Journal of Pharmaceutical and Biomedical Analysis 134 (2017) 11–17

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Comparison of miRNA signature versus conventional biomarkers before and after off-pump coronary artery bypass graft

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ARTICLE INFO

Article history:
Received 6 August 2016
Received in revised form 24 October 2016
Accepted 9 November 2016
Available online 10 November 2016

Keywords:
Cardiac biomarkers
MicroRNAs
Off-pump coronary artery bypass surgery

ABSTRACT

Circulating levels of microRNAs (miRNAs) and their expression patterns are supposed to serve as signatures for diagnosis or prognosis of cardiovascular events. The present study aimed at determining if there is any correlation between the release pattern of 2 miRNAs and the plasma levels of conventional biomarkers cardiac troponin I (cTnI), creatine kinase (CK) and uric acid (UA) in patients undergoing their first off-pump coronary artery bypass graft (OCABG). Seventy OCABG patients (69% men, aged 59.2 ± 8.2 years) were enrolled. Emergencies, re-operations, abnormal preoperative serum cTnI and combined procedures were excluded from this study. Pre-operative mean ejection fraction was 45.8 ± 8.6%, the average number of grafts was 3 ± 0.87/patient, and the internal mammary artery was used for all. Beside conventional clinical assays, we performed real-time quantitative PCR to analyze the circulating levels of miR-155, miR-126 and miR-499 at 1 day before surgery as well as 4 days after surgery. Importantly, there was no report of myocardial infarction in our patients, pre- or post-operatively. In contrast to conventional biomarkers cTnI and CK, circulating levels of miRNAs decreased significantly ($P < 0.01$) after revascularization surgery. A significant positive correlation was seen between the cTnI and miR-499 ($r \simeq 0.53$, $P < 0.01$) and between miR-126 and UA ($r \simeq 0.5$, $P < 0.01$). Time course study of circulating miR-499, miR-126 and miR-155 in cardiac surgery clarified their advantage and correlations to the traditional biomarkers cTnI, total CK, CK-MB and UA. Our results suggest that this signature is a novel, early biomarker which indicates myocardial ischemia in cardiac surgery. It could be postulated that the application of these miRNAs may be considered for monitoring of response to pharmacological interventions aimed at reducing cardiac ischemia, especially in OCABG candidates.

1. Introduction

Circulating levels of miRNAs have been investigated in several cardiovascular diseases, such as stable and unstable coronary artery disease, acute and chronic heart failure, arrhythmias and thromboembolism. Vascular or myocardial-derived miRNAs are supposed to be used as novel biological markers of cardiovascular injury and vasomotion disorders that are involved in pathogenesis of cardiovascular, metabolic, and inflammatory diseases. Circulating levels of miRNAs are thought to reflect a balance between cell stimulation, proliferation, apoptosis, and cell death. This study aimed to reveal any association between plasma concentrations of cardiac conventional biomarkers and miRNA signature miR-155, miR-126 and miR-499 in patients who are candidate for CABG, and to assess this signature for risk stratification [1–4].

It has been shown that in patients with CAD or acute coronary syndrome, miRNA signatures are expressed differently when compared to healthy subjects [3–6]. Data attribute these differen-

Abbreviations: miRNAs, microRNAs; cTnI, cardiac troponin I; CK, creatine kinase; UA, uric acid; OCABG, off-pump coronary artery bypass graft; CAD, coronary artery disease; CRP, C-reactive protein; ECs, endothelial cells; SDF-1, stromal cell-derived factor 1; EF%, ventriclular ejection fraction.

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http://dx.doi.org/10.1016/j.jpba.2016.11.014
0731-7085/

tially expressed signatures to the severity of endothelial function in atherosclerosis and myocardial injury during ischemia [1–3,7]. Indeed, patients with higher levels of circulating miRNAs are supposed to have a higher risk for future cardiovascular events [1,6,7]. This applies specifically for those miRNAs which are mostly derived from the activated or apoptotic cells in cardiovascular system [1,3,8]. For example, miR-155, miR-126 and miR-499, mostly derived from activated or apoptotic endothelial cells or leukocytes in cardiovascular system, are supposed to play key roles in regulation of several key signaling pathways, such as transforming growth factor-ß, toll-like receptor-4 and hypoxia-inducible factor-1α (HIF-1 α) pathway and regulating a number of human cardiac ion channels including the L-type Ca^{2+} channel and the K^{+} channels [4–8,9–12].

It is supposed that increased levels of miR-155 and miR-126 may explain pro-inflammatory and pro-thrombotic state of atherosclerosis, and may indicate an urgent need for reparative procedures for re-establishment of perfusion in coronary arteries [2,6,8].

