



# SNPs and interaction analyses of *myocilin*, *optineurin*, and *apolipoprotein E* in primary open angle glaucoma patients

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**Purpose:** To evaluate the association of *myocilin* (*MYOC*), *optineurin* (*OPTN*), and *apolipoprotein E* (*APOE*) genes and their interactions in primary open angle glaucoma (POAG).

**Methods:** A cohort of 400 unrelated POAG patients (294 high tension glaucoma, HTG, and 106 normal tension glaucoma, NTG) and 281 unrelated control subjects were recruited. All coding exons and splicing junctions in *MYOC* and *OPTN* were screened for sequence alterations. Common polymorphisms in *APOE* were genotyped. Single genes were investigated by univariate and haplotype analysis, and gene-gene interactions by logistic regression and stratified analysis. Multiple comparisons were corrected by the Bonferroni method. Bioinformatics analysis was performed to assess the conservation of mutation sites across species and to predict putative motifs and secondary structures in mutated proteins.

**Results:** Disease-causing mutations in *MYOC* and *OPTN* were identified in 1.75% and 1% of POAG patients, respectively. Most of these mutations were highly conserved across species, many predicted to create new motifs or change protein secondary structures. No individual *MYOC* polymorphisms significantly contributed to HTG or NTG. A haplotype containing the minor allele of the *MYOC* IVS2+35A>G increased NTG risk ( $p=0.0001$ ). Three *OPTN* polymorphisms, T34T, IVS5+38T>G, and IVS8-53T>C increased NTG risk ( $p<0.0008$ ), while IVS5+38T>G increased HTG risk ( $p=0.0006$ ). One haplotype that contains the minor alleles of 3 *OPTN* polymorphisms, T34T, IVS5+38T>G, and IVS7+24G>A, increased NTG risk ( $p=0.0002$ ). *APOE*  $\epsilon 4$  carriers had a decreased NTG risk ( $p=0.007$ ). Possible gene-gene interactions were found between *MYOC*, *OPTN*, and *APOE*.

**Conclusions:** Disease-causing mutations in *MYOC* and *OPTN* accounted for only a small proportion of Chinese POAG patients. Common polymorphisms in *MYOC*, *OPTN*, and *APOE* might interactively contribute to POAG, indicating a polygenic etiology.

Glaucoma is a group of diseases causing optic neuropathy and is characterized by optic disc cupping and loss of visual field. It is the second leading cause of blindness worldwide, estimated to affect about 70 million people, with 6.7 million of these being bilaterally blind [1]. Primary open angle glaucoma (POAG; OMIM 137760) is a major primary type of glaucoma.

POAG is genetically heterogeneous [2]. Seven chromosomal loci have been identified: *GLC1A*, *GLC1B*, *GLC1C*, *GLC1D*, *GLC1E*, *GLC1F*, and *GLC1G* [3-7]. Putative loci on chromosomes 2p14, 14q11, 14q21-22, 17p13, 17q25, and 19q12-14 have been reported in adult-onset POAG in a genome-wide scan study involving 113 sib-pairs [8]. A genome-wide scan on 146 POAG families of African descent suggested possible linkage to 2q33.3-37.3 and 10p12-13 [9], although a study of 8 Finnish POAG families revealed no association with these sites [10]. A genome-wide scan in 25 families with juvenile-onset POAG identified possible glaucoma loci on 9q22 and 20p12 [11]. The first POAG associated gene was identified as *myocilin* (*MYOC*; OMIM 601652) in the *GLC1A* lo-

cus. In Caucasians about 2%-4% of POAG cases are due to *MYOC* mutations [12], although it can be as high as 36% in juvenile hereditary POAG families [13]. In our previous studies, the prevalence of *MYOC* mutations is about 1.1%-1.5% in Chinese POAG patients [14,15]. The second gene for POAG was identified as *optineurin* (*OPTN*; OMIM 602432) in *GLC1E*. Mutations in *OPTN* had been found in 16.7% of 54 families with hereditary and adult-onset POAG and 12% of 124 sporadic patients with POAG, most of them with intraocular pressure (IOP) less than 22 mm Hg [16]. We found *OPTN* mutations accounting for 1.6% of sporadic Chinese POAG patients [17]. However, later studies reported no glaucoma causing mutations in *OPTN* among Caucasian POAG patients, one on 801 patients of variable age onset [18] and the other 86 adult-onset patients [19]. No *OPTN* mutation was found in 313 sporadic Japanese patients, 148 of them with normal tension glaucoma (NTG) [20]. Prevalence of *OPTN* mutations that cause glaucoma is still to be confirmed.

