

RESEARCH ARTICLE

The relationship among angiotensinogen genes polymorphisms and hs-CRP and coronary artery disease

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Objective: To assess the association of gene polymorphisms of angiotensinogen (AGT), the key factor in rennin-angiotensin-aldosterone system (RAAS), with high-sensitivity C-reactive protein (hs-CRP) and coronary artery disease (CAD).

Methods: The current study recruited the patients who were hospitalized and assessed by coronary angiography for suspected CAD. The patients with documented CAD served as CAD group (n = 492) while the patients without documented CAD (n = 87) served as control group. We compared laboratory data and CAD risk factors between the two groups. Furthermore, we analyzed the association of AGT M235T, G217A, G152A, G-6A, A-20C genotypes with coronary artery stenosis and in-stent restenosis.

Results: There were significantly differences between two patient groups in sex, smoking history, diabetes mellitus, carotid atherosclerosis, lower limb arteriosclerosis, hs-CRP, blood glucose, and the level of high-density lipoprotein (HDL; $P < 0.05$). In CAD group, hs-CRP levels increased with increasing number of coronary artery branches (1, 2, or ≥ 3 ; $P < 0.01$), and Gensini integral was positively correlated with hs-CRP levels ($r = 0.361$, $P < 0.01$). Frequencies of genotype and allele distribution in individual angiotensinogen loci (M235T, G217A, G152A, G-6A, A-20C) did not differ in two patient groups. Following stratification of patients according to hs-CRP levels (< 1 mg/L, 1-3 mg/L, and > 3 mg/L), the distribution frequency of allele M235T was statistically different among the groups ($P < 0.05$).

Conclusion: In CAD patients, M235T among several AGT gene polymorphisms is associated with elevated hs-CRP levels with AGT C allele as the significant factor for patients with hs-CRP level of more than 1 mg/L.

KEYWORDS

angiotensinogen, coronary artery disease, genes polymorphisms, high-sensitivity C-reactive protein, rennin-angiotensin-aldosterone system

Zhu and Lin contributed equally to this work.

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1 | INTRODUCTION

Coronary artery disease (CAD) is a common cardiovascular disease, with a high mortality in acute coronary events. Approximately 17 million people die from CAD annually worldwide.¹ CAD presents a series of clinical symptoms characterized by precordial pain caused by coronary artery stenosis, myocardial insufficiency, myocardial anoxia caused by coronary artery lipid deposition, spasm, atherosclerosis, and increased myocardial oxygen consumption. Etiology studies have shown that CAD is a chronic complex disease mediated by multiple genes and interactions between genes and environment, of which organism inflammation reaction may lead to vascular endothelial damage, which plays an important role in the process of atherosclerosis and promotes the onset of CAD.²

Angiotensinogen (AGT) encodes the precursor of angiotensin (Ang) that is the only known renin substrate. AGT forms Ang II under the action of renin and angiotensin-converting enzyme (ACE). In renin-angiotensin system (RAS), Ang II regulates vasoconstriction, water-electrolyte metabolism, aldosterone biosynthesis, important for angiotasis, cardiac, and vascular remodeling. Binding of Ang II to Ang II type 1 receptor (AT1R) stimulates vascular endothelial cells (VEC) to increase release of chemotactic factor, promote neutrophil aggregation, and induce the onset of atherosclerosis. Ang II also stimulates the growth of vascular smooth muscle cells, increases the proliferation of myocardial cells and intima, increases the activity of sympathetic nerve system, increases the resistance of the coronary artery, and even induces arrhythmia. It also contracts coronary artery, and even rupture the plaque, leading to local thrombosis.³ Therefore, the relationship between the RAS gene AGT and the pathological process of CAD has obtained wide attention.

