

ORIGINAL ARTICLE

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

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Abstract

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, $P = 2.81 \times 10^{-33}$), we observed one SNP showing significant association to POAG (*CDC7-TGFBR3* rs1192415, $OR_{G-allele} = 1.13$, $P_{meta} = 1.60 \times 10^{-8}$). 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.

Introduction

Glaucoma is the leading cause of irreversible visual impairment and blindness, affecting >60 million people worldwide, and it is estimated that the number of affected individuals will reach 80 million in 2020 (1–3). Primary open angle glaucoma (POAG) is the most prevalent form of glaucoma in most populations and is characterized by progressive retinal ganglion cell (RGC) loss that causes characteristic structural changes of the optic nerve with associated with visual field loss in the face of an open drainage angle in the eye. POAG has a strong genetic component that has been well documented (4). Indeed, several susceptibility loci have been identified for POAG through the use of linkage and association studies (5). Genes known to contribute to glaucoma include myocillin (*MYOC*), optineurin (*OPTN*), TANK-binding kinase 1 (*TBK1*) and WD repeat domain 36 (*WDR36*) (6–12). However, mutations in these genes account for no >5–10% of all POAG cases in the general population (5).

It is likely that POAG, as a complex trait, results from the interactions of multiple genes and environmental factors (13–15). Genome-wide association studies (GWAS) have provided further insights into the genetic basis of POAG (16). The first GWAS on POAG was conducted on 1263 POAG cases and 34 877 controls from Iceland. Genome-wide significant association was detected at the *CAV1-CAV2* locus on Chromosome 7q31 and subsequently was replicated in a multi-ethnic sample collection from Sweden, the UK, Australia, Hong Kong and China (17). This was rapidly followed by five other GWAS studies, which utilized either advanced or non-advanced POAG cases derived from populations

of European or East Asian ancestries (18–22). These latter studies led to the discovery of nine additional genetic regions associated with POAG disease risk (*TMCO1*, *CDKN2B-AS1*, *SIX1-SIX6*, an intergenic region on chromosome 8q22, *ABCA1*, *GAS7*, *AFAP1*, *GMDS* and *PMM2*). Several of these genetic loci have been replicated in ethnically diverse populations, demonstrating them to be bona fide POAG associations with global implications (23–25).

Similar in concept and laboratory chemistry to the whole-genome genotyping chip design, the exome array approach evaluates putative functional coding variants selected from the exome sequences of >12 000 individuals (26). In addition, the exome array also contains >5000 common variant SNPs from GWAS arrays with a minor allele frequency (MAF) exceeding 5% which can serve as ancestry informative markers. Exome array genotyping allows us to specifically explore the possible contribution of potentially functional coding variants in POAG disease susceptibility.

Results

Common genetic variants in *CDKN2B-AS1* and *TGFBR3-CDC7* are associated with POAG

We conducted a two-stage Exome Chip discovery and replication on POAG cases and normal controls. For the discovery stage (Stage 1), genotyping was performed using the Illumina Infinium HumanExomeBeadChip (v1.0) on a total of 3822 POAG cases and 10 426 normal controls drawn from seven countries (Table 1). In addition to the $\approx 247\,000$ SNP markers present on the standard

Table 1. Sample collections of POAG cases and controls for Stages 1 (discovery) and 2 (replication)

