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# Circulating p53-Responsive MicroRNAs Are Predictive Indicators of Heart Failure After Acute Myocardial Infarction

# **Short Communication**

Sen Matsumoto,\* Yasuhiko Sakata,\* Shinichiro Suna, Daisaku Nakatani, Masaya Usami, Masahiko Hara, Tetsuhisa Kitamura, Toshimitsu Hamasaki, Shinsuke Nanto, Yukio Kawahara, Issei Komuro

<u>Rationale</u>: Despite a recent decline of in-hospital mortality attributable to acute myocardial infarction (AMI), the incidence of ischemic heart failure (HF) in post-AMI patients is increasing. Although various microRNAs have been proposed as diagnostic indicators for AMI, no microRNAs have been established as predictors of ischemic HF that develops after AMI.

<u>Objective</u>: We attempted to identify circulating microRNAs that can serve as reliable predictors of ischemic HF in post-AMI patients.

<u>Methods and Results</u>: Using sera collected a median of 18 days after AMI onset, we screened microRNAs in 21 patients who experienced development of HF within 1 year after AMI and in 65 matched controls without subsequent cardiovascular events after discharge. Among the 377 examined microRNAs, the serum level of only miR-192 was significantly upregulated in AMI patients with development of ischemic HF. Because miR-192 is reported to be p53-responsive, the serum levels of 2 other p53-responsive microRNAs, miR-194 and miR-34a, also were investigated. Interestingly, both microRNAs were coordinately increased with miR-192, particularly in exosomes, suggesting that these microRNAs function as circulating regulators of HF development via the p53 pathway. Furthermore, miR-194 and miR-34a expression levels were significantly correlated with left ventricular end-diastolic dimension 1 year after AMI.

<u>Conclusions</u>: In the sera of post-AMI patients who experienced development of de-novo HF within 1 year of AMI onset, the levels of 3 p53-responsive microRNAs had been elevated by the early convalescent stage of AMI. Further investigations are warranted to confirm the usefulness of these circulating microRNAs for predicting the risk of development of ischemic HF after AMI. (*Circ Res.* 2013;113:322-326.)

Key Words: exosomes ■ heart failure ■ microRNA ■ myocardial infarction ■ tumor suppressor protein p53

A lthough recent advances in the management of acute myocardial infarction (AMI), including primary percutaneous coronary intervention strategies and evidence-based therapies, have resulted in a substantial decline in mortality, the number of post-AMI patients who survive AMI but experience development of ischemic heart failure (HF) is increasing worldwide.<sup>1</sup> Therefore, the identification of biomarkers that can predict risk of HF development in post-AMI patients is needed for optimizing management and treatment strategies. To date, several types of biomarkers, such as N-terminal probrain natriuretic peptide and cardiac troponin T, have been shown to predict cardiovascular events after AMI; however, it remains inconclusive whether these biomarkers can predict future HF in post-AMI patients.<sup>2</sup>

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MicroRNAs are small endogenous noncoding RNAs that regulate gene expression posttranscriptionally. Recently, various cell types were found to release microRNAs in

\*These authors contributed equally to this work.

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Nonstandard Abbreviations and Acronyms			
ami	acute myocardial infarction		
Hf	heart failure		

membrane-bound vesicles termed exosomes, which circulate stably in the bloodstream.<sup>3–5</sup> Although the physiological significance of circulating microRNAs is not fully understood, they have attracted attention as potential diagnostic and prognostic biomarkers for various diseases, particularly cancer.<sup>3,4</sup>

With respect to cardiovascular disease, several cardiac microRNAs, including miR-1, miR-133a, and miR-208a, have been detected in the serum in the acute phase of AMI and thus represent potentially useful diagnostic markers for AMI.<sup>5,6</sup> However, these microRNAs are most likely released from necrotic heart tissue into the blood directly and are not encapsulated within exosomes and, therefore, have short half-lives. For this reason, these cardiac microRNAs are unlikely to be predictive of future HF development in post-AMI patients.<sup>6</sup> The aim of the present study was to identify circulating microRNAs that can serve as predictors of HF development in patients who survive the acute stage of AMI.

#### **Methods**

We retrospectively analyzed the records of patients registered in the Osaka Acute Coronary Insufficiency Study, which has been described elsewhere.<sup>7</sup> The study protocol was approved by the ethics committee of each participating hospital, and written informed consent was provided by each patient at the time of registration. On the basis of the results of an initial screening (Online Table I), we performed a second screening to examine the microRNA profiles of an increased number of matched patients (HF group, n=21; control group, n=65; Online Table II). Detailed methods are described in the Online Data Supplement. Results were analyzed by the Mann–Whitney *U* test or Student *t* test. Correlations were tested using Pearson correlation coefficient. Statistical significance was set as P<0.05, P<0.01, or P<0.001.

