ORIGINAL ARTICLE

E2-2 Protein and Fuchs's Corneal Dystrophy

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ABSTRACT

BACKGROUND

Fuchs's corneal dystrophy (FCD) is a leading cause of corneal transplantation and affects 5% of persons in the United States who are over the age of 40 years. Clinically visible deposits called guttae develop under the corneal endothelium in patients with FCD. A loss of endothelial cells and deposition of an abnormal extracellular matrix are observed microscopically. In advanced disease, the cornea swells and becomes cloudy because the remaining endothelial cells are not sufficient to keep the cornea dehydrated and clear. Although rare genetic variation that contributes to both early-onset and typical late-onset forms of FCD has been identified, to our knowledge, no common variants have been reported.

METHODS

We performed a genomewide association study and replicated the most significant observations in a second, independent group of subjects.

RESULTS

Alleles in the transcription factor 4 gene (*TCF4*), encoding a member of the E-protein family (E2-2), were associated with typical FCD ($P=2.3\times10^{-26}$). The association increased the odds of having FCD by a factor of 30 for persons with two copies of the disease variants (homozygotes) and discriminated between case subjects and control subjects with about 76% accuracy. At least two regions of the *TCF4* locus were associated independently with FCD. Alleles in the gene encoding protein tyrosine phosphatase receptor type G (*PTPRG*) were associated with FCD ($P=4.0\times10^{-7}$), but the association did not reach genomewide significance.

CONCLUSIONS

Genetic variation in *TCF4* contributes to the development of FCD. (Funded by the National Eye Institute and others.)

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Fuchs's corneal dystrophy (FCD) is a progressive, bilateral condition characterized by dysfunction of the corneal endothelium, leading to reduced vision. The prevalence of FCD has been estimated at about 5% among persons over the age of 40 years in the United States.[1] Corneal edema that is associated with FCD may progress after cataract surgery and is the most common indication for the 42,000 corneal transplantations that take place each year in the United States. Enthusiasm for refractive surgery is decreased among patients who are at high risk for FCD.[2] Thus, the ability to diagnose FCD before symptoms develop and knowledge of the biologic pathways leading to the disorder are important for the approximately 10 million patients worldwide who undergo refractive or cataract surgery each year.

The vision loss in patients with FCD results from a loss of corneal transparency associated with the irregularity of inner corneal layers in early disease and edema of the cornea in advanced disease. Ultrastructural features of FCD include loss and attenuation of endothelial cells, with thickening and excrescences of the underlying basement membrane.[3] These excrescences, called guttae, are the clinical hallmark of FCD and become more numerous with progression of the disease. As the endothelial layer develops confluent guttae in the central cornea, the cells are no longer able to keep the cornea dehydrated and clear (Fig. 1).

FCD is thought to be a genetically complex trait that has been observed in 38% of the first-degree relatives of probands.[4] The condition is often divided into early-onset familial disease and age-related disease. Early-onset FCD is rare and has been linked to mutations in the *COL8A2* gene, encoding the α_2 subunit of collagen VIII, a component of the endothelial basement membrane.[5] The two types of FCD may be different diseases, since guttae are not typically present in the early-onset type.[6]

Rare autosomal dominant mutations leading to age-related FCD have been found in three genes. FCD develops in patients with vitreoretinal degeneration due to mutations in the gene encoding potassium inwardly-rectifying channel subfamily J member 13 (*KCNJ13*).[7,8] Mutations in the sodium-borate cotransporter gene *SLC4A11* on chromosome 20p12 have been reported to cause FCD, and mutations in this gene were previously shown to cause congenital hereditary endothelial dystrophy, a very rare form of congenital corneal endothelial dysfunction.[9] Rare variation in the gene encoding zinc finger E-box binding homeobox 1 protein (*ZEB1*) on chromosome 10p11.2 was identified in 4 of 192 subjects with FCD.[10] Mutations in *ZEB1* had previously been found in posterior polymorphous corneal dystrophy, a clinically distinct and rare corneal endothelial disorder.[10] Variation in these genes does not seem to contribute substantively to the risk of FCD but does implicate these biologic pathways in the disease process.

