

# Mitochondrial Polymorphism A10398G and Haplogroup I Are Associated With Fuchs' Endothelial Corneal Dystrophy

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Submitted: October 28, 2013

Accepted: May 26, 2014

Citation: Li YJ, Minear MA, Qin X, et al; for the FECD Genetics Consortium. Mitochondrial polymorphism A10398G and Haplogroup I are associated with Fuchs' endothelial corneal dystrophy. *Invest Ophthalmol Vis Sci*. 2014;55:4577-4584. DOI:10.1167/iov.13-13517

**PURPOSE.** We investigated whether mitochondrial DNA (mtDNA) variants affect the susceptibility of Fuchs endothelial corneal dystrophy (FECD).

**METHODS.** Ten mtDNA variants defining European haplogroups were genotyped in a discovery dataset consisting of 530 cases and 498 controls of European descent from the Duke FECD cohort. Association tests for mtDNA markers and haplogroups were performed using logistic regression models with adjustment of age and sex. Subset analyses included controlling for additional effects of either the *TCF4* SNP rs613872 or cigarette smoking. Our replication dataset was derived from the genome-wide association study (GWAS) of the FECD Genetics Consortium, where genotypes for three of 10 mtDNA markers were available. Replication analyses were performed to compare non-Duke cases to all GWAS controls (GWAS1,  $N = 3200$ ), and to non-Duke controls (GWAS2,  $N = 3043$ ).

**RESULTS.** The variant A10398G was significantly associated with FECD (odds ratio [OR] = 0.72; 95% confidence interval [CI] = [0.53, 0.98];  $P = 0.034$ ), and remains significant after adjusting for smoking status (min  $P = 0.012$ ). This variant was replicated in GWAS1 ( $P = 0.019$ ) and GWAS2 ( $P = 0.036$ ). Haplogroup I was significantly associated with FECD (OR = 0.46; 95% CI = [0.22, 0.97];  $P = 0.041$ ) and remains significant after adjusting for the effect of smoking (min  $P = 0.008$ ) or rs613872 ( $P = 0.034$ ).

**CONCLUSIONS.** The 10398G allele and Haplogroup I appear to confer significant protective effects for FECD. The effect of A10398G and Haplogroup I to FECD is likely independent of the known *TCF4* variant. More data are needed to decipher the interaction between smoking and mtDNA haplogroups.

**Keywords:** mitochondrial haplogroup, genetic association, oxidative stress, *TCF4*, smoking

Fuchs endothelial corneal dystrophy (FECD), first described by Fuchs in 1910,<sup>1</sup> is characterized by progressive and nonregenerative loss of corneal endothelium. As endothelial cells significantly diminish as the disease advances, patients develop corneal edema with a loss of corneal clarity and progressively lose visual acuity.<sup>1-4</sup> This disorder affects as many as 4% of the United States population over the age of 40 years,<sup>5,6</sup> and it occurs predominantly in women, who comprise approximately 75% of cases.<sup>7,8</sup> While the pathogenesis of FECD remains poorly understood, genetic and environmental factors are thought to contribute to its causation and severity.<sup>6,9-12</sup>

Genetic predisposition is considered the best predictor for FECD given that many FECD cases show familial clustering.<sup>8,13</sup> To date, genetic studies of FECD have reported mutations in *COL8A2* (multiple interval mapping [MIM], 120252; chr1p34.3), *SLC4A11* (MIM, 610206; chr20p13), *ZEB1* (also known as *TCF8*; MIM, 609141; chr10p11.22), *LOXHD1* (MIM, 613072;

chr18q21.1), and *AGBL1* (MIM, 615496; chr15q25.3),<sup>14-21</sup> as well as the association of a single nucleotide polymorphism (SNP) rs613872 in transcription factor 4 (*TCF4*; MIM, 602272; chr18q21.2) with FECD.<sup>22</sup> In particular, the association between rs613872 and FECD has been replicated by multiple studies.<sup>23-27</sup> In contrast to the significant evidence implicating a genetic component to FECD, knowledge of environmental risk factors for the disease is limited. In the Reykjavík eye study, Zoega et al.<sup>28</sup> evaluated environmental risk factors associated with the formation of cornea guttae, the key diagnostic criterion for FECD, and reported that smoking more than 20 pack-years increased the risk of corneal guttae formation, whereas higher weight and body mass index decreased the risk of corneal guttae. Recently, Zhang et al.<sup>12</sup> reported that smoking history increases the risk for advanced FECD (grades 4-6). Together, these studies suggest that cigarette smoking may be a potential environmental risk factor for FECD.