#### Results

To identify microRNAs with altered expression in accordance with HF development after the convalescent stage of AMI, we initially compared the expression level of individual microRNAs in sera collected a median of 18 days after AMI onset using a high-throughput array between HF and control groups (n=7, respectively). Among 377 microRNAs, the expression levels of 14 microRNAs were found to significantly differ between the 2 groups when U6 small nuclear RNA, miR-766, or let-7d was used as an internal control (Online Table I). The serum levels of the 14 microRNAs were further examined in a second screening. Two of the microRNAs, miR-485-3p and miR-518d-3p, were below the limit of detection of the assay. However, the expression level of miR-192 was significantly upregulated in the HF group, whereas those of the remaining 11 microRNAs were comparable between the 2 groups (Figure 1A; Online Figure I).

Because miR-192 is reported to be p53-responsive, the levels of 2 p53-responsive microRNAs, miR-194 and miR-34a,<sup>8,9</sup> also were measured. As anticipated, the serum levels of miR-194 and miR-34a also were upregulated in the AMI patients who experienced development of ischemic HF (Figure 1B). Furthermore, the miR-192, miR-194, and miR-34a expression



Figure 1. Upregulation of 3 specific microRNAs in sera of the heart failure (HF) group. The expression levels of miR-192 (A) and of miR-194 and miR-34a (B) in sera of the HF (black bars, n=21) and control groups (white bars, n=65) are displayed after normalization to U6 snRNA. Similar results were obtained when miR-766 or let-7d was used as an internal control. \*P<0.05, \*\*P<0.01.

levels were significantly correlated with one another, suggesting that the 3 microRNAs were coordinately upregulated in a single cascade, such as the p53 signaling pathway. In particular, a high correlation (r=0.86) was detected between miR-194 and miR-34a (Online Figure II). We also examined the serum levels of miR-208 and miR-499, which are candidate markers for severe myocardial damage and necrosis,<sup>10</sup> but neither microRNA was detected in the serum of either the HF group or the control group.

To investigate whether miR-192, miR-194, and miR-34a were released into the serum within exosomes or had directly leaked from necrotic heart tissue, we separated collected serum



Figure 2. Circulating miR-34a, miR-192, and miR-194 are highly enriched in serum exosomes. A, Western blot analysis against CD63 in the exosome (Exo) and supernatant fractions (Sup) of the serum. B, Expression levels of miR-192, miR-194, and miR-34a in the serum exosome fraction (black bars, n=4) and supernatant fraction (white bars, n=4) are displayed after normalization to U6 snRNA. C, Expression levels of miR-192, miR-194, and miR-34a in the serum Exo fraction and Sup fraction obtained from the heart failure group (gray bars, n=4) and control group (white bar, n=4) are shown. \*P<0.05.



Figure 3. p53-responsive microRNAs were upregulated after p53 activation and influenced cell viability. Treatment of rat H9c2 cells with doxorubicin (Dox) increased p53 protein level (A), followed by an increase of caspase 3/7 activity (B) and a decrease of cell viability (C). Along with p53 activation, intracellular (D) and subsequently extracellular (E) levels of miRNA-192, miRNA-194, and miRNA-34a were upregulated. Dox treatment after knockdown of all 3 microRNAs, but not individual microRNA, in H9c2 cells increased cell viability not in 24 hours, but in 36 hours (F), whereas coculture with media containing 3 p53-responsive microRNAs decreased cell viability in the presence of Dox (G). n=3-5 for each experiment (B-G). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

into exosome and supernatant fractions. Western blot analysis against CD63, an exosome marker protein, confirmed the purity of the 2 fractions (Figure 2A). The levels of the 3 microR-NAs were then quantified in each fraction, demonstrating that all 3 microRNAs were highly enriched in the exosome fraction (Figure 2B). We further compared the expression levels of these microRNAs in each fraction between the HF and control groups. Although no significant differences were detected in the supernatant fraction, the expression levels of miR-192 and miR-194 in the exosome fraction of the HF group were significantly higher than those of the control group (Figure 2C). A similar tendency also was observed for miR-34a, suggesting that the 3 microRNAs were mainly released into the serum within exosomes. Using cultured myoblasts derived from embryonic rat heart, we confirmed that both intracellular and extracellular levels of miR-192, miR-194, and miR-34a were increased after p53 activation, and that knockdown of these 3 microRNA increased cell viability, whereas transfer of extracellular microRNAs via the culture media decreased cell viability of myoblasts (Figure 3; Online Figure III).



