Circulating MicroRNAs are Promising Novel Biomarkers of Acute Myocardial Infarction

Rongrong Wang, Ning Li, Yinhui Zhang, Yuqin Ran and Jielin Pu

Abstract

Objective Recent studies have revealed that microRNAs (miRNAs) are involved in the regulation of cardiac development, physiologic, and pathologic processes via post-transcriptional control of gene expression. The stable circulating miRNAs offer unique opportunities for the early diagnosis of several diseases. In this study, we examined the circulating miR-133 and miR-328 levels from patients with acute myocardial infarction (AMI).

Patients and Methods Twenty-eight control subjects and fifty-one consecutive AMI patients were enrolled. The plasma and whole blood samples from AMI patients were obtained within 24 hours (n=51) and 7 days (n=6) after the onset of AMI symptoms. The circulating miR-133 and miR-328 levels were analyzed using quantitative real-time PCR.

Results The miR-133 levels in plasma from AMI patients exhibited a 4.4-fold increase compared with control subjects (p=0.006). Moreover, the increased miR-133 levels in whole blood were comparable with those in plasma samples. In contrast, the miR-328 levels in plasma and whole blood of AMI patients were markedly increased by 10.9-fold and 16.1-fold, respectively, compared to those in control subjects (p=0.033 and p <0.001). The elevated circulating miR-133 and miR-328 levels were recovered to the control levels at 7 days after AMI. In addition, there was a correlation between circulating miR-133 or miR-328 levels and cardiac troponin I. Furthermore, circulating miR-133 or miR-328 showed no significant changes in AMI patients with tachyarrhythmia (n=24) or bradyarrhythmia (n=26) compared to those in patients without arrhythmias. Receiver operating characteristic curve analysis revealed that the areas under the curve of miR-133 or miR-328 in plasma and whole blood were 0.890, 0.702 and 0.810, 0.872, respectively (all p<0.05).

Conclusion The miR-133 and miR-328 levels in plasma and whole blood in AMI patients were increased compared to those in control subjects. These miRNAs may represent novel biomarkers of AMI.

Key words: acute myocardial infarction, biomarker, miR-133, miR-328

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Introduction

MicroRNAs (miRNAs) comprise a class of 19-25 nucleotides long noncoding RNAs that regulate gene expression via degradation or translational inhibition of their target mRNAs and play important roles in a wide range of physiologic and pathologic processes (1). Recent studies have revealed that miRNAs, especially muscle-specific miR-1, miR-133, and miR-499 as well as cardiac-specific miR-208, are key modulators of both cardiovascular development and angiogenesis (2). In fact, miRNA-specific expression profiles have been involved in the pathological conditions of several cardiovascular diseases, e.g. acute myocardial infarction (AMI), cardiac hypertrophy, heart failure, and cardiac arrhythmias (3-6). Intriguingly, some reports have suggested that miRNAs are present in human peripheral blood in a consistent, reproducible, and stable manner (7). More importantly, a series of evidences have demonstrated that at least in some pathological conditions, such as cancer, heart fail-
ure, and liver damage, circulating miRNAs may in part reflect tissue damage (8-10). This discovery opens up the possibility of using miRNAs as non-invasive biomarkers of cardiac diseases, especially AMI.

AMI is a serious clinical condition with high morbidity and mortality. Some biomarkers, such as creatine kinase-MB, cardiac myoglobin, and troponins, have been widely applied in clinical diagnosis. However, exploring new approaches that can complement and improve current strategies for AMI diagnosis is continuous. The muscle-specific miR-133 has been demonstrated to regulate heart development, and to be dysregulated in the hypertrophic and failing hearts (11). Recent studies revealed that miR-133 is dysregulated in samples of infarcted tissue and remote myocardium in patients with MI (12). In contrast, miR-328 is not a muscle-specific or cardiac-specific miRNA. However, it can be detected in the blood samples of AMI patients. Recently, Lu et al reported that miR-328 contributes to adverse electrical remodeling in atrial fibrillation (AF) (13).

