·Basic Research·

The association between matrix metalloprotease-9 gene polymorphisms and primary angle-closure glaucoma in a Chinese Han population

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Received: 2013-10-20 Accepted: 2014-02-21

Abstract

• AIM: To examine the association between the single nucleotide polymorphisms (SNPs) of matrix metalloprotease-9 *(MMP-9)* gene and primary angle-closure glaucoma (PACG) in a Chinese Han population.

• METHODS: DNA samples were extracted from peripheral-blood mononuclear cells of 214 PACG patients and 224 healthy controls. Genotyping of rs3918249, rs3918254, rs17577 and rs3787268 in *MMP-9* was performed using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis and the direct sequencing technique. The association between these genetic polymorphisms and risk of PACG was estimated by χ^2 test.

• RESULTS: The distributions of rs3918249, rs3918254, rs17577 and rs3787268 genotypes among cases and healthy controls were compatible with that from Hardy-Weinberg equilibrium (HWE, *P*>0.05).The increased frequency of CC and CT genotypes of rs3918254 were observed in PACG patients compared to healthy controls [*P*=0.006, *P* corrected (Pcorr) =0.048]. The haplotype analysis showed that the CCGG haplotype was nominal associated with PACG (*P*=0.015),however, the significant was lost when the Bonferroni correction was used (Pcorr=0.105).

• CONCLUSION: Our results revealed that rs3918254 in *MMP-9* may be a susceptible locus to PACG in China, people with the CC and CT genotypes of rs3918254 are more susceptible to PACG. The susceptibility to PACG in Chinese Han patients may be not influenced by SNPs rs3918249, rs3787268 and rs17577 in *MMP-9*.

• **KEYWORDS:** matrix metalloprotease-9 gene; primary angle-closure glaucoma; single nucleotide polymorphisms
DOI:10.3980/j.issn.2222-3959.2014.03.02

Gao XJ, Hou SP, Li PH. The association between matrix metalloprotease-9 gene polymorphisms and primary angle-closure glaucoma in a Chinese Han population. *Int J Ophthalmol* 2014;7(3): 397-402

INTRODUCTION

Glaucoma is considered to be the second leading cause of irreversible blindness diseases [1]. It is estimated to affect about 79.6 million people in 2020; among them, about 11.2 million patients are bilaterally blind[2]. Two major forms of glaucoma are primary glaucoma and secondary glaucoma. The primary glaucoma is classified to primary open angle glaucoma (POAG) and primary angle-closure glaucoma (PACG). The incidence of the two kinds of primary glaucoma is different around the world. PACG is the main type in the Asian population[3]; however, it is very rare in the west countries. PACG has been estimated to cause more blindness than POAG worldwide [4]. In Singapore, China and India, closured angle is responsible for the most bilateral glaucoma-caused blindness[5-7].

PACG patients have some common anatomic structures, such as the shallow anterior chamber, increased thickness of the lens, narrow anterior chamber angle, short axial length, and hypermetropic refraction error [8,9]. The risk factors for developing PACG include age, female gender, and family history [10]. Siblings of Chinese patients with PAC or PACG have almost a 50% probability of having narrow angles and are more than 7 times more likely to have narrow angles than the general population[11].

The relationship between gene and PACG remains unclear. Some tissue remodeling genes, including frizzled-related protein (MFRP), extracellular matrix metalloprotease-9 (*MMP-9*), methylenetetrahydrofolate reductase (MTHFR), and hepatocyte growth factor (HGF), have been reported to be associated with PACG [12-16]. Even though heat shock protein 70 (HSP70) does not regulate tissue remolding directly, the gene regulates the expression of matrix

metalloproteinases (MMPs) and is thought to be associated with PACG[12,17].

