

Effect of Trinucleotide Repeat Expansion on the Expression of *TCF4* mRNA in Fuchs' Endothelial Corneal Dystrophy

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PURPOSE. CTG trinucleotide repeat (TNR) expansion is frequently found in *transcription factor 4 (TCF4)* in Fuchs' endothelial corneal dystrophy (FECD), though the effect of TNR expansion on FECD pathophysiology remains unclear. The purpose of this study was to evaluate the effect of TNR expansion on *TCF4* expression in corneal endothelium of patients with FECD.

METHODS. Peripheral blood DNA and Descemet membrane with corneal endothelium were obtained from 203 German patients with FECD. The CTG TNR repeat length in *TCF4* was determined by short tandem repeat (STR) assays and Southern blotting using genomic DNA. Genotyping of rs613872 in *TCF4* was performed by PCR. *TCF4* mRNA levels in corneal endothelium were evaluated by quantitative PCR using three different probes. Control corneal endothelial samples were obtained from 35 non-FECD subjects.

RESULTS. The STR assay and Southern blotting showed that 162 of the 203 patients with FECD (80%) harbored CTG trinucleotide repeat lengths larger than 50. Quantitative PCR using all three probes demonstrated that *TCF4* mRNA is significantly upregulated in the corneal endothelium of patients with FECD, regardless of the presence of TNR expansion. However, the length of the TNR tended to show a positive correlation with *TCF4* expression level. No correlation was shown between the genotype of *TCF4* SNP, rs613872, and the level of *TCF4* expression.

CONCLUSIONS. Our findings showed that *TCF4* mRNA is upregulated in the corneal endothelium of patients with FECD. Further studies on the effects of *TCF4* upregulation on corneal endothelial cell function will aid in understanding the pathophysiology of FECD.

Keywords: Fuchs' endothelial corneal dystrophy, *TCF4*, trinucleotide repeat

Fuchs' endothelial corneal dystrophy (FECD) is characterized by a loss of corneal endothelial cells that is associated with a clinically phenotypical hallmark of guttae, which are excrescences of Descemet membrane formed by abnormal deposition of extracellular matrix. As the corneal endothelium maintains corneal transparency by regulating the degree of hydration in the corneal stroma, severe corneal endothelial cell loss due to FECD induces corneal haziness, resulting in vision loss.^{1,2}

FECD is a hereditary disease with a typically autosomal dominant pattern of inheritance, though the genetic basis is not fully elucidated.^{1,2} In 2012, Wieben and colleagues³ reported that expansion of a trinucleotide repeat (TNR) in the third

intron of *TCF4* was strongly associated with FECD, and the sensitivity and specificity for identifying FECD in their patient cohort was 79% and 96%, respectively.³ They postulated that FECD is a TNR disorder within the non-coding region, with similarities to Friedreich's ataxia, myotonic dystrophy type 1, and fragile X syndrome.³ Subsequently, other researchers have replicated this strong association in other independent cohorts such as Caucasian, Indian, Chinese, and Japanese.⁴⁻⁹

Simple sequence repeats exist throughout the human genome, but expansion of the TNR was discovered as causal genetic basis in patients with spinal and bulbar muscle atrophy in 1991.¹⁰ Since the first discovery of TNR expansion disease,



more than 20 disorders have been identified, all of which are neurologic or neuromuscular degenerations.¹¹⁻¹³ TNR expansion disease can be categorized into two groups: TNR expansion disease occurring in coding regions or TNR expansion disease occurring in noncoding regions. Diseases with CAG repeat expansion in coding regions induce polyglutamine tracts, which generate toxic proteins. By contrast, TNR expansion in noncoding regions in triplet repeat disorders can suppress or enhance gene transcription of the surrounding gene, produce antisense RNA, alter the RNA splicing pattern by sequestration of splicing factors, result in intron retention, and/or have repeat-associated non-ATG (RAN) translation, and eventually play an essential role in pathophysiology.¹³⁻¹⁹

Elucidation of the effect of TNR expansion on expression of the gene harboring the expansion is beneficial for understanding the pathophysiology of triplet repeat diseases; however, the effect of TNR expansion on the transcription of *TCF4* is not well elucidated in FECD. In the current study, we collected peripheral blood samples from 398 German patients with FECD, as well as the Descemet membrane and associated corneal endothelium when these patients underwent Descemet membrane endothelial keratoplasty (DMEK). We subsequently evaluated whether expression levels in the corneal endothelium were altered by TNR expansion in *TCF4* in samples derived from 203 of these FECD subjects.

