의학박사 학위논문

Exploring the Novel Susceptibility Gene Variants for Primary Open-Angle Glaucoma in Korean Cohorts

한국인 코호트에서 원발 개방각녹내장 연관 새로운 유전자 변이 발굴 연구

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지도교수 박 기 호

이 논문을 의학 박사 학위논문으로 제출함

2020 년 5 월

서울대학교 대학원

의학과 안과학 전공

김 용 우

김용우의 박사 학위논문을 인준함

2020 년 7 월

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ABSTRACT

Primary open-angle glaucoma (POAG) can develop even within normal ranges of intraocular pressure, and this type of glaucoma (so-called ‘normal-tension glaucoma [NTG]’) is highly prevalent in East Asia including Korea and Japan. We conducted exome chip analysis to identify low-frequency and rare variants associated with POAG from the primary cohort (309 POAG patients and 5,400 control, all Koreans). For replication, Korean (310 POAG patients and 5,612 controls) and Japanese (565 POAG patients and 1,104 controls) cohorts were further investigated by targeted genotyping. For known POAG-related gene variants in other ethnicities, representative POAG-related single nucleotide polymorphisms (SNPs) from six loci (*CDKN2B-AS1, SIX1/SIX6, ATOH7, CDC7-TGFBR3, CAV1, TMCO1*) were selected and genotyped from discovery (POAG = 309, heathy = 5,400) and replication cohorts from Korea (POAG = 310, healthy = 5,612 and POAG = 221, healthy = 6,244, respectively). SNP rs116121322 in *LRRC27* showed nominally significant association with POAG in the discovery cohort (OR = 29.85, P = 2.2E–06). This SNP was validated in the Korean replication cohort but only in the NTG subgroups (OR = 9.86, P = 0.007). Japanese replication cohort did not show significant association with POAG (P = 0.44). However, the meta-analysis in the entire cohort revealed significant association of rs116121322 with POAG ($OR_{combined}$ = 10.28, $P_{combined}$ = 1.4E−07). The LRRC27 protein expression was confirmed from human trabecular meshwork cells. For

gene-based testing, *METTL20* showed a significant association in POAG ($P_{combined}$ = 0.002) and in the subgroup of NTG ($P_{combined}$ = 0.02), whereas *ZNF677* were significantly associated with only in the subgroup of high-tension glaucoma ($P_{combined}$ = 1.5E−06). In terms of previously known POAG-related variants, rs1900004 in *ATOH7* (OR = 1.29, *P* = 0.0024); rs1063192 (OR = 0.69, *P* = 0.0006), rs2157719 (OR = 0.63, *P* = 0.0007), and rs7865618 (OR = 0.63, *P* = 0.0006) in *CDKN2B-AS1*, and rs10483727 in *SIX1/SIX6* (OR = 0.68, *P* = 7.9E–05) were nominally associated with the risk of POAG. The replication cohorts revealed significant associations with rs2157719 (OR = 0.72, *P* = 0.0135), rs1063192 (OR = 0.63, *P* = 0.0007) and rs7865618 (OR = 0.52, *P* = 0.0004) in *CDKN2B-AS1*. A mega-analysis from the entire Korean population revealed significance with rs1063192 (OR = 0.77, *P* = 6.0E–05), rs2157719 (OR = 0.63, *P* = 0.0007) and rs7865618 (OR = 0.58, *P* = 1.9E–06) in *CDKN2B-AS1* and with rs10483727 in *SIX1/SIX6* (OR = 0.79, *P* = 9.4E–05), with the same direction of effect between the discovery association and the replication sample. Our findings may provide further genetic backgrounds into the pathogenesis of POAG, especially for the patients who have lower baseline intraocular pressures.

Keywords: primary open-angle glaucoma; normal-tension glaucoma; high-tension glaucoma; exome chip analysis; single nucleotide polymorphism

Student I.D. Number: 2018-30085

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LIST OF ABBREVIATIONS

ADPKD: autosomal dominant polycystic kidney disease

ATOH7: atonal bHLH transcription factor 7

BSA: bovine serum albumin

CAV1: caveolin 1

C/D: cup-to-disc ratio

CDC7-TGFBR3: cell division cycle 7 - transforming growth factor beta receptor 3

CDKN2B-AS: cyclin-dependent kinase inhibitor 2B antisense

CDKN2B: cyclin-dependent kinase inhibitor 2B

DAPI: 4′,6-diamidino-2-phenylindole

ETF: electron transfer flavoprotein

GCIPL: ganglion cell-inner plexiform layer

GENIE: Gene-environmental interaction and phenotype

GLAU-GENDISK: GLAUcoma GENe DIscovery Study in Korea

GWAS: Genome-wide association studies

HEXA: KoGES_health examinee

HTG: high-tension glaucoma

HTMC: Human trabecular meshwork cells

H-PEACE: Health and Prevention Enhancement

HRP: horseradish peroxidase

IOP: intraocular pressure

IVL: involucrin

KNIH: Korea National Institute of Health

KoGES: Korean Genome and Epidemiology Study

LRRC27: encoding leucine-rich repeat-containing protein 27

MAF: minor allele frequencies

METTL20: mitochondrial lysine-specific methyltransferase 20

NBK: National Biobank of Korea

NTG: normal-tension glaucoma

OR: odds ratio

PBS: phosphate-buffered saline

PCA: principal component analysis

PCR: polymerase chain reaction

PC1: polycystin-1

PC2: polycystin-2

PKD1L2: encoding polycystic kidney disease 1-like 2

POAG: Primary open-angle glaucoma

PVDF: polyvinylidene difluoride

p53: 53-kDa protein

RGC: retinal ganglion cell

RNFL: retinal nerve fiber layer

SNP: single nucleotide polymorphism

TMCO1: transmembrane and coiled-coil domains 1

VF: visual field

ZNF677: encoding zinc finger protein 677

INTRODUCTION

Glaucoma threatens the vision of more than 60 million people globally. Primary open-angle glaucoma (POAG) is the most common sub-type of glaucoma[1], and reversing the elevation of intraocular pressure (IOP), the most prominent risk factor for the development of POAG, remains the only therapeutic target for the management of POAG.[2] However, POAG can develop even within the normal range of IOP, resulting in so-called normal-tension glaucoma (NTG) or low-pressure glaucoma.[3-6]

Previous population-based studies have shown that NTG comprises the majority of open-angle glaucoma in East Asians; in contrast, the proportion of NTG is lower in Caucasians or individuals of African descent. The proportions of NTG were 92% in the Tajimi Study (Japan)[7], 90% in the Handan Eye Study (China)[8], and 77% in the Namil Study (South Korea).[9] Although the pathogenesis of NTG development is not fully understood, these geographical and ethnic differences in the prevalence of NTG imply that genetic variations may play a role.

Previous twin and familial studies have reported heritabilities of POAG ranging from 0.26 to 0.81.[10-12] A recent phenome-wide heritability study estimated the array-based heritability to be 26.0% for glaucoma.[13] Furthermore, the prevalence of POAG varies from race to race. The risk for POAG is 3 times greater in individuals

of African descents than of European ancestry.[1] In light of these facts, it is worth to explore the genetic determinants of POAG development from various ethnicities.

Genome-wide association studies (GWAS) have been increasingly applied to investigate the molecular basis of POAG pathogenesis.[14,15] Multiple genes have been reported to be associated with POAG at the genome-wide level, including *CAV1/CAV2*, *ATOH7*, *TMCO1*, *CDC7-TGFBR3*, *MPP7*, *CDKN2B-AS1*, and *SIX1/SIX6*, from various populations in Europe, the United States of America, India, Japan, and China.[16-24] *CDKN2B-AS1* and *SIX1/SIX6* have been shown to be significantly associated with NTG in individuals of European ancestry[25,26], Han Chinese population[27], and Japanese population.[19,28,29] The latest update on the GWAS results of POAG has been described elsewhere.[30]

Given the high prevalence of NTG in East Asians, it is worth further exploring the genetic architecture associated with glaucoma risk in this ethnic group. Accordingly, in this study, we performed an exome chip analysis for POAG and the relevant gene variants have been validated in East Asian cohorts. We also have genotyped 8 previously known single nucleotide polymorphisms (SNPs) (rs1063192, rs2157719, and rs7865618 in *CDKN2B-AS1*; rs10483727 in *SIX1/SIX6*, rs1900004 in *ATOH7*, rs1192415 in *CDC7-TGFBR3*, rs4236601 in *CAV1*, and rs4656461 in *TMCO1*) that are available from exome-chip analysis data. Among them, 4 POAG-related SNPs (rs1063192 and rs7865618 in *CDKN2B-AS1*; rs10483727 in *SIX1/SIX6*, and rs1900004 in *ATOH7*) were further validated from another Korean population-based cohort and explored in terms of their risk of

POAG. The aims of the present study were to identify the novel genetic loci associated with POAG in East Asian populations and to investigate the difference in genetic associations according to the baseline IOP.

MATERIALS AND METHODS

This study was undertaken as a part of the GLAU-GENDISK (GLAUcoma GENe DIscovery Study in Korea) project, which is an ongoing prospective study designed in 2011. The primary objective of the GLAU-GENDISK project was to investigate and identify novel genetic susceptibility loci for various types of glaucoma in a Korean population. The secondary objectives included establishing the genotype-phenotype relationships in glaucoma patients and constructing new disease prediction models. The Japanese POAG patients were further recruited from the institutes related to Tohoku University and the control subjects were recruited from the Tohoku Medical Megabank Organization.

The present study was approved by the Seoul National University Hospital Institutional Review Board and followed the tenets of the Declaration of Helsinki (1964). Written informed consent was obtained from each of the enrolled participants. The Institutional Review Board of the Tohoku Graduate School of Medicine approved the secondary use of the genomic array data. All methods were performed in accordance with the relevant guidelines and regulations.

