The assessment of the relationship between variations in the apelin gene and coronary artery disease in Turkish population

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Abstract

Objective: Apelin is a novel endogenous peptide with inotropic and vasodilatory properties and is the ligand for the angiotensin receptor-like 1 (APJ) receptor. The aim of the study was to investigate the association of 2 single-nucleotide polymorphisms (SNPs) in the apelin gene with susceptibility to coronary artery disease (CAD) in the Turkish population.

Methods: The present observational case-control study consisted of 244 subjects (134 angiographically proven CAD patients and 110 healthy controls) aged 30-65 years. The association of 2 SNPs (rs3115758 and rs3115759) in the apelin gene and CAD risk was investigated. Real-time polymerase chain reaction (RT-PCR) was used to analyze the 2 SNPs in both the CAD and the healthy subjects. Allele and genotype frequencies between patients and control groups were compared using the Chi-square (χ^2) test. The relationships of the 2 polymorphisms with the presence of CAD were determined with multiple binary logistic regression analysis after adjustment for CAD risk factors.

Results: TT and AA risk genotypes of the rs3115758 and rs3115759 variants in the apelin gene were found to be significantly related with the risk of CAD with the same power (OR: 6.36, 95% CI: 1.41-28.6) (p=0.007). After adjustments for traditional CAD risk factors, the homozygous TT genotype for rs3115758 and AA genotype for rs3115759 increased the CAD risk, both with an OR of 5.91.

Conclusion: Genetic variants in the apelin gene are significantly associated with the risk of CAD in the Turkish population.

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Keywords: apelin, single nucleotide polymorphism, coronary artery disease

Introduction

Coronary artery disease (CAD) is the leading cause of mortality and morbidity worldwide (1, 2). Recently, many epidemiological studies demonstrated that in addition to conventional risk factors of CAD, including age, male sex, hypertension, diabetes mellitus, obesity, hypercholesterolemia, smoking, and family history, both lifestyle and environmental factors increase the susceptibility to CAD (3). Apart from these factors, genetic predisposition is also thought to play an important role in the pathogenesis of CAD, as illustrated by twin and family studies (4). Although large population studies have reported that multiple genetic variations contribute to the inherited risk of CAD, the exact identity of the candidate genes and the quantity of their effect on disease pathogenesis are not well known.

The apelin gene (APLN) in humans is located on chromosome Xq25-26.1, which possesses one intron within its open reading frame of ~6 kb and encodes a 77-amino-acid prepropeptide that is cleaved into a mature 36-amino-acid peptide (5-7). Apelin mRNA expression was found in the gastrointestinal tract, adipose tissue, brain, lung, kidney, liver, skeletal muscle, and cardiovascular system (8). In the cardiovascular system, it has been detected in endothelial cells of large conduit arteries, coronary vessels, and the endocardium of the right atrium (8). Apelin was shown to be a powerful inotrope and a vasodilator, dilating intact vessels using an endothelial nitric oxide (NO)-dependent pathway (9-11). Since its discovery, the apelin-APJ pathway has emerged as an important regulator of cardiovascular homeostasis that may play a role in the pathophysiology of various cardiac diseases and represents an exciting target for the development of new therapies. Recent studies reported that apelin signaling may be involved in the regulation of blood pressure, cardiac contractile function, fluid balance, angiogenesis, and inhibition of apoptosis (12).

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The interactive effects of genetic defects in apelin-APJ pathway on the susceptibility of CAD in hypertensive Chinese subjects were demonstrated by Jin et al. (13). They have demonstrated apelin levels to be lower in stable CAD patients compared to healthy controls (14). In Kozani study (15), apelin levels in acute coronary syndrome and chronic ischemic heart disease patients were found to be significantly lower than in the control group. Moreover, a relationship, independent of other risk factors, was demonstrated between apelin level and the extent of CAD in this study (15). In a large population-based study, apelin serum concentration was demonstrated to be decreased in heart failure patients with systolic left ventricular dysfunction (16). Falcon et al. (12) investigated the role of the G212A and A445C apelin-APJ polymorphisms in hypertensive Italian subjects with CAD and found no difference between patient and control groups in terms of allele and genotype frequencies. In the same study, the frequency of the G212 allele was demonstrated to be increased in hypertensive CAD patients compared with normotensive subjects. Since there is a lack of information about the relationship between variants in the apelin gene and CAD in the Turkish population in the literature, we aimed to investigate this association. To the best of our knowledge, this is the first study evaluating this association in this population.