In the present study, we examined the miR-133 and miR-328 levels in plasma or whole blood in patients with AMI using quantitative real-time polymerase chain reaction (PCR). We found that the circulating miR-133 and miR-328 levels in AMI patients were significantly increased compared to those in control subjects, suggesting that circulating miR-133 and miR-328 might be promising biomarkers of AMI.

Patients and Methods

Patient selection

Twenty-eight control subjects and fifty-one consecutive AMI patients admitted to Fu Wai Hospital (Beijing, China) from March 2009 to December 2009 were enrolled in this study. The diagnosis of AMI was based on the latest developed standard definition of MI (14). Briefly, plasma creatine kinase-MB levels increased to twice the upper limit of normal or cardiac troponin I levels were greater than 0.1 ng/mL in conjunction with at least one of the following criteria: chest pain lasting >20 minutes or diagnostic serial electrocardiographic (ECG) changes consisting of new pathological Q waves or ST-segment and T-wave changes. All of the patients were diagnosed as AMI for the first time. The control subjects were subjected to coronary angiography to exclude coronary heart disease (CHD). The exclusion criteria for all subjects were previous history of cardiac diseases (e.g. MI, heart failure, cardiac arrhythmias, pacing, and cardiomyopathy), known malignancy, renal replacement therapy, surgery or skeletal muscle damage within the previous months which would affect the expression of muscle-specific miRNAs, and the lack of the informed consent. All participants underwent clinical evaluation, including medical and family history reviews, physical examination, 12-lead ECG and echocardiography examinations. The present study conformed to the guiding principals of the Declaration of Helsinki. The Institutional Human Investigations committee approved all of the research protocols and written informed consent was obtained from all participants.

Blood samples collection

Whole blood samples (4 mL) were collected from AMI patients with K2-EDTA-coated tubes within 24 hours (n=51) and at 7 days (n=6) after the onset of syndromes. Subsequently, 3 mL of whole blood was centrifuged at 1000 g for 10 minutes, and then the plasma (about 1 mL) was aliquoted into an RNase-free tube. All plasma and whole blood samples were stored at -80°C before RNA extraction.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from plasma or whole blood sample using Trizol LS (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, 3 mL or 1.5 mL of Trizol LS was used for 1mL plasma or 0.5 mL of whole blood followed by phase separation. Subsequently, RNA was precipitated by adding 2 mL or 1 mL of isopropanol to the collected aqueous phase. The pellet was resuspended in 10μL of RNase-free water. The concentration of RNA was determined by a UV visible spectrophotometer and 0.2 μg total RNA was subsequently reverse transcribed to single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. The RT primers were as follow: miR-133: 5’-GTCGTATCCAGTGTCGTGTTAGTGCGCAAATTGCACTGGAGCACCAGCTG-3’; miR-328: 5’-GTCGTATCCAGTGTCGTGTTAGTGCGCAGTTGCACCTGAGACTACGGAA-3’; U6: 5’-CGCTTCA CGAATTTGCGTTCAT-3’.

Quantitative real-time PCR was performed on ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA). The SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) was used for relative quantification of miRNAs with U6 as an internal control. The primers used for quantitative real-time PCR were as follow: miR-133: forward 5’-GGGTTTGGTCCCCTTCAA-3’, reverse 5’-AGTGCAGTGCTGGTGTGGCAGTC-3’; miR-328: 5’-CTGGGCCC TTCTGTCGCC-3’, reverse 5’-CAGTGCGTGTCGTGGAGTGTCAT3’, U6: 5’-CGCTTACAGAATTGCGTGTCAT-3’. The PCR cycles consisted of denaturation at 95°C for 15 seconds, and annealing as well as extension at 60°C for 1 minute for 40 cycles. Each sample was analyzed two times. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeds the given threshold. The obtained data were translated into log2 scale (15). The 2-ΔΔCt method was used to analyze the relative expression of miRNAs.

