Receptor for advanced glycation end products gene polymorphisms in cardiac syndrome X

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Abstract. Endothelial and microvascular dysfunction serve important roles in the formation and pathogenesis of cardiac syndrome X (CSX). Expression of receptor for advanced glycation end products (RAGE) is suggested to be increased in several conditions, including diabetes, inflammation and vascular diseases. In the present study, *RAGE* gene polymorphisms in patients with CSX and healthy controls were investigated. A total of 114 patients, diagnosed with CSX using coronary angiography results following complaints of angina and objective ischemia, and 103 healthy controls participated in the study. Whether there was a difference in genotype distributions of *RAGE* gene -374T/A, -429T/C and Glys82Ser polymorphisms between patients with CSX and healthy controls was investigated. Following DNA isolation from blood samples of the participants, the polymorphic regions were examined by quantitative polymerase chain reaction, and the genotyping results were statistically analyzed. When the genotypic distributions of -374T/A, -429T/C and Gly82Ser polymorphisms were investigated in patients with CSX and healthy controls, no statistically significant differences were identified between the two groups (P>0.05). Likewise, no statistically significant differences were observed in the allelic distributions of all 3 polymorphic regions (P>0.05). To the best of our knowledge, the present study also investigated the association between CSX and *RAGE* gene polymorphisms for the first time. No statistically significant differences in *RAGE* gene polymorphisms between the CSX and control groups were observed. We hypothesized that significant results may be obtained by increasing the numbers of patients and healthy controls in future studies.

Introduction

Cardiac syndrome X (CSX) is defined as an angina-like pain despite positive treadmill exercise tests and normal coronary arteriograms (1-3). The pathogenesis of CSX is not completely understood and diagnosis of the syndrome is based on eliminating other potential causes of chest pain (4). Previous studies have suggested that coronary microvascular dysfunction may be one of the primary underlying causes of ischemic heart disease, and of chest pain without any significant coronary obstruction (5). In addition, myocardial ischemia, which is a result of microvascular and endothelial dysfunctions, is also considered to be one of the causative factors of angina (6). Coronary microvascular dysfunction is suggested to be the primary reason for the visible symptoms in a number of patients experiencing angina attacks without a significant cardiac or systemic disease (7).

Inflammation is known to be involved in endothelial and vascular dysfunction. Additionally, previous studies have suggested that inflammation may serve a significant role in the pathogenesis of CSX (8,9). The role of microvascular dysfunction in CSX has been studied by examining the response to vasoactive stimuli that occurs in coronary flow (1), and it is considered to be the primary pathological cause in numerous patients (10).

Advanced glycation end products (AGE) are spontaneously modified structures or newly formed compounds of proteins, lipids or nucleic acids with non-enzymatic glycation and glycosylation in oxidation accelerating environments (11). The receptor for AGE (RAGE), which is a member of the immunoglobulin superfamily and a transmembrane multiligand receptor, is primarily active in the response to acute and chronic stress (12,13). Upregulation of RAGE is observed in diabetes, vascular, inflammatory and neurodegenerative diseases, and it is hypothesized to serve a role in the pathogenesis and complications of these diseases (14,15). RAGE is generally expressed in vascular endothelial and epithelial cells, smooth muscles, macrophages and fibroblasts (16). Under normal physiological conditions, RAGE is usually expressed in small

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amounts in adult tissues, while its expression is upregulated in pathological vascular conditions. The receptor is suggested to serve an important role in the progression of coronary artery disease (17). In addition, previous studies have suggested that different *RAGE* gene polymorphisms are associated with a number of diseases including coronary artery disease, heart failure, nephropathy, chronic renal failure and diabetes (18).

To the best of our knowledge, no experiments have been conducted to investigate the association between CSX disease and *RAGE* gene polymorphisms. Therefore, the present study compared patients with CSX and control subjects from the Turkish population in the terms of 429T/C, -374T/A and G82S single nucleotide polymorphisms (SNPs), which have been previously evaluated in a number of studies due to their associations with a number of cardiovascular diseases (19-21).

Materials and methods

Patient selection. The present study was approved by Ethical Committee of Cerrahpaşa Medical Faculty (approval no. 2015/279734) and was performed in accordance with Helsinki Declaration of World Health Organization. Only patients and controls, who agreed to give written informed consent, were included.