Study Population

All subjects included in this analysis were of Asian descent. The participants in this study included 622 patients with POAG and 213 healthy controls who were

enrolled in the GLAU-GENDISK, 10,799 healthy controls from the population-based cohorts in the Korean Genome and Epidemiology Study (KoGES)[31], 221 patients with POAG and 6,244 healthy controls from the GENIE (Gene-environmental interaction and phenotype) study, a subcohort of the H-PEACE (Health and Prevention Enhancement) study[32], and 565 patients with POAG and 1,104 healthy controls from Japan. Patients with POAG in Korea were recruited from Seoul National University Hospital, Seoul National University Boramae Hospital, and Healthcare System Gangnam Center in Korea. The data for healthy Korean controls were further provided by the National Biobank of Korea (NBK). The NBK secured biospecimens from the general population from various cohorts organized by the Korea National Institute of Health (KNIH).[31] Exome chip analysis data and epidemiological survey data from each participant (including lifestyle, medical history, physical activity, food consumption, disease-related blood test results, and body measurements) were obtained from healthy populations from the population-based cohorts in the KoGES, including the KoGES_Ansan and Ansung study and the KoGES_health examinee (HEXA) study.[31] All participants were proven to have healthy eyes based on the survey.

Detailed information from the H-PEACE and GENIE studies has been published elsewhere.[32] Among the total of 91,336 healthy examinees from Seoul National University Hospital Healthcare System Gangnam Center, 17,455 who provided informed consent and donated blood samples were enrolled into the GENIE. Of these, 6,465 participants with genotype data were finally included in the study.

For exploration of POAG-related novel gene variant, the Korean POAG patients and healthy controls were randomly classified to the primary cohort (312 POAG patients and 5,400 controls) and replication cohort #1 (310 POAG patients and 5,612 controls). The Japanese POAG patients and healthy controls were assigned as replication cohort #2.

POAG was defined as the presence of glaucomatous optic disc changes with corresponding glaucomatous visual field (VF) defects and an open angle confirmed by gonioscopic examination. Glaucomatous optic disc changes were defined as neuroretinal rim thinning, notching, excavation, or retinal nerve fiber layer (RNFL) defects. Glaucomatous VF defects were defined as (1) glaucoma hemifield test values outside the normal limits, (2) three or more abnormal points with a probability of being normal of $P < 5\%$, of which at least one point has a pattern deviation of $P < 1\%$, or (3) a pattern standard deviation of $P < 5\%$. The VF defects were confirmed on two consecutive reliable tests (fixation loss rate $\leq 20\%$, false-positive and false-negative error rates $\leq 25\%$). The baseline IOP value was defined as the mean of at least two measurements before initiation of IOP-lowering treatment. Based on the baseline IOP values, high-tension glaucoma (HTG) eyes were defined as POAG eyes with a baseline IOP of greater than 21 mmHg, and NTG eyes were defined as POAG eyes with baseline IOP of less than or equal to 21 mmHg.

In the GENIE cohort, POAG was defined as the presence of glaucomatous optic disc changes as well as RNFL defect. Glaucomatous optic disc changes were

defined as vertical cup-to-disc ratio (C/D) greater than 0.7, neuroretinal rim thinning (superior or inferior rim width less than 0.1 times disc diameter), notching, or excavation. Eyes with RNFL defects but without glaucomatous optic disc changes (n = 152) were excluded from the study. The baseline IOP value was defined as the mean of at least two measurements before initiation of IOP-lowering treatment.

Patients with POAG in GLAU-GENDISK cohort underwent a complete ophthalmic examination, including a visual acuity assessment, slit-lamp biomicroscopy, gonioscopy, Goldmann applanation tonometry, refractions, dilated fundus examination, disc stereophotography, and red-free fundus photography using a digital fundus camera (VX-10; Kowa, Nagoya, Japan) and standard automated perimetry (Humphrey C 24-2 SITA-Standard visual field; Carl Zeiss Meditec, Inc., Dublin, CA, USA). The central corneal thickness (Pocket II; Quantel Medical, Clermont-Ferrand, France) and axial length (AXIS-II Ultrasonic Biometer; Quantel Medical S.A., Bozeman, MT, USA) were measured. A 200 × 200 optic disc cube scan was performed using Cirrus HD-OCT (Carl-Zeiss Meditec), and the average peripapillary RNFL thickness was measured with the built-in analysis algorithm (software version 6.0; Carl Zeiss Meditec). A 200 × 200 macular cube scan was performed to obtain the macular ganglion cell-inner plexiform layer (GCIPL) thickness.

POAG patients from Japan also underwent a complete ophthalmic examination including a visual acuity assessment, slit-lamp biomicroscopy, gonioscopy,

Goldmann applanation tonometry, refractions, stereoscopic fundus camera photographs (nonmyd WX, Kowa Company, Nagoya, Japan), central corneal thickness (CASIA, Tomey Cooperation, Nagoya, Japan), axial length (IOLMaster, Carl Zeiss Meditec), standard automated perimetry (Humphrey C 24-2 SITA-Standard visual field; Carl Zeiss Meditec), and OCT scan (3D OCT 2000, Topcon, Tokyo, Japan).[29]

The present study excluded participants with a diagnosis or history of any secondary glaucoma, a history of ocular trauma, a history of systemic or ocular infection, or a history of systemic or ocular use of glucocorticoids.

Exome Chip Analysis

Study samples from primary cohort were processed on a HumanExome Bead-Chip 12v1-1 system (Illumina, Inc.; San Diego, CA, USA), which included 244,651 markers focused on protein-altering variants. Details regarding SNP content and selection strategies can be found at the exome array design webpage (http://genome.sph.umich.edu/wiki/Exome_Chip_Design). Genotype calling was performed using Illumina's GenTrain version 2.0 clustering algorithm with GenomeStudio software (V2011.1). Cluster boundaries were determined using Illumina's standard cluster file. After additional visual inspection of SNPs with call rates of less than 0.99 and SNPs with minor allele frequencies (MAF) of less than 0.002, 244,552 of 244,651 (99.96%) attempted markers were successfully genotyped with call rates greater than 98% (average call rate: 99.92%). In total,

309 of 312 patients were successfully genotyped (call rate > 98%).

The obtained control dataset from NBK, processed with the HumanExome Bead-Chip 12v1-1 system, passed the quality control criteria (call rate > 98%). Individuals who had POAG or NTG were excluded, as were related individuals whose estimated identity-by-state values were high (> 0.50). We carried out principal component analysis (PCA) to avoid artifactual results due to family relatedness. The possible population stratification in this study using PCA was examined using HelixTree.

After excluding monomorphic SNPs, 63,880 SNPs were used for statistical analysis. SNP genotype frequencies were examined for Hardy-Weinberg equilibrium using the chi-squared statistics and all were found to be consistent (P > 0.05). Data were analyzed using an unconditional logistic regression to calculate an odds ratio (OR) as an estimate of the relative risk of POAG associated with SNP genotypes. To determine the association between the genotype and haplotype distributions, a logistic analysis was performed controlling for age and sex as covariates to eliminate or reduce any confounding factors that could influence the findings.

For gene-based testing, we used the SKAT-O test[33], which encompassed burden tests and SKAT as special cases.[34] SKAT-O has been shown to perform well under a range of scenarios, including scenarios in which protective, deleterious, and null variants are present and those in which a large number of variants are causal and associated in the same direction.[33]

Validation Analysis

Targeted genotyping of 6 SNPs (rs116121322 in *LRRC27*, rs138980799 in *IVL*, rs191590289 in *METTL20*, rs140732889 in *ZNF677*, and rs4889261 and rs13339342 in *PKD1L2*) in the POAG samples from replication cohort #1 was carried out by the Taqman assay (Applied Biosystems, Carlsbad, CA, USA)[35]. The Taqman assay was performed according to following steps: 1) preparation of approximately 20 ng of purified genomic DNA; and 2) preparation of genotyping mixture consisting of 2X genotyping master mix, 20X SNP genotyping assay, DNAse-free water and template DNA; and 3) polymerase chain reaction (PCR) containing 40 cycles of denaturation and annealing/extension steps.[35] When completed PCR, genotypes of DNA samples were analyzed on the ABI prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Genotyping quality control was performed in 10% of the samples by duplicate checking (rate of concordance in duplicates > 99.5%) The call rate was 99.5% in all of the 6 SNPs and was therefore considered informative. Study samples from replication cohort #2 were processed on a Japonica array (Toshiba, Tokyo, Japan), a custom-designed array optimized for the Japanese population based on the information from the reference panel from 1,070 Japanese.[29] SNP quality control and the imputation procedure were performed by using a 1,070 Japanese whole-genome panel as previously reported.[24] For meta-analysis of datasets from Korea and Japan, the basic meta-analysis function in PLINK was applied. Fixed-effect

meta-analysis *P* value and fixed-effect OR were estimated.

Study samples from GENIE were processed on KoreanChip ver.1.0 (Axiom Customized Genome-Wide Human Assay, Affymetrix platform, DNA Link, Inc., Seoul, Korea), a custom-designed array optimized for the Korean population. SNP genotype data were directly matched from those of GLAU-GENDISK. Among 8 SNPs, only 4 (rs1063192 and rs7865618 in *CDKN2B-AS1*; rs10483727 in *SIX1/SIX6*, and rs1900004 in *ATOH7*) were available for the replication analysis in the GENIE cohort.

Cell Culture

Human trabecular meshwork cells (HTMC) were purchased from Sciencell Research Laboratories (San Diego, CA, USA) and cultured in trabecular meshwork cell medium (Sciencell). The cells were passaged by trypsinization every 3–4 days.

Western Blot Analysis

For Western blot analysis, the collected HTMCs lysed using a RIPA buffer with protease inhibitor cocktail (Sigma, St Louis, MO, USA). Protein extracts were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dried milk and incubated overnight with LRRC27 rabbit polyclonal antibody (1:500, Novus Biologicals, Littleton, USA) or β-actin mouse monoclonal antibody (1:500, Sigma)

at 4℃. Then the membranes were washed and incubated with mouse anti-rabbit IgG or goat anti-mouse IgG (1:1000; Life Technologies, Eugene, OR, USA) for 1 hour. The immunoreactive bands were detected by chemiluminescent horseradish peroxidase (HRP) substrates (Thermo Scientific, Rockford, USA).

Immunofluorescence

HTMCs were fixed in 10% neutral buffered formalin for 10 minutes and washed with phosphate-buffered saline (PBS). Cells were blocked with bovine serum albumin (BSA) solution for 30 minutes and incubated with primary antibody overnight at 4 ℃. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit (Molecular Probes, Eugene, OR, USA) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Stained cells were examined by fluorescence microscope (DMI4000B; Leica, Germany).

RESULTS

Patients and Control Demographics

We recruited 619 patients with POAG and 11,012 healthy controls from NBK. Replication samples of 565 POAG patients and 1,104 healthy controls were further recruited from Japan. Subjects' demographics are provided in **Table 1**.

