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Original Article

Angiotensinogen gene M235T and angiotensin II-type 1 receptor gene A/C1166 polymorphisms in chronic obtructive pulmonary disease

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Abstract: Chronic obstructive pulmonary disease (COPD) occurs irreversibly and is characterized by progressive airflow obstruction. Renin angiotensin system (RAS) has many different key enzymes and receptors that have a role for different systemic processes. We aimed to determine genotype and allele frequencies of angiotensinogen (AGT) M235T and angiotensin II-type 1 receptor (AT1-R) A/C1166 polymorphisms in patients with COPD. This study was performed on 56 unrelated COPD patients and 29 healthy subjects. DNA samples for each individual were isolated from peripheral blood by phenol/chloroform method, analyzed by polymerase chain reaction and enzymatic digestion methodologies. The distribution for each of AGT genotypes were 23.2% for MM (13), 75.0% for MT (42) and 1.8% for TT (1) in the COPD group; 37.9% for MM (11), 34.5% for MT (10) and 27.6% for TT (8) in the control group. The distribution of AGT genotypes was found significantly different between groups (X^2 = 18.604; df = 2; P = 0.000). The frequencies for each of the AT1-R genotypes were found as 53.6% for AA (30), 42.9% for AC (24), 3.6% for CC (2) in the COPD group; 55.2% for AA (16), 41.4% for AC (12) and 3.4% for CC (1) in the control group. The distribution of AT1-R genotypes did not change significantly between groups. Allele frequencies of interested genes were not significantly different between groups. We suggest that AGT polymorphism may play a role for the development of COPD. We believe these data can be served for large scale population genetics research, considering the frequency of AGT and AT1-R genes and alleles in COPD patients in the Turkish population.

Keywords: Angiotensinogen, M235T polymorphism, angiotensin-II type 1 receptor, A/C1166 polymorphism, chronic obstructive pulmonary disease

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by abnormally an excessive inflammatory response of the lung parenchyma to inhaled irritants and toxins. It causes an irreversible obstruction of the small airways. This disease is rapidly increasing, especially in developed countries, and it is expected that it will be one of the most important causes of mortality worldwide in the near future [1, 2]. Different researchers have reported that there are possible correlations between several genetic polymorphisms and the development of COPD [3-5].

The Renin Angiotensin System (RAS) is one of the main regulatory systems for blood pressure and fluid homeostasis. It has also a role in cardiovascular remodeling and vascular tone. This system contains many proteins such as angiotensinogen (AGT), angiotensin converting enzyme (ACE), ACE2, angiotensin II (AngII) and several receptors such as Ang-II Type 1 Receptor (AT1-R) and Ang-II Type 2 Receptor (AT2-R) [6]. In enzymatic cascade, AGT is cleaved by renin (REN) to form AngI. After that, AngI is further cleaved by ACE to produce AngII, which is the main effector of the RAS [6, 7]. The effects of the AngII are transmitted through its specific receptors. Angiotensin II receptor 1 (AT1-R) is responsible for the vasoconstriction in response to AngII [8]. Both of polymorphisms for the AGT gene M235T and for the AT1-R gene A1166C have been identified within the RAS [9]. The AGT

M235T single nucleotide polymorphism (SNP) is a methionine (Met) to threonine (Thr) amino acid substitution at codon 235, designated the M and T alleles, respectively [10]. A functional role for the AGT M235T polymorphism has been reported that the 235T rare variant accounts for higher AGT level in circulating blood than those homozygous for the M235. Heterozygotes have an intermediate level of AGT [7, 11]. In the literature, links between AT1-R polymorphism and the pathological states have not been examined in detail, although the AT1-R mediates most of the physiological effects of AngII. In one of these rare studies it has been reported that there is a possible association of the AT1-R C allele with hypertension in Lebanese hypertensive patients [12]. It is also known that AT1-R C allele is associated with greater response to AngII at lower concentrations [13].

