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Original Article

Serum microRNA-499 and microRNA-208a as biomarkers of acute myocardial infarction

Junjie Xiao[1,2], Bo Shen[3], Jin Li[1,2], Dongcao Lv[1,2], Yingying Zhao[4], Fei Wang[4], Jiahong Xu[5]

[1]Regeneration Lab and Experimental Center of Life Sciences, Shanghai University, Shanghai 200444, China; [2]Shanghai Key Laboratory of Bio-Energy Crops, School of Life Science, Shanghai University, Shanghai 200444, China; [3]Shanghai Entry-exit Inspection and Quarantine Bureau, Shanghai 200135, China; [4]Tongji University School of Medicine, Shanghai 200065, China; [5]Department of Cardiology, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China

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Abstract: Objective: Acute myocardial infarction (AMI) is a most serious cardiovascular disease with high morbidity and mortality. Novel biomarkers for AMI are explored continuous. MicroRNAs (miRNAs, miRs) are present in the circulation in a consistent, stable, and reproducible manner, attracting major interest of using circulating miRNAs as biomarkers. In plasma, miR-208a and miR-499 are considered to be the best candidate for AMI diagnosis. However, serum has slightly higher miRNA yields compared to plasma and the majority of archived samples are stored in form of serum, marking interesting to determine whether miR-208a and miR-499 in serum can be used as biomarkers for AMI. Methods: AMI was induced by coronary ligation and the serum and heart tissues were collected. The levels of miR-208a and miR-499 in serum and heart tissues were determined using TaqMan-based miRNA quantitative real-time polymerase chain reactions (qRT-PCRs). Results: Serum miR-208a was increased by 36-fold and 51-fold while miR-499 was elevated by 103-fold and 95-fold at 4 h and 24 h after AMI. Moreover, the expression level of miR-499 was significantly decreased in the myocardial infarct zone comparing to the remote zone or the sham group while miR-208a remained unchanged. Conclusion: Serum miR-499 and miR-208a might be potential biomarkers for AMI. miR-499 might be released from damaged heart to the circulation.

Keywords: Serum, microRNA, acute myocardial infarction, biomarker

Introduction

Acute myocardial infarction (AMI) represents a most common cardiovascular emergency with worldwide high morbidity and mortality [1, 2]. A rapid diagnosis of AMI is critical for clinicians to risk stratify the patients and also guarantees the immediate initiation of reperfusion therapy [2]. Thus, exploring novel biomarkers for AMI is continuous [3].

MicroRNAs (miRNAs, miRs) are endogenous, 20-25 nucleotide long non-coding RNAs that function as posttranscriptional regulators of gene expression by specifically interaction with certain mRNAs by inducing their degradation or repressing their translation [4]. According to the latest miRBase database 20.0, more than 2000 mature miRNAs have been identified in humans and at least one-third of human protein-encoding genes seem to be miRNA regulated [5]. miRNAs participate in a variety of essential biological processes, including proliferation, differentiation, apoptosis, necrosis, autophagy, development, and ageing [6-8]. Accumulating evidences have demonstrated that aberrant expression of miRNAs in tissues contributes to various diseases, including cancer, vascular disease, and cardiovascular disease [9, 10]. In addition, many miRNAs exhibit a tissue-specific distribution manner, igniting wide interest in their diagnostic potential [9]. Intriguingly, considerable reports have indicated that miRNAs are present in the serum and plasma in a consistent, stable, and reproducible manner, attracting considerable interest of using circulating miRNAs as biomarkers [4, 11-15].

With the hypothesis that muscle or heart specific miRNAs can be released into the circulation from the injured heart, miR-1, miR-133,

miR-208, and miR-499 have been found to be consistently elevated in plasma of AMI [16-20]. Besides in plasma, miR-1 has also been reported to be increased in serum of AMI [3]. Among above four miRNAs, miR-208a and miR-499 are considered to be the best candidate for AMI diagnosis [21, 22]. Using plasma for determining miRNAs has the disadvantages such as frequent presence of platelets reported, which increases platelet-specific miRNAs, and further work-up necessary as well [23]. By contrast, serum has slightly higher miRNA yields compared to plasma and the majority of archived samples are stored in form of serum, marking interesting to determine whether miR-208a and miR-499 in serum can be used as biomarkers for AMI [3, 23].

