Correlation of Severity of Fuchs Endothelial Corneal Dystrophy With Triplet Repeat Expansion in TCF4

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IMPORTANCE The CTG18.1 triplet repeat expansion in TCF4 has recently been found to be a common functional variant contributing significant risk to the development of Fuchs endothelial corneal dystrophy (FECD) in Eurasian populations.

OBJECTIVES To determine the effect of the expanded CTG18.1 allele of TCF4 on FECD severity and to correlate CTG triplet repeat allele length to the severity of FECD.

DESIGN, SETTING, AND PARTICIPANTS In a cross-sectional analysis, we studied 139 index cases (probands and unrelated individuals) with FECD recruited from a cornea referral practice at the University of Texas Southwestern Medical Center, Dallas, from April 2010 through February 2015. The triplet repeat polymorphism CTG18.1 was genotyped using a combination of short tandem repeat analysis, triplet repeat–primed polymerase chain reaction assay, and Southern blot analysis. Severity of FECD was graded using a modified Krachmer grading system (severity scale of 0-6 based on extent of confluent guttae).

MAIN OUTCOMES AND MEASURES The CTG triplet repeat length of the largest allele was compared with the Krachmer grade of FECD severity, keratoplasty proportion, and central corneal thickness in the white subset.

RESULTS Eighty-five of 122 white index cases with FECD (69.7%) harbored the triplet repeat expansion. The mean (SD) Krachmer grade was 5.61 (0.76) in the group with the repeat expansion compared with 5.11 (1.05) in the group without the expanded repeats ($P = .01$). Forty-seven participants with the repeat expansion (55.3%) had undergone keratoplasty at the time of recruitment, compared with 13 (35.1%) of those without the expansion ($P = .0497$). There was a positive correlation of Krachmer grade to triplet repeat number ($P = .002$) and a nominal association of the keratoplasty proportion with triplet repeat number ($P = .04$). The mean (SD) central corneal thickness was 605.9 (50.5) μm in the group with the expanded repeats compared with 581.3 (50.5) μm in the group without the expansion ($P = .04$).

CONCLUSIONS AND RELEVANCE The Krachmer grade of disease severity was greater in FECD cases with the CTG18.1 triplet repeat expansion in TCF4 than in those without the expansion. The CTG triplet repeat allele length was positively correlated with the Krachmer grade of severity. The TCF4 triplet repeat expansion resulted in a more severe form of FECD, with clinical and surgical therapeutic implications.
common, adult-onset Fuchs endothelial corneal dystrophy (FECD; OMIM 136800) is a degenerative disorder of the corneal endothelium. This inherited disease affects 5% of the US population older than 40 years. With more than 14,000 corneal transplant procedures performed for FECD in 2013, the disorder represents the leading indication for keratoplasty in the United States. The hallmark of the disease is premature senescence of the endothelium with increased variability in cell size and shape accompanied by diffuse thickening of the Descemet membrane, the underlying basement membrane of the endothelial cells. Guttae, focal excrescences of the Descemet membrane seen by slitlamp biomicroscopy, are pathognomonic for the disorder when they reach central confluence in the cornea. In FECD, progressive decline in endothelial cell density leads to corneal edema and scarring with commensurate loss of vision.

Although FECD has been characterized as an autosomal dominant trait, the disorder is a complex genetic trait with locus heterogeneity. Incomplete penetrance and phenocopies within large FECD pedigrees have made the dissection of underlying genetic loci challenging. Early-onset corneal endothelial dystrophy is a rare disorder caused by heterozygous mutations in COL4A2 (OMIM 120252). Rare heterozygous mutations in SLC4A11 (OMIM 610206), TCF8 (OMIM 189909), LOXHD1 (OMIM 613267), and AGBLI (OMIM 615523) have been found only in a small number of common, adult-onset FECD cases.