Methods

Study groups

The present observational case-control study consisted of 244 Turkish subjects (134 CAD patients and 110 healthy controls), aged 30-65 years, who were enrolled between September 2012 and August 2013 for this study due to symptoms of angina, dyspnea, and chest discomfort at the time of diagnosis. CAD was defined as 50% stenosis in the left main coronary artery or multiple significant stenoses (≥70%) in more than one coronary artery as documented by coronary angiography, history of prior cardiac bypass surgery, history of prior percutaneous coronary intervention, or acute myocardial infarction (AMI). Early-onset CAD was defined as clinical symptoms of CAD occurring at ≤55 years of age in males or ≤65 years of age in female patients. The diagnosis of AMI was confirmed with the 2012 European Society of Cardiology Universal Definition of Myocardial Infarction Guidelines (17). Grading of coronary artery stenosis was carried out by two independent cardiologists who did not participate in this study.

CAD risk factors, such as age, gender, body mass index (BMI), history of diabetes, and hypertension, were identified from the information obtained through the patient's health questionnaire. Subjects with systolic blood pressure (SBP) ≥140 mm Hg or diastolic blood pressure (DBP) ≥90 mm Hg and/or those using antihypertensive medication were classified as hypertensive according to current guidelines (18). Hyperlipidemia was diagnosed in subjects when the 12-hour fasting blood total cholesterol (TC) was ≥220 mg/dL, low-density lipoprotein (LDL) was ≥100 mg/dL, and triglyceride (TG) was ≥150 mg/dL and/or in subjects taking antihyperlipidemic drugs according to current guidelines (19). Diabetes mellitus in subjects was defined as fasting glucose levels ≥126 mg/dL by two consecutive fasting plasma glucose measurements or hemoglobin (Hb) A1c ≥6.5% or those who were being treated with oral anti-diabetic drugs or insulin according to current guidelines (20). The height and weight were recorded, and BMI was calculated as the ratio of weight in kilograms divided by the square of the height in meters. Obesity was defined as having a BMI of ≥30 according to the World Health Organization (WHO) classification (21).

The control group consisted of 110 healthy subjects from the same geographical area, who were admitted to the outpatient clinic with mild-moderate chest pain and had a positive exertional test without any stenosis in their coronary arteries, as documented by coronary angiography. Exclusion criteria were age >55 years in male subjects and >65 years of age in female subjects, auto-immune disease, severe kidney and hepatic diseases, cancer, pregnancy, and subjects who had contraindications for heparin and contrast agents.

The study complies with the Declaration of Helsinki, and the trial protocol was approved by the local Ethics Committee. Written informed consent was obtained from all participants who were included in this study.

DNA extraction and genotyping

While selecting these single-nucleotide polymorphisms, both the results of population genetics on the apelin gene, found in the ENSEMBLE genome database, and the literature support were taken into consideration. Moreover, significant results were found for these single-nucleotide polymorphisms in Asian populations (13, 22-24).

Ten milliliters of blood per patient was collected for biochemical analyses. Peripheral venous blood sample was collected from each subject into tubes containing the anticoagulant EDTA for DNA isolation and real-time PCR procedure. Samples were stored in aliquots at -20°C until they were analyzed. Also, polymerase chain reaction (PCR) and reverse hybridization methods were used to genotype samples for analyses of 2 SNPs (rs3115758 and rs3115759). A LightCycler 480® system was used to perform SNP genotyping using hybridization probes consisting of 3'-fluorescein and a 5ı-LightCycler® red-labeled pair of oligonucleotide probes (TIB MOLBIOL GmbH, Berlin, Germany). The primers for the rs3115758 polymorphism were ggAggACATATTTTATATgTAACAAT and gAgAATgTTgAgCATACACTCTA, and the probes were AATCATgCTTAgCCgAAgggA-FL and 640-CCgAACAggAgTAAAAAATggTCCCAA p. For the rs3115759 polymorphism, the primers were AgATgTTTAAATgTCgAATTATTg and AATgTgACTgCTTCTgCAT, and the probes were ggCTgCTTTTCAACTgTTgA-FL and 640-CATATggTTAgTATg AggAATgACAgTAgggTp. Genotyping was performed in a 20-µL volume containing 2.0 µL of LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany), 1.0 µL Reagent Mix, 3.0 mM $MgCl_2$, and 50 ng of genomic DNA. The quality of SNP genotyping was ensured by independently repli-

cating the genotyping of randomly selected samples. The results from the quality control were in 100% agreement with the initial genotyping results.

