Association of Genetic Variants in the TMCO1 Gene with Clinical Parameters Related to Glaucoma and Characterization of the Protein in the Eye

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PURPOSE. Glaucoma is the leading cause of irreversible blindness worldwide. Primary open angle glaucoma (POAG) is the most common subtype. We recently reported association of genetic variants at chromosomal loci, 1q24 and 9p21, with POAG. In this study, we determined association of the most significantly associated single nucleotide polymorphism (SNP) rs4656461, at 1q24 near the TMCO1 gene, with the clinical parameters related to glaucoma risk and diagnosis, and determined ocular expression and subcellular localization of the human TMCO1 protein to understand the mechanism of its involvement in POAG.

METHODS. Association of SNP rs4656461 with five clinical parameters was assessed in 1420 POAG cases using linear regression. The TMCO1 gene was screened for mutations in 95 cases with a strong family history and advanced disease. Ocular expression and subcellular localization of the TMCO1 protein were determined by immunolabeling and as GFP-fusion.

RESULTS. The data suggest that individuals homozygous for the rs4656461 risk allele (GG) are 4 to 5 years younger at diagnosis than noncarriers of this allele. Our data demonstrate expression of the TMCO1 protein in most tissues in the human eye, including the trabecular meshwork and retina. However, the subcellular localization differs from that reported in other studies. We demonstrate that the endogenous protein localizes to the cytoplasm and nucleus in vivo and ex vivo. In the nucleus, the protein localizes to the nucleoli.

Conclusions. This study shows a relationship between genetic variation in and around TMCO1 with age at diagnosis of POAG and provides clues to the potential cellular function/s of this gene. (Invest Ophthalmol Vis Sci. 2012;53:4917–4925) DOI: 10.1167/iovs.11-9047

Glaucoma refers to a group of neurodegenerative ocular diseases united by a clinically characteristic optic neuropathy. Open-angle glaucoma (OAG) is common, with a prevalence of approximately 3% in people older than 50 years.1 The disease has long been known to have a genetic component. First-degree relatives of affected patients exhibit a 9-fold increased relative risk of developing primary OAG (POAG) compared with the general population.2 The Glaucoma Inheritance Study in Tasmania (GIST) revealed that a positive family history is found in approximately 60% of cases of POAG when family members are examined,3 and the disease is more severe in people with a family history of glaucoma, compared with those with “sporadic” glaucoma.4 Mutations in the myocilin (MYOC) gene are the most common reported genetic cause of POAG and account for approximately 3% to 5% of cases.5 Mutations in optineurin (OPTN) and WDR36 genes have also been implicated.3 Recent genome-wide association studies have identified common variants at several new genetic loci are associated with POAG, including CAV1 and CAV2 on chromosome 7q31; CDKN2B-AS1, CDKN2A, and CDKN2B on 9p21; and TMCO1 on 1q24.6,7 The mechanism of disease association is not yet clear for any of these genetic associations and very little is known about the normal function of TMCO1 and its role in the eye.

The TMCO1 gene encodes the Transmembrane and coiled-coil domains-1 protein which belongs to the DUF841 superfamily of unknown function.8 The gene is located approximately 6 Mb upstream of the known POAG gene MYOC on chromosome 1. The protein sequence is highly conserved across mammalian species.9 The gene is expressed in a variety of human adult and fetal tissues at varying levels,9 including ocular tissues.6 Immunohistochemistry revealed cytoplasmic labeling in the rat retinal ganglion cell layer.6

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The green fluorescent protein (GFP)-fusion of TMCO1 has been reported to localize to endoplasmic reticulum and Golgi apparatus in COS7 cells, and to the mitochondria in porcine PK-15 cells. These reports suggest that the subcellular localization of the protein may differ depending on cell type. A homozygous frame-shift mutation in the TMCO1 gene has been shown to cause a rare recessive syndrome in the Amish consisting of craniofacial dysmorphism, skeletal anomalies, and mental retardation, now known as TMCO1 defect syndrome. There are no reports of glaucoma in this family. Our recent study failed to find any coding mutations in POAG patients carrying two risk haplotypes at the TMCO1 locus, indicating that coding mutations in TMCO1 are unlikely to account for the association observed with POAG.

The most associated single nucleotide polymorphism (SNP) at the 1q24 locus (rs4656461) is just 3' to TMCO1 and the second ranked SNP rs7518099 is within intron 2 of this gene and is in almost complete linkage disequilibrium with rs4656461. In addition, the highest ranked imputed SNP, rs7524755 is within the 3' UTR of TMCO1. All the associated SNPs are within the same linkage disequilibrium block as TMCO1 with no other transcripts in the block. The common haplotype across the gene, which is efficiently tagged by the most associated SNP, increases the risk of POAG. This association was discovered in a cohort of POAG patients with severe blinding disease, replicated in a second similar cohort and another cohort with less-severe glaucoma. From these data, however, it is as yet unclear whether TMCO1 genetic variation can be used to pinpoint a distinct subtype of POAG or if TMCO1 mutations might contribute to some cases of familial OAG.

In the present study, we further investigated the TMCO1 gene for association with clinical traits relevant to glaucoma risk and diagnosis, and screened for mutations in patients with a strong family history of POAG. In addition, we investigated the detailed expression pattern of TMCO1 in human eye and determined the subcellular localization of the protein in relevant cell lines and in vivo in the eye.

**Methods**

**Patient Recruitment and Data Collection**

Participants were drawn from the Australian & New Zealand Registry of Advanced Glaucoma (ANZRAG), the GIST, the Blue Mountains Eye Study (BMES), and patients attending the eye clinic at Flinders Medical Centre (Adelaide, Australia). All participants were included in the study reporting the association of TMCO1 with POAG and the cohorts are described in more detail in that report. This cohort was also examined for genotype-phenotype relationships at another POAG locus on chromosome 9p21 in an independent study. POAG was defined by best-corrected visual acuity worse than 6/60 due to POAG, or a reliable 24-2 Visual Field with a mean deviation of worse than –22 dB or at least 2 of 4 central fixation squares affected with a Pattern SD of less than 0.5% and a strong family history of POAG were selected for re-sequencing of the protein coding regions of the TMCO1 gene. Family history was defined as three or more relatives diagnosed with glaucoma. This was self-reported in ANZRAG but determined by ophthalmic examination of extended families in GIST. Each exon and flanking intron of the TMCO1 gene was sequenced as previously described. Sequences were compared with the TMCO1 reference sequence (GeneBank Accession NM_019026) using Sequencher V4.10.1 (GeneCodes Corporation, Ann Arbor, MI).

**Sequencing of the TMCO1 Gene**

Ninety-five participants from the ANZRAG and GIST with advanced POAG (defined as best-corrected visual acuity worse than 6/60 due to POAG, or a reliable 24-2 Visual Field with a mean deviation of worse than −22 dB or at least 2 of 4 central fixation squares affected with a Pattern SD of less than 0.5%) and a strong family history of POAG were selected for re-sequencing of the protein coding regions of the TMCO1 gene. Family history was defined as three or more relatives diagnosed with glaucoma. This was self-reported in ANZRAG but determined by ophthalmic examination of extended families in GIST. Each exon and flanking intron of the TMCO1 gene was sequenced as previously described. Sequences were compared with the TMCO1 reference sequence (GenBank Accession NM_019026) using Sequencher V4.10.1 (GeneCodes Corporation, Ann Arbor, MI).

