ASSOCIATION OF ANGIOTENSINOGEN T174M AND M235T GENE VARIANTS WITH DEVELOPMENT OF HYPERTENSION IN TURKISH SUBJECTS OF TRAKYA REGION

A. Ay Basak¹, T. Sipahi¹, S. Ustundag², Z. Ozgen², M. Budak¹, S. Sen², S. Sener²
Trakya University, School of Medicine, Department of Biophysics, Edirne, Turkey¹
Trakya University, School of Medicine, Department of Nephrology, Edirne, Turkey²
Correspondence to: Tammam Sipahi
E-mail: tammam@trakya.edu.tr

ABSTRACT
Genetic determinations of human essential (primary) hypertension are discussed by reviewing the candidate genes. Angiotensinogen (AGT) gene, coding the precursor of potent vasoactive hormone angiotensin II, in renin-angiotensin-system (RAS) has been reported to be associated with the onset of hypertension. The aim of this study was to investigate the role of variation in the 174 and 235 sites in exon 2 in AGT gene in the developing of primary hypertension in Turkish subjects from Trakya region. Our study involved 136 subjects, 84 hypertensive and 52 gender and age matched controls. T174M and M235T polymorphisms of the AGT gene were investigated using allele specific polymerase chain reaction (PCR) assay, and restriction fragment length polymorphism (RFLP).

The frequency of genotypes of the variant T174M in the patients with primary hypertension was TT=%73.8, TM=%26.2, and MM=%0.0, that were not different from the controls TT=%73.1, TM=%25.0, and MM=%1.9. And for M235T, the genotype frequencies in patients with primary hypertension were MM=%19.0 MT=%54.8, and TT=%26.2, which were again not significantly different from that of the controls MM=%26.9 MT=%46.2 and TT=%26.9.

In conclusion this study shows that T174M and M235T variants of the AGT gene were not associated with primary hypertension in Turkish subjects from Trakya region.

Keywords: Renin angiotensin system, angiotensinogen gene; T174M gene polymorphism; M235T gene polymorphism; essential (primary) hypertension

One frequently studied gene has been AGT, a logical candidate gene, given the strong correlation of plasma AGT levels and blood pressure (2, 14). Mature AGT, consisting of 452 amino acid residue, is produced in many tissues. The majority of serum AGT is synthesized in the liver (3, 22). Hence, the AGT level was much the same in carriers of allele M and in homozygotes TT of T174M (13).

AssoCIATIon of AngIoTensInogen T174M And M235T gene VArIAnTs wITh deVelopMenT of hyperTensIon In TurKIsh subjeCTs of TRAKyA regIon

Introduction
Primary hypertension is a common, polygenic (19), complex disorder, resulting from interaction of several genes with each other and with a number of factors such as inflammation (24), obesity (24, 35), cigarette smoking and alcohol intake (17, 21, 24), sodium sensitivity and insulin resistance (29), high salt intake (6), sedentary lifestyle (20, 36), stress (5), and dyslipidemia (41). Since the genetic alterations responsible for inherited primary hypertension remain elusive, currently most studies focus on the genes coding for proteins that regulate blood pressure as their physiological role makes them prime suspects (16, 19). Such proteins are present in renin-angiotensin-system (RAS), steroid-hormone metabolism, and renal sodium transporters (19).

The RAS may be the most important of the endocrine system that affects the control of blood pressure (16, 19). Some studies reported a possible influence of polymorphisms of genes encoding for components of the RAS such as the angiotensinogen (AGT) (30, 33), the angiotensin converting enzyme (ACE) (10, 28), and the angiotensin II type 1 receptor (ATIR) on the onset of hypertension (15, 38), while others did not (26, 27, 34).
The aim of this study was to investigate the association between the AGT T174M and M235T gene variants and primary hypertension in Turkish subjects from Trakya region, and to realize the fact that the association in one population could not be extrapolated to another population.