C-reactive protein (CRP) is an acute phase inflammatory response protein synthesized in the liver. In homeostatic condition, CRP levels are low, but can increase by 1000 times when infection, trauma, surgery, and other inflammatory events occur for 7-12 days.⁴ In prospective studies, early warning effect of high-sensitivity C-reactive protein (hs-CRP) on CAD risk is higher than that of traditional biochemical markers (such as TC, HDL-C, LDL-C, and Lp (a)). Meanwhile, only hs-CRP and TC/HDL-C are independent risk factors⁵ in multivariate analysis of other CAD risk factors. Three categories of hs-CRP levels (<1, 1-3 and >3 mg/L) have been used to stratify cardiovascular risk. Because some lipid levels fail to effectively alert coronary artery cases, the use of both TC and hs-CRP will increase the opportunity to predict coronary artery events.⁶

In this study, we analyzed the relationship of AGT gene in RAS system and hs-CRP levels with Chinese Han population CAD patients in Zhejiang, Taizhou, China, to provide the basis for molecular prediction of the degree of vascular lesions in CAD patients.

2 | MATERIAL AND METHODS

2.1 | Patients

This study prospectively recruited the patients who were hospitalized for suspected coronary artery disease and tested by

coronary angiography at Taizhou hospital during January 2010 and December 2016. Baseline characteristics of the enrolled patients should be detailed recorded, including age, gender, smoking history, drinking history, hypertension, diabetes, chronic obstructive pulmonary disease (COPD), valvular heart disease, atrial fibrillation, cardiac dysfunction, carotid atherosclerosis, and the level of serum troponin, TG, HDL, LDL, apo A1, apo B, lipoprotein a, and hs-CRP. (Routine biochemistry test were performed on Beckman Coulter All 5800 (American) by Turbidimetric inhibition immune assay, and the level of troponin were tested on Beckman DKI 800 (American) by Double antibody sandwich method, and the level of hs-CRP were measured on OTTOMAN (China) by Particle enhanced immunoturbidimetric method.) Exclusion criteria: including secondary hypertension, the history of aorta surgery, pulmonary or connective tissue disease, cardiomyopathy, congestive heart failure (CHF), congenital heart disease, active cancer, infective endocarditis, syphilis, rheumatic heart disease (RHD), medium or severe valve disease, diabetes, and chronic kidney disease. All the patients were recruited from the Taizhou Zhejiang province Chinese Han population, none of them were related to each other, and all subjects signed informed consent. After filtrated by these precondition, a total of 579 patients were included, among them 492 were diagnosed as CAD according to the World Health Organization guidelines,⁷ and 87 were diagnosed as non-CAD.

2.2 | Evaluation of coronary artery stenosis degree

Coronary angiography (CAG) was conducted by at least two experienced clinicians. At least two senior clinicians evaluate CAG and the degree of coronary artery stenosis independently. Then, we used the numbers of lesion vessels and the Gensini Score to quantify the degree of coronary artery stenosis as previously reported.⁸

2.3 | AGT genotyping

2.3.1 | Blood collection and DNA extraction

The 2 mL of peripheral venous blood was drawn from each patient. Genomic DNA was extracted from peripheral blood leukocytes using DNA Extraction kit according to manufacture's instruction (GK1042; Shanghai Generay Biotech Co., Ltd, Shanghai, China), dissolved with appropriate volume of TE buffer (pH8.0), and were measured by nucleic acid analyzer. Only samples with a ratio of DNA at 1.7-2.0 were used as for polymerase chain reaction (PCR).

2.3.2 | Primer synthesis

Specific primers of five AGT single-nucleotide polymorphisms (SNP), including M235T, G217A, G152A, G-6A, A-20C were designed using Primer Premier 5.0 (Table 1).

TABLE 1 The primer sequence of AGT gene SNP sites

SNP site	Sense(5'-3')	Anti-sense(5'-3')	Tm (°C)	Fragment size (bp)
M235T	TCATGGTGGTGGGCGTGTT	CCAGGAGATGTGGGTTTC	54	618
G217A	CCTGCAAACCTTCGGTAAATG	AGCGGAAGAAGGAAGACCTGACCAT	54	531
A-20C				
G-6A				
G152A				

AGT, angiotensinogen; SNP, single-nucleotide polymorphisms; Tm, temperature.

