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ARTICLE

Trinucleotide repeat expansion in the transcription factor 4 (*TCF4*) gene in Thai patients with Fuchs endothelial corneal dystrophy

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Abstract

Purpose To evaluate the association of single nucleotide polymorphisms (SNPs) and the intronic expansion of a trinucleotide repeat (TNR) in the *TCF4* gene with Fuchs endothelial corneal dystrophy (FECD) in a Thai population.

Methods In total, 54 Thai FECD patients and 54 controls were recruited for the study. Five SNPs (rs613872, rs2123392, rs17089887, rs1452787, and rs1348047), previously reported to be associated with FECD, were genotyped by direct sequencing. The repeat length was determined by direct sequencing of PCR-amplified DNA (a short tandem repeat; STR assay) and by triplet repeat primed PCR (TP-PCR).

Results Only one of the 54 patients with FECD harboured rs613872 (1.9%). Four SNPs (rs2123392, rs17089887, rs1452787, and rs1348047), which are not rare polymorphisms in the Thai population, were found in approximately half of the patients. Of the 54 patients, 21 (1 homozygous and 20 heterozygous patients; 39%) harboured a TNR ≥ 40, while 33 patients (61%) harboured a TNR < 40.

Conclusions The association of TNR expansion in *TCF4* with FECD is shown for the first time in the Thai population. The intronic TNR expansion identified in various ethnic groups underlines the importance of expansion as a potent pathophysiological cause of FECD.

Introduction

Fuchs endothelial corneal dystrophy (FECD) is a bilateral corneal disease characterised by a progressive loss of corneal endothelial cells that typically begins after the age of 40 [1, 2]. The corneal endothelium is responsible for maintaining corneal transparency by regulating the amount of water in the corneal stroma; therefore, a severe loss of corneal endothelial cells results in vision loss due to corneal haziness.

FECD initially showed an autosomal dominant inheritance pattern with variable penetrance and expressivity in investigations of large families conducted in the 1970s and 1980s [3–5]. Early screening studies for functional candidate genes and investigations of causal genes of other corneal endothelial dystrophies identified four genes (*SLCA411*, *TCF8*, *LOXHD1*, and *AGBL1*) that were associated with late-onset

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FECD (the typical form of FECD) [6–11]. However, these genes were not frequently encountered in the general population of FECD patients; therefore, researchers have continued searching for a more common genetic cause.

In 2010, a genome-wide association study (GWAS) reported a significant association between the intronic single nucleotide polymorphism (SNP) in the transcription factor 4 (*TCF4*) and late-onset FECD [12]. Subsequently, Wieben et al. reported a strong association between FECD and a cytosine–thymine–guanine (CTG) repeat expansion in the non-coding region of *TCF4* and found a sensitivity and specificity of ≥50 repeats of 79% and 96%, respectively, for identifying FECD patients in their cohort [13]. Subsequent studies have replicated these novel findings in various populations, although the frequency of the repeat expansion in FECD tends to be lower in non-Caucasian (i.e., Chinese, Indian, and Japanese) cohorts [14–19]. These studies have provided evidence to support the concept, proposed by Wieben et al. [13], that FECD is a trinucleotide repeat (TNR) expansion disorder.

This common genetic basis of FECD has revealed a novel therapeutic target for the development of new therapies, such as pharmacotherapy and gene therapy. However, the TNR expansion is not always identified in patients with FECD, probably due to the heterogeneity of this disease. Therefore, we were motivated to investigate the *TCF4* variants of FECD in a patient cohort from Thailand. In the current study, we examined the previously reported SNPs found in various ethnic groups, as well as the CTG repeat expansion, to provide baseline genetic data for FECD in Thailand.

Materials and methods

Ethics statement

The human tissues used in this study were handled under the guidelines based on the ethical principles of the Declaration of Helsinki. This study was performed according to a protocol approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 543/55), Doshisha University, and Kyoto Prefectural University of Medicine. Informed consent was obtained from the patients with FECD who participated in the study.

Preparation of genomic DNA

The 54 patients with FECD and 54 control subjects were recruited between February 2013 and September 2018 at King Chulalongkorn Memorial Hospital. All FECD cases were examined with slit-lamp biomicroscopy and diagnosed by cornea specialists. Noncontact specular microscopy (CEM530; Nidek Co., Ltd., Aichi, Japan) was also used to confirm guttae. The inclusion criteria for the FECD cases were: (1) Presence of bilateral guttae, with or without corneal oedema; or (2) Presence of unilateral guttae, with or without corneal oedema, and the presence of bullous keratopathy in the fellow eye; as well as (3) Absence of history of endotheliitis, previous peripheral iridotomy, cataract surgery, or any intraocular surgery; and (4) Presence of a minimum grade of 1 (12 or more central nonconfluent guttae), based on noncontact specular microscopy grading, as proposed by Krachmer et al. [4], or histopathologic evidence of Descemet's membrane thickening and guttae for patients who had undergone keratoplasty. The control subjects were selected based on the following criteria: (1) >60 years old, (2) no guttae observed by slit-lamp microscopy, and (3) absence of previous peripheral iridotomy, cataract surgery, or any intraocular surgery. After obtaining informed consent, peripheral blood leucocytes were obtained from the patients and genomic DNA was extracted using a Puregene blood kit (Qiagen, Hilden, Germany). The amount and quality of DNA were analysed by UV spectrophotometry (NanoDrop; NanoDrop Technologies, DE).

Measurement of SNPs

The primers used for PCR are listed in Supplementary Table 1. PCR reactions were performed with Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) under the following conditions: denaturation at 94 °C for 30 s, 32–35 cycles of annealing at 54–60 °C for 30 s, and elongation at 72 °C for 30 s. The PCR products were separated by electrophoresis on 2% agarose gels in Tris-acetate buffer, stained with ethidium bromide, and analysed with an LAS4000S luminescence imager (Fuji Film, Tokyo, Japan). The PCR products were extracted from the agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Direct sequencing was carried out with a TaqDyeDeoxy Terminator Cycle Sequencing Kit and a SeqStudio Genetic Analyzer (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions.

Evaluation of the CTG repeat length

PCR reactions were performed with Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) under the following conditions: denaturation at 94 °C for 30 s, 32–35 cycles of annealing at 54–60 °C for 30 s, and elongation at 72 °C for 30 s. Primers used for PCR were a sense primer (5′-CA GATGAGTTTGGTGTAAGATGCATTTG-3′) and an anti-sense primer (5′-ACAAGCAGAAAGGGGGCTGCA AAGC-3′). The PCR products were separated by electrophoresis on 2% agarose gels in Tris-acetate buffer, stained

with ethidium bromide, and analysed with an LAS4000S luminescence imager (Fuji Film, Tokyo, Japan). The 305-bp bands were then extracted from the agarose gels using the Wizard SV Gel and PCR Clean-Up System. Direct sequencing was performed with a TaqDyeDeoxy Terminator Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Triplet repeat primed PCR (TP-PCR) was also performed to detect the CTG repeat lengths, as previously reported [14]. Briefly, the PCR was performed with the following conditions: 20 ng of genomic DNA, 0.2 µmol/L of locus-specific (forward) fluorescent primer P1, 0.006 µmol/L of repeat-specific (reverse) primer P4, 0.2 µmol/L of primer P3, 200 µmol/L dNTPs, 1 mmol/L $MgCl_2$, and 1.25 U of TksGflex DNA Polymerase (Takara Bio Inc., Otsu, Japan). The primers were those previously reported by Mootha and colleagues. PCR was performed under the following conditions: initial denaturation of 9 min at 95 °C, 10 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and elongation at 68 °C for 4 min, followed by 25 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 45 s, elongation at 68 °C for 4 min 15 s, and final elongation at 68 °C for 10 min. The TP-PCR products were mixed with GeneScan 500 LIZ dye Size Standard (Applied Biosystems, Foster City, CA), subjected to capillary electrophoresis on an ABI 3730 Genetic Analyzer, and analysed with a SeqStudio Genetic Analyzer.

