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Association of rs613872 and Trinucleotide Repeat Expansion in the *TCF4* Gene of German Patients with Fuchs Endothelial Corneal Dystrophy

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Abstract

Purpose: To investigate single nucleotide polymorphisms (SNPs) and trinucleotide repeat (TNR) expansion in the *transcription factor 4 (TCF4)* gene in a large cohort of German patients with Fuchs endothelial corneal dystrophy (FECD).

Methods: Genomic DNA was obtained from 398 patients with FECD and from 58 non-FECD controls. Thirty-seven previously reported SNPs were evaluated by genotyping. The 398 FECD samples were analyzed for TNR expansions by short tandem repeat (STR) assays and Southern blotting. The possible associations between TNR length and clinical parameters (age, sex, visual acuity, and central corneal thickness) were analyzed in 132 patients.

Result: The SNPs in *COL8A2*, *TCF8*, *LOXHD1*, and *AGBL1* showed no heterogeneity in 36 cases, although *SLCA411* showed 3 nonsense mutations. SNPs were detected for *TCF4*

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(rs613872, rs2123392, rs17089887, rs1452787, and rs1348047), but only rs613872 showed a significant association with FECD ($P=9.93 \times 10^{-12}$). Overall, 315/398 (79%) patients harbored TNR lengths >50, while no non-FECD control harbored TNR lengths >50. The *TCF4* SNP rs613872 genotype was TT:39 (67%), TG:18 (31%), and GG:1 (2%) in non-FECD controls; TT:39 (47%), TG: 38 (46%), and GG:6 (7%) in FECD cases harboring TNR <50; and TT:23 (8%), TG: 224 (79%), and GG:38 (13%) in FECD cases harboring TNR >50 ($P=2.93 \times 10^{-25}$). No significant association was detected between clinical parameters and TNR length.

Conclusions: Our large German cohort demonstrated a significant association between the risk allele G in rs613872 and FECD, irrespective of TNR expansion, although this risk allele was more frequent in FECD cases with TNR expansion than without.

Keywords

Fuchs endothelial corneal dystrophy; *TCF4*; SNP

INTRODUCTION

Fuchs endothelial corneal dystrophy (FECD) is a bilateral, progressive, and hereditary corneal disease. Hallmarks of FECD are the formation of excrescences of Descemet's membrane (basement membrane) due to excessive deposition of extracellular matrix and the loss of corneal endothelial cells. In its advanced stage, FECD is characterized by corneal stromal edema, which results in severe vision loss, and by eye pain due to epithelial edema.¹

FECD is a genetically heterogeneous disease, typically exhibiting an autosomal dominant hereditary pattern with variations in penetrance and expressivity.² Linkage analysis in large families shows that FECD is associated with *FCD1*, *FCD2*, *FCD3*, and *FCD4* loci on chromosomes 13, 18, 5, and 9, respectively.^{3–6} A missense mutation of *COL8A2*, which is a major component of Descemet's membrane, is causative for the rare form of early onset FECD but not for the common form of late onset FECD.⁷ Early genetic analyses of late onset FECD revealed an association of four genes (*SLCA411*, *TCF8*, *LOXHD1*, and *AGBL1*) and the disease phenotype.^{6, 8–12} However, those genetic mutations were rarely identified in other cohorts of patients with FECD¹³, suggesting the existence of other causative genetic factors for the majority of late onset FECD cases.

In 2010, a genome-wide association study (GWAS) demonstrated a significant association between late onset FECD and a single nucleotide polymorphism (SNP), rs613872, in the fourth intron of transcription factor 4 (*TCF4*) on chromosome 18.¹⁴ Subsequent researchers replicated these findings, and several other SNPs in *TCF4* were also identified in other populations.^{15–19} More recently, a strong association was found between FECD and the expansion of a CTG trinucleotide repeat (TNR) in the third intron of *TCF4*,²⁰ and subsequent studies have replicated this association in other independent cohorts.^{18, 21–25}