2.3.3 | Determination of AGT polymorphism

AGT gene polymorphism were determined by polymerase chain reaction-restriction fragment length polymorphism and confirmed by direct sequencing. Genomic DNA (0.1 µg) was amplified by PCR under the conditions included preheating at 95°C for 2 minutes for initial denaturation, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at their own annealing temperature (as per list enclosed) for 45 seconds, and extension for 1 minute at 72°C, with a final extension for 10 minutes at 72°C. PCR products were confirmed using 1.8% agarose gel electrophoresis, ethidium bromide staining (Figure 1), and predicted product size as reported previously.⁸

2.4 | Statistical analysis

Genetic linkage disequilibrium and haplotype were analyzed by SHEsis software (<http://analysis.bio-x.cn/myAnalysis.php>). Gene frequency was used to calculate the genotypes and allele frequency of the two groups (Composition ratio, CR), allele frequency = $(2 \times \text{homozygous} + \text{heterozygote}) / (2 \times \text{the participants})$, the comparison of frequency was evaluated by χ^2 test. Means \pm SD of quantitative data were calculated and compared by student's *t* test. Throughout $P < 0.05$ was considered significant. All calculations were performed using SPSS18.0 statistical package.

3 | RESULTS

3.1 | Baseline characters and laboratory testing

There were statistically significant differences in gender, age, smoking history, co-morbidities (diabetes, carotid atherosclerosis, lower extremity atherosclerosis), and the levels of hs-CRP, blood glucose and HDL levels (Table 2). However, we did not see statistical difference between CAD and non-CAD patients in alcohol drinking history, hypertension, cerebral infarction, creatinine, serum troponin, triglyceride, total cholesterol, LDL, apolipoprotein A1, apolipoprotein B, or lipoprotein. In CAD patients, the levels of CRP varied significantly depending on the number of branches of coronary artery (1, 2, or ≥ 3 ; $P = 0.000$). There was a trend for positive association of CRP levels with the numbers of coronary artery branches (Table 3). Gensini integral was also positively correlated with hs-CRP levels ($P = 0.000$).

3.1.1 | Genotype and allele distribution frequency analysis

DNA sequencing confirmed the specificity of PCR products for G217A, G152A, G-6A and A-20C site as well as M235T loci (Figure 2). In Hardy-Weinberg balance test, genotype distribution of each SNP

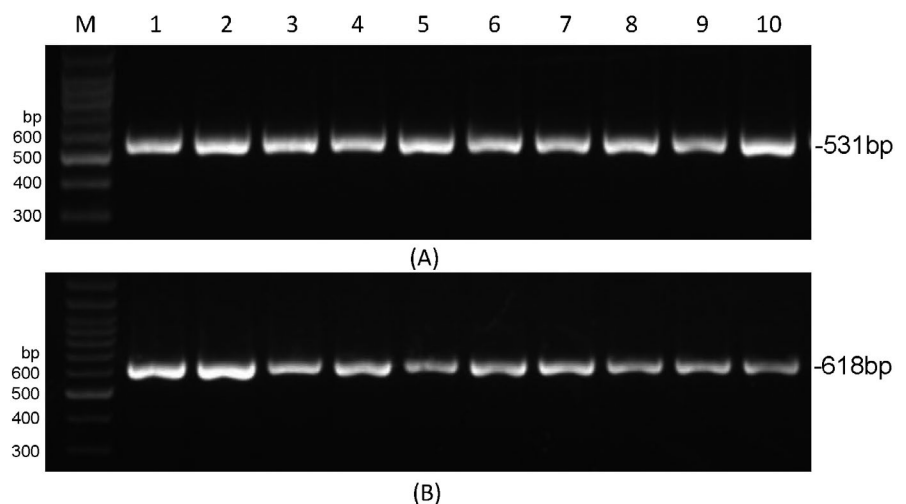


FIGURE 1 The specificity of AGT gene PCR product was analyzed by gel electrophoresis. A, PCR product fragment containing four SNP sites, G217A, a-20c, g-6a, and G152A. B, PCR product fragment containing M235T SNP site. Lane M represents DNA Marker; Lane 1-10 are PCR amplification products of different DNA samples.