**Subcellular Localization**

A GFP-TMCO1 fusion construct was generated by cloning the open-reading frame of the protein in pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA). For this, TMCO1 cDNA was amplified from the human retina as previously described and cloned in-frame with GFP at EcoRI and XbaI sites in the vector. In-frame cloning was confirmed by sequencing. Expression of the fusion protein was demonstrated in transiently transfected HEK293A cells by Western blotting as previously described, except that protein extraction was performed in radioimmunoprecipitation assay buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, Protease Inhibitor Cocktail [Roche Diagnostics Australia Pty Ltd., Sydney, NSW, Australia], 57 μM phenylmethysulfoxylfluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 20 mM sodium fluoride). For subcellular localization, 3 × 10^5 SH-SY5Y human neuroblastoma cells were seeded onto glass coverslips in six-well tissue culture plates. The cells were cultured in a 1:1 mixture of Dulbecco’s...
modified Eagle’s medium and Ham’s F12 medium (GIBCO, Invitrogen Australia Pty Ltd., Mulgrave, Victoria, Australia) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, in a humidified atmosphere at 37°C and 5% CO₂. On the following day, the cells were transfected with either GFP-TMCO1 fusion construct or empty vector using Lipofectamine 2000 (Invitrogen Australia Pty Ltd.) as per the manufacturer’s protocol. Approximately 48 hours after transfection, the cells were incubated with 1 μM BODIPY-TR-Ceramide (Molecular Probes, Invitrogen Australia Pty Ltd.) for 2 hours to label the Golgi apparatus or with 0.25 μM MitoTracker Red CM-H₂XRos (Molecular Probes) for 30 minutes to label mitochondria. After incubation, the cells were washed several times with PBS, fixed in 4% paraformaldehyde/PBS, and mounted on microscope slides in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). Confocal microscopy was performed on a Leica TCS SPS inverted Spectral confocal microscope equipped with LAS AF software (Leica Microsystems Pty Ltd., North Ryde, Australia).

**Immunolabeling**

For immunohistochemical labeling of the TMCO1 protein in human eye, the eye tissue was obtained from deceased donors through the Eye Bank of South Australia, following the guidelines of the Southern Adelaide Health Service/Flinders University HREC. The tissue was obtained within 12 hours of donor death and average donor age was 70 years. The tissue from donors without a history of ocular disease, such as keratoconus, pterygium, refractive surgery, inflammation, tumor, and glaucoma, was obtained. Specificity of the rabbit anti-human TMCO1 antibody (Sigma-Aldrich, Pty Ltd., Castle Hill, NSW, Australia) used for immunolabeling was demonstrated in human optic nerve by Western blotting as previously described. For immunolabeling, the eye tissue was fixed in buffered-formalin and embedded in paraffin; 4-μm-thick paraffin-embedded sections of the human eye were immunolabeled for the TMCO1 protein as previously described except for the following variations. After hybridization with the rabbit anti-human TMCO1 primary antibody (1:1000), the sections were hybridized with the NovoLink Polymer complex reagent (Leica Microsystems, Bannockburn, IL) and visualized with Chromogen substrate coloration (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin and mounted in DepX (Merck KGaA, Darmstadt, Germany). Light microscopy was performed on an Olympus BX41 microscope attached with a digital DP20/DP70 camera using CellSens Standard Photography software (Olympus Corporation, Tokyo, Japan).

Double immunofluorescent labeling in human eye sections was performed as previously described. Visualization of nucleolin, coilin, and SC35 was achieved using a three-step procedure (primary antibody, biotinylated secondary antibody, streptavidin-conjugated AlexaFluor 594), whereas TMCO1 was labeled by a two-step procedure (primary antibody, secondary antibody conjugated to AlexaFluor 488). In brief, tissue sections were deparaffinized. Next, antigen retrieval was achieved by microwave treatment in 10 mM citrate buffer (pH 6.0). Tissue sections were then blocked in PBS containing 3% normal horse serum and subsequently incubated overnight at room temperature in the appropriate combination of primary antibodies. On the following day, sections were incubated with biotinylated anti-mouse secondary antibody (1:250) for the three-step procedure plus the anti-rabbit secondary antibody conjugated to AlexaFluor 488 (1:250; Molecular Probes) for the two-step procedure for 30 minutes, followed by streptavidin-conjugated AlexaFluor 594 (1:500; Molecular Probes) for 1 hour. Sections were then mounted using antifade mounting medium and examined under a confocal fluorescence microscope. The following antibodies were used: rabbit anti-human TMCO1 (ARP49429; Aviva Systems Biology, San Diego, CA), mouse anti-human nucleolin (ab15541; Abcam, Cambridge, MA), mouse anti-human coilin (ab11822; Abcam), and mouse anti-SC35 (ab11826; Abcam).