Materials and Methods

Reagents and chemicals
All reagents for PCR Amplification and Gel Electrophoresis were purchased from Fermentas Life Sciences (ELİPS), Istanbul, Turkey. All other chemicals were bought from Sigma and Merck (BO&GA), Istanbul, Turkey, and were of the highest purity available.

Patients and Blood Pressure
This research was designed as a case-control study. 84 untreated and uncomplicated primary hypertensive patients (34 females and 50 males), who were defined as those with multiple blood pressure readings ≥ 140/90 mmHg with mean age 41.7 ± 8.6 years, and age and sex matched 52 healthy subjects (21 females and 31 males) with mean age 39.6 ± 8.2 years, were enrolled. Healthy was defined as being free from hypertension and other diseases and not taking any drugs.

Blood pressure was measured after 5 minutes resting, twice with a conventional mercury sphygmomanometer in a sitting position with a 2 minutes interval between measurements, and the mean of the two readings was taken. All patients were found to have mild to moderate hypertension. This definition is consistent with the clinical definition of high blood pressure according to WHO/ISH hypertension guidelines (39). To eliminate the decline in renal function and overt atherosclerosis, patients with corrected endogen creatinine clearance < 85 ml/min/1.73 m², overt proteinuria detectable by 5% sulfosalicylic acid, myocardial infarction or stroke and unstable angina pectoris were excluded. Pregnancy, diabetes mellitus (according to second hour of oral glucose tolerance test), liver and renal disease, malignancy, acute, chronic infection and taking multiple vitamins were also considered as exclusion criteria.

Approval for the study was obtained from the Ethics Committee of Trakya University School of Medicine.

DNA Extraction
Peripheral blood (2 ml) was collected into tubes containing ethylenediamine-tetraacetic acid (EDTA) as an anticoagulant. DNA was isolated from Peripheral blood, containing EDTA, by E.Z.N.A. (EaZy Nucleic Acid Isolation) blood DNA kits (Omega Bio-tek, Doraville, USA).

DNA purity and quantity were assessed by absorbance values in spectrophotometer (Shimadzu UV-1208) and checked by 0.3% agarose gel electrophoresis (4, 32).

 Determination of T174M and M235T Genotypes
Amplification of Genomic DNA by Polymerase Chain Reaction (PCR)
To determine the T174M and M235T genotypes of the hypertensive and normotensive groups, a genomic DNA fragments (Fig. 1) on exon 2 of the AGT gene were amplified by PCR in a 25 µl PCR reaction mixture containing 200ng of DNA, deoxynucleotide triphosphates (0.2 mM of each), upstream and downstream oligonucleotide primers (0.5 nmol), 75 mM tris-hcl (ph 8.8), 20 mM (NH₄)₂SO₄, 0.01% Tween 20, and 1.25 U of Taq DNA polymerase (Fermentas Life Sciences). The reactions contained 2.5 mM MgCl₂ for T174M and 2 mM MgCl₂ for M235T respectively. For T174M the PCR primers with the sequences reported by Niu et al. (26) were used. And for M235T the PCR primers with the sequences reported by Russ A.P. et al. were used (31). The PCR antisense primer for M235T contains two mismatches (CT→AC).

DNA amplification were performed with a Techne (TechGene) DNA Thermal Cycler with 5 min of denaturation at 95°C, followed by 37 cycles with DNA denaturation for 30 sec at 95°C, annealing for 45 sec at 68°C, and extension for 45 sec at 72°C for T174M, and 5 min of denaturation at 95°C,
followed by 35 cycles with DNA denaturation for 1 min at 95°C, annealing for 1 min at 68°C, and extension for 1 min at 72°C for M235T, followed by 10 min of extension at 72°C in both protocols. The PCR products were electrophorized on 2% agarose gels, stained with ethidium bromide, and checked under ultraviolet light. In all PCR experiments several reactions containing no DNA were included to control the possibility of contamination.