Linkage studies have identified multiple chromosomal loci associated with common age-related FCD, including chromosomes 5q33.1–q35.2, 9p22.1–9p24.1, 13ptel–13q12.13, and 18q21.2–q21.32.[10-14] However, genetic variation contributing to FCD within these loci has not been identified. Since the genetic basis of only a small proportion of FCD cases is known, we performed a genomewide association study to identify loci contributing to typical age-related FCD.

METHODS

SUBJECTS

We recruited subjects for the discovery group from our clinics and examined their eyes, using specular biomicroscopy (Table 1). Subjects were considered to have FCD if the clinical description of the cornea was consistent with grade 1 guttae or higher on a published scale (ranging from 0 to 6, with 0 indicating no guttae and 6 indicating confluent guttae with corneal edema) or if either cornea had been transplanted because of FCD.[4] Subjects who were 60 years of age or older were considered to be controls if no guttae were observed. We excluded subjects with guttae graded as less than 1 or in whom the corneal endothelium could not be evaluated. All subjects reported being of European descent. The distribution of subjects according to Krachmer grade is presented in the Supplementary Appendix, available with the full text of this article at NEJM.org.

The study was conducted in accordance with the provisions of the Declaration of Helsinki, and the protocol was approved by the institutional review board at each institution. The study was conducted in accordance with the protocol as amended. All subjects provided written informed consent.

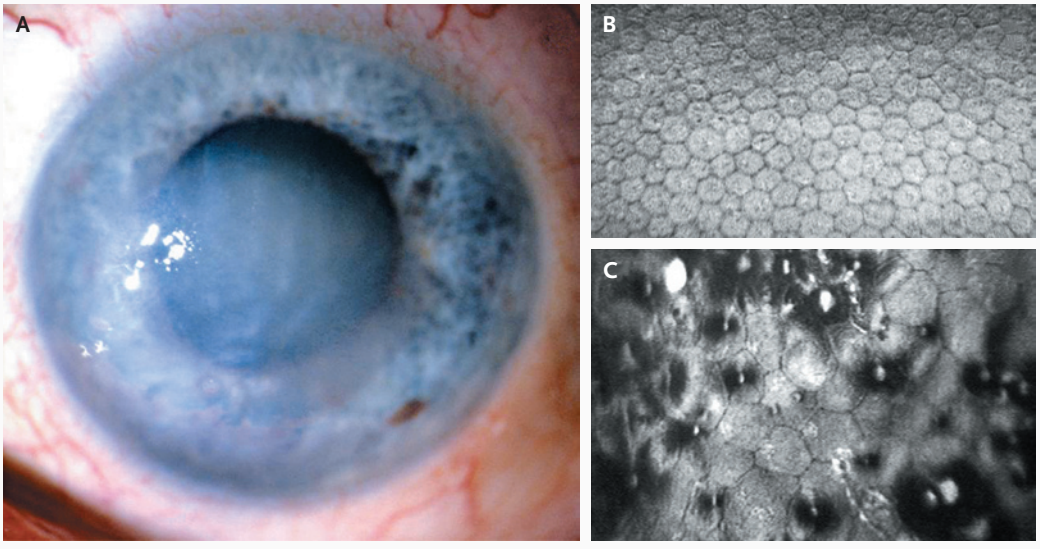

Figure 1. Features of Fuchs's Corneal Dystrophy (FCD).

In Panel A, a clinical photograph shows severe corneal edema caused by FCD, with an associated loss of corneal clarity. In Panel B, a confocal photomicrograph of the corneal endothelium in a control subject shows the normal appearance of the endothelial monolayer, with a regular mosaic of small, densely packed cells. In Panel C, a confocal photomicrograph of the corneal endothelium in a patient with FCD shows larger but fewer endothelial cells. The dark areas are subendothelial deposits called guttae.