After a genome-wide association study of adult-onset FECD highlighted the association of single-nucleotide polymorphisms across the transcription factor 4 gene (TCF4; OMIM 602272), including most notably single-nucleotide polymorphism rs613872, expanded trinucleotide repeats at the intronic CTG18.1 locus of TCF4 were also reported to be associated with the trait. The triplet repeat expansion was found to be more predictive of the disorder with greater sensitivity and specificity than rs613872. In our previous studies, we found single-nucleotide polymorphism rs613872 and the CTG18.1 triplet repeat polymorphism to be in tight linkage disequilibrium and calculated that 1 copy of the expanded CTG18.1 allele confers more than a 30-fold increase in the risk for development of FECD in white individuals. Cosegregation of the triplet repeat expansion with FECD in 15 of 29 examined white pedigrees with complete penetrance added evidence that the triplet repeat expansion was likely a functional variant. After transethnic studies including haplotype analysis and replication of the association with an odds ratio of 66.5 for each expanded CTG18.1 allele in a Singapore Chinese cohort with FECD, we concluded that the repeat expansion is a shared, common functional variant for FECD susceptibility in Eurasian populations rather than a tagged polymorphism in linkage disequilibrium with another causal variant.

Simultaneous reports in early 2015 documented the presence of CUG RNA nuclear foci in the corneal endothelium of patients with FECD with the CTG18.1 triplet repeat expansion, implicating toxic RNA as the mechanism of disease in this common disorder. The RNA nuclear foci, a hallmark of toxic gain-of-function RNA, had been previously seen only in rare, neurodegenerative disorders caused by simple repeat expansions. Rather than a primary mechanism of haploinsufficiency of TCF4, the triplet repeat expansion at the CTG18.1 locus may mediate endothelial dysfunction via aberrant gene splicing as a result of the mutant CUG RNA transcripts sequestering the splicing factor muscleblind-like 1.

These recent findings have established FECD as a common repeat expansion disorder. It has been speculated that the number of patients with FECD may far eclipse the number of individuals with all the other hereditary diseases caused by simple repeat expansions combined. A clinical description of patients with FECD with triplet repeat expansion in TCF4 is currently lacking in the literature. In this study, we aimed to compare and contrast the clinical phenotypic characteristics of patients with FECD with triplet repeat expansion in TCF4 vs those without the expansion. Additionally, we studied the effect of the triplet repeat expansion on FECD severity and correlated the triplet repeat allele length to severity of disease.

Methods

Study Participants

All study participants were recruited at the cornea referral practice at the University of Texas Southwestern, Dallas, from April 2010 through February 2015 after they provided written informed consent. Index FECD cases (probands and unrelated individuals with FECD) from our cohort were included in this study. A standardized questionnaire was used to ascertain demographic information, family history, smoking history, and ocular history including glaucoma. Each patient who had undergone keratoplasty in 1 or both eyes at the time of recruitment was noted. All participants underwent a complete eye examination including slitlamp biomicroscopy by a cornea fellowship–trained ophthalmologist (V.V.M.). Inclusion criteria for FECD cases included the presence of slitlamp examination findings of grade 2 or higher on the modified Krachmer FECD grading scale: grade 0 indicated no central guttae; grade 1, up to 12 scattered central guttae; grade 2, 12 or more scattered central guttae; grade 3, 1- to 2-mm confluent central guttae; grade 4, 2- to 5-mm confluent central guttae; grade 5, greater than 5-mm confluent central guttae without stromal edema; and
grade 6, greater than 5-mm confluent central guttae with stromal edema and/or histopathologic confirmation of the diagnosis after keratoplasty.\textsuperscript{13} All Krachmer grades were determined by a single investigator (V.V.M.). Postkeratoplasty eyes were classified as Krachmer grade 6. The Krachmer grade of the more severely affected eye was used in data analysis; thus, all the data are represented by single-eye Krachmer grades. Presence or absence of map-dot-fingerprint dystrophy (MDFD), also known as epithelial basement membrane dystrophy, was systematically noted on slitlamp examination. Central corneal thickness (CCT) measurements were performed at the center of the cornea using a Corneo-Gage Plus ultrasonic pachymeter (Sonogage). The average of 3 separate measurements was used as the CCT. Postkeratoplasty eyes were excluded in CCT analysis. The study protocol was approved by the institutional review board of the University of Texas Southwestern Medical Center and was in compliance with the tenets of the Declaration of Helsinki.