MMP-9 is an enzyme, which is also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B (GELB). The MMP-9 is important in remodeling of the extra-cellular matrix (ECM) during homeostasis and remodeling, which is one of tightly regulated family of zinc dependent enzyme[18]. Studies have established that MMPs were involved in reducing resistance to the aqueous humor outflow within the eye [19-21]. Human and animal studies have indicated that intraocular *MMP-1, 2, 3* and *9* were expressed in the aqueous humor outflow pathway (aqueous humor and iridocorneal angle tissue) [20,22]. In humans, particularly changes in *MMP-2* and *9* have been involved with the process of glaucoma [23,24]. Compare to healthy eyes, *MMP-2* and *9*are highly expressed in glaucomatous eyes [25,26]. This change has been found in the aqueous humor, iridocorneal angle tissue and Tenon's capsule in patients with POAG, PACG and exfoliation glaucoma (ExG)[23,24,27,28]. Above all, *MMP-9* may play an important role in PACG. We, therefore, aimed to assess the association of *MMP-9* with PACG in Chinese Han population.

MATERIALS AND METHODS

This study was approved by the ethics committees of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and was adhered to the tenets of the Declaration of Helsinki. The written informed consent was obtained from each participant or his or her legal guardian.

Materials Blood samples were collected from 214 PACG patients and 224 gender-, ethnically-matched unrelated healthy Chinese Han people. All the patients and controls were recruited from Department of ophthalmology at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China).

PACG was considered as the presence of glaucomatous optic neuropathy with compatible visual field loss, associated with a closed angle on indentation gonioscopy. A closed angle was considered as the presence of at least 180 degrees of angle in which the trabecular meshwork was not visible on gonioscopy. The typical glaucomatous optic nerve injury was considered as a cup/disc (C/D) ratio over 0.5 by funduscope examination. The typical glaucomatous visual defect (paracentral scotoma, arcuate scotoma, nasal step *etc*) was examined by Humphrey Field Analyzer 30-2 pattern (Humphrey Field Analyzer, Zeiss/Humphrey Systems, Dublin, California, USA).

Each subject was provided a complete ophthalmic examination. Ophthalmological examinations included visual acuity, slit lamp biomicroscopy, interocular pressure (IOP) measurement, ophthalmoscopic observations, A-mode ultrasonoscope, perimetry. All the patients were selected by the criteria which was refer to Cong *et al*[29]: 1) age >thirty years old; 2) narrow anterior chamber angle; at least, 180 degrees of closed angle; 3) IOP level over 21 mmHg; 4) typical glaucomatous visual defect by Humphrey Field Analyzer 30-2 pattern (Humphrey Field Analyzer, Zeiss/Humphrey Systems, Dublin, California, USA); 5) typical glaucomatous optic never injury, C/D ratio over 0.5 by funduscope examination; 6) secondary glaucoma was excluded, such as uveitis, trauma or lens subluxation. The control group includes two hundred and twenty-four healthy people who have the same examinations with the case group. The inclusion criteria of the healthy controls included the following: 1) no symptoms or signs of PACG; 2) no glaucoma history; 3) no glaucoma family history; 4) no ocular disease.

Single Nucleotide Polymorphisms Selection Four single nucleotide polymorphisms (SNPs) including rs3918254, rs3787268, rs17577, rs3918249 were enrolled in this study as candidate SNPs. The choice of candidate SNPs was based on the study reported by Awadalla *et al*[13]. In that study, SNPs rs3918249, rs17576 were found to be significant associated with PACG; SNPs rs3918254, rs3787268, rs17577 were not associated with PACG. Haplotype blocks were analyzed using Hapmap data by Haploview 4.0. The results showed that SNPs rs3918261, rs4810482, rs3918254 were tag SNPs (Figure 1) of *MMP-9*. The ratio of genotyping of tag SNP rs3918261 was lower than 60%. It may influence the results of our study, so the result of this SNP locus was not involved in the statistics. From Haplotype blocks, we know that tag SNP rs3918261 and SNP rs2274756 are completely linked (Figure 1). Tag SNP rs3918261 can be represented by SNP rs2274756 which has merged into rs17577 (http://www.ncbi.nlm.nih.gov/snp/?term=rs2274756). So rs17577 was also detected in our study.

Tag SNP rs4810482 and SNP rs17576 are in the same linkage block and they are strongly linked (Figure 1). However, SNP rs17576 were not associated with the PACG risk in Chinese population[29]. The linkage block including tag SNP rs4810482 maybe not associated with PACG in China. So this tag SNP was not involved in our study.