MATERIALS AND METHODS

Ethics Statement

Institutional review board approvals for research involving human subjects were obtained from the Friedrich-Alexander University Erlangen-Nürnberg, Doshisha University, Kyoto Prefectural University of Medicine, and the Mayo Clinic. The human tissue was handled under the guidelines based on the tenets of the Declaration of Helsinki. Non-FECD human donor corneas were obtained from SightLife (Seattle, WA, USA).

Study Participants

The 398 patients with FECD who were scheduled for DMEK were recruited between October 2013 and September 2015 at the Department of Ophthalmology, Friedrich-Alexander University Erlangen-Nürnberg. After informed consent, peripheral blood samples were collected and Descemet membranes with corneal endothelial cells (CECs) were obtained during DMEK. For samples utilized in this study, genomic DNA obtained from peripheral blood required a DNA concentration of ≥ 3.0 $\mu\text{g}/\text{mL}$ and total RNA isolated from CECs required an RNA integrity number (RIN) ≥ 5.2 . Of the samples from the 398 patients, 203 fulfilled the quality criteria for genomic DNA and total RNA isolation. As controls, genomic DNA was isolated from donor corneas (corneal stroma) of 35 non-FECD subjects, and cDNA was synthesized from the CECs of the same 35 non-FECD subjects.

Preparation of Genomic DNA From Peripheral Blood and Donor Corneas

Genomic DNA was isolated from 200 μL of peripheral blood of FECD subjects or non-FECD donor corneal stromas with a commercial DNA kit (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany). Briefly, peripheral blood and stromas were lysed with protease K, the lysate was applied to spin columns (DNeasy Mini Spin Columns; Qiagen), and the columns were washed with ethanol and then with buffer to elute the genomic

DNA. The amount and quality of each isolated DNA sample were analyzed by UV spectrophotometry (NanoDrop; NanoDrop Technologies, Wilmington, DE, USA). As a control, genomic DNA was isolated from corneal stromas of the 35 non-FECD donor corneas.

Preparation of Total RNA and Synthesis of cDNA From Corneal Endothelium

An RNA extraction kit (RNeasy Mini Kit; Qiagen) was used to extract total RNA from the corneal endothelium obtained from the 203 FECD patients during DMEK. Briefly, Descemet membranes with corneal endothelium were lysed, and the lysate was applied to spin columns (Qiagen) with ethanol. The total RNA was eluted from columns, and cDNA was synthesized using a master mix (SuperScript VILO Master Mix; Thermo Fisher Scientific Inc., Waltham, MA, USA). As a control, Descemet membranes with corneal endothelium were peeled from the 35 non-FECD donor corneas, and cDNA was synthesized from the extracted total RNA.

Evaluation of CTG TNR Length

The CTG TNR length in *TCF4* was evaluated by a short tandem repeat (STR) assay, as described previously.⁴ Briefly, PCR was performed for the CTG repeat region using one fluorescently labeled primer. The size of the products was determined by capillary electrophoresis on a DNA analyzer (ABI 3730xl; Applied Biosystems, Foster City, CA). Samples that showed a single signal by STR were further analyzed by Southern blotting (described previously)³ using unamplified genomic DNA to determine if these samples carried a large repeat.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was performed with a real-time PCR system (QuantStudio 3; Applied Biosystems). All samples were analyzed in duplicate with a program of 95°C for 20 seconds and 40 cycles of 95°C for 1 second and 60°C for 20 seconds. For quantification, standard curves were run in parallel using serial dilutions (5-5³ copies) of cDNA of cultured immortalized human CECs, as described previously.²⁰ Ratios relative to *GAPDH* were calculated for normalization of gene expression levels. In this study, qPCR was based on a hydrolysis probe system (TaqMan; Thermo Fisher Scientific, Inc.). The hydrolysis probes (Thermo Fisher Scientific, Inc.) were for *TCF4*, Hs 00971338_m1; Hs00162613_m1; Hs00972432_m1; and predevelopment human *GAPDH* (Thermo Fisher Scientific, Inc.).