	Study subjects		P value ^a
	n-CAD (N = 87)	CAD (N = 492)	
Sex (M/F)	49/38	346/146	0.010
Age (y)	60.7 ± 9.4	65.24 ± 10.69	<0.001
Smoking, N (%)	33 (37.9)	255 (51.8)	0.011
Drinking, N (%)	22 (25.3)	114 (23.2)	0.379
Diastolic (mm Hg)	82.89 ± 13.72	81.35 ± 13.58	0.332
Systolic (mm Hg)	137.14 ± 20.46	137.18 ± 22.78	0.986
Diabetes mellitus, N (%)	8 (9.2)	110 (22.4)	0.002
Carotid atherosclerosis, N (%)	37/86 (43.0)	295/442 (66.7)	0.000
Lower limb atherosclerosis, N (%)	36/85 (42.4)	313/483 (64.8)	0.000
Stroke, N (%)	13 (14.9)	53 (10.8)	0.171
Creatinine (μmol/L)	87.13 ± 17.15	86.22 ± 21.72	0.712
Blood glucose (mmol/L)	5.32 ± 1.06	6.23 ± 2.29	0.000
TG (mmol/L)	1.63 ± 0.96	1.83 ± 1.29	0.168
TC (mmol/L)	4.87 ± 1.05	4.66 ± 1.11	0.103
ApoA1	1.21 ± 0.33	2.20 ± 17.02	0.589
ApoB	0.99 ± 0.28	1.41 ± 6.35	0.539
Lp (a)	234.45 ± 201.03	248.28 ± 205.53	0.562
HDL (mmol/L)	1.40 ± 0.29	1.28 ± 0.33	0.001
LDL (mmol/L)	2.74 ± 0.80	2.69 ± 1.56	0.720
CRP (mg/L)	0.320 (0.310, 1.810)	1.555 (0.320, 5.375)	0.000

apo A1, apolipoprotein A1; apo B, apolipoprotein B; CAD, Coronary artery disease; CRP, C-reactive protein; HDL: high-density lipoprotein; LDL, low-density lipoprotein; Lp (a), lipoprotein a; TC, total cholesterol; TG, triacylglycerol.

^aP < 0.05, test by Mann-Whitney U test.

TABLE 2 Clinical data and biochemical characteristics of study subjects

TABLE 3 The collection between the level of CRP and the increase of coronary artery branches in CAD group

	Coronary artery branches		
	1 (N = 85)	2 (N = 136)	≥3 (N = 251)
CRP	163.12 ^a	235.44 ^a	261.93 ^a
H value	33.640		
P value	<0.01 ^b		

CRP, C-reactive protein.

^aThe mean rank.

^bThe difference were assessed by the Kruskal-Wallis test.

AGT gene locus in non-CAD patients meets the Hardy-Weinberg balance requirements ($P > 0.05$). Site genes (M235T, G217A, G152A, G-6A, and A-20C) were not statistically different between two patient groups ($P > 0.05$) (Tables 4 and 5). Following CAD risk stratification by hs-CRP levels (<1 mg/L, 1-3 mg/L, and >3 mg/L), a significant difference was noted for M235T allele distribution frequency among patient groups ($P = 0.043$). The C allele frequency distribution in CRP < 1 mg/L and ≥ 1mg/L groups was statistically significant difference ($P < 0.05$).

3.1.2 | Linkage imbalance and haplotype analysis

AGT gene M235T, G217A, G152A, A-20C, and G-6A site were analyzed by SHEsis, linkage imbalance relationship showed that the M235T and G-6A site allelic distribution presented as strong linkage disequilibrium ($D' = 0.897$, $r^2 = 0.670$). Meanwhile, G152A and A-20C site alleles presented as a loose linkage imbalance ($D' = 0.941$, $r^2 = 0.227$; Figure 3).

4 | DISCUSSION

In the current study, we found no differences in the genotype and allele distribution frequency of AGT gene, M235T, G217A, G152A, G-6A, and A-20C site between CAD and non-CAD patients. However, after adjusted for serum hs-CRP level, the independent risk factor for CAD, we identified M235T and C allele as a risk element and a risk factor, respectively, for CAD.

