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ORIGINAL ARTICLE – OPEN ACCESS

Investigation of Angiotensinogen M235T and T174M Gene Polymorphisms in Coronary Artery Disease

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ABSTRACT

Objective: Coronary artery disease (CAD) is a multifactorial disorder and is caused by both environmental and genetic factors. As the alterations in angiotensinogen (AGT) gene lead to changes in angiotensin II and plasma levels of AGT, variants of this gene may play a role in CAD pathogenesis. This study aimed to investigate the relationship between CAD and polymorphisms of AGT gene at M235T and T174M regions. Moreover, the associations of potential risk factors with these gene regions and CAD were investigated.

Materials and Methods: In total, the study enrolled 214 cases with CAD and 200 controls. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to detect polymorphisms at M235T and T174M. PCR products were electrophoresed on 2% agarose gel, with ethidium bromide, and were then examined under ultraviolet light. Subsequently, RFLP was used to detect gene polymorphisms. A multiple binary logistic regression model was used to investigate the association of risk factors with both CAD and AGT variants.

Results: The number of TT polymorphisms at M235T were significantly higher in the case group than in control group. However, there were no significant differences between cases and controls regarding T174M gene polymorphisms. The presence of hypertension, low high-density lipoprotein level, alcohol consumption, and family history were associated with CAD.

Conclusion: TT polymorphisms at the M235T region in AGT can be an influential factor in the development of CAD.

Keywords: Coronary artery disease, angiotensinogen, M235T, T174M, polymorphism

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INTRODUCTION

Globally, cardiovascular diseases are the leading cause of morbidity and mortality, and coronary artery disease (CAD) is the most common type of cardiovascular disease. CAD is caused by both environmental and genetic factors (1, 2). Epidemiological studies have shown that diabetes, smoking, high-fat diet, sedentary lifestyle, age, and cholesterol are the major risk factors for CAD. In addition, genetic factors reportedly contribute to CAD development (3, 4). To understand the etiology of CAD, it is necessary to identify the potential mechanisms underlying its development, its risk factors, and the candidate genes that may contribute to its pathogenesis (5, 6). In addition, it has been shown that polymorphisms in the components of renin angiotensin system (RAS), which regulates sodium homeostasis, vascular remodeling, and blood pressure, play important roles in CAD progression and development (7, 8).

Recent advancements in molecular biology have helped identify several polymorphisms that may alter the protein functions in vascular homeostasis (9). The gene polymorphisms that have been identified for all RAS components might be responsible for the hyperactivity of this system. The primary polymorphic gene components of RAS are angiotensinogen (AGT), angiotensin I-converting enzyme (ACE), and angiotensin II type 1 receptor (AT-1) (10). Variations in these components possibly play a crucial role in the pathogenesis of CAD, hypertension, and myocardial infarction (MI) (9, 11). *In vivo* and cell culture studies have demonstrated the role of angiotensin II in the maintenance of vascular structure as well as the role of ACE inhibitors in reducing recurrent MI risk (1, 12). Angiotensin II is the most important effector molecule of RAS and plays a crucial role in the regulation of blood pressure. AGT, a human liver protein, is converted to angiotensin I, the prohormone of angiotensinogen II, through renin. Subsequently, angiotensin I is converted to angiotensin II, which induces cardiac hypertrophy, fibrosis, and vasoconstriction (13, 14).

As alterations in AGT induces changes in the levels of angiotensin II and plasma AGT, which have a potent vasoconstrictor effect, these genetic variants possibly have a role in the pathogenesis of CAD (15, 16). The AGT gene is found in 1q42-43, which contains five exons (17). Reportedly, single-nucleotide gene polymorphisms have been detected at the second exon of AGT, via a methionine to threonine substitution at codon 235 (M235T) and a threonine to methionine substitution at codon 174 (T174M) (18–20). Therefore, these two codon regions of AGT have gained much attention.

In this study, we aimed to investigate (1) the correlation of CAD with AGT polymorphisms at M235T and T174M regions; (2) the relationship of risk factors, including sex, age, hypertension, diabetes, smoking, alcohol consumption, family history, and high-density lipoprotein (HDL) and triglyceride levels, with these gene regions; and (3) associations between these risk factors and CAD.

MATERIALS and METHODS

Study Population

This retrospective case-control study was conducted in the Biophysics Department of the Trakya University Faculty of Medicine. A total of 414 subjects were included in the study, and all of them were informed according to the Helsinki Declaration. In addition, the study was approved by the Trakya University Medical School Ethics Committee on Non-Interventional Clinical Investigations (approval date: 05/12/2012; issue number: TÜTF-GOKAEK 2012/192). Patients who visited the Trakya University Medical Faculty Cardiology Clinic or Emergency Polyclinic with a complaint of chest pain, who were subsequently referred to undergo coronary angiography, and were diagnosed with CAD according to angiography findings (≥50% stenosis in at least one major coronary artery) were determined as cases. In contrast, patients who were not diagnosed with CAD based on angiography results (smooth coronary arteries or <50% stenosis in major coronary arteries) were determined as controls. Patients aged <25 years who did not undergo coronary angiography and those who were diagnosed with malignancy were excluded. A stratified sampling method was used to select subjects. Using this approach, 214 cases (154 males and 60 females) and 200 controls (133 males and 67 females) were selected based on sex stratification.

Collection of Blood Samples and Laboratory Analysis

For both cases and controls, blood samples (2 ml) were collected using vacuum tubes containing ethylenediaminetetraacetic acid. Subsequently, DNA samples were isolated using a DNA isolation kit (Roche USA). Polymerase chain reaction (PCR) and restriction fragment length polymorphism were used to determine polymorphisms at the T174M and M235T regions of AGT from the isolated DNA. For M235T, 25 µL of PCR mixture was used, which contained 200 ng of DNA, 0.2 mM of each deoxynucleotide triphosphate, 0.5 nmol of forward (5′-CCGTTTGTGCAGGGCCTGGCTCTCT-3′) and reverse (5′-CAGGGTGCTGTCCACACTGGACCCC-3′) oligonucleotide primers, 1X Taq buffer, 1.5 mM MgCl2, and 1.25 U Taq DNA polymerase. For T174M, the 25 µL PCR mixture used contained 200 ng of DNA, 0.2 mM of each deoxynucleotide triphosphate, 0.5 nmol of forward (5′-TGGCACCCTGGCCTCTCTCTATCT-3′) and reverse (5′-CAGCCTGCATGAACCTGTCAATCT-3′) oligonucleotide primers, 1X Taq buffer, 1.5 mM MgCl2, and 1.25 U Taq DNA polymerase. The amplification protocol was as follows: for M235T, an initial denaturation at 95°C for 4 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min; for T174M, an initial denaturation at 94°C for 4 min, 38 cycles of denaturation at 94°C for 15 s, annealing at 64°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products obtained were electrophoresed on 2% agarose gels and were then stained with ethidium bromide. Finally, the formation of products was assessed under ultraviolet (UV) light.

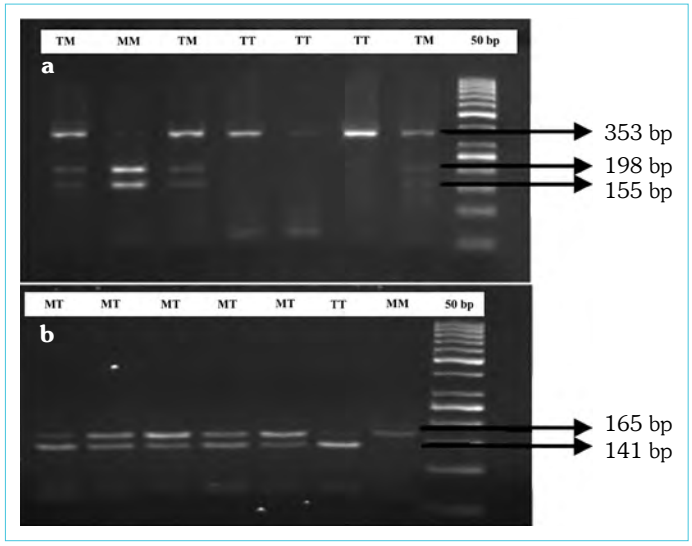

Figure 1. The restriction fragment length polymorphism results of the case and control PCR products that were electrophoresed on 2.5% gel and examined under ultraviolet light. (a) T174M region of AGT. (b) M235T region of AGT

For the M235T and T174M regions, PCR products were cut using the restriction enzyme TT111I (5′... GACN ↓ NNGT C... 3′, 3′... CTGNN ↑ NCA G... 5′) and NcoI (5′ C ↓ CATG G... 3′, 3′... GGTAC ↑ C... 5′), respectively, at 37°C for 4 h. These restriction digest products were loaded onto 2% agarose gel prepared with ethidium bromide, and the bands formed under UV light were observed. Finally, it was determined whether T174M and M235T regions of AGT has M or T alleles (Fig. 1a, b).