Table 1. Demographics of Primary Open-Angle Glaucoma (POAG) Cases and Controls

	Primary Cohort (Korea)			Replication Cohort #1 (Korea)			Replication Cohort #2 (Japan)		
	POAG (*n* = 309)	**Control (*n* = 5,400)**	***P*-value**	**POAG (*n* = 310)**	**Control (*n* = 5,612)**	***P*-value**	**POAG (*n* = 565)**	**Control (*n* = 1,104)**	***P*-value**
Genotyping	Exome chip	Exome chip		TaqMan Assay	Exome chip		Japonica array	Japonica array	
Age, y	56.1 ± 13.6	52.6 ± 8.4	< 0.001*	54.7 ± 13.5	52.1 ± 8.9	0.001*	64.5 ± 11.7	59.7 ± 14.1	<0.001*
Age range, y	21–88	39–70	-	20–83	21–84	-	35–94	35–88	-
Female, %	50.2	54.6	0.15†	45.2	52.4	0.015†	44.4	51.1	0.01†

POAG: primary open-angle glaucoma, HTN: hypertension, DM: diabetes mellitus. Mean ± standard deviation, *Comparison performed using Student's t-test, †Comparison performed using chi-square test

When POAG cases were stratified by baseline IOP, 503 cases were NTG (mean baseline IOP, 15.2 ± 3.0 mmHg), and 116 cases were HTG (mean baseline IOP, 24.0 ± 6.8 mmHg) from Korea, and 446 cases were NTG (mean baseline IOP, 16.1 ± 2.7 mmHg) and 119 cases were HTG (mean baseline IOP, 27.5 ± 6.9 mmHg) from Japan. There were no differences in age and an axial length between the two groups (all $P > 0.05$). However, a higher proportion of patients with NTG eyes were women, and lower central corneal thickness measurements were observed in NTG cases (**Table 2**). Clinical characteristics of NTG and HTG patients from each cohort are provided in **Table 2**.

Table 2. Clinical Characteristics of High-Tension Glaucoma (HTG) and Normal-Tension Glaucoma (NTG)

	Primary Cohort (Korea)			Replication Cohort #1 (Korea)			Replication Cohort #2 (Japan)		
	HTG (*n* = 55)	**NTG (*n* = 254)**	***P*-value**	**HTG (*n* = 61)**	**NTG (*n* = 249)**	***P*-value**	**HTG (*n* = 119)**	**NTG (*n* = 446)**	***P*-value**
Age, y	54.1 ± 15.3	56.5 ± 13.2	0.29*	54.7 ± 15.6	54.7 ± 13.0	0.99*	64.1 ± 10.7	64.4 ± 12.0	0.78*
Female, %	**36.4**	**53.1**	**0.035†**	39.3	46.6	0.38†	**36.1**	**47.5**	**0.034†**
Baseline IOP, mmHg	**24.8 ± 8.1**	**15.2 ± 3.2**	**< 0.001***	**23.3 ± 5.3**	**15.2 ± 2.9**	**<0.001***	**27.5 ± 6.9**	**16.1 ± 2.7**	**< 0.001***
CCT, μm	**545.0 ± 30.7**	**532.0 ± 34.3**	**0.010***	**547.0 ± 30.4**	**534.0 ± 33.9**	**0.010***	**520.0 ± 35.5**	**509.3 ± 34.4**	**0.012***
AXL, mm	24.7 ± 1.6	24.5 ± 1.6	0.47*	24.9 ± 2.4	24.8 ± 1.7	0.88*	25.4 ± 1.8	25.2 ± 1.8	0.47*
Average RNFLT, μm	**64.0 ± 11.6**	**70.9 ± 11.4**	**<0.001***	**63.6 ± 14.9**	**70.8 ± 12.1**	**0.002***	76.8 ± 13.2	80.1 ± 13.9	0.051*
MD, dB	**–15.2 ± 10.1**	**–8.8 ± 6.9**	**< 0.001***	**–13.6 ± 9.0**	**–7.0 ± 6.1**	**<0.001***	-14.8 ± 9.1	-13.1 ± 8.5	0.12*

HTG: high-tension glaucoma, NTG: normal-tension glaucoma, IOP: intraocular pressure, CCT: central corneal thickness, AXL: axial length, RNFL: retinal nerve fiber layer, MD: mean deviation. Mean ± standard deviation, *Comparison performed using Student's t-test, †Comparison performed using chi-square test

For the validation of previously known POAG-related variants, 309 POAG patients and 5,400 healthy controls for discovery analysis (GLAU-GENDISK#1) along with 310 POAG patients and 5,612 healthy controls (GALU-GENDISK#2) and 221 POAG patients and 6,244 healthy controls (GENIE) for replication analysis were enrolled. The demographic information on the enrolled subjects is provided in **Table 3**. The POAG patients were significantly older than the healthy controls in all of the populations. There was no significant difference in gender in GLAU-GENDISK#1 or #2, but GENIE had more females in the control group (42.0%) than among POAG patients (28.1%, $P < 0.001$). The average IOP was 16.9 ± 5.8 mmHg for GLAU-GENDISK#1, 16.6 ± 4.6 mmHg for GLAU-GENDISK#2, and, for GENIE, 13.1 ± 3.7 mmHg and 12.4 ± 2.9 mmHg among POAG patients and healthy controls, respectively.

Table 3. Demographics for Validation for Previously Known POAG-related Variants

	Primary Cohort (GLAU-GENDISK#1)			Replication Cohort #1 (GLAU-GENDISK#2)			Replication Cohort #2 (GENIE)		
	POAG (*n* = 309)	**Control (*n* = 5,400)**	***P*-value**	**POAG (*n* = 310)**	**Control (*n* = 5,612)**	***P*-value**	**POAG (*n* = 221)**	**Control (*n* = 6,244)**	***P*-value**
Age, y	56.1 ± 13.6	52.6 ± 8.4	< 0.001*	54.7 ± 13.5	52.1 ± 8.9	0.001*	52.2 ± 8.8	50.6 ± 9.0	0.009*
Age range, y	21–88	39–70	-	20–83	21–84	-	34–91	20–85	-
Female, %	50.2	54.6	0.15†	45.2	52.4	0.015†	28.1	42.0	<0.001†
IOP, mmHg	16.9 ± 5.8	-	-	16.6 ± 4.6	-		13.1 ± 3.7	12.4 ± 2.9	0.009

POAG: primary open-angle glaucoma; GLAU-GENDISK: GLAUcoma GENe DIscovery Study in Korea; GENIE, Gene-environmental interaction and phenotype; IOP: intraocular pressure. Mean ± standard deviation, *Comparison performed using Student's t-test, †Comparison performed using chi-square test

Novel Single-Variant Associations with POAG

The MAF distribution from exome chip analysis was highly skewed towards very low-frequency variants, with 81.3% (*n* = 198,878) of variants successfully genotyped as monomorphic and 8.1% (*n* = 19,850) as having a MAF less than 0.05, and 10.6% (*n* = 25,824) as having a MAF greater than 0.05. There was no significant stratification detected using all SNPs (**Figure 1**), and the genomic inflation factor (lambda) was 1.08, showing no significant dispersion of test statistics from the expected distribution (**Figure 2**).

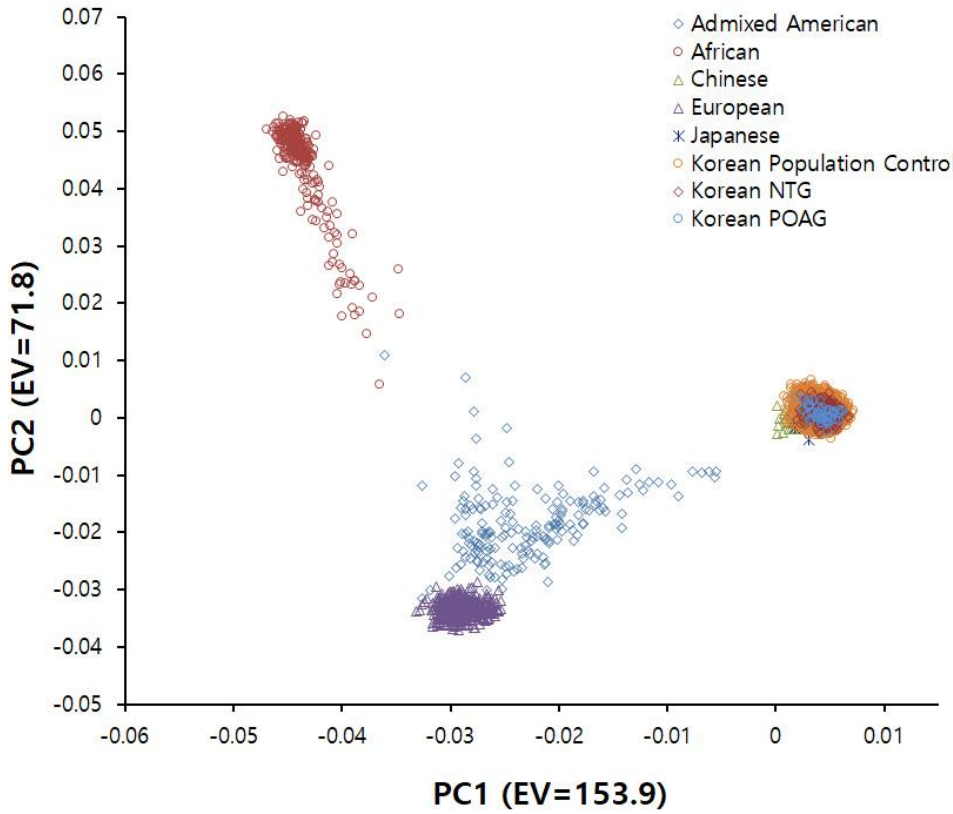

Figure 1. Principal component analysis of study participants. First and second principal components of our study samples and 1000 genomes project samples.

