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Determination of the adrenomedullin gene variations in patients with coronary artery disease

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Ethics Committee Approval
The study was approved by the Manisa Celal Bayar University Faculty of Medicine Local Ethics Committee, dated July 2, 2014 and numbered 20478486261.
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Coronary artery disease is characterized by atherosclerosis in the vessel wall. The protein encoded by the adrenomedullin (ADM; OMIM No. 103275) gene is a preprohormone, post-translationally modified to form two biologically active peptides. These peptides are involved in a myriad of functions including vasodilation, bronchodilation, regulation of hormone secretion, growth modulation, promotion of angiogenesis, and antimicrobial activity. This study was designed to explore the correlation between the development of atherosclerosis and variations in the *ADM* gene.
Methods: The study analyzed 62 cases with atheromatous plaques and 46 cases without such plaques. Genomic DNA was extracted from peripheral blood. Variations in the *ADM* gene were determined by Sanger sequencing.
Results: In this study, four different variants were found in the *ADM* gene, including c.-2140T>C (rs3814700), c.248+91T>G (rs545190978), c.150C>G (rs5005), and c.261C>T (rs767028428 C>T). The allele frequencies of these ADM gene polymorphisms in patients were as follows: T at 93% and C at 7% for rs3814700 (T>C); T at 99.2% and G at 0.08% for rs545190978 (T>G); C at 99.2% and G at 0.08% for rs5005 (C>G); and C at 99.2% and T at 0.08% for rs767028428 (C>T).
Conclusion: It was found that there was no significant difference in allele frequencies and genotypes of these polymorphic regions between the patients and the control group.

Keywords: coronary artery disease, atherosclerosis, vasodilation, *ADM* gene

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Introduction

Coronary artery disease (CAD) is a progressive and systemic inflammatory disease, where atherosclerosis with occlusive plaque formation is the underlying cause. This includes pathologies affecting the coronary arteries that supply blood to the heart. In developed countries, CAD is a leading cause of death. An important event in atherosclerosis is the formation of an atheroma or occlusive atheromatous plaque due to lipid accumulation and intimal thickening [1,2]. The role of several enzymes in the cardiovascular system and atherosclerotic plaque formation is considered protective against the disease. Adrenomedullin (ADM) with properties such as vasodilation, natriuresis, anti-apoptosis, and nitric oxide stimulation is believed to inhibit atherosclerosis by suppressing vascular smooth muscle cell proliferation. It has significant effects concerning vascular pathologies [3]. ADM is a peptide synthesized by endothelial cells with a vasoprotective effect on the cardiovascular system. Differences in ADM plasma levels have been noted due to its vasoprotective effect in various cardiovascular diseases [4]. High levels of ADM are found in atherosclerosis [5]. Consisting of 2,717 bases, the ADM gene is located on the p15.4 arm of chromosome 11. The ADM gene consists of four exons, three introns, and several TATA, CAAT, and GC regions in the 5 UTR regions that have bounds with five transcription factors [6]. Sequence changes in the ADM gene's promoter region are known to affect ADM and cause various pathologies [7]. Plasma ADM levels are associated with a single nucleotide polymorphism (SNP) in the ADM gene. Proadrenomedullin (proADM) was recognized as a novel biomarker reflecting vascular function [8]. ADM gene polymorphisms are thought to influence its expression, possibly leading to a genetic predisposition to various systemic diseases. Ong et al. reported that rs11042725 change was a risk factor for dysglycaemia, and other ADM gene sequence changes were predicted to carry the same risk [9]. In the study by Cheung et al., they correlated plasma ADM levels with IL-6 levels and linked the ADM gene rs4910118 polymorphism with lower adrenomedullin levels. The study proposed that ADM gene sequence variations could be utilized to predict cardiovascular risk [10]. It is hypothesized that functional polymorphisms of the ADM gene may make individuals susceptible to the development of atherosclerosis.

The objective of this study was to explore the correlation between gene sequence variations and the onset of atherosclerosis in ADM.

Materials and methods

Study population

Manisa Celal Bayar University Faculty of Medicine's Department of Cardiology included a total of 108 patients, over 40 years of age, with pre-diagnosis of CAD in a study. The study consisted of 62 patients with atheroma plaque and 46 patients who had normal angiography results. Each patient was analyzed for demographic characteristics and risk factors such as smoking, diabetes mellitus, dyslipidemia, hypertension, and family history of CAD. The Ethics Committee of the Manisa Celal Bayar University Faculty of Medicine approved this study on July 2, 2014, with the approval number 20478486261.

Determination of ADM gene changes

Genomic DNA isolation was conducted using an Invitrogen DNA isolation kit procedure with peripheral venous blood samples. Both patient and control DNAs were obtained following the appropriate protocol. The purity range and the quantities of DNA samples from the isolation were assessed using a NanoDrop device. Standard 50 µl PCR reactions were formed using PCR components, drawing from 30 ng/µl cases from the DNA samples. Two pairs of primer designs were created to augment the promoter and exonic regions of the ADM gene, and these designs were applied in the PCR studies (promoter region primers: F:5′-CTAGCTGTCCTTGACCTCCC-3′, R:5′-ATCGAATGTCCTGAAGCCCA-3′; exonic region primers: F1: 5′-GCTCGACTCTCTTTCTTCT-3′, R1:5′- GAAGTCCTTCGTCCCGGG-3′). Primer3Plus, an online software primer design program, was used for the primers [11]. The PCR was conducted in a 50 µl reaction which included 150 ng DNA, 10× PCR buffer, 2.5mM MgCl2, 20 µM dNTPs, forward primer (10 pmol/ µl), reverse primer (10 pmol/ µl), and 5U/ µl hot start Taq polymerase. Amplification conditions were as follows: initial activation at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 1 min and 45 s, and a final extension step at 72°C for 10 min. To assess the amplification products from the PCR, we conducted a 1.5% agarose gel electrophoresis alongside a 100 base pair (bp) DNA Ladder. Samples underwent sequencing if PCR product formation occurred. DNA purification preceded sequencing, following the appropriate protocol.

Sequence study and analysis

Purification was carried out using Exosap. The sequencing was conducted following the appropriate protocol. For the PCR sequence, 2 µl of the purified product was incorporated. Relevant primers (F and R primers) were also diluted and added. The primary dilution process involved adding 16 µl distilled water and 4 µl primer, priming with 1/10 primers to contribute 2 pmol. Following this, a mixture of 2 µl 5× buffer, 2 µl dNTP and ddNTP (dideoxynucleotide triphosphate), featuring BigDye to detect the study's DNA region using fluorescent nucleotides, and 2 µl dH_2O, prepared by adding a 6 µl mix, was introduced. The PCR program for the sequence entailed: heating for 10 s at 96°C, 5 s at 50°C, 4 min at 60°C, and repeating these steps 25 times before holding for 1 h at 4°C and indefinitely at 100°C. DNA sequencing pertains to determining the sequence of nucleotide bases (adenine, guanine, cytosine, and thymine) in a DNA molecule. The sequencing is aligned to a single primer direction but can be ordered separately from the two directions, forward and reverse primer.

Sequence evaluation

The resulting sequence data was analyzed using the MEGA5.1 sequencing program. This program aligns the sequences with each other. This comparison was carried out for 108 samples. As a result, the transformed regions (base) were determined, and repeated PCR was performed to confirm the sequence analysis and the sequence analysis was repeated. In

addition, new changes and sequence results for polymorphic regions were checked from graphs.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) was utilized for the statistical analysis of the study's results. Complementary statistical methods (mean, standard deviation) were employed to evaluate the data. Differences in frequencies and distributions between groups were assessed by the chi-squared test. Furthermore, allelic distributions and frequencies were evaluated using the chi-squared test, with percentages and p-values being calculated. Values of p <0.05 were deemed statistically significant. Additionally, the odds ratio method was used to compute the risk values for genotype-allele distribution frequency and disease risk factors.

Results

The study included a total of 108 individuals, consisting of 62 patients (32 males and 30 females) and 46 controls (24 males and 22 females). Both groups underwent an assessment for coronary risk factors. Significantly higher instances of hypertension, dyslipidemia, and family history were observed in the patient group compared to the control group. Table 1 presents the data collected relative to coronary risk factors.

Table 1: Percentages of risk factors forming the study group

Risk factors	Patient group (n=62)	Control group (n= 46)	OR (Odds Ratio) (95%CI)	*P*-value	Total (n=108)
DM	25	10	1.40 (1.03-1.91)	0.061	35
HT	39	18	1.51 (1.06-2.15)	0.019	57
DL	26	6	1.71 (1.28-2.91)	0.001	32
family history	20	6	1.50 (1.11-2.02)	0.024	26
smoking	15	11	1.00 (0.68-1.47)	135	16

DM: diabetes mellitus, HT: hypertension, DL: dyslipidemia

The research determined ADM genotypes amongst patients with coronary angiography atheroma plaques, and a separate control group without such plaques. The researchers observed the presence of *ADM* gene: NM_001124.3: c.-2140T>C variation (rs3814700) within the promoter region. Another variant, *ADM* gene: NM_001124.3: c.248+91T>G (rs545190978), was detected within the 5′UTR regions.

Similarly, the *ADM*: NM_001124.3: c.150C>G variation (rs5005) was identified within exon 3. This particular change also prompts a switch from the serine amino acid at position 50 to arginine. Lastly, the *ADM*: NM_001124.3: c.261C>A: p.(Ala87=) variant was found within exon 4, although this substitution does not alter the amino acid sequence (alanine to alanine).

Genotype and allele frequencies of case controls are summarized in Tables 2, 3, 4, and 5. The distribution of rs3814700 genotype frequencies in the patient group was 87.1% (54/62) for TT and 12.9% (8/62) for TC. In the control group, the distribution of genotypes was 93.5% (43/46) for TT and 6.5% (3/46) for TC. There were no significant differences in rs3814700 genotype frequencies when comparing the patient group and controls (*P*=0.278) as Table 2 shows. T and C allele frequencies of the rs3814700 polymorphism were 93% and 7% in the patient group, and 96% and 4% in the control group, respectively (*P*=0.764).

The distribution of rs5005 genotype frequencies in the patient group was 98.4% (61/62) for CC and 1.6% (1/62) for CG. In the control group, the distribution of genotypes was 100% (46/46) for CC, and 0% (0/46) for CG. There were no significant differences in the rs5005 genotype frequencies when comparing the patient group to the controls (*P*=0.386) (Table 5). The C and G allele frequencies of the rs5005 polymorphism were 99.2% and 0.08% in the patient group, and 100% and 0% in the control group, respectively. There were no significant differences in the rs5005 allele frequencies when comparing the patient group and the controls (*P*=0.387) (Table 3).

Similarly, the distribution of rs545190978 genotype frequencies in the patient group was 98.4% (61/62) for TT and 1.6% (1/62) for TG. Meanwhile, in the control group, the genotype distribution was 100% (46/46) for TT and 0% (0/46) for TG. The comparison of rs545190978 genotype frequencies between the patient and control groups did not reveal any significant differences (*P*=0.386). (Table 4). The T and G allele frequencies of rs545190978 polymorphism were 99.2% and 0.08% in the patient group, and 100% and 0% in the control group, respectively. There were no significant differences in rs545190978 allele frequencies between the patient and control groups (*P*=0.387) (Table 4).

The distribution of rs767028428 genotype frequencies in the patient group was 98.4% (61/62) for CC and 1.6% (1/62) for CT. In the control group, the genotype distribution was 100% (46/46) for CC and 0% (0/46) for CT. There were no significant differences in rs767028428 genotype frequencies between the patient and control groups (*P*=0.386) (Table 4). C and T allele frequencies of rs767028428 polymorphism were 99.2% and 0.08% in the patient group, and 100% and 0% in the control group respectively. There were also no significant differences in rs767028428 allele frequencies between the patient and control groups (*P*=0.387) (Table 4).

Table 2: ADM gene (rs3814700-promoter) polymorphism genotype frequencies

rs3814700	Patient group (62)	Control group (46)	OR (Odds Ratio) (95%CI)	*P*-value
Genotype frequency and distribution				
TT	54 (87.1%)	43 (93.5%)		
TC	8 (12.9%)	3 (6.5%)	0.76 (0.51-1.14)	**0.278**
Allele frequency and distribution				
T allele	116 (93%)	89 (96%)		
C allele	8 (7%)	3 (4%)	0.77 (0.53-1.13)	**0.291**

Table 3: ADM gene (rs5005-exon 3) polymorphism genotype frequencies

rs5005	Patient group (62)	Control group (46)	OR (Odds Ratio) (95%CI)	*P*-value
Genotype frequency and distribution				
CC	61 (98.4%)	46 (100%)		
CG	1 (1.6%)	0 (0%)	0.57 (0.48-0.67)	**0.386**
Allele frequency and distribution				
C allele	123 (99.2%)	92 (100%)		
G allele	1 (0.08%)	0 (0%)	0.57 (0.50-0.64)	**0.387**

Table 4: ADM gene (rs545190978-5'UTR) polymorphism genotype frequencies

rs545190978	Patient group (62)	Control group (46)	OR (Odds Ratio) (95%CI)	*P*-value
Genotype frequency and distribution				
TT	61 (98.4%)	46 (100%)		
TG	1 (1.6%)	0 (0%)	0.57 (0.48-0.67)	**0.386**
Allele frequency and distribution				
T allele	123 (99.2%)	92 (100%)		
G allele	1 (0.08%)	0 (0%)	0.57 (0.50-0.64)	**0.387**

Table 5: ADM gene (rs767028428-exon 4) polymorphism genotype frequencies

rs767028428	Patient group (62)	Control group (46)	OR (Odds Ratio) (95%CI)	*P*-value
Genotype frequency and distribution				
CC	61 (98.4%)	46 (100%)		
CT	1 (1.6%)	0 (0%)	0.57 (0.48-0.67)	**0.386**
Allele frequency and distribution				
C allele	123 (99.2%)	92 (100%)		
T allele	1 (0.08%)	0 (0%)	0.57 (0.50-0.64)	**0.387**

Discussion

The polymorphisms of the *ADM* gene may influence ADM expression and predispose individuals to various systemic diseases. Variations in the *ADM* gene are suggested to contribute to the development of atherosclerosis due to ADM's roles in processes such as inhibiting vascular smooth muscle cells and enabling vasodilation in vessels. A cohort study involving 1370 patients with acute coronary syndrome found adrenomedullin to be an accurate biomarker for predicting acute coronary syndrome and comorbid cardiovascular disease in patients with chronic kidney disease and preserved renal function [12]. Another study indicated that ADM expression is higher in patients with coronary heart disease [13].

The four different polymorphisms were detected in the promoter, 5'-untranslated region (5′UTR), exon 3, and exon 4 of the *ADM* gene. This study's findings suggest no statistically significant disparity between the patient and control groups in terms of allele and genotype frequencies and polymorphism regions. The *ADM* promoter region presented a higher frequency of the rs3814700 polymorphism in the patient group; however, no statistically significant outcomes were attained. The A>G change (rs3814700) at position 1984, previously reported, is associated with blood pressure, and plasma ADM levels are higher in healthy controls with the G allele [14]. The absence of plasma ADM levels in our study precludes the evaluation of this polymorphism's effect on ADM levels in individuals. The frequency of the polymorphism observed in this study compares to the prevalence of the polymorphism in the healthy European population. Given the insufficient number of cases and the unknown ethnic origin, we believe that the determination of the ADM rs3814700 polymorphism in healthy populations will yield more precise data on the actual polymorphism's frequency in our country.

The rs767028428 polymorphism, detected in the fourth exon of the *ADM* gene, is a synonymous mutation that does not alter the encoded protein. The current literature review reveals no publications on this polymorphism in relation to the *ADM* gene and atherosclerosis. According to the available database data, this polymorphism's clinical significance remains uncertain. Given our study's limited number of control cases, we believe that a larger sample size would provide more precise data on this polymorphism.

The rs5005 polymorphism, identified in exon 3 of the *ADM* gene, has been cited in the literature as a variant of uncertain clinical significance. The detected change is a non-synonymous change that results in the substitution of a small, polar amino acid for a large, basic amino acid at position 50 in the encoded protein. The effect of this change on protein folding remains unknown. Given this polymorphism's low prevalence in other populations and the fact that the patient group is the only one with a detected polymorphism, it is critical to evaluate the polymorphism in conjunction with ADM levels to determine its clinical significance and assess ADM expression in patients with this polymorphism.

In their 2023 study, Chang et al. [15] proposed that a long-acting adrenomedullin analog may prove beneficial in managing hypertension and preventing organ damage related to vascular ischemia in pre-eclampsia patients. The intramyocardial injection of Recombinant Adeno-Associated Viral Vector PR39/Adrenomedullin co-expression has been shown to increase angiogenesis and reduce apoptosis in a rat myocardial infarction model [16].

Limitations

As a result, in the present study, further analysis is needed in terms of the polymorphisms determined by the sequence analysis. To determine the role of adrenomedullin in the pathogenesis of atherosclerosis, ADM expression, and plasma levels should be evaluated simultaneously.

Conclusion

No significant differences were found in the allele and genotype frequencies of the ADM gene between the patient and control groups. A potential new gene therapy-based approach for clinical use is proposed.

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