A common variant near TGFBR3 is associated with primary open angle glaucoma

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A common variant near TGFBR3 is associated with primary open angle glaucoma


†These authors contributed equally to this work.
†A list of members of each consortium appears in the Supplementary Material, Note.
‡These authors also contributed equally to this work.

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Primary open angle glaucoma (POAG), a major cause of blindness worldwide, is a complex disease with a significant genetic contribution. We performed Exome Array (Illumina) analysis on 3504 POAG cases and 9746 controls with replication of the most significant findings in 9173 POAG cases and 26 780 controls across 18 collections of Asian, African and European descent. Apart from confirming strong evidence of association at CDKN2B-AS1 (rs2157719 [G], odds ratio [OR] = 0.71, 95% confidence interval [CI] = 0.64–0.79), we observed one SNP showing significant association to POAG (CDC7-TGBFR3 rs1192415, ORG-allele = 1.13, Pmeta = 1.60 × 10−3). This particular SNP has previously been shown to be strongly associated with optic disc area and vertical cup-to-disc ratio, which are regarded as glaucoma-related quantitative traits. Our study now extends this by directly implicating it in POAG disease pathogenesis.
Table 1. Sample collections of POAG cases and controls for Stages 1 (discovery) and 2 (replication)

<table>
<thead>
<tr>
<th>Collection</th>
<th>N cases</th>
<th>N controls</th>
<th>Ethnicity</th>
<th>Age of cases</th>
<th>Age of controls</th>
<th>Collection comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singapore</td>
<td>850</td>
<td>2347</td>
<td>Singapore Chinese</td>
<td>71.6 ± 10.1</td>
<td>58.88 ± 9.6</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Japan</td>
<td>923</td>
<td>640</td>
<td>Japanese</td>
<td>65.3 ± 13.1</td>
<td>71.9 ± 5.8</td>
<td>Previously GWAS in Nakano et al. (25)</td>
</tr>
<tr>
<td>USA-African-Americans</td>
<td>590</td>
<td>636</td>
<td>African American</td>
<td>65.4 ± 12.3</td>
<td>54.8 ± 9.8</td>
<td>New recruitment</td>
</tr>
<tr>
<td>China-Beijing</td>
<td>587</td>
<td>461</td>
<td>Northern Chinese</td>
<td>58.5 ± 12.5</td>
<td>Population-based controls</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>375</td>
<td>2962</td>
<td>Southern Chinese</td>
<td>62.3 ± 15.3</td>
<td>Population-based controls</td>
<td>Previously described for replication in Thorleifsson et al. (17)</td>
</tr>
<tr>
<td>South India</td>
<td>121</td>
<td>716</td>
<td>Indian</td>
<td>60.9 ± 12.2</td>
<td>51.0 ± 6.5</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Vietnam</td>
<td>58</td>
<td>1984</td>
<td>Vietnamese</td>
<td>63 ± 7.1</td>
<td>Population-based controls</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Total discovery</td>
<td>3504</td>
<td>9746</td>
<td></td>
<td></td>
<td></td>
<td>2206 cases are new discovery samples</td>
</tr>
<tr>
<td>Stage 2 replication</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singapore-2</td>
<td>520</td>
<td>5473</td>
<td>Singapore Chinese</td>
<td>71.1 ± 10</td>
<td>Population-based controls</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Japan-2</td>
<td>935</td>
<td>996</td>
<td>Japanese</td>
<td>64.3 ± 14.0</td>
<td>57.5 ± 13.9</td>
<td>N = 411 used for replication in Nakano et al. (25)</td>
</tr>
