Increased MicroRNA-1 and MicroRNA-133a Levels in Serum of Patients With Cardiovascular Disease Indicate Myocardial Damage

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Background—Recently, elevation of circulating muscle-specific microRNA (miRNA) levels has been reported in patients with acute myocardial infarction. However, it is still unclear from which part of the myocardium or under what conditions miRNAs are released into circulating blood. The purpose of this study was to identify the source of elevated levels of circulating miRNAs and their function in cardiovascular diseases.

Methods and Results—Serum levels of miRNA (miR)-1 and miR-133a were increased significantly in patients not only with acute myocardial infarction but also with unstable angina pectoris and Takotsubo cardiomyopathy without elevation of serum creatine phosphokinase or cardiac troponin. MicroRNA microarray analysis of the heart from a mouse model of myocardial infarction indicated that the levels of miR-1, miR-133a, miR-208a, and miR-499 were significantly reduced in the infarcted myocardium. In situ hybridization of miR-133a also showed that miR-133a levels were very low in the infarcted and peri-infarcted myocardium. It has been shown that circulating miRNAs are localized inside exosomes, which are released after Ca\(^{2+}\) stimulation. We stimulated H9c2 cardiomyoblasts with A23187 and measured miR-133a levels in the exosome fraction of the culture medium. A23187 induced a dose-dependent release of miR-133a, and significant elevation was observed only at concentrations where dead cells were detected. We also found that miR-133a–containing exosomes reduced the luciferase activity of 293FT cells transfected with an miR-133a sensor vector.

Conclusions—These results suggest that elevated levels of circulating miR-133a in patients with cardiovascular diseases originate mainly from the injured myocardium. Circulating miR-133a can be used as a marker for cardiomyocyte death, and it may have functions in cardiovascular diseases. (Circ Cardiovasc Genet. 2011;4:446-454.)

Key Words: circulating microRNA • myocardial infarction • cell death • calcium ionophore

MicroRNAs (miRNAs) are endogenous, single-stranded, 22-nucleotide noncoding RNAs. MicroRNAs are generally regarded as negative regulators of gene expression through inhibition of translation and/or promotion of mRNA degradation by base-pairing to complementary sequences within the 3′ untranslated region (3′UTR) of protein-coding mRNA transcripts.1 The first miRNA assigned to a specific function was lin-4, which targets lin-14 during temporal pattern formation in Caenorhabditis elegans.2 Since then, a variety of miRNAs have been discovered. More than 500 miRNAs have been cloned and sequenced in humans, and the estimated number of miRNA genes may be as high as 1000 in the human genome.3 Each miRNA regulates dozens to hundreds of distinct target genes; thus miRNAs are estimated to regulate the expression of more than one-third of human protein-coding genes.4

Clinical Perspective on p 454

The implications of miRNAs in the pathological process of the cardiovascular system have been recognized recently, and research on miRNAs in relation to cardiovascular disease has become a rapidly evolving field.5,6 It is known that miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, and miR-499 are muscle-specific miRNAs. Among them, miR-133b and miR-206 are expressed only in skeletal muscle and miR-208a is expressed only in cardiac muscle. Recent studies have shown that several miRNAs are present in circulating blood and that they are present in exosomes7 and micropar...
It was also reported that the levels of circulating miRNAs are associated with specific disease conditions, including heart failure. Recently, circulating miRNAs have been reported in patients with myocardial infarction (MI). From these results, it has been hypothesized that miRNAs in the systemic circulation may reflect tissue damage, and, for this reason, they can be used as a biomarker of such diseases.

We first measured the levels of circulating miR-1 and miR-133a associated with cardiovascular diseases and could detect that the levels of circulating miR-1 and miR-133a are elevated early after the onset of chest pain, when there was no elevation in serum creatine phosphokinase (CK or CKP) or cardiac Troponin T (cTnT). We also detected the elevation of miR-133a not only in patients with acute coronary syndrome (ACS) but also in those without ACS (non-ACS). Next, we tried to determine the tissue distribution of miR-133a after MI, and the results suggested that not only the infarcted region but also the border zone is the source of circulating miR-133a. Further in vitro analysis showed that stimulation of H9c2 cells with the calcium ionophore A23187 can release miRNA only when cell death was detected. Our data also indicated that miR-133a in the exosome fraction is transferable and functional. Thus, these results suggest that circulating miRNAs can be released from the myocardium in association with cellular damage, and serum miR-133a can be used as a biomarker for myocardial injury.

**Methods**

**Study Population**

Seventy-one patients admitted to Department of Cardiovascular Medicine in Kyoto University Hospital during July 2009 to March 2011 were analyzed retrospectively. This study was approved by the Institutional Review Board of Kyoto University Graduate School and Faculty of Medicine. Written informed consent was given by all patients or their families in accordance with the Declaration of Helsinki. Diagnosis was based on the final diagnosis at discharge, which relied on the treating physician’s diagnosis. Diagnostic criteria for acute MI are described in the online-only Data Supplement Methods section. Diagnosis of Takotsubo cardiomyopathy was based on the criteria described previously.