**TABLE 1.** Demographic Data for FECD Cases and Controls for the Discovery and Replication Datasets

| Dataset           | Description  | Cases        | Controls      | Total         |
|-------------------|--|--------------|---------------|---------------|
| Discovery         |  |              |               |               |
| Discovery dataset | All FECD cases   | 530          | 498*          | 1028          |
|                   | Female, <i>n</i> (%)                                       | 379 (71.51%) | 282 (56.63%)  | 661 (64.30%)  |
|                   | Age, mean (SD)   | 69.5 (11.0)  | 65.3 (10.0)   | 67.5 (10.7)   |
| Grade3+           | FECD cases with grade $\geq 3$                             | 457          | 498           | 955           |
|                   | Female, <i>n</i> (%)                                       | 332 (72.65%) | 282 (56.63%)  | 614 (64.29%)  |
|                   | Age, mean (SD)   | 70.0 (10.8)  | 65.3 (10.0)   | 67.5 (10.7)   |
| <i>TCF4</i>       | Subjects with <i>TCF4</i> rs613872 genotype data available | 529          | 494           | 1023          |
| Ever smoker       | Status of current or past smoking                          | 435          | 428           | 863           |
| Current smoker    | Status of smoking at the time of enrollment                | 234          | 119           | 353           |
| Replication       |  |              |               |               |
| GWAS1             | CWRU cases vs. all GWAS controls                           | 857          | 2343          | 3200          |
|                   | Female, <i>n</i> (%)                                       | 565 (65.93%) | 1353 (57.75%) | 1918 (59.94%) |
|                   | Age, mean (SD)   | 70.6 (10.4)  | 69.3 (6.0)    | 69.6 (7.5)    |
| GWAS2             | CWRU cases vs. non-Duke controls                           | 857          | 2186          | 3043          |
|                   | Female, <i>n</i> (%)                                       | 565 (65.93%) | 1271 (58.14%) | 1836 (60.34%) |
|                   | Age, mean (SD)   | 57.6 (10.4)  | 69.2 (5.8)    | 69.6 (7.4)    |

\* 337 samples from Duke POAG Genetics group.

Aside from nuclear DNA, few studies have examined the role of mitochondrial DNA (mtDNA) in the pathogenesis of FECD.<sup>11,29-31</sup> In a serial analysis of gene expression (SAGE) study of donor corneal endothelial cells,<sup>29,30</sup> several mtDNA genes were found to be the most abundantly expressed genes among all expressed genes identified (table 3 in the study of Gottsch et al.<sup>30</sup>). Another SAGE comparison between healthy donor corneas and FECD-affected corneas concluded there were more downregulated genes than upregulated genes in FECD corneas. Particularly, mitochondrial transcripts account for the majority of the downregulated genes, including cytochrome b, and NADH dehydrogenase subunits 1, 2, and 4 (Table 3 in the study of Gottsch et al.<sup>29</sup>). A case study of an FECD patient with other health complications, such as sensorineural hearing loss, diabetes, cardiac conduction defects, ataxia, and hyperreflexia, identified missense substitutions in lymphocyte mtDNA at mt15257 (G to A, in the cytochrome b subunit of complex III) and mt4216 (T to C, in

the ND1 subunit of complex I).<sup>32</sup> Jurkunas et al.<sup>11</sup> reported a set of significantly down-regulated antioxidant genes, including superoxide dismutase 2 (*SOD2*) in mitochondria, expressed in the corneal endothelium of FECD. Therefore, they proposed that the mitochondrial genome may be a specific target for oxidative stress in FECD, and that it may be involved in the pathogenesis of FECD.

Human mtDNA, a circular molecule, encodes 37 mitochondrial genes, which include 22 transfer RNAs (tRNAs), 2 ribosomal RNAs, and 13 protein-coding genes for respiratory chain subunits that are essential for cellular energy production.<sup>33</sup> As a byproduct of energy production, mitochondria also generate most of the endogenous reactive oxygen species (ROS) of the cell.<sup>33</sup> Therefore, mtDNA is particularly susceptible to oxidative damage and has a higher mutation rate than nuclear DNA due to its proximity to high ROS production and its limited DNA-repair capacity.<sup>34</sup> Additionally, corneal endothelial cells contain a large number of mitochondria<sup>35</sup> to

**TABLE 2.** SNPs Used to Define Nine European mtDNA Haplogroups

| Marker, Location, and Haplogroup |           |            |           |           |           |             |             |           |            |             |
|----------------------------------|-----------|------------|-----------|-----------|-----------|-------------|-------------|-----------|------------|-------------|
| mtDNA SNP                        | G1719A    | G4580A     | T7028C    | G8251A    | G9055A    | A10398G     | A12308G     | G13368A   | A13708G    | G16391A     |
| SNP rsID                         | rs3928305 | rs28357975 | rs2015062 | rs3021089 | -         | rs2853826   | rs2853498   | rs3899498 | rs28359178 | rs34301918  |
| Gene locus                       | 16S rRNA  | ND2        | COI       | COII      | ATPase6   | ND3         | tRNA Leu    | ND5       | ND5        | D-loop      |
| Corresponding SNP in array*      | -         | -          | -         | -         | -         | MitoG10399A | MitoA12309G | -         | -          | MitoG16393A |
| Amino acid                       | -         | Met        | Ala       | Gly       | Ala > Thr | Thr > Ala   | -           | Gly       | Ala > Thr  | -           |
| Synonymous?                      | -         | Yes        | Yes       | Yes       | No        | No          | -           | Yes       | No         | -           |
| H                                | -         | -          | C         | -         | -         | A           | -           | -         | -          | -           |
| I                                | A         | -          | T         | A         | -         | G           | -           | -         | -          | A           |
| J                                | -         | -          | T         | -         | -         | G           | -           | -         | A          | -           |
| K                                | -         | -          | T         | -         | A         | G           | G           | -         | -          | -           |
| T                                | -         | -          | T         | -         | -         | A           | -           | A         | -          | -           |
| U                                | -         | -          | T         | -         | -         | A           | G           | -         | -          | -           |
| V                                | -         | A          | T         | -         | -         | A           | -           | -         | -          | -           |
| W                                | -         | -          | T         | A         | -         | A           | -           | -         | -          | -           |
| X                                | A         | -          | T         | -         | -         | A           | -           | -         | -          | -           |

