

A translational study of circulating cell-free microRNA-1 in acute myocardial infarction

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A B S T R A C T

miRNAs (microRNAs) participate in many diseases including cardiovascular disease. In contrast with our original hypothesis, miRNAs exist in circulating blood and are relatively stable due to binding with other materials. The aim of the present translational study is to establish a method of determining the absolute amount of an miRNA in blood and to determine the potential applications of circulating cell-free miR-1 (microRNA-1) in AMI (acute myocardial infarction). The results revealed that miR-1 is the most abundant miRNA in the heart and is also a heart- and muscle-specific miRNA. In a cardiac cell necrosis model induced by Triton X-100 *in vitro*, we found that cardiac miR-1 can be released into the culture medium and is stable at least for 24 h. In a rat model of AMI induced by coronary ligation, we found that serum miR-1 is quickly increased after AMI with a peak at 6 h, in which an increase in miR-1 of over 200-fold was demonstrated. The miR-1 level returned to basal levels at 3 days after AMI. Moreover, the serum miR-1 level in rats with AMI had a strong positive correlation with myocardial infarct size. To verify further the relationship between myocardial size and miR-1 level, an IP (ischaemic preconditioning) model was used. The results showed that IP significantly reduced circulating miR-1 levels and myocardial infarct size induced by I/R (ischaemia/reperfusion) injury. Finally, the levels of circulating cell-free miR-1 were significantly increased in patients with AMI and had a positive correlation with serum CK-MB (creatinine kinase-MB) levels. In conclusion, the results suggest that serum miR-1 could be a novel sensitive diagnostic biomarker for AMI.

INTRODUCTION

miRNAs (microRNAs) are endogenous non-coding single-stranded RNAs of ~22 nucleotides and constitute a novel class of gene regulators [1]. Analogous to the first RNA revolution in the 1980s, when Cech's group

discovered the enzymatic activity of RNA [1a], the more recent discoveries of RNAi (RNA interference) and miRNA may represent the second RNA revolution [2]. Although the first miRNA, lin-4, was discovered in 1993 [3,4], their presence in vertebrates was only confirmed in 2001 [5]. Currently, approx. 800 miRNAs

Key words: acute myocardial infarction, biomarker, ischaemic preconditioning, ischaemia/reperfusion injury, microRNA, serum.
Abbreviations: AMI, acute myocardial infarction; CK-MB, creatine kinase-MB; i.p., intraperitoneally; I/R, ischaemia/reperfusion; IAR, ischaemic area at risk; IP, ischaemic preconditioning; LAD, left anterior descending coronary artery; miRNA, microRNA; qRT-PCR, quantitative real-time PCR; snoRNA, small nucleolar RNA; TnI, troponin I; TnT, troponin T; TTC, triphenyltetrazolium chloride; VSMC, vascular smooth muscle cell.

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have been cloned and sequenced in humans, and the estimated number of miRNA genes is as high as 1000 in the human genome [6]. The mature miRNAs bind to the 3'-UTR (untranslated region) of their mRNA targets and negatively regulate gene expression via degradation or translational inhibition. Functionally, an individual miRNA is important as a transcription factor because it is able to regulate the expression of its multiple target genes [7]. As a group, miRNAs are estimated to regulate over 30% of the genes in a cell [8]. It is thus not surprising that miRNAs are involved in the regulation of almost all major cellular functions, including apoptosis and necrosis. Accordingly, miRNAs may be involved in many diseases, including cardiovascular disease, as described in our recent review articles [9,10].

Tissue- and cell-specific expression is one important characteristic of miRNA expression [11]. Indeed, one miRNA may be highly expressed in one tissue or one cell, but has no or low expression in other tissues or cells. For example, miR-1 is reported to be a muscle- or heart-specific miRNA [11], whereas miR-145 is a VSMC (vascular smooth muscle cell)-specific miRNA, as we have described in recently [12]. The tissue-specific miRNA expression and tissue expression signatures of diseases have provided a great diagnostic opportunity for diverse diseases [10].

Recent studies have revealed that miRNAs exist in circulating blood [13,25]. In contrast with our original hypothesis, cell-free miRNAs are relatively stable due to binding with other materials such as exosomes in circulating blood [13]. Moreover, cancer tissue miRNAs are able to be released into circulating blood and serum or plasma cell-free miRNAs can be used as novel biomarkers for diverse cancers [13]. However, a quantitative method to measure the absolute amount of a miRNA in blood has not been well-established due to a lack of stable control RNAs in blood, especially under disease conditions. More importantly, the roles of the circulating cell-free miRNAs in patients with cardiovascular diseases are currently unclear. In the present study, a quantitative method to determine the serum level of miR-1 was established based on qRT-PCR (quantitative real-time PCR) technology. The potential applications of serum miR-1 in AMI (acute myocardial infarction) were also determined by the translational study from cells to animal and humans.

