Original Article

Circulating miR-499 are novel and sensitive biomarker of acute myocardial infarction

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Background: miRNAs are known to play a crucial role in cardiac development, and the expression of miRNA is altered in the diseased heart. The aim of this study was to investigate the value of plasma microRNA-499 (miR-499) as a novel biomarker for early diagnosis of acute myocardial infarction (AMI).

Methods: Enrolled in this study were 227 patients with chest pain on presentation to the departments of emergency and cardiology of Wuxi Second People's Hospital between October 2011 and May 2014. Additional 100 healthy individuals who received physical examination in the same hospital during the same period were used as control. Plasma was collected at admission, and the abundance of miR-499 was measured using reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: MiR-499 was significantly elevated in 142 patients diagnosed with AMI as compared with 85 patients in non-AMI group and 100 subjects in healthy control group. Plasma miR-499 were already detectable in the plasma as early as 1 h after onset of chest pain in AMI patients, and continued to increase gradually without any sign of decreasing tendency within 9 h in AMI patients. miR-499 was highly positively correlated with the serum creatine kinase-MB (CK-MB) and cTnI. The area under the curve (AUC) of miR-499 for the diagnosis of AMI was 0.86, with an optimal cut-off value of 4.79, sensitivity of 80%, and specificity of 80.28%.

Conclusions: miR-499 was shown to substantially increase the diagnostic accuracy of CK-MB and cTnI in the diagnosis of AMI, and therefore it may prove to be a useful marker for early diagnosis of AMI.

Keywords: microRNA (miR); acute myocardial infarction (AMI); early diagnosis; biomarker

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Introduction

Acute chest pain is the leading cause of hospital admission worldwide. It is critically important to accurately determine whether a patient with acute chest pain is suffering from acute myocardial infarction (AMI) without delay (1,2). Generally speaking, the diagnosis of AMI is mainly based on patient's medical history, presenting symptoms and signs, electrocardiography (ECG) and laboratory markers. However, the patient's medical history and sings is largely affected by the cognitive ability of the patient and the expressive ability of the clinician. For instance, some patients are unconscious when they are sent to hospitals and their family members cannot remember the exact onset time. ECG is an important basis for the diagnosis of AMI, but interpretation of the ECG is quite subjective. In addition, some AMI patients have no ECG manifestations. In contrast, laboratory markers are more favored by clinicians. Troponin (cTnI/T) and creatine kinase-MB (CK-MB) are generally accepted as the most reliable biomarkers, but neither can absolutely and accurately confirm or exclude the diagnosis of AMI (3,4). It is therefore necessary to

seek more sensitive and specific novel biomarkers for the diagnosis of AMI (5).

microRNAs (miRs), non-coding single-stranded small RNA consisting of 21-23 nucleotides, are negative regulators of gene expression by binding to 3'UTR of mRNA (6,7). miRNAs used to be considered existing in cells only for some time. However, recent studies discovered that miRNAs are stably present in human serum/plasma (8,9). Most importantly, these circulating miRNAs can serve as potential biomarkers for various diseases, such as cancer (10), tissue injury (11), autoimmune diseases (12) and other common clinical diseases.

More recent studies have demonstrated that miRNAs play an important role in AMI. miR-1, miR-133a, miR-208 and microRNA-499-5p (miR-499-5p) were found to be cardio- or skeletal-muscle specific and could be used as biomarkers for the diagnosis of MI (13,14). Animal models have demonstrated that miR-499 specifically expressed in cardiomyocytes and was a potential marker of myocardial injury (15). A milder elevation with 6-fold of miRNA-499 was observed in viral myocarditis while 100-fold was observed in AMI (16), indicating that the level of plasma miR-499 was not affected by inflammation and it could reflect myocardial damage. miRNA array analysis showed that plasma miR-499 concentrations increased in AMI group while it were below the limit of detection in other patient groups (17). These clinical studies, preliminary, have indicated that miR-499 hold potential as a new cardiac biomarker.

Therefore, the present study was designed to determine the diagnostic value of circulating miR-499 in the assessment of suspected AMI patients, measure the plasma levels of cardio specific miR-499 following MI, and evaluate its usefulness as a biomarker of early cardiac cell death.

Materials and methods

Patients and subjects

A total of 227 consecutive patients with acute chest pain within 12 h admitted to departments of emergency and cardiology of Wuxi Second People's Hospital (Wuxi, China) between October 2011 and May 2014 were recruited in the study. The inclusion criteria were based on the universal definition of MI (18). Briefly, patients with AMI were clinically diagnosed by biochemical markers (cTnI >0.1 ng/mL), acute ischemic-type chest pain, ECG change, and coronary angiography.

Patients were excluded if the chest pain caused by trauma, drugs, medical intervention, patients with terminal kidney failure who required dialysis or received intravenous thrombolytic before the initial blood samples were obtained. In addition, 100 adult healthy volunteers with normal ECG findings and no history of cardiovascular disease during the same period were enrolled as normal controls.

Whole blood samples (3-5 mL) were collected and extracted from citrated tubes within 2 h after hospitalization and stored at –80 ℃ for miRNA analysis. Finally, 0.4 mL of plasma was exactly used for RNA extraction. The protocol was approved by the local ethics committee and all study participants signed an informed consent form. Clinical characteristics are shown in *Table 1*.

Detection of miR-499

Total RNA was extracted from plasma samples using the mirVana PARIS kit (Ambion, Applied Biosystem, Foster City, USA) with enrichment for small RNAs. Reverse transcription of miRNA was performed using the miScript reverse transcription kit (Applied Biosystem, Inc.). MiR-499 was quantitated by TaqMan miRNA quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay according to the manufacturer's instructions (Applied BioSystems, Inc.). U6RNA was used as a miRNA internal control.

Data were analyzed with automatic setting for assigning baseline. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeds the given threshold. Ct values greater than 40 as obtained from real-time PCR were treated as 40. The plasma levels of miRNA were detected and analyzed independently by two investigators who were blinded to the clinical data of patients. Data obtained by real-time PCR were translated in log2 (relative level).

cTnI and CK-MB testing

Serum cTnI and CK-MB was assayed with BECKMAN DXI800 system and OLYMPUS AU5421 system, respectively. Both assays were performed according to the manufacturer's instructions. The upper limit for the normal reference range was set at 0.01 ng/mL and 24 U/L, respectively.

Statistical analysis

Experimental data are expressed as mean ± standard deviation

Table 1 Clinical characteristics of all the patients

Characteristic	Healthy control (N=100)	non-AMI (N=85)	AMI (N=142)	P value
Age(years)	66.97±11.06	66.45±10.61	64.86±12.84	0.347
Sex (male/female)	80/20	59/26	102/40	0.211
Current smokers (No./total No.)	53/100	31/85	79/142	0.015
Hypertension (No./total No.)	60/100	59/85	91/142	0.411
Hyperlipemia (No./total No.)	31/100	18/75	37/142	0.546
TC (mg/dL)	3.72±0.10	4.03±0.13	4.03±0.08	0.045
TG (mg/dL)	1.65±0.10	1.46±0.10	1.72±0.07	0.135
HDL	1.21±0.04	1.08±0.04	1.09±0.03	0.028
LDL	2.47±0.09	2.57±0.11	2.65±0.07	0.263
Body mass index (kg/m^2)	23.11±3.11	23.09±3.01	23.75±2.86	0.144
Diabetes mellitus	31/100	29/85	40/142	0.638
History of CVD	4/100	5/85	15/142	0.130
Creatinine	81.45±19.65	83.61±32.15	87.80±44.12	0.362
Creatine kinase MB	18.20±4.70	18.38±8.78	80.42±104.30	0.000
TroponinI	0.12±0.21	2.16±8.00	17.64±30.41	0.000
Time since chest-pain onset		5.32±3.96	5.55±3.98	0.670

($\bar{x}\pm s$). Before analysis, all data were subjected to a normality test (Shapiro-Wilk). Kruskal-Wallis H test or one-way ANOVA was used for comparison of data between the three groups, and LSD-t test was used for inter-group comparison. Receiver operating characteristic (ROC) curves were used for evaluation of diagnostic accuracy of miR-499, CK-MB and cTnI. Spearman correlation test was used to analyze correlations between miR-499, cTnI and CK-MB. Statistical analyses were performed with the SPSS statistical package. A value $P<0.05$ was considered statistically significant.