<tr>
<td>USA-African-American</td>
<td>497</td>
<td>304</td>
<td>African-American</td>
<td>69.1 ± 11.0</td>
<td>66.7 ± 13.1</td>
<td>New recruitment</td>
</tr>
<tr>
<td>South India-2</td>
<td>453</td>
<td>2496</td>
<td>Indian</td>
<td>62.5 ± 9.9</td>
<td>58.9 ± 10.1</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Korea</td>
<td>400</td>
<td>454</td>
<td>Korean</td>
<td>59.0 ± 11.8</td>
<td>40.3 ± 14.1</td>
<td>New recruitment</td>
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<tr>
<td>Saudi Arabia</td>
<td>236</td>
<td>655</td>
<td>Middle Eastern</td>
<td>60.8 ± 12.7</td>
<td>54.4 ± 11.7</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Malaysia</td>
<td>132</td>
<td>2540</td>
<td>Malay</td>
<td>65.1 ± 8.2</td>
<td>58.7 ± 11.0</td>
<td>New recruitment</td>
</tr>
<tr>
<td>China-Beijing 2</td>
<td>115</td>
<td>251</td>
<td>Northern Chinese</td>
<td>54.2 ± 12.4</td>
<td>71.53 ± 7.16</td>
<td>New recruitment</td>
</tr>
<tr>
<td>UK</td>
<td>336</td>
<td>6090</td>
<td>European</td>
<td>71.4 ± 10.8</td>
<td>Population-based controls</td>
<td>New recruitment</td>
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<tr>
<td>China-Shantou</td>
<td>247</td>
<td>289</td>
<td>Southern Chinese</td>
<td>52.9 ± 19.4</td>
<td>75.7 ± 6.1</td>
<td>Previously described for replication in Thorleifsson et al. (17)</td>
</tr>
<tr>
<td>Germany</td>
<td>56</td>
<td>142</td>
<td>European</td>
<td>67.9 ± 11.4</td>
<td>78.4 ± 8.9</td>
<td>New recruitment</td>
</tr>
<tr>
<td>Vietnam-2</td>
<td>76</td>
<td>245</td>
<td>Vietnamese</td>
<td>52.4 ± 17.4</td>
<td>51.3 ± 17.8</td>
<td>New recruitment</td>
</tr>
<tr>
<td>France</td>
<td>80</td>
<td>75</td>
<td>European</td>
<td>75.6 ± 8.5</td>
<td>73.5 ± 8.3</td>
<td>New recruitment</td>
</tr>
<tr>
<td>China-Shanghai, Chengdu 2</td>
<td>181</td>
<td>286</td>
<td>Southern Chinese</td>
<td>54.7 ± 16.5</td>
<td>84.7 ± 11.7</td>
<td>Previously described in Chen et al. (19)</td>
</tr>
<tr>
<td>China-Shanghai, Chengdu</td>
<td>608</td>
<td>1005</td>
<td>Southern Chinese</td>
<td>49.6 ± 17.0</td>
<td>62.9 ± 12.1</td>
<td>Previously described in Chen et al. (19)</td>
</tr>
<tr>
<td>USA (NEIGHBOR)</td>
<td>2170</td>
<td>2347</td>
<td>European descendant</td>
<td>66.4</td>
<td>68</td>
<td>Previously described in Wiggs et al. (22)</td>
</tr>
<tr>
<td>USA (GLAUGEN)</td>
<td>976</td>
<td>1140</td>
<td>European descendant</td>
<td>63.6</td>
<td>65.5</td>
<td>Previously described in Wiggs et al. (22)</td>
</tr>
<tr>
<td>Australia (ANZRAG)</td>
<td>1155</td>
<td>1992</td>
<td>European descendant</td>
<td>60.5 ± 14.3</td>
<td>55.6 ± 14.4</td>
<td>Previously described in Gharahkhani et al. (20)</td>
</tr>
<tr>
<td>Total replication</td>
<td>9173</td>
<td>26780</td>
<td></td>
<td></td>
<td></td>
<td>3425 cases are new replication samples</td>
</tr>
<tr>
<td>Total all samples</td>
<td>12677</td>
<td>36526</td>
<td></td>
<td></td>
<td></td>
<td>5631 cases are new in this report</td>
</tr>
</tbody>
</table>

*Population-based controls are ascertained from large-scale studies and do not have demographic data available. Based on many well-described examples, both by others and us, the frequency of POAG in the general population is uncommon (i.e. <5%). In this regard, the false-negative rate for POAG status in the population-based controls is likely to be low and thus the effect of loss of statistical power is negligible.