**Blood Sampling From Patients**

Venous blood samples were obtained from patients. The venous blood was put into a commonly used test tube containing polyethylene resin for serum separation (Terumo). We let the tube stand for at least 10 minutes at room temperature. Then, the tube was centrifuged at 12,000 g for 10 minutes to obtain serum. The serum was aliquoted and stored at −80 °C until assayed.

**Quantification of Circulating miRNAs and Cardiac TnT in Serum**

Total RNA was extracted from 350 μL of serum, using TRIzol LS reagent (Invitrogen), and was dissolved in 10 μL of diethylpyrocarbonate water. The quantity and quality of total RNA were determined with the use of a spectrophotometer (GeneQuant pro, GE Healthcare). MicroRNAs were quantified by means of quantitative reverse-transcriptase–polymerase chain reaction (qRT-PCR) TaqMan MicroRNA Assays (Applied Biosystems) in accordance with the manufacturer’s instructions. Ten nanograms of total RNA was used to synthesize miRNA-specific cDNA. Amplification of each sample was duplicated, and the PCR was run for 40 cycles, using an ABI Prism 7900HT sequence detection system (Applied Biosystems). The Ct value was defined as the cycle number at which the

**Table. Characteristics of Patients With Non-ACS and ACS**

<table>
<thead>
<tr>
<th></th>
<th>Non-ACS (n=42)</th>
<th>ACS (n=29)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>69.2±2.2</td>
<td>69.7±2.4</td>
<td>0.7566</td>
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<tr>
<td>Male sex, n (%)</td>
<td>24 (57.1)</td>
<td>23 (79.3)</td>
<td>0.0522</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>121±3</td>
<td>130±5</td>
<td>0.1486</td>
</tr>
<tr>
<td>Diastolic</td>
<td>67±2</td>
<td>77±4</td>
<td>0.0236</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>76.8±3.9</td>
<td>77.4±3.9</td>
<td>0.6035</td>
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<tr>
<td>Physical data</td>
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<td></td>
</tr>
<tr>
<td>Height, cm</td>
<td>159.8±1.7</td>
<td>163.9±2.0</td>
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</tr>
<tr>
<td>Body weight, kg</td>
<td>57.2±2.2</td>
<td>62.9±3.2</td>
<td>0.1805</td>
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<tr>
<td>Body mass index</td>
<td>22.2±0.7</td>
<td>23.5±0.9</td>
<td>0.4827</td>
</tr>
<tr>
<td>Laboratory data</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WBC/μL</td>
<td>6333±456</td>
<td>9441±724</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC ×1012/μL</td>
<td>3.98±0.12</td>
<td>4.34±0.13</td>
<td>0.0522</td>
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<tr>
<td>Hemoglobin, g/dL</td>
<td>12.2±0.4</td>
<td>13.4±0.4</td>
<td>0.0407</td>
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<tr>
<td>Platelets ×109/μL</td>
<td>210.4±13.6</td>
<td>171.6±14.2</td>
<td>0.1003</td>
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<tr>
<td>AST, IU/L</td>
<td>24.1±1.5</td>
<td>108.7±34.0</td>
<td>0.0009</td>
</tr>
<tr>
<td>ALT, IU/L</td>
<td>18.5±1.9</td>
<td>33.3±6.2</td>
<td>0.0287</td>
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<td>γ-GTP, IU/L</td>
<td>38.5±4.1</td>
<td>43.7±9.3</td>
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<td>LDH, IU/L</td>
<td>218.8±10.5</td>
<td>413.3±83.3</td>
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<tr>
<td>CK, IU/L</td>
<td>106.3±12.0</td>
<td>1108±538.2</td>
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<td>CK-MB, IU/L</td>
<td>13.4±1.3</td>
<td>74.2±31.1</td>
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<tr>
<td>Total protein, g/dL</td>
<td>6.8±0.1</td>
<td>6.9±0.2</td>
<td>0.6879</td>
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<td>Albumin, g/dL</td>
<td>4.0±0.1</td>
<td>3.9±0.1</td>
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<td>T-Bil, mg/dL</td>
<td>0.8±0.1</td>
<td>0.7±0.1</td>
<td>0.721</td>
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<tr>
<td>C-reactive protein, mg/dL</td>
<td>0.9±0.3</td>
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<tr>
<td>BUN, mg/dL</td>
<td>20.6±1.8</td>
<td>18.6±1.4</td>
<td>0.7255</td>
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<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.2±0.2</td>
<td>1.1±0.2</td>
<td>0.9626</td>
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<tr>
<td>eGFR, mL/min/1.73 m²</td>
<td>64.8±4.3</td>
<td>68.8±4.3</td>
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<tr>
<td>Hemoglobin A₁₀₀, %</td>
<td>5.8±0.1</td>
<td>6.2±0.4</td>
<td>0.6868</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>166.8±6.1</td>
<td>194.8±10.2</td>
<td>0.0123</td>
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<td>Triglyceride, mg/dL</td>
<td>109.0±13.4</td>
<td>72.0±7.7</td>
<td>0.0525</td>
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<td>Glucose, mg/dL</td>
<td>118.5±10.2</td>
<td>186.1±21.7</td>
<td>&lt;0.0001</td>
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<td>Amylase, IU/L</td>
<td>81.1±5.7</td>
<td>87.9±10.9</td>
<td>0.8379</td>
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<td>Na, mEq/L</td>
<td>139.2±0.6</td>
<td>139.3±0.7</td>
<td>0.7653</td>
</tr>
<tr>
<td>K, mEq/L</td>
<td>4.2±0.1</td>
<td>4.0±0.1</td>
<td>0.194</td>
</tr>
<tr>
<td>Cl, mEq/L</td>
<td>102.1±0.7</td>
<td>102.0±0.5</td>
<td>0.4467</td>
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<tr>
<td>Ca, mg/dL</td>
<td>8.5±0.2</td>
<td>8.6±0.1</td>
<td>0.6546</td>
</tr>
</tbody>
</table>