Statistical analysis

The data were represented as mean±standard deviation (SD) unless otherwise indicated. The quantitative data were evaluated whether they followed the normal distribution by...
Table 1. Clinical Characteristics of the AMI Patients and the Control Subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AMI patients (n=51)</th>
<th>Control subjects (n=28)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60.06±11.53</td>
<td>57.86±10.36</td>
<td>0.400</td>
</tr>
<tr>
<td>Male/Female (n/n)</td>
<td>43/8</td>
<td>19/9</td>
<td>0.089</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.13±3.21</td>
<td>27.23±3.92</td>
<td>0.086</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>9 (17.65)</td>
<td>8 (28.57)</td>
<td>0.261</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>34 (66.67)</td>
<td>13 (46.42)</td>
<td>0.080</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>26 (50.98)</td>
<td>16 (57.14)</td>
<td>0.602</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>26 (50.98)</td>
<td>19 (67.86)</td>
<td>0.150</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.62±17.62</td>
<td>126.32±19.19</td>
<td>0.076</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73.20±12.87</td>
<td>79.32±12.53</td>
<td>0.085</td>
</tr>
<tr>
<td>Heart rate (beats/minutes)</td>
<td>76.79±15.64</td>
<td>73.27±15.00</td>
<td>0.325</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.26±1.04</td>
<td>4.53±0.83</td>
<td>0.002*</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.89±0.76</td>
<td>1.86±0.75</td>
<td>0.863</td>
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<tr>
<td>LDL (mmol/L)</td>
<td>3.09±0.84</td>
<td>2.57±0.67</td>
<td>0.007*</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.23±0.60</td>
<td>1.07±0.22</td>
<td>0.177</td>
</tr>
<tr>
<td>WBC (×10^9/L)</td>
<td>11.10±3.23</td>
<td>6.76±2.03</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Cr (µmol/L)</td>
<td>75.02±15.70</td>
<td>71.61±12.14</td>
<td>0.324</td>
</tr>
<tr>
<td>CK (IU/L)</td>
<td>1742.70±1629.83</td>
<td>71.44±33.09</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CK-MB (IU/L)</td>
<td>254.58±188.65</td>
<td>9.89±3.37</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Tn I (ng/mL)</td>
<td>14.81±13.85</td>
<td>0.02±0.02</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>NT-pro-BNP (fmol/mL)</td>
<td>1241.66±1149.53</td>
<td>543.72±149.16</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ET-1 (fmol/mL)</td>
<td>0.77±0.40</td>
<td>0.58±0.15</td>
<td>0.012*</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>7.00±4.00</td>
<td>2.98±2.74</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>


Results

Baseline clinical characteristics of the study population

There were no statistical differences between the control subjects and the AMI patients for any of the considered variables except for TC and LDL, which were more prevalent in AMI patients. In addition, other biomarkers, such as white blood cell, creatine kinase, creatine kinase-MB, cardiac troponin I, N-terminal pro-brain natriuretic peptide, endothelin-1, and high sensitivity C-reactive protein were significantly increased compared to that in control subjects, consistent with previous reports (Table 1).

Circulating miR-133 or miR-328 levels were significantly increased in AMI patients

We detected the miR-133 and miR-328 levels in plasma and whole blood from AMI patients to determine whether the circulating miRNAs levels were actually responsible for the onset of AMI. As depicted in Fig. 1, miR-133 was markedly increased by 4.4-fold in plasma from AMI patients compared to that from control subjects (p=0.006). A similar finding was obtained using whole blood samples (p=0.005). Moreover, the miR-328 levels in plasma and whole blood were also increased by 10.9-fold and 16.1-fold, respectively, in AMI patients compared to that in control subjects (p=0.033 and p<0.001). In addition, the circulating miR-133 and miR-328 levels were determined in six out of the fifty-one AMI patients at 7 days after AMI (subacute phase). The elevated circulating levels of miR-133 and miR-328 were recovered to the control levels in the subacute phase (Fig. 2).

Simultaneous miR-133, miR-328 and cardiac troponin I levels

To investigate the correlation between circulating miR-133 or miR-328 levels and cardiac troponin I, miR-133 or miR-328 and troponin I were measured in the same blood...
Figure 1. Expression levels of miR-133 in plasma (A) and whole blood (B) as well as miR-328 in plasma (C) and whole blood (D) from AMI patients are significantly higher than those from control subjects. The miRNAs expression ratio (the Ct of miR-133 or miR-328/the Ct of U6) was transformed to log₂ scale. The lower ratio value represents the higher expression level. Dark lines represent the means of the miRNAs expression ratio in log₂ scale. AMI: acute myocardial infarction.

