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Value of the expression of miR-208, miR-494, miR-499 and miR-1303 in early diagnosis of acute myocardial infarction

Ping Li[1], Si-Yao Li[1], Ming Liu*, Jiang-Wen Ruan, Zheng-Dong Wang, Wen-Chao Xie

Department of Cardiology, Yulin First People's Hospital (The Sixth Affiliated Hospital of Guangxi Medical University), Yulin, Guangxi 537000, China

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ABSTRACT

Aims: This study aims to investigate the value of the expression of miR-208, miR-494, miR-499 and miR-1303 in the early diagnosis of acute myocardial infarction (AMI).

Main methods: Patients were divided into two groups: AMI group ($n = 41$), and Stable angina pectoris (SAP) group ($n = 32$). Peripheral venous blood was sampled from these patients at the time of admission (T0), 6 h after onset (T6) and 12 h after onset (T12), while blood was sampled once from healthy subjects who underwent physical examination in the same time period (control group, $n = 10$). The expression of miR-208, miR-494, miR-499 and miR-1303 in serum were detected by real-time quantitative polymerase chain reaction (qRT-PCR), and differences in miRNA expression among these three groups of patients were analyzed.

Key findings: Serum miR-208, miR-494, miR-499 and miR-1303 expression levels at different time points were significantly higher in the AMI group than in the SAP group and control group. The differences among these groups were statistically significant ($P < 0.05$), while the difference between the SAP group and control group was not statistically significant ($P > 0.05$). Variation trend: The miRNA levels above began to increase at T0 in the AMI group, the peak levels of miR-208, miR-494 and miR-499 appeared before T12, and the peak level of miR-1303 appeared between T6 and T12, or after T12.

Significance: miR-208, miR-494, miR-499 and miR-1303 were not superior to hs-cTnI as myocardial markers in the diagnosis of early acute myocardial infarction.

1. Introduction

At present, coronary heart disease (CHD) has become one of the main causes of mortality in China, among which acute myocardial infarction (AMI) takes the highest proportion [20]. The early and accurate diagnosis of AMI is the most effective approach to reduce mortality and improve prognosis. At present, cardiac markers commonly used in the clinical diagnosis of AMI include creatine kinase-MB (CK-MB), cardiac troponin T (cTnT), cardiac troponin I (cTnl), myoglobin (Myo), etc. [36]. The most commonly used marker is hs-cTnI [23]. The disadvantage of hs-cTnI is the time limit. That is, hs-cTnI can only be detected within 3–4 h after the myocardial infarction. Therefore, a new type of highly sensitive, specific and early detectable myocardial biomarker is urgently needed.

In recent years, microRNAs (miRNAs) have gained increasing attention. miRNA in the blood circulation of cancer patients can also be used for diagnosis and prognosis [7,17,25]. The value of miRNAs in diagnosing AMI has also been confirmed by recent studies [29]. It has been reported that miRNA208 could affect proliferating cardiomyocytes and myocardial hypertrophy [19] and miRNA499 could promote the differentiation of cardiac progenitor cells into myocytes [35]. miRNA494 was demonstrated could protective myocardium against ischemia-induced cardiac injury [31]. Therefore, these cardiac specific or cardiac-enriched miRNAs might be useful biomarkers for screening AMI. At present, most studies on miRNA in myocardial infarction show that miRNA-208 and miRNA-499 are up-regulated after myocardial infarction [3,8,9,19,35]. But there is no clear conclusion on when the upregulation of miRNA-494 and its change rule after myocardial infarction. Studies have proposed that miRNA-1303 is also related to myocardial infarction [32], but there are few related studies and no consistent conclusion.

In this study, the expression of the above miRNAs in the serum of patients with acute myocardial infarction at different times was detected, to explore when the expression of these miRNAs was abnormal

* Corresponding author at: Department of Cardiology, Yulin First People's Hospital (The Sixth Affiliated Hospital of Guangxi Medical University), No.495 Education Middle Road, Yuzhou District, Yulin, Guangxi 537000, China.
E-mail address: liuming_1966@163.com (M. Liu).
[1] Equal contributors co-first author.