Materials and methods

Generation of AMI model in mice

C57/BL6 male mice aged 10-12 weeks, purchased from Shanghai SLAC Laboratory Animal CO. LTD were used in this study. AMI were generated by left anterior descending coronary artery (LAD) ligation in mice. In brief, mice anesthetized with 1-1.5% isoflurane underwent open-chest coronary artery ligation. The left coronary artery about 2 mm under the left auricle was occluded in AMI group while in the sham group, the needle was passed around the artery without ligation. After ligation, the chests of the mice were closed and the mice were allowed to recover. AMI was confirmed with triphenyltetrazolium chloride (TTC) staining combined by echocardiography. This study was approved by the local ethical committees and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996).

Serum and tissue sampling and RNA isolation

The blood samples from mice were collected via tail vein and then placed at room temperature for 1 h. After that, the samples were centrifuged at 4000 rpm for 20 min at 4°C and the supernatant (serum) was transferred to RNase/DNase-free tubes and stored at -80°C until RNA isolation. The total RNA was isolated from the serum using a mirVana PARIS isolation kit (Ambion, Austin, Texas) according to the manufacturer's instructions for serum samples without enrichment for small RNAs. Briefly, 200 μL of serum was used to extract the total RNA. Caenorhabditis elegans miR-39 (cel-miR-39) of 50 pmol/L was added as the spike-in control after the equal volume of denaturing solution was added. Each sample was eluted in 80 μL of RNAse-free water. Cardiac tissues from AMI and sham mice were immediately flash frozen in liquid nitrogen, and stored at -80°C until RNA isolation using a mirVana PARIS isolation kit (Ambion, Austin, Texas) according to the manufacturer's instruction similarly as described above.

Determination of miR-499 and miR-208a level

The expression levels of miR-499 and miR-208a were determined using TaqMan-based miRNA quantitative real-time polymerase chain reactions (qRT-PCRs) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The 15-μL RT reaction master mix contains 0.15 μL of 100 mM dNTPs, 1 μL of MultiScribe RT, 1.5 μL of 10 × RT buffer, 0.19 μL of RNase inhibitor, 3 μL of RT primer, 5 μL of RNA sample and 4.16 μL of ddH_2O. For RNA from tissue samples, the concentration was diluted to 2 ng/μL. qRT-PCR was carried out using the 7900HT Fast Real-Time PCR System on 20 μL of PCR master mix containing 1 μL of TaqMan assay, 2 μL of RT products, 10 μL of TaqMan 2 × Universal PCR Master Mix, and 7 μL of ddH_2O. The qRT-PCR reactions were performed in triplicate, and the signal was collected at the end of every cycle. The Ct values from qRT-PCRs larger than 40 were treated as 40. As no endogenous stable miRNAs for correction the expression level of miRNAs in serum are widely accepted, we chose to correct for spikes by using cel-miR-39, which lacks sequence homology to mice miRNAs as previous described. For the tissue samples, snoRNA202 was used as the endogenous control.

Statistical analysis

Relative miRNA expression was presented using the $2^{-\Delta\Delta Ct}$ method. Data were presented as mean ± SEM. Comparisons between two groups were performed with an independent samples *t*-test. All analyses were performed using SPSS 17.0, and all statistical tests were two-sided. *P* values less than 0.05 were considered to be statistically significant.

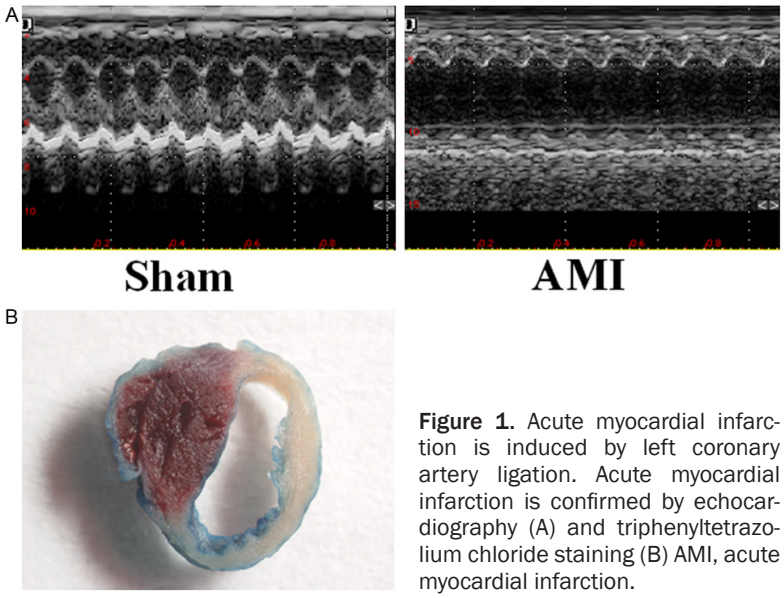

Figure 1. Acute myocardial infarction is induced by left coronary artery ligation. Acute myocardial infarction is confirmed by echocardiography (A) and triphenyltetrazolium chloride staining (B) AMI, acute myocardial infarction.

