Genome-Wide Analysis of Central Corneal Thickness in Primary Open-Angle Glaucoma Cases in the NEIGHBOR and GLAUGEN Consortia

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Purpose. To investigate the effects of central corneal thickness (CCT)-associated variants on primary open-angle glaucoma (POAG) risk using single nucleotide polymorphisms (SNP) data from the Glaucoma Genes and Environment (GLAUGEN) and National Eye Institute (NEI) Glaucoma Human Genetics Collaboration (NEIGHBOR) consortia.

Methods. A replication analysis of previously reported CCT SNPs was performed in a CCT dataset ($n = 1117$) and these SNPs were then tested for association with POAG using a larger POAG dataset ($n = 6470$). Then a CCT genome-wide association study (GWAS) was performed. Top SNPs from this analysis were selected and tested for association with POAG. cDNA libraries from fetal and adult brain and ocular tissue samples were generated and used for candidate gene expression analysis.

Results. Association with one of 20 previously published CCT SNPs was replicated: rs12447690, near the *ZNF469* gene ($P = 0.001$; $\beta = -5.08$ μm/allele). None of these SNPs were significantly associated with POAG. In the CCT GWAS, no SNPs reached genome-wide significance. After testing 50 candidate SNPs for association with POAG, one SNP was identified, rs7481514 within the neurotrimin (*NTM*) gene, that was significantly associated with POAG in a low-tension subset ($P = 0.00099$; Odds Ratio [OR] = 1.28). Additionally, SNPs in the *CNTNAP4* gene showed suggestive association with POAG (top SNP = rs1428758; $P = 0.018$; OR = 0.84). *NTM* and *CNTNAP4* were shown to be expressed in ocular tissues.

Conclusions. The results suggest previously reported CCT loci are not significantly associated with POAG susceptibility. By performing a quantitative analysis of CCT and a subsequent analysis of POAG, SNPs in two cell adhesion molecules, *NTM* and *CNTNAP4*, were identified and may increase POAG susceptibility in a subset of cases. (*Invest Ophthalmol Vis Sci.* 2012;53:4468–4474) DOI:10.1167/iovs.12-9784

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The cornea is a highly collagenous, transparent tissue that is a continuation of the sclera, which posteriorly supports the optic nerve. A reduction in central corneal thickness (CCT) has been observed in a number of systemic and ocular diseases, including Ehlers-Danlos syndrome, osteogenesis imperfecta, keratoconus, and brittle cornea syndrome.[1–4] Reduced CCT has also been associated with an increased risk of primary open-angle glaucoma (POAG), the second leading cause of blindness worldwide.[5,6] In POAG, CCT has been shown to be a risk factor independent of other known risk factors, including advanced age and elevated intraocular pressure (IOP), and has been linked with increased disease severity and rapid disease progression.[5–8]

Due to the strong association observed between CCT and POAG, elucidating the genetics of CCT may lead to identification of novel POAG risk genes, and could improve the understanding of the molecular mechanism driving this association. CCT has previously been shown to be a highly heritable, normally distributed quantitative trait with heritability estimates as high as 95%.[5] Several studies have been successful in identifying CCT loci through candidate gene and genome-wide approaches. An Australian and UK cohort study first identified the *ZNF469* gene, an uncharacterized zinc-finger protein, and *FOXO1*, a member of the forkhead family of transcription factors, in a genome-wide analysis of CCT.[2] The *ZNF469* locus has been replicated in follow-up studies in Croatian, Scottish, and multiple Asian populations.[3,4,9] Consistent with the belief that CCT is a complex quantitative trait with multigenic inheritance, these studies have also identified numerous other loci in genomic regions containing collagen V alpha-1 (*COL5A1*), collagen VIII alpha-2 (*COL8A2*), A-kinase anchor protein 13 (*AKAP13*), inhibitor of Bruton's tyrosine kinase (*IBTK*), leucine-rich repeat kinase 1 (*LRRK1*), as well as multiple intergenic regions.[3,4,9]

Although these genome-wide studies have successfully identified genetic variants associated with CCT, they have been performed in primarily nonglaucomatous population cohorts. Because of the strong correlation of reduced CCT and increased POAG risk, it is possible that POAG cases have additional variants associated with CCT compared with normal populations, and that these variants may explain the underlying molecular mechanism driving the association between reduced CCT and POAG risk. Identification of complex disease genes through quantitative endophenotypes has many advantages, and has been successful in POAG with other quantitative risk factors.[10–12] In this study, an investigation of the effects of previously reported CCT-associated variants and a genome-wide analysis of CCT was performed in a primarily POAG case population. The effects of these SNPs were then tested using a larger POAG case-control population.

