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Original Article

Development of a New Framework for Health Assessment in Patients With Coronary Artery Disease by Using microRNA-197 in Iranian Adults

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ABSTRACT

Background: Coronary artery disease (CAD) refers to stenosis or obstruction in a part or all of a coronary artery due to atherosclerosis or clotting. This study aimed to evaluate the possible efficacy of the serum microRNA-197 (miR-197) as an indicator of diagnosis in patients with CAD.

Methods: In this study, 100 patients with CAD who had angiography and vascular transplantation were selected and evaluated. The expression level of miR-197 was determined via the real-time RT-PCR technique and the SYBR Green method. For the analysis of the miRNA expression level and the significance of the patient sample, the *t* test was used. Additionally, the Pearson correlation coefficient test was utilized to determine the relationship between the expression levels of miRNAs and CAD severity.

Results: A positive correlation was observed between miR-197 expression and CAD severity. The average expression of 0.78 in the control sample was increased to 2.76 according to the severity of involvement in the patient. In other words, the relative expression of miR-197 in the CAD $^{+}$ group was significantly increased compared with the control group (P<0.004).

Conclusions: It appears that miR-197 can be considered an indicator of coronary endothelial cell function, and it is possible to use it as a biomarker for the prognosis, control, or treatment of CAD. ***(Iranian Heart Journal 2022; 23(1): 17-24)***

KEYWORDS: miR-197, Coronary artery disease, Real-time RT-PCR, U6 snRNA

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Coronary artery disease (CAD) refers to narrowing or obstruction in all or a part of a coronary artery due to the process of atherosclerosis or the presence of a clot. [1,2] There are several ways to diagnose, detect, track, and control CAD. In this regard, the use of anticoagulants, percutaneous coronary intervention, coronary stenting, coronary artery bypass graft surgery, and the transplantation of parts of the saphenous vein

can be considered. These treatments are, however, invasive, dangerous, and costly. [3, 4] Therefore, it seems necessary to search for new methods for the identification of susceptible individuals with stenosis. To that end, a study was conducted on microRNAs (miRNAs), an important family in regulating the expression of genes. [5] Noncoding RNAs with a length of 19 to 24 nucleotides that regulate the expression of target cell messenger RNAs (mRNAs), miRNAs are involved in various cellular bioassays, including differentiation, proliferation, apoptosis, metabolism, regulation of gene expression, and even neoplasia. [6, 7]

Several studies have been conducted on the possible role of miR-197 in cardiac disorders. The results of an investigation showed an increased miR-197 level in the bloodstream of patients with severe symptoms of coronary artery stenosis; in addition, significant elevations of miR-197 were associated with coronary artery inflammatory reaction and platelet activation. [8]

Interleukin-22 (IL-22), produced by Th1, Th17, and Th22 cells, plays an important role in the pathogenesis of T-cell-mediated inflammatory diseases. This interleukin can increase the expression of miR-197 by binding STAT3 phosphoric to the miR-197 sequence promoter, as well as the IL-22 receptor itself, a direct target for miR-197. Consequently, miR-197 controls the IL-22 inflammatory signal. [9]

Another study investigated the possible relationship between miR-197 and inflammatory factors and found a 2-way regulatory pathway between inflammatory signals IL16/STAT3 and miR-197. Based on the results of that investigation, IL-16 could induce the expression of STAT3 at the level of protein synthesis (not at the mRNA level), and there was a bilateral regulatory relationship between miR-197 and IL-16/STAT3 inflammatory signals in the cell. [10]

In several studies, the interaction between this miRNA and various tumor suppressors has also been studied. For example, this miRNA, through its direct effect on the 2 NOXA and BMF proteins, has reduced the process of induction of P53 and facilitates cell proliferation. Therefore, increasing the expression of this miRNA not only induces cellular proliferation but also causes the death of the programmed cell to stop. [11]

