Analysis of the Posterior Polymorphous Corneal Dystrophy 3 Gene, TCF8, in Late-Onset Fuchs Endothelial Corneal Dystrophy

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PURPOSE. Because the endothelial (posterior) corneal dystrophies share common pathologic features and result from primary endothelial dysfunction, it is possible that a proportion of them could be clinical manifestations of different mutations of the same gene. The aim of our study was to determine whether mutations of the TCF8 gene, recently implicated in posterior polymorphous dystrophy, may also play a role in the development of the more common Fuchs endothelial corneal dystrophy (FEDC).

METHODS. Genomic DNA was extracted from leukocytes of peripheral blood, and the nine exons of the TCF8 gene were PCR amplified and subjected to bidirectional sequencing and analysis. Samples from 74 unrelated Chinese patients (55 women, 19 men) with a diagnosis of late-onset FEDC and 93 age- and race-matched controls were studied.

RESULTS. The affected probands ranged in age from 52 to 91 years (mean age, 65.1 years); 8 had familial FEDC and 66 had sporadic FEDC. The authors found two mutations in the coding region of the TCF8 gene: a novel missense mutation in one patient c.2087A→G in exon 7 (Asn696Ser) and a silent mutation in exon 2 c.192T>G (D64D).

CONCLUSIONS. The identification of a novel missense mutation in only one of the patients implied that TCF8 does not play a significant role in the pathogenesis of FEDC in this Chinese population. (Invest Ophthalmol Vis Sci. 2008;49:184–188) DOI:10.1167/iovs.07-0847

Fuchs endothelial corneal dystrophy (FEDC; Mendelian Inheritance in Man [MIM] 136800) is a bilateral, often asymmetric, progressive disorder affecting the corneal endothelium1 in approximately 4% of the population older than 40 years.2 From onset, it typically progresses slowly due to impaired endothelial dysfunction, eventually leading to stromal edema and impaired vision.3 The only effective treatment for restoring vision is surgical replacement either by penetrating keratoplasty4 or, more recently, by a selective posterior lamella procedure such as deep lamellar endothelial keratoplasty (DLEK)5 or Descemet stripping endothelial keratoplasty (DSEK).6 FEDC is a common indication for allograft transplantation,7 and although penetrating keratoplasty is a successful transplantation procedure, visual rehabilitation is slow because of delayed stromal wound healing, suture-related complications, and surgically induced astigmatism.8 Some of these complications are now circumvented by newer procedures such as DLEK9 and DSEK,10 but there is still the requirement of allograft material and, hence, the risk for allograft rejection.

FEDC has been described in the literature as an autosomal dominant inherited condition.10–12 It may be classified as an early-onset variant (familial FEDC) or a late-onset (classic) variant. The latter may be subclassified as late-onset familial FEDC or the more common late-onset sporadic FEDC.13 During the past 10 years, some progress has been made in ascertaining the genetic basis of FEDC. The much rarer early-onset variant is caused by mutations in the COL8A2 gene.14,15 This gene encodes the α2 subtype of collagen VIII, a major component of Descemet membrane. Evidence that these mutations are involved in the more common late-onset form of FEDC is not convincing.15,14,16 Recently, two genetic loci, FCD117 and FCD218 were identified for late-onset FEDC. FCD1 was mapped in a single dominant inherited (white) family, and linkage analysis showed the presence of another locus, FCD2, in three other unrelated families. Until the genes located at these loci have been identified, candidate gene analysis poses a sensible method of identifying genes for this condition.

The association between posterior polymorphous dystrophy (PPCD; MIM 122000) and FEDC arises from their mutual clinical characteristics.24–26 The association between posterior polymorphous dystrophy (PPCD; MIM 122000) and FEDC arises from their mutual clinical characteristics.24–26 The association between posterior polymorphous dystrophy (PPCD; MIM 122000) and FEDC arises from their mutual clinical characteristics.24–26 The association between posterior polymorphous dystrophy (PPCD; MIM 122000) and FEDC arises from their mutual clinical characteristics.24–26 The association between posterior polymorphous dystrophy (PPCD; MIM 122000) and FEDC arises from their mutual clinical characteristics.24–26

In this study, we report, for the first time, the results of mutation analysis of the TCF8 gene in 74 unrelated patients with late-onset FEDC. We were unable to identify any of the

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previously identified pathogenic mutations associated with PPCD, but we did find a novel coding mutation in one patient that led to an amino acid sequence change in a conserved region of the protein. Hence, we conclude that TCF8 did not play a major role in the pathogenesis of FECD in this Chinese population.