Materials and Methods

Study Population

Genome-wide genotype data were available from two separate cohort studies: the Glaucoma Genes and Environment (GLAUGEN) study (dbGaP Study Accession: phs000308.v1.p1, available at http://www.ncbi.nlm.nih.gov/projects/gap), which is part of the Gene, Environment Association Study consortium, and the National Eye Institute (NEI) Glaucoma Human Genetics Collaboration (NEIGHBOR) study.[13,14] The GLAUGEN study included Caucasian POAG cases and controls selected from three different sub studies: the Nurses' Health Study, the Health Professionals Follow-up Study, and the Genetics Etiologies of Primary-Open Angle Glaucoma Study. This study was approved by the institutional review boards of the Massachusetts Eye and Ear infirmary, the Harvard University School of Public Health and the Brigham and Woman's Hospital. The NEIGHBOR study consists of Caucasian POAG cases and controls collected from 12 different study sites. Each individual study was approved by their respective institutional review boards (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental). All human subjects' research adhered to the tenets of the Declaration of Helsinki. POAG cases for both studies were defined as individuals with glaucomatous optic neuropathy with reproducible visual field tests or a cup-to-disc ratio of greater than or equal to 0.7 in at least one eye. POAG cases were grouped into two subsets based on IOP for stratified analyses. A low-tension subset was defined by an IOP of less than or equal to 21 mmHg at the time of sample collection, with no history of IOP greater than 21 mmHg (either by self report or by chart review). A total of 705 POAG cases met the low-tension definition while 1629 POAG cases with a history of any IOP greater than 21 mmHg were included in the high-tension subset. POAG controls had normal IOP (< 21 mmHg), no evidence of visual field loss, and a normal cup-to-disc ratio (< 0.6).

Table 1. Population Characteristics of the CCT Dataset

CCT Dataset	*n*	Mean (SD)
Total	1117	
Age (y)		61.6 (12.58)
Sex (% male)		48.9%
CCT (μm)		
*POAG control	144	557.7 (33.80)
*POAG case	973	545.9 (35.85)
*Standard pachymeter	1010	545.6 (35.33)
*Portable pachymeter	107	565.0 (35.60)
*POAG cases, low IOP subset	307	541.8 (36.10)
*POAG cases, high IOP subset	625	548.4 (35.87)

* Student's *t*-test shows statistically significant differences were observed when comparing CCT distributions between: POAG cases versus controls; CCT measurements made by a standard pachymeter versus a portable model; and POAG cases within the low- versus high-tension subsets.

CCT Association Analysis

CCT measurements were available for 851 individuals, both POAG cases and POAG controls, from the NEIGHBOR study, and 325 individuals, all POAG cases, from the GLAUGEN study (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental). For the CCT analysis, the mean CCT from both eyes was used as the analysis outcome variable because a strong correlation between right eye CCT and left eye CCT ($R^2 = 0.92$ in GLAUGEN; $R^2 = 0.83$ in NEIGHBOR) was observed. Ten outliers in GLAUGEN and 16 outliers in NEIGHBOR, which were defined by large left-right differences (beyond 2.5 SD of the overall distribution of left-right differences) were removed from the analysis. A lack of left-right eye correlation could be due to true right and left eye CCT differences, measurement artifacts, or unknown corneal defects. Additionally, individuals with known corneal diseases, noncaucasian ancestry, and one distribution outlier with an extremely small average CCT value (< 374 μm) were removed. Population substructure was assessed by principal components analysis performed using Eigenstrat[15] and genetic outliers were removed from the analysis (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental). After data cleaning, the CCT dataset consisted of 1,117 individuals, including 668 POAG cases and 144 POAG controls from the NEIGHBOR study, and 305 POAG cases from the GLAUGEN study (Table 1).

