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RESEARCH

The relationship between ghrelin and ghrelin leu72met polymorphism in coronary artery disease

Koroner arter hastalığında ghrelin ve ghrelin leu72met polimorfizmi arasındaki ilişki

Hatice Yıldırım Yaroğlu[1], Buğra Özkan[2], Şenay Balcı[3], Nil Doğruer Ünal[4], Sema Erden Ertürk[5], Dilek Çiçek Yılmaz[2], Lülüfer Tamer[3]

[1]Mersin University Health Science Institute Stem Cell and Regenerative Medicine, Mersin, Turkey
[2]Mersin University Faculty of Medicine, Departments of Cardiology, [3]Department of Medical Biochemistry, Mersin, Turkey
[4]Mersin University Faculty of Pharmacy, Department of Biochemistry, Mersin, Turkey
[5]Mersin University Vocational School of Medical Services, Mersin, Turkey

Abstract

Purpose: Ghrelin represents a hormone, which is defined as an endogenous ligand bound to the growth hormone-releasing hormone receptor (GHS-R1a). However, the mechanisms that underlie ghrelin's impacts on cardiovascular diseases have not been completely detected. For this reason, we aimed to research the relationship between serum ghrelin and ghrelin gene polymorphism in coronary artery disease (CAD).

Materials and Methods: The study group consisted of 88 patients diagnosed with a minimum of one coronary artery stenosis over 70%, and the control group comprised 81 individuals without coronary artery lesions. An autoanalyzer was used to analyze fasting blood glucose (FBG) and lipid parameter levels. Ghrelin levels were examined with an enzyme-linked immunosorbent assay (ELISA) kit.

Results: Ghrelin levels were found to be 2.2 ng/ml in the control group and 2.1 ng/ml in the CAD group. No statistical relation in ghrelin Leu72Met genotypes were detected between the control and patient groups.

Conclusion: Serum ghrelin levels were higher in the control group than in the CAD group. Whether ghrelin levels and Leu72Met polymorphism have protective effects in CAD must be revealed in an extensive study group with other polymorphisms and ghrelin expression in the ghrelin gene.

Keywords:. Ghrelin, coronary artery disease, leu72met, polymorphism

Öz

Amaç: Ghrelin, büyüme hormonu salgılayan hormon reseptörüne (GHS-R1a) bağlı endojen bir ligand olarak tanımlanan bir hormondur. Bununla birlikte, ghrelin'in kardiyovasküler hastalıklar üzerindeki etkilerinin altında yatan mekanizmalar tam olarak tespit edilememiştir. Bu nedenle koroner arter hastalığında (KAH) serum ghrelin düzeyleri ile ghrelin gen polimorfizmi arasındaki ilişkiyi araştırmayı amaçladık.

Gereç ve Yöntem: Çalışmaya %70'in üzerinde en az bir koroner arter stenozu olan 88 hasta ve koroner arter lezyonu olmayan 81 birey dahil edildi. Açlık kan şekeri (AKŞ) ve lipid parametreleri düzeylerini analiz etmek için otoanalizör kullanıldı. Ghrelin düzeyleri enzim-ilişkili immunosorbent assay (ELISA) kiti ile çalışıldı.

Bulgular: Ghrelin düzeyleri kontrol grubunda 2,2 ng/ml,KAH grubunda 2,1 ng/ml olarak bulundu. Kontrol ve hasta grupları arasında ghrelin Leu72Met genotiplerinde istatistiksel bir ilişki saptanmadı.

Sonuç: Serum ghrelin düzeyleri kontrol grubunda KAH grubuna göre daha yüksek bulundu. Ghrelin düzeylerinin ve Leu72Met polimorfizminin KAH'da koruyucu etkisinin olup olmadığı, diğer polimorfizmler ve ghrelin genindeki ghrelin ekspresyonu ile kapsamlı bir çalışma grubunda ortaya konmalıdır.

