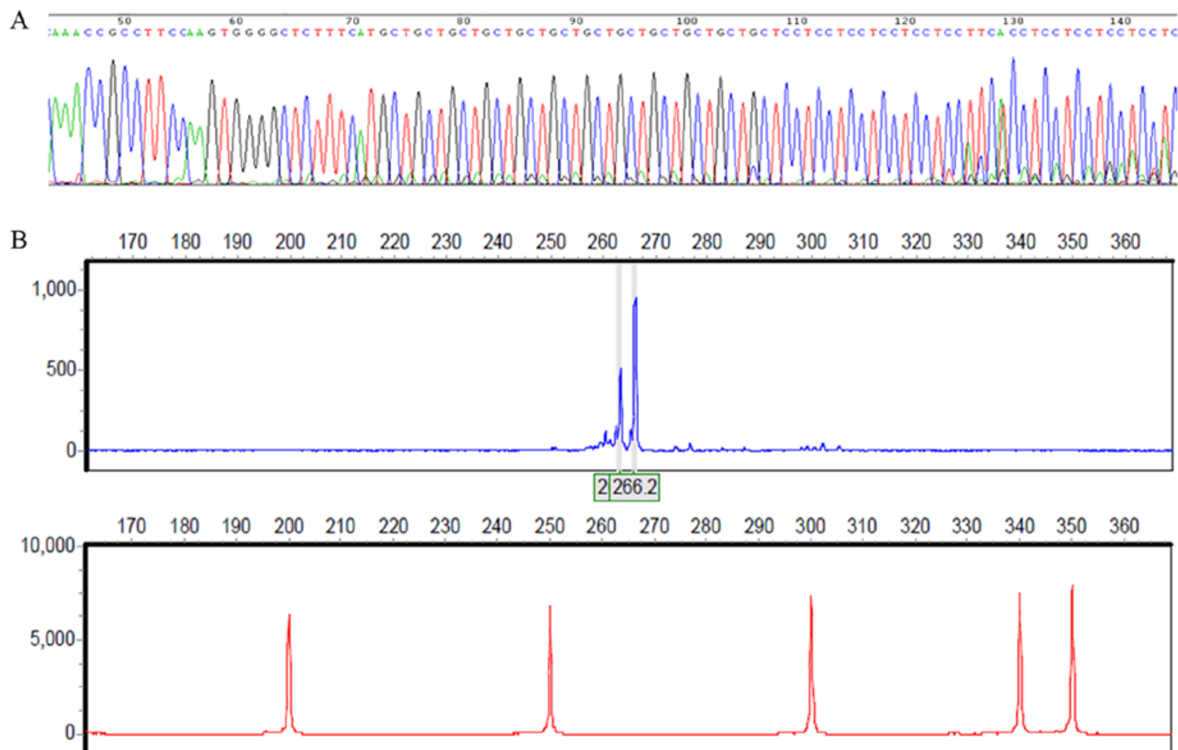

Figure 5. Analysis of TGC trinucleotide repeat expansion (rs193922902) of *TCF4*. (A) Sanger DNA sequencing of DNA samples of proband showed heterozygous for allele $(TGC)_{11}$ and $(TGC)_{12}$. (B) STR analysis of PCR amplicons of proband verified heterozygous for allele $(TGC)_{11}$ and $(TGC)_{12}$.

married into this family (rs1348047, 16/28; rs1452787, 14/28; and rs2123392, 11/28) (Table II), none of these 6 SNPs from dbSNP co-segregated with the disease.

One TGC trinucleotide repeat expansion (rs193922902) of TCF4, a repeat length >50 of which is known to play a pathogenic role in the majority of FCD cases and is considered to be a predictor of disease risk (16), was also detected in the present study. The direct sequencing of the proband indicated that it contained one 11- and one 12-repeat allele (Fig. 5A), and this result was further confirmed by STR analysis (Fig. 5B). The expanded repeat was not found in any of the subjects in our pedigree (0/84) (Table II), which indicated that this TGC trinucleotide expansion did not play a pathogenic role in this specific FCD family.