*Apolipoprotein E* (*APOE*; OMIM 107741) had been reported to be a potent modifier gene for POAG [21]. The promoter polymorphism -219T>G was associated with increased optic nerve damage, while -491A>T interacted with -1000C>G to increase IOP in POAG patients. In another study the *APOE*  $\epsilon 4$  allele increased risk of NTG in 70 Tasmania patients [22]. However, two recent studies on Caucasian POAG patients, one involving 155 NTG patients [23] and the other with 137

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mixed POAG and NTG patients [24], did not reveal an association between the *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotype with glaucoma.

To date, *MYOC* is the undisputed disease-causing gene for POAG but most studies showed that it accounts for only a minor number of POAG patients. Glaucoma-causing effects of *OPTN* and *APOE* have been inconsistently reported. In this study, we evaluated the association of *MYOC*, *OPTN* and *APOE* genes and their possible interactions in POAG in a large Chinese sample.

## METHODS

**Study subjects:** Unrelated patients with POAG were recruited from the Eye Clinic of the Prince of Wales Hospital, Hong Kong. POAG was defined as meeting all of the following criteria: exclusion of secondary causes (e.g., trauma, uveitis, or steroid-induced glaucoma), anterior chamber angle open (grade III or IV gonioscopy), characteristic optic disc changes (e.g., vertical cup-disc ratio >0.5, disk hemorrhage, or thin or notched neuroretinal rim), and characteristic visual field changes with reference to Anderson's criteria for minimal abnormality in glaucoma [25]. Visual acuity was determined using Snellen eye chart, IOP by applanation tonometry, and visual field by Humphrey's perimeter with the Glaucoma Hemifield Test. Patients with congenital glaucoma were excluded. Unrelated control subjects were recruited from people who attended the clinic for conditions of senile cataract, floaters, refractive errors, or itchy eyes. They did not have systemic diseases and

were excluded from glaucoma using the same criteria of diagnosis as the POAG patients after going through the same procedure of ophthalmic examination. The project was approved by the Ethics Committee for Human Research, the Chinese University of Hong Kong. Informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study, in accordance with the tenets of the Declaration of Helsinki.

A total of 400 unrelated POAG patients were included in this study, 250 males and 150 females. The highest IOP ranged from 10 to 67 mm Hg (mean $\pm$ SD: 26.3 $\pm$ 9.2 mm Hg): 294 (73.5%) patients were high tension glaucoma (HTG) with highest IOP greater than or equal to 22 mm Hg, and 106 (26.5%) normal tension glaucoma (NTG) having highest IOP <22 mm Hg. Age at diagnosis ranged from 8 to 91 years (mean $\pm$ SD: 61.0 $\pm$ 17.2 years): 80 (20%) were juvenile-onset POAG with age at diagnosis <35 years, and 320 (80%) were adult-onset POAG with age at diagnosis greater than or equal to 35 years. There were 281 unrelated control subjects, 180 males and 101 females, age ranging from 50 to 90 years (mean $\pm$ SD: 69.8 $\pm$ 9.8 years), and IOP<22 mm Hg. All study subjects were Hong Kong Chinese. Genomic DNA was extracted from 200  $\mu$ l venous whole blood using Qiamp Blood Kit (Qiagen, Hilden, Germany). A subset of these patients had been previously reported: 187 POAG patients for *MYOC* mutations [15] and 119 POAG patients for *OPTN* mutations [17].

