Original Research

Single-nucleotide polymorphisms of *TLR4* and *GAS7* linked to primary open-angle glaucoma among patients of Shenyang, China

Jiao Jie[1] and Tengfei Wu[2]

[1]Department of Ophthalmology, The Fourth People's Hospital of Shenyang, Shenyang 110031, P.R. China;
[2]Department of Laboratory Animal Science, China Medical University, Shenyang, Liaoning 110122, P.R. China
Corresponding author: Jiao Jie. Email: Jiaojieerping@163.com

Impact Statement

At present, a shortage of evidence for relevant studies was reported among the Chinese populace. The study focused on the investigation of the relationship of *TLR4* (rs7873784, rs77358523, rs752998, and rs7868859) and *GAS7* (rs9900085, rs74629981, rs8072311, and rs11656696) polymorphisms with POAG in Shenyang, China. What is more, to further screen the candidate genes *GAS7* and *TLR4* for risk alleles associated with the development of POAG and to provide a genetic basis and targets for exploring their potential pathogenic mechanisms.

Abstract

The potential for adverse outcomes and classifications of glaucoma differ among race, country, gender, and family medical history. Nearly, 50 represent candidate genes are considered as potential contributors to the happening for the primary open-angle glaucoma (POAG) since the advent of GWASs. Our investigation is the first to report the Toll-like receptor 4 (*TLR4*) and growth arrest-specific 7 (*GAS7*) among people in Shenyang, China; to investigate whether single-nucleotide polymorphisms (SNPs) in (*TLR4*) or *GAS7* gene are risk factors for POAG among people in Shenyang, China; and also to explore their potential pathogenic mechanisms. POAG patients from July 2015 to June 2019 at Shenyang Fourth People's Hospital were selected. A total of 218 POAG patients and 252 controls were enrolled. Eight potentially functional SNPs of *TLR4* (rs7868859, rs7873784, rs77358523, and rs752998) and *GAS7* (rs8012311, rs11656696, rs74629981, and rs9900085) were genotyped. Multifactor analysis was conducted to evaluate the correlation between *TLR4*, *GAS7*, and POAG. The allele frequency of rs7873784 of *TLR4* demonstrated that the GC ($P = 0.030$), CC ($P = 0.040$), and GC + CC genotypes ($P = 0.009$) were significantly higher compared with CC genotype for POAG patients than that for controls. The rs8072311 and rs9900085 of *GAS7* gene also were significantly associated with POAG. Haplotype analysis found that the C-A-T-A haplotype (order: rs7873784-rs77358523-rs752998-rs7868859) of *TLR4* gene and the two haplotypes A-C-C-A and C-C-A-C of *GAS7* (order: rs9900085-rs74629981-rs8072311-rs11656696) were associated with an elevated susceptibility to POAG ($P < 0.05$). In this study, rs7868859 of *TLR4* and rs8012311 and rs9900085 polymorphisms of *GAS7* were first identified to be related to POAG among people in Shenyang, China.

Keywords: Primary open-angle glaucoma, haplotype, *TLR4*, *GAS7*, Chinese, disease

***Experimental Biology and Medicine* 2024; 248: 2319–2324. DOI: 10.1177/15353702231214254**

Introduction

Primary open-angle glaucoma (POAG) is well-known as a highly dangerous and blinding eye disease and this particular form of glaucoma is highly prevalent, making up 68.6% of total glaucoma instances.[1] The prevalence of POAG varies among countries and races. The prevalence of POAG is 4.2% in the African group, 2.3% in the Asian group, and 1.3% in the Caucasian group. The prevalence of POAG in China is about 1.5–3%. Due to the difference in population size, 52% of the POAG population live in Asian countries, with China and India accounting for 39%, seriously affecting the visual health of the Asian population.[2]

The pathogenesis of POAG is not clear yet. Most scholars believe that POAG is a multifactorial disease, with complication disease induced by various genetic and environmental elements.[3] Specifically, the likelihood and different categories of glaucoma differ based on ethnicity, nation, sex, and familial medical background, suggesting that genetic elements might have a significant impact on the advancement of POAG. Therefore, it is important for studying and screening genes that are linked to the development of POAG, which can be pathogenic or susceptible, as this will aid in the timely prevention, diagnosis, and therapy of POAG.

After researching genome-wide association study (GWAS), nearly 50 genes represent candidate genes that may

Table 1. Summary of SNPs and HWE test in control group.