**ACS** indicates acute coronary syndrome; WBC, white blood cell; RBC, red blood cell; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; LDH, lactate dehydrogenase; CK, creatine phosphokinase; CK-MB, creatine phosphokinase-MB fraction; T-Bil, total bilirubin; BUN, blood urea nitrogen; and eGFR, estimated glomerular filtration rate.

All values are expressed as mean±SEM. Body mass index is weight in kilograms divided by the square of the height in meters.
fluorescence (ΔRn) exceeded the threshold. The threshold was 0.20, which was defined as the default setting. To evaluate the miRNA expression levels, PCR was carried out in duplicate, and the average of the Ct values was converted into $2^{-\Delta\Delta Ct}$. If the Ct value could not be determined because the PCR was run for up to 40 cycles and the miR expression level was below the detection limit, the Ct value was treated as 40 (ie, miR expression level $= 2^{-40}$).

Cardiac TnT levels in the serum were measured with the use of an electrochemiluminescence method (ECLIA; Roche).

**Mouse MI Model**

All mice used in the present study were C57BL/6 male mice. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. MI was induced by ligating the left anterior descending coronary artery, as described previously.16

**MicroRNA Microarray Analysis of Mouse Hearts and MicroRNA Quantification**

MicroRNA expression profiles were examined using a commercially available miRNA microarray analysis system (3D-Gene, Toray, Tokyo). MicroRNAs were quantified by means of qRT-PCR TaqMan MicroRNA Assays (Applied Biosystems), in accordance with the manufacturer’s instructions. Each miRNA level was normalized by using U6 small nuclear RNA calculated by the $2^{-\Delta\Delta Ct}$ method.17

**In Situ Hybridization of miR-133a**

miR-133a in situ hybridization was performed on 10-μm frozen sections of mouse hearts as described previously, with slight modifications.18 Detailed methods are described in the online-only Data Supplement Methods section.

**Figure 1.** MicroRNA (miR)-1 and miR-133a levels increased in the serum of patients with acute coronary syndrome (ACS). A and B, Expression levels of miR-1 (A) and miR-133a (B) in the serum of patients with non-ACS versus ACS. *P*<0.0005, **P*<0.0001. The levels of circulating miRNAs decreased over time in the serum of patients with ACS. C and D, miR-1 (C) and miR-133a (D) expression levels are shown according to the time of blood sampling after onset. Dots with daggers indicate samples without elevation of creatine phosphokinase or cardiac troponin T. Horizontal lines indicate the median.

**Figure 2.** miR-1 and miR-133a levels increased in the serum of patients with a variety of cardiovascular diseases. A and B, Expression levels of miR-1 (A) and miR-133a (B) in the serum of patients with cardiovascular diseases. NSTEMI indicates non-ST-segment elevation–myocardial infarction; STEMI; ST-segment elevation–myocardial infarction; PAD, peripheral artery disease; and AAA, abdominal aortic aneurysm. Horizontal lines indicate the median.
Cell Culture and Evaluation of Cell Viability

Total lactate dehydrogenase activity in the culture medium was measured with the use of a lactate dehydrogenase cytotoxicity assay kit (Cayman Chemical Co), in accordance with the manufacturer’s instructions. Detailed methods are described in the online-only Data Supplement Methods section.

Exosome Isolation From the Media of H9c2 Cells and Quantification of Exosomal MicroR-133a

H9c2 cells were cultured overnight in a 15-cm dish (2 × 10^6 cells). After washing with PBS 3 times, the medium was changed to Dulbecco modified Eagle Medium without fetal bovine serum. Cells were incubated for 1 hour and stimulated with A23187 for 1 hour. Exosomes were collected from the medium by means of the conventional centrifugation method of Savina et al,19 with some modifications. Briefly, the culture medium was collected, centrifuged at 800 g for 10 minutes to pellet the cells, and then centrifuged at 12 000 g for 30 minutes to remove cellular debris. Exosomes were isolated from the supernatant by centrifugation at 110 000 g for 2 hours. To extract total RNA from the exosome fraction, the pellet was suspended in 1 mL of TRIzol reagent (Invitrogen). The pellet of total RNA was dissolved in 10 μL of diethylpyrocarbonate water. The quantity and quality of total RNA were determined with the use of a spectrophotometer (GeneQuant pro, GE Healthcare). One hundred nanograms of total RNA was used to synthesize miR-133a-specific cDNA, using TaqMan MicroRNA Assays (Applied Biosystems) in accordance with the manufacturer’s instructions, and the miR-133a expression level was evaluated in the same manner as the calculation for serum miRNA levels described above (miR-133a expression level=2 ^{−ΔΔCt}).