On the other hand, plasma levels of biomarker UA and cardiac risk indicator CRP have been associated with endothelial dysfunction and platelet activation which collectively predict poor outcomes in CABG [13–17,18]. Both UA and CRP have been associated with generation of free radicals which impair endothelial function and lead to platelet activation and higher levels of inflammatory markers [13,16,17–20]. From the literature, it may indirectly be assumed that circulating levels of miRNA signature miR-126, miR-155 and miR-499 could be associated with circulating levels of UA and CRP [3,4,21–24]. Concomitantly, in the context of ischemia condition, circulating levels of miRNAs may also correlate with the cardiac biomarkers cTnI, CK and CK-MB [3,6–8,13–15].

Therefore, beside conventional clinical assays, the present study performed real-time PCR assays to analyze the relative expression levels of miRNA signature miR-126, miR-155 and miR-499 in plasma of CAD patients undergoing OCABG surgery to confirm the aforementioned relationships.

2. Materials and methods

2.1. Instruments, materials and reagents

The Medtronic Octopus device for heart surgery was sourced from Medtronic Inc. (Minneapolis, Min, USA); the cTnI-assay Stratus II system was purchased from Dade Behring Inc., Germany; the ADVIA 1650 General Chemistry Analyzer was from Siemens (Germany); EDTA-coated Venoject tubes were obtained from Terumo Corporation, Japan; the cTnI-electrochemiluminescence immunoassay (ECLIA) kit and Elecsys 2010 system were from Roche Diagnostics Inc. (Basel, Switzerland); serum total CK and CK-MB immunoinhibitory assay kits were also made by Roche Diagnostics Inc.; the mirVana PARIS kit was obtained from Ambion Inc. (Warrington, UK); DNA-free kit was sourced from Ambion Inc. (USA); qRT-PCR using BR SYBR-green supermix for iQ was purchased from Quantabio (MA, USA) and used in a MyiQ iCycler device from Bio-Rad Inc. (USA); RNA extract (15 μL) was used per 20 μL reaction to generate cDNA using the miScript kit obtained from Qiagen (USA). Molecular data were analyzed using the iCycler device, Bio-Rad Inc. (USA).

2.2. Participants and study design

The study population included 70 adult patients referred for elective OCABG surgery to the Afshar Hospital affiliated to Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Patients aged 40–79 years and were consecutively enrolled in a cross sectional setting. Emergency cases, reoperations, abnormal preoperative cTnI and combined procedures were excluded from the study. Patients were also excluded if found ineligible for OCABG (e.g. hemodynamic instability, target distal vessels <1.5 mm, vessels inadequately visualized, or heavily calcified vessels). Those patients with severe lung, renal or aortic disease, or with history of prior OCABG, or with any other severe cardiac or medical condition didn't enter the study. Baseline clinical data including medical history, cardiac risk factors, operative details, New York Heart Association functional class, and the European System for Cardiac Operative Risk Evaluation (EuroSCORE) were collected pre-operatively by an experienced full-time physician. Blood samples were collected on the day before CABG surgery and on day 4 post-surgery. All samples were immediately centrifuged for 10 min at 850g, and plasma stored at −80 °C for miRNA analysis [25].

2.3. Surgical procedure

Anesthesia and medications were according to a general procedure previously described [18]. The revascularization was performed on the beating heart using the Medtronic Octopus device, using intraluminal shunt during procedure to decrease the acute ischemic insult to myocardium. Most patients had at least one arterial graft (left internal thoracic artery, LITA). In all patients, the left anterior descending (LAD) was revascularized first, using the LITA, then the left-sided grafts followed by the right-sided grafts. Patients were fully heparinized at completion of harvest of the conduits. In all patients, hypertension was treated with vasodilators nitroglycerin and nitroprusside. All patients received standardized postoperative care [18].

2.4. Ethics statement

The study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and conducted according to the Declaration of Helsinki. In addition, an informed written consent form was signed by each patient prior to the cardiac surgery.

2.5. Serum biomarker assays

The levels of cTnI, total CK and CK-MB in serum were measure at 1 day before operation and also at day 4 post-operatively. Serum cTnI was assayed with the Stratus II system according to the manufacturer's instructions. Activities of CK and CK-MB were measured with an immune-inhibitory assay at 37 °C, by means of an N-acetylcysteine-activated system. Serum UA levels were measured using an enzymatic photometric assay.

2.6. Plasma RNA extraction for miRNA analysis

Venous blood samples from all subjects were collected in EDTA tubes, and total RNA extracted from 100 μL of plasma using the mirVana PARIS kit according to the manufacturer's instructions (without enrichment for small RNAs) before elution in 50 μL of nuclease-free water. Subsequently, potential genomic DNA contamination was eliminated using DNA-free kit.