These genetic analyses of causal genes in several cohorts and ethnicities have confirmed the presence of the *TCF4* repeat expansion in the majority of Caucasian populations studied thus far, but the expansion occurs in a minority of Asian^{18, 26} and African-American²⁷ patients with FECD. In the present study, we conducted a “semi-comprehensive” analysis of the

causal genetic mutations in a large German cohort of patients with FECD. We evaluated the 37 previously reported SNPs in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, *AGBL1*, and *TCF4*, as well as TNR expansion in 398 German patients with FECD who were scheduled for Descemet membrane endothelial keratoplasty (DMEK). We also investigated the potential relationship between TNR length and several clinical parameters related to the severity of FECD.

MATERIALS AND METHODS

Ethics statement

The human tissue used in this study was handled under guidelines based on the ethical principles of the Declaration of Helsinki. This study was performed according to a protocol approved by the ethical review committee of the Friedrich-Alexander University Erlangen-Nürnberg, Doshisha University, Kyoto Prefectural University of Medicine, and the Mayo Clinic. Informed consent was acquired from all patients with FECD. Normal human donor corneas were obtained from SightLife™ (<http://www.sightlife.org/>, Seattle, WA).

Study Participants

The 493 patients with late-onset FECD (German subjects of Caucasian descent) who were scheduled for DMEK at the Friedrich-Alexander University Erlangen-Nürnberg were recruited between October 2013 and September 2015, and peripheral blood samples were obtained. Of the 493 participants, the peripheral blood leukocytes of 398 had the genomic DNA concentrations above 3.0 µg/ml required for this study and were used for subsequent genetic analyses. The samples were numerically coded at the Friedrich-Alexander University Erlangen-Nürnberg, and genomic DNA was analyzed at Doshisha University and Mayo Clinic in a pseudonymized fashion. As control, genomic DNA was isolated from donor corneas (corneal stroma) of 58 non-FECD subjects of Caucasian descent. The potential effect of TNR length on clinical parameters, such as age at the time of surgery, sex, visual acuity at the time of surgery, and central corneal thickness at the time of surgery, were evaluated in 132 patients with FECD.

Preparation of Genomic DNA

Genomic DNA was isolated from 200 µL of peripheral blood by using the DNeasy® Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. As a control, genomic DNA was similarly isolated from 58 residual corneal rims of non-FECD donor corneas used for corneal transplantation. The amount and quality of each isolated DNA sample were analyzed with a UV spectrophotometer (NanoDrop; NanoDrop Technologies, DE, USA), and the samples were stored at −80°C until use.

Genotyping

The 37 previously reported SNPs in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, *AGBL1*, and *TCF4* in the 36 randomly selected FECD cases were genotyped by PCR using the primers shown in Supplemental Table 1. In addition, rs613872 (SNPs in *TCF4*) was genotyped in all 398 FECD cases. The PCR reactions were performed with Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) under the following conditions: denaturation at 94°C for 30 seconds

(one cycle), annealing at 54–60°C for 30 seconds and elongation at 72°C for 30 seconds (32–35 cycles). The PCR products were separated by electrophoresis on 2% agarose gels in Tris-acetate buffer, stained with ethidium bromide, and analyzed 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 performed with a Taq Dideoxy Terminator Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Evaluation of CTG Repeat Length

The CTG repeat length in *TCF4* was determined by a short tandem repeat (STR) assay, as described previously.²⁰ Briefly, a fluorescently labeled primer was used for PCR of the CTG repeat region. The sizes of the PCR products were evaluated by capillary electrophoresis on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Samples demonstrating a single signal from the STR assay, which is too large for detection by the PCR-based STR assay, were subjected to Southern blotting on unamplified DNA, as described previously.²⁰