CCT distribution differences were assessed among a variety of clinical variables, including POAG case status, CCT measurement device, DNA source (blood versus cheek), DNA extraction method

(Qiagen, DNAzol, GENTRA), history of laser trabeculoplasty, and incisional glaucoma filtration surgery using the Student's *t*-test calculated in SAS (version 9.2; SAS Institute Inc., Cary, NC).

Genotyping was performed using the Human660W-Quad v1 BeadChip (Illumina, Inc., San Diego, CA) array for both the NEIGHBOR and GLAUGEN studies. SNPs with a missing call rate of greater than 2% and SNPs out of Hardy-Weinberg equilibrium in the control population ($P < 10^{-4}$) were removed from the analysis. The final dataset contained 480,304 cleaned SNPs shared between the two studies. SNPs were tested for association with average CCT using linear regression performed in PLINK,[16] adjusted for age, sex, instrument used to measure CCT, and POAG case/control status.

POAG Association Analysis

Candidate SNPs were tested for association with POAG risk in the full NEIGHBOR and GLAUGEN datasets. After quality control filtering, including removal of sex mismatches, duplicate samples, and population outliers, the final dataset contained 2125 POAG cases and 2251 POAG controls from the NEIGHBOR study, and 964 POAG cases and 1130 POAG controls from the GLAUGEN study. In the NEIGHBOR study, logistic regression models included age, sex, collection site, and four principal components as covariates. In the GLAUGEN study, the method of DNA extraction, the specimen type (blood versus cheek), and site were found to be independently associated with both DNA quality and POAG case/control status, and were included in logistic regression models along with age, sex, and six principal components. A formal meta-analysis of the two studies was conducted in PLINK.[16] POAG risk was assessed in the overall dataset, the low-tension subset, and the high-tension subset.

Gene Expression Analysis

cDNA was prepared from human, adult and fetal brain and ocular tissue samples to determine the presence of candidate gene expression. Adult and fetal brain cDNA libraries were generated from the Human Total RNA Master Panel II (Clontech Laboratories, Inc., Mountain View, CA). A fetal ocular sample from a 24 week gestation period was obtained from Advanced Biosciences Resources (Alameda, CA) and was preserved in RNAlater (Qiagen, Inc., Valencia, CA) within minutes of collection. An adult eye was provided from the North Carolina Eye Bank (Winston-Salem, NC). This sample was collected 5 hours and 10 minutes postmortem and was preserved in RNAlater (Qiagen). The cornea, optic nerve, and sclera wall was dissected from each eye. The cornea was isolated using a 5-mm biopsy punch at the center of the cornea, optic nerve samples were collected using dissection scissors, and the scleral tissue was isolated using a 7-mm biopsy punch centered behind the fovea. To reduce retinal contamination of the scleral sample, a second 5-mm biopsy was taken from the center of the 7-mm punch after complete removal of the retina. mRNA was extracted from each tissue sample independently using a total RNA extraction kit (Ambion mirVana; Ambion, Austin, TX). Quality control for each sample included measuring RNA concentration and RNA subunit ratios at 260/280 nm using Nanodrop technology (Invitrogen, Carlsbad, CA). cDNA was generated from each mRNA library using a synthesis kit (SuperScript III First-Strand Synthesis kit; Invitrogen).

The brain, cornea, optic nerve, and sclera cDNA libraries were used to perform PCR assays to determine the presence or absence of contactin-associated protein-like 4 (*CNTNAP4*) and neurotrimin (*NTM*) gene expression. The PCR assays contained 2 μL cDNA, 1 × PCR buffer, 200 μmol/each dNTP, 1.5 mM $MgCl_2$, 200 ng of the forward primer, 200 ng of the reverse primer, and 3 U of Taq polymerase (Platinum Taq DNA polymerase; Invitrogen). Primers were designed using Primer3[17] and spanned intron-exon boundaries. *CNTNAP4* was amplified using the forward primer 5′ AGG ATA CTG CAC TGG CAG GT 3′ and reverse primer 5′ TGC TGA TAA ATG CGA ACA GC 3′ and *NTM* was amplified using the forward primer 5′ TGG CTT TGT GAG TGA AGA CG 3′ and the reverse primer 5′ AGG CCA CGC AAG TGT AGT TC 3′. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a control to check for cDNA quality, and was amplified using the forward primer 5′ CGA CCA CTT TGT CAA GCT CA 3′ and the reverse primer 5′ AGG GGT CTA CAT GGC AAC TG 3′. The products were amplified using a touchdown PCR protocol under the following conditions: 94°C for 3 minutes; 2 cycles of 94°C for 5 seconds, 61°C for 30 seconds, and 72°C for 30 seconds repeated using an incremental 2°C decrease in annealing temperature until 51°C was reached; followed by 30 cycles with a 51°C annealing temperature; and a final extension at 72°C for 3 minutes. The PCRs were purified (PureLink Quick Gel Extraction Kit; Invitrogen) and the products were verified by sequencing at Eton Bioscience, Inc. (Durham, NC). The results were visualized on an ethidium bromide stained, 1.5% agarose gel.