**Materials and Methods**

The study protocol had the approval of the institutional review boards and ethics committees of the participating centers in Singapore and Hong Kong and was in compliance with the tenets of the Declaration of Helsinki.

**Patient Identification and DNA Collection and Preparation**

All patients underwent complete ophthalmic examination, including funduscopy, slit lamp examination, and confocal specular microscopy. FECD was diagnosed based on the presence of more than 5 mm confluent central corneal endothelial guttae in each eye (Kochmer grade 4 or higher), or it was confirmed histopathologically based on the findings of penetrating keratoplasty. A detailed history, including family history and duration of onset of symptoms, was recorded for all subjects. Written informed consent to participate in the research was obtained from each study participant after the nature and possible consequences of study enrollment were explained.

**Mutation Screening of TCF8 Gene in FECD**

Genomic DNA was extracted from leukocytes of the peripheral blood (Nucleon Blood Extraction Kit, Amersham, UK), and the coding regions of the TCF8 gene were amplified by PCR with custom-designed primers. Sequences of the oligonucleotide primers used are shown in Table 1. All primers were designed so that they would be positioned on intronic segments at least 60 nucleotides on either side of the intron-exon boundary to ensure complete reading of the exons.

**Table 1. PCR Primers and Conditions Used for TCF8 Gene Amplification**

<table>
<thead>
<tr>
<th>Exon/Size (bp)</th>
<th>Primer Sequences 5′→3′</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (96)</td>
<td>F: AAGCCGGCAGGGTTGCTGTA A: CTGACATGGGACCAAACAG</td>
<td>58.7</td>
</tr>
<tr>
<td>2 (201)</td>
<td>F: GTTACCTCTTCCTGCTGTGTT G: TCCTCTCCACTCAGCCTACC</td>
<td>59.3</td>
</tr>
<tr>
<td>3 (60)</td>
<td>F: GAGCAAGAGGAGTGGAGAAGA A: GACTGCTGCAATTTGAGGTCT</td>
<td>57.5</td>
</tr>
<tr>
<td>4 (162)</td>
<td>F: CTTAGTTGGAAGTGGCTTGTC R: CCTATAGTAGAGCAGGTTCC</td>
<td>58.9</td>
</tr>
<tr>
<td>5 (203)</td>
<td>F: GTGGTAGCAATATCTGTGG R: ACCGCTGACATAGACAGTCC</td>
<td>55.4</td>
</tr>
<tr>
<td>6 (106)</td>
<td>F: GACGCTGCTAATTGAGGTCT R: GCCATTTGGGAGAAAGGGCC</td>
<td>57.5</td>
</tr>
<tr>
<td>7 (181)</td>
<td>F: CATGGTCCTGAACAGAGTGAT R: TTGCGTCCTAGGGGCTGTA</td>
<td>60.4</td>
</tr>
<tr>
<td>Int 1: TGCACTGAAATCTGTCGACG</td>
<td>57.5</td>
<td></td>
</tr>
<tr>
<td>Int 2: GACCTAAGAGCAGCCTAGTCA</td>
<td>57.5</td>
<td></td>
</tr>
<tr>
<td>R: TTGGCTCTACGGGACTGATA</td>
<td>57.5</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; Int, internal primer.

* Exon 7 was amplified as a large fragment by flanking primers (F and R) and were sequenced with additional internal primers (Int 1 and Int 2) because of its large size.

**Results**

Seventy-four unrelated patients with a diagnosis of late-onset FECD were enrolled in the study. The affected probands ranged in age from 52 to 91 years, and their average age was 65.1 years (SD 12.2 years). There was no statistical difference in the demographic information between the FECD and the control group (mean age, 68.9 [SD 4.58] years; range, 62–87 years; P = 0.56). The study group consisted of 55 women and 19 men. All the patients were Chinese. Eight patients had a family history of FECD, and 66 had late-onset sporadic FECD. Of the eight probands with a family history, one had an affected nephew, two had an affected parent, two had an affected son, one had an affected sister, and one had two affected sons.

