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Thymosin beta-4 A/T polymorphism and acute coronary syndrome risk

Timozin beta-4 A/T polimorfizmi ve akut koroner sendrom riski

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Abstract

Aim: Acute coronary syndrome (ACS) describes all the clinical conditions due to myocardial infarction that is caused by decreased blood flow in the coronary artery. Thymosin beta-4 (Tβ4) plays a significant role in the recovery of damaged tissues and promoting the survival of cardiomyocytes in ACS. In this study, it was aimed to determine the Tβ4 A/T (rs75112573) variation in ACS and its effects on the disease.

Methods: This was a prospective case-control study. Forty-eight patients with ACS and 45 healthy controls were recruited for this study. Genetic analysis was performed using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP).

Results: The AT genotype (p<0.001, X2:12.40, OR:5.42, 95% CI:2.02-14.53) and the A allele (p<0.001, X2:17.22, OR:6.66, 95% CI:2.61-16.98) frequency was found significantly higher in the patient group, while in the control group the TT genotype was statistically higher (p<0.001, X2:17.22, OR:2.13, 95% CI:1.44-3.16). LDL-cholesterol levels in the patient group (p<0.001, 95% CI:30.12-55.90), and HDL-cholesterol levels in the control group (p<0.001, 95% CI:5.30-15.34) were significantly higher. In the patient group, total cholesterol and HDL-cholesterol levels were found significantly higher in AT genotype carriers compared to the AA genotype carriers (p=0.036, 95% CI:0.59-17.25), while VLDL-cholesterol levels were higher in the AA genotype carriers compared to the AT (p=0.011, 95% CI:6.73-49.86), and TT (p=0.018, 95% CI:4.95-49.49) genotype carriers.

Conclusion: It can be concluded that carrying the Tβ4 A/T (rs75112573) gene polymorphism AT genotype and the A allele may increase risk of ACS.

Keywords: Thymosin beta-4 A/T, polymorphism, acute coronary syndrome, PCR-RFLP

Öz

Amaç: Akut koroner sendrom (ACS), koroner arter kan akımının azalması sonucu miyokard iskemisinin neden olduğu klinik tabloların tamamını ifade eder. Thymosin beta-4 geni (Tβ4) hasarlı dokuların iyileşmesinde ve ACS'de kardiyomiyositlerin canlı kalmasında önemli rol oynamaktadır. Bu çalışmada ACS'li hastaların Tβ4 genindeki A/T (rs75112573) varyasyonunun tespit edilmesi ve akut koroner hastalığına olan etkilerinin belirlenmesi amaçlanmıştır.

Yöntemler: Bu prospektif bir vaka kontrol çalışmasıdır. Çalışmaya ACS'li 48 hasta ve 45 sağlıklı kontrol birey dahil edildi. Genetik analiz, polimeraz zincir reaksiyonu/restriksiyon parça uzunluk polimorfizmi (PCR / RFLP) yöntemleri kullanılarak yapıldı.

Bulgular: Kontrol grubu ile karşılaştırıldığında hasta grubunda AT genotipi (p<0.001, X2:12.40, OR:5.42, %95 CI:2.02-14.53) ve A alleli (p<0.001, X2:17.22, OR:6.66, %95 CI:2.61-16.98) taşıma sıklığı anlamlı olarak yükselmiştir. Kontrol grubunda ise TT genotip sıklığının hasta grubuna göre istatistiksel olarak yüksek olduğu gözlemlenmiştir (p<0.001, X2:17.22, OR:2.13, %95 CI:1.44-3.16). Hasta grubumuzun LDL-kolesterol seviyesi (p<0.001, %95 CI:30.12-55.90), kontrol grubumuzun ise HDL-kolesterol seviyesi yüksek bulunmuştur (p<0.001, %95 CI:5.30-15.34). Hasta grubunda AT genotipi taşıyanlarda AA genotipi taşıyanlara göre kolesterol ve HDL-kolestrol düzeyleri anlamlı derecede yüksek olarak bulunmuştur (p=0.036, %95 CI:0.59-17.25). VLDL-kolestreol düzeyleri ise hasta grubunda AA genotipi taşıyanlarda, AT (p=0.011, %95 CI:6.73-49.86) ve TT (p=0.018, %95 CI:4.95-49.49) genotipi taşıyanlara göre anlamlı şekilde yükselmiştir.