**Sequence analysis of *MYOC*:** The coding regions of

TABLE 1. *MYOC* POLYMORPHISMS OBSERVED IN THIS STUDY

Location	Sequence change	Codon change	Allele frequency (percentage)			Genotype frequency		
			HTG (n=588)	NTG (n=212)	Control (n=562)	HTG (n=294)	NTG (n=106)	Control (n=281)
Exon 1	c.34G>C	G12R	3 (0.5)	1 (0.5)	4 (0.7)	0/ 3/291	0/ 1/105	0/ 4/277
Exon 1	c.49G>T	A17S	0 (0.0)	0 (0.0)	1 (0.2)	0/ 0/294	0/ 0/106	0/ 1/280
Exon 1	c.57G>T	Q19H	2 (0.3)	0 (0.0)	0 (0.0)	0/ 2/292	0/ 0/106	0/ 0/281
Exon 1	c.136C>T	R46X	6 (1.0)	2 (0.9)	7 (1.2)	0/ 6/288	0/ 2/104	0/ 7/274
Exon 1	c.227G>A	R76K	38 (6.5)	16 (7.5)	51 (9.1)	0/ 38/256	0/16/ 90	1/49/231
Exon 1	c.271C>T	R91X	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 1	c.284T>C	L95P	0 (0.0)	0 (0.0)	1 (0.2)	0/ 0/294	0/ 0/106	0/ 1/280
Exon 2	c.624C>G	D208E	2 (0.3)	0 (0.0)	2 (0.4)	0/ 2/292	0/ 0/106	0/ 2/279
Exon 3	c.734G>A	C245Y	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 3	c.898G>A, c.900G>A	E300K	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 3	c.938C>T	S313F	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 3	c.1058C>T	T353I	9 (1.5)	3 (1.4)	4 (0.7)	0/ 9/285	0/ 3/103	0/ 4/277
Exon 3	c.1412A>G	Y471C	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 1	c.369C>T	T123T	2 (0.3)	0 (0.0)	4 (0.7)	0/ 2/292	0/ 0/106	0/ 4/277
Exon 1	c.384G>C	R128R	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 1	c.402A>G	Q134Q	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 3	c.780A>G	A260A	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Exon 3	c.864C>T	I288I	1 (0.2)	0 (0.0)	3 (0.5)	0/ 1/293	0/ 0/106	0/ 3/278
Exon 3	c.1464C>T	A488A	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Promoter	-83G>A	-	37 (6.3)	17 (8.0)	50 (8.9)	0/ 37/257	0/17/ 89	1/48/232
Intron 2	IVS2+35A>G	-	119 (20.2)	55 (25.9)	91 (16.2)	9/101/184	7/41/ 58	3/85/193
3'UTR	1515+73G>C	-	5 (0.9)	0 (0.0)	6 (1.1)	0/ 5/289	0/ 0/106	0/ 6/275

187 HTG patients had been previously reported [15] and from them R91X, E300K, and Y471C were each found in one patient. The numbers under "Genotype frequency" are the counts of homozygotes, heterozygotes, and wildtype.

*MYOC*, exons 1-3, including splicing junctions, were screened for sequence alterations using polymerase chain reaction (PCR), high-throughput conformation sensitive gel electrophoresis (HTCSGE), and a cost saving sequencing protocol [15] on an ABI 377XL automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequence data were aligned using Sequence Navigator analysis software (version 1.0.1; Perkin-Elmer Applied Biosystems) and compared to the published *MYOC* gene sequence (GenBank AB006688).

*Sequence analysis of OPTN*: All coding exons, 4-16, of *OPTN* and their splicing junctions were screened for sequence alterations by PCR, HTCSGE, and direct DNA sequencing [17]. Sequence data were compared to the published *OPTN* gene sequence (GenBank AF420371).

*Polymorphisms of APOE*: The promoter polymorphisms -491A>T, -427T>C, and -219T>G, and the  $\epsilon 2/\epsilon 3/\epsilon 4$  genotype on exon 4 were investigated by PCR followed by restriction endonuclease assays and polyacrylamide gel electrophoresis [26,27].

*Crosschecking of sequence studies*: More than one quarter of the PCR products in each of the sequence studies on *MYOC*, *OPTN*, and *APOE*, with no regard of the respective HTCSGE or restriction endonuclease assay results, were selected at random for direct DNA sequencing. Complete matching of results was obtained.

*Statistical analysis*: SAS (release 8.2; SAS Institute, Cary,

NC) was used for statistical analyses. Hardy-Weinberg equilibrium for each polymorphism was tested by  $\chi^2$  test. A  $\chi^2$  test or Fisher's exact test was used to compare allele or genotype frequencies of HTG or NTG patients with control subjects. Expectation-maximization (EM)-based haplotype frequency estimations and permutation-based hypothesis testing procedures were performed in HTG, NTG and control subjects using the GENECOUNTING program (version 1.3) [28].