For immunolabeling of endogenous TMCO1 in SH-SY5Y cells, 3 × 10⁵ cells were seeded onto glass coverslips in six-well plates. Three days later, labeling was performed as previously described. The cells were hybridized with the rabbit anti-TMCO1 primary antibody (1:1000; Sigma) followed by hybridization with the Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibody (1:1000; Molecular Probes). For double labeling, the cells were hybridized with the anti-TMCO1 antibody and mouse antinucleolin monoclonal antibody (1:500; Abcam). The anti-TMCO1 antibody was detected with Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibody and antinucleolin with Alexa Fluor 594 conjugated anti-mouse IgG secondary antibody (1:500; Molecular Probes). The single- and double-labeled cells were mounted, and confocal microscopy was performed as described previously.

**RESULTS**

**Characterization of the Glaucoma Phenotype Associated with TMCO1 Genetic Variants**

Genotype information for SNP rs4656461 and clinical characterization of the disease were available for 1420 individuals. Descriptive statistics of each measured clinical trait are given in Table 1. For some individuals, the data for some of the clinical traits was not available. Each clinical trait was assessed for association with SNP rs4656461 (Table 2). Age at diagnosis was significantly decreased in carriers of the POAG risk allele (G) at this SNP (P = 0.004). Vertical cup/disc ratio was increased (P = 0.017); however, this result did not survive Bonferroni correction for the five traits assessed and the effect size estimate was small. Exclusion of 20 patients carrying pathogenic mutations in the MYOC gene did not significantly alter the results.

To explore the relationship between SNP rs4656461 and age at diagnosis further, multiple linear regression was conducted, including additional clinical covariates in the model. Carriers of POAG risk alleles were diagnosed more than 3 years earlier than patients with no risk alleles at this locus (P = 0.024, Table 3). Highest recorded IOP also contributed significantly to the model with a higher IOP also leading to a slightly younger age at diagnosis (0.26 years, P = 0.0002). Exclusion of patients with MYOC mutations improved the significance of the observed association with age at diagnosis. The B coefficient increased to −3.54 (P = 0.011), indicating a slightly stronger relationship when these generally young age at diagnosis patients were removed. In the multivariate analysis, the association with IOP was still significant but slightly attenuated with the exclusion of MYOC mutations.

**TABLE 1. Clinical Characteristics of the Cohort**

<table>
<thead>
<tr>
<th>Clinical Trait</th>
<th>n</th>
<th>Mean ± SD or % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest recorded IOP, mm Hg</td>
<td>1275</td>
<td>26.5 ± 10.0</td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
<td>1141</td>
<td>62.4 ± 14.0</td>
</tr>
<tr>
<td>Mean deviation, dB</td>
<td>658</td>
<td>−17.8 ± 9.4</td>
</tr>
<tr>
<td>Cup/disc ratio</td>
<td>1146</td>
<td>0.87 ± 0.13</td>
</tr>
<tr>
<td>Central corneal thickness, μm</td>
<td>693</td>
<td>518 ± 41</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>1389</td>
<td>46.6% (647)</td>
</tr>
</tbody>
</table>

**TABLE 2. Association of SNP rs4656461 with Each Clinical Trait under a Dominant Model**

<table>
<thead>
<tr>
<th>Clinical Trait</th>
<th>B</th>
<th>SE</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest recorded IOP</td>
<td>0.07</td>
<td>0.60</td>
<td>0.908</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>−2.56</td>
<td>0.88</td>
<td>0.004†</td>
</tr>
<tr>
<td>Mean deviation</td>
<td>−0.58</td>
<td>0.77</td>
<td>0.465</td>
</tr>
<tr>
<td>Cup/disc ratio</td>
<td>0.02</td>
<td>0.008</td>
<td>0.017</td>
</tr>
<tr>
<td>Central corneal thickness</td>
<td>−1.43</td>
<td>3.29</td>
<td>0.665</td>
</tr>
</tbody>
</table>

P < 0.01 is considered significant and is indicated in bold.
Table 3. Multiple Regression for Age at Diagnosis