\* SNPs available in the Illumina HumanOmni 2.5-4 array bead chip. The map position is off for one to two base pairs from the conventional designation because the Homo\_sapiens\_MT assembly was used in array bead chip instead of GRCh37.p10, which was used for the conventional mitochondrial haplogroup markers (refer to the NCBI dbSNP database). rsID, reference sequence ID in dbSNP; -, not applicable. Haplogroup SNP definitions taken from the study of Torroni et al.<sup>37</sup>

TABLE 3. Haplogroup Frequencies in Discovery Dataset

| Haplogroup | FECD,<br>N = 530<br>N (%) | Controls,<br>N = 498<br>N (%) | Total,<br>N = 1028<br>N (%) |
|------------|---------------------------|-------------------------------|-----------------------------|
| H          | 220 (41.5)                | 195 (39.2)                    | 415 (40.4)                  |
| I          | 12 (2.3)                  | 24 (4.8)                      | 36 (3.5)                    |
| J          | 42 (7.9)                  | 49 (9.8)                      | 91 (8.9)                    |
| K          | 30 (5.7)                  | 43 (8.6)                      | 73 (7.1)                    |
| T          | 69 (13.0)                 | 52 (10.4)                     | 121 (11.8)                  |
| U          | 81 (15.3)                 | 71 (14.3)                     | 152 (14.8)                  |
| V          | 17 (3.2)                  | 16 (3.2)                      | 33 (3.2)                    |
| W          | 19 (3.6)                  | 15 (3.0)                      | 34 (3.3)                    |
| X          | 16 (3.0)                  | 7 (1.4)                       | 23 (2.2)                    |
| Others*    | 24 (4.5)                  | 26 (5.2)                      | 50 (4.9)                    |

\* Others, Other 10 marker combinations not defined by the nine European haplogroups.

generate the energy required for the active transport of water and ions from the stroma to maintain the corneal deturgescence required for visual acuity.<sup>36</sup> Therefore, it is plausible that these cells generate high levels of ROS, leading to increased oxidative stress.

Since mtDNA is transmitted maternally without undergoing recombination, it has been used widely in evolutionary studies. As a result, mtDNA haplogroups have been well established for different ethnic groups, and this has led to our understanding of human evolutionary patterns across different continents. These mtDNA haplogroups also can serve as good surrogates for investigating the role of the mitochondrial genome in influencing disease risk. The nine most common European mtDNA haplogroups are defined by 10 SNPs,<sup>37</sup> and some have been reported to be associated with several human diseases, including Alzheimer disease,<sup>38</sup> Parkinson disease,<sup>39,40</sup> and various ocular diseases, such as age-related macular degeneration (AMD),<sup>41</sup> primary open angle glaucoma (POAG),<sup>42</sup> and keratoconus.<sup>43</sup> We, therefore, hypothesized that mtDNA variants and haplogroups might contribute to FECD genetic susceptibility. We investigated the role of European mtDNA haplogroups in FECD risk using patients of European ancestry, and also examined whether their effect of mtDNA on FECD risk changes in the presence of the rs613872 risk allele or cigarette smoking, two known risk factors for FECD.

## MATERIALS AND METHODS

### Ethics Statement

Our study was performed in accordance with the Declaration of Helsinki, and the guideline of the institutional review boards (IRB) at the Duke University Medical Center and at Case Western Reserve University (CWRU). The Fuchs research groups at sites obtained the appropriate IRB approval for research on human subjects before initiating recruitment of study participants, and all individuals provided written, informed consent. The discovery dataset included control participants from the Duke University POAG Genetics group,<sup>44</sup> which also were recruited under the approval of the Duke IRB and consented to allow their biological samples to be used by other research studies.

### Study Participants

All FECD cases underwent detailed ophthalmic examination, including slit-lamp biomicroscopy, to determine FECD severity as described previously.<sup>23</sup> Grading of disease severity was

determined using a slightly modified version of the Krachmer scale classification system.<sup>13</sup> We defined cases based on equivalent grading between Duke and CWRU; that is, grade  $\geq 2$  for Duke subjects is equivalent to grade  $\geq 3$  for CWRU subjects. Controls were required to have a normal cornea during slit-lamp examination and to be 45 years of age or older.

This study included a discovery dataset from the Duke FECD genetic study (described previously<sup>23</sup>) and a replication dataset extracted from the FECD Genetics Consortium's ongoing genome wide association study (GWAS, see Table 1 for sample sizes). A description of the GWAS design can be found on the National Institutes of Health (NIH) dbGaP website (available in the public domain at <http://www.ncbi.nlm.nih.gov/gap>; study accession, phs000421.v1.p1). Only individuals of European ancestry were included. The discovery dataset consists of 530 unrelated FECD cases and 498 unrelated controls. All 530 cases and 161 of the control subjects were recruited by the FECD clinical team at the cornea clinic in the Duke University Eye Center; the other 337 control samples were subjects without glaucoma from the Duke POAG genetic study. All POAG control subjects had detailed eye examinations and showed no corneal abnormalities at the time of POAG study enrollment.