Statistical Analysis

Statistical analysis was performed with the R statistical package (version 3.4.1 for Windows, Vienna, Austria). Differences between two groups were detected with Welch's t-test for age data and the Chi-square test for gender data. The differences among the three groups were statistically analyzed by analysis of variance (ANOVA) and the Chi-square test or Fisher's exact test. Bonferroni correction was applied when the allele frequencies and genotype frequencies were compared between 1) control and FECD harboring TNR < 50; 2) control and FECD harboring TNR > 50; and 3) FECD harboring TNR < 50 and FECD harboring TNR > 50 (significance threshold, $P = 0.05$ divided by the number of groups: $P = 0.017$). Spearman's rank correlation coefficient was used to determine any significant correlations between the TNR length and clinical parameters (age, visual acuity, and central corneal thickness at the time of surgery). Multivariate logistic regression analysis was applied to analyze the association between TNR > 50 and several clinical parameters (age, sex, visual acuity, and central corneal thickness at the time of surgery). A P-value of <0.05 was considered statistically significant. Results are expressed as mean $\pm$ standard deviation (SD).

RESULTS

The demographics of the participants in this study are shown in Table 1. Consistent with previous reports,²⁸ the proportion of females (61%) was higher in the FECD group than in the non-FECD controls (38%). The mean age was 58.2 ± 11.9 (range, 18 to 92) years in the control group and 69.5 ± 9.6 (range, 32 to 92) in the FECD group.

The SNPs in *COL8A2*, *TCF8*, *LOXHD1*, and *AGBL1* showed no heterogeneity in the 36 randomly selected FECD cases, but 3 nonsense mutations were detected in rs34460295, rs3827075, and rs3803956 of *SLCA411* (Table 2). Variations were also detected in several SNPs in *TCF4*, including rs613872, rs2123392, rs17089887, rs1452787, and rs1348047.

However, only rs613872 showed a significant association with FECD when compared with the non-FECD controls ($P=9.93\times 10^{-12}$). The mean age of the 36 FECD cases used for genotyping was 73.1 ± 6.4 (range, 60 to 86) years and the sex distribution was 17 males and 19 females.

Given our finding that only rs613872 in *TCF4* was associated with FECD, we further evaluated the association between rs613872 and CTG repeat expansion in *TCF4* (Table 3). STR assay and Southern blotting results demonstrated that 315 of the 398 patients (79%) harbored CTG trinucleotide repeat lengths > 50 (8 homozygous and 307 heterozygous patients), while 83 of the 398 patients (21%) did not harbor TNR lengths > 50 (Table 1). The genotypes of rs613872 were TT:39 (67%), TG:18 (31%), and GG:1 (2%) in non-FECD controls; TT:39 (47%), TG: 38 (46%), and GG:6 (7%) in FECD patients harboring TNR lengths < 50 ; and TT:23 (8%), TG: 224 (79%), and GG:38 (13%) in FECD patients harboring TNR lengths > 50 . Fisher's exact test showed a significant trend among the three groups: non-FECD control, FECD harboring TNR < 50 , and FECD harboring TNR > 50 ($P=2.93\times 10^{-25}$). The difference between FECD harboring TNR < 50 and FECD harboring trinucleotide repeat > 50 was also statistically significant according to Fisher's exact test ($P=9.88\times 10^{-14}$). The allele of *TCF4* SNP rs613872 was T:96 (83%) and G:20 (17%) in the non-FECD controls; T:116 (70%) and G:50 (30%) in FECD harboring TNR lengths < 50 ; and T:270 (43%) and G:360 (57%) in FECD harboring TNR lengths > 50 . The Chi-square test showed significance among all three groups: non-FECD control, FECD harboring TNR < 50 , and FECD harboring TNR > 50 ($P=1.91\times 10^{-19}$) and between two groups: FECD harboring TNR < 50 and FECD harboring TNR > 50 ($P=9.95\times 10^{-10}$).

We also examined the potential correlation between TNR length and the severity of FECD by using Spearman's rank correlation coefficient to compare several clinical parameters defined at time of surgery. TNR length was not correlated with age ($p=0.054$) (Figure 1A), visual acuity ($p=0.28$) (Figure 1B), or central corneal thickness ($p=0.63$) (Figure 1C). In addition, none of these parameters showed any significant association with the CTG TNR length when analyzed by multivariate logistic regression (age: $p=0.36$; sex: $p=0.25$; visual acuity: $p=0.51$; central corneal thickness; $p=0.16$) (Table 4).

