Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans

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Aims microRNA (miRNA) is reported to be present in the blood of humans and has been increasingly suggested as a biomarker for diseases. We aim to determine the potential of cardiac-specific miRNAs in circulation to serve as biomarkers for acute myocardial infarction (AMI).

Methods and results By verifying their tissue expression patterns with real-time polymerase chain reaction (PCR) analysis, muscle-enriched miRNAs (miR-1, miR-133a, and miR-499) and cardiac-specific miR-208a were selected as candidates for this study. With miRNA microarray and real-time PCR analyses, miR-1, miR-133a, and miR-499 were present with very low abundance, and miR-208a was absent in the plasma from healthy people. In the AMI rats, the plasma levels of these miRNAs were significantly increased. Especially, miR-208a in plasma was undetected at 0 h, but was significantly increased to a detectable level as early as 1 h after coronary artery occlusion. Further evaluation of the miRNA levels in plasma from AMI patients (n = 33) demonstrated that all four miRNA levels were substantially higher than those from healthy people (n = 30, P < 0.01), patients with non-AMI coronary heart disease (n = 16, P < 0.01), or patients with other cardiovascular diseases (n = 17, P < 0.01). Notably, miR-208a remained undetectable in non-AMI patients, but was easily detected in 90.9% AMI patients and in 100% AMI patients within 4 h of the onset of symptoms. By receiver operating characteristic curve analysis, among the four miRNAs investigated, miR-208a revealed the higher sensitivity and specificity for diagnosing AMI.

Conclusion Elevated cardiac-specific miR-208a in plasma may be a novel biomarker for early detection of myocardial injury in humans.

Keywords microRNA • MiR-208a • Blood • Acute myocardial infarction • Biomarker

Introduction

Acute myocardial infarction (AMI) is the world’s leading cause of morbidity and mortality. An early and correct diagnosis may warrant immediate initiation of reperfusion therapy to potentially reduce the mortality rate.1 Biomarkers, used to establish a diagnosis in patients with AMI, have emerged largely from targeted analyses of known myocardial proteins and become more and more important for diagnosis of AMI.2,3 Current biomarkers such as creatine kinase–MB isoenzymes, cardiac myoglobin, and troponins have been widely applied in clinical diagnosis.4 Among these, cardiac troponins are currently considered as the ‘gold standard’ for AMI diagnosis.5 However, the exploration of new biomarkers with high sensitivity and specificity in early diagnosis of AMI never stop.

MicroRNAs (miRNAs) are a class of 19–25-nucleotide non-coding RNAs that have been implicated in regulating diverse...
cellular processes, such as proliferation, differentiation, development, and cell death.6–8 Though the biological functions of miRNAs are not fully understood, it is clear that some miRNAs are present in a tissue- or cell-specific manner.9 Some cardiac-specific miRNAs, including miR-1, miR-133a, and miR-208a, play important roles in maintaining development and function.10–12 Recent reports show that miRNAs are also present in various biological fluids including blood,13,14 and the levels of individual miRNA and specific miRNA signatures are linked to the diagnosis and prognosis for diseases.15,16 We hypothesized that the heart-specific miRNAs might release into the circulation during AMI and could be used to detect and monitor the myocardial injury. In this study, we detected the cardiac-specific miRNAs in blood from rat models of AMI and from AMI patients. Our results suggested that the elevated cardiac-specific miRNAs in plasma from AMI patients could be potential biomarkers for the diagnosis of AMI.

**Methods**

An expanded Methods section is provided in the Supplementary material online.

**Population**

We assessed 33 consecutive AMI and 33 non-AMI patients with chest distress and pain admitted to Department of Cardiology in Shanghai Changhui Hospital between September 2008 and May 2009. The inclusion criteria for patients with AMI were based on the newly developed universal definition of MI.2 Briefly, these patients with AMI were clinically diagnosed by biochemical markers (cardiac troponin I, cTnI > 0.1 ng/mL), acute ischaemic-type chest pain, electrocardiogram change, and coronary angiography. Importantly, cTnI was measured from an initial blood sample and a second sample obtained at 12 h after admission. The duration of chest pain from onset to emergency in AMI patients was within 12 h (4.8 ± 3.5 h). The non-AMI patients with distress and chest pain were separated by the outcomes of coronary angiography, including 16 patients with coronary heart disease (CHD) and 17 patients with other cardiovascular diseases (non-CHD). Patients were excluded if they had received intravenous thrombolytic or anticoagulant therapy before the initial blood samples were obtained. In addition, 30 adult healthy volunteers (normal electrocardiographic finding and no history of cardiovascular disease, 20 men, 10 women; 60.7 ± 7.3 years) were enrolled in this study.