Collection	N cases	N controls	Ethnicity	Age of cases	Age of controls ^a	Collection comment
Singapore	850	2347	Singaporean Chinese	71.6 ± 10.1	58.88 ± 9.6	New recruitment
Japan	923	640	Japanese	65.3 ± 13.1	71.9 ± 5.8	Previously GWAS in Nakano <i>et al.</i> (25)
USA-African-Americans	590	636	African American	65.4 ± 12.3	54.8 ± 9.8	New recruitment
China-Beijing	587	461	Northern Chinese	58.5 ± 12.5	Population-based controls	New recruitment
Hong Kong	375	2962	Southern Chinese	62.3 ± 15.3	Population-based controls	Previously described for replication in Thorleifsson <i>et al.</i> (17)
South India	121	716	Indian	60.9 ± 12.0	51.0 ± 6.5	New recruitment
Vietnam	58	1984	Vietnamese	63 ± 7.1	Population-based controls	New recruitment
Total discovery	3504	9746				2206 cases are new discovery samples
Stage 2 replication						
Singapore-2	520	5473	Singaporean Chinese	71.1 ± 10	Population-based controls	New recruitment
Japan-2	935	996	Japanese	64.3 ± 14.0	57.5 ± 13.9	N = 411 used for replication in Nakano <i>et al.</i> (25)
USA-African-American 2	497	304	African-American	69.1 ± 11.0	66.7 ± 13.1	New recruitment
South India-2	453	2496	Indian	62.5 ± 9.9	58.9 ± 10.1	New recruitment
Korea	400	454	Korean	59.0 ± 11.8	40.3 ± 14.1	New recruitment
Saudi Arabia	236	655	Middle Eastern	60.8 ± 12.7	54.4 ± 11.7	New recruitment
Malaysia	132	2540	Malay	65.1 ± 8.2	58.7 ± 11.0	New recruitment
China-Beijing 2	115	251	Northern Chinese	54.2 ± 12.4	71.53 ± 7.16	New recruitment
UK	336	6090	European	71.4 ± 10.8	Population-based controls	New recruitment
China-Shantou	247	289	Southern Chinese	52.9 ± 19.4	75.7 ± 6.1	Previously described for replication in Thorleifsson <i>et al.</i> (17)
Germany	56	142	European	67.9 ± 11.4	78.4 ± 8.9	New recruitment
Vietnam-2	76	245	Vietnamese	52.4 ± 17.4	51.3 ± 17.8	New recruitment
France	80	75	European	75.6 ± 8.5	73.5 ± 8.3	New recruitment
China-Shanghai, Chengdu 2	181	286	Southern Chinese	54.7 ± 16.5	84.7 ± 11.7	Previously described in Chen <i>et al.</i> (19)
China-Shanghai, Chengdu	608	1005	Southern Chinese	49.6 ± 17.0	62.9 ± 12.1	Previously described in Chen <i>et al.</i> (19)
USA (NEIGHBOR)	2170	2347	European descendant	66.4	68	Previously described in Wiggs <i>et al.</i> (22)
USA (GLAUGEN)	976	1140	European descendant	63.6	65.5	Previously described in Wiggs <i>et al.</i> (22)
Australia (ANZRAG)	1155	1992	European descendant	60.5 ± 14.3	55.6 ± 14.4	Previously described in Gharahkhani <i>et al.</i> (20)
Total replication	9173	26 780				3425 cases are new replication samples
Total all samples	12 677	36 526				5631 cases are new in this report.

^aPopulation-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) $P \geq 10^{-6}$. 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 ($N_{\text{meta-analysis}} = 7$ collections). A quantile–quantile plot derived from the meta-analysis P-values showed no significant dispersion of test statistics from the expected distribution ($\lambda_{GC} = 1.042$; 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 = 1.84×10^{-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], OR = 0.71, $P_{\text{meta}} = 2.81 \times 10^{-33}$) (Supplementary Material, Table S2 and Fig. S4). 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_{het} between Asians and Europeans = 0.0042) (Fig. 1C). A recently reported GWAS on POAG showed strong association with common SNP markers mapping to AFAP1 (rs4478172) (20). 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 -value for meta-analysis = 4.25×10^{-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).