Genotyping

Genotyping of rs613872 in *TCF4* was performed by PCR using the following primers: forward primer-5'-actgtcaagcactaagcaaa gagg-3', reverse primer- 5'-cccagtaggggtgtgatgatg-3'. PCR reactions were carried out with Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) under the following conditions: 1 cycle of denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds. Upon completion of the 35 cycles, a final elongation was performed at 72°C for 5 minutes. The PCR products were separated by electrophoresis on 2% agarose gels in Tris-acetate buffer, stained with ethidium bromide, imaged with a luminescence imager (LAS4000S; Fuji Film, Tokyo, Japan), and then extracted from the agarose gels using the a commercial gel and a PCR cleanup system (Wizard SV Gel; Promega, Madison, WI, USA). A terminator cycle sequencing

TABLE. Demographic Data of Patients With FECD and non-FECD Controls

	CTG Repeat			P Value
	Control (n = 35)	No Expansion CTG < 50 (n = 41)	Expansion CTG ≥ 50 (n = 162)	
Age, y	58.0 ± 13.1 (21, 92)	68.9 ± 10.2 (42, 90)	69.8 ± 9.4 (34, 87)	<0.01*
Sex				<0.01†
Male	28	11	74	
Female	7	30	88	

Data represent the mean ± SD (minimum, maximum).

* P value of Student's *t*-test for case and control age comparisons.

† P value of Fisher exact test for case and control sex comparisons.

kit and a DNA sequencer (Taq DyeDeoxy and 373A DNA sequencer; Applied Biosystems) were used for direct sequencing.

Statistical Analysis

Statistical analysis was performed with the R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria). Possible confounding effects of sex and age of the subjects were assessed using the correlations between the case and control samples with the Student's *t* test or the Fisher exact test. Wilcoxon rank sum test was used to detect difference of expression of *TCF4* level between sexes. The statistical significance for the comparison of multiple sample sets was determined with the Steel-Dwass test. Spearman's rank correlation coefficient was used to determine any significant correlations between the CTG repeat length and expression of *TCF4* mRNA level. Results are expressed as mean ± SD. Statistical significance was defined as *P* value < 0.05.

RESULTS

TNR Expansion in Patients With FECD

We first determined the lengths of the CTG TNR expansions in *TCF4* by analyzing genomic DNA from peripheral blood samples of 203 patients with FECD and from control corneal stromas from 35 non-FECD subjects. The STR assays and Southern blotting demonstrated that all 35 non-FECD control subjects had CTG TNR lengths <50, whereas 162 of 203 patients with FECD (80%) harbored CTG TNR lengths ≥50. The remaining 41 patients with FECD (20%) had TNR lengths <50 (Table). The mean subject age was 58.0 ± 13.3 (range, 21 to 92) years in the control group, 68.9 ± 10.3 (range, 42 to 90) in the FECD group without TNR expansion, and 69.8 ± 9.4 (range, 34 to 87) in the FECD group with TNR expansion. Consistent with previous reports,² the female ratio was higher

in the FECD subjects (11 males and 30 females with CTG < 50, and 74 males and 88 females with CTG ≥ 50).

Expression Levels of *TCF4* in the Corneal Endothelium of Patients With FECD

We used qPCR to evaluate the expression level of *TCF4* in the corneal endothelium of patients with FECD. As *TCF4* has multiple alternatively spliced isoforms, we used three probes (Thermo Fisher Scientific, Inc.) for qPCR that are all contained within the canonical *TCF4* transcript (Refseq: NM_001083962) that encodes TCF4-B (Fig. 1). The expression level of *TCF4* determined by Hs00971338 was significantly higher in patients with FECD with or without CTG TNR expansion >50 than in non-FECD subjects (control: 7.2 ± 4.8; FECD without expansion: 22.3 ± 29.9; and FECD without expansion: 47.4 ± 46.3; *P* < 0.01). The *TCF4* level was also significantly higher in FECD with expansion than in FECD without expansion (*P* < 0.01; Fig. 2A). The expression level of *TCF4* determined by Hs00162613 was significantly higher in patients with FECD with or without CTG TNR expansion >50 than in non-FECD subjects (control: 2.2 ± 1.3, FECD without expansion: 4.5 ± 1.6, and FECD without expansion: 4.3 ± 1.3; *P* < 0.01); however, no statistically significant difference was observed in *TCF4* levels between FECD with expansion and FECD without expansion (Fig. 2B). The expression level of *TCF4* determined by Hs00972432 was higher in FECD with or without CTG TNR expansion >50 than in non-FECD subjects (control: 2.8 ± 3.1, FECD without expansion: 6.7 ± 4.9, and FECD without expansion: 10.8 ± 8.2), but only the *TCF4* level in FECD with expansion showed statistical significance when compared with the control subjects (*P* < 0.01). Consistent with the *TCF4* level determined by Hs00971338, the *TCF4* level determined by Hs00972432 was significantly higher in FECD with expansion than in FECD without expansion (*P* < 0.01; Fig. 2C).