<table>
<thead>
<tr>
<th>Clinical Trait</th>
<th>B</th>
<th>SE</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest recorded IOP</td>
<td>-0.26</td>
<td>0.07</td>
<td>0.0002</td>
</tr>
<tr>
<td>Mean deviation</td>
<td>-0.02</td>
<td>0.09</td>
<td>0.841</td>
</tr>
<tr>
<td>Cup:disc ratio</td>
<td>8.55</td>
<td>7.16</td>
<td>0.253</td>
</tr>
<tr>
<td>Central corneal thickness</td>
<td>-0.03</td>
<td>0.02</td>
<td>0.078</td>
</tr>
<tr>
<td>Sex</td>
<td>1.78</td>
<td>1.36</td>
<td>0.193</td>
</tr>
<tr>
<td>rs4656461 G allele carriers</td>
<td>-3.13</td>
<td>1.39</td>
<td>0.024</td>
</tr>
</tbody>
</table>

$R^2 = 0.068$. $P < 0.01$ is considered significant.

carriers ($B = -0.243$, $P = 0.001$). The mean age at diagnosis by genotype of rs4656461 is shown in Table 4. Consistent with the multivariate model, the mean age at diagnosis was decreased 2 to 3 years for each POAG risk allele in the total cohort. This is also evident when MYOC carriers are excluded. No significant association of rs4656461 with age at diagnosis was observed in the MYOC mutation carriers (ANOVA $P = 0.971$). The borderline association with cup:disc ratio was not significant after inclusion of IOP, age at diagnosis, central corneal thickness (CCT), and sex in the model ($P = 0.066$, data not shown).

Of 320 carriers of the rs4546461 risk allele (G), 60% ($n = 195$) reported a family history of glaucoma. By comparison, only 51% of noncarriers (331 of 643) reported a family history. This demonstrates an enrichment for family history of glaucoma among TMCO1 risk allele carriers (odds ratio = 1.47, 95% confidence interval [1.12–1.93], $P = 0.007$). To identify heritable rare variants that may contribute to the POAG phenotype, 95 patients with a strong family history of the disease were selected for resequencing of the gene. Although several previously reported SNPs, present in the general population, were observed, no novel pathogenic mutations were detected in the protein coding regions of the gene in this subset of individuals.

Subcellular Localization of TMCO1 and Expression in Mammalian Eye

To explore the subcellular localization of the TMCO1 protein in cell lines relevant to the retina, GFP-TMCO1 fusion was transiently expressed in SH-SYSY human neuroblastoma cells. Before that, expression of the fusion protein by the fusion construct in HEK293A cells was demonstrated by Western blotting (Fig. 1A). In SH-SYSY cells, the fusion protein was found uniformly distributed in the cytoplasm, or concentrated around the nucleus, or aggregated on one side of the nucleus (Fig. 1B and data not shown). However, labeling of these cells with the Golgi apparatus or mitochondrial marker did not show any colocalization of the protein with either organelle (Fig. 1B, top and middle panels). In GFP-expressing control cells, the protein distributed throughout the cell and, as expected, did not colocalize with the Golgi apparatus or mitochondria (Fig. 1B, bottom panels). A similar localization pattern of the GFP-TMCO1 fusion was seen in SRA 01/04 lens epithelial cells (data not shown).

For determining expression and in vivo localization of TMCO1 in the human eye, immunolabeling was performed in human ocular sections with the anti-TMCO1 antibody. Specificity of the antibody was demonstrated by detection of the expected approximately 21-kDa protein band in human optic nerve by Western blotting (Fig. 2A). In human ocular sections, positive immunolabeling was observed in the iridocorneal angle, trabecular meshwork, ciliary body, and retina (Fig. 2B).

In the trabecular meshwork, positive immunolabeling was primarily seen as 1 to 2 dots in the nucleus along with cytoplasmic labeling (Figs. 2B-b, 2B-c). A similar pattern of nuclear labeling and cytoplasmic labeling was observed in cells in the ciliary body (Figs. 2B-d, 2B-e). In the retina, dot-like nuclear and positive cytoplasmic labeling was observed in the photoreceptor, bipolar, and ganglion cell layers (Figs. 2B-f, 2B-g). Cytoplasmic labeling was more prominent in the photoreceptor outer segments and ganglion cells than in bipolar cells. These data suggest that the TMCO1 protein is expressed in most ocular tissues and corroborate the previous finding of TMCO1 transcript in these tissues.\(^6\) Cytoplasmic labeling correlates with that previously reported by us in rat retinal ganglion cells.\(^6\) Nuclear and cytoplasmic labeling of the protein in the eye indicates that TMCO1 is likely a multifunctional protein with functions both in the cytoplasm and nucleus. However, as all nuclear proteins are produced in the cytoplasm and then translocated to the nucleus, it is possible that the cytoplasmic labeling of TMCO1 represents its production rather than function in this compartment.

