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Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction

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ABSTRACT

Recent studies have revealed the role of microRNAs (miRNAs) in a variety of basic biological and pathological processes and the association of miRNA signatures with human diseases. Circulating miRNAs have been proposed as sensitive and informative biomarkers for multiple cancers diagnosis. We have previously documented aberrant up-regulation of *miR-1* expression in ischemic myocardium and the consequent slowing of cardiac conduction. However, whether *miR-1* could be a biomarker for predicting acute myocardial infarction (AMI) is unclear. In the present study, we recruited 159 patients with or without AMI for quantification of *miR-1* level in plasma using real-time RT-PCR method. We performed Wilcoxon rank sum and signed rank tests for comparison. Univariable linear regression and logistics regression analyses were performed to assess the potential correlation between *miR-1* and known AMI markers. We also conducted receiver–operator characteristic curve (ROC) analysis to evaluate the diagnostic ability of *miR-1*. We found that: *miR-1* level was significantly higher in plasma from AMI patients compared with non-AMI subjects and the level was dropped to normal on discharge following medication. Increased circulating *miR-1* was not associated with age, gender, blood pressure, diabetes mellitus or the established biomarkers for AMI. However, *miR-1* level was well correlated with QRS by both univariable linear and logistics regression analyses. The area under ROC curve (AUC) was 0.7740 for separation between non-AMI and AMI patients and 0.8522 for separation AMI patients under hospitalization and discharge. Collectively, our results revealed that circulating *miR-1* may be a novel, independent biomarker for diagnosis of AMI.

Despite that advanced medications have been performed for the prevention of ischemic heart disease, the sudden cardiac death remains the primary cause of mortality for patients with acute ischemic arrhythmias [1]. Acute-phase reaction is an important pathogenesis of myocardial infarction. Several biochemical or proteomic biomarkers have been found to be associated with increased cardiovascular events and even death [2–4]. Yet the development of clinically validated detection markers represents an unmet challenge for acute myocardial infarction (AMI). New approaches that can complement and improve current strategies for AMI diagnosis are urgently needed.

MicroRNAs (miRNAs) are small RNAs that regulate expression of proteins by acting on the 3′UTRs of target genes encoding these proteins [5–7]. The expression profile of miRNAs was found to be tissue-/cell-specific. And aberrant expression of miRNA can directly reflect disease status. Several studies reported that miRNAs are stable in circulation [8]. And findings from recent studies have led to a notion that circulating miRNAs are useful markers for the diagnosis of multiple cancers [9,10] and drug-induced liver injury [11]. Our previous study has found that *miR-1* is over-expressed in ischemic myocardium of a rat model of AMI and of individuals with coronary artery disease as well. This pathologic over-expression of *miR-1* induces and exacerbates arrhythmogenesis, owing to its ability to impair cardiac conduction by post-transcriptionally

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repressing *KCNJ2* that encodes the inward rectifier K^+ channel subunit Kir2.1 and *GJA1* encoding connexin 43 gap junction channels [12,13]. In light of the sustained presence of miRNAs in circulation of cancer patients as appealing biomarkers, we hypothesized that *miR-1* may be released from damaged cardiac cell to blood stream resulting in elevated level of circulating *miR-1* in the case of AMI. Accordingly, we established a procedure to measure the circulating *miR-1* concentration and assessed its value as a potential molecular marker for patients with AMI.

Materials and methods

Participants. Between September 2008 and August 2009, we studied 159 patients at ages of 30–75, with 93 AMI patients and 66 healthy subjects from the First Affiliated Hospital, the Second Affiliated Hospital of Harbin Medical University (Harbin, China) and Fuwai Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (Peking, China). AMI was diagnosed based on combination of several parameters: ischemic symptoms plus increased cardiac troponin I (cTnI) and creatine kinase-MB (CKMB), pathological Q wave. ST-segment elevation or depression defined by the European Society of Cardiology/American College of Cardiology [14,15]. Baseline ECG was recorded in all patients. Written consents were obtained from all subjects studied and the study protocol was approved by the ethics committee of the Harbin Medical University.

Isolation of human plasma. For miRNA detection, whole blood (WB) samples (2.5 mL per patient) were collected from subjects via a direct venous puncture into tubes containing sodium citrate, centrifuged at 1000*g* for 5 min, and then the supernatant (plasma) was carefully transferred into an RNase-free tube for extraction of RNA.

RNA isolation and real-time quantitative RT-PCR (qRT-PCR). Total RNA was isolated from 1 mL plasma using phenol/chloroform extraction procedures as described before [10].

cDNA synthesis was performed according to the manufacturer's instructions (Reverse Transcription System, Cat. #A3500, Promega) as described previously [16–18]. The SYBR Green PCR Master Mix Kit (Applied Biosystems, Cat. #4309155) was used in real-time PCR for relative quantification of miRNAs in our study with U6 as an internal control. qRT-PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems). The RT primers used were (1) *miR-1*: GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTG GATACGACTACATAC; (2) *miR-133*: GTCGTATCCAGTGCGTGTCGTGG AGTCGGCAATTGCACTGGAT ACGACCAGCTG; (3) U6: CGCTTCACGA ATTTGCGTGTCAT. The PCR primers for the study included (1) *miR-1* forward: GGGGTGGAATGTAAAGAA and *miR-1* reversed: TGCGTGTCGTGGAGTC; (2) *miR-133* forward: GGGTTTGGTCCCCTT CAA and *miR-133* reversed: AGTGCGTGTCGTGGAGTC; (3) U6 forward: GCTTCGGCAGCACATATACTAAAAT and U6 reversed: CGCTTC ACGAATTTGCGTGTCAT.

Statistical analysis. Data were described as means ± SD and median for general characteristics of subjects. Wilcoxon rank sum test was performed to compare the expression of *miR-1* and *miR-133* between AMI patients and non-AMI subjects. The comparison of miRNA levels within AMI patients was made between two time points: at the time of hospitalization and at the time of discharge from hospital after efficient treatment for 2 weeks, using Wilconxon signed rank test.

Univariable linear and logistics regression analyses were taken to evaluate the relationships between *miR-1* and clinically related indexes QRS, ST segment, cTnI, CKMB, and blood pressure (BP). Age and sex were considered as controlled variables.

Receiver–operator characteristic (ROC) analyses were also performed with circulating *miR-1* levels plotted against AMI. Area under ROC curve (AUC) was estimated to assess the predictive power. In our study, AUC values indicate the ability of circulating *miR-1* to distinguish AMI and non-AMI subjects or AMI and discharged subjects.

SAS 9.1 software (Serial No. 989155. Institute, Inc., China) was used for all statistical analyses. Two-tailed *P* values with composite results $P < 0.05$ were considered statistically significant.

Results

Baseline clinical characteristics of study population

On admission, the ages of AMI patients were 58.2 ± 10.2 years and of control group were 55.1 ± 9.6 years ($P > 0.05$). The number of males in both groups was greater than that of females (female/male = 27/39 in control group and 26/67 in AMI group). There were no significant differences between AMI and Ctl subjects in BP, hyperlipidemia and diabetes mellitus (Table 1).

miR-1 levels in plasma

Circulating levels of *miR-1*, as indicated by the median Ct values, were significantly higher in AMI than in non-AMI patients (median: Ctl = 32.11, AMI = 28.35; Z value = 8.1185, $P < 0.0001$, Fig. 1A). Strikingly, the increased circulating level of *miR-1* was restored back to the control value when the AMI patients were discharged from hospitals after having received medication for 2 weeks (median: AMI = 28.23, discharge = 32.43; $S = 454.5$, $P < 0.0001$ by Wilcoxon rank signed test, Fig. 1A).

As illustrated in Fig. 1B, no significant differences of *miR-133* level were observed among three groups ($P > 0.05$ by Wilcoxon rank sum test and Wilcoxon rank sign test).

A Ct value $\geqslant 30$ in a RNA sample of 0.5 μg was taken as an indication of absence of the target miRNA. For example, HEK293 cell line is known to be devoid of endogenous *miR-1* and the Ct value obtained from 1 μg RNA sample isolated from HEK293 cells is $\geqslant 30$ [16,17]. Using this cutoff criterion, we found that out of 66 non-AMI subjects, only less than 8 had Ct value <30 (12%). By comparison, over half of the AMI patients (73%) had Ct values <30, but at the time of discharge their Ct values were restored back to >30 (Fig. 1C).