Anahtar kelimeler: Ghrelin, koroner arter hastalığı, leu72met, polimorfizm

Address for Correspondence: Hatice Yildirim Yaroğlu, Stem Cell and Regenerative Medicine, Health Science Institute, Mersin University, Mersin, Turkey. E-mail: haticeyildirim@mersin.edu.tr
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INTRODUCTION

Coronary artery disease (CAD) represents a complicated disease generally associated with traditional risk factors, involving hypercholesterolemia, diabetes mellitus, and hypertension. Genetic predisposition is considered to originate from the interaction between environmental determinants, e.g., nutrition, smoking, and physical mobility[1]. Nevertheless, CAD is not related to these risk factors in some individuals and other genetic causes are known to lead to coronary atherosclerosis[2]. The chronic inflammatory response to the oxidative modification of low-density lipoprotein (LDL) occurs. Consequently, inflammatory cells, e.g., macrophages and T lymphocytes, accumulate in the subendothelial area. These cells are involved in the progress of atherosclerosis and atherogenesis[3]. Ghrelin represents a hormone, which is defined as an endogenous ligand bound to the growth hormone-releasing hormone receptor (GHS-R1a). It is a 28-amino acid (aa) lipopeptide hormone primarily released by the stomach fundus[4]. The pituitary, hypothalamus, thyroid gland, salivary gland, kidneys, small intestine, heart, alpha, beta, and epsilon cells of the pancreas, immune system, lung, central nervous system, gonads, placenta, breast, and teeth also synthesize ghrelin[5-9]. Researchers have demonstrated that ghrelin has protective effects against atherosclerosis by hindering the pro-inflammatory response, prohibiting redox-related cellular signals, and endothelial dysfunction[10-12].

Numerous diseases such as colorectal cancer, breast cancer, type 2 diabetes, metabolic syndrome, and obesity have been connected with ghrelin polymorphisms[13-16]. Few research has examined the correlation between ghrelin gene polymorphisms and CAD. The mechanisms that underlie ghrelin's impacts on cardiovascular diseases have not been completely detected. The hypothesis of this study is to investigate whether there is a relationship between levels of ghrelin and ghrelin Leu72Met Polymorphism in CAD. CAD is a major concern for the community, as genetic differences are likely to exist in different populations. The Leu72Met polymorphism and the risk of CAD in the Turkish population have not yet been examined in any studies. With our findings, this study will contribute to the literature by determining the possible role of CAD in our society. Therefore, we aimed to research whether there is a correlation between ghrelin with CAD.

MATERIALS AND METHODS

This study was carried out at Mersin University, Faculty of Medicine, Department of Cardiology and Medical Biochemistry.

Sample

A total of 169 patients who underwent coronary angiography with a femoral intervention using the standard Judkins technique by Dilek Çiçek Yılmaz and Buğra Özkan in the Cardiology Department of Mersin University were enrolled in this research.

The study group consisted of 88 patients diagnosed with a minimum of one coronary artery stenosis over 70%, and the control group comprised 81 individuals without coronary artery lesions. Patients with a history of coronary artery bypass graft surgery, inflammatory disease, infectious disease, hematological disease, autoimmune disease, kidney disease, liver disease, malignancy, and those on continuing immunosuppressive therapy were excluded from the study. Power analysis was used to determine the number of samples in the study. According to the results of the analysis performed by Medical Informatics and Biostatistics specialist Sema Erden Ertürk, the sample size was calculated as 96 for each group in this study (alpha=0.05, test power=0.80). Six people who did not meet the inclusion criteria in the patient group were excluded from the study, while 15 people in the control group refused to participate in the study.

Procedure

Genetic analysis, serum levels of ghrelin and lipid parameters were performed in the Medical Biochemistry Department of Mersin University by Hatice Yıldırım Yaroğlu, Şenay Balcı, Nil Doğruer Ünal and Lülüfer Tamer. Confirmation for the current research was accepted by our local Mersin University Clinical Research Ethics Committee which was appropriated by the specifications of the Declaration of Helsinki (03. 23.2017, 2017/88). All persons who participated in the study were told about the study and their written consent was taken. All data collected during this study were kept confidential in terms of the reliability of the records and the privacy of the patients included in the study and were

not shared anywhere. The patient data recorded in our study were safely stored in the hospital archive.