Figure 2. The increased plasma and whole blood levels of miR-133 (A, B) or miR-328 (C, D) were restored to the control levels at 7 days after AMI. The miRNAs expression ratio (the Ct of miR-133 or miR-328/the Ct of U6) was transformed to log₂ scale. The lower ratio value represents the higher expression level. AMI: acute myocardial infarction.

samples obtained 5.24 ± 1.38 hours after AMI (T0), and at 20 hours and 7 days after T0 in five AMI patients. Cardiac troponin I which was significantly increased at 5.24 ± 1.38 hours after AMI remained increased until 20 hours after T0, and it was restored to the normal value at 7 days after T0 (Fig. 3A). In contrast, both miR-133 and miR-328 levels in plasma or whole blood samples were already at their peak values at T0. The elevated circulating miR-133 and miR-328 were decreased 20 hours after T0 and returned to the control levels at 7 days after T0 (Fig. 3B-3E). These data indicated a correlation between circulating miR-133 or miR-328 levels and cardiac troponin I. However, the miR-133 and miR-328 may exhibit a faster time course and peak before cardiac troponin I.
Changes in circulating miRNAs levels in AMI patients with or without cardiac arrhythmias

We further compared the circulating miR-133 and miR-328 levels in the AMI patients with or without cardiac arrhythmias. There were 24 patients (47.06%) with tachyarrhythmia including 8 patients with atrial tachyarrhythmia, 11 patients with ventricular tachyarrhythmia, and 5 patients with both atrial and ventricular tachyarrhythmia, and 26 patients (50.98%) with sinus bradycardia. The statistical data showed no significant differences for miR-133 and miR-328 levels between patients with or without arrhythmias (all p>0.05).

Circulating miR-133 or miR-328 expression levels as potential predictors of AMI

To evaluate the predictive merit of circulating miR-133 and miR-328 for AMI, ROC analysis was performed. As shown in Fig. 4, the areas under ROC curve (AUC) of miR-133 in plasma and whole blood were 0.890 (95% confidence interval (CI) 0.772-0.965, p<0.001) and 0.702 (95% CI 0.577-0.827, p=0.005), respectively. Similarly, the AUCs of miR-328 in plasma and whole blood were 0.810 (95% CI 0.705-0.916, p<0.001) and 0.872 (95% CI 0.769-0.976, p<0.001), respectively. It was suggested that circulating miR-133 and miR-328, which had both high sensitivity and specificity, might be used as the potential biomarkers for the diagnosis of AMI.

Discussion

It has been reported that muscle-specific miR-1, miR-133, and miR-499 as well as cardiac-specific miR-208a levels in plasma from AMI patients were significantly higher than those in healthy subjects, CHD patients without AMI, or patients with other cardiovascular diseases (15). However, the amplitudes of the increase were different among the 4 types of miRNAs, the highest increase was the miR-133 (16). Since miR-1, miR-499, and miR-208a are only marginal expression levels, these miRNAs may not be the ideal biomarkers for the diagnosis of AMI. Therefore, we chose miR-133 for one of the candidate miRNAs. As shown in Fig. 1, miR-133 was markedly increased in plasma and whole blood from AMI patients compared to that in con-
controls, consistent with previous studies (15, 16). Moreover, ROC analysis revealed that the AUCs of miR-133 in plasma and whole blood samples were 0.890 and 0.702, indicating that miR-133 may be a clinically practicable biomarker for AMI diagnosis. However, in the earliest study in 2009, Ai et al reported that the miR-133 level did not vary between the individuals with or without AMI (17). The possible reasons for the different results from our study might include (i) in the present study the muscle damage that might influence the muscle-specific miR-133 was excluded; (ii) the time from the onset of symptoms to sampling the circulating blood might be different; (iii) relative small patient numbers of the studies.