Results

The CCT between POAG cases and controls was significantly different ($P = 0.0002$), with a mean (SD) of 557.7 (33.80) μm in POAG controls and 545.9 (35.85) μm in POAG cases (Table 1). A significant difference ($P = 0.008$) in the distribution of CCT was observed between the low-tension compared with high-tension POAG cases (Table 1). The high-tension subset had a mean CCT of 548.4 (35.87) μm, while the low-tension POAG cases had a mean CCT of 541.8 (36.10) μm. The instrument used to measure CCT (ultrasound table unit versus a portable handheld unit; DGH Technology, Inc., Exton, PA) had a significant effect on mean CCT ($P < 0.0001$; Table 1). Within the CCT dataset, history of laser trabeculoplasty and glaucoma filtration surgery was available for 422 POAG cases. There was no difference in CCT between individuals with or without a history of laser trabeculoplasty ($P = 0.86$) or glaucoma filtration surgery ($P = 0.62$).

Twenty previously published CCT SNPs (Table 2) were tested for association with CCT in the NEIGHBOR/GLAUGEN CCT dataset ($n = 1117$). Using a Bonferroni multiple testing correction significance threshold of $0.05/20 = 0.0025$, one SNP, rs12447690 upstream of *ZNF469* ($P = 0.001$, $\beta = -5.08$ μm/allele) showed significant evidence of association with CCT. Three additional SNPs showed suggestive evidence of association: rs7044529 in *COL5A1* ($P = 0.003$, $\beta = -6.58$ μm/allele), rs1536478 in the *RXRA/COL5A1* intergenic region ($P = 0.004$, $\beta = 4.47$ μm/allele), and rs1538138 near *IBTK* ($P = 0.004$, $\beta = -4.98$ μm/allele) (Table 2). The direction and the effect size of these four variants are consistent with previous reports.[2–4,9] These 20 SNPs were then tested for association with POAG case/control status in the larger NEIGHBOR/GLAUGEN meta-analysis in the overall dataset ($n = 6470$), low-tension ($n = 4086$), and high-tension ($n = 4966$) subsets. Using the same significance threshold as above (0.0025), none of these SNPs showed any evidence of association with POAG (Table 2).

Next, a CCT genome-wide association study (GWAS) was performed in order to identify novel candidate SNPs using the CCT dataset. No SNP met a genome-wide significance threshold of 1×10^{-7} (Fig. 1), either in the overall dataset or when the analysis was confined to only POAG cases. The top five most significant SNPs (Fig. 1, and see Supplementary Material and Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental) were located on chromosome (Chr.) 2 at *PRKCE* (rs10205024; $P = 1.68 \times 10^{-6}$; $\beta = -7.49$ μm/allele); on Chr. 16 at the *CNTNAP4* gene (rs9939043; $P = 2.70 \times 10^{-6}$; $\beta = 7.15$ μm/allele); on Chr. 20 at the *HAO1* gene (rs2423322; $P = 4.48 \times 10^{-6}$; $\beta = -9.03$ μm/allele); in an intergenic region on Chr. 7 at the *EMID2/MYL10* locus (rs17135662; $P = 8.69 \times 10^{-6}$; $\beta = 8.31$ μm/allele); and on Chr. 11 at the *NTM* gene (rs7108536; $P = 1.00 \times 10^{-5}$; $\beta = -6.78$ μm/allele).