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after myocardial infarction and when they returned to normal, and to evaluate the value of these miRNAs in the early diagnosis of myocardial infarction compared with the traditional myocardial markers.

2. Subjects and methods

2.1. Subjects

2.1.1. Patients source

Patients who visited Yulin First People's Hospital from October 2015 to October 2016 due to typical chest pain within 3 h were randomly included into the present study. According to the patient's clinical symptoms and related test results, and indications for coronary angiography, and based on the 2010 Diagnostic Criteria for Myocardial Infarction and Stable Angina. Patients who met the enrollment criteria were divided into two groups: AMI group ($n = 41$), and Stable angina pectoris (SAP) group ($n = 32$). During the same period, age- and gender-matched healthy subjects without cardiovascular disease were enrolled as the control group ($n = 10$). All patients were admitted to the hospital for the first time. No surgery or drug treatment was performed before the first blood sampling. These patients were informed of the contents of the trial, and provided an informed consent. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Yulin First People's Hospital (The Sixth Affiliated Hospital of Guangxi Medical University). No approval number is applicable.

2.1.2. Inclusion criteria

AMI group: Within 3 h after the chest pain, a diagnosis of AMI was drawn based on the 2010 Guidelines for the Diagnosis of Myocardial Infarction [5]: (1) typical clinical symptoms, such as chest pain and vasovagal response; (2) electrocardiogram (ECG) revealing ischemic changes, such as ST-segment elevation or new pathological Q waves; (3) changes in myocardial marker levels, preferably the troponin recommended in the latest guidelines.

SAP group: (1) the presence of typical labor angina symptoms, which can be relieved when rest or orally taking nitrate drugs; (2) the absence of an increase in myocardial marker levels; (3) ECG has no obvious change, or has only changes induced by ST-segment depression and T-wave; (4) stenosis of the coronary artery $> 50\%$, as revealed by coronary angiography.

Control group: healthy subjects without previous cardiovascular disease, who underwent a health examination.

2.1.3. Exclusion criteria

Patients who recently had severe diseases, such as thrombotic diseases, surgery, trauma, various acute infections, malignant tumors, allergic diseases, autoimmune diseases, severe liver and kidney dysfunction, heart failure and cerebrovascular accidents, were excluded.

2.2. Research methods

2.2.1. Sample acquisition

Peripheral venous blood samples were collected from patients in the AMI and SAP groups after the onset of chest pain at the following time points: at the time of admission (T0), at 6 h after onset (T6), and at 12 h after onset (T12). Peripheral venous blood samples were also collected from healthy controls. Blood was sampled using anticoagulant-free tubes. After centrifugation, the supernatant was obtained and placed into different epoxy resin (EP) tubes. Then, the samples were stored in a refrigerator at $-80\,^{\circ}C$ for subsequent evaluation.

2.2.2. Clinical data acquisition

The following hospitalization data of these patients, including their basic information, were collected: age, gender, admission number, clinical diagnosis, hypertension, hyperlipidemia, history of diabetes, and history of smoking. In addition, the expression levels of hs-cTnI after the onset of chest pain were also obtained at different time points (T0, T6 and T12).

2.2.3. Detection of miRNA

The current study examined a number of miRNAs from clinical blood and healthy volunteers. These included miR-208, miR-494, miR-499 and miR-1303. As a first step, Plasma miRNA were extracted using miRcute serum/plasma miRNA isolation kit (Tiangen, Beijing, China) following the manufacturer's instructions and quantified using NanoDrop ND-1000 spectrophotometer (Nanodrop technologie, DE, USA). The first-strand cDNA was synthesized using miRcute Plus miRNA first-strand cDNA kit (Tiangen, Beijing, China). Amplifications were performed using a miScript SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and qRT-PCR was run on Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). U6 snRNA was used as an internal standard and the expression levels of different miRNA were quantitatively analyzed using the $2^{-\Delta\Delta CT}$ approach, respectively. Each sample was run in triplicate.

2.2.4. Hs-cTnI detection

High-sensitivity cTnI was assayed from frozen samples of all patients using the Architect STAT High-Sensitivity Troponin-I assay (Abbott Laboratories, Abbott Park, Illinois, US) with a limit of detection of 1.2 ng/L and a 99th percentile in the healthy population of 26.2 ng/L.

2.3. Statistics analysis

Data were analyzed using statistical software SPSS 17.0. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm SD$), the qRT-PCR results were visually presented as a Ct value, and the level of each miRNA was expressed as relative quantification ($2^{-\Delta\Delta Ct}$). The expression levels of miRNAs were compared among different time points in the same group using analysis of variance with a completely randomized design, compared between two groups using independent sample *t*-test, and compared among multiple groups using Chi-square test. The correlation between each miRNA and hs-cTnI was evaluated using simple linear correlation analysis. $P < 0.05$ was considered statistically significant. The receiver operating characteristic (ROC) curve was used to analyze the value of each miRNA for the early diagnosis of AMI.

3. Results

3.1. Comparison of basic clinical data among groups

Basic, data such as age, gender, place of residence, history of hypertension, history of hyperlipidemia, history of diabetes mellitus and history of smoking, were compared between the experimental group and control group. The differences in age, gender, residence and history of diabetes mellitus between these two groups were not statistically significant ($P > 0.05$), while the differences in history of hypertension, history of hyperlipidemia and history of smoking between these two groups were statistically significant ($P < 0.05$) (Table 1). In order to exclude the impact of these factors on miRNA, multiple regression analysis was conducted. The results revealed that miRNAs were not affected by history of hypertension, history of hyperlipidemia, and history of smoking ($P > 0.05$).

3.2. miRNA levels at different time points in various groups.

3.2.1. Expression level

(1) Plasma miR-208, miR-494, miR-499 and miR-1303 levels at all time points were significantly higher in the AMI group than in the SAP

Table 1
Comparison of clinical basic data.

Characteristics	AMI group	SAP group	Control group	*P* value
Age(years)	62.95 ± 11.04	63.16 ± 10.63	59.50 ± 12.87	0.639
Male/female(n/n)	30/11	19/13	6/4	0.421
Residence(town/village)	13/28	15/17	3/7	0.362
Hypertension(%)				
Hyperlipidemia(%)	24(58.53%)	16(50.00%)	1(10.00%)	0.023
Diabetes(%)	26(63.41%)	11(34.38%)	5(50%)	0.048
Smoking(%)	9(21.95%)	4(12.50%)	0	0.189
	16(39.02%)	5(15.63%)	1	0.036

group, and the differences were statistically significant ($P < 0.05$).
(2) Plasma miR-208, miR-494, miR-499 and miR-1303 levels at all time points were significantly higher in the AMI group than in the healthy control group ($P < 0.05$). But, the differences in plasma miR-208, miR-494, miR-499 and miR-1303 levels at all time points between the SAP group and healthy control group were not statistically significant ($P > 0.05$).
(3) The differences in plasma miR-208, miR-494, miR-499 and miR-1303 levels among all time points were not statistically significant ($P > 0.05$, Table 2).

3.3. Dynamic evolution

(1) The rule of changes in miR-208, miR-494 and miR-499 levels in the AMI group and SAP group: these levels immediately increased at T0, peaked within 12 h, and began to decrease at T12, but had not decrease to normal levels (Fig. 1).
(2) The rule of change in miR-1303 level in the AMI group, when compared to the SAP group: this level immediately increased at T0, may reach the peak from T6 to T12, and may also reach the peak after T12 (Fig. 1).

3.4. The correlation between miR-208, miR-494, miR-499 and miR-1303 with hs-cTnI

The correlation analysis results revealed that the following: (1) there was a positive correlation between miR-208 and hs-cTnI, and the correlation coefficient was 0.18 ($P = 0.038$); (2) there was a positive correlation between miR-494 and hs-cTnI, and the correlation coefficient was 0.223 ($P = 0.013$); (3) there was a positive correlation between miR-499 and hs-cTnI, and the correlation coefficient was 0.227 ($P = 0.012$); (4) there was a positive correlation between miR-1303 and hs-cTnI, and the correlation coefficient was 0.23 ($P = 0.011$).

Table 2
Comparison of miRNAs expression levels ($\bar{x} \pm s$).