Figure 4. Circulating levels of miR-194 and miR-34a are correlated with left ventricular diastolic dimension (LVDd) measured  $\approx$ 1 year after acute myocardial infarction (AMI) onset. Each dot represents the serum expression levels of miR-192 (left), miR-194 (middle), and miR-34a (right) relative to U6 snRNA ( $-\Delta$ Ct) and LVDd that was measured  $\approx$ 1 year after the onset of AMI. Values for the heart failure group are indicated by black dots (n=12) and those of the control group are indicated by open dots (n=46). CI indicates confidence interval.

Finally, we analyzed whether the serum levels of the 3 p53responsive microRNAs were correlated with left ventricular diastolic dimension, a clinical parameter of cardiac remodeling. The expression levels of miR-194 and miR-34a, but not miR-192, were positively correlated with left ventricular diastolic dimension, which was measured for 58 patients by echocardiography ≈1 year (median, 402 days) after the onset of AMI (miR-194: r=0.33, P=0.01; miR-34a: r=0.38, P=0.003; and miR-192: r=0.09, P=0.52; n=58; Figure 4). Furthermore, the serum levels of miR-194 (r=-0.28; P=0.03) and miR-34a (r=-0.27; P=0.04), but not miR-192 (r=-0.21; P=0.10), were correlated with left ventricular ejection fraction measured ≈1 year after AMI onset. These correlations were not altered after consideration of the history of HF-related medications as a potential confounding factor (Online Table III). This finding suggested that the serum levels of these p53-responsive microRNAs could predict left ventricular remodeling after the convalescent stage of AMI.

#### Discussion

In this study, we demonstrated that serum levels of the microRNAs, miR-192, miR-194, and miR-34a, are upregulated as early as a median of 18 days after AMI onset in patients who survived AMI but experienced development of HF within 1 year. Furthermore, circulating miR-194 and miR-34a levels in the convalescent stage of AMI were associated with left ventricular diastolic dimension. Thus, these p53-responsive microRNAs may be useful for stratifying the risk of future ischemic HF events and cardiac remodeling in post-AMI patients.

Because the expression levels of the 3 identified microRNAs were well-correlated with each other, it is conceivable that their expression was coordinately upregulated in a single cascade. One of the most likely candidates for an inducer of this coordinated expression is the tumor suppressor p53, because all 3 microRNAs are reported to be induced by the direct binding of p53 to the promoter regions of the corresponding genes.<sup>8,9</sup> In particular, it was recently demonstrated that miR-34a is induced in a p53-dependent manner, and that cardiomyocyte apoptosis can be suppressed through the blockade of miR-34a

processing in a mouse model of AMI.<sup>11</sup> We confirmed that these 3 microRNAs are induced by p53 activation in cultured myoblasts and are subsequently released from cells. Furthermore, transfer of these extracellular microRNAs accelerated cell death (Figure 3). Taken together with our recent finding that p53 accumulation in the myocardium in response to pressure overload or after MI plays an essential role in HF progression in mice,<sup>12,13</sup> our present results suggest that activation of p53 and the increased expression of the p53-responsive microRNAs, miR-192, miR-194, and miR-34a, are likely involved in the pathogenesis of HF after AMI. However, it remains undetermined whether circulating p53-responsive microRNAs were derived from infarct or ischemic heart tissues.

Interestingly, circulating miR-208b and miR-499, which are both reported to reflect severe myocardial damage or necrosis,<sup>10</sup> were not detected in the serum of either HF patients or control patients in the present study. Thus, detection of p53-responsive microRNAs may have reflected ongoing myocardial damage associated with the development of future HF, which was qualitatively different from necrosis.

It was recently reported that miR-34 family members are upregulated in the heart in response to stress, including AMI, and that systemic injection of miR-34 anti-miR significantly attenuated cardiac remodeling and dysfunction in a mouse AMI model.<sup>14</sup> Accordingly, modification of the p53 pathway using microRNAbased technologies may be a novel therapeutic approach to prevent ischemic HF in post-AMI patients, particularly for those with increased levels of circulating p53-responsive microRNAs.

In conclusion, we identified 3 p53-responsive microRNAs that were upregulated in the serum of post-AMI patients who experienced development of HF within 1 year of AMI onset. Further investigations with the increased number of samples and in other cohorts are required to confirm the present findings and future clinical applications of miR-192, miR-194, and miR-34a as predictive indicators of HF.

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#### None.