GENOMEWIDE ASSOCIATION STUDY

Archived samples of genomic DNA were used to perform a collaborative genomewide association study.[15] The genotyping was performed at the Center for Inherited Disease Research with the use of age-related macular degeneration as the phenotype. We recoded the available subjects, described above, for FCD (Table 1). Each affected case subject was matched to two control subjects on the basis of age and sex. After applying quality-control filters, we evaluated the autosomal genotype distributions from the Illumina 370K Beadchip panel, using log-additive genotype models, as implemented in SAS software, version 9.1. Genotype distributions along the X chromosome were not associated with FCD, regardless of whether the minor allele was coded as 1, 1.5, or 2 copies in male subjects.

REPLICATION STUDIES

The single-nucleotide polymorphisms (SNPs) with the highest association with FCD were selected for a test of replication in an independent group of subjects. The replication group consisted of 150 case subjects, each of whom was matched to a single control subject according to age and sex. The genotyping was performed with the use of TaqMan assays (Applied Biosystems), as described previously.[16]

IMPUTATION, SINGLE SNP, AND HAPLOTYPE STUDIES

We imputed SNPs across loci associated with FCD, as described previously.[17] We performed single SNP analyses on genotype distributions, using imputed genotype dosage. Imputation is a method that allows use of the known pattern of coinheritance of SNPs (linkage disequilibrium) to predict the genotype of nearby variants that were not actually genotyped in the original genomewide association study. We calculated linkage disequilibrium (r^2) between SNPs, using the imputed dosage. We then performed haplotype studies, using the most likely imputed genotype and score.[18] Manual haplotype inspection was performed with Haploview, and three-SNP sliding-window approaches were implemented in haplo.stats with the use of R statistical software.[18,19] In the sliding-window approach, the overall association between haplotypes composed of the first three SNPs and disease is determined. The process is repeated for the second, third, and fourth SNPs and for each

Table 1. Association between Fuchs's Corneal Dystrophy and the Most Significant Single-Nucleotide Polymorphism on Chromosome 18 (rs613872 in Intron 3 of *TCF4*) among Case and Control Subjects.*

Variable	Discovery Group			Replication Group			Combined Group		
	Case (N=130)	Control (N=260)	P Value†	Case (N=150)	Control (N=150)	P Value†	Case (N=280)	Control (N=410)	P Value†
Allele			1.01×10^{-12}			1.79×10^{-13}			2.34×10^{-26}
T	0.63	0.86		0.57	0.85		0.60	0.85	
G	0.37	0.14		0.43	0.15		0.40	0.15	
Genotype			4.25×10^{-10}			2.51×10^{-9}			1.29×10^{-18}
TT	0.35	0.74		0.24	0.70		0.29	0.73	
GT	0.56	0.23		0.66	0.29		0.61	0.25	
GG	0.08	0.03		0.10	0.01		0.10	0.02	

* Case subjects were matched with control subjects on the basis of age and sex. The mean (±SD) age was 77±9 years in the discovery group and 74±8 years in the replication group. The male-to-female ratio was 0.34 in the discovery group and 0.44 in the replication group. The odds ratios for one copy of the risk allele (heterozygotes, GT) were 4.22 (95% confidence interval [CI], 2.69 to 6.63) in the discovery group, 8.90 (95% CI, 4.34 to 18.27) in the replication group, and 5.47 (95% CI, 3.75 to 7.99) in the combined group. The distributions are additive on the log scale. Thus, the odds ratio for two copies of the risk allele (homozygotes, GG) is the square of the odds ratio for heterozygotes.
† P values are for the comparison between case subjects and control subjects.

subsequent group of three contiguous SNPs. We performed conditional analyses of SNPs and haplotypes and estimated population attributable fractions, as described previously[20] (for details, see the Supplementary Appendix).

RESULTS

GENOMEWIDE ASSOCIATION AND REPLICATION STUDIES

The full set of results that were obtained through genomewide analysis is available in the Genotypes and Phenotypes database at www.ncbi.nlm.nih.gov/gap (accession number, phs000246.v1.p1). The genomewide analysis of specimens from the discovery group (Table 1) revealed one region that had a significant association with FCD (Fig. 2). This region spans the gene encoding transcription factor 4 (*TCF4*), which is located on chromosome 18q21.2. The association across this region was confirmed in an independent group of subjects (Table 1). Each additional risk allele of the SNP that was most highly associated with FCD (rs613872) increased the risk of disease by a factor of 5.47 (Table 1).