Table 2. Top Ten SNPs Associated with POAG in the Low-Tension Subset

			CCT Analysis ($n = 1117$)		POAG Analysis Overall Dataset ($n = 6470$)	
SNP[2–4,9]	Chr.	Locus	Δ µm/Minor Allele	*P*	OR	*P*
rs3767703	1	*Col8A2*	−2.37	0.37	0.95	0.51
rs7550047	1	*Col8A2*	−2.78	0.27	0.98	0.78
rs96067	1	*Col8A2*	−0.83	0.66	0.94	0.20
rs11694554	2	*Col4A3*	3.82	0.10	1.14	0.25
rs1538138	6	*IBTK*	-4.98	0.004	0.96	0.42
rs1324183	9	*9p23*	−3.66	0.04	1.09	0.09
rs1409832	9	*RXRA/COL5A1*	−3.31	0.06	1.09	0.07
rs1536478	9	*RXRA/COL5A1*	4.47	0.004	0.99	0.87
rs1536482	9	*RXRA/COL5A1*	−1.69	0.29	1.04	0.38
rs7044529	9	*COL5A1*	-6.58	0.003	0.99	0.86
rs1034200	13	*AVGR8*	2.9	0.08	0.94	0.31
rs2721051	13	*FOXO1*	−4.69	0.04	1.02	0.75
rs2755237	13	*FOXO1*	−1.66	0.40	1.06	0.28
rs1828481	15	*AKAP13*	0.11	0.94	0.96	0.29
rs4965359	15	*LRRK1*	−2.82	0.07	0.99	0.81
rs6496932	15	*PDE8A/AKAP13*	−2.35	0.22	1.05	0.30
rs7172789	15	*AKAP13*	0.23	0.88	0.95	0.25
rs930847	15	*LRRK1*	2.15	0.23	0.97	0.47
rs12447690	16	*ZNF469*	-5.08	0.001	1.04	0.33
rs9938149	16	*ZNF469*	−3.84	0.01	1.05	0.24

The 50 SNPs most strongly associated with CCT were selected from the GWAS results based on a *P* value cut off of 1×10^{-4} and tested for association with POAG risk using the overall NEIGHBOR/GLAUGEN meta-analysis and meta-analyses stratified by IOP. Using a significance threshold of $P \leq 0.001$ (Bonferroni correction of 0.05/50), one SNP at the *NTM* gene locus (rs7481514, $P = 0.00099$, OR = 1.28) was significantly associated with POAG in the low-tension subset (Table 3). As