Analysis with univariable linear and logistics regression models ruled out the correlation between age/gender and circulating *miR-1* concentration ($P > 0.05$, Table 2). We then further excluded the possible influence from variables, such as diabetes mellitus, sys-

Table 1
Characteristics of subjects.

Characteristics	Ctl ($n = 66$)	AMI ($n = 93$)	*P* value
Ages (yr)			
Mean	55.1 ± 9.6	58.2 ± 10.2	>0.05
Range	30–71	36–75	
Median	53	59	
Sex			
Man	39	67	>0.05
Woman	27	26	
Systolic pressure (mm Hg)			
Mean	142.4 ± 17	139 ± 28	>0.05
Range	110–195	90–220	
Median	140	138	
Diastolic pressure (mm Hg)			
Mean	88.2 ± 11	85.8 ± 16.1	>0.05
Range	68–117	50–120	
Median	88	85	
Diabetes, *n* (%)	11 (20.7)	11 (13.9)	>0.05
Hyperlipidemia, *n* (%)	14 (26)	23 (29)	>0.05

Data are presented as means (±SD) or number (%).

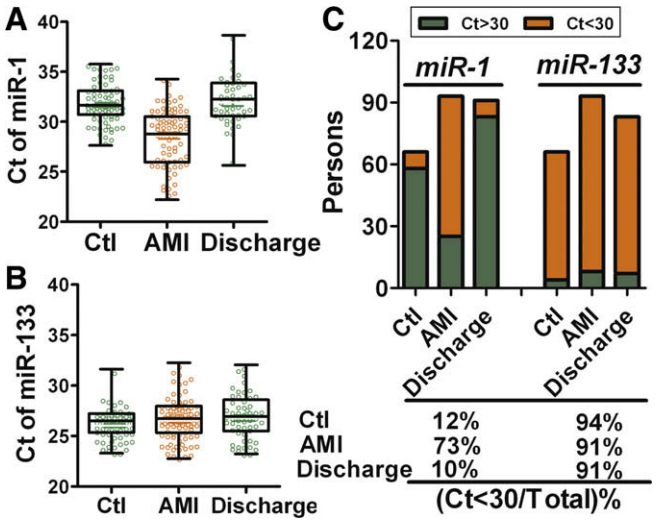

Fig. 1. Circulating levels of *miR-1* and *miR-133* in plasma from hospitalized and discharged AMI patients. Increased *miR-1* levels in plasma (A) as indicated by the decreased Ct values in hospitalized AMI patients compared with Ctl group (Wilcoxon rank sum test: $P < 0.0001$) or discharged AMI patients paired-compared with AMI (Wilconxon signed rank test: $P < 0.0001$). No significant difference of Ct for *miR-133* in plasma (B) among three groups (Wilcoxon rank sum test and Wilconxon signed rank test: $P > 0.05$). (C) The percentage of *miR-1* and *miR-133* with Ct < 30. Ct: cycle threshold of PCR amplification. $n = 66$ for Ctl, $n = 93$ for AMI; $n = 83$ for paired hospitalize and discharged AMI patients. Ctl: control; AMI: acute myocardial infarction.

tolic pressure, and diastolic pressure in our study population (Table 2).

Association of circulating miR-1 with abnormal QRS

With the univariable linear regression model, QRS was found significantly correlated with *miR-1* level. Using the Ct cutoff criterion, QRS was also found significantly correlated with *miR-1* level by univariable logistics regression model (Table 3).

With respect to other AMI related biomarkers, we found no significant correlations between *miR-1* and ST segment or cTnI/CKMB by either univariable linear or logistics regression models (Table 3).

miR-1 expression as a potential predictor of acute myocardial infarction

ROC analysis was performed to evaluate the predictive power of circulating *miR-1* for AMI. When comparison was made between AMI and non-AMI, the area under ROC curve (AUC) was 0.7740 (95% confidence interval; CI = 0.7065–0.8414, $P < 0.0001$). When comparison was made between AMI at the time of hospitalization and AMI at the time of discharge, AUC was 0.8522 (95% CI = 0.7908–0.9136, $P < 0.0001$) (Fig. 2).