Illumina Exome array (26), we also included an extra 25 000 coding frame SNP markers obtained from exome sequencing of 2000 individuals of East Asian descent. Stringent quality control (QC) filters were applied to both SNPs and samples: per SNP call rate ≥99%, per-sample call rate ≥95%, non-monomorphic SNPs and non-significant deviation from Hardy–Weinberg equilibrium (HWE) P ≥ 10^{-6}. Samples under suspicion of cross-contamination and biologically related samples were removed by verification of extreme heterozygosity and identical by descent/identical by state information, if applicable. We further performed principal component analysis (PCA) to verify that cases and controls were well-matched ancestrally (Supplementary Material, Fig. S1) (27–32). As a result, a total of 3504 POAG cases and 9746 controls passing QC filters were included for association analysis using unconditional logistic regression, with adjustments for the principal components (PCs). From these datasets, a total of 2206 POAG cases represent entirely new patient collections, which have not been previously reported (Supplementary Material, Table S1). Each study-specific point estimate was then summarized using fixed-effects meta-analysis (Nmeta-analysis = 7 collections). A quantile–quantile plot derived from the meta-analysis P-values showed no significant dispersion of test statistics from the expected distribution (λGC = 1.042; Supplementary Material, Fig. S2) suggesting that the association results were not confounded by cryptic population stratification. Using additive effect models, we observed experiment-wide significant
(\(P = 0.05/272,000\) SNPs = \(1.84 \times 10^{-7}\)) evidence of association at CDKN2B-AS1 (rs2157719 [G], per-allele OR = 0.73, \(P = 1.10 \times 10^{-7}\)) (Supplementary Material, Fig. S3).

All SNP markers showing \(P < 0.0005\) in the discovery stage were followed up in a validation stage (Stage 2) comprised of up to 9173 POAG cases and 26,780 controls. A total of 21 SNPs at 20 independent loci were brought forward for validation genotyping using Sequenom MassARRAY iPLEX or in silico look-ups if genome-wide genotyping data were available (see Supplementary Material, Table S2).

CDKN2B-AS1 rs2157719 once again showed significant evidence of association in the meta-analysis of all replication collections (OR = 0.70, \(P = 2.48 \times 10^{-27}\)) as well as meta-analysis of all samples tested (rs2157719 [G], \(P_{\text{meta}} = 2.81 \times 10^{-33}\)) (Supplementary Material, Table S2). No heterogeneity of effect between Asians (OR = 0.72, \(P = 1.15 \times 10^{-15}\)) and Europeans (OR = 0.69, \(P = 5.54 \times 10^{-19}\)) were detected for this marker (Fig. 1A), consistent with the multiple previous reports describing association at this locus (18,22,25). Apart from CDKN2B-AS1 rs2157719, a second SNP marker (CDC7-TGFBR3 rs1192415) showed clear evidence of replication in Stage 2 (OR = 1.12, \(P = 1.04 \times 10^{-5}\), Fig. 1B and Supplementary Material, Table S2). On meta-analysis with the exome-chip discovery findings, genome-wide significant evidence of association was observed for rs1192415 (OR = 1.13, \(P_{\text{meta}} = 1.6 \times 10^{-8}\)) (Fig. 1B; Supplementary Material, Table S2). Although the risk allele ranged from between 11 and 34% across all ethnic groups studied for this marker, the association observed appeared to be uniform and consistent across most groups with little overall heterogeneity (\(I^2\) index < 20%, Supplementary Material, Table S3). For rs1192514, the association appeared stronger in Asians (OR = 1.17, \(P = 1.48 \times 10^{-7}\)) compared with Europeans (OR = 1.10, \(P = 0.01\)) (Fig. 1B), although the difference was not statistically significant (\(P\text{\_het} = 0.17\)). One other marker (FNDC3B rs4894796) was nominally significant in the replication stage (OR = 0.95, \(P = 0.02\)) and remained suggestively associated with POAG in the overall meta-analysis (OR = 0.93, \(P = 1.40 \times 10^{-5}\)) (Fig. 1C; Supplementary Material, Table S2). However, the association appeared to be nearly entirely driven by the Asian POAG collections (OR = 0.89) compared with the Europeans (OR = 0.99, \(P\text{\_het\ between Asians and Europeans} = 0.0042\)) (Fig. 1C). Looking up on our exome dataset, we successfully genotyped rs7437940 (\(r^2 = 0.18\), \(D' = 0.97\) with rs4478172) which also mapped within AFAP1. We note significant association at this AFAP1 marker (rs7437940: Stage 1 \(P = 1.94 \times 10^{-5}\), Stage 2 \(P = 0.08\), \(P\text{-value for meta-analysis} = 4.25 \times 10^{-6}\) (Supplementary Material, Table S2), which supports the previous report (20).

