CTG18.1 Expansion is the Best Classifier of Late-Onset Fuchs’ Corneal Dystrophy Among 10 Biomarkers in a Cohort From the European Part of Russia

Liubov O. Skorodumova,1 Alexandra V. Belodedova,2 Olga P. Antonova,2 Elena I. Sharova,1 Tatiana A. Akopian,1 Oksana V. Selezneva,1 Elena S. Kostryukova,1 and Boris E. Malyugin2

1Federal Research and Clinical Center of Physical-Chemical Medicine, Moscow, Russia
2S. Fyodorov Eye Microsurgery Complex Federal State Institution, Ministry of Health of Russian Federation, Moscow, Russia

Correspondence: Liubov O. Skorodumova, Laboratory of Human Molecular Genetics, Federal Research and Clinical Center of Physical-Chemical Medicine, 1a Malaya Pirogovskaya Street, Moscow, 119435, Russia; l.skorodumova@rcpcm.org.

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Purpose. To assess the occurrence and diagnostic performance of nine single-nucleotide variants (SNVs) in the TCF4, SLC4A11, LOXHD1, and AGBL1 genes and the CTG18.1 trinucleotide repeat expansion in a Russian cohort of Fuchs’ endothelial corneal dystrophy (FECD) patients.

Methods. This retrospective case-control study included 100 patients diagnosed with FECD (cases) and 100 patients with cataracts (controls). Blood DNA was used to perform PCR and subsequent Sanger sequencing of rs613872 and rs17595731 in TCF4, c.79-100delTC, rs267607065, rs267607064, and rs267607066 in SLC4A11, rs113444922 in LOXHD1, and rs181958589 and rs185919705 in AGBL1. The number of CTG18.1 trinucleotide repeats was determined by a combination of conventional PCR or triplet primed PCR with fragment analysis.

Results. At least one rs613872 marker allele was found in 78% of FECD patients and 21% of controls, and at least one rs17595731 marker allele was found in 14% and 2%, respectively. CTG18.1 trinucleotide expansion (>40 repeats) was detected in 72% of FECD patients and 5% of controls. Marker alleles of the tested SNVs in SLC4A11, LOXHD1, and AGBL1 were not found in our FECD cohort. One FECD patient carried the marker allele of the rs181958589 SNV. Analysis of the diagnostic performance of individual markers in TCF4 and their combinations showed that the CTG18.1 repeat expansion was the best classifier for FECD (AUC = 0.84).

Conclusions. Patients carrying CTG18.1 repeat expansion constituted a high proportion of the Russian FECD cohort; therefore, this marker is suitable for development of diagnostic and therapeutic approaches.

Keywords: corneal dystrophy, Fuchs dystrophy, TCF4, molecular markers, microsatellite expansion
polymorphism (SNP) rs613872 with FECD was discovered in the Genome-Wide Association Study (GWAS) performed by Baratz et al.12 Later, this association was confirmed in a number of studies involving more than a thousand people.13-21 Other SNPs in the TCF4 gene are associated with FECD but to a lesser extent, namely: rs17595731, rs9954153, and rs2286812.12,14,15 Wieben et al.12 found another important relationship between FECD and the expansion of trinucleotide repeats in the TCF4 gene intron CTG18.1: The expansion of trinucleotide repeats appeared to be a more specific marker of FECD than rs613872 (96% vs. 79%). Mootha et al.18 have shown the segregation of appeared to be a more specific marker of FECD than rs613872 (96% vs. 79%). Mootha et al.18 have shown the segregation of appeared to be a more specific marker of FECD than rs613872 (96% vs. 79%). Mootha et al.18 have shown the segregation of appeared to be a more specific marker of FECD than rs613872 (96% vs. 79%). Mootha et al.18 have shown the segregation of the expansion of CTG18.1 repeats.23,27 Thus, on the basis of its high specific and sensitivity, the expansion of CTG18.1 trinucleotide repeats in the TCF4 gene intron is the most promising marker and potent driver of FECD so far.