DISCUSSION

Linkage and mapping studies in FECD families have identified associations between coding mutations and FECD, including mutations in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, and *AGBL1*.^{6, 8-12} In the current study, none of the previously identified mutations in *COL8A2*, *TCF8*, *LOXHD1*, and *AGBL1* were found in a small subset of our cohort, although three nonsense mutations were identified in *SLCA411*. This latter gene is associated with congenital hereditary endothelial dystrophy (CHED2) and has been shown to be associated with FECD in Chinese, Indian, and Caucasian populations.^{8, 9} We found three SNPs (rs34460295, rs3827075, and rs3803956) in *SLCA411* in our cases, but these SNPs cause nonsense mutations and are not rare polymorphisms, according to the database of 1000 Genomes Project. Accordingly, our results indicate that mutations in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, and *AGBL1* are very rare in German patients with FECD.

A previous GWAS and replication study by Baratz and colleagues that included 280 cases and 410 controls indicated a strong association between an intronic variant in *TCF4*, rs613872, and FECD.¹⁴ The minor allele (G) was present more frequently in FECD cases (G=0.40) than in non-FECD controls (G=0.15), and the odds ratio for one copy of the risk allele G (heterozygous) was 5.5 and for two copies (homozygous) was 30.¹⁴ Subsequent studies confirmed the association between rs613872 and FECD, but mainly in Caucasian cohorts.^{15, 19} In a previously reported German cohort, 33 (79%) of 42 unrelated patients with FECD had heterozygous TG and 4 (10%) had homozygous GG. By contrast, 65 (70%) of 93 non-FECD controls had homozygous TT and 21 (23%) had heterozygous TG.²⁴ These results were in agreement with the findings presented here for a larger cohort. However, rs613872 is monomorphic in several ethnic cohorts, such as Southern Chinese and Indian populations, and populations of Chinese descent in Singapore. Instead, variations in *TCF4* SNPs other than rs613872 have been identified in Chinese and Indian FECD populations, suggesting the existence of ethnic variations of SNPs in this gene.^{16–18} In our analysis of rs613872 and three other SNPs identified in the Chinese and Indian studies, only rs613872 was associated with FECD in German patients. Although our control corneal tissues were obtained from Caucasian donors but were not limited to German subjects, our findings confirmed that the minor allele (G) was found frequently (G=0.52) in German patients with FECD, in agreement with other studies on Caucasian cohorts.

Wieben and colleagues reported a strong association between the expansion of CTG TNR in *TCF4* and FECD.²⁰ Their evaluation of 66 FECD cases and 63 unaffected controls showed that a sensitivity and specificity of >50 repeats identifying FECD of 79% and 96%, respectively, with an even higher specificity than that reported previously for rs613872.²⁰ This strong association between *TCF4* TNR expansion and FECD was replicated in an independent Caucasian cohort, in which repeat expansion was found in 88 of 120 (73%) cases and in 7 of 100 (7%) control subjects.²¹ This association has again been confirmed recently in another large cohort of 574 FECD cases and 354 controls, in which a repeat length > 40 was identified in 62% FECD cases and 3.7% controls.²² In a German cohort, 33 (79%) of 42 cases exhibited TNR expansion > 50, while 10 (11%) of 93 cases exhibited an expanded allele.²⁴ The association of TNR expansion with FECD was replicated in other populations, although their reported frequencies of repeat expansion in FECD cases were lower than in Caucasian cohorts: 44% in Chinese, 34% in Indian, and 26% in Japanese.^{18, 21, 23} This suggests that the frequency of harboring TNR expansion varies depending on ethnicity. Here, our results for this large German cohort showed that 79.4% of the FECD cases have TNR expansion > 50, confirming that the frequency of German patients who harbor *TCF4* TNR expansion is strikingly similar to that of other Caucasian cohorts.