The FECD GWAS consisted of 3996 unrelated individuals, which included 677 individuals (518 cases and 159 controls) from the Duke FECD genetic study, 1440 individuals (885 cases and 554 controls) recruited from CWRU, and 1879 controls from the genetic variation of refractive error substudy of the Age-Related Eye Disease Study (AREDS) from dbGaP (available in the public domain at <http://www.ncbi.nlm.nih.gov/gap>; phs000429.v1.p1). The AREDS samples selected for use in the FECD GWAS had no prior cataract surgery or corneal dystrophies. To form two replication datasets, we used FECD cases from CWRU and controls from either all GWAS controls (GWAS1 subset, 857 cases and 2343 controls) or all non-Duke GWAS controls (GWAS2 subset, 857 cases and 2186 controls). All samples selected for replication datasets also were of European ancestry, and all controls were 45 years of age or older.

We created additional subsets by stratifying the Duke discovery dataset using grade level, *TCF4* SNP rs613872 genotype, and cigarette smoking status. The Grade 3+ subset contained 457 FECD cases with grade  $\geq 3$  and all 498 controls. The *TCF4* subset contained 529 FECD cases and 494 controls that had rs613872 genotype data available. For cigarette smoking, we investigated two binary cigarette smoking statuses: current smoker, whether a participant was currently smoking cigarettes at the time of study enrollment; and ever smoker, whether a participant had ever smoked cigarettes (either currently or in the past as a former smoker). The current smoker subset contained 234 FECD cases and 119 controls, whereas the ever smoker subset contained 435 FECD cases and 428 controls (Table 1). There were more controls in the ever smoker subset because POAG controls have ever smoking status data available, but lack current versus former smoking status data.

### Mitochondrial Haplogroups and Genotyping

European mtDNA haplogroups can be differentiated by 10 SNPs.<sup>37</sup> Table 2 summarizes the conventional mtDNA marker names, the current dbSNP rsIDs if available, the corresponding marker names from the GWAS genotyping array if available, and how the nine common European mtDNA haplogroups are defined by the alleles of the 10 mtDNA SNPs. For the discovery dataset, all 10 SNPs were genotyped with custom-designed TaqMan allelic discrimination assays (Life Technologies, formerly Applied Biosystems, Inc., Foster City, CA, USA), which

**TABLE 4.** Association Results for Single mtDNA SNP Markers and Haplogroups in the Discovery Dataset

| SNP/Haplogroup | Allele | MAF  | P            | OR   | 95% CI     |
|----------------|--------|------|--------------|------|------------|
| G1719A         | A      | 0.07 | 0.919        | 0.98 | 0.59, 1.61 |
| G4580A         | A      | 0.03 | 0.871        | 1.06 | 0.52, 2.18 |
| T7028C         | C      | 0.40 | 0.832        | 0.97 | 0.75, 1.26 |
| G8251A         | A      | 0.07 | 0.078        | 1.56 | 0.95, 2.55 |
| G9055A         | A      | 0.09 | 0.569        | 0.88 | 0.57, 1.36 |
| A10398G        | G      | 0.22 | <b>0.034</b> | 0.72 | 0.53, 0.98 |
| A12308G        | G      | 0.22 | 0.546        | 0.91 | 0.67, 1.24 |
| G13368A        | A      | 0.12 | 0.137        | 0.74 | 0.50, 1.10 |
| G13708A        | A      | 0.11 | 0.595        | 0.90 | 0.60, 1.34 |
| G16391A        | A      | 0.03 | 0.071        | 1.88 | 0.95, 3.74 |
| I vs. H        |        |      | <b>0.041</b> | 0.46 | 0.22, 0.97 |
| J vs. H        |        |      | 0.561        | 0.87 | 0.54, 1.39 |
| K vs. H        |        |      | 0.104        | 0.65 | 0.39, 1.09 |
| T vs. H        |        |      | 0.766        | 0.91 | 0.50, 1.67 |
| U vs. H        |        |      | 0.224        | 1.30 | 0.85, 1.98 |
| V vs. H        |        |      | 0.705        | 1.08 | 0.73, 1.58 |
| W vs. H        |        |      | 0.847        | 0.93 | 0.45, 1.94 |
| X vs. H        |        |      | 0.814        | 1.09 | 0.53, 2.25 |
| Other vs. H    |        |      | 0.117        | 2.14 | 0.83, 5.54 |

Nominal significant is indicated in bold.

use unlabeled PCR primers and two allele-specific probes containing the TaqMan minor groove binding group (MGB) probe, and either a FAM and VIC dye label in a 384-well plate format. The PCR reactions were performed with TaqMan Universal PCR Master Mix on the GeneAmp PCR System 9700 (Applied Biosystems, Inc.), and the ABI7900HT Fast PCR System (Applied Biosystems, Inc.) was used for reading allelic discrimination calls. Quality control (QC) samples, including two Centre d'Etude du Polymorphisme Humain (CEPH) pedigree individuals, one no-template sample, and two duplicate samples (one male, one female), were contained within each quadrant of each 384-well plate.

The FECD GWAS samples were genotyped using the Illumina HumanOmni2.5-4v1\_H array. Genotyping was performed by the Center for Inherited Disease Research (CIDR) at Johns Hopkins University (Baltimore, MD, USA), and rigorous QC checks were conducted by the Genetics Coordinating Center at the University of Washington (Seattle, WA, USA). Only three of the 10 European haplogroup markers are present on this chip: A10398G (MitoG10399A), A12308G (MitoA10309G), and G16391A (MitoG16393A). The minor base pair position differences are due to different genetic map assemblies for mtDNA markers used in the haplogroup literature<sup>45</sup> and the Illumina SNP array (refer to NCBI dbSNP, Table 2).