Next, we investigated the identity of the discrete subnuclear regions immunopositive for TMCO1 in the cells in ocular tissues. We hypothesized that these subnuclear regions represent nucleoli, Cajal body, or nuclear speckles. Nucleolin is a protein that frequently localizes to the nucleoli and is therefore used as a nucleolar marker;\(^16\) however, it is also known to localize to the perinucleolar compartment.\(^17\) The coilin protein marks the Cajal body and SC35 the speckles in the nucleus.\(^18\) To determine whether TMCO1 localizes to the nucleolus, Cajal body, or speckles in the cells in ocular tissues, we individually assessed its colocalization with nucleolin, coilin, and SC35 proteins. Double labeling with the anti-TMCO1 and antineucleolin antibodies revealed colocalization of TMCO1 and nucleolin in the nucleus to discrete subcellular regions in the cells in human ocular sections. Representative colocalization in the retinal ganglion cells can be seen in Figure S. The two proteins were also observed in the cytoplasm in ocular cells where they colocalized with each other. However, double labeling with anti-TMCO1 and anticoilin antibodies and with anti-TMCO1 and anti-SC35 antibodies revealed that TMCO1 did not colocalize with coilin or SC35 in the nucleus or cytoplasm (Fig. 3). These data indicate that subnuclear TMCO1 in the cells in ocular tissues localizes to nucleoli.

Finally, we investigated whether endogenous TMCO1 in SH-SYSY cells mimics in vivo localization in the human eye. Immunolabeling with the anti-TMCO1 antibody revealed distribution of the protein in the cytoplasm and nucleus in a

Table 4. Mean Age (Years) at Diagnosis by rs4656461 Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All Patients</th>
<th>MYOC Carriers</th>
<th>Non-MYOC Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean Age</td>
<td>SD</td>
</tr>
<tr>
<td>AA</td>
<td>769</td>
<td>63.3</td>
<td>14.1</td>
</tr>
<tr>
<td>AG</td>
<td>351</td>
<td>60.9</td>
<td>13.7</td>
</tr>
<tr>
<td>GG</td>
<td>21</td>
<td>58.7</td>
<td>14.4</td>
</tr>
</tbody>
</table>