Ten additional genomic regions showed an association with FCD, but these associations were not significant at the genomewide level in the discovery set (threshold for significance, $P=5\times10^{-8}$). We tested 12 SNPs from these 10 regions for association in the replication set, using the selection criteria described in the Supplementary Appendix. Only one of these loci, which houses the gene encoding protein tyrosine phosphatase receptor type G (*PTPRG*), was also associated with FCD in the replication group. The association between FCD and *PTPRG* genotypes in the combined subject groups was strong ($P=1.30\times10^{-6}$) but did not reach genomewide significance (see Tables S4 and S5 in the Supplementary Appendix). Additional tests of replication are required to determine whether the association with *PTPRG* genotypes is valid.

SINGLE SNP ASSOCIATION ACROSS THE *TCF4* LOCUS

We genotyped five SNPs in the genomic region spanning *TCF4* in the replication group and confirmed the association with FCD (for details, see the "Replication studies" section and Tables S1 and S2 in the Supplementary Appendix). We used 152 SNPs that were genotyped across the *TCF4* locus in the original genomewide association study to impute a total of 720 SNPs in order to understand how the association with FCD varied across this region.

Figure 3 shows that many SNPs across the *TCF4* locus are associated with FCD. Furthermore, although many of the SNPs are highly correlated, the low coinheritance between rs613872 and some of the other associated SNPs suggests that multiple variants are independently associated with FCD. We confirmed this observation using stepwise re-

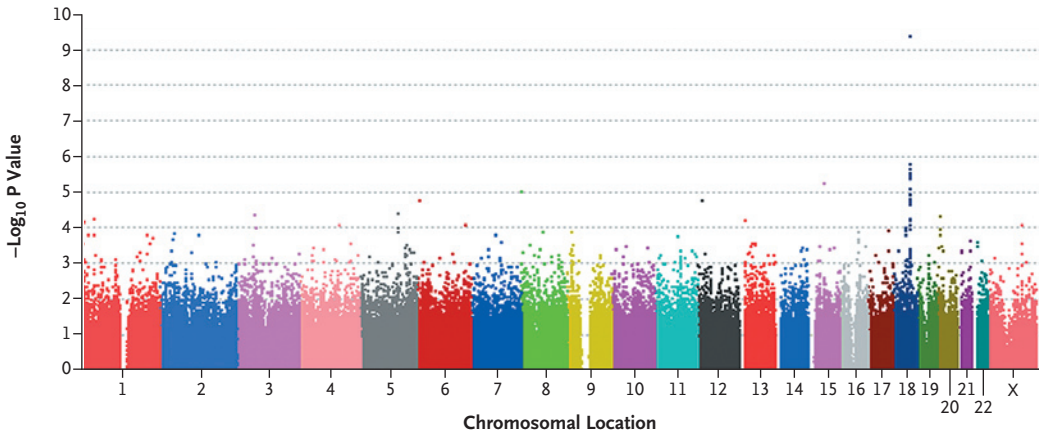

Figure 2. Association between 338,727 Single-Nucleotide Polymorphisms (SNPs) and Fuchs's Corneal Dystrophy (FCD).
The strength of association between individual SNPs and FCD across the genome is shown. The negative log of the P values of association between genotyped SNPs and FCD in the discovery group is plotted against chromosomal location. The association reaches genomewide significance at one region on chromosome 18 that spans the locus encoding transcription factor 4 (*TCF4*).

gression and implicated four SNPs as being independently associated with FCD (Table S5 in the Supplementary Appendix).

HAPLOTYPES ACROSS THE *TCF4* LOCUS

Genotypes from adjacent or nearby SNPs can be used to estimate the common chromosomal segments or haplotypes of DNA (as defined by variable markers) that are present in the population. These haplotypes help to identify regions along the *TCF4* locus that are independently associated with FCD. We observed that the haplotypes that were most strongly associated with FCD spanned exon 6 and that haplotypes from other regions of the locus were independently associated with FCD after conditioning (Fig. 4, and Tables S6, S7, and S8 and Fig. S5 in the Supplementary Appendix). The individual haplotypes that were most strongly associated with FCD ($P<1.0\times10^{-8}$) had a prevalence of 11 to 17% among case subjects and 1 to 4% among control subjects. Thus, uncommon haplotypes in multiple regions of *TCF4* had a large effect on the risk of FCD.