Expression of POAG-associated genes in ocular tissues

We examined the mRNA expression of CDC7, TGFBR3 and FNDC3B in multiple eye tissues. Expression of all three genes was observed in tissues relevant to POAG such as the trabecular meshwork, optic disc and nerve. In contrast to TGFBR3 and FNDC3B, which were expressed in all tested ocular tissues, CDC7 expression was absent in the iris, ciliary body and choroid (Fig. 2).
We also investigated the localization of FNDC3B protein in normal ocular tissues, focusing our attention on cornea and the tissues of the outflow pathway. FNDC3B could be immunolocalized to cells in the trabecular meshwork and all three layers of the cornea (Fig. 2). Immunolocalization of TGFBR3 and CDC7 could not be similarly investigated due to lack of availability of specific antibodies.
We next proceeded to conduct gene-based tests on mutational load to further investigate the role of low-frequency variants in POAG for all patient collections in the discovery stage. Gene-based tests are an alternative to single-marker tests for association, which are often underpowered to detect association with rare variants. To more directly address the impact of low frequency, non-synonymous genetic variants, we considered only the variants with MAF of <5% (33). As a result, we were able to assess a total of 7822 genes having at least two such variants using the sequence kernel association optimal test (34). We note two genes (**MYO18A** and **SULF2**) which showed nominal evidence of association on burden test ($P = 6.26 \times 10^{-7}$ and $P = 5.78 \times 10^{-5}$, respectively), but these findings are primarily driven by the small Vietnamese collection ($N = 58$ cases and $N = 1984$ controls). Removal of this dataset resulted in a marked reduction of the burden test association results (Supplementary Material, Table S4).

**Figure 2.** Analysis of **FNDC3B**, **TGFBR3** and **CDC7** expression in ocular tissues. (A) The **FNDC3B**-specific 162 bp and **TGFBR3**-specific 152 bp amplification product was observed in all analyzed ocular tissues. **CDC7**-specific 242 bp product was observed in sclera, cornea, trabecular meshwork, retina, optic disc and optic nerve. The ubiquitously expressed gene, **ACTB** was used as the normalizing control. A no template sample acted as the negative control (NC) to ensure non-contamination of the RT–PCR reaction mix. The variable M denotes molecular-weight marker. (B) Immunoblot of whole cell lysates from NPCE, retinal pigment epithelial (ARPE19) and HTM cells, probed for **FNDC3B** and β-actin, as a loading control. Positions of the ~133 and ~44 kDa forms of **FNDC3B** are indicated. All ocular cells analyzed expressed the ~133 kDa protein, while ARPE19 cells expressed a smaller ~44 kDa isoform of **FNDC3B**. (C) Immunolocalization of **FNDC3B** in human eye tissues. Strong immunofluorescence labeling of **FNDC3B** (green) was seen in the ciliary muscle (CM) (top row). In the trabecular meshwork (TM, middle row), **FNDC3B** (green) labeling was relatively weaker. Scale bar: 40 µm. **FNDC3B** positive immunoreactivity was also observed in cornea epithelial (*) and cornea endothelial cells (white arrows) (bottom row). Nuclei were stained with DAPI (blue). Scale bar: 100 µm.

**Analysis of rare variants from the exome-chip discovery collection**

We next proceeded to conduct gene-based tests on mutational load to further investigate the role of low-frequency variants in POAG for all patient collections in the discovery stage. Gene-based tests are an alternative to single-marker tests for association, which are often underpowered to detect association with rare variants. To more directly address the impact of low frequency, non-synonymous genetic variants, we considered only the variants with MAF of <5% (33). As a result, we were able to assess a total of 7822 genes having at least two such variants using the sequence kernel association optimal test (34). We note two genes (**MYO18A** and **SULF2**) which showed nominal evidence of association on burden test ($P = 6.26 \times 10^{-7}$ and $P = 5.78 \times 10^{-5}$, respectively), but these findings are primarily driven by the small Vietnamese collection ($N = 58$ cases and $N = 1984$ controls). Removal of this dataset resulted in a marked reduction of the burden test association results (Supplementary Material, Table S4).
Discussion