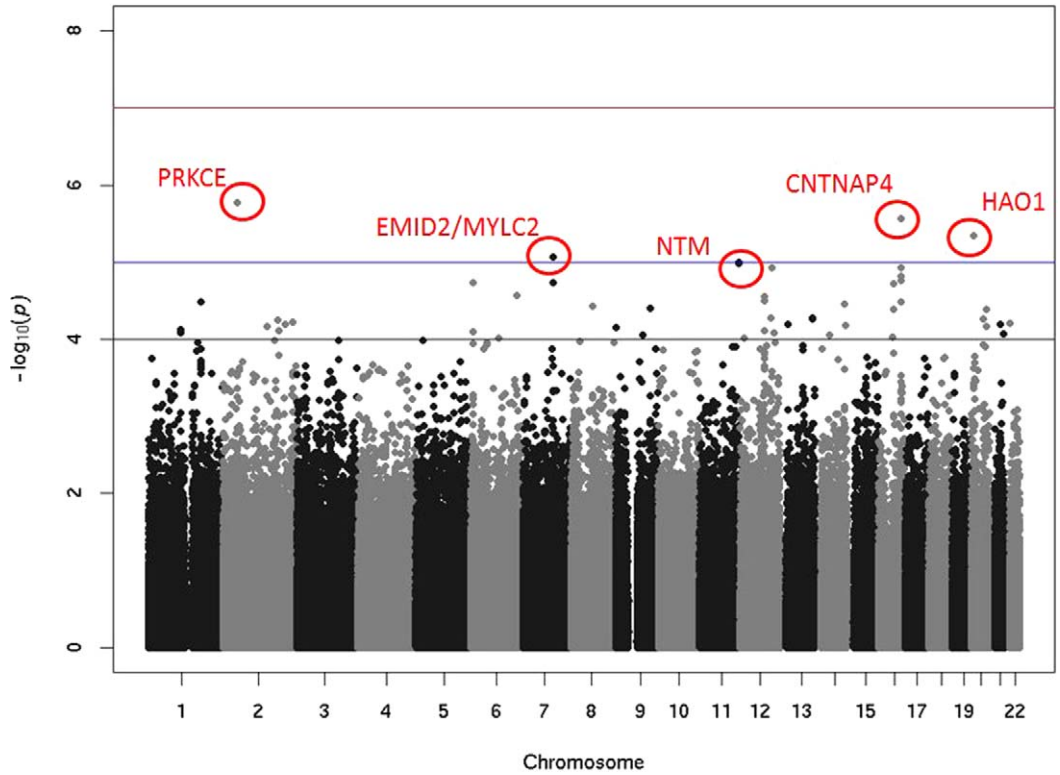

Figure 1. Manhattan plot showing the results of the linear regression analysis for CCT in the NEIGHBOR/GLAUGEN CCT dataset. The *red line* (*upper*) indicates the genome-wide significance threshold ($P = 1 \times 10^{-7}$), the *blue line* (*middle*) indicates the suggestive significance threshold ($P = 1 \times 10^{-5}$), and the *black line* (*lower*) indicates the threshold level selected for follow up POAG association analysis ($P = 1 \times 10^{-4}$). A quantile-quantile plot of the resulting *P* values is shown in Supplementary Figure S2 (see Supplementary Material and Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental).

Table 3. Top Ten SNPs Associated with POAG in the Low-Tension Subset

				CCT GWAS ($n = 1117$)		POAG risk ($n = 4086$)	
SNP	**Locus**	**CHR**	**Allele**	**Δ μm/Minor Allele**	***P***	**OR**	***P***
rs7481514	*NTM*	11	A	−6.89	1.03×10^{-5}	1.28	0.00099
rs7108536	*NTM*	11	G	−6.78	1.00×10^{-5}	1.25	0.0017
rs1428758	*CNTNAP4*	16	G	6.61	1.74×10^{-5}	0.84	0.018
rs9939043	*CNTNAP4*	16	A	7.15	2.70×10^{-6}	0.85	0.022
rs6701037	*TNN*	1	C	−6.11	3.31×10^{-5}	0.85	0.024
rs4959388	*LY86-AS1*	6	G	7.07	1.85×10^{-5}	1.18	0.031
rs1109739	*16q12*	16	A	−8.48	4.13×10^{-5}	0.81	0.038
rs2866710	*CNTNAP4*	16	G	6.84	1.17×10^{-5}	0.86	0.051
rs2052866	*CNTNAP4*	16	A	6.51	3.27×10^{-5}	0.86	0.054
rs1428759	*CNTNAP4*	16	A	6.78	1.53×10^{-5}	0.87	0.072

expected, each copy of the minor allele is associated with reduced CCT and an increased risk of POAG. A second SNP at the *NTM* locus was marginally associated in the low-tension subset (rs7108536, $P = 0.0017$, OR = 1.25). The two *NTM* SNPs are in high linkage disequilibrium ($r^2 = 0.87$). An additional five SNPs at the *CNTNAP4* gene showed suggestive association, although they did not meet the multiple testing corrected level of significance (Table 3). The most significant *CNTNAP4* SNP was rs1428758 ($P = 0.018$, OR = 0.84). The strong linkage disequilibrium in this region (see Supplementary Material and Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9784/-/DCSupplemental) suggests these signals are probably not independent. The effect of these five SNPs is also in the expected direction, with each copy of the minor allele being associated with an increase in CCT and a protective effect on POAG risk. No SNP was associated with POAG in the overall dataset or the high-tension subset.

CNTNAP4 and *NTM* gene expression was evaluated in human adult and fetal brain, optic nerve, cornea, and sclera tissues (Fig. 2). *CNTNAP4* was shown to be expressed in the adult brain, optic nerve, and cornea, but no evidence of *CNTNAP4* expression in the adult sclera sample was observed. Similarly, evidence of *CNTNAP4* expression was observed in the fetal brain, optic nerve, and cornea tissues, but not in the fetal sclera sample. *NTM* expression was seen in all of the tissues examined in both the adult and fetal stage tissues (Fig. 2).