Type	Time	AMI group(n = 41)	SAP group(n = 41)	Control group(n = 10)
miRNA-208	TOh	3.71 ± 3.39●※	1.07 ± 0.19	1.00 ± 0.00
	T6 h	12.16 ± 1.90●※	1.03 ± 0.59	
	T12 h	8.30 ± 7.76●※	1.15 ± 0.51	
miRNA-494	TOh	5.13 ± 5.04●※	1.22 ± 0.74	1.26 ± 0.81
	T6 h	13.31 ± 12.70●※	1.52 ± 1.41	
	T12 h	8.69 ± 8.91●※	2.02 ± 5.37	
miRNA-499	TOh	5.14 ± 5.50●※	1.19 ± 0.55	1.21 ± 0.82
	T6 h	15.55 ± 16.29●※	1.52 ± 1.46	
	T12 h	11.78 ± 10.33●※	1.30 ± 1.26	
miRNA-1303	TOh	4.22 ± 3.10●※	1.30 ± 0.99	0.82 ± 0.61
	T6 h	10.54 ± 9.53●※	1.68 ± 1.93	
	T12 h	12.14 ± 13.74●※	1.29 ± 1.25	

●Indicates statistical difference from healthy controls, $P < 0.05$; ※Indicates statistically significant differences between the same time window and the SAP group, $P < 0.05$.

3.5. ROC curve analysis of miR-208, miR-494, miR-499 and miR-1303 with hs-cTnI in AMI in the AMI group when compared to the SAP group.

The ROC analysis results revealed that the following: (1) The AUC of miR-208 was 0.868 (95% confidence interval [CI]: 0.818–0.917); (2) the AUC of miR-494 was 0.839 (95% CI: 0.786–0.892); (3) the AUC of miR-499 was 0.852 (95% CI: 0.802–0.901); (4) the AUC of miR-1303 was 0.884 (95% CI: 0.840–0.929); (5) the AUC of hs-cTnI in AMI was 0.992 (95% CI: 0.979–1.000) (Figs. 2–5).

4. Discussion

miRNAs are small non-coding single-stranded RNAs that regulate gene expression [13], and regulate the translation and/or degradation of mRNA in an incomplete complementary base-pairing manner. miRNAs play a regulatory role in cell differentiation, growth, proliferation and apoptosis, and is involved in the pathophysiological process of various diseases [2,21]. The first miRNA was discovered in *Caenorhabditis elegans* by Lee et al. [15]. With the development of science and technology, miRNA has gained increasing attention. The latest miRNA center database has revealed nearly 2500 miRNAs in the human genome. In human, at least approximately 30% of proteins are encoded by miRNA-regulated genes [14]. A latest study proposed that miRNAs can be detected in circulating blood and urine [27]. These extracellular miRNAs exist in highly stable forms, and can resist repeated freezing/thawing, strong acid and strong alkaline environment, and the actions of RNA enzymes in circulating blood [10,22]. Another study revealed that the expression of miR-208, miR-494, miR-499 and miR-1303 were upregulated or downregulated after myocardial infarction, suggesting that the above-mentioned miRNAs may be new markers for myocardial infarction. Therefore, in the present study, the levels of the above-mentioned miRNAs in AMI patients at different time points were detected by qRT-PCR to explore the value of these miRNAs as biomarkers for early AMI.

In the present study, the plasma miR-208, miR-494, miR-499 and miR-1303 levels in AMI patients, SAP patients and healthy controls were detected. The four above-mentioned miRNAs had the same results: plasma levels at T0, T6 and T12 were significantly higher in AMI patients than in SAP patients, while in the SAP group, these plasma levels were not significantly elevated at the different time points, and there were no significant differences in levels of the above-mentioned miRNAs at different time points between the SAP group and healthy control group.

