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Differential Expression of Let-7 MicroRNA in Patients with Coronary Artery Disease: Possible Potential for Early Diagnosis, Treatment, and Prevention

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ABSTRACT

Background: Coronary artery disease (CAD) and its numerous consequences significantly reduce the quality of life for millions of people worldwide.

Objectives: This study investigated the differential expression of let-7 microRNA between CAD patients and healthy peers, which can be used as an early diagnostic, therapeutic, and preventive tool.

Methods: In this case-control study, participants were selected through convenient sampling and categorized into CAD-positive (n = 25) and CAD-negative (n = 25) groups. Blood samples were obtained, and the level of let-7 miRNA was measured. Various other parameters, including demographic variables, blood pressure, and results of routine blood tests besides smoking status, educational level, overweight, diabetes status, and history of coronary atherosclerosis, were recorded. Familial history of hypertension, diabetes, atherosclerosis, and sudden death were also registered. Different statistical methods including independent sample t, Mann-Whitney, and chi-squared tests were used to compare variables. A *P* value of less than 0.05 was considered significant.

Results: Patients with CAD were older, with a higher frequency of the male sex. Fasting blood sugar (P = 0.002), triglyceride (P < 0.001), HDL-cholesterol (P = 0.038), triglyceride-glucose index (P < 0.001), TG/HDL-cholesterol ratio (P < 0.001), and LDL/HDL ratio (P = 0.011) were significantly higher in the case group. Also, smoking (P = 0.001), illiteracy (P = 0.005), dyslipidemia (P = 0.048), and a history of coronary atherosclerosis (P = 0.022) were more prevalent in the CAD patients. Differential expression of let-7 microRNA between groups was at the borderline of statistical significance (P = 0.058).

Conclusion: Let-7 microRNA was expressed higher in patients with CAD, which may be helpful in the early diagnosis of atherosclerotic plaques and can possibly be used in designing therapeutic and preventive approaches.

1. Introduction

Coronary artery disease (CAD) and its major consequence, myocardial infarction, has been a serious health problem in recent years due to its heavy burden on healthcare systems throughout the world (1). The development of an atherosclerotic plaque within the coronary arteries is determined by sophisticated interactions between intrinsic and extrinsic factors (2). A developed plaque increases the risk of life-threatening ischemic events (3, 4). Early diagnosis prior to clinical symptoms through inexpensive and minimally invasive methods results in designing and applying preventive and therapeutic plans and can help decline myocardial infarction (MI) incidence and relevant complications.

All aspects of cardiovascular biology are somehow related to non-coding RNAs (5). MicroRNAs (miRNA) are a group of small non-coding RNAs whose primary function is gene expression regulation in multiple biological processes. This regulation is usually performed via induction of impairment in protein synthesis by either degradation or repression of

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mRNAs (6). miRNAs are differentially expressed in the biofluids of patients with different cardiovascular disorders (5). They modulate the expression of chemokines that are involved in the proper functioning of endothelial cells, smooth muscle cells, and macrophages. Given that all these cell types are critically implicated in the growth and development of atherosclerotic plaques, the progression of atherosclerosis is widely influenced by miRNAs (7-9). The contribution of several miRNAs to the development of atherosclerosis has been documented previously (10). Indeed, it was shown that circulating miRNAs are good predictors of coronary events in patients with CAD (11).

Let-7 miRNA was one of the first non-coding RNAs discovered even before the emergence of long non-coding RNAs (12, 13). The let-7 miRNA family was the second group of miRNAs discovered in *Caenorhabditis elegans*. Recent investigations found its high expression in the cardiovascular system. The association of the let-7 miRNA family with cardiovascular disease has also been reported (14). Members of this family become overexpressed in different cardiovascular disorders such as heart hypertrophy, cardiac fibrosis, dilated cardiomyopathy, myocardial infarction, arrhythmia, angiogenesis, atherosclerosis, and hypertension. Intriguingly, the differentiation of embryonic stem cells to cardiac lineage is related to the activity of let-7 miRNA, which is closely associated with target genes of TLR4, LOX-1, Bcl-xl, and AGO1 (14). Therefore, miRNAs' potential for early atherosclerosis diagnosis has opened a promising window (2).