Plasmids

miR-133a sensor vector was constructed as described previously.20 Detailed methods are described in the online-only Data Supplement Methods section.

Statistical Analysis

Data are presented as mean±SE unless otherwise described. Horizontal lines indicate the median in the scatterplots of the miR expression level. For statistical comparisons, the Mann-Whitney test (2 groups), Pearson χ² test (2 groups), or ANOVA (n groups) with Turkey post hoc tests were used as appropriate. For 2-group comparisons of in vitro experiments only, the Student t test was performed. A probability value <0.05 was considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 5 or JMP version 7 statistical packages.

Results

Serum Levels of miR-1 and miR-133a Increased Rapidly in Patients With ACS

Quantitative RT-PCR methodologies have been widely applied to miRNA research, especially in assessing the low level of certain serum miRNAs. To date, the most widely used and successful approach in terms of specificity and sensitivity is a 2-step approach, using looped miRNA-specific reverse transcription primers and TaqMan probes.21 Therefore, we applied this system to determine the circulating levels of miRNAs in our patients. In contrast to tissue or cellular miRNAs, of which the expression levels can be normalized against U6, circulating miRNAs are reported to have no such housekeeping miRNA that can be used as an internal control for miRNA normalization.22 First, we examined the levels of miR-17–5p, miR-454, and miR-1249, which have been used as internal controls of circulating miRNAs.10,11,23 However, miR-17–5p expression levels were significantly higher in patients with non-ACS than in those
with ACS (online-only Data Supplement Figure IA). Moreover, miR-454 and miR-1249 were undetectable in some samples, as shown in online-only Data Supplement Figure IB and IC, respectively. Therefore, these data indicate that there is no suitable internal calibrator for evaluating circulating miRNA in serum. Thus, we measured and evaluated the levels of miRNAs in serum as described in the Methods section.

During our study, we enrolled 29 patients with ACS and 42 patients with non-ACS. The characteristics of the patients are shown in the Table. Patients with ACS had significantly higher levels of diastolic blood pressure, white blood cell count, hemoglobin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, CK, CK-MB, total cholesterol, and glucose. Clinical characteristics of the ACS patients are shown in online-only Data Supplement Table I. We examined serum levels of miR-1, miR-133a, and miR-208a in these patients by TaqMan qRT-PCR analysis. miR-1 and miR-133a expression levels were detectable in 40.9% and 80.0% of patients, respectively. On the other hand, miR-208a expression levels were detectable in a few patients only (5.6%). The results with ACS and non-ACS patients are indicated in Figure 1A and 1B. The levels of miR-1 and miR-133a were significantly higher in ACS patients compared with non-ACS patients. Next, we plotted these data according to the time after onset of ACS. As shown in Figure 1C and 1D, the serum levels of these miRNAs peaked before 3 hours after chest pain was reported and decreased gradually thereafter. The dots with daggers indicate the samples with no elevation of CPK and cTnT. miR-1 expression levels decreased rapidly and were almost not detectable 15 hours after the onset of chest pain. On the other hand, miR-133a expression levels decreased gradually compared with those of miR-1.

Serum Levels of miR-133a Are Elevated in Patients With Unstable Angina Pectoris and Takotsubo Cardiomyopathy

To investigate whether serum levels of miR-1 and miR-133a are elevated in other cardiovascular diseases, we categorized the patients according to specific diseases (Figure 2A and 2B). Although the sample sizes were small, the serum level of miR-133a increased significantly in patients with unstable angina pectoris (UAP) (n=8, P<0.05) and Takotsubo cardiomyopathy (n=6, P<0.005), which is also known as apical ballooning, compared with other patients without ACS or Takotsubo cardiomyopathy, as shown in online-only Data Supplement Figure II. There was no elevation of CPK or cTnT in UAP patients.

Serum miR-133a Level Was Sensitive for Myocardial Injury Compared With miR-1 Level

Next, we performed receiver operating characteristic analysis to examine if circulating miRNAs could be used as diagnostic biomarkers for ACS. Figure 3A and 3B show receiver operating characteristic analysis of miR-1 and miR-133a, respectively. Areas under the curve of miR-1 and miR-133a were 0.777 and 0.932, respectively. Furthermore, we evaluated the correlation between miR-1 or miR-133a and highly sensitive cTnT in serum. As shown Figure 3C and 3D, miR-1 and miR-133a levels significantly correlated with the cTnT levels in serum. These data suggest that miR-1 and miR-133a levels indicate myocardial damage, and the miR-133a level in serum was more sensitive compared with the miR-1 level for the diagnosis of ACS.

Expression Levels of miR-1 and miR-133a Decreased in Infarcted Myocardium in Mice

To evaluate the expression changes of miRNAs in infarcted myocardium, we measured their expression levels in infarcted hearts by miRNA microarray analysis. We extracted total
RNA from infarcted and noninfarcted regions in a mouse model of MI at 24 hours after coronary ligation (4 samples were mixed). Figure 4A shows that the levels of miR-1, miR-133a, miR-208a, and miR-499, which are specifically expressed in the myocardium, were reduced in infarcted myocardium compared with samples from a sham operation. We also confirmed the reduced expression levels of miR-1, miR-133a, miR-208a, and miR-499 by TaqMan qRT-PCR analysis (Figure 4B). It was also observed that the serum levels of miR-1 and miR-133a were elevated significantly in this mouse model of MI (online-only Data Supplement Figure III), suggesting that the serum elevation of these miRNAs can be used as an early diagnostic marker of ACS.