In the present study, changes in the four above-mentioned miRNAs in the AMI group were observed, and the levels of miR-208, miR-494 and miR-499 were higher in AMI patients than in healthy controls at T0. These gradually increased from T0 to T6, and decreased at T12, but remained higher than those in healthy controls. This result has proven that the peaks appeared before T12, the most significant increase occurred from T0 to T6, and the peaks were likely to appear at around T6. Furthermore, miR-1303 level was higher in AMI patients than in healthy controls at T0, which rapidly increased from T0 to T6, and continued to increase at T12. This suggests that the peak of the miR-

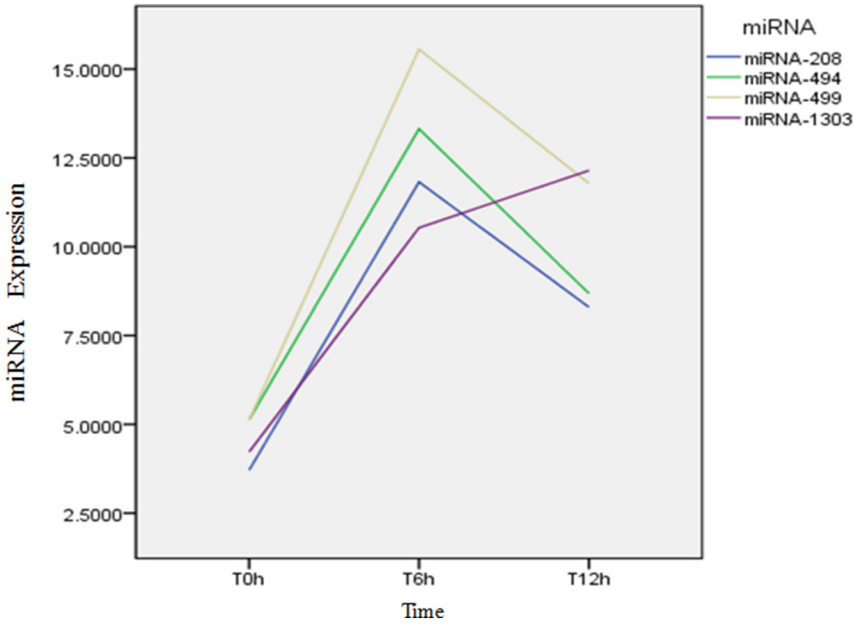

Fig. 1. Dynamic evolution of miRNAs in different time after onset of AMI.

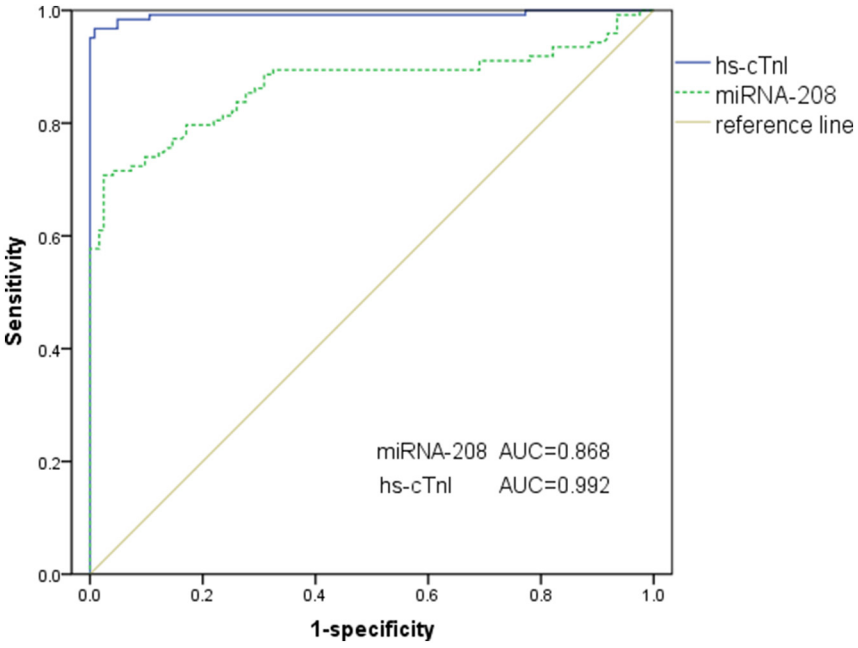

Fig. 2. ROC curve analysis of mir-208 and hs-ctni in the experimental group after onset of AMI.

1303 level may appear from T6 to T12, or appear after T12.

