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Identification of a novel *TCF4* isoform in human corneal endothelium

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Abstract

PURPOSE—Alternative splice isoforms of *TCF4*, a gene implicated in Fuchs corneal dystrophy, have been identified in multiple human tissues outside the eye. We aimed to identify the transcriptional profile of *TCF4* in corneal endothelium.

METHODS—We extracted RNA from donor corneal endothelium and performed rapid amplification of cDNA ends (RACE). We tested the expression pattern of one newly identified isoform (7b) in a panel of cDNA derived from multiple human tissues and included cDNA from corneal endothelial and RPE cell lines. To further delineate the differential expression of *TCF4* splice variants that span CTG18.1, we analyzed the expression of six alternative splice isoforms that are transcribed from either exon 2 or 3 in RNA extracted from corneal endothelium of three normal donors and a corneal endothelial cell line.

RESULTS—We identified 11 different isoforms in control corneal endothelial tissue, including one isoform (7b) not reported previously. This isoform is enriched in the corneal endothelium and placenta specifically compared to other tissues in a panel of human cDNA.

CONCLUSIONS—We demonstrate the complex expression profile of *TCF4* in human corneal endothelium, and reveal the expression of alternative splice variants of *TCF4*.

INTRODUCTION

The corneal endothelium is a single monolayer that lines the posterior cornea. It contributes barrier and pump functions through maintenance of tight junctions and solute pumps which contribute to corneal clarity. Loss of the corneal endothelium, such as occurs in Fuchs Corneal Dystrophy (FCD), results in corneal edema.^{1,2} This condition, the most frequent indication for endothelial keratoplasty performed in the United States,³ demonstrates early changes in the corneal endothelium and progresses to affect the entire cornea in later stages of the disease.

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The significance of *TCF4* in corneal endothelial function is evidenced by its disruption in FCD. Following the identification of four FCD loci, *FCD1-4*^{3–6} and causal mutations in *SLC4A11*, *TCF8*, *LOXHD1*, and *AGBL1*,^{6–9} *TCF4* was implicated in Fuchs dystrophy pathogenesis through a strong association with expansion of a trinucleotide (CTG) repeat^{10–13} a pattern replicated in multiple cohorts of varying ethnic backgrounds.^{14–16} However, next generation sequencing of the complete *TCF4* gene was unable to detect any variant that could explain the genetic etiology for FCD in these cases.¹⁷ Our recent analysis of a large cohort of late-onset FCD cases confirmed a strong association with *TCF4* trinucleotide repeat expansion in hundreds of cases and controls.¹⁸ Further functional studies have identified the sequestration of splicing proteins by the expansion of CTG repeats that results in missplicing of several *TCF4* transcripts and RNA toxicity in Fuchs dystrophy.^{19,20}

Given the discrete contribution of the *TCF4* locus to a late-onset disorder, we wanted to determine the transcriptional profile of this locus in the corneal endothelium.

METHODS

Donor cornea specimens

Three donor corneas (2 males, 1 female) above age of 60 were obtained from Keralink Eye Bank (Baltimore, MD), with a maximum of 24 hour death-to-preservation time, containing not less than 1800 corneal endothelial cells per mm². Corneas were transported in Optisol and Descemet membrane was removed for RNA isolation.

Genotyping of *CTG18.1* allele

The trinucleotide repeats in intron 3 of *TCF4* were determined by short tandem repeat (STR) and triplet repeat primed PCR assays as described.¹⁸ The PCR products were run on ABI3730 and fragment analysis performed with Gene Mapper software (Applied Biosystems Inc.).

RNA Isolation and 5' RACE

Corneal endothelium isolated by dissection of normal donor cornea was lysed and homogenized in 250µl of Trizol (Thermo Fisher Scientific). 750µl of Trizol was added post-homogenization and followed manufacturer's RNA extraction protocol. Finally, RNA was resuspended in 30µl of RNase-free water.

RNA from an immortalized corneal endothelial (CE) cell line²³ was extracted from 1×10^6 cells using Trizol and resuspended in 50µl of RNase-free water. To identify the isoforms of *TCF4*, RNA extracted (Trizol, Sigma) from the corneal endothelium of a postmortem eye (male, 70 years of age) obtained from the eye bank (Keralink, Baltimore, MD) with 14 hours death-to-preservation time and seven days from preservation to use. 5' RACE was performed with 0.5 micrograms of RNA as a starting material as per manufacturer's instructions using the GeneRacer Kit (Thermo Fisher Scientific, Waltham, MA). The cDNA obtained by RACE was used as a template for PCR reactions with a forward primer (provided in the kit) that binds the ligated 5' RNA adapter and *TCF4* specific reverse primers (5'-GTCTGCTGAGGAGTGTGATGGATAG-3' and 5'-

CATAGACTGAAGATGGCAAACCTGG-3') that span exon 11 and exon 8-9. The two independent PCR reactions were ligated with pCR4TOPO vector. The bacterial colonies were screened by digesting miniprep DNA with EcoRI, and the positive clones were sequenced with M13 primers by Sanger sequencing. The sequences were compared with the RefSeq and predicted isoforms in UCSC genome browser.