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**Disclosures** 

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## **Novelty and Significance**

#### What Is Known?

- A number of patients survive acute myocardial infarction (AMI) but experience development of chronic ischemic heart failure (HF).
- MicroRNAs are noncoding small RNAs that are released from various cell types and circulate in the bloodstream.
- Accumulation of p53, a multifunctional protein, after AMI plays an essential role in HF development in mice.

#### What New Information Does This Article Contribute?

- Serum levels of 3 p53-responsive microRNAs, miR-192, miR-194, and miR-34a, are higher in the early convalescent stage of AMI (median 18 days after onset) in patients with development of HF within 1 year.
- Serum levels of miR-194 and miR-34a correlate with left ventricular diastolic dimension, a clinical parameter of cardiac remodeling, measured 1 year after AMI.

 Cultured myoblasts exposed to miR-192, miR-194, and miR-34a released from other cells have decreased viability.

Although measurements of circulating microRNAs have been reported to be useful for the diagnosis of AMI and HF, it is not clear whether microRNA levels are reliable predictors of ischemic HF that develops after AMI. We found that 3 p53-responsive microR-NAs are upregulated during the early convalescent stage of AMI in the sera of patients who experienced development of HF within 1 year after discharge. Moreover, serum levels of these microR-NAs were positively correlated with left ventricular diastolic dimension 1 year after AMI. These findings suggest that circulating p53-responsive microRNAs may be clinically useful as predictive indicators of HF in post-AMI patients.

## **Supplemental Material**

## **Detailed Methods**

## **OACIS** registry

The Osaka Acute Coronary Insufficiency Study (OACIS) is a prospective, multicenter observational study designed to collect and analyze demographic, procedural, and outcome data and blood samples in patients with acute myocardial infarction (AMI) at 25 collaborating hospitals in Osaka, Japan, as previously described<sup>1-3</sup>. Briefly, in accordance with the guideline of each participating hospital, research cardiologists and specialized research nurses or coordinators recorded data on sociodemographic variables, medical histories, therapeutic procedures, and clinical events during hospitalization, as well as follow-up clinical data at 3 and 12 months after the occurrence of AMI and annually thereafter. Information was obtained from hospital medical records and by direct interviews with patients, their family members, and their treating physicians. The study protocol was approved by the ethics committee of each participating hospital, and written informed consent was provided by each patient at the time of registration for blood sampling and analysis.

#### Patients

Among 8,603 patients with AMI who were registered in OACIS between 1998 and 2009, we first selected 4160 consecutive patients who fulfilled the following criteria: 1) survival discharge, and 2) written informed consent for serum analysis was obtained at the time of registration. Among the 4160 patients, 218 were hospitalized for HF during a median of 1069 days after survival discharge. In the first screening phase, we randomly selected 7 patients who developed HF within one year after discharge (HF group) and an additional 7 patients who did not experience any cardiovascular events throughout a 2-year follow-up period (control group), using propensity score-based matching of age, sex, diabetes mellitus, hypertension, dyslipidemia, smoking, previous MI, Killip class >II at AMI onset , infarction size, reperfusion therapy rates, and medication at the time of serum collection to adjust for potential baseline differences between the two groups. In the second screening, we increased the number of patients (HF group; n = 21, control group; n = 65) and again matched the backgrounds of each group (**Online Table II**) to validate the results obtained in the first screening.

#### Serum collection

Fasting blood samples were collected into serum separator tubes in the convalescent phase of AMI (median; 18 days after AMI onset) and were then incubated at 4°C for 4 h. After centrifugation at 4,000 rpm for 15 min, aliquots of serum were transferred to new tubes and stored at -80°C until use.

#### **Exosome isolation**

Serum exosomes were isolated using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA) according to the manufacturer's protocol with a minor modification. Briefly, 250  $\mu$ l of serum was centrifuged at 5,700 rpm for 15 min at 4°C to remove cell debris, and the resulting supernatant was then transferred to new sterile tubes, to which 63  $\mu$ l of ExoQuick Exosome Precipitation Solution was added. After overnight incubation at 4°C, the tubes were centrifuged at 4,000 rpm for 30 min at 4°C to obtain the exosome-containing pellet, which was then resuspended in 25  $\mu$ l phosphate buffered saline (exosome fraction). Half of the supernatant fraction (~150  $\mu$ l) and the exosome fraction were stored at -20°C until use.