The protocol of this study was carried out according to the principles of the Declaration of Helsinki and approved by the Medical Ethics Committee in Shanghai Changhui Hospital. Written informed consent was obtained from all the participants before enrolment.

**Myocardial infarction rat model**

Acute myocardial infarction was induced by coronary artery ligation, as described previously, with a modification.17 Blood samples were drawn before the operation and at 1, 3, 6, 12, and 24 h after the ligation. All experimental protocols complied with the guidelines of the institutional animal care and use committee.

**Plasma collection and storage**

Blood samples for miRNA detection were collected from the patients in the emergency department or the cardiac catheterization laboratory and were processed within 1 h of collection by two-step centrifugation. The supernatant was transferred to RNase/DNase-free tubes and stored at − 80°C.

**RNA preparation**

Total RNA in plasma were isolated by using TRI Reagent BD (MRC, TR126) following the instructions from the manufacturer with modification. The Caenorhabditis elegans miRNA (cel-miR-39) was synthesized for the spiked-in control.18

**Quantitative reverse transcriptase–polymerase chain reaction (real-time polymerase chain reaction) analysis**

MiRNAs were quantified by using TaqMan miRNA quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) assay according to the protocol of the manufacturer (Applied BioSystems, Inc.). The data were analysed with automatic setting for assigning baseline; the threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence exceeds the given threshold. The Ct values from real-time PCR assays greater than 40 were treated as 40. The plasma levels of miRNA were detected and analysed by two investigators who were blinded to the clinical data of patients. The data obtained by real-time PCR were translated in log2 (relative level).

**Statistical analysis**

The quantitative data were evaluated whether they followed the normal distribution by the Shapiro–Wilks test. The basis to declare a certain parameter as normally distributed was \( P = 0.20 \). For the data that did not fit the normal distribution, the Kruskal–Wallis test was performed. For the data of normal distribution, Levene’s test of homogeneity of variance was further performed. When the data fitted the homogeneity of variance, one-way ANOVA was applied, and for the data that did not fit the homogeneity of variance, the Kruskal–Wallis test was performed. The receiver operating characteristic (ROC) curves were established for discriminating AMI patients from the ones with chest pain. The qualitative data were compared with Fisher’s exact test. All P-values are two-sided and less than 0.05 was considered a statistically significant difference. All statistical calculations were performed by the SPSS 16.0.

**Results**

**Detection of microRNAs specifically expressed in the human heart**

On the basis of cloning and microarray experiments in our laboratory with RNAs of human heart as well as a survey of previously reported miRNA profiling results in other species,18,19 four miRNAs that are highly or specifically expressed in the heart were enrolled in the present study: miR-499 and miR-208a are exclusively expressed in heart, whereas miR-1 and miR-133a are highly expressed both in heart and in skeletal muscle. First, we performed northern blot analysis of various tissues from rat. As expected, miR-499 and miR-208a were detected only in heart, whereas miR-1 and miR-133a were highly expressed both in skeletal muscle and heart (see Supplementary material online, Figure S1), which is in agreement with previous reports.11,19,20 Then, real-time PCR analysis, a more sensitive method, was performed and it demonstrated that miR-1 and miR-133a exhibited high expressed levels both in skeletal muscle and heart. The levels of these two miRNAs in skeletal muscle were three- to four-fold higher than in heart (Figure 1A). In addition, miR-499 was mainly expressed in heart and could also be detected in skeletal muscle, although the
level was less than that in heart. Remarkably, miR-208a was detected only in heart and not in skeletal muscle. All four miRNAs were either barely detectable or only reflected a trace amount in the rest of the tissues. Additionally, we detected these four miRNA levels in various tissues from humans, and found that they displayed a similar expression pattern in humans too (Figure 1B).