VARIATION ACROSS THE *TCF4* LOCUS AND FCD

We used the four genotyped SNPs (rs17595731, rs613872, rs9954153, and rs2286812) that were independently associated with FCD across the *TCF4* locus to construct a risk model, using the discovery group (for details, see the Supplementary Appendix). The model was based on multiple logistic-regression analyses with additive linear effects for each of the four SNPs. The risk model distinguished between case subjects and control subjects with 76% accuracy, with the use of the concordance statistic. Application of this model to the replication group produced a similar result, distinguishing case subjects from control subjects with 78% accuracy. In our preliminary analyses, the effect of *TCF4* variants on the risk of FCD appeared to increase with the severity of the disease.

DISCUSSION

We identified two regions of the genome that appear to contribute to FCD. The first region spans the *TCF4* locus and was significantly associated with FCD at the genomewide level. The second region, spanning the *PTPRG* locus, was strongly associated with FCD, but the association did not reach genomewide significance. Genetic variation across the *TCF4* locus may explain the linkage signal with FCD previously observed on chromosome 18q21.[11] The high impact on disease risk suggests that a pathway regulated by E2-2, the protein encoded by *TCF4*, is a major contributor to FCD. So far, we have not identified variation in the coding region of *TCF4* that is associated with FCD, and we do not know which genetic variation in this region directly drives the associations

