

Original Article

Correlation between serum exosome derived miR-208a and acute coronary syndrome

Shaojie Bi¹, Chunyan Wang², Yanwu Jin³, Zhaopeng Lv¹, Xiaowei Xing¹, Qinghua Lu¹

¹Department of Cardiology, Second Hospital of Shandong University, Jinan, China; ²Department of Emergency, Second Hospital of Shandong University, Jinan, China; ³Department of Anesthesiology, Second Hospital of Shandong University, Jinan, China

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Abstract: Objective: To explore the correlation of miRNAs with clinical characteristics of ACS. Methods: 50 ACS patients and 50 healthy controls were randomly selected. On the basis of miRNA expression levels, ACS patients were classified as low miRNA expression group (fold change: <3) and high miRNA expression group (≥ 3). Results: miR-208a expression increased markedly in the serum exosomes of ACS patients, and miR-208a expression in the serum of ACS patients was also significantly higher than that in healthy controls. However, the sensitivity of serum miR-208a was inferior to that of exosome miR-208a. Analysis of clinical characteristics showed the mean age of 500 ACS patients was 62.35 ± 9.70 years, and there were 300 patients in low miR-208a expression group and 200 patients in high miR-208a expression group. When compared with low miR-208a expression group, patients with high miR-208a expression were older, and had higher Killip class, higher CK-MB peak, higher cTnT peak and elevated LDL ($P < 0.05$). Within 1-year follow up, 32 patients died including 10 in low miR-208a expression group with the mortality of 3.3% and 22 in high miR-208a expression group with the mortality of 11.0%. Kaplan-Meier survival analysis revealed that the 1-year survival rate reduced significantly in patients with high miR-208a expression. Conclusion: miRNA-208a expression is significantly up-regulated in the serum exosomes of ACS patients and is crucial for the diagnosis of ACS.

Keywords: Exosomes, miR-208a, acute coronary syndrome, marker

Introduction

Acute coronary syndrome (ACS) and its complications are one of major diseases significantly threatening the human health and increase the family and social burden [1]. ACS includes unstable coronary artery disease (UAP), non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation acute myocardial infarction (STEMI), which share pathophysiological processes: plaque rupture, thrombosis and subsequent complete or incomplete obstruction of blood vessels. Thus, for ACS patients, the pathological progression of coronary artery lesions is very rapid, and to identify markers for the early diagnosis and prognosis is crucial for the clinical therapy of ACS [2]. Traditionally, the severity of coronary heart diseases is determined according to the extent of coronary stenosis and the revascularization. From the view of pathophysiological view, the

stability of atherosclerotic plaques is valuable for prediction of the occurrence and prognosis of ACS. In recent years, studies have revealed that miRNAs are involved in the regulation of atherosclerotic plaque stability [3]. miRNAs are a group of small molecular non-encoding RNA with 18-25 nt in length and widely distributed in eukaryotic cells. miRNAs may degrade target mRNAs or inhibit the translation of target mRNAs to regulate the proliferation, differentiation and apoptosis of cells, involve the development, metabolism and occurrence and development of cancers. Thus, miRNAs are closely related to the occurrence and development of diseases and have been regarded biomarkers for some diseases such as tumors, muscular injury and inflammatory injury [4-6]. Studies have shown that some miRNAs (such as miR-499 and miR-133) may be rapidly released into circulation following myocardial injury and serve as markers for myocardial injury. However, the

Table 1. Quantitative real-time RT-PCR primers

Gene	Primer (5'-3')
miR-208a	GUCCAGUUCAGGAUCCCU
U6	F-CTCGCTTCGGCAGCACA
	R-AACGCTTCACGAATTTGCGT

specificity and sensitivity of these markers are still superior to those of traditional markers [7, 8]. Exosomes have been a hot topic in studies of life science. Exosomes are a group of vesicles with 40-100 nm in size. In 2013, three scientists were awarded Nobel Prize in Physiology or Medicine due to the discovery of mechanisms underlying the regulation of vesicular trafficking [9]. It has been confirmed that exosomes naturally exist in different body fluids (such serum and urine) and contain different substances such as mRNA, miRNA and proteins. Exosomes have been a new hot topic in the screening of markers for cardiovascular diseases and tumors [10, 11]. Whether it is possible to identify miRNAs of serum exosomes closely related to the ACS is still unclear and has never been reported.