Discussion

To date, progress toward identifying the underlying genetic components of FCD has been limited to the analysis of a few genes, including *SLC4A11*, *ZEB1*, *LOXHD1* and *COL8A2* (4,10,12,13,20,21). Several genome-wide linkage studies have additionally provided evidence of linkage to several different chromosomal loci, namely FCD1, FCD2, FCD3 and FCD4, on chromosomes 13, 18, 5 and 9, respectively (10,24-26), that appear to influence familial FCD. Thus, it appears that locus heterogeneity may exist for FCD, whereby mutations in several genes on different chromosomes may produce a common disease phenotype. Significant progress toward understanding non-familial FCD was made using GWAS. Baratz *et al* identified an SNP on chromosome 18q21, rs613872, in an intron of a gene encoding (*TCF4*) (MIM 602272) and showed a significant genome-wide association with FCD susceptibility in Europeans (14). This finding was further validated by Li *et al* (27) in another independent study. Although rs613872 was not found to be present in Singaporean Chinese FCD subjects, 2 other SNPs (rs17089887 and rs17089925) (15) and one TGC trinucleotide repeat expansion (rs193922902) (16) of the *TCF4* gene were reported to be significantly associated with FCD in the Chinese subjects.

Despite these insights, knowledge regarding the genetic basis of FCD in the China mainland population has remained limited, possibly due to the varying prevalence of FCD in different ethnic populations. The prevalence of FCD is generally considered to be approximately 4% in individuals above 40 years of age in the United States and accounts for the second most common indication for corneal transplants performed in the United States in patients over the age of 60 years (28,29). The prevalence of FCD in other countries and areas has been confirmed by studies that have examined indications for PK at various institutions worldwide; prevalences of 15.4, 7.1 and 4.7% have been reported in populations from the UK (30), Singapore (31) and Australia (32), respectively, while studies in China suggest a relatively lower prevalence of FCD, namely, 4.5% in Taiwan (33) and <3.9% in both the northern and eastern mainland of China (34,35). Combined with clinical experience in the US that suggests a significantly decreased prevalence of FCD among individuals of African-American, Latin-American, or Asian origin, a greater understanding of the genetic basis of FCD in patients of different ethnic origins will shed more light on the molecular mechanisms of the disease.

To the best of our knowledge, the present study is the first study on a Chinese mainland population to focus on the genetic

basis of the multi-generational Chinese pedigree with LO FCD that was previously reported by our group (36). In the present study, we performed a sequence analysis of the *SLC4A11*, *ZEB1*, *LOXHD1*, *COL8A2* and *TCF4* genes in this LO FCD pedigree.

Screening of the *SLC4A11* gene revealed 14 known dbSNP variants; among these, an intronic variant, is a known SNP from dbSNP (rs372201212, MAF: G=0.0014/7), its minor allele (G) was detected in 4 of 8 affected members of this FCD pedigree, II-1, II-5, III-9 (II-1's daughter), and III-19 (II-5' son) (4/16), and 3 of 20 healthy descendants in this family, III-3 (II-1's son, III-9's older brother), IV-4 (II-1's granddaughter, III-3's daughter) and IV-16 (II-5's grandson, III-19's son) (3/40). Although this variant was not identified in any unaffected individuals who married into this family (0/28) or in the unrelated healthy controls (0/382), it may not be considered pathogenic as it did not co-segregate with the disease in this FCD pedigree. However, we cannot rule out the possibility that this variant has an association with FCD; if we consider the late onset of the disease and the fact that IV-4 and IV-16 were 20 and 14 years old, respectively, when the blood samples were collected in 2009, the disease status in these younger individuals is uncertain, as they may not have been old enough to manifest the disorder and may not clinically exhibit the disease, suggesting that this variant may be correlated with FCD in this family. Further analysis of this known SNP from dbSNP (rs372201212) in the *SLC4A11* gene in larger numbers of Chinese patients with FCD may elucidate the significance of this gene in corneal endothelial dystrophies.

Similarly, 2 intron variants of the *LOXHD1* gene were identified in this FCD pedigree: c.5332-126C>T and c.1809+155G>A (GenBank reference ID: NM_144612.6). These 2 variants have not been previously reported in FCD patients and were absent from dbSNP. Neither of these variants was identified in the unaffected family members or in the healthy controls (n≥100). As these variants were each found in only a single case in this FCD pedigree (c.5332-126C>T in II-3, and c.1809+155G>A in II-5), they are likely examples of *de novo* mutations.

