

Circulating microRNAs as Novel Biomarkers for Atherosclerosis

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Abstract. *Background/Aim: In this study, we determined the expression of selected circulating microRNAs (miRNA) and their potential roles as biomarkers in patients with atherosclerosis and a control group. Materials and Methods: In order to obtain insight into miRNA expression levels in atherosclerosis, we analyzed miRNA expression levels by real-time polymerase chain reaction (RT-PCR) in case (n=89) and healthy control (n=93) groups. Receiver operating characteristic curve analysis was performed to assess the diagnostic capability of miRNAs. Results: miRNA221 and miRNA221 expression levels were significantly lower in patients than controls (p=0.011 and p=0.004, respectively). Receiver operator curve analysis demonstrated that expression levels of miRNA221 [area under curve (AUC)=0.623, p=0.0086] and miRNA222 (AUC=0.654, p=0.0006) were significantly different between groups. There were positive correlations between miRNA122a and triglyceride (p=0.046) and very-low-density lipoprotein (p=0.029) levels. Conclusion: miRNA221 and miRNA222 could be convenient biomarkers for diagnosis of atherosclerosis.*

Atherosclerosis is the predominant cause of mortality and morbidity in developed countries despite therapeutic improvements in interventional surgery and pharmacologic treatment strategies (1). According to World Health Organization (WHO) reports, 17.7 million people die each year because of cardiovascular diseases (CVD) (2). Thus, new strategies are needed for understanding underlying

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mechanisms of atherosclerosis to discover new therapeutic strategies and novel diagnostic biomarkers.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs of 18-22 nucleotides in length that negatively regulate gene expression by interfering transcription or inhibit translation at the post-transcriptional level (3). It has been shown that 30% of protein-coding genes are controlled by miRNAs (4). Owing to their stable characteristics, miRNAs have emerged as potential biomarkers. Furthermore, miRNAs can easily be obtained from body fluids by non-invasive methods (5). They play crucial roles in diverse physiological and pathological cell process including proliferation, cell differentiation, migration, and apoptosis, as well as angiogenesis, cardiogenesis, endothelial and myocyte growth and lipoprotein metabolism (6, 7). Although many studies showed relationships between miRNA processes and atherosclerosis, effective diagnostic therapeutic alternatives have not been validated yet.

In this study, we aimed to investigate the relationships between miRNA expressions and their potential therapeutic role as biomarkers in atherosclerosis.

Materials and Methods

Study population. Patient and control groups were selected after detailed clinical examinations at the Cardiovascular Surgery Departments of Marmara University and Derince Training and Research Hospital. The control group (n=93) consisted of individuals who presented for check-ups while the patients with atherosclerosis (n=89), defined as having coronary artery disease (CAD) were documented by angiography. Angiographic inclusion criteria were: $\geq 50\%$ stenosis of at least one major coronary vessel because of atherosclerosis. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the study protocol was approved by the Yeditepe University Medical Faculty Ethics Committee (file no: 03.06.2017/428) on research on humans. After obtaining informed consent of each participant, blood samples were collected in EDTA-containing tubes.

Sample preparation. Serum was obtained from whole blood samples which had been collected in 5 ml plain serum separator tubes. Serum samples were centrifuged at $1500 \times g$ for 10 min then the

specimen was decanted into a new sterile tube and frozen at -80°C until miRNA assessments were conducted.

miRNA isolation from 200 μl serum was performed using a miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The optical density of miRNA isolated from samples was measured with NanoDrop2000 (ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription from isolated miRNA samples to cDNA was performed with miScript II RT Kit (Qiagen). Transcribed miRNA concentration was evaluated and equalized using Qubit miRNA Assay Kit standard protocol on a Qubit 3.0 Fluorometer (ThermoFisher Scientific).

miRNA selection. Bioinformatics analysis of data compiled from several medical databases, including miRBase, TargetScanHuman, miRanda.org and Embase, was used for selection of the miRNAs. The examined miRNAs (*miRNA221*, *miRNA222*, *miRNA143*, *miRNA145*, *miRNA133a*, *miRNA133b*, *miRNA122a*, *miRNA494*, *miRNA499a*) in this study were chosen to provide a comprehensive approach for atherosclerosis.