Considering the different genetic background between Chinese and Caucasian, the associated SNP rs3918249 was also involved in our study. In a word, the four SNPs rs3918254, rs3787268, rs17577, rs3918249 were enrolled in this study as candidate SNPs.

DNA Extraction Genomic DNA was extracted from peripheral-blood mononuclear cells (PBMCs), using Promega DNA Mini Blood kit (Promega Corp. Madison, WI, USA). DNA samples were collected into a 1.5 mL Eppendorf tube and stored at -20℃ till use.

Genotyping The genotyping of *MMP-9* (rs3918249, rs3918254, rs17577 and rs3787268 in dbSNP) was detected by polymerase chain reaction fragment length polymorphism

Table 1 Primers and restriction enzymes of rs3918249, rs3918254, rs17577 and rs3787268 used for PCR-RFLP

Name	SNP	Restriction enzyme	Primer sequence (5'→3')	Product length (bp)
rs3918249	C/T	BfaI	F: 5' CCCTGGGTGGTCAGAAAG 3' R: 5' GGCTGGGCTCAAACTCCT 3'	153bp
rs3918254	C/T	KpnI	F: 5' CCACCGCCAACTACGAC 3' R: 5' CCATTGGAAGTCAGGGATAG 3'	223bp
rs17577	A/G	STYI	F: 5'GGCTCAGCACCTGTCTCCT 3' R: 5'GCTTTTTCTTCCTCGCTCAG 3'	225bp
rs3787268	A/G	TAQI	F: 5'GGCCATAGAGGATGTCGCTTAAATC 3' R: 5'TCTCAGTGAAGCCTCCTCTGGC 3'	110bp

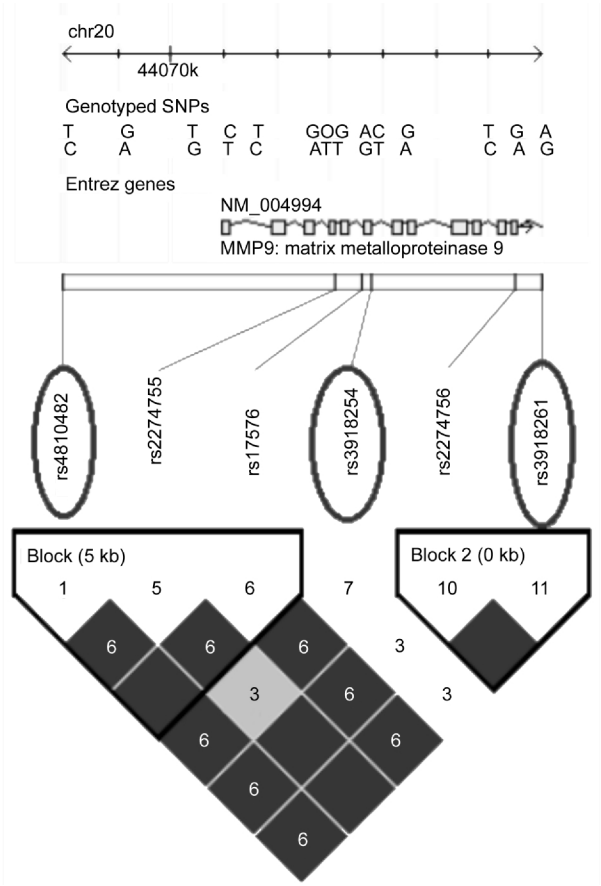

Figure 1 Haplotype blocks´ structure of the tag SNPs of *MMP-9* analyzed using Hapmap data. The circled SNPs rs4810482, rs3918254, rs3918261 are the tag SNPs of *MMP-9*

(PCR-RFLP) analysis. Four pairs of primers (Table 1) were designed by primer premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). PCR reactions underwent the following conditions: 5min at 95℃, followed by 37 cycles at 95℃ for 30s, annealing at different temperatures (63℃ for rs3918249, 60℃ for rs3918254 and rs17577, 62℃ for rs3787268) for 30s, 72℃ for 30s, and then for 5min at 72℃ . PCR products of rs3918249, rs3918254, rs17577 and rs3787268 were respectively digested with four units of BfaI, KpnI, STYI, and TAQI (MBI Fermentas, Burlington, ON, Canada) restriction enzymes (Table 1) in a 10-μL reaction volume at 37℃ (except rs3787268 with TAQI in 65℃) for 3-16h. Then, the samples were put into 4% agarose gel, which was stained by GoldView (SBS Genetech, Beijing, China), to do electrophoresis reaction. Ten percent of PCR products were randomly selected to directly sequence to confirm the PCR-RFLP results (Majorbio Biotech Co. Ltd., Shanghai, China).