Statistical Analysis

Outliers were detected and parametric assumptions were assessed. Shapiro–Wilk test was used to test the normality assumption of data. Student's t-test was used to compare two independent groups for numerical variables. A Pearson chi-square test was used to determine the relationship among categorical variables. Multiple binary logistic regression analysis with stepwise selection was used to determine the relationship of risk factors with both CAD and AGT M235T and T174M polymorphisms. Numerical variables were reported as means±standard deviations, whereas categorical variables were reported as frequencies and percentages. Results with $p<0.05$ were considered statistically significant. All data were analyzed using the IBM SPSS 21.0 statistical software.

RESULTS

The mean age of individuals in the CAD group was 63.1±12.2 years and in the control group was 54.1±14.3 years. Assessment of the demographic and the clinical results of the case and control groups revealed a significant association of CAD with risk factors, such as age, hypertension, diabetes, smoking, alcohol consumption, family history, total cholesterol, and HDL (Table 1). The genotype and allele frequencies in the M235T and T174M gene regions of both the groups are summarized in Table 2. In total, 11 values were missing in the case group. The genotype distributions for M235T (TT, MM, and MT) were significantly different between the two groups ($p=0.038$). In con-

Table 1. Demographic and clinical characteristics of the control and case groups

	Control (n=200)	Case (n=214)	p
Sex			0.228
Male	133 (66.5%)	154 (72.0%)	
Female	67 (33.5%)	60 (28.0%)	
Age (year)	54.1±14.3	63.1±12.2	<0.001*
Hypertension	91 (45.7%)	142 (66.5%)	<0.001*
Diabetes	47 (23.4%)	81 (37.9%)	0.002*
Smoking	50 (25.1%)	95 (44.4%)	<0.001*
Alcohol consumption	20 (9.9%)	49 (23.0%)	<0.001*
Family history	24 (12.0%)	52 (24.2%)	0.001*
Total cholesterol (mg/dl)	188.7±38.8	177±50.3	0.011*
HDL (mg/dl)	42.6±13.2	39.4±10.6	0.007*
LDL (mg/dl)	121.3±32.3	119.3±43.7	0.599
Triglyceride (mg/dl)	143.5±93.3	148.3±97.8	0.613

*: Statistically significant at p<0.05. Descriptive statistics expressed as means±standard deviations and frequencies (percentages). HDL: High-density lipoprotein; LDL: Low-density lipoprotein

Table 2. Genotype and allele frequencies of M235T and T174M in the case and control groups

	Control (n=200)	Case (n=203)	p
M235T			
TT	7 (3.5%)	20 (9.9%)	
MM	75 (37.5%)	70 (34.5%)	0.038*
MT	118 (59.0%)	113 (55.7%)	
T allele	132 (33.0%)	153 (37.7%)	0.164
M allele	268 (67.0%)	253 (62.3%)	
T174M			
TT	161 (80.5%)	166 (79.0%)	
MM	5 (2.5%)	11 (5.2%)	0.350
TM	34 (17.0%)	33 (15.7%)	
T allele	356 (89.0%)	365 (86.9%)	0.357
M allele	44 (11.0%)	55 (13.1%)	

*: Statistically significant at p<0.05. Descriptive statistics expressed as frequencies (percentages)

trast, there were no significant difference between two groups regarding allele frequencies for M235T (p=0.164). Furthermore, there were no significant differences between the groups for genotype and allele frequencies in the T174M region (p=0.350 and p=0.357, respectively).