Although other groups have identified the relations of gene polymorphisms in RAS with different clinical settings [14-16], we could not observe any report about AGT M235T and AT1-R A/C1166 polymorphisms in patients with COPD. We believe eliminating RAS polymorphisms in COPD can provide benefits to improve clinical treatments and help us understand COPD pathophysiology and development of comorbidities. Therefore, we aimed to determine the genotype and allele frequencies of AGT M235T and AT1-RA/C1166 polymorphisms in patients with COPD.

Materials and methods

Study design

The study was performed on 56 unrelated COPD patients who were treated at the Department of Thoracic Medicine, Medical Faculty, at Dumlupınar University in Kütahya, Turkey. The diagnosis of COPD was established on the basis of criteria proposed by Global Initiative for Chronic Obstructive Lung Disease (GOLD). The control group consisted of 29 healthy age-matched subjects. The COPD group consisted of 15 females and 41 males, and the control group consisted of 10 females and 19 males. Participants of each group were chosen among the Turkish population. The procedures were explained to all subjects, and a written informed consent was obtained from each individual. The study protocol conformed to the ethical guidelines of Declaration of Helsinki and was approved by the Ethics Committee of Afyon Kocatepe University.

Detection of ANG gene M235T and AT1-R gene A/C1166 polymorphisms

Blood samples were collected in tubes containing EDTA from patients and healthy subjects. In total, 85 genomic DNA from peripheral blood leukocytes were isolated by standard phenol/chloroform extraction method as described previously [17].

AGT amplification of DNA was performed by PCR and M235T polymorphism determined by enzyme digestion methodologies. Genomic DNA was subjected to 30 cycles of specific amplification of exon 2 of the AGT gene in 50 µl of a buffer that contained 50 mmol/l KCl, 5 mmol/l Tris-HCl, 0.01% gelatin, 1.5 mmol/l $MgCl_2$, 125 µmol/l NTPs, 10 pmol forward primer 5'-CCG TTT GTG CAG GGC CTG-3' and reverse primer 5'-TGC TGT CCA CAC TGG ACC CC-3', and 0.5 U Taq polymerase at 94°C for 1 min, 61°C for 1 min, 72°C for 1 min followed by digestion of 165 bp PCR product with restriction enzyme Tth 111I for 2 h at 65°C. Digested products were separated on a 1, 5% agarose gel. Diagnostic fragments were visualized by ethidium bromide staining and UV transillumination. The heterozygous form (MT) was indicated by the appearance of two fragments of 165 bp and 141 bp, while the homozygous form revealed only one band of 141 bp (TT) and individuals lacking this mutation showed only one band of 165 pb (MM) [18].

AT1-R polymorphism was determined using PCR and enzymatic digestion methods. The sequence of the sense and antisense primers were 5'-ATA ATG TAA GCT CAT CCA CCA AGA AG-3' and 5'-TCT CCT TCA ATT CTG AAA AGT ACT TAA-3', respectively. Reactions were performed on 1 µl genomic DNA in a final volume of 50 µl containing 100 pmol each primer, 1.5 mmol/l $MgCl_2$, 75 mmol/l Tris-HCl (pH 9.0), 20 mmol/l $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 0.2 mmol/l of each dNTP, and 0.5 U Taq polymerase. The amplification profile included an initial denaturation step at 94°C for 3 min and 30 cycles of denaturation at 96°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 45 s with a final extension time of 5 min. PCR products were digested with the AfI II restriction endonuclease. The heterozygous form was indicated by the appearance of two fragments of 139 bp and 166 bp (AC). The homozygous form revealed only one band of 139 bp (CC) and individuals lacking this mutation showed only one band of 166 bp (AA) [13].

Table 1. Hardy-Weinberg equilibrium of the AGT gene M/T polymorphism and AT1-R gene A/C polymorphism

		COPD		Control	
	Genotype	Expected	Observed	Expected	Observed
Common homozygotes	MM	20.6	13	8.8	11
Heterozygotes	MT	26.7	42	14.3	10
Rare homozygotes	TT	8.6	1	5.8	8
		$X^2 = 18.33$, $P < 0.05$		$X^2 = 2.66$, $P > 0.05$	
	Genotype	Expected	Observed	Expected	Observed
Common homozygotes	AA	31.5	30	16.7	16
Heterozygotes	AC	21.0	24	10.6	12
Rare homozygotes	CC	73.5	2	1.7	1
		$X^2 = 1.14$, $P > 0.05$		$X^2 = 0.49$, $P > 0.05$	

Table 2. Genotype and allele frequencies of the AGT gene M/T polymorphism and A/C AT1-R gene polymorphism

	COPD		Control	
	n	%	n	%
Genotype Frequency				
AGT M/T polymorphism				
MM	13	23.2	11	37.9
MT	42	75.0	10	34.5
TT	1	1.8	8	27.6
Total	56		29	
$X^2 = 18.604$; df = 2; P = 0.000				
AT1-R A/C polymorphism				
AA	30	53.6	16	55.2
AC	24	42.9	12	41.4
CC	2	3.6	1	3.4
Total	56		29	
$X^2 = 0.02$; df = 2; P = 0.990				
Allele Frequency				
AGT M allele	68	60.7	32	55.2
AGT T allele	44	39.3	26	44.8
$X^2 = 0.485$; df = 1; P = 0.486				
AT1-R A allele	64	69.6	44	75.9
AT1-R C allele	28	30.4	14	24.1
$X^2 = 0.700$; df = 1; P = 0.403				

COPD = chronic obstructive pulmonary disease; AGT = angiotensinogen; AT1-R = angiotensin II receptor I; df = degrees of freedom.

Statistical analysis

Statistical analyses were done by SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) 16.0 package program. All data are given as mean ± standard error of the mean (SEM).

Statistical significance of the observed genotype frequencies was evaluated according to the Hardy-Weinberg rule compared to the expected genotype frequencies. The Chi-square test was used for comparison of genotype and allele frequencies between groups. All *P* values < 0.05 were accepted as statistically significant.

Results

The mean age was found as 67.9 ± 1.5 in the COPD group and 59.6 ± 4.2 in the control group. The frequency of genotype for AGT gene M/T polymorphism in the COPD group showed a significant deviation from the Hardy-Weinberg equilibrium ($P < 0.05$). The frequency of the genotype for the AGT gene M/T polymorphism in the control group did not show a significant deviation from the Hardy-Weinberg equilibrium ($P > 0.05$) (**Table 1**). The frequencies for each of the AGT genotype were found as 23.2% for MM (13), 75.0% for MT (42) and 1.8% for TT (1) in the COPD group; 37.9% for MM (11), 34.5% for MT (10) and 27.6% for TT (8) in the control group. The distribution of AGT genotypes was found significantly different between groups ($X^2 = 18.604$; df = 2; P = 0.000) (**Table 2**). The frequency of MT genotype was significantly higher in the patients than the control group (odds ratio for MT = 5.7; 95% CI = 2.3-19.3; P = 0.000). The frequency of TT genotype was significantly higher in the control group than the patients (odds ratio for TT = 0.048; 95% CI = 0.024-0.297; P = 0.000). The allele frequencies for the AGT gene in the COPD patients and the control subjects were shown in **Table 2**. The distribution for the AGT gene M alleles were found (68) 60.7% in the COPD group and (32) 55.2% in the control group; T alleles were found (44) 39.3% in the COPD group and (26) 44.8% in the control group. There was not statistically significant difference between the groups for allele frequency ($X^2 = 0.485$; df = 1; P = 0.486) (**Table 2**).

The frequency of genotype for the AT1-R gene A/C polymorphism in COPD and control groups did not show a significant deviation from the Hardy-Weinberg equilibrium ($P > 0.05$) (**Table**

1). The frequencies for each of the AT1-R genotype were found as 53.6% for AA (30), 42.9% for AC (24), 3.6% for CC (2) in the COPD group; 55.2% for AA (16), 41.4% for AC (12) and 3.4% for CC (1) in the control group. The statistical analyses of the data indicated there were no statistically significant differences between the control and COPD groups for AT1-R genotypes (X^2 = 0.02; df = 2; *P* = 0.990) (**Table 2**). The allele frequencies for the AT1-R gene in the COPD patients and control subjects were shown in **Table 2**. The distribution of the AT1-R gene A alleles were found (64) 69.6% in the COPD group and (44) 75.9% in the control group; C alleles were found (28) 30.4% in the COPD group and (14) 24.1% in the control group. There was no statistically significant difference between the groups for allele frequency (X^2 = 0.700; df = 1; P = 0.403) (**Table 2**).