### Statistical Analysis

The number and percentage of females, and the average (SD) age at enrollment were computed for the discovery and replication datasets. Since mtDNA is a haploid genome, each marker was coded as a binary variable based on the nucleotide that an individual carries: 1 for the minor allele, 0 for the common allele. Minor allele frequency (MAF), therefore, is equivalent to the proportion of samples carrying the minor allele. Logistic regression was applied to test for association between FECD and either all 10 mtDNA SNP markers in the discovery dataset or three mtDNA SNP markers in the replication dataset; these models included covariate adjustment for age and sex. The odds ratio (OR) and its 95% confidence interval (CI) were obtained for each marker along with a *P* value.

For haplogroup association analyses, each subject was assigned a haplogroup based on the allele combinations listed in Table 2. Haplogroup frequencies were computed in the discovery dataset for cases and controls. The same logistic regression model with covariate adjustment of age and sex was applied to test association between each haplogroup and FECD. Since haplogroup is specified as a categorical variable with more than two levels, we tested each haplogroup against a common reference haplogroup, Haplogroup H, which is the most common European haplogroup<sup>45</sup> (40.4% in our dataset). This approach allowed us to evaluate the effects of all haplogroups consistently. The haplogroup analyses were performed across the entire discovery dataset and across all four subsets (Grade 3+, *TCF4*, current smoker, and ever smoker). To examine whether the known FECD risk factors *TCF4* rs613872 and cigarette smoking have an effect on the level of FECD risk conferred by mtDNA markers or haplogroups, conditional logistic regression analyses also were performed by including the appropriate additional covariate, either *TCF4* rs613872 for the *TCF4* subset, current smoker status for the current smoker subset, or ever smoker status for the ever smoker subset, to the original logistic regression model.

All statistical analyses performed for the discovery dataset were performed using SAS software, release 9.3 (SAS, Inc., Cary, NC, USA). The analyses for GWAS1 and GWAS2 were performed using PLINK<sup>46</sup> (available in the public domain at <http://pngu.mgh.harvard.edu/~purcell/plink/>).

### RESULTS

The sample sizes of the discovery and replication datasets are listed in Table 1 along with demographic data. A total of 1028 samples was included in the discovery dataset with comparable sample sizes between cases and controls (530 vs. 498). Among cases, 457/530 met the grade  $\geq 3$  criteria (Grade 3+ subset). As expected, there were more females than males, particularly among FECD cases (71.5% females). The average age was 69.5 (SD = 11.0) years old for cases and 65.3 (SD = 10.0) years old for controls. The *TCF4* subset is nearly the same size as the full dataset, except five subjects from the full dataset have missing *TCF4* rs61872 genotype data. The data available in the current smoker subset are much smaller than that for ever smoker subset due to the inclusion definition of these two categories, and the POAG controls having only ever smoking status data available.

Since samples from the Duke cohort also were included in the FECD GWAS, the minor difference between two replication datasets (GWAS1 and GWAS2) is the inclusion or exclusion of Duke control subjects (*N* = 157). Therefore, GWAS2 is a completely independent dataset from the discovery dataset, because Duke controls were excluded, while GWAS1 has a larger sample size to increase the statistical power, but including a small number of shared Duke controls. Similar to the discovery dataset, the replication dataset also had more females than males among FECD cases (65.93% females). The average ages also are comparable between cases and controls in the replication dataset (70.6 vs. 69.3 years in GWAS1, Table 1).

Haplogroup frequencies are summarized in Table 3 for cases and controls from the discovery dataset. The frequencies of haplogroups in our controls (Table 3) did not differ appreciably from those in a previous report of the North American control population.<sup>45</sup> As expected, Haplogroup H is the most common haplogroup in our dataset (40.4%), followed by Haplogroups U (14.8%) and T (11.8%, Table 3). As for the marker-specific MAFs derived from all subjects, G4580A and G16391A are the rarest alleles with MAF = 3%, followed by G8251A with MAF = 7% (Table 4).

