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RESEARCH ARTICLE

A genomewide association study on individuals with occludable angles identifies potential risk loci for intraocular pressure

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Abstract. Glaucoma is a heterogeneous group of optic neuropathies and is one of the leading causes of irreversible blindness worldwide. Primary angle closure glaucoma (PACG) is a major subtype, prevalent mostly in east and south Asia, where occludable anterior chamber angle is considered as a primary risk factor, which in turn could be responsible for high intraocular pressure (IOP) and subsequent neurodegeneration of retinal ganglion cells. Clinically, IOP is considered as a major risk factor for glaucoma and viewed as an important endophenotype to promote the disease severity. To investigate the comprehensive genomic insights, we conducted a genomewide association study (GWAS) on IOP in individuals with occludable angle (< 15 degrees), thus anatomically predisposed to PACG. After performing GWAS on IOP, we identified 25 genomewide suggestive significant loci ($P < 1e^{-05}$, $n = 240$) of which, six were in complete linkage disequilibrium with the *ABCA4* genic region. We successfully replicated the most significant discovery, SNPs of *ABCA4* (rs2065712) in a separate cohort of 89 individuals ($P = 1.16e^{-09}$). We identified multiple SNPs in *ABCA4* to be associated with IOP. Also, we obtained genes harbouring significantly associated SNPs, included in relevant biological pathways that could potentially be involved in IOP variation and glaucomatous neurodegeneration.

Keywords. quantitative trait genomewide association study; intraocular pressure; occludable anterior chamber angle; *ABCA4* gene; taqman assay; bioinformatics.

Introduction

Primary angle closure glaucoma (PACG) is a major subtype of glaucoma, prevalent in Asia, especially in India and China (Quigley and Broman 2006). Several anatomical and physiological factors are involved in the pathogenesis of PACG which could raise intraocular pressure (IOP) followed by optic neuropathy and subsequent blindness (Ramakrishanan *et al*. 2010). Nowadays, major treatment modality in glaucoma in terms of lowering of IOP includes either increasing the drainage of aqueous humor or decreasing aqueous humor formation (Weinreb *et al*. 2016). Therefore, investigating genetic risk factors, genes or related pathways that influence IOP might open new avenues in therapeutic intervention for PACG. Earlier, twin studies on Chinese families reported high

Sudipta Chakraborty and Anshul Sharma contributed equally to this work.

SC performed genomewide and TaqMan genotyping, conducted all the statistical and bioinformatics analysis, drafted, and edited the original manuscript. AnS collected the samples with clinical data and extracted the DNA. IB helped in performing the genomewide genotyping experiments. SP provided codes for statistical analyses. CB helped in initial data analysis. VG helped in recruitment of individuals in the current study cohort. AM supervised the genomewide genotyping experiments. SB supervised all the statistical and bioinformatics analyses, helped in conceiving and designing the study, reviewing, and editing the manuscript draft. ArS conceived the study, coordinated the overall subject recruitment with complete clinical records, sample collection, DNA isolation and edited the manuscript. RS conceived the study, led diagnosis, clinical evaluation, and recruitment of individuals for the current study cohort, set the clinical parameters for selecting study subjects and edited the manuscript. MA conceived the study, led the genomewide genotyping experiments, supervised analyses and interpretation of results, and drafted the manuscript. All the authors read and approved the final version of the manuscript for publication.

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heritability estimates for IOP that supports the role of genetic effects among the families in the segregation of IOP (van Koolwijk *et al*. 2007). Also, shallow anterior chamber depth allows pupillary block and forward bowing of the iris that could block the trabecular meshwork and raise intraocular pressure leading to glaucomatous neurodegeneration and corresponding visual-field deficits (Weinreb *et al*. 2016). From this perspective, IOP as a quantitative trait (QT) plays an important role in detecting genomic risk factors associated with PACG (Bassi *et al*. 2019), that would help in localizing genetic susceptibility factors and understanding subsequent molecular events responsible for PACG disease biology. Earlier studies have identified several risk loci influencing IOP, but they were not distinctly reported in occuldable angle individuals, anatomically predisposed to PACG (Choquet *et al*. 2018; MacGregor *et al*. 2018). This study includes subjects with only anatomically predisposed eyes having an occludable narrow angle. The rationale behind this approach was to identify the genes that are involved in elevation of IOP in angle closure individuals with subsequent glaucomatous neurodegeneration. Thus, we conducted QT-GWAS analysis on intraocular pressure that we believe could shed some light on the disease biology of PACG.