Results

The mean age of the Thai patients with FECD was 62.7 ± 13.6 years (range, 21–85 years) (10 male and 44 female) and the mean age of controls was 68.2 ± 6.5 years (range, 60–87 years) (12 male and 42 female). No statistically significant differences were observed in the subject age and male-to-female ratio between the FECD and control groups. The rs613872, rs2123392, rs17089887, rs1452787, and rs1348047 SNPs of *TCF4*, which were previously reported to be correlated with FECD, were evaluated in Thai patients with FECD. The results revealed that these patients differed from the previously reported Caucasian cohorts as they rarely harboured rs613872 (2/54). By contrast, the other four SNPs (rs2123392, rs17089887, rs1452787, and rs1348047) were found in approximately half of the Thai patients. The frequencies of these SNPs were not statistically different between the FECD cases and the controls, and they were consistent with those in East Asian (EAS) populations according to the database of the 1000 Genomes Project Phase 3, based on Ensembl v80 GRCh37 (Table 1).

Direct sequencing and TP-PCR demonstrated that no control subjects harboured a CTG trinucleotide repeat (TNR) higher than 40 repeats. Conversely, 21 of the 54 patients (39%; 1 homozygous and 20 heterozygous) harboured CTG TNR lengths greater than 40, while 33 patients of the 54 patients (61%) did not harbour TNR lengths greater than 40 ($P < 0.001$). The mean age of the 5 male and 16 female patients harbouring ≥40 CTG TNR was 65.3 ± 9.6 years (range, 39–78 years), while the mean age of the 5 male and 28 female patients harbouring <40 CTG TNR was 61.1 ± 15.6 years (range, 21–85 years). No statistically significant difference was observed in either age or gender among control subjects, patients with or without CTG TNR expansion higher than 40 repeats (Table 2). In agreement with the previous study, the distribution of the TNR length was bimodal, and fourteen patients harboured ≥100 CTG TNR (Fig. 1). One patient with FECD (a 66-year-old female) was homozygous for the CTG TNR expansion, and she was considered to be a Krachmer grade 2 and exhibited a transparent cornea. Clinical manifestations of the representative Thai patients with FECD harbouring CTG TNR < 40 and ≥40 are shown in Supplementary Fig. 1.

Discussion

Baratz et al. used GWAS to investigate 280 FECD cases and 410 controls and identified the minor allele (*G*) in rs613872 more frequently in the FECD cases ($G = 0.40$) than in non-FECD controls ($G = 0.15$); the odds ratios for this minor allele were 5.5 and 30 for being heterozygous and homozygous, respectively [12]. Subsequent reports in independent cohorts replicated the association between

Table 1 Genotyping of the reported single nucleotide polymorphisms (SNPs) in *TCF4*

Chr position	rs ID	Mutation	1000 Genomes (EUR) $n = 1006$	1000 Genomes (EAS) $n = 1008$	Control $n = 54$	Case $n = 54$	*P* value
18:55543071	rs613872	T > G	G = 0.1769	G = 0.0040	0/108	2/108	0.155
18:55547634	rs2123392	T > C	C = 0.3588	C = 0.3611	45/108	41/108	0.578
18:55541025	rs17089887	T > C	C = 0.0885	C = 0.4395	46/108	47/108	0.891
18:55539976	rs1452787	A > G	G = 0.2972	G = 0.3591	45/108	41/108	0.578
18:55382827	rs1348047	G > T	T = 0.2734	T = 0.4950	55/108	47/108	0.276

