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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 $\chi^2$ 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 $\chi^2$ 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 $\alpha$ 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 $\alpha$ 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 $\chi^2$ 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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## References

[1] H.A. Quigley, Number of people with glaucoma worldwide, Br. J. Ophthalmol. 80 (1996) 389–393.
[2] D. Gupta, Glaucoma diagnosis and management, Lippincott Williams & Wilkins, Philadelphia, 2005.
[3] Y. Shiose, Y. Kitazawa, S. Tsukahara, T. Akamatsu, K. Mizokami, R. Futa, H. Katsushima, H. Kosaki, Epidemiology of glaucoma in Japan – a nationwide glaucoma survey, Jpn. J. Ophthalmol. 35 (1991) 133–155.
[4] A. Iwase, Y. Suzuki, M. Araie, T. Yamamoto, H. Abe, S. Shirato, Y. Kuwayama, H.K. Mishima, H. Shimizu, G. Tomita, Y. Inoue, Y. Kitazawa, The prevalence of primary open-angle glaucoma in Japanese: the Tajimi Study, Ophthalmology 111 (2004) 1641–1648.
[5] V. Raymond, Molecular genetics of the glaucomas: mapping of the first five "GLC" loci, Am. J. Hum. Genet. 60 (1997) 272–277.
[6] M. Sarfarazi, Recent advances in molecular genetics of glaucomas, Hum. Mol. Genet. 6 (1997) 1667–1677.
[7] E.M. Stone, J.H. Fingert, W.L. Alward, T.D. Nguyen, J.R. Polansky, S.L. Sunden, D. Nishimura, A.F. Clark, A. Nystuen, B.E. Nichols, D.A. Mackey, R. Ritch, J.W. Kalenak, E.R. Craven, V.C. Sheffield, Identification of a gene that causes primary open angle glaucoma, Science 275 (1997) 668–670.
[8] T. Rezaie, A. Child, R. Hitchings, G. Brice, L. Miller, M. Coca-Prados, E. Heon, T. Krupin, R. Ritch, D. Kreutzer, R.P. Crick, M. Sarfarazi, Adult-onset primary open-angle glaucoma caused by mutations in optineurin, Science 295 (2002) 1077–1079.
[9] S. Monemi, G. Spaeth, A. DaSilva, S. Popinchalk, E. Ilitchev, J. Liebmann, R. Ritch, E. Heon, R.P. Crick, A. Child, M. Sarfarazi, Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1, Hum. Mol. Genet. 14 (2005) 725–733.
[10] K. Fujikawa, T. Iwata, K. Inoue, M. Akahori, H. Kadotani, M. Fukaya, M. Watanabe, Q. Chang, E.M. Barnett, W. Swat, VAV2 and VAV3 as candidate disease genes for spontaneous glaucoma in mice and humans, PLoS One 5 (2010) e9050.
[11] J.H. Zhao, D. Curtis, P.C. Sham, Model-free analysis and permutation tests for allelic associations, Hum. Hered. 50 (2000) 133–139.
[12] F. Faul, E. Erdfelder, A.G. Lang, A. Buchner, G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences, Behav. Res. Methods 39 (2007) 175–191.
[13] S.J. Chanock, T. Manolio, M. Boehnke, E. Boerwinkle, D.J. Hunter, G. Thomas, J.N. Hirschhorn, G. Abecasis, D. Altshuler, J.E. Bailey-Wilson, L.D. Brooks, L.R. Cardon, M. Daly, P. Donnelly, J.F. Fraumeni Jr., N.B. Freimer, D.S. Gerhard, C. Gunter, A.E. Guttmacher, M.S. Guyer, E.L. Harris, J. Hoh, R. Hoover, C.A. Kong, K.R. Merikangas, C.C. Morton, L.J. Palmer, E.G. Phimister, J.P. Rice, J. Roberts, C. Rotimi, M.A. Tucker, K.J. Vogan, S. Wacholder, E.M. Wijsman, D.M. Winn, F.S. Collins, Replicating genotype–phenotype associations, Nature 447 (2007) 655–660.
[14] K.N. Rao, I. Kaur, R.S. Parikh, A.K. Mandal, G. Chandrasekhar, R. Thomas, S. Chakrabarti, Variations in NTF4, VAV2, and VAV3 genes are not involved with primary open-angle and primary angle-closure glaucomas in an indian population, Invest. Ophthalmol. Vis. Sci. 51 (2010) 4937–4941.
[15] X.R. Bustelo, Vav proteins, adaptors and cell signaling, Oncogene 20 (2001) 6372–6381.