In this study, for the first time, we simultaneously analyzed the occurrence of 10 genetic variants reported to be associated with sporadic late-onset FECD in a European Russia cohort. These variants included the SNPs rs613872 and rs17595731 and the CTG18.1 trinucleotide repeat expansion in TCF4, four variants in the SLC4A11 gene (namely, c.99-100delTC, rs267607065, rs267607064, and rs267607066), a causal mutation in the LOXHD1 gene (namely, rs113444922), and two mutations in the ABG1L gene (rs181958589 and rs185919705). In addition, the individual and combined specificity of TCF4 gene markers were estimated for Russian FECD patients.

MATERIALS AND METHODS

Ethical Statements

This study was approved by the Institutional Review Boards of S. Fyodorov Eye Microsurgery Complex Federal State Institution (FEMCFSI) and was performed in compliance with the tenets of the Declaration of Helsinki.

FECD Patients and Control Subjects

Patients recruited in the FEMCFSI were those with complaints on decreased visual acuity associated with the development of age-related cataracts and patients referred for consultation with pseudophakia or endothelial corneal dystrophy after cataract surgery. Patients were diagnosed with FECD based on the results of thorough ophthalmic examination with special focus on corneal biomicroscopy. The main subjective complaints of the patients were vision decrease and glare. Vision fluctuations were also characteristic for the early FECD with blurred vision typically occurring in the morning hours. Anterior segment slit lamp examination (SL-50; Opton, Munich, Germany) was performed in all cases. Presence of the corneal guttae was the main diagnostic criteria. We checked for biomicroscopy evidence of corneal guttae and their extent, as well as the signs of epithelial and stromal edema. Advanced disease was characterized by the presence of clinically obvious corneal edema as well as the epithelial and sub epithelial bullae. Central corneal thickness was assessed with optical coherent tomography (Visante OCT; Carl Zeiss, Jena, Germany). It is known that corneal pachymetry measures are of limited utility given the wide variation of corneal thickness in normal subjects. However, we used the central corneal thickness threshold of 640 microns for consideration to perform combined procedures (cataract phacoemulsification, IOL implantation, and endothelial keratoplasty) as it was recommended by Seitzman et al.28 Mean central endothelial cells density, pleomorphism, and polymegathism as well as the presence of guttae, were evaluated using confocal microscopy (Confoscan 4; Nidek, Aichi, Japan).

FECD stage was scored according to the Volkov and Dronov classification (1978).29 The latter stratifies the disease into 5 stages: I - endothelial (endothelial changes appear as centrally located single or confluent guttae), II - stromal (development of edema in the stroma and corneal epithelium), III - epithelial (bullaous), IV - neovascular, and V - terminal (fibrotic). Patients diagnosed with FECD (≥45 years old) were included in the FECD group (n = 100). All of the FECD group participants were from the European part of Russia. Most were from the Central Federal District (n = 88), others were residing in Southern Federal District (n = 7) and Volga Federal District (n = 5).

Thirty-five FECD patients had undergone cataract phacoemulsification with intraocular lens (IOL) implantation. In 23 FECD patients, endothelial keratoplasty (DMEK or DSAEK) was performed. In 31 patients, simultaneous phacoemulsification, IOL implantation, and endothelial keratoplasty were carried out. Central descemetoherisis without endothelial replacement was performed in 11 FECD patients.

The control group (n = 100) was recruited among patients referred for routine phacoemulsification and IOL implantation of age-related cataracts (≥45 years old). Patients with severe eye comorbidity (glaucoma, medium- to high-degree myopia, retinal or corneal dystrophy, etc.) were not included. Acceptable comorbidities included mild myopia, pseudoxflio- lysis syndrome, and cataracts in patients <65 years old. During the preparation for the planned surgical procedure, the following examinations were carried out in the control group: refractometry, visual acuity measurement with and without correction, perimeter, tonometry, biomicroscopy, ophthalmoscopy, and ultrasonic and optical biometry. All patients from the control group were from the European part of Russia: the Central (n = 97), the Northwestern (n = 1), the North Caucasian (n = 1), and the Volga Federal Districts (n = 1).

Surgery was carried out before genetic studies for all participants. Venous blood (4–6 mL) was collected from each participant in Vacutainer tubes with EDTA (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were stored at −20°C prior to the genetic study.

DNA Extraction

DNA was isolated from thawed blood samples with the Wizard Genomic DNA Purification Kit (Promega Corp., Fitchburg, WI, USA) or Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA was resuspended in a low TE buffer to a final concentration of 10 ng/μl.