The study was designed as a cohort study, and was conducted from September 2015 to January 2016; a total of 217 subjects were included. Patients were admitted to the Cardiology Clinic of Bakırköy Dr. Sadi Konuk Training and Research Hospital (Istanbul, Turkey) with complaints of angina and objective ischemia. If they were diagnosed with CSX according to results of coronary angiography, they were included in the study cohort (n=114, 76 females and 38 males; average age, 52.0±11.9 years). The control group was formed of 103 consecutive subjects with similar demographic data but without CSX diagnoses.

Exclusion criteria were as follows: Previous diagnosis of coronary artery disease, valvular heart disease, congestive heart failure, cardiomyopathy, pericarditis or myocarditis; abnormality in liver enzyme tests; creatinine >2 mg/dl; presence of active infection; and diagnosis of pulmonary thromboembolism or acute coronary syndrome.

The diagnosis of CSX was performed according to 2006 Stable Angina Guidelines of European Society of Cardiology (22). As suggested in the guidelines, patients were diagnosed with CSX if they met the following criteria: Angina being induced by typical exercise; positive ECG in exercise stress or evidence of ischemia by other screening method; and normal epicardial coronary arteries in angiography.

The control group was recruited from the subjects admitted to the cardiology clinic with angina pain, but with no ischemic change following an exercise test and a risk of cardiovascular events <10%, according to Framingham risk score (23).

Detection of objective ischemia. All the patients underwent an exercise test, which was modified according to Bruce's protocol (24). Based on the results of ECG exercise records, after 60-80 milliseconds from J point, at least 2 derivations

and at least 1 mm horizontal or downsloping ST segment depression of ischemic change were considered as abnormal. Patients exhibiting evidence of objective ischemia in the exercise test underwent coronary angiographies using the Judkins technique during right femoral artery bypass.

Genotyping. Blood samples were collected in K3-EDTA tubes. Genomic DNA isolation was performed using peripheral blood from 200 μl blood samples, according to the manufacturer's protocol of the Invitrogen PureLink® Genomic DNA Mini kit (cat. no. K1820-02; Invitrogen; Thermo Fisher Scientific, Inc.). Absorbance (A_{260}/A_{280}) values of the extracted DNA samples were calculated using the NanoDrop 2000 system, and the minimum acceptable concentration level was determined as 1.8±0.2. The DNA samples were stored in -20°C until further use.

RAGE gene -374T>A (rs1800624); -429T>C (rs1800625) and Gly82Ser (rs2070600) polymorphisms were detected by quantitative polymerase chain reaction using the Applied Biosystems 7500 Fast system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and gene fragment-specific TaqMan probes (Thermo Fisher Scientific, Inc.; cat. no. 4351379), according to a VIC- and FAM-labeled allelic discrimination method for each polymorphism: rs1800624 (assay ID., C__3293837_1_); rs1800625 (assay ID., C__8848033_1_); and rs2070600 (assay ID., C__15867521_20). The primers were as follows: rs1800624, forward, 5'-CCCATCTTGATTGCGCAAAGTT-3' and reverse, 5'-TCAAAAAACATGAGAAACCCCAGAA-3' with the reference gene, 5'-CCAGACTGTTGTCTGCAAGGGTGCA[A/T]TTGGGCCTGCATCATGAAGGCAAGG-3'; rs1800625, forward, 5'-CCCATCTTGATTGCGCAAAGTT-3' and reverse, 5'-TCAAAAAACATGAGAAACCCCAGAA-3' with the reference gene, 5'-GGAACAGGAGAGAAACCTGTTTGGA[A/G]CTTCGTGAAAGAAAATCATTTTTTT-3'; rs2070600, forward, 5'-TGGTCTCCTTTCCATTCCTGTTCA-3' and reverse, 5'-CCGTCACTCTGCCTCACAGTCC-3' with the reference gene, 5'-CCGACAGCCGGAAGGAAGAGGGAGC[C/T]GTTGGGAAGGACACGAGCCACACTG-3'. The qPCR conditions were denaturation for 10 min at 95°C, followed by 35 cycles of denaturation for 15 sec at 95°C, annealing and extension for 1 min at 60°C, and a final extension for 1 min at 60°C. The $2^{-\Delta\Delta Cq}$ method was used to quantify the results (25).