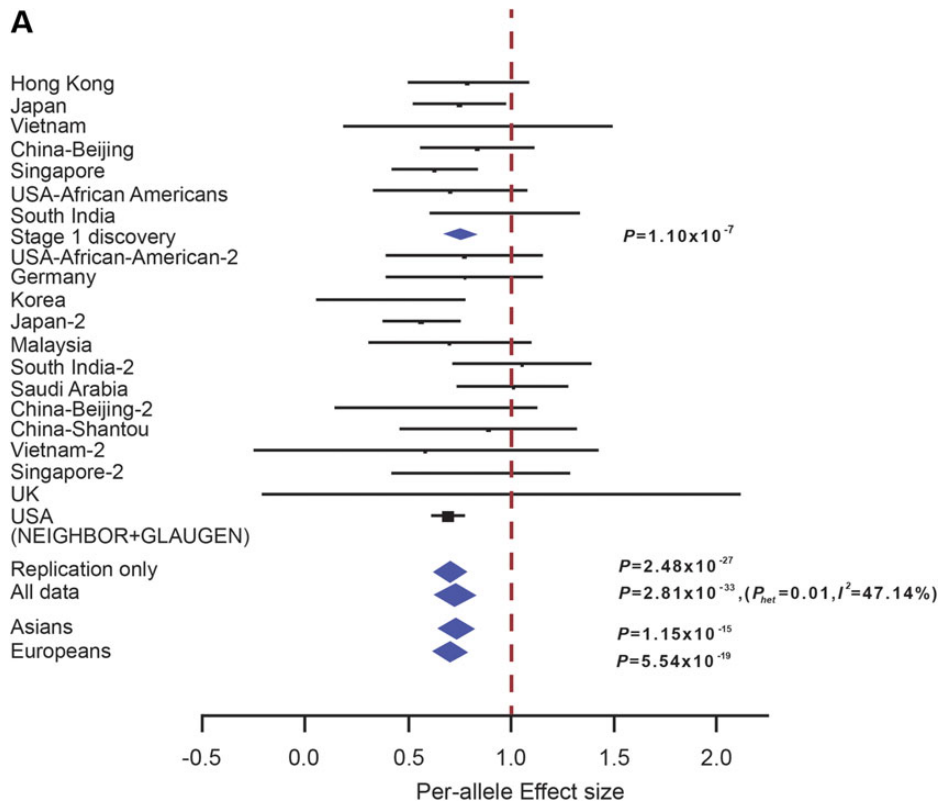


Figure 1. Forest plots showing evidence of association between SNPs: (A) CDKN2B-AS1 rs2157719, (B) CDC7/TGFBR3 rs1192415 and (C) FNDC3B rs4894796. The vertical line represents a per-allele odds ratio of 1.00. The oblongs represent point estimates (referring to the per-allele odds ratio), with the height of the oblongs inversely proportional to the standard error of the point estimates. Horizontal lines indicate the 95% confidence interval for each point estimate. Meta-analysis of Stages 1 and 2, OR, P_{meta} and I^2 was labeled on the right-hand side for corresponding analysis. For rs4894796 genotyping, see Supplementary Material, Information for sample collections.