Mootha and colleagues in their U.S. cohort showed a more frequent occurrence of *TCF4* TNR expansion in cases with the G risk allele of rs613872.²¹ In the current study, we also showed a significant correlation between this G risk allele and *TCF4* CTG repeat expansion in our FECD cases. The likely explanation for the strong association of FECD with both rs613872 and TNR expansion is that only TNR expansion is causative for FECD, whereas the risk allele G of rs613872 is only in linkage disequilibrium. This hypothesis is supported by the observation that the association of the TNR expansion with FECD is stronger than the association with SNPs demonstrated in other studies.^{16–18, 20–22} In addition, studies of ex

vivo and cultured endothelial tissues have shown the occurrence of RNA toxicity, repeat associated non-ATG translation, and altered TCF4 expression, which are mechanisms consistent with CTG repeat-induced disease.^{29–31} In our current study, no significant association was detected between the CTG repeat length and several clinical parameters related to the severity of FECD. This suggests the existence of a threshold of TNR length for disease induction, but the length is not related to disease severity. However, further studies on additional clinical parameters (e.g. disease progression speed, guttae formation) are necessary to determine the threshold levels of TNR length and the pathological phenotype.

Recently, Afshari and colleagues performed a GWAS on 1,404 FECD cases and 2,564 controls of European ancestry, followed by a replication study using independent data sets with 671 FECD cases and 778 controls.³² They showed that three novel SNPs in *KANK4* (rs79742895), *LAMC1* (rs3768617), and *LINC00970/ATP1B1* (rs1200114), in addition to *TCF4* (rs784257), are associated with FECD with genome-wide significance. Calculation of the genetic risk scores of these SNPs revealed a significant increase in the predictive value of *TCF4* SNP by adding the three novel SNPs, thereby supporting the concept of FECD as a multifactorial disease.³² We have not performed confirmatory studies regarding those SNPs, but future subsequent replicate studies should further our understanding of the pathology of this disease.

In summary, we verified that potentially causative genetic mutations in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, and *AGBL1* are rare and that TNR expansion and SNP rs613872 in *TCF4* are strongly associated with FECD in the German population. In addition, TNR expansion is frequently identified in patients harboring the risk allele G of rs613872. Our findings further substantiate a significant role for TNR expansion in FECD and should encourage future research efforts to investigate the role of *TCF4* TNR expansion in the pathogenesis of FECD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Correlation between CTG trinucleotide repeat (TNR) length and the severity of Fuchs endothelial corneal dystrophy (FECD)

(A) CTG TNR length was evaluated by analyzing the genomic DNA from peripheral blood and the patient age at the time of surgery was plotted. No significant correlation was noted between the CTG TNR length and the age at the time of surgery, based on the Spearman's rank correlation coefficient ($p=0.054$).

(B) No significant correlation was detected between the CTG TNR length and visual acuity at the time of surgery, based on the Spearman's rank correlation coefficient ($p=0.28$).

(C) No significant correlation was detected between the CTG TNR length and central corneal thickness at the time of surgery, based on the Spearman's rank correlation coefficient ($p=0.63$).

Table 1.

Demographic data of control and Fuchs endothelial corneal dystrophy (FECD) cases with and without CTG trinucleotide repeat (TNR) expansion in *TCF4*

	Control (n=58)	No expansion CTG < 50 (n=83)	Expansion CTG ≥ 50 (n=315)	P value
Age, yr				2.53×10^{-9} ^a
	58.2±11.9 (18, 92)	69.1±10.2 (42, 92)	69.5±9.4 (32, 90)	
Sex				3.33×10^{-5} ^b
Male	36 (62%)	20 (24%)	135 (43%)	
Female	22 (38%)	63 (76%)	180 (57%)	

^a P value calculated by using the analysis of variance (ANOVA).

^b P value calculated by using the Chi-square test.