Statistical analysis. Data were analyzed with the SPSS software v22.0 for Windows (IBM Corp.). Descriptive data are presented as the mean ± standard deviation, and median (range), frequency and ratio. The distribution of the variables was analyzed by Kolmogorov-Smirnov test. Qualitative data were analyzed Mann-Whitney U and independent Student's t-tests. Quantitative data were measured using the χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Demographic patient data. The comparisons between demographic, clinical and laboratory data of the patients are summarized in Tables I and II. In Table I, demographic characteristics of the CSX and control groups were compared based on age, sex, BMI and smoking habits; no significant

Table I. Patient demographic data.

Categories	Patients (n=114)			Controls (n=103)			
	Mean ± SD	Median (range)	n (%)	Mean ± SD	Median (range)	n (%)	P-value
Age	52.0±11.9	52 (17-75)		50.3±10.5	51 (22-73)		0.123
BMI	24.8±3.0	24 (19-32)		25.0±2.2	25 (20-30)		0.335
Sex							
Female			76 (67)		-	61 (59)	0.256
Male			38 (33)		-	42 (41)	
Smoking			36 (32)		-	30 (29)	0.695
T2DM			27 (24)		-	8 (8)	0.001
Family history			47 (41)		-	17 (17)	<0.001
HT			31 (27)		-	13 (13)	0.008

Age and BMI data were analyzed using Mann-Whitney U test, all other data using a χ^2 test. BMI, body mass index; T2DM, type 2 diabetes mellitus; HT, hypertension; SD, standard deviation.

Table II. Clinical laboratory results.

Serum markers	Patients		Controls		
	Mean ± SD	Median (Min-Max)	Mean ± SD	Median (Min-Max)	P-value
FPG	105.5±30.3	98 (74-266)	93.3±17.4	89 (71-196)	<0.001
ALT	20.8±15.1	18 (7-152)	23.6±14.9	20 (8-91)	0.075
AST	21.1±7.9	20 (6-74)	21.9±10.7	19 (11-103)	0.968
GGT	25.1±17.2	21 (9-156)	26.3±13.2	24 (7-74)	0.088
HDL-C	47.7±11.6	47 (24-79)	49.3±17.1	46 (30-179)	0.817
LDL-C	123.5±40.2	121 (46-254)	122.7±32.6	118 (44-224)	0.962
TG	142.9±83.9	123 (41-603)	131.5±71.6	113 (17-452)	0.373
Total cholesterol	199.5±44.5	199 (101-343)	202.2±38.4	205 (114-318)	0.718
Uric acid	5.0±2.6	5 (2-28)	4.9±1.4	5 (1-8)	0.631

Total cholesterol data were compared using an independent Student's t-test, all other data were analyzed using the Mann-Whitney U test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FPG, fasting plasma glucose; GGT, γ-glutamyl transferase; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; SD, standard deviation.

differences were observed among the groups. In addition, when CSX and control groups were compared based on family history, the presence of type 2 diabetes mellitus, and the presence of hypertension, statistically significant differences were identified (P<0.05; Table I).

No significant differences between CSX and control groups regarding clinical parameters including aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, total cholesterol and uric acid were observed (P>0.05). Conversely, the fasting plasma glucose levels in the CSX group were significantly increased compared with the control group (P<0.05; Table II).

Genotyping data. Genetic distributions of the subjects are summarized in Table III. No significant difference was observed when the CSX and control groups were compared on the basis of genotypic distributions of different *RAGE* genes, including the genotypic distribution of rs1800624 and rs1800625 (P>0.05; Table III). Similarly, no significant differences were observed between the CSX and control groups in the distribution of rs2070600 genotype (P>0.05; Table III). In addition, when the allele distributions of the polymorphisms in patients were compared with the control group, no significant difference was identified (Table IV).

Discussion

The results of the present study indicated that *RAGE* gene rs1800624, rs1800625 and rs2070600 polymorphisms were not statistically different between CSX and control groups. Mostly microvascular dysfunction has been suggested to serve a role in the pathogenesis of CSX. Inflammation is

Table III. Genotyping results of *RAGE* polymorphisms.