Another study reported that epigenetic, transcriptional, and metabolic alterations were modulated by pioglitazone through the miRNA/mRNA networks, previously not associated with pulmonary arterial hypertension/right ventricular dysfunction. Further, pre-miR-197 and pre-miR-146b repressed the genes that drive FAO (Cpt1b and Fabp4) in primary cardiomyocytes. [12]

Kawasaki disease is the main cause of acquired heart disease in children. A previous investigation suggested the serum exosomal miR-197 level as a diagnostic biomarker for Kawasaki disease. Based on the results of that study, the miR-197 set could distinguish patients with Kawasaki disease from other febrile patients, as well as healthy individuals, in a single pass, with a minimal rate of false positives and negatives. [13]

Cardiometabolic risk factors are heritable, and they cluster in individuals. A previous investigation revealed that cardiometabolic risk factors were associated with multiple shared and unique mRNA and miRNA signatures. Moreover, miR-197-3p was associated with 4 cardiometabolic traits, and this implicated transcript might play causal roles in cardiometabolic risks or be the downstream consequences of cardiometabolic risk factors in the transcriptome. [14]

Extensive research has also been conducted on the molecular and regulatory roles of miR-197 in all types of neoplasia, from breast cancer and colorectal cancer to pancreatic cancer. [15-17]

Accordingly, it seems necessary in the framework of an investigation to compare the expression level of miR-197 between patients with coronary artery disease (CAD)

and healthy subjects with a view to finding potential biomarkers for prognosis, primary diagnosis, and treatment.

METHODS

Study Area

This research, conducted in Imam Ali Cardiac and Vascular Research Center, Kermanshah, Iran, from February 16, 2018, through December 2019, assessed the expression level of miR-197 in patients referred for angiography (percutaneous coronary intervention) and coronary artery bypass graft surgery due to CAD and healthy individuals.

Study Population

This study was performed on 100 patients with CAD and coronary artery stenosis with varying degrees of vascular involvement as the case group (CAD^+) and 30 individuals without coronary artery stenosis as the control group (CAD−).

All blood samples from the CAD^+ patients were collected in nuclease-free tubes containing the sterile EDTA anticoagulant (ethylenediaminetetraacetic acid). The samples were immediately stored at −80 °C until the test. In addition, the demographic characteristics of the patients were registered.

Extracting miRNAs and Primer Design

The extraction of miRNAs was carried out on peripheral blood plasma samples using the Favorgen miRNA isolation kit (Cat No, FAMIK001), capable of extracting small RNAs of less than 200 bp (including miRNAs). This method was based on the selectable coupling of the RNA molecules of different sizes to the fiber matrix based on a silica-based fiber matrix in the presence of a chaotropic salt at various times. The purity and concentration of these miRNAs were measured using a spectrophotometer ultraviolet (absorption =260 nm and 280 nm) using the NanoDrop (BioTek Instruments, Inc, US). The RNA samples showed high purity (>1.9 at A260/280). The miRNAs were then stored at −80 °C until the complementary DNA (cDNA) synthesis stage. An NCBI nucleotide database was used to obtain a favorable primer whose target areas had the least similarity with other sequences. Thereafter, the protected areas were determined with the Multiple Alignment and MEGA4 software, and primers were designed with the Allele ID7 software. The sequence of the primers used for miR-197 consisted of forward: 5´-TGA TGA CCC CAG GTA ACT CT-3´and reverse: 5´-GCG AGC ACA GAA TTA ATA-3´. Additionally, the U6 snRNA primer as the housekeeping gene consisted of forward: 5'-CTC GCT TCG GCA GCA CA-3´ and reverse 5'- TGG TGT CGT GGA GT-3´.