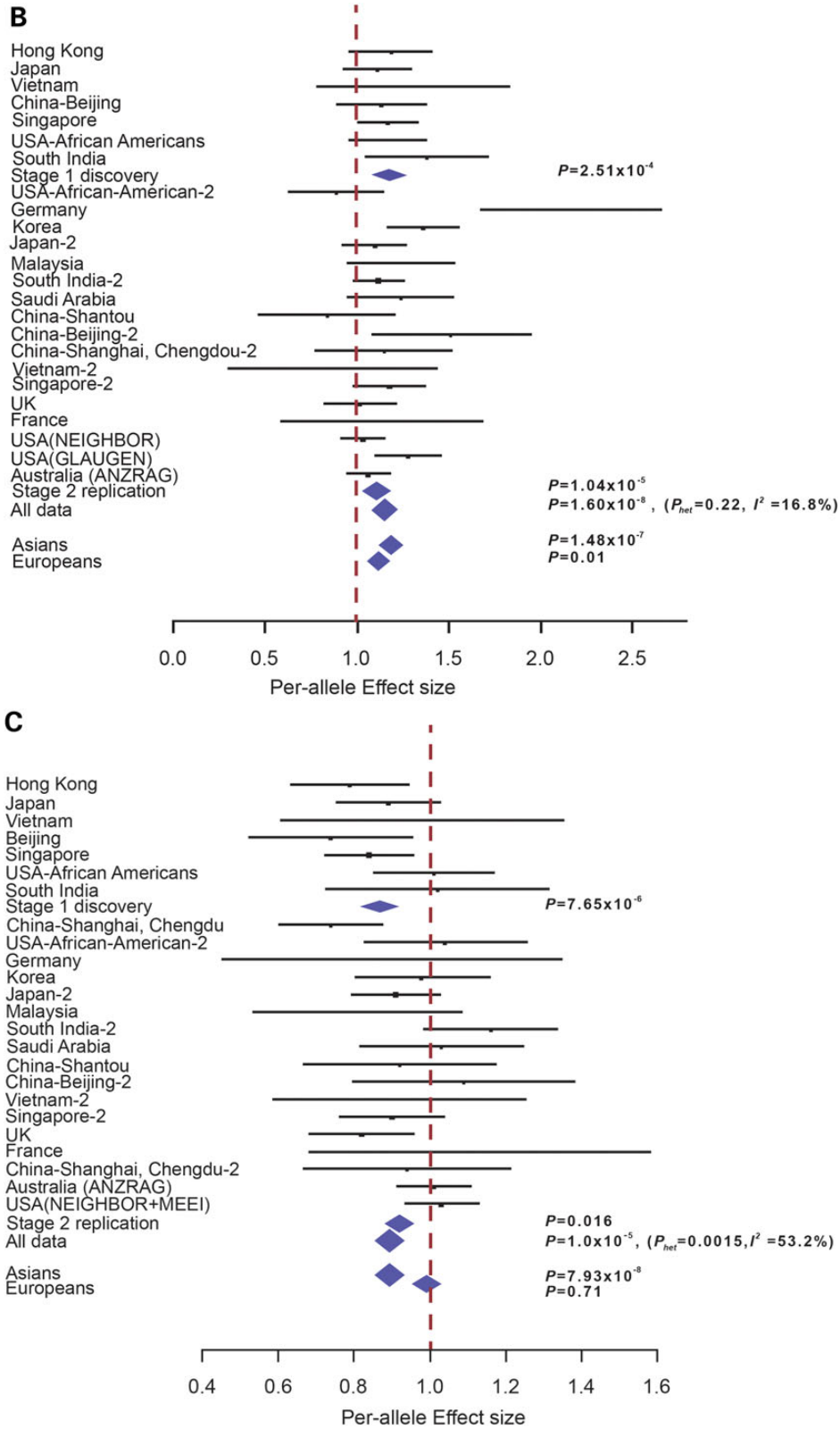


Figure 1 Continued

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.