**TABLE 5.** Summary of Significant Results From Different Subsets of the Discovery Dataset

| Subset         | Variable*      | P         | OR    | CI          |
|----------------|----------------|-----------|-------|-------------|
| Discovery      |                |           |       |             |
| Grade 3+       | A10398G        | 0.020     | 0.68  | 0.49, 0.94  |
|                | Haplogroup I   | 0.029     | 0.40  | 0.18, 0.91  |
| <i>TCF4</i>    | Haplogroup I   | 0.034     | 0.35  | 0.13, 0.92  |
|                | Haplogroup X   | 0.028     | 3.44  | 1.15, 10.34 |
|                | rs613872       | <0.000001 | 5.43  | 4.16, 7.07  |
| Current smoker |                |           |       |             |
| A10398G        | A10398G        | 0.012     | 0.51  | 0.30, 0.86  |
|                | Current smoker | 0.258     | 0.69  | 0.36, 1.31  |
| T7028C         | T7028C         | 0.007     | 0.50  | 0.31, 0.83  |
|                | Current smoker | 0.227     | 0.67  | 0.35, 1.28  |
| G16391A        | G16391A        | 0.034     | 4.50  | 1.12, 18.0  |
|                | Current smoker | 0.287     | 0.71  | 0.37, 1.34  |
| Haplogroup I   | Haplogroup I   | 0.008     | 0.15  | 0.04, 0.61  |
|                | Haplogroup K   | 0.007     | 0.31  | 0.13, 0.72  |
| Current smoker | Current smoker | 0.181     | 0.64  | 0.33, 1.24  |
|                | Ever smoker    |           |       |             |
| A10398G        | A10398G        | 0.024     | 0.68  | 0.48, 0.95  |
|                | Ever smoker    | 0.655     | 1.07  | 0.8, 1.42   |
| G16391A        | G16391A        | 0.029     | 2.35  | 1.09, 5.08  |
|                | Ever smoker    | 0.735     | 1.05  | 0.79, 1.4   |
| Haplogroup I   | Haplogroup I   | 0.013     | 0.34  | 0.15, 0.79  |
|                | Haplogroup K   | 0.044     | 0.56  | 0.32, 0.99  |
| Ever smoker    | Ever smoker    | 0.677     | 1.06  | 0.8, 0.66   |
|                | Replication    |           |       |             |
| GWAS1          | A10398G        | 0.019     | 0.79  | 0.65, 0.96  |
|                | GWAS2          | A10398G   | 0.036 | 0.81        |

FECD with grade  $\geq 2$  was tested for all subset except Grade 3+ subset, which tests the FECD with grade 3 or above.

\* In each model, the independent variables used were age, sex, and the variable(s) listed in the table.

Table 4 summarizes the association results of mtDNA markers and haplogroups in the discovery dataset. Among mtDNA variants, only A10398G showed significant association with FECD ( $P = 0.034$ ). The 10398G allele was associated with a decreased risk (protective effect) of FECD (OR = 0.72; 95% CI = 0.53, 0.98). The A10398G marker remains significantly associated with higher graded FECD in the Grade 3+ subset ( $P = 0.020$ ). Of the three markers that were present in our GWAS data within the replication dataset, we identified significant association between 10398G and FECD in GWAS1 (OR = 0.79; CI = 0.65, 0.96;  $P = 0.019$ ) and GWAS2 (OR = 0.81; CI = 0.66, 0.98;  $P = 0.035$ ; Table 5). The other two markers (A12308G and G16391A) did not show evidence of significant association with FECD in the GWAS replication subsets.

Of the nine common European haplogroups, only Haplogroup I was significantly associated with FECD in the entire discovery dataset ( $P = 0.041$ ) and in the Grade 3+ subset ( $P = 0.029$ ; Tables 4, 5) relative to Haplogroup H. Haplogroup I also showed a protective effect on FECD risk, with an OR = 0.46 (95% CI = 0.22, 0.97) in the discovery dataset and an OR = 0.40 (95% CI = 0.18, 0.91) in the Grade 3+ subset.

When we control for the effect of rs613872 (the *TCF4* subset), we did not detect significant association in the *TCF4* subset at the single marker level, but as expected *TCF4*

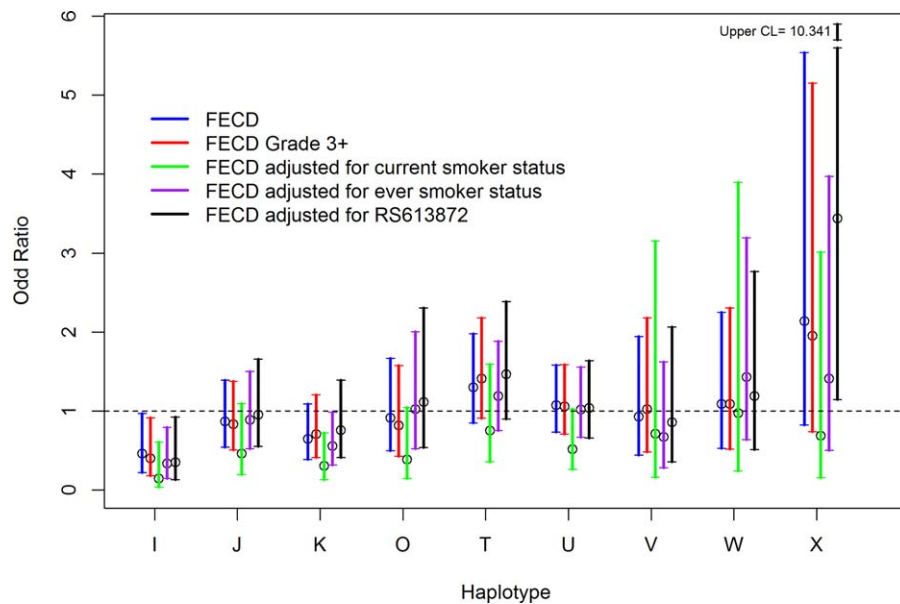
rs613872 is associated strongly with FECD ( $P < 0.000001$ ). However, for haplogroup association tests, Haplogroup I ( $P = 0.034$ ) and Haplogroup X ( $P = 0.028$ ) are significantly associated with FECD. Haplogroup I is again associated with a lower risk of FECD (OR = 0.35; 95%CI = 0.13, 0.92), but Haplogroup X increases the risk of FECD (OR = 3.44; 95%CI = 1.15, 10.34; Table 5).