Statistical analysis

The present study had a case-control design. Continuous data were expressed as mean±standard deviation (SD). The normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Differences between continuous variables were evaluated by the independent- samples t-test or the Mann-Whitney U test, depending on the shape of the distribution curves. Statistical analysis for the comparison of variables between the patient and control groups was performed using student's t-test. 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. The relationships of the 2 polymorphisms with the presence of CAD were determined with the odds ratio (OR) and 95% confidence interval (CI) for the risk alleles by multiple binary logistics regression analysis after adjustment for CAD risk factors. All statistical analyses were performed using SPSS for Windows (SPSS for Windows, version 17.0. Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

Baseline demographic, clinical, and laboratory characteristics of the patient and control groups are presented in Table 1. There were 134 CAD patients and 110 healthy controls enrolled in this study. The mean ages of the CAD patients and controls were 57.43±3.43 and 58.35±5.67 years, respectively. No significant differences were observed between patients and controls in terms of age, gender, BMI, uric acid, and triglycerides (all p values >0.05). However, CAD patients had significantly greater SBP (p<0.001), DBP (p<0.001), hemoglobin A1c (p<0.001), creatinine (p=0.039), serum total cholesterol (p=0.003), and LDL cholesterol (all p<0.001) than controls. Moreover, HDL cholesterol levels were found to be significantly lower in CAD patients as compared to healthy subjects (p<0.001). Also, the prevalence of hypertension and obesity was found to be significantly higher in patients compared with the control group (all p values <0.001).

The genotype and allele frequencies of 2 SNPs (rs3115758 and rs3115759) in CAD patients and healthy controls are presented in Table 2. The genotypes frequencies of rs3115758 and rs3115759 were in accordance with Hardy-Weinberg equilibrium among the patients and controls (p>0.05). Significant differences were observed in the genotype and allele frequencies of rs3115758 and rs3115759 variants between CAD patients and healthy controls (p=0.007 and p=0.002, respectively). For rs3115758 and rs3115759 variants in the apelin gene, both the risk genotypes TT and AA were associated with an increased risk of CAD with the same power (both OR of 6.36, 95% CI 1.41-28.6).

Table 1. Demographic, clinical, and laboratory characteristics of CAD patients and controls

	CAD (n=134)	Control (n=110)	*P*
Age, years	58.35±5.67	57.43±3.43	0.118
Gender F/M, n, %	51/83 (38.1/61.9)	43/67 (39.1/60.9)	0.869
Hypertension, n (%)	88 (65%)	45 (40.9%)	<0.001
Obesity, n (%)	32 (23.8%)	15 (13.6%)	<0.001
BMI, kg/m^2	25.55±3.37	25.54±3.4	0.970
SBP, mm Hg	134.89±29.33	119.26±14.7	<0.001
DBP, mm Hg	82.16±16.45	72.81±11.22	<0.001
HbA1c, %	6.42±0.77	5.93±0.29	<0.001
Creatinine, mg/dL	1.03±0.37	0.96±0.17	0.039
Uric acid, mg/dL	6.89±31.52	4.12±0.83	0.359
Total cholesterol, mg/dL	204.69±50.58	187.04±40.64	0.003
Triglycerides, mg/dL	149.41±56.42	138.77±72.44	0.199
HDL cholesterol, mg/dL	39.64±6.35	46.68±11.26	<0.001
LDL cholesterol, mg/dL	140.11±39.0	111.96±28.69	<0.001

BMI - body mass index; DBP - diastolic blood pressure; HbA1c - hemoglobin A1c; HDL - high-density lipoprotein; LDL - low-density lipoprotein; SBP - systolic blood pressure

Table 2. Genotypes and allele frequencies for APLN1 rs3115758 and APLN1 rs3115759 in CAD patients and controls

APLN1 rs3115758 Genotype	Control (n=110) (%)	CAD (n=134) (%)	OR (95% CI)	*P*
GG	100 (90.9)	110 (82.1)	Reference	
GT	8 (7.3)	10 (7.5)	1.13 (0.43-2.99)	0.8
TT	2 (1.8)	14 (10.4)	6.36 (1.41- 28.6)	0.007
Allele				
G	208 (94.5)	230 (85.8)	Reference	
T	12 (5.5)	38 (14.2)	2.86 (1.45-5.62)	0.002
APLN1 rs3115759 Genotype				
GG	100 (90.9)	110 (82.1)	Reference	
GA	8 (7.3)	10 (7.5)	1.13 (0.43-2.99)	0.8
AA	2 (1.8)	14 (10.4)	6.36 (1.41- 28.6)	0.007
Allele				
G	208 (94.5)	230 (85.8)	Reference	
A	12 (5.45)	38 (14.2)	2.86 (1.45-5.62)	0.002

OR - odds ratio

Furthermore, both the T risk allele of rs3115758 and the A risk allele of rs3115759 were found to be present in 14.2% of CAD patients. Both the T allele in rs3115758 and the G allele in rs3115759 increased the risk of CAD 2.86 times (95% CI 1.45-5.62) as compared to controls (p=0.002).

The ratio of heterozygote (GT) and homozygote (TT) mutants of the rs3115758 variation in the apelin gene was found to be increased in hypertensive patients compared to normotensive subjects (13.3% vs. 4.7% and 10.7% vs. 4.7%, respectively; p=0.01). Furthermore, the co-existence of the GG+TT genotypes

was found to be significantly higher in hypertensives compared to controls (24% vs. 9.5%; p=0.002). The co-existence of the heterozygote and homozygote forms (GT+TT) of this variant increased the risk of hypertension significantly (OR=3.02, 95% CI 1.44-6.32, p=0.002).

The ratio of heterozygote (GA) and homozygote mutant (AA) genotypes of the rs3115759 variation in the apelin gene was found to be significantly higher in hypertensive patients compared to normotensive subjects (13.3% vs. 4.7% and 10.7% vs. 4.7%, respectively; p=0.01). The co-existence of the GA+AA genotypes was demonstrated to be significantly higher in hypertensive patients compared to normotensive subjects (24% vs. 9.5%; p=0.002). The co-existence of heterozygote and homozygote forms of this variant also resulted in a significant increase in the risk of hypertension (OR=3.02, 95% CI 1.44-6.32, p=0.002).

The possible selective effects of the 2 SNPs on CAD were investigated using multiple binary logistic regression analysis after adjusting for some risk factors, such as hypertension, diabetes. The TT variant of rs3115758 (p<0.03), AA variant of rs3115759 (p<0.03), diabetes (p<0.001), and hypertension (p<0.001) were found to be important risk factors for CAD (Table 3). After age and gender adjustments, the logistic regression analysis revealed that having a homozygous TT and AA genotype for rs3115758 and rs3115759, respectively, increased the risk of CAD with the same OR of 5.91 (p<0.03, OR=5.91, 95% CI 1.15-30.1).

Table 3. Results of multiple binary logistic regression analysis

Covariates	*P*	OR	95% CI
rs3115758 TT genotype	0.03	5.91	1.15-30.1
rs3115759 AA genotype	0.03	5.91	1.15-30.1
Diabetes	<0.001	0.04	0.01-0.14
Hypertension	<0.001	0.17	0.08-0.36
OR - odds ratio			

Discussion

This is the first study to investigate the relationship between variations in the apelin gene and CAD in the Turkish population in the literature. In this study, 2 SNPs (rs3115758 and rs3115759) were found to be associated with CAD in the Turkish population with the same power. Furthermore, the TT variant of rs3115758, AA variant of rs3115759, diabetes, and hypertension were found to be significant independent predictors for CAD in a multiple binary logistic regression analysis after adjusting for some risk factors.

Jin et al. (13) sought to investigate the association of 5 promising polymorphisms (rs3761581, rs56204867, rs7119375, rs10501367, rs9943582) in the apelin/APJ pathway with CAD among 1702 hypertensive Chinese patients. Single-locus analyses exhibited no significant differences in the genotype/allele frequencies of the examined polymorphisms between CAD patients and controls (p=0.05). In our study, as in this study, 2 SNPs in the apelin gene that contributed to the development of CAD were analyzed, and both polymorphisms (rs3115758 and rs3115759) were associated significantly with CAD. Also, in our study, as in this study, the CAD and control groups were classified as evidenced by coronary angiography, and age/gender adjustment is provided.

Falcone et al. (12) evaluated the possible relationship between the G212A and A445C APJ polymorphisms and CAD in 664 Italian patients and 183 healthy controls by restriction fragment length polymorphism (RFLP-PCR). There were no differences between the two groups in terms of allelic and genotypic frequencies (12). In the CAD population, there was an increased frequency of the G212 allele in patients with hypertension as compared to patients without hypertension. They hypothesized that the presence of the A allele may cause a gain in function of the apelin/APJ system, which is associated with a lower risk of hypertension. In our study, as opposed to this study, both polymorphisms (rs3115758 and rs3115759) were associated significantly with CAD. In addition, the heterozygous (GT) and homozygous mutant (TT) genotypes of rs3115758 and the heterozygous (GA) and homozygous mutant (AA) genotypes of rs3115759 of the apelin gene were significantly higher in hypertensive patients as compared to normotensive patients. In our study, the mutant T and A alleles of these 2 polymorphisms may be associated with the development of hypertension through the apelin/APJ pathway and indirectly with CAD.

Zhang et al. (22) selected 3 single-nucleotide polymorphisms (SNPs) that could capture all common variants in the APLN gene region and genotyped them in 1892 type 2 diabetic patients and 1808 normal glucose regulation controls. None of the SNPs or haplotypes showed evidence of an association with type 2 diabetes; however, rs2235306 was nominally associated with fasting plasma glucose levels in the male subjects with normal glucose regulation (p=0.04). No significant difference was observed between any SNP and other variables. It suggests that APLN genetic variants may contribute to clinical features related to glucose metabolism in the Chinese population. Unlike this study, the relationship of variations in the apelin gene with CAD was studied in our study. The apelin gene variant rs3115759 AA risk genotype was significantly associated with an increased risk of CAD (OR: 6.3, 95% CI:1.41-28.6) (p=0.006). The GA and AA genotypes of the rs3115759 variant, taken together, showed significant differences between patients and controls (p=0.048). The GA + AA genotype combination was associated with a significant increase in the risk of CAD (OR: 2.182, 95% CI: 0.994-4.787) (p=0.048).

Four tagging APLN SNPs (rs3115757, rs2235310, rs3761581 and rs2235307) were genotyped in 1627 Chinese subjects by Liao et al. (23). They treated primary adipocytes with high glucose plus insulin because of a close relation between insulin resistance and obesity. The minor homozygote CC of the rs3115757 SNP was associated with a high BMI in women, but this genetic effect was not present in men. Accordingly, genetic association and functional studies suggest that genetic variants in APLN may influence apelin expression and are associated with the

susceptibility of obesity phenotypes. In our study, the rate of obesity in the CAD group was higher, too. Thus, the relationship between apelin and CAD indirectly suggests that there may be other risk factors associated.

Li et al. (24) analyzed 1015 Han Chinese from 248 families with essential hypertension, using the powerful and highly reliable family-based association tests, and each individual was genotyped for 6 SNPs in the apelin gene. The analysis showed that 2 SNPs (rs3761581 and T-1860C) within apelin conferred a significant association with hypertension and its related phenotypes, even after correcting for age and gender. Likely, in our study, apelin gene variations of the rs3115758 heterozygous (GT) and homozygous mutant (TT) genotypes and rs3115759 heterozygous (GA) and homozygous mutant (AA) genotypes were in a significantly higher proportion of hypertensive patients according to normotensive patients.

Zhang et al. (22) analyzed 3 SNPs (rs2235307, rs2235306, rs3115759) in APLN and genotyped them in 3156 diabetic patients and 3736 nondiabetic individuals. In diabetic patients, no significant associations of the three SNPs with hypertension were observed, but rs2235306 was associated with hypertension in non-diabetic males after adjusting for covariates (p=0.03), while rs2235307 and rs3115759 displayed no evidence of association in either gender. They concluded that common variants in APLN are not associated with the prevalence of hypertension in either diabetic or non-diabetic subjects and that common variants of APLN may not play a major role in the regulation of blood pressure. In contrast to this study, both SNPs in the apelin gene showed significantly higher rates in hypertensive compared to normotensive patients in our study. In addition, with regard to the selective effects of these 2 SNPs of the apelin gene, examined through multiple binary logistic regression analysis after correction of other CAD risk factors, the rs3115758 (TT) and rs3115759 (AA) genotypes were found to be significant independent predictors of the development of CAD.

Study limitations

The present study focused on both CAD patients and control subjects. The limitations of this study included a small sample size and data from a single center.

Conclusion

Nevertheless, to our knowledge, this is the first study to demonstrate an association between common SNPs (rs3115758 and rs3115759) in the apelin gene and CAD in a Turkish population. Complex cardiovascular diseases, such as CAD, have variable gene-to-gene and gene-to-environment interactions in different populations. Multiple and different SNPs in the apelin gene should be studied prospectively in a wider population to fully understand the molecular mechanism underlying the association and to assign a patho-physiological role to apelin gene variants in the etiopathology of CAD in Turkish populations.

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