Effect of CTG Repeat Length or Genotype on *TCF4* Expression Level

We evaluated whether the CTG TNR length or the genotype of the single nucleotide polymorphism (SNP) rs613872 in *TCF4* correlated with *TCF4* expression levels in FECD. Spearman's correlation coefficient by rank test revealed a weak positive correlation between CTG TNR length in genomic DNA from peripheral blood and the expression level of *TCF4* in the corneal endothelium determined by Hs00971338 ($\rho = 0.24$, *P* < 0.01) and Hs00972432 ($\rho = 0.22$, *P* < 0.01; Figs. 3A, 3C). Conversely, the CTG TNR length did not show significant correlation with expression level of *TCF4* determined by Hs00162613 (Fig. 3B). We also evaluated the effect of other potent confounders, including sex and age, on the expression level of *TCF4*. The expression level of *TCF4* (normalized by expression level of *GAPDH*) in the corneal endothelium, determined by Hs00971338, was 39.1 ± 49.7 in males and

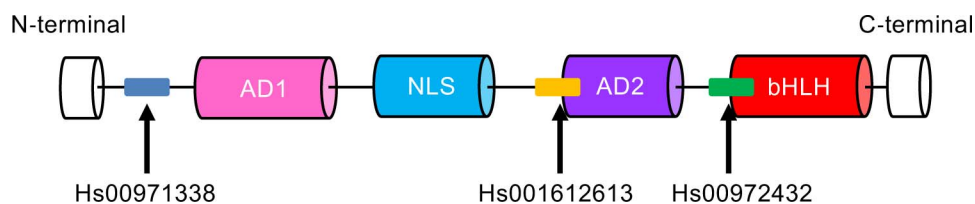


FIGURE 1. Schematic image of *TCF4* and the genomic regions recognized by hydrolysis probes. Hs00971338 recognized the N-terminal side of active domain 1 (AD1), Hs00162613 recognized the N-terminal side of active domain 2 (AD2), and Hs00972432 recognized the N-terminal side of basic helix-loop-helix (bHLH) of *TCF4*.