2.7. Relative expression analysis of miRNAs by real-time PCR technique

Relative expression of miR-126, miR-155 and miR-499 were quantified by RT-qPCR using BR SYBR-green supermix for iQ in a MyiQ iCycler device [9,10], according to the manufacturer protocol. Fixed volumes of starting plasma and buffer for the elution of RNA from each sample were put into the RT reaction in each assay for technical consistency. The RNA extract (15 μL) was used

Table 1
Clinical and some laboratory characteristics of 70 patients at the time of admission.

Number of Patients	Age [year]	Sex	BMI	Hypertension	No. ofGrafts	EF [%]	WBC [10^6/μL]
70	40–70	49 M 21F	24–26	68%	2–3	40–50	7.2–12.1
Mean ± SD	59.2 ± 8.2	69%M31%F	24.1 ± 4.1	68%	3 ± 0.87	44.8 ± 8.6	9.8 ± 3.1

BMI = body mass index; EF = left ventricular ejection fraction; WBC = white blood cells count.

Table 2
Biochemical characteristics of 70 patients at the time of admission (range and mean ± SD).

Total cholesterol [mg/dL]	HDL cholesterol [mg/dL]	FBS [mg/dL]	Urea[mg/dL]	Cr [mg/dL]	UA [mg/dL]	CK [IU/L]	CK-MB[U/dL]	cTnI[ng/mL]
147–210	30–48	98–260	35–56	1.02–1.5	3.3–7.8	45–96	14–20	0.1–0.6
177 ± 35	45.2 ± 12.8	158 ± 70.6	39.7 ± 9.5	1.16 ± 0.18	5.2 ± 0.6	86 ± 42.5	17.2 ± 4.1	0.2 ± 0.3

UA = uric acid; FBS = fasting blood sugar; Cr = creatinine; cTnI = cardiac troponin I; CK-MB = creatine kinase-MB isoenzmye.

per 20 μL reaction to generate cDNA using the miScript kit which is designed to specifically detect mature miRNAs. The 20 μL reaction mix was then diluted × 4 in nuclease-free water, and 2 μL of cDNA was added per RT-qPCR. Data were analyzed with the iCycler device with automatic setting for assigning baseline. All RT-qPCR data were standardized to 5s rRNA which had been validated as an internal standard in patients and fulfilled the following criteria: detectable in all samples, low dispersion of expression levels and null association with CAD. All reactions were run in triplicate, and miRNA expressions were normalized to small RNA U6, with the similar efficiency of miRNAs. Data of RT-qPCR were demonstrated by nominal CT value (normalized to U6). Plasma levels of miRNAs were transformed into quantities using the method $2^{-\Delta Ct}$, and fold changes in miRNA expression were calculated by $2\hat{|}-^{\Delta\Delta Ct}|$ method. Higher nominal CT value means lower miRNA expression level. In addition, miRNA relative expression was calculated using the same method.

2.8. Statistical analysis

All values are presented as mean ± standard deviation. Results of cardiac enzymes, hemodynamic parameter changes, and RT-qPCR results between time intervals were analyzed by paired *t*-test. Spearman correlation coefficients were used to examine the relationship between miRNAs and cardiac biomarkers. $P < 0.05$ was considered as indicative of statistically significant difference.

3. Results and discussion

3.1. Patients' demographics before revascularization surgery

The patients' clinical and laboratory characteristics at admission are shown in Tables 1 and 2. They had a normal (Gaussian) distribution. There was no report of myocardial infarction in our patients, pre- or post-surgery.

3.2. Increased levels of myocardial biomarkers cTnI & CKs after OCABG

Serum concentrations of cTnI and CKs in all patients were within the normal range preoperatively. Both biomarkers showed increase after OCABG surgery. However, uric acid levels fell (Fig. 1).