The G allele at this SNP is the risk allele for glaucoma. Analysis on MYOC mutation carriers and noncarriers is also shown.
**FIGURE 1.** (A) Fusion protein encoded by the GFP-TMCO1 construct. Cell lysates from HEK 293A cells transiently transfected with the fusion construct (GFP-TMCO1) or pE8GFP-C1 vector (GFP) were analyzed by Western blotting with the anti-GFP antibody. The band of approximately 50 kDa seen in the TMCO1-GFP lane corresponds with the expected size of the fusion protein and that of approximately 28 kDa in the GFP lane corresponds with the size of GFP. The greater than 28-kDa protein band seen in the TMCO1-GFP lane may represent fusion protein degradation. Molecular sizes of the protein standards are indicated in kilodaltons. (B) GFP-TMCO1 fusion localization in SH-SY5Y human neuroblastoma cells. Cells transiently transfected with the fusion construct (GFP-TMCO1) or pE8GFP-C1 vector (GFP) (green channel) were either stained with BODIPY-TR-Ceramide for labeling the Golgi apparatus (Golgi) or MitoTracker Red CM-H2XRos for labeling mitochondria (Mito) (red channel), nuclei counterstained with DAPI (blue channel), and visualized by confocal microscopy. GFP-TMCO1 can be seen in the cytoplasm (green). However, in overlayed images from the three channels (Merge) it neither colocalizes with the Golgi apparatus nor mitochondria. In cells expressing GFP as a control, the protein distributed in the nucleus and cytoplasm.
FIGURE 2. (A) Endogenous TMCO1 protein in human optic nerve. Protein lysate from optic nerve tissue were analyzed by Western blotting with the rabbit anti-TMCO1 antibody. The approximately 21-kDa band corresponds with the expected size of the TMCO1 protein. Molecular sizes of the protein standards are indicated in kilodaltons. (B) Immunolabeling of TMCO1 in the human eye. Paraffin sections of human eye were immunolabeled with the anti-TMCO1 antibody and imaged by light microscopy. Positive immunolabeling can be seen as red signal in the (a) iridocorneal angle, (b, c) trabecular meshwork, (d, e) ciliary body, and (f, g) retina. Nuclei are stained blue. Pigment in panels a, c, and e is marked by the letter “p.” Immunolabeling of TMCO1 as an intense spot in the nucleus can be seen in cells in the trabecular meshwork (c), ciliary body (e), and retina (g). Cytoplasmic immunolabeling is also visible in these tissues. Images are, a at ×10; b, d, f at ×40; and c, e, g at ×600, original magnification. The presented results are representative of experiments on eyes from four independent donors.
punctate fashion in these cells (Fig. 4, bottom left panel). The
signal was stronger in the nucleus compared with the
cytoplasm. In addition, in a small proportion of cells, instead
of being evenly distributed in the nucleus, the protein was
concentrated to discrete subnuclear regions as seen in vivo in
the eye (Fig. 4, top left panel). To determine if these TMCO1-
positive subnuclear regions represent nucleoli, we assessed
colocalization of TMCO1 with nucleolin in SH-SY5Y cells.
Double labeling with the anti-TMCO1 and antinucleolin
antibodies revealed TMCO1 localization as described previous-
ly, and localization of nucleolin to discrete subnuclear regions
(Fig. 4, second column). In cells with subnuclear localization of
TMCO1, the protein showed complete colocalization with
nucleolin in some cells (Fig. 4, top row), as observed in vivo,
and exhibited incomplete colocalization in other cells (Fig. 4,
middle row). In cells where TMCO1 was evenly distributed in
the nucleus, the protein did not colocalize with nucleolin (Fig.
4, bottom row). Double labeling with anti-TMCO1 and
anticolin and with anti-TMCO1 and anti-SC35 antibodies
showed that TMCO1 does not colocalize with collin or SC35
in SH-SY5Y cells (data not shown). From these data, TMCO1
appears to shuttle between the cytoplasm and nucleus and
transiently localize to the nucleolus or the perinucleolar
compartment.

**DISCUSSION**

Genetic variation in and around the TMCO1 gene at 1q24 has
been shown to be associated with POAG. The function of this
gene is not well elucidated and it is not yet known how this
gene contributes to glaucoma. This study has shown that
POAG patients carrying the glaucoma risk alleles at SNP
r4656461 tend to be diagnosed with the disease several years
earlier than patients without these alleles. However, this study
was not able to show association of r4656461 with IOP,
cup:disc ratio, mean deviation, or central corneal thickness.
Thus, apart from a slightly earlier onset, TMCO1-related

glaucoma does not appear to be a clinically distinct subtype
of POAG. These SNPs may be severity factors for POAG, which
could influence the age at onset of disease. This idea is
consistent with our previous work showing an increased odds
ratio for association in patients with blinding POAG compared
with patients with less severe POAG. In patients with a family
history of advanced glaucoma, there may be a tendency for
increased monitoring and thus earlier diagnosis (pseudo-
anticipation). This phenomenon may contribute to the current
findings, given the observation of an increased family history in
risk allele carriers. This is a limitation of our approach;
however, the same association with age of diagnosis was not
observed at the CDKN2B-AS1 gene in this same cohort. Thus,
we believe pseudo-anticipation does not have a major affect on
the current findings.

**MYOC**

Glaucoma is also well known to have a younger age
of onset, but within this small group of patients, the TMCO1
gene does not appear to be modifying this risk, although the
power of this analysis is minimal in this cohort with a limited
number of participants carrying both MYOC mutations and
TMCO1 risk alleles. The inclusion of patients with MYOC
mutations in the current analysis also does not account for the

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**FIGURE 3.** Immunolabeling of TMCO1 with nucleolin, collin, and SC35 in human retina. Confocal microscopy was performed on sections
immunolabeled with anti-TMCO1 (green) antibody together with either antinucleolin, anticolin, or anti-SC35 antibodies (red). Nuclei (blue) were
stained with DAPI. Images from the three color channels overlayed (merge) for determining colocalization. **Top row,** TMCO1 localized to discrete
regions in the nucleus colocalizes with nucleolin (highlighted by arrows). **Middle row,** TMCO1 does not colocalize with collin (green and red
separated in merged image). **Bottom row,** TMCO1 does not colocalize with SC35 (green and red distinct in merged image). Scale bar: 5 μm.
younger age of diagnosis observed in TMCO1 risk allele carriers. The methods used for the collection of age at diagnosis data do have some limitations. Where complete clinical records were not available to confirm the age of definitive glaucomatous visual loss, self-reported age at treatment initiation was used as a surrogate. However, it is unlikely that any bias introduced by this limitation would be distributed differently across genotypes. Nevertheless, replication of the observed association in independent POAG cohorts is required to confirm the current findings.

By re-sequencing of 95 patients with a strong family history of POAG, coding variants do not appear responsible for the disease phenotype in these families deemed likely to have highly penetrant dominant mutations. This is not surprising given that the amino acid sequence of this protein is completely conserved among all mammals investigated to date. This implies a crucial role for this ubiquitously expressed protein. The absence of coding variants in POAG patients in this study suggests genetic variation in regulatory elements may be responsible for the observed association signal. Further in-depth and functional analysis of the locus is required to determine this.

As an initial step toward functional analysis of the TMCO1 gene, we determined subcellular localization of the encoded protein and protein expression in the human eye. Expression of the protein in all the ocular tissues is consistent with its reported ubiquitous expression. Localization of the GFP-TMCO1 fusion in cultured mammalian cells in this study contradicted the previous reports. Iwamuro et al. reported localization of the human TMCO1-GFP fusion to the endoplasmic reticulum and Golgi apparatus in COS-7 cells but did not determine colocalization with the organelle-specific markers. Zhang et al., however, reported localization of the GFP fusion of porcine TMCO1 to mitochondria in PK-15 porcine kidney cells and colocalization with the mitochondrial marker. In this study, we observed neither colocalization of human GFP-TMCO1 with the endoplasmic reticulum and Golgi apparatus, nor mitochondria in cultured human neuroblastoma and lens epithelial cells. In addition, the fusion protein did not colocalize with the Golgi apparatus or distribute to the
endoplasmic reticulum in COS-7 cells under our experimental conditions (data not shown), which further contradicts findings in these cells by Iwamuro et al.10 Furthermore, we observed cytoplasmic and nuclear localization as inclusions of endogenous TMCO1 in the human ocular tissues. In SH-SY5Y neuroblastsoma cells also, endogenous TMCO1 was found in the cytoplasm and nucleus and in some cells as nuclear inclusions. Together, our data suggest that GFP-TMCO1 fusion localizes differently from endogenous TMCO1. This may be because TMCO1 protein is smaller than GFP (approximately 21 vs. 28 kDa); thus, the latter leads to aberrant localization of the former or due to overexpression of the fusion protein. Grossly different localization patterns of the GFP fusion and endogenous protein seen in this study highlight that ectopically expressed GFP fusions may not always reflect biological localization of the protein of interest.

The functional significance of the cytoplasmic localization of endogenous TMCO1 is as yet unclear. However, the conspicuous subnuclear localization in vivo in the human eye and ex vivo in some SH-SY5Y cells, and colocalization with nucleolin is very interesting. Nucleolin, the major component of the nucleolus, also localizes to the perinucleolar compartment, nucleoplasm, and the cell surface.17,19 The nucleolus is conventionally involved in ribosome biogenesis but also has nonconventional roles, including regulation of tumor suppressor and oncogene activities, nuclear export, and control of aging.6,21 From the present data, it is not certain that TMCO1 function at diagnosis of POAG. It also suggests that SH-SY5Y cells are a suitable model for investigating TMCO1 function. Further longitudinal studies will investigate whether TMCO1 genotyping can predict severity or progression of glaucoma.

The TMCO1 Gene and Glaucoma

References