**In Situ Detection and In Vitro Study of miR-133a Revealed That miR-133a Expression Decreased in the Infarcted Region and Border Zone**

To determine which part of the myocardium is the source of the circulating serum miRNAs, we carried out in situ hybridization for miR-133a in the heart at 24 hours after coronary ligation. The purple dots represented the miR-133a signal, and a strong signal of miR-133a was detected in the interventricular septum and right ventricle (online-only Data Supplement Figure IV-A). TTC staining demonstrated that the interventricular septum and right ventricle were viable (online-only Data Supplement Figure IV-B). A strong signal of miR-133a was detected in the noninfarcted region (Figure 5A and 5D). However, the intensity of the signal decreased in the border zone (Figure 5B and 5E), and the signal was absent in the infarcted region (Figure 5C and 5F). Oligomycin and iron-chelator deferoxamine, which form a hypoxic mimic, decreased the expression levels of miR-133a in rat H9c2 cells after 24 and 60 hours of incubation (online-only Data Supplement Figure V). Therefore, it may be the case that miR-133a translation is reduced and/or degradation is enhanced in hypoxic conditions. However, it is also possible that miR-133a could be released into circulating blood not only from infarcted myocardium but also from the border zone, and we conducted further in vitro experiments.

**MicroRNA Can Be Released by Stimulation With a Calcium Ionophore**

Our results from in situ hybridization of miR-133a and serum elevation of miR-133a in patients with Takotsubo cardiomyopathy indicated that there is a possibility that the living myocardium may be the source of circulating miR-133a. It is also known that circulating miRNAs are included in exosomes, which are released after the stimulation with Ca$^{2+}$.

Thus, we examined whether miR-133a is released from living H9c2 cells after stimulation with A23187. We stimulated cultured H9c2 cells with A23187 for 1 hour and then collected the culture medium. Exosomes were collected in accordance with a conventional centrifugation method, and total RNA was prepared from the exosomes. Figure 6A shows that A23187 dose-dependently induced the release of miR-133a from H9c2 cells into the culture medium. However, we could not detect the elevation of miR-133a when cells were stimulated with A23187 at concentrations of 0.1 or 0.25 μmol/L, when there was no induction of cell death (Figure 6B and 6C). We also tried to address the involvement of cell death in the release of miR-133a from cells; we examined whether Z-VAD-FMK, a pan-caspase inhibitor, could reduce cellular injury after 1 hour of incubation with A23187. However, caspase inhibitor could not suppress such early cellular injury or miR-133a release from cells.

**MicroRNA Released by Calcium Ionophore Treatment Is Functional**

We further examined whether the miRNA released by A23187 treatment was functional. We generated a miR-133a sensor vector, as indicated in the Methods section (Figure 7A). The function of this sensor vector was evaluated in 293FT cells cotransfected with miR-133a expression vector and the sensor vector (Figure 7B). After collection of exosomes, as indicated in the previous experiment, we added these exosomes to 293FT.
cells expressing the miR-133a sensor vector (Figure 7C). As shown in Figure 7D, only exosomes prepared from H9c2 cells stimulated with A23187 reduced the luciferase activity. These results indicate that A23187 can release miR-133a–containing exosomes, and it is also possible that circulating miRNAs after MI may be transferred into other cells or organs to induce their specific functions.

Discussion

We report that (1) the levels of circulating miR-1 and miR-133a are elevated early after the onset of chest pain when there was no elevation in CPK or cTnT, (2) elevated levels of miR-133a were also observed in patients with UAP and Takotsubo cardiomyopathy, (3) miRNA microarray analysis and in situ hybridization indicated that miRNAs are released from infarcted and peri-infarcted myocardium, (4) miR-133a contained in the exosome fraction is released in association with cell death, and (5) miR-133a in the exosome fraction is transferable and functional.

Several recent reports have indicated that miR-1, miR-133a, miR-208, and miR-499 are upregulated after MI and can be used as biomarkers of acute MI.11,12,14 However, miR-208a and miR-499 were not detectable in serum of patients with acute MI as long as the described method was used. One reason for this may be the expression levels of these miRNAs are lower than the detectable limit. Moreover, there were also some discrepancies between miR-1 and miR-133a levels. miR-1 levels were declined rapidly in serum of patients with ACS compared with miR-133a levels (Figure 1C and 1D) and were less sensitive for the diagnosis of ACS (Figure 3C and 3D). Because it is reported that in vitro studies each miRNA has a different distribution in the medium, such as in exosomes and microvesicular bodies,25 the mechanism of release of miR-1 and miR-133a from injured myocardium may be different.