Table 2 Demographic data of subjects with Fuchs endothelial corneal dystrophy (FECD) with or without cytosine-thymine-guanine (CTG) trinucleotide repeat (TNR) expansion

	Control ($n = 54$)	No expansion CTG < 40 ($n = 33$)	Expansion CTG ≥ 40 ($n = 21$)	*P* value
Age, years				0.012[a]
	68.2 ± 6.5 (60,87)	61.1 ± 15.6 (21,85)	65.3 ± 9.6 (39,78)	
Sex				0.663[b]
Male	12	5	5	
Female	42	28	16	

Data represent the mean ± SD (minimum, maximum)

[a]*P* value of analysis of variance (ANOVA)

[b]*P* value of Chi-square test

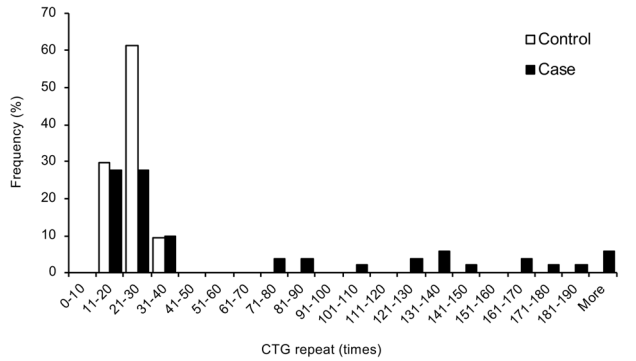

Fig. 1 Frequency histogram of the cytosine–thymine–guanine (CTG) repeat length. The CTG trinucleotide repeat (TNR) length of the longest allele determined by direct sequencing and triplet repeat primed PCR (TP-PCR) in 54 subjects with Fuchs endothelial corneal dystrophy (FECD) and 54 non-FECD Thai controls is shown as a histogram. The distribution of TNR length in FECD cases was bimodal, and 14 patients harboured ≥100 CTG repeats

rs613872 and FECD. In addition to rs613872, other SNPs were identified in some populations, especially from non-Caucasian cohorts, indicating ethnic variation of SNPs in *TCF4* [16, 20–23]. No previous study has examined SNPs in *TCF4* in FECD in Thailand; therefore, we first looked for the reported SNPs in our Thai cohort. We found that only two among the 54 FECD subjects were polymorphic with regard to rs613872. Four other reported SNPs (rs2123392, rs17089887, rs1452787, and rs1348047) were found in our cases, but these SNPs are not rare polymorphisms, according to our findings in the controls and the 1000 Genomes Project database. We found that none of the reported SNPs in *TCF4* showed an association with FECD in the Thai population.

The first report showing TNR expansion in *TCF4*, conducted in a Caucasian population, revealed that 52 of 66 (79%) FECD cases harboured CTG TNR ≥ 50, whereas only 2 of 63 (3%) controls had CTG TNR ≥ 50 [13]. Replication studies in an independent Caucasian cohort showed that 73% of the patients with FECD and 7% of the control subjects had CTG TNR expansion [14]. In a large hospital-based study in US, 62% of 574 FECD cases and 3.7% of 354 controls harboured TNR length >40 [18]. This association between TNR and FECD was also replicated in non-Caucasian cohorts, but the proportion of the patients harbouring TNR was lower than that observed in Caucasians. For example, in a Chinese cohort, 25 of 57 (44%) cases and 2 of 121 (1.7%) control subjects harboured CTG TNR lengths >50 [15], whereas these numbers were 15 of 44 (34%) cases and 5 of 97 (5.2%) control subjects in an Indian cohort [16], and 12 of 47 (26%) cases and 0 of 96 (0%) control subjects in a Japanese cohort [17]. In the current study, 21 of 54 patients (39%) and 0 of 54 controls (0%) in our Thai cohort harboured CTG TNR lengths ≥40. Our study in the Thai population provided further evidence that the harbouring of a CTG TNR expansion in *TCF4* patients with FECD is a global finding, although the proportion was again lower in our Thai population when compared with the previously reported proportion in Caucasians.