2. Objectives

This study investigated the differential expression of let-7 miRNA in patients with CAD compared to healthy peers. The findings of this study can be an advancing step toward using a simple blood test for early diagnosis of CAD patients.

3. Methods

From December 2019 to February 2021, in a hospital affiliated with Shiraz University of Medical Sciences (Shiraz, Iran), patients referred for coronary angiography by interventional cardiologists based on clinical findings were included. Exclusion criteria were previous heart disease (except coronary atherosclerosis), chronic kidney disease, obstructive pulmonary disorders, and other apparent inflammation. This case-control study was approved by the Ethics Committee of Shiraz University of Medical Sciences. All participants were asked to sign an informed consent form.

Prior to the angiography, 5 ml blood samples were obtained in EDTA tubes, labeled anonymously, and kept in the refrigerator until further analysis. In the catheterization laboratory, a single interventional cardiologist performed angiography according to the guidelines of the American College of Cardiology (15). According to the angiography report, patients who had coronary stenosis constituted the CAD-positive group (n = 25), and individuals with normal coronary arteries were assigned to the CAD-negative group (n = 25).

Also, a variety of different parameters in a predefined questionnaire were obtained by a trained nurse. These included demographic variables, systolic and diastolic blood pressure, TyG index (insulin resistance), total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides (TG), TG/HDL-C ratio, LDL/HDL ratio, creatinine, blood urea nitrogen (BUN), and body mass index. Moreover, smoking status, education level, and the existence of overweight, dyslipidemia, diabetes, and a history of coronary atherosclerosis were sought. Family history of hypertension, diabetes, atherosclerosis, and sudden death were recorded as well.

In the molecular lab, blood samples were thawed on ice, and total RNA was extracted by Iraizol® (EX6101-RNX Plus Solution for total RNA isolation, Sinaclon, Iran) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were examined by a Nanodrop spectrophotometer. In the next step, cDNA was synthesized with the RNA as the template by the AddScript cDNA Synthesis Kit (Addbio, South Korea). Finally, a real-time PCR master mix (RNA biotechnology company, Iran) was used to quantify the let-7 miRNA level with the aid of sequence-specific primers. U6 was used as the internal control.

Statistical analyses: The Shapiro-Wilk test was used to check the normality of the variables. Continuous and categorical variables are expressed as mean ± standard deviation (SD) and number (percentage). Different statistical tests, including independent sample t, Mann-Whitney, and chi-square, were used to compare variables between groups as appropriate in SPSS V16 for Windows (SPSS, Inc., Chicago, IL, USA). The method of choice for analysis of gene expression was $2^{\wedge-\Delta\Delta Ct}$, which presents the fold change of gene expression in the case versus the control group.

4. Results

Out of 25 patients in the case group, two were omitted due to data missing. Table 1 compares continuous variables between the case and control groups. Patients in the case group were significantly older and had higher fasting blood sugar. Assessment of lipid profile shows the worse condition of dyslipidemia in the case group in terms of TyG index (as a marker for insulin resistance), HDL-C, TG, TG/HDL-C ratio, and LDL/HDL ratio. Also, creatinine levels were significantly higher in this group. Notably, blood pressure (neither systolic nor diastolic), total cholesterol, LDL-C, BUN, and BMI were not different between the case and control individuals.

The comparison of categorical variables (Table 2) demonstrates that case patients were more likely to be male, smokers, illiterate, and suffering from dyslipidemia. However, the prevalence of diabetes and overweight were not remarkably different between the two groups.

While there were no significant differences in the family history of hypertension, diabetes, and sudden death between the two groups, the family history of atherosclerosis was the only one that was higher in the case group (Table 3).

Analysis of differential gene expression revealed that although the expression level of let-7 miRNA was higher in the case group, the P-value was at the borderline (P = 0.058) and was not statistically significant.