Although cTnT is a robust diagnostic and prognostic marker in the setting of suspected ACS, there are still perceived limitations of cTnT. Because of the kinetics of cTnT release from the disrupted cellular cytoskeleton, early diagnostic studies of troponin indicated that a delay of at least 6 hours from symptom onset was necessary to support acceptable assay sensitivity. Moreover, detectable quantities of cTnT are released only in the setting of irreversible myocardial injury, for example, myocardial necrosis, thereby leaving the patients with unstable angina, which by definition indicates myocardial ischemia without necrosis, undiagnosed with cTnT. In this report, we observed that the level of serum miR-133a was elevated within 2 hours after the onset of chest pain. The rapid elevation of circulating miR-133a after MI and its elevation in patients with UAP or Takotsubo cardiomyopathy underscore the clinical use of assays of circulating miRNAs. However, it should be noted that the newer generations of cTnT assays undergoing evaluation appear to decrease the limit of detection by an additional 10- to 100-fold compared with the current generation of assays,26 and the limitations for cTnT may be less than at present.

Currently, reverse transcription and amplification is inevitable for the measurement for miRNA. Because the optimal treatment of many acute cardiovascular diseases relies more on early recognition and early initiation of therapy compared with many noncardiac conditions, a greater importance is placed on the rapidity and sensitivity of the diagnosis. Therefore, advances in technology are expected for the measurement of circulating miRNAs.

Figure 6. miR-133a in exosomes is released into the culture media when H9c2 cells are stimulated with a calcium ionophore, and the release is associated with cell death. A, miR-133a levels in exosomes in the medium increased significantly when H9c2 cells were stimulated with 1 μmol/L A23187 (n=5 to 6). B, Cell injury rates were determined using a lactate dehydrogenase assay (n=3 to 7). *P<0.05; **P<0.001; ***P<0.0001.
It is still unclear how miRNAs are released from cardiomyocytes into circulating blood. We studied changes in the expression profiles of cardiac miRNAs by using an miRNA microarray technique in a mouse model of MI. The expression levels of cardiomyocyte-specific miRNAs, such as miR-1, miR-133a, miR-208, and miR-499, were reduced in the infarcted region. Moreover, in situ hybridization of miR-133a clearly showed that the infarcted region does not contain miR-133a in cardiomyocytes. The signals of miR-133a also disappeared in several cardiomyocytes in the border zone.

We attempted to determine whether there is a possibility that the surviving cardiomyocytes can release miRNAs in vitro. Previous reports suggested that living cells can release exosomes after stimulation with a calcium ionophore. Therefore, we stimulated H9c2 cardiomyoblasts with A23187 and attempted to detect miR-133a in the exosome fraction from the culture medium. A23187 dose-dependently increased the expression levels of miR-133a; however, significant upregulation was observed only at concentrations in which cell death occurred. These results suggest that circulating miRNAs released after MI may originate from dead cells after MI.

It has been shown recently that exosomes7 and microparticles8 can be released from cells and subsequently internalized by other cells. Therefore, it is possible that miRNAs released after MI can affect or be used in the heart or distant organs. Thus, we also tried to see whether circulating miR-133a might have any function in an in vitro assay. After the collection of exosomes from A23187-stimulated H9c2 cells, we added these exosomes to cells expressing a miR-133a sensor vector. As shown in the results, only exosomes prepared from H9c2 cells stimulated with A23187 reduced luciferase activity, and it was indicated that A23187 can induce the release of exosomes that contain miR-133a from H9c2 cells. These results suggest that after MI, circulating miRNAs may be taken up into other cells or organs for the induction of specific functions. Because both miR-1 and miR-133a appear to play a role in the regulation of cardiac hypertrophy,27 their capture by adjacent surviving myocardium may be utilized to suppress inadequate hypertrophy when the blood flow and energy supply is limited. Further research is required to identify targets of miRNAs released from the injured myocardium.

**Acknowledgments**

We thank N. Sowa for providing excellent technical assistance. We also thank members of the clinical research unit for the collection of samples.

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Promotion of Science and the Ministry of Education, Culture, Sports, Science, and Technology (to Drs Kimura, Kita, Horie, and Ono).

Disclosures

None.

References


CLINICAL PERSPECTIVE

Recently, it was reported that levels of muscle-specific microRNA (miRNA or miR) increased in the plasma or serum of patients with acute myocardial infarction (MI). However, it is still poorly understood from where or under what conditions miRNAs are released into the blood stream. We first show that muscle-specific miR-1 and miR-133a increased in the serum of patients with acute coronary syndrome, and these microRNA levels were elevated in the early phase after the onset of acute MI, when there was no increase of serum cardiac Troponin T. The expression levels of these microRNAs were correlated with the serum cardiac Troponin T levels. We also indicated that the miR-133a levels increased in the serum of patients with not only acute MI but also unstable angina pectoris and Takotsubo cardiomyopathy. Next, we attempted to determine the tissue distribution of miR-133a in a mouse model of MI and revealed that not only the infarcted region but also the border zone is the source of circulating miR-133a. Furthermore, in vitro experiments indicated that stimulation of calcium ionophore increased miR-133a release from cardiac myoblasts only at concentrations in which cell death was observed and the released miRNA was functional. Taken together, our data suggest that circulating miR-133a, which is derived from injured myocardium, can be used as a sensitive, early diagnostic biomarker for myocardial damage. Additionally, because released microRNAs can regulate gene expression in other cells, the present study may provide a new insight into the function of miRNA in the pathophysiology of MI.
Increased MicroRNA-1 and MicroRNA-133a Levels in Serum of Patients With Cardiovascular Disease Indicate Myocardial Damage
Yasuhide Kuwabara, Koh Ono, Takahiro Horie, Hitoo Nishi, Kazuya Nagao, Minako Kinoshita, Shin Watanabe, Osamu Baba, Yoji Kojima, Satoshi Shizuta, Masao Imai, Toshihiro Tamura, Toru Kita and Takeshi Kimura