Logistic regression analysis was used to search for gene-gene interactions. Disease status was set as dependent variable (HTG or NTG=1, control=0), and gene polymorphisms as independent variables (homozygote=2, heterozygote=1, wildtype=0). Logistic regression models were built using various gene polymorphisms along with their interaction items. A stepwise regression approach was used to optimize the analysis. To reduce false-positive interactions, stratified analysis was used to verify the gene-gene interactions identified from logistic regression analysis. We stratified the study subjects according to the genotype of one gene and performed analysis of the other gene in different strata defined by the genotype of the former gene. The Breslow-Day test was used to test the homogeneity of the odds ratios (ORs) in different strata. The confidence intervals (CIs) for ORs were calculated by the Mantel-Haenszel method. An  $\alpha$  level of 0.05 was chosen; the Bonferroni method was used to adjust for multiple comparisons.

TABLE 2. *OPTN* POLYMORPHISMS OBSERVED IN THIS STUDY

Location	Sequence change	Codon change	Allele frequency (percentage)			Genotype frequency		
			HTG (n=588)	NTG (n=212)	Control (n=562)	HTG (n=294)	NTG (n=106)	Control (n=281)
Exon 5	c.573T>C	I88T	1 (0.2)	1 (0.5)	0 (0.0)	0/ 1/293	0/ 1/105	0/ 0/281
Exon 5	c.603T>A	M98K	100 (17.0)	42 (19.8)	88 (15.7)	10/80/204	3/36/67	7/74/200
Exon 5	c.619G>C	E103D	0 (0.0)	1 (0.5)	0 (0.0)	0/ 0/294	0/ 1/105	0/ 0/281
Exon 14	c.1767A>G	H486R	0 (0.0)	1 (0.5)	0 (0.0)	0/ 0/294	0/ 1/105	0/ 0/281
Exon 16	c.1944G>A	R545Q	21 (3.6)	7 (3.3)	19 (3.4)	1/19/274	0/ 7/ 99	0/19/262
Exon 4	c.412G>A	T34T#	86 (14.6)	46 (21.7)	61 (10.9)	10/66/218	6/34/ 66	2/57/222
Exon 4	c.457C>T	T49T	7 (1.2)	1 (0.5)	9 (1.6)	0/ 7/287	0/ 1/105	0/ 9/272
Exon 6	c.754G>A	V148V	0 (0.0)	1 (0.5)	1 (0.2)	0/ 0/294	0/ 1/105	0/ 1/280
Exon 7	c.907T>C	P199P	0 (0.0)	0 (0.0)	1 (0.2)	0/ 0/294	0/ 0/106	0/ 1/280
Exon 7	c.916G>A	T202T	0 (0.0)	0 (0.0)	1 (0.2)	0/ 0/294	0/ 0/106	0/ 1/280
Intron 5	IVS5+38T>G*	-	73 (12.4)	53 (25.0)	29 (5.2)	15/43/236	16/21/ 69	2/25/254
Intron 6	IVS6+50delT	-	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Intron 6	IVS6-5T>C	-	50 (8.5)	32 (15.1)	56 (10.0)	7/36/251	6/20/ 80	4/48/229
Intron 6	IVS6-10G>A	-	29 (4.9)	14 (6.6)	25 (4.4)	0/29/265	0/14/ 92	0/25/256
Intron 7	IVS7+24G>A	-	37 (6.3)	15 (7.1)	24 (4.3)	0/37/257	0/15/ 91	1/22/258
Intron 8	IVS8+20G>A	-	11 (1.9)	10 (4.7)	7 (1.2)	0/11/283	0/10/ 96	0/ 7/274
Intron 8	IVS8-53T>C**	-	21 (3.6)	16 (7.5)	12 (2.1)	3/15/276	0/16/ 90	0/12/269
Intron 8	IVS8-84G>A	-	1 (0.2)	0 (0.0)	0 (0.0)	0/ 1/293	0/ 0/106	0/ 0/281
Intron 13	IVS13+21C>G	-	3 (0.5)	1 (0.5)	3 (0.5)	0/ 3/291	0/ 1/105	0/ 3/278
Intron 15	IVS15+10G>A	-	9 (1.5)	1 (0.5)	8 (1.4)	0/ 9/285	0/ 1/105	0/ 8/273
Intron 15	IVS15-48C>A	-	9 (1.5)	0 (0.0)	3 (0.5)	1/ 7/286	0/ 0/106	0/ 3/278