The human AGT gene is located in the q42.2 region of chromosome 1 with a length of about 12KB and consists of 5 exons and 4 introns. So far, many SNP variants have been identified in

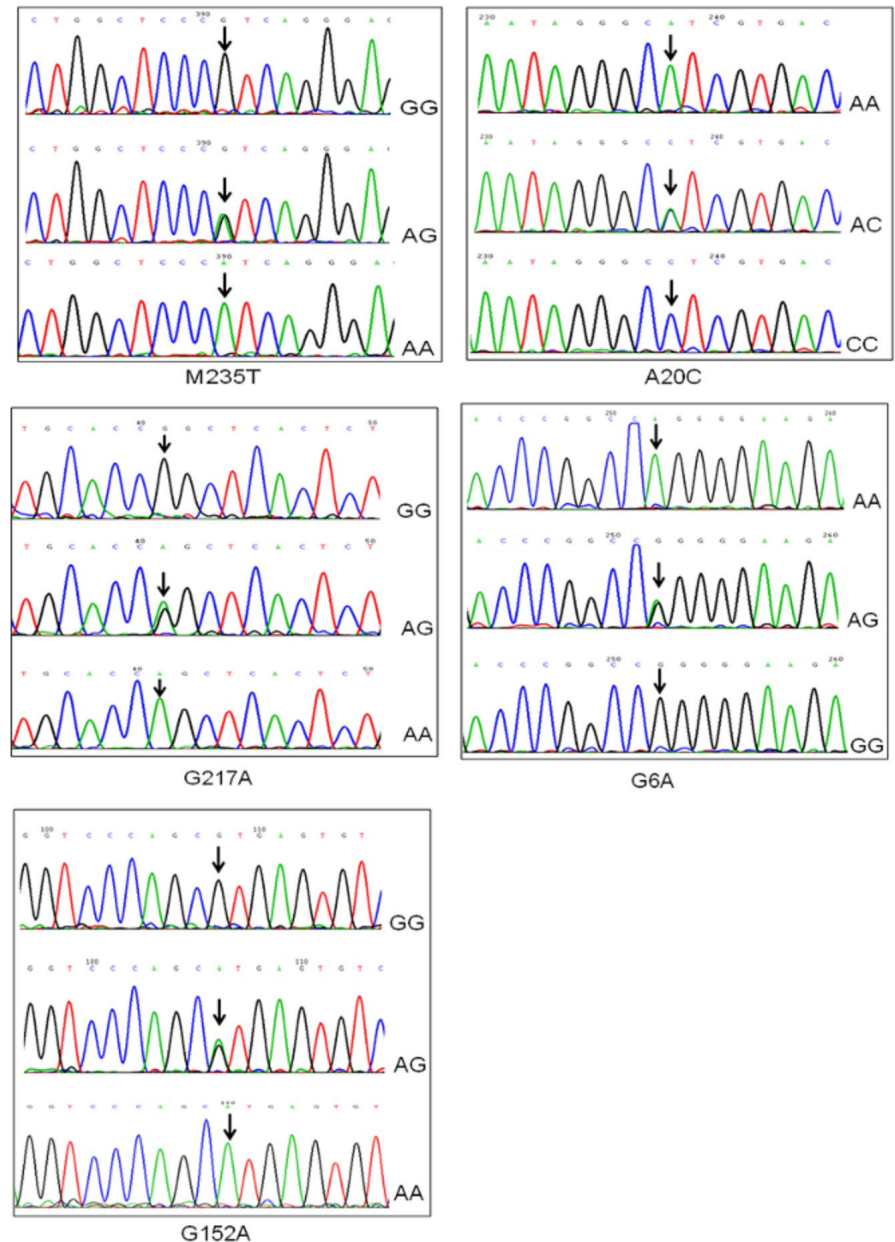


FIGURE 2 Representative images of polymorphisms for Angiotensinogen (AGT) (M235T, G217A, G152A, G-6A, A-20C), and each arrow indicates a single-nucleotide polymorphism of them