Table 1. Comparison of Continuous Variables between the Case and Control Groups

	Total (n = 48)	Control (n = 25)	Case (n = 23)	*P*	Reference Range
Age	59.5 ± 11.4	54.9 ± 12.5	64.5 ±7.7	0.002	-
Systolic blood pressure (mmHg)	119 ± 12	121 ± 11	117 ± 12	0.249	< 120 mmHg
Diastolic blood pressure (mmHg)	74 ± 8	74 ± 8	75 ± 9	0.668	< 80 mmHg
FBS (mg/dl)	104 ± 20	95.6 ± 16	113.3 ± 20.3	0.002	70 – 99 mg/dL
TyG index (Insulin resistance)	8.85 ± 0.61	8.47 ± 0.46	9.27 ± 0.46	<0.001	Normal cut-off value is 8
Total cholesterol (mg/dl)	173 ± 37	163 ± 27	184 ± 44	0.059	< 200 mg/dL
HDL-C (mg/dl)	44 ± 10.1	46.9 ± 10.9	40.9 ± 8.3	0.038	Optimal: ≥ 60 mg/dL
LDL-C (mg/dl)	102 ± 30	94.5 ± 22.7	109.5 ± 36	0.096	< 100 mg/dL
TG (mg/dl)	157 ± 91	110 ± 52	208 ± 98	< 0.001	< 150 mg/dL
TG/HDL-C ratio	3.9 ± 2.5	2.6 ± 1.5	5.3 ± 2.5	< 0.001	Ideal: ≤ 2.0 High: 4.0 - 6.0
LDL/HDL ratio	2.4 ± 0.9	2.1 ± 0.7	2.7 ± 0.9	0.011	Ideal: < 2.0 Good: < 5.0
Cr (mg/dl)	0.96 ± 0.17	0.91 ± 0.17	1.01 ± 0.16	0.039	For adult men, 0.74 to 1.35 mg/dL For adult women, 0.59 to 1.04 mg/dL
BUN (mg/dl)	18 ± 7	20 ± 5	16 ± 7	0.096	7 – 20 mg/dl
BMI (kg/m^2)	25.7 ± 4.03	26.8 ± 3.8	24.5 ± 4.5	0.078	18.5 – 24.9 kg/m^2

Abbreviations: FBS; fasting blood sugar, HDL-C; high-density lipoprotein-cholesterol, LDL-C; low-density lipoprotein-cholesterol, TG; triglycerides, Cr; creatinine, BUN; blood urea nitrogen, BMI; body mass index. Data are presented as mean ± SD. Bold values imply statistical significance ($P < 0.05$).

Table 2. Comparison of Categorical Variables between the Case and Control Groups

	Total (n = 48)	Control (n = 25)	Case (n = 23)	Odds Ratio# (CI)	*P* (Mantel-Haenszel)	*P (Pearson Chi-Square)*
Sex (male)	29 (60%)	10 (40%)	19 (83%)	7.13 (1.86-27.28)	0.004	0.003
Smoking	19 (40%)	4 (16%)	15 (65%)	9.84 (2.50-38.78)	0.001	< 0.001
Education (illiterate)	27 (56%)	9 (36%)	18 (78.3%)	6.40 (1.77-23.11)	0.005	0.003
Overweight	26 (54%)	14 (56%)	12 (52.2%)	0.86 (0.28-2.67)	0.790	0.790
Dyslipidemia	22 (46%)	8 (32%)	14 (61%)	3.31 (1.01-10.83)	0.048	0.045
Diabetes	10 (21%)	3 (12%)	7 (30%)	3.21 (0.72-14.35)	0.127	0.116
History of coronary atherosclerosis	9 (19%)	1 (4%)	8 (35%)	12.80 (1.45-112.85)	0.022	0.006

Mantel-Haenszel Common Odds Ratio Estimate is used. Abbreviations: CI; confidence interval, Data are presented in numbers (%). Bold values imply statistical significance ($P < 0.05$).