These are consistent with results of most present studies. miR-208 is a heart specific miRNA, is encoded within the MHC gene, and is a positive regulator of the MHC gene expression [28,34]. The mechanisms of miR-208 may be as follows: after myocardial infarction, miR-208 is released into peripheral circulating blood from the damaged cardiomyocytes in the form of microbubbles, and inhibits the expression of NLK, which is an effective target molecule for cardiomyocyte apoptosis (an evolutionarily conserved ERK-2/MAPK-like kinase). NLK can not only promote the apoptosis of cardiomyocytes induced by AngII, but also promote the proliferation and differentiation of cardiac fibroblasts. In addition, a study proposed that decreasing miR-208 can increase cardiomyocyte apoptosis, which mainly affects the reactive oxygen species (ROS) in cardiomyocytes, and this effect could be achieved mainly by inhibiting the protein expression of the p21 gene [18]. miR-494 is a miRNA derived from chromosome 4q32.31 [26]. Wang et al. [31] revealed that the role of miR-494 is not only to support apoptosis, but also to resist apoptosis. Its regulatory mechanism is as follows: miR-494 regulates three pro-apoptotic gene proteins (PTEN, ROCK1 and CaMKIIδ) and two anti-apoptotic gene proteins (FGFR2 and LIF) by complementary pairing with the 3′ untranslated region (UTR) of its mRNA. However, the final result is to activate the Akt pathway, thereby playing a cardiovascular protective role. MiR-499 is closely correlated to cardiomyocyte differentiation [1]. MiR-499 is a miRNA encoded by the intron of myosin gene Myh7b, while the Myh7b protein is almost independently produced by the heart [9]. Recent studies in China, such as studies conducted by Han et al. [11] and Zhang et al. [37], have proposed that the following: the level of miR-499 in circulating blood is significantly higher in AMI patients than in non-AMI patients and healthy controls. Xiao et al. [33] revealed that the expression of miR-

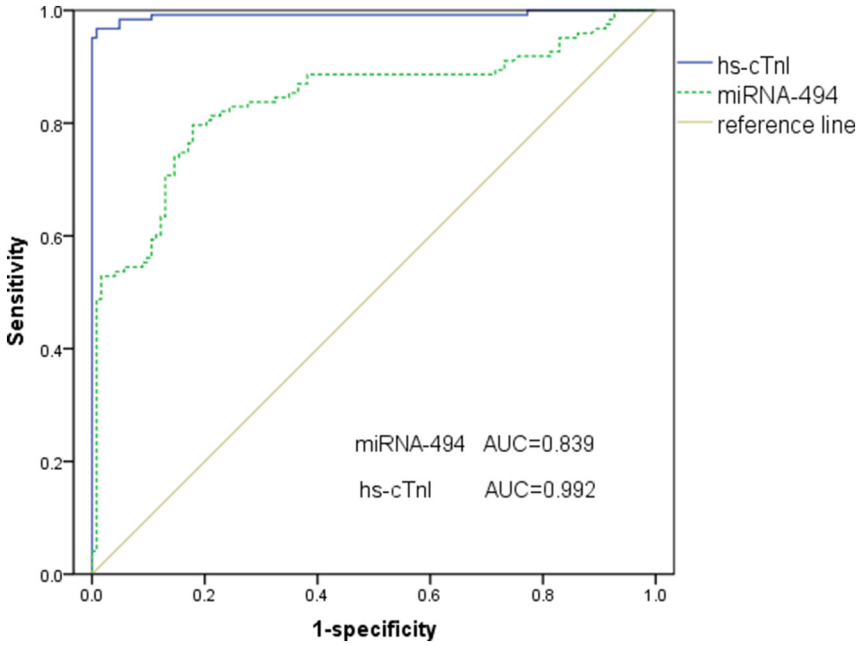

Fig. 3. ROC curve analysis of mir-494 and hs-ctni in the experimental group after onset of AMI.