**Determination of T174M Polymorphism by NcoI Restriction Enzyme**

The PCR products were digested with NcoI (Takara Bio Inc, Japan) according to the manufacturer’s instructions, obtaining for the 174M variant (presence of thymine at position 520; C↑CAGGG) a 155 bp and a 198 bp fragments, and a 353 bp for the 174T variant (presence of cytosine at position 520; CCACCGG) (Fig. 1) (25, 26). General reaction mixture was electrophorized on 2.5% agarose gels and stained with ethidium bromide (Fig. 2).

**Determination of M235T Polymorphism by Tth111I Restriction Enzyme**

The specific mismatches incorporated into the antisense primer create Tth111I (Takara Bio Inc, Japan) site if the 235T variant (presence of cytosine at position 704; GAGCN↓NN GTG) a 141 and a 24 bp fragments, and for the 235M variant (presence of thymine at position 704; GATNNNGTG) a 165 bp (no restriction fragments) (Fig. 1) (31, 37). Mixture was electrophorized on 2.5% agarose gels and stained with ethidium bromide (Fig. 3).

**Statistical Analysis**

Statistical analyses were performed with the SPSS 15.0 software and STATA program, version 8. Data are presented as mean ± SD, median, or as percent frequency. Normality of distribution was checked by using the Kolmogorov-Smirnov test. Independent-Samples T test or Mann-Whitney U test was used to evaluate differences in continuous variables. Chi-square test was performed to determine the differences dichotomized variables between control and patient group and female and male patients. Allele frequencies were calculated from the genotypes of all subjects. Hardy-Weinberg equilibrium was assessed by χ² analysis. Allele and genotype frequencies were compared by standard contingency table analysis using chi-square test. P < 0.05 was considered statistically significant.

**Results and Discussion**

At baseline the two groups were similar with regard to gender and age. Essential hypertension patients had higher family history of hypertension, systolic and diastolic blood pressures, and mean arterial pressure (p < 0.001), Risk factors for hypertension such as smoking, triglycerides and total cholesterol were not significantly higher in the hypertension group than the control group (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Control Group (n:52)</th>
<th>p</th>
<th>HT Group (n:84)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>21/31</td>
<td>NS</td>
<td>34/50</td>
</tr>
<tr>
<td>Family history of HT (%)</td>
<td>51.9</td>
<td>&lt;0.001</td>
<td>87.2</td>
</tr>
<tr>
<td>Current Smoker (%)</td>
<td>43.1</td>
<td>NS</td>
<td>27.7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.6±8.2</td>
<td>NS</td>
<td>41.7±8.6</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>118±9</td>
<td>&lt;0.001</td>
<td>155±11</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>75±13</td>
<td>&lt;0.001</td>
<td>98±7</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>90±7</td>
<td>&lt;0.001</td>
<td>117±7</td>
</tr>
<tr>
<td>FFBG (mg/dl)</td>
<td>89±10</td>
<td>0.002</td>
<td>95±10</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>126±69</td>
<td>NS</td>
<td>160±115</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>184±39</td>
<td>NS</td>
<td>197±57</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48±13</td>
<td>NS</td>
<td>47±12</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>110±38</td>
<td>NS</td>
<td>118±52</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. HT, hypertensives; SBP and DBP, systolic and diastolic blood pressures; MAP, mean arterial pressure; FFBG, fasting blood glucose; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; NS, non significant.
All genotype distributions are in Hardy-Weinberg equilibrium. Genotypic distribution of T174M and M235T polymorphisms and allelic frequencies in patients and control subjects are presented in Table 2 and Table 3, respectively. There was no statistically significant difference in either the genotypic distribution or allelic frequency between the patient and control groups for T174M and M235T polymorphisms.