MATERIALS AND METHODS

AMI, IP (ischaemic preconditioning) and I/R (ischaemia/reperfusion) injury in animal models

AMI, IP and I/R injury in rats were induced by LAD (left anterior descending coronary artery) ligation, as described in our recent study [14]. In brief, 10-week-old

male Sprague-Dawley rats (weighing 250–300 g) were anaesthetized with ketamine [80 mg/kg of body weight, i.p.(intraperitoneally)] and xylazine (5 mg/kg of body weight, i.p.). Under sterile conditions, an anterior transmural AMI was created by occlusion of the LAD with a silk suture. Sham-operated rats served as controls. Sham operation involved an identical procedure, except the suture was passed around the vessel without LAD occlusion. IP was achieved via four cycles of 5 min of LAD occlusion/5 min of reperfusion cycles. I/R injury was induced in rat hearts via 1 h of LAD occlusion/3 h of reperfusion. The animals were divided into three study groups. Group 1 was for the time course study of serum miR-1. In this group, the blood samples were obtained via tail vein from rats before (0 h) and at 1 h, 3 h, 6 h, 12 h, 24 h, 3 days, 7 days, 14 days, 21 days and 28 days after AMI. Eight rats were used in the time course study. In addition, eight sham-operated rats were used as the controls. Group 2 had 12 rats that were used to study the relationship between serum miR-1 and myocardial infarct size induced by I/R injury. Group 3 was used to study the effect of IP on serum miR-1 and myocardial infarct size induced by I/R injury, in which six sham-operated rats, six I/R rats and six IP°I/R rats finished the experiment.

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Medicine & Dentistry of New Jersey-New Jersey Medical School, and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1985).

Measurement of infarct size

Myocardial infarct size was determined by pathological staining, which is the gold standard for AMI. At the end of experiments, rats were anaesthetized and 6 ml of 10% Evans Blue dye was injected into the vena cava to define the area that was not supplied by LAD. The myocardial IAR (ischaemic area at risk) was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2-mm-thick slices. The slices were incubated in 1% TTC (triphenyltetrazolium chloride) at 37°C for 10 min to identify the non-infarcted and infarcted areas. TTC staining was displayed as a red colour. The infarcted area was defined as the TTC-unstained area (white colour). Infarct size was expressed as a percentage of the IAR [14].

Blood sample collection, serum miRNA isolation and establishing the quantitative method for miR-1 assay

Before and at different times after AMI, IP, I/R injury or sham surgery, the blood samples were collected from a tail vein. The samples were placed for 1 h at room temperature (26°C) and were then centrifuged

at 1600 g for 20 min at 4°C. Serum samples were carefully transferred into plain propylene tubes and stored at 70°C until miRNA isolation. miRNAs were isolated in 200 µl of serum using miRNAs Isolation Kit solution supplied by RNA Bioscience, according the manufacturer's instructions. In brief, miRNAs in serum were first denatured by denaturing solution (Solution A) and were separated by phase-separation solution (Solution B). Then, the samples were processed using precipitation solution (Solution C), washing solution (Solution D) and dissolving solution (Solution E). miR-1 was measured by qRT-PCR with a Roche Lightcycler 480 Detection System using the primer [12] 5'-GT-CGTATCCAGTGCGTGTCTGGAGTCGGCAAT-TGCACTGGATACGACTACATAC-3' (Applied Biosystems). The same isolation and assay were performed using a series of concentrations of standard miR-1 (synthesized by IDT, Coralville, IA, U.S.A.) to make a standard curve. The absolute amount of miR-1 was calculated by software based on serum sample qRT-PCR numbers and the standard curve, and are expressed as pmol/l.

miRNA expression signature assay

To verify the specific expression of miR-1 in the heart, miRNAs were isolated from rat heart, aorta and lung using mirVana miRNA isolation kit (Ambion) and an miRNA expression signature was performed by miRNA microarray analysis using a chip containing 341 mature miRNAs (Chip ID miRat 12.0 version; LC Sciences), as described in our recent studies [15–17]. In addition, the miRNAs in rat heart, aorta, lung, liver, brain, small intestine and kidney were also isolated for miR-1 assay.

Cell culture

Primary cultures of neonatal rat cardiac ventricular myocytes were performed as described previously [14,18]. In brief, hearts from 1–2-day-old Sprague-Dawley rats were removed after hypothermia anaesthesia immersion in ice water and placed in ice-cold 1× PBS solution. After repeated rinsing, the atria were removed, and the ventricles were minced with scissors. The minced tissue and ventricular cells were dispersed by digestion with collagenase type IV (0.45 mg/ml), 0.1% trypsin, and 15 µg/ml DNase I. Cardiomyocytes (0.33×10^6 cells/ml) were cultured in cardiac myocyte culture medium [Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 4 µg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/l BSA (fraction V), 3 mmol/l pyruvic acid, 15 mmol/l Hepes, 100 µmol/l ascorbic acid, 100 µg/ml ampicillin, 5 µg/ml linoleic acid, 1% penicillin, 1% streptomycin and 100 µmol/l 5-bromo-2'-deoxyuridine], and seeded into six-well plates.

Necrosis model of cultured cardiac myocytes and miR-1 release assay

The necrosis model of cultured cardiac myocytes was induced by Triton X-100 as described previously [19]. Briefly, rat cardiac myocytes in six-well plates cultured with 10% (v/v) fetal bovine serum were washed three times with 0.01 M PBS to remove medium and serum. Then, the cells were treated for 20 min at room temperature with different concentrations of Triton X-100 (0.25, 1 and 2%) diluted in 0.1% sodium citrate. As a negative control, the cells were incubated with PBS alone (vehicle). After treatment, the culture supernatant from each well was collected for the miR-1 assay. In addition, in the 2% Triton X-100-treated group, the supernatants were kept at 37°C for 6, 12 and 24 h for the stability assay of miR-1.

Clinical study

AMI was defined as (i) chest pain characteristic of myocardial ischaemia for 30 min or more, (ii) ST segment elevation within 6 h of chest pain at least 0.1 mV in at least two leads of the ECG, and (iii) confirmation of the diagnosis of AMI by elevated by CK-MB (creatine kinase-MB) isoenzyme in serum, which was at least twice the normal range. Blood samples from patients without the confirmation of AMI were not used in the present study. In total, 31 patients with AMI were used [18 men and 13 women; mean age, 57 ± 0.1 years (range, 45–71 years)]. Of these, 20 had coronary angiography data showing at least one-vessel disease. Among the patients studied, 11 had hypertension and were being treated with antihypertensive medication, an AngII (angiotensin II) receptor inhibitor and/or β -blocker; and eight patients had hyperlipidaemia and were being treated with simvastatin. No patients with diabetes were included in the present study. Blood samples were obtained within 24 h of AMI. The time interval between the onset of typical chest pain and blood samples was 8.5 ± 3.82 h. Blood samples obtained from 20 age- and gender-matched healthy volunteers were used as controls. All the blood samples were placed for 1 h at room temperature and were centrifuged at 1600 g for 20 min at 4°C. Serum miR-1 levels were determined as described above. CK-MB was determined using an immunoinhibition assay method (Roche Diagnostics; normal range, 0–25 units/l).

The protocol was approved by the Institutional Review Board at Guangdong Provincial People's Hospital, and all subjects gave informed consent.

Statistics

All results are presented as means \pm S.E.M. For relative gene expression, the mean value of the vehicle control group was defined as 100% or 1. Two-tailed unpaired Student's *t* tests and ANOVA were used for statistical evaluation of the data. Linear regression analysis was

used to determine the relationship between myocardial infarct size and serum miR-1, and the relationship between serum CK-MB and serum miR-1. The SigmaStat statistical analysis program was used for data analysis. A P value <0.05 was considered significant.

RESULTS

miR-1 is a heart-selective miRNA and is the most abundant miRNA in normal rat hearts

Microarray analysis of miRNAs in the heart revealed that miR-1 was the most abundant miRNA in normal rat hearts; however, the expression of miR-1 in aorta and lung samples was almost undetectable [14]. The average microarray signal of miR-1 in heart was 51191.30, whereas in lung and aorta the signals were only 62.12 and 31.23 respectively. To verify further the heart selectivity of miR-1 expression, miR-1 levels in rat liver, brain, small intestine and kidney were determined by qRT-PCR. No significant miR-1 expression was found in these tissues (results not shown). The results are consistent with reports from other research groups [20–22].

miR-1 is released by necrotic cardiac myocytes *in vitro*

As shown in Figure 1(A), the necrosis of cardiac myocytes was induced by Triton X-100. Accordingly, miR-1 was released into the culture medium (Figure 1B) at 20 min after treatment with Triton X-100. The amount of miR-1 release was related to the number of necrotic cardiac myocytes, as increased miR-1 levels were found with the increasing concentrations of Triton X-100 (Figure 1B). Moreover, the released miR-1 was stable in culture solution for 24 h at least, as shown in Figure 1(C).

Establishing a quantitative method to assay miR-1 in blood

The linear range was determined by analysis of synthesized standard miR-1, normal rat serum miR-1 and serum miR-1 from rats following AMI. C_T values of the standard curve were plotted against the logarithmic concentration of the serial dilutions of the isolated miR-1. The amplification plot of different concentrations of miR-1 is shown in Figure 2(A). A representative standard curve of these assays, and the correlation coefficient of the analysis ($R^2 = 0.99$) and efficient amplification (slope = -3.4573) for all of the experiments are shown in Figure 2(B). The assay was linear over a range of 0.01 to 10 pmol/l miR-1.