Blood sampling

Ten ml of venous blood taken into tubes not containing any anticoagulant was centrifuged at 3000 rpm for 15 minutes after 12 hours of fasting. The serum obtained was stored at –20 °C until being studied. Using a Cobas 501 autoanalyzer (Roche Diagnostics, Manheim, Germany), the serum levels of triglyceride (TG), total cholesterol (TC), LDL, and high-density lipoprotein (HDL) were detected by employing an enzymatic colorimetric (CHOD/PAP) method, while fasting blood glucose (FBG) levels were measured with a hexokinase method. Serum ghrelin levels were used by an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Human GHRL ELISA Kit catalog no: E-EL-H1919).

DNA extraction and genotyping

To perform genetic analysis, peripheral blood was drawn into tubes containing ethylenediaminetetraacetic acid (EDTA). DNA extraction was performed from circulating leukocytes by utilizing a high pure PCR template preparation kit (Roche Diagnostics, GmbH, Mannheim, Germany, catalog no: 1 796 828), and ghrelin gene polymorphisms (https://www.ncbi.nlm.nih.gov/snp/rs696217) were detected by utilizing LightCycler and RS696217 GHRL mutation detection kits (TIB MOLBIOL) by real-time polymerase chain reaction (RT-PCR) with a LightCycler instrument (Roche Diagnostics, Manheim, Germany). The PCR conditions used were as follows: 10.4 μmol distilled water, 1 μmol/l reagent mix, 1.6 μmol $MgCl_2$ (25 mM), 50 ng of genomic DNA, and 2μl of the LightCycler FastStart DNA Master Hybridization mix in a final volume of 20 μl. The cycling programs for ghrelin Leu72Met polymorphism are presented in Table 1.

Table 1. Program for the amplification and melting curve analysis of ghrelin Leu72Met polymorphism

Steps	Substeps	Target temperature	Incubation time (seconds)	Temperature transition rate
Denaturation		95 °C	600	4.6
Amplification	Denaturation	95 °C	10	4.6
	Annealing	60 °C	10	2.4
	Extension	72 °C	30	4.6
Melting		95 °C	30	4.6
		40 °C	120	2.0
		75 °C	0	-

C: centigrade decree

Statistical analysis

IBM Statistical Package for the Social Sciences Version 20.0 package program was used for statistical analysis (IBM SPSS Inc. Free Download, Chicago, Illinois, USA). Descriptive statistics such as percentage, mean, and standard deviation were evaluated by conducting the normal distribution compliance test, Kolmogorov-Smirnov test, independent samples t-test, and binary logistic regression. In statistical analysis, the significance value was taken as $p < 0.05$. When the test of conformity to normal distribution was applied to the data, it was determined that the data met the normality assumption ($p > 0.001$), and the independent samples t-test was conducted to compare the two group means in terms of ghrelin. In our study, the www.e-picos.com website was used for Hardy-Weinberg equilibrium analysis. A $p<0.05$ value was admitted as statistically significant in the analyses conducted.

RESULTS

Eighty-eight subjects (36 female and 52 male) with CAD and 81 control subjects (41 female and 40 male) were registered in the current research. The mean (±SD) age was 66±11.2 in patients and 65±12.3 in

control subjects. Table 2 presents the characteristics of the patients and controls. There was no relationship between sex, age, diabetes, hypertension, TG, and FBG in the groups (p>0.05). Distinctions were revealed between the control and patient groups regarding HDL, LDL, and TC, (p<0.05). HDL, LDL, and TC, levels were significantly higher in the control group in comparison with the CAD group. We think that this is caused by the use of statin-group drugs with cholesterol-lowering effects in the CAD group. Serum ghrelin levels were higher in the control group than in the CAD group, but it was not statistically significant (p>0.05) (Figure 1).