The present study supports the association of POAG a locus that has been previously implicated with vertical cup-disc ratio, a glaucoma-associated endophenotype. This locus is defined by rs1192415, a common SNP marker mapping to the intergenic region between CDC7 and TGFBR3 (allele [G], OR = 1.13, \( P_{\text{meta}} = 1.6 \times 10^{-10} \)). We were also able to strongly corroborate the established association of POAG with CDKN2B-AS1. CDC7-TGFBR3 rs1192415 has previously been reported to be strongly associated with overall optic disc area, which together with VCDR is a glaucoma-related quantitative trait \((35,36)\). Examination of this locus in >4700 POAG cases and >90,000 controls of European descent in a recent study showed direction of effect consistent with our data \((OR = 1.06, P = 0.12)\) \((37)\). Indeed, in the European section of our study \((N = 4773 \text{ cases and } 11786 \text{ controls})\), we note a similar effect size \((OR = 1.10, P = 0.01)\) in keeping with that shown by Springelkamp and others. The effect of this locus appeared to be much stronger in Asians \((OR = 1.17, P = 1.48 \times 10^{-3})\), leading to a genome-wide significant association upon meta-analysis of all collections. Each copy of the rs1192415 minor allele is associated with a relatively modest increase in POAG risk of 1.13 at exome-wide significance. The consistency of the effect across 21 of 24 POAG collections \(P_{\text{meta}} = 0.22, i^2 \text{ index for heterogeneity} = 16.9\%)\, lend further credence to the observed association.

It is of note that the G allele of the rs1192415 is associated with increase in disc area and therefore a larger VCDR. This may indicate a lower threshold for diagnosing glaucoma in those individuals harboring the risk allele \((38)\). However, as the diagnosis of POAG in this study was not based solely upon the presence of an increased cup-disc ratio but also compatible visual field loss, it is unlikely that selection bias has influenced the result obtained with rs1192415. We note that FNDC3B was one of 16 loci associated with central corneal thickness (sentinel SNP being rs4894535) in a meta-analysis conducted on >20,000 individuals of European and Asian descent \((39)\). That study also showed marker rs4894535 to be associated with POAG in 2979 cases and 7399 controls of European descent \((OR = 0.83, P = 5.6 \times 10^{-4})\). We were able to confirm association of rs4894535 with POAG in 8160 cases and 13,175 controls with readily available DNA for genotyping across 13 collections in this study \((OR = 0.91, P = 0.0018; \text{ Supplementary Material, Table S5})\, thus lending further support for this association.

It is noteworthy that the FNDC3B SNP we report here \(rs4894796\) was nominally associated with POAG in the overall meta-analyses \((OR = 0.93, P = 1.4 \times 10^{-5})\). In particular, the association at rs4894796 was observed to be particularly strong in Asians \((OR = 0.89, P = 7.93 \times 10^{-5})\) compared with Europeans \((OR = 0.99, P = 0.71)\). Unsurprisingly, rs4894796 was found within a different linkage disequilibrium (LD) block than the SNP associated with central corneal thickness and POAG \(rs4894535\). The low LD between rs4894796 and rs4894535 suggests that the association signal at the latter SNP is not likely to be driven by rs4894796. An in-depth inspection of the LD patterns of this genetic region between European and East Asian populations, which currently appear visually identical according to available heat maps \((http://www.hapmap.org)\, may be useful to better understand the degree of LD dissimilarity between populations and its bearing on the results obtained for FNDC3B. Of note, a further SNP \(rs6445055\) at FNDC3B showed genome-wide significant association for intraocular pressure \((IOP) (P = 4.9 \times 10^{-8})\), but only marginal association was seen for POAG per se \((P = 0.03, OR = 0.92)\) in 4284 POAG cases and 95,560 controls of European descent \((21)\). As the FNDC3B SNP associations with POAG are still suggestive rather than affirmative it is presumptive at this stage to speculate that it could be one of the links for IOP-dependent mechanisms of POAG.