Polymorphisms	Allele	Patients n (%)	Controls n (%)	P-value
RAGE rs1800624	AA	40 (35)	38 (37)	0.471
	AT	54 (47)	53 (51)	
	TT	20 (18)	12 (12)	
RAGE rs1800625	CC	4 (4)	1 (1)	0.213
	TC	11 (10)	16 (16)	
	TT	99 (87)	86 (83)	
RAGE rs2070600	GG	108 (95)	95 (92)	0.453
	GA	5 (4)	6 (6)	
	AA	1 (1)	2 (2)	

Data were analyzed using χ^2 test. RAGE, receptor for advanced glycation end products; SD, standard deviation.

indicated to have an important role in the formation of both endothelial and microvascular dysfunctions in CSX (8,26). An increase in C-reactive protein (CRP) levels is a marker of inflammation in the presence of injury, infection or pain (27). Arroyo-Espliguero *et al* (28) indicated that significantly increased levels of CRP were detected in patients with CSX compared with controls. It has also been established that inflammation alters the bioavailability of nitric oxide and has an important role in the pathogenesis of vascular dysfunction (29).

Even though cardiovascular diseases are polygenic conditions, genetic mutations may be involved in familial cardiovascular risk factors (30). Therefore, family history should be considered in the diagnosis of CSX. Besides, dyslipidemia, hypertension and obesity are cardiovascular risk factors, which may also increase the risk of type 2 diabetes (31); type 2 diabetes mellitus was suggested as a contributing factor to the risk of vascular complications when patients with the condition were compared with non-diabetic individuals (32). Impaired endothelial function is observed also in patients with type 2 diabetes mellitus (33). These data supported the observations of the present study, which demonstrated that even though healthy individuals and patients exhibited similar demographic attributes, there were statistically significant differences between the two groups in terms of family history, hypertension and type 2 diabetes mellitus (P<0.05). In previous studies, when patients with CSX were compared with healthy individuals, it was revealed that the patients with CSX had an elevated inflammatory response (8,9). Based on these data, we investigated the association between CSX and 3 different cytokines, including pro-inflammatory cytokines, IL-6 and TNF-α, and an anti-inflammatory cytokine IL-10 in a previous study. The results indicated that patients with CSX exhibited increased serum IL-6 and IL-10 levels, but decreased serum TNF-α levels compared with the control group. Conversely, no significant differences in *IL-6*, *IL-10* and *TNF-α* gene polymorphisms were observed between patients with CSX and healthy controls (34).

In other respects, RAGE is suspected to initiate a proinflammatory process and to cause the development of endothelial dysfunction by increasing oxidative stress at a microvascular level. As a result, vasoreactivity may be impaired and lead to ischemia at a microvascular level in the coronary artery system. Furthermore, RAGE may cause fibrosis at microvascular levels and attribute to CSX pathogenesis by decreasing the ability of the small arteries to dilate in the myocardium, and by causing an increase in the levels of microvascular resistance. A previous study demonstrated an increase of microvascular resistance in patients with CSX (4).

Data from previous studies suggest that CSX has a complex mechanism (4,8,9) and RAGE may be involved in the pathogenesis of the disease. To the best of our knowledge, there are no studies examining RAGE levels in patients with CSX, emphasizing the requirement for new studies to investigate the topic.

RAGE is known to interact with certain members of the S100/Calgranulin Family and high mobility group Box-1 proteins; accumulation of these ligands is common in inflammation, cancer or neurodegenerative disorders (35). Binding of such ligands to RAGE causes chemotaxis and oxidative stress, and an enhanced production of pro-inflammatory molecules by triggering the self-expression of RAGE (36). These RAGE-mediated signals are involved not only in diabetes, atherosclerosis or Alzheimer's disease, but also in cardiac ischemia and acute peritoneal inflammation (12). Therefore, oxidative and carbonyl stresses cause an inflammatory response by damaging a number of biological compounds including protein, lipids and nucleic acids (11). In addition, in the tissues that are exposed to oxidative stress, RAGE contributes to vascular inflammation and atherosclerotic changes by interacting with AGE (13). These interactions lead to numerous biological responses, including cell migration and proliferation, and the activation of proinflammatory and prothrombotic pathways (37). The upregulation of these pathways and RAGE-mediated oxidative stress generation results in tissue damage (15). Expression of both proinflammatory cytokines, and transcription factor NF-κB, which is activated as a result of ligand-receptor binding, also imply that the RAGE-AGE interaction affects the inflammatory response (38). A previous study reported the RAGE-AGE interaction in activation of cell-cell signaling and in gene expression, indicating that RAGE expression serves a significant role in the pathogenesis of vascular and heart diseases (39). SNPs of -429/C and -374T/A; and SNP in exon 3 of the gene (Gly82Ser) are among the most frequently studied polymorphisms in the *RAGE* gene (38). In addition, polymorphisms of -429T/C and 374T/A that have been identified in the *RAGE* promoter gene are considered to have effects on RAGE mRNA and protein expression (40). A previous study suggests that the AA genotype of the -374T/A polymorphism exhibits a protective effect against coronary atherosclerosis (41). Conversely, the G82S polymorphism has been observed in the ligand-binding section, affecting the ligand binding affinity, which may cause a number of diseases associated with an increase in oxidative stress and inflammation through RAGE activation (37). *In vitro* studies have demonstrated that the presence of the -429C allele increases the transcriptional activity of the gene and

Table IV. Allelic distribution results of *RAGE* polymorphisms.