Table 1. SNP list of IOP.

	CHR	SNP	Allele	*P*-value	Gene
1	3	rs2047975	A	3.81e−08	*GALNT15*
2	16	rs369868	G	4.34e−08	
3	2	rs10498177	A	8.63e−07	*DOCK10*
4	2	rs13405414	C	1.17e−06	*ZNF385B*
5	11	rs1043418	A	1.28e−06	*CAPN5*
6	11	rs7948226	A	1.70e−06	*LRP5*
7	6	rs1192039	A	1.66e−06	*MIR30C2*
8	5	rs11952823	A	1.59e−06	*LOC105377706*
9	**1**	**rs2065712**	**C**	**1.55e−06**	***ABCA4***
10	13	rs754592	A	5.34e−06	*LOC101927437*
11	1	rs1538025	A	5.50e−06	*LOC105373224*
12	11	rs11601477	A	6.18e−06	*GALNT18*
13	1	rs12079787	G	6.31e−06	*LOC440704*
14	16	rs1078346	C	6.31e−06	*C16orf47*
15	19	rs2930902	G	6.55e−06	*MED16*
16	1	rs16832542	C	6.83e−06	*LINC01720*
17	6	rs9458994	G	6.99e−06	
18	19	rs1688268	A	7.56e−06	*HSD17B14*
19	**1**	**rs4147856**	**C**	**8.80e−06**	***ABCA4***
20	19	rs10420354	A	9.27e−06	*CYP4F12*
21	18	rs1431786	A	9.88e−06	*DCC*
22	**1**	**rs3789379**	**G**	**9.96e−06**	***ABCA4***
23	**1**	**rs7531001**	**A**	**9.96e−06**	***ABCA4***
24	**1**	**rs2275031**	**A**	**9.96e−06**	***ABCA4***
25	**1**	**rs2275032**	**C**	**9.96e−06**	***ABCA4***

SNPs with (P <1e^{-05}) are presented. Genomic positions are according to GRCh37/hg19; CHR, chromosome. The SNPs of ABCA4 are shown in bold font.

Materials and methods

Phenotype and subject selection

Subjects were recruited based on gonioscopic narrow angles (≤15 degrees). To conduct a GWAS, we compared older anatomically suspect controls having narrow angle (PACS) with early onset PACG cases. It helps to increase the detection power of genetic component that is responsible for the shift from a narrow angle to the appearance of peripheral anterior synechiae, which eventually damages the trabecular meshwork by rising intraocular pressure and causing glaucomatous neuropathy (Chakraborty *et al*. 2021). Goldmann applanation tonometry was used to measure the IOP. For each patient, there were three baseline IOP readings measured from three consecutive visits. A single mean IOP was calculated from these three baseline IOPs. A total of 240 and 89 individuals with occludable narrow angles participated in the study as a discovery cohort and replication cohort, respectively. The detailed exclusion and inclusion criteria are provided in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/. The age and gender distributions of the study participants in both discovery and replication cohort are shown in figure 1 in electronic supplementary material.

Genotyping

DNA samples from individuals were extracted using QIAamp DNA kits. Genotyping was performed using the Illumina platform (Illumina, USA) by whole genome DNA microarray (OmniExpress-24 v1.2 BeadChip) comprising 713,599 loci. Raw data were analysed, and genotype calls were made using GenomeStudio.

Quality checking

The raw genotype data were extracted for further statistical analyses using stringent quality control measures.