ZEB1 is a zinc finger E-box binding homeobox 1 gene (MIM 189909) and is also known as human zinc finger *TCF8*, which maps to chromosome 10p11.2, comprises 9 exons and encodes a transcription factor that is organized into multiple functional domains starting with N-terminal zinc finger clusters (172-292), followed by a homeodomain (581-640), a repression domain (754-901), C-terminal zinc finger clusters (905-981) and an acidic activation domain (1011-1124) (37). The structure of *ZEB1* allows for a wide range of functions as each zinc finger has different DNA-binding specificities and effects on gene expression (38). Mutations in the *ZEB1* transcripts have been shown to produce a wide range of ocular phenotypes (39). It was estimated that changes in this gene may account for approximately 50% of all PPCD cases (40), and its mutations also account for LO FCD (12). Through *ZEB1* screening, we identified 3 continuous indels located at the junction of the 5'-UTR and the adjacent 5' end of exon 1 of the *ZEB1* gene in the cases in this FCD pedigree, and these 3 indels covered the region from -86 to -42 (numbering system based on the cDNA sequence with +1 corresponding to the A of the ATG TSS in the Ref Seq: NM_030751.5). A schematic illustration of the *ZEB1* genomic DNA and the location of these 3 continuous indels relative to exon 1 is shown in Fig. 4C, and these 3 continuous indels, including 34 bp Indel 1 (containing 23 bp of the 5'-UTR region and 11 bp of the 5' end of exon 1), 7 bp Indel 2 (containing 7 bp of exon 1), and 4 bp Indel 3 (containing 4 bp of exon 1). In addition, according to the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=488683917_eMaJPXBAmxFX5D7tqTNSeva4dT39), the bases affected by these 3 indels are relatively well conserved through evolution and also lie within transcription factor binding sites, and these regions are also enriched with H3K27AC, which is often found near active regulatory elements. In the case of Indel 1, the splice site variation affects the first splice site and may likely cause mis-splicing of the pre-mRNA transcript. This variation would lead to either exon skipping or intron retention, which would consequently result in an altered protein structure. Therefore, we hypothesized that a different haplotype of *ZEB1* will alter the mRNA structure or influence splicing efficiency. Further studies are needed to determine what effect, if any, these indels may have on *ZEB1* gene function and FCD pathogenesis; however, the presence of these 3 indels in the 14 healthy spouses who married into this family, the unaffected family members, and the healthy controls (n≥100) suggests that these 3 indels are not likely to be pathogenic.

In the screening of the *COL8A2* gene, neither of the previously reported pathogenic mutations of *COL8A2* (p.L450W and p.Q455K or Q455V) (4,21,23) was observed in this family, and none of the novel mutations were identified in the *COL8A2* gene in this LO FCD pedigree of Chinese descent. Our results are not surprising, as several studies have been published demonstrating a lack of *COL8A2* mutations in LO FCD (22).

Variations in candidate genes of FCD, deemed pathogenic on the basis of their absence in control chromosomes, were later identified as common polymorphisms in other ethnic populations (21-23), due to the fact that frequency of gene variant may depend greatly on the population screened. The MAF of 8 coding variants across *SLC4A11* (p.R161R, p.S213S and p.T833T) and *COL8A2* (p.A35A, p.R155Q, p.L335L, p.G495G and p.T502M) genes in healthy controls with Chinese ancestry (n≥100) were compared with MAF data from the 1000 Genomes database (Table II). The statistical analysis demonstrated that 4 MAF of *COL8A2* gene (p.A35A, p.L335L, p.G495G and p.T502M) in Chinese healthy controls tested in the present study were all significantly higher than the data from 1000 Genomes (P<0.01) (Table II), indicated that these varations were rare in occidental populations (21), which may account for the absence of these minor allele distribution in control chromosomes in previous study (21-23).

Since the publication of the initial GWAS results indicating a significant association of an intronic SNP in *TCF4*, rs613872, with FCD (14), several studies across different cohorts from diverse populations have demonstrated that polymorphisms near *TCF4* were consistently linked to an increased prevalence of FCD (15,27). These SNPs of *TCF4* were detected in this Chinese LO FCD pedigree, and the analysis revealed that the SNP rs613872, which is most highly associated with FCD in Caucasians, was not found to be present in this Chinese FCD family (0/84), and only one individual carrying the heterozygous variant of rs613872 was detected in the healthy controls (1/382), the MAF of healthy controls tested in the present study is significantly lower when compared with the data from 1000 Genomes (P<0.01) (Table II). This result is