Gene	Location	SNPs	Consequence	Allele	MAF	HWE *P* in control group
TLR4	9q33.1	rs7873784	3′-UTR	G/c	0.120	0.43
		rs77358523	3′-UTR	A/g	0.175	0.15
		rs752998	3′-UTR	G/t	0.169	0.83
		rs7868859	3′-UTR	A/g	0.243	0.85
GAS7	17p13.1	rs9900085	3′-UTR	A/c	0.430	0.58
		rs74629981	5′-UTR	C/t	0.107	0.12
		rs8012311	5′-UTR	C/a	0.180	0.61
		rs11656696	Intronic[a]	C/a	0.471	0.68

SNP: single-nucleotide polymorphisms; MAF: minor allele frequency; HWE: Hardy–Weinberg equilibrium; *TLR4*: Toll-like receptor 4; UTR: untranslated regions; *GAS7*: growth arrest-specific 7.
[a]The SNP was reported in previous genome-wide association study.

be engaged in the pathogenesis of POAG.[4] However, only OPTN, MYOC, and WDR36 are known to be the causative genes of POAG, and these genes are relatively under-represented in the causative population. There are other unknown genetic influences on the pathogenesis of POAG, and more genetic studies on POAG are still needed to find new pathogenic genes.

Toll-like receptor 4 (*TLR4*) is one of renowned constituents in the TLR group, involved in the identification of pathogens and the control of innate immunity and is expressed in the retina, lymphocytes, and monocytes. In recent years, an increasing number of studies have found evidence that POAG's pathogenesis could potentially involve *TLR4*. Researches have uncovered proof that *TLR4* is linked to the vulnerability of POAG in populations from Mexico and Japan.[4–6] However, no genetic association of *TLR4* with POAG has been found in Saudi populations.[7]

Growth arrest-specific 7 (*GAS7*) belongs to the GAS gene family,[8] which is abundantly present around the ciliary body, trabecular meshwork, sieve plate, optic nerve, and retina of the eye.[9] The relationship between *GAS7* and POAG was first found by Wiggs *et al*.[10] Later on, their association was also found successively in some populations in developed countries;[3,8,11,12] however, there is no significant relationship between *GAS7* and POAG in the Saudi population.[12]

Insufficient evidence exists for pertinent studies conducted on the Chinese population. The study focused on the investigation of the relationship of *TLR4* (rs7873784, rs77358523, rs752998, and rs7868859) and *GAS7* (rs9900085, rs74629981, rs8072311, and rs11656696) polymorphisms with POAG in Shenyang, China. What is more, to further screen the candidate genes *GAS7* and *TLR4* for risk alleles associated with the development of POAG and to provide a genetic basis and targets for exploring their potential pathogenic mechanisms.

Materials and methods

Study population

POAG patients from July 2015 to June 2019 at Shenyang Fourth People's Hospital were selected. This research utilized a case-control study methodology. A total of 218 POAG patients and 252 controls were enrolled. All participants accepted with a comprehensive ophthalmologic examination, including gonioscopy, slit-lamp examination, fundus examination, intraocular pressure (IOP), and the best-corrected visual acuity. In our hospital, doctors can provide a full range of services, including screening, treatment, and emergency care. These POAG patients are initially treated with medication and laser therapy, and surgery is considered if the IOP is poorly controlled with progressive visual field narrowing.

The inclusion criteria were as follows: (1) anterior chamber angle open (Shaffer Level III or Level IV); (2) a characteristic glaucomatous visual field defect; (3) a characteristic glaucomatous optic neuropathy; and (4) no record of angle closure, secondary, or congenital glaucoma. The inclusion criteria for the control group were as follows: (1) patients who underwent examinations, screenings, and medical examinations at our hospital during the same period without a history of glaucoma, ocular hypertension, and other major eye diseases and (2) similar age and gender ratio to POAG patients. Exclusion criteria: patients with systemic diseases, such as a tumor, were also excluded from the controls. The informed written consent was provided. The entire study was approved by the ethics committee at our hospital.

Selection of single-nucleotide polymorphisms and genotyping

For the first time, POAG-related candidate genes *TLR4* and *GAS7* were selected to be studied in a population of Shenyang. Biallelic single-nucleotide polymorphism (SNP) genotypes of Chinese Han in Beijing were downloaded from Ensembl Project (http://asia.ensembl.org/Homo_sapiens/Tools/VcftoPed?db=core), and Haploview 4.2 software was used for analysis (https://www.broadinstitute.org/haploview/haploview). The inclusion criteria of candidate SNPs were as follows: (1) SNPs with function, such as missense variations; (2) SNPs with potential function, such as located in 5′- or 3′-untranslated regions (UTRs) or promoter regions; (3) minor allele frequency >0.1; and (4) SNPs have been reported in previous GWASs. As a result, a total of eight SNPs in *TLR4* and *GAS7* were included in this study (Table 1).