Allele frequencies in the M235T and T174M regions are provided in Figure 2. Multivariate binary logistic regression analysis was performed using risk factors that were significantly associated with CAD in univariate analyzes. According to the results of binary logistic regression analysis, hypertension (odds ratio [OR]=2.523, 95% confidence interval [CI]: 1.441–4.418), alcohol consumption (OR=2.498, 95% CI: 1.093–5.712), and family history (OR=2.357, 95% CI: 1.077–5.155) were the risk factors for CAD, whereas HDL had a protective effect against CAD (OR=0.975, 95% CI: 0.952–0.999) (Table 3). The relationships between the risk factors and gene polymorphisms at M235T and T174M (MM, MT, and TT) have been summarized in Tables 4 and 5. Accordingly, MM polymorphism at M235T was associated with hypertension (OR=2.910, p=0.003), diabetes (OR=2.172, p=0.039), and smoking (OR=2.365, p=0.027), whereas MT polymorphism was associated with hypertension (OR=1.986, p=0.014), smoking (OR=3.096, p<0.001), alcohol consumption (OR=4.645, p=0.004), and family history (OR=2.367, p=0.016). At the T174M region, MT polymorphism was associated only with family history (OR=4.909, p=0.042) and TT polymorphism was associated with hypertension (OR=2.430, p<0.001), diabetes (OR=1.840, p=0.013), smoking (OR=2.702, p<0.001), and alcohol consumption (OR=3.037, p=0.006). Using the significant risk factors indicated in Tables 4 and 5, multiple binary logistic regression analysis was performed to further investigate the relationships.

We found that the MM polymorphism at M235T was associated only with hypertension (OR=3.467, 95% CI: 1.525–7.882), whereas MT polymorphism was associated with hypertension (OR=2.230, 95% CI: 1.016–4.896), alcohol consumption (OR=4.110, 95% CI: 1.267–13.337), and family history (OR=2.843, 95% CI: 1.042–7.752). Finally, for T174M, TT polymorphism was associated with hypertension (OR=2.838, 95% CI: 1.557–5.172) and alcohol consumption (OR=3.650, 95% CI: 1.474–9.039). These results have been summarized in Table 6.

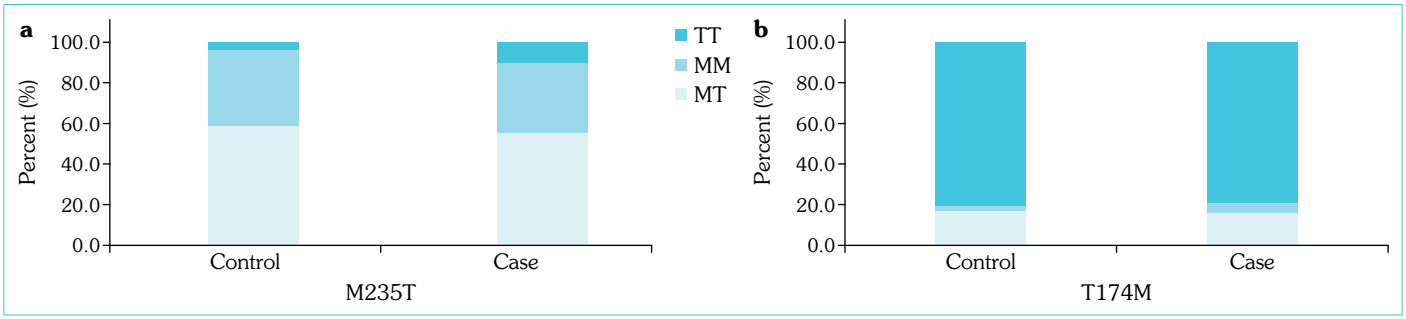

Figure 2. Stacked bar graphs for allele distributions in AGT regions. (a) Allele distributions in M235T. (b) Allele distributions in T174M

Table 3. Results of multiple binary logistic regression analysis for risk factors

	Coeff.	St. error	p	OR	95% CI	
					Lower	Upper
Intercept	0.839	0.558	0.133	2.314		
Hypertension	0.926	0.286	0.001*	2.523	1.441	4.418
HDL	-0.025	0.012	0.042*	0.975	0.952	0.999
Alcohol	0.916	0.422	0.030*	2.498	1.093	5.712
Family history	0.857	0.399	0.032*	2.357	1.077	5.155

*: Statistically significant result at p<0.05; Coeff.: Estimated beta coefficients; St. error: Standard error; OR: Odds ratio; HDL: High density lipoprotein; CI: Confidence interval

DISCUSSION

CAD is a multifactorial disease and both environmental and genetic factors play important roles in its etiology. In this regard, both candidate genes and risk factors that contribute to CAD pathogenesis can help us understand the etiology of the disease. In recent years, a number of studies have been performed to delineate this, particularly regarding the association of CAD with the M235T and T174M regions of AGT.