Results

Serum miR-499 and miR-208a are elevated in AMI

As shown in **Figure 1**, AMI was successfully induced as confirmed by TTC staining and echocardiography. Serum miR-208a was increased by 36-fold and 51-fold at 4 h and 24 h after AMI, respectively (**Figure 2A**). Serum miR-499 was elevated by 103-fold and 95-fold at 4 h and 24 h after AMI, respectively (**Figure 2B**). These results indicated that serum miR-499 and miR-208a might be potential biomarkers for AMI.

miR-499 is decreased in the myocardial infarct zone in AMI while miR-208a remains unchanged

The expression level of miR-499 was significantly decreased in the myocardial infarct zone comparing to the remote zone or the sham group (**Figure 3A**). However, the expression level of miR-208a remained unchanged in the myocardial infarct zone comparing to the remote zone or the sham group (**Figure 3B**). These findings suggest that miR-499 might be released from damaged heart to the circulation.

Discussion

Due to the acute occlusion of a coronary artery, MI reflects cardiomyocyte death as a consequence of cardiac ischemia [24]. Circulation troponin (Tn) I and T are currently recognized as the standard biomarkers for the diagnosis of AMI [2, 25, 26]. However, more novel biomarkers with high specificity and sensitivity are highly needed [20, 27-29].

Ideally, a biomarker of AMI should fulfill the features as follows [2, 20, 30, 31]. Firstly, it should be specific or abundant expressed in heart. Secondly, its expression level in circulation under normal conditions should be extremely low or undetectable. Finally, in AMI, it can be quickly released into the circulation from the damaged heart and stably expressed for some time. miRNAs are found to be present in circulation in a remarkably stable form, which can withdraw multiple freeze-thaw cycles and are resistant against RNase-mediated degradation as well. miR-499 and miR-208a are both cardiac or muscle specific miRNAs and are considered to be the best candidate for AMI diagnosis among circulating miRNAs [16, 17, 19, 22]. They have been reported to be elevated in plasma of AMI [16, 17, 32], however, their expressions in serum of AMI are unclear. Considering the advantages of using serum for determining miRNAs, it would be interesting to know whether miR-499 and miR-208a in serum can be used as biomarkers for AMI. To the best of our knowledge, this is the first report providing direct evidence that serum miR-499 and miR-208a are elevated in AMI, indicating these two miRNAs could be potential biomarkers of AMI.

To better understanding the source of miR-499 and miR-208a in serum during AMI, we determined their expression levels in the myocardial infarct zone, remote zone, and the sham group. We found that miR-499 was significantly decreased in the myocardial infarct zone whether comparing to the remote zone or the sham group, indicating that miR-499 might be released from damaged heart to the circulation. In a recent study, circulating miRNAs in acute coronary syndrome (ACS) was checked by measuring the concentration gradients of

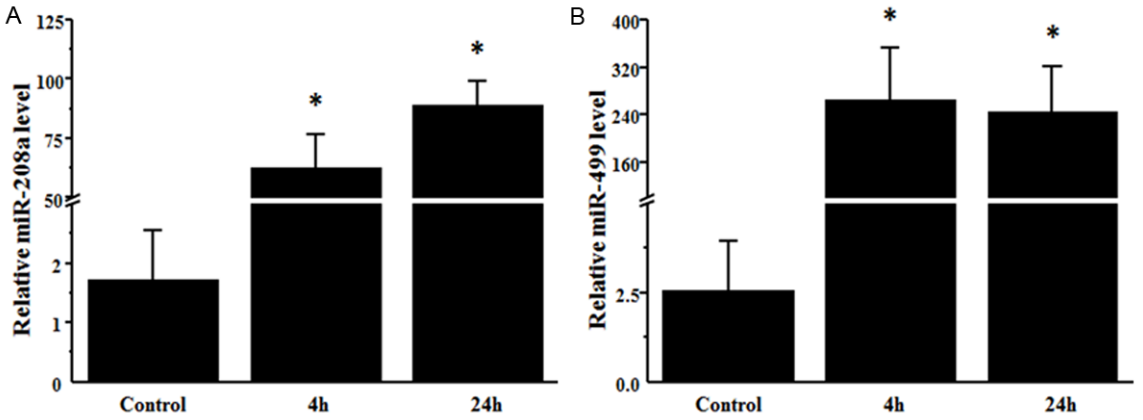

Figure 2. Serum miR-499 and miR-208a are elevated in acute myocardial infarction. Serum miR-208a (A) and miR-499 (B) are elevated at 4 h and 24 h after acute myocardial infarction.

