Expression Level of MicroRNA-122 in Serum Samples of Patients With Atherosclerosis

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Abstract- Atherosclerosis is another type of arteriosclerosis, characterized by the hardening of arteries, playing a significant role in various cardiovascular diseases. It stands as one of the leading causes of mortality in contemporary societies, especially in advanced countries experiencing higher levels of stress. The primary feature of atherosclerosis is the accumulation of excessive lipid deposits (plaques), obstructing blood flow and leading to strokes and various cardiovascular diseases. While various factors contribute to plaque formation, both environmental and genetic factors play crucial roles. In the cross-sectional study, 80 patients with atherosclerosis, referred to Rajaee Hospital in Tehran and Madani Hospital in Tabriz, along with 80 healthy individuals for comparative analysis, were selected. All relevant information and serum levels of important biomarkers were measured, followed by statistical analysis. MicroRNA extraction was performed using Trizol solution, and CDNA was synthesized by adding polyadenine tails to all microRNA samples. The obtained CDNA was utilized for real-time PCR with LNA primers. The results, normalized using Spike RNA, indicated a significant 90% decrease in the serum levels of these microRNAs in patients with atherosclerosis. The findings suggest that microRNA miR-122 could serve as a promising biomarker for diagnosing atherosclerosis.

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Introduction

MicroRNAs (miRNAs) constitute a group of small non-coding RNAs, typically consisting of 19 to 23 nucleotides, that exert their influence on gene expression (1). Since their discovery in 1993, these RNAs play crucial and diverse roles across various organisms (2). Altered expression profiles of miRNAs are associated with various diseases, making miRNAs potential biomarkers for disease onset (3). Atherosclerosis is the most common heart disease (4-14). Among various specific cellular and molecular responses, endothelial dysfunction plays a pivotal role in the initiation and progression of the disease. These events often coincide with oxidative stress occurrences (15). Increased production of reactive oxygen species and low-density lipoprotein oxidation has been identified throughout the advancement of atherosclerosis (3). Additionally, microRNAs have been demonstrated to act as crucial gene expression regulators, controlling post-transcriptional modification of cellular responses and functions (16).

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Genetic studies of atherosclerosis indicate a direct correlation between altered miRNA expression profiles and the pathophysiology of this disease, providing potential miRNA candidates for the development of novel therapeutic strategies (17). Various genes and different mutations have been reported to contribute to atherosclerosis, but the role of microRNAs is currently under scrutiny. While most miRNAs are localized within cells, some of them, known as circulating or extracellular miRNAs, are present in extracellular environments such as various biological fluids and cell culture media (18). Misregulation of miRNA expression has been implicated in diseases, and miRNA-based therapies are under investigation (19).

This research aims to determine the level of microRNA-122 in the serum of individuals with atherosclerosis. By quantifying circulating miR-122, this research investigates to elucidate its potential role in the disease's pathogenesis and assess its value as a biomarker for atherosclerosis.

Materials and Methods

Patients

This research was conducted by examining individuals suffering from atherosclerosis who visited the specialized Cardiac Care Center at Shahid Madani Hospital in Tabriz, as well as Shahid Rajaei Heart Hospital in Tehran. In this study, 80 men and women diagnosed with atherosclerosis, were enrolled with predefined inclusion criteria: age range of 45 to 78 years, willingness to cooperate in the research, and no history of other chronic diseases. The exclusion criteria included insufficient blood samples for the study, chronic smoking (more than 3 cigarettes per day for over 48 months), alcohol consumption, diabetes (type 1 and 2), and adherence to specific dietary patterns such as a vegetarian diet. Additionally, 80 healthy individuals were included in the study. The sample size was determined using Equation 1.

$$\text{Equation1: } n = \frac{\frac{z^2 pq}{d^2}}{1 + \frac{q}{N}\left(\frac{z^2 pq}{d^2} - 1\right)}$$

RNA extraction

Prior to the commencement of the study, 5 ml blood samples were collected from all participants and stored at 70° C. Subsequently, fasting blood sugar (FBS), High-density lipoprotein (HDL), Low-density lipoprotein (LDL), Total cholesterol (TC), and Triglyceride (TG) were measured using an autoanalyzer. The baseline and endpoint data, including age, weight, and body mass index (BMI), were recorded for both the experimental and control groups.