This study aimed to compare the miRNA expression profiles of serum exosomes between ACS patients and healthy controls; screen miRNAs with high expression from the serum exosomes of ACS patients; further validate the specificity of these miRNAs in ACS patients; investigate the correlation of these miRNAs with clinicopathological features of ACS patients. Our findings may provide evidence for the early diagnosis and prognosis of ACS.

Subjects and methods

Subjects

From February 2012 to December 2013, a total of 500 patients diagnosed with ACS in our hospital were recruited into present study, and 200 healthy subjects receiving coronary angiography served as controls. Inclusion criteria for ACS: ACS was diagnosed according to the Guideline of American College of Cardiology (ACC) and American Heart Association (AHA), chest pain occurred within 24 h, and revascularization was not performed. Inclusion criteria in control group: subjects had no history of cardiovascular diseases, electrocardiography, chest X ray, detection of liver and kidney func-

tion, biochemical detection were done, and concomitant infection and tumors were excluded.

Collection of clinicopathological features

General information: gender, age; Medical history: hypertension, dyslipidemia, diabetes mellitus, smoking, drinking, and family history of coronary heart diseases (CHD). Indicators on admission: Percutaneous coronary intervention (PCI) was done depending on the diagnosis on admission, and findings were collected from PCI. Detection of blood pressure, heart rate, kidney function, liver function, electrolytes, glucose, lipids and uric acid troponin T (cTnT) and creatine kinase isoenzyme (CK-MB) and routine blood test were performed.

Sample collection and processing

Venous blood was collected and allowed to stay at room temperature for 60 min. After centrifugation at 3000 rpm for 10 min, the supernatant was collected and stored at -80°C for use.

Separation and identification of exosomes

Serum was harvested and centrifuged at 3000 rpm for 15 min. The supernatant was transferred to an aseptic tube, followed by addition of ExoQuick Exosome Precipitation Solution (SBI). Incubation was done for 30 min. Following addition of ExoQuick/supernatant, centrifugation was done at 1500 rpm for 30 min. The supernatant was removed, and centrifugation was done again for 5 min at 1500 rpm to remove residual ExoQuick solution. Protein lysis buffer was added, and BCA method was employed to determine the protein concentration of exosomes. Western blot assay was performed to detect the CD63 expression.

Extraction of RNA from exosomes and miRNA microarray assay

mirVana™ miR isolation kit was used to extract RNA from exosomes and the RNA concentration was measured. The RNA was then subjected to miRNA microarray assay.

Q-PCR

RNA was extracted, and UV spectrophotometry was done to detect the purity and concentration of RNA, which was then used for reverse

Table 2. microRNA microarray assay and screening of miR-208a

	miRNAs	ACS	
		FC	P value
Up-regulated Exo-miRNAs in ACS	miR-21	2.314	0.432
	miR-208a	4.513	0.003
	miR-208b	2.544	0.032
	miR-323	3.347	0.021
	miR-199a	2.844	0.043

transcription into cDNA. SYBR® Premix Ex Taq™ II (Perfect Real Time) kit was used for fluorescence quantitative PCR, and Primer 5.0 software was employed for the primer designing (**Table 1**).

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. Quantitative data are expressed as mean $\pm$ standard deviation. Qualitative data are expressed as percentage. A value of $P < 0.05$ was considered statistically significant. Comparisons between two groups were done with t test, and categorical data were compared with chi square test. COX regression analysis was employed to evaluate the correlation between risk factors and mortality. Kaplan-Meier method was used to analyze the survival. Survival curve was delineated.