Sonuç: Tβ4 A/T (rs75112573) gene polymorphism için AT genotipi ve A alleli taşımanın ACS riskini artırabileceği sonucuna ulaşılabilir.

Anahtar kelimeler: Timozin beta-4 (Tβ4) A/T, polimorfizm, akut koroner sendrom, PCR-RFLP

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Introduction

Cardiovascular diseases are the most significant reason of general morbidity and mortality in industrialized and developing countries [1]. Acute coronary syndrome (ACS) is the leading case of cardiovascular diseases, the most common reason of administration to emergency response service or hospitals, especially the coronary care units, and death in both Turkey and worldwide, although many innovations and advances have been made in the diagnosis and treatment lately [2-4]. ACS comprises all clinical conditions that cause myocardial ischemia, which is identified as reduced blood flow in the coronary artery, including unstable angina, ST-segment elevation myocardial infarction or non-ST-segment elevation myocardial infarction [5, 6].

Thymosin beta-4 (Tβ4) is an actin binding protein, containing 43 amino acids and weighing 5kDa, coded by the TMSB4X gene. Tβ4, which has a preventive effect against many pathological conditions, play important roles in repair of damaged tissues and perpetuating cardiomyocytes in acute coronary syndrome [7, 8]. Moreover, it also shows functions as enabling endothelial cell migration, acceleration of angiogenesis, slowing down the inflammatory response, and avoiding apoptosis and oxidative damage [9]. Some studies suggested that Tβ4 might be a significant factor in regulation of myocardial infarction since it is found in high concentrations in platelets and wound fluids [10]. Recently, it has been shown that Tβ4 is expressed in developing hearts, induces cardiomyocytes and migration of endothelial cells, and ultimately play an important role in cardiac vessel development [11, 12].

To date, there have been no studies present conducted on the genetic variants of Tβ4. In this study, it was aimed to determine the Tβ4 gene A/T (rs75112573) variant in ACS patients and its effects on the disease.

Material and methods

Subject Selection

This research has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the Istanbul Medical Faculty Ethical Committee, Istanbul University (#2012/1669-1263). The sample size to be used in the study was determined with power analysis. According to the results of this analysis, the minimum sample size required to detect a significance difference using this test should be at least 40 individuals in each group (in total 80 individuals), considering type I error (α) of 0.05, power (1-β) of 0.8 and effect size of 0.9.

The Tβ4 A/T (rs75112573) gene polymorphism was investigated in 45 healthy subjects (28 women, 17 men) who did not have any heart disease and 48 patients (22 women, 26 men) diagnosed with ACS admitted to the Department of Cardiovascular Surgery, Medicana Bahcelievler Hospital, Istanbul, Turkey, between January 2013 and January 2014.

The patient group was randomly selected patients who were diagnosed with ACS in the hospital which the samples were collected. ST segment elevated q positive or non ST segment elevated but enzyme positive patients are included into the group. Unstable angina pectoris (USAP) patients were excluded. All patients who are diagnosed with acute coronary syndrome had at least one vessel disease detected angiographically. The mentioned control group was created by the people who are working at the same hospital via a survey. Especially the lack of family history was the main criteria to maintain the survey. While creating the control group, the age range was held constant with the patient group.

The biochemical values of individuals with ACS included in the study were obtained from routine laboratory tests and are shown in Table 1.

Table 1. Clinical details of the patient and control groups.

Parameters	Patient Group (n=48)	Control Group (n=45)	P
LDL-cholesterol (mg/dL)	130.10±32.7	87.09±29.9	<0.001
Blood urea nitrogen (mg/dL)	21.25±14.2	-	-
HDL-cholesterol (mg/dL)	39.90±9.4	50.22±14.6	<0.001
Cholesterol (mg/dL)	213.12±40.0	167.96±35.4	<0.001
VLDL-cholesterol (mg/dL)	43.15±24.8	30.17±18.6	<0.001
Triglyceride (mg/dL)	153.29±93.1	27.85±22.1	<0.001
Creatinine (mg/dL)	1.20±1.2	-	-
Urea (mg/dL)	42.97±27.8	-	-
Presence of diabetes Mellitus (%)	58.3	-	-
Presence of hypertension (%)	81.2	-	-
Glucose (mg/dL)	155.98±85.4	-	-

LDL: Low density lipoprotein, HDL: High density lipoprotein, VLDL: Verl low density lipoprotein.