Analysis of *TCF4* isoforms

The expression of *TCF4* isoform 7b, exons 16-19 representing total *TCF4*, and *GAPDH* was tested in a panel of cDNA from multiple human tissues (Human MTC Panel I, Clontech, Cat. No. 636742), corneal endothelial and retinal pigment epithelial (RPE) cell lines; PCR primers are listed in Table S1. The following PCR cycle was used to amplify *TCF4* exons 16-19 and *GAPDH*: 95°C for 2min; and cycled 35 times at 95°C for 20sec; 58°C for 25sec; 68°C for 1min; 68°C for 2min as a final extension with LaTaq polymerase (Takara Bio). *TCF4* isoform 7b was amplified at 95°C for 2min; cycled 35 times at 95°C for 20sec; 64°C for 25sec; 68°C for 40sec; 68°C for 2min as final extension. Real-time PCR analysis to test the relative expression of isoform 7b was carried out with Power Sybr Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) in a reaction volume of 10µl and 250nM final concentration of each primer. The threshold C_T of *TCF4* isoform 7b was normalized with *GAPDH* tested with TaqMan assay (Table S1). The relative fold change was calculated based $2^{-(dC_T)}$ of normalized expression.

To analyze isoforms for which a predesigned TaqMan assay was not available, the Custom TaqMan assay design tool (Thermo Fisher Scientific, Waltham, MA) along with bioinformatics analyses (Custom Plus) were used. The 25bp sequence for the location of probes and assay IDs have been listed in Table S1. PCR reactions with appropriate TaqMan assay probes and primers mixed with cDNA and 2X TaqMan Gene Expression master mix (Thermo Fisher Scientific, Waltham, MA) were performed in duplicate. The dC_T of each *TCF4* isoform specific TaqMan assay was derived by normalizing with the average C_T of *GAPDH*. The relative expression (dCt) of each *TCF4* isoform was expressed as $2^{-(dC_T)}$. This value from one control donor sample was converted to a fold change of 1.0 and the fold change of other samples were calculated relative to 1.0.

Results

We aimed to identify the isoforms of *TCF4* that are expressed in corneal endothelium. Alternative splice isoforms of *TCF4* have been identified in multiple human tissues,²⁴ excluding those from the eye.

Therefore, we extracted RNA from donor corneal endothelium and performed rapid amplification of cDNA ends (RACE). Since the previously-reported alternatively spliced isoforms of *TCF4* differed significantly on the 5' exons, we used a forward primer that binds 5' RACE adapter and two reverse primers within exon 11 and exon 8-9 in independent PCR reactions (Methods). Subsequent to sequencing and analysis of 83 bacterial colonies resultant of the transformation of the multiple PCR products specific to *TCF4*, we identified a total of 11 different transcripts that included seven mutually exclusive 5' exons; some of them shared the same 5' exon but different downstream exons, as exemplified by isoforms

2a and 3a (Fig 1 and S1). Out of 11 isoforms, the first exon of the seven transcripts (2a, 2b, 3a, 3b, 3c, 3d, 4a, 7a, and 9a) is annotated as an exon according to the UCSC genome browser. We compared sequences of 5' ends of the isoforms of *TCF4* with that of the primer sequences reported by Sepp and colleagues,²⁴ and identified isoform 7c that was reported in this study. We also identified isoform 7b that is not annotated as an isoform in the UCSC Genome Browser and not reported in the literature.

We tested the expression pattern of isoform 7b in a panel of cDNA derived from multiple human tissues and included cDNA from corneal endothelial and RPE cell lines. RT-PCR of cDNA from these multiple sources suggested an elevated expression of *TCF4* isoform 7b in specifically the corneal endothelium as well as placenta (Fig 2A) whereas the amplification of exons 16-19 of *TCF4* suggestive of the total pool of *TCF4* transcripts showed equal expression similar to *GAPDH*. The differential expression of isoform 7b was further confirmed by real-time PCR analysis of the cDNA samples (Fig 2B) and suggested a similar pattern of expression as observed in Fig 2A. It is to be noted that the expression of this isoform is 10-fold higher in CE and compared to its nearest neighboring cell type (RPE) further suggesting the specificity of its expression.

