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Original Article

Characterization of the apelin -1860T>C polymorphism in Turkish coronary artery disease patients and healthy individuals

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Abstract: To evaluate the association between the apelin -1860T>C polymorphism and plasma apelin levels in Turkish patients with coronary artery disease (CAD). A total of 276 individuals were enrolled in the present study, including 158 patients with CAD and 118 individuals without CAD as controls. The presence of the apelin -1860T>C gene polymorphism and plasma apelin levels were determined using polymerase chain reaction/restriction fragment length polymorphism and enzyme-linked immunosorbent assay, respectively. Significance was set at $p \leq 0.05$ for all statistical analyses. The genotype and allele frequencies of interested genes were significantly different between groups ($\chi^2=10.2$; df=2; p=0.006 and $\chi^2=13.4$; df=1; p=0.000, respectively). Frequency of CC genotype and the C allele of -1860T>C site was significantly higher in CAD patients compared to healthy controls. We found that individuals with the TC and CC genotypes were associated with an increased risk of CAD when compared with the TT genotype in CAD patients, and the adjusted ORs (95% CI) were 6.50 (1.27-33.0) and 6.39 (1.77-23.0), respectively. Plasma apelin levels were significantly lower in CAD patients compared to control group. Apelin level of CAD patient group having CC genotype of -1860T>C site was significantly lower compared to those having TT genotypes, but it was not statistically significant ($p > 0.05$). The homozygous CC genotype of apelin gene is associated with high risk of CAD. Apelin gene polymorphism -1860T>C is a significant predictor of predisposition to CAD in in Turkish population.

Keyword: Apelin -1860T>C gene, coronary artery disease, polymorphism

Introduction

Apelin is an adipocytokine and an endogenous ligand for Angiotensin-like 1 Receptor (APJ). Apelin peptides and the apelin receptor represent a relatively new therapeutic axis for the potential treatment of cardiovascular disease. Increased apelin expression has been found in coronary vessels, cardiomyocytes, large conduit vessels [1] vascular smooth muscle cells, and endothelial cells [2]. Apelin have play an important role in some functions such as positive inotropism [3], endothelium-dependent vasodilation, angiogenesis [4], cardiac contractility, apoptosis [5], reduction of vascular wall inflammation [6] and arterial blood pressure [7].

With CAD, plaque first grows within the walls of the coronary arteries until the blood flow to the heart's muscle is limited. The traditional risk factors for coronary artery disease are high low density lipoprotein (LDL) cholesterol, low high density lipoprotein (HDL) cholesterol, high blood pressure, family history, diabetes, smoking, being post-menopausal for women and being older than 45 for men. Obesity may also be a risk factor [8].

The present study is the first investigation to examine the association between apelin -1860T>C gene polymorphism and plasma apelin levels in Turkish CAD patients. The aim of this study was to determined polymorphisms in apelin -1860T>C gene, plasma apelin levels and

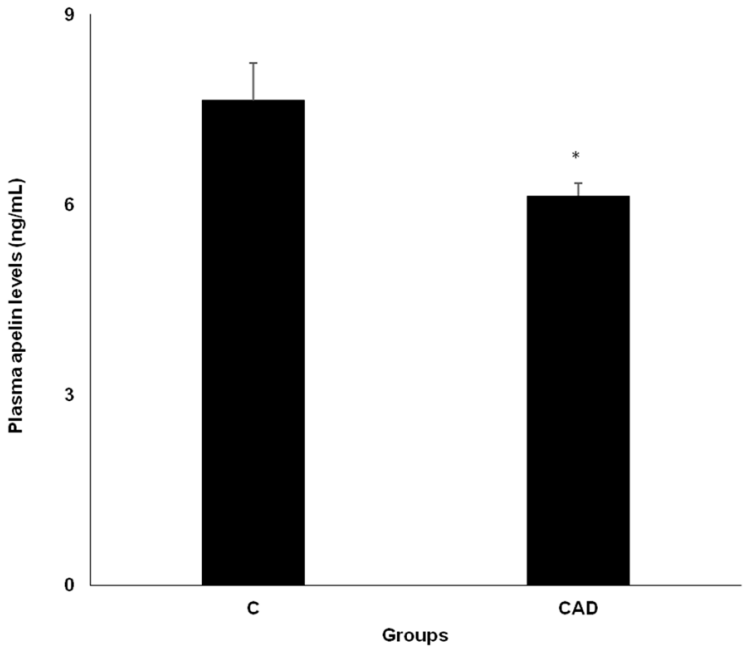

Figure 1. Plasma apelin levels of the control (C) and CAD patients. Data were expressed as mean ± standard error. *p ≤ 0.05 vs control group.