Serum miR-1 is a novel biomarker for AMI

As shown in Figure 3(A), AMI was induced by LAD ligation as demonstrated using a pathological staining

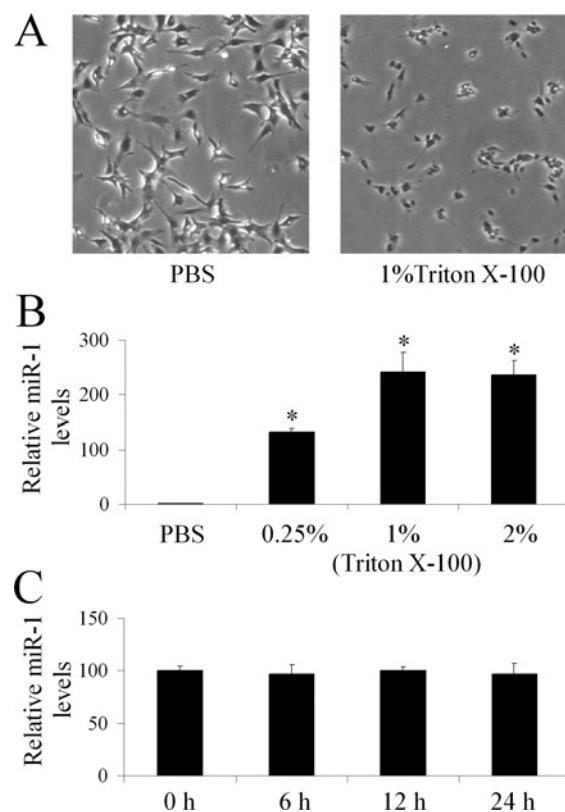


Figure 1 miR-1 released by necrotic cardiac myocytes *in vitro*

(A) Representative images from vehicle (PBS)- or Triton X-100-treated cardiac cells. (B) The relative levels of miR-1 in the culture medium of rat cardiac cells treated with vehicle (PBS) or different concentrations of Triton X-100 for 20 min as determined by qRT-PCR. The miR-1 level in vehicle-treated group is expressed as 1. Values are means \pm S.E.M. ($n = 6$); * $P < 0.05$ compared with PBS control. (C) The stability assay of miR-1 in cultured medium. Cardiac cells were treated with 2% Triton X-100 for 20 min. Then, the cultured medium was collected and was kept at 37 °C for up to 24 h. The miR-1 levels were determined by qRT-PCR at 0, 6, 12 and 24 h after medium collection. Values are means \pm S.E.M. ($n = 6$).

method. In serum from the sham group (normal), the miR-1 level was low and was barely detectable using the highly sensitive qRT-PCR method (0.016 ± 0.008 pmol/l). Interestingly, serum miR-1 levels were significantly increased to 3.67 pmol/l in rats at 6 h after AMI (Figure 3C). To determine the time course of changes in miR-1 after AMI, serum miR-1 levels were determined before (0 h), and at 1 h, 3 h, 6 h, 12 h, 24 h, 3 days, 7 days, 14 days, 21 days and 28 days after AMI in eight rats. As shown in Figures 3(B) and 3(C), compared with normal controls (sham and 0 h groups), serum miR-1 levels were rapidly increased, peaking at 6 h after AMI, at which an increase in miR-1 levels over 200-fold was demonstrated. At 3 days after AMI, the serum miR-1 level returned to basal levels.

