European Journal of Therapeutics

DOI: 10.58600/eurjther-337

Original Research

Signature of Serum miR-199a/b in Coronary Artery Bypass Graft Surgery

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ABSTRACT

Objective: microRNAs (miRNAs) have important potential as biomarkers in the diagnosis and prognosis of ischemia/reperfusion (I/R) injury in coronary artery bypass grafting surgery (CABG). This study investigated the relationship between preoperative (pre-op) and postoperative (post-op) cardiac parameters and miRNA expressions in CABG.

Methods: We analyzed a total of 94 individuals (CABG, n= 46 and healthy control, n=48). Quantitative real-time polymerase chain reaction (qRT) was performed to determine plasma miRNA expressions (miR-21, miR-181a, miR-199a, miR-199b, and miR-320a-5p) in triplicates: before surgery, 1 hour after surgery, and 24 hours after surgery. The target genes and pathways of miRNA were determined using bioinformatic analysis. The biomarker potentials of miRNAs were evaluated with receiver operating characteristic (ROC) curve analysis.

Results: All miRNAs were significantly downregulated ($p < 0.05$). Troponin I, LVEF, CPK, and CK-MB were found to be statistically significant for operation groups ($p < 0.05$). miRNA expressions and cardiac markers were associated with troponin I and/or CK-MB. In ROC analyses, miR-199a was a good diagnostic marker. CREBRF and ZNF704 genes may be a target for these miRNAs.

Conclusions: Downregulation of miR-199a has a regulatory role in ischemia/reperfusion. They may contribute to CABG pathology through these two genes involved in signaling cascades to turn on protein response and ion binding.

Keywords: coronary artery, microRNA, diagnostic marker, ischemia, reperfusion.

INTRODUCTION

Coronary artery disease (CAD) is a disease characterized by reduced blood flow to the heart muscle due to atherosclerosis, affecting the structure and functions of the heart (1). Types include st/unstable angina, myocardial infarction, and sudden cardiac death (2). There are various risk factors responsible for the root of the disease and the epigenetic basis has a very important place. While the methods used in the diagnosis of the disease detect recent or instantaneous changes, searching for epigenetic markers can make it possible to go to the point and time where these processes started. Most interventions to reduce disease risk are based on cardiac markers and imaging systems. However, there is limited evidence to identify people at low risk or without symptoms. Therefore, from the onset of symptoms, even earlier methods of diagnosis, including genetic markers, may provide significant advances. Ischemia develops due to a lack of oxygen as a result of insufficient perfusion of organs and tissues as a result of decreased arterial or venous blood flow (3). Lack of energy and accumulation of toxic metabolites cause cell death (4). Damage to ischemic tissue due to reperfusion is more serious than the damage caused by ischemia. The cellular structures most susceptible to I/R are proteins, nucleic acids, and membrane lipids (5).

Although significant advances have been made in understanding the mechanisms responsible for myocardial I/R injury, it is difficult to match the findings clinically. miRNAs control a variety of cellular activities by degradation or inhibition of translation of the target mRNA. However, they can be tissue-specific or expressed in more than one tissue. Many studies have shown that miRNAs have an important role in cardiac processes and determine their fate by regulating cell death and regeneration after myocardial infarction (6). There are many known pathways, responsible genes, and epigenetic marker candidates in the miRNA mechanism. Evaluation of the reflections of these markers on clinical parameters and their diagnostic or therapeutic potential

How to cite: Kandilli E, Görücü Yılmaz Ş, Yardımcı M, Nacak M, Benlier N. Signature of Serum Mir-199a/B in Coronary Artery Bypass Graft Surgery. Eur J Ther. 2023; 29(1): 49-57.

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Received: 31.08.2021 • **Accepted:** 27.06.2022

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may enable them to be presented as descriptive and therapeutic in CABG. In this study, we evaluated the regulation of five miRNAs and their relationship with parameters in cardiac processes in the sera of healthy, pre-and post-operative individuals. We demonstrated the contribution of these miRNAs to the pathophysiology of the disease and their diagnostic potential. Finally, we identified the targets and pathways of miRNAs functional in CABG using bioinformatic analyses.

METHODS

Population Data

Ethics approval was obtained from the Medical Ethics Committee (Protocol number: 276). Consent to participate in the study was obtained from the patient and control individuals and the study protocol conforms to the ethical guidelines of the 2013 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. The study included 46 CABG patients (female = 26 and male = 20) and 48 healthy volunteers (female = 32 and male = 16) who applied to the University Research and Practice Hospital, Department of Cardiovascular Surgery. The average age of healthy individuals is 62.6±11.2, and the mean age of individuals with CABG was 42.4±11.2. The duration of intensive care and post-op hospital stay, weight, the number of bypasses, cardiopulmonary bypass, and cross-clamp times of the patients were recorded. The criteria for inclusion of volunteers in the patient group; Male and female patients aged 18-65 years, diagnosed with coronary artery disease, at risk of CAD, and undergoing CABG. Inclusion criteria of healthy volunteers in the control group; Healthy males and females between the ages of 18-65, who do not have diseases such as coronary artery disease, hypertension, diabetes, and kidney failure.

Plasma Sampling

5 ml peripheral blood samples were obtained from healthy controls and patients requiring cardiopulmonary bypass operation before the operation (pre-op), 1 hour after the operation (post-op 1), and 24 hours after the operation (post-op 24). Blood samples collected in tubes with 7.5% EDTA and gel tubes were immediately centrifuged at 2000xrpm for 15 minutes for RNA isolation and biochemical analysis (LDH, BUN, glucose, troponin I, CPK, CK-MB). The 2 ml supernatant was taken into Eppendorf tubes (coded for each patient) and stored at –80 °C until RNA was obtained.

miRNA Expression Analysis

MiRNAs that are effective in vivo and in vitro cardiac processes were investigated bioinformatically and selected according to their match scores. Importantly, a new miRNA target and pathway has been proposed that was not previously demonstrated in bioinformatics analyses. Total RNAs were obtained according to the commercial miRNA isolation kit protocol (Qiagen, miRNeasy Mini Kit, 217004). Cell-miR-39 (Qiagen, MS00080247) was used as an internal control (for the isolation stage) to quantify the expression values of miRNAs (by adding the spike-in before). 2-ΔΔCt method was used to calculate the fold change (Fc) of gene expression between the patient and control group (7).