In the patient group, the G allele frequency was 0.915, and the C allele frequency was 0.085, with the population in Hardy-Weinberg equilibrium (p: 0.38). In the control group, the G allele frequency was 0.895 and the T allele frequency was 0.105, with the population in Hardy-Weinberg equilibrium (P:0.29) (data not shown). Table 3 demonstrates the frequency of ghrelin Leu72Met heterozygous (GT) and wild genotypes (GG). No statistical correlation in ghrelin Leu72Met genotypes were detected between the control and patient groups (p>0.05) (Table 3).

Table 2. Characteristics of CAD and controls

Characteristics	CAD (n=88)	Controls (n=81)	p
	n (%)	n (%)	
Male	52 (59)	40 (49.4)	0.21
Female	36(41)	41(50.6)	
Diabetes (Type 2)			
-	36(41)	39(48)	0.36
+	52(59)	42(52)	
Hypertension			
-	23(26)	26(32)	0.39
+	65(74)	55(68)	
	Mean±sd	Mean±sd	
Age	66±11.2	65±12.3	0.58
FBG	98±8.91	101±17.9	0.120
TC	156.60±28.8	182±39.1	0.001
TG	152.12±49.9	157±70.9	0.597
HDL	44.68±9.86	48.7±12.7	0.019
LDL	89.45±29.8	103±34.6	0.006

Continuous variables are given as mean±standard deviation (Mean±sd)
n: Number of samples, p: Significance between the groups, CAD:Coronary artery disease, FBG: Fasting blood glucose, TC: Total cholesterol,TG: Triglyceride, HDL: high-density lipoprotein, LDL: low-density lipoprotein.

Table 3: Association of the CAD and control groups with the ghrelin Leu72Met genotype

Ghrelin Leu72Met	CAD (88)	Control (n=81)	P	OR (95%)
Wild (GG)	73 (83%)	64 (79%)	0.514	reference
Heterozygous (GT)	15 (17%)	17 (21%)		0.774(0.358-1.673)

Wild genotypes are used as a reference.CAD:Coronary artery disease

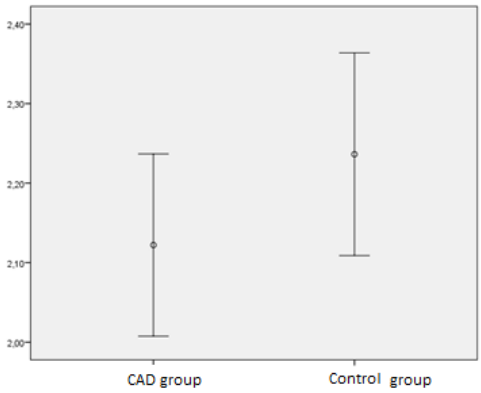

Figure 1. Serum ghrelin levels in coronary artery patients and control groups

CAD: Coronary artery disease

DISCUSSION

Recently, it has been proven that non-traditional risk factors are as important as traditional risk factors in the development of CAD. The most important risk factors are increased plasma and tissue oxidized LDL levels[3-17]. Ghrelin downregulates the expression of anorectic and proinflammatory cytokines from human monocytes and T cells and downregulates chemokines from the human endothelium. Some research has determined that ghrelin has anti-inflammatory effects both in vitro and in vivo[10]. The mRNA of ghrelin has been reported in the aorta and heart[18-19]. In volunteer patients who received intravenous ghrelin injections, it has been observed that ghrelin lowers heart rate without altering arterial pressure[18]. It was reported that ghrelin decreased blood pressure, increased cardiac index and volume in volunteer subjects who received intravenous ghrelin injections. One of the cardiovascular effects of the hormone, especially the vasodilatation of vessels, is noteworthy. The intracerebroventricular (ICV) injection of ghrelin to the nucleus tractus solitarii in rats reduced sympathetic activity and heart rate as well as lowered blood pressure[20]. Growth hormone secretagogue (GHS) and ghrelin delivery caused the increased volume of the left ventricle. Healthy heart development was detected after GHS administration in the pituitary glands. Furthermore, ghrelin eliminates the vasoconstrictor effect of endothelin-1 in the arteries[21].