This is one of the largest studies on the genetics of POAG, yet the power to detect genes with small effects was limited. One crucial reason for this apparent loss of power could be the scope of the genetic content which was used for interrogation \((as \text{ exomic content only comprises } <2\% \text{ of the entire human genome, much useful data could be missed if the bulk of the true positive genetic associations for POAG lie in the non-coding regions of the genome})\. The fact that our discovery cohort was not mono-ethnic may also have reduced the power to detect ethnic specific variants of small effect sizes due to significant differences in allele frequencies between ethnic groups. The phenotypic heterogeneity and the lack of standardized clinical criteria across the cohorts may also have contributed to a loss in power. The level of IOP is commonly used to subdivide POAG into two subtypes: POAG with high IOP \((>21 \text{ mmHg})\, named high-tension glaucoma, HTG) and NTG with normal IOP \((<21 \text{ mmHg})\). In our cohorts, we had differing number of these subtypes with some cohorts having more NTG or HTG than others. In this study, we also did not subdivide POAG subjects into HTG and NTG to discover subtype-specific variants, focusing instead on identifying genetic variants for the overarching phenotype of POAG. However, when CDC7-TGFBR3 rs1192415 and FNDC3B rs4894796 were analyzed within NTG and HTG subgroups, they did not show differences in strength of association by subtype \((data \ not \ shown)\.

All three loci we report in this study \((CDKN2B-AS1, TGFBR3-CDC7 \ and \ FNDC3B)\) contain genes which may contribute to the regulation of transforming growth factor-\(\beta\) (TGF-\(\beta\)) signaling. TGF-\(\beta\) has been implicated previously in glaucomatous optic nerve damage and RGC death \((40,42)\). The TGF-\(\beta\)-family includes TGF\(\alpha_1\), TGF\(\alpha_2\) and TGF\(\alpha_3\), all of which bind to TGF-\(\beta\) receptor type-2 \(TGFBR2\). All TGF-\(\beta\) family members are dimeric polypeptide growth factors that inhibit the progression of cell cycle, which in turn may lead to terminal differentiation or apoptosis \((40,43,44)\). TGF-\(\beta\) also modulates developmental and repair processes in several tissues. TGF-\(\beta\) signaling has been implicated in a wide variety of diseases including inflammation, autoimmune disorders, fibrosis, cancer, cataracts as well as glaucoma \((40,43,44)\). The most strongly POAG-associated locus, CDKN2B-AS1, has been shown to regulate the transcription of cyclin-dependent kinase inhibitor 2A and 2B \((CDKN2A \ and CDKN2B)\) \((45)\, which inhibit cell proliferation via the TGF-\(\beta\)-pathway by inducing G1-phase cell cycle arrest \((44)\). Burdon et al. reported the up-regulation of CDKN2A and CDKN2B in response to elevated IOP \((18)\). Collectively, these data suggest a link between the most well-recognized physiological risk factor of POAG and a downstream molecular response that may lead to RGC death. In this context, both CDC7 and TGFBR3 are also of interest and could have relevance to glaucomatous optic nerve damage and RGC death.

CDC7 encodes a cell division cycle protein with kinase activity that also interacts with CDKN2A. TGFBR3 is a TGF-\(\beta\)-super family co-receptor, and is the most abundant of all TGF-\(\beta\) receptors \((46)\). Through protein crystallography, murine TGF-3 ZP domain (ZP-3) has been recently identified as a novel major TGF-\(\beta\)-binding site \((47)\). It has been suggested that TGFBR3 may serve to enhance the binding of TGF-\(\beta\) ligands to TGF-\(\beta\) type II receptors by binding TGF-\(\beta\) and presenting it to TGFBR2, the receptor for all three TGF-\(\beta\) ligands. A linkage between FNDC3B, an oncogene and TGF-\(\beta\) signaling was also reported recently by Cai et al. Overexpression of FNDC3B was shown to induce epithelial-to-mesenchymal transition and activate several cancer pathways, including PI3-kinase/
Akt, Rb1 and TGF-β signaling (48). FND3C also induced expression of all three TGF-β ligands and promoted TGFBR1 cell-surface localization (48). This connection of POAG-associated genes/loci with the TGF-β signaling pathway therefore lends further credence to the hypothesis of TGFβ pathway involvement in glaucoma.

However, despite these attractive speculations on the genes vicinal to the associated SNPs, it is possible that these variants may also affect distant as yet unidentified target genes. Definitive evidence for the involvement of these genes in the pathogenesis of POAG awaits confirmation in other datasets, as well as the identification and characterization of functional variants.