Statistical Analysis Statistical analysis was performed using the SPSS software (version 17.0, SPSS, Inc., Chicago, IL, USA). Haploview 4.0 was used to examine the linkage disequilibrium (LD) of the tested four SNPs. Hardy-Weinberg equilibrium (HWE) was tested by the χ^2 test. The Chi-square test and Fisher's exact test were used in the study. The P values were corrected (Pc) with the Bonferroni correction by multiplying with the number of analyses. Pc<0.05 was considered as significance.

RESULTS

Four hundred thirty-eight Chinese Han people were involved in the study, including 214 PACG cases [average age (mean±SD)=61.86±9.46] and 224 healthy controls [average age (mean±SD)=61.92±6.03]. The baseline characteristics of study participants were showed in Table 2. All the genotype distributions did not deviate from Hardy-Weinberg equilibrium (P>0.05). The results of genotyping were confirmed by the directly sequence of samples. The results of directly sequence were showed in Figure 2.

The CC and CT genotypes frequency of rs3918254 showed a significant increase in PACG patients compared to healthy controls (P=0.006, Pc=0.048). The odds ratio (OR) of CC and CT genotypes is 4.397 and 95% confidence interval (95%CI) is between 1.455 and 13.289 (Table 3). However, we did not find the association between other three SNPs rs3918249, rs3787268, rs17577 and PACG. Haplotype analysis was also performed in this study (Figure 3). The haplotype analysis showed the CCGG haplotype had a nominal significant difference with PACG (P=0.015), however, it did not survive after correction by multiple testing (Pc=0.105) (Table 4).

In this study, a power calculation was also performed by online software (http://stat.ubc.ca/~rollin/stats/ssize/caco.html) using the prevalence data of China population reported by Song *et al*[30]. We presumed that the prevalence in China is 0.0142 for PACG[30]. The samples size (214 cases and 224 controls) can reach 70% power to detect a 4.397 odds ratio (OR) value at the 5% significance level.

Table 2 Baseline characteristics of study participants

Groups	*n*	Sex		Age (a)		Anterior chamber depth (mm)	
		M *n* (%)	F *n* (%)	$\bar{x} \pm s$	Range	Right eye	Left eye
PACG	214	64 (29.91)	150 (70.09)	61.86±9.46	40-85	1.95±0.40	2.02±0.40
Control	224	70 (31.25)	154 (68.75)	61.92±6.03	50-83	N/A	N/A

Table 3 Frequencies of alleles and genotypes of MMP-9 polymorphisms in PACG patients and controls

SNP	Genotype/Allele	PACG *n* (%)	Controls *n* (%)	χ^2	*P*	*P*c	OR (95CI)
rs3918249		*n*=213	*n*=221				
	CC	91(42.7)	104(47.1)	0.747	0.387	NS	0.846 (0.579-1.236)
	CC+CT	200 (93.8)	207 (93.7)	0.010	0.920	NS	1.041 (0.477-2.269)
	C *vs* T	291 (68.3)	311 (70.4)	0.430	0.512	NS	0.908 (0.680-1.212)
rs3918254		*n*=214	*n*=220				
	CC	125 (58.4)	122 (55.5)	0.387	0.534	NS	1.128 (0.771-1.650)
	CC+CT	210 (98.1)	203 (92.3)	8.085	[1]0.006	0.048	4.397 (1.455-13.289)
	C *vs* T	335 (78.3)	325 (73.9)	2.313	0.128	NS	1.275 (0.932-1.743)
rs3787268		*n*=213	*n*=220				
	AA	19 (8.90)	23 (10.5)	0.291	0.590	NS	0.839 (0.443-1.590)
	AA+AG	131 (61.5)	131 (59.5)	0.173	0.677	NS	1.085 (0.738-1.596)
	A *vs* G	150 (35.2)	154 (35.0)	0.004	0.948	NS	1.009 (0.764-1.334)
rs17577		*n*=214	*n*=224				
	AA	4 (1.90)	3 (1.30)	0.195	[1]0.719	NS	1.403 (0.310-6.344)
	AA+AG	48 (22.4)	46 (20.5)	0.233	0.629	NS	1.119 (0.709-1.766)
	A *vs* G	52 (12.1)	49 (10.9)	0.315	0.574	NS	1.126 (0.744-1.705)