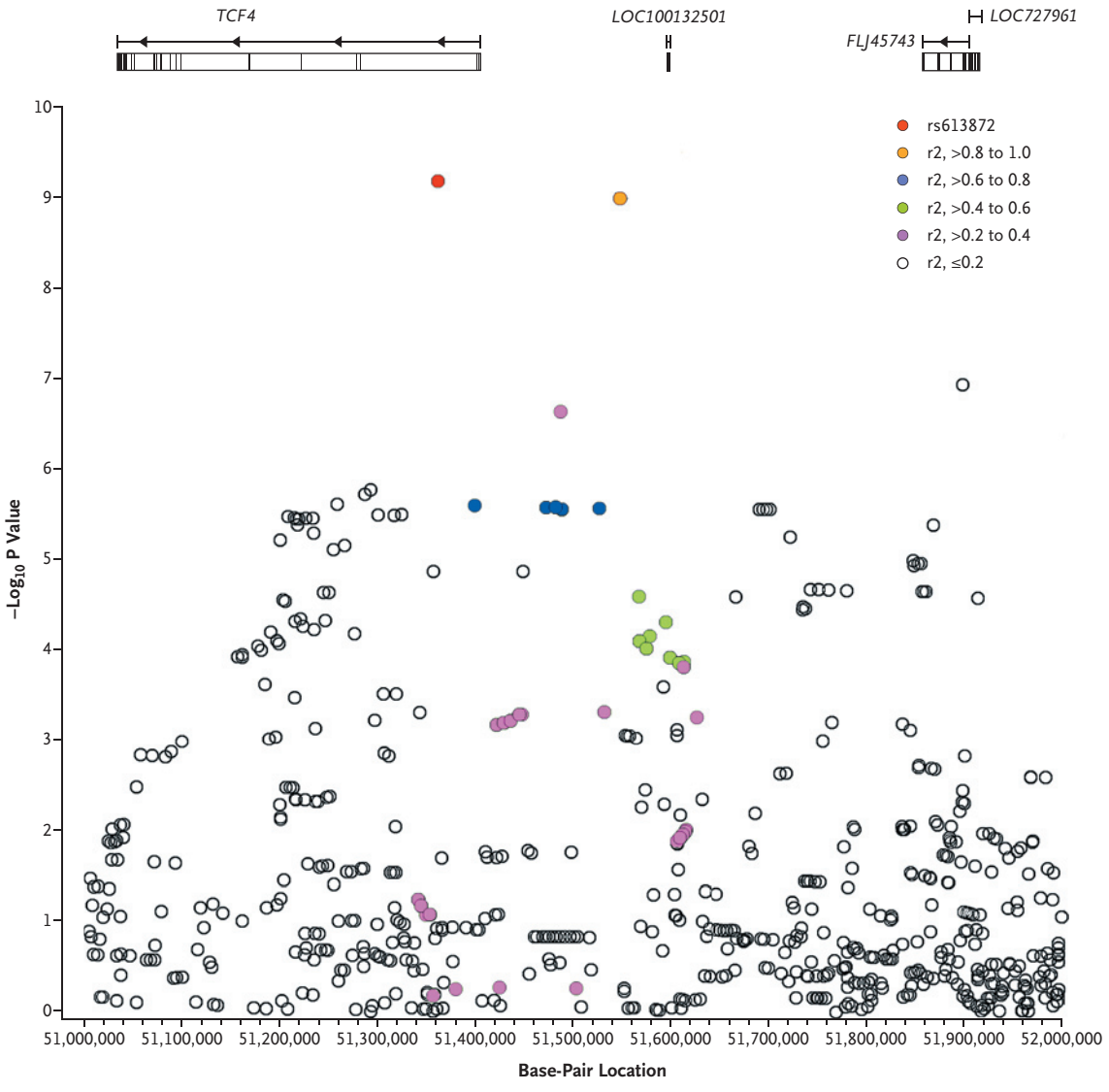

Figure 3. Association between Single-Nucleotide Polymorphisms (SNPs) and Fuchs's Corneal Dystrophy (FCD) across 1 Million Base Pairs of the Chromosome 18 Locus That Spans *TCF4*.

The top of the figure shows the intron–exon structure of *TCF4* and upstream hypothetical transcripts (e.g., FLJ45743). These transcripts do not contain open reading frames and are not thought to be functional. The circles show the level of association (y axis) and base-pair location (x axis) for each SNP. The colors show the extent of linkage disequilibrium (r^2) between the most highly associated SNP (rs613872) and the other 719 imputed SNPs across this region. The strong association between FCD and SNPs that are not highly correlated with rs613872 (open black circles) suggests that more than one variant may contribute to the risk of disease.

that we have observed. It is possible that risk is determined by noncoding variants that influence the forms or extent of expression of E2-2.

The protein encoded by *TCF4* has had many names but is usually referred to as E2-2. The protein product of the gene encoding transcription factor 7–like 2 (*TCF7L2*) is also called T-cell factor 4 (TCF4), which has created confusion in the literature. E2-2 is a member of the ubiquitously expressed class I basic helix–loop–helix (bHLH) transcription factors that are involved in cellular growth and differentiation.[21] Homodimers or het-

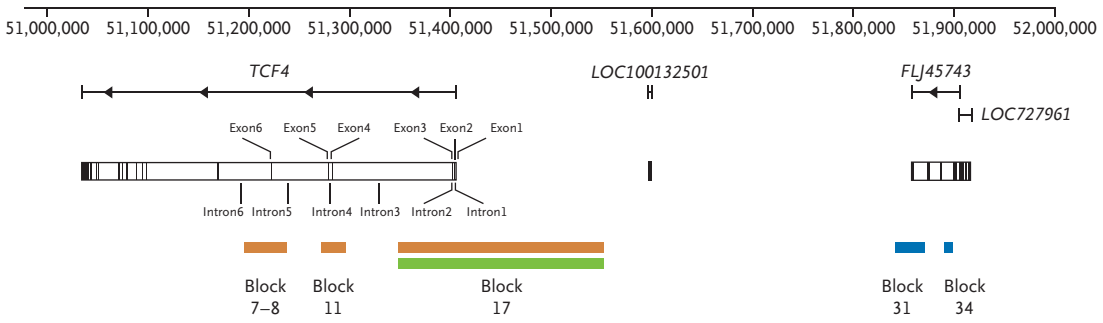

Figure 4. Regions along the *TCF4* Locus Showing an Independent Association with Fuchs's Corneal Dystrophy (FCD).