When exosomes were extracted from culture media, we treated culture media with RNase cocktail (Life Technologies Co., Carlsbad, CA) at a final concentration of 5 U/mL RNase A and 200 U/mL RNase T1, then incubated them at 37°C for 30 min. Then, 500  $\mu$ l of culture media was centrifuged at 5,700 rpm for 15 min at 4°C to remove cell debris, and the resulting supernatant was then transferred to new sterile tubes, to which 500  $\mu$ l of ExoQuick Exosome Precipitation Solution was added. After overnight incubation at 4°C, the tubes were centrifuged at 4,000 rpm for 30 min at 4°C to obtain the exosome-containing pellet, which was then resuspended in 25  $\mu$ l phosphate buffered saline (exosome fraction) as previously described<sup>4</sup>.

#### **RNA** isolation

Total RNA in the serum was isolated using a mirVana PARIS kit (Life Technologies Co.) according to the manufacturer's protocol. Briefly, 1 ml of the serum was thawed on ice and lysed with an equal volume of 2x Denaturing Solution for 5 min. After an equal volume of acid phenol chloroform was added, the sample tube was centrifuged at 12,000 rpm for 5 min at 4°C. The aqueous phase was carefully transferred to a new tube, and 1.25 volumes of 100% ethanol were then added. The sample was then passed through a mirVana PARIS column followed by washing three times with 75% ethanol, and total RNA was then eluted in 100  $\mu$ l of preheated (95°C) nuclease-free water and stored at -80°C until use.

Total RNAs in the serum exosome and supernatant fractions were extracted using ISOGEN II (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Briefly, the supernatant (~150  $\mu$ l) and exosome solution (25  $\mu$ l) were diluted to 400  $\mu$ l with nuclease-free water and then lysed with 1 ml of ISOGEN II at room temperature for 15 min. After centrifugation at 12,000 rpm for 15 min at 4°C, 1 ml of the supernatant was transferred to a new tube and mixed with 1 ml isopropanol and 7.5  $\mu$ g/ml glycogen. After incubation on ice for 30 min, the sample tubes were centrifuged at 12,000 rpm for 15 min at 4°C, and then washed twice with 500  $\mu$ l 75% ethanol. Finally, the isolated RNAs from

the exosome and supernatant fractions were dissolved in 25  $\mu$ l and 12.5  $\mu$ l of RNase free water, respectively, and stored at -80°C until use. When total RNAs were extracted from cell culture media using ISOGEN II, a total of 2.5 fmol of synthetic C. elegans microRNA-39 (Qiagen, Hilden, Germany) was added to media as an external standard as previously described<sup>4</sup>.

### Reverse transcription (RT) and preamplification

RT and preamplification steps were performed using a TaqMan MicroRNA RT kit, Megaplex RT Primers, and Megaplex PreAmp Primers (Life Technologies Co.) according to the manufacturer's protocols. Briefly, 3  $\mu$ l of total RNA was mixed with 0.80  $\mu$ l 10x Megaplex RT primers, 0.20  $\mu$ l 100 mM dNTPs, 0.80  $\mu$ l 10x RT buffer, 0.90  $\mu$ l 25 mM MgCl<sub>2</sub>, 2 U RNase Inhibitor, 75 U MultiScribe Reverse Transcriptase, and 0.20  $\mu$ l nuclease-free water, and then reverse transcribed using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) and the following conditions: 40 cycles of 2 min at 16°C, 1 min at 42°C, and 1 s at 50°C, and for 5 min at 85°C. Subsequently, 2.5  $\mu$ l of the RT product was mixed with 12.5  $\mu$ l 2x TaqMan PreAmp Master Mix, 2.5  $\mu$ l 10x Megaplex PreAmp primers, and 7.5  $\mu$ l nuclease-free water, and was then preamplified for 10 min at 95°C, for 2 min at 55°C and for 2 min at 72°C, and 12 cycles for 15 s at 95°C and for 4 min at 60°C.

#### **MicroRNA** profiling

Expression of 377 microRNAs was screened using a TaqMan Human MicroRNA A Array (version 2.0; Life Technologies Co.). Briefly, 9.0  $\mu$ l preamplified RT product was mixed with 441  $\mu$ l nuclease-free water and 450  $\mu$ l 2x TaqMan Universal Master Mix II no UNG. Quantitative PCR was performed for 10 min at 95°C, following by 40 cycles of 15 min at 95°C and 1 min at 60°C using a 7900HT Fast Real-Time PCR system (Life Technologies Co.). The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeded a given threshold, and the expression level of each microRNA was normalized to that of U6 snRNA, miR-766 or let-7d using the calculated mean Ct of each sample ( $\Delta$ Ct). The data were analyzed with SDS Relative Quantification Software (version 2.3) using the automatic settings. The relative quantification of microRNA was calculated by the equation of  $2^{-\Lambda_{Ct}}$ , where  $\Delta$ Ct = mean Ct<sub>target</sub> - mean Ct<sub>internal control</sub>. All microRNAs were considered to be expressed when Ct values were less than 40.