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Supplemental Methods

Diagnosis for acute myocardial infarction

Acute myocardial infarction (MI) on initial presentation was diagnosed if there was 20 minutes or more of chest pain and (1) a serial rise and fall of creatine phosphokinase (CPK) and CK-MB fraction, or a cardiac troponin T (cTnT) level $\geq 0.1$ ng/mL; the decision limit in effect during hospitalization; (2) new Q-wave formation during the initial 24 hours after presentation; or (3) coronary arteriogram with documented acute occlusion of a major coronary vessel within 24 hours of admission. ST-segment elevation (STEMI) was determined by ST-segment elevation of $>0.2$ mV in at least 2 contiguous electrocardiogram (ECG) leads. Unstable angina was diagnosed in patients who were without CPK, CK-MB, and cTnT elevation or ECG criteria for acute MI, and the treating physician diagnosed acute coronary syndrome (ACS)-related chest pain.

In situ hybridization of miR-133a

A mouse was perfused with 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) and the heart was removed. The sections were re-fixed in 4% PFA/PBS at 4 °C for 10 min. After
fixation, the specimens were incubated in 15% sucrose/PBS at 4 °C for 1 hour and in 30% sucrose/PBS overnight at 4 °C. The next day, the tissue samples were embedded in Tissue-Tek® OCT (Sakura, Japan) compound. We used LNA-modified DNA oligonucleotide probes (mmu-miR-133a, product # 39460-00; Scramble-miR, negative control, # 99004-00; Exiqon, Vedbaek, Denmark). Probes were labeled enzymatically using a 3’-end digoxigenin (DIG) labeling kit (Roche) in accordance with manufacturer’s instructions and purified using illustra® MicroSpin® G-25 Columns (GE Healthcare). miR-133a in situ hybridization was performed on 10 μm frozen sections of mouse hearts as described previously with slight modifications 18. Tissue sections were dried for 20 min, washed, and fixed in 4% PFA/ PBS at 4 °C for 10 min. The heart tissue sections were treated in 1 μg/mL proteinase K for 12 min, washed, and re-fixed in 4% PFA/PBS at 4 °C for 10 min. Subsequently, the sections were washed and acetylated in 1.15% triethanolamine and 0.25% acetic anhydride. After post-acetylation washing, the sections were prehybridized in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM pH 8.0 Tris-HCl, 5 mM EDTA, 10 mM pH 8.0 NaPO₄, 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mg/mL yeast RNA) for 4 hours. The digoxigenin (DIG)-labeled detection probes (20 nM) were added to the slides, and the heart tissues sections were covered with plastic coverslips (HybriSlip™, Grace Bio-Labs) and hybridized with the probes overnight at 55 °C for miR-133a. The next day, the slides were soaked in 5× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate) and
the coverslips were removed. Post-hybridization stringency washing in 50% formamide/1× SSC containing 0.1% Tween-20 was performed twice at 51 °C for 1 hour each, followed by washing in 0.2× SSC and PBS. After blocking for 1 hour in blocking solution (0.5% Roche Blocking reagent, 10% goat serum, 0.1% Tween-20, 1× PBS), the sections were incubated in blocking solution containing alkaline phosphatase conjugated anti-DIG Fab fragments (1/4000, Roche). The slides were then washed in PBS containing 0.1% Tween-20 and treated in NTM buffer (100 mM Tris-HCl pH 9.5, 5 M NaCl, 1 M MgCl₂). Color development was performed in Developer solution (1-Step™ NBT/BCIP [Pierce] and 2 mM levamisol [Sigma]). Unless otherwise indicated, all washing steps were conducted in PBS and all incubations were performed at room temperature. Images were captured using a microscope (BZ-8100, Keyence).

Triphenyltetrazolium chloride (TTC) staining

A removed heart was sliced into three separate sections. The middle section was incubated in 1.5% triphenyltetrazolium chloride (TTC) at 37 °C for 15 min. After TTC staining, viable myocardium appeared red.

Cell culture and evaluation of cell viability

A rat cardiomyoblast cell line, H9e2, and 293FT cells were obtained from the American
Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO2 incubator. Oligomyocin and Z-VAD-FMK, a Caspase inhibitor I, were purchased from Calbiochem. Deferoxamine was purchased from Sigma. H9c2 cells were stimulated with calcium ionophore A23187 (Sigma) or indicated agent, and cultured in serum-free medium (SFM). In Z-VAD-FMK experiment, H9c2 cells were pre-incubated in Z-VAD-FMK contained medium for 1 hour. Total LDH activity in the culture medium was measured using a LDH Cytotoxicity Assay Kit™ (Cayman Chemical Company) in accordance with the manufacturer’s instructions. Cell viability was evaluated using a MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma) assay. Briefly, H9c2 cells were stimulated with A23187 in SFM for 1 hour and labeled with MTT at a final concentration of 0.5 mg/mL for at least 4 hours at 37 °C. Viability was then evaluated by measuring the absorbance at 570 nm using an Elx800 Microplate Reader (BIO-TEK Instruments Inc.).