Our findings revealed a significant relationship between TT polymorphism at the M235T region and CAD. This result was in accordance with the results reported by Katsuya et al. (1), Winkelmann et al. (9), Ragia et al. (10), Freitas et al. (21), Gardemann et al. (22), and Perez et al. (23). However, Ichihara et al. (11), Babunova et al. (16), Kuo et al. (18), and Nair et al. (24) found no significant relationship between polymorphisms at the M235T region and CAD. Our results revealed that polymorphisms in the T174M gene region were not associated with CAD. This result is consistent with the results reported by Ichihara et al. (11), Wang (14), Babunova et al. (16), Xu et al. (15), Ilhan et al. (25), and Nair et al. (24). However, it is contradictory to the result reported by Li et al. (3), Gardemann et al. (22), and Spiridonova et al. (26) who found a significant association between T174M gene polymorphisms and CAD. Previous meta-analyzes (15, 27) demonstrated a weak relationship between M235T gene polymorphism and CAD. However, when the meta-analyzes are limited to large studies only, the relationship was no longer observed. Furthermore, there was no significant relationship between T174M gene polymorphism and CAD (15, 27). Another meta-analysis of studies enrolling Chinese patients revealed that M235T and T174M gene polymorphisms were associated with CAD. However, the effects of gene variants on CAD and their mechanisms still remain unclear (3). Sui and Gao (13) conducted a meta-analysis to investigate the relationship between M235T polymorphism in AGT and acute MI; they found no significant relationship between M235T variant and MI. In another meta-analysis (14), a significant relationship was noted between T174M polymorphism in AGT and CAD; however, following stratification by races, a significant relationship was observed between gene polymorphism and CAD in Caucasians, but not in Asians.

Table 4. Relationship between risk factors and M235T region polymorphisms

Risk factors	MM					MT					TT				
	Case		Control		p	Case		Control		p	Case		Control		p
	n	%	n	%		n	%	n	%		n	%	n	%	
Gender															
Male	50	49.5	51	50.5	0.654	82	51.9	76	48.1	0.182	15	71.4	6	28.6	0.557
Female	20	45.5	24	54.5		31	42.5	42	57.5		5	83.3	1	16.7	
Hypertension															
Yes	45	63.4	26	36.6	0.003*	70	57.9	51	42.1	0.014	14	82.4	3	17.6	0.366
No	22	37.3	37	62.7		38	40.9	55	59.1		6	66.7	3	33.3	
Diabetes															
Yes	25	61.0	16	39.0	0.039*	37	56.9	28	43.1	0.079	8	80.0	2	20.0	0.529
No	41	41.8	57	58.2		70	44.0	89	56.0		11	68.8	5	31.2	
Smoking															
Yes	27	64.3	15	35.7	0.027*	49	66.2	25	33.8	<0.01*	4	57.1	3	42.9	0.226
No	35	43.2	46	56.8		50	38.8	79	61.2		13	81.2	3	18.8	
Alcohol															
Yes	12	63.2	7	36.8	0.577	25	86.2	4	13.8	0.004*	6	85.7	1	14.3	0.320
No	50	56.2	39	43.8		74	57.4	55	42.6		11	64.7	6	35.3	
Family history															
Yes	11	64.7	6	35.3	0.160	29	67.4	14	32.6	0.016*	4	75.0	1	25.0	0.659
No	52	46.4	60	53.6		70	46.7	80	53.3		14	70.0	6	30.0	

*: Statistically significant result at p<0.05. Descriptive statistics expressed as frequency (percentage)

Table 5. Relationship between risk factors and T174M region polymorphisms

Risk factors	MM					MT					TT				
	Case		Control		p	Case		Control		p	Case		Control		p
	n	%	n	%		n	%	n	%		n	%	n	%	
Gender															
Male	9	69.2	4	30.8	0.931	20	48.8	21	51.2	0.922	122	53.0	108	47.0	0.204
Female	2	66.7	1	33.3		13	50.0	13	50.0		44	45.4	53	54.3	
Hypertension															
Yes	7	87.5	1	12.5	0.347	22	61.1	14	38.9	0.139	106	62.0	65	38.0	<0.01*
No	4	66.7	2	33.3		10	41.7	14	58.3		53	40.2	79	59.8	
Diabetes															
Yes	5	83.3	1	16.7	0.475	11	68.8	5	31.3	0.052	60	60.0	40	40.0	0.013
No	6	66.7	3	33.3		20	40.8	29	59.2		97	44.9	119	55.1	
Smoking															
Yes	5	100.0	0	0.0	0.118	11	52.4	10	47.6	0.940	65	66.3	33	33.7	<0.01*
No	5	62.5	3	37.5		19	51.4	18	48.6		78	42.2	107	57.8	
Alcohol															
Yes	2	100.0	0	0.0	0.488	7	70.0	3	30.0	0.470	33	80.5	8	19.5	0.01*
No	8	80.0	2	20.0		23	57.5	17	42.5		110	57.6	81	42.4	
Family history															
Yes	7	87.5	1	12.5	0.185	9	81.8	2	18.2	0.04*	30	63.8	17	36.2	0.066
No	4	57.1	3	42.9		22	47.8	24	52.2		115	49.1	119	50.9	