We were intrigued by this complex alternative splicing and determined if all of the above-identified transcripts would be capable of producing a relevant protein. Analysis of these isoforms in UCSC Genome Browser suggested potential translation start sites within exon 2 and the first codon of exons 3 and 7. The first exons of isoforms 4a and 9a possess a translation start site as well and are in-frame with the first codons on exons 4 and 9. Therefore, analysis of all the above-identified transcripts suggest that they can be potentially translated into functional proteins. Moreover, analysis of possible open reading frames (ORFs) in the first exon isoform 7b revealed translation start site resulting in a protein sequence MVMAE followed by an in-frame first codon in exon 7 suggest yet another functional transcript of *TCF4*.

To further delineate the differential expression of *TCF4* splice variants that span CTG18.1, we analyzed the expression of six alternative splice isoforms that are transcribed from either exon 2 or 3 in RNA extracted from corneal endothelium of three normal donors and a corneal endothelial cell line immortalized with hTert. The length of CTG in the normal tissues and the cell lines assayed by TPPCR were in the normal range [(CTG)_{n<40}]. Custom TaqMan assays were designed to specifically distinguish between the six isoforms being tested (Table S2). We also included an additional TaqMan assay that detected the last two exons of *TCF4*, which helps detect the complete pool of *TCF4* transcript. We detected the expression of all six *TCF4* isoforms in all donor corneas (controls) and corneal endothelial cell line. The relative expression of the six isoforms in the controls suggested a similar level of expression of isoforms 2a and 3a followed by 3c and 3d and comparatively very low levels of 2b and 3b (Fig 3). The relative expression of the total pool of *TCF4* transcript assayed with a primer and probe that spans exons towards the 3' end of the transcripts common to most *TCF4* isoforms showed increased variability within normal donor control RNA (0.36-1.00 fold change) and corneal endothelial cell line (Control-4) expressed the least with a relative fold change of 0.23.

DISCUSSION

In this study, we have identified multiple isoforms of *TCF4* in the corneal endothelium, including a novel isoform that was expressed in normal control endothelium and its detection replicated in a corneal endothelial cell line.

TCF4 spans a 443kb genomic fragment on chromosome 18, and the full-length transcript (NM_001243226) is comprised of 20 coding exons. The diversity of *TCF4* expression emerges not only from the alternatively spliced transcripts with variable lengths, but also from generating multiple first exons that vary with each isoform, adding complexity to the expression.²⁴ Some of these isoforms share the same first exon but may be different with their composition of downstream exons. Sepp and colleagues have identified a range of *TCF4* isoforms and analyzed the expression of each of these isoforms in multiple human tissues, although not the eye, suggesting tissue specificity of certain isoforms.²⁴

We demonstrate that, consistent with what has been observed in other tissues, *TCF4* expression in the corneal endothelium follows a complex pattern. In this study we have identified *TCF4* isoforms from the corneal endothelium of a 70-year old donor while the susceptibility and progression of FCD starts typically in the fourth to fifth decade of life. It is possible that the expression of *TCF4* and the processing of its alternative splice variants may undergo modifications with age. We sequenced a total of 83 clones and terminated further screening of clones due to the redundancy of the identified transcripts. It is likely that some of the known transcripts were not sequenced with this approach. Investigations to probe RNA-seq results and analyze transcriptomic reads for isoforms in FCD specimens with known trinucleotide repeat expansion may be warranted to expand our understanding of the role of *TCF4* in the pathogenesis of FCD.

In conclusion, we have analyzed the transcriptional profile of *TCF4* in corneal endothelium, and show that multiple isoforms of *TCF4* are expressed therein. The complexity of *TCF4* expression requires further probing to understand the effect of the protein function and its role in FCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Identification of *TCF4* isoforms in the corneal endothelium. Schematic representation of partial *TCF4* gene with exons 1-11

The eleven isoforms of *TCF4* identified by 5' RACE of RNA from corneal endothelium are labeled with the number of the succeeding exon followed by an alphabet.

A **B**

Figure 2. Tissue-specific expression of *TCF4* isoform 7b

(A) RT-PCR performed on human cDNA panel from the indicated tissue sources representing the expression of *TCF4* and *GAPDH*. Amplification of exon 16-19 of *TCF4* represents the complete pool of *TCF4* transcripts whereas the top panel shows the expression of isoform 7b. CE (corneal endothelial cell line), Control (positive control provided in the kit), RPE (retinal pigment epithelial cell line). (B) Real-Time PCR analysis of isoform 7b representing the relative expression normalized to *GAPDH* in the samples tested in (A).

Figure 3. Differential expression of the six isoforms of *TCF4* in control RNA

The differential expression of the indicated isoforms of three controls donor corneal endothelium and one CE cell line were calculated with respect to the total *TCF4* expression of a control donor and the fold change represents the relative change compared to the expression level of isoform 2A in a control donor corneal endothelium. The horizontal line represents the mean of the fold change of the RNA samples tested.