Peripheral blood was drawn from the investigator and used to acquire whole DNA by the Wizard™ Genomic DNA extraction kit (No. A1120, Promega, Madison, WI, USA). Genotyping was conducted by the TaqMan SNP

Table 2. The demographics and clinical characteristics of POAG patients and controls.

	Cases ($n=218$)	Controls ($n=252$)	*P*-value
Age	59.06 ± 9.65	58.98 ± 9.97	0.923
Males (*n* %)	141 (64.7%)	161 (63.9%)	0.859
Intraocular pressure	28.18 ± 5.30	15.09 ± 2.30	<0.05
Cup-to-disk ratio	0.75 ± 0.13	0.31 ± 0.06	<0.05

POAG: primary open-angle glaucoma.

genotyping assay on a 7500 Real-Time polymerase chain reaction (PCR) System (Applied Biosystems, Foster City, CA, USA). The PCR amplification mix was as follows: 20 µL of predesigned SNP-genotyping assay mixture (Applied Biosystems), 8 µL of genomic DNA, and 12 µL of ddH_2O. The PCR reaction was performed with a standard method.

Statistical analysis

SPSS 23.0 was performed in statistical analysis. The Hardy–Weinberg equilibrium of the control group was examined to assess population representativeness. The association between eight SNPs and POAG was analyzed by multiple-factor logistic regression. Haplotype frequencies were calculated by SHEsis statistical analysis platform (http://analysis.bio-x.cn/myanalysis.php; test level $\alpha = 0.05$).

Results

Clinical characteristics of study subjects

A total of 218 POAG patients and 252 controls were included with 64.7% of males in the POAG group and 63.9% of males in the control group with mean age of 59.06 ± 9.65 years in patients and mean age of 58.98 ± 9.97 years in controls. The comparison between them by gender and age had no statistical significance ($P < 0.05$). The IOP of cases and controls was 28.18 ± 5.30 mmHg and 15.09 ± 2.30 mmHg, respectively. The cup-to-disk ratio of cases was elevated compared with that of controls (0.75 ± 0.13 versus 0.31 ± 0.06, $P < 0.05$), as shown in Table 2.

Associations between candidate SNPs and POAG

All SNPs were in Hardy–Weinberg equilibrium among controls (Table 1). The associations between SNPs and POAG of the population of Shenyang, China were shown in Table 3. The rs7873784 of *TLR4* demonstrated a remarkable association with the risk of POAG. The frequencies for the rs7873784 GC ($P = 0.030$), CC ($P = 0.040$), and GC + CC genotypes ($P = 0.009$) were significantly higher compared with GG genotype for POAG patients than that for controls. However, there was no significant association between rs7868859, rs77358523, as well as rs752998 and POAG. There was a notable correlation between rs8072311 and rs9900085 of the *GAS7* gene and POAG. The odds ratios (ORs) of rs8072311 for POAG with CA, AA, and CA + AA genotypes compared with CC homozygotes were 1.564 (95% confidence interval [CI], 1.057–2.316, $P = 0.025$), 2.400 (95% CI, 1.062–5.425, $P = 0.031$), and 1.664 (95% CI, 1.145–2.418, $P = 0.007$), respectively. For rs9900085, the patients with AC, CC, and AC + CC genotypes had a higher risk of POAG than that of the AA genotype.

Haplotypes of *TLR4* and *GAS7* genes in cases and controls

The results of the haplotypes of *TLR4* and *GAS7* were shown in Table 4. In the population of Shenyang, China, there was a notable correlation between the C-A-T-A haplotype (order: rs7873784-rs77358523-rs752998-rs7868859) of the *TLR4* gene and a heightened susceptibility to POAG. The two haplotypes A-C-C-A and C-C-A-C of *GAS7* (order: rs9900085-rs74629981-rs8072311-rs11656696) have a higher risk of POAG (both $P < 0.05$). No correlation was observed between the likelihood of POAG and other haplotypes.

Discussion

Irreversible blindness is primarily caused by POAG, and its incidence has been steadily rising over the past few decades.[13] The pathogenesis of POAG has been demonstrated to be influenced significantly by genetic factors. However, the genetic etiology remains unknown. Therefore, it is necessary to search for POAG-related pathogenic genes and genetic hotspot variants. POAG has an important underlying genetic basis, and many loci related to POAG have been reported by single gene analysis and GWAS. Further investigation is still needed to explore the dependence of risk factors and genetic factors on ethnic and geographic variations.