consistent with previous research (15), in which rs613872 was not found to be present in Chinese FCD subjects, as well as with the data from the Human Genome Diversity Project, in which the minor (risk) allele, G, of rs613872 was found to be rare in populations from Africa, Eastern Asia, and Central and South America and more frequent in European, Middle Eastern, and Southern Asian populations (41). Two other SNPs (rs17089887 and rs17089925), which have been reported to be significantly associated with FCD in Singaporean Chinese (15), and a TGC trinucleotide repeat expansion (rs193922902) of *TCF4*, a repeat length >50 of which plays a pathogenic role in the majority of FCD cases (16), were also detected in this Chinese LO FCD pedigree. The results revealed that none of these 3 SNPs co-segregated with the disease. Therefore, we investigated whether the three SNPs that were marginally associated with FCD in Singaporean Chinese (rs1348047, rs1452787 and rs2123392) (15) had some association with FCD in this Chinese LO FCD pedigree, and the results revealed that all three risk alleles were present in both the 8 cases and the 14 healthy individuals who married into this family. This finding led to the conclusion that none of these known SNPs provide strong evidence of pathogenesis in this specific muti-generational LO FCD Chinese family; however, because only seven fragments containing 7 SNPs were sequenced in the present study, we still cannot rule out the possibility that additional variants in other regions of the *TCF4* gene that were not assessed in the present study may be present that could confer phenotypic changes.

In conclusion, to identify and exclude known mutations and SNPs associated with FCD, we screened our LO FCD pedigree for all known exons and adjacent splice sites in the previously reported FCD genes that were associated with either LO FCD (*SLC4A11*, *ZEB1*, *LOXHD1* and *TCF4*) or EO FCD (*COL8A2*). Twenty-seven variants (including 22 known dbSNP variants and 5 variants absent from dbSNP) were detected. None of these variants provided strong evidence of pathogenesis, making it unlikely that SNPs or mutations in them caused FCD in this specific pedigree. The possibility of pathogenic changes occurring within the promoter, intronic, or untranslated non-coding regions of these genes playing a role in the pathogenesis of FCD has not been excluded in this study. The fact that we did not detect any pathogenic variants in these genes in our pedigree is likely a combination of the fact that these genes carry a low genetic load in FCD and that we screened only one LO FCD pedigree, a small sample that is underpowered for detecting variants that occur at relatively low frequencies. Clearly, a genome-wide linkage scan to identify linkage to one of the previously described FCD loci or to identify a novel locus for FCD will need to be performed in this multi-generational Chinese pedigree with LO FCD. Our observation, nevertheless, expands the current knowledge regarding the genetic status of Chinese ancestry patients with FCD.