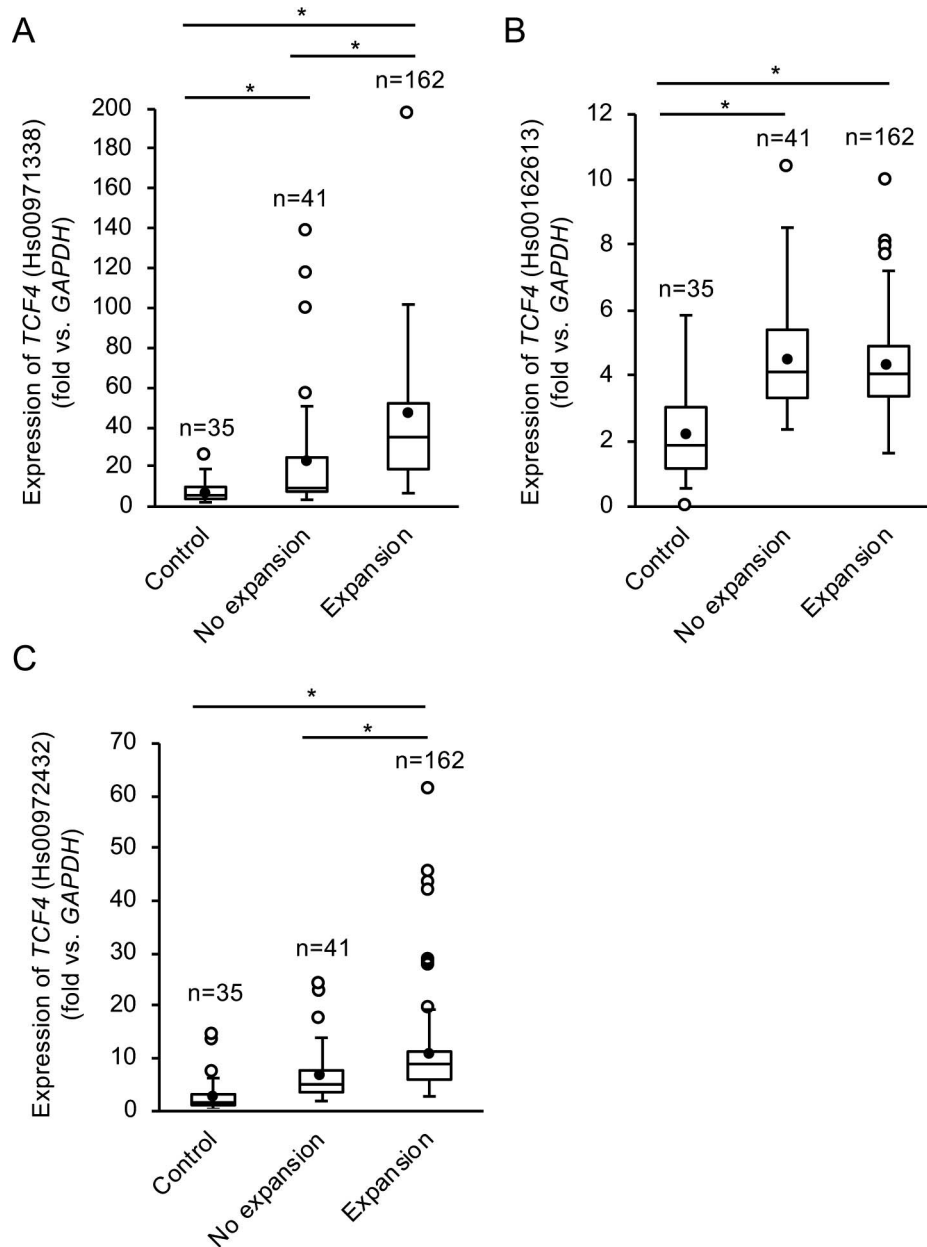


FIGURE 2. Expression of *TCF4* mRNA in corneal endothelium of patients with FECD. **(A)** Total RNA was extracted from the corneal endothelium of the 203 patients with FECD and 35 non-FECD subjects, and cDNA was synthesized. The expression level of *TCF4* determined by Hs00971338 was significantly higher in FECD with or without a CTG expansion of a trinucleotide repeat (TNR) expansion larger than 50 when compared with non-FECD subjects. In addition, *TCF4* level was significantly higher in FECD with expansion than in FECD without expansion. * $P < 0.01$. **(B)** Expression level of *TCF4* determined by Hs00162613 was significantly higher in FECD with or without a CTG TNR expansion larger than 50 than in non-FECD subjects. * $P < 0.01$. **(C)** Expression level of *TCF4* determined by Hs00972432 was higher in FECD with or without CTG-TNR expansion larger than 50 when compared to non-FECD subjects, but *TCF4* level showed a statistically significant difference only in FECD with expansion when compared to the control subjects. The *TCF4* level determined by Hs00972432 was significantly higher in FECD with expansion than in FECD without expansion. * $P < 0.01$. The statistical significance was determined with the Steel-Dwass test.

35.4 ± 36.2 in females ($P = 0.77$). Likewise, expression level of *TCF4* was similar level between sexes, at 3.9 ± 1.6 in males and 4.1 ± 1.4 in females, determined by Hs00162613 ($P = 0.29$), and 8.1 ± 6.7 in males and 9.7 ± 8.6 in females ($P = 0.10$), determined by Hs00972432. Since Wirgenes and colleagues previously reported that age showed a very weak positive correlation with *TCF4* mRNA levels in blood,²¹ we also evaluated the correlation between age and the expression level of *TCF4* in the corneal endothelium. Spearman's correlation coefficient by rank test did not show a significant correlation

with the expression level of *TCF4*, determined by Hs00162613 and Hs00972432, though the expression level determined by Hs00971338 revealed a weak positive correlation with marginal statistical significance (Supplementary Fig. S1).