119 POAG patients (72 HTG and 47 NTG) had been previously reported [17]. E103D and H486R had been each found in one NTG patient. The numbers under "Genotype frequency" are the counts of homozygotes, heterozygotes, and wildtype. The sharp (#) indicates that when compared to controls, a higher frequency of allele A carriers was found in NTG (OR=2.3, 95% CI: 1.4, 3.7; p=0.0008). The single asterisk indicates that when compared with controls, the frequencies of allele G carriers were higher in HTG (OR=2.3, 95% CI: 1.4, 3.8; p=0.0006) and NTG (OR=5.0, 95% CI: 2.9, 8.9; p<0.0001). The double asterisk indicates that the frequency of allele C carriers was higher in HTG (OR=4.0, 95% CI: 1.8, 8.7; p=0.0002) than in controls.

**Bioinformatics analysis:** Firstly, to assess whether the normal residues at disease-causing mutation sites were conserved across species, the following protein entries in Swiss-Prot or TrEMBL were selected: MYOC\_Human (Q99972), MYOC\_Monkey (Q863A3), MYOC\_Bovine (Q9XTA3), MYOC\_Mouse (Q70624), MYOC\_Rat (Q9R1J4), MYOC\_Rabbit (Q866N2), MYOC\_Pig (AAN59763), MYOC\_Cat (AAS68633), OPTN\_Human (Q96CV9), OPTN\_Macmu (Q861Q8), OPTN\_Macfa (Q95KA2), OPTN\_Ponpy (Q5R923), OPTN\_Pig (Q7YS99), OPTN\_Rat (Q8R5M4), OPTN\_Mouse (Q8K3K8), OPTN\_Chick (Q90Z16) and OPTN\_Zebrafish (Q5RI56). The sequences were aligned by the multiple alignment tool ClustalW [29]. Secondly, to predict putative motifs in the mutated human protein sequences, the ScanProsite tool was used including patterns with the high probability of occurrence [30]. Finally, the PSIPRED program was applied to predict the secondary structure of the mutated human proteins [31].

## RESULTS

### Univariate analysis of individual polymorphisms in MYOC:

A total of 22 sequence polymorphisms of *MYOC* were identified (Table 1). All sequence polymorphisms followed Hardy-Weinberg equilibrium. Q19H, R91X, C245Y, E300K, S313F, and Y471C were disease-causing mutations, accounting for 1.75% (7/400) of POAG patients. Except for Q19H, which was identified in two patients, all the others were found only in one patient. All disease-causing mutations were found only in HTG patients. There was no significant association of all these polymorphisms with HTG or NTG ( $p > 0.0023$ , the Bonferroni corrected significance level; Table 1).

**Univariate analysis of individual polymorphisms in OPTN:** A total of 21 polymorphisms of *OPTN* were identified (Table 2). All sequence polymorphisms followed the Hardy-Weinberg equilibrium. I88T, E103D, and H486R were dis-

ease-causing mutations, accounting for 1% (4/400) of POAG patients. I88T was identified in one HTG patient and one NTG patient, respectively, the other two mutations were each found in only one NTG patient. The frequency of allele G carriers for IVS5+38T>G was higher in HTG (OR=2.3, 95% CI: 1.4, 3.8;  $p=0.0006$ ) than in controls. When compared with controls, higher frequencies were found in NTG for T34T (OR=2.3, 95% CI: 1.4, 3.7;  $p=0.0008$ ), IVS5+38T>G (OR=5.0, 95% CI: 2.9, 8.9;  $p < 0.0001$ ) and IVS8-53T>C (OR=4.0, 95% CI: 1.8, 8.7;  $p=0.0002$ ). There was no significant association of other polymorphisms with HTG or NTG ( $p > 0.0024$ , the Bonferroni corrected significance level; Table 2).

**Univariate analysis of individual polymorphisms in APOE:** All four informative polymorphisms of *APOE*, -491A>T, -427T>C, -219T>G, and  $\epsilon 2/\epsilon 3/\epsilon 4$  genotype investigated in this study followed the Hardy-Weinberg equilibrium (Table 3). Compared to control subjects, when analyzed with non- $\epsilon 4$ -carriers (genotypes 2/3+3/3), the frequency of  $\epsilon 4$ -carriers (genotypes 2/4+3/4+4/4) was significantly lower in NTG (OR=0.4, 95% CI: 0.2, 0.8;  $p=0.007$ ). The distributions of -219T>G, -427T>C, and -491A>T were not statistically different between patients with HTG or NTG and control subjects ( $p > 0.0125$ , the Bonferroni corrected significance level, Table 3).