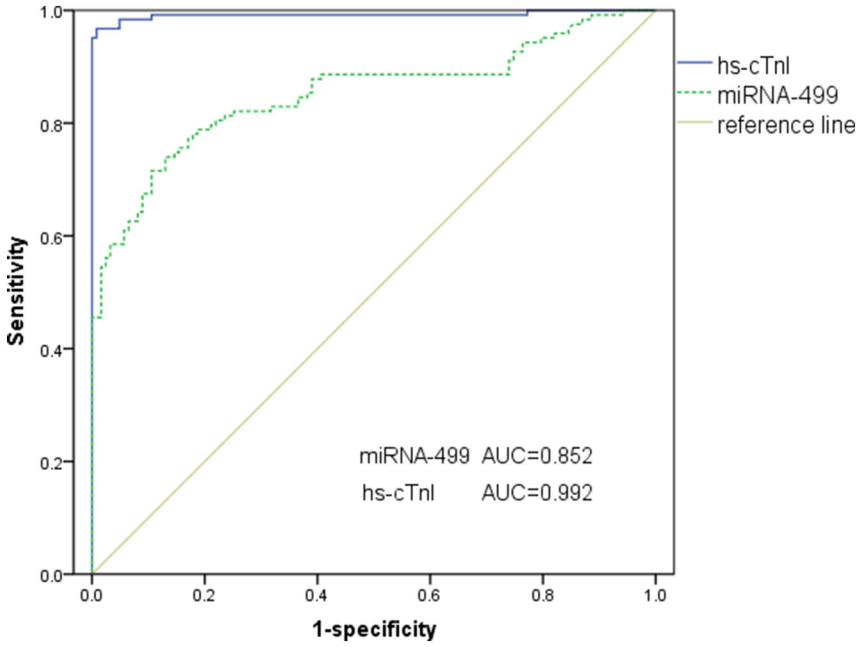

Fig. 4. ROC curve analysis of mir-499 and hs-ctni in the experimental group after onset of AMI.

499 is elevated in the myocardial infarction area of rats with ligation of the coronary artery (left anterior descending branch). Furthermore, they found that this was significantly decreased in the non-infarction area, and significantly decreased in the sham operation group. This suggests that miR-499 may be released from damaged cardiomyocytes into the blood circulation after ligation. The mechanism may be as follows: miR-499 protects the myocardium by inhibiting the expression of Pdcd4 and PACS2, thereby inhibiting cardiomyocyte apoptosis [30]. At present, there are few studies on miR-1303 in China. Chaud et al. [4] revealed that the expression of miR-1303 was downregulated after cardiomyocyte apoptosis. However, this is not consistent with the results of the present study. Furthermore, there are few studies on the detection of miR-1303 after myocardial infarction. Differences in the results of different studies may be due to the differences in detection methods, selected primers, external control genes, or sample quality. Hence, the specific mechanism of miR-1303 in the pathogenesis of AMI needs to be further studied.

In the present study, the correlation analysis results revealed that the four above-mentioned miRNAs were positively correlated with traditional cardiac marker hs-cTnI, but the correlation coefficients were small. This suggests that these were not completely overlapped with hs-cTnI in terms of diagnostic information, and that these contain diagnostic information other than hs-cTnI, which needs further exploration. The ROC curve analysis and comparison revealed that all four miRNAs and hs-cTnI have great diagnostic value for AMI in patients with typical chest pain. However, hs-cTnI had the highest accuracy rate, and its diagnostic value was better than the four above miRNAs. But, some studies have revealed that hs-cTnI is influenced by renal function.