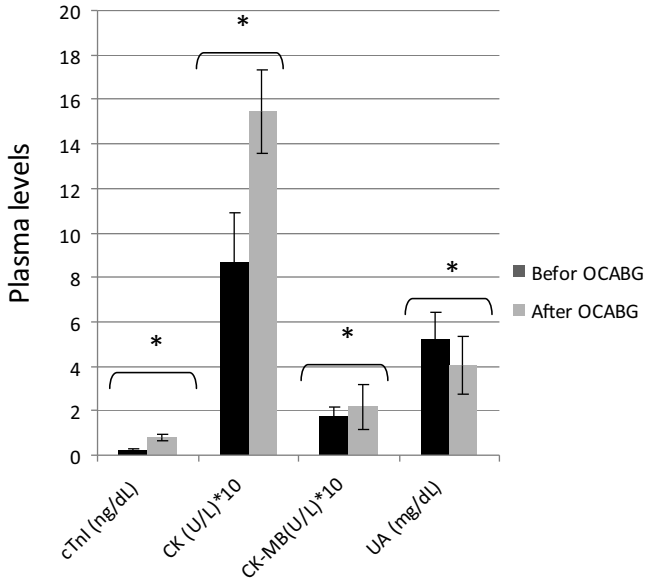

Fig. 1. Plasma profiles of myocardial biomarkers cTnI [concentrations in ng/dL] and CKs [serum-enzyme activity in U/L], beside endothelial biomarker uric acid [UA in mg/dL], on day 1 pre- and on day 4 post-revascularization surgery in 70 patients undergoingt off-pump CABG. Data represent mean values [n = 70]. Error bars represent standard deviation of the mean values [SD].*: $p < 0.05$ for differences between pre- and post-surgery mean values. cTnI = cardiac troponin I; CK = total creatine kinase; CK-MB = creatine kinase-MB isoenzmye; UA = uric acid.

3.3. Decreased levels of miR-499/155/126 after OCABG

As is seen in Fig. 2C, there was a significant reduction in plasma levels of these miRNAs in all patients on day 4 post-revascularization surgery ($p < 0.01$).

The present study provides the first insights into the role of circulating miR-499, miR-126 and miR-155 (here collectively called miRNA signature) in cardiac surgery. Time course study of circulating levels of this miRNA signature in cardiac injury clarified their priority to the traditional biomarkers cTnI, CK, CK-MB and UA for identifying cardiac ischemia in heart surgery. Data analysis identified that circulating levels of miRNA signature appeared earlier than the protein traditional markers during ischemia condition and in myocardium stress. Studies have demonstrated that there are significant expression profile changes in a bunch of miRNAs in ischemic tissues and peripheral blood. Moreover, some miRNAs perform bold biological functions of protecting or damaging cardiac cells in myocardial ischemia through different pathways, which particularly suggests miRNAs as potential candidates in the field of cardiac theranostics **(1–4)**.

3.4. Plasma levels of miR-499 and cTnI specifically correlated

The present work provides the first new insight into the expression profile changes of miRNA signature during OCABG and its correlation to the cardiac injury biomarkers. Plasma concentrations of myocardial biomarker cTnI showed a mild positive correlation with miR-126 ($r \sim 0.30$, $P < 0.01$), while depicting a more strong positive correlation with miR-499 ($r \sim 0.507$, $P < 0.005$) (Fig. 3A).