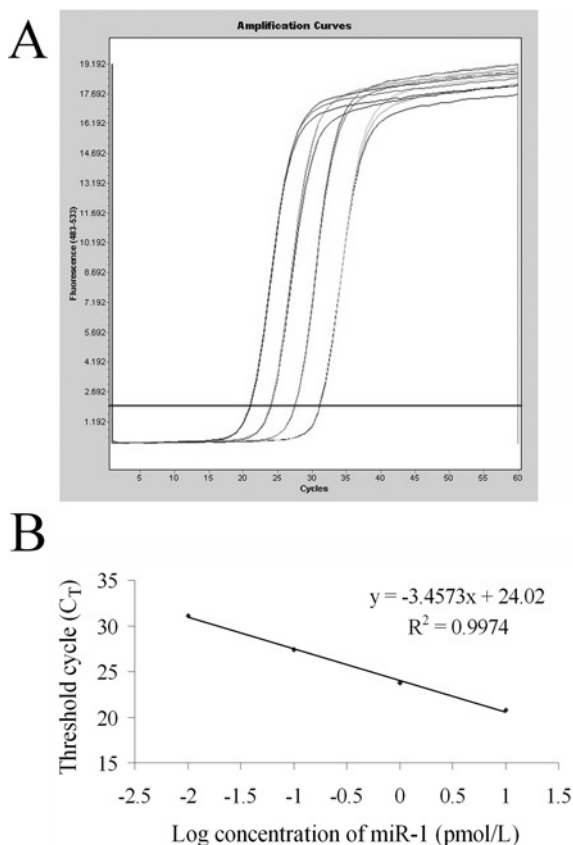


Figure 2 Establishing a quantitative method for the serum miR-1 assay

(A) Amplification chart of different concentrations of miR-1 (from left to right: 10, 1, 0.1 and 0.01 pmol/l). (B) Standard curve of the miR-1 concentration in serial dilutions compared with the cycle number (C_T). Each dot represents the result of triplicate PCR amplification for each dilution.

Serum miR-1 levels are associated with myocardial infarct sizes

To determine the relationship between serum miR-1 levels and myocardial infarct sizes, the infarct sizes induced by I/R injury and serum miR-1 levels were determined in 12 rats. As shown in Figure 4, a strong positive correlation was demonstrated between the two variables ($r = 0.88$; $P < 0.05$).

IP reduces myocardial infarct size and serum miR-1 levels

To determine further the relationship between serum miR-1 levels and myocardial infarct size, IP was performed on rat hearts prior to I/R injury. As shown in Figures 5(A) and 5(B), AMI was induced by I/R injury. Accordingly, serum miR-1 levels were significantly increased in rats following I/R injury (Figure 5C). As expected, myocardial infarct size was significantly reduced by IP (Figures 5A and 5B). Interestingly, serum

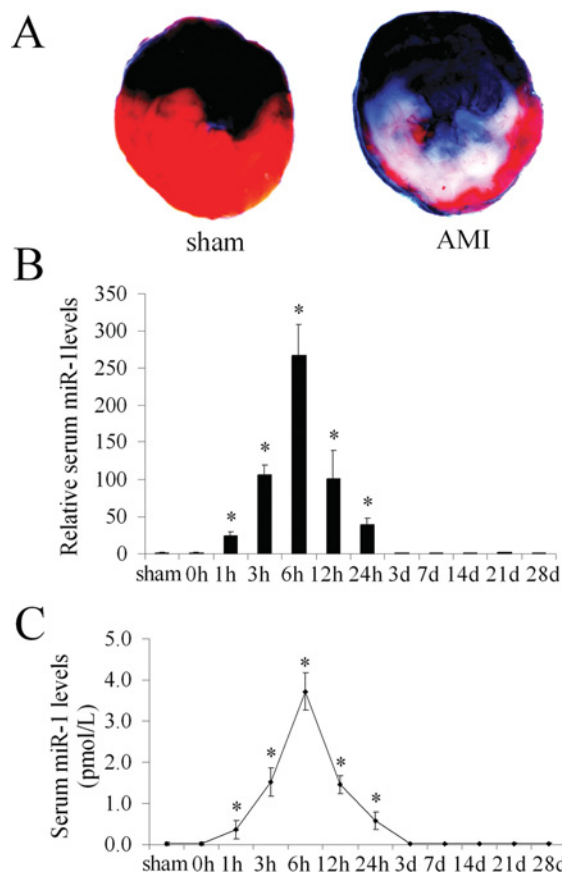


Figure 3 Circulating cell-free miR-1 in rats with or without AMI

(A) AMI in the rat heart was induced by LAD ligation and the infarct size was determined by the pathological staining of heart slices with Evans Blue. The region without Evans Blue staining is the myocardial IAR. The red colour is TTC staining. The area not stained with TTC within the IAR is the infarcted area. Infarct size is expressed as a percentage of the IAR. (B) The relative serum miR-1 levels in eight sham-operated control rats, and in eight rats before (0 h) and at different time points after AMI. The mean value of serum miR-1 before AMI was expressed as 1. Values are mean \pm S.E.M. ($n = 8$); $*P < 0.05$ compared with the group before AMI (0 h). (C) The absolute amount of serum miR-1 sham-operated control rats, and in rats before (0 h) and at different time points after AMI is shown. Values are mean \pm S.E.M. ($n = 8$); $*P < 0.05$ compared with the group before AMI. d, day.

miR-1 levels induced by I/R injury were also inhibited by IP (Figure 5C).