A study of a population in northeastern China found that the 3′-UTR of *TLR4* was related to a higher likelihood of developing POAG. One study reported that carriers carrying the rs7873784C allele had a larger potential of POAG than those carrying the wild-type gene. Haplotype analysis showed that there were seven major haplotypes constructed from the four SNPs (order: rs7873784-rs77358523-rs752998-rs7868859). Only the C-A-T-A haplotype, constructed by rs7873784 C allele, was associated with a higher risk of POAG (OR = 3.327). In contrast, all five haplotypes, constructed by rs7873784 G allele, showed no significant link to an increased likelihood of POAG. Those data of genotype and haplotype demonstrated that rs7873784 could be used as a predictive marker of POAG. In addition, this study found no significant association between rs7868859, rs77358523, and rs752998 and the risk of POAG.

SNPs in *TLR4* could influence the risk of multifactorial disorders, including several inflammatory disorders, immune disorders, and glaucoma. D299G (rs4986790), T399I (rs4986791), and rs2149356 are three most studied SNPs in different glaucoma populations.[3] Navarro-Partida *et al.*[5] found that rs4986791 and rs4986790 of *TLR4* put Mexicans at increased likelihood of POAG. Nevertheless, this finding is in contrast to studies in Saudi Arabian populations, where there was no association between rs4986791 and POAG in Saudi Arabs.[14] Many studies reported that rs2149356 was related to an increased likelihood of developing POAG in different races, such as Mexican,[15] China,[16] and Japan.[6] And multiple SNPs (rs1927914, rs10759930, rs1927911,

Table 3. Genotype distributions and associations between SNPs and POAG.

Gene	rs No.	Genotype	Cases	Controls	OR (95% CI)	*P*-value
TLR4	rs7873784	GG	146	196	–	–
		GC	61	51	1.606 (1.045–2.466)	0.030*
		CC	11	5	2.953 (1.004–8.685)	0.040*
		GC + CC	72	56	1.726 (1.146–2.600)	0.009*
	rs77358523	AA	143	175	–	–
		AG	61	66	1.131 (0.749–1.708)	0.558
		GG	14	11	1.558 (0.686–3.536)	0.286
		AG + GG	75	77	1.192 (0.809–1.756)	0.374
	rs752998	GG	139	173	–	–
		GT	68	71	1.192 (0.799–1.779)	0.390
		TT	11	8	1.711 (0.670–4.371)	0.257
		GT + TT	79	79	1.245 (0.848–1.826)	0.263
	rs7868859	AA	116	145	–	–
		AG	92	93	1.237 (0.848–1.804)	0.270
		GG	10	14	0.893 (0.383–2.084)	0.793
		AG + GG	102	107	1.192 (0.827–1.716)	0.346
GAS7	rs9900085	AA	52	85	–	–
		AC	115	119	1.580 (1.028–2.428)	0.037*
		CC	51	48	1.737 (1.029–2.933)	0.038*
		AC + CC	166	167	1.625 (1.082–2.440)	0.019*
	rs74629981	CC	164	199	–	–
		CT	45	47	1.162 (0.735–1.837)	0.521
		TT	9	6	1.820 (0.635–5.219)	0.259
		CT + TT	54	53	1.236 (0.803–1.904)	0.335
	rs8072311	CC	119	168	–	–
		CA	82	74	1.564 (1.057–2.316)	0.025*
		AA	17	10	2.400 (1.062–5.425)	0.031*
		CA + AA	99	84	1.664 (1.145–2.418)	0.007*
	rs11656696	CC	65	75	–	–
		CA	104	122	0.984 (0.644–1.501)	0.939
		AA	49	55	1.028 (0.618–1.709)	0.915
		CA + AA	153	177	0.997 (0.671–1.483)	0.990

SNP: single-nucleotide polymorphism; POAG: primary open-angle glaucoma; OR: odds ratio; CI: confidence interval; *TLR4*: Toll-like receptor 4; *GAS7*: growth arrest-specific 7.
*$P < 0.05$.

Table 4. Haplotypes of *TLR4* and *GAS7* gene in cases and controls.

