Association of angiotensin-converting enzyme and angiotensinogen T174M gene polymorphisms with coronary artery disease

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Objectives: The aim of this study was to estimate the frequencies of some DNA polymorphisms of two genes of the renin–angiotensin system (RAS), T174M angiotensinogen gene and insertion–deletion polymorphism in angiotensin-converting enzyme gene, in CAD patients and control groups. For this purpose 158 coronary artery disease and 68 control subjects have been analyzed.

Methods: Blood was drawn and DNA extracted. Angiotensin-converting enzyme (I/D) gene polymorphism was analyzed by polymerase chain reaction (PCR) and AGT gene polymorphisms by restriction fragment length polymorphism-PCR.

Results: The relative frequencies of the T and M alleles of the T174M did not significantly differ between patients without or with single-, double- or triple-vessel disease and between subjects without or with myocardial infarction (MI). In contrast, D allele was significantly higher in patients with double/triple vessel disease and in CAD patients who had a history of MI. An interaction between both angiotensinogen gene polymorphisms and ACE gene polymorphism were not observed when the D allele of ACE and the T allele of M174T were combined was combined

Conclusions: The present study strengthens the hypothesis of an association of both angiotensinogen and angiotensin converting enzyme gene polymorphisms with the extent of coronary heart disease.

Key words: ACE I/D polymorphism, AGT T174M polymorphism, CAD


Introduction

Coronary artery disease (CAD) is a major health problem in many industrialized countries. This disease contributes to significant morbidity and mortality. Therefore, for medical science, the study of the molecular genetic basis of this disease is an important issue. Most of the study is aimed at structural organization of genes that play an important role in coagulation and renin–angiotensin cascades and lipid homeostasis. The genes encoding components of the renin-angiotensin system (RAS) present attractive candidates as risk factors for cardiovascular disease. Angiotensinogen (AGT) and angiotensin-I converting enzyme (ACE) are the key components of the RAS. An important function of ACE is the conversion of angiotensin-I to angiotensin-II and the other function is inactivation of kinins. AGT is the major substrate for renin and is converted by renin into angiotensin-I. Recently, both plasma ACE and AGT concentrations have been found to be strongly genetically determined.

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The renin-angiotensin and kallikrein-kinin systems have systemic and local effects on vascular tone and blood pressure. Angiotensin-converting enzyme and angiotensinogen play important roles within these systems. Therefore, ACE and AGT are candidate genes that influence vascular tone and have potentially detrimental effects on coronary arteries. With the use of ACE inhibitors, the role of ACE in the events leading to a myocardial infarction (MI) has been documented clinically, and in epidemiologic studies. The association of the ACE deletion (D) allele has been confirmed to correlate with elevated levels of ACE in the circulation.

Although AGT is known to influence vascular tone, its potential effects on CVD have not been well defined. Several variations have been identified in AGT gene, and one of this molecular variant of AGT also constitutes the cytosine-thymine transition at nucleotide 521, which causes a sense mutation from threonine to methionine at amino acid 174 (T174M).

In this study we analyzed the AGT T174M and ACE I/D polymorphisms in CAD patients with or without MI and association with the extent of CAD. The results will help us to understand the role of the polymorphisms of the AGT genes in the manifestation of CAD.

Methods

Subjects

Our study population consisted of 226 subjects (188 male, 38 female) who had undergone coronary angiography in the Firat University, Elazig, Turkey, because of symptoms possibly related to coronary artery disease. 68 person has normal coronary angiography and selected as control subjects. Relevant history, cardiovascular risk factors, and current treatment were obtained from each patient using a standard questionnaire, and the data were validated with reference to hospital case records. Acute myocardial infarction was defined for this study according to the World Health Organization (WHO) criteria based on symptoms, ECG findings and cardiac enzyme abnormalities. Acute myocardial infarction was classified as ST segment elevation and/or new bundle branch block and non-ST elevation based on the presence or absence of >1 mm of ST segment elevation on two or more continuous leads on the initial ECG. About coronary risk factors such as body mass index (kg/m²), family history, arterial hypertension (≥ 140 mmHg systolic blood pressure, ≥ 90 mmHg diastolic blood pressure), smoking history and hyperlipidemia (total cholesterol >240 mg/dl and triglycerides >250 mg/dl) were also evaluated. Written informed consent was obtained from all subjects and a local ethical committee approved the study protocol.