Results

Description and clinical features of the patients

Of the 227 patients who were initiated enrolled in this study, AMI was confirmed in 142 patients. In the non-AMI patients, stable angina was diagnosed in 13 patients, unstable angina in 18 patients, hypertensive heart disease in 18 patients, heart failure (Grade III and IV) in 10 patients, myocarditis in 4 patients, pericarditis in 2 patients, myocardial bridge in 2 patients, aortic dissection in 2 patients, pulmonary embolism in 2 patients, penetrating abdominal aortic ulcer in one patient, and ischemic cardiomyopathy in one patient. *Table 1* shows clinical characteristics of all the patients.

Increased circulation concentrations of miR-499 after AMI

MiR-499 was measured in the plasma of 142 AMI patients, 85 non-AMI patients and 100 healthy controls (*Figure 1A*). In the healthy control, miR-499 almost had undetectable concentrations, and was at a low level in the non-MI chest pain group. Compared to the healthy control population, circulating miR-499 levels were higher in patients with AMI. Furthermore, circulating levels of miR-499 were markedly elevated in AMI patients than any subgroup of the non-AMI patients (*Figure 1B*).

Simultaneous miR-499 plasma levels in acute myocardial infarction patients

Plasma miR-499 level was measured at admission in the 142 AMI patients. Plasma miR-499 level increased gradually at different time points after the onset of chest pain. It was detectable within 1 h after the onset of chest pain and continued to increase gradually without any sign of decreasing tendency within 9 h (*Figure 2*). While positive CK-MB and cTnI were detected 2 and 2-4 h after chest pain respectively. Ten AMI patients were early founded by mir-499 detection who were otherwise missed by troponin and 48 by CK-MB.

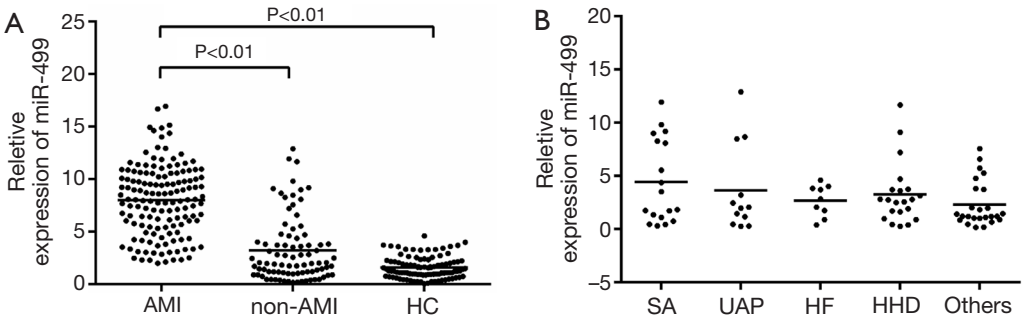

Figure 1 (A) The circulation concentrations of miR-499 in AMI group, non-AMI group and healthy control group; (B) the circulation concentrations of miR-499 in subgroups of the non-AMI patients. miR-499, microRNA-499; AMI, acute myocardial infarction; SA, stable angina; UAP, unstable angina; HF, heart failure; HHD, hypertensive heart disease.