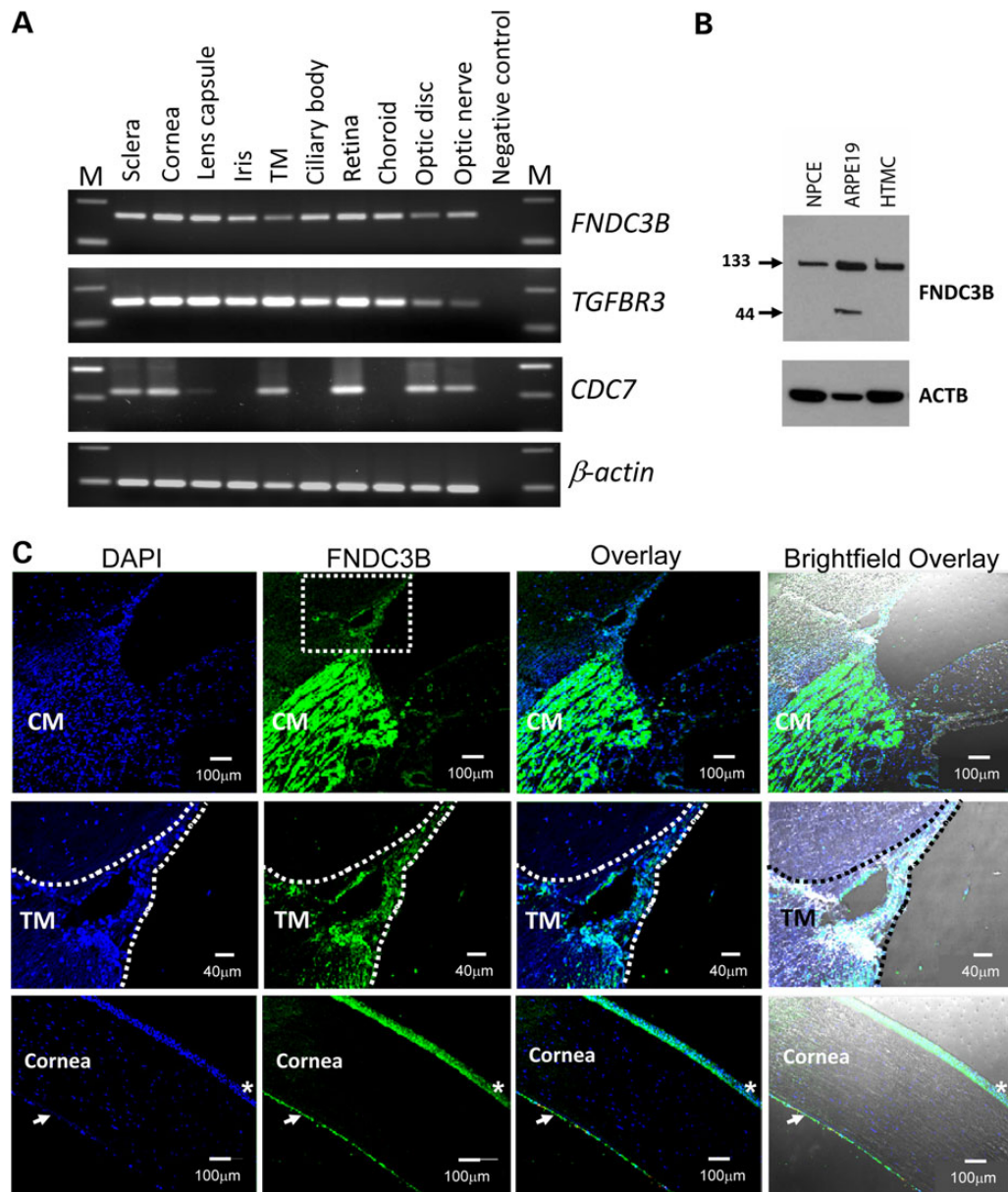


Figure 2. Analysis of *FNDC3B*, *TGFBR3* and *CDC7* expression in ocular tissues. (A) The *FNDC3B*-specific 162 bp and *TGFBR3*-specific 152 bp amplification product was observed in all analyzed ocular tissues. *CDC7*-specific 242 bp product was observed in sclera, cornea, trabecular meshwork, retina, 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). Scale bar: 100 μ m. In the trabecular meshwork (TM, middle row), *FNDC3B* (green) labeling was relatively weaker. Scale bar: 40 μ m. *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 μ m.

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, $P_{\text{meta}} = 1.6 \times 10^{-8}$). 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 ($N = 4773$ cases and 11786 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, $P = 1.48 \times 10^{-7}$), 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 ($P_{\text{het}} = 0.22$, I^2 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 5810 cases and 13 175 controls with readily available DNA for genotyping across 13 collections in this study (OR = 0.91, $P = 0.0018$; 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, $P = 7.93 \times 10^{-8}$) 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 exomic content only comprises <2% 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 mmHg; named high-tension glaucoma, HTG) and NTG with normal IOP (<21 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- β (TGF- β) signaling. TGF- β has been implicated previously in glaucomatous optic nerve damage and RGC death (40–42). The TGF- β family includes TGF β 1, TGF β 2 and TGF β 3, all of which bind to TGF- β receptor type-2 (TGFBR2). All TGF- β 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- β also modulates developmental and repair processes in several tissues. TGF- β 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- β 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- β super family co-receptor, and is the most abundant of all TGF- β receptors (46). Through protein crystallography, murine TGF- β 3 ZP domain (ZP-C) has been recently identified as a novel major TGF- β -binding site (47). It has been suggested that *TGFBR3* may serve to enhance the binding of TGF- β ligands to TGF- β type II receptors by binding TGF- β and presenting it to TGFBR2, the receptor for all three TGF- β ligands. A linkage between *FNDC3B*, an oncogene and TGF- β 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). FNDC3B 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 TGF β 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 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, pseudoexfoliation, 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 variability 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 before PC analysis was conducted. 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 ancestral 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 ($\lambda_{GC} = 1.042$), 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-TGFB3rs1192415 and FNDC3Brs4894796 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-TGFB3rs1192415, and FNDC3Brs4894796 using 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, $P < 2 \times 10^{-7}$, was considered to be statistically significant. Heterogeneity of the meta-analyses was calculated by measuring I^2 .

Expression analysis of genes

Expression of CDC7, TGFB3 and FNDC3B was assessed by semi-quantitative reverse transcription-PCR (RT-PCR) using gene-specific primers (CDC7-forward 5'-TTTCTCCCCAGCGTGACC-3', CDC7-reverse 5'-GCAATTTCTCTCAGGTCCTAC-3'; TGFB3-forward 5'-TCTCCTCAGTCCACATCCAC-3', TGFB3-reverse 5'-TGC

TGATGAAAACCTGGACCAC-3'; FNDC3B-forward 5'-AGCATCATCT TCCCACACA-3', FNDC3B-reverse 5'-AAGAAGGAGGGCTGTTG 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'-CCAACCGCGA GAAGATGA-3' and reverse 5'-CCAGAGCGGTACAGGGATAG-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 (APRE19), human Trabecular Meshwork cell line (HTM) was purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and the human non-pigmented ciliary epithelial cell line (NPCE) is a kind gift from Prof. Miguel Coca-Prados from Yale School of Medicine. Cell lysates were obtained by lysing individual cell lines with lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM NaVO₄, 10 mM NaF, 0.4 mM 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-horseradish 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, Westgrove, 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 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Supplementary Material

Supplementary Material is available at HMG online.

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