Serum miR-1 levels are significantly increased in patients with AMI

As shown in Figure 6(A), the serum miR-1 levels from 31 patients within 24 h of AMI were significantly increased compared with healthy controls. Among them, a nearly 100-fold increase in serum miR-1 was found in patients at 6 h after AMI. The release of miR-1 into the circulation was very rapid, as the increase in serum miR-1 was found

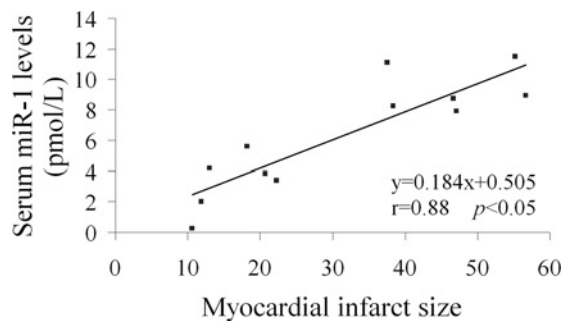


Figure 4 Relationship between serum miR-1 levels and myocardial infarct sizes in rats with AMI

AMI was induced by I/R injury in 12 rats, and the infarct sizes and serum miR-1 levels were determined in rats at 3 h after reperfusion. A strong positive correlation was demonstrated between the two variables ($r = 0.88$; $P < 0.05$).

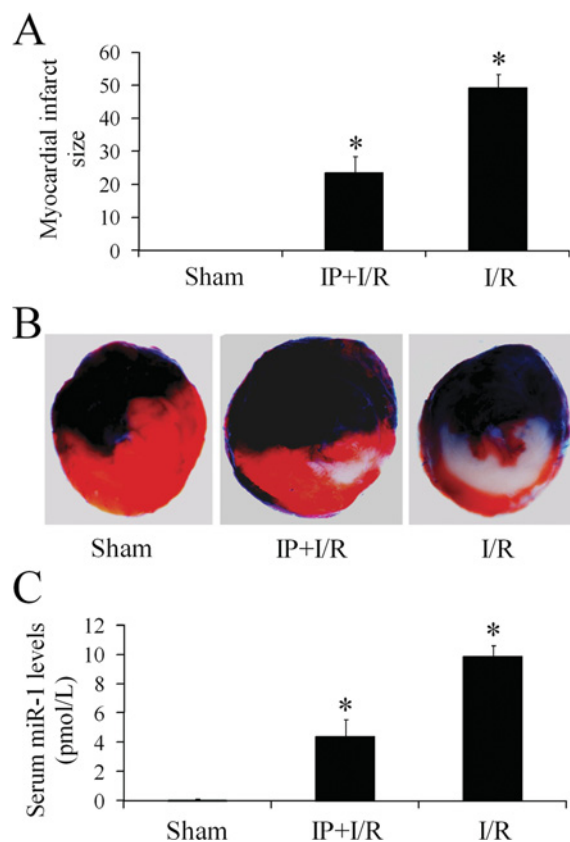


Figure 5 IP reduces myocardial infarct size and serum miR-1 level

(A) Myocardial infarct size induced by I/R injury was reduced by IP. Values are means \pm S.E.M. ($n = 6$); $*P < 0.05$ compared with I/R group. (B) Representative heart slice images from rats in the sham-operated, IP+I/R and I/R alone groups. The region without Evans Blue staining is myocardial IAR. The red colour is TTC staining. The area not stained with TTC within the IAR is the infarcted area. (C) The increase in serum miR-1 induced by I/R injury is inhibited by IP. Values are means \pm S.E.M. ($n = 6$); $*P < 0.05$ compared with I/R group.

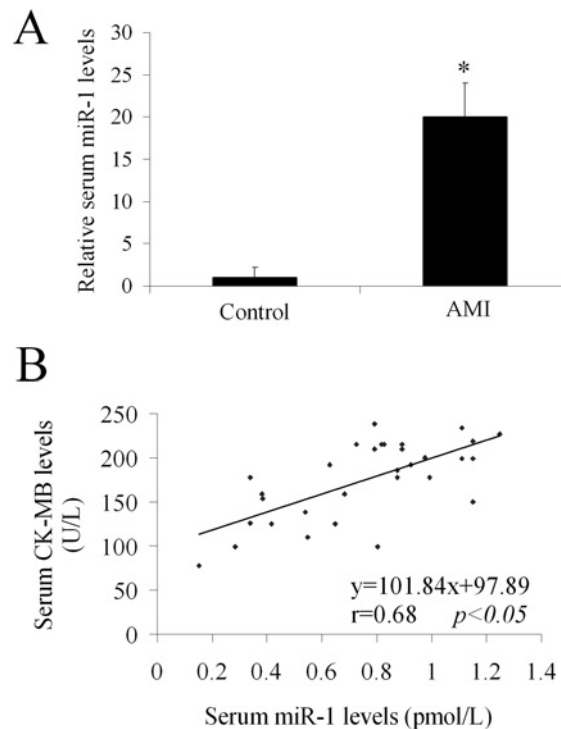


Figure 6 Serum miR-1 is increased in patients with AMI

(A) Serum miR-1 levels were determined from patients ($n = 31$) within 24 h of AMI. The serum from age-matched healthy controls ($n = 20$) was used as the control group. Values are means \pm S.E.M.; $*P < 0.05$ compared with the control group. (B) The relationship between serum miR-1 levels and CK-MB levels in patients ($n = 31$). A positive correlation was demonstrated between the two variables ($r = 0.68$; $P < 0.05$).

in patients within hours of presentation. No increase in serum miR-1 levels was found in patients at 3 and 7 days after AMI (results not shown). To investigate the potential relationship between serum miR-1 levels and myocardial infarct sizes in humans, CK-MB levels in these patients were also determined. As shown in Figure 6(B), a positive correlation was demonstrated between serum miR-1 and CK-MB levels ($r = 0.68$; $P < 0.05$).

DISCUSSION

Recent studies have revealed that miRNAs are critical regulators in the pathogenesis of many diseases, including disorders of the cardiovascular system [9,10]. The tissue- and cell-specific expression of miRNAs indicate that miRNAs may be used as biomarkers of tissue and cell identification. Moreover, different diseases have different miRNA expression profiles [10]. The unique tissue miRNA expression signatures provide novel diagnostic tools for diseases as has already been well demonstrated in cancer studies [23]. However, diseased tissues are often difficult to obtain under most disease conditions

such as cardiovascular disease. The fortunate part of this misfortune is that miRNAs are able to be released into circulating blood from tissues [13]. More importantly, recent reports have revealed that circulating miRNAs released from tissues are stable due to binding with other materials [24]. Therefore circulating cell-free miRNAs can be used as novel biomarkers for diverse cancers [25]. In this respect, miRNAs in the peripheral blood have been proven to be useful biomarkers for diseases such as cancer [26] and liver injury [27].

miRNAs are highly expressed in the cardiovascular system. Moreover, recent studies, including ours, have revealed that miRNA expression in the cardiovascular system may also be tissue- and cell-specific [15,16]. For example, miR-145 is selectively expressed in VSMCs, but has no or very low expression in endothelial cells [12]. In the present study, using microarray analysis and qRT-PCR, we identified that miR-1 is the most abundant miRNA in heart and it is a heart-specific miRNA. In addition, recent studies have demonstrated that cardiac tissue miR-1 is involved in the pathogenesis of cardiac diseases, such as cardiac hypertrophy, heart failure [28,29] and AMI [30,31]. However, whether cardiac miR-1 can be released into the circulation during AMI is currently unclear.

Establishing a method to determine the absolute amount of an miRNA in blood is the prerequisite for blood miRNA study. Unlike tissue, which has many good markers such as U6 for normalization and calculating the amount of tissue miRNAs, blood lacks this kind of internal control for miRNA normalization. Although there are some reports using RNAs such as 18S, 5S, snoRNA (small nucleolar RNA) U38B, snoRNA U43, snRNA (small nuclear RNA) U6 and other miRNAs as the internal controls for normalization, the levels of these control makers can often change, especially under pathological conditions. After several years targeting the critical drawbacks in blood miRNA arrays, we have identified that using the serum (plasma or blood) volume as normalization (pmol/l) is the best method to determine the levels of miRNAs in blood. The amount of molecules per ml or per litre of serum (plasma or blood) is also the standard method of evaluating the blood levels of other molecules in the clinic. As described in the Materials and methods section of the present study, we have developed a method of determining the absolute amount of an miRNA for circulating cell-free miR-1 based on qRT-PCR technology. Our unpublished results also revealed that it is suitable for other miRNA measurement in solutions, such as serum or culture medium (Y. Cheng, J. Yang and C. Zhang, unpublished work).

In the present study, we first performed an *in vitro* study to determine whether the damaged cardiac cells can release miR-1 into the culture medium and if the released miR-1 is stable in cell culture solution. The results showed that cardiac miR-1 is able to be released

and the released amount is associated with the extent of cardiac cell damage. Moreover, the released miR-1 is stable in the cell culture solution at least for 24 h.

Although there are only trace amounts of miR-1 in normal rat and human serum, it can still be detected using highly sensitive qRT-PCR technology. We hypothesized that the very low level of miR-1 may represent a novel metabolism pathway of miRNAs. It is reported that miRNAs are able to be packaged into exosomes within the cells and these miRNA-packaged exosomes are able to be released into circulating blood [24]. As miR-1 is the most abundant miRNA in the normal heart, the trace amount of miR-1 released into the circulating blood under physiological conditions may be responsible for the low basal serum level of miR-1. However, after AMI, rat serum miR-1 levels were quickly increased. The increase in serum miR-1 reached as high as 200-fold, which occurred at 6 h after AMI. The time course study revealed that, at 3 days after AMI, the levels of miR-1 returned to basal levels. In addition, the levels of serum miR-1 had a strong positive correlation with the myocardial infarct sizes. The relationship between serum miR-1 and myocardial infarct sizes was verified further by using a well-established IP model. The results suggested that serum miR-1 is a novel early sensitive biomarker for AMI.