Synthesis of cDNAs From miRNAs

The synthesis of cDNAs from miRNAs was performed in 2 steps. The first step involved the application of the poly-A tail to the 3´ end of miRNAs with a poly-A polymerase enzyme. The polyadenylation reaction consisted of a 10X buffer, the poly-A enzyme, ATP, and RNA, with a final volume of 20 µL. Next, the reaction was incubated at 37 °C for 10 minutes. In the next step, the synthesis of cDNAs was performed by using 2 primers: 1 reverse primer (viz, oligo dT-VN as the primer adapter [QIAGEN]) and another direct primer as the specific primer similar to the miRNAs sequence (QIAGEN). For this purpose, the dNTP mix, (M-MLV) the RT enzyme, the RNase inhibitor, the DEPC water, and the corresponding buffer were used. Eventually, the single-stranded DNA of the miRNA supplement was made up to a final volume of 20 µL at 42 °C for 60 minutes. After cDNA synthesis, the temperature was adjusted for 5 minutes at 95 °C to disable the RT enzyme and incubate and complete the reaction.

Real-Time Polymerase Chain Reaction (RT-PCR) Measurement

In the next step, the RT-PCR test was employed to measure the miRNA expression level using 2 primers. The forward primer was similar to the specific sequence of miR-197 and the reverse primer was the complement of the unique Oligo dT-VN primer. Each sample was measured at 1 µL from cDNA to a 10 ng concentration and duplicated in the AB Applied Biosystems StepOne Real-Time PCR System. For the normalization of the assay at each stage, U6 snRNA was used.

The reaction to measure this miR-197 in the final volume of 20 µL consisted of 10 µL of the SYBR Green master mix, which was comprised of the Hot Start Taq DNA Polymerase, Mgcl2, dNTP, dUTP, and the corresponding buffer; 1 µL of a forward primer; 1 µL of a reverse primer; 8 µL of cDNA (diluted); and 0.4 µL of the dye fluorescence color ROX. For each sample, the duplicate was used.

The temperature scheduled for the cyber green method consisted of 10 minutes at 95 °C for the activation of the enzyme; then, 40 cycles of proliferation, composed of 10 seconds at 95 °C, 60 seconds at 57 °C for annealing, and 30 seconds at 72 °C for the extension.

In each run, a sample was evaluated as a negative control to determine the Master Mix contamination and internal and positive controls. Optical data from the device were entered into the LinReg PCR software, version 11. For each sample (inclusive: internal control, normal, and target) the amplification plot, CT (the cycle that interrupts the curve of the threshold line), ΔCT (Ct difference between the specimen desired and the internal control), ΔΔCT (ΔCT difference between the specimen or the control patient and caliber samples), fold change ($2^{-\Delta\Delta CT}$), the melting curve, and the mean PCR efficiency were determined. Finally, based on the Livak formula, the expression ratio of miR-197 in the patient sample was determined in comparison with the normal sample.

Statistical Analysis

The data were analyzed with the SPSS vol.16 software. The diagnostic value of serum miRNA was found to differentiate patients from control subjects. The data analyses were performed using the Pearson correlation analysis to determine the relationship between miR-206 expression and CAD severity. The *t* test was also used to determine the significance of the expression of miR-206 in the 4 groups with CAD, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

The expression levels of miR-197 in all the patient samples (CAD^+) showed a significant increase in comparison with the healthy group (CAD−) ($P<0.02$) (Table 1).

Further, according to the results in 100 patients, the expression of miR-197 was significantly and directly related to the percentage and number of involved vessels, indicating that the expression of miR-197 with respect to CAD increased from 1.08 to 2.76, as compared with the control group (0.78). Out of the total CAD^+ patients, 28 patients had single-vessel disease, 17 double-vessel disease, 52 triple-vessel disease, and 3 major coronary artery stenosis. The mean serum level of the expression of miR-197 was 1.08 in patients with single-vessel disease (mean age =58 y; 95% confidence interval [CI], 0.31 to 0.88), 1.64 in patients with double-vessel disease (mean age =60 y; 95% CI, 0.33 to 0.96), 2.14 in patients with triple-vessel disease (mean age =65 y; 95% CI, 0.34 to 1.04), and 2.76 in patients with multiple-vessel disease (mean age= 64 y; 95% CI, 0.55 to 1.42). The differences between the groups constituted statistical significance based on the Pearson correlation coefficient ($P=0.001$).