The demographic and clinical characteristics of the primary hypertension group classified according to gender were shown in Table 4. The female and male groups were similar with regard to family history of hypertension, smoking, systolic blood pressures, mean arterial pressure, fasting blood glucose, and total cholesterol. About the other clinical characteristics they were considered statistically significant.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>T174M Genotype Frequency (%)</th>
<th>T174M Allele Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group (%)</td>
<td>TT 73.1, TM 25.0, MM 1.9</td>
<td>T 85.6, M 14.4</td>
</tr>
<tr>
<td>HT Group (%)</td>
<td>TT 73.8, TM 26.2, MM 0</td>
<td>T 86.9, M 13.1</td>
</tr>
</tbody>
</table>

* Non significant between groups

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>M235T Genotype Frequency (%)</th>
<th>M235T Allele Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group (%)</td>
<td>MM 26.9, MT 46.2, TT 26.9</td>
<td>M 50.0, T 50.0</td>
</tr>
<tr>
<td>HT Group (%)</td>
<td>MM 19.0, MT 54.8, TT 26.2</td>
<td>M 46.4, T 53.6</td>
</tr>
</tbody>
</table>

* Non significant between groups

Distributions of T174M and M235T genotype and allele frequencies in the HT group classified according to gender were shown in Tables 5 and 6. No significant difference between both groups was observed.

The renin-angiotensin system is an important component of blood pressure regulation, playing roles in saltwater homeostasis and vascular tone, and has been suspected to be involved in hypertension. Genetic linkage of human hypertension to the angiotensinogen locus was obtained in several studies, which have shown a high prevalence of the M174 and T235 variants among patients with hypertension (1, 14, 33). In other studies, researches have shown no differences in the allele frequencies and genotype distributions of AGT T174M and M235T gene polymorphisms between the control and essential hypertensive groups (8, 18, 37). In our study we used the candidate gene approach to determine whether the gene encoding the angiotensinogen, the precursor of potent vasoactive hormone angiotensin II, is involved in human primary hypertension in Turkish subjects from Trakya region. To avoid the risks, especially in the aged population, which may mask the effects of a single genetic factor such as a high-salt diet, smoking and obesity, we examined young subjects with hypertension. These subjects may possess strong genetic backgrounds for primary hypertension. So, we screened 84 hypertensives (mean age 41.7±8.6) and 52 age matched controls (mean age 39.6±8.2), we could not identify any association between the AGT T174M and M235T gene variants and primary hypertension. Our study was the first study in Trakya region which aims to investigate the association between the AGT T174M and M235T gene variants and primary hypertension. The present finding for these two genetic polymorphisms did not confirm the previous study in the Turkish population, in which a presence of genetic influence on hypertension was found (1). The study; which was made by Agachan et al. in Istanbul, consisted of subjects from the hypertension outpatient clinic of Marmara University Hospital. In Agachan et al. study hypertensive group consisted...
of a larger subject group than in ours (109 subjects), but contained aged population (mean age 51.73±9.72).

Conclusions
In conclusion this study shows that T174M and M235T variants of the AGT gene were not associated with primary hypertension in Turkish subjects from Trakya region. However, the present study is limited in terms of the small sample of subjects. Therefore, further studies, involving larger samples of hypertensive young subjects, are needed to clarify the role of T174M and M235T gene polymorphisms in hypertensives Turkish patients from Trakya region.

REFERENCES

TABLE 5
Distributions of T174M genotype and allele frequencies in the HT group classified according to gender

<table>
<thead>
<tr>
<th>T174M Genotype Frequency*</th>
<th>T174M Allele Frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>TM</td>
</tr>
<tr>
<td>Female (%)</td>
<td>67.6</td>
</tr>
<tr>
<td>Male (%)</td>
<td>78.0</td>
</tr>
</tbody>
</table>

*Non significant between groups

TABLE 6
Distributions of M235T genotype and allele frequencies in the HT group classified according to gender

<table>
<thead>
<tr>
<th>M235T Genotype Frequency*</th>
<th>M235T Allele Frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>MT</td>
</tr>
<tr>
<td>Female (%)</td>
<td>23.5</td>
</tr>
<tr>
<td>Male (%)</td>
<td>28.0</td>
</tr>
</tbody>
</table>

* Non significant between groups