the AGT gene. Some genetic studies have shown that AGT polymorphism is closely related to CAD, of which there are more studies on the M235T site. It has been reported⁹ that the AGT M235T polymorphism was associated with the risk of systemic arterial hypertension (SAH) in Caucasian Brazilians, and it is an independent risk factor. Tarek et al¹⁰ found that M235T site TT genotype increased the susceptibility to premature coronary artery disease (PCAD) in a Egypt population, independent of smoking, hypertension, diabetes, total cholesterol, triglyceride, and LDL. However, Al-Hazzani et al¹¹ have shown no comparable MM and MT gene frequency of AGT M235T site in Saudi CAD and non-CAD patients. The differences in published studies may be attributed to population genetic background and race. In addition, in patients that carry the 235T allele, plasma AGT levels were 10%-20% higher than those in non-carriers, with the highest levels of AGT expression in patients with TT genotype.¹² It was

speculated that the molecular variation of the AGT gene M235T may affect the expression of AGT and/or Ang II. Besides, G-6A and A-20C mutations in AGT gene 5' end can increase the transcription level of AGT gene and increase its gene expression, the mutations at the two sites were linkage imbalance with M235T, indicating that M235T may be only a genetic marker, G-6A and A-20C may be the direct cause of increased plasma AGT levels. We failed to demonstrate the genotype and allele distribution frequency of AGT gene polymorphisms (M235T, G217A, G152A, G-6A, and A-20C) between CAD and non-CAD patients, although it has been¹³ reported that AGT gene M235T may be a susceptible factor for myocardial infarction in a Chinese population, which may be related to the difference of the subjects.

CRP is an acute phase response protein can be secreted by liver and cells in atherosclerotic plaques, it is the most widely studied inflammatory marker and is related to the activation of

TABLE 4 The relationship between the genotype distribution of AGT gene polymorphism and CAD

SNP	Genotype	CAD (%)	n-CAD (%)	OR (95% CI)	χ^2 value	P value
M235T	C/C	23 (62.2%)	123 (72.4%)	/	2.846 ^a	0.241
	C/T	11 (29.7%)	42 (24.7)			
	T/T	3 (8.1)	5 (2.9%)			
G217A	G/G	46 (68.7%)	151 (59.9)	/	1.737 ^a	0.42
	A/G	18 (26.9%)	88 (34.9%)			
	A/A	3 (4.4%)	13 (5.2%)			
G152A	G/G	61 (91.0)	236 (93.6%)	1.451 (0.545-3.864)	0.560	0.454
	A/G	6 (9.0%)	15 (6.0%)			
	A/A	0 (0.0%)	1 (0.4%)			
A-20C	A/A	54 (80.6%)	195 (77.4%)	0.824 (0.420-1.615)	0.32	0.572
	A/C	13 (19.4)	55 (21.8%)			
	C/C	0 (0.0%)	2 (0.8%)			
G-6A	A/A	44 (65.7%)	171 (67.9%)	/	3.154 ^a	0.207
	A/G	16 (23.9%)	69 (27.4%)			
	G/G	7 (10.4%)	12 (4.8%)			

CAD, coronary artery disease; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphisms.

^aThe distribution was analyzed by the $R \times C$ chi-square test.

TABLE 5 The relationship between the allele frequency of AGT gene polymorphism and CAD

SNP site	Allele	CAD (%)	n-CAD (%)	OR (95% CI)	χ^2 value	P value ^a
M235T	C	57 (77.0%)	288 (84.7%)	1.652 (0.891-3.061)	2.508	0.108
	T	17 (23.0%)	52 (15.3%)			
G217A	G	110 (82.1%)	390 (77.4%)	1.340 (0.822-2.183)	1.384	0.239
	A	24 (17.9%)	114 (22.6%)			
G152A	G	128 (95.5%)	487 (96.6%)	1.343 (0.519-3.745)	0.372	0.524
	A	6 (4.5%)	17 (3.4%)			
A-20C	A	121 (90.3%)	445 (88.3%)	0.810 (0.430-1.526)	0.425	0.514
	C	13 (9.7)	49 (11.7%)			
G-6A	A	104 (77.6%)	411 (81.5%)	1.275 (0.801-2.028)	1.054	0.305
	G	30 (22.4%)	93 (18.5%)			

CAD, coronary artery disease; CI, confidence interval; OR, odds ratio.