Population stratification analyses

Population stratification was checked by principal component analyses using PLINK 2.0 to detect a systematic difference in allele frequencies between subpopulations in genotyped samples. The top 40 principal components (PC) were further reduced to two values by multidimensional reduction scaling (MDS). MDS1 and MDS2 values were used as covariates in terms of population stratification adjustment for the GWAS analysis.

GWAS analysis and covariate adjustment

We performed linear regression of mean IOP of all individuals with the following covariates: age, sex, MDS1 and MDS2 as ancestry principal components. We have taken

care to correct the distortion in IOP distribution by using a log-transformation to remove skewness. All the analyses were performed on log-transformed IOP values. Subsequently, we conducted a quantitative GWAS for IOP using a linear regression model under the assumption of additive allelic effects of the SNP dosages in PLINK v.1.9 to assess genetic associations.

Annotation of associated significant SNPs

The official list of GWAS hits maintained on the NHGRI GWAS catalogue lists all the associations reported in GWAS publications with $P<1e^{-05}$ (Buniello *et al*. 2019). Based on the genomic positions of the GRCh37/hg19 assembly, the SNPs with $P < 1e^{-05}$ on genotype dataset were mapped to autosomal genes by a 25-kb flanking region and was used to capture proximal regulatory and functional elements affecting gene regulation.

In silico analyses

We prioritized the SNPs/genes based on statistical significance and number of SNPs present in linkage disequilibrium using bioinformatics tools and databases. Further, the gene lists ($P < 1e^{-04}$) were subjected to pathway enrichment analysis though gene ontology database to obtain meaningful biological inferences in connection with IOP of PACG pathobiology.

Replication of prioritized SNPs in an independent external cohort

We have genotyped rs2065712 (intronic SNP in *ABCA4*) in a separate replication cohort using TaqMan SNP genotyping assay in QuantStudio 7 (Thermo). We obtained all the assigned genotype calls and clusters. Undetermined genotypes were excluded from further analysis. For a total of 89 individuals, genotypes were extracted using TaqMan Genotyper software 1.4. Linear regression model was fitted on IOP to genotype rs2065712 in R programming software.

Power calculation

Our GWAS had low power if viewed with the lens of a conventional GWAS because we recruited individuals having specifically defined phenotype. In a separate replication cohort, we performed a power calculation considering the effect size of the prioritized SNP (rs2065712; *ABCA4*) and number of individuals considered for the replication, which is depicted in figure 2 in electronic supplementary material. This study was conducted after obtaining approval from Institutional Ethics Committees of AIIMS, New Delhi and NIBMG, Kalyani, India. The samples were collected from patients with written inform consent.

Data availability

The genomewide genotyping data have been deposited in the centralized data storage services of NIBMG. To gain access, interested individuals should contact corresponding authors.

Results

We performed a QT-GWAS in a total of 240 subjects having occludable anterior chamber angle. The distribution of mean IOP in both eyes of the participants is shown in figure 3 in electronic supplementary material. Linear regression was performed for the genotype on IOP, by adjusting covariates, namely age, sex, MDS1, MDS2. None of the covariates were statistically significant ($P < 0.5$) (see table 2 in electronic supplementary material). The Manhattan plot (figure 1a) was drawn from the genomewide P values after conducting the QT-GWAS on IOP. The Q–Q plot of expected vs observed –log P values showed minor tail inflation, which is indicative of genomewide significance beyond the suggestive threshold (figure 4 in electronic supplementary material). In IOP, 25 variants were found to be associated below genomewide suggestive significant level ($P <1e^{-05}$). With a 25 kb of flanking regions, most of these genetic variants map to their respective genes based on GRCh37/hg19 position, listed in table 1. Further, we prioritized the genomic variations in the *ABCA4* gene based on LD analysis, and the bioinformatics analysis were based on biological relevance. The regional association plot showed that all the markers of *ABCA4* (rs2065712, rs4147856, rs3789379, rs7531001, rs2275031 and rs2275032) were in complete LD ($r^2 > 0.8$) (figure 1, b&c). To replicate our GWAS findings, we used the TaqMan assay to genotype our sentinel SNP (rs2065712) in the *ABCA4* gene followed by linear regression analysis on the replication cohort, which further confirmed that the C allele of rs2065712 was significantly associated with high IOP ($P = 1.16e^{-09}$). In both discovery and replication cohorts, the C allele of rs2065712 in the *ABCA4* gene appears to be as the risk allele for high IOP (figure 2, a&b), (table 2). We also performed regression analysis to our subgroups PACG and PACS. In both groups, P values were significant (table 3 in electronic supplementary material). EyeIntegrationv1.05 hosted by the National Eye Institute (NIH, USA) showed that *ABCA4* is mostly expressed in retinal tissues than other eye tissues (figure 2c). Additionally, ocular tissue expression database showed even higher expression of *ABCA4* in retinal tissues with respect to other eye tissues (table 4 in electronic supplementary material). The regulomeDB score for six significant SNPs of *ABCA4* gene were checked for their predicted functional significance as putative sites (table 5 in electronic supplementary material). Biological pathways enriched in IOP is obtained from genes directly derived from associated genetic variants ($P < 1e^{-04}$) (table 3). The most significantly associated pathway is the