We observed a consistent protective effect for the 10398G allele after controlling for current smoker status (OR = 0.51,  $P = 0.012$ ) and ever smoker status (OR = 0.68,  $P = 0.024$ , Table 5). Similarly, Haplogroup I still shows a protective effect for FECD after controlling for current smoker status (OR = 0.15,  $P = 0.008$ ) and ever smoker status (OR = 0.34,  $P = 0.013$ ). Although smoking status itself was not a significant covariate in subset analyses, the effects of A10398G and Haplogroup I on FECD appeared to be slightly stronger than those derived from the models that did not control for the effect of smoking. Additionally, T7028C was significantly associated with FECD after adjusting for current smoker status ( $P = 0.007$ ), as was G16391A after adjusting for either smoking status ( $P = 0.034$  for current smoker and  $P = 0.029$  for ever smoker). Finally, Haplogroup K showed the same protective effect as Haplogroup I with OR = 0.31 (95% CI = 0.13, 0.72;  $P = 0.007$ ) in the current smoker subset and OR = 0.56 (95% CI = 0.32, 0.96;  $P = 0.04$ ) in the ever smoker subset (Table 5). The comparison of OR and 95% CI among all haplogroups for all subsets from the discovery cohort is depicted in the Figure.

## DISCUSSION

Although evidence suggests a potential role for the mitochondrial genome in FECD,<sup>11,29,31,32</sup> less is known about the effect of variants in the mitochondrial genome on genetic susceptibility for this age-related corneal disorder. To our knowledge, this is the first study to investigate the association between mtDNA markers or European mtDNA haplogroups and FECD using large discovery ( $N = 1028$ ) and replication (max  $N = 3200$ ) datasets of ethnically matched FECD case and control samples. Based on evidence derived from discovery and replication datasets (GWAS1 and GWAS2), we concluded that A10398G is associated with FECD and its minor 10398G allele decreases the risk of FECD. This significant protective effect was verified further in the higher graded FECD subset (grade  $\geq 3$ ) and in two subsets examining either current or ever smoking status. With respect to mitochondrial haplogroups, our analyses using different subsets and models consistently identify Haplogroup I as being associated with a decreased risk of FECD, an effect that is strengthened when we controlled for the effect of cigarette smoking (see Figure).

The 10 SNPs that define European haplogroups include seven coding markers (Table 2). The A10398G marker is of particular interest, as it is a common nonsynonymous SNP (MAF = 22%) that changes the penultimate amino acid from threonine (Thr) to alanine (Ala) in the mitochondrially-encoded NADH dehydrogenase 3 (*MTND3*) gene. The *MTND3* gene is one of seven mtDNA encoded subunits contributing to approximately 41 polypeptides of the respiratory Complex I,<sup>33</sup> the first step in the electron transport chain of mitochondrial oxidative phosphorylation that generates usable energy for the cell. The *MTND3* subunit is located in the hydrophobic protein fragment of Complex I (MIM \*516000), which serves as the electron donor to ubiquinone. The A10398G variant probably is one of the most studied mitochondrial polymorphisms.<sup>39,47-49</sup> Prior studies have suggested that the G allele of A10398G has a lower potential to generate ROS.<sup>39</sup> Given that excessive ROS production can induce oxidative stress,<sup>50,51</sup> and that increased oxidative stress



**FIGURE.** The OR and 95% CI for each haplogroup from five sets of analyses: (1) Full dataset, (2) Grade 3+ subset, where FECD cases have grade  $\geq 3$ , (3) *TCF4* subset, adjusting for the effect of rs613872, (4) current smoker subset, adjusting for current smoking status, and (5) ever smoker subset, adjusting for ever smoked status.

can affect FECD risk,<sup>11,52</sup> our finding of 10398G allele associated with a decreased risk of FECD makes functional sense. We hypothesized that the 10398G allele functions to protect mitochondria from oxidative stress, thereby decreasing the risk of developing FECD. Further studies in the mitochondria of corneal endothelial cells from FECD patients carrying the 10398G allele are needed to confirm this hypothesis.

Haplogroup I is determined by the minor alleles of five mtDNA markers (1719A, 7028T, 8251A, 10398G, 16391A; Table 2). The most frequent minor alleles are 7028T (40%) and 10398G (22%). Since C7028T is a synonymous variant, the protective effect observed in Haplogroup I is likely driven by the functional A10398G variant. Furthermore, it is not surprising to see a stronger effect (higher OR) in our Grade 3+ subset, since the number of FECD cases carrying Haplogroup I will be even smaller as the number of FECD cases is reduced in this analysis.

The *TCF4* SNP rs613872 is by far the strongest and most consistently associated FECD genetic risk marker<sup>23-27</sup> and we have noted previously that this association exists within our datasets.<sup>23,27</sup> For this reason, we also examined whether *TCF4* rs613872 affects the association between haplogroups and FECD. Despite the fact that *TCF4* rs613872 is significantly associated with an increased risk of FECD, Haplogroup I remains associated with a decreased risk of FECD after controlling for the effect of rs613872. Therefore, we hypothesized that Haplogroup I acts independently from *TCF4* in contributing to the pathogenesis of FECD. Although our *TCF4* subset analysis also identified Haplogroup X as a risk factor for FECD ( $P = 0.028$ ), we have reservations regarding this finding because of the low frequency (2.2%) and large OR confidence interval (95% CI = 1.15, 10.34) for Haplogroup X; therefore, replication in a larger dataset is needed.