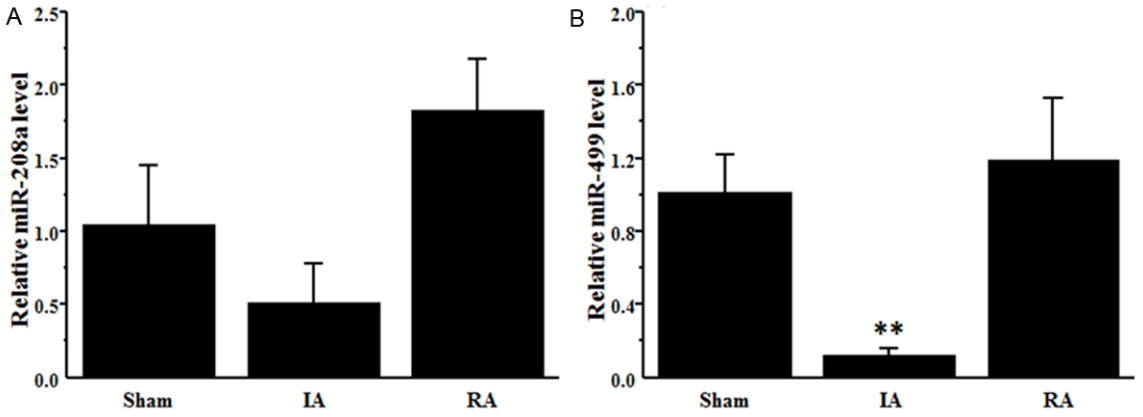

Figure 3. miR-499 is decreased in the myocardial infarct zone in acute myocardial infarction while miR-208a remains unchanged. miR-208a (A) was unchanged in the myocardial infarct zone in acute myocardial infarction while miR-499 (B) was decreased. IA, infarct area; RA, remote area.

miRNAs across the coronary circulation. A significant increase in the circulating levels of miR-499 across the coronary circulation was observed in troponin-positive ACS compared with patients of coronary artery diseases, further supports that miR-499 is released into the coronary circulation in AMI [33]. Interestingly, miR-208a remains unchanged in the myocardial infarct zone though tend to be decreased in the present study, which might be due to its relative low abundance in the heart.

Several potential limitations of this study should be highlighted. Firstly, whether the serum expression levels of miR-499 and miR-208a are well correlated well with TnI, TnT and the infarct size are unclear in the present study. However, plasma expression levels of miR-499 and miR-208a have been widely proven to be correlated well with TnI, TnT and the infarct size in AMI as well [3, 16, 17, 32, 34]. Secondly, further work is required to determine the additive benefit of serum miR-499 and miR-208a in algorithms for risk stratification, and therapeutic management of AMI in a prospective manner. Thirdly, it is unclear how miRNAs are released from heart into circulating blood. Accumulating evidences have indicated that exosomes, microparticles, or apoptotic bodies might be involved in [9, 12, 35, 36]. Finally, whether serum miR-499 and miR-208a in AMI are solely wastes or have any potential biological functions such as extracellular communicators in cell-to-cell communication still need to be clarified in the further study.

In conclusion, we have found that serum miR-208a and miR-499 were elevated in AMI, making them potential novel biomarkers for the diagnosis of AMI. As serum miR-208a and miR-

499 provide new non-invasive windows to the damaged heart, the possibility of using serum miRNA-based assays to diagnose AMI is opened.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Junjie Xiao or Dr. Jiahong Xu, Experimental Center of Life Sciences and Regeneration Lab, School of Life Science, Shanghai University, 333 Nan Chen Road, Shanghai 200444, China. Tel: 0086-21-66138131; Fax: 0086-21-66138131; E-mail: junjiexiao@shu.edu.cn (JJ Xiao); jiahongxu_tj@hotmail.com (JH Xu)

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