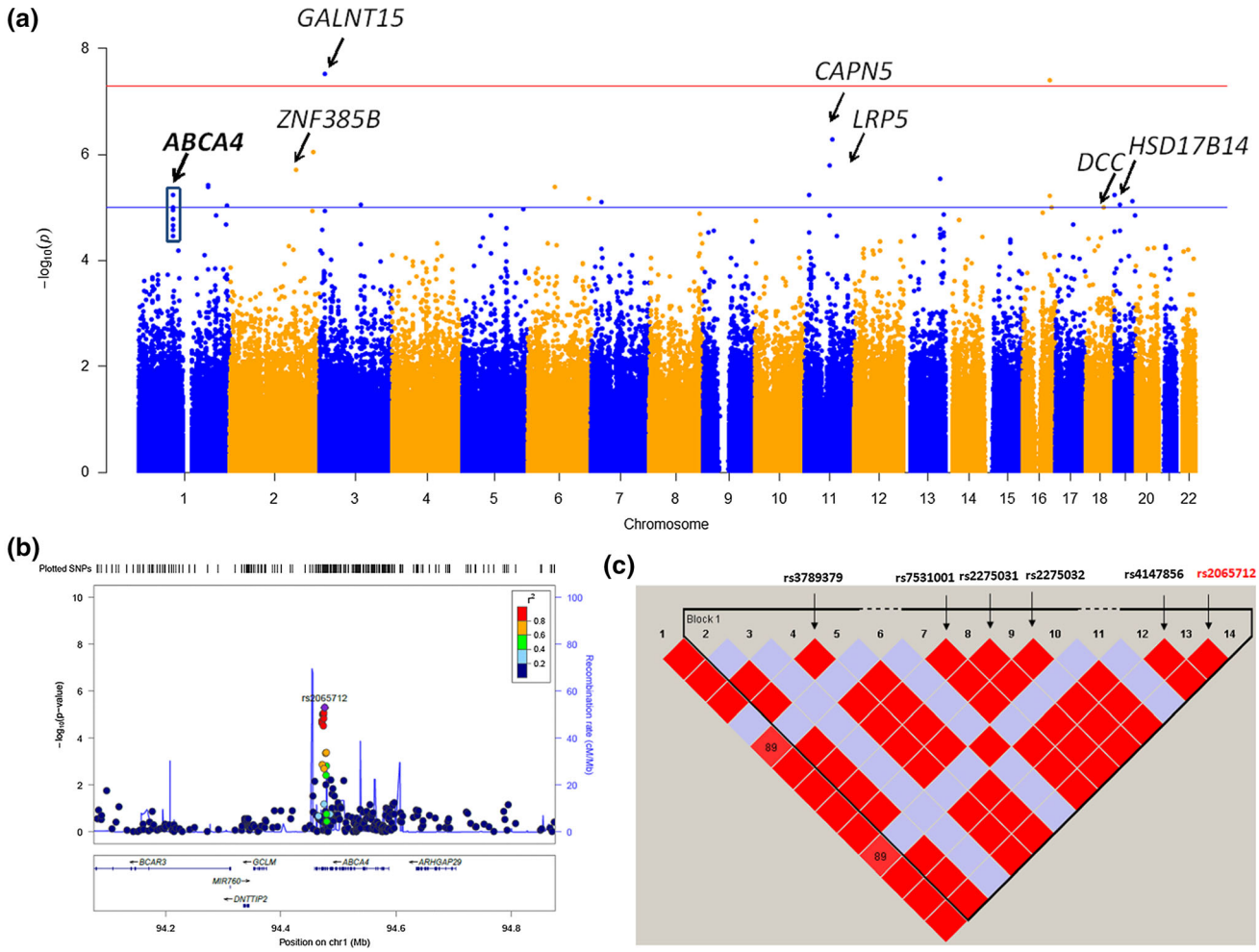

Figure 1. (a) Manhattan plots were drawn after performing GWAS on genotyped SNPs on IOP. SNP markers are plotted according to chromosomal location on the *x* axis, with the –log10 *P* values on the *y* axis. The blue horizontal line ($P < 1 \times 10^{-5}$) denotes the suggestive threshold for genomewide significance. The red horizontal line ($P < 1 \times 10^{-7}$) denotes the threshold for genomewide significance. (b) Regional association plots data are shown for the *ABCA4* locus around rs2065712. The genotyped SNP with the most significant association is denoted by a blue diamond. The *x* axis represents base-pair positions along the chromosome (human genome Build 37). The left *y* axis on the left represents –log10 *P* values and the right *y* axis represents the recombination rate. (c) LD plot of genomewide suggestive significant six SNPs of *ABCA4* showing their distribution in a single LD blocks. The sentinel SNP (rs2065712) and other five SNPs are in red and black, respectively.

negative regulation of interleukin-8 biosynthetic process ($P = 0.000087$). Apart from ABCA4, we also found other GWAS significant hits, their functional relevance are listed in table 6 in electronic supplementary material.

Discussion

Our QT-GWAS on IOP in the occludable angle identified 25 loci in Indian population. The fine mapping analyses showed much higher number of significantly associated SNPs in the *ABCA4* genic region. *ABCA4* is a retina specific ATP-binding cassette transporter, which is expressed exclusively in retinal photoreceptor cells. Earlier reports suggested that *ABCA4* is involved in retinal degeneration, cone–rod dystrophy, stargardt macular dystrophy, and retinitis pigmentosa (Burke and Tsang 2011; Lenis *et al*. 2018). The top significant locus (rs2065712) for IOP is in linkage disequilibrium with many SNPs in that specific region of *ABCA4*, which was further validated in a separate replication cohort of 89 individuals, showing a significant association with the allele C. To the best of our knowledge, this is the first report of genomic association of IOP with genic region of *ABCA4*. Additionally, we have performed extensive text mining to check biological relevance of genes harbouring other significantly associated genomic variants with IOP.

From the pathway enrichment analyses, our most significant pathways indicate towards negative regulation of interleukin-8 biosynthetic process. Earlier studies reported elevation of interleukin-8 as a significant risk factor in detection and management of glaucoma (Chono *et al*. 2018). Previous investigations also suggested that presence