Screening for the TCF8 gene in the affected patients did not reveal any of the previous pathogenic mutations associated with posterior polymorphous dystrophy (c.2916_2917delTG, c.2087A > G, c.1578_1579insG). A novel missense mutation substituting aspartagine by serine at codon 696 (Asn696Ser) caused by a c.2087A > G mutation within exon 7 was identified in a sporadic FECD case of Chinese ethnicity (Fig. 1A). This mutation, located in a conserved region of the protein sequence (Fig. 1B), was not found in any of our 93 healthy controls. We did not find this gene to be polymorphic in the Chinese population. The other single nucleotide polymorphisms identified were a silent mutation (D64D) in exon 2 (c.192T > C) and sequence variations in intron 2 (IVS2–83T>C, IVS2–101G>A) and intron 6 (IVS6+79C>T).

We did not identify any of the coding changes in the COL8A2 gene that have been associated with either late-onset sporadic FECD (R304Q and R434H) or early-onset familial FECD (L450W and Q455K).

**TCF8 in Fuchs Endothelial Corneal Dystrophy**

PCR reactions were performed in a 50-μL mixture containing 10 mM Tris HCl (pH 8.9), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each deoxyribose nucleoside triphosphate, 25 pmol each primer (100 μM concentration), 0.2 μL DNA polymerase (HotstarTaq; Qiagen GmbH, Hilden, Germany), and 4 μL (50–100 ng) genomic DNA. Thermal cycling was performed (DNA Thermocycler 9700; Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation for 15 minutes at 95°C; 40 cycles of 95°C for 30 seconds, annealing variable temperature 55.4°C to 60.4°C (Table 1) for 30 seconds, and extension 72°C for 30 seconds; and final extension 72°C for 5 minutes.

PCR products were analyzed on an agarose gel to confirm the product size, and they were purified using PCR clean-up columns (GFX; Amersham, Piscataway, NJ). Sequence variations were identified by automated bidirectional sequencing (BigDye Terminator Mix version 3.1; Applied Biosystems) according to manufacturer’s protocols. Samples were denatured at 96°C for 1 minute and cycled 25 times at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Primers for sequence reactions were the same as those for the PCR reaction. Removal of unincorporated nucleotides and purification of PCR products (BigDye Terminator Mix version 3.1; Applied Biosystems) were performed by ethanol precipitation at 4°C. The samples were resuspended in highly deionized formamide (Hi-Di; Applied Biosystems) before sequencing. An automated DNA sequencer (ABI PRISM 3100; Applied Biosystems) was used. Sequence alterations were recorded based on TCF8 cDNA sequence, with +1 corresponding to the A of the ATG translation initiation codon in reference sequence NM_011546.

To identify and exclude known mutations causative of FECD, we screened our panel for coding changes in the COL8A2 gene that were associated with either late-onset sporadic FECD (R304Q and R434H) or early-onset familial FECD (L450W and Q455K). PCR fragments were generated using previously described primers and conditions.

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DISCUSSION

PPCD was mapped to a new region, PPCD3, on chromosome 10, and the gene has been recently cloned. The PPCD gene encodes for the two-handed zinc-finger homeodomain transcription factor 8 (TCF8; GenBank accession number NM_030751, which is also known as AREB6, BZP, Nil-2α, ZEB1, ZFHEP, Zfxh1A, and δEF-1). The structure of TCF8 allows for a wide range of functions because each zinc finger has different DNA-binding specificities and effects on gene expression. Information from the BodyMap database (http://bodymap.ims.u-tokyo.ac.jp/dbSNP) indicates that TCF8 transcripts are localized to the corneal endothelial layer, and mutations in TCF8 transcripts have been shown to produce a wide range of ocular phenotypes. These findings advocate its suitability as a possible candidate gene for corneal endothelial diseases.

Before this study was conducted, we were unaware of any published reports of the screening of the TCF8 gene in a large number of FECD patients from any ethnic origin. Identification of TCF8 as the PPCD3 gene was possible through screening of a large family. In that family, one member with FECD was found not to have any mutation within the TCF8 gene. In a screen of 74 FECD patients, we identified a single heterozygous TCF8 gene mutation (Asn696Ser) in one FECD patient that was absent in 93 age- and race-matched controls. Clinically, the patient had features consistent with FECD confirmed by slit lamp biomicroscopy and guttata confirmed by specular microscopy (Fig. 2); no clinical features of PPCD were observed. The Asn696Ser mutation was identified in exon 7 of TCF8. This exon seems to be a hot spot area of the TCF8 gene with respect to pathogenic mutations. In the original PPCD family, most mutations were identified in this region. More recently, four more novel mutations in PPCD patients have been identified within this exon.