Results

miRNA microarray assay and screening for miR-208a

miRNA microarray assay showed the expression of following miRNAs increased markedly in ACS patients when compared with healthy controls: miR-21, miR-208a, miR-208b, miR-323 and miR-199a. Of these miRNAs, the increase in miR-208a expression was the most obvious (**Table 2**).

Validation of miR-208a expression by Q-PCR

Q-PCR was performed to detect the miR-208a expression in the serum, exosomes and exosome-depleted supernatant in ACS patients. Results showed miR-208a was mainly expressed in the exosomes (**Figure 1A**). Further Q-PCR was performed to validate the expression of miR-208a in healthy controls and ACS

patients. Results revealed that miR-208a expression increased significantly in the serum exosomes of ACS patients, and the miR-208a expression in the serum of ACS patients was markedly higher than that in healthy controls. However, the sensitivity of miR-208a in serum was inferior to that in exosomes (**Figure 1B**).

Correlation between miR-208a and clinical features

Of 500 patients, there were 300 patients in low miR-208a expression group and 200 in high miR-208a expression group. Analysis of clinical features showed patients in high miR-208a expression group were older and had significantly increased Killip class, elevated CK-MB peak, increased cTnT peak and elevated LDL ($P < 0.05$) when compared with low miR-208a expression group (**Table 3**).

Kaplan-Meier survival curve

During the 1-year follow up period, a total of 32 patients died, of whom there were 10 in low miR-208a expression group with the mortality of 3.3% and 22 in high miR-208a expression group with the mortality of 11.0%. Kaplan-Meier survival analysis showed the 1-year survival rate in high miR-208a expression group was significantly lower than that in low miR-208a expression group ($\chi^2 = 16.498$, $P < 0.001$) (**Figure 2**).

Discussion

ACS is a common, severe clinical syndrome and significantly threatens the health of patients. Thus, the early recognition and timely therapy are helpful to improve the prognosis of ACS and reduce the risk for complications [12]. Our results showed miRNA-208a expression was significantly up-regulated in the serum exosomes of ACS patients. In addition, ACS patients with high miRNA-208a expression (fold change ≥ 3) were older and had higher Killip class, elevated CK-MB peak, increased cTnT peak and elevated LDL when compared with low miRNA-208a expression group. The survival rate of patients with high miRNA-208a expression also reduced markedly. Thus, we speculate that exosomal miRNA-208a is crucial for the early diagnosis and prognosis of ACS.