Table 3. Comparison of Family History between the Control and Case Groups

	Total (n = 48)	Control (n = 25)	Case (n = 23)	*P*
Hypertension	17 (35%)	7 (28%)	10 (43.5%)	0.266
Diabetes	13 (27%)	5 (20%)	8 (35%)	0.254
Atherosclerosis	23 (48)	8 (32%)	15 (62%)	0.024
Sudden death	10 (21%)	3 (12%)	7 (30%)	0.162

Data are presented in numbers (%). Bold values imply statistical significance ($P < 0.05$).

Table 4. Expression levels of let-7 miRNA in the case and control groups

	Control	Case	P	P*	P**	P***
Let-7 miRNA	0.16 ± 0.28	0.39 ± 0.50	0.058	0.350	0.563	0.712

*Adjusted for age, sex, and BMI. ** Adjusted for age, sex, BMI, diabetes, dyslipidemia, and hypertension. *** Adjusted for age, sex, BMI, diabetes, dyslipidemia, hypertension, smoking status, and history of atherosclerosis.

Also, three models of analyses incorporating different combinations of confounding variables were considered. However, no statistical significance was revealed (Figure 1 and Table 4).

5. Discussion

The gradual growth of an atherosclerotic plaque within the arterial wall eventually restricts the blood supply to the myocardium, augmenting the risk of an ischemic event. Although post-MI strategies like pharmacotherapy or coronary revascularization methods improve cardiac

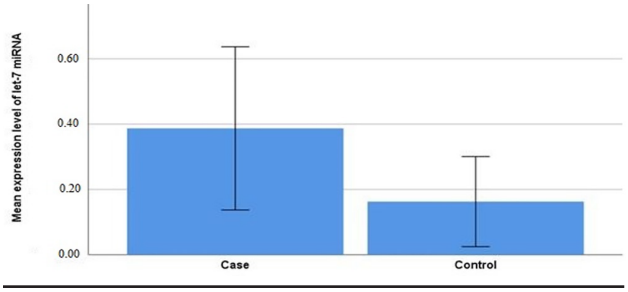

Figure 1. Expression Levels of Let-7 miRNA in the Case and Control Groups.

function to some degree, atherogenesis still remains the fundamental problem (16). Indeed, about 70% of the cardiomyocytes undergo necrosis during the first hours after MI (17). The resulting malfunctions seem to be lifelong and are not restored by the current therapeutic approaches. This shows the value of a biomarker that is capable of diagnosing the presence of atherosclerotic plaques prior to major cardiovascular events.

In humans, only 2% of the genome is translated into proteins, and the remaining majority codes for different types of non-coding RNAs (18, 19). Although non-coding RNAs do not transform into proteins, they are extensively involved in diverse biological processes (2). Based on the size of non-coding RNAs, they are categorized into long and small (5). Long non-coding RNAs (>200 nucleotides) have been subjected to less selective pressure, and hence, they have less conservative sequences (20, 21). On the other hand, miRNAs (20-25 nucleotides) are the evolutionary conserved ones. The general function of miRNAs is the posttranscriptional silencing of target mRNA (22, 23). The level of miRNA expression, which reflects the extent of its activity, is easily measurable in different biological fluids such as blood, saliva, and urine by routine laboratory tests (24, 25).

All stages of atherosclerosis are controlled by miRNAs (26, 27). The current study provides insights into the potential diagnostic or even prognostic potential of circulating let-7 miRNA in atherosclerosis. In particular, the footprints of let-7 family members are found in different stages of atherosclerosis, including cell proliferation, angiogenesis, and immunotolerance (28, 29). Let-7 miRNA is also involved in inflammatory activation of macrophages (30). Through minimally invasive blood sampling, the expression level of let-7 miRNA was compared between CAD patients and healthy peers in our study. Molecular measurements showed that the let-7 miRNA expression level was higher in CAD patients, though statistically insignificant. Similar to our finding, let-7 miRNA had higher expression in patients with heart failure among 11 circulating miRNAs, though statistically insignificant. Most of the examined miRNAs were expressed at lower levels, which may be due to their increased uptake by recipient cells or diminished release from the producers. This downregulation was declared to be associated with high levels of inflammatory-, angiogenic-, and endothelial impairments in different manifestations of atherosclerosis like CAD, transient ischemic attack, stroke, and peripheral arterial disease. The authors stated that downregulated miRNAs are good predictors of cardiovascular readmission after heart failure. However, it should be noted that some miRNAs remained unchanged or overexpressed (e.g., miR-16-5P and miR-423-5p) in patients with CAD (31).