Gene	Haplotype	Cases	Controls	OR (95% CI)	*P*-value
TLR4	G-A-G-A	85 (39.0%)	106 (42.1%)	0.942 (0.721–1.232)	0.665
	G-A-G-G	26 (11.9%)	42 (16.7%)	0.702 (0.482–1.021)	0.063
	G-A-T-A	17 (7.80%)	26 (10.3%)	0.775 (0.493–1.219)	0.269
	G-G-G-A	26 (11.9%)	32 (12.7%)	0.974 (0.656–1.446)	0.897
	C-A-G-A	17 (7.80%)	18 (7.14%)	1.165 (0.718–1.899)	0.535
	G-A-T-G	12 (5.50%)	7 (2.78%)	1.976 (0.988–3.950)	0.050
	C-A-T-A	7 (3.20%)	3 (1.20%)	3.327 (1.204–9.190)	0.014*
GAS7	C-C-C-C	53 (24.3%)	63 (25.0%)	1.030 (0.762–1.393)	0.847
	A-C-C-C	35 (16.0%)	54 (21.4%)	0.743 (0.532–1.039)	0.082
	A-C-A-C	26 (11.9%)	25 (9.92%)	1.353 (0.893–2.049)	0.153
	A-C-C-A	26 (11.9%)	44 (17.5%)	0.665 (0.458–0.965)	0.031*
	C-C-C-A	20 (9.17%)	19 (7.54%)	1.286 (0.807–2.050)	0.290
	C-C-A-C	10 (4.59%)	5 (1.98%)	2.640 (1.227–5.681)	0.010*

TLR4: Toll-like receptor 4; *GAS7*: growth arrest-specific 7; OR: odds ratio; CI: confidence interval.
*$P < 0.05$.

rs12377632, and rs7037117) in the *TLR4* gene are associated with POAG.[6,17] However, there are some inconsistent results about the *TLR4* gene and POAG. Suh *et al*.[18] showed that polymorphisms (rs10759930, rs1927911, rs2149356, rs1927914, rs12377632, rs7037117, rs11536889, and rs7045953) are not associated with normal tension glaucoma. Chen *et al*.[19] found a non-significant association between rs7037117 and POAG in the southern Chinese population, but not in the northern Chinese population. The conflicting results may be used for the different populations and races.

The first association of *GAS7* with POAG was seen in a US population. The relevant mechanisms are not clear yet. Up to now, only a few SNPs were reported to be associated with POAG, such as rs9913911,[20,21] rs9897123, rs8080535,[1] and rs11656696.[11,12] rs11656696 is the most studied SNP in different glaucoma populations with different results. van Koolwijk *et al*.[8] first identified the strongest association rs11656696 with IOP. But another study[7] illustrated that in the Saudi population, rs1165661996 did not show any association with POAG or its endophenotypic characteristics, and it was not considered as a risk factor of POAG.

Our study explored *GAS7* in the population of Shenyang, China. The rs8012311 and rs9900085 were first identified to be associated with a high risk of POAG in this study. The result of genotypes and haplotypes showed that rs9900085 C allele and rs8072311 A allele were the risk factors of POAG. The patients with AC or CC genotypes of rs9900085 and CA or AA genotypes of rs8072311 had a higher risk of POAG than those with CC of rs9900085 and AA of rs8072311. The similar relationship was also reported in the dominant allele model. The haplotype analysis illustrated that the individuals with the A-C-C-A haplotype, constructed with minor allele of rs9900085, and C-C-A-C haplotype, constructed with minor allele of rs8072311, had an increased prostate cancer risk.[22] The results of genotype distributions and haplotype analysis suggested that *GAS7* gene is the candidate POAG risk gene. No significant distinction was observed between the cases and controls for rs11656696 and rs74629981, aligning with the Saudi population.

In conclusion, this study is intended to be the first validation of a case-control study of the population of Shenyang. The polymorphisms of rs7868859 of *TLR4* and rs8012311 and rs9900085 of *GAS7* were related to POAG in the population of Shenyang, China. In this article, we only focused on the genetic characteristics of POAG in Shenyang, China, and we need to collect a larger sample size in a larger population in the future. More loci and samples in various populations are required to confirm our results and determine the function of *TLR4* and *GAS7* genes during the development of POAG.

AUTHORS' CONTRIBUTIONS

JJ was in charge of designing the project, performed the experiment, collected the data, and wrote the article. TW analyzed the data. All authors reviewed the article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The research received approval from the ethics committee at Shenyang's Fourth People's Hospital.

ORCID ID

Jiao Jie https://orcid.org/0009-0000-1207-2923

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(Received May 3, 2023, Accepted August 30, 2023)