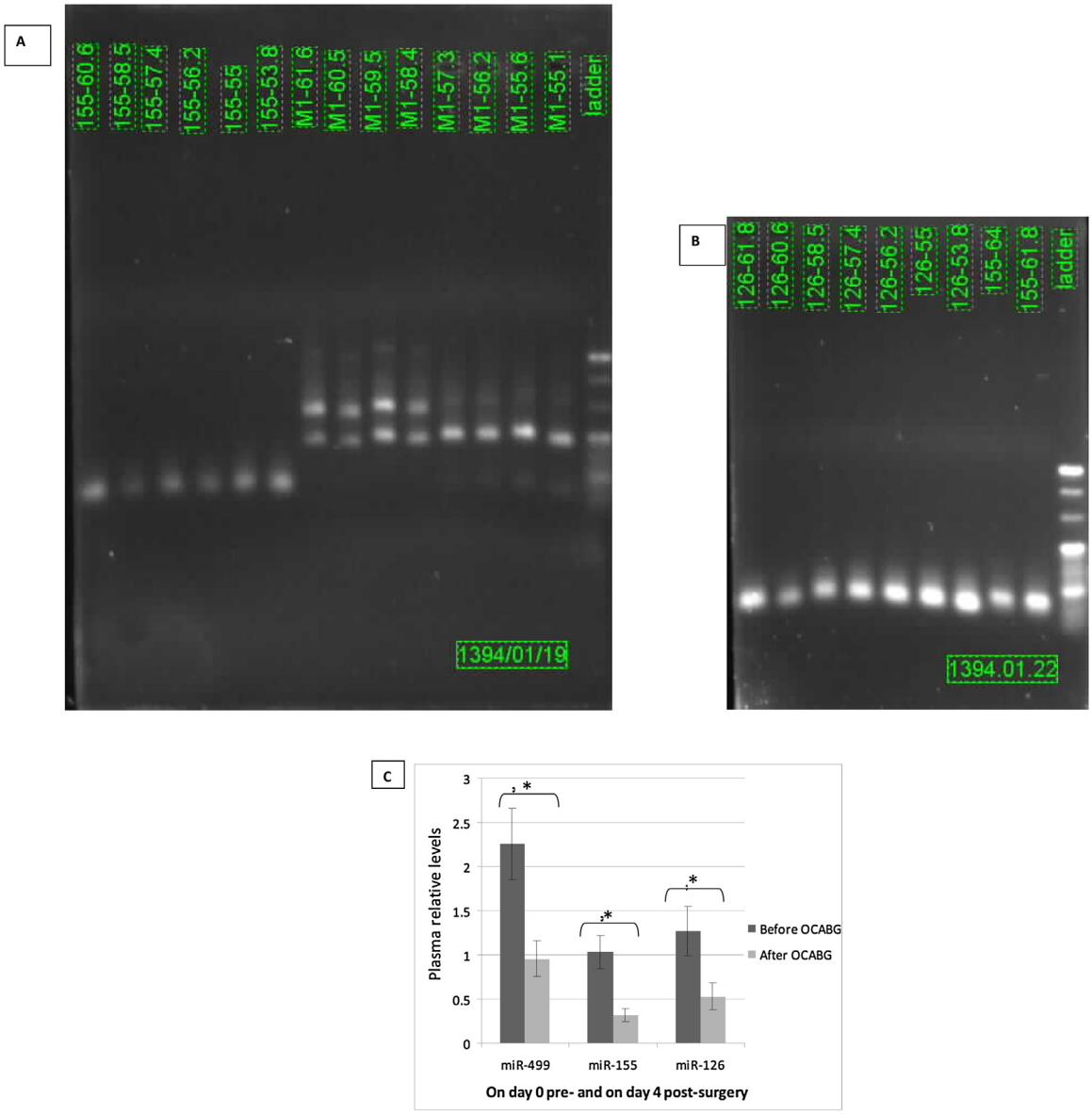

Fig. 2. Relative levels of miR-499, miR-155 and miR-126 in plasma of 70 patients undergoing off-pump CABG. Gel electrophoresis was performed to check RT-qPCR analysis for miRNA expression [A & B]. Plasma relative levels of each miRNA were obtained at two time points on the day before OCABG and on day 4 after OCABG, and compared [C]. Plasma levels of miRNAs were transformed into quantities using the formula $2^{-\Delta Ct}$. Data represent mean values [n = 70]. Error bars represent standard deviation of the mean values ± [SD]. $p < 0.05$, *: $p < 0.01$ for differences between pre- and post-surgery mean values.

Consistent evidence has indicated that miR-499 is produced almost exclusively in the heart and the myocardium [5,18]. Thus, miR-499 can be regarded as a robust biomarker for myocardial injury and function. Plasma levels of miRNAs in patients before revascularization surgery might be ascribed to ischemia conditions, reflecting some aspects of atherosclerosis lesions in CAD patients.

3.5. Plasma levels of miR-126 and miR-499 showed positive correlation

A relatively strong correlation between miR-499 and miR-126 was found ($r \sim 0.66$; $P < 0.01$) (Fig. 3B). Data indicate that circulating levels of miRNAs miR-499, miR-126 and miR-155 are associated with atherosclerotic lesions, and in vulnerable CAD patients are derived from the apoptotic cardiac cells which systemically activate endothelium and stimulate platelets. Differentially expressed miRNA signature miR-155, miR-126 and miR-499 reflects several aspects of inflammation, hypoxia and apoptosis in CAD patients [1–8].

3.6. Plasma levels of miR-126 and UA specifically correlated

Our data indicated a rather strong correlation between miR-126 and UA ($r \sim 0.5$; $P < 0.05$) (Fig. 3A). Both of them are signaling products of atherosclerotic lesions, and can be considered as biomarkers for endothelial injury and function [2,9]. Regardless of how miRNAs enter the bloodstream, miR-155 and miR-126 have been specifically secreted in response to stress such as hypertension and low-grade inflammation in humans, and may be clinical prognostic indicators of target organ damage. There are evidences for a relationship between circulating levels of miR-155 and 126 and severely narrowed coronary arteries in CAD patients [3,8,15,21,26]. Accordingly, during vascular inflammatory reactions or in ischemia/reperfusion injuries, miR-126 is originated from activated endothelial cells (ECs) and is taken up by other cells in other tissues including those in kidney vessels [2,8,22].