Genotyping

Primers for the DNA regions of interest were designed and tested for specificity using Premier Primer programs (Premier Biosoft) and Primer-BLAST (http://imagej.nih.gov/ij/; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, USA; Table 1).30 Primer sequences for trinucleotide analysis CTG-P3, CTG-P4 were taken from a relevant publication.18

SNVs were genotyped using Sanger sequencing of PCR products. Gene Pak PCR MasterMix Core kit (IsoGene Lab. Ltd., Moscow, Russia) was used for amplification under the following conditions: reaction volume of 20 μl, DNA input of 50 ng, and final primer concentration of 0.5 μM each. Sequencing was conducted using the BigDye Terminator v3.1
null hypothesis was rejected at a significance level of 0.05. Surgical procedures were divided into those that involved the corneal endothelium (DSAEK, DMEK or descemetorhexis) or not (phacoemulsification with IOL implantation of age-related cataracts alone).

**Results**

**Demographics of Study Participants**

We included 100 unrelated patients with sporadic late-onset FECD and 100 unaffected control subjects from the European part of Russia (Table 2). The mean age of the control group was higher in order to reduce the number of patients in whom FECD had not yet manifested, as done by Kuot et al.\textsuperscript{14} According to the Volkov and Dronov classification, 53 FECD patients had grade I, 20 were graded as II, 43 as grade III, and four as grade IV. None of the patients from the FECD cohort had grade V.

**Genotyping of TCF4 Gene Variants**

All of the participants from the FECD and control groups were genotyped for three markers in TCF4: rs613872, rs17595731, and the CTG18.1 trinucleotide repeat expansion. The occurrences of heterozygous and homozygous genotypes of the analyzed variants in TCF4 are provided in Table 3. Table 4 shows data on the distribution of the patients according to the Volkov and Dronov classification and their CTG18.1 expansion status. Primary data on repeat sizing is shown in Figure 1. Data on the number of CTG18.1 trinucleotide repeats in FECD and control cohort are summarized in Figure 2.
Genotyping of SLC4A11, LOXHD1 and AGBL1 Variants in FECD Patients

Variants in the SLC4A11, LOXHD1, and AGBL1 genes were assessed in 100 FECD patients. None of the four SLC4A11 gene variants (c.99-100delTC, rs267607065, rs267607064, or rs267607066) were detected in this investigated group of Russian FECD patients. The same was found with the rs113444922 variant in LOXHD1 and rs185919705 in AGBL1. Among the investigated variants, we found only one heterozygous rs181958589 genotype in one patient. This patient also had one expanded CTG18.1 allele and one marker allele in rs613872.

Diagnostic Performance of TCF4 Gene Variants

We evaluated the diagnostic performance of rs613872 and rs17595731 and the CTG18.1 trinucleotide repeat expansion in this Russian cohort using the main diagnostic parameters. As all three variants were genotyped in each study participant, we could assess not only individual markers but also their combinations. A patient was considered to have a marker signal if he or she had at least one marker (expanded) allele.

<p>| TABLE 3. TCF4 Gene Variants rs613872 and rs17595731 and the CTG18.1 Expansion Occurrence in the Cohort of Patients From the European Part of Russia |</p>
<table>
<thead>
<tr>
<th>Variant</th>
<th>Genotype</th>
<th>FECD Patients, n (%)</th>
<th>Controls, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs613872</td>
<td>G/G</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>G/T</td>
<td>70</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>22</td>
<td>79</td>
</tr>
<tr>
<td>rs17595731</td>
<td>G/G</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G/C</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>86</td>
<td>98</td>
</tr>
<tr>
<td>CTG18.1</td>
<td>X/X*</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>X/S†</td>
<td>67</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>S/S</td>
<td>28</td>
<td>95</td>
</tr>
</tbody>
</table>

* X = expanded allele.
† S = short allele.

<p>| TABLE 4. Distribution of the FECD Patients Grade and CTG18.1 Trinucleotide Repeat Expansion Status |</p>
<table>
<thead>
<tr>
<th>FECD Grade (Volkov &amp; Dronov Classification)</th>
<th>CTG18.1 Expansion Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Nonexpanded CTG18.1</td>
<td>14</td>
</tr>
<tr>
<td>Expanded CTG18.1</td>
<td>19</td>
</tr>
</tbody>
</table>

rs113444922 variant in LOXHD1 and rs185919705 in AGBL1. Among the investigated variants, we found only one heterozygous rs181958589 genotype in one patient. This patient also had one expanded CTG18.1 allele and one marker allele in rs613872.
Data on the sensitivity (SE), specificity (SP), accuracy (ACC), positive predictive value (PPV), negative predictive value (NPV), area under the curve (AUC), balanced accuracy (BAD), odds ratio (OR) and risk ratio (RR) are listed in Table 5.

The highest individual sensitivity was with the rs613872 marker and the lowest was with the rs17595731 marker. The combination of rs613872 and rs17595731 provided the highest overall sensitivity. The most specific marker was rs17595731, but it had the lowest accuracy. Among the individual markers, CTG18.1 had the highest accuracy, PPV, AUC, BAD, OR, and RR. No combination of TCF4 gene markers improved the values of these complex parameters.

We evaluated the association of the CTG18.1 repeat status (expanded or nonexpanded) with the type of surgical procedure (whether it involved corneal endothelium), as the CTG trinucleotide repeat expansion status in patients with FECD can increase the likelihood of corneal transplantation.33,34 We found a tendency between the involvement of corneal endothelium in the surgical procedure and the CTG trinucleotide repeat expansion status in our FECD cohort, but it did not reach the level of significance (Table 6). We did not find an association between the FECD grade and CTG trinucleotide repeat expansion status.

DISCUSSION

The prevalence of markers of late-onset FECD may vary among different ethnicities. Thus, it is vital to determine the proportion of patients carrying each marker in an unexplored population before the development of optimal diagnostic or therapeutic approaches. The Russian Federation is a multiethnic country; thus, it is important to understand not only the occurrence of markers in some ethnic group but also to have information about the background population of all ethnicities. A similar situation was found in studies on the occurrence of FECD markers in the American population: in some studies, the ethnicity was not specified.16,17,19,35 FECD patients hospitalized at the FEMCFSI are mostly from the Central, Southern, and Volga Federal Districts, and thus, these were included in our study. All of these Federal Districts are situated in the European part of Russia, so we specified that we investigated the occurrence of FECD markers in the background population of this part of Russia.

We defined the occurrences of 10 genetic variants in the TCF4, SLC4A11, LOXHD1, and AGBL1 genes in 100 late-onset sporadic FECD patients from the European part of Russia to understand what marker or combination of markers can be the
The most frequent genetic variants of FECD were rs613872 and CTG18.1 in the TCF4 gene. All of the samples with expanded CTG18.1 allele also had at least one G allele of rs613872. This is in agreement with the haplotype analysis of this two variants reported by Mootha et al.18 According to complex diagnostic parameters, CTG18.1 trinucleotide repeat expansion occurred to be more specific marker than rs613872. This was also the case in the publications previously mentioned here.18,22 According to complex diagnostic parameters, CTG18.1 trinucleotide repeat expansion is considered to be the best classifier among all tested markers of late-onset FECD in the patients from the European part of Russia. Thus, the determination of CTG18.1 trinucleotide repeat expansion is suitable for a diagnostic test design. The development of antisense therapy against transcripts containing expanded CTG18.1 repeats may be helpful for this population.24,26

The most distinct difference in the occurrence of FECD markers is for TCF4 variants between American/European populations and Indian, Chinese, and Japanese populations (Table 7). Based on the results of our study, we can conclude that the occurrence of marker alleles in TCF4 gene variants in patients from the European part of Russia is very close to those of American and European FECD patients. It would be of great interest to investigate the occurrence of TCF4 markers in the FECD patients from all other Federal Districts of Russia.

Pathogenic variants in AGBL1 were found within the expected frequency of 1% to 2% suggested by Riazuddin et al.8 The investigated pathogenic SLC4A11 variants were estimated to occur in 4% of FECD patients and the LOXHD variants in 1% to 2%.7,45,44 However, they were absent in this cohort of Russian FECD patients. Marker alleles were not found in 21 FECD patients; thus, exome sequencing for those particular patients is warranted.

**References**