Polymorphisms	Allele	Patients, n (%)	Controls, n (%)	P-value
RAGE rs1800624	A	134 (58.8)	129 (62.6)	0.978
	T	94 (41.2)	77 (37.4)	
RAGE rs1800625	C	19 (8.3)	18 (8.7)	0.982
	T	209 (91.7)	188 (91.3)	
RAGE rs2070600	G	221 (96.9)	196 (95.1)	0.478
	A	7 (3.1)	10 (4.9)	

Data were analyzed using χ^2 test. RAGE, receptor for advanced glycation end products.

increases RAGE expression by 2-fold. Consequently, in the pathogenesis of heart disease, polymorphic regions have been closely examined as potential risk factors (39).

A number of studies have focused on polymorphisms within the *RAGE* gene, examining the effects on diabetic complications and the inflammatory response in athero-sclerosis (15). In a cerebral cortex ischemia mouse model, RAGE was expressed in both macrophages and neurons, which led to neuronal cell death and inflammation (12). In addition, a previous study has indicated that RAGE may be involved in the initiation and progress of atherosclerosis (41). Bucciarelli *et al* (14) demonstrated an association between RAGE and cardiac dysfunction, through the observation of ischemic myocardial damage in *RAGE*-null mice.

In the study by Falcone *et al* (41), the -374T/A polymorphism within the *RAGE* gene was indicated to exhibit protective effects against cardiac death, angina and myocardial infarction in diabetic patients. The association between the 82G/S polymorphism and ischemic stroke was studied by Cui *et al* (18), and their data suggested that in the subjects with the 82S/S genotype, the risk of ischemic stroke was significantly increased compared with the individuals with 82G/S and 82G/G genotypes (18). The results of a meta-analysis, consisting of 17 studies with 4,343 patients and 5,402 controls, which examined the -429T/C, -374T/A and G82S SNPs in the *RAGE* gene, were consistent with the results of the present study, which demonstrated that there were no statistically significant associations between polymorphisms and cardiovascular diseases (42). In a similar study examining the association between coronary artery disease and -374T/A and G82S *RAGE* polymorphisms in Turkish subjects, no significant difference was identified between patients and controls (43). Likewise, no significant difference was observed in the allele distributions of all 3 polymorphic regions between patients and control group in the present study.

In conclusion, the results of the present study indicated that there was no significant differences in *RAGE* polymorphisms or allele distribution between patients with CSX and controls in the terms of CSX pathogenesis. To the best of our knowledge, this is the first study that aimed to examine and evaluate polymorphisms of the *RAGE* gene in a Turkish CSX population. Conversely, there were certain limitations to the study: As CSX is a rare disorder, the number of patients participating in the study was limited. The study

could also be developed further to evaluate the association between RAGE and several interacting parameters like AGE, S100/calgranulines and high-mobility group box-1 proteins by ELISA, and their roles in CSX. In addition, other polymorphic loci in the *RAGE* gene could be genotyped to determine the effect of those polymorphisms in CSX. Therefore, in order to comprehensively determine the contribution of *RAGE* gene polymorphisms in CSX pathogenesis, additional studies with more participants are required, and the RAGE levels in patients with CSX may also be quantified.

Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Authors' contributions

AGA, SÖ and BÖ designed the study. BD and BÖ designed and performed data collection. BÖ and DÖ performed the experiments. BO, BD and DÖ wrote the manuscript, and AGA and SÖ were responsible for the final edits of the manuscript.

Ethics approval and consent to participate

The study was approved by Ethics Committee of Cerrahpaşa Medical Faculty (approval no. 2015/279734) and was performed in accordance with Helsinki Declaration of World Health Organization. All the individuals who participated in the study provided written informed consent.

Patient consent for publication

All the individuals who participated in the study provided written informed consent for the publication of any associated data.

Competing interests

The authors declare that they have no competing interests.

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