On the other hand, UA and CRP are important causal agents in endothelial dysfunction, and impair nitric oxide production by

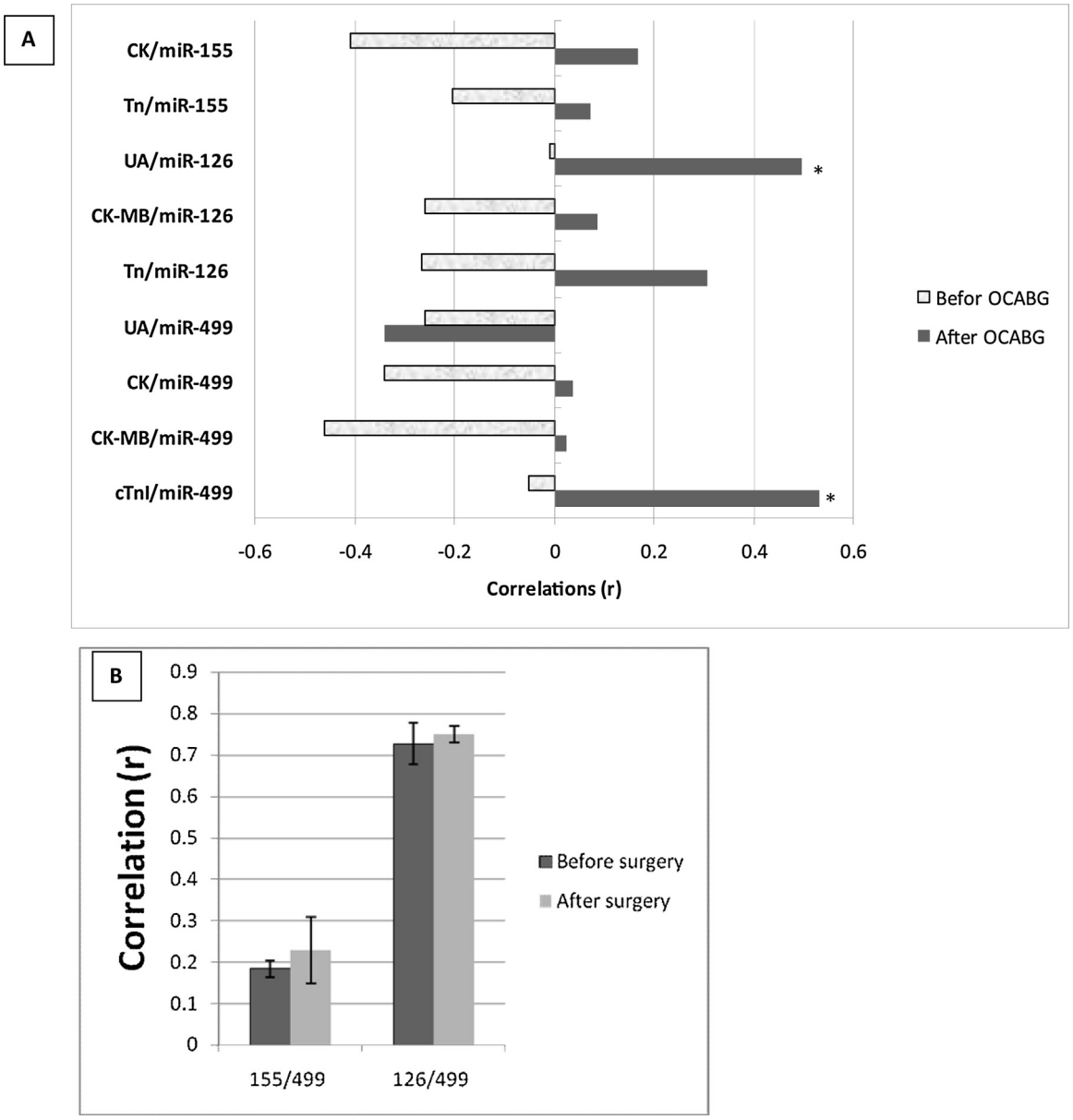

Fig. 3. The correlations between plasma relative levels of miRNAs and circulating levels of biomarkers cTnI, CKs and UA on the day before surgery and on day 4 post-revascularization, in 70 patients undergoing off-pump CABG [A & B]. The plasma levels of miR-499 and miR-126 exhibit more strong positive correlation with cTnI and UA, respectively, than others [A]. * $P < 0.05$ represents a significant correlation between variables, pre- and post-operation [n = 70].

uncoupling of endothelial nitric oxide synthase, thereby hampering regeneration in vascular system [17,20–22,27–30]. Assuming from the literature, increased levels of myocardium-derived miR-499, leukocyte-derived miR-155 and endothelium-derived mir-126 may concomitantly be associated with circulating levels of UA and CRP [3,4,16–18,24].

In patients with established CAD, an association has been clearly established between UA levels and outcome of surgical revascularization. There is a relationship between UA and another major risk factor CRP, and both share similar effects on platelet activation and endothelial function [15–17]. During ischemia, there is a decrease in adenosine triphosphate production due to a decrease in tissue blood flow. By a fall in energy, the cell is no longer able to maintain normal ion gradients across the cell membrane, which leads to swelling and calcium-dependent protease activation, with resultant shift from xanthine dehydrogenase into xanthine-oxidase which successively oxidizes purine bases into xanthine and finally, into UA [16–18].