The extraction of total serum microRNA was carried out using TRIzol solution according to the protocol. Briefly, the remaining cells were centrifuged at 12000 rpm for 15 minutes to extract total RNA. Initially, 200 µl blood serum were mixed with 200 µl TRIzol solution and kept at room temperature for ten minutes. Subsequently, the samples were centrifuged at 10000×g for 10 minutes and the clear supernatant was transferred to a new tube. Then, 100 µl sodium acetate were added followed by centrifugation at 10000×g for 10 minutes at room temperature. The resulting supernatant was transferred to a new tube, and RNA pellets were resuspended in 2.5 M lithium chloride and absolute ethanol, followed by centrifugation for 10 minutes at 10000×g. The liquid was poured off, and the resulting pellet, after drying, was resuspended in 20 µl of DEPC (Diethylpyrocarbonate)-treated water.

The Nanodrop instrument was used to determine RNA concentration, employing spectrophotometry with high speed and accuracy in nucleic acid concentration measurement. This instrument measures the OD of the sample at 260 nm wavelength to determine RNA concentration, where each OD unit at 260 nm corresponds to 40 ng/ml of single-stranded RNA.

cDNA synthesis and amplification

For this purpose, the cDNA synthesis kit (CinnaGen) was used and the components listed in Table 1. The mixture was gently shaken and then incubated at 37° C for 30 minutes. After 5 minutes at 95° C in thermocycler (Bio-Rad), the samples were incubated according to the table 2. After completing cDNA synthesis, 20 µl of RNase-free water was added for Real-Time PCR reaction, and stored at -20°C. RNA SPIKE was used as an internal control. Specific LNA primers were designed using oligo 7.

Table 1. Amplification of microRNAs via PCR

Components for PCR			
	Required ingredients		**Amount required**
1	Poly A polymerase buffer		4.0 µl
2	rNTP (10 mM)		1.0 µl
3	MgCl2 (optional) 25 mM		1.0 µl
4	*x* µl of total RNA		(30 ng – 1 µg)
5	Specific primers		5'-ccAttGtcAca^{m}Ctc^{m}Ca-3'
6	DEPC-treated water		Up to 20µl
Thermocycler setup for PCR			
	Time required		**Temperature of incubation**
1	denaturation	5 minutes	95 °C
2	Elongation (40)	15 minutes	25 °C
3		30 minutes	42 °C
4		5 minutes	95 °C

Table 2. Demographic information of subjects

Property	**Control**		**Case**		***P*****
TG (mg/dl)	196		348		0.03
TC (mg/dl)	217		248		0.04
LDL (mg/dl)	135		177		0.04
HDL (mg/dl)	58		42		0.05
FBS (mg/dl)	82		93		0.05
LDL/HDL	2.32		4.21		0.2
	Healthy men	**Healthy women**	**Case men**	**Case women**	
Age (years)	61±0.4	58±0.6	59±0.7	61±0.8	0.31
Weight (kg)	77±0.3	64±0.5	79±0.2	67±0.8	0.48
Height (cm)	168±0.6	160±0.3	167±0.1	159±0.7	0.33
BMI (kg/m^2)	27±0.4	25±0.2	28±0.4	26±0.8	0.38

***P*<0.05. BMI, body mass index; HDL, High-density lipoprotein; FBS, fasting blood sugar; LDL, Low-density lipoprotein; TC, Total cholesterol; and TG, Triglyceride

Data analysis

REST software was utilized for computing fold change values using the 2-ΔΔCt method. Additionally, the software was employed to calculate the significance level of gene expression changes (*P*) and assess the efficiency of PCR and primers by utilizing the results from consecutive replicates.

Statistical analyses were conducted using SPSS 18.0 software to analyze the data. Initially, the normality of the data distribution was assessed using the Kolmogorov-Smirnov (K-S) test. Quantitative data were expressed as mean±standard deviation. The intra-comparison of means was performed using the paired t-test, and the comparison between multiple groups was conducted using ANOVA. It is worth noting that statistical significance was considered when $P \leq 0.05$.

Results

Demographic information

The measurements of FBS, HDL-C, LDL-C, TC, and TG factors, and LDL/HDL ratio in both patient and control groups are presented in Table 2. As observed, there were noticeable changes in FBS, HDL-C, LDL-C, TC, and TG levels in both patient and control groups. As seen, LDL/HDL ratio significantly varied in both patient and control groups and lead to atherosclerosis ($P<0.05$) (Table 2).