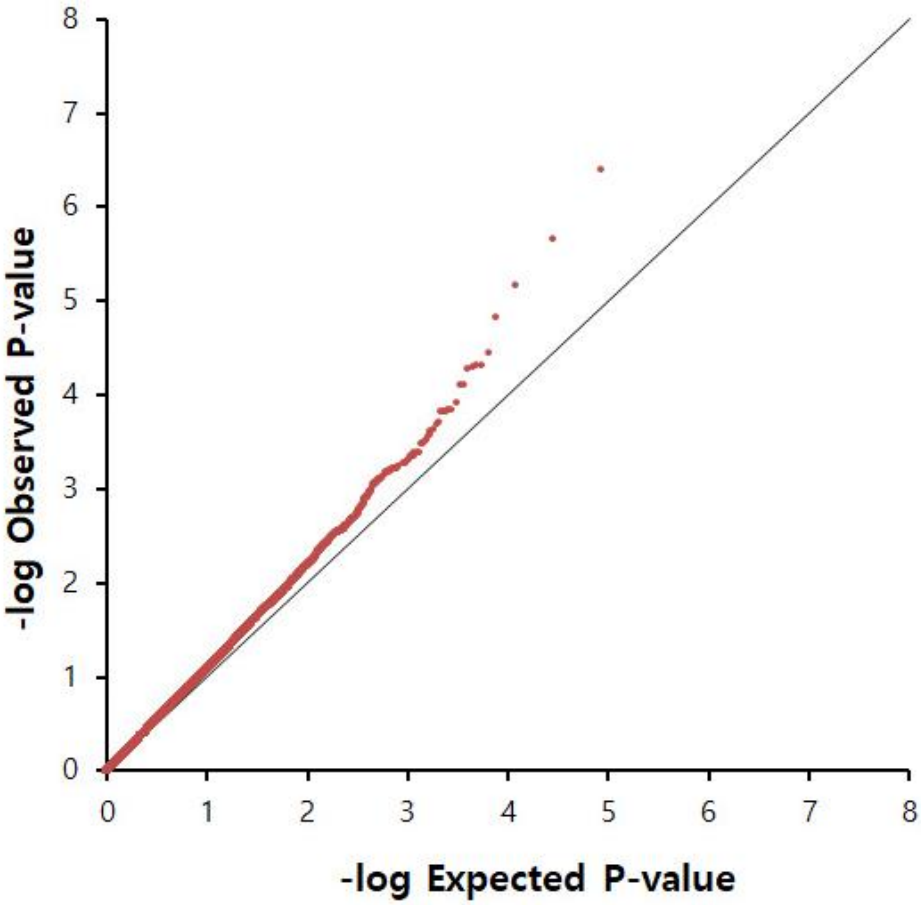

Figure 2. Quantile-quantile plots of *P* values in the association results of the exome chip analysis on the Korean population. Under the null hypothesis, the plots would be expected to follow the red line (y = x). The genomic inflation factor (lambda) was 1.08, showing no significant dispersion of test statistics from the expected distribution.

The strongest association for the risk of POAG was provided by rs138980799 (OR = 83.24, *P* = 4.0E–07) in *IVL*, which causes an H162R amino acid change, with exome-wide statistical significance. Additionally, nominally significant associations were observed for rs116121322 (OR = 29.85, *P* = 2.2E–06) in *LRRC27*, rs191590289 (OR = 3.42, *P* = 6.9E–06) in *METTL20*, rs140732889 (OR = 20.72, *P* = 5.1E–05) in *ZNF677*, and rs4889261 (OR = 0.33, *P* = 5.4E–05) and rs13339342 (OR = 0.34, *P* = 7.8E−05) in *PKDIL2*; however these associations did not reach statistical significance after Bonferroni corrections for multiple testing (**Tables 4 and 5, and Figure 3**).

Table 4. Association of rs116121322 in *LRRC27* with Primary Open-Angle Glaucoma

rsID	Gene	Chr	AA change	Alleles	Population	Stage	MAF Case	MAF Control	OR (95% CI)	*P*-value
rs116121322	LRRC27	10	V189I	G>A	**POAG**	**Primary (KOR)**	**0.00971**	**0.00037**	**29.85 (7.99–111.59)**	**2.2E-06**
						Replication#1 (KOR)	**0.00647**	**0.00054**	**10.40 (2.82–38.32)**	**0.002**
						Replication#2 (JPN)	0.00090	0.00241	0.43 (0.05–3.78)	0.44
						Combined (KOR)	**0.00809**	**0.00046**	**17.79(7.19-44.01)**	**1.6E-07***
						Meta-analysis (KOR+JPN)			**10.28**	**1.4E-07***
					NTG	**Primary (KOR)**	**0.00787**	**0.00037**	**18.75 (4.47-78.69)**	**0.0004**
						Replication#1 (KOR)	**0.00604**	**0.00054**	**9.86 (2.35–41.31)**	**0.007**
						Replication#2 (JPN)	0.00135	0.00241	0.67 (0.08-5.88)	0.72
						Combined (KOR)	**0.00697**	**0.00046**	**14.13 (5.21–38.37)**	**2.3E-05***
					HTG	**Primary (KOR)**	**0.01818**	**0.00037**	**76.78 (13.32-442.58)**	**0.0004**
						Replication#1 (KOR)	0.00820	**0.00054**	15.18 (1.77–130.02)	0.06
						Replication#2 (JPN)	0.00000	0.00241	NA	NA
						Combined (KOR)	**0.01293**	**0.00046**	**28.91 (7.85–106.46)**	**3.8E-04***

Chr: chromosome, AA: amino acid, MAF: minor allele frequency, POAG: primary open-angle glaucoma (i.e., NTG + HTG), NTG: normal-tension glaucoma, HTG: high-tension glaucoma, OR: odds ratio, KOR: Korea, JPN: Japan, NA: not applicable. **P*-values adjusted by Benjamini-Hochberg method to compensate for multiple comparison

Table 5. Candidate Low-Frequency Variants Associated with Primary Open-Angle Glaucoma at $P < 10^{-4}$

rsID	Gene	Chr	Position	AA change	Alleles	Stage	MAF Case	MAF Control	OR (95% CI)	*P*-value
rs138980799	IVL	1	152882758	H162R	A>G	**Primary (KOR)**	**0.00971**	**0.00009**	**83.24 (9.74–711.44)**	**4.0E-07**
						Replication#1 (KOR)	0.00000	0.00018	NA	NA
						Replication#2 (JPN)	0.00000	0.00000	NA	NA
						Combined (KOR)	**0.00485**	**0.00014**	**27.40 (6.70–111.97)**	**1.6E-05***
						Meta-analysis (KOR+JPN)			NA	NA
rs191590289	METTL20	12	31820716	D194V	A>T	**Primary (KOR)**	**0.03560**	**0.01048**	**3.42 (2.13–5.48)**	**6.9E-06**
						Replication#1 (KOR)	0.00485	0.01186	0.41 (0.13–1.31)	0.08
						Replication#2 (JPN)	0.00000	0.00000	NA	NA
						Combined (KOR)	**0.02023**	**0.01119**	**1.83(1.21−2.78)**	**0.012***
						Meta-analysis (KOR+JPN)			NA	NA
rs140732889	ZNF677	19	53741442	G180R	C>T	**Primary (KOR)**	**0.00809**	**0.00037**	**20.72 (5.42−79.27)**	**5.1E-05**
						Replication#1 (KOR)	0.00000	0.00053	NA	NA
						Replication#2 (JPN)	0.00000	0.00000	NA	NA
						Combined (KOR)	**0.00406**	**0.00045**	**9.60 (3.22–28.60)**	**0.001***
						Meta-analysis (KOR+JPN)			NA	NA
rs4889261	PKD1L2	16	81213378	L711P	G>A	**Primary (KOR)**	**0.01618**	**0.04630**	**0.33 (0.18-0.63)**	**5.4E-05**
						Replication#1 (KOR)	0.05519	0.04840	1.13 (0.79–1.61)	0.51
						Replication#2 (JPN)	0.03680	0.02959	1.24 (0.83–1.85)	0.29
						Combined (KOR)	0.03566	0.04736	0.73(0.54-1.00)	0.048*
						Meta-analysis (KOR+JPN)			0.89	0.45*
rs13339342	PKD1L2	16	81219187	R636H	C>T	**Primary (KOR)**	**0.01618**	**0.04574**	**0.34 (0.18-0.64)**	**7.8E-05**
						Replication#1 (KOR)	0.05663	0.04758	1.18 (0.83–1.68)	0.36
						Replication#2 (JPN)	0.03680	0.02959	1.24 (0.83–1.85)	0.29
						Combined (KOR)	0.03641	0.04667	0.76(0.56-1.03)	0.07*
						Meta-analysis (KOR+JPN)			0.91	0.45*

Chr: chromosome, AA: amino acid, MAF: minor allele frequency, OR: odds ratio, KOR: Korea, JPN: Japan, NA: not applicable. **P*-values adjusted by Benjamini-Hochberg method to compensate for multiple comparison.

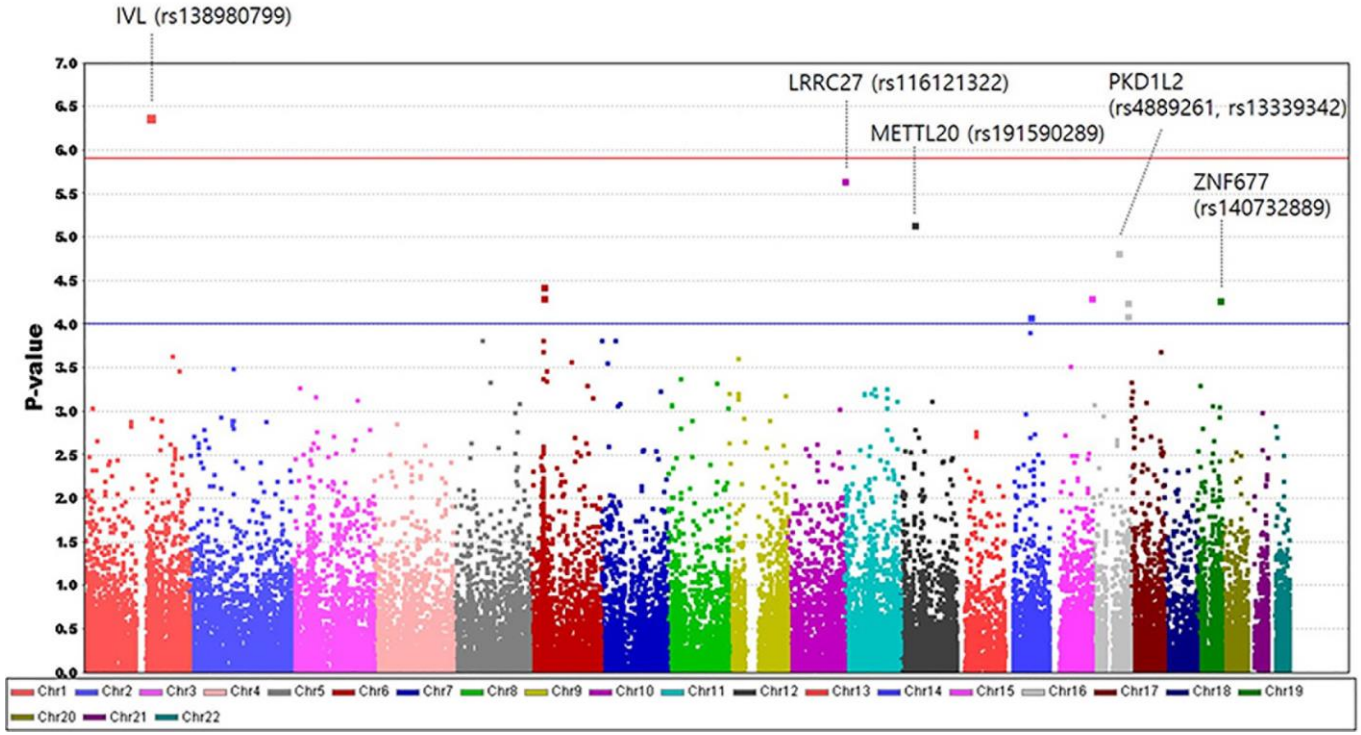

Figure 3. Manhattan plot of the associations of primary open-angle glaucoma from an analysis of quality control passed 63,880 single nucleotide polymorphisms on a custom HumanExome BeadChip v1.0 (Illumina, Inc.). The red line represents *P* = 7.8E–07, the level set significant after Bonferroni correction.

The 6 SNPs mentioned above were further validated from the replication cohort #1 (Korea) and cohort #2 (Japan). In replication cohort #1 (Korea), only the SNP rs116121322 in *LRRC27*, which causes a V189I amino acid change, revealed a significant association with POAG (OR = 10.40, $P = 0.002$). However, this SNP did not show any statistical significance in replication cohort #2 (Japan) (OR = 0.43, $P = 0.44$). The combined OR in Korean population was 17.79 (adjusted P = 1.6E–07), and the meta-analysis with the Japanese cohort still exhibited a significant association with POAG (OR = 10.28, adjusted P = 1.4E–07, **Table 5**).

The SNPs rs138980799 in *IVL*, rs191590289 in *METTL20*, rs140732889 in *ZNF677*, and rs4889261 and rs13339342 in *PKDIL2* did not reach statistical significance with POAG from both replication cohort #1 (Korea) and cohort #2 (Japan). However, the combined analysis from Korean population showed significant association with POAG from the SNPs rs138980799 in *IVL* ($OR_{combined}$ = 27.40, adjusted P = 1.6E–05), rs191590289 in *METTL20* ($OR_{combined}$ = 1.83, adjusted $P = 0.012$), and rs140732889 in *ZNF677* ($OR_{combined}$ = 9.60, adjusted P = 0.001, **Table 5**).

Single-Variant Association Differences between Patients with NTG and HTG

The rs116121322 in *LRRC27* was associated with NTG in primary cohort and replication cohort #1 (Korea) but not in replication cohort #2 (Japan). However, the combined analysis in entire Korean cohorts showed a significant association with

NTG ($OR_{combined}$ = 14.13, adjusted *P* = 2.3E–05, **Table 4**). The SNPs rs138980799 in *IVL* and rs191590289 in *METTL20* were associated with NTG in the primary cohort and from a combined analysis of entire Korean cohorts but found to be monomorphic in replication cohort #2 (**Table 6**). The significant findings of SNPs rs4889261 and rs13339342 in *PKD1L2* from exome chip analysis did not reach statistical significance in validation analysis from replication cohorts #1 and #2 (**Table 6)**.

Table 6. Comparison of Associations between Normal-Tension Glaucoma (NTG) and High-Tension Glaucoma (HTG) for Candidate SNPs.

rsID	Gene (near gene)	Stage	MAF Control	NTG			HTG		
				MAF Case	OR (95% CI)	*P*-value	MAF Case	OR (95% CI)	*P*-value
rs138980799	IVL	Primary	**0.00009**	**0.01181**	**101.74 (11.78–878.74)**	**1.5E-07**	0.00000	NA	NA
		Rep#1	0.00018	0.00000	NA	NA	0.00000	NA	NA
		Rep#2	0.00000	0.00000	NA	NA	0.00000	NA	NA
		Combined (KOR)	**0.00014**	**0.00598**	**33.78 (8.24–138.51)**	**1.0E-05***	0.00000	NA	NA
rs191590289	METTL20	Primary	**0.01048**	**0.03346**	**3.16 (1.87-5.37)**	**0.0002**	**0.04545**	**4.85 (1.89–12.45)**	**0.007**
		Rep#1	0.01186	0.00605	0.51 (0.16–1.63)	0.21	0.00000	NA	NA
		Rep#2	0.00000	0.00000	NA	NA	0.00000	NA	NA
		Combined (KOR)	**0.01119**	**0.01992**	**1.81 (1.13–2.88)**	**0.04***	0.02155	2.02 (0.81–4.99)	0.17*
rs140732889	ZNF677	Primary	0.00037	0.00197	4.50 (0.48–41.98)	0.26	**0.03636**	**89.07 (21.43–370.22)**	**7.2E-07**
		Rep#1	0.00053	0.00000	NA	NA	0.00000	NA	NA
		Rep#2	0.00000	0.00000	NA	NA	0.00000	NA	NA
		Combined (KOR)	0.00045	0.00100	2.41 (0.30–19.15)	0.46	**0.01724**	**39.93 (12.10–131.73)**	**3.8E-05***
rs4889261	PKD1L2	Primary	**0.04630**	**0.01969**	**0.41 (0.22–0.77)**	**0.002***	0.00000	NA	NA
		Rep#1	0.04840	0.06073	1.25 (0.85–1.83)	0.27	0.03279	0.66 (0.24–1.79)	0.38
		Rep#2	0.02960	0.03514	1.16 (0.72–1.87)	0.54	0.04011	1.37 (0.76-2.49)	0.30
		Combined (KOR)	0.04736	0.03992	0.82 (0.60–1.14)	0.35*	**0.01724**	**0.35 (0.13–0.95)**	**0.013***
rs13339342	PKD1L2	Primary	**0.04574**	**0.01969**	**0.42 (0.22-0.79)**	**0.002**	0.00000	NA	NA
		Rep#1	0.04758	0.06250	1.31 (0.90–1.91)	0.17	0.03279	0.67 (0.25–1.82)	0.40
		Rep#2	0.02960	0.03514	1.16 (0.72–1.87)	0.54	0.04011	1.37 (0.76–2.48)	0.3008
		Combined (KOR)	0.04667	0.04083	0.86 (0.62–1.18)	0.41*	**0.01724**	**0.36 (0.13–0.96)**	**0.013***

MAF: minor allele frequency, NTG: normal-tension glaucoma, HTG: high-tension glaucoma, OR: odds ratio, Rep: replication. **P*-values adjusted by Benjamini-Hochberg method to compensate for multiple comparison.

The SNPs rs116121322 in *LRRC27* ($OR_{combined}$ = 28.91, adjusted *P* = 3.8E–04) and rs140732889 in *ZNF677* ($OR_{combined}$ = 39.93, adjusted *P* = 3.8E–05) showed significant association with HTG from primary cohort and combined analysis form entire Korean cohorts **(Tables 4 and 6)**. The rs191590289 in *METTL20* was significantly associated with HTG only in primary cohort (OR = 4.85, *P* = 0.007) but not in replication cohorts. The SNPs rs4889261 ($OR_{combined}$ = 0.35, adjusted *P* = 0.013) and rs13339342 ($OR_{combined}$ = 0.36, adjusted *P* = 0.013) in *PKD1L2* showed only marginal significance from combined analysis of entire Korean cohorts (**Table 6**).

Gene-based Association Analysis

Since the majority of individual variants are very rare (median MAF =0.0084), we assessed the burden of 63,880 variants across 13,923 genes. *METTL20* (N172S, D188N, and D194V) showed an exome-wide significant association with POAG (adjusted *P* = 0.006) from the primary cohort but did not reach statistical significance in replication cohort #1. The combined analysis showed a significant association with POAG (*P* = 0.002). This gene revealed a significant association with NTG from entire Korean cohorts (*P* = 0.02) but not in HTG eyes. *ZNF677* (G180R and Y347T) was significantly associated with HTG eyes but not with NTG eyes, with exome-wide statistical significance from the primary cohort (adjusted *P* = 3.6E–05) and in entire Korean cohorts (*P* = 1.5E–06) (**Table 7**).

Table 7. Gene-based rare variant association analysis using SKAT-O method.

Diagnosis	Gene Name	SNPs	Stage	Glaucoma	Control	*P*-value
			Primary	**8.41%**	**2.93%**	**0.006***
POAG	METTL20	N172S, D188N, D194V	Replication#1	2.26%	3.33%	0.38
			Combined	**5.49%**	**3.13%**	**0.002**
			Primary	7.48%	2.93%	0.69*
NTG	METTL20	N172S, D188N, D194V	Replication#1	2.41%	3.33%	0.70
			Combined	**5.17%**	**3.13%**	**0.02**
			Primary	**7.3%**	**0.1%**	**3.6E-05***
HTG	ZNF677	G180R, Y347T	Replication#1	0.00%	0.12%	0.67
			Combined	**0.81%**	**0.12%**	**1.5E-06**

POAG: primary open-angle glaucoma, NTG: normal-tension glaucoma, HTG: high-tension glaucoma, SNP: single nucleotide polymorphism. **P*-value adjusted by Bonferroni correction.

LRRC27 Protein Expression in Human Trabecular Meshwork Cells

To determine whether *LRRC27* gene is a candidate for involvement in the pathogenesis of glaucoma, the expression of LRRC27 protein from human trabecular meshwork cells (HTMC) was confirmed by western blot and immunofluorescence analysis (**Figures 4 and 5**). The LRRC27 proteins were confirmed to be expressed at the cytosol of HTMCs.

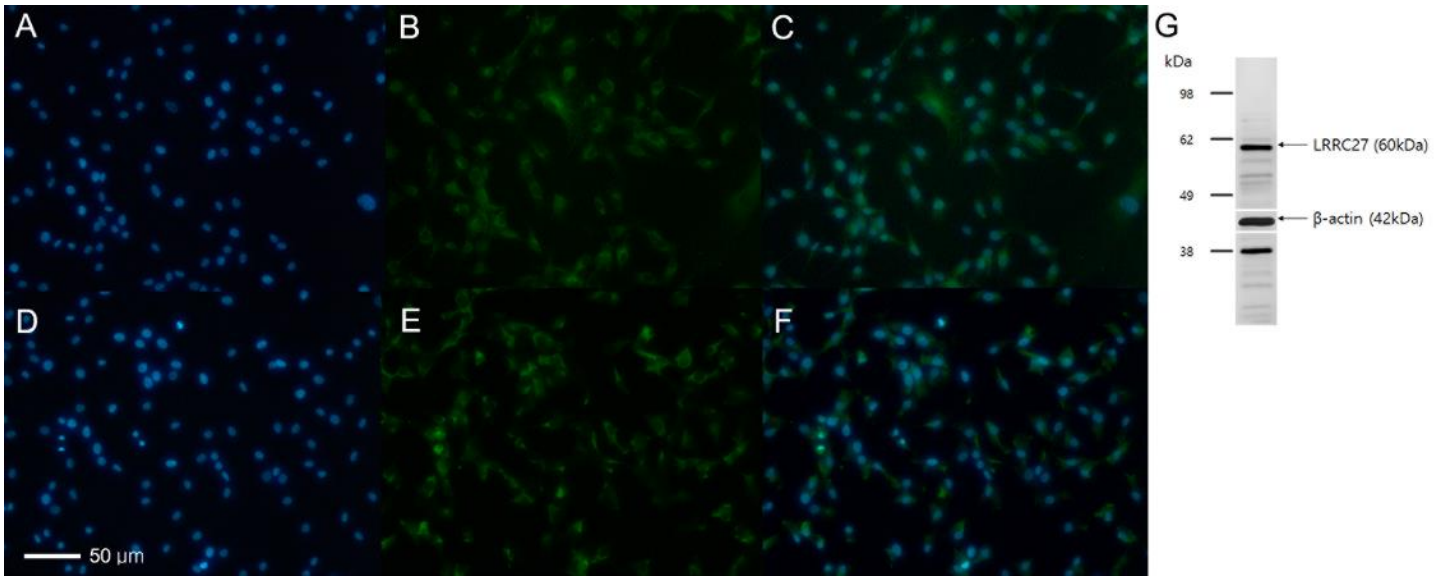

Figure 4. Expression of LRRC27 protein from human trabecular meshwork cells (HTMC). (A) DAPI, (B) LRRC27, (C) Merged image of A and B, (D) DAPI, (E) α-SMA, the marker for HTMC (positive control), (F) Merged image of D and E, (G) Western blot analysis demonstrated expression of LRRC27 protein (60kDa). The β-actin was used as positive control. The blots from LRRC27 and β-actin were cropped from different gels and grouped together (Cropped region marked as white line). Full-length blots/gels are presented in **Figure 5**.

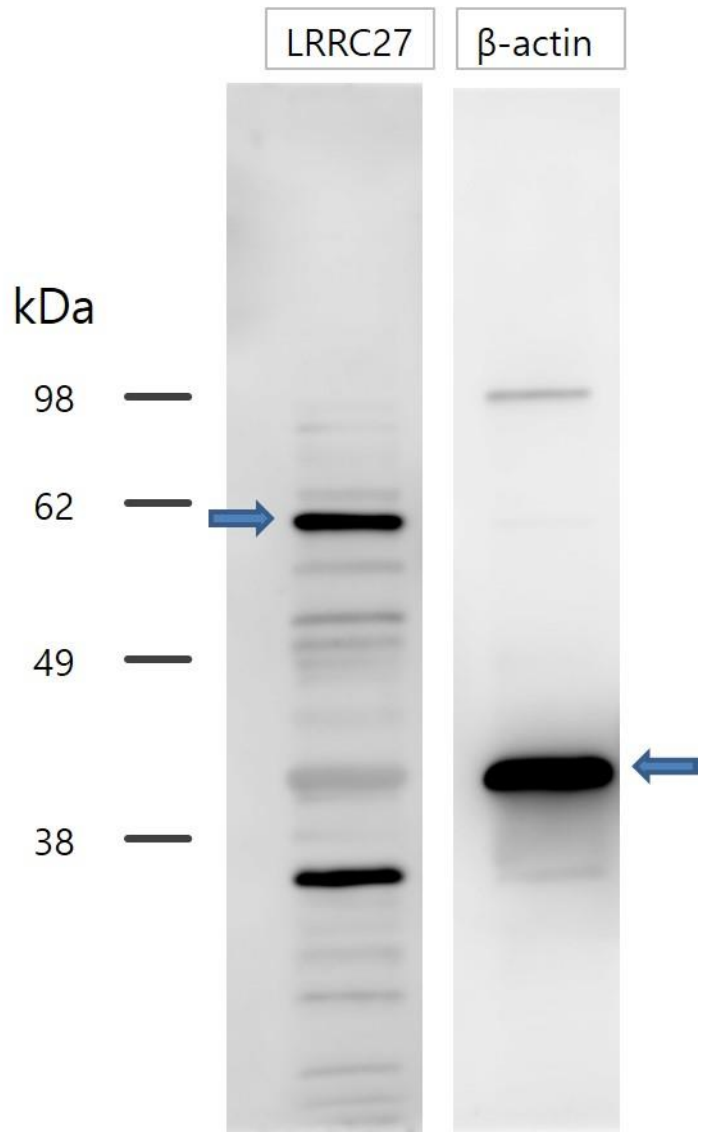

Figure 5. Full-length gels and blots for LRRC27 and β-actin.

Clinical Characteristics of LRRC27 Mutant POAG Patients

The *LRRC27* mutation was observed from six patients. Four cases were NTG and the other two cases were HTG. All of the cases were bilateral glaucoma, and two patients reported their family history of glaucoma. The average onset age of POAG diagnosis was 48.7 ± 20.5 years (range: 18 – 73 years-old), and average baseline IOP of worse-MD eye was 18.2 ± 4.6 mmHg (range: 12 – 24 mmHg). The average MD of worse-MD eye was –13.80 ± 5.6 dB (range: –7.23 – –23.53 dB). Clinical features of *LRRC27* mutants in the present cohort are provided in **Figure 6**.

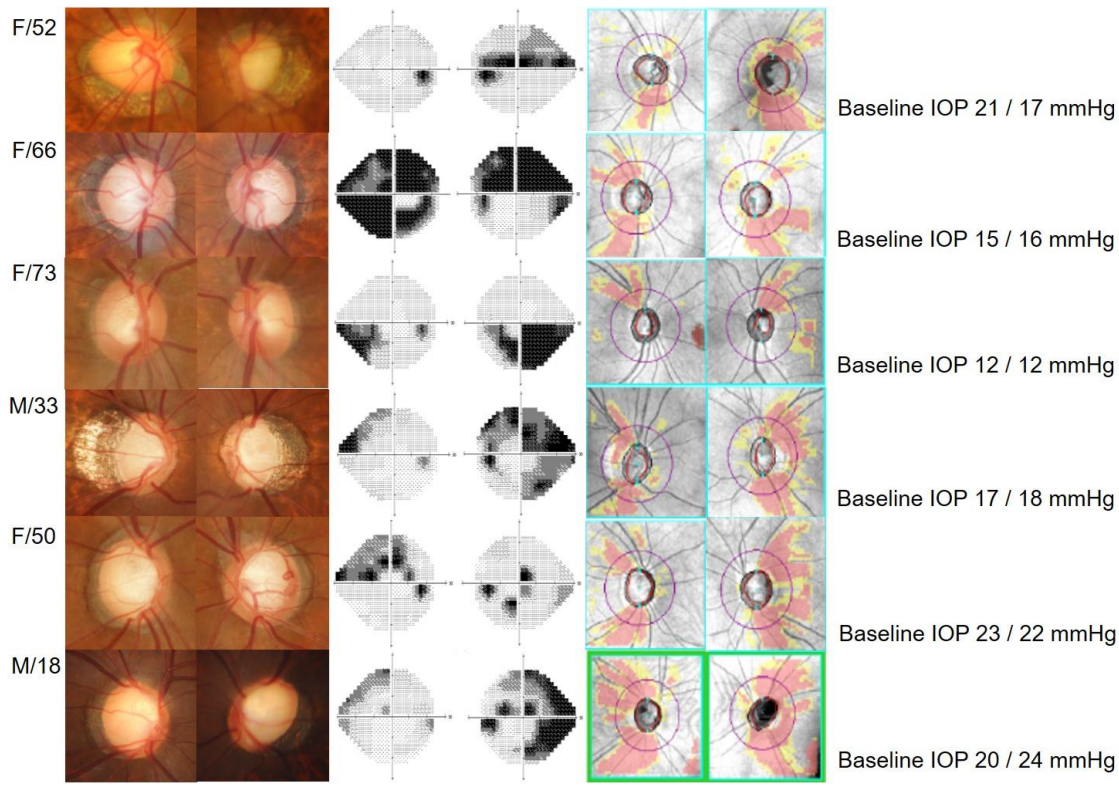

Figure 6. Clinical Features of *LRRC27* Mutants with Primary Open-Angle Glaucoma

Clinical features, including age, gender, baseline intraocular pressure (IOP), optic disc photograph, automated perimetry, and spectral-domain optical coherence tomography (SD-OCT) scans, of *LRRC27* mutants with primary open-angle glaucoma (POAG) (6 patients) are provided.

Validation for Previously Known POAG-related SNPs

From the exome-chip analysis, rs1900004 in *ATOH7* (OR = 1.29, *P* = 0.0024); rs1063192 (OR = 0.69, *P* = 0.0006), rs2157719 (OR = 0.63, *P* = 0.0007), and rs7865618 (OR = 0.63, *P* = 0.0006) in *CDKN2B-AS1*, and rs10483727 in *SIX1/SIX6* (OR = 0.68, *P* = 7.9E–05) were nominally associated with the risk of POAG. In contrast, rs1192415 in CDC7-TGFBR3 (*P* = 0.11) was not associated with the risk of POAG (**Table 8**). The SNPS rs4236601 in *CAV1* (*P* = 0.25) and rs4656461 in *TMCO1* (*P* = 0.86) were not associated with POAG, and, since their minor allele frequency was found to be less than 0.05, no further validation analysis was conducted.

Table 8. Associations of *CDKN2B-AS1, SIX1/SIX6, ATOH7* and *CDC7-TGFBR3* loci with Primary Open-Angle Glaucoma

Gene	dbSNP	GLAU-GENDISK#1 (case: 309, control: 5,400)		GLAU-GENDISK#2 (case: 310, control: 5,612)		GENIE (case: 221, control: 6,244)		Mega-Analysis	
		OR	P-value	OR	P-value	OR	P-value	OR	P-value
CDKN2B-AS1	rs1063192	**0.69 (0.56–0.86)**	**0.0006**	0.96(0.79–1.18)	0.70	**0.63 (0.48-0.82)**	**0.0007**	**0.77 (0.68-0.87)**	**6.0E–05**
CDKN2B-AS1	rs2157719	**0.63 (0.47–0.83)**	**0.0007**	**0.72(0.55–0.94)**	**0.0135**	NA	NA	**0.67 (0.55–0.82)**	**3.3E–05**
CDKN2B-AS1	rs7865618	**0.63 (0.47–0.83)**	**0.0006**	NA	NA	**0.52 (0.37-0.75)**	**0.0004**	**0.58 (0.47-0.73)**	**1.9E–06**
SIX1/SIX6	rs10483727	**0.68 (0.56–0.83)**	**7.9E-05**	0.88(0.73–1.06)	0.18	0.85 (0.68-1.06)	0.16	**0.79 (0.71-0.89)**	**9.4E–05**
ATOH7	rs1900004	**1.29 (1.10–1.53)**	**0.0024**	1.00(0.85–1.18)	0.99	0.96 (0.79-1.17)	0.71	1.09 (0.99-1.20)	0.10
CDC7-TGFBR3	rs1192415	1.00 (0.79–1.27)	0.97	1.21(0.96–1.51)	0.11	NA	NA	1.10 (0.94–1.30)	0.24

GLAU-GENDISK: GLAUcoma GENe DIscovery Study in Korea; GENIE, gene-environmental interaction and phenotype; ATOH7: Atonal BHLH Transcription Factor 7; CDC7-TGFBR3: Cell Division Cycle 7-Transforming growth factor beta receptor III; CDKN2B-AS1: Cyclin-dependent kinase 4 inhibitor B antisense RNA 1; SIX1: Sineoculis homeobox homolog 1; SIX6: Sineoculis homeobox homolog 6

In the replication analysis from GLAU-GENDISK#2, rs2157719 (OR = 0.72, *P* = 0.0135) in *CDKN2B-AS1* was significantly associated with the risk of POAG. However, no other SNPs were associated with the risk of POAG (**Table 8**). In the replication analysis from GENIE, rs1063192 (OR = 0.63, *P* = 0.0007) and rs7865618 (OR = 0.52, *P* = 0.0004) in *CDKN2B-AS1* were nominally associated with the risk of POAG. However, rs10483727 in *SIX1/SIX6* (*P* = 0.16) and rs1900004 in *ATOH7* (*P* = 0.71) were not associated with the risk of POAG (**Table 8**).

The mega-analysis for the entire population revealed rs1063192 (OR = 0.77, *P* = 6.0E–05), rs2157719 (OR = 0.63, *P* = 0.0007) and rs7865618 (OR = 0.58, *P* = 1.9E–06) in *CDKN2B-AS1* and rs10483727 in *SIX1/SIX6* (OR = 0.79, *P* = 9.4E–05) to be significantly associated with the risk of POAG, with the same direction of effect between the discovery association and replication sample (**Table 8**).

DISCUSSION

In this study, we performed an exome-wide association study for POAG in a Korean population, and reviewed their results from Japanese population, in which the prevalence of NTG is very high compared with that in other regions or races. Our data identified one novel candidate gene variant (rs116121322 in *LRRC27*) associated with the risk of POAG, which revealed statistical significance from Korean population and meta-analysis from Korean and Japanese population. In addition, the present study investigated, for the first time, the genetic association of previously known POAG-related loci from two large Korean population-based cohorts. The present data demonstrated that SNPs from *CDKN2B-AS1* and *SIX1/SIX6* were significantly associated with the risk of POAG, while those from *ATOH7, CDC7-TGFBR3, CAV1*, and *TMCO1* were not.

The SNP rs116121322 in *LRRC27* (encoding leucine-rich repeat-containing protein 27) was significantly associated with POAG. The SNP rs116121322 is a missense variant or non-coding transcript variant; however, its clinical significance has not yet been clearly elucidated. The gene *LRRC27* has 14 transcripts, of which 5 are containing an open reading frame. The cDNA of *LRRC27* is expressed in various tissues, including the fetal eye, lens, anterior segment, optic nerve, and retina, while the level of expression is highest from sex organs (testis followed by fallopian tube).[36,37] As the cDNA of *LRRC27* been confirmed to be expressed in ocular tissues including optic nerve and retina, the gene variant may be expected to alter the physiology of the optic nerve. The present data further confirmed the

expression of LRRC27 protein from HTMCs. From the findings that this gene has a high expression of mRNA at sex organs, altered metabolism of sex-hormones or altered sex organ-related functions may have increased the risk for glaucoma development. Evidence is increasing that the retina and optic nerve are sex hormone-sensitive tissues.[38-40] In addition, epidemiological studies reveal higher prevalence of glaucoma in men than women.[1,41,42] Interestingly, MAF of rs116121322 for POAG and healthy control differed by gender in our cohorts (male, 0.62 % vs. 0.05 %, female, 1.02 % vs. 0.04%) (**Table 9**).

Table 9. Gender Difference of Minor Allele Frequency for rs116121322 in *LRRC27* in Korean Population

rsID	Gene	Chr	AA change	Alleles	Diagnosis	Gender	Stage	MAF Case	MAF Control	OR (95% CI)	*P*-value
rs116121322	*LRRC27*	10	V189I	G>A	POAG	Male	Primary	0.00649	0.00000	-	-
							Replication#1	0.00588	0.00095	5.69 (1.07–30.32)	0.08
							Combined (KOR)	0.00620	0.00049	13.43 (3.50–51.48)	9.0E–04
						Female	Primary	0.01290	0.00069	15.16 (3.54–64.88)	9.1E–04
							Replication #1	0.00719	0.00017	39.77 (3.46–456.60)	0.004
							Combined (KOR)	0.01020	0.00043	20.59 (6.05–70.10)	1.0E–05

Chr: chromosome, AA: amino acid, MAF: minor allele frequency, OR: odds ratio, POAG: primary open-angle glaucoma, KOR: Korea

Further functional investigation to explore the role of this gene variant on optic nerve head tissues and its relationship with gender is needed. Unlike expectations, this SNP was not associated with POAG in Japanese cohorts. Considering the low MAF of rs116121322, relatively small population size of healthy controls of Japan than those of Korea (1,104 vs. 11,012) may have biased the association results.

In this study, the SNP rs138980799 in *IVL* (involucrin) was significantly associated with NTG, although failed to be validated from the replication cohorts. The *IVL* gene encodes a protein, involucrin, that is expressed in keratinocytes in the epidermis and other stratified squamous epithelia, including the cornea.[43] This protein is synthesized during terminal differentiation and becomes cross-linked to membrane proteins through the catalytic function of transglutaminase, contributing to the formation of a cell envelope that protects corneocytes.[44] Involucrin expression is known to be altered in response to environmental biomechanical changes, i.e., when corneal keratinocytes are exposed to nonphysiological substrate elasticity.[45] Considering the close relationship between corneal biomechanics and POAG development and/or progression, our results provide insights into the role of involucrin in the pathogenesis of POAG.

Gene-based analysis revealed that the combination of SNPs in *METTL20* was significantly associated with NTG but not with HTG. METTL20 is a mitochondrial lysine-specific methyltransferase that targets the β subunit of electron transfer flavoprotein (ETF) and modulates its activity.[46] ETF transfers electrons to the ubiquinone pool of the mitochondrial respiratory chain via quinone oxidoreductase. Alterations in METTL20 expression may impair the mitochondrial respiratory

chain and induce oxidative stress, which is strongly implicated in the pathogenesis of POAG.[47,48] Recently, our group reported a spectrum of mitochondrial DNA variants in a patient with NTG.[49] The current study may further support that mitochondrial dysfunction may be a genetic risk factor for the development of POAG.

The SNP rs140732889 in *ZNF677* (encoding zinc finger protein 677) was significantly associated with HTG but not with NTG from the exome-chip analysis. Gene-based analysis showed consistent results with a combination of variants in *ZNF677*. The *ZNF677* gene is a tumor suppressor in non-small cell lung cancers; however, the clinical implications of the SNP rs140732889 have not yet been reported.[50] Tumor-suppressor genes are often associated with the pathogenesis of POAG. For example, polymorphisms in the *TP53* gene, a prototypical tumor-suppressor gene encoding a 53-kDa protein (p53), have been shown to be associated with POAG.[51] The *INK4* locus at chromosome 9p21, which encodes three tumor-suppressor genes (*CDKN2A*, *ARF*, and *CDKN2B*), has been reported to be associated with POAG and with retinal ganglion cell (RGC) susceptibility in mice.[52,53] Given that POAG is characterized by dysregulation of RGC apoptosis, the role of tumor-suppressor genes in POAG pathogenesis should be investigated further in the clinical setting.

Our current findings revealed that the SNPs rs4889261 and rs13339342 in *PKD1L2* (encoding polycystic kidney disease 1-like 2) were marginally associated with HTG. This gene encodes a member of the polycystin protein family, which includes polycystin-1 (PC1). Aberrant expression of this gene is known to cause

autosomal dominant polycystic kidney disease (ADPKD).[54] PC1 localizes to the apical primary cilia and interacts with polycystin-2 (PC2) to form a receptor complex that transduces Ca^{2+} signals in response to renal flow and maintains the cilia in a differentiated state.[55-57] Dysfunctions in primary cilia signaling can give rise to retinal degeneration, including Bardet-Biedl syndrome or Lowe syndrome.[58-60] Recently, Luo et al.[61] demonstrated that the primary cilia in trabecular meshwork cells respond to pressure changes and mediate subsequent signal transduction to regulate the aqueous humor balance. Thus, the PC1 dysfunction may have deteriorated the ability of primary cilia to sense the IOP, resulting in impaired signal transduction.