### CTG18.1 Polymorphism Genotyping
Genomic DNA was extracted from leukocytes of peripheral blood samples with the Nucleon blood extraction kit (Amerham Biosciences). The CTG18.1 trinucleotide repeat polymorphism was genotyped using short tandem repeat and triplet repeat–primed polymerase chain reaction (PCR) assays as previously described.\textsuperscript{18-20} For the short tandem repeat assay, primers flanking the repeat region were used for PCR amplification with 1 primer labeled with 6-carboxyfluorescein on the 5′ end. The triplet repeat–primed PCR assay was performed using the same flanking 5′-6-carboxyfluorescein-labeled primer paired with repeat sequence–targeted primers for PCR amplification as previously described.\textsuperscript{19} The PCR amplicons were subsequently loaded on an ABI 3730xl DNA analyzer (Applied Biosystems) and the results were analyzed using ABI GeneMapper software version 4.0 (Applied Biosystems). Southern blot analysis was performed to measure large triplet repeat expansions using digoxigenin-labeled probes as previously described.\textsuperscript{21}

### Statistical Analysis
For the CTG18.1 trinucleotide repeat polymorphism, we dichotomized alleles such that those with 40 or more CTG triplet repeats were considered a mutant, expanded allele and those with fewer triplet repeats were considered a normal allele as we have done in previous reports.\textsuperscript{19,20} Best-corrected visual acuity (logMAR) and CCT of the eye with the greater Krachmer grade from each patient are reported and were used for statistical analysis. Comparisons of the demographic and clinical phenotypic features between CTG18.1 expansion carrier groups were performed by a 2-sample t test for continuous traits and by Fisher exact test for binary traits. Spearman rank correlations between Krachmer grade, CCT, and repeat length were calculated. We also fit ordered logistic regression models to test association of the repeat length with Krachmer grade, CCT, and keratoplasty proportion adjusting for age and sex. The alleles were separated into 4 groups based on the CTG triplet repeat length of the largest allele: fewer than 40 (n = 37), 40 to 84 (n = 23), 85 to 120 (n = 35), and more than 120 (n = 27) CTG triplet repeats. We used R version 3.1.3 statistical software (R Foundation) for statistical analysis. P < .05 was considered statistically significant.

### Results
#### Demographic Characteristics of Study Participants
We recruited 139 index cases with FECD. The demographic information of the entire study cohort stratified by ethnicity is presented in Table 1. The majority of participants were white (n = 122 [87.8%]), and 98 participants (70.5%) were female. Of the 139 patients with FECD, 64 (46.0%) had undergone keratoplasty in at least 1 eye at the time of recruitment. Of the 122 white index patients with FECD, 85 (69.7%) harbored the triplet repeat expansion. Also among these white patients with FECD, 21 (17.2%) were noted to have slitlamp findings of MDFD. Among the nonwhite patients, 14 were African American and 3 were South Asian Indian. Two African American patients...
had the expanded risk allele and no patients of Indian descent had the expansion.