Discussion

CCT is an important quantitative risk factor for POAG, but whether CCT directly influences POAG susceptibility remains unknown. One way to improve understanding of this relationship is to identify genes that are associated with both traits. This study replicated the association of previously published CCT genetic variants in a POAG population, and showed that despite the well established genetic association observed between these loci and CCT, there was no evidence that they influence POAG risk. Two POAG candidate genes were identified, *NTM* and *CNTNAP4*, first by association with CCT, and second by association analysis with POAG risk. These genes are good biological candidates for future research in the etiology of POAG.

In our dataset, POAG cases have significantly lower CCT compared with controls and within the POAG cases, the low-tension subset has significantly lower mean CCT compared with the high-tension subset. Consistent with these results, several studies have suggested CCT is reduced in normal-tension glaucoma as compared with POAG.[18–20] One possible explanation for this observation is that thinner corneas may be representative of a thinner, weaker posterior eye structure, which may increase susceptibility to optic nerve stress caused by elevated or fluctuating IOP.

In this study, we investigated the effects of previously published CCT SNPs and replicated the association of rs12447690 near the *ZNF469* gene, an uncharacterized zinc-finger protein.[2–4] Mutations in this gene are known to cause brittle cornea syndrome, a systemic connective tissue disorder characterized by a high risk of corneal tearing.[21] The association of this SNP with CCT has been replicated in multiple populations, and there is strong evidence that this gene is involved with normal CCT variation.[2–4] SNPs within two genes, *COL5A1* and *IBTK,* were previously identified in multiple population-based CCT studies.[3,4,9] There was also evidence that these SNPs are associated with CCT in this study's primarily POAG case population. *COL5A1* is a logical candidate gene for cornea thickness variation as the corneal stroma is largely composed of regularly arranged collagen fibers. Mutations in this gene have been identified in Ehlers-Danlos syndrome patients who have reduced cornea thickness.[22] *IBTK* was recently identified in a CCT meta-analysis conducted in a multiethnic Asian population cohort study.[9] This group hypothesized that *IBTK* may regulate corneal thickness during developmental stages and embryogenesis through its negative regulation of *BTK* kinase activity or its effect on nuclear factor-kappa-B (NF-κB) activation.

The remaining 16 published CCT SNPs did not show evidence of association with CCT. This may be due to the limited sample size of the CCT dataset ($n = 1117$) which had an estimated 66% power, assuming a minor allele frequency of 0.25 and an effect size of –6.00 Δ CCT μm/allele,[23] to detect association with CCT. Thus, based on this estimate, false negative findings for those 16 loci cannot be ruled out. However, after examining the potential association of all 20 CCT-associated loci with POAG in a larger dataset, there was no evidence that any of these well established CCT genes results in increased POAG susceptibility. It is expected that the overall POAG dataset has greater power to detect genotypic effects because of the increased sample size ($n = 6470$), although the true power of this dataset is unknown because the putative effect sizes of these loci on POAG risk remain unknown.

In the CCT GWAS, no SNPS met genome-wide significance thresholds. As with the replication analysis, this may be due to a lack of statistical power resulting from sample size limitations or due to the fact that this study population consists mainly of POAG cases, which may have different genetic determinants of CCT than population controls.

To determine if any of the top SNPs identified in the CCT analysis also influence POAG risk, candidates SNPs were