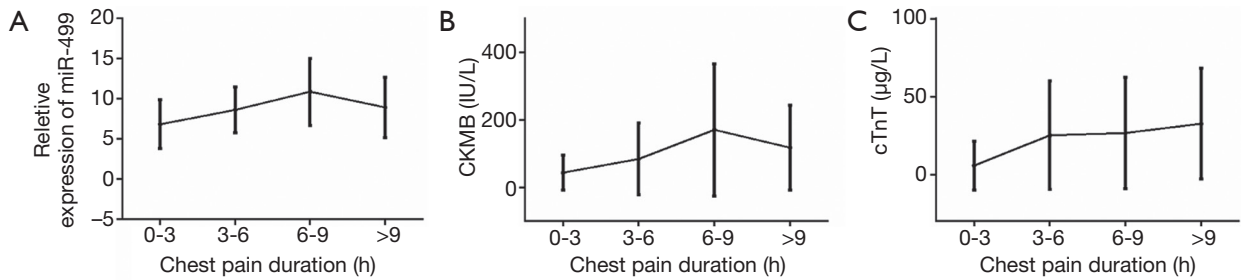

Figure 2 The time courses of miR-499, CK-MB and cTnI level in patients with different onset time. CK-MB, creatine kinase-MB; miR-499, microRNA-499; AMI, acute myocardial infarction.

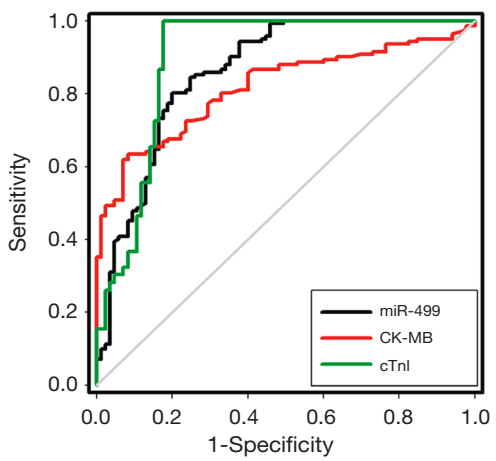

Figure 3 ROC curve analysis for discrimination between AMI group and non-AMI group. CK-MB, creatine kinase-MB; AMI, acute myocardial infarction.

Evaluation of miR-499 in plasma as new biomarkers for acute myocardial infarction

To investigate the character of miR-499 as a potential biomarker of AMI, ROC analysis was performed on data from all 227 patients with chest pain, including 142 AMI patients and 85 non-AMI patients. The ROC curves of miR-499 reflected separation between AMI and non-AMI group, with an area under curve (AUC) of 0.86 [95% confidence interval (CI), 0.81-0.91], compared with cTnI with an AUC of 0.90 (95% CI, 0.85-0.95), CK-MB with an AUC of 0.82 (95% CI, 0.76-0.87), respectively (*Figure 3*). These results showed that miR-499 had a diagnostic value in AMI patients, but its diagnostic accuracy was lower than that of cTnI (P<0.05).

Correlations between miR-499 with cTnI and CK-MB

To evaluate the significance of increased levels of circulating miR-499 following myocardial infarction, we analyzed whether the levels of miR-499 correlated with clinical parameters CK-MB and cTnI. We found that the level of miR-499 correlated significantly with circulating troponinI (P=0.000, r=0.488) (*Figure 4A*), CK-MB (P=0.000, r=0.495) (*Figure 4B*).

Discussion

In the present study, our results showed that cardiac-enriched

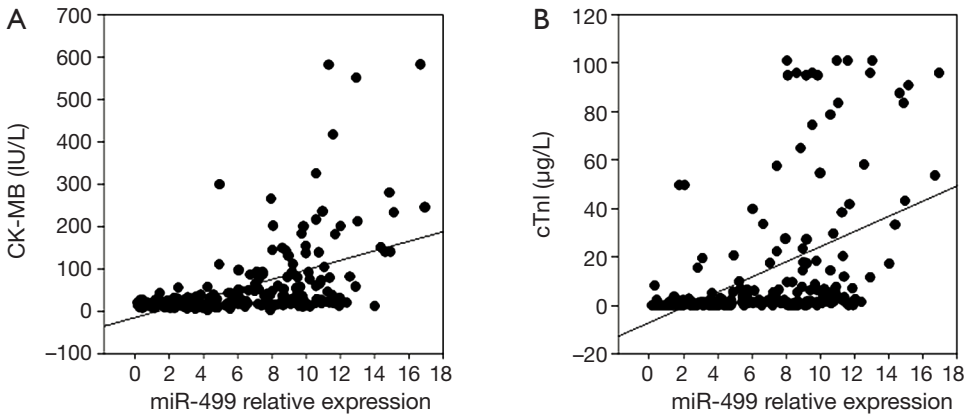

Figure 4 Correlation of the miR-499 with cTnI (A) and CK-MB (B) in AMI patients. CK-MB, creatine kinase-MB; AMI, acute myocardial infarction.