Materials and Methods

Sample collections

Ethics statement

Ethics approval was obtained from the Centralized Institutional Review Board for the Singaporean patient sample and data collection and for the conduct of this study. All study protocols for patient and control sample collection were approved by the respective relevant Medical Ethics Committees of each participating site. All studies were conducted under the tenets of the Declaration of Helsinki with written informed consent obtained from all participants.

POAG and health controls subjects inclusion criteria

POAG cases in this study were defined by the following criteria: the presence of glaucomatous optic neuropathy (defined as loss of neuroretinal rim with a vertical cup: disc ratio of >0.7 or the presence of glaucomatous optic neuropathy (de

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POAG and health controls subjects inclusion criteria

POAG cases in this study were defined by the following criteria: the presence of glaucomatous optic neuropathy (defined as loss of neuroretinal rim with a vertical cup: disc ratio of >0.7 or an inter-eye asymmetry of >0.2 and/or notching attributable to glaucoma) with compatible visual field loss, open angles on gonioscopy, and absence of secondary causes of glaucomatous optic neuropathy. POAG patients with a mean IOP without treatment that is consistently <21 mmHg on diurnal testing are classified as having NTG, whereas those with a mean IOP without treatment that is consistently >21 mmHg are classified as having HTG. Patients who were unable to give informed consent, or with secondary glaucoma due to trauma, uveitis, neovascularization, pigtrom exfoliation, pigment dispersion, etc., were excluded from this study.

Controls in this study were recruited in a hospital-based or population-based manner. Hospital-based controls were all generally over the age of 40 years and confirmed to have no sign of glaucoma or other major eye diseases except for mild cataract and mild refractive errors (defined as |SE| < 3D) by an ophthalmic examination. These subjects at time of recruitment had IOP of <21 mmHg with open angles, healthy optic nerves, normal visual fields, and no family history of glaucoma. Population-based controls were ethnically matched healthy individuals over the age of 40 years, unless indicated otherwise (see Table 1; Supplementary Material for more details of the samples used in this study).

Genotyping and data QC

Study participants in the discovery stage were genotyped using the Illumina’s Infinium HumanExomeBeadChip (Version 1.0) + Semi-Custom BeadChip (ILLUMINA Inc.) that contains ~250 000 SNPs of base content and an additional 25 000 East Asian-specific polymorphisms located on the coding frame. Stringent QC filters were applied after the laboratory work in genotyping completed. SNP markers that had missingness exceeding 5%, gross departure from HWE (P-value 1e–6) or were monomorphic were excluded from subsequent analysis. Likewise, individual samples with an overall call rate <95% were excluded. Samples were subjected to biological relationship verification by using the principle of variable in allele sharing according to the degree of relationship. Identity-by-state information was derived by PLINK. Those individuals who showed evidence of cryptic relatedness were removed prior to analysis. In addition, samples showing gender discrepancies between the clinical gender and genetically inferred gender were removed. A total of 1008 samples were excluded after rigorous application of QC filters (568 sample exclusions were due to PCA ancestral outliers, 246 were excluded due to per-sample call rate <95%, 168 were excluded due to first-degree familial relationships detected from the discovery stage exome-chip genotyping, and 26 were excluded due to suspicions of sample contamination). PC analysis was undertaken using EIGENSTRAT to account for spurious associations resulting from ancestry differences of individual SNPs (49). PCs showing significant effect on univariate analysis were used to correct for any underlying population substructure. We adjusted for the top three PCs (PC1–PC3) for Singapore, Hong Kong, Japan, USA-African Americans, China-Beijing and Vietnam. The Indian POAG collection was adjusted for the top 10 PCs (PC1–PC10), as there was more population substructure in this collection. After adjustment, we observed minimal evidence of genomic inflation (λGC = 1.042), thereby suggesting that this well-described method of controlling for population stratification was adequate in our study. Genotyping clouds for the key SNPs CDKN2B-AS1 rs2157719, CDC7-TGFBR3rs1192415 and FND3Crs4894796 were directly visualized (Supplementary Material, Fig. S5) to ensure good quality.