Stability is important for a circulating disease biomarker. We found that incubation of serum at room temperature for up to 4 h, or subjecting it to up to several freeze-thaw cycles, had no significant effect on endogenous miR-1 level, although the serum contained high levels of RNase activity; an observation consistent with recent reports [13,32]. Thus serum miR-1 is a stable biomarker. The reasons why miRNAs are stable in blood are still not completely clear; however, binding with other materials either in blood or in released cells, such as exosomes, may be one of the critical factors [24]. Indeed, in contrast with the endogenous binding of miR-1, we found that exogenously added mature miR-1 to serum was quickly degraded both *in vitro* and *in vivo* (results not shown).

Finally, in the clinical study, we found that serum miR-1 in patients with AMI was also quickly increased within hours of presentation. In patients within 24 h of AMI, there was an increase in miR-1 serum levels of over 20-fold. In addition, there was a positive relationship between serum miR-1 and CK-MB. The result suggested that serum miR-1 may also be related to myocardial infarct size in humans. In addition, on the basis of the animal study, the time course change in miR-1 was similar to that of CK-MB in AMI, but is different from that of TnT (troponin T). In patients at 3 and 7 days after AMI, the serum level returned to the basal level (results not shown). Thus miR-1 may be an early biomarker for AMI.

The present study does have some limitations. First, the qRT-PCR method to measure miR-1 in serum takes longer than the method to determine CK-MB or troponin

[TnT or TnI (tyroponin I)], and does not allow for easy and rapid detection of microRNAs for clinical routine. However, the development of a highly sensitive quick assay for RNA is an intense area of research. In fact, progress has been made in RNA arrays, such as fluorescent and ELISA methods. Breakthroughs in the research area may overcome the pitfalls in the future. The second limitation was that there were only 31 patients in the clinical study. There were not enough patients to determine the time course changes of miR-1 and the relationship between serum miR-1 level and severity, as well as the effect of therapeutic intervention on AMI. In addition, a large number of AMI patients need to be used to determine the diagnostic standard compared with other biomarkers such as TnT and TnI. The third limitation is that skeletal muscle also expresses miR-1 [22,33]. Thus damage of skeletal muscle should be excluded when miR-1 is used in the diagnosis of AMI, because miR-1 is also expressed there. A strategy to compensate for this is to select more circulating miRNAs that have different expression levels between heart and skeletal muscle.

The search for new biomarkers of cardiovascular diseases, including AMI, remains a large and growing area. Currently, all of the clinically applied circulating biomarkers are peptides or proteins determined by biochemical or immunoassay techniques. The advances in molecular biology and technology have fuelled an interest in nucleotide-based biomarkers that may enhance diagnostic or therapeutic effectiveness. An ideal nucleotide blood biomarker of AMI should be abundant, be preferentially (or exclusively) produced in heart and be present at low concentrations in the blood. Upon AMI, such nucleotides should be released into the circulating blood, where they are relatively stable and can be quickly detected by molecular technologies. The present study revealed that serum miR-1 matches the above requirements.

During the preparation of the present report, we were very happy to note that several new studies in this research area have been published. In an animal study, another cardiac-selective miRNA, circulating miR-208, was shown to be related to cardiac injury [34]. In a much larger group with appropriate controls, and patients with ($n=93$) or without AMI ($n=66$), circulating miR-1 levels were quantified using real-time RT (reverse transcription)-PCR and receiver-operator characteristic curve analysis was performed to evaluate the diagnostic value of miR-1 [35]. The authors concluded that circulating miR-1 may be a novel marker for the diagnosis of AMI. More recently, Wang et al. [36] reported that the levels of circulating miR-1, miR-133a, miR-499 and miR-208a were increased in both rats and patients with AMI. These new reports support our viewpoint that miRNA-based technology is able to reflect cardiac injury. The present translational study has provided new information regarding the direct release of miR-1 from injured cardiac cells, the time course study of circulating

miR-1 in AMI, and the effect of therapy on the level of circulating miR-1.

In the present study, we have established, for the first time, a quantitative method to determine the absolute amount of a miRNA in blood (solutions) at pmol/l levels. It will be important not only for cardiovascular diseases, but also for other diseases. To our knowledge, this is the first translational study to determine the role of circulating cell-free miRNA in cardiovascular diseases, especially in AMI. The results suggest that miR-1 can be used as a highly sensitive early biomarker of AMI. Using circulating cell-free miRNAs as diagnostic biomarkers may represent a new revolution in cardiology.

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