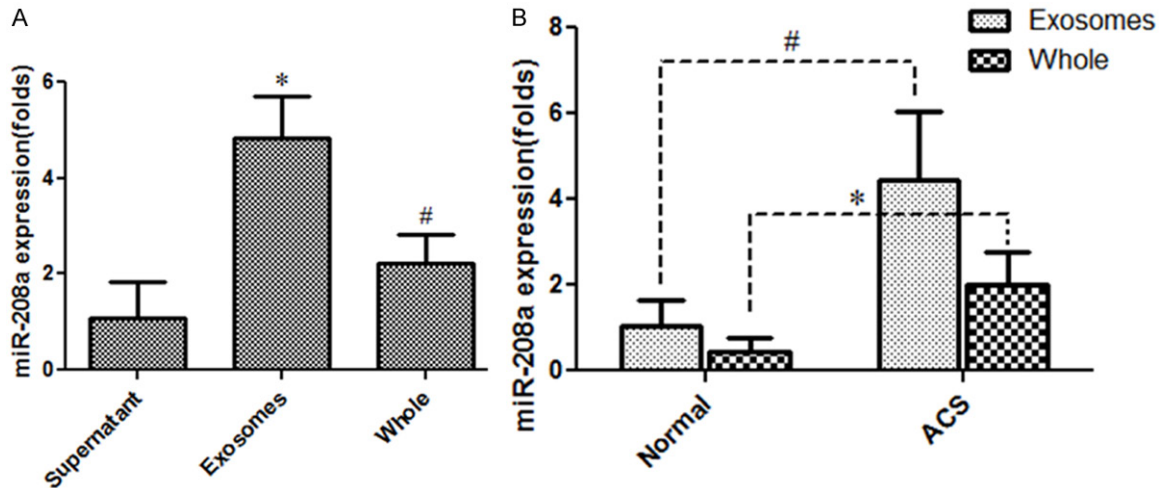


Figure 1. Q-PCR was performed to confirm the miR-208a expression. A: miR-208a expression in the serum exosomes, exosome-depleted supernatants, and serum was determined by qRT-PCR; B: miR-208a expression in exosomes and serum of ACS patients and healthy controls was determined by qRT-PCR. * $P < 0.01$; # $P < 0.001$.

Table 3. Correlations of clinicopathologic characteristics with exosomal miR-208a expression

	Low miR-208a (n=300)	High miR-208a (n=200)	P
Age (yr)	58.3±9.8	66.7±11.3	<0.001
Male (n)	184	193	0.13
Hypertension	102 (34.0%)	98 (49.0%)	0.08
Diabetes	54 (18.0%)	30 (15.0%)	0.16
Family history of CHD	46 (15.3%)	52 (26.0%)	0.25
History of myocardial infarction	46 (15.3%)	28 (14.0%)	0.52
Killip class >1	3 (1.0%)	30 (15.0%)	<0.001
CK-MB peak (μg/L)	65.1±50.4	164.2±72.6	<0.001
cTnT peak (μg/L)	2.2±3.1	5.3±3.0	<0.001
TC (mmol/L)	4.5±2.0	5.1±1.6	0.26
LDL (mmol/L)	2.6±1.1	3.3±0.9	0.02
HDL (mmol/L)	0.9 ± 0.4	0.9±0.1	0.42
TG (mmol/L)	1.5±1.3	2.0±1.4	0.08

Exosomes are a group of vesicles either released from the cell when multivesicular bodies fuse with the plasma membrane or released directly from the plasma membrane. Exosomes possess a large amount of intracellular biological information and contain some molecules such as protein, lipids, mRNA and microRNAs which are closely related to the origin and functions of exosomes [13]. Exosomes are protected by the lipid bilayer and thus not susceptible to the interference and influence of enzymes in peripheral blood and can stably exist in peripheral blood. Thus, the mRNA, miRNA and pro-

teins of exosomes remain relatively stable [14]. In addition, exosomes extracted from blood can be used for the detection of molecular markers, which reduces the sample complexity and is helpful for the detection of markers with low abundance. Riccardo et al [15] investigated the serum exosomes of non-small cell lung cancer and found that there were differentially expressed miRNAs and proteins which could be used for the screening and diagnosis of lung cancer (sensitivity: 96%; specificity: 76%). In the present study, the miR-208a expression was detected in the serum, exosomes and exosome-free supernatant, and results showed miR-208a expression in exosomes was significantly higher than that in serum and exosome-free supernatant. Thus, detection of molecular markers in exosomes is superior to that in serum.

miR-208a is a specifically expressed miRNA in the heart. miR-208a can regulate the expression of myosin heavy chain (MHC) gene, is closely related to the differentiation of cardiac embryonic stem cells and involves in the myocardial fibrosis and hypertrophy via regulating subunits α and β of MHC. There is evidence

