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), lipoygenase homology domains 1 (LOXHD1), collagen, type VIII, alpha 2 (COL8A2) and transcription factor 4 (TCF4)] were previously reported to be implicated in the pathogenesis of FCD, and 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 gggaggctggagagggtGGGGGGGAAGG); Indel 2 (c.-52_46delinsGGAGGG); 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.

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 to 3 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

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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, rs63872, 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 rs63872 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 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 MgCl₂, 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 5 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'-CGACTCACATATA GGGAGACGCGC-3' and reverse, 5'-AAGAATCATCGA 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    | GCCCGGTCCCTTCCTCTCT    | GCCAAAAGCATTCAGACACTAG | 553               | 136                    |
|        | 2,3  | CGGCTAGGGAATGCTGGAGA   | GGACAGCGGGAGGATCTCT    | 631               | 153, 50                |
|        | 4,5  | CCCGCTGTCCTCCTCCTCT    | GCAGTGCCTCAGCCTCCTCT  | 720               | 232, 82                |
|        | 6    | GCCGGCCCAACCAACTTT     | CCGGTGTTTGAATAGGATAG   | 556               | 124                    |
|        | 7,8  | GGCGAGACACCTTCCTCTCT   | CCCGCTGTGTGTCTGCTCA    | 688               | 219, 94, 126           |
|        | 9B, 10, 11 | TCCCCAGCAAAACCTCCTCTCT | TGGGGAGCAAAATGGTGAG   | 687               | 114, 133               |
|        | 12   | TGGCCTTTATGCCTTTTCAC   | CACCGGACACACACTACCTT   | 493               | 74                     |
|        | 12, 14, 15A | CCCCCGGAGCCCTTTCTCTCT | GCCGGCCCAACAGTTCTCG   | 755               | 253, 107, 169          |
|        | 15B, 16, 17A | TCCCCGGGAAATCAGAGGAGGT | GCAGCGATAGGAGAGGAGAGG | 791               | 174, 196               |
|        | 17B, 18 | GCCCGTGACCTCGAGAGT    | CCCGCCCAATCTCCGACAC   | 617               | 170                    |
|        | 19   | TGGGCTGGAGAATGGTCTG    | GCCAGTACAGGAGGACAGGT  | 557               | 70                     |
| ZEB1   | 1    | CCGGCCGGTCCTACGAAACAGA | CGGAGGGGCGAGAGGACACTT | 413               | 58                     |
|        | 2    | TGGTCTGAAAATCCTGGCTCTG | TCTTTTACCTACAGCATTTG  | 1,115             | 20                     |
|        | 3    | TCTTTTCATGTTTGGGAGATT | TGAATCTGTGTGTGGAGATG   | 789               | 60                     |
|        | 4    | GGCGGCTGCTAAGGCAAGAAT | AAGGCGAGGCAAGAACACCT  | 994               | 162                    |
|        | 5    | AGCCCGGATTTGAACTGCTT   | TTTCTTGAGGAGGAGATGTTG | 512               | 203                    |
|        | 6    | CAAAACAACCATGAGCTGCA    | TCTAAGGGTCTTACATGGTTA | 674               | 106                    |
|        | 7A   | CAGTTCGTCACAAGAGCTGATC | TGGCCTAGGTGCTGAAAGCT  | 791               | 1,811*                 |
|        | 7B   | CACCATTAGCGCAACCTTCT   | TGAGGTTCTATTGGCAAGTT  | 876               |                        |
|        | 7C   | TGAAAAAGTGAAGCCTGGACAG | GGGCTGATCCTTCAAGGTT   | 702               |                        |
|        | 7D   | GGAGCTGCTGACAGGAGAACA  | GGGCATCGCAAGGGCTCTT    | 743               |                        |
|        | 8    | TTGCGTGTCCTTGCCTTCTCTC | GCCGAGATTGAGGAGGTG    | 640               | 181                    |
|        | 9    | AACCTCCTCCTCCTTACATG   | GGCCACCCGGAGATTGGT    | 900               | 593                    |
| LOXHD1 | 1    | AGAAGGCCAGAGGGAACAG   | ATGGGTAATACGAGGAAA    | 439               | 130                    |
|        | 2    | GTTGTGCTGGAAAGATATTAC | CTGTTGCTGGTGGAGA      | 844               | 115                    |
|        | 3    | TCTCTCCTCTGATCCAC      | CACACCTGATGATCCCATTT  | 508               | 81                     |
|        | 4    | ACCGAGGTTCGAGGAA       | GCAGAGACGGCGA         | 405               | 185                    |
|        | 5    | GGAGTAGTGTATGTGATGTTG  | TTCTGTTCTTCTGCTTTG   | 650               | 99                     |
|        | 6    | AAGGAGTTCTGGTAGGCTGAA  | CTGAAATCTGTAGAAGAGTG  | 657               | 149                    |
|        | 7    | AAGTAAATCGCCAGATGCA    | TTCAGGAGCAGGAGA       | 473               | 124                    |
|        | 8    | ATTCTTAGCGCAACCGG    | GGAAATCATAACCCAAAGA   | 822               | 251                    |
|        | 9, 10 | TGGGTGATACCTACTTTG    | ATCCCTTCCTCCTTTCTCTC | 1,116             | 136, 161               |
|        | 11, 12 | GTTTATGTCTTGGAGGAGT  | ACTTGGAGGATGGCTTTT    | 1,127             | 87, 136                |
|        | 13   | GGAAGGTGACGCCCCAGAT    | TCCAGGATGACACAGAG     | 546               | 155                    |
|        | 14   | GACGCGGATGTGGTTGG     | ATGGGAGCGTGGTTCTT     | 913               | 161                    |
|        | 15   | TCAATCTCAGCAAGAC      | GCAGGAGCGAGAATCCT    | 452               | 77                     |
|        | 16   | ATTTCACGCTTCTTCTCTC   | TCTTATGCTCTTACCTCTCTC | 857               | 197                    |
|        | 17   | CCCCTCTGTTGCTTCTAC    | CCTTGGGCTCTAGTGT     | 677               | 193                    |
|        | 18   | GCTGTGAATGCTGTCTCTCT  | GTTGGTGGCTTACAGT     | 737               | 161                    |
|        | 19, 20 | CTGGGCTTTTCTGTGGG     | GTTGGTGTCTGCTGCTTTG  | 1,423             | 463, 155               |
|        | 21   | TCCAGCAAACCTCTCTCTCT  | GTCTTCTTACCAGACTACC  | 513               | 134                    |
|        | 22   | CAGGCAAATATGCTAATGGG  | GAAGGAGGAAGAAGATAGGA | 428               | 164                    |
|        | 23   | GGCCTACAGATACAGGAG    | GCCAAACACTAAACACCA   | 640               | 105                    |
|        | 24, 25 | AACTCCAACATGAAACCA   | CGAGGAGGAAAGAACCAAA  | 1,441             | 129, 165               |
|        | 26   | GGAGATAGGAGGAAGGTTG   | AGGAGGAGCAGGGTGAG    | 897               | 182                    |
|        | 27, 28 | GGCGAGCAGAGGAGCAT     | GGCGAGGAGAAGAACCAAG  | 1,984             | 117, 163               |
|        | 29   | TGGCAGTAGATTAGTGA     | AGGCAAGGGCCAAG       | 661               | 155                    |
|        | 30, 31 | GAGTGGTGTGATGTTGGG    | ATCTGGTATGATGGGG     | 608               | 210, 136               |
|        | 32   | GAAACCTACCAAGAATGT    | GTGGCTCAACCAAGAATGT  | 1,003             | 209                    |
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 χ² 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; p.S213S, 5/16; p.T833T, 5/16).
Figure 2. Pedigree of a Chinese family with late-onset (LO) Fuchs corneal dystrophy (FCD) with genotypes of 11 variations 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 variations 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.