Cigarette smoking is a probable environmental risk factor for FECD, as it has been reported to affect the risks of developing corneal guttae<sup>28</sup> and advanced FECD.<sup>12</sup> Interestingly, when we adjusted for the effect of cigarette smoking, either current smoker or ever smoker status, we observed stronger association signals for A10398G and Haplogroup I. In addition, Haplogroup K became significantly associated with

FECD with the same direction of effect as Haplogroup I. Among nine haplogroups, Haplogroups I, J, and K are the only haplogroups that contain the 10398G allele (Table 2); however, it is known that these three haplogroups belong to different mitochondrial clades,<sup>53</sup> implying that the 10398G allele is an independent mutation in these three branches and is likely the main driver for the protective effect to FECD observed in Haplogroups I and K. Since smoking theoretically could contribute to increased oxidative stress,<sup>54,55</sup> it is possible that carriers of the 10398G allele, Haplogroup I, and/or Haplogroup K have lower ROS production to prevent cellular oxidative stress than others carrying different haplogroups or mtDNA SNPs, resulting in a lower risk of developing FECD.

Although we replicated statistically significant genetic association of the mitochondrial 10398G allele with FECD, our study contains several limitations. First, the significant  $P$  values identified in this study ranged between 0.01 and 0.05 despite reasonably large sample sizes. For single marker tests, ideally we should correct for multiple testing of 10 markers by requiring the significance threshold to be  $P < 0.005$  using the most stringent criteria of Bonferroni correction. While the individual dataset does not meet this criterion, we are confident of the genuine association of A10398G with FECD based on the consistent findings from replication and subsets analyses. Furthermore, if we conduct a meta-analysis using Fisher's method<sup>56</sup> for A10398G, the meta- $P$  values for discovery and replication datasets are 0.0054 if GWAS1 is used and 0.0094 if GWAS2 is used. It is promising that meta- $P$  values are close to the boundary of the Bonferroni significance level. Considering the consistent pattern of nominal significant findings in discovery and replication datasets, it is likely that we will identify nominal significant results in additional independent datasets, and the meta- $P$  will likely reach the Bonferroni significance threshold. As for the haplogroup analyses, haplogroups are equivalent to the alleles of a single multiallelic marker. Therefore, a nominal significance threshold is reasonable.

Second, since only three of 10 mtDNA markers for European haplogroups are present on the GWAS SNP bead array used in the replication dataset, we were unable to evaluate whether the association between Haplogroup I and

FECD would replicate in a larger dataset. Unfortunately, due to the limitation on accessing majority of samples in GWAS1 particularly all control samples from AREDS, we were unable to genotype the other seven mtDNA markers to make full comparison. Given that A10398G is a main contributor to the Haplogroup I and that its significant association with FECD was replicated in our analyses, we believed that it is likely that we would be able to replicate the Haplogroup I association if the other markers were available. Further, the multiple secondary analyses using the subsets from the discovery dataset have identified the Haplogroup I association consistently, which provide additional supporting evidence for Haplogroup I.

Third, the relationship between mtDNA and cigarette smoking status is important as it may provide an explanation of the underlying mechanism of how oxidative stress contributes to FECD. The reason why we did not detect significant association between smoking status and FECD in our dataset may be due to the low number of individuals for which we have cigarette smoking data (current smoker  $N = 353$ , or ever smoker  $N = 863$ ), particularly among controls. Therefore, we should not exclude the role of cigarette smoking for FECD given that our sample size is smaller than the one used by Zhang et al.<sup>12</sup> Finally, our current data do not have sufficient power to test for gene-gene interactions (e.g., mtDNA and *TCF4*) or gene-environment interactions (e.g., mtDNA and cigarette smoking).

In summary, our analyses suggested that the 10398G allele and Haplogroup I decrease the risk of developing FECD in patients with European ancestry. These data supported the theory that oxidative stress has a role in the pathogenesis of FECD. While these findings need replication in additional independent datasets, the results of this study suggested that future research in the area of mitochondrial-mediated oxidative stress in FECD may lead to the development of novel nonsurgical therapeutic and/or preventive strategies for FECD. Our study presented an important step toward understanding the effect of mtDNA risk variants on FECD.

### Acknowledgments

The authors thank the study participants and their family members for generously agreeing to participate in the study, as well as Elmer Balajonda and Shera Watson from Duke, and the clinical teams from the FECD Genetics Multi-Center Study Group in Case Western Reserve University for their assistance with recruitment of study participants. Finally, the authors thank the genotyping and data cleaning efforts for the GWAS data from CIDR at Johns Hopkins University, and the Genetics Coordinating Center at University of Washington.

Supported by NIH National Eye Institute (NEI) Grants R01EY016514 (GKK), R01EY023196 (SKI and NAA), R01EY015543 (RRA), R01EY019126 (MAH), and R01EY013315 (MAH).

Disclosure: **Y.-J. Li**, None; **M.A. Minear**, None; **X. Qin**, None; **J. Rimmler**, None; **M.A. Hauser**, None; **R.R. Allingham**, None; **R.P. Igo**, None; **J.H. Lass**, None; **S.K. Iyengar**, None; **G.K. Klintworth**, None; **N.A. Afshari**, None; **S.G. Gregory**, None

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## APPENDIX

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