Phenotypic Characteristics of White Patients With FECD

We performed further analysis on the white subset of index FECD cases as they represented the vast majority of the reported cohort (n = 122). Phenotypic characteristics of white patients with FECD with the CTG18.1 triplet repeat expansion are compared with those without the repeat expansion in Table 2. A majority of the patients were female in both the CTG18.1 expansion-positive group (54 of 85 patients [63.5%]) and the CTG18.1 expansion-negative group (29 of 37 patients [78.4%]). There was no difference in the prevalence of MDFD in the 2 groups (13 [15.3%] with the expansion vs 8 [21.6%] without the expansion; P = .44). The mean (SD) Krachmer grade was 5.61 (0.76) in the group with the triplet repeat expansion compared with 5.11 (1.05) in the group without the expanded repeats (P = .01). Among the patients with the triplet repeat expansion, 47 (55.3%) had undergone keratoplasty at the time of recruitment in at least 1 eye in comparison with 13 (35.1%) of those without the expansion (P = .0497). The mean (SD) CCT was 605.9 (50.5) μm in the group with the expanded repeats compared with 581.3 (50.5) μm in the group without the expansion (P = .04).

Correlation of CTG Triplet Repeat Length to Disease Severity

There was a positive correlation of Krachmer grade of FECD severity to triplet allele length (Figure). The Spearman rank correlation coefficient was estimated to be 0.27 (P = .002) (Figure), and an ordinal regression adjusting for age and sex led to P = .007. There was an association of keratoplasty proportion with triplet allele length (P = .04). The proportions of patients who had undergone keratoplasty at the time of recruitment were 31.5%, 55.6%, 45.7%, and 69.6% in the groups with CTG triplet repeat allele lengths of less than 40, 40 to 84, 85 to 120, and more than 120 CTG triplet repeats, respectively. The ordinal regression model suggested a positive correlation between CCT and triplet allele length (P = .04); however, the Spearman rank correlation coefficient was estimated to be only 0.14 (P = .21) (eFigure in the Supplement). The CCT was found to be positively correlated with the Krachmer grade (P = .04).

CTG18.1 Expanded Allele Dosage

A comparison of the phenotypic characteristics of the 8 white patients with 2 expanded CTG18.1 alleles vs the 77 white patients with 1 expanded allele found no differences in the Krachmer grade (P = .58), keratoplasty proportion (P = .29), or CCT (P = .43) between the 2 groups.

Discussion

As CTG18.1 triplet repeat expansion in TCF4 is a common functional variant contributing significant risk to the development of FECD in Eurasian populations,20 it is clinically relevant to ascertain the effect of the expansion on the trait's...
severity and assess the role of CTG triplet repeat allele length on severity of disease.

Among the 122 white index patients, 85 (69.7%) harbored the triplet repeat expansion at CTG18.1, suggesting that this locus may contribute to a significant portion of the genetic burden of disease in this ethnicity. Previous association studies performed on white cohorts with FECD report the triplet repeat expansion to be present in 73% to 80% of their patients. In these association studies, 3% to 7% of controls had expansions without evidence of guttae on slit-lamp examination. In contrast, only 2 of the 14 African American patients with FECD had evidence of triplet repeat expansion. Although the association of the expansion with the trait has been reported in 34% of patients from a cohort with FECD in India, we did not detect the expanded polymorphism in the 3 South Asian Indian patients examined. The FECD trait appears to be more severe in white individuals compared with the other ethnic groups (Table 1), but additional studies are warranted. The reported cohort with FECD also reflects a strong female bias for the disorder (Table 1). Larger, population-based studies would be ideal to determine unbiased estimates of the prevalence, penetrance, and effect of the CTG18.1 triplet repeat expansion on the FECD phenotype in various ethnicities.

The Krachmer grade of disease severity is greater in FECD cases with the CTG18.1 triplet repeat expansion in TCF4 (Table 2). Patients with FECD with the triplet repeat expansion had a higher mean Krachmer grade, 5.61, than their counterparts without the repeat expansion, who had a mean Krachmer grade of 5.11 (P = .01). A greater proportion of patients with FECD with the triplet repeat expansion (55.3%) had undergone keratoplasty at the time of recruitment compared with those without the expansion (35.1%) (P = .0497). Although the keratoplasty proportion may be subject to some ascertainment bias, we feel that this parameter provides some additional evidence of increased clinical severity.