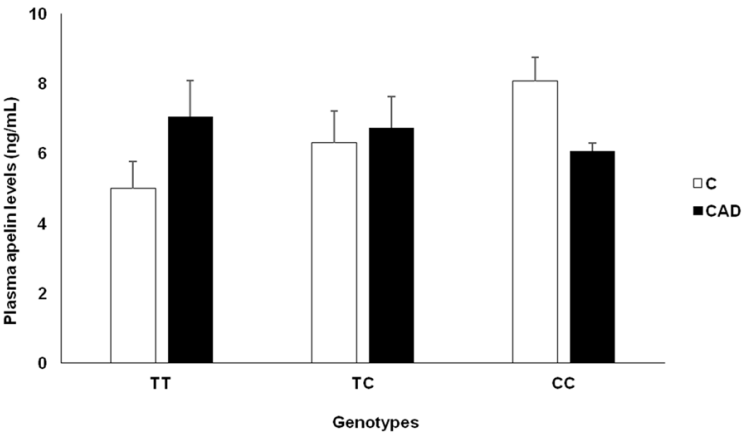

Figure 2. Plasma apelin levels of the C and CAD patients with three genotypes

explored their association in Turkish patient with CAD and healthy controls.

Methods

Study population

A total of 276 study subjects including 158 CAD patients (117 males and 41 females) (mean age ± standard error; 64.2 ± 0.95) and sex, age and ethnicity matched healthy controls, 118 (64 males and 54 females) (mean age ± standard error; 61.5 ± 0.99) were enrolled in the current study. CAD was diagnosed on the basis of the patients' clinical history, a physical examination and coronary angiography, according to the World Health Organization criteria [9]. The patients as well as controls enrolled were from Turkey. Written informed consents were taken from all individual participants included in the study. The study protocol conformed to the ethical guidelines of Declaration of Helsinki and was approved by the Ethics Committee of Uludağ University (Bursa, Turkey).

Biochemical investigation

Blood samples were taken after a 10-h overnight fast before angiography and centrifuged at 1000 g for 10 min, then plasma specimens were stored at -80°C until analysis. Plasma apelin levels were analyzed with human ELISA assay kit using the chemiluminescence method (Cusabio, China) according to the instruction of manufacturer by an ELISA microplate reader (Spectro Star Nano, Bmg Labtech, Germany).

Genotyping of apelin -1860T>C polymorphism

DNA extraction: Genomic DNA was extracted from peripheral blood leukocytes using GeneJET RNA Purification Kit (Thermo, Cat No: # K0722) according to the manufacturer's protocol. DNA was quantified by using a Maestro Nano Micro-Volume spectrophotometer (Maestrogen Inc., Las Vegas, NV) and the quality was assessed by running on an agarose gel.

Genetic analysis and PCR amplification

Genotyping of the apelin -1860T>C reported to be associated with alteration in plasma apelin levels (**Figure 2**) was performed using poly-

Tablo 1. Distribution of Apelin -1860T>C gene polymorphism in control and CAD

Apelin T1860C Genotypes	Control (n=118) (%)	CAD (n=158) (%)	OR (95% CI)	p
TT	13 (11%)	3 (1.9%)	Reference	-
TC	6 (5.1%)	9 (5.7%)	6.50 (1.27-33.0)	0.018
CC	99 (83.9%)	146 (92.4%)	6.39 (1.77-23.0)	0.001
	χ^2=10.2 df=2 p=0.006			
Allele				
T	32 (13.6%)	15 (4.7%)	Reference	-
C	204 (86.4%)	301 (95.3%)	3.14 (1.66-5.96)	0.0004
	χ^2=13.4 df=1 p=0.000			

OR - odds ratio

Tablo 2. Allele/Genotype frequencies and test of Hardy-Weinberg (HW) equilibrium

	Controls		CAD	
f(T)	0.135		0.047	
f(C)	0.865		0.953	
	O	E	O	E
TT	13	2.16	3	0.36
TC	6	27.6	9	14.2
CC	99	88.1	146	143.3
	χ^2=72.3, p=0.000		χ^2=21.6, p=0.000	

f= observed frequency of each allele (T or C); O= observed genotype numbers; E= expected genotype numbers under a Hardy-Weinberg (HW) equilibrium assumption; X^2= Chi-square values; p= probability of difference.

merase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [10]. PCR analysis was performed in the genomic DNAs isolated by using proper primers for the Apelin -1860T>C area (forward primer 5'-GGG GAA CAG TGA AGG GAG AAT GGT-3' and reverse primer 5'-AGA AGC GGG TCC TGA AGT TGT TTG -3') [10]. A 0.5 ng/mL of genomic DNA sample obtained from the peripheral leukocytes was added to the reaction mixture. The mixture also included 0.5 nmol/L forward primer, 0.5 nmol/L reverse primer, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 10X PCR buffer, 0.025 U/mL Taq DNA polymerase. A total of 50 µL of PCR volume was used in the study. The procedure was as follows: denaturation at 94°C for 5 min and 94°C for 40 sec, annealing at 58°C for 40 sec, extension at 72°C for 40 sec for a total of 30 cycles and waiting at 72°C for 10 min in a step wise manner. Samples were kept at 4°C until analysis.