DNA Isolation and Analysis of Polymorphism

In EDTA containing tubes, 10ml of venous blood samples were obtained from the participants. Samples were stored at -20°C until the genomic DNA isolation was performed using the salting out method [13]. Primers used for the polymerase chain reaction (PCR) amplifications of the regions of the Tβ4 A/T polymorphism are given in Table 2. Reaction volumes were set for a total of 25 µl as 16.5 µl apyrogenic water, 2.5 µl MgCl2 free (10X) buffer, 1.7 µl MgCl2 (25mM), 1.5 µl dNTP (10 mM), 1.5 µl mix of forward (10 pmol) and reverse primers (10 pmol), 0.3 µl Taq polimerase (5U/µl) and 1 µl 200 ng/µl genomic DNA sample. PCR mixes were prepared on ice and in a sterile cabin.

For the Tβ4 A/T polymorphism, the PCR reaction conditions were set as following the initial denaturation of 95ºC for 5 minutes, 94 ºC for 45 sec, 65 ºC for 45 sec and 72 ºC 45 sec for 35 cycles and a final elongation duration of 5 min at 75 ºC. PCR yields were controlled on 3% agarose gel electrophoresis.

In order to determine the Tβ4 A/T polymorphism, obtained PCR yields were digested with the Tsp45I restriction enzyme. Digested yields were separated on 3% agarose gel electrophoresis and genotyped after being viewed under UV light. The obtained PCR and restriction yields and genotyping of the polymorphisms are shown in Table 2.

Table 2. PCR–RFLP-based evaluation of the Tβ4 A/T (rs75112573) polymorphism.

Primers	Restriction enzymes	Interpretation (bp)
F: 5'-GTCACAGGAATCGTACCCCT-3' R: 5'-ATTTTTGCAACAGCAGCGCA-3'	Tsp45I	TT: 113+79 TA: 193+113+79 AA: 193

(F: Forward primer, R: Reverse primer)

Evaluation of the Tsp45I Restriction Enzyme Digestion Results

Following digestion, fragments of 193, 113 and 79bp were observed. A single band of 193bp was evaluated as AA (homozygote variant), 113 and 79bp TT (homozygote wildtype) and all three as (heterozygote variant).

Statistical Analysis

The statistical analysis was performed using SPSS version 16.0 (SPSS inc. Chicago, USA). Values of $p<0.05$ were considered as statistically significant. Distributions of the genotype and allele frequencies between patient and control

groups were evaluated using the Chi-square and Fisher's exact test. Demographic data between the patient and control groups were compared using the Student's T and Anova tests. Allele frequencies were calculated according to the gene counting method.

Results

Clinical parameters of the patient group are given in Table 2. In the patient group, total cholesterol (p<0.001, 95% CI:29.62-60.71), LDL-cholesterol (p<0.001, 95% CI:30.12-55.90), VLDL-cholesterol (p<0.001, 95% CI:3.88-21.87) and triglyceride (p<0.001, 95%CI:97.96-152.90) levels were statistically higher than the control group. On the other hand, HDL- cholesterol (p<0.001, 95%C I: 5.30-15.34) levels were higher in the control group than the patient group.

When the patient and control groups were evaluated in terms of the Tβ4 A/T polymorphism genotype and allele distributions, the TT genotype frequency was statistically higher in the control group (p<0.001, X2:17.22, OR:2.13, 95% CI:1.44-3.16). In the patient group, the AT genotype (p<0.001, X2:12.40, OR:5.42, 95% CI:2.02-14.53) and A allele presence (p<0.001, X2:17.22, OR:6.66, 95% CI:2.61-16.98) were significantly higher (Table 3).

Table 3.The Tβ4 A/T (rs75112573) genotype/allele distributions in the patient and control groups.

		Patient Group (n=48) (n (%))	Control Group (n=45) (n (%))	P
Genotype	TT	18 (37.5)	36 (80)	<0.001
	AT	24 (50)	7 (15.6)	<0.001
	AA	6 (12.5)	2 (4.4)	-
Allele	T	60 (62.5)	79 (87.7)	-
	A	36 (37.5)	11 (12.3)	<0.001

In the patient group, the AT genotype carriers had significantly higher HDL- cholesterol levels compared to the AA carriers (p=0.036, 95% CI:0.59-17.25). Total cholesterol levels, however, were higher in the AA genotype carriers than AT carriers (p=0.038, 95% CI:2.15-73.68). Moreover, VLDL-cholesterol levels were significantly higher in the AA carriers than AT (p=0.011, 95% CI:6.73-49.86) and TT (p=0.018, 95% CI:4.95-49.49) carriers. When we compared according to the alleles, total cholesterol (p=0.048, 95% CI:0.41-72.82) and VLDL (p=0,009, 95% CI:7.44-48.21) levels were significantly lower in T allele carriers (Table 4) and in the control group, total cholesterol levels were lower in A allele carriers (p=0.048, 95% CI:0.18-51.64) (Table 5).