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

Table 2.Genotyping of reported SNPs in *COL8A2*, *SLCA411*, *TCF8*, *LOXHD1*, *AGBL1*, and *TCF4*

Gene	Chr position	rs ID	Mutation	1000 Genomes (EUR)	Case
<i>COL8A2</i>				n=2012	n=36
	1:36099217	rs75864656	C>T	T=0.0010	0/72
	1:36098770	rs369487110	C>T	T=0.0000	0/72
	1:36098380	rs201235688	C>T	T=0.0020	0/72
	1:36098332	rs80358192	A>C,G		0/72
<i>SLCA411</i>	20:3237580-1		TCdel		0/72
	20:3234816	rs778688114	C>G,T		0/72
	20:3234760	rs200940928	A>G	C=0.0000	0/72
	20:3234249	rs34460295	G>A	A=0.0249	14/72
	20:3234173	rs3827075	T>A,G	G=0.3956	22/72
	20:3233935	rs3803956	G>A	A=0.1779	16/72
	20:3231375	rs193080010	G>A	A=0.0000	0/72
	20:3231346	rs760889152	C>T		0/72
	20:3230970	rs139297339	C>A,T	T=0.0010	0/72
	20:3230954	rs267607065	G>A		0/72
	20:3230934	rs78274653	C>T	T=0.0000	0/72
	20:3229694	rs201595005	C>T	T=0.0000	0/72
	20:3229632	rs755379986	C>G,T		0/72
	20:3229620	rs754745672	C>T		0/72
	20:3229410	rs201613216	C>T	T=0.0010	0/72
	20:3229223	rs376848818	G>A		0/72
	20:3228952	rs267607064	G>A		0/72
	20:3228687	rs267607066	C>T		0/72
	20:3228366	rs58757394	G>A	A=0.0089	0/72
<i>TCF8</i>	10:31461211	rs80194561	A>C	C=0.00	0/72
	10:31521280	rs781750314	C>G		0/72
	10:31521764	rs19944415	A>C,G	C=0.00,G=0.00	0/72
	10:31521854	rs118020901	A>C	C=0.0119	0/72
	10:31524042	rs78449005	C>G	G=0.0010	0/72
<i>LOXHD1</i>	18:46591948	rs113444922	G>A		0/70
	18:46577732	rs141932807	C>T	T=0.0000	0/72
	18:46521131	rs372241056	A>G		0/72
<i>AGBL1</i>	15:86674435	rs185919705	C>T	T=0.0050	0/72
<i>TCF4</i>	18:55543071	rs613872	T>G	G=0.1769	36/72

Gene	Chr position	rs ID	Mutation	1000 Genomes (EUR)	Case
	18:55547934	rs2123392	T>C	C=0.3588	9/72
	18:55541025	rs17089887	T>C	C=0.0885	12/72
	18:55539976	rs1452787	A>G	G=0.2972	5/72
	18:55382827	rs1348047	G>T	T=0.2734	21/72

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Table 3.

CTG trinucleotide repeat (TNR) expansion and rs613872 genotype of Fuchs endothelial corneal dystrophy (FECD) cases and controls

	Control (n=58)	No expansion CTG < 50 (n=83)	Expansion CTG > 50 (n=315)	P value
Genotype				$2.93 \times 10^{-25}^a$
TT	39 (67%)	39 (47%)	23 (8%)	
TG	18 (31%)	38 (46%)	224 (79%)	
GG	1 (2%)	6 (7%)	38 (13%)	
Allele				$1.91 \times 10^{-19}^b$
T	96 (83%)	116 (70%)	270 (43%)	
G	20 (17%)	50 (30%)	360 (57%)	

^aP value calculated by using the Fisher's exact test.

^bP value calculated by using the Chi-square test.

Data represent the number of samples (%).

Table 4.

Multivariate analysis of the associations between CTG trinucleotide repeat (TNR) expansion and patient age, sex, visual acuity, and central corneal thickness at the time of surgery

Variables	Odds ratio	95% confidence interval	P value
Age 71 (vs. < 71)	1.55	0.61-3.91	0.36
Sex Female (vs. Male)	0.57	0.22-1.48	0.25
Visual acuity (logMAR) 0.52 (vs. < 0.52)	0.73	0.29-1.84	0.51
Central corneal thickness 635 (vs. < 635)	1.97	0.76-5.10	0.16