*: Statistically significant result at p<0.05. Descriptive statistics expressed as frequency (percentage)

Table 6. Results of multiple binary logistic regression analysis for risk factors of the MM genotype of M235T, MT genotype of M235T, and TT genotype of T174M

Genotype	Variable	Coeff.	St. error	p	OR	95% CI	
						Lower	Upper
MM genotype of M235T	Intercept	-0.288	0.289	0.319	0.750		
	Hypertension	1.243	0.419	0.003*	3.467	1.525	7.882
MT genotype of M235T	Intercept	-0.125	0.342	0.716	0.883		
	Hypertension	0.802	0.401	0.046*	2.230	1.016	4.896
	Alcohol	1.413	0.601	0.019*	4.110	1.267	13.337
	Family history	1.045	0.512	0.041*	2.843	1.042	7.752
TT genotype of T174M	Intercept	-0.120	0.234	0.608	0.887		
	Hypertension	1.043	0.306	0.001*	2.838	1.557	5.172
	Alcohol	1.295	0.463	0.005*	3.650	1.474	9.039

*: Statistically significant at p<0.05. Coeff.: Estimated beta coefficients; St. error: Standard error; OR: Odds ratio; CI: Confidence interval

Moreover, studies have shown that the M235T homozygotes at AGT (MM and TT) are associated with risk factors, such as diastolic blood pressure, systolic blood pressure, total cholesterol, body mass index, family history, smoking, alcohol consumption, diabetes, hypertension, and hypercholesterolemia (1, 9, 10, 21–23, 27, 28). Lanz et al. (20) found no significant relationships between risk factors, such as sex, age, diabetes, hypertension, total cholesterol, HDL, LDL, triglycerides, and smoking, and the M235T genotypes of AGT; however, they found that atherosclerosis was significantly associated with the T allele of M235T. Pilbrow et al. (29) found no significant relationships between the M235T polymorphism of AGT and hypertension, smoking, diabetes, heart failure, MI, and cholesterol. Similarly, they found no significant associations between T174M polymorphism and hypertension, smoking status,

diabetes mellitus, MI, and cholesterol. However, the T174M gene polymorphism was associated with heart failure (29). Our study results showed that in the M235T gene region, MM polymorphism was associated with hypertension and MT polymorphism was associated with hypertension, alcohol consumption, and family history. In addition, in the T174M gene region, TT polymorphism was associated with hypertension and alcohol consumption.

Our study has several limitations. As the CAD group received cholesterol-lowering drugs, total cholesterol, LDL, HDL, and triglyceride levels may have been affected. In addition, the control group did not include healthy subjects but patients suspected of having CAD. These patients were included in the control group as blood samples cannot be obtained from healthy volunteers owing to ethical reasons.

CONCLUSION

Our study results showed that the presence of hypertension, low HDL level, alcohol consumption, and family history were associated with CAD. In addition, TT polymorphism in the M235T region of AGT may be associated with CAD.

Future studies may be conducted to investigate other potential risk factors for CAD, including C-reactive protein, lipoprotein-a, homocysteine, and fibrinogen.

Ethics Committee Approval: The Trakya University Medical School Ethics Committee on Non-Interventional Clinical Investigations granted approval for this study (date: 05.12.2012, number: 2012/192).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – BEY, NS, TG, OP, TS; Design – BEY, NS, TG, OP, TS; Supervision – BEY, NS, TG, OP, TS; Resource – BEY, NS, TG, TS; Materials – BEY, OP; Data Collection and/or Processing – BEY, NS, OP; Analysis and/or Interpretation – BEY, TG, TS; Literature Search – BEY, TG, TS; Writing – BEY, NS, TG, OP, TS; Critical Reviews – BEY, NS, TG, OP, TS.

Conflict of Interest: The authors have no conflict of interest to declare.

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