Real-time polymerase chain reaction (PCR) analysis of miRNA. Nine different custom primers for *miRNA221-3p*, *miRNA222-3p*, *miRNA143-3p*, *miRNA145-3p*, *miRNA133a-3p*, *miRNA133b-3p*, *miRNA122a-3p*, *miRNA494-3p* and *miRNA499a-5p*, which were embedded in PCR plate, were used to demonstrate miRNA expression levels (Qiagen). Three housekeeping assay genes, namely *mirRNAU6*, *mirSNORD61*, and *SNORD68*, were used for determine delta cycle threshold (ΔCt) values of each primers (Qiagen). Ct values were obtained by miScript SYBR Green PCR Kit (Qiagen) on an Applied Biosystems 7500 Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). The miRNA expression levels were calculated with Livak formula ($2^{-\Delta\Delta\text{Ct}}$) (8). ΔCt , ($2^{-\Delta\Delta\text{Ct}}$) and normalized expression levels [$\log_2[2^{-\Delta\Delta\text{Ct}}]$] were calculated using Microsoft Excel (Microsoft Corporation, Redmond, MA, USA).

Statistical analysis. Values are expressed as the mean \pm standard deviation (SD). Chi square and Fisher's exact tests were used to compare demographic information. Student's *t*-test was used to examine the significance of differences between the case and control groups. miRNA expression levels were analyzed by Student's *t*-test. Correlations were determined using Pearson correlation test, and univariate linear regression analysis methods. Statistical analysis was performed using SPSS Ver. 23 software (IBM Corp., Armonk, NY, USA). The diagnostic value of circulating miRNAs determined by receiver operator curve (ROC) analysis using MedCalc version 15.0 (MedCalc Software, Ostend, Belgium) was used for ROC analysis with 95% confidence interval. Reported *p*-values are two sided and a significance level of $p < 0.05$ was considered to indicate statistical significance.

Results

We determined the expression levels of circulating miRNAs in 89 patients with atherosclerosis and 93 healthy controls without evidence of CAD. The mean age of the patients and healthy controls was 58.97 ± 13.79 and 57.07 ± 9.80 years, respectively ($p = 0.08$). There were no significant differences between two groups in terms of body mass index, body

surface area, plasma cholesterol, triglyceride (TG), low-density lipoprotein and very low-density lipoprotein (VLDL). Regarding clinical risk factors, there were differences were observed in family history, diabetes and hypertension and high-density lipoprotein levels.

It was found that *miRNA221*, *miRNA222*, *miRNA133b*, *miRNA143* and *miRNA122a* were down-regulated, whereas *miRNA145*, *miRNA133a*, *miRNA494* and *miRNA499a* were up-regulated (Figure 1A). Logistic regression analysis of circulating miRNA expression levels in patients with atherosclerosis and control group are given in Table I. From the risk estimate analysis, altered levels of *miRNA221* ($p = 0.011$) and *miRNA222* ($p = 0.004$) were statistically significant.

Although there were statistically significant differences for only *miRNA221* and *miRNA222* expression levels, all miRNAs underwent ROC analysis to reveal results in detail. Area under the curve (AUC) results indicated that *miRNA221* and *miRNA222* not surprisingly achieved highest ROC values. *miRNA221* reached a specificity of 49.43% and a sensitivity of 76.27% (AUC=0.623, $p = 0.0086$) and *miRNA222* had a specificity of 54.02% and a sensitivity of 69.49% (AUC=0.654, $p = 0.0006$) (Figure 1B and C). Our results indicate that *miRNA221* and *miRNA222* could be eligible candidate biomarkers for diagnosis of atherosclerosis.

Determining lipid profiles and miRNA expression levels indicated that there was positive correlation between *miRNA122a* and TG ($p = 0.046$) and VLDL ($p = 0.029$) levels. Owing to limited study populations, these analyses were performed in all participants ($n = 182$). Univariate linear regression analysis of miRNA expression and TG and VLDL levels are shown in Table II.