**Plasmids**

miR-133a sensor vector was constructed as described previously. Detailed methods are described in the Supplemental Methods. Unmodified pMIR-REPORT™ vector was used as the sensor control vector. pRL-TK™ Renilla reniformis luciferase (RL) plasmid was purchased
from Promega. An expression vector for miR-133a was generated using a BLOCK-iT™ Pol II
miR RNAi Expression Vector Kit in accordance with the manufacturer’s instructions
(Invitrogen).

_Dual-luciferase assay_

For the sensor vector efficacy study, 0.05 μg of FL reporter gene (miR-133a sensor or sensor
control), 0.2 μg of miR-133a expression vector, and 0.02 μg of pRL-TK™ RL plasmid for
normalizing transfection efficiency were transfected into 293FT cells. After 2 days incubation,
both luciferase activities were measured using a dual-luciferase reporter assay system (Toyo Ink
Co.). For the exosome miRNA functional study, 0.02 μg of FL reporter gene and 0.02 μg of RL
plasmid were tranfected. At 24 hours after transfection, the exosome pellet of H9c2 cells was
re-suspended in SFM, and the medium of the 293FT cells was changed to exosome pellet
re-suspended media. After 2 days incubation, both luciferase activities were measured as
described above.
### Supplemental Table 1. Clinical characteristics of acute coronary syndrome patients.

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Mean ± S.E. 69.7 ± 2.4 8.8 ± 1.6 3032 ± 761 231.3 ± 49.8

HFABP, heart-type fatty acid binding protein; cTnT, cardiac troponin T; CPK, creatine phosphokinase; CK-MB, creatine phosphokinase-MB fraction; M, male; F, female; MI, myocardial infarction; STEMI, ST-elevation MI; NSTEMI, non-ST-elevation MI; UAP, unstable angina pectoris; N.A., not applicable; N.D., not determined.
Supplemental Figure Legends

Supplemental Figure 1.

miR-17-5p (A), miR-454 (B), and miR-1249 (C) are not suitable as normalizers for evaluating miRNA expression levels in serum. Comparisons of RNA levels in serum of patients with non-acute coronary syndrome (non-ACS) and ACS using Taqman microRNA RT-PCR. miR-17-5p: non-ACS patients, n = 7, ACS patients, n = 5; miR-451: non-ACS patients, n = 16, ACS patients, n = 13; miR-1249: non-ACS patients, n = 7, ACS patients, n = 5. *, p<0.05.

Supplemental Figure 2.

miR-133a levels were up-regulated in the serum of patients with unstable angina pectoris (UAP) and Takotsubo cardiomyopathy (Takotsubo CM). A. In UAP patients, miR-133a levels were increased significantly in the serum compared with non-ACS patients and non-Takotsubo CM patients. B. In Takotsubo CM patients, miR-133a levels were also increased in the serum compared with non-ACS patients. *, p<0.05; **, p<0.005.

Supplemental Figure 3.

Serum levels of miR-1 (A) and miR-133a (B) in mouse serum after myocardial infarction. Significant elevation was observed at 1 hour after coronary ligation. n = 3-5. *, p<0.05.
Supplemental Figure 4.

*In situ* hybridization of miR-133a and triphenyltetrazolium chloride (TTC) staining of a mouse myocardial infarction model. **A.** Short axis view of miR-133a *in situ* expression. **B.** Transverse section of an infarcted heart after TTC staining.

Supplemental Figure 5.

Mature miR-133a levels decreased after stimulation of oligomycin or deferoxamine (DFO). When H9c2 cells were stimulated with oligomycin of 20 μmol/L for 24 hours (**A**) or DFO of 200 μmol/L for 60 hours (**B**), cell viability significantly decreased. Stimulating of oligomycin (**C**) or DFO (**D**), mature miR-133a levels were down-regulated in H9c2 cells evaluating Taqman microRNAs RT-PCR. *n = 3.* *, p<0.05; ***, p<0.0005.
Supplemental Fig. 1.

A. miR-17-5p

B. miR-454

C. miR-1249
Supplemental Fig. 2.

A  Unstable angina

B  Takotsubo cardiomyopathy

miR-133a expression level

non-ACS  non-Takotsubo CM  UAP

non-ACS  non-Takotsubo CM  Takotsubo CM
Supplemental Fig. 3.

(A) miR-1

(B) miR-133a

miR-1 expression level (A.U.)

miR-133a expression level (A.U.)

Sham MI Sham MI Sham MI
1 hr 3 hr 6 hr 1 hr 3 hr 6 hr

* n.s. n.s. * n.s.
Supplemental Fig. 5.

**A**

![Graph showing cell viability](image)

**B**

![Graph showing miR-133a/U6](image)