Biochemical parameters (Cholesterol, Triglyceride) were assayed by using Olympus AU 600 Otoanalyzer (Olympus Optical Co Ltd, Japan). Method for CK and CK-MB based on the rate of increase of absorbance at 340/660 nm for CK and 340 nm for CK-MB due to the formation of NADPH is directly proportional to the activity of CK and CK-MB in the sample. Reference intervals for CK <171U/l for males and < 145 for females and for CK-MB is 24 U/l. The test is linear within an enzyme activity range of 10-2000 U/l. The lowest detectable level was estimated at 3 U/l for CK and 1 U/l for CK-MB. The lowest detectable level represents the lowest measurable level of CK and CK-MB that can be distinguished from zero.

Coronary vessels with at least 50% stenosis were defined as diseased. The severity of coronary heart disease (CHD) was also estimated by calculating the Gensini score.

Isolation of DNA

Whole blood specimens were collected in tubes containing K3-EDTA, and DNA was prepared from DNA isolation kit (High Pure PCR Template Preparation kit, Catalog no: 1 796 828).

Polymerase chain reaction (PCR) for AGT gene polymorphism

For T174M polymorphism genotyping, 20 µl of the PCR product was digested with 1U of Nco I restriction endonuclease overnight at 37°C according to Caulfield et al. Digested fragments were detected by electrophoresis in %3 agarose gel containing ethidium bromide. Following digestion, the homozygous threonine genotype (TT/T) appears as a single band of 354 bp and the M/M genotype as 260 bp and 94 bp.

Polymerase chain reaction (PCR) for ACE gene polymorphism

A genomic DNA fragment on intron 16 of the ACE gene was amplified by polymerase chain reaction (PCR) using a flanking primer pair and a primer pair that recognizes insertion specific sequence. Reactions were performed with 10 pmol of each primer: forward primer 5’-CTG GAG ACC ACT CCC ATC CIT TCT-3’ and reverse primer 5’-GAT GTG GTG GCC
ATC TTC GTC AGA T-3' in a final volume of 50 ml containing 3 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.5 mM of each dNTP (MBI Fermentas), and 1 unit of Taq polymerase (MBI Fermentas). Amplification was carried out in a DNA Thermal Cycler (MJ Research Techne) for 30 cycles with denaturation steps at 94°C for 1 min., annealing at 58°C for 1 min., and extension at 72°C for 2 min. PCR products were separated on 3% agarose gel, and DNA was visualized by ethidium bromide staining. Genotype interpretation was based on the length of the PCR products, 190 and 490 base pairs for the deletion (D) and insertion (I) alleles respectively. We confirmed the accuracy of the genotyping results in the DD homozygotes by using an insertion-specific primer. To increase the specificity of DD genotyping, PCR amplifications were performed with an insertion specific primer pair (5’ TGG GAC CAC AGC GCC CGC CAC TAC 3’; 5’ TCG CCA GCC CTC CCA TGC CCA TAA 3’) with 25 µl of the reaction mixture with 1 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 67°C (annealing), and 2 min at 72°C (extension).

**Statistical methods**

Statistics were carried out on SPSS 10.0 version. The statistic significance of differences on mean levels of variables among groups was examined by analysis of variance (ANOVA). Data of total cholesterol, HDL cholesterol, LDL-cholesterol and triglyceride were presented as mean ± standard deviations. Qualitative data were compared between groups by the χ² test. Allele frequencies were estimated by counting and Hardy-Weinberg's equilibrium was checked by a χ² test. A value of P<0.05 was considered to represent a statistically significant result.

**Results**

**Clinical characteristics of the patients**

158 cases (mean age 58.64±9.3 years, 16.5% women) and 68 randomly selected age- and gender-matched community controls (mean age 53.76±10.57 years, 17.6% women) were included in the study. Table 1 shows the main characteristics of our case and control groups. Hypertension (chi-square 0.355; P>0.05), smoking (chi-square 0. 307; P>0.05) were not significantly different in the group of patients compared with the group of controls. The control group presented lower levels of total cholesterol (P<0.0001) and. LDL-cholesterol (P<0.05) and the control group presented higher levels of HDL cholesterol (P<0.0001).

By means of coronary angiography, the study population was divided into subjects without any angiographically detectable CAD or with coronary arterial stenoses less than 50% (no vessel disease; n: 68) and individuals with single vessel disease (n: 62), double or triple vessel disease (n: 96) (Table 1). The serum total cholesterol levels were significantly higher in individuals with single vessel disease (P<0.001), double or triple vessel disease (P<0.0001) than in normal controls. The highest level of serum LDL cholesterol level was observed in individuals with double or triple vessel disease compared with control subjects (P<0.05). Age, triglyceride, cigarette consumption and hypertension were almost identical between the groups of the total study population.