Restriction enzyme digestion

10 µL of the PCR amplification product for studied apelin -1860T>C were digested with XhoI (New England Biolabs, Shanghai, China) in a 20 µL volume mixture containing 2 µL 1X CutSmart Buffer. The reaction mixture was incubated at 37°C during the night. The RFLP products mixing with 2 µl loading buffer for apelin -1860T>C were separated and electrophoresed in 5% ethidium bromide-stained 2.5% agarose gel at 100 mV for 1 h in 100 mL TBE buffer. The size of the restriction fragments for RFLP reactions products were determined using 100 bp DNA ladder (Invitrogen, USA). The differences in polymorphic allele were directly typed under ultraviolet light.

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 analysis for the comparison of variables between the patient and control groups was performed using student's t-test. Both parametric and nonparametric tests were used for evaluation of the data. The Fisher exact test was used for the comparison of categorical variables, as well as to test the departure of the genotype frequencies from Hardy-Weinberg equilibrium (HWE). Allele and genotype frequencies between patients and control subjects were compared using the chi-square (χ^2) test, from which the odds ratio (OR) and the 95% confi dence interval (95% CI) were calculated. All p values ≤0.05 were accepted as statistically significant.

Results

Position apelin -1860T>C

Three different genotypes and allele frequencies of the apelin -1860T>C polymorphism were TT, TC and CC genotypes and T and C alleles, respectively. Allele frequencies and genotypes of the apelin -1860T>C polymorphism in CAD patients and healthy controls are shown in

Table 1. Three (1.9%) of CAD and 13 (11%) of control were homozygote TT, 9 (5.7%) of CAD and 6 (5.1%) of control were heterozygote TC, whereas homozygote CC was found in 146 (92.4%) of CAD and 99 (83.9%) of control at this position. The distribution of apelin -1860T>C genotypes was found significantly different between groups (χ^2=10.2; df=2; p=0.006) (**Table 1**). For -1860T>C variants in the apelin gene, the risk genotypes CC and CT were associated with an increased risk of CAD (odds ratio for CC=6.39; 95% CI=1.77-23.0; p=0.001 and odds ratio for CT=6.50; 95% CI=1.27-33.0; p=0.018). Furthermore, The T allele was encountered in 4.7% (15) of the CAD and 13.6% (32) of the controls. The C allele was seen in 95.3% (301) of the CAD and 86.4% (204) of the controls. Distribution of the allele was found significantly different between groups (χ^2=13.4; df=1; p=0.000). The C allele in apelin -1860T>C gene increased the risk of CAD 3.14 times (95% CI 1.66-5.96, p=0.0004) as compared to controls (**Table 1**).

Hardy-weinberg (HW) equilibrium

The frequency of apelin -1860T>C gene polymorphism genotypes in controls and CAD showed a significant deviation from Hardy-Weinberg equilibrium (p=0.000) (**Table 2**).

Plasma apelin levels

Plasma apelin level in the CAD group was significantly lower than controls (6.13 ± 0.21 ng/mL vs. 7.64 ± 0.59 ng/mL; p=0.000) (**Figure 1**). When we compared the plasma apelin levels and apelin -1860T>C gene polymorphism of the CAD patients with TT genotype was 7.06 ± 1.04 ng/mL. It was 6.73 ± 0.91 ng/mL and 6.07 ± 0.22 ng/mL in the patients with TC and CC genotypes, respectively. At apelin -1860T>C no significant difference was observed among the genotypes with respect to plasma apelin level. Although at apelin -1860T>C site, in CAD group, plasma apelin level was lower in CC genotype compared to TT or TC genotypes, but, it was not statistically significant (**Figure 2**).

When we compared the plasma apelin levels and apelin -1860T>C gene polymorphism of the C group with TT genotype was 5.00 ± 0.78 ng/mL. It was 6.31 ± 0.91 ng/mL and 8.07 ± 0.69 ng/mL in the patients with TC and CC genotypes, respectively. There were not significant changes of the plasma apelin level and three genotypes in the C groups (p=0.223). In addition, plasma apelin level was higher in CC genotype compared to TT or TC genotypes in the C groups but, it was not statistically significant (**Figure 2**).

Discussion

CAD, a common complex disease, is the leading cause of death in the Western World. CAD causes 40% of all deaths in Turkey, and it is the most common cause of death among European men under 65 years old and the second most common cause in women [11]. Apelin was isolated in 1998 by Tatemoto et al. as a 36-amino acid peptide from bovine stomach extracts [12]. Apelin, which is an adipokine with a DNA sequence that is similar to angiotensin II [13, 14].

Various apelin levels have been demonstrated in different studies. It is difficult to suggest a standard normal level of apelin. El-Mesallamy HO et al. [15] found that the mean apelin level in the control group was 1.11 ng/mL. Li et al. [16] reported an apelin level of 1.98 ng/mL in the healthy group. Ellinor et al. [17] reported a mean apelin level of 304 pg/mL in the normal population. Kadoglou et al. [18] reported that the apelin level in the healthy control group was 2.99 ± 1.52 ng/mL. We found that the plasma apelin level in the control group was 7.64 ± 0.59 ng/mL. The relationship between CAD and apelin is not clear. Elevated plasma apelin concentrations were shown in obese subjects [19, 20], whereas apelin concentrations decreased after obese subjects lost their weight by diet control [21]. A reduced serum level of apelin was associated with a higher risk of acute coronary syndrome [16]. Akboga et al. [22] showed that plasma apelin was increased in patients with good coronary collaterals and higher plasma apelin level was related to better coronary collateral development [22]. Yokoyama et al. [23] found that plasma apelin level was decreased in patients with cardiac diseases, especially in those with CAD and it may be associated with coronary atherosclerosis [23]. Bilik et al. [24] showed that patients with isolated coronary artery ectasia have decreased plasma apelin levels compared with the control group [24]. In the present study, plasma apelin levels were measured in CAD (6.13 ± 0.21 ng/mL) and found significantly lower compared

with controls (7.64 ± 0.59 ng/mL). This study's results are compatible with the aforementioned study.

It was shown for the first time that the -1860T>C polymorphism of the apelin gene was significantly related to plasma apelin levels. In this study, it was aimed to analyze the distribution of the apelin -1860T>C gene polymorphisms and plasma apelin levels in patients with CAD. In this study, we found a strong association between apelin -1860T>C genotype and patients with CAD compared with healthy controls (p=0.000). Individuals with the CC genotype have approximately six times greater risk of CAD compared with those carrying the TT genotype (adjusted OR=6.39). However, there was a significant variation in apelin -1860T>C allele distributions between patients with CAD and controls (p=0.000). The apelin -1860T>C polymorphism was first found in the Han Chinese population by Li et al. [25]. The apelin -1860T>C is a newly identified Single nucleotide polymorphism (SNP) and it is located in the promoter region of the apelin gene [20]. Kidoya et al. [26] showed that the minor allele frequency of the -1860T>C SNP is 0.347 in the Chinese [26]. Jia et al. [27] found that apelin -1860T>C genotype and allelic frequencies did not differ significantly between the participants with hypertension and controls and the apelin gene polymorphism is not associated with the risk of hypertension in older Chinese individuals. In addition, they demonstrated that older Chinese women with the apelin TT genotype had a greater response to the angiotensin II receptor antagonist losartan than patients with the CC and CT genotypes [27].

In Kozani study [16], apelin levels in acute coronary syndrome and chronic ischemic heart disease patients were found to be significantly lower than in the control group. In a large population-based study, apelin serum concentration was demonstrated to be decreased in heart failure patients with systolic left ventricular dysfunction [28]. In this study, we found that plasma apelin concentration in CAD patients was lower than that control group (p=0.000) (**Figure 1**). In addition, although plasma apelin level of the CAD patients with CC genotype was lower and C groups with CC genotype was higher, It was not statistically significant. There was no significant difference between the plasma apelin levels of the C groups and CAD patients with different genotypes (p=0.223 and p=0.667 respectively) (**Figure 2**). There is a lack of information about the relationship between variants in the apelin -1860T>C gene and plasma apelin levels in the literature that can be compared with the results of this study. The presence of the CC genotype in CAD was associated with lower plasma apelin levels. Conversely, The presence of the CC genotype in C groups was associated with higher plasma apelin levels, but it was not significant. Our findings suggest us that plasma apelin level can be independent from apelin -1860T>C polymorphism, which should be confirmed with further studies performed in larger populations.

Conclusion

To the best of our knowledge, this study is the first case-control study on the association of the -1860T>C of apelin with CAD in a Turkish population. The results showed that the frequency of CC genotype significantly increased in CAD patients, compared with controls, suggesting that CC could be a risk factor for patients with CAD, which provided the evidence again that variation of apelin may be involved in susceptibility to CAD. In addition C allele carriers may have more risk than T allele individuals for development of CAD. Plasma apelin level of the CAD patients with CC genotype was lower. Patients with CAD who have CC genotype and C allele may be evaluated from the population because of decreased plasma apelin level, which may have deleterious effects on CAD pathophysiology. Our results suggested that the -1860T>C variation in apelin may be a genetic risk factor for CAD and further functional analysis to elucidate the role of apelin in CAD is needed.

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Disclosure of conflict of interest

None.

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