On the contrary, another study reported the downregulation of three miRNAs, including let-7f, in patients with coronary atherosclerotic plaques (16). Also, expression of let-7f was lower in patients with diabetes, who are prone to atherosclerosis. Intriguingly, the let-7 level became normal after 12 months of antidiabetic therapy (32). Other than diabetes, it was shown that other cardiovascular risk factors like high plasma glucose and cigarette smoking decrease let-7f (33, 34). In our population, although the prevalence of diabetes was not different between groups, fasting blood sugar and smoking were more common in CAD patients. It was shown that overexpression of let-7 family members reduces ROS production and oxidizes LDL, leading to apoptosis decline in endothelial cells. Also, let-7 is effective in decreasing senescence (35). In return, oxidized LDL downregulates let-7 in the endothelial cells. Experimental administration of let-7 decreases proliferation rate while increasing the migration of endothelial cells (36). All in all, members of the let-7 family protect the endothelium from being dysfunctional.

Because miRNAs play vital roles in intercellular communication and modulation of inflammatory processes in physiological or pathological conditions, they are suitable candidates for detecting plaque growth even in early phases (37, 38). Both circulating and cellular miRNA levels are in association with clinical subtypes of acute coronary syndrome (39, 40). In one study, three miRNAs including miR-21, -92a, and -99a were highly expressed in atherosclerotic plaques. Noteworthy, they were closely related to the activity of genes involved in atherogenesis. Hence, they could be potent targets for reducing the severity of atherosclerosis (16). For instance, since miR-92a modulates the proliferation of endothelial cells and inflammation (41), prevention of atherosclerosis and ischemic injury seems feasible via locked nucleic acid-modified antisense miR-92a (42).

The discovery of miRNAs has entirely changed how we look at gene expression and its regulation. However, there is a long road to completely elucidate the mechanisms of regulation made by miRNAs as well as their exact functions. Once the role of miRNAs is clearly elucidated during atherosclerosis, a better understanding of the most important contributors of plaque growth and rupture would be in hand, which in turn helps to design more efficient medications and precise therapeutic strategies to decrease the huge burden of MI (16). Given that microRNA expression profiles differ between subjects with atherosclerotic plaques and healthy peers, a suitable strategy to inhibit the progression of atherosclerotic lesions would be interfering with miRNA expression. However, the cost-effectiveness of this approach remains in doubt (16).

The limitations of the present study should be acknowledged. The sample population was relatively small, which is the cause of statistical insignificance in differential gene expression of let-7 miRNA between patients with CAD and healthy peers. Also, assigning someone to the case or control group was a clinical decision made by an interventional cardiologist and mainly depends on the gross observation of coronary arteries besides the physician's experience, whereas our study deals with the expression of a miRNA that even tiny and microscopic plaques may change. This may lead to imprecise groupings of participants. It would be better if patients with CAD were further classified based on the stenosis severity, and differential gene expression was compared among the subgroups.

5.1. Conclusion

The expression of let-7 miRNA was higher in patients

with coronary atherosclerotic plaques. This differential gene expression helps to provide a substrate for early diagnosis, treatment, and even designing preventive strategies.

5.2. Ethical Approval

This study was approved under the ethical approval code of IR.SUMS.REC.1398.003 by the Ethics Committee of Shiraz University of Medical Sciences.

5.3. Informed Consent

All participants signed an informed consent form.

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Authors' Contribution

M.J.Z. and I.R.J. contributed substantially to the concept and design of the study. A.K.A., M.D., and Z.J. had roles in data acquisition and analyses. I.R.J. drafted the first version. All the authors revised the manuscript and approved the final version.

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The authors have no financial interests related to the material in the manuscript

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