**Table 1**

<table>
<thead>
<tr>
<th>Gender (F/M)</th>
<th>CAD (total of patients) n: 158</th>
<th>Single Vessel n: 62</th>
<th>Double/ Triple vessel n: 96</th>
<th>CAD with history of MI n: 80</th>
<th>CAD without history of MI n: 78</th>
<th>Control n: 68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.6±9.3</td>
<td>58.7±9.8</td>
<td>58.6±9.1</td>
<td>58.1±9.1</td>
<td>58.2±9.6</td>
<td>53.8±10.6</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>46.8</td>
<td>48.4</td>
<td>45.8</td>
<td>47.5</td>
<td>53.8</td>
<td>41.2</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>53.2</td>
<td>48.4</td>
<td>56.3</td>
<td>45.0</td>
<td>61.5</td>
<td>47.1</td>
</tr>
<tr>
<td>Total-C (mg/dL)</td>
<td>223.8±38.1</td>
<td>218.2±33.4</td>
<td>227.5±40.3</td>
<td>219.3±30.1</td>
<td>227.9±44.9</td>
<td>186.1±25.9</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>146.4±34.4</td>
<td>143.5±37.7</td>
<td>148.3±32.4</td>
<td>141.8±28.6</td>
<td>151.1±39.4</td>
<td>128.8±29.1</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>42.7±7.6</td>
<td>44.1±7.6</td>
<td>41.8±7.6</td>
<td>41.6±8.5</td>
<td>43.9±6.6</td>
<td>49.3±9.6</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>178.5±76.7</td>
<td>162.9±47.1</td>
<td>188.4±89.8</td>
<td>173.9±87.7</td>
<td>183.1±64.3</td>
<td>158.2±49.5</td>
</tr>
</tbody>
</table>

* P<0.0001, compared with control  
* P<0.05, compared with control  
* P<0.001, compared with control  
* P<0.01, compared with control
According to MI history, all subjects were divided into three groups: coronary artery disease with a history of myocardial infarction (n: 80), coronary artery disease without a history of myocardial infarction (n: 78) and normal controls (Table 1). The serum total cholesterol levels were significantly higher in individuals who had a history of MI in CAD patients than in normal controls (P<0.001). The highest level of serum HDL cholesterol level was observed in individuals with control subjects.

**Distribution of AGT and ACE genotype and alleles within the study population**

Genotypic and allelic frequencies of AGT and ACE in the study groups are shown in Table 2 and Table 3. There was no deviation from Hardy-Weinberg equilibrium for the polymorphisms considered. The TT genotype frequency was higher in the CAD patients compared with control subjects (83.5% vs. 73.5%), and the difference was statistically significant (P = 0.05, OR = 1.76; CI = 0.03–1.09). In contrast the frequency of the MM genotype was more in controls than in the CAD patients (11.8% vs. 2.5%) and the difference was statistically significant (P < 0.05). The relative frequencies of the genotypes of the T174M gene variation did not significantly differ between patients without or with single-, double- or triple-vessel disease. The TT and TM genotype frequency was higher in the CAD patients with a history of MI compared with control subjects (82.5% vs. 73.5%), and the difference was statistically significant (P = 0.05). The T allele frequency was higher in the CAD patients than in controls (90.5% vs. 88.2%).

### Table 2

**Genotypic frequencies of AGT and ACE in patients and in controls**

<table>
<thead>
<tr>
<th></th>
<th>TT</th>
<th>TM</th>
<th>MM</th>
<th>DD</th>
<th>ID</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAD</strong></td>
<td>122 (83.5%)</td>
<td>22 (13.9%)</td>
<td>4 (2.5%)</td>
<td>86 (54.4%)</td>
<td>60 (38.0%)</td>
<td>12 (7.6%)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>50 (73.5%)</td>
<td>10 (14.7%)</td>
<td>8 (11.8%)</td>
<td>32 (47.1%)</td>
<td>18 (26.5%)</td>
<td>18 (26.5%)</td>
</tr>
<tr>
<td><strong>Single vessel</strong></td>
<td>52 (87.1%)</td>
<td>63 (9.7%)</td>
<td>2 (3.2%)</td>
<td>26 (41.9%)</td>
<td>32 (51.6%)</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td><strong>Double/triple vessel</strong></td>
<td>78 (81.3%)</td>
<td>16 (16.7%)</td>
<td>2 (2.1%)</td>
<td>60 (62.5%)</td>
<td>28 (29.5%)</td>
<td>8 (8.3%)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>50 (73.5%)</td>
<td>10 (14.7%)</td>
<td>8 (11.8%)</td>
<td>32 (47.1%)</td>
<td>18 (26.5%)</td>
<td>18 (26.5%)</td>
</tr>
</tbody>
</table>