Consistent with previous reports confirming the "G" risk allele in rs613872,²² genotyping results showed that the genotype of this *TCF4* SNP was TT:30 (15%), TG:140 (69%), and GG:33 (16%) in German FECD cases, while it was TT:27 (77%), TG:7 (20%), and GG:1 (3%) in the control subjects ($P < 0.01$). No correlation was found between the genotype of *TCF4*

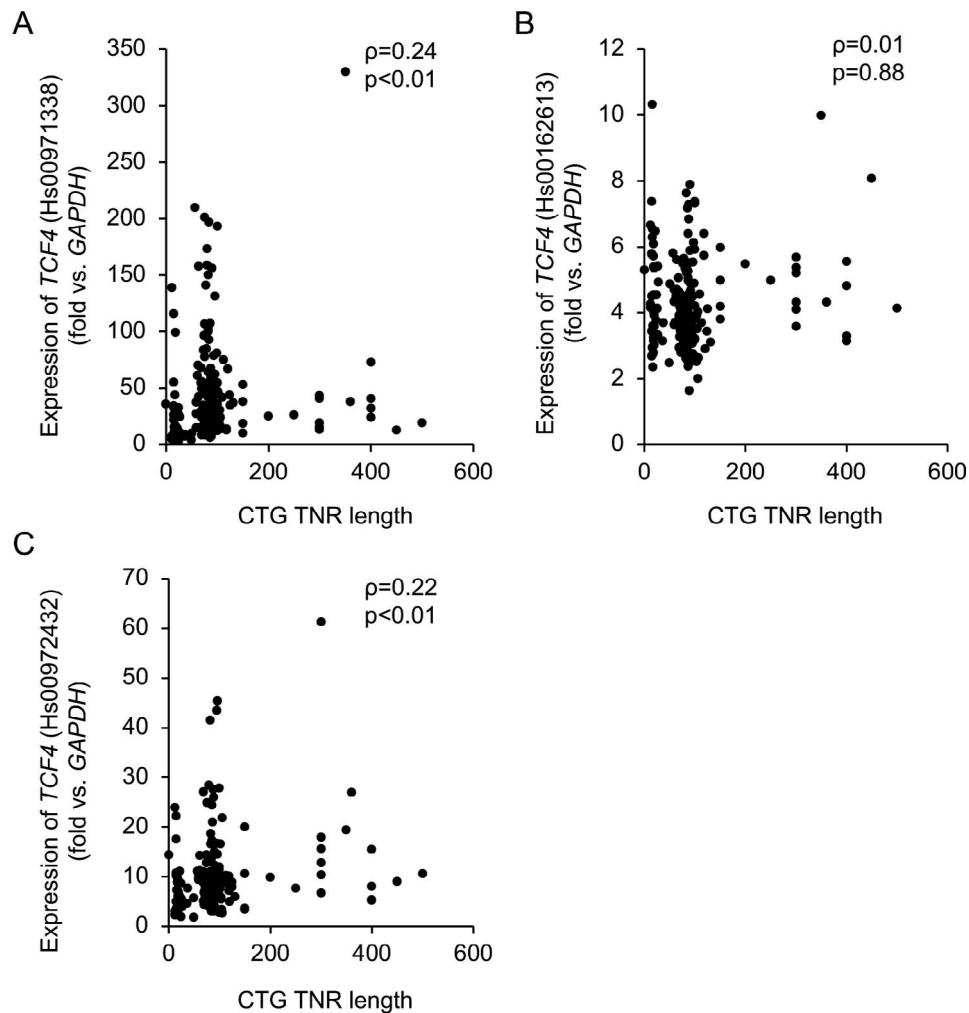


FIGURE 3. Correlation between CTG-TNR length and expression of *TCF4* mRNA. (A) CTG TNR length was evaluated by analyzing the genomic DNA from peripheral blood and expression levels were plotted for *TCF4* mRNA in the corneal endothelium of patients with FECD. Spearman's correlation coefficient by rank test revealed a weak positive correlation between CTG-TNR length and expression level of *TCF4* determined by Hs00971338 ($\rho = 0.24$, $P < 0.01$). (B) CTG-TNR length did not show a significant correlation with the expression level of *TCF4* determined by Hs00162613 ($\rho = 0.01$, $P = 0.884$). (C) CTG-TNR length showed a weak positive correlation with the expression level of *TCF4* determined by Hs00972432 ($\rho = 0.22$, $P < 0.01$). Correlation was determined by a rank test using Spearman's correlation coefficient.

SNP rs613872 and expression level of *TCF4* determined by all three probes (Figs. 4A–C).