^aThe distribution was analyzed by the $R \times C$ chi-square test.

circulating monocytes, cytokine release, endothelial dysfunction, smooth muscle cell migration, and extracellular matrix remodeling.^{4,14} CRP is not only an inflammatory marker, but plays a direct regulatory role in the process of atherosclerosis.¹⁵ Consistently, the current study showed a statistically significant difference in the serum levels of hs-CRP between the CAD and non-CAD groups. Increasingly, studies have shown that the circulating hs-CRP is closely related to coronary artery events, including myocardial infarction, stroke, lower extremity arterial disease, sudden cardiac arrest, and other events.¹⁶ CRP as independent predictor has been considered to be more valuable than LDL. In previous study, it has been already proved that there was a good correlation between hs-CRP levels and the severity of coronary artery anatomical lesion,¹⁷ which is consistent with the correlation we found

between the coronary score and the hs-CRP level in CAD patients ($r = 0.358$, $P = 0.000$). Interestingly, the interaction of AngII and CRP in the vascular wall has been confirmed. AngII induced CRP production by vascular smooth muscle cells via the AT1 receptor pathway accompanying by the activation of MAPK signaling pathway.³ AngII stimulated human aortic endothelial cells (hAECs) and promoted the expression of CRP mRNA and protein.¹⁸ In the current study, we further found following stratification of patients according to hs-CRP levels, the distribution frequency of allele M235T was statistically different among the groups. The C allele frequency distribution in CRP < 1 mg/L and ≥ 1 mg/L groups was statistically significant difference. Combined with the previous reports, our study suggests that M235T may confer susceptibility to CAD via AngII-CRP related pathway.

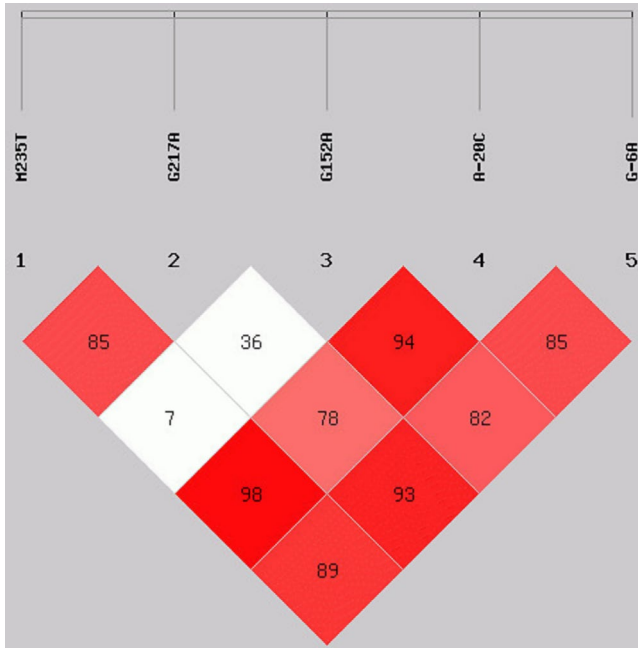


FIGURE 3 The Hardy-Weinberg balance test showed collection among the genotype distribution of each SNP locus of AGT gene (M235T, G217A, G152A, G-6A, A-20C), and linkage imbalance relationship showed that the M235T and G-6A site allelic distribution presented as strong linkage disequilibrium ($D' = 0.897$, $r^2 = 0.670$)

5 | CONCLUSION

In CAD patients, the severity of vascular lesions was positively associated with hs-CRP levels. Despite no difference in AGT M235T, G152A, G-6A, A-20C polymorphism between CAD and non-CAD patients, AGT gene M235T was associated with high hs-CRP levels. Thus, our study suggests that M235T may confer susceptibility to CAD.

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