## **Real-Time PCR**

Real-time PCR was performed using the 7900HT Fast Real-Time PCR system. Briefly, 1.3  $\mu$ l preamplified RT product was mixed with 7.7  $\mu$ l nuclease-free water, 1.0  $\mu$ l 20x TaqMan Assay and 10  $\mu$ l 2x TaqMan Universal Master Mix II no UNG. We utilized U6 snRNA (or miR-766, let-7d when mentioned) as an internal standard and synthetic C. elegans microRNA-39 as an external standard for

RNAs extracted from cell cluture media, respectively. Quantitative PCR was carried out in duplicate, as described above.

#### **Protein extraction**

Ten µl of the exosome fraction was mixed with 50 µl lysis buffer (20 mM Tris-HCl (pH 7.9), 25% Glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). The sample was then subjected to four freeze-thaw cycles, followed by centrifugation at 15,000 rpm for 20 min at 4°C, and the resulting supernatant was then transferred to a new tube and stored at -80°C until use. Protein concentrations were determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Inc.).

## Antibodies

The primary antibodies used were as follows: mouse monoclonal anti-CD63 (BD Biosciences, San Diego, CA), rabbit polyclonal anti-p53 (Abcam) and mouse monoclonal anti- $\alpha$ -Tubulin (Sigma-Aldrich).

#### Western blot analysis

Twenty µg of proteins extracted from the exosome and supernatant fractions were mixed with sodium dodecyl sulfate (SDS) sample buffer (58 mM Tris-Cl (pH 6.8), 2% SDS, 5% glycerol, 0.002% bromophenol blue, and 100 mM DTT) and denatured by boiling for 5 min at 95°C. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 30 min in running buffer (0.1% SDS, 25 mM Tris, and 192 mM glycine) and transferred to polyvinylidene fluoride (PVDF) membranes at 100 V for 90 min in transfer buffer (25 mM Tris, 192 mM glycine, and 15% methanol). The membrane was blocked with 0.25% nonfat dry milk in phosphate buffered saline containing 0.1% Tween 20 (PBST), and then incubated with primary antibodies for 10 min at room temperature using SNAP i.d. (Millipore, Billerica, MA). After incubating the membranes for 10 min at room temperature with secondary antibodies, if necessary, followed by three washes with PBST, antigen-antibody complexes were visualized by chemiluminescence using ECL-Plus (GE Healthcare UK Ltd., Buckinghamshire, England).

#### Cell culture

The myoblast cell line H9c2, derived from embryonic rat heart<sup>5</sup>, were obtained from American Type Culture Collection, Manassas, VA. H9c2 and HEK293 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids

solution (Life Technologies Co.), 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin and 250 ng/mL amphotericin B (Life Technologies Co.). Cells were grown at 37°C in a 5% CO<sub>2</sub> humidified incubator. For p53 activation, 1.0  $\mu$ M of doxorubicin (Sigma-Aldrich) was added to culture media after 48h of serum removal as previously described<sup>6</sup>.

#### Knockdown of microRNAs in cardiac myoblasts

The Anti-miR miRNA Inhibitors for miR-192, miR-194 and miR-34a were obtained from Life Technologies Co. Transfection of Anti-miR miRNA Inhibitors was performed in a 96 well plate with Lipofectamine RNAiMAX (Life Technologies Co.). In brief, 3.6 pmol of Anti-miR miRNA Inhibitor was mixed with 0.2  $\mu$ l of Lipofectamine RNAiMAX in 10  $\mu$ l of Opti-MEM medium (Life Technologies Co.) and then the mixture was added to each well. After 48 hours, 1.0  $\mu$ M of doxorubicin was added to induce p53-responsive microRNAs by p53 activation.

#### Establishment of stable HEK293-derived stable cell lines that over-expressed microRNAs

To generate expression constructs for pri-miR-192, pri-miR-194 and pri-miR-34a tagged with ZsGreen1, a fragment of human chromosomal DNA encompassing the region transcribed to yield each pri-miRNA was amplified using human genomic DNA (Novagen, Darmstadt, Germany) as a template and then inserted into the pmR-ZsGreen1 vector (Clontech, Mountain View, CA) as previously described<sup>7</sup>. The resultant clone and a selectable marker for puromycin resistance (Linear Puromycin Marker; Clontech) were co-transfected into HEK293 cells. Transfected cells were grown in the presence of 1.0  $\mu$ g/ml puromycin (Sigma-Aldrich), and individual clones that expressed ZsGreen1 were isolated and analyzed for each microRNA expression by real-time PCR.