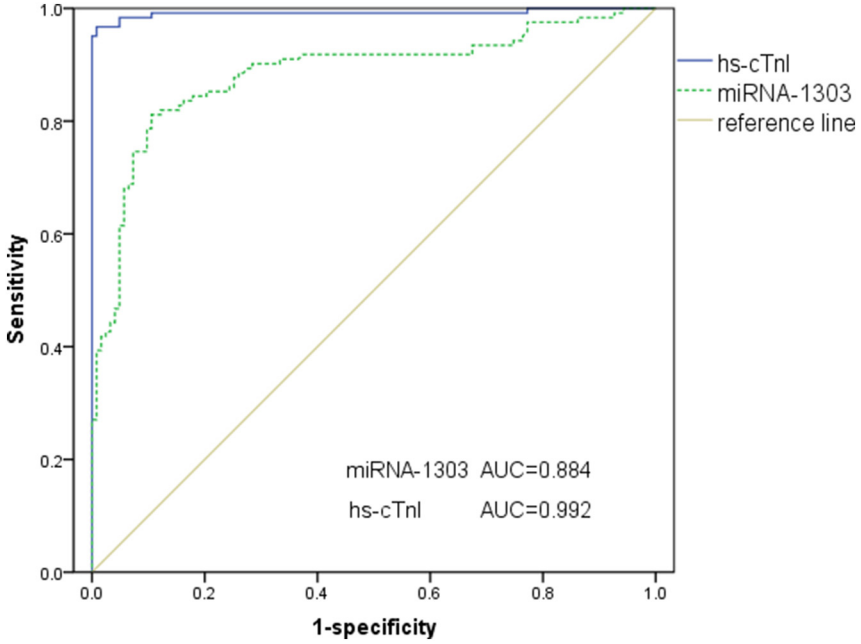

Fig. 5. ROC curve analysis of mir-1303 and hs-ctni in the experimental group after onset of AMI.

Collinson et al. pointed out that [6,12] hs-cTnI was significantly increased in patients with end-stage nephropathy. In addition, Corsten et al. [7] pointed out that the expression of miR-208 and miR-499 in AMI patients were not influenced by renal function. Therefore, under some specific situations, such as chest pain, for patients with renal dysfunction, the diagnostic efficiency of miRNAs was higher than that of hs-cTnI.

Our studies have several limitations. First, the sample size of control group was small. In this study, we mainly wanted to prove the diagnostic value of four miRNAs in people with typical chest pain. The blood samples of healthy volunteers were just used as blank samples in order to exclude the influence of venipuncture and other environmental factors on expression of miRNAs, and provide a baseline to make the results more intuitive. Although we did not draw our conclusions from comparisons between AMI patients and healthy volunteers, the samples size of control group was also one of the limitations of the study. Second, the miRNA detection was prospective and double-blinded in our study, while hs-cTnI is not blinded to the clinicians. This would be influenced by subjective factors to a great extent, causing a false increase in the diagnostic accuracy of hs-cTnI, to a certain extent.

In summary, based on the results of the present study and related studies at home and abroad, it was revealed that plasma miR-208, miR-494, miR-499 and miR-1303 levels at all time points were significantly higher in AMI patients than in non-AMI patients, and these have a certain correlation with hs-cTnI. Furthermore, it was found that the above miRNAs are effective for diagnosing AMI, but are not superior to hs-cTnI. The order of diagnostic efficiency from highest to lowest was as follows: miR-1303, miR-208, miR-499, and miR-494. Hence, miR-1303 has the highest diagnostic efficiency. There are few studies on miR-1303. At present, the specific mechanism of miRNA-1303 in AMI remains unclear. Li et al. [16] revealed that miR-1303 was highly expressed in neuroblastoma, and can promote the proliferation of neuroblastoma cells. However, its specificity needs to be further explored. The AUC of miR-208 was merely second to that of miRNA-1303, but the sensitivity and specificity are relatively high. At present, a large number of studies have revealed that this was highly expressed in myocardial tissues. In addition, the results of a large number of studies at home and abroad are consistent with the results of the present study, and the sample size was adequately large. miRNA-1303 is expected to become a new cardiac marker for clinical application.

5. Conclusion

miR-208, miR-494, miR-499 and miR-1303 were highly expressed in AMI and correlated with hs-cTnI to some extent. In patients with typical chest pain, miR-208 had the highest myocardial specificity and high efficacy to diagnose AMI, but the diagnostic efficacy was not superior to that of the traditional hs-cTnI.

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Abbreviations

AMI: Acute myocardial infarction
SAP: stable angina pectoris
hs-cTnI: High-sensitive cardiac troponin I
miR: microRNA
PCR: polymerase chain reaction
RT-PCR: reverse transcription-polymerase chain reaction
ELISA: enzyme-linked immuno sorbent assay
cDNA: complementary DNA
CK-MB: Creatine kinase MB
Myo: myohemoglobin
cTnT: cardiac troponin T
cTnI: cardiac troponin I