Initially, information regarding age, weight, and BMI were recorded for both the patient and control groups. As indicated in Table 3, the average BMI of the patient group was higher than that of the normal individuals. Therefore, it can be concluded that obesity is one of the significant factors in the occurrence of atherosclerosis. The physical characteristics of the patients and control individuals in this study include BMI categories: less than 18.5 kg/m² as underweight, between 18.5-24.9 kg/m² as normal weight, between 25-29 kg/m² as overweight, and greater than 29 kg/m² as obese.

Key indices of the participants in the control and healthy groups are presented in Figure 1. By comparing the patient groups with the control group, a noticeable

increase in the BMI of the patient group compared to the control group is observed. Therefore, it is concluded that obesity can be an important factor in atherosclerosis. Previous results have indeed confirmed this, indicating that individuals with atherosclerosis may not necessarily be obese but may have excess weight. This suggests that obesity itself can be one of the factors contributing to the occurrence of atherosclerosis. However, this may be related to social conditions and lifestyle.

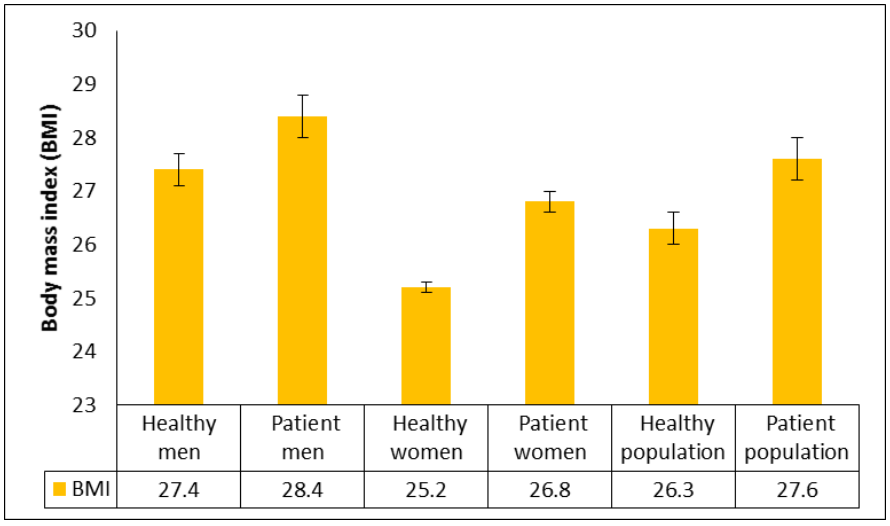

Figure 1. Comparing BMI (body mass index) of atherosclerosis patients and healthy controls with considering gender distribution

Expression levels of miR-122

The fold change in the expression level of miR-122 in the patient group was 90% lower than that in the control group and this down-regulation had a mean factor of 0.113 (S.E. range is 0.014-0.915) with *P*(H1)< 0.001. Moreover, there was no significant difference in the serum level of microRNA-122 of women and men neither in individuals with normal weight nor overweight subjects. Therefore, it can be considered as a risk factor for atherosclerosis and can be utilized for early diagnosis (Figure 2) (Table 3).

Table 3. Expression levels of miR-122

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	*P*(H1)	Result
Spike RNA	REF	1.0	1.000				
mir-122	TRG	1.0	0.113	0.014 - 0.915	0.003 - 6.549	<0.001	DOWN
Spike RNA men	REF	1.0	1.204				
mir-122 men	TRG	1.0	0.104	0.016-0.677	0.003-3.350	<0.001	DOWN
Spike RNA women	TRG	1.0	0.089				
mir-122 women	REF	1.0	0.830	0.012-0.624	0.002-2.357	<0.001	DOWN

P (H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. C.I, Confidence interval; REF, Reference; TRG, Target

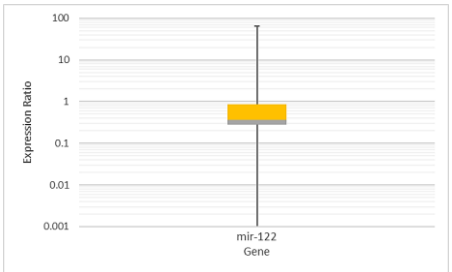

Figure 2. Expression level of miR-122 in blood samples of patients and controls

Considering the potential impact of gender on the serum level of this microRNA, the analysis of the serum

level of the microRNA in the patient and control groups was separately evaluated in males and females. The results demonstrated a significant increase in the serum level of this microRNA. The expression level of miR-122 in the serum of female patients was 0.08, and in male patients, it was 0.1, showing a significant difference in both groups. These results suggest a more confident association with the increase in the serum level of this microRNA in patients.