The explanation may be that miR-126 signals are required for endothelial repair through its transfer from apoptotic/activated ECs into the plasma [3]. In the context of ischemia, circulating level of miR-126 is associated with systemic level of stromal cell-derived factor 1 (SDF-1) and mobilization of progenitor cells into the peripheral circulation. In response to ischemia, ECs present in the ischemic tissue express and secrete elevated levels of SDF-1. SDF-1 or CXCL12 is up-regulated in ECs in a HIF-1α-dependent fashion to facilitate the homing of progenitor cells from the peripheral circulation to the site of injury. Plasma concentration of miR-126 has also been associated with secretion of brain natriuretic peptide and the degree of heart failure [2,9,22].

3.7. Higher levels of miR-499 in CRP-positive patients

The patients were divided into two subgroups based on initial serum CRP status: positive and negative (assuming 12 mg/L as the cut-off between positive and negative). There was no considerable

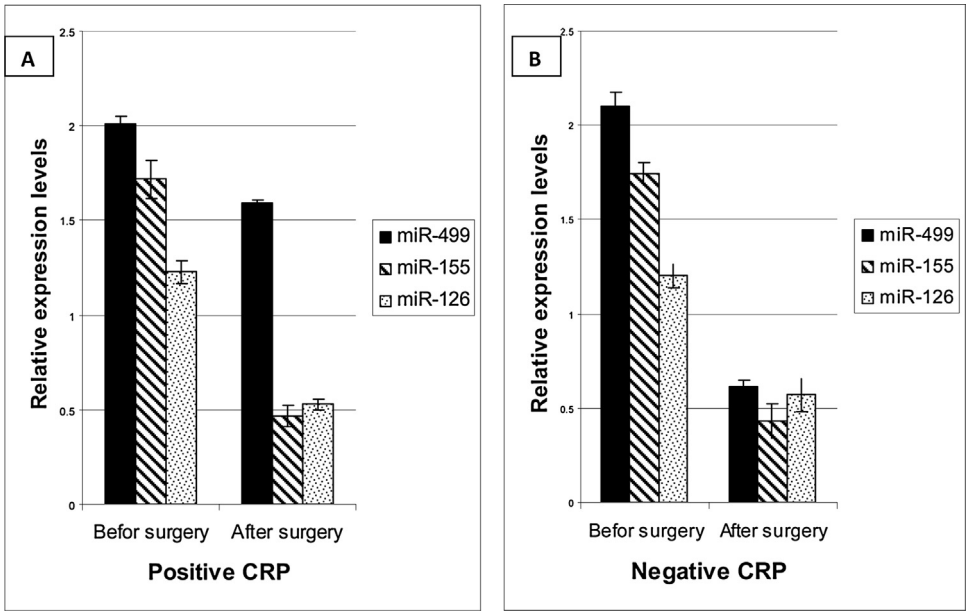

Fig. 4. Expression profiles of miRNAs in plasma of 70 patients undergoing off-pump CABG, were more divided into two baseline subgroups; one positive for CRP [A] and the other negative for CRP [B], as illustrated here. There were no considerable difference between two subgroups positive [n = 26], or negative for CRP [n = 44], regarding relative levels of miRNA expression except for miR-499. However, plasma levels of miR-499 were significantly higher in samples positive for CRP [A] [n = 26], compared to samples negative for CRP [B] [n = 44], $p < 0.01$. Data represent the mean values. Error bars represent standard deviation of the mean values [SD]. $p < 0.05$ shows significant difference between the mean values, pre- and post-revascularization surgery, in general.

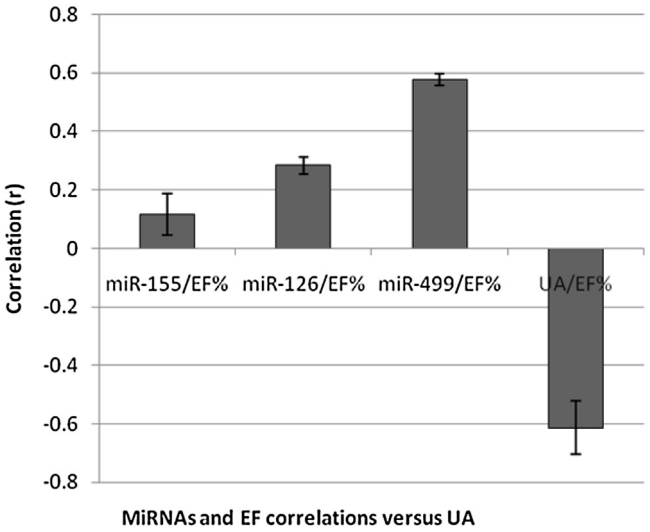

Fig. 5. Correlation between miRNA expression levels and cardiac contractility in plasma of 70 patients undergoing off-pump CABG. In contrast to UA, miRNA expression levels exhibited a positive correlation with ventricular ejection fraction [EF%]. There was a significant ($p < 0.05$) difference between miR-499, miR-126 and miR-155 in this regard. Error bars represent standard deviation of the mean values [SD].