Discussion

COPD is a highly prevalent chronic disease and characterized by heterogeneous chronic airway inflammation and obstruction. It is well known that progressive airway obstruction in COPD is largely irreversible. In this disease inflammation affects the whole respiratory tract and it also has systemic features [19, 20]. RAS is a complex system. Mainly, this system plays a critical role in the regulation of blood pressure and fluid homeostasis. Gene polymorphisms of RAS components have been identified in different populations and also in several different clinical cases [14-16].

AGT, which is synthesized in the liver, is one of the most significant component of the RAS. It plays an important role in salt and water homeostasis [21]. The AGT gene M235T polymorphism has been associated with elevated plasma levels of AGT, with 235TT homozygotes having more plasma level of AGT, approximately 15% more than in 235 MM individuals. It has been reported that there is a possible correlation between M235T polymorphism and hypertension, coronary heart disease and atrial fibrillation [16, 22]. Recent studies have implied the AGT M235T gene variant is associated with inflammation as well [21]. Meta-analysis suggests that M235T polymorphism is related with increased risk of heart failure in Caucasians [22]. An association between AGT M235T polymorphism and myocard infarction risk in Asians but not in the overall population has been reported [10]. It has been accepted that plasma AGT level was increased in subjects carrying the 235T allele in Caucasians, but not in Asians [23]. Researchers reached different conclusions about gene polymorphisms in different populations. These outcomes could because of variations in genetic and environmental backgrounds across the numerous populations [12]. Recently, we have reported that ACE, which is one of the RAS component, gene I/D polymorphism, does not show a significant difference between COPD and control subjects who are from the Aegean part of Turkey [24]. We did not observe any report about AGT M235T polymorphism in COPD. In the present study, we observed the distribution of AGT genotypes was significantly different between COPD and the control subjects, although the frequency of genotype for AGT gene M235T polymorphism in COPD group showed a significant deviation from the Hardy-Weinberg equilibrium. This be a result of genetic drift. We assume that rare TT genotype causes genetic drift in COPD population. Patients with COPD who have TT genotype may be evaluated from the population because of elevated AGT level, which may have deleterious effects on COPD pathophysiology. We observed that, the MT genotype is significantly higher in COPD, compared to the control group. This can cause a higher plasma level of AGT in COPD, compared to control; but to confirm it, we need to conduct further studies. On the other hand, the TT genotype was found significantly higher in the control, compared to COPD. It is highly possible that T allele disappears in the COPD population because of its deleterious effects. We think it is also could be a sign that there is a relationship between the TT genotype and development of COPD. Most probably, COPD patients with TT genotype reach the final stage of their life span earlier than other subjects with the MM and MT genotypes.

AT1-R C allele has been found to be associated with hypertension in Lebanese hypertensive patients [12]; however, it has been determined that the AT1-R gene CC genotype is not a risk factor for myocardial infarction in patients in a South Indian population [25]. Previously, we have reported AT1-R polymorphisms as possible risk factors for the development of hypertension in acromegaly. The same study suggests that AT1-R A/C1166 genotype may be used as a genetic marker for developmental

progress of acromegaly [16]. In the literature, we could not observe any study, which explains the relation between AT1-R A/C polymorphism and COPD. Our results comprise the first report about the frequency for AT1-R A/C1166 genotype in COPD in the Turkish population. We believe further studies are needed in different populations to explain the clear function of interested polymorphism on development of COPD.

Conclusion

We conclude that there seems to be a possible relation between AGT gene M235T SNP and COPD. However, we could not observe any significant difference for AT1-R A/C1166 polymorphism between COPD and control groups. We believe that AGT gene M235T SNP can be useful for outcome predictions during diagnostic processes and can be helpfull in finding new treatment strategies. Our results may be taken as a basis for large-scale studies on AGT and AT1-R genotypes in COPD. We suppose that further studies are needed to clarify the exact role of obtained polymorphisms on COPD development.

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Disclosure of conflict of interest

None.

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