Serum exosome microRNA panel as a noninvasive biomarker for molecular diagnosis of fulminant myocarditis

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Exosome-derived microRNAs (miRNAs) are potential diagnostic biomarkers. However, little is known about their effectiveness as diagnostic biomarkers of fulminant myocarditis (FM). This study aimed to explore serum exosomal miRNAs as potential biomarkers for FM diagnosis. Peripheral blood samples were collected from 99 patients with FM, 32 patients with nonfulminant myocarditis (NFM), and 105 healthy controls (HCs). The miRNA expression profiles of serum exosomes were determined using next-generation sequencing, and differentially expressed miRNAs were further analyzed by quantitative reverse transcriptase polymerase chain reaction. A logistic regression model was constructed using a training cohort (n = 120) and then validