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Molecular genetic analysis of primary open-angle glaucoma, normal tension glaucoma, and developmental glaucoma for the *VAV2* and *VAV3* gene variants in Japanese subjects

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ABSTRACT

The *VAV2* and *VAV3* genes have been implicated as being causative for primary open angle glaucoma (POAG) in the Japanese. We studied 168 unrelated Japanese patients with primary open-angle glaucoma (POAG), 163 unrelated Japanese patients with normal tension glaucoma (NTG), 45 unrelated Japanese patients with developmental glaucoma (DG), and 180 ethnically matched normal controls, to determine whether variants in the vav 2 guanine nucleotide exchange factor (*VAV2*) and vav 3 guanine nucleotide exchange factor (*VAV3*) genes are associated with POAG, NTG, or DG in the Japanese. Genomic DNA was extracted from peripheral blood leukocytes, and variants in the *VAV2* and *VAV3* genes were amplified by polymerase chain reaction (PCR) and directly sequenced. Two variants were identified: rs2156323 in *VAV2* and rs2801219 in *VAV3*. The variants and the prevalence of POAG, NTG, and DG in unrelated Japanese patients indicated that the variants were not involved in the pathogenesis of POAG, NTG, or DG.

1. Introduction

Glaucoma is a complex, heterogeneous disease characterized by a progressive degeneration of the optic nerve axons, and it is the second highest cause of blindness, affecting approximately 70 million people worldwide [1]. Because the optic nerve axons degenerate in eyes with glaucoma, visual field defects develop. Primary open-angle glaucoma (POAG), the most common type of glaucoma, is associated with elevated intraocular pressure (IOP). Patients with POAG who have IOP in the normal range (<22 mmHg) are classified as having normal tension glaucoma (NTG) [2]. The prevalence of NTG is significantly higher among the Japanese than among Caucasians [3,4].

Although the precise molecular basis of POAG has not been established, it is most likely a genetically heterogeneous disorder caused by the interaction of multiple genes and environmental factors [5,6]. Several genetic loci that contribute to susceptibility to POAG have been identified. To date, at least 15 loci (GLC1A to GLC1O) have been linked to POAG, and three genes have been identified: the myocilin (*MYOC*) gene [7], the optineurin (*OPTN*) gene [8], and the WD-repeat domain 36 (*WDR36*) gene [9]. The *MYOC* gene encodes for myocilin and is mutated in juvenile-onset primary open-angle glaucoma. The optineurin (*OPTN*) gene is mutated in families with autosomal dominant, adult-onset POAG, including some with normal tension glaucoma. The *WDR36* gene is a relatively new causative gene for adult-onset POAG. However, several studies have reported that the *OPTN* and *WDR36* variants do not predispose subjects to POAG/NTG [10–15].

Recently, the genes for vav 2 guanine nucleotide exchange factor (*VAV2*) (OMIM 600428) and vav 3 guanine nucleotide exchange factor (*VAV3*) (OMIM 605541) were reported to cause POAG in the Japanese [10]. The authors provided functional evidence suggesting that *Vav2*- and *Vav2/Vav3*- deficient mice had a spontaneous glaucoma phenotype resulting in progressive iridocorneal changes and elevated IOPs. In addition, a genome-wide association study (GWAS) that screened for glaucoma susceptibility loci using single nucleotide polymorphism (SNP) analysis identified intronic SNPs in *VAV2* (rs2156323) and *VAV3* (rs2801219) as candidates for genes associated with POAG in Japanese glaucoma patients.

An accurate diagnostic test for pre-symptomatic individuals at risk for glaucoma is needed, and screening for the *VAV2* and *VAV3* genes may identify pre-symptomatic cases in the general population. Thus, the purpose of this study was to determine

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whether variants in the *VAV2* and *VAV3* genes contribute to POAG, NTG, and developmental glaucoma (DG) in Japanese patients.

2. Patients and methods

2.1. Patients

One hundred and sixty eight unrelated Japanese patients with POAG (89 men and 79 women; mean age 63.6 ± 14.4 years), 163 unrelated Japanese patients with NTG (86 men and 77 women; mean age 61.8 ± 13.7 years), and 45 unrelated Japanese patients with DG (18 men and 27 women; mean age 30.7 ± 10.7 years), who were diagnosed in the ophthalmology clinic at the Tohoku University Hospital, Sendai, Japan, were studied. The percentages of patients from each of the different regions of Japan were as follows: 70% of the patients were from the northern region, 20% were from the eastern region, and <10% were from the western region of Japan.

The purpose and procedures of the experiment were explained to all the patients, and their informed consent was obtained. The procedures used conformed to the tenets of the Declaration of Helsinki, and the Tohoku University Institutional Review Board approved this study.

Routine ophthalmic examinations were performed on all the patients. The criteria for classifying a patient as having POAG were the following: (1) applanation IOP >22 mmHg in each eye; (2) glaucomatous cupping in each eye, including a cup-to-disc ratio >0.7; (3) visual field defects, determined by Goldmann and/or Humphrey perimetry, that are consistent with glaucomatous cupping in at least one eye; and (4) an open anterior chamber angle. The criteria for NTG were the following: (1) applanation IOP less than 22 mmHg in both eyes at each examination; and (2) the same characteristics as the POAG group. Patients with glaucoma due to secondary causes, e.g., trauma, uveitis, or steroid use, were excluded.

Control subjects (95 men and 85 women; mean age 68.0 ± 7.7 years) were characterized by the following characteristics: (1) IOP <22 mmHg; (2) normal optic discs; and (3) no family history of glaucoma. To decrease the chance of including individuals with pre-symptomatic glaucoma in this group, we studied individuals who were older than 60.

2.2. Sample preparation and variant screening

Genomic DNA was extracted from peripheral blood leukocytes and purified using the Qiagen QIAamp Blood Kit (Qiagen, USA). The SNPs rs2156323 (*VAV2*) and rs2801219 (*VAV3*) and their flanking regions were amplified by polymerase chain reaction (PCR) using 0.5 μM intronic primers in an amplification mixture (25 μl) containing 0.2 mM dNTPs and 0.5 U Ex Taq polymerase (Takara), with the addition of 30 ng of template DNA at an annealing temperature of 60° C. The oligonucleotides for amplification and sequencing were selected using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/, Massachusetts Institute of Technology, Cambridge, MA).

The PCR fragments were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA) and were sequenced with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, California, USA) on an automated DNA sequencer (ABI PRISM™ 3100 Genetic Analyzer, Perkin-Elmer).

2.3. Statistical analyses

Differences in the genotype frequencies among the cases and controls were tested using Fisher's exact test, depending on the cell counts. Odds ratios (approximating relative risk) were calculated to measure the association between the WDR36 genotype and the POAG/NTG phenotype, and the effects of the mutant allele were assumed to be dominant (wild/wild vs. wild/mutant and mutant/mutant combined). For each odds ratio, a *P* value and the 95% confidence intervals were calculated. The inferred haplotypes and LD, expressed as *D'* [11] and quantified between all pairs of biallelic loci, were estimated using the SNPAlyze program version 4.0 (Dynacom, Yokohama, Japan). The significance of the associations was determined by contingency table analysis using the chi-square test or Fisher's exact test. The Hardy–Weinberg equilibrium was analyzed using the gene frequencies obtained by simple gene counting and the chi-square test with Yates' correction for comparing observed and expected values. For general stand-alone statistical power analysis, we used G*Power [12]. G*Power computes the power values for given sample sizes, effect sizes, and alpha levels (post hoc power analyses), and the sample sizes for given effect sizes, alpha levels, and power values (a priori power analyses).

3. Results

3.1. Allelic frequencies for rs2156323 SNP in VAV2 and rs2801219 SNP in VAV3

Two variants were identified: rs2156323 in *VAV2* and rs2801219 in *VAV3*. The allelic frequencies for rs2156323 in *VAV2* and rs2801219 in *VAV3* for POAG, NTG, DG, and the control subjects are presented in Table 1. The allele frequencies of rs2156323 in *VAV2* in the POAG, the NTG, and the DG groups were not significantly different from the control group (minor allele frequency 0.051, 0.049, 0.022 vs. 0.036, respectively; $P = 0.35$, 0.40, and 0.51, respectively). The allele frequency of rs2801219 in *VAV3* was also not significantly higher in the two groups than in the control group (minor allele frequency 0.211, 0.236, 0.244 vs. 0.197, respectively; $P = 0.64$, 0.22 and 0.32, respectively). The SNP adhered to the Hardy–Weinberg expectations ($P > 0.05$).

3.2. Genotype frequencies for rs2156323 SNP in VAV2 and rs2801219 SNP in VAV3

The genotype frequencies for rs2156323 in *VAV2* and rs2801219 in *VAV3* are listed for the POAG, NTG, DG, and control subjects in Table 2. For rs2156323 in *VAV2*, the genotype frequency was not statistically higher in the POAG ($P = 0.25$), the NTG ($P = 0.29$), and the DG ($P = 0.62$) groups than in the control group (Table 2). For rs2801219 in *VAV3*, the genotype frequency was not statistically higher in the POAG ($P = 0.90$), the NTG ($P = 0.07$), and the DG

Table 1
VAV2 and *VAV3* SNPs allele frequencies in patients with POAG, NTG and in controls in Japanese.

SNP	VAV2 (rs2156323 A/G)		*P*-value
Allele	G	A	
POAG ($n = 168$)	0.949	0.051	0.35
NTG ($n = 163$)	0.951	0.049	0.40
DG ($n = 45$)	0.978	0.022	0.51
Control ($n = 180$)	0.964	0.036	
	VAV3 (rs2801219 A/C)		
	A	C	
POAG ($n = 168$)	0.789	0.211	0.64
NTG ($n = 163$)	0.764	0.236	0.22
DG ($n = 45$)	0.756	0.244	0.32
Control ($n = 180$)	0.803	0.197	

The significance of the association was determined by a contingency table analysis using the χ^2 test.

Table 2
Frequency of genotypes *VAV2* and *VAV3* gene in patients with POAG, NTG and in controls in Japanese.

	POAG (n = 168)	NTG (n = 163)	DG (n = 45)	Control (n = 180)
VAV2 (rs2156323 A/G)				
G/G	151 (89.9%)	147 (90.2%)	43 (95.6%)	168 (93.3%)
G/A	17 (10.1%)	16 (9.8%)	2 (4.4%)	11 (6.1%)
A/A	0 (0%)	0 (0%)	0 (0%)	1 (0.6%)
P value*	0.25	0.29	0.80	
VAV3 (rs2801219 A/C)				
A/A	108 (64.3%)	92 (56.4%)	25 (55.6%)	119 (66.1%)
A/C	49 (29.2%)	65 (39.9%)	18 (40.0%)	51 (28.3%)
C/C	11 (6.5%)	6 (3.7%)	2 (4.4%)	10 (5.6%)
P value*	0.90	0.07	0.32	

Data presented are number of patients, unless otherwise indicated. The asterisk indicates that the significance of the association was determined by a contingency table analysis using the χ^2 test.

(P = 0.32) groups than in the control group (Table 2). The SNP adhered to the Hardy–Weinberg expectations (P > 0.05).

3.3. Dominant and recessive model for rs2156323 SNP in VAV2 and rs2801219 SNP in VAV3

The homozygotes for the rs2156323 SNP A/A were 0% in the glaucoma subjects, and 0.6% in the control subjects (P > 0.05; Table 2). We analyzed the dominant and recessive model for the rs2801219 SNP in *VAV3* (Table 3). There was also no significant difference between the subgroups of glaucoma and SNP rs2801219 in *VAV3*. However, in the NTG group, P was 0.06 for the dominant model.

4. Discussion

Obtaining evidence that candidate genes and gene variants are significantly associated with a specific disease is more biologically meaningful when the same associations are also found in different ethnic populations. The significant associations would then indicate that these genes play a role in the pathogenesis of the disease [13]. Our findings showed that the alleles rs2156323 (*VAV2*) and rs2801219 (*VAV3*) were not significantly associated with POAG in Japanese patients. These risk alleles were also not significantly associated with POAG or primary angle closure glaucoma (PACG) in Indian cohorts [13]. It has also been reported that the genotype frequencies at these loci were not significantly different among POAG, PACG, and control subjects in Indian cohorts [14].

The finding that *Vav2*-deficiency alone resulted in a glaucoma phenotype in mice suggested that the absence of *Vav2* is associated with the development of glaucoma in mice. However, our findings demonstrated that there was no significant association between the *VAV2* SNP and POAG, NTG, and DG. In addition, the *VAV2* SNP rs2156323 was not associated with these glaucoma phenotypes. Functionally, the *Vav2/Vav3*-deficient ($Vav2^{-/-}Vav3^{-/-}$) mice had buphthalmos and iridocorneal changes that altered the aqueous outflow that lead to elevated intraocular pressure. The optic nerve head cupping resembled that found in developmental glaucoma and PACG. Thus, we hypothesized that *VAV2* and *VAV3* could be major candidate genes for developmental glaucoma in humans. However, our results showed that DG, POAG and NTG were not significantly associated with alleles rs2156323 (*VAV2*) and rs2801219 (*VAV3*) (Tables 1 and 2). We also compared the DG group with an age-matched young control group (n = 60; 30.4 ± 6.2 years). Neither the frequencies of the A allele of VAV2 rs2156323 (DG 0.022 and young controls 0.075; P = 0.09, chi-square test), nor the C allele of rs2801219 (DG 0.244 and young controls 0.175; P = 0.22, chi-square test) was significantly different from young controls (data not shown).

There is a possibility that the lack of significant associations at these loci in our POAG cases could have been due to clinical heterogeneity.

Another possibility for the lack of significant associations is the sample size because small sample sizes are known to cause a type II error. There were 45 DG cases and 180 controls for a total of 225 subjects. For an effect size = 0.3 (medium), an α error probability of 0.05, and a degree of freedom (Df) = 2, the power (1 – beta err probability) is 98.6%. However, for an effect size = 0.1 (small), an α error probability of 0.05, and a Df = 2, the power is only 24.9%. To obtain a power of 80% under the same conditions, the total sample size must be 964 cases.

The Vav family of proteins consists of a group of signal transduction molecules with oncogenic potential that play important roles in development and cell signaling. The best known function of the Vav proteins is their role as GDP/GTP exchange factors that activate Rho guanosine triphosphatases (GTPases) in a phosphorylation-dependent manner [15]. In addition to their function as exchange factors, the evidence increasingly suggests that Vav proteins can mediate other cellular functions, most likely as adaptor molecules. Deregulation of the GDP/GTP exchange is one possible mechanism for the alterations that lead to iridocorneal angle closure. Thus, we suggest that *VAV2* and *VAV3* may still be candidate genes for PACG, and the association between Vav2/Vav3 and PACG deserves further study.

In summary, the variants rs2156323 in the *VAV2* gene and rs2801219 in the *VAV3* gene do not appear to be major risk factors for the pathogenesis of glaucoma in the Japanese. However, Vav2/

Table 3
Frequency of genotypes in dominant or recessive model in *VAV3* gene in patients with POAG, NTG and in controls in Japanese.

VAV3 (rs2801219 A/C)		POAG (n = 168)	NTG (n = 163)	DG (n = 45)	Control (n = 180)
Dominant	A/A	108 (64.3%)	92 (56.4%)	25 (55.6%)	119 (66.1%)
	A/C + C/C	60 (35.7%)	71 (43.6%)	20 (44.4%)	61 (33.9%)
P value*		0.72	0.06	0.19	
Recessive	A/A + A/C	157(93.5%)	157 (96.3%)	43 (95.6%)	170 (94.4%)
	C/C	11(6.5%)	6 (3.7%)	2(4.4%)	10(5.6%)
P value*		0.70	0.41	0.77	

Data presented are number of patients, unless otherwise indicated. The asterisk indicates that the significance of the association was determined by a contingency table analysis using the χ^2 test.

Vav3-deficient mice can still serve as useful models for the study of spontaneous glaucoma, and investigations into the development of the phenotype may provide information on the pathogenesis of glaucoma in humans.

Disclosure

D. Shi, None; Y. Takano, None; M.T. Nakazawa, None; Mengkegale, None; S. Yokokura; None, K. Nishida, None; N. Fuse, None.

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Association of *HK2* and *NCK2* with Normal Tension Glaucoma in the Japanese Population

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Abstract

Although family studies and genome-wide association studies have shown that genetic factors play a role in glaucoma, it has been difficult to identify the specific genetic variants involved. We tested 669 single nucleotide polymorphisms (SNPs) from the region of chromosome 2 that includes the GLC1B glaucoma locus for association with primary open-angle glaucoma (POAG) and normal tension glaucoma (NTG) in the Japanese population. We performed a two-stage case-control study. The first cohort consisted of 123 POAG cases, 121 NTG cases and 120 controls: the second cohort consisted of 187 POAG cases, 286 NTG cases, and 271 controls. Out of six SNPs showing significant association with POAG in the first round screening, seven SNPs were tested in the second round. Rs678350 in the *HK2* gene coding sequence showed significant allelic ($p = 0.0027$ in Stage Two, 2.7XE-4 in meta-analysis) association with POAG, and significant allelic ($p = 4.7$XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association with NTG. Although alleles in the *TMEM182* gene did not show significant association with glaucoma in the second round, subjects with the A/A allele in *TMEM182* rs869833 showed worse visual field mean deviation ($p = 0.01$). Even though rs2033008 in the *NCK2* gene coding sequence did not show significant association in the first round, it had previously shown association with NTG so it was tested for association with NTG in round 2 ($p = 0.0053$ in Stage Two). Immunohistochemistry showed that both *HK2* and *NCK2* are expressed in the retinal ganglion cell layer. Once multi-testing was taken into account, only *HK2* showed significant association with POAG and NTG in Stage Two. Our data also support previous reports of *NCK2* association with NTG, and raise questions about what role *TMEM182* might play in phenotypic variability. Our data suggest that *HK2* may play an important role in NTG in the Japanese population.

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Introduction

Glaucoma is a complex, heterogeneous disease characterized by a progressive degeneration of the optic nerve fibers, and is the second highest cause of blindness worldwide affecting approximately 70 million people [1]. The most common type of open-angle glaucoma, primary open-angle glaucoma (POAG), is associated with elevated intraocular pressure (IOP) [2], and another less-common subgroup of open-angle glaucoma, called low-tension glaucoma (LTG) [3,4] or normal tension glaucoma (NTG) is associated with IOP that does not rise outside of the normal range [5]. The prevalence of NTG is reported to be higher among the Japanese than among Caucasians [6,7]. This is an important medical and public health problem because simple screening programs based on detection of elevated IOP are not effective in a population where NTG is highly prevalent. Thus, an accurate diagnostic test for presymptomatic detection of individuals at risk for glaucoma, especially NTG in Japan, is urgently needed.

Open-angle glaucoma is a genetically heterogeneous disorder attributed to the interaction of multiple genes and environmental factors [8,9]. More than 15 POAG loci have been identified by linkage, and five open-angle glaucoma genes located within those loci have been identified [10,11]. More recently, genome-wide association studies (GWAS) using high-density single nucleotide polymorphism (SNP) arrays have been used to identify genetic risk factors involved in the common, complex forms of open-angle glaucoma that do not show classical Mendelian inheritance

patterns. Burdon et al. identified susceptibility loci at TMCO1 and CDKN2B-AS1 that contribute to severe forms of glaucoma [12]. Ramdas et al. used meta-analysis of data from six separate studies to find significant evidence that three common variants of CDKN2B, ATOH7 and SIX1 are associated with POAG [13]. Wiggs et al. found significant evidence that genetic variants in CDKN2B-AS1 and a gene desert on 8q22 are associated with optic nerve damage in glaucoma [14].

Based on a linkage study involving 6 Caucasian families in the UK, the *GLC1B* locus for adult-onset open-angle glaucoma was identified at chromosome 2cen–q13 [15]. The patients in these families had clinical characteristics of low to moderate IOP, disease onset in their late 40 s, and a good response to medical therapy, and those phenotypes mimic the majority of Japanese NTG cases. Thus, the screening of the gene around GLC1B locus may be useful for diagnosis of POAG and NTG in the general Japanese population.

The purpose of this study was to screen for candidate genes for POAG and NTG on chromosome 2, around the GLC1B (glaucoma 1, open angle, B) locus in unrelated Japanese patients, using high density SNP scanning and case-control association. Here we report one gene that shows significant association with POAG and NTG, support for a previously reported association with NTG, and a gene for which genotype is predictive of severity of mean deviation on the visual field test.

Results

A Two-stage Case-control Study of SNPs on Chromosome2

To identify a gene associated with glaucoma we did a high-density scan of the region around GLC1B on chromosome 2 by screening 669 SNPs on chromosome 2 in a two-stage case-control study design (Figure 1). We were especially interested in whether any SNPs that fall within genes in the GLC1B region might be associated with POAG or NTG (Table 1). Among genes from this region we found fourteen SNPs that show significant evidence of association with POAG, and nine SNPs that show significant evidence of association with NTG. Four of the SNP alleles which show significant evidence of association are identical between the POAG and NTG subjects.

We identified six SNPs in Stage One that showed evidence of association with POAG (rs1239066, rs1529385, rs869833, rs960011, rs1027003) and two SNPs (rs678350, rs2033008) that showed association with NTG (Figure 2 and Table 2). The SNPs rs869833 and rs960011 are located within the *TMEM182* gene, which contains 5 exons and 229 amino acids. The amino acid sequence of *TMEM182* predicts an evolutionarily-conserved novel transmembrane protein, which consists of four putative membrane-spanning regions indicative of an integral membrane topology. The SNP rs678350 is located within the *Hexokinase 2 (HK2)* gene which contains 18 exons and 917 amino acids. The SNP rs678350 exists on intron1 of *HK2* gene. The *HK2* gene produces a protein product localizes on the outer membrane of mitochondria and plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. There are no known genes closely neighboring the SNP rs1239066 and rs1027003. We have checked the SNPs near rs1529385 within LOC129293 and TMSB10 and did not find get any positive polymorphisms.

We selected three genes for second stage mutation screening. *TMEM182* and *HK2* genes were selected because they contain SNPs that showed significant evidence of association in Stage One in this study. Even though SNPs in the *NCK2* gene showed significant evidence of association (p = 0.014), the *NCK2* gene was selected for second stage mutation screening based of the previous report that it is associated with NTG [16].

The SNP rs1239066 showed significant evidence of association in both Stage One and Stage Two screenings. In the meta-analysis it showed significant evidence of association (POAG, NTG; P = 0.001, 0.005), as did rs1027003 (POAG; P = 0,010) (Table 2).

HK2 Variants Detected in this Study

The SNP rs678350 in the *HK2* gene coding sequence showed significant allelic (p = 0.0027 in Stage Two, 2.7XE-4 in meta-analysis) association with POAG, and significant allelic (p = 4.7XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association

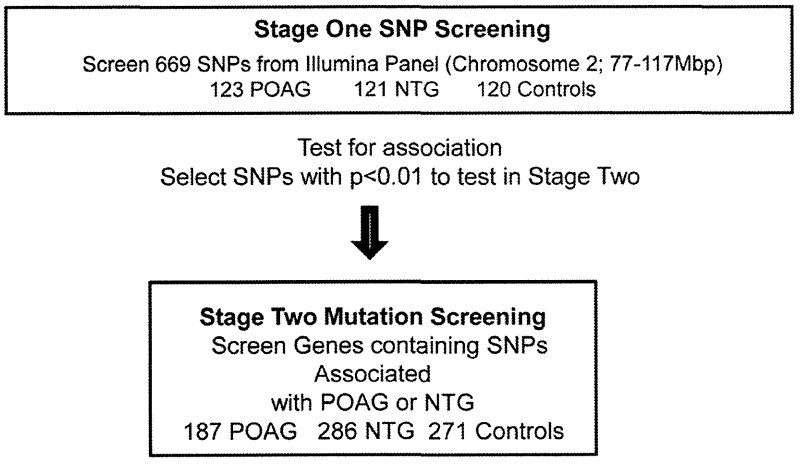

Figure 1. Experimental Study Design. The study used a first stage of SNP screening in one population to identify SNPs and genes to be tested in a second population through SNP association testing and mutation screening of genes containing SNPs associated with glaucoma. Stage Two tested SNPs for association in a second population and did mutation screening in that second population in genes containing SNPs that showed significant association with POAG or NTG (p<0.01) in Stage One of our study or that had been previously reported to show significant evidence of association [16].
doi:10.1371/journal.pone.0054115.g001

Table 1. Stage one Test of SNPs in GLC1B-Region Genes for Association with POAG or NTG.

Genomic Information			POAG			NTG			Control
rs number	Location	Gene Symbol	MAF	Odds ratio (CI)	p value*	MAF	Odds ratio (CI)	p value*	MAF
rs741788	2p13	*DCTN1*	0.455	1.47 (1.02–2.12)	0.038	0.455	1.47 (1.02–2.11)	0.040	0.363
rs909177		*DCTN1*	0.455	1.44 (1.00–2.08)	0.047	0.463	1.49 (1.03–2.14)	0.032	0.367
rs740277		*DCTN1*	0.455	1.44 (1.00–2.08)	0.047	0.463	1.49 (1.03–2.14)	0.032	0.367
rs678350	2p13	*HK2*	0.333	1.50 (1.01–2.23)	0.043	0.371	1.77 (1.19–2.62)	0.004	0.250
rs651071		*HK2*	0.199	0.60 (0.40–0.92)	0.018	0.256	0.84 (0.56–1.25)	0.383	0.292
rs1807090		*HK2*	0.268	1.59 (1.03–2.44)	0.034	0.238	1.35 (0.87–2.10)	0.181	0.188
rs1239066	2p12		0.293	3.04 (1.95–4.72)	0.009	0.269	1.55 (1.01–2.38)	0.045	0.192
rs53915	2p12-p11.1	*CTNNA2*	0.199	0.64 (0.42–0.98)	0.039	0.260	0.91 (0.61–1.36)	0.641	0.279
rs1529385	2p11.2	*LOC129293 (C2orf89)*	0.053	0.39 (0.20–0.77)	0.005	0.087	0.66 (0.37–1.20)	0.173	0.125
rs1053561	2p11.2	*TGOLN2*	0.114	0.53 (0.32–0.88)	0.012	0.178	0.89 (0.56–1.40)	0.609	0.196
rs1562322	2p11.2	*LOC51255 (RNF181)*	0.321	0.88 (0.61–1.29)	0.520	0.256	0.64 (0.43–0.95)	0.027	0.349
rs3024831	2p12-p11.2	*SFTPB*	0.250	0.92 (0.61–1.38)	0.675	0.190	0.65 (0.42–0.99)	0.045	0.267
rs6875	2q11.2	*RW1 (TMEM131)*	0.008	0.21 (0.05–0.98)	0.030	0.025	0.66 (0.23–1.88)	0.431	0.038
rs1982336		*RW1 (TMEM131)*	0.008	0.23 (0.05–1.05)	0.027	0.029	0.75 (0.28–2.05)	0.576	0.038
rs718159		*RW1 (TMEM131)*	0.008	0.21 (0.05–0.98)	0.028	0.029	0.76 (0.28–2.07)	0.587	0.038
rs222	2q11.2	*INPP4A*	0.183	0.69 (0.44–1.06)	0.091	0.161	0.59 (0.38–0.93)	0.021	0.246
rs1530028	2q11.2	*FLJ45273 (LONRF2)*	0.228	0.66 (0.44–0.99)	0.045	0.306	0.99 (0.67–1.46)	0.952	0.308
rs1030902	2q11.2	*ALS2*	0.225	0.65 (0.44–0.98)	0.039	0.314	1.03 (0.70–1.51)	0.892	0.308
rs1369482	2q11.2	*NPAS2*	0.244	0.65 (0.43–0.96)	0.030	0.298	0.85 (0.58–1.24)	0.398	0.333
rs871656	2q12	*IL1R1*	0.337	0.70 (0.49–1.01)	0.058	0.322	0.66 (0.45–0.95)	0.025	0.421
rs878539	2q11.2	*SLC9A2 (NHE2)*	0.463	1.52 (1.06–2.18)	0.024	0.422	1.28 (0.89–1.85)	0.185	0.363
rs869833	2q12.1	*TMEM182*	0.467	1.66 (1.15–2.40)	0.006	0.376	1.14 (0.79–1.65)	0.490	0.346
rs960011		*TMEM182*	0.415	0.57 (0.40–0.82)	0.001	0.512	0.85 (0.60–1.21)	0.232	0.554**
rs2033008	2q12	*NCK2*	0.293	0.76 (0.51–1.11)	0.147	0.252	0.62 (0.42–0.91)	0.015	0.354
rs1027003	2q12		0.110	3.16 (1.46–6.88)	0.002	0.058	1.58 (0.67–3.71)	0.295	0.038
rs1474220	2q12.3	*GCC2*	0.106	0.59 (0.35–1.00)	0.050	0.136	0.79 (0.48–1.30)	0.353	0.167
rs899259	2q13	*EDAR*	0.098	0.66 (0.38–1.14)	0.134	0.075	0.49 (0.28–0.90)	0.019	0.142
rs1509414	2q13	*BENE(MALL)*	0.037	0.40 (0.18–0.88)	0.020	0.041	0.45 (0.21–0.98)	0.039	0.096
rs1567366	2q13	*NPHP1*	0.561***	1.34 (0.94–1.92)	0.105	0.576***	1.44 (1.01–2.07)	0.045	0.488
rs2119112	2q14.2	*MARCO*	0.110	0.57 (0.34–0.95)	0.029	0.107	0.55 (0.33–0.93)	0.025	0.179

*chi-square test.
**minor allele frequency in stage 2 control was 0.494.
***minor allele in control was major allele in POAG and NTG subjects.
MAF; minor allele frequency, CI; confidence interval.
doi:10.1371/journal.pone.0054115.t001

with NTG (Table 2). The rs678350 showed also a significant difference in genotype frequency (p = 0.0046 and 0.0039) in the POAG and NTG groups (Table 3). In the second round, we screened the *HK2* coding sequence and intron-exon boundaries for mutations in POAG and NTG patients. After direct sequencing, we found 2 coding SNPs; p.Gln142His (A/T at the third nucleotide; rs2229621) in exon 4 and p.Arg844Lys (G/A at the second nucleotide; rs2229629) in exon 17. The allelic frequency of the p.Gln142His (A/T) variant was significantly higher in the NTG group than in the control group (p = 0.025), but it was not higher in the POAG group than in the control group (p = 0.181). The genotype frequency of the p.Gln142His (A/T) variant (dominant model) was significantly higher in the NTG group than in the control group (p = 0.019) but we did not find evidence that the frequency in the POAG group was different from the control group (p = 0.179). There was no evidence of a significant difference between POAG and NTG for p.Arg844Lys. No other mutation was found. We tested the LD block and found no linkage disequilibrium between SNPs rs678350 and rs2229621 (D′ = 0.08). We tested the correlation between the phenotypes POAG or NTG and the genotypes screened in the second stage screening of HK2, and found no association with any phenotypes including age at diagnosis, maximum IOP under medication, and MD value of the visual field (Table 4). None of the polymorphisms showed deviation from Hardy–Weinberg equilibrium ($P<0.05$).

NCK2 Variants Detected in this Study

The SNP rs2033008 in the *NCK2* gene showed a significant difference in allelic frequency (p = 0.015 in Stage One, p = 0.0053 in Stage Two, and 2.2XE-4 in meta-analysis) between controls and NTG, but not between control and POAG status (p = 0.147 in Stage One, 0.35 in Stage Two, 0.12 in meta-analysis) (Table 2 and

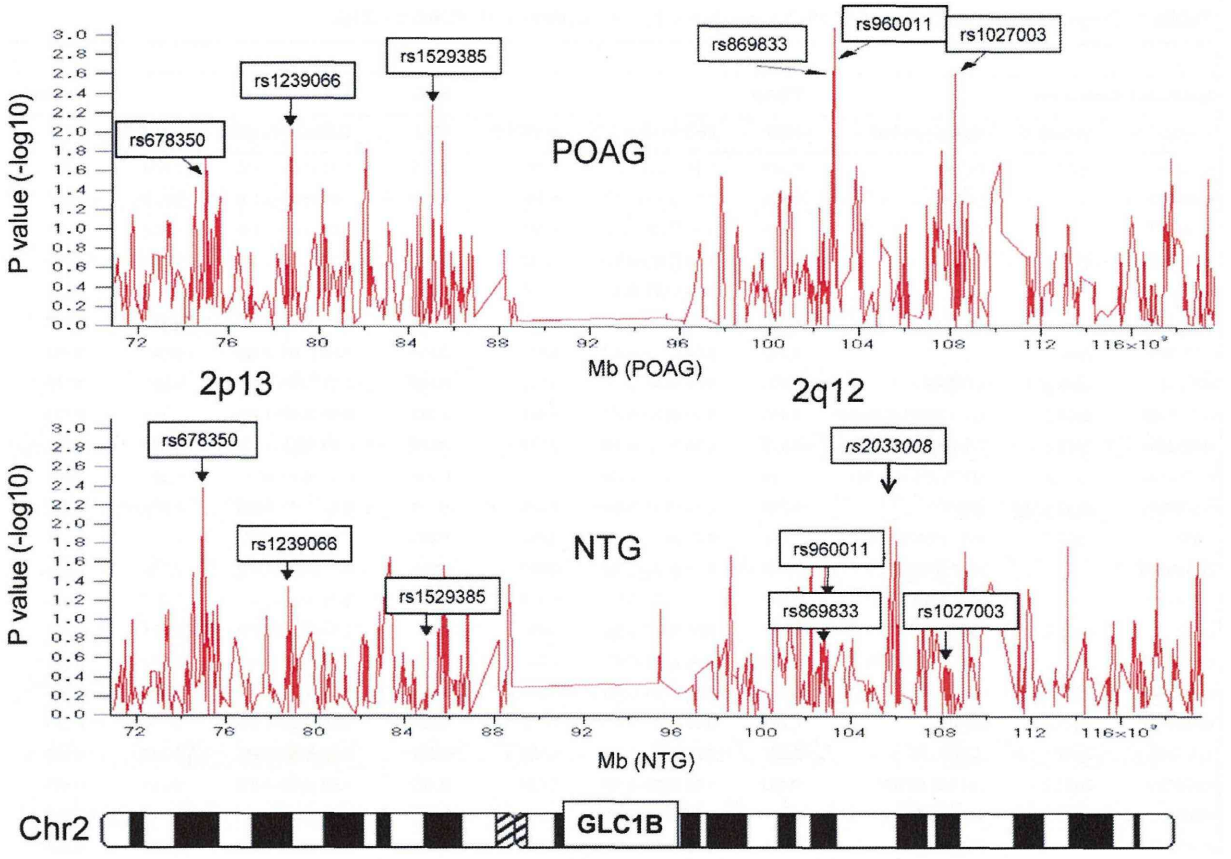

Figure 2. High-density scan of the GLC1B region on chromosome 2 to identify candidate glaucoma genes. Six SNPs that showed significant evidence of association with POAG or NTG ($p<0.01$) and the previously-reported candidate *NCK2* gene are shown. Vertical line shows p value (−log10), and horizontal line shows chromosomal location (kb).
doi:10.1371/journal.pone.0054115.g002

Table 5). The odds ratio for association with NTG supports a model in which *NCK2* is associated with NTG; OR = 0.69 (0.53–0.89), but the odds ratio for association with POAG was not significant; 0.87 (0.65–1.16) in Stage Two. Only this rs2033008 polymorphism in Stage One showed deviation from Hardy–Weinberg equilibrium ($p = 0.030$).

We screened the sequence of the *NCK2* coding sequence and intron-exon boundaries for mutations in POAG and NTG patients and found 1 synonymous coding base change: Thr14Thr (ACC >ACT) in one NTG subject. Although SNP rs2033008 showed significant association but the Thr14Thr variant showed no statistical difference in allele frequency between NTG and normal subjects ($p = 0.33$). The Thr14Thr heterozygotes (A/A) in the NTG subjects have the worse Mean Deviation value of the visual field compared with human reference sequence (T/T) ($p = 0.05$) (Table 6).

The single NTG subject with the Thr14Thr variant was a 50 year old woman whose father also had NTG. Her age at diagnosis was 40 years old. Her initial IOP was 15 mmHg in each eye. The mean deviation (MD) of the visual field test was −3.87 dB in the right eye, and −1.97 dB in the left eye, reflecting a mild NTG phenotype.

TMEM182 Variants Detected in this Study

We found no mutations in *TMEM182* coding sequence and intron-exon boundaries, for mutations in POAG and NTG patients. After finding association for SNPs rs869833 and rs960011 in the POAG and NTG subjects in Stage One, we did not find this association confirmed in Stage Two SNP testing (Table 7). When we tested for correlation between Stage Two case endophenotypes and TMEM182 genotype, we found association with the MD value of the visual field in POAG subjects. The POAG subjects homozygous for the A/A allele of SNP rs869833 have worse Mean Deviation value of the visual field test compared with those who carry the G/G genotype in Stage Two subjects ($p = 0.01$) (Table 8).

Immunohistochemistory of the *HK2* and *NCK2*

Representative immunohistochemistry (IHC) photographs with *Hk2*, *Nck2*, astrocyte marker (GFAP) and retinal ganglion cell marker (C38) on the retinas of untreated mice were shown (Fig. 3, A, B). *Hk2* and *Nck2* were strongly immunoreactive in the ganglion cell layer. C38 signals co-localized with *Hk2* or *Nck2* in the ganglion cell layer, as indicated by arrows (Fig. 3 A, B). *Hk2* expression is only located in the ganglion cell layer. *Nck* is expressed in the ganglion cell layer, inner nuclear layer, and outer plexiform layer, with the highest level of expression in the ganglion cell layer.

Discussion

HK2 in POAG and NTG subjects

The rs678350 in the *HK2* gene coding sequence showed significant allelic ($p = 0.043$ in Stage One, $p = 0.0027$ in Stage

Table 2. Stage One and Stage Two Association Test Results.

Stage One Screening

		POAG			NTG			CNTL
SNP	Minor Allele	MAF	Odds ratio	p value*	MAF	Odds ratio	p value*	MAF
rs1239066	C	0.293	3.04 (1.95–4.72)	0.009	0.269	1.55 (1.01–2.38)	0.045	0.192
rs1529385	T	0.053	0.39 (0.20–0.77)	0.005	0.087	0.66 (0.37–1.20)	0.173	0.125
rs869833	G	0.467	1.66 (1.15–2.40)	0.006	0.376	1.14 (0.79–1.65)	0.490	0.346
rs960011	T	0.415	0.57 (0.40–0.82)	0.001	0.512	0.85 (0.60–1.21)	0.232	0.554
rs1027003	G	0.110	3.16 (1.46–6.88)	0.002	0.058	1.58 (0.67–3.71)	0.295	0.038
rs678350	G	0.333	1.50 (1.01–2.23)	0.043	0.371	1.77 (1.19–2.62)	0.004	0.250
rs2033008	A	0.293	0.76 (0.51–1.11)	0.147	0.252	0.62 (0.42–0.91)	0.015	0.354

Stage Two Screening

		POAG			NTG			CNTL
SNP	Minor Allele	MAF	Odds ratio	p value*	MAF	Odds ratio	p value*	MAF
rs1239066	C	0.246	1.70 (1.09–2.65)	0.019	0.240	1.64 (1.06–2.51)	0.024	0.161
rs1529385	T	0.122	1.42 (0.73–2.77)	0.305	0.072	0.79 (0.37–1.68)	0.543	0.089
rs869833	G	0.409	0.98 (0.75–1.28)	0.855	0.421	1.03 (0.81–1.30)	0.834	0.415
rs960011	T	0.497	1.13 (0.86–1.47)	0.363	0.481	1.06 (0.84–1.34)	0.641	0.467
rs1027003	G	0.049	1.10 (0.42–2.90)	0.840	0.047	1.04 (0.30–2.83)	0.944	0.045
rs678350	G	0.334	1.58 (1.18–2.11)	0.0027	0.337	1.60 (1.23–2.08)	4.7XE-4	0.242
rs2033008	A	0.297	0.87 (0.65–1.16)	0.348	0.250	0.69 (0.53–0.89)	0.0053	0.327

Meta-analysis

		POAG			NTG			CNTL
SNP	Minor Allele	MAF	Odds ratio	p value*	MAF	Odds ratio	p value*	MAF
rs1239066	C	0.264	1.65 (1.22–2.23)	0.001	0.248	1.52 (1.14–2.04)	0.005	0.178
rs1529385	T	0.082	0.73 (0.46–1.16)	0.20	0.081	0.71 (0.45–1.14)	0.16	0.109
rs869833	G	0.432	1.17 (0.95–1.45)	0.16	0.408	1.06 (0.87–1.30)	0.54	0.393
rs960011	T	0.465	0.89 (0.72–1.10)	0.28	0.490	0.99 (0.81–1.20)	0.92	0.494
rs1027003	G	0.084	2.17 (1.20–3.92)	0.010	0.053	1.32 (0.69–2.53)	0.42	0.041
rs678350	G	0.334	1.56 (1.23–1.96)	2.7XE-4	0.347	1.65 (1.32–2.05)	1.0XE-5	0.244
rs2033008	A	0.295	0.83 (0.66–1.04)	0.12	0.251	0.66 (0.53–0.82)	2.2XE-4	0.335

MAF; Minor allele frequency, CNTL; Control.
*Fisher's exact test.
doi:10.1371/journal.pone.0054115.t002

Table 3. Stage Two *HK2* SNPs Allele Frequencies in Japanese POAG, NTG and Control Subjects.

	Allele frequency				Genotype			
rs678350	**A**	**G**	**Odds ratio (CI)**	**p value***	**A/A**	**A/G**	**G/G**	**p value***
POAG	0.666	0.334	1.58 (1.18–2.11)	0.0027	82/187	85/187	20/187	0.0046
NTG	0.663	0.337	1.60 (1.23–2.08)	4.7XE-4	133/286	113/286	40/286	0.0039
Control	0.758	0.242			161/271	89/271	21/271	
	Allele frequency				**Genotype**			
rs2229621/Q142H	**A**	**T**	**Odds ratio (CI)**	**p value***	**A/A**	**A/T**	**T/T**	**p value***
POAG	0.738	0.262	1.24 (0.92–1.69)	0.181	102/187	72/187	13/187	0.179
NTG	0.719	0.281	1.36 (1.04–1.79)	0.025	146/286	119/286	21/286	0.019
Control	0.777	0.223			165/271	91/271	15/271	

*Fisher's exact test; dominant model.
G/G or T/T is mutant homozygote, A/G or A/T is heterozygote, and A/A is wild homozygote.
doi:10.1371/journal.pone.0054115.t003

Two, 2.7XE-4 in meta-analysis association with POAG, and significant allelic (p = 0.004 in Stage One, p = 4.7XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association with NTG (Table 2). The rs678350 polymorphism showed a significant case-control difference in genotype frequency ($p = 0.0046$ and 0.0039) in the POAG and NTG groups (Table 3). However, there was no association of this SNP with glaucoma endophenotypes including age at diagnosis, maximum intra ocular pressure under medication, and MD value of the visual field. So the *HK2* gene may contribute to disease susceptibility to POAG and NTG, but may not account for all of the phenotypic variability between individuals whose glaucoma results from variants in this gene. Our association findings suggest that the *HK2* gene that contains this polymorphism might play a role in POAG and NTG in the Japanese population, but it remains to be seen whether rs678350 is actually causative, perhaps through altering transcription or splicing, or whether another allele(s) in this gene or its regulatory region might actually be causing the disease. There remains a possibility that the p.Gln142His (A/T) SNP in *HK2* may play a role in disease pathology, but our study can only show association, not causation. Because none of our subjects come from the original families used to map the GLC1B locus, we can only draw conclusions regarding the possible role of this gene in the Japanese population, but this finding raises questions about whether this could be the GLC1B gene.

The HK2 gene product plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. The *HK2* gene localizes to the outer membrane of mitochondria. Since reduced glucose-6-phosphate content in muscle has been demonstrated in pre-non-insulin-dependent diabetes mellitus (pre-NIDDM) and NIDDM subjects, *HK2* was investigated as a promising candidate gene for noninsulin-dependent diabetes mellitus (NIDDM; OMIM125853) [17,18]; however, those studies concluded that mutations of the *HK2* gene, including a common p.Gln142His polymorphism is not a major etiologic factor for NIDDM in the Finnish [17,18,19,20], British [19], and Danish [20] populations. In brain, mitochondrial-hexokinase activity plays a key antioxidant role protecting against oxidative stress (ROS) [21], and complements the classical antioxidant enzymes that protect against oxidative stress [22]. Hexokinase antagonizes the release of mitochondrial cytochrome C activation of Akt, which is recognized as a potent inhibitor of apoptosis. *HK2* is probably associated with an anti-oxidative reaction and inhibition of apoptosis through Bax/Bak-mediated cytochrome *c* release [23]. Leber's hereditary optic neuropathy (LHON) -associated mitochondrial DNA mutations were found in Japanese patients with POAG [24], so it is reasonable to consider a gene whose product plays a role in mitochondria as a candidate gene for other phenotypes involving optic neuropathy.

NCK2 genes in POAG and NTG subjects

The *NCK2* gene, which was previously reported to be associated with NTG [16], encodes a member of the NCK family of adaptor proteins, and the adaptor protein which associates with tyrosine-phosphorylated growth factor receptors of their cellular substrates. SH2/SH3 domain-containing adapter proteins, such as the NCK family, play a major role in regulating tyrosine kinase signaling [25]. Previously, microsatellite marker D2S176 within the GLC1B locus showed significant association with NTG in the Japanese population, and D2S176 is located 24 kb from the *NCK2* gene [16]. Brain-derived neurotrophic factor (BDNF) binds to and activates the TrkB tyrosine kinase receptor to regulate cell differentiation and survival in the nervous system. BDNF

Table 4. Correlation between the POAG or NTG Endophenotypes and *HK2* SNPs Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
rs678350 genotype	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****
POAG	61.6	55.3	59.0	0.83	24.0	23.6	21.9	0.56	−14.82	−15.64	−11.94	0.53
NTG	57.5	54.9	56.9	0.99	17.1	16.0	17.6	0.64	−10.72	−12.10	−7.14	0.37
rs2229621 genotype	**A/A**	**A/T**	**T/T**	**p value****	**A/A**	**A/T**	**T/T**	**p value****	**A/A**	**A/T**	**T/T**	**p value****
POAG	58.5	58.0	55.5	0.93	24.4	22.9	25.5	0.94	−16.42	−11.94	−17.65	0.65
NTG	57.8	55.2	57.4	0.91	16.1	17.5	16.9	0.63	−11.93	−10.34	−11.16	0.95

*IOP; intraocular pressure (under medication).
**Dunnett's test.
G/G or T/T is mutant homozygote, A/G or A/T is heterozygote, and A/A is wild homozygote.
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stimulation promotes interaction of *Nck2* with TrkB in cortical neuron [26]. And BDNF signaling in glia is known to play important roles in neural protection and regeneration, particularly in conversion of Muller glia to photoreceptors [27]. In our study, it is interesting that the *NCK2* variant rs2033008 showed a significantly difference from the control population in the NTG group, where the disease pathology seems to be focused on the retinal ganglion cells and the optic nerve, but not in the POAG group, where a substantial disease component localizes to the anterior chamber of the eye (Table 5). Thus although our study falls short of achieving a level of significance needed to identify *NCK2* de novo as a glaucoma gene and this polymorphism showed deviation from Hardy–Weinberg equilibrium ($p = 0.030$ in Stage One), our data do support the prior finding of significant allele frequency differences between NTG cases and normal controls in the Japanese population [16]. It is unclear whether this deviation from Hardy-Weinberg equilibrium in Stage One might represent the absence of some alleles from this population because they are associated with diagnoses specifically excluded from this study, such as ocular hypertension.

Immunohistochemistory of the *Hk2* and *Nck2*

Although KH2 and NCK2 had previously been detected in retina, more precise localization to specific cell types is needed to begin understanding how the gene products might play a role in disease pathology. In the representative IHC photographs, antibody against *Hk2* was strongly immunoreactive in the ganglion cell layer (GCL). The *Hk2* protein localizes to the outer membrane of mitochondria, and interestingly the *Hk2* protein appears in the GCL. *Nck2*, which interacts with BDNF, is expressed in ganglion cell layer (GCL), inner nuclear layer (INL) and outer plexiform layer (OPL), and most expressed in GCL. This localization makes it highly conceivable that the *Hk2 gene products* could each play a role in glaucoma, and there is possibility that *Nck2* could have relationship with glaucoma.

TMEM182 in POAG and NTG

SNPs rs869833 and rs960011 in the TMEM182 gene showed significant association with POAG and NTG in Stage One that was not confirmed in the second stage. The primary amino acid sequence of *TMEM182* predicts an evolutionarily-conserved novel transmembrane protein, which consists of four putative membrane-spanning regions indicative of an integral membrane topology. The *TMEM182* protein sequence lacks homologies with previously-defined protein families. However, the pro-inflammatory cytokine TNFα down-regulates *TMEM182* transcript expression in adipocytes [28]. Its transcript expresses in white adipose tissues, heart, muscle, and lower relative levels of *TMEM182* transcript are found in kidney, testis, and brain. Identification of the intracellular signaling pathway involved in the TNFα-mediated decrease might be one clue offering insights into association between POAG and *TMEM182* function. Failure to confirm the association with POAG in the second stage could be attributable to clinical heterogeneity, but this result still needs to be confirmed in a second population. Nakano et al. demonstrated heterogeneity in the Japanese POAG population when their genome-wide association study of 1,575 Japanese POAG and normal subjects identified significant evidence of association with 6 SNPs on Chromosome 1, 10, 12 [29]. They did not report evidence of association with SNPs on Chromosome 2.

Table 5. Stage Two *NCK2* SNP rs2033008 Allele Frequencies in Japanese POAG, NTG and Controls Subjects.

	Allele frequency				Genotype			
	T	**A**	**Odds ratio (CI)**	**p value***	**T/T**	**T/A**	**A/A**	**p value****
POAG	0.703	0.297	0.87 (0.65–1.16)	0.35	89/187	85/187	13/187	0.069
NTG	0.750	0.250	0.69 (0.53–0.89)	0.0053	159/286	111/286	16/286	0.0056
Control	0.673	0.327			130/271	105/271	36/271	

*Fisher's exact test,
**Chi-square test.
A/A is mutant homozygote, T/A is heterozygote, and T/T is wild homozygote.
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Table 6. Correlation between the POAG or NTG Endophenotypes and *NCK2* SNP rs2033008 Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
Genotype	**T/T**	**T/A**	**A/A**	**p value****	**T/T**	**T/A**	**A/A**	**p value****	**T/T**	**T/A**	**A/A**	**p value****
POAG	55.8	61.0	54.2	0.94	23.7	24.2	25.0	0.87	−15.38	−14.94	−12.28	0.63
NTG	56.8	56.0	52.2	0.70	16.7	17.0	16.0	0.79	−9.83	−10.95	−16.28	0.05

*IOP; intraocular pressure (under medication).
**Dunnett's test.
A/A is mutant homozygote, T/A is heterozygote, and T/T is wild homozygote.
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Thus these SNPs on Chr2 might be the variants for which our study is not well-powered, or clinical heterogeneity might be complicating our ability to detect the association in our limited sample size.

In our data set, *HK2* shows the strongest evidence of association with NTG in the Japanese population out of all of the genes that have SNPs represented on the screening panel we used.

Even when a single simple Mendelian locus causes a disease, variants in other genes may contribute to phenotypic variability, and phenotypic complexity along with locus and allele heterogeneity can complicate the problem of identifying the underlying causes of the disease. Our findings raise questions about whether additional genes in this region may be contributing to phenotypic heterogeneity within the NTG and POAG populations. The MD values of the visual field in these studies indicate middle to advanced stages of the disease, with the range of values possibly resulting from a combination of genetic complexity and genetic heterogeneity.

On the other hand, we also have to consider the importance of apparent gene deserts. The SNP rs1239066 shows significant evidence of association in both Stage One and Stage Two screenings, and in the meta-analysis (POAG, NTG; P = 0.001, 0.005). Significant evidence was also found for rs1027003 (POAG; P = 0,010) (Table 2), but neither one is in the immediate vicinity of a known gene. Wiggs et al. found significant evidence that genetic variants in a gene desert on 8q22 are associated with optic nerve damage in glaucoma [14]. So additional follow up studies will need to explore this gene desert region to determine whether any functional sequences there are playing a role in the disease.

Glaucoma is a complex disease, and it involves genetic variants that confer moderate to low effect sizes (e.g., OR = 1.2–1.5). The OR which was identified in the first stage with the P value cutoff of 0.01 was 1.68. This cutoff value was a bit strict to exclude the false positives.

Further investigations of the structure and function of the *HK2*, *NCK2* and *TMEM182* proteins would be helpful in understanding the pathogenesis of POAG and NTG. Our data suggest that *HK2* may play an important role in NTG in the Japanese population; although our data suggest that *HK2* might be the GLC1B gene, a firm conclusion on the subject awaits screening of members of the families originally used to map the GLC1B locus.

Patients and Methods

Ethics statement

This study was approved by the Institutional Review Board of Tohoku University, Keio University, Tokyo Metropolitan Police Hospital, Niigata University, Ideta Eye Hospital, and all procedures were conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent after an explanation of the purpose and procedures to publish these case details.

Patient Recruitment and Characteristics

The samples used in the first screening (Stage One) were collected in Keio University hospitals, Tokyo Metropolitan Police Hospital, Niigata University, and Ideta Eye Hospital, and the

Table 7. Stage Two *TMEM182* SNP Allele Frequencies in Japanese POAG, NTG and Control Subjects.

	Allele frequency			Genotype			
rs869833	**A**	**G**	**p value***	**A/A**	**A/G**	**G/G**	**p value****
POAG	0.590	0.410	0.855	61/187	99/187	27/187	0.095
NTG	0.579	0.421	0.834	94/286	143/286	49/286	0.269
Control	0.585	0.415		100/271	117/271	54/271	
	Allele frequency			**Genotype**			
rs960011	**C**	**T**	**p value***	**C/C**	**C/T**	**T/T**	**p value****
POAG	0.503	0.497	0.363	45/187	98/187	44/187	0.479
NTG	0.520	0.480	0.641	76/286	145/286	65/286	0.782
Control	0.533	0.467		79/271	131/271	61/271	

*Fisher's exact test,
**Chi-square test.
G/G or T/T is mutant homozygote, A/G or C/T is heterozygote, and A/A or C/C is wild homozygote.
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Table 8. Correlation between the POAG or NTG Endophenotypes and *TMEM182* SNPs Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
rs869833 Genotype	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****
POAG	54.8	61.1	56.0	0.95	24.7	23.8	23.3	0.80	−17.5	−15.0	−9.95	0.01
NTG	55.8	55.7	59.9	0.40	16.9	16.9	16.4	0.84	−12.60	−9.62	−12.21	0.98
rs960011Genotype	**C/C**	**C/T**	**T/T**	**p value****	**C/C**	**C/T**	**T/T**	**p value****	**C/C**	**C/T**	**T/T**	**p value****
POAG	55.5	57.3	62.0	0.29	24.8	24.4	23.2	0.73	−14.2	−18.5	−9.44	0.37
NTG	53.8	56.4	60.1	0.14	17.1	17.3	15.7	0.16	−10.77	−11.02	−11.76	0.88

*IOP; intra ocular pressure (under medication),
**Dunnett's test.
G/G or T/T is mutant homozygote, A/G or C/T is heterozygote, and A/A or C/C is wild homozygote.
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samples used in the second screening (Stage Two) were collected at Tohoku University.

Routine ophthalmic examinations were performed on all subjects. Individuals were included as POAG cases if they fulfilled the following inclusion criteria: 1) applanation IOP greater than 22 mm Hg in each eye; 2) spherical equivalent more than −8 diopter; 3) glaucomatous cupping in each eye including cup-to-disc ratio greater than 0.7; 4) visual field loss measured by Goldmann perimetry or Humphrey automated field analyzer (Carl Zeiss Meditec, Dublin, CA) in Stage One. The severity of the visual field defects was scored from 1 to 5 according to previously reported criteria ref. The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry or the classification was based on results of Humphrey automated field analyzer [30,31]. Kozaki's classification is widely used in Japan. In Stage Two, all of the visual field loss were measured by Humphrey automated field analyzer according to Anderson-Pattela classification [30] consistent with the glaucomatous cupping in at least one eye; and 5) open anterior chamber angles; and exclusion of secondary causes (e.g., trauma, uveitis, or steroid-induced glaucoma). The criteria for NTG were the same as for POAG except that NTG subjects showed applanation IOP less than 22 mm Hg in both eyes at each examination. Baseline clinical parameters including age, gender, spherical equivalent base line visual acuity (VA), IOP measured by Goldmann applanation tonometry were recorded at the time of first diagnosis of POAG or NTG in each patient. Mean deviation (MD) values indicative of visual field damage were obtained by the Swedish interactive threshold algorithm (SITA)-standard strategy of the 30-2 program of HFA (Carl Zeiss Meditec, Dublin, Californuia, USA). MD was used on reliable visual field test results (<20% fixation errors, <33% false-positive results, and <33% false-negative results). Control subjects had these characteristics: IOP less than 22 mm Hg, normal optic discs, and no family history of glaucoma. To decrease the chance of enrolling individuals with pre-symp-

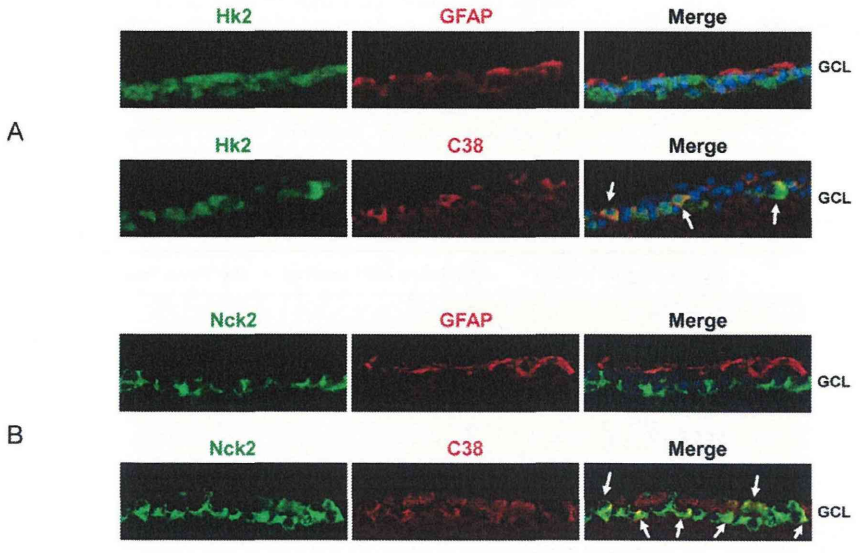

Figure 3. *Hk2* and *Nck2* Immunohistochemistory. Representative IHC photographs showing Hk2, Nck2, astrocyte maker (GFAP) and RGC marker (C38) in the retinas of untreated mice. Arrows indicated the co-localization area.
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tomatic glaucoma, we limited this group to individuals older than 60 years.

Two Stage Screening Protocol

The two-stage screening strategy is shown in Figure 1. The first stage screened used 669 SNPs from the GLC1B region, using the Illumina panel #8 (Chromosome 2; 77–117 Mbp) (Illumina, San Diego, CA, USA) carried by Illumina (San Diego, CA, USA), and each SNP was tested for association with POAG or NTG. The first stage used a cohort of 368 unrelated Japanese individuals: 123 POAG cases (63 men and 60 women), 121 NTG cases (61 men and 60 women) and 120 normal subjects (61 men and 59 women). Mean age of POAG cases was 56.9±11.5 years. Mean age of NTG cases was 54.0±12.3 years. Mean age of controls was 70.3±10.2 years. The visual field scores were 2.8±1.0 in POAG cases and 2.7±0.9 in NTG cases (Table 9). Single-nucleotide polymorphisms (SNPs) with a call rate <90% were excluded from the analysis. All of the polymorphisms showed no deviation from Hardy–Weinberg equilibrium ($P>0.05$) except rs2033008 in Stage One ($p = 0.030$).

The second stage screened SNPs that showed significant evidence for association in the first round in this study ($p<0.01$) and in the NCK2 gene, which previously showed association with NTG [16]. The odds ratio which was identified in the first stage with the P value cutoff of 0.01 was 1.68. Second stage screening was carried out using sequencing of DNA PCR amplified by polymerase chain reaction from genomic DNA samples from a population of 473 unrelated Japanese individuals, including 187 POAG cases (119 men and 68 women), 286 cases NTG (139 men and 147 women), and 271 control subjects (145 men and 126 women). Mean age of POAG cases was 57.8±12.0 years. Mean age of NTG cases was 56.4±13.3years. Mean age of controls was 69.7±9.3 years. Maximum intra ocular pressure under medication were 23.5±5.3 mmHg in POAG subjects and 16.8±2.4 mmHg in NTG subjects. Mean deviation (MD) value of the visual field test was −15.0±9.0 dB in POAG cases and −11.0±7.1 dB in NTG cases (Table 9).

Sample Preparation

Genomic DNA was extracted from leukocytes of the peripheral blood. It was purified by the Qiagen QIAamp Blood Kit (Qiagen, Valencia, CA, USA).

Mutation Screening

Mutation screening was carried out in genes that contained SNPs that showed significant evidence of association in the first stage (HK2 and TMEM182) plus the previously-reported NCK2 gene [16]. All of the exons of the *HK2*, *NCK2*, *TMSB10* and *TMEM182* genes, and positive SNPs were amplified by a polymerase chain reaction (PCR) using 0.5 μM concentration of primers in an amplification mixture (25 μl) containing 0.2 mM dNTPs and 0.5 U Ex Taq polymerase (Takara Bio, Shiga, Japan) with 30 ng template DNA. Oligonucleotides for amplification and sequencing were selected using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/ provided in the public domain by the Massachusetts Institute of Technology, Cambridge, MA). Primers for amplification and sequencing of coding sequence were placed in introns far enough from the intron/exon junctions to allow for visualization of the splice site sequences. The PCR fragments were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA), sequenced by the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) on an automated DNA sequencer (ABI PRISM™ 3100 Genetic Analyzer, Perkin-Elmer, Waltham, MA, USA).

Statistical Analysis

The significance of association was determined by contingency table analysis using Fisher's exact test or Chi-square test, depending on cell counts. In estimation of genotype-phenotype correlation, we used Dunnett's test to compare group means of those carrying the mutant variant being tested against the group means of those carrying the normal, reference sequence. Odds ratios (approximating to relative risk) were calculated as a measure of the association between the allele frequency and the phenotype of POAG/NTG, estimated using the SNPAlyze program version 7.0 (Dynacom, Yokohama, Japan). Hardy–Weinberg equilibrium was analyzed using gene frequencies obtained by simple gene counting and the chi-square test with Yates' correction for comparing observed and expected values.

Immunohistochemistry of the *HK2* and *NCK2*

Murine retinas were fixed with 4% PFA at 4°C overnight and then cryoprotected in phosphate buffered saline (PBS) with 20% sucrose. Cryosections (thickness 10 μm) were mounted on the slides and incubated with blocking buffer (10% goat serum, 0.5% gelatin, 3% BSA and 0.2% Tween 20 in PBS). Next, they were incubated with primary antibodies at 4°C overnight. Primary

Table 9. Clinical Characteristics of Subjects studied in Stage One and Two Screening.

Endophenotype		Age at diagnosis (y.o.)	Maximum IOP* (mmHg)	The Visual Field Score**
Stage One	POAG	56.9±11.4	25.3±5.6	2.8±1.0
	NTG	54.0±12.2	16.0±2.3	2.7±0.9
	Control	70.3±10.2	13.9±2.2	
Stage Two	POAG	57.8±12.0	23.5±5.3	−15.0±9.0 (dB)
	NTG	56.4±13.3	16.8±2.4	−11.0±7.1 (dB)
	Control	69.7±9.3	13.9±2.2	

*IOP; intra ocular pressure (under medication).

**The Visual Field Score was evaluated by Humphrey MD value or Goldmann perimetry (Stage One) and Humphrey MD value (Stage Two).

In Stage One, the severity of the visual field defects was scored from 1 to 5 according to previously reported criteria. The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry or the classification was based on results of visual field perimetry (Humphrey Field Analyzer; Carl Zeiss Meditec, Dublin, CA). Kozaki's classification is widely used in Japan.

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antibodies used were Mouse anti-Glial Fibrillary Acidic Protein (GFAP) (1:200; MAB360; Chemicon, Millipore, MA, USA), mouse anti-C38 (1:200; provided by Dr. Jun Kosaka), rabbit anti-NCK2 (1:200; ab14590; Abcam), or rabbit anti-HK2 (1:200; 2867S; Cell Signaling Technology, MA, USA). The sections were washed three times with PBST (PBS containing 0.2% Tween 20) and then incubated with secondary goat anti-rabbit IgG antibody (1:200; A11008 Invitrogen, Carlsbad, CA, USA) tagged with Alexa 488 or goat anti-mouse IgG A11030; Invitrogen, Carlsbad, CA, USA) tagged with Alexa 546 for 1 hour. The slides were washed three times and mounted with Vectashield mounting medium (H1000; Vector, Burlingame, CA).

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Author Contributions

Conceived and designed the experiments: T. Funayama YM KN NF. Performed the experiments: DS T. Funayama YT AS KY MM AM TN NF. Analyzed the data: DS T. Funayama YM JER NF. Contributed reagents/materials/analysis tools: T. Funayama YT AS NY T. Fukuchi HA HI TN. Wrote the paper: DS KY JER NF.

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CLINICAL INVESTIGATION

Development of a new strategy of visual field testing for macular dysfunction in patients with open angle glaucoma

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Abstract
Purpose To explore methods of automated visual field (VF) examination for the assessment of macular function.
Method We used a VF examination (AP-7000 automatic perimeter, Kowa, Japan) to examine macular function in 53 eyes from 29 patients with open angle glaucoma. We measured the mean total deviation (c-MD) of 16 points in the central VF located in a 2-degree-interval 4 × 4 array with various stimulus sizes (Goldmann sizes III, II, and I). The retinal nerve fiber layer (RNFL) thickness, ganglion cell complex (GCC), and ganglion cell layer plus inner plexiform layer (GCL + IPL) were measured with the 3D OCT-2000 System (Topcon, Japan). The c-MDs of various stimulus sizes were compared with the OCT parameters using the Spearman rank correlation.
Results The average examination time was 93.5 ± 23.5 s and the c-MD values were −11.8 ± 8.2 (stimulus size III), −11.9 ± 9.5 (stimulus size II), and −12.3 ± 9.6 dB (stimulus size I). The c-MD (stimulus size III) and averaged total deviations of the Humphrey Field Analysis 10-2 program were significantly correlated ($\rho = 0.91$). The C-MD values for stimulus size III were significantly correlated with the OCT parameters (RNFL: $\rho = 0.59$; GCC: $\rho = 0.65$; and GCL + IPL: $\rho = 0.64$). The correlation coefficient between the c-MD and the GCC was better for stimulus sizes II and I ($\rho = 0.69$) than for stimulus size III ($\rho = 0.65$).
Conclusion The C-MD values for the 16 measured central VF points were significantly correlated with macular structure, and the smaller stimulus sizes of the automated VF test had a higher correlation coefficient of within 8°.

Keywords Macular function · Open angle glaucoma · Ganglion cell complex · Retinal nerve fiber layer

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Introduction

Glaucoma is characterized by glaucomatous optic neuropathy and a corresponding progressive degeneration of the retinal ganglion cells (RGCs). It is the second highest cause of blindness worldwide, affecting approximately 70 million people [1, 2]. One of the most significant risk factors for glaucoma progression is high intraocular pressure (IOP), and therapies to lower IOP have, therefore, become well established as treatments for primary open angle glaucoma (POAG) [3, 4]. Glaucoma progresses with age [5], and the increasing frequency of visual impairment is becoming a serious issue in Japan as the country's population ages.

In Asia, normal tension glaucoma (NTG) is more common than POAG [6–8], and IOP-lowering treatment is of more limited efficacy in this type of the disease. NTG eyes undergo significantly more damage nasally and inferiorly to the fixation point [9, 10]. Additionally, as we have previously demonstrated, the myopic disc type is significantly more common in patients with an advanced stage of NTG [11] and in patients with decreased visual acuity [12] when patients are classified by optic disc type [13, 14]. We found that these patients showed significant thinning of the temporal circumpapillary retinal nerve fiber layer thickness (cpRNFLT), including the papillomacular bundle. Decreased visual acuity in such patients may be related

to decreased tissue blood flow (filling defect) in the temporal optic disc [12]. Recently, patients with strong fluctuations in ocular perfusion pressure have been shown to have significant progression of visual field loss in the central 10° [15]. Decreased macular function is thus implicated in glaucoma, and decreased ocular perfusion pressure may be related to its pathogenesis.

Macular functions, including retinal sensitivity and visual acuity, are very important for quality of life even in patients with glaucoma [16]. Optical coherence tomography (OCT), which measures cpRNFLT, has proven, since its introduction by Huang et al. [17], to be useful in detecting and following the progression of glaucoma. Recently, spectral domain (SD) technology has led to a significant improvement in segmentation algorithms, and advances by several research groups have enabled us to visualize each retinal layer in the macular area, including the ganglion cell complex (GCC) and the ganglion cell layer plus inner plexiform layer (GCL + IPL) [18, 19]. Measurement of macular GCC thickness has the same performance in glaucoma diagnosis as cpRNFLT [20, 21], even in myopic patients [7]. Another tool used in glaucoma assessment is the Humphrey Field Analyzer (HFA). The 30-2 test pattern of the HFA has only 4 points within the central 4.2° (radius) of fixation, making the 10-2 test pattern a better choice to understand glaucomatous visual field damage in the central area of vision. However, using both test patterns on the same day can be fatiguing to patients because of the amount of time required. This means that, although glaucoma specialists can now use OCT to quickly and simply assess the structure of the macula, an equivalently quick and simple option to assess function is currently unavailable.

In this study, we sought to develop a method that allows evaluation of macular function with a shorter testing time and the results correlate well with those of both the 10-2 program of the HFA (HFA 10-2) and the OCT-measured retinal macular layer thickness. We evaluated parafoveal threshold testing (PTT), a new macular visual field examination with the AP-7000 automatic perimeter (Kowa Company, Nagoya, Japan). First, we investigated the correlation between the mean central total deviation (c-MD) at 16 test points, measured with PTT, and the average total deviation (TD) at 16 central test points, measured with the HFA 10-2. Second, we changed the stimulus size (Goldmann size III, II, or I) and compared the correlation of the measurement parameters taken from a macular OCT map (RNFL, GCC, and GCL + IPL) with the PTT c-MD values with different spot sizes. This method of measuring the macular visual field is quicker and less fatiguing for patients, enabling us to assess macular function more easily during the routine management of glaucoma patients.

Patients and methods

Inclusion criteria

This retrospective, cross-sectional study comprised 53 eyes of 29 Japanese adult patients with OAG. The cases were a continuous series, and all were introduced to the neuroprotective treatment unit of our department from March 2012 to September 2012. All the patients had visual field loss close to the fixation point, decreased visual acuity, and damage to the papillomacular bundle, and exhibited glaucomatous optic neuropathy. The inclusion criteria were: (1) diagnosis of OAG, including PAOG; (2) a spherical equivalent refractive error of >-8.00 D; and (3) a glaucomatous visual field meeting the Anderson-Patella classification. The exclusion criteria were: (1) decimal visual acuity <0.3 and (2) macular disease such as macular edema, macular degeneration, or premacular fibrosis.

The baseline clinical parameters recorded for each patient were age, sex, refractive error, and IOP. The baseline best-corrected visual acuity (BCVA) was measured with a standard Japanese decimal visual acuity chart and converted to the logarithm of the minimum angle of resolution (logMAR) for statistical analysis. IOP was measured with Goldmann applanation tonometry. Demographic data for the glaucoma patients of this study are listed in Table 1.

The study adhered to the tenets of the Declaration of Helsinki, and the protocols were approved by the Clinical Research Ethics Committee of Tohoku University Graduate School of Medicine.

AP-7000 automated perimeter

The AP-7000 is an instrument used to measure the visual field. It is functionally equivalent to the HFA. The hardware specifications for the AP-7000 for common static perimetry are the same as those for the HFA. The stimulus size is based on the Goldmann perimeter (sizes I to V). The examination distance is 300 mm. The maximum stimulus intensity is 10,000 asb. The stimulus presentation time is 0.2 s. The background intensity is 10 cd/m^2. Eye fixation is monitored with the Heijl–Krakau method. The AP-7000 has a fast-threshold strategy named "Quick." Basically, "Quick" is a staircase strategy with a single threshold crossing and a fixed step size of 3 dB. To more accurately detect abnormalities, a step size of 2 dB instead of 3 dB is adopted within a certain range of sensitivity based on a normative database.

Visual field analysis

Sixteen points in the central visual field were examined by means of PTT, all located in a 2°-interval 4 × 4 array (Fig. 1a, b). Various stimulus sizes (Goldmann sizes III, II,

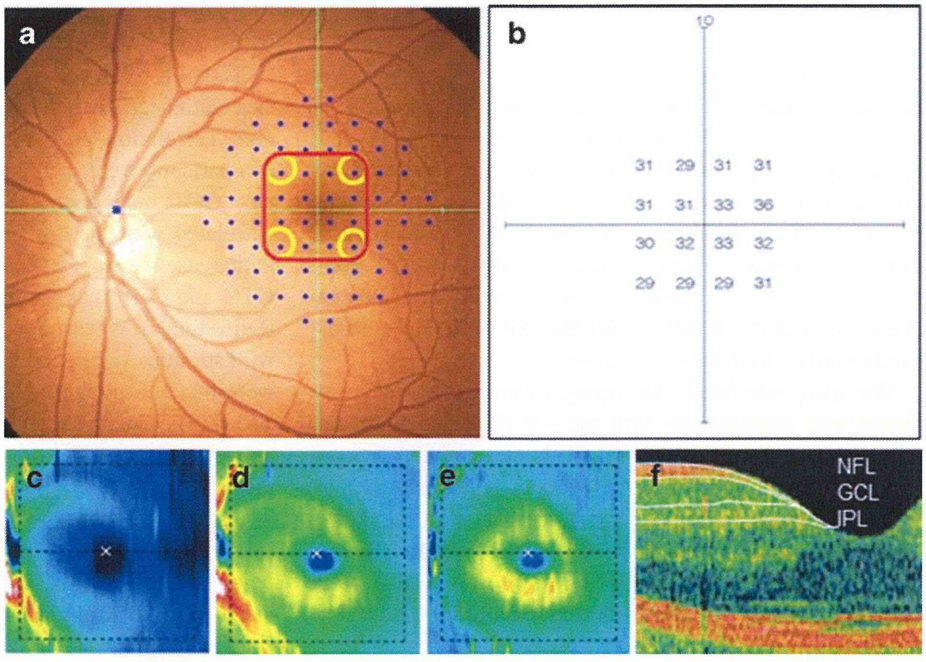

Fig. 1 Correspondence between parafoveal threshold testing (PTT) and the HFA 10-2. **a** Correspondence of the 16 PTT test points (red square) and the central 16 HFA 10-2 test points (68 blue dots). **b** Results from 16 PTT points. **c** Macular OCT map of the RNFL, **d** GCC, and **e** GCL + IPL from the 3D OCT-2000. OCT map with layer information. RNFL indicates nerve fiber layer; GCL, ganglion cell layer; and **f** IPL, inner plexiform layer

Table 1 Demographic data for the glaucoma patients of this study

	Mean ± standard deviation (range)
Male/female, eyes	30/23 (57 %/43 %)
Age, years	59.9 ± 12.9 (19–89)
Spherical equivalent, D	−3.1 ± 2.9 (−7.8 to 2.6)
Visual acuity, logMAR	0.10 ± 0.34 (−0.18 to 0.34)
MD of HFA 30-2, dB	−13.9 ± 8.4 (−29.4 to −0.3)
MD of HFA 10-2, dB	−18.8 ± 8.9 (−34.5 to −3.6)
Baseline IOP, mmHg	13.7 ± 2.7 (7–19)

MD mean deviation, *IOP* intraocular pressure

and I) were used and the c-MD values, measured. Standard automated perimetry was performed with the HFA. We used the Swedish interactive threshold algorithm (SITA)-standard strategy of the 10-2 program (Carl Zeiss Meditec, Dublin, CA, USA). The stimulus size of the HFA 10-2 was size III. HFA was performed within 3 months of the OCT measurement. Only reliable measurements of the visual field were included in the analysis (<20 % fixation errors, <33 % false-positive results, and <33 % false-negative results).

OCT macular map

The macular RNFL, GCC, and GCL + IPL thicknesses were measured using 3D OCT-2000 software (version 8.00; Topcon Corporation, Tokyo, Japan), with macular cube scans (a 6 × 6-mm square corresponding to a 20° square of the retina in the macular area) centered on the fovea. The thickness of each retinal layer was calculated automatically by the embedded 3D OCT software. Our analysis used the average thickness of the retinal layers in the area corresponding to the 16 central test points from the AP-7000 (an 8° square) (Fig. 1c–f). Although we found RGC and RNFL displacement in the fovea [22], the central 8° square was still suitable for analysis of the macula [22]. If the image quality score was <60, the image was excluded.

Correlation between structure and function in the parafovea

To analyze the right and left eyes together, all left eyes were flipped across the vertical midline to give the appearance of right eyes. We investigated the correlation of c-MDs measured with stimulus sizes III, II, and I with OCT-measured RNFL, GCC, and GCL + IPL, respectively.

Statistical analysis

The Spearman rank correlation was used to determine the correlations among the structural examination (RNFL, GCC, and GCL + IPL measured with the 3D OCT-2000), the results of the functional examination (c-MD and HFA 10-2 MD), and visual acuity. The significance level was set at $P < 0.05$. Statistical analysis was performed with JMP software (Pro version 9.0.2; SAS Institute Japan, Tokyo, Japan). A Z test was used to find the correlation coefficient level, with >1.96 set as the significance level.

Results

To determine whether the central 16 test points that we measured within a 8° square were valuable for clinical use in the management of glaucoma patients, we first compared the correlation between AP-7000 (c-MD) and average HFA 10-2 TDs in 16 corresponding test points. C-MD levels measured with a Goldmann size III stimulus were significantly correlated with average TDs ($\rho = 0.908$, $P < 0.0001$; Fig. 2). These data suggest that the results of the visual sensitivity tests with the AP-7000 are significantly similar to those of the HFA.

We also measured the times taken to complete a visual field examination with the AP-7000 and with the HFA 10-2. The mean time taken to measure the central 16 test points with the AP-7000 was 93.5 ± 23.5 s, while that with the SITA Standard test with the HFA 10-2 was 452.9 ± 63.5 s in the same patients. These data indicate that significantly less time was needed with the AP-7000 to complete an examination of macular function.

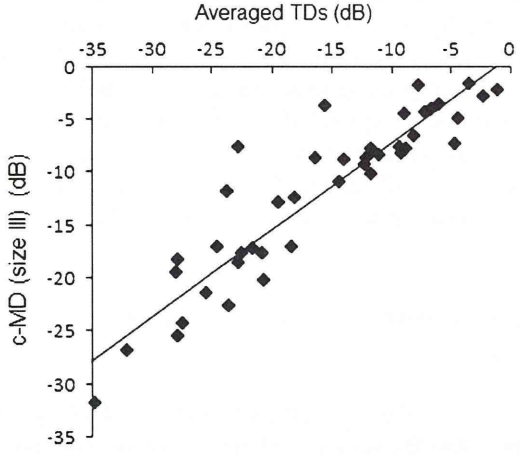

Fig. 2 Correlation between 16 PTT points and corresponding 16 HFA 10-2 points. Blot graph showing the correlation between 16-point PTT c-MD and average HFA 10-2 TD in the corresponding 16 test points. The formula of the correlation was $y = 0.82x + 0.92$, and the correlation coefficient was $\rho = 0.908$ ($P < 0.0001$)

Next, we compared c-MDs measured with stimulus sizes III, II, and I with the thicknesses of the RNFL, GCC, and GCL + IPL, respectively, from the OCT macular map. Correlation coefficients obtained from a single Spearman rank correlation analysis are listed in Table 2. All the comparisons were significantly correlated. From the macular OCT map data, the GCC thickness was the most correlated with the c-MD. A smaller stimulus size tended to increase the slope of the correlation formula between the GCC and c-MD, as shown in the blot graph (Fig. 3a–c). RNFL thickness was most correlated with results measured with stimulus size I, and GCC and GCL + IPL thicknesses were most correlated with results measured with stimulus size II. These data suggest that the power of the correlation between structure and function is different for each layer of the macula.

Finally, we compared macular function (c-MDs measured with stimulus size I, II, or III in the 16 central test points, as well as the entire area of the HFA 10-2) and visual acuity. Correlation coefficients obtained from a single Spearman rank correlation analysis are listed in Table 3. All the correlation coefficients were significant (Table 2). A Z test revealed that the only correlation coefficient showing a significant difference was between the entire area of the HFA 10-2 and the central area of the AP-7000, comprising the 16 central test points.

Discussion

In this study, we devised and tested a new strategy to evaluate macular function with the AP-7000 automated perimeter and found that it produced results comparable to those of the HFA. The average examination time for a central 4 × 4 set of test points was 93.5 ± 23.5 s. The C-MD results were significantly correlated with the average HFA 10-2 TD and the OCT-measured macular layer thickness. These data suggest that this new strategy can be used to quickly and accurately evaluate glaucoma patients with macular lesions.

The c-MD from the central 16 test points (within 8°) was significantly correlated with retinal layer thickness from

Table 2 Correlation coefficients for 16-point PTT and HFA 10-2

	PTT 16 pt			HFA 10-2
	III	II	I	Averaged TD (16 test points)
RNFL	$\rho = 0.591$*	$\rho = 0.636$*	$\rho = 0.638$*	$\rho = 0.589$*
GCC	$\rho = 0.651$*	$\rho = 0.693$*	$\rho = 0.691$*	$\rho = 0.672$*
GCL + IPL	$\rho = 0.642$*	$\rho = 0.684$*	$\rho = 0.677$*	$\rho = 0.659$*

PTT parafoveal threshold testing, *HFA* Humphrey Field Analyzer, *TD* total deviation, *RNFL* retinal nerve fiber layer, *GCC* ganglion cell complex, *GCL + IPL* ganglion cell layer plus inner plexiform layer

* $P < 0.0001$

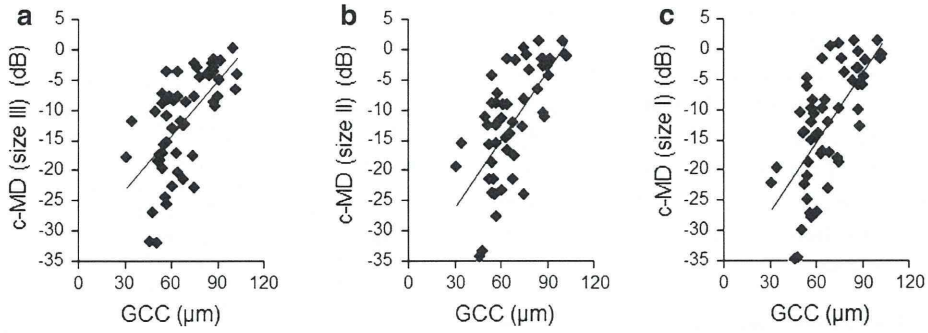

Fig. 3 Correlation between structure and function in the parafovea. *Blot graph* showing the correlation between GCC thickness and 16-point PTT c-MD with simulated stimulus sizes **a** III, **b** II, and **c** I. The formulas of correlation between GCC and the c-MD of stimulus sizes III, II, and I were $y = 0.30x - 32$ (III), $y = 0.38x - 38$ (II), and $y = 0.39x - 39$ (I), respectively

Table 3 Correlation coefficients between visual field and visual acuity

		Number of test points	ρ	*P* value
HFA 10-2	Central area	16	−0.421	<0.001
	Entire area	68	−0.302	0.019
PPT 16 pt	Size III	16	−0.474	<0.001
	Size II	16	−0.488	<0.001
	Size I	16	−0.463	<0.001

ρ correlation coefficient, *HFA* Humphrey Field Analyzer, *PTT* parafoveal threshold testing

the OCT macular map. The thicknesses of the macular RNFL [23], GCC [24], and GCL + IPL [22] were significantly correlated with macular function in glaucoma patients. This indicates that this new strategy using the AP-7000 can serve as a useful examination of macular function.

The value of this new system for central visual field testing is its speed and high correlation with the average HFA 10-2 TD and with OCT-measured macular layer thickness. Routine visual field tests often employ the 30-2 or 24-2 test of the HFA, but not the 10-2 test. The use of both on the same day is prohibitively time-consuming and fatiguing to the patient. Evaluation of the macula with OCT has many benefits, including its objective and quantitative nature and its less-burdensome examination procedure. However, OCT is a simple anatomical measurement of the retinal layer's thickness, and is, therefore, not a perfect alterative to a functional examination of the macula. Additionally, there is presently no useful electrophysiological examination of macular RGCs. Therefore, our new strategy is worthwhile in many situations arising in the management and follow-up of glaucoma patients.

The correlation of stimulus size with macular OCT parameters differed for Goldmann stimulus sizes I, II, and III. The correlation coefficient between macular structure and function for stimulus sizes I and II was larger than that for stimulus size III. For the central examination points of the HFA 30-2, a stimulus size of III covers an area of the retina containing approximately 150 RGCs. This makes the density of RGCs significantly higher than it would be in the peripheral retina [25]. The area that the Goldmann spots stimulate increases 4-fold with each step (size I: 0.25 mm^2; size II: 1 mm^2; size III: 4 mm^2), and, theoretically, smaller stimulus sizes should stimulate a smaller number of RGCs. In addition, we found that the correlation coefficient for stimulus size I was highest for the RNFL, but for stimulus size II, it was higher for the GCC and the GCL + IPL. Because of the macula's anatomy [26], the fovea has no RGCs or RNFL. We found that the anatomical displacement of the RNFL was more pronounced in the parafoveal area than in the GCC or GCL + IPL, which may have affected the correlation coefficient. It is hard to judge the best stimulus size for the macular area by considering only the higher correlation coefficient for the smaller stimulus size. It is also necessary to consider the effect of the stimulus size on the dynamic range of visual sensitivity, the fluctuation range of the threshold, and the opacity of the optic media.

This study had several limitations. First, it involved only a small number of glaucoma patients. Second, factors such as age, axonal length, glaucoma stage, and myopia were reported to be associated with bias in the correlation between structure and function [27, 28]. To decrease the influence of these factors on the results, we excluded glaucoma patients with high myopia (less than −8 D). Third, since this study was retrospective, macular assessment in the glaucoma patients had an inbuilt selection bias because macular lesions are not always involved in the disease process of glaucoma. Fourth, glaucoma patients with focal damage outside the examination area (the 16 central test points of the AP-7000) were outside the scope of this evaluation method.