**Bioinformatics analysis of disease-causing mutations:** In the *MYOC* gene, 6 disease-causing mutations were identified. The normal residues at these mutation sites were highly conserved across species including human, monkey, bovine, rabbit, mouse, rat, pig, and cat, with an exception that the mutation Q19H was not conserved in mouse. Q19H is located within the signal peptide (residues 1-32). The nonsense mutation R91X truncates the MYOC protein into a polypeptide with only 58 amino acids, creating a new casein kinase II phosphorylation site (residues 88-91: TkaX). The missense mutations C245Y, E300K, S313F, and Y471C are located within the

TABLE 3. *APOE* POLYMORPHISMS OBSERVED IN THIS STUDY

Polymorphism	Allele	Allele frequency (percentage)			Genotype	Genotype frequency (percentage)		
		HTG (n=588)	NTG (n=212)	Control (n=562)		HTG (n=294)	NTG (n=106)	Control (n=281)
-491A>T	T	28 (4.8)	5 (2.4)	15 (2.7)	TT	3 (1.0)	1 (0.9)	1 (0.4)
	A	560 (95.2)	207 (97.6)	547 (97.3)	TA	22 (7.5)	3 (2.8)	13 (4.6)
					AA	269 (91.5)	102 (96.2)	267 (95.0)
-427T>C	C	5 (0.9)	1 (0.5)	5 (0.9)	CC	1 (0.3)	0 (0.0)	0 (0.0)
	T	583 (99.1)	211 (99.5)	557 (99.1)	CT	3 (1.0)	1 (0.9)	5 (1.8)
					TT	290 (98.6)	105 (99.1)	276 (98.2)
-219T>G	G	222 (37.8)	69 (32.5)	187 (33.3)	GG	35 (11.9)	14 (13.2)	24 (8.5)
	T	366 (62.2)	143 (67.5)	375 (66.7)	GT	152 (51.7)	41 (38.7)	139 (49.5)
					TT	107 (36.4)	51 (48.1)	118 (42.0)
$\epsilon 2/\epsilon 3/\epsilon 4^*$	4	39 (6.6)	8 (3.8)	52 (9.3)	4/4	1 (0.3)	0 (0.0)	0 (0.0)
	2	63 (10.7)	16 (7.5)	48 (8.5)	2/4	5 (1.7)	0 (0.0)	8 (2.8)
	3	486 (82.7)	188 (88.7)	462 (82.2)	3/4	32 (10.9)	8 (7.6)	44 (15.7)
					2/3	58 (19.7)	16 (15.1)	40 (14.2)
					3/3	198 (67.4)	82 (77.4)	189 (67.3)

The asterisk indicates that, compared to control subjects, when analyzed with non- $\epsilon 4$ -carriers (genotypes 2/3+3/3), the frequency of  $\epsilon 4$ -carriers (genotypes 2/4+3/4+4/4) was significantly lower in NTG (OR=0.4, 95% CI: 0.2, 0.8;  $p=0.007$ ).

olfactomedin-like domain (residues 245-504). C245Y creates a new tyrosine sulfation site (residues 238-252: sgegdgtgYgelvwvg) and was predicted to change the MYOC secondary structure from coiled-coil to  $\beta$ -strand at residue 247. The E300K mutation was predicted to change the protein secondary structure from  $\beta$ -strand to coiled-coil at residues 288-289 and 327. Similarly, S313F would change the secondary structure from  $\beta$ -strand to coiled-coil at residues 314 and 319.

In *OPTN*, 3 disease-causing mutations were identified. The normal residues at the mutation sites of E103D and H486R were highly conserved across species including human, monkey, mouse, rat, orangutan, pig, chick, and zebrafish. I88T was conserved only across human, rat, and orangutan. Mutations I88T and E103D are located within the region that interacts with RAB8 (residues 58-209) while H486R was within the region that interacts with Huntingtin (residues 411-577). Moreover, the *OPTN* secondary structure is predicted to change from  $\alpha$ -helix to coiled-coil at residues 101-102 due to the E103D mutation.