Figure 3 shows that this microRNA does not differ significantly between overweight and normal-weight individuals, suggesting its uniform applicability for rapid disease detection in all individuals.

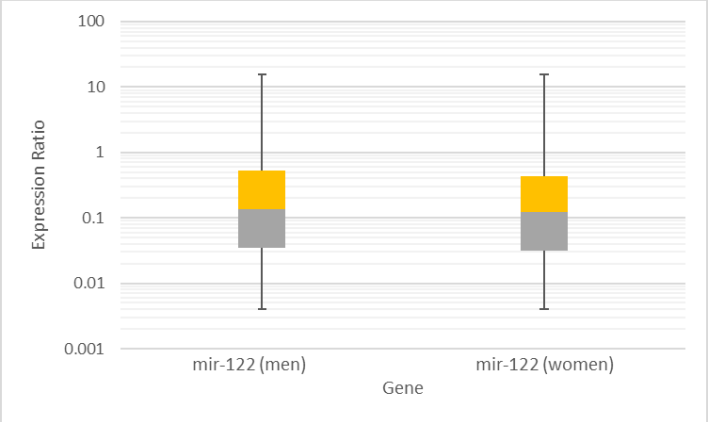

Figure 3. Comparing expression level of miR-122 in women and men

Discussion

Early detection of atherosclerosis can be crucial to prevent its development and progression, with elevated blood cholesterol levels and obesity being among the factors associated with atherosclerosis (20,21). Inflammation is also recognized as another significant factor, as reported in numerous studies (22). As indicated by the results of this research, a significant increase in cholesterol levels was observed in atherosclerosis patients, aligning with findings from other studies such as Badimon *et al*., (23) and Vorkas *et al*., (24). Furthermore, this study revealed a meaningful correlation between the occurrence of the disease and individuals' BMI, indicating a higher prevalence in obese individuals. These results are consistent with findings of a study by Bastin e*t al*, where a significant association between obesity and various heart diseases was reported (25). Therefore, these markers can be utilized for disease diagnosis, although their predictive value might be limited, as these factors become distinguishable only after the disease has developed.

Common diagnostic methods such as blood tests for cholesterol, triglycerides, and proteins, as observed in the results section, show a significant increase in many of these factors in both male and female patient groups. The use of genetic factors, especially microRNAs, is currently under investigation as a robust molecular marker for rapid disease detection and early prevention in case of symptoms. There are various diagnostic methods available, but the investigation of early detection methods using microRNAs has become an intriguing subject for research (26).

MicroRNAs are a class of non-coding RNAs involved in various cellular processes. Recently, alterations in the expression of these molecules have been reported in numerous diseases. The microRNA miR-122, for instance, plays a role in preventing the development of atherosclerosis. An increase in the level of this microRNA not only does not lead to disease but also, by enhancing the migration of immune cells CD4, contributes to the elimination of plaques in the arteries (27-29). Therefore, the results of this study demonstrated a nearly 100% reduction in the level of miR-122 in the patient group compared to the control group. Understanding the mechanism of miR-122 suggests that this microRNA prevents the onset of the disease, and its levels significantly increase in atherosclerosis.

The study highlights the significant downregulation of microRNA-122 in individuals suffering from atherosclerosis. The cross-sectional analysis involved 80 atherosclerosis patients and 80 healthy controls, measuring various biomarkers and demographic factors. The results revealed a 90% decrease in serum levels of microRNA-122 among patients, with this reduction being uniform across different genders and weight categories.

This notable decreases positions microRNA-122 as a promising biomarker for early atherosclerosis detection, emphasizing its potential utility in clinical settings regardless of the patient's gender or body weight. Additionally, the study reinforces the association between higher BMI and atherosclerosis, pointing towards the importance of managing obesity to mitigate the risk of this cardiovascular condition. Overall, these findings contribute to a deeper understanding of the molecular underpinnings of atherosclerosis and pave the way for improved diagnostic approaches leveraging microRNA-122.

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Ethical Approval

No animal or human subjects were used by the authors in this study.

Availability of Data and Materials

The datasets produced and/or processed in the course of the present Research could be provided by the respective author on a reasonable request. The information is not made publicly available because of privacy and ethical limitations.

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