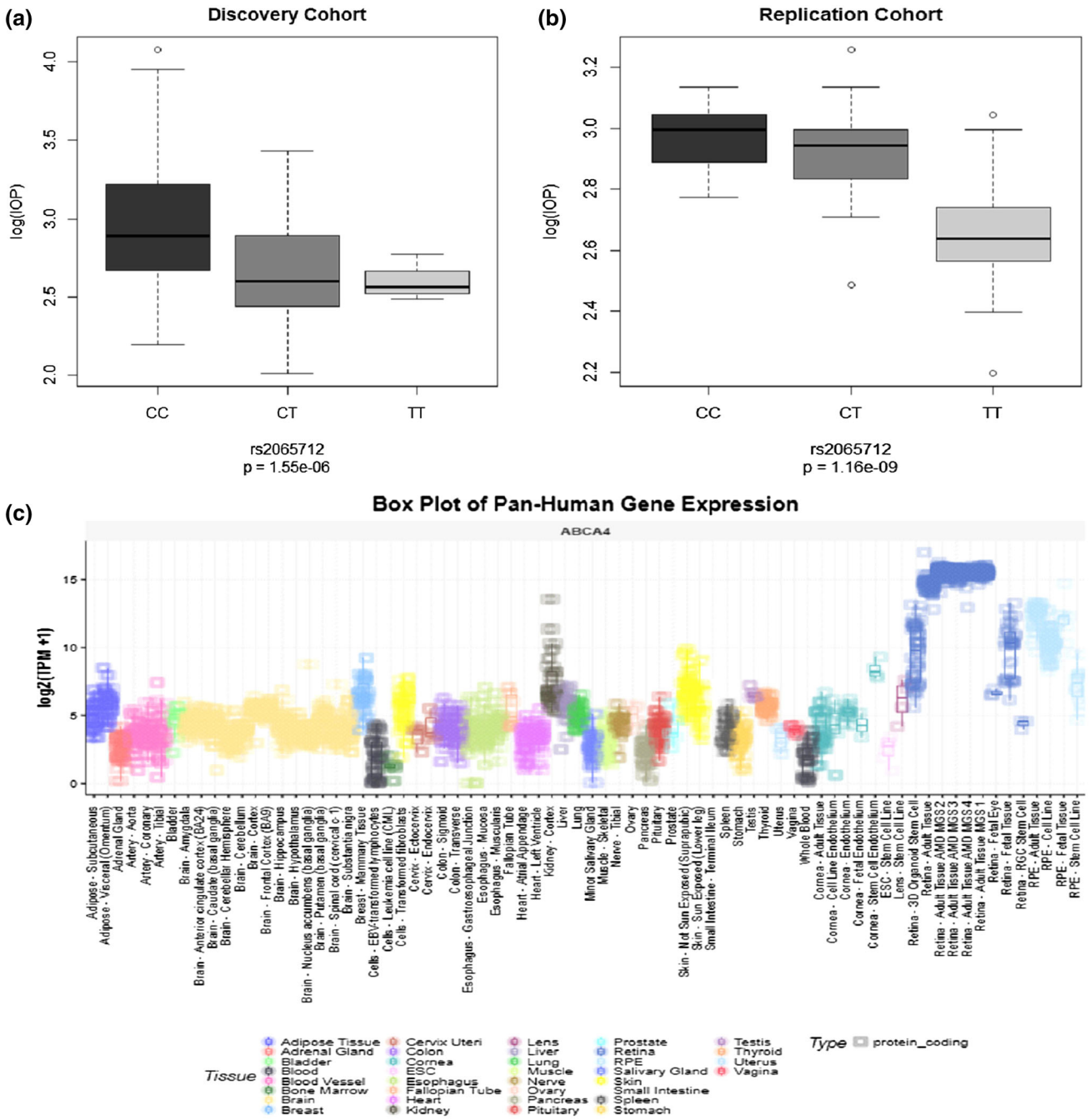

Figure 2. The box plot showing relationships between SNP genotype of rs2065712 (in *ABCA4*) on IOP from linear regression analysis (a) discovery cohort, (b) replication cohort. The genotypes of rs2065712 are indicated on the *x*-axis; and the corresponding log(IOP) trait value is shown on the *y* axis. The C allele leads to higher IOP value than T allele in both sample cohort. (c) The expression of *ABCA4* in the whole-body tissues (from eyeIntegration).