**Haplotype analysis of common polymorphisms in MYOC:** Six common polymorphisms with a prevalence of more than 1% in the study subjects were included in the haplotype analysis of *MYOC*. They were -83G>A, R46X, R76K, IVS2+35A>G, T353I, and 1515+73G>C. Global test showed that the difference of overall haplotype frequency profiles was significant between NTG and controls ( $p=0.03$ ). The frequency of one haplotype that contains the minor allele of IVS2+35A>G and the major alleles in the other 5 loci was significantly higher in NTG than in controls ( $OR=2.0$ ;  $p=0.0001$ , Bonferroni corrected significance level 0.003).

**Haplotype analysis of common polymorphisms in OPTN:** Twelve common polymorphisms with a prevalence of more than 1% in the study subjects were included in the haplotype analysis of *OPTN*. They were T34T, T49T, M98K, IVS5+38T>G, IVS6-5T>C, IVS6-10G>A, IVS7+24G>A, IVS8+20G>A, IVS8-53T>C, IVS15+10G>A, IVS15-48C>A,

and R545Q. When compared to controls, global tests showed a significant difference of overall haplotype frequency profiles in NTG ( $p<0.0001$ ). The frequency of one haplotype that contains the minor alleles of 3 *OPTN* polymorphisms (T34T, IVS5+38T>G, and IVS7+24G>A) and the major alleles in the other 9 loci was higher in NTG than in controls ( $OR=8.0$ ;  $p=0.0002$ , Bonferroni corrected significance level 0.0007).

**Haplotype analysis of common polymorphisms in APOE:** All four polymorphisms investigated in this study were included in the haplotype analysis of *APOE*. Global test showed that the difference of overall haplotype frequency profiles was marginally significant between NTG and controls ( $p=0.04$ ). However, all the permutation-based  $p$  values for individual haplotypes were higher than the Bonferroni corrected significance level of 0.003.

**Analysis of gene-gene interactions:** Logistic regression analysis identified two pairs of significant gene-gene interactions for HTG and three pairs for NTG. Among them, one pair of interaction was found between *MYOC* and *OPTN* while the others were between *APOE* and *MYOC* or *OPTN*. Stratified analysis further verified all these significant gene-gene interactions (Table 4).

## DISCUSSION

As far as we know, this is the first report on a systemic evaluation of the association and interactions of *MYOC*, *OPTN*, and *APOE* in POAG based on a large sample. According to Alward's criteria [32], coding sequence changes were assumed to be disease-causing mutations if they are (1) expected to alter the amino acid sequence of the corresponding protein, and (2) more commonly observed in patients with POAG than in controls. To meet the latter criterion, a polymorphism needs to be completely absent from the control population or significantly more common in the POAG population. Based on such requirements, disease-causing mutations in *MYOC* and *OPTN* were identified in 1.75% and 1%, respectively, of our unrelated POAG patients. Therefore, disease-causing mutations in *MYOC* and *OPTN* accounted for only a small proportion of Chinese POAG patients. Bioinformatics analysis showed that most of these disease-causing mutations were highly conserved across species, indicating these residues to be structurally or functionally essential and important. Many are predicted to create new motifs or change protein secondary structures. Further experimental investigation is required to define the actual changes. By using transfected COS-7 cells we recently found that the C245Y mutant *MYOC* protein formed homomultimeric complexes that migrated at molecular weights larger than their wildtype counterparts and these mutant complexes remained sequestered intracellularly. This cysteine to tyrosine mutation may cause the protein to fail to fold or oligomerize correctly [33]. Further functional studies of the other mutations reported in this study would help decipher the mechanisms by which these mutants cause glaucoma.