Discussion

This study established the measurement of the muscle-specific miRNA *miR-1* in plasma as an important approach for the blood-based detection of human AMI. Particularly notable is that the present study found for the first time that *miR-1* level is significantly elevated in the blood of AMI patients relative to non-AMI subjects that have virtually no detectable *miR-1* in circulation, and the increased *miR-1* level is well correlated with abnormal QRS widening in AMI. The elevated *miR-1* level in AMI patients on admission is recovered to normal value at the time of discharge following medications. These results are also in agreement with our previous findings in ischemic myocardium from a rat model of AMI and from patients with coronary artery disease [12]. We therefore conclude that circulating *miR-1* may be an independent biomarker for diagnosis of AMI and the associated ischemic arrhythmias.

To avoid possible bias from patient selection, subjects with ages <30 or >75 were excluded from the present study, and the ratio of gender was also well balanced between AMI and non-AMI groups. Both linear and logistics regression analyses further demonstrated that age and gender did not influence *miR-1* level in plasma, suggesting *miR-1* a potential biomarker for broad population. Fair stability of miRNAs in blood has been documented by previous studies [8–10] and the present study confirmed this notion.

In the present study, the *miR-1* values for controls and AMI patients overlap to a certain extent. In order to evaluate the predictive ability of *miR-1*, we compared the *miR-1* levels of AMI patient between hospitalization and discharge. Strikingly, the increased *miR-1* level in most patients was recovered. The data acquired from this type studies could have been biased by non-AMI related disease states. To rule out this possibility, we measured the level of another muscle-specific miRNA *miR-133* and found that the *miR-133* level in the circulation between AMI and non-AMI subjects was indifferent, indicating the uniqueness of blood *miR-1* as an AMI marker.

The existing biochemical markers for acute coronary syndrome include cTn, CKMB, myoglobin, CRP, and the natriuretic peptides BNP and NT-proBNP [3,4]. Among the aforementioned markers, CKMB, cTnI and myoglobin have been considered the outdated markers [2,15]. An important note is that a majority of existing biomarkers, if not all, are proteins or peptides, and these traditional proteomic biomarker discovery–validation pipelines often encounter bottlenecks at the point of antibody generation and quantitative assay development for validation of biomarker candidates. Rapid and accurate detection and quantification of these biomarkers in blood may represent a further challenge, though a multiplicity of assays have been developed for the detection of these proteins. Here we propose for the first time miRNAs as molecular markers for AMI patients. The advantage of using *miR-1*, or using miRNA in general, as a biomarker, is that it can be precisely quantified using qRT-PCR that provides exceptionally high sensitivity and specificity of detection in a minimally invasive way. Moreover,

Table 2
Correlation between circulating *miR-1* and various variables determined by univariable linear and logistics regression analyses.

Variable	Univariable linear model				Univariable logistics model			
	Estimate	SE	*t* value	*P* value	Estimate	SE	Wald. χ^2	*P* value
Age	−0.0248	0.02724	−0.91	0.3654	−0.0326	0.0214	2.3271	0.1271
Sex	0.02729	0.60333	0.05	0.9640	0.4462	0.4899	0.8296	0.3624
SP (mm Hg)	−0.0016	0.01050	−0.15	0.8785	−0.0055	0.00832	0.4517	0.5015
DP (mm Hg)	0.00186	0.01828	0.10	0.9193	0.00127	0.0142	0.0080	0.9287

Note. Dependent variable Y: *miR-1*; independent variable: clinical variables.
For univariable logistics model, we set a cutoff value for *miR-1*, 30 Ct numbers were set as cutoff value. *miR-1* level was coded as 0 when <30 Ct, otherwise marked as 1. DP = diastolic pressure; SP = systolic pressure.

Table 3
Correlation between circulating *miR-1* and AMI related variables by univariable linear and logistics regression analyses.

Variable	Univariable linear model				Univariable logistics model			
	Estimate	SE	*t* value	*P* value	Estimate	SE	Wald. χ^2	*P* value
QRS (ms)	−0.03432	0.01123	−2.47	0.0092	−0.0668	0.0204	10.6325	0.0010
ST segment (mv)	−0.25987	0.91467	−0.28	0.7772	0.04850	0.05870	0.1074	0.7243
CKMB (U/L)	0.00156	0.00305	0.51	0.6106	−0.0012	0.00248	0.2329	0.6294
cTnI (ng/mL)	0.03527	0.03608	0.98	0.3326	0.01220	0.0264	0.2138	0.6438

Note. Dependent variable Y: *miR-1*; independent variable: clinical variables.
For univariable logistics model, we set a cutoff value for *miR-1*, 30 Ct numbers were set as cutoff value. *miR-1* level was coded as 0 when <30 Ct, otherwise marked as 1.