For a long time, most of the published studies were mainly focused on using muscle-specific and cardiac-specific miRNAs as biomarkers for AMI, and other miRNAs which were not muscle specific or cardiac specific have been overlooked. MiR-328 is ubiquitously distributed in many tissues (13) and has been involved in many pathological conditions, such as Alzheimer disease (18), zonation morphogenesis (19), drug-resistance of breast cancer (20), the malignant progression of gliomas (21), and so on, via post-transcriptional regulation of target protein expression. In addition, Lu et al revealed that miR-328 was markedly increased in the left atrial samples from dogs with AF established by right atrial tachypacing (13). The present study showed that miR-328 levels in the plasma and whole blood samples were also significantly higher in AMI patients than that in control subjects. ROC analysis revealed that the AUCs of miR-328 in plasma and whole blood were 0.810 and 0.872, suggesting that the increased miR-328 level might be associated with AMI. However, the reason why miR-328 is increased in AMI patients needs further investigation.

Moreover, the present data showed that circulating miR-133 or miR-328 levels were correlated with the myocardial damage marker, cardiac troponin I, indicating that the miR-133 and miR-328 may be released from damaged myocardium into circulating blood when heart injury occurs. However, the miR-133 and miR-328 exhibited faster peaks than cardiac troponin I, consistent with previous reports (15, 16, 27). Thus, increased circulating miR-133 or miR-328 might reflect the myocardial damage in AMI patients. Additionally, compared to cardiac troponin I, the priority factors for using miR-133 or miR-328 as biomarkers for AMI might include: (i) miR-133 and miR-328 are detected in circulating blood in a remarkably stable form, which can withstand enzymatic degradation, repetitive freeze-
miR-328 might contribute to the adverse atrial electric re-
ting potential (24-26). More recently, Lu et al reported that miR-
channel and pacemaker channel in myocardial cells, all of

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The authors state that they have no Conflict of Interest (COI).

References
1. Kloosterman WP, Plasterk RH. The diverse functions of micro-
RNAs in animal development and disease. Dev Cell 11: 441-450,
2006.
3. Shan ZX, Lin QX, Fu YH, et al. Upregulated expression of miR-
1/miR-206 in a rat model of myocardial infarction. Biochem Bio-
5. Divakaran V, Mann DL. The emerging role of microRNAs in car-
6. Yang B, Lu Y, Wang Z. Control of cardiac excitability by micro-
7. Chen X, Bu Y, Ma L, et al. Characterization of microRNAs in se-
um: a novel class of biomarkers for diagnosis of cancer and other
11. Meder B, Katus HA, Rottbauer W. Right into the heart of
12. Bostjancic E, Zidar N, Stajer D, Glavac D. MicroRNAs miR-1,
miR-133a, miR-133b and miR-208 are dysregulated in human myocar-
verse electrical remodeling in atrial fibrillation. Circulation 122:
2375-2387, 2010.
Task Force for the Redefinition of Myocardial Infarction. Univer-
sal definition of myocardial infarction. Eur Heart J 28: 2525-2537,
2007.
novel potential biomarker for early diagnosis of acute myocardial
tial novel biomarker for acute myocardial infarction. Biochem Bio-
18. Boissonneault V, Plante I, Rivest S, Provost P. MicroRNA-298 and
microRNA-328 regulate expression of mouse beta-amyloid precur-

2009.
zonation morphogenesis by targeting CD44 expression. PLoS One 3:
e2420, 2008.
20. Pan YZ, Morris ME, Yu AM. MicroRNA-328 negatively regulates
the expression of breast cancer resistance protein (BCRP/ABCG2)
functional characterization of microRNAs involved in the mali-
22. Dimmeler S, Zeiher AM. Circulating microRNAs: novel biomark-
23. Abbas NA, John RI, Webb MC, et al. Cardiac troponins and re-
nal function in nondiabetes patients with chronic kidney disease. Clin
functional characterization of microRNAs involved in the mali-
K+ channel expression contributing to QT prolongation in diabetic
contributes to re-expression of pacemaker channel genes HCN2
and HCN4 in hypertrophic heart. J Biol Chem 283: 20045-20052,
2008.
of troponin T measurements in acute myocardial infarction. Circu-

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