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).
| Gene          | Chr position | rs ID        | mRNA                | Amino acid change | Functional consequence | 1000 Genomes MAF | 8 Cases MAF | 14 Unrelated spouses MAF | Healthy controls MAF |
|--------------|--------------|--------------|---------------------|-------------------|------------------------|------------------|------------|--------------------------|----------------------|
| **SLC4A11**  | 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                | G=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           | G=0.4798/2402          | 5/16 (0.3125)    | 13/28 (0.4643) |
|              | 20:3233935   | rs3803956    | c.639G>A            | p.S213S           | T=0.1663/833           | 2/16 (0.1250)    | 5/28 (0.1786) |
|              | 20:323504    | rs372201212  | c.777+10T>C         | nc                | G=0.0014/7             | 4/16 (0.2500)    | 0/28 (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                | T=0.4477/2242          | 0/16 (0.0000)    | 16/28 (0.5714) |
|              | 20:3231073   | rs3803954    | c.1091-19T>C        | nc                | G=0.0696/335           | 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           | T=0.0901/450           | 5/16 (0.3125)    | 6/28 (0.2143) |
|              |              |              |                     |                   |                        |                 |            |                           |                      |
| **ZEB1**     | 10:31319149  | NA           | c.-86_-53delggaggggt| utr 5 prime, ex | NA                     | Del=16/16 (1.0000)| 28/28 (1.0000)| 368/370 (0.9946) |
|              | 10:31319183  | NA           | c.-52_-46delGGGGAGGG| ex                | NA                     | Del=9/16 (0.5625)| 14/28 (0.5000)| 183/370 (0.4946) |
|              | 10:31319190  | NA           | c.-45_-42delGGGGAGGG| ex                | NA                     | Del=5/16 (0.3125)| 12/28 (0.4286)| 162/370 (0.4378) |
|              | 10:3150273   | 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**   | 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**   | 1:36100138   | rs57985157   | c.105G>A            | p.A35A            | T=0.0966/484           | 5/16 (0.3125)    | 12/28 (0.4286) |
|              | 1:36099217   | rs75864656   | c.464G>A            | p.R155Q           | T=0.0377/188           | 3/16 (0.1875)    | 3/28 (0.1071) |
|              | 1:36098676   | rs79833067   | c.1005C>G           | p.L335L           | C=0.0413/207           | 0/16 (0.0000)    | 6/28 (0.2143) |
|              | 1:36098196   | rs35495320   | c.1485G>A           | p.G495G           | T=0.1815/909           | 12/16 (0.7500)   | 19/28 (0.6786) |
|              | 1:36098176   | rs117860804  | c.1505C>T           | p.T502M           | A=0.0587/204           | 7/16 (0.4375)    | 6/28 (0.2143) |