Based on our findings, comorbid MDFD is not a specific finding for CTG18.1-mediated FECD. There was no difference of MDFD prevalence in the white patients with FECD with the triplet repeat expansion (15.3%) vs those without the expansion (21.6%) (P = .44). Our detection of MDFD by slitlamp examination may be an underestimate based on the classic article by Eagle et al that documented characteristic epithelial and basement membrane/extracellular matrix findings of MDFD in 62.8% of FECD cases as well as 48.9% of pseudophakic or aphakic bullous keratoplasty buttons examined by light microscopy.

We found that the CTG triplet repeat number at this locus correlates with the Krachmer grade of FECD severity (P = .002) (Figure). Additional support that the triplet repeat allele length affects the severity was its nominal association with the keratoplasty proportion (P = .04). Nearly 70% of the patients harboring expansions of greater than 120 CTG repeats had undergone keratoplasty at the time of recruitment. Although this study documents increased severity with longer CTG18.1 triplet repeat expansions in FECD, the correlation between repeat expansion length and disease severity has been well established in numerous neurodegenerative disorders caused by simple repeat expansions. In myotonic dystrophy 1 (OMIM 160900) caused by CTG repeat expansions in the 3′ untranslated region of DMPK (OMIM 605377), longer alleles have been long known to result in greater disease severity and earlier onset. Given that the early symptoms of FECD may be more insidious in comparison with the neurodegenerative disorders caused by repeat expansions, studies on the correlation of CTG repeat length to age at onset in FECD may be more challenging and may require a longitudinal study design.

We found a positive correlation between repeat expansion length and FECD Krachmer grade, but this relationship was not found to be a simple linear correlation (Figure). It is possible that limitations with our study design such as recruitment of a cohort with FECD from a tertiary care cornea practice resulting in a predominance of patients with moderate or advanced FECD may have contributed to our inability to detect a linear correlation. Our parameters of disease severity also have their own inherent limitations. We need further refinements in the Krachmer grading system, as the use of simple integral values from 1 to 6 may not be adequate to discriminate between the advanced FECD cases. Previous distribution analysis has shown that the CCT ranges from 460 to 640 μm in white individuals, suggesting that this parameter may not be an ideal measure for grading FECD severity in cross-sectional studies. Until better tools are developed, the widely adopted Krachmer grading system remains the method of choice for assessing severity of FECD in large studies.

Somatic instability of the CTG18.1 locus may be a valid physiological explanation as to why the correlation of the largest triplet allele length in peripheral leukocytes to corneal endothelial disease may not be a simple linear relationship. Trinucleotide repeat tracts show high levels of somatic instability in terminally differentiated somatic cells that increases with age in both noncoding triplet repeat expansion disorders such as myotonic dystrophy 1 as well as in coding triplet repeat expansion disorders such as Huntington disease. Studies on the somatic instability and further expansion in corneal endothelium may explain why only this tissue in the body is apparently so susceptible to disease in middle age. Additionally, intergenerational instability of triplet repeat expansion was not evaluated in this study but would be a relevant topic of investigation in the future.

Although patients with 2 expanded CTG18.1 alleles had a higher keratoplasty proportion and Krachmer grade, these values did not reach statistical significance. Our previous familial studies have shown that the expanded allele cosegregates with the FECD trait in most white pedigrees, compatible with an autosomal dominant trait with variable penetrance. Therefore, 1 expanded allele may be sufficient to cause the trait. Although our study indicates that there is no additive effect in the presence of 2 expanded alleles, larger studies are certainly warranted to investigate the effect of CTG18.1 expansion dosage on the clinical phenotype.
Conclusions

In this study, the mean Krachmer grade of disease severity was greater in FECD cases with the CTG18.1 triplet repeat expansion in TCF4 than in those without the repeat expansion. Additionally, the CTG triplet repeat allele length was positively correlated with the Krachmer grade of severity. The TCF4 triplet repeat expansion resulted in a more severe form of FECD, with clinical and surgical therapeutic implications.