### Table 3

**Allelic frequencies of ACE and AGT in patients and in controls**

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>M</th>
<th>D</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAD</strong></td>
<td>90.5%</td>
<td>9.5%</td>
<td>73.4%</td>
<td>26.6%</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>80.9%</td>
<td>19.1%</td>
<td>60.3%</td>
<td>39.7%</td>
</tr>
<tr>
<td><strong>Single vessel</strong></td>
<td>96.8%</td>
<td>3.2%</td>
<td>67.7%</td>
<td>32.3%</td>
</tr>
<tr>
<td><strong>Double/triple vessel</strong></td>
<td>97.9%</td>
<td>88.2%</td>
<td>2.1%</td>
<td>11.8%</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>77.1%</td>
<td>60.3%</td>
<td>22.9%</td>
<td>39.7%</td>
</tr>
<tr>
<td><strong>CAD with history of MI</strong></td>
<td>100%</td>
<td>0%</td>
<td>81.3%</td>
<td>18.8%</td>
</tr>
<tr>
<td><strong>CAD without history of MI</strong></td>
<td>94.9%</td>
<td>5.1%</td>
<td>65.4%</td>
<td>34.6%</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>88.2%</td>
<td>11.8%</td>
<td>60.3%</td>
<td>39.7%</td>
</tr>
</tbody>
</table>

*P<0.05, compared with control
**P<0.005, compared with control
According to ACE genotypes, DD genotype was higher in CAD patients compared with controls (P = 0.05), and in patients with double/triple vessel disease, DD genotype was higher compared with patients without history of MI and controls (P = 0.05, P = 0.01). D allele frequency was higher in the CAD patients, patients with double/triple vessel and patients with a history of MI compared with control subjects, and the difference was statistically significant (P = 0.05).

Potential interactions between both AGT and ACE gen polymorphisms

We analyzed a possible synergistic effect between the AGT and ACE polymorphisms (Table 4). In CAD and control subjects, interactions between both AGT and ACE gene variations were not observed. Subjects an interaction between both AGT and ACE gene polymorphisms was also not observed when the D allele of ACE and the T allele of M174T were combined (data not shown). The frequency of ACE DD + AGT TT carriers was not significantly different in the overall patient group compared with controls [83.7% vs 75.0%]; P > 0.05; Table 4].

Discussion

ACE and angiotensinogen are key components of the RA system, which is a powerful regulatory system with a major influence on salt and water metabolism and blood pressure. The present investigation was performed to analyse the relationships of the angiotensinogen AGT T174M and ACE gene polymorphisms with CHD and to search association with the extent of severity of coronary atherosclerosis among these polymorphisms.

ACE (I/D) polymorphism in CHD: Cambien et al\textsuperscript{18} first described an association between the DD genotype of the ACE gene polymorphism and MI. Other studies found an association with CHD,\textsuperscript{13} MI,\textsuperscript{19} hypertrophic cardiomyopathy,\textsuperscript{20} coronary artery restenosis,\textsuperscript{18} dilated cardiomyopathy\textsuperscript{21} and parental history of MI.\textsuperscript{22} In the present study, DD genotype and D allele frequency was higher in CAD patients compared with healthy subjects (P<0.05). However, conclusions derived from these studies remain controversial due to the lack of association between DD genotype and CHD in other reports.\textsuperscript{17,23,24} These findings stress the necessity of considering ethnic factors in the assessment of genetic risk identifiers. The DD cause faster and more potent vasoconstriction due to the rapid local conversion of AI into AII.\textsuperscript{25} Therefore vasoconstriction, vascular wall damage, inflammation and repairing process could be also related to genotype interactions of these genes. In a case-control study involving 697 Caucasian patients, Ludwig et al.\textsuperscript{9} defined the presence of CAD by at least a > 60% stenosis in any major coronary vessel, while the absence of CAD was defined by < 10% stenosis. The analysis was focused on the ACE I/D polymorphism and no relationship was observed between the genotype and the presence or absence of CAD. However, Nakai et al.\textsuperscript{8} found a positive relationship between the number of stenosed coronary arteries and the presence of the ACE D allele in 178 Japanese who had angiographically defined CAD. The frequency of the D allele was high in the 29 patients with triple-vessel disease (0.71) compared with the 95 patients with single vessel disease (0.54, P<0.01). Agreement with this study, in our study, the highest frequencies of D allele was observed in CAD patients with double or triple vessel disease and also in CAD patients with a history of MI compared with controls [0.77 (in Table 4 The relations between both AGT and ACE gene variations