Detection of cardiac-specific microRNAs in plasma of healthy people

Recent works have revealed that numerous mature miRNAs are present in plasma.13,14 We performed miRNA microarray and real-time PCR to detect miRNA levels in plasma from four healthy subjects. About 170 miRNAs could be detected by microarray in all four samples of plasma. The top 40 miRNAs with detection signal intensity are presented in Figure 2. About 100 miRNAs present in human plasma are consistent with recent reports with their levels variable.21 Especially, the signal from miR-451 was top ranked in all four healthy people, and miR-16 was also detected with high level of expression. These results were consistent with previously reported miRNA profiling results by Solexa deep sequencing.14 We noticed that only miR-133a had a low level presence in the plasma; miR-1, miR-499 and miR-208a were undetectable. Real-time PCR analysis also revealed that miR-451 and miR-16 were detected with high abundance (the average Ct values were 19.89 and 22.37 respectively), while miR-133a was detected with low level (the average Ct value was 33.68). In addition, miR-1 and miR-499, which were undetectable by microarray, could be detected with a marginal level of expression by the real-time PCR (the average Ct values were 35.29 and 36.78, respectively). However, miR-208a could be detected neither by microarray nor by real-time PCR (the Ct value was beyond 40). The Ct value in the real-time PCR and the signal intensity of the microarray from the six miRNAs is compared in Table 1. The results demonstrate that miR-1, miR-133a, and miR-499 are present with low levels, whereas miR-208a is absent in plasma from healthy people.

Cardiac-specific microRNAs are increased in plasma from rats with acute myocardial infarction

To investigate whether cardiac miRNAs can be detected with their increase in circulating blood after myocardial infarction, we established an AMI rat model by coronary artery ligation and evaluated the levels of circulating miRNAs. Blood samples were collected from the rats at various time points (0, 1, 3, 6, 12, 24 h after coronary artery ligation). Real-time PCR analysis showed that the levels of miR-1, miR-133a, and miR-499 in plasma were increased at 1–3 h, peaked at 3–12 h and decreased at 12–24 h after coronary artery ligation (Figure 3A). Notably, the level of miR-208a was undetectable at 0 h, but significantly increased to a detectable level at as early as 1 h, peaked at 3 h, then began to decrease at 6–12 h and reduced to the undetectable level at 24 h after coronary artery occlusion. The level of miR-16 was also observed to increase, though to a lesser extent, after coronary artery occlusion. However, the level of liver-specific miR-122 did not change considerably during the whole procedure.
A skeletal muscle injury occurred while performing the surgical procedure of thoracotomy. MiR-1, miR-133a, and miR-499 are expressed in skeletal muscle, and the increased level of these miRNAs in plasma might be due to skeletal muscle damage. We further conducted experiments with the following three groups (n = 6, each group): rats received thoracotomy followed by coronary artery ligation (ligation group), rats received thoracotomy only (sham-op group), and rats received anesthetic agent only (non-op group). Blood samples were drawn 3 h after coronary artery ligation. As expected, the levels of miR-1, miR-133a, miR-499, miR-208a, and miR-16 were higher in plasma from the ligation group compared with those from the non-op group (Figure 3B). Essentially, the levels of miR-1, miR-133a, miR-499, and miR-16 in plasma from the sham-op group were also observed to be substantially higher compared with those from the non-op group. The level of muscle-specific miR-206 in plasma was higher for both the ligation group and the sham-op group than that for the non-op group, whereas no significant change of liver-specific miR-122 was observed among the three groups. These observations suggested that skeletal muscle injury during surgery also effectively released the muscle-born miRNAs into the blood. However, the level of miR-208a in plasma was undetectable either in the sham-op group or in the non-op group and was only increased in the ligation group (Figure 3B), indicating that cardiac miR-208a is a more specific marker, among these miRNAs, for cardiac damage.

Blood cardiac-specific microRNA levels are increased in patients with acute myocardial infarction

We further investigated the miRNA levels in plasma from AMI patients to determine whether the level of cardiac miRNAs in circulating blood can actually be responsible for the onset of AMI in patients. The clinical characteristics of all the patients are shown in Table 2. No significant difference in age and sex was observed among the three groups. As shown in Figure 4, real-time PCR analysis demonstrated that miR-1, miR-133a, and miR-499 were detected with higher levels in plasma from the AMI group compared with those from the healthy group (P < 0.01). However, there were no statistically significant differences in the levels of these three miRNAs among the healthy, non-CHD, and CHD groups. Significantly, miR-208a could not be detected in any of the plasma samples from the healthy, non-CHD, or CHD group, but was obviously detectable in plasma from most of the AMI patients (90.9%, 30/33; Figure 4A). It should also be noticed that there were no significant differences in the levels of two other miRNAs, miR-16 and miR-451, among all four groups.

To further determine whether the elevated miRNAs in plasma from AMI patients present any changes after medical treatment,
Figure 3  Increase of microRNAs in plasma from rats with acute myocardial infarction. (A) The plasma was collected from the rats at different times after coronary artery occlusion (n = 6). Total RNA was isolated, reverse-transcribed and subjected to real-time polymerase chain reaction analysis. Levels of microRNAs were expressed as fold increase (logarithmic scale) relative to those from the corresponding rats at 0 h. (B) The plasma was collected from rats of non-operation (non-op), sham-operation (sham-op), and ligation groups at 3 h after coronary artery occlusion (n = 6, each group). †Ct values greater than 40 were treated as 40 when normalizing data. *P < 0.05 and **P < 0.01.

<table>
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<tr>
<th>Characteristic</th>
<th>Total patients (n = 66)</th>
<th>Patients with AMI (n = 33)</th>
<th>Patients without AMI</th>
<th>P1</th>
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<tr>
<td></td>
<td></td>
<td>Patients with AMI (n = 33)</td>
<td>Patients without AMI</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>CHD (n = 16)</td>
<td>Non-CHD (n = 17)</td>
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<tr>
<td>Age (years)</td>
<td>63.9 ± 8.9</td>
<td>63.5 ± 10.1</td>
<td>63.4 ± 6.7</td>
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<td>Male/female (n/n)</td>
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<td>23/10</td>
<td>10/6</td>
<td>12/5</td>
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<td>Current smoking, n (%)</td>
<td>25 (37.9)</td>
<td>12 (36.4)</td>
<td>7 (43.8)</td>
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<td>DM, n (%)</td>
<td>15 (22.7)</td>
<td>8 (24.2)</td>
<td>4 (25.0)</td>
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<td>Hypertension, n (%)</td>
<td>31 (47.0)</td>
<td>17 (51.5)</td>
<td>6 (37.5)</td>
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<td>Hyperlipidaemia, n (%)</td>
<td>32 (48.5)</td>
<td>20 (60.6)</td>
<td>8 (50.0)</td>
<td>4 (23.5)</td>
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<td>Fasting glucose (mmol/L)</td>
<td>5.54 ± 1.02</td>
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<td>5.76 ± 1.21</td>
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<td>SBP (mmHg)</td>
<td>129.5 ± 13.6</td>
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<td>132.5 ± 13.9</td>
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<td>DBP (mmHg)</td>
<td>78.6 ± 8.7</td>
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<td>75.9 ± 7.3</td>
<td>81.8 ± 8.2</td>
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<td>TC (mmol/L)</td>
<td>4.87 ± 1.83</td>
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<td>4.68 ± 1.11</td>
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<td>TG (mmol/L)</td>
<td>1.89 ± 1.71</td>
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<td>1.73 ± 1.08</td>
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<td>HDL (mmol/L)</td>
<td>0.99 ± 0.27</td>
<td>0.91 ± 0.19</td>
<td>1.00 ± 0.30</td>
<td>1.12 ± 0.33</td>
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<td>LDL (mmol/L)</td>
<td>2.91 ± 1.29</td>
<td>3.15 ± 1.66</td>
<td>2.68 ± 0.60</td>
<td>2.67 ± 0.82</td>
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<td>WBC (×10^9/L)</td>
<td>8.35 ± 2.49</td>
<td>9.61 ± 2.30</td>
<td>7.01 ± 2.22</td>
<td>7.16 ± 1.85</td>
<td>&lt;0.001</td>
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<td>Cr (μmol/L)</td>
<td>72.4 ± 15.6</td>
<td>71.2 ± 14.1</td>
<td>77.8 ± 19.6</td>
<td>69.7 ± 13.4</td>
<td>0.530</td>
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</table>

DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, total glyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cell; Cr, creatinine. P1: comparison between patients with AMI and without AMI. P2: comparison among patients with AMI, with CHD, and non-CHD.
a follow-up investigation was performed in 5 out of the 33 patients with AMI. The five patients received PCI and conventional pharmacological treatment. After 2 months, all five patients showed apparent clinical improvements, including recovery symptoms associated with AMI. The blood was collected for plasma miRNAs detection. The plasma levels of miR-1, miR-133a, miR-499, and miR-208a were found to be lower than their respective plasma levels during the onset of AMI. Particularly, miR-208a was reduced to undetectable level at the time of follow-up (Figure 4B).

**Evaluation of microRNAs in plasma as new biomarkers for acute myocardial infarction**

To investigate the characters of these miRNAs as potential biomarkers of AMI, ROC analysis was performed on data from all 66 patients with chest pain, including 33 AMI patients and 33 non-AMI patients. The ROC curves of miR-208a, miR-499, miR-1, and miR-133a reflected strong separation between AMI and non-AMI groups, with an area under curve (AUC) of 0.965 (95% confidence interval 0.920–1.000), 0.822 (95% confidence interval 0.717–0.927), 0.847 (95% confidence interval 0.751–0.943) and 0.867 (95% confidence interval 0.771–0.963), respectively, compared with initial cTnI with an AUC of 0.987 (95% confidence interval 0.966–1.000) (Figure 5A). Furthermore, plasma level of miR-208a could detect individuals with AMI with 90.9% sensitivity at 100% specificity, although the levels of miR-499, miR-1, and miR-133a in plasma were less sensitive (36.4, 33.3, and 15.2%, respectively) for AMI diagnosis (Figure 5B). Our results demonstrated that among the four miRNAs investigated, miR-208a displayed the higher sensitivity and specificity for diagnosis of AMI (Figure 5B). Our results demonstrated that among the four miRNAs investigated, miR-208a displayed the higher sensitivity and specificity for diagnosis of AMI.

Figure 4 Elevation of cardiac-specific microRNAs in plasma from acute myocardial infarction patients. (A) The plasma was collected from patients with acute myocardial infarction (n = 33), patients with coronary heart disease but without acute myocardial infarction (coronary heart disease, n = 16), patients with other cardiovascular disease (non-coronary heart disease, n = 17) and healthy volunteers (healthy, n = 30). Total RNA was isolated, reverse-transcribed and subjected to real-time polymerase chain reaction analysis. Dark lines represent mean or median values where appropriate (**P < 0.01). (B) The plasma was collected from five patients with acute myocardial infarction after receiving 2 months of medical treatment.
**Discussion**

The present work has led us to identify that a set of miRNAs can be clinically practicable biomarkers for AMI diagnosis. In the AMI animal model, we found that the plasma levels of miRNAs, especially the cardiac-specific miR-208a, were significantly increased after AMI, suggesting that cardiac miRNAs could release into circulating blood when heart injury occurred. This notion is supported by a recent study in an animal model with drug-induced heart damage.22 We further determined the cardiac miRNA levels in plasma from healthy people and AMI patients. Our result that the miR-208a was undetectable in plasma from healthy people, but could be detected in plasma from AMI patients revealed for the first time that monitoring the plasma levels of miR-208a could also be applied in clinical diagnosis of AMI. Compared with the result in the AMI animal model, we noticed that the plasma levels of cardiac miRNAs in AMI patients were much lower than those in AMI rats, which are more difficult to detect. Having tested popular RNA-isolation methods, we established an optimized protocol that could provide the highest yield and consistent reproducible results, especially in the case of the low abundant miRNAs in blood samples.