#### Cell viability and caspase activity assays

Cell viability and caspase 3/7 activity were analyzed with CellTiter-Fluor Cell Viability Assay and Caspase-Glo 3/7 Assay kits (Promega) according to the manufacturer's instruction. In brief, 20 µl of CellTiter-Fluor Reagent was added to cells, and then incubated for 30 minutes at 37°C by orbital shaking. For caspase activity assay, an equal volume of Caspase-Glo 3/7 Reagent was further added to each well, and then incubated for 30 minutes at room temperature. Cell viability was assessed by using a fluorometer (400 nm/505 nm; SH-9000Lab, CORONA ELECTRIC Co.), and Caspase-3/7 activity was assessed by a luminometer (SH-9000Lab, CORONA ELECTRIC Co.).

#### **Co-culture experiments**

Co-culture experiments were performed as previously described<sup>8</sup> using well inserts for 24-well plates with a 0.4 µm pore sized filter (Thin Cert<sup>TM</sup>) (Greiner, Frickenhausen, Germany). H9c2 cells were

seeded into the lower well and cultured for 24 hours prior to the co-culture experiments. Then, either HEK293 or HEK293-derived stable cell lines that over-expressed microRNAs were seeded into the upper well. After incubation for 24 hours, 0.01 µM of doxorubicin was added to activate p53.

## Statistical analysis

Variables are displayed as the mean  $\pm$  standard deviation (SD) and qualitative data are presented as percentages. Patient characteristics were assessed using the Student's *t* test, and chi-square test. Results were analyzed by the Mann-Whitney *U* test or Student's *t* test. Correlations were tested using Pearson's correlation coefficient, and r values are presented as Pearson product-moment correlation coefficients. All P values are two-sided and statistical significance was set as P<0.05 (\*), P<0.01 (\*\*) or P<0.001 (\*\*\*). All statistical analyses were performed using SPSS version 11.0 (SPSS Japan, Inc., Tokyo, Japan).



# **Online Figures and Figure Legends**

Online Figure I. Comparison of the serum expression of 11 out of 14 microRNAs selected in the first screening between the heart failure and control groups. Expression levels of 11 microRNAs in sera of the HF group (black bars, n=21) and control group (white bars, n=65) are displayed as the mean  $\pm$  SEM after normalization to U6 snRNA, which was used as an internal control. No significant differences in expression were observed between the two groups.



**Online Figure II. Correlation among microRNAs with elevated serum levels.** Each dot represents the serum expression level of miR-192, miR-194, or miR-34a relative to U6 snRNA (- $\Delta$ Ct). miR-194 vs. miR-192 (*left panel*), miR-34a vs. miR-192 (*middle panel*) and miR-34a vs miR-194 (*right panel*) (n = 58).



**Online Figure III. Establishment of HEK293-derived stable cell lines that over-expressed p53-responsive microRNA. A,** The relative expression levels of the indicated microRNAs in each cell line are displayed after normalization to U6 snRNA (n=3). **B,** The extracellular expression levels of the indicated microRNAs in culture media are displayed after normalization to synthetic C. elegans microRNA-39 (n=3).

microRNA	Control	Heart Failure Group	Control Group	Alteration*	P value
		(Mean $\Delta Ct$ , n = 7)	(Mean $\Delta Ct$ , n = 7)		
miR-125a-5p	let-7d	3.1640	-0.4510	Down	0.020
miR-15b	let-7d	-2.2600	0.4680	Up	0.025
miR-20a	let-7d	1.2865	6.3040	Up	0.015
miR-30b	let-7d	-3.2670	-0.1380	Up	0.022
miR-146a	miR-766	-0.2330	-1.4240	Down	0.035
miR-192	miR-766	4.0990	5.5555	Up	0.040
miR-19a	miR-766	1.5530	3.0280	Up	0.048
miR-200c	miR-766	5.4790	2.0940	Down	0.025
miR-345	miR-766	5.7100	2.9590	Down	0.034
miR-374b	miR-766	2.6970	-0.0785	Down	0.022
miR-485-3p	miR-766	5.2855	-0.3520	Down	0.034
miR-518d-3p	miR-766	6.9290	4.4440	Down	0.034
miR-125a-5p	U6 snRNA	5.9820	3.7270	Down	0.020
miR-215	U6 snRNA	4.4980	7.0930	Up	0.020
miR-532-5p	U6 snRNA	2.7960	6.5370	Up	0.039

Online Table I. Candidate microRNAs with Altered Levels in Sera of the Heart Failure Group.

Alteration; Up-regulation (Up) or down-regulation (Down) of microRNA expression in the heart failure group.