The location of introns and exons in the most common transcript from *TCF4* is shown at the top. Additional transcripts are shown, but these are not thought to represent genes at this time. The physical locations of the six haplotype blocks that contain individual haplotypes highly associated with FCD ($P<1.0\times10^{-8}$) are shown. Two groups of haplotype blocks (orange and blue) were independently associated with FCD. Haplotype block 17 (orange–green pattern) contains one haplotype that is highly correlated with the orange pattern and a second haplotype that appears to contribute to FCD independently of the other blocks. It is not known whether the block 31 and 34 regions (blue) regulate *TCF4* or have an effect on FCD that is independent of *TCF4*. The first six exons are absent in some splice variants of *TCF4* and do not encode a known protein domain. Additional information is available in the Supplementary Appendix.

erodimers with other classes of bHLH proteins bind Ephrussi (E-box) promoter sequences within target genes, either suppressing or activating tissue-specific gene transcription.[22-24] Loss of function of one copy of E2-2 through coding mutations or genomic deletions gives rise to the Pitt–Hopkins syndrome, a form of syndromic mental retardation characterized by seizures and episodic hyperventilation.[25] FCD is not known to develop in patients with this syndrome.

E2-2 is expressed in the developing corneal endothelium and is an attractive candidate for FCD (Fig. S6 in the Supplementary Appendix). E2-2 is implicated in both the development and suppression of cancer.[26] It represses the expression of the cell-adhesion protein E-cadherin, resulting in the loss of cellular polarity and cell-to-cell contact. E2-2 also has an important role in the epithelial-to-mesenchymal transition,[27] which is a normal aspect of cell migration in embryogenesis and in promoting tumor-cell invasion and metastasis.[27,28] Another protein (ZEB1) that is implicated in FCD also binds E-box promoter sites and is involved in the epithelial-to-mesenchymal transition through E-cadherin repression.[29] *ZEB1* expression is up-regulated by E2-2, and because mutations that inactivate ZEB1 are thought to contribute to FCD, these observations suggest the possibility that the *TCF4* variants associated with FCD could confer risk by altering the expression of *ZEB1*.

PTPRG belongs to the protein tyrosine phosphatase (PTP) family, members of which regulate a wide array of cellular functions, including growth, differentiation, cell mobility, gene expression, cellular adhesion, ion-channel control, and oncogenesis.[30-34] Ligands regulate the phosphatase activity of PTPRG by binding to its extracellular domain.[35] It has yet to be determined whether PTPRG interacts with ZEB1 or E2-2.

The biologic pathways that are implicated by the contribution of *TCF4* and probably *ZEB1* and *PTPRG* variants to typical FCD suggest several mechanisms of pathogenesis. One mechanism could be a decrease in the number of endothelial cells through senescence, deficient proliferation, or migration. The diminished proliferation of progenitor cells and premature senescence observed in a mouse model of *Zeb1* deficiency[36] could lead to a loss of corneal endothelial cells, as seen in patients with FCD. If the variants in the *TCF4* locus that are associated with FCD decrease ZEB1 activity, perhaps early senescence or muted formation of progenitor cells gives rise to disease. However, the human corneal endothelium is traditionally considered a nonreplicating tissue layer, and this hypothesis does not explain the extracellular deposits that characterize FCD.[37] Nevertheless, there is some evidence of corneal endothelial proliferation in patients with FCD, such as the expression of stem-cell markers and mitotic figures in the corneal periphery.[38,39] Alternatively, increased mesenchymal characteristics, with enhanced extracellular matrix deposition, would probably ensue if the risk variants increased E2-2 activity.

Another mechanism could be increased cellular stress through abnormal development of the basement membrane, abnormal ion-channel regulation, or premature senescence. Support for the role of these processes comes from observations of increased endoplasmic reticulum stress, the unfolded-protein response, markers of senescence, and apoptosis in the corneal endothelium in patients with FCD.[40-42]

In conclusion, our findings suggest that genetic variation in the *TCF4* locus substantively contributes to the risk of FCD. The genetic risk appears to localize to multiple regions of the *TCF4* locus. The prevalence of the risk haplotypes is relatively low (a few to several percent), but the presence of these haplotypes confers a high risk of FCD.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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