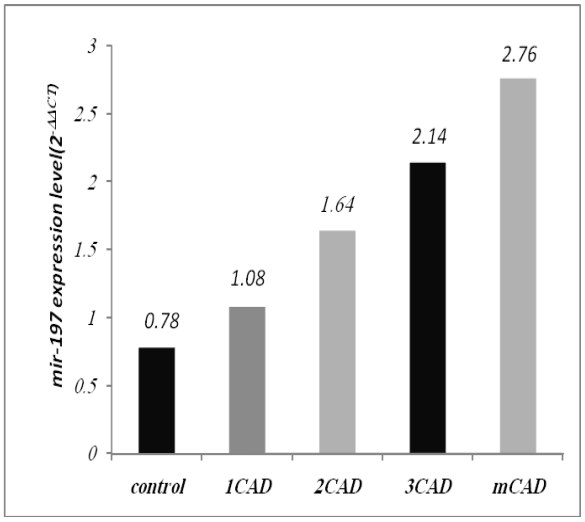

Figure 1. The diagram illustrates the relative expression level of miR-197 according to the number of coronary arteries involved.

1CAD, Single-vessel disease; 2CAD, Double-vessel disease; 3CAD, Triple-vessel disease; MCAD, Multivessel disease

Concerning biochemical variables, the mean cholesterol level was 153±22 mg/dL in the control group (CAD−) and 190±45 mg/dL in the case group (CAD^{+}). Despite the rise in cholesterol levels, the results of the *t* test showed no significant difference regarding the mean serum cholesterol level between the control and case groups ($P<0.407$).

Table 1. Expression levels of miR-197 and CAD types

Types of CAD	Expression Levels of miR-197	*P*-value
Single-vessel disease	1.08	<0.059
Double-vessel disease	1.64	<0.012
Triple-vessel disease	2.14	<0.005
Multivessel disease	2.76	<0.004
Healthy controls	0.78	_

CAD, Coronary artery disease

Table 2. Comparison of biochemical data between the CAD+ and CAD− patients

Factors	CAD^{+}	CAD−	*P*-value
Sex (male/female)	87/13	16/14	-
Age (y)	57±9	55±8	0.364
BMI (kg/m^2)	27.78±3.45	27/45±2.09	0.228
TC (mg/dL)	190±45	153±22	0.407
LDL (mg/dL)	129±31	100±13	0.733
HDL (mg/dL)	33±3	30±2	0.278
TG(mg/dL)	188±49	145±18	0.320

BMI, Body mass index; TC, Total cholesterol; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; TG, Triglyceride

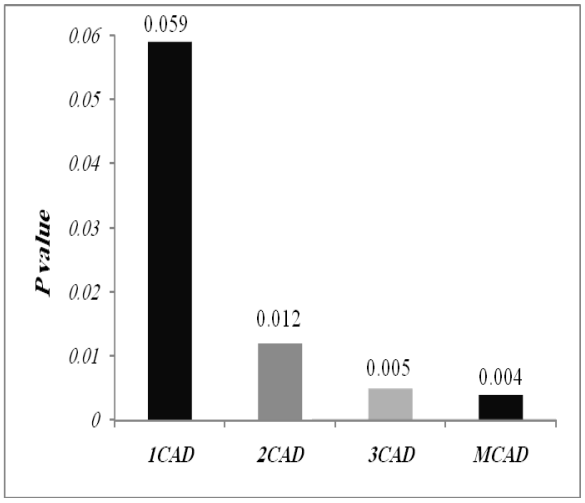

Figure 2. The image depicts the distribution of *P*-values and significant levels of the clinical data of patients with CAD based on the number of vessels involved.