circulating miR-499 was hardly detectable in cohort of healthy controls, very close to the detection limit of the assay. However, miR-499 increased in some patients with non-AMI cardiac disease whose troponin was negative on initial presentation, including angina pectoris and myocarditis. Although the mechanisms of this phenomenon remain unclear, the packaging of miR-499 into microvesicles may be the explanation. miRNAs were considered a new type of signaling molecule, and they may have direct or indirect biological effects that can deliver multiple messages at a time or regulate numerous target genes independent of cell contact or adhesion in the same time (19,20). As a result, the release of miR-499 can be affected by any form of cellular stress including cardiac necrosis. These observations in this study suggest that miR-499 may be a new potential biomarker to distinguish angina pectoris and myocarditis from other diseases in early phases.

On the basis of previous studies, we further observed the time courses of miR-499, CK-MB and cTnI level in AMI patients with different onset time. Comparable to cTnI and CK-MB, miR-499 are present in the plasma as early as 1 h after the onset of chest pain, and continued to increase even after 9 h following the onset of chest pain, while CK-MB and cTnI were detected 2 h after chest pain, indicating that miR-499 level could be used to detect AMI earlier. This supported the evidence that miR-499 can be a very early biomarker of AMI.

Receiver operating characteristic analysis further indicated that miR-499 has considerable diagnostic efficiency. These results are partially supported by a very recent report that miR-499 level was significantly higher in plasma from AMI patients than non-AMI subjects (21). What's more, miR-499 could early find patients missed by troponin or CK-MB. It indicates that the miR-499 profiling system may work as a potential replenishment of the currently available biomarkers for the clinical diagnosis of AMI. Based on ROC curves, we screened the cut-off values to define one that could best discriminate AMI patients from patients with non AMI chest pain, and found that miR-499 >12.93 may serve as a determinant for the diagnosis of AMI, these important cut-off values may help provide evidence for future research in this area.

Cardiac troponins are considered the gold standard of biomarkers for the diagnosis of myocardial infarction at present. MiR-499 and cTnT have a certain correlation, indicating that they can be used as markers of acute cardiomyocyte cell death. On the other hand, it suggested that the diagnostic information of miR-499 does not completely overlap with that of troponin and CK-MB, miR-499 may provide more diagnostic information than troponin. In addition, cTnT has the ability to evaluate the prognosis of AMI, so the positive correlation also indicates that it can evaluate the prognosis. A recent study elegantly demonstrated that in some patients undergoing catheterization, the concentration of several miRs (such as miR-499) in transcoronary was increased (22). So we included all patients and healthy control at the first presentation and collected blood before any treatment. We also found that plasma miR-499 could provide more diagnostic information than CK-MB and cTnI, and therefore combination of miR-499 with CK-MB and/or cTnI may improve the diagnostic accuracy.

Some problems need to be solved before miR-499 can be used as a clinical marker for AMI, including technical problems in detecting miR-499 timely. However, compared with cTnT and other conventional markers, miR-499

has unique advantages, for instance, it is not affected by renal function (21) and has high stability *in vitro* (14). In addition, cTnT and CK-MB are currently detected by immunological methods and susceptible to interference of some specimen factors such as giant troponin (23) and troponin antibodies (24). Thus, miR-499 is still an expected marker of myocardial injury.

In summary, this study confirms the value of circulating miR-499 as a marker for the diagnosis of AMI, devoid of the deficit of CK-MB and cTnI. It is necessary to continue with the research in this area to provide new evidence for explaining the value of miR-499 in the diagnosis and prognostic prediction of AMI.

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