difference in the relative levels of miRNA expression between the two subgroups, except for miR-499. Plasma levels of miR-499 were significantly higher in samples positive for CRP; $p < 0.05$ (Fig. 4A &B).

It is known that CRP accelerates disturbed flow-induced atherosclerosis by enhancing the inflammatory responses, such as inducing the production of pro-inflammatory cytokines [17,18,29,30]. In addition, there seems to be a relationship between plasma levels of CRP and miR-155 and miR-126 in patients with severely narrowed coronary arteries [3,4,18].

3.8. Ventricular ejection fraction (EF%) positively correlated with miRNA levels

In the present study, the ventricle contractility (EF%) showed positive correlations with plasma levels of miRNA signature (miR-499: $r \simeq 0.58$, miR-126: $r \simeq 0.28$, and miR-155: $r \simeq 0.11$, $P < 0.05$). Compared to miR-499, the endothelium- and leukocyte-enriched miRNAs (miR-126 and miR-155, respectively) showed less correlation with EF%. There was also a negative correlation between circulating levels of UA and EF% ($r \simeq -0.6$, $P < 0.05$) (Fig. 5).

It has been reported that elevated levels of UA in circulation are associated with endothelial dysfunction and peripheral arterial disease [15,16]. Plasma miRNA levels act more than being merely a biomarker, and regulate gene expression in cardiovascular system [1–8]. In particular, some of them are highly abundant in cardiovascular system, such as miR-499, miR-155 and miR-126 which have been related to the degree of cell rupture and death caused by a given insult. On the other hand, their extracellular presence reflects response to stress such as low-grade inflammation, and has been associated with regulation of the gene expression in cardiovascular system. Therefore, profiling of miRNA signature in the circulation and its association with cardiac function may uncover miRNA regulatory roles in cardiovascular system [3,4,21,22].

4. Conclusion

Circulating levels of miRNAs are thought to reflect a balance between cell stimulation, proliferation, and cell death. Increased levels of plasma miRNAs have been detected in several disorders such as arrhythmias, thromboembolism, atherosclerosis and dyslipidemia [4,9–12,26]. Some special miRNAs are highly abundant in the heart (e.g. miR-133 and miR-499) and their circulating levels have been related to the degree of cardiac cell rupture and death caused by a given insult [3,6,11,12,14]. Among all miRNAs, miR-155, miR-126 and miR-499 signature has been in particular associated with several aspects of atherosclerosis such as inflammation, hypoxia and endothelial apoptosis. Understanding the role of these miRNAs in CAD conditions sheds light on the current con-

cepts of atherogenesis and may provide novel treatment options for cardiovascular diseases [1–5,7–9,21].

In general, the results obtained here comply with previous studies and imply an association between circulating levels of miRNAs and cTnI, UA and CRP in high-risk coronary patients **(16–18, 21, 22)**.

Generally, the present study has detected, for the first time, an actual influence of treatment (in this case, OCABG) on circulating levels of miRNAs in CAD patients. It suggests that regulation of miRNA levels may be a new therapeutic approach for improving endothelial dysfunction during the course of cardiovascular diseases, with prognostic implications. In contrast to traditional cardiac biomarkers in serum which are affected by thoracotomy, injections, traumas to the chest wall and other musculoskeletal injuries, the serum levels of miRNAs may be a better indicator of myocardial/endocardial ischemia per se. So, further investigations may be recommended to establish practical application of miRNA signatures for monitoring of the course of disease, early diagnosis of changes, and assessment of response to any kind of pharmacologic or non-pharmacologic intervention in patients with heart problems.

The present study provides new insights into circulating levels of miRNA signature; miR-499, 126 and 155 in cardiac surgery. The time course study of circulating levels of this signature in cardiac surgery clarified their correlation and priority to the traditional biomarkers cTnI, CK, CK-MB and UA, in identifying cardiac ischemia in OCABG surgery. It could be postulated that these miRNAs may be considered for monitoring of response to pharmacological interventions aimed at reducing cardiac ischemia, especially in OCABG candidates.

Conflicts of interest

There are no conflicts of interest.

Acknowledgment

We thank the staff in the Afshar heart hospital, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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