CAD, Coronary artery disease; 1CAD, Single-vessel disease; 2CAD, Double-vessel disease; 3CAD, Triple-vessel disease; MCAD, Multivessel disease

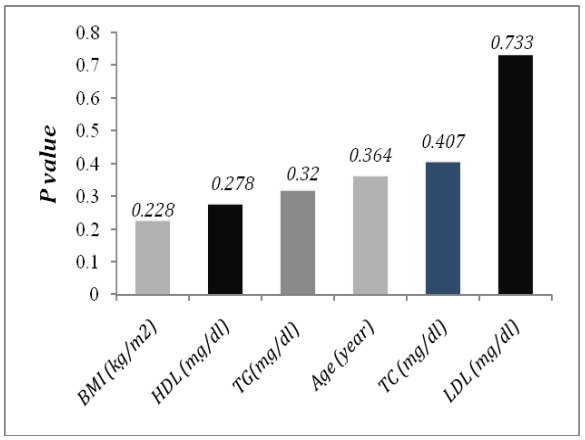

Figure 3. The image illustrates the distribution of *P*-values and significant levels of the biochemical variables and their correlations with coronary artery disease.

BMI, Body mass index; TC, Total cholesterol; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; TG, Triglyceride

DISCUSSION

Over the past few years, researchers have focused on miRNAs at the cellular and molecular levels and their potential roles in various disorders and diseases. [18] In this study, we sought to evaluate the possible efficacy of the serum miR-197 as an indicator of diagnosis in patients with CAD. The most precise method for checking this category of miRNAs is the SYBR Green color, which can bind to 2-stranded DNA, to provide a suitable fluorescence signal, and to show proliferation during RT-PCR. [19, 20]

Our comparison of CAD− and CAD^{+} cases revealed different expression levels of miR-197 and different pathological coronary artery outcomes (the percentage of obstruction to the number of vessels involved). We performed the Pearson correlation analysis to investigate the relationship between miR-197 expression and CAD and obtained a correlation coefficient of between 0.26 and 0.30, indicating a positive correlation between the increase in the severity of vascular lesions and the increase in the miR-197 expression level. The elevation in the expression of miR-197 had a significant and direct correlation with the severity of CAD. Additionally, our *t* test for determining the significance of the miR-197 expression level and the severity of CAD in the 4 groups of CAD yielded a statistically significant difference (P<0.02). Some risk factors are considered important vis-à-vis CAD development and are, therefore, routinely evaluated in patients with cardiovascular disease. The factors include total cholesterol, low-density lipoprotein, triglyceride, body mass index, and age. In the present investigation, with the exception of high-density lipoprotein, which showed a decrease and an inverse correlation, the other parameters exhibited a positive correlation and an increase in regard to the expression level of miR-197. However, despite the increased expression level of these routine parameters in the 4 groups of patients, with the involvement of 1, 2, 3, and multiple coronary arteries, this increase was not statistically significant. It may be

concluded that despite the relative increase in the expression level of miR-197 and even a small rise in the level of the aforementioned common risk factors, coronary artery lesions can be observed. Therefore, there seems to be a significantly positive correlation between the miR-197 biomarker and CAD development.

CONCLUSIONS

The increase and specific changes in miR-197 in coronary artery lesions may well reflect changes in the endothelial function of coronary arteries in patients with CAD. This miRNA can, therefore, be considered a potential biomarker for detecting, tracking, and controlling the status of coronary artery endothelial cells in this category of patients. Nonetheless, the more important point is that the expressive and meaningful expression of miR-197 may be an important indication for diagnosis before any clinical-pathological symptoms appear in cardiac patients.
Further research on the entire miRNA panel, including miR-197, can assist us in the early detection of this type of cardiovascular disorder.

Conflict of Interest

The authors hereby declare no conflicts of interest.

Ethical approval: This study was approved by the Ethics Committee of Islamic Azad University, North Tehran Branch, Tehran, Iran. The study protocol was also approved by the Ethics Committee of Behavioral Research, Kermanshah University of Medical Sciences, Iran.

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