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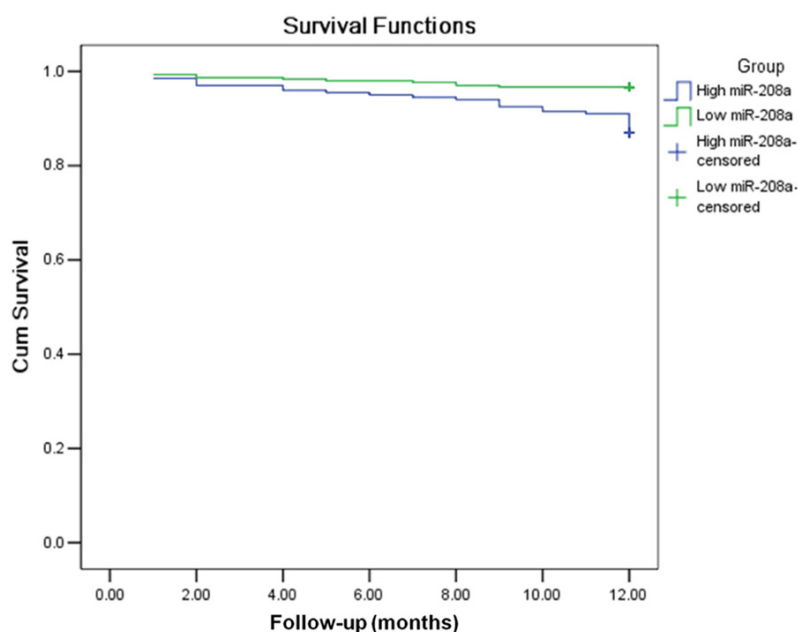


Figure 2. Kaplan-Meier survival analysis of patients in low miR-208a expression group and high miR-208a expression group.

showing that the expression of miR-208a and miR-208b in STEMI patients increased significantly when compared with healthy controls and chest pain patients without coronary lesions [16]. Wang et al [17] found miR-208a was undetectable in non-AMI patients, but detectable in 90.9% of AMI patients and 100% of patients with AMI within 4 h. In the present study, Q-PCR was employed to detect the serum miR-208a expression in ACS patients and healthy controls. Results showed miR-208a expression in the serum of ACS patients increased markedly, which was consistent with previously reported. Of interest, the miR-208a expression in the exosomes was higher than that in the serum of ACS patients. This suggests that exosomal miR-208a has a higher sensitivity, which was for the first time reported.

In addition, on the basis of fold change of exosomal miR-208a expression, ACS patients were classified as low miR-208a expression group (fold change: <3) and high miR-208a expression group (≥ 3). Analysis of clinical features showed patients with high miR-208a expression were older, and had higher Killip class, elevated CK-MB peak, increased cTnT peak and elevated LDL ($P < 0.05$). In addition, within 1-year follow up period, 32 patients died of

whom there were 10 in low miR-208a expression group with the mortality of 3.3% and 22 in high miR-208a expression with the mortality of 11.0%. Kaplan-Meier survival analysis showed the 1-year survival rate in high miR-208a expression group was significantly lower than that in low miR-208a expression group. This suggests that miR-208a is closely related to the clinical characteristics and survival of ACS patients and may serve as an important marker for the early diagnosis and prognosis of ACS.

There were still limitations in the present study. The sample size was small and the correlation of miR-208a with

other parameters was not evaluated. In addition, ACS patients were not sub-divided and the exosomal miR-208a expression was not compared in early ACS patients and late ACS patients. In our future studies, more patients will be recruited to validate our findings; the relationship between exosomal miR-208a and other routinely used parameters will be evaluated to elucidate the superiority of miR-208a as a marker of ACS; the exosomal miR-208a expression will be detected in different ACS subgroups and patients with early and later ACS, to improve the integrity of our study.

Taken together, our results show exosomal miR-208a expression in the peripheral blood of ACS patients increases significantly and is closely related to the clinical characteristics and survival of ACS patients. Thus, we speculate that miR-208a is important for the early diagnosis and prognosis of ACS.

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Disclosure of conflict of interest

None.

Address correspondence to: Qinghua Lu, Department of Cardiology, Second Hospital of Shandong University, Jinan, China. E-mail: luqianghua_doc@163.com

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