There are few studies on the relationship between ghrelin levels and CAD. In the present research, ghrelin levels were detected to be 2.2 ng/ml in the control group and 2.1 ng/ml in the CAD group ($p>0.05$). We found that patients with CAD had lower serum ghrelin levels than the control group. Similar to our findings, Kadoglou et al. demonstrated significantly lower ghrelin levels in both the acute coronary syndrome (ACS) and stable CAD groups compared to the control group[22]. Zhang et al. reported no difference in ghrelin levels between patients with ACS and stable CAD[23]. Hedayatzadeh-Omran et al. found no association between ghrelin levels in heart failure patients and the control group[24]. Moreover, in our study, the frequency of the ghrelin Leu72Met heterozygous genotype was 17% in CAD patients and 21% in the control group. In contrast, the frequency of the ghrelin Leu72Met wild genotype was 83% in CAD patients and 79% in controls. No significant difference in the ghrelin Leu72Met genotypes were detected between the control and patient groups (OR: 0.774 95% CI: (0.358-1.673) ($p>0.05$)).

The distribution of the Leu72Met genotype varies between populations. The frequency of the Met72 allele was determined to be 7.8% in Danes, 12.9% in Finns, 18.1% in Koreans, 20% in Japanese, 4.3% in Italians, and 22% in Chinese[25]. The Leu72Met genotype distribution in Turkey has not been studied. Research has proven the connection of Leu72Met polymorphism with diabetes, obesity, metabolic syndrome, cancer, and kidney dysfunction. Steinle et al. showed that Leu72Met polymorphism was correlated with metabolic syndrome, low HDL and high triglyceride levels, whereas Miraglia et al. determined that it was associated with obesity. In line with the literature review, we found only three publications related to ghrelin Leu72Met polymorphism and CAD[26,27]. Among these studies, Zhang et al. indicated that the Leu72Met polymorphism of the ghrelin gene took an essential part in the progression of CAD in patients with heart failure[23]. Hedayatizadeh-Omran et al. found a significant association of ghrelin genotypes and ghrelin serum levels with heart failure and CAD, whereas Thang et al. demonstrated that Leu72Met polymorphism was not related to CAD[24,25]. Similar to the research by Thang et al., we did not reveal a significant difference between Leu72Met polymorphism and CAD at the end of our study.

The minor allele percentage of the Leu72Met polymorphism (rs696217) has been reported as 0.078 Globally and 0.081 in Europeans. In this study, while the mutant (TT) genotype was not detected in the patient and control groups, the percentage of minor alleles being carried were found to be 0.085 in the patient group. It means that this polymorphism is also not seen in our population. In addition, population diversity, disease and geographic differences lead to differences in polymorphism studies. Furthermore, ghrelin levels were not found to be significantly low, since there were no mutant (TT) genotypes in our study group.

This current study has some limitations which have to be noticed. A low number of participants is the major shortcoming of our study and it should be supported by wide-ranging studies. If the number of our study group was sufficient, we could detect a significant relationship between ghrelin gene polymorphism and CAD.

In our study, there was no difference between ghrelin genotypes in both CAD and control groups, and the ghrelin level was found to be higher in the control group than in the CAD. In order to determine whether ghrelin has a protective role in CAD, more comprehensive, multicenter and prospective studies with increased sample size are needed comparing both ghrelin expression and ghrelin levels in addition to other mutations in the ghrelin gene.

In conclusion, whether ghrelin levels and Leu72Met polymorphism have a significant effect on CAD should be demonstrated in a comprehensive study group with other polymorphisms in the ghrelin gene and ghrelin expression.

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