[1]Fisher's exact test; PACG: Primary angle-closure glaucoma; NS: Not significant; *P*c: Bonferroni corrected *P* value; OR: Odds ratio; 95CI: 95 Confidence interval.

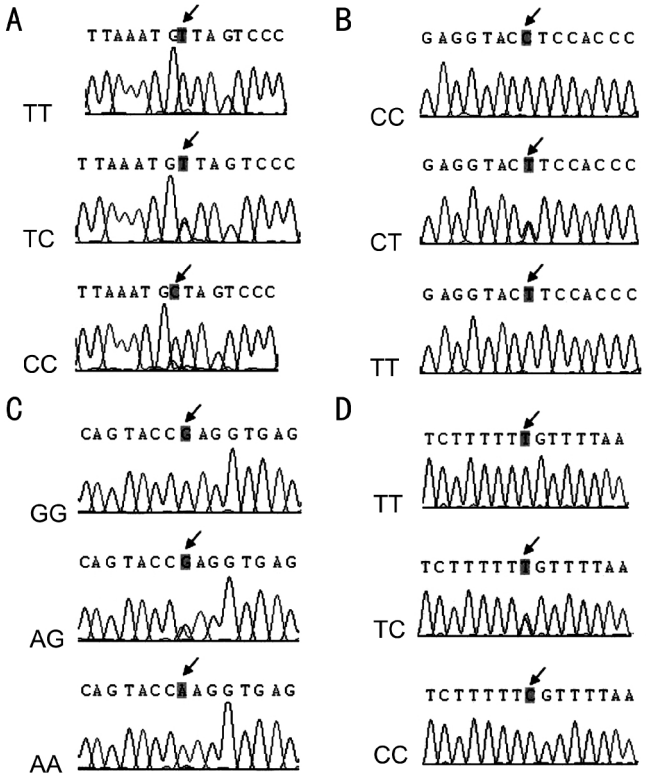

Figure 2 The maps represent the results of directly sequence
A: The maps represent the TT, TC and CC genotypes of rs3918249; B: The maps represent the CC, CT and TT genotypes of rs3918254; C: The maps represent the GG, AG and AA genotypes of rs17577; D: The maps represent the TT, TC and CC genotypes of rs3787268.

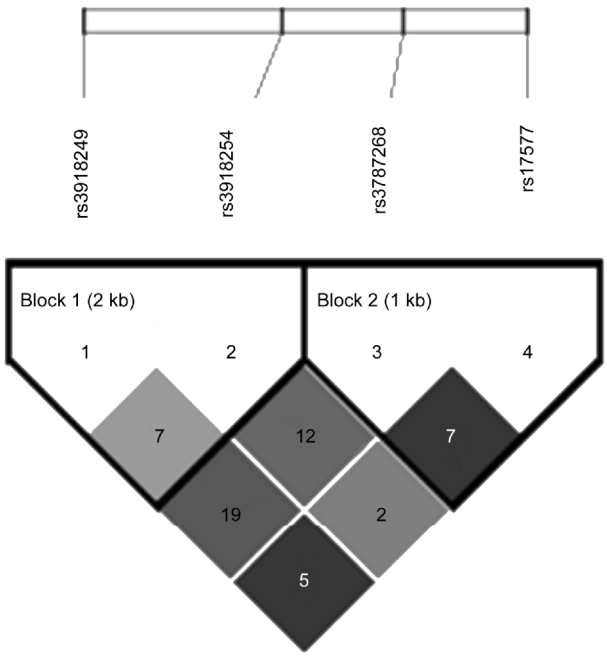

Figure 3 Structure of linkage disequilibrium (LD) of Han population studied in this text. The colour of the box represents D´ value, the number in the box represents the r^2 value.

