Circulating microRNAs as Novel Biomarkers for Atherosclerosis

SEDA GULEC YILMAZ, SELIM ISBIR, ATIKE TEKELI KUNT and TURGAY ISBIR

Departments of 1Molecular Medicine, Institute of Health Sciences and 4Medical Biology, Faculty of Medicine, Yeditepe University, Istanbul, Turkey; 2Department of Cardiovascular Surgery, Marmara University Pendik Education and Research Hospital, Istanbul, Turkey; 3Department of Cardiovascular Surgery, Ankara Numune Education and Research Hospital, Ankara, Turkey

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 miRNA222 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 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. The angiographic inclusion criteria were: ≥50% stenosis of at least one major coronary vessel because of atherosclerosis (n=89), defined as having coronary artery disease (CAD) were documented by angiography. Angiographic inclusion criteria were: ≥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 × g for 10 min then the...
miRNA selection. Bioinformatics analysis of data compiled from several medical databases, including miRBase, TargetScanHuman, microrna.org and Embase, was used for selection of the miRNAs. The examined miRNAs (miRNA221, miRNA222, miRNA494, miRNA499a, miRNA143, miRNA145, miRNA133a, miRNA 133b, miRNA 122a, miRNA 494, miRNA 499a) 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, miRNA145-5p, miRNA133a-3p, miRNA 133b-3p, miRNA 122a-3p, miRNA 494-3p and miRNA 499a-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 \( 2^{-\Delta\Delta Ct} \) (8). \( \Delta Ct \) (2−\( \Delta\Delta Ct \)) and normalized expression levels \( \log_{2} (1 + \Delta A C T) \) were calculated using Microsoft Excel (Microsoft Corporation, Redmond, MA, USA).

Statistical analysis. Values are expressed as the mean±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 (IBMCorp., Armonk, NY, USA). The diagnostic value of circulating miRNAs determined were performed using NanoDrop2000 (Thermoscientific, 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 (Thermoscientific).

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 (Thermoscientific, Waltham, MA, USA). 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 miRNA222a 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.
In the past decades, a great number of miRNAs have been recognized for their role in pathological processes of atherosclerosis. Because of the important role of vascular endothelial cells (VECs) on vascular system homoeostasis 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

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).
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. miRNA22a 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 antagonirs. 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.

References
1. Wang T, Palucci D, Law K, Yanagawa B, Yam J and Butany J: Atherosclerosis: pathogenesis and pathology. Diaig Histopathol 18: 461-467, 2012.
2. http://www.who.int/cardiovascular_diseases/en/
3. Carthew RW and Sontheimer EJ: Origins and Mechanisms of miRNAs and siRNAs. Cell 136(4): 642-655, 2009.
4. Wang Z, Luo X, Lu Y and Yang B: miRNAs at the heart of the matter. J Mol Med 86(7): 771-783, 2008.
5. Li C, Pei F, Zhu X, Duan DD and Zeng C: Circulating microRNAs as novel and sensitive biomarkers of acute myocardial Infarction. Clin Biochem 45(10-11): 727-732, 2012.
6. Santovito D, Mezzetti A and Cipollone F: MiR-122 regulates LDL receptor and cholesterol pathways in cell lines. Cell 122(2): 87-98, 2006.
7. Wojciechowska A, Braniewska A and Kozar-Kaminska K: MicroRNA in cardiovascular biology and disease. Adv Clin Exp Med 26(5): 865-874, 2017.
8. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) Method. Methods 25(4): 402-408, 2001.
9. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercantanti A, Hammond S and Rainaldi G: MicroRNAs modulate the angiogenic properties of HUVECs. Blood 108(9): 3068-3071, 2006.
10. Song KH, Li T, Owsley E and Chiang JY: A putative role of microRNA-122 in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res 79(4): 581-588, 2008.
11. Iliopoulos D, Drosatos K, Hiyama Y, Goldberg IJ and Zannis VI: MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. J Lipid Res 51(6): 1513-1523, 2010.
12. Sang H, Li T, Owseley and Chiang JY: A putative role of microRNA in the regulation of cholesterol 7alpha-hydroxylase expression in human hepatocytes. J Lipid Res 51(8): 2223-2233, 2010.

Received December 20, 2017
Revised January 18, 2018
Accepted January 29, 2018