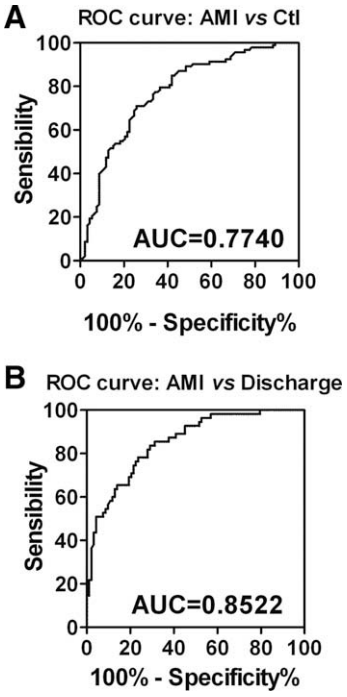

Fig. 2. Receiver–operator characteristic (ROC) curve analyses for onset and relieved AMI patients. (A) ROC curve for the onset of AMI patients; (B) ROC curve for relieved AMI patients. AUC = area under receiver–operator characteristic curve.

qRT-PCR also allows absolute quantification of miRNAs down to the level of copy number within a cell or per certain amount of RNA sample, potentially providing precise cutoff concentrations for the purpose of diagnosis. For example, in this study we used Ct = 30 as an effective cutoff to distinguish AMI and non-AMI with an initial total RNA sample of 0.5 μg. Thus, the discovery–validation pipeline for miRNA biomarkers is expected to be more efficient. Our finding that there is a lack of correlation between circulating *miR-1* and the known biomarkers of AMI including cTnI and CKMB indicates that *miR-1* might be a unique independent biomarker for AMI.

miR-1 is known to be a muscle-specific miRNA, being expressed abundantly in cardiac and skeletal muscles with little expression in other tissues [19–21]. The source of *miR-1* in the blood stream of AMI patients is presumably solely from the heart. The appearance of *miR-1* in circulation in AMI patients suggests a release of *miR-1* from necrotic myocytes.

Aberrant *miR-1* expression has been associated with arrhythmogenesis under several pathological settings of the heart in the previous studies from our laboratories and from others as well [12,17–21]. One of the mechanisms for the arrhythmogenic potential of *miR-1* is its ability to target the genes encoding key proteins, connexin 43 and Kir2.1, which play critical role in maintaining cardiac conduction. In our previous study on miRNAs in a rat model of AMI, we demonstrated that up-regulation of *miR-1* caused widening of QRS complex indicating an impairment of ventricular conduction and actual slowing of cardiac conduction velocity [12]. Strikingly, in this study the increase in circulating *miR-1* level is also found to be well correlated with the broadening of QRS duration in AMI patients (Table 3). This finding clearly suggests that appearance of *miR-1* in circulation is accompanied by a functional impairment of cardiac conduction. And *miR-1* as a biomarker would provide invaluable information for management of patients with AMI and the associated sudden cardiac death. Indeed, increase in QRS duration has been a long-recognized characteristic for AMI hearts [22] and has been believed to be related to the size of infarct area [23]. A recent study reported that an increase in QRS duration by 20-ms increments is associated with increasing 30-day mortality rate in AMI patients [24].

Limitations

It should be noted that the consideration of circulating *miR-1* as a biomarker for AMI is at present based on our results from a relatively small sample size and larger clinical studies are definitely required to establish the case. Nonetheless, the present study lays the groundwork for future efforts to identify and develop *miR-1* (perhaps also other miRNAs) as a novel class of blood-based biomarkers for AMI. Further, since the *miR-1* level was determined in the AMI patients on their first presentation and on discharge 2 weeks after treatment, the potential prognostic value of circulating *miR-1* could not be evaluated. Yet the fact that circulating *miR-1* was restored back to normal in AMI patients on discharge may give an insinuation for the potential of *miR-1* as a prognostic marker as well. This issue undoubtedly merits future investigation.

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