DISCUSSION

Although PACG is thought to be caused by the spatial and anatomical relationships between the lens and the anatomy of the angle, the iris and the lens, genetic factors are also considered to play an important role in this disease. Some

Table 4 Haplotype ferquences in PACG patients and controls

Haplotype	Freqence	χ^2	*P*	*P* correct
CCAG	0.326	0.261	0.609	NS
TCGG	0.259	3.475	0.062	NS
CTGG	0.197	0.116	0.734	NS
CCGA	0.106	0.311	0.577	NS
CCGG	0.051	5.871	0.015	0.105
TTGG	0.025	3.392	0.660	NS
TCAG	0.017	0.248	0.619	NS

NS: Not significant.

studies have been performed to investigate the risk genes of PACG [12,14-16,31]. It has been reported that the first-degree relatives of PACG patients are at a 6 to 9 fold increased risk of developing PACG.

The *MMP-9* gene is located on chromosome 20q11.2-q13.1, which contains 13 exons. *MMP-9* protein plays an important role in extracellular matrix remodeling by cleaving denatured collagen and type IV collagen in the basement membrane[18]. Additionally, some SNPs of *MMP-9* had been demonstrated to be associated with PACG [13,14,29,32]. Wang *et al* [32] found SNP rs2664538 in *MMP-9* was significantly associated with acute PACG patients in Taiwanese. Cong *et al* [29] found a positive association between SNP rs2250889 in *MMP-9* and PACG. In addition, there was significant association between SNPs rs17576, rs3918249 and PACG in a Caucasian population and in a Pakistan population [13,14]. Recently, Awadalla *et al* [13] examined five SNPs (rs3918249, rs17576, rs3918254, rs3787268, rs17577) of *MMP-9* in a Caucasian population and found a new risk SNP rs3918249in *MMP-9* to PACG patients. These five SNPs of *MMP-9* have not been studied in Chinese Han population except rs17576, which was not associated with PACG in southern China[29]. So, rs3918249, rs3918254, rs17577 and rs3787268 were selected as candidate SNPs, which had not been studied in Chinese.

Since one candidate gene has numerous SNPs and only a few of them may be involved in the development of the disease, it is extremely important to select the appropriate SNPs. We consider the following factors to chose SNPs: 1) rs3918249, rs3918254, rs17577 and rs3787268 of *MMP-9* were associated with PACG in other ethnic populations, however, no reports was performed in Chinese population; 2) The tag SNPs were chosen in this study.

Our results showed that the frequencies of CC and CT genotypes of rs3918254 were increased in PACG patients comparing to healthy controls. The OR of CC and CT is 4.397, suggesting that people with CC and CT genotypes may be more susceptible to PACG. The other three SNPs of *MMP-9*, rs3918249, rs3787268 and rs17577 did not find an associate result. The haplotype analysis showed the CCGG haplotype had a nominal significant difference with PACG, and it needs to perform the further studies. The reason why our results were not agreed with Caucasian study may arise from genetic heterogeneity. rs17576 in *MMP-9* showed significant difference between PACG patients and healthy controls in Caucasian population[13] and Pakistan population[14], but lack of association in southern China[29]. So, more studies need to perform to prove the relationship between *MMP-9* gene and PACG.

As a case-control study, we restricted our study population to minimize confounding by ethnic variation. And the number of volunteers enrolled in this study (214 PACG patients and 224 gender-, ethnically-matched healthy controls) was large enough to avoid a bias of the results.

Some limitations also involve in the study. The present study only examined the association between the *MMP-9* polymorphisms and PACG but did not refer to other related genes. Our study does not exclude the possibility that other SNPs of *MMP-9* are associated with this disease. More studies are needed to clarify this issue.

In conclusion, SNP rs3918254, but not SNPs rs3918249, rs3787268 and rs17577, in *MMP-9* was associated with increasing PACG risk in Chinese Han population.

ACKNOWLEDGEMENTS

The authors would like to thank all donors enrolled in the present study.

Foundation: Supported by National Natural Science Foundation of China (No.81270990; No.81070723)

Conflicts of Interest: Gao XJ, None; **Hou SP,** None; **Li PH,** None.

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