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).
Table II. Continued.

| Gene  | Chr position | rs ID         | mRNA     | Amino acid change | mRNA functional consequence | 1000 Genomes | Healthy controls | Unrelated spouses | Healthy controls |
|-------|--------------|---------------|----------|-------------------|-----------------------------|--------------|------------------|-------------------|------------------|
|       |              |              |          |                   |                             | n=16         | n=28             | n=16             | n=16             |
| TCF4  | 18:55382876  | rs1149047     | NM_003199.2 | 816 (0.5000)      | in                          | T=a.2780/1392| 10/16 (0.6250)  | 16/28 (0.5714)   | 3/16             |
|       |              |              |          |                   |                             |              |                  |                   |                  |
|       |              | rs1149478     | NM_003199.2 | 2143 (0.0000)     | in                          | T=0.0020/146  | 0/16 (0.0000)   | 0/16 (0.0000)    | 0/16 (0.0000)   |
|       |              | rs1149479     | NM_003199.2 | 316 (0.1875)      | in                          | T=0.1569/786 | 0/16 (0.0000)   | 0/16 (0.0000)    | 0/16 (0.0000)   |
|       |              | rs1149480     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |
|       |              | rs1149481     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |
|       |              | rs1149482     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |
|       |              | rs1149483     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |
|       |              | rs1149484     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |
|       |              | rs1149485     | NM_003199.2 | 1336 (0.0825)     | in                          | T=0.0313/109  | 9/16 (0.5625)   | 9/28 (0.3214)    | 11/28 (0.3929)  |

There were 8 cases and 14 unrelated spouses (in whom DNA collection and genetic analysis were performed), 16 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; ... 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 (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.

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_003751.5): 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 GGAGGGG. 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 unrelated, ethnically matched, healthy control subjects (Indel 1: Del, 368/370; Indel 2: Del, 142/187; and Indel 3: Del, 125/161) (Table II) (Fig. 2), as well as in unrelated, ethnically matched, healthy control subjects (Indel 1: Del, 368/370; Indel 2: Del, 142/187; and Indel 3: Del, 125/161) (Table II) (Fig. 2). These 3 indels were 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).
strained 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 (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.

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 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 (rs37220112); and LOXHD1: c.5332-126C>T and c.1809+155G>A (both absent from dbSNP). The underline marks the variant nucleotide.
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, 20/28; p.R155Q, 23/28; 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.
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), 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=0.2708/1356; 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 rs1452787 (7/16), and 7 out of 8 cases carried the risk allele (G) of rs1348047 (7/16). All 8 cases carried the risk allele (C) of rs1452787 (8/16).
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’s son/116, and 3 of 20 healthy descendents 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 RefSeq; 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_eMaJPXBAmxFX5D7qTNSae4dT39), 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 p.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) genes in healthy controls with Chinese ancestry were rare in occidental populations (21), which may account for the absence of these minor alleles 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 homozygous 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 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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