DISCUSSION

Diseases with TNR expansion in non-coding regions typically cause a loss of gene function or toxic effects at the mRNA level, but their pathophysiology varies depending on the type and the location of the TNR.^{12,13} For example, myotonic dystrophy 1 harbors CTG repeats within the 3' UTR in the myotonic dystrophy protein kinase (*DMPK*) gene. These CTG repeats in *DMPK* gene lead to the formation of transcript aggregates in the nucleus (referred to as RNA foci) that sequester RNA-binding proteins, resulting in a spliceopathy of downstream effector genes.²³ In Friedreich's ataxia, a GAA repeat expansion in the first intron of the frataxin gene suppresses the frataxin mRNA and protein expression, leading to impaired mitochondrial iron transport.^{24–26} Likewise, a CGG repeat expansion in the 5' UTR of the fragile X mental retardation gene (*FMR1*)²⁷ represses transcription by CpG hypermethylation of the *FMR1* promoter.²⁸ However, suppression of gene expression by repeats is not always identified among triplet repeat expansion

diseases. In spinocerebellar ataxia type 12, overexpression of the *PPP2R2B* gene²⁹ is linked to the length of a CAG TNR expansion, suggesting this is involved in the underlying pathophysiology.³⁰ These reports encouraged us to conduct the current study to answer the simple but fundamental question: “Does CTG repeat expansion in FECD alter the expression of *TCF4* mRNA level in the corneal endothelium?”

Studies reporting the expression of *TCF4* in patients with FECD have resulted in contrasting conclusions. For example, Mootha and colleagues³¹ reported no alterations in *TCF4* levels in the corneal endothelium of FECD patients when compared to control subjects. Likewise, Oldak and colleagues³² analyzed the corneal endothelium from 40 FECD cases and 23 control subjects, and showed similar expression levels of *TCF4* in both groups and stable *TCF4* levels regardless of the risk allele of rs613872. By contrast, Foja and colleagues⁹ reported decreased *TCF4* levels in the corneal endothelium of FECD patients in their examination of 6 FECD patients with TNR expansion >50 and 5 controls with TNR expansion <50.

In the current study, we demonstrated a significantly higher expression of *TCF4* at the mRNA level in the corneal endothelium of patients with FECD compared to normal