Our univariate analysis showed that when compared with controls, carriers with T34T or IVS8-53T>C in *OPTN* were more susceptible to NTG (Table 2). When compared with controls, carriers with IVS5+38T>G in *OPTN* conferred suscep-

**TABLE 4. ANALYSIS OF GENE-GENE INTERACTIONS USING COMMON POLYMORPHISMS**

HTG patients (n=294) compared to control subjects (n=281)		
Significant interactions between polymorphisms	Logistic regression	Stratified analysis
T353I (MYOC) and IVS15+10G>A (OPTN)	0.030	0.016
IVS5+38T>G (OPTN) and -491A>T (APOE)	0.042	0.033
NTG patients (n=106) compared to control subjects (n=281)		
Significant interactions between polymorphisms	Logistic regression	Stratified analysis
R545Q (OPTN) and $\epsilon 2/\epsilon 3/\epsilon 4$ (APOE)	0.019	0.003
-83G>A (MYOC) and $\epsilon 2/\epsilon 3/\epsilon 4$ (APOE)	0.019	0.011
IVS2+35A>G (MYOC) and -219T>G (APOE)	0.037	0.036

Logistic regression analysis was used to test for interaction between polymorphisms. A stratified analysis (Breslow-Day tests for homogeneity of the odds ratios) was used to verify the significant interactions found in logistic regression analysis. The table shows  $p$  values for these tests.

tibility to both NTG and HTG (Table 2). The *APOE*  $\epsilon$ 4 carriers had a decreased NTG risk (Table 3). Haplotype analysis revealed that one haplotype that contains the minor allele of the *MYOC* polymorphism, IVS2+35A>G, increased NTG risk. Another haplotype that contains the minor alleles of 3 *OPTN* polymorphisms, T34T, IVS5+38T>G, and IVS7+24G>A, significantly increased NTG risk by 8 fold. These findings indicate that besides disease-causing mutations, noncoding or silent sequence changes in *MYOC* and *OPTN* may also modify the susceptibility of POAG.

We simultaneously analyzed the common polymorphisms in *MYOC*, *OPTN*, and *APOE* using a logistic regression approach to hunt for possible gene-gene interactions. Several interactions among *MYOC*, *OPTN*, and *APOE* were identified. Stratified analysis further verified these interactions. It has been reported that the *APOE* polymorphism -491A>T interacted with the polymorphism -1000C>G in *MYOC*, and *APOE* was a potential modifier for POAG [21]. In the present study, we did not investigate -1000C>G, which we recently showed to have similar frequencies in 212 POAG patients and 221 controls [34]. However, we identified another two interactions between *MYOC* and *APOE*, -83G>A with  $\epsilon$ 2/ $\epsilon$ 3/ $\epsilon$ 4 and IVS2+35A>G with -219T>G. We found two interactions between *OPTN* and *APOE* (Table 4), further supporting the modifier effects of *APOE* on POAG. In addition, we found a potential interaction between *MYOC* and *OPTN*, T353I with IVS15+10G>A. There was some evidence to suggest that *MYOC*-T353I, together with other genetic and environmental risk factors including family history, hypertension, and cigarette smoking, might increase POAG risk [35]. In the present study, the polymorphism *MYOC*-T353I individually remained no significant contribution to POAG risk but there was a significant interaction with *OPTN*-IVS15+10G>A. Our findings demonstrated that common polymorphisms in *MYOC*, *OPTN*, and *APOE* might interactively contribute to POAG, indicating a polygenic etiology.

Because rare sequence polymorphisms are not informative for both haplotype analysis and logistic regression analysis, in the present study we only included in these two kinds of multivariable analyses common polymorphisms which prevalence is not less than 1% in our study subjects. Meanwhile, we did not match cases and controls in age since only elderly individuals could reasonably serve as normal controls for POAG. However, we found similar results when we analyzed our data by considering age as an independent variable in the logistic regression model (data not shown). It indicated that age was not a confounding factor in our study. In the present study we defined POAG as having both characteristic optic disc changes and visual field damages. It was usually acceptable to diagnose POAG as having a milder cup-disc ratio threshold (>0.5) but with a definite and reliable visual field defect [36]. In our study all patients with a cup-disc ratio between 0.5 and 0.7 had a glaucomatous visual field defect. We have considered using cup-disc ratio >0.7 as a criterion for POAG. But our subgroup analysis involving only patients with cup-disc ratio >0.7 showed similar results with patients with cup-disc ratio >0.5 (data not shown). In addition, noncoding

polymorphisms both in *MYOC* and *OPTN* affected susceptibility to glaucoma. Whether these *MYOC* polymorphisms resulted in altered expression levels of *MYOC*, or the *OPTN* polymorphisms caused transcription complications, that lead to production of proteins with altered function or stability, remain to be investigated. Further functional studies using either transgenic cell lines or animal models are needed to confirm the gene and gene-gene interactive effects of the sequence polymorphisms.

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