Complementary DNA (cDNA) Reactions for Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase reactions were performed according to the kit protocol. The mixture prepared in a total volume of 280 µl (for 69 samples + 1 positive sample) contained 112 l 5xmiScript Hispec buffer, 56 µl 10xmiScript Nucleics mix, 56 µl DNase-Rnase free water, and 56 µl transcriptase mix (Qiagen, 205311). The reaction mixture was dispensed into the pico plate as 48 µl and then, 3.5 µl of cDNA followed by 3.5 µl of RNA were added to each well. Finally, thermal cycling conditions were incubated at 37°C for 60 min and 95°C for 5 min, and total cDNA was obtained. Expressions were performed for *hsa-miR-21-5p* (Qiagen, MS00009079), *hsa-miR-181a-5p* (Qiagen, MS00008827), *hsa-miR-199a-5p* (Qiagen, MS00006741), *hsa-miR-199b-5p* (Qiagen, MS00006741), and *hsa-miR-320a-5p* (Qiagen, MS00014707) relative to the endogenous control miRTC (miRNA reverse transcription control, Qiagen miScript II RT Kit, 218161).

Dynamic Array

Dynamic arrays were prepared in 6 steps: 1- PRIME the dynamic array, 2- Assay plate preparation, 3- Preparation of samples, 4- Loading sample into a dynamic array, 5- Array loading to Dynamic Array in IFC, 6- Analysis of Dynamic array in Biomark (BioMark™ 96.96 Dynamic Array). After chip priming, samples premixed with master mix were pipetted into separate sample inlets of the dynamic array, and then assay plates were prepared. The reaction mixture was prepared in a total volume of 25 µl with 2.5 µl of primer (100 µM), 3.8 µl of nuclease-free water, 6.25 µl of microfluid universal primer, 13 µl of 2xAssay Loading Reagent. The samples were prepared for RT-PCR. The reaction mixture was prepared as 255 µl qPCR Master Mix, 25.5 µl 20xDNA Binding Dye, 59.5 µl nuclease-free water in a total volume of 340 l. The reactions were placed in the Dynamic Array IFC controller and analyzed using a gene expression program and SybrGreen probe technology. Thermal cycling conditions were set as follows: 2 min at 50°C, 30 min at 70°C, 10 min at 25°C for the thermal mix step, 10 min at 95 °C for starting temperature, 15 sec at 94 °C for

Table 1. Clinical and demographic data of CABG patients and controls

Clinical characteristics	Reference (adult)	Control (n = 48) (mean±SD)	CABG (n=46) (mean ± SD)	P– value
Age (year)	NA	42.3 ± 11.2	62.6 ± 11.2	0.001*
LDH (U/L)	140–280	192.4 ± 23.8	228.2 ± 66.0	0.001*
BUN (mg/dL)	7–20	13.8 ± 9.9	8.6 ± 8.7	0.001*
Fasting blood glucose (mg/dL)	70–100	94.7 ± 9.9	100.3 ± 8.7	0.002*

* P-values <0.05 are indicated in bold. CABG; coronary artery bypass grafting, LDH; lactate dehydrogenase, BUN; blood urea nitrogen, n; the number of individuals.

Table 2. Expression analysis of five miRNAs in control and pre-op (Control= 48, CABG = 46)

miRNAs	Groups	*Mean ΔCt*	*SD*	*P-value*
miR-21-5p	Control	2.4	1.4	**0.001***
	pre-op	3.7	2.4	
miR-181a-5p	Control	9.1	2.2	**0.001***
	pre-op	11.6	3.5	
miR-199a-5p	Control	7.6	2.5	**0.001***
	pre-op	11.0	3.7	
miR-199b-5p	Control	8.2	1.6	**0.001***
	pre-op	10.6	3.5	
miR-320a-5p	Control	2.0	1.6	**0.001***
	pre-op	3.6	3.1	

* P-values <0.05 are indicated in bold. Pre-op; before from operation, post-op; after from operation. SD; Standart Deviation

denaturation, 30 sec at 55 °C for bonding, 30 sec at 70 °C for elongation, and finally between 60-95 °C for melting temperature.

Receiver Operating Characteristic (ROC) Curve Analysis

The area under the ROC curve (AUC) determines the accuracy of the test in distinguishing between patients and non-patients. The potent miRNA/miRNAs were determined by determining the sensitivity, specificity, and AUC values of five miRNAs before and after the operation.

Bioinformatic Analysis

miRNA::target gene analysis was performed to determine the miRNA target genes and the pathways. The miRDB database (8) and DIANA TOOLS (9) were used for specific targets and matching scores of miRNAs. Genes with a match score of 80% and above were selected for further analysis. Target Scan database was used to detect conserved sequences between miRNA and mRNA (10, 11). Gene ontology (GO) analyses were performed with miR2GO (12). The Reactome database was used for pathways (13, 14). Finally, the proteins with which these genes interact were analyzed for their specific effects (STRING v11.0) (15).

Statistical Analysis

Compliance of numerical data between patient and control with normal distribution was tested with the Shapiro Wilk test. The Mann-Whitney U test was used to compare the variables that were not normally distributed in the two groups. In investigating the regulation of five miRNAs for control, pre-op and post-op patient groups; Repeated measures analysis of variance, LSD multiple comparison tests, Friedman 2-way analysis of variance, and all pairwise multiple comparison tests were used for normally distributed and non-normally distributed variables. The relationship between cardiac processes and miRNA regulation was determined by the Spearman correlation coefficient. In the analysis, using the SPSS package program (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) (16), it was considered significant when $p < 0.05$.

Main Points:

- MicroRNA-199a-5p is strongly associated with ischemia/reperfusion.
- In CABG patients, miR-21-5p levels may vary in correlation with cardiac markers.
- miR-199a-5p can be evaluated as a marker in determining the severity of coronary artery disease.
- miR-320a, miR-199b-5p, and miR-181a-5p levels may have a role in activating inflammation-related pathways involved in the pathogenesis of coronary artery disease.

RESULTS

Analysis of Demographic, Clinical, and Operation Data

LDH was found to be significant in patients as an expected outcome ($p < 0.05$). Serum BUN levels are significant ($p < 0.05$). The plasma glucose levels were also found to be high in our patient group ($p < 0.05$) (Table 1). In patients, intensive care duration (hours); 49.9±8.8, post-op hospital duration (days); 5.5±0.8, weight (kg); 5.5±0.8, cardiopulmonary bypass duration (min); 54.174 ± 9.481, aortic cross-clamp duration (min); 37.7±7.4, number of bypasses; 3.3±0.9 were determined. No relationship was found between miRNA expressions and demographic and clinical data.

Evaluation of Parameters in Cardiac Processes

Troponin I and CK-MB were found to be statistically significant in the analysis of cardiac parameters in the pre-op patient and control groups (Table 2). The mean values for troponin 1 and CK-MB were higher in the patient group than in the controls ($p = 0.001$).

Table 3. Pre-op vs control cardiac parameters

Cardiac parameters	Groups	n	Mean±SD	P- value
Troponin I	Control	48	0.02±0.1	0.001*
	Pre-op	46	0.9±2.8	
CPK	Control	48	79.1±40.3	0.812
	Pre-op	46	88.0±57.1	
CK-MB	Control	48	18.1±4.7	0.001*
	Pre-op	46	27.9±17.6	

* P-values <0.05 are indicated in bold. CPK; creatine phosphokinase, CK-MB; creatine Kinase myocardial band, n; the number of individuals.

Table 4. miRNA expression analysis in between the operation groups (CABG = 46)

miRNAs	Groups	Mean±SD	P-value
miR-21-5p	Pre-op	3.7±2.4	**0.001***
	Post-op 1	3.9±2.6	**0.001***
	Post-op 24	3.9±2.3	**0.001***
miR-181a-5p	Pre-op	11.6±3.5	**0.001***
	Post-op 1	10.4±3.6	**0.001***
	Post-op 24	10.7±3.4	**0.005***
miR-199a-5p	Pre-op	11.0±3.7	**0.001***
	Post-op 1	10.5±3.8	**0.001***
	Post-op 24	11.7±3.4	**0.001***
miR-199b-5p	Pre-op	10.6±3.5	**0.001***
	Post-op 1	10.3±3.3	**0.001***
	Post-op 24	10.8±3.2	**0.001***
miR-320a-5p	Pre-op	3.6±3.1	**0.004***
	Post-op 1	3.9±3.6	**0.002***
	Post-op 24	3.6±3.2	**0.004***

* P-values <0.05 are indicated in bold. Significant according to the Mann-Whitney u test.

CPK, which is defined by the increase in its activity at 4-6 hours after MI, is evaluated together with the increase in CK-MB. It is a maximum of 10-20 hours after chest pain. Although CPK was higher than controls, it was not significant in the analysis.

In univariate and multivariate analysis of pre-op and post-op cardiac parameters, it was observed that troponin I was significantly higher in post-op 1 (14.5±69.03) (p = 0.001) than pre-op (0.9±2.8) and post-op 24 (4.6±7.7). Troponin was detected significant in multiple analyses (pre-op vs post-op 24, p = 0.001; pre-op vs post-op 1, p = 0.001) except for post-op 24 vs post-op (p = 0.348). In the comparison of CPK values, an increase was observed in post-op 1 (429.1±335.2) and post-op 24 (451.1±253.7) compared to pre-op (88.0±57.1) (p = 0.001). In multiple analysis, it was found to be statistically significant according to pre-op/post-op 24 (p = 0.001), pre-op/post-op 1 (p = 0.001), but not significant compared to post-op 24/post-op 1 (p = 0.835). CK-MB values were found to be statistically significant in post-op 1 (63.4±64.5) compared to both pre-op (27.9±17.6) and post-op 24 (55.5±52.0) (p < 0.05). CK-MB values were found to be significant in the comparative analysis of the groups. A distinction can be made between ejection fraction (EF) and systolic-diastolic heart failure, how much the heart contracts with each beat. The normal EF is between 50-70 %. In our patient group, this value is below normal. LVEF analysis showed an increase in post-op 1 (56.39.0) and post-op 24 (56.4±9.0) compared to pre-op (55.6±9.5) and were not significant. LVEF values below the normal level in pre-o started to increase in post-op 1 and showed improvement in post-op 24.

miRNA Expression Analysis

Expression analyzes of five miRNAs in the patient-control group are given as averages using delta Ct values. Five miRNAs were found to be downregulated in pre-op in CBAG patients diagnosed with MI (p < 0.05) (Table 3). In analyzes of each miRNA expression in pre-op and post-op, five miRNAs were significant in post-op 1 and post-op 24 compared to pre-op (p < 0.05) (Table 4)

Correlations Between Cardiac Markers and miRNA Expressions

miR-21-5p

In comparative analysis, there was a correlation between pre-op miR-21-5p expression levels and pre-op troponin values (r=0.34, p=0.01), and a negative correlation between troponin values measured at post-op 24 and post-op 1 (r= -0.38, p=0.00). When evaluated before and after the operation, there is a relationship between increased troponin I levels (95% CI=-10.9-24.2, SD=7.1, SE = 4.1) and upregulation of miR-21 (95% CI=3.6-4.0, SD=0.07, SE = 0.04) (p < 0.001). CPK (95% CI=-183,05-828.5, SD=203.6, SE = 117.6) and miR-21-5p levels increased to post-op 1 and post-op 24 (p < 0.001). LVEF was low in post-op 1 and higher in post-op 24 (95% CI=55.1-57.1, SD=0.4, SE=0.2), while miR-21-5p (95% CI=3.6-4.0, SD=0.07, SE=0.04) was upregulated but not statistically significant (p = 0.06). A statistical significance was determined between the expression of CK-MB and miR-21 (p < 0.001). CK-MB is high in post-op 1, low in post-op 24 (95% CI=2.6-95.2, SD=18.6, SE=10.8), and miR-21-5p is upregulated.

miR-181

In the patient group, there was a negative correlation between miR-181 values measured pre-op and cardiac troponin values measured 1 hour and 24 hours later (r= -0.3, p=0.02; r= -0.3, p=0.01). Troponin I level is high in post-op 1, miR-181a is downregulated (95% CI=-10.9-24.2, SD=7.1, SE=4.1), troponin I is low in post-op 24, and vice versa, miR-181 is upregulated (95% CI=9.3-12.4, SD=0.6, SE=0.4) (p = 0.01). A statistically significant difference was found between miR-181 (p < 0.001) and CPK levels. No correlation was detected between miR-181 and EF (p = 0.596). A correlation was found between post-op 1 of miR-181a and pre-op, post-op 1 of CK-MB (r=0.3, p=0.03; r=0.3, p=0.03) (Table 5). CK-MB is high in post-op 1, low in post-op 24 (95% CI=2.6-95.2, SD=18.6, SE=10.8), and vice versa in miR-181 (p = 0.002).

miR-199a-5p

In the patient group, there was a moderate negative correlation between miR-199a-5p values 24 hours after the operation and

Table 5. Comparative analysis of Troponin I and CK-MB for miR-21-5p (CABG = 46)

			Troponin I			CK-MB		
			Pre-op	Post-op 1	Post-op 24	Pre-op	Post-op 1	Post-op 24
miR-21-5p	Pre-op	r	0.3	0.0	0.0	0.3	0.0	-0.2
		P-value	**0.02***	0.64	0.68	0.09	0.96	0.29
	Post-op 1	r	0.1	-0.2	-0.1	0.2	0.0	-0.3
		P-value	0.33	0.23	0.57	0.12	0.76	0.08
	Post-op 24	r	0.1	-0.4	-0.1	0.1	0.1	0.0
		P-value	0.49	**0.01***	0.59	0.53	0.60	0.83
miR-181a-5p	Pre-op	r	0.2	-0.05	0.1	0.1	0.0	-0.05
		P-value	0.20	0.72	0.68	0.71	0.99	0.72
	Post-op 1	r	0.1	-0.3	0.0	0.3	0.3	0.0
		P-value	0.3	0.03	0.93	**0.04***	**0.03***	0.62
	Post-op 24	r	-0.0	-0,4	-0.1	0.0	0.2	-0.1
		P-value	0.92	**0.01***	0.70	0.94	0.23	0.69
miR-199a-5p	Pre-op	r	0.2	0.0	0.1	-0.1	0.0	-0.1
		P-value	0.15	0.63	0.41	0.74	0.81	0.38
	Post-op 1	r	0.1	0.0	-0.03	-0.0	0.2	-0.03
		P-value	0.44	0.61	0.82	0.87	0.18	0.81
	Post-op 24	r	-0.0	-0.5	-0.2	-0.1	-0.05	-0.2
		P-value	0.14	**0.00***	0.15	0.36	0.74	0.24
miR-199b-5p	Pre-op	r	0.1	0.0	0.0	-0.04	-0.05	-0.2
		P-value	0.37	0.92	0.75	0.77	0.80	0.20
	Post-op 1	r	0.2	0.0	0.1	0.0	0.2	-0.1
		P-value	0.12	0.92	0.75	0.77	0.70	0.20
	Post-op 24	r	0.0	-0.3	-0.1	-0.03	0.1	-0.02
		P-value	0.77	**0.03***	0.66	0.83	0.39	0.86
miR-320a-5p	Pre-op	r	0.3	0.0	0.0	0.2	0.0	-0.2
		P-value	**0.03***	0.99	0.83	0.21	0.97	0.30
	Post-op 1	r	0.2	-0.2	-0.1	0.2	0.2	-0.1
		P-value	0.19	0.10	0.47	0.19	0.16	0.58
	Post-op 24	r	0.0	-0.2	0.0	-0.03	0.2	-0.02
		P-value	0.82	0.15	0.91	0.81	0.13	0.85

* P-values <0.05 are indicated in bold. r; correlation value

cardiac troponin values measured 1 hour after the operation (r=-0.5, p=0.001). While troponin I level increased in post-op 1, miR-199a-5p is downregulated (95% CI=-10.9-24.2, SD=7.1, SE=4.1). miR-199a-5p is upregulated while Trponin I decreases in post-op 24 (95% CI=9.5-12.6, SD=0.6, Std. Er.=0.4) (p = 0.016). Statistically significant difference was found between miR-199a-5p (p < 0.001) and CPK levels. No corelation between EF values and miR-199b-5p expression levels (p = 0.588). There is significant relationship between miR-199a-5p and CK-MB (p = 0.002). CK-MB is high in post-op 1, low in post-op 24, and miR-199a-5p is downregulated in post-op 1 and upregulated in post-op 24.

miR-199b-5p

While miR-199b-5p was associated with post-op 24 troponin levels and there is a negative correlation between groups (r=-0.3, p=0.034). miR-199b is downregulated while troponin I rises in post-op 1 (95% CI=-10.9-24.2, SD=7.1, SE=4.1). miR-199a-5p is upregulated while troponin I decrease in post-op 24 (95% CI=9.9-11.2, SD=0.3, SE=0.1) (p = 0.003). Statistically significant difference was found between miR-199b-5p (p < 0.001) and CPK. No statistical significance was found between miR-199b-5p and LVEF (p = 0.593). CK-MB is high in post-op 1, low in post-op 24, and miR-199b-5p is downregulated in post-op 1 and upregulated in post-op 24 (p < 0.001).

miR-320a

There was a positive correlation between pre-op and pre-op troponin values for miR-320a-5p in the patient group (r=0.3, p=0.031). Troponin I and miR-320a levels increase in post-op 1 (95% CI=-10.9-24.2, SD=7.1, SE=4.1) and decrease in post-op 24 (95% CI=3.2-4.1, SD=0.2, SE=0.1) (p = 0.001). Statistically significant difference was found between miR-320a and CPK levels (p < 0.001). LVEF and miR-320 expressions are not related (p = 0.344). There is no significant relationship between miR-320a-5p expression and CK-MB values (Table 5).

Table 6. miRNA target genes according to database analysis

hsa-miR-21-5p	*YOD1, PRDM11, FASLG, ZNF367, VCL, SKP2, TGFBI, PLAG1, IL12A, **CREBRF***, RAB6D, KRIT1, PELI1, RBPJ, RALGPS2, ADGRG2, GATAD2B, PBRM1, SCML2, RSAD2, PPP1R3B, PLEKHA1, FGF18, SPRY1, FAM13A, GPATCH2L, STAT3, BCL7A, SKI, YAP1, MALT1, ZBTB41, KLF3, MBNL3, CCL1, NKIRAS1, TIAM1, OSR1, PAN3, KDM7A, CASKIN1, PDCD4, GID4, HSD17B4, MAP3K1, PDZD2, UBE2D3, AKAP12, CPEB3, RECK, CCL20, PPP1R3A, NTF3, TIMP3, ANGPTL5, BCL11B, JAG1, FAM3C, MEI4, EPM2A, SLC30A10, BTG2, SYT15, MPRIP, NFIA, KLHL15, CFAP300, GLCCI1, SPRY2, LRRC57, STAG2, KDM1B, GRAMD2B, RMND5A, C7, ALX4, RASA1, SOX5, RNF103, GLIS2, NEGR1, ARHGAP24, NIPAL1, LTV1, ANKS1B, TMEM170A, HIPK3, ELF2, EPHA4, PPP1R3D, **ZNF704***, RAD51AP1, CLDN8, EHD1, ATXN10, MCMDC2, ITCH, MATN2, NPPB, RASA2, CLIC2, SMARCD1, OLFM3, USP15, MAST4, KCNJ10, NIPAL2, PCSK6, TPRG1L, WWP1, ARL1, LPA, GABRB2, CSRNP3, STK40, NFIB, UBR3, CHIC1, CUX1, RASGRP1, LATS1, FDX1, MINDY2, BCL11A, SPEF2, PITX2, SMAD7, PLAA, UBE2D1, THRB, BAHD1, MED21, FRMD3, RP2, TENT5A, KHDC1, CDH7, DLGAP1, RBMS3, KHDC1L, ZDHHC17, BEST3, STK38L, MSH2, KLF6, PTPN20, CNOT6, SOS2, DUSP8, KBTBD6*
hsa-miR-181a-5p	***CREBRF***, C2CD5, ZNF594, ZNF268, ZNF439, DDX3X, ZNF781, PRTG, TRIM2, SESN3, TNPO1, ZFP90, ZNF37A, ZNF780B, KMT2A, FIGN, ZFP36L2, S1PR1, OSBPL3, ZNF780A, PDE5A, DMXL2, GLS, ZIC3, ZNF844, PPIP5K2, PROX1, BEND3, CLIP1, NWD2, LGALSL, ACVR2B, TENT4B, NEXMIF, ZNF559, REPS2, TRIM71, GPD1L, RNF217, SPRY4, TBC1D1, CBX7, KMT2C, ARF6, PLCL2, GABRA1, SPP1, PTBP3, NEK7, ZNF426, AP1G1, KCNQ5, CDYL, IL2, CDON, RLF, SSX2IP, TMEM94, LARP4, TADA2B, BTBD3, SCD, ADAM11, CEP97, GIGYF1, ZNF597, SIPA1L2, POU2F1, **ZNF704***, INO80D, UBE2B, ST8SIA4, TMEFF1, MARK1, ZNF440, ZNF302, ATP2B1, DNAJC13, SPIRE1, NELFA, PITPNB, ZFP36L1, SLC4A10, SLC25A37, DOCK4, TMEM87B, ARSJ, CXCL9, MB21D2, ARHGEF3, MTF2, PCDHA1, PLAG1, PNRC2, ZFP14, AKIRIN1, MFSD6, TRAK1, LMO1, KATNBL1, OSBPL8, GPD2, ZBTB4, ABHD18, TGFBR1, PHF20L1, TOM1L1, LATS1, UBP1, ANKRD44, TCERG1, SIK3, LIN28B, HOXC8, CPNE2, THRB, ADCY9, KIF3A, PPFIA1, KAT2B, PCDHA8, PCDHAC1, HIPK3, ZNF800, HIC2, ATXN3, BIRC6, ZNF584, ZNF468, GSKIP, NOVA1, PCDHAC2, PCDHA13, GATA6, SOWAHA, PCDHA10, PCDHA5, CPD, PCDHA6, CREB1, CTTNBP2NL, PCDHA11, PCDHA3, OOSP2, TCF7L2, RALGAPB, MGAT2, RBBP7, NUS1, TGFBRAP1, PPP3R1, PCDHA4, PRLR, SLAIN2, BRD1, NAALADL2, PCDHA7, PCDHA2, ZNF124, ZNF700, PAX9, PCDHA12, LCLAT1, USP42, PI4K2B, ATM, PEAK1, SALL4, EPHA4, PIAS1, CLASP1, NMBR, TMEM64, KIF1B, KCNH1, E2F5, PSPC1, CHIC1, BHLHE40, FNDC3B, ATXN7, TXNDC12, FUT9, TOGARAM1, WDR82, ADGRB3, KIF3B, KLHL2, G3BP2, OXGR1, PER3, SEC24C, AKT3, NOTCH2, AMER2, GSE1, PRKCD, KLHL29, EPC2, MIER3, IPO8, IL1A, SLITRK1, MAMDC2, ZNF479, ACVR2A, ZNF773, FAM126B, DCBLD2, ELMSAN1, IQCJ-SCHIP1, PAX5, NIPAL4, PHTF2, HMBS, PARP11, ZNF189, SEMA4G, YTHDC2, ZIC2, PCDHA9, C2orf69, KLF6, ZNF266, AFG3L2, EYS, TPRX1, KLF15, BCLAF1, SLC7A11, UBE2D3, BAZ2B, ATP2B3, CNTN4, LYRM1, NCOA2, FAM122B, HSP90B1, RFC1, ONECUT2, SS18L1, PRRC2C, ZNF140, USP33, HRH1, MBOAT2, TMEM144, EXOC5, SYT16, TMEM165, CRIM1, HEY2, ETNK1, ETS1, NR1D2, PAFAH1B2, NR6A1, CLVS1, ZBTB43, PKNOX2, CHMP1B, MAN2A1, LIN28A, ACER3, KPNB1, ADARB1, SAMHD1, SLC35F3, FAM3C, CARM1, GPBP1, KRBOX4, RAI1, CTDSPL, FOXP1, TRDMT1, IGF2BP2, GTSE1, SERTAD2, CBFA2T3, FGD4, RAB3IP, CDKN3, B4GALT1, RNF34, ATP1B1, LRBA, ATP2B2, DNAJC3, SMAP1, CNKSR2, BCL2, SLC2A3, IPMK, RPS6KB1, CCNJ, ZDHHC7, DYNC2H1, CALCR, RPS6KA3, CNKSR3, MYBL1, OTUD4, ENTPD6, LIMCH1, AP1S3, RASSF2, FSBP, SRSF7, LIG4, SPECC1L, ZFP1, CPOX, TTC39B, AGO4, CDC73, EN1, MPP5, QKI, NLN, KIAA1549L, BCL2L11, NCALD, KDM5A, ETV6, ZNF563, RORA, NIPBL, ST6GALNAC5, AHCTF1, PRR27, BLOC1S6, HAO1, GOLGA1, HOXD1, GHITM, TBC1D4, ZNF586, ZNRF2, TNFRSF11B, NSUN7, PDE3A, GOLGA8M, BRAP, PCSK1, LNPK, ZBTB2, KLHL5, NKAIN2, RAB30, DUSP6, HOXA1, UNC80, NR2C2, MUC22, ENAH, YLPM1, RAD54B, PAK5, PDAP1, ARMH4, LOX, SACM1L, FNDC3A, SLC4A8, STARD4, POLQ, DARS, CPEB4, ZFP62, FAM160A1, SIN3B, JARID2, RIMKLB, ADAMTS18, PNISR, GCC2, PAWR, ACSL4, OGFRL1, MS4A1, ZEB2, TMLHE, ARMC8, ZDHHC17, DCN, MEGF9, SLC5A9, LPCAT2, TREML4, VPS41, ESR1, BOLL, RNF169, KCNA1, ZNF527, ABTB2, TNF, ADAMTS6, GRB10, N4BP2, ATP8B2, CNOT2, LRRN1, SLC12A5, KCNJ10, TNFSF4, OTOGL, SCHIP1, PHACTR4, PPP2R3A, AASDHPPT, FBXO34, KANK1, PARM1, PBX3, DCLK1, FSD1L, GPRIN3, TBC1D14, MICU3, SLC25A36, UBL3, PHLDA1, MBNL2, ZDHHC3, FAM135A, TNS1, ZFAND6, APOO, CCNDBP1, GOLGA8J, SYNPR, APBA1, GOLGA8H, FNIP2, NOTCH4, SEC24A, LAMA1, SIX4, TM9SF4, MLXIP, GRM5, KPNA1, ASPH, TAB3, RASSF8, CCNK, GPR137C, ZNF544, FAM19A2, LEMD3, ATP2A2, SLC38A11, TAOK1, OSBPL2, CCL8, RLIM, GOLGA8R, RAB8B, MTPN, MAPK1IP1L, DERL1, PSG11, MTX3, TNRC6B, GOLGA8K, SRGAP2, BAG4, ACAP2, SSB, COX15, TBPL1, CDK17, FKBP1A, DNAJA4, ZFAND4, NR4A3, RAD21, MUC7, PTPDC1, CECR2, ADAMTS5, RAB3GAP1, RASSF1, EYA3, BMP2K, HECA, IKZF5, TESMIN, GOLGA8O, MAP2K1, CALM1, TBCEL, GOLGA8N, ITGA3, ASAH2B, RECK, NAA50, TRDN, ATXN1, NLK, DLG2, MYCBP2, ADO, ATMIN, XPO7, AKIRIN2, PHIP, PAPOLG, RAB3C, CA8, SH2B3, ASTN1, ANKRD13C, PRDM4, UNC5D, GOLGA6L4, ARL5A, E2F7, MCC, IPO7, CDH8, RYR3, AKAP6, GRIK2, LCTL, CCP110, JADE2, AP1AR, BRWD1, SELENOT, C1orf109, LRRC8D, NAB1, STXBP5, CHD1, AK9, ANO1, ESM1, DEPTOR, KLHL42, UBE2D1, DEK, CDC40, TMED4, PLAU, CFAP161, ADCY1, HEATR3, PLEKHJ1, PTPN4, AGFG1, NPEPPS, IGDCC3, CHMP2B, GOT2, QSER1, CPSF6, PTPN22, LPCAT1, PHF3, TAB2, ZNF121, TCFL5, IRS2, YTHDF3, PDGFRA, NAP1L1, SLC35E1, NUCKS1, MTURN, PNMA2, NRXN1, TNFAIP1, HOXA11, C14orf28, MORC3, UBE3C, DDX60L, CDC5L, WNK1, PAM, MYO1E, FBXO33, RNF182, MAGOHB, ADRA1A, ZNF44, TMEM131, MED26, CD69, PIK3R3, TMF1, PSRC1, MBTPS2, SLC10A7, GALNT4, RIOX2, WSB1, MAP3K3, BACH2, MAB21L3, IL25, ZNF823, CAMSAP2, DISC1, ETFBKMT, MSANTD3-TMEFF1, CBLB, HCN2, POC1B-GALNT4*

hsa-miR-199a-5p	*DDR1, SLC25A23, NAA40, ZNF763, MYRF, CLCN3, SLC24A3, CELSR1, LIN7C, HAPLN1, FZD4, MAP3K11, RAD23B, GCNT2, SULF1, AKAP1, KL, ARHGAP12, ZNF189, CDCA7L, FAM222B, GPR63, ZNF773, RASSF2, MICAL3, ZNF426, BCAM, ZNF439, MARCH8, ZBTB20, GNG5, ZNF544, ITGA3, ZNF440, MAB21L1, TSPAN6, ARHGAP21, GPRC5A, **ZNF704***, ZFYVE27, RBM47, ECE1, TUBG1, ZNF516, PAXBP1, SIRT1, ZFAND4, NSG1, CLIP1, ACVR2B, WAPL, VPS26A, M6PR, PDPN, HMCN1, NFIL3, NPAS2, FER, ANK3, SHOC2, CCNL1, KPNA4, TPR, ASRGL1, SOS2, AUTS2, CCDC88C, FZD6, ZNF563, PLXNA2, PPARGC1A, TXNL1, POU3F2, FLRT3, SORCS3, INO80D, BTRC, CSDC2, TMEM245, RASSF3, NINL, LXN, AGAP1, ZNF418, GPR89A, HSPA5, TAB3, ZNF589, PIK3CD, ZNF479, PLEKHF2, ABHD17C, ZNF776, ATG14, P3H2, UBAP1, CYP51A1, GSK3B, ATP13A2, SACS, ZNF584, BICC1, TGFB2, SMARCD1, CECR2, ATXN7, SUN1, FAM19A2, HSPA12A, AP1G1, CCDC120, RALGAPA1, ANKRD23, KLHL29, MINDY3, IPO8, ZNF468, **CREBRF***, SLF2, FAM126B, WDR76, NLK, ZNF329, RFX3, ITGA8, USP31, RGMA, NTNG1, TAF9B, SERPINE1, MARCH7, NCSTN, CELF2, EIF5B, PLXND1, ADD3, ZFP90, ZNF559, PAN3, KDM3B, DENND6A, PPFIBP1, CLEC2D, RNF38, HIF1A, GPR89B, CCNJ, TMEM220, PAX3, ABCA1, BEND3, MGAT3, CRYBG3, KIAA1109, RNF11, ETS1, GIT1, ZNF579, ARF6, ZNF559-ZNF177, FBXO4, IL36B, ARRB2, CCDC43, ZNF195, MGAT4B, CEP350, RBPMS, NUDT5, RAB7A, PDE4D, PODXL, TST, PI4KA, USPL1, CACUL1, COL5A3, AFTPH, RBM24, YIPF6, TMEM135, CACNB2, GAS2L2, NPY2R, MPP5, RCSD1, ZNF547, TVP23C-CDRT4, WDTC*
hsa-miR-199b-5p	*DDR1, SLC25A23, NAA40, MYRF, CLCN3, SLC24A3, CELSR1, ZNF763, LIN7C, HAPLN1, FZD4, MAP3K11, RAD23B, GCNT2, SULF1, AKAP1, ZNF773, ARHGAP12, ZNF189, CDCA7L, GPR63, RASSF2, KL, MICAL3, ZNF426, BCAM, ZNF439, MARCH8, ZBTB20, GNG5, ZNF544, ITGA3, FAM222B, ZNF440, MAB21L1, TSPAN6, ARHGAP21, GPRC5A, **ZNF704***, ZFYVE27, RBM47, ECE1, TUBG1, ZNF516, PAXBP1, SIRT1, ZFAND4, NSG1, CLIP1, ACVR2B, WAPL, VPS26A, M6PR, PDPN, HMCN1, NFIL3, NPAS2, FER, ANK3, SHOC2, CCNL1, KPNA4, UBAP1, TPR, ASRGL1, SOS2, AUTS2, CCDC88C, FZD6, ZNF563, PLXNA2, PPARGC1A, TXNL1, POU3F2, FLRT3, SORCS3, INO80D, BTRC, CSDC2, TMEM245, RASSF3, NINL, LXN, AGAP1, ZNF418, GPR89A, HSPA5, TAB3, ZNF589, PIK3CD, ZNF479, PLEKHF2, ABHD17C, ZNF776, ATG14, P3H2, CYP51A1, GSK3B, ATP13A2, SACS, ZNF584, BICC1, TGFB2, SMARCD1, CECR2, ATXN7, SUN1, FAM19A2, HSPA12A, AP1G1, CCDC120, RALGAPA1, ANKRD23, KLHL29, MINDY3, IPO8, ZNF468, **CREBRF***, SLF2, FAM126B, WDR76, NLK, ZNF329, RFX3, ITGA8, USP31, RGMA, NTNG1, TAF9B, SERPINE1, MARCH7, NCSTN, CELF2, EIF5B, PLXND1, ADD3, ZFP90, ZNF559, PAN3, KDM3B, DENND6A, PPFIBP1, CLEC2D, RNF38, HIF1A, GPR89B, CCNJ, TMEM220, PAX3, ABCA1, BEND3, MGAT3, CRYBG3, KIAA1109, RNF11, ETS1, GIT1, ZNF579, ARF6, ZNF559-ZNF177, FBXO4, IL36B, ARRB2, CCDC43, ZNF195, MGAT4B, CEP350, RBPMS, NUDT5, RAB7A, PDE4D, TST, PI4KA, USPL1, CACUL1, COL5A3, AFTPH, RBM24, YIPF6, TMEM135, CACNB2, GAS2L2, NPY2R, MPP5, RCSD1, TVP23C-CDRT4*
hsa-miR-320	*ZC3H12B, RIMKLB, CLCN3, DUSP18, SOX4, KCNB1, TMEM178B, GFRA1, TNRC6B, LMLN, MAP3K9, HOXA10, RAB29, CHRM2, CCR5, ANKRD13C, PTGER4, SSTR3, RASSF5, ZSWIM6, TTC7B, MON2, MANEAL, UBR3, ANKRD37, SMARCD1, CNDP2, POU2F1, FBXW11, **ZNF704***, ZSLC16A4, CD300E, NR6A1, SDK1, XG, TIA1, MMP2, RAD54L2, NAV1, RORA, NIPBL, KIAA1211L, NFASC, PARM1, ERICH5, CENPA, CACNA1E, UGGT2, LOC403312, ERBB4, FXYD3, XKR7, TNS1, MPDZ, TNFRSF19, TBK1, TRAF3, SV2B, ARMC2, NMT1, VAPB, AGAP1, GAREM1, PRICKLE2, LARP1, TMEM267, ABCC4, SRP19, FGF13, SLC9A7, RPL36A-HNRNPH2, PAX2, AFTPH, XYLT1, SH3TC2, SNED1, RFX7, RAPGEF4, STAT5B, HTR5A, ATL3, NMD3, EEF1A1, TAOK1, COG3, ZNF366, HAO1, FABP7, SORCS1, CLMP, ZNF287, PPBP, NEUROG2, PRDM16, AAK1, KDM3B, GRAMD1B, INSR, CREB5, TRIM9, NFATC2, FAM84B, CENPBD1, PKNOX2, MCM6, C6orf136, **CREBRF***, SMCR8, C1orf115, CA8, ST8SIA5, WARS2, ETS1, RIDA, ZSWIM1, NUDT15, ST3GAL3, STK35, TMPRSS15, INTS14*

* The *ZNF704* and *CREBRF* genes are common targets for the five miRNAs.

ROC Curve Analysis of Five miRNAs

miR-21-5p (AUC=0.77, CI=0.68-0.86), miR-181a-5p (AUC=0.78, CI=0.69-0.86) and miR-320a-5p (AUC=0.78, CI=0.69-0.86) were determined as weak, miR-199a-5p (AUC=0.81, CI=0.71-0.88) and miR-199b-5p (AUC=0.80, CI=0.72-0.88) were determined as good (control vs pre-op).

In Silico Analysis for miRNA Targets and Pathways

Target genes with a matching score between 80-100% were selected. We analysed 469 genes for miR-21-5p, 1408 genes for miR-181a-5p, 562 genes for miR-199a-5p, 556 genes for miR-199b-5p and 607 genes were analysed for miR-320a-5p (Table 6). In comparisons for miRNA target genes, CREB3 Regulatory Factor (CREBRF) and Zinc Finger Protein 704 (ZNF704) genes targeted by all five miRNAs were detected (Table 7). The association of target genes with miRNAs was analyzed by gene ontology (GO). Parameters used in this association with miRpath 3.0; micro T-CDS (v5.0) was chosen as p-value threshold = 0.05, MicroT threshold = 0.8. In the analysis of miRNA pathways, we determined that CREBRF is responsible for the metabolism of proteins, as a CREB3 factor activating gene in the formation of unfolded protein response, and it binds to CREB. ZNF704 shows DNA binding transcription factor activity. The proteins they work with were identified using the protein interaction database (STRING V.11.00). According to these results; CREBRF is associated with CREB3 and ZNF704 with TRIM28 for its potential effects on CABG.

DISCUSSION

In this study, cardiac parameters and miRNA expressions (miR-21-5p, miR- 199a-5p, miR-199b-5p, miR-181a-5p ve miR-320a-5p) were investigated in the I/R status of patients who underwent CABG. Surgery was compared in plasma samples with healthy controls, as well as between preoperative, postoperative, and intraoperative hours. The target genes of miRNAs and the pathways

Table 7. miRNA::target mRNA matching specificity data

miRNAs	Target mRNA	Target end	Site type	Position (nucleotide)
miR-21-5p	CREBF	3'-UTR	7mer	1221-1227
	ZNF704	3'-UTR	7mer	354-360
miR-181a-5p	CREBF	3'-UTR	8mer	736-743
	ZNF704	3'-UTR	8mer	4357-4364
			8mer	11162-11169
			7mer	12207-12213
miR-199a-5p	CREBF	3'-UTR	7mer	**3259-3266***
	ZNF704	3'-UTR	7mer	**10021-10027***
			7mer	**12361-12367***
			7mer	**12572-12578***
miR-199b-5p	CREBF	3'-UTR	8mer	**3259-3266***
	ZNF704	3'-UTR	7mer	**10021-10027***
			7mer	**12361-12367***
			7mer	**12572-12578***
miR-320a-5p	CREBF	3'-UTR	8mer	2831-2838
	ZNF704	3'-UTR	7mer	9279-9286

*The same target position of miR-199a-5p and miR-199b-5p (they are miRNAs belonging to the miR-199a/b family strengthens the target similarity) for *CREBRF* and *ZNF704* genes.

in which these genes are functional in CABG were determined by bioinformatic analyses. These analyses aimed to investigate the relationship between clock-related blood values of cardiac parameters and miRNA expressions, determine their behavior in CABG, their contribution to I/R, and cardiac remodeling, and reveal the potential for early diagnosis in these patients with MI.

miRNAs overexpressed in the cardiovascular system, such as miR-16 and miR-499, have been suggested as markers in CAD (17). Targeting the 3'-UTR of PTEN, miR-21 is functional in I/R. While these in vitro studies showed negative regulation of miR-21 in the I/R, in in vivo studies miR-21 is down-regulated in the ischemic region (18). miR-21 was also downregulated in our group. Differently, in the analyzes in which we determined the operation status, it is upregulated in the first 1 hour and the next 24 hours after the operation. Post-operative analyzes in plasma show that miR-21 increases with time and can be detected 24 hours after the operation, therefore, it may be related to cardiac markers in this process both time and expression level. We suggest that the expression can be used for prognosis and perioperative biomarker even after the operation.

The significant increase in plasma miR-181a levels at 6 and 12 hours after the onset of MI and the correlation with CK-MB, cardiac troponin I, and ROC analyses indicate that this miRNA has diagnostic value in MI (19). In our study, miR-181a levels are downregulated. Downregulation of miR-181a in patients diagnosed with MI is likely to be associated with injury, overlapping with preoperative elevated and abnormal clinical presentation. Therefore, the stability and continuity of miR-181a in plasma and its association with troponin, CK-MB, CPK, and EF are very important for monitoring the process.

MiR-199a, which is among the cardiomyocyte-specific miRNAs, stands out with the regulation of the SIRT1 gene in heart tissue. The downregulation of this miRNA is associated with high levels of biochemical markers associated with inflammation, angiogenesis, and endothelial dysfunction that play a role in the development and progression of atherosclerosis (20, 21). Here we propose for the first time that CREBRF and ZNF704 and their downregulation of cardiac parameters may be effective in CABG. Post-op upregulation of miR-199b suggests that this miRNA may be entitled to cardiac remodeling. Bioinformatics analyzes also show that this task is done through CREBRF and ZNF704. In addition, its correlation with troponin I indicate that it can be an effective miRNA in post-op. Based on ROC analysis results, two miRNAs are potential candidates for determining postoperative prognosis.

miR-320 plays a role in the correction of I/R-induced cardiac injury (22). The closest findings to our study are the investigation of miR-34a, miR-15a, and miR-320a gene expressions before and 24 hours after surgery in plasma samples of patients undergoing cardiopulmonary bypass. In this study, it was determined that the miR-320a level increased during surgery (23). In contrast to this study, we examined I/R in CABG patients with MI. Similarly, we found high levels of miR-320 in post-op. The expression of miR-320 after I/R injury and its upregulation after bypass graft surgery show that it can be a marker that can be monitored in this process, and most importantly, it can be a treatment target in I/R injury.

As a result of our bioinformatic analyzes, we have achieved two common targets that may be effective in CABG: CREBRF and ZNF704 for miR-199a/b, and their co-operating proteins, CREB3 and TRIM28 are involved in cardiac processes in CBAG. In addition, it is functional in diagnosis, treatment, and prognosis. These

miRNAs are important tools for the management of I/R injury and their diagnostic potential in MI-diagnosed CABG.

LIMITATIONS

This is a case-control study with relatively small sample size. The molecular processes, oscillations, transports, targets of miRNAs, and the mechanisms mediating these processes have not been fully elucidated. Interventional studies are needed to detect circulating miRNAs and make them available in routine laboratories. In addition, profiling of circular and tissue-specific miRNAs will strengthen the association studies of CABG and miRNAs.

CONCLUSION

In our study, the expression levels of miR-21-5b, miR-199a-5p, miR-199b, miR-181a, and miR-320a were lower in patients with coronary artery disease compared to the control group. Moreover, miR-199a-5p was higher in patients with coronary artery disease compared to the first hour and 24th hour after the operation. Accordingly, it can be said that the decrease in the levels of these miRNAs may pave the way for the pathogenesis of coronary artery disease. miR-199a can be used to predict future adverse events, optimize patient care, and improve the patient clinic in coronary artery bypass graft surgery patients. Clinicians need to know the morbidity and mortality of patients, and miR-199a-5p has the potential to address this need as an epigenetic marker.

Acknowledgments: We sincerely thank the Gaziantep University Research Projects Unit for their support.

Conflict of interest: The authors declare that they have no competing interest.

Funding: The authors declared that this study has received no financial support.

Authors' contributions: All authors contributed equally to the article. All authors read and approved the final version.

Ethics Committee Approval: This study was approved by Gaziantep University Medical School Medical Ethics Committee with the decision numbered 2016/276 (Date: 17 October 2016, Protocol Number: 276) and supported by Gaziantep University Scientific Research Projects Unit (TF. DT.17.11).

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