For Stage 2 (replication stage), genotyping was performed using the Sequenom MassArray platform (www.sequenom.com). Samples recruited at the latter stage of replication were genotyped for the key SNPs CDC7-TGFBR3rs1192415, and FND3Crs4894796 using the pre-developed Taqman Assays (Applied Biosystems, Foster City, CA, USA; www.appliedbiosystems.com)

Statistical analysis

We contrasted the genotypes between POAG cases and healthy controls via single-SNP analysis using unconditional logistic regression fitted for genotype trend effects (1-degree-of-freedom score test). To do this, the PLINK software [version 1.07] (50) was used for modeling within a logistic regression framework, adjusting for age, gender and genetic ancestry (reflected by PCs). Manhattan and LD plots were created using Haploview [version 3.2] (51). Q-Q and regional association plots were created using the software R [www.r-project.org ] (52). Meta-analysis summarizing the results across all cohorts was performed using both fixed and random-effects modeling weighted in an inverse-variance manner (53). This method weighs each study according to effective sample size and cohort-specific MAF of the associated variants. To avoid an otherwise unacceptable number of false positive signals as an artifact of multiple testing, the threshold for exome-wide significance, P < 2 × 10–7, was considered to be statistically significant. Heterogeneity of the meta-analyses was calculated by measuring I².

Expression analysis of genes

Expression of CDC7, TGFBR3 and FND3C was assessed by semi-quantitative reverse transcription–PCR (RT–PCR) using gene-specific primers (CDC7-forward 5‘-TTTCTCCCAGGGTGACCC-3’, CDC7-reverse 5‘-GCAATTTTCTCAGGCTCTAC-3’, TGFBR3-forward 5‘-TCTTCTACGTCACATCCAC-3’, TGFBR3-reverse 5‘-TGC
TGATGAAAATGGGAC-3'; FNDC3B-forward 5'-AGCACTCATCT TCCCCACACA-3', FNDC3B-reverse 5'-AAAGGAGGGCCTGTG AGG-3') on total RNA extracted from a variety of ocular tissues (cornea, sclera, retina and retinal pigment epithelium, iris, lens capsule and optic nerve) as described earlier (54). We used the ubiquitously expressed ACTB gene (forward 5'-CCAAACCCCGGA GAAGATGCA-3' and reverse 5'-CCAGGGGCTACAGGGATAG-3') as amplification and normalizing control.

**Western blotting**

Cell lines obtained from American Type Culture Collection (Manassas, VA, USA) were the human retinal pigment epithelial cell line (A191), human Trabecular Meshwork cell line (HTM) was purchased from ScienCellResearch Laboratories (Carlsbad, CA, USA) and the human non-pigmented ciliary epithelial cell line (NPC) is a kind gift from Prof. Miguel Coca-Prados from Yale School of Medicine. Cell lysates were obtained by lysing individual cell lines in lysis buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 M NaF, 0.4 M EDTA and 10% glycerol). SDS-PAGE resolved proteins were transferred to Hybond-C Extra nitrocellulose membranes (Amersham Life Science Inc., Arlington Heights, IL, USA). Membranes were blocked and blotted by 5% nonfat milk, 0.1% Tween 20 in Tris-buffered saline (20 mM Tris–HCl, pH 7.6, 150 mM NaCl) for 1 h before incubation with FNDC3B for 1 h (1:250) (Sigma-Aldrich Corp., St. Louis, MO, USA). Actin-heralded peroxidase (HRP) (1:50 000) from Santa Cruz Biotechnology (Dallas, TX, USA). The bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Biosciences, Pittsburgh, PA, USA), and visualized by Luminata Forte Western HRP substrate (Millipore, Bedford, MA, USA).

**Immunofluorescence confocal microscopy**

Immunofluorescence confocal microscopy was performed on antigen retrieved 4 µm paraffin sections. Blocking of tissue sections was performed with blocking buffer (5% nonfat milk, 5% FBS, 0.1% PBS-Tween; 1× pen/strep) for 1 h at RT. FNDC3B antibody (Sigma-Aldrich Corp.) was diluted (1:50) into blocking buffer and incubated overnight at 4°C. Secondary FITC (1:300)-labelled anti-rabbit antibody (Jackson Laboratories, West Grove, PA, USA) was also diluted in blocking buffer and incubated at RT for 1 h followed by application of Vectashield with 4′,6-diamidino-2-phenyl-indole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Coverslips were then used to overlay the sections and stored in the dark at 4°C until viewing with Olympus Fluoview 1000 confocal microscopy (Olympus Optical Co. Ltd., Tokyo, Japan).

**Supplementary Material**

Supplementary Material is available at HMG online.

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