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References

1. Fuchs E: Fuchs E Dystrophia epithelialis corneae. Albrecht Von Graefes Arch Klin Exp Ophthalmol 76: 478-508, 1910.
2. Adamis AP, Filatov V, Tripathi BJ and Tripathi RC: Fuchs' endothelial dystrophy of the cornea. Surv Ophthalmol 38: 149-168, 1993.
3. Krachmer JH, Purcell JJ Jr, Young CW and Bucher KD: Corneal endothelial dystrophy. A study of 64 families. Arch Ophthalmol 96: 2036-2039, 1978.
4. Gottsch JD, Sundin OH, Liu SH, Jun AS, Broman KW, Stark WJ, Vito EC, Narang AK, Thompson JM and Magovern M: Inheritance of a novel COL8A2 mutation defines a distinct early-onset subtype of fuchs corneal dystrophy. Invest Ophthalmol Vis Sci 46: 1934-1939, 2005.
5. Waring GO III, Bourne WM, Edelhauser HF and Kenyon KR: The corneal endothelium. Normal and pathologic structure and function. Ophthalmology 89: 531-590, 1982.
6. Bergmanson JP, Sheldon TM and Goosey JD: Fuchs' endothelial dystrophy: A fresh look at an aging disease. Ophthalmic Physiol Opt 19: 210-222, 1999.
7. Thompson RW Jr, Price MO, Bowers PJ and Price FW Jr: Long-term graft survival after penetrating keratoplasty. Ophthalmology 110: 1396-1402, 2003.
8. Anshu A, Price MO, Tan DT and Price FW Jr: Endothelial keratoplasty: a revolution in evolution. Surv Ophthalmol 57: 236-252, 2012.
9. Ham L, Dapena I, van Luijk C, van der Wees J and Melles GR: Descemet membrane endothelial keratoplasty (DMEK) for Fuchs endothelial dystrophy: review of the first 50 consecutive cases. Eye (Lond) 23: 1990-1998, 2009.
10. Riazuddin SA, Vithana EN, Seet LF, Liu Y, Al-Saif A, Koh LW, Heng YM, Aung T, Meadows DN, Eghrari AO, *et al*: Missense mutations in the sodium borate cotransporter SLC4A11 cause late-onset Fuchs corneal dystrophy. Hum Mutat 31: 1261-1268, 2010.
11. Vithana EN, Morgan P, Sundaresan P, Ebenezer ND, Tan DT, Mohamed MD, Anand S, Khine KO, Venkataraman D, Yong VH, *et al*: Mutations in sodium-borate cotransporter SLC4A11 cause recessive congenital hereditary endothelial dystrophy (CHED2). Nat Genet 38: 755-757, 2006.
12. Riazuddin SA, Zaghloul NA, Al-Saif A, Davey L, Diplas BH, Meadows DN, Eghrari AO, Minear MA, Li YJ, Klintworth GK, *et al*: Missense mutations in TCF8 cause late-onset Fuchs corneal dystrophy and interact with FCD4 on chromosome 9p. Am J Hum Genet 86: 45-53, 2010.
13. Riazuddin SA, Parker DS, McGlumphy EJ, Oh EC, Iliff BW, Schmedt T, Jurkunas U, Schleif R, Katsanis N and Gottsch JD: Mutations in LOXHD1, a recessive-deafness locus, cause dominant late-onset Fuchs corneal dystrophy. Am J Hum Genet 90: 533-539, 2012.
14. Baratz KH, Tosakulwong N, Ryu E, Brown WL, Branham K, Chen W, Tran KD, Schmid-Kubista KE, Heckenlively JR, Swaroop A, *et al*: E2-2 protein and Fuchs's corneal dystrophy. N Engl J Med 363: 1016-1024, 2010.
15. Thalamuthu A, Khor CC, Venkataraman D, Koh LW, Tan DT, Aung T, Mehta JS and Vithana EN: Association of *TCF4* gene polymorphisms with Fuchs' corneal dystrophy in the Chinese. Invest Ophthalmol Vis Sci 52: 5573-5578, 2011.
16. Wieben ED, Aleff RA, Tosakulwong N, Butz ML, Highsmith WE, Edwards AO and Baratz KH: A common trinucleotide repeat expansion within the transcription factor 4 (TCF4, E2-2) gene predicts Fuchs corneal dystrophy. PLoS One 7: e49083, 2012.
17. Gottsch JD, Zhang C, Sundin OH, Bell WR, Stark WJ and Green WR: Fuchs corneal dystrophy: Aberrant collagen distribution in an L450W mutant of the *COL8A2* gene. Invest Ophthalmol Vis Sci 46: 4504-4511, 2005.
18. Yellore VS, Rayner SA, Emmert-Buck L, Tabin GC, Raber I, Hannush SB, Stulting RD, Sampat K, Momi R, Principe AH and Aldave AJ: No pathogenic mutations identified in the *COL8A2* gene or four positional candidate genes in patients with posterior polymorphous corneal dystrophy. Invest Ophthalmol Vis Sci 46: 1599-1603, 2005.
19. Hemadevi B, Srinivasan M, Arunkumar J, Prajna NV and Sundaresan P: Genetic analysis of patients with Fuchs endothelial corneal dystrophy in India. BMC Ophthalmol 10: 3, 2010.
20. Vithana EN, Morgan PE, Ramprasad V, Tan DT, Yong VH, Venkataraman D, Venkatraman A, Yam GH, Nagasamy S, Law RW, *et al*: SLC4A11 mutations in Fuchs endothelial corneal dystrophy. Hum Mol Genet 17: 656-666, 2008.