Table 4. Relationship between the Tβ4 A/T (rs75112573) polymorphism and plasma lipid levels in the patient group (n=48).

		Cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	VLDL-cholesterol (mg/dL)	Triglyceride (mg/dL)	p
Genotype	TT (n=18)	210.28±43.9	38.67±9.5	131.39±36.3	40.28±19.0	19.83±9.6	-
	AT (n=24)	207.25±35.8	42.42±9.4	125.62±30.7	39.21±18.3	32.54±27.0	<0.05
	AA (n=6)	245.17±34.5	33.50±5.2	144.17±29.3	67.50±46.7	33.17±23.4	<0.05
Allele	T (n=42)	208.55±39.0	40.81±9.5	128.10±32.9	39.67±18.9	27.10±22.1	<0.05
	A (n=30)	214.83±38.2	40.63±9.4	129.33±30.9	44.87±27.8	32.67±26.0	-

Discussion

Cardiovascular diseases are the leading cause of mortality in Turkey and worldwide. In Europe, cardiovascular diseases are responsible for 45% of female deaths under the age of 75 and it is 38% for men [14, 15]. The term ACS describes all the clinical syndromes from unstable angina pectoris to ST – segment elevation and non-ST- segment elevation myocardial infarction [16]. Tβ4 is excessively expressed during fetal development and in cardiovascular systems following injury such as myocardial infarction, and induces endogenous stem cell arrival to the site of injury by increasing neovasculogenesis and paracrine signals to support wound healing. Therefore, recently, many studies have been published on the fact that Tβ4 decreases the infarct area and preserves cardiac function [17, 18]. In these studies, it has been shown that Tβ4 prevents damage to the heart muscle and coronary arteries by providing cardiac protection following heart diseases [19], induces angiogenesis in cardiovascular sites [20], prepares the ischemic and epicardium-derived progenitor cells to differentiate to cardiomyocytes [21], promotes cardiac cell migration [11] and also prevents inflammation in ACS with its anti-inflammatory effects [22].

Table 5. Relationship between the Tβ4 A/T (rs75112573) polymorphism and plasma lipid levels in the control group (n=45).

		Cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	VLDL-cholesterol (mg/dL)	Triglyceride (mg/dL)	p
Genotype	TT (n=36)	173.14±37.2	49.92±15.2	90.86±32.0	32.06±19.0	162.17±95.3	-
	AT (n=7)	144.86±17.4	56.00±9.7	70.71±12.1	17.43±7.9	89.43±38.5	-
	AA (n=2)	155.50±5.0	35.50±10.6	76.50±9.2	43.00±24.0	217.0±121.6	-
Allele	T (n=43)	168.53±36.2	50.91±14.5	87.58±30.5	29.67±18.5	150.3±121.6	-
	A (n=9)	147.22±15.9	51.44±12.9	72.00±11.3	23.11±15.7	117.78±78.3	<0.05

There was only a single study investigating Tβ4 in ACS that was conducted on the Turkish population. However, this study was not a genetic study and authors evaluated the endogenous Tβ4 levels before and after primary percutaneous coronary intervention (PCI) in patients administered with ST-segment elevation acute myocardial infarction. They reported that Tβ4 levels were elevated to the point similar to the control group after PCI [9].

To this date, there have been no studies conducted on Tβ4 genetic variants to our knowledge, which makes the presented study the first. Our results indicate that since the TT genotype frequency was statistically higher in the control group, the TT genotype may decrease the risk of ACS. Moreover, the AT genotype and the A allele frequencies being higher in the patient group suggest that the AT genotype and the A allele may disrupt the function of Tβ4, promoting the survival of cardiomyocytes in ACS, therefore increase the risk of ACS.

Consequently, we believe that the low number of individuals in our study affected our results, and further studies with larger sample groups are needed to exactly clarify the role of the Tβ4 polymorphism in the pathogenesis of ACS. But even so there were a limited number of participants included in the study; we think that our findings will contribute to the understanding of the molecular mechanisms of ACS.

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