The CDKN2B-AS (cyclin-dependent kinase inhibitor 2B antisense) is a long noncoding antisense RNA that regulates the expression of CDKN2B (cyclin-dependent kinase inhibitor 2B).[62,63] The CDKN2B induces apoptosis by inhibiting CDK4/6 (cyclin-dependent kinase 4 and 6), which promotes cell cycle progression.[64] The minor allele of *CDKN2B-AS* can reduce the expression of CDKN2B and thus has a protective effect on POAG development by inhibiting apoptosis. Since the *CDKN2B-AS1* variant was first identified from GWAS on the Australian POAG population[17], this locus was further replicated from other ethnic groups including Japanese[19,65,66], Chinese[27], U.S. Caucasians[65,67], Europeans[22], and an Afro-Caribbean population[68]. The carriers of *CDKN2B-AS1* minor alleles are associated with smaller vertical cup-to-disc ratio and low IOP as compared with the major allele carriers.[69,70] In this regard, it has been suggested that *CDKN2B-AS1* locus may be related with the risk of POAG independent of IOP elevation. The present study is consistent with the previous findings, as the majority of the POAG

populations had their IOP within a normal range.

The SNP rs10483727 is located in the intergenic region between the *SIX1* and *SIX6* loci.[26] The human *SIX* genes are known to regulate eye development[71], and expression of *SIX6* has been isolated in the eye and pituitary gland.[72] The SNP rs10483727 also has been identified as associated with greater vertical C/D and IOP.[23,73] The risk allele near *SIX6* also has been shown to be associated with thinner RNFL in studies in Singapore and Japan.[66,74] This locus has been demonstrated to be associated with POAG in Asian[27,66,75], U.S. Caucasians[67] and Europeans[68] but not in African ethnic groups.[68,76,77] A recent meta-analysis specifically detected SNP rs10483727 in East Asian and Caucasian cohorts but not in South Asian or African cohorts.[78]

The variants in *ATOH7, CDC7-TGFBR3, CAV1*, and *TMCO1* were not associated with the risk of POAG in the present Korean population. These SNPs have been found to be associated with POAG mostly in Caucasians,[16,17,22,65,79] though negative association results also have been reported in other ethnic groups including Chinese[27], South or Middle East Asians[65,80] and Africans[68,76,77]. The present positive and negative association results for the 8 SNPs in *CDKN2B-AS1, SIX1/SIX6, ATOH7, CDC7-TGFBR3, CAV1*, and *TMCO1* are summarized in **Table 10**.

Table 10. Summary of Positive and Negative Results of Association Studies of *CDKN2B-AS1, SIX1/SIX6, ATOH7, CDC7-TGFBR3, CAV1,* and *TMCO1* for Primary Open-Angle Glaucoma

		***CDKN2B-AS1* (rs1063192, rs2157719, rs7865618)**		***SIX1/SIX6* (rs10483727)**		***ATOH7* (rs1900004)**		***CDC7-TGFBR3* (rs1192415)**		***CAV1* (rs4236601)**		***TMCO1* (rs4656461)**	
		positive	**negative**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**
Asian	**Japan**	1-3		3-5				5	2		5-7		5
	China	8	2	8			8		2	7, 9		8	
	South		2, 10		10	10			2	10		10	
	Middle East			11			12				13		
Caucasian	**U.S.A.**	2, 14		14			14	2		15, 16			
	U.K.		2						2		9		
	Australia	17									9	18, 19	
	Iceland									9			
	Others	20		20		20			20				
African	**U.S.**		2		21				2				21
	Barbados	22			22		22		22		22		
	Africa		23		21, 23		23				23		21, 23, 24

The numbers in the table refer to the corresponding reference numbers provided below. ATOH7: Atonal BHLH Transcription Factor 7; CAV1: Caveolin 1; CDC7-TGFBR3: cell division cycle 7-transforming growth factor beta receptor 3; CDKN2B-AS: cyclin-dependent kinase inhibitor 2B antisense; SIX1: Sineoculis homeobox homolog 1; SIX6: Sineoculis homeobox homolog 6; TMCO1: Transmembrane and Coiled-Coil Domains 1; U.S.A: United States of America; U.K.: United Kingdom

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Ethnic-specific genetic associations can be found in other novel POAG-related loci, such as *LHPP, HMGA,* and *MEIS2*.[81] These loci were associated with POAG in a Japanese population but failed to be found in either a European or an African population. The different genetic associations among ethnic groups suggest that the mechanisms of POAG development may vary from race to race. Further replication studies are needed for other ethnic groups, such as Hispanic/Latino groups, before more information will be available on the role of genetic variation in the development of POAG.

The present study has several limitations. First, relatively small POAG population may have weakened the statistical power to detect associations of low-frequency variants. This may have precluded identifying significant associations from our study population. To overcome this limitation, gene-based analysis, the SKAT-O method, was used to reduce the number of tests performed and enhance the detection ability for low-frequency variants. Second, the design of the exome chip was based on pooled exome sequencing data, in which the majority of included individuals were European Americans. These data may have lacked information regarding some important rare alleles in Asian populations. Third, it did not validate other known POAG-related loci. The samples from GLAU-GENDISK and GENIE were genotyped by array chip analysis, and thus it was difficult to include the whole set of known POAG-related SNPs. For the same reason, the SNPs rs2157719 in *CDKN2B-AS1* and rs1192415 in *CDC7-TGFBR3* could not be validated in the GENIE cohort. Lastly, the present study did not investigate the correlation of genotype with glaucoma endophenotypes including IOP, C/D, rim area, and RNFL thickness.

In conclusion, the present study, for the first time, identified novel low-frequency variants associated with POAG risk in East Asian population by exome chip analysis. The SNP in *LRRC27* gene was significantly associated with POAG and confirmed to be expressed in HTMC. *LRRC27* mutants showed early-onset, advanced bilateral glaucoma. Gene-based testing demonstrated *METTL20* gene to be associated with NTG while *ZNF677* gene is associated with HTG. The SNPs rs1063192, rs2157719, and rs7865618 in *CDKN2B-AS1* and rs10483727 in *SIX1/SIX6* were significantly associated with POAG in the Korean population. The *CDKN2B-AS1* and *SIX1/SIX6* loci need further thorough exploration to elucidate the molecular mechanism of POAG development in Asian populations, including Koreans. In contrast, the SNPs in *ATOH7*, *CDC7-TGFBR3*, *CAV1* or *TMCO1* did not show significant associations with POAG in the Korean population. Our current findings may provide further genetic and pathophysiological pathways for understanding the pathogenesis of POAG.

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초록

원발 개방각녹내장은 정상 안압 범위에서도 발생할 수 있으며, 소위 정상안압녹내장으로 불리는 이러한 유형의 녹내장은 한국과 일본을 비롯한 동아시아국가에서 유병율이 매우 높다. 본 연구에서는 원발 개방각녹내장과 연관되어 있는 저빈도/희귀 유전 변이를 탐색하기 위해 일차 코호트 (원발 개방각녹내장 309명, 정상군 5400명, 모두 한국인)에서 엑솜칩 분석을 수행하였다. 검증을 위해 한국인 코호트 (원발 개방각녹내장 310명, 정상군 5612명)와 일본인 코호트 (원발 개방각녹내장 565명, 정상군 1104명)에서 유전자형을 추가 분석하였다. 타인종에서 원발 개방각녹내장과 연관된 것으로 보고된 대표적인 6개 유전자 (*CDKN2B-AS1, SIX1/SIX6, ATOH7, CDC7-TGFBR3, CAV1, TMCO1*) 단일염기다형성 (SNP)을 선정하여 일차코호트 (원발 개방각녹내장 309명, 정상군 5400명, 모두 한국인)와 검증코호트 (#1: 원발 개방각녹내장 310명, 정상군 5612명, #2: 원발 개방각녹내장 221명, 정상군 6244명, 모두 한국인)에서 유전자형을 분석하였다. 일차 코호트에서 *LRRC27* 유전자의 SNP rs116121322이 유의하게 원발 개방각녹내장과 연관되었다 (OR = 29.85, P = 2.2E-06). 이 SNP는 한국인 코호트에서는 추가 검증 되었으나 (OR = 9.86, P = 0.007) 일본인 코호트에서는 원발 개방각녹내장과 유의한 연관이 없었다 (P = 0.44). 그러나 전체 코호트에서 수행한 메타분석에서는

원발 개방각녹내장과 유의한 연관을 보였다 ($OR_{combined}$ = 10.28, $P_{combined}$ = 1.4E–07). 또한 LRRC27 단백질이 인간섬유주세포에서 발현되는 것을 확인하였다. 유전자 수준의 분석에서, *METTL20* 유전자가 원발 개방각녹내장 ($P_{combined}$ = 0.002) 과 정상안압녹내장군 ($P_{combined}$ = 0.02)에서 유의한 연관을 보였고 *ZNF677* 유전자는 고안압녹내장군에서 유의한 연관을 보였다 ($P_{combined}$ = 1.5E–06). 타인종에서 원발 개방각녹내장과 연관된 것으로 보고된 유전자 변이 중에서 *ATOH7* 유전자의 rs1900004 (OR = 1.29, P = 0.0024), *CDKN2B-AS1* 유전자의 rs1063192 (OR = 0.69, P = 0.0006), rs2157719 (OR = 0.63, P = 0.0007), rs7865618 (OR = 0.63, P = 0.0006) 그리고 *SIX1/SIX6* 유전자의 rs10483727 (OR = 0.68, P = 7.9E–05)가 원발 개방각녹내장과 유의한 연관을 보였다. 검증코호트에서는 *CDKN2B-AS1* 유전자의 rs2157719 (OR = 0.72, P = 0.0135), rs1063192 (OR = 0.63, P = 0.0007), rs7865618 (OR = 0.52, P = 0.0004)가 유의한 연관을 보였다. 전체 한국인 코호트 통합 분석에서 *CDKN2B-AS1* 유전자의 rs1063192 (OR = 0.77, P = 6.0E–05), rs2157719 (OR = 0.63, P = 0.0007), rs7865618 (OR = 0.58, P = 1.9E–06), 그리고 *SIX1/SIX6* 유전자의 rs10483727 (OR = 0.79, P = 9.4E–05)가 유의하게 원발 개방각녹내장과 연관되었다. 본 연구를 통해 원발 개방각녹내장, 특히 기저안압이 정상범위인 환자들의 유전적 병인에 대한 이해를 높일 것으로 판단된다.

주요어: 원발 개방각녹내장, 정상안압녹내장, 고안압녹내장, 엑솜칩 분석, 단일염기다형성

학번: 2018-30085