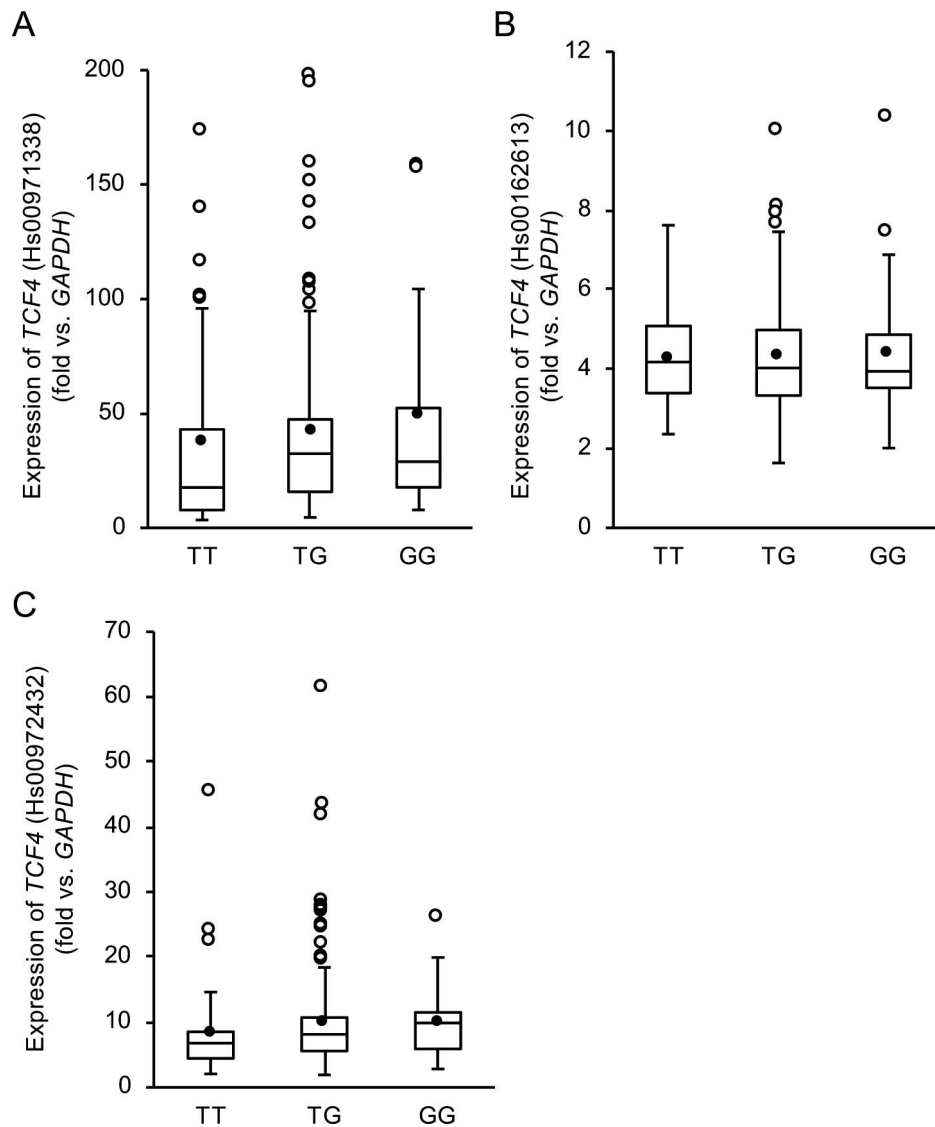


FIGURE 4. Correlation between the genotype of *TCF4* SNP, rs613872, and *TCF4* expression level. Genotyping of rs613872 in *TCF4* was performed by PCR. No statistically significant correlation was revealed by the Steel-Dwass test between the genotype of *TCF4* SNP rs613872 and the expression level of *TCF4* determined by three probes: (A) Hs00971338, (B) Hs00162613, and (C) Hs00972432.

controls. However, *TCF4* has multiple alternatively spliced variants and the variants expressed in corneal endothelium have not been elucidated. Therefore, we examined the splicing variants of *TCF4* at three different loci that correlated with conserved motifs in the TCF4 protein sequence, and we found upregulation of *TCF4* transcription in the patients with FECD, even in the patients without TNR expansion. Interestingly, the CTG repeat length, but not the presence of risk allele G in rs613872, tended to correlate with the *TCF4* mRNA level. Further studies will be necessary to address whether expansion upregulates *TCF4* transcription and if the increase in *TCF4* transcription has toxic effects in the corneal endothelium.

TCF4 encodes numerous transcripts, but the probeset Hs00971338, located proximally (5') to the TNR, will only amplify the canonical *TCF4* transcript encoding TCF4-B. Interestingly, the qPCR results showed that levels of the TCF4-B transcript are significantly elevated in FECD cases with repeat expansions when compared to cases without an expansion and controls. This finding may indicate a differential

effect of the FECD-associated TNR in *TCF4* on the expression of the different *TCF4* transcripts in CECs.

TCF4 is a basic helix-loop-helix (bHLH) transcription factor that plays an important role in various developmental processes.³³ One large-scale genome-wide association study (GWAS) identified *TCF4* as the first gene to show a strong association with schizophrenia.³⁴ *TCF4* variants have also shown associations with primary sclerosing cholangitis and Pitt-Hopkins syndrome, as well as with FECD.³³ The role of *TCF4* in the context of disease pathogenesis still remains unclear, but involvement in the regulation of the epithelial-mesenchymal transition (EMT) was described in certain cell types. For instance, the overexpression of *TCF4* in kidney cells induced EMT by upregulation of EMT-related markers.³⁵ In human neuroblastoma cells, genome-wide expression profiling showed that knockdown of *TCF4* altered multiple signaling pathways related to the EMT and to transforming growth factor- β (TGF- β), an essential mediator of the EMT.³⁶ We established a cell model from patients with FECD and showed that TGF- β upregulates EMT-related genes, thereby inducing

excessive extracellular matrix (ECM) protein production, which is a hallmark of FECD.²⁰ More recently, we reported that upregulation of TGF- β signaling in FECD induces a chronic overload of ECM proteins to the endoplasmic reticulum in an immortalized cell model of FECD.^{37,38} The overload of ECM protein results in accumulation of unfolded protein and triggers the intrinsic apoptotic pathway through the unfolded protein response.³⁷ These findings suggested that upregulation of *TCF4* can be linked to the underlying pathology of FECD, including upregulation of TGF- β signaling and chronic overloading of ECM proteins in the endoplasmic reticulum.

In conclusion, we have demonstrated that *TCF4* mRNA is upregulated in patients with FECD, regardless of the presence or absence of TNR expansion, but the length of the TNR in cases with expansion tended to correlate with the *TCF4* expression level. These findings suggest the possibility that upregulation of *TCF4* plays a central role in FECD. However, further studies are needed to identify the "missing link" between the increases in *TCF4* mRNA and the proposed pathophysiology that appears to involve upregulation of TGF- β signaling, disruption of protein loading in the endoplasmic reticulum.

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