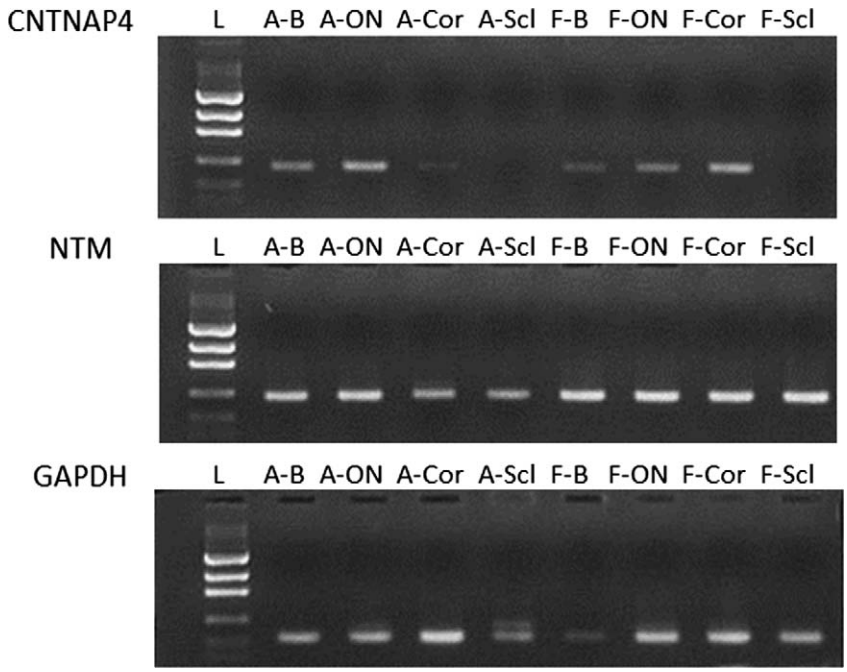

Figure 2. Agarose gel image of the PCR assays showing *CNTNAP4* (*top*), and *NTM* (*middle*) and *GAPDH* (*lower*) expression in brain and ocular tissue samples. The lanes from left to right contain size standard ladder (L; band sizes 100, 200, 400, 800, 1200, and 2000 base pairs), adult brain (A-B), adult optic nerve (A-ON), adult cornea (A-Cor), adult sclera (A-Scl), fetal brain (F-B), fetal optic nerve (F-ON), fetal cornea (F-Cor), and fetal sclera (F-Scl).

selected and tested for association with POAG in the overall dataset and two subsets, low tension and high tension, stratified by a history of elevated IOP. Stratification by IOP may provide a more clinically homogenous population resulting in reduced genetic heterogeneity within the dataset. In the low-tension subset of POAG cases, neurotrimin (*NTM*) was significantly associated with increased POAG risk and, as expected, a decrease in CCT. The size of these effects is consistent with published reports suggesting a decrease of 40 μm of CCT is associated with a 1.7-fold increased risk of POAG.[6] *NTM* is a member of the immunoglobulin domain-containing IgLON superfamily of glycosyl-phosphatidylinositol (GPI)-anchored cell adhesion molecules. *NTM* can be found in both membrane-bound and soluble forms and has known roles in mediating cell-cell interactions and regulating neurite outgrowth.[24–26] It has high homology with the well characterized neuronal cell adhesion molecule (NCAM), which has also been shown to have a role in neurite outgrowth. NCAM was found to be constitutively expressed in human corneal endothelium cells, although the role of neuronal cell adhesion molecules within the cornea remains unknown.[27] Previous reports found *NTM* to be expressed during development, continuing into adulthood, and an abundant expression has been observed in the ganglion cell layer of the retina.[26] It is possible that *NTM* plays a role in glaucoma susceptibility by influencing neuron outgrowth during development. Therefore, the presence of *NTM* expression was examined in relevant tissues from a developing fetus and *NTM* was shown to be expressed in the brain, optic nerve, cornea, and sclera with similar expression observed in the adult sample. An adult brain sample was included as a positive control since it has been shown that *NTM* is expressed primarily in the central nervous system.[27] This expression work provides further evidence that *NTM* is a viable candidate for future POAG research.

Contactin-associated protein-like 4 (*CNTNAP4*) was also identified as a candidate gene for glaucoma risk in the low-tension POAG subset. Although the statistical evidence for association with this gene is modest, like *NTM*, it is a neural cell adhesion molecule and is important in cell-cell interaction within the nervous system.[28] *CNTNAP4* is located within a region linked to keratoconus, a disease defined by the progressive thinning of the cornea.[29] In addition, SNPs within *CNTNAP2*, a gene closely related to *CNTNAP4*, has previously been associated with pseudoexfoliation glaucoma.[30,31] *CNTNAP4* was found to be expressed in the brain, optic nerve, and cornea during development and in adulthood. Together, NTM and CNTNAP4 suggest that neural cell adhesion molecules could constitute a novel pathway relating reduced CCT to POAG risk.

CCT is a readily measurable and highly heritable trait, and like many other quantitative traits, it is likely to be mediated by many common loci of modest effect sizes. Rare variants within the CCT loci may result in profound structural changes in the cornea, but more common variants might produce the CCT variation that is observed on a population level. Our results show that these common variants are not associated with POAG. Nonetheless, CCT genomics research offers promise to reveal new insight into the genetic architecture of POAG, as suggested by the *NTM* association.

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