Our results from clinical samples in patients demonstrated that the levels of miR-1, miR-133a, miR-499, and miR-208a in blood from patients with AMI are elevated compared with those from patients without AMI. Receiver operating characteristic analysis further indicated that these four miRNAs might be good biomarkers for AMI diagnosis. These results are partially supported by a very recent report that miR-1 level was significantly higher in plasma from AMI patients compared with non-AMI subjects.23 However, previous expression profiling results revealed the presence of miR-1, miR-133a, and miR-499 in skeletal muscle such that miR-1 and miR-133a expression levels in skeletal muscle were even higher than those in the heart. Our results from animal experiments indicated that elevated plasma levels of these three miRNAs might be due to skeletal muscle injury, suggesting that miR-1 and miR-133a, and miR-499 could also be released from wounded skeletal muscle during surgery procedure and thus lack absolute cardio-specificity. Instead, miR-208a is expressed in a cardiac-specific fashion, suggesting that the circulating level of miR-208a can be minimally affected by non-cardiac tissue injury. In addition, miR-208a appears absent in plasma from healthy people and non-AMI patients. To further evaluate the specificity of miR-208a, we detected the miR-208a level in plasma from patients with non-cardiovascular diseases, including acute kidney injury (n = 8), chronic renal failure (n = 11), stroke (n = 11), and trauma (n = 9). Real-time PCR analysis demonstrated that the miR-208a remained undetectable in the plasma from all these patients (see Supplementary material online, Figure S2). In contrast, miR-208a can be significantly detected in plasma from 30 out of 33 (90.9%) AMI patients. Receiver operating characteristic analysis also demonstrated that miR-208a provided the highest sensitivity and specificity as a new diagnostic test. Accordingly, among these four miRNAs, miR-208a is a more reliable biomarker for AMI diagnosis.

The level of cTnI in blood begins to rise after 4–8 h of myocardial injury.24 Our animal experimental data showed that an apparent elevation of miRNAs was observed within 1 h of coronary ligation. More strikingly, we noticed that, in three AMI patients enrolled in the present study, the level of miR-208a became detectable within 1–4 h of chest pain, when the cTnI level was still detected below the cut-off value. In the cells, cTnI is mainly bound to the myofibrils; only 2.8–4.1% of cTnI is cytosolic.24 MiRNAs are bound to protein complex which is predominantly cytosolic. This may affect their release kinetics when cell damage occurs. Our results suggest that miRNAs may leak into blood during the early stage of myocardial injury. Recent reports indicated that miRNAs are steadily present in circulation.1314

**Figure 5** Evaluation of plasma microRNAs for the diagnosis of acute myocardial infarction. (A) Receiver operating characteristic curves were drawn with the data of plasma microRNAs from 66 patients with chest pain. AUC, the area under curve. (B) Sensitivity and specificity of plasma microRNA levels in the diagnosis of acute myocardial infarction. The dashed line indicates a 100% specificity threshold. Filled circle, acute myocardial infarction patients; inverted triangle, non-acute myocardial infarction patients.
Microvesicles, also named exosomes, ectosomes, or microparti-
cles, are small vesicles of endocytic origin released by normal
healthy or damaged cells and are reported to be present in the
peripheral blood.\(^{25}\) More evidence reveals that miRNAs are
present in microvesicles.\(^{16,26,27}\) It remains to be seen whether
cardiac-origin miRNAs released from damaged myocardium are
in the form of microvesicles when they enter into circulating blood.

The present study provides the first clinical evidence of circulat-
ing miR-208a as a marker of cardiac damage. However, research
limitations exist in our study, including that the sample size is
small. Therefore, additional investigations with larger cohorts of
healthy people and patients are needed to extensively evaluate
the miRNAs as practical biomarkers in comparison with other
cardiac markers, as well as the false-positive rate. It may warrant
further development of approaches for simple and rapid detection
of miRNAs in blood. Our work also suggests that miRNAs in blood
may be sensitive and specific biomarkers for early monitoring of
cardiovascular diseases, including myocarditis and heart failure,
and for the evaluation of myocardial protection during cardiac
surgery.

**Supplementary material**

Supplementary material is available at European Heart Journal
online.

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