The location of the Asn696Ser mutation in a conserved region of the protein, though outside the homeobox domain of TCF8, suggested that this site may be important to protein function. No structural information is yet available for this portion of the protein, making it difficult to assess the effect of this mutation on the structure of the protein. The Asn696 residue is in a low-complexity region of the protein, with a number of serine and proline residues that are likely to be phosphorylated. According to the phosphorylation prediction site at http://www.cbs.dtu.dk/services/NetPhos/, the substitution of asparagine with a serine residue also increases the phosphorylation potential of the Asn696 residue.
likelihood of phosphorylation at this site. It is unknown whether this increase in phosphorylation will have a profound effect on the TCF8 function as a transcription factor. All TCF8 mutations detected in PPCD patients thus far have been deletions, insertions, or nonsense coding changes predicted to truncate the protein. Given that these mutations will lead to loss of function of the protein, haploinsufficiency has been suggested as the disease mechanism associated with the PPCD phenotype. No TCF8 missense mutations have been identified in PPCD patients. The identification of our heterozygous missense mutation in a FECD patient, therefore, suggested a possible genotype-phenotype correlation for TCF8 mutations, with loss-of-function mutations associated with PPCD and missense mutations with FECD. This, however, remains an interesting hypothesis until the consequences of Asn696Ser mutation on TCF8 function and the mechanism of disease are identified through further functional analysis.

DNA variations in candidate genes of FECD, deemed pathogenic on the basis of their absence in control chromosomes, were later identified as common polymorphisms in other populations. The heterozygous missense mutation in the COL8A2 gene, R155Q, is one such example; it was described as a pathogenic mutation in a UK sample with late-onset FECD because it was not found in 184 control chromosomes. This mutation was later found in healthy Japanese controls, at the same frequency as in patients with FECD, indicating that it is likely to be a common polymorphism in the Japanese population. We cannot exclude the possibility of an increased prevalence of our mutation (Asn696Ser), identified in only one Chinese FECD sample, in other ethnic groups. This warrants the screening of TCF8 gene in FECD patients of other ethnic origins.

The incidence of affected family members of FECD probands may vary from 24.1% to 50%. Most of our patients did not show any preponderance to a familial trait (10.8% in our series), possibly because of several reasons. Ethnic genetic differences may exist in the penetrance of affected genes, as seen in the low incidence of FECD in Japanese patients. Krachmer et al. showed that the proportion of affected relatives increased, depending on the age and sex of the relatives. Many relatives of the patients we examined were young, which accounted for the low incidence of classified affected cases. We were also unable to examine all the elderly family members of some of our probands. Mutation screening has shown that the diagnosis of dominantly inherited dystrophy can occur in cases of spontaneous pathogenic mutations. We may find that many cases previously classified as sporadic may, in fact, be familial as these family members age.

The Asn696Ser mutation was identified in a patient with no recorded family history of FECD; this may represent a de novo pathogenic mutation likely to segregate in dominant fashion in subsequent generations. We were unable to demonstrate cosegregation of the mutation with disease in the family of this patient, primarily because of the nonavailability of parental DNA as a result of death and of the noncompliance of some family members for genetic analysis. We hope to follow up with family members of this patient for future clinical and DNA analyses. Mutations in the TCF8 gene have been associated with the expression of COL4A3 in the corneal endothelium of PPCD patients. We also hope to examine the expression of this protein when we are able to obtain a specimen of the patient’s endothelium after corneal transplantation.

In summary, we were able to identify a novel protein-altering mutation in the TCF8 gene in a Chinese patient with FECD that was not present in 93 control chromosomes analyzed. Our results suggest that TCF8 does not play a significant role in the pathogenesis of FECD in this Chinese population. We cannot, however, exclude the possibility of pathogenic changes within the promoter, intronic, or untranslated non-coding region of TCF8 playing a role in the pathogenesis of FECD, though there is no evidence to support this. Screening of the TCF8 gene in FECD patients of other ethnicities is required for a conclusion to be made of the pathogenic involvement of TCF8 in FECD.

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References