<table>
<thead>
<tr>
<th></th>
<th>ACE</th>
<th>DD</th>
<th>Patients</th>
<th>Controls</th>
<th>ID</th>
<th>Patients</th>
<th>Controls</th>
<th>II</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT</td>
<td>TT</td>
<td>72 (83.7%)</td>
<td>24 (75.0%)</td>
<td>52 (86.7%)</td>
<td>12 (66.7%)</td>
<td>8 (66.7%)</td>
<td>14 (77.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>12 (14.0%)</td>
<td>4 (12.5%)</td>
<td>8 (13.3%)</td>
<td>6 (33.3%)</td>
<td>2 (14.0%)</td>
<td>0 (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>2 (2.3%)</td>
<td>4 (12.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (16.7%)</td>
<td>4 (22.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* P<0.05, compared with control

\* P<0.005, compared with control
Double/Triple vessel) vs 0.60 (in control) and 0.81 (in CAD with history of MI) vs 0.60]) (P<0. 05). More recently, Gardemann et al.26 found no difference in D-allele frequency among patients with single-, double-, or triple-vessel disease. In some case-control studies, patients were characterized according to the presence of angiographically defined CAD,27-32 but no analysis between the severity or extent of the coronary lesions and any of these RAS genetic polymorphisms was mentioned.

It is interesting to note that ACE is expressed in T lymphocytes33 that accumulate and participate in the inflammatory reaction at the site of coronary plaque disruption.34 In addition, ACE activity that is induced by experimental vascular injury35 could interfere with in situ fibrinolysis by degrading bradykinin, which is a potent mediator of release of tissue-plasminogen activator.36 Finally, it is tempting to speculate that increased RAS activity mediated by one or several of these polymorphisms could enhance coronary vasoconstriction, which has been found to be an important contributor to the pathogenesis of ischemic heart disease.37 Recent results obtained in Japanese CAD patients suggest that the DD genotype could represent a genetic susceptibility for coronary artery spasm.38

AGT polymorphisms in CHD: Research on angiotensinogen (AGT) gene started after 1992, when structural features of AGT gene T174M and M235T resulting in its higher concentration in plasma were discovered.13 The AGT gene has been mapped on chromosome 1q42-43 and implicated in essential hypertension through both genetic linkage and allelic association by Jeunemaitre et al.,13 initially in both Utah and French Caucasians. They studied M235T and T174M variants in exon 2 and reported that subjects carrying M235T genotypes with or without the T174M variant (0.14 and 0.33) were more associated with hypertensives than with controls (0.09 and 0.28). Since then, several studies have been carried out to show the association of M235T and T174M genotypes of AGT with CHD:29, 31,40-44 and hypertension,45-51 wherein positive as well as negative reports are documented. In the present study, in CAD patients TT Genotype frequency was higher compared with control subjects (0.83 vs. 0.73, P<0.05). Cong et al.41 have examined 140 CAD patients with diagnosed arterial stenosis for M235T and T174M frequencies. They found a reliable increase of 174TT in the group with CVD as compared to the control group. The frequencies of the T and M alleles in the present study sample show similarities to those of previous investigations.52-54 In controls, AGT T174M T allele frequencies of 0.8852, 0.82,53 0.8854 and 0.81 (present study) were calculated and we did not find significant association between mutant variant of the AGT genotype and the extent of severity of coronary artery disease with these patient groups.

Genotype combinations of ACE (I/D) and AGT T174M polymorphisms in CHD. The analysis of gene-gene interaction with ACE carried out by the authors showed that 174TT is a CVD risk factor for the patients with II genotype of ACE gene and BMI, 27 kg/m2.41 In contrast with this study, in our present study interactions between both AGT and ACE gene variations were not observed in the overall patient group, there was not a significantly different in the frequency of ACE DD + AGT TT carriers compared with healthy subjects (83.7% vs. 75.0%), P>0.05).

In conclusion, we failed to detect an association of angiotensinogen T174 M polymorphism with the extent of severity of coronary artery disease, but the present study finds an evidence to support a genetic association between the ACE I/D polymorphism and the extent of severity of CAD. Further studies are necessary to elucidate the genetic risk factors for severity of CAD and MI.

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