In the current study, we utilised TP-PCR to determine very large TNR expansions, as these large expansions were difficult to detect by direct sequencing of PCR products. However, measuring very large repeats is challenging, and mosaicism within the leucocyte population further complicates these measurements. Therefore, the possibility of underestimation of patients harbouring TNR length ≥40 should be kept in mind due to the difficulty of obtaining accurate measurements of very large repeats.

TCF4 is a member of the basic helix-loop-helix (bHLH) family of transcription factors that are expressed in various tissues, such as brain, muscle, liver, lung, testis, and cornea [24]. Associations with *TCF4* have been identified for primary sclerosing cholangitis and Pitt-Hopkins syndrome, as well as FECD [25]. However, probably because of the varied roles played by TCF4 in fundamental cellular events, the mechanistic cascade that determines the contribution of TCF4 to the phenotypes of these diseases remains unclear. Du and colleagues reported that poly(CUG)n RNA accumulated as RNA foci in the corneal endothelial cells of patients with FECD, and induced RNA toxicity and mis-splicing [26]. The involvement of repeat-associated non-ATG translation, a protein translation mechanism that has no requirement for an initiating ATG, was also suggested to explain TNR expansion in *TCF4* as part of the pathophysiology of FECD [27]. A recent study has shown that antisense oligonucleotide targeting $(CAG)_7$ could reduce the gain-of-function RNA toxicity caused by TNR in TCF4, supporting TNR as a potent therapeutic target [28]. Our investigations of the pathophysiology of FECD have resulted in the establishment of a cellular model for the corneal endothelium of patients with FECD. We have

also demonstrated that the genes that induce the epithelial–mesenchymal transition (EMT) play an important role in the production of extracellular matrix materials, such as type 1 collagen and fibronectin, which are components of guttae [29]. Sobrado et al. have also reported that overexpression of TCF4 in Madin–Darby canine kidney cells generated motile and highly invasive phenotypes that acquired EMT-related markers, and they proposed that TCF4 was an important regulator of the EMT [30]. Pathway analysis also showed that knockdown of *TCF4* in neuroblastoma cells altered the expression of EMT-related genes [31]. However, the possibility that TCF4 plays a role in regulating the EMT has not been clearly elucidated in the corneal endothelium. Therefore, further studies of TCF4 function, focusing on the possible connection with the regulation of the EMT, are anticipated to elucidate the pathophysiology of FECD.

In conclusion, the findings of an association between SNPs in *TCF4* and FECD differed depending on the ethnic background of the patients. The CTG repeat in intron 3 of *TCF4* was always identified in all ethnic groups studied thus far, but with some variations in frequency. Here, we provide additional evidence that the CTG TNR in *TCF4* is associated with FECD in the Thai population. To the best of our knowledge, this is the first genetic analysis of FECD in Thailand.

Summary

What was known before

- A significant association between the SNP in the *TCF4* and late-onset FECD was shown in the Caucasian population.
- FECD cases frequently harbour intronic TNR expansion in *TCF4* with CTG repeat ≥50 in the Caucasian population, but the proportion of the patients harbouring TNR was lower in non-Caucasians.
- Ethnic variation of SNPs and TNR expansion in *TCF4* is indicated, and the genetic analysis of FECD in Thai cases has not yet reported.

What this study adds

- Only 2 among the 54 FECD subjects in the Thai cohort were polymorphic with regard to rs613872.
- Twenty-one of 54 patients with FECD (39%) and 0 of 54 non-FECD controls (0%) in the Thai cohort harboured CTG TNR length ≥40.
- This study is the first genetic analysis of FECD in Thailand which provided additional evidence that CTG repeat in *TCF4* was present in all ethnic groups studied thus far with some variations in frequency.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

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