21. Biswas S, Munier FL, Yardley J, Hart-Holden N, Perveen R, Cousin P, Sutphin JE, Noble B, Batterbury M, Kielty C, *et al*: Missense mutations in *COL8A2*, the gene encoding the alpha2 chain of type VIII collagen, cause two forms of corneal endothelial dystrophy. Hum Mol Genet 10: 2415-2423, 2001.
22. Kobayashi A, Fujiki K, Murakami A, Kato T, Chen LZ, Onoe H, Nakayasu K, Sakurai M, Takahashi M, Sugiyama K and Kanai A: Analysis of *COL8A2* gene mutation in Japanese patients with Fuchs' endothelial dystrophy and posterior polymorphous dystrophy. Jpn J Ophthalmol 48: 195-198, 2004.
23. Mok JW, Kim HS and Joo CK: Q455V mutation in *COL8A2* is associated with Fuchs' corneal dystrophy in Korean patients. Eye (Lond) 23: 895-903, 2009.
24. Sundin OH, Jun AS, Broman KW, Liu SH, Sheehan SE, Vito EC, Stark WJ and Gottsch JD: Linkage of late-onset Fuchs corneal dystrophy to a novel locus at 13pTel-13q12.13. Invest Ophthalmol Vis Sci 47: 140-145, 2006.
25. Sundin OH, Broman KW, Chang HH, Vito EC, Stark WJ and Gottsch JD: A common locus for late-onset Fuchs corneal dystrophy maps to 18q21.2-q21.32. Invest Ophthalmol Vis Sci 47: 3919-3926, 2006.
26. Riazuddin SA, Eghrari AO, Al-Saif A, Davey L, Meadows DN, Katsanis N and Gottsch JD: Linkage of a mild late-onset phenotype of Fuchs corneal dystrophy to a novel locus at 5q33.1-q35.2. Invest Ophthalmol Vis Sci 50: 5667-5671, 2009.
27. Li Y-J, Minear MA, Rimmler J, Zhao B, Balajonda E, Hauser MA, Allingham RR, Eghrari AO, Riazuddin SA, Katsanis N, *et al*: Replication of TCF4 through association and linkage studies in late-onset Fuchs endothelial corneal dystrophy. PLoS One 6: e18044, 2011.
28. Klintworth GK: Corneal dystrophies. Orphanet J Rare Dis 4: 7, 2009.
29. Mannis MJ, Holland EJ, Beck RW, Belin MW, Goldberg MA, Gal RL, Kalajian AD, Kenyon KR, Kollman C, Ruedy KJ, *et al*; Cornea Donor Study Group: Cornea Donor Study Group: Clinical profile and early surgical complications in the Cornea Donor Study. Cornea 25: 164-170, 2006.
30. Rahman I, Carley F, Hillarby C, Brahma A and Tullo AB: Penetrating keratoplasty: indications, outcomes, and complications. Eye (Lond) 23: 1288-1294, 2009.
31. Tan DT, Janardhanan P, Zhou H, Chan YH, Htoon HM, Ang LP and Lim LS: Penetrating keratoplasty in Asian eyes: The Singapore Corneal Transplant Study. Ophthalmology 115: 975-982.e1, 2008.
32. No authors listed: The Australian Corneal Graft Registry: 1990 to 1992 report. Aust N Z J Ophthalmol 21 (Suppl 2): 1-48, 1993.
33. Chen WL, Hu FR and Wang IJ: Changing indications for penetrating keratoplasty in Taiwan from 1987 to 1999. Cornea 20: 141-144, 2001.
34. Xie L, Song Z, Zhao J, Shi W and Wang F: Indications for penetrating keratoplasty in north China. Cornea 26: 1070-1073, 2007.
35. Zhang C and Xu J: Indications for penetrating keratoplasty in East China, 1994-2003. Graefes Arch Clin Exp Ophthalmol 243: 1005-1009, 2005.
36. Huo L, Hui T, Tao S, Hong D and Yan X: A pedigree of Fuchs Corneal Dystrophy. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 27: 231-232, 2010 (In Chinese).
37. Ikeda K, Halle JP, Stelzer G, Meisterernst M and Kawakami K: Involvement of negative cofactor NC2 in active repression by zinc finger-homeodomain transcription factor AREB6. Mol Cell Biol 18: 10-18, 1998.
38. Ikeda K, Kawakami K, Stelzer G, Meisterernst M and Kawakami K: DNA binding through distinct domains of zinc-finger-homeodomain protein AREB6 has different effects on gene transcription. Eur J Biochem 233: 73-82, 1995.
39. Moroi SE, Gokhale PA, Schteingart MT, Sugar A, Downs CA, Shimizu S, Krafchak C, Fuse N, Elner SG, Elner VM, *et al*: Clinicopathologic correlation and genetic analysis in a case of posterior polymorphous corneal dystrophy. Am J Ophthalmol 135: 461-470, 2003.
40. Krafchak CM, Pawar H, Moroi SE, Sugar A, Lichter PR, Mackey DA, Mian S, Nairus T, Elner V, Schteingart MT, *et al*: Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells. Am J Hum Genet 77: 694-708, 2005.
41. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM and Haussler D: The human genome browser at UCSC. Genome Res 12: 996-1006, 2002.