Variables	Heart Failure Control Group		P value	
	Group			
	(n=21)	(n=65)		
Age (years; mean±SD), N=86	69 ± 9	$69 \pm 10$	0.794	
Men (%), N=86	81.0	66.2	0.277	
Body mass index (kg/m <sup>2</sup> ;	$24.7 \pm 3.5$	$23.8\pm3.7$	0.255	
mean±SD), N=80				
Diabetes mellitus (%), N=85	47.6	37.5	0.449	
Hypertension (%), N=82	75.0	66.1	0.585	
Dyslipidemia (%), N=83	57.1	48.4	0.615	
Smoking (%), N=85	71.4	54.7	0.209	
Previous MI (%), N=84	4.8	17.5	0.279	
STEMI <sup>§</sup> (%), N=85	85.7	85.9	1.000	
Anterior MI (%), N=86	66.7	56.9	0.458	
Killip class $\geq$ II on admission (%),	33.3	30.2	0.791	
N=84				
Peak CK (IU/L; median (IQR)),	3620	2210	0 224	
N=82	(869, 7954)	(1086, 4743)	0.221	
Peak CK $\ge$ 3000 (IU/L) (%), N=82	52.4	36.1	0.207	
Reperfusion therapy rates (%), N=86	90.5	93.8		
Infarct-related artery				
LMT (%), N=82	5.3	7.9	1.000	
LAD (%), N=82	68.4	47.6	0.125	
LCX (%), N=82	10.5	12.7	1.000	
RCA (%), N=82	10.5	27	0.215	
Final TIMI III (%), N=75	88.9	93	0.626	
Multivessel disease (%), N=83	60.0	36.5	0.074	
TIMI risk score, N=71	$6.89 \pm 2.52$	$6.75 \pm 2.96$	0.864	
eGFR at discharge (mL/min/1.73m <sup>2</sup> ), N=78	$61.7 \pm 22.0$	59.4 ± 25.9	0.739	
GRACE risk score, N=51	$124.2 \pm 23.0$	$114.6 \pm 29.2$	0.288	
hs-CRP at discharge (mg/dL; median	0.560	0.375	3)	
(IQR)), N=80	(0.098, 2.013)	(0.170, 1.283)		
hs-TnT at discharge (ng/mL; median	0.045	0.039	0.293	

Online Table II. Baseline Characteristics of Subjects Evaluated in the Second Screening.

(IQR)), N=78	(0.025, 0.53)	(0.016, 0.16)	
Statin (%), N=85	52.4	42.2	0.456
ACEI <sup><math>\dagger</math></sup> or ARB <sup><math>\#</math></sup> (%), N=85	81.0	68.8	0.404
β-blocker (%), N=85	61.9	54.7	0.619
Anti-platelet therapy (%), N=85	100	100	-

STEMI; ST-segment elevation MI. ACEI; angiotensin-converting enzyme inhibitor. ARB; angiotensin receptor blocker. SD; standard deviation. IQR; interquartile range.

Treatment for			Pearson's	95%CI		n-value
adjustment			correlations	orrelations		p-value
	LVEF	miR-34a	-0.28	-0.51	-0.02	0.03
		miR-194	-0.27	-0.50	-0.01	0.04
RASI		miR-192	-0.22	-0.45	0.05	0.10
KA51	LVDd	miR-34a	0.39	0.14	0.59	0.003
		miR-194	0.33	0.08	0.55	0.01
		miR-192	0.09	-0.17	0.34	0.49
	LVEF	miR-34a	-0.28	-0.51	-0.02	0.03
		miR-194	-0.27	-0.50	-0.01	0.04
Data blashara		miR-192	-0.21	-0.44	0.06	0.12
Beta-blockers	LVDd	miR-34a	0.38	0.13	0.58	0.003
		miR-194	0.33	0.08	0.54	0.01
		miR-192	0.09	-0.18	0.34	0.51
	LVEF	miR-34a	-0.26	-0.49	0.00	0.04
		miR-194	-0.25	-0.48	0.01	0.06
Stating		miR-192	-0.21	-0.44	0.06	0.12
Statilis	LVDd	miR-34a	0.36	0.11	0.57	0.005
		miR-194	0.30	0.05	0.52	0.02
		miR-192	0.07	-0.19	0.33	0.58

**Online Table III.** Correlations of LVDd or LVEF and microRNA expression after consideration of medications.

CI; Confidential Interval. RASI; Angiotensin-converting enzyme inhibitor (ACEI) or Angiotensin receptor blocker (ARB). LVDd; left ventricular end-diastolic dimension. LVEF; left ventricular ejection fraction.

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