Discussion

miRNAs are thought to be novel negative gene regulators having crucial roles in biological and pathological processes such as proliferation, migration, oxidative stress and apoptosis. miRNAs show their effects not only by mRNA degradation but also translational repression of proteins. It has been shown that miRNAs participate in pathological processes including cancer, diabetes and obesity, as well as atherosclerosis (9). Circulating miRNAs have great interest as potential clinical biomarkers due to their highly stable and accessible features. Furthermore, miRNAs can easily be sampled from body fluids such as blood, saliva, urine with non-invasive methods (10).

There are several animal and *in vitro* studies regarding miRNAs as biomarkers in atherosclerosis (11), although few human data have been provided so far. The present study demonstrated that *miRNA221* and *miRNA222* expression levels were significantly lower in patients with atherosclerosis, and therefore could be eligible risk factors for atherosclerosis.

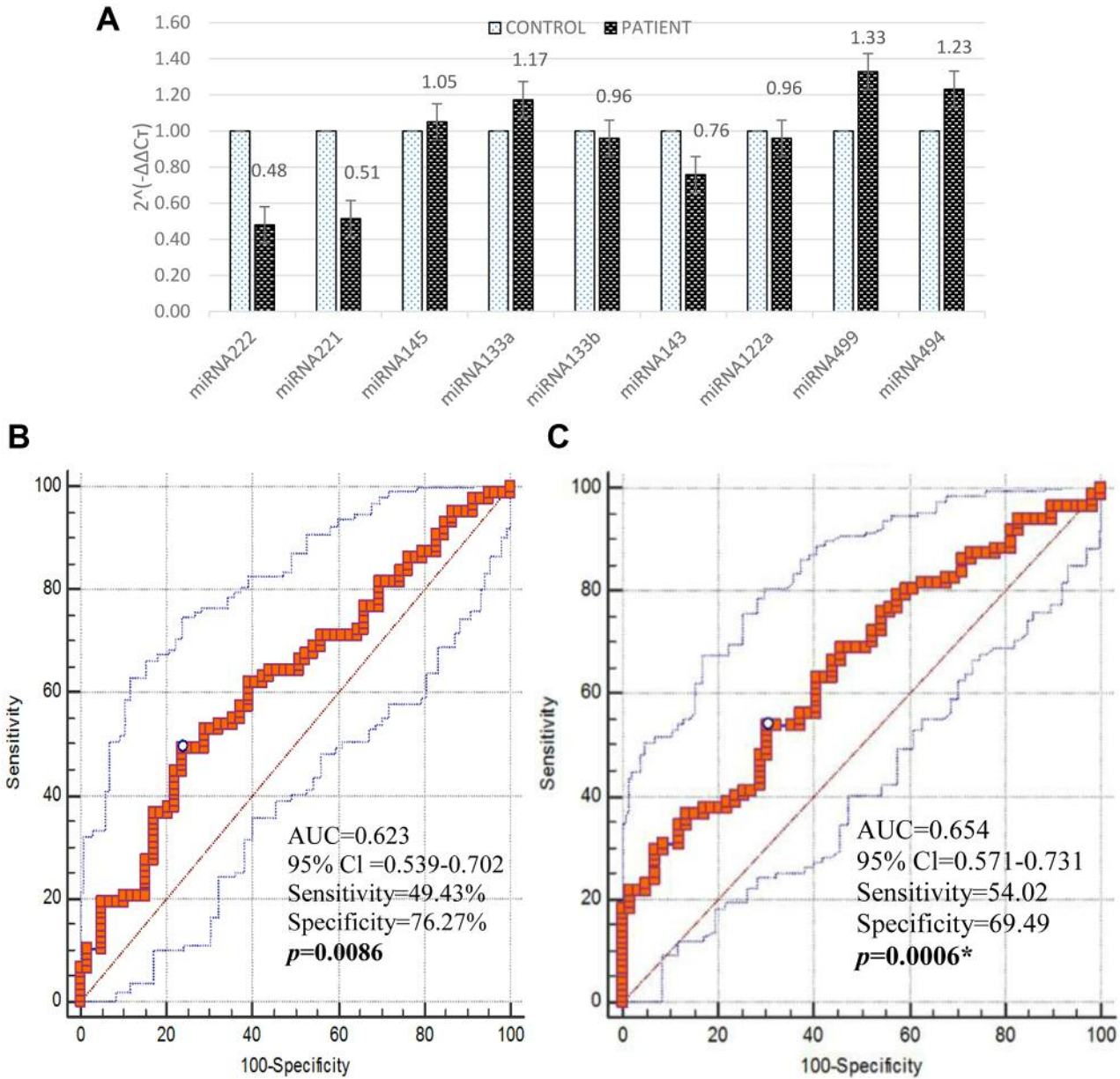


Figure 1. A: The expression of circulating miRNAs in patients with atherosclerosis. B, C: Receiver operator characteristic curves for linear regression of expression levels of circulating miRNA221 (B) and miRNA222 (C).

In the past decades, a great number of miRNAs have been recognized for their toll pathological processes of atherosclerosis. Because of the important role of vascular endothelial cells (VECs) on vascular system homeostasis as regulators of proliferation, angiogenesis, platelet aggregation, cell adhesion, specific class of miRNAs were identified as affecting physiological regulation of VECs (11). *miRNA221* and *miRNA222* are members of same miRNA family, named antiangiogenic gene regulators, and are highly expressed in

VECs. Decreased expression levels of *miRNA221* and *miRNA222* in serum samples demonstrated that limited cell proliferation had a crucial role in epithelial dysfunction in atherosclerosis. *miRNA221* and *miRNA222* modulate angiogenic activity of VECs *via* controlling proliferation factors such as human proto-oncogen c-KIT and receptor for stem cell factor (12). It has also been shown that *miRNA221* and *miRNA222* regulate c-KIT protein expressions at the post-transcriptional level without mRNA degradation, thus

Table I. Logistic regression analysis of circulating miRNA expression levels in patients with atherosclerosis versus a control group.

miRNA	Fold change $\log_2(-\Delta\Delta C_T)$	OR	SE	95% Confidence interval	p-Value
<i>miRNA221</i>	-0.72	2.82	0.133	0.549-0.926	0.011*
<i>miRNA222</i>	-0.65	2.52	0.145	0.494-0.872	0.004*
<i>miRNA122a</i>	-0.06	1.20	0.115	0.756-1.188	0.642
<i>miRNA133a</i>	-0.02	0.72	0.128	0.755-1.248	0.816
<i>miRNA133b</i>	-0.29	0.76	0.155	0.751-1.376	0.914
<i>miRNA143</i>	0.08	0.94	0.127	0.648-1.068	0.148
<i>miRNA145</i>	0.05	0.93	0.129	0.763-1.268	0.900
<i>miRNA494</i>	0.21	0.90	0.137	0.932-1.596	0.147
<i>miRNA499a</i>	0.20	0.87	0.188	0.756-1.578	0.638

SE: Standard error. OR: Odds ratio *Statistically significant difference.

Table II. Univariate linear regression analysis of circulating miRNA expression and triglyceride (TG) and very low-density lipoprotein (VLDL) levels in the whole study population (n=182).

Analysis of	Constant (B)	SE	95% Confidence interval	p-Value
miRNA vs. TG				
<i>miRNA221</i>	151.83	12.31	127.48-176.18	0.841
<i>miRNA222</i>	145.38	9.60	126.39-164.36	0.542
<i>miRNA122a</i>	131.55	11.01	109.78-153.32	0.046*
<i>miRNA133a</i>	147.15	6.60	133.47-160.84	0.726
<i>miRNA133b</i>	143.93	8.51	127.14-161.02	0.998
<i>miRNA143</i>	147.86	6.69	134.62-161.09	0.407
<i>miRNA145</i>	157.46	11.35	135.00-179.91	0.466
<i>miRNA494</i>	124.00	20.28	138.89-164.12	0.169
<i>miRNA499a</i>	158.42	13.28	131.88-184.96	0.335
miRNA vs. VLDL				
<i>miRNA221</i>	29.86	1.30	27.28-32.43	0.878
<i>miRNA222</i>	30.19	1.31	27.58-32.80	0.447
<i>miRNA122a</i>	29.84	1.23	27.40-32.29	0.038*
<i>miRNA133a</i>	29.11	1.12	26.88-31.35	0.464
<i>miRNA133b</i>	28.68	1.63	25.43-31.95	0.637
<i>miRNA143</i>	29.90	1.27	27.38-32.42	0.344
<i>miRNA145</i>	30.10	1.26	27.60-32.59	0.568
<i>miRNA494</i>	29.65	1.28	27.11-32.18	0.108
<i>miRNA499a</i>	29.95	1.68	26.58-33.31	0.128

SE: Standard error. *Statistically significant.

they can modulate proliferation of vascular cells (13). Liu *et al.* demonstrated interesting data regarding *miRNA221* and *miRNA222*, they showed that these miRNAs have cell-specific functions. *miRNA221* and *miRNA222* have inverse effects on VECs and vascular smooth muscle cells (VSMCs). Gene expression of proto-oncogene *c-KIT* was very high in VECs while it was significantly lower in VSMCs. Therefore, altered *miRNA221* and *miRNA222* expression levels appear to have opposing effects on VSMCs and ECs (14). *miRNA221* and *miRNA222* have regulatory roles in VSMC proliferation in atherosclerosis (15). Zhang *et al.* demonstrated that

patients with atherosclerosis had significantly lower *miRNA221* and *miRNA222* expression levels than healthy controls. They showed that down-regulated *miRNA221* and *miRNA222* expressions had crucial roles in the atherosclerotic process and suggested that these two miRNA could be potential candidate biomarkers for CAD (16).

The advantages presented by miRNAs show they are not only potential and useful biomarkers but also useful therapeutic targets. *miRNA122a* constitutes 70% of all miRNAs expressed in adult liver cells, and is therefore described as a mammalian liver-specific microRNA (17). Esau *et al.* demonstrated that

miRNA122 plays important roles on lipid regulation by targeting six different mRNA which have direct or indirect effects on lipid metabolism. It has been shown that cholesterol synthesis rates decreased significantly in mice when *miRNA122* was inhibited by antisense oligonucleotides. *miRNA122* interference resulted in reduction of plasma cholesterol levels and hepatic fatty acid synthesis, while it increased in hepatic fatty-acid oxidation. Their results showed that *miRNA122* could be a therapeutic target for lipid homeostasis (18). Iliopoulos *et al.* reported that inhibition of *miRNA122* had similar effects on lipid regulation in human hepatocyte-derived cell lines. Their data revealed an association between *miRNA122* and lipogenic genes, up-regulation of *miRNA122* inhibited gene regulation that cause TG accumulation in liver by increased lipogenesis and suppressed β -oxidation. Moreover, reduced expression levels of *miRNA122* caused decrements in sterol regulatory element-binding protein 1c (*SREBF-1c*) gene, which regulates LDL receptor and cholesterol pathways in cell lines (19). In the current study, we demonstrated that *miRNA122a* positively correlated with lipid profiles. Complementary to our results, another study was performed by Song *et al.*, with silencing of *miRNA122* by antagomirs. Inhibition of *miRNA122*-stimulated bile acid synthesis, therefore serum cholesterol and TG levels decreased (20).

In conclusion, our results demonstrated potential data for association between *miRNA221*, *miRNA222* and atherosclerosis. Although metabolic effects of *miRNA221* and *miRNA222* were vague, altered expression levels in serum of patients with atherosclerosis indicated that they could be candidate novel circulating biomarkers for diagnosis. Besides, expression level of *miRNA122a* has a significant impact on serum TG and VLDL levels, because of positive correlations between them. These results should be supported with more detailed future studies.

Conflicts of Interest

The Authors declare that there are no financial disclosures or conflict of interest associated with this study.

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