of IL-8 in the aqueous humor may have critical role to play in IOP elevation in open angle glaucoma (Chono *et al*. 2018). Primarily, two different categories of biological pathways emerged: one is inflammatory pathways, and the other is body fluids and blood pressure related pathways. Both these types of pathways have already been reported to modulate the aqueous humor formation and outflow (Bodh *et al*. 2013). Our analysis is also indicative of association of cytokine and chemokine-mediated regulation with IOP. Although, the current study provides insights on association of IOP pertaining to narrow iridocorneal angle in an Indian cohort at genomewide level, a few limitations are noteworthy to mention in this study. The sample size is relatively small in comparison with conventional GWAS, due to stringent inclusion criteria. In the current study, we were interested specifically to look

Table 2. Illustrates the effect size corresponding confidence interval with *P*-value of the prioritized SNP rs2065712 of ABCA4 in the both cohort.

Cohort	(rs2065712) allele	Effect size	95% CI	*P*-value
Discovery	C	4.992	(2.78–7.19)	1.55e−06
Replication	C	3.382	(2.77–4.38)	1.16e−09

Table 3. The top enriched significant (*P*-value < 0.01) pathways by 94 genes for IOP using gene ontology (GO) database.

GOBPID	*P*-value	Odds ratio	Exp. count	Obs. count	Size	Term
GO:0045415	0.000087	382.905	0.016	2	3	Negative regulation of interleukin-8 biosynthetic process
GO:0048870	0.002958	2.939	5.017	12	922	Cell motility
GO:0038034	0.004546	9.900	0.337	3	62	Signal transduction in absence of ligand
GO:0036323	0.005442	2.146	0.005	1	1	Vascular endothelial growth factor receptor-1 signaling pathway
GO:0070947	0.005442	7.214	0.005	1	1	Neutrophil mediated killing
GO:0086024	0.005442	8.364	0.005	1	1	Adrenergic receptor signaling pathway
GO:1904694	0.005442	3.660	0.005	1	1	Negative regulation of vascular smooth muscle contraction
GO:0042035	0.005889	8.980	0.370	3	68	Regulation of cytokine biosynthetic process
GO:0042107	0.008310	7.879	0.419	3	77	Cytokine metabolic process
GO:1903523	0.008657	15.909	0.141	2	26	Negative regulation of blood circulation
GO:0051270	0.010097	2.992	3.069	8	564	Regulation of cellular component movement
GO:0003095	0.010854	9.255	0.011	1	2	Pressure natriuresis
GO:0045079	0.01085	18.77	0.010	1	2	Negative regulation of chemokine biosynthetic process
GO:0032677	0.01294	12.717	0.174	2	32	Regulation of interleukin-8 production
GO:0045085	0.01623	93.488	0.016	1	3	Negative regulation of interleukin-2 biosynthetic process

for genetic association in IOP pertaining to individuals, who were already predisposed to occludable angles, which is one of the major risk factors of PACG. This design required prior measurement of gonioscopic angles of <15 degrees to be included in our sample cohort, with no prior history of surgery. Building a cohort with such restrictions makes it difficult to recruit individuals in the current study. To date, all the GWAS on IOP are carried out on individuals with no specific selection based on any ocular parameter. In this study, however, we were interested to investigate genomic association of IOP in the occludable angle individuals. This could be a reason that our results do not corroborate with other reported GWAS variants which were earlier reportedly associated with IOP.

In conclusion, we identified 25 SNPs beyond the genomewide suggestive threshold to be associated with IOP in narrow occuldable angle individuals. Among these, 24% SNPs (six of 25) located in the *ABCA4* genic region are in linkage disequilibrium. Bioinformatics analyses prioritizes *ABCA4* gene. The sentinel SNP (rs2065712) in *ABCA4* has been successfully replicated in a separate cohort. This rs2065712 genotype is associated with the IOP that indicates qualitative interaction (i.e., complementary mechanism of action) more in PACG cases that needs to be further investigated in the future. In addition, we have identified a few interesting pathways through the enrichment analysis that are biologically relevant with IOP. We believe that these genetic variants and gene-set-based pathways associated with IOP will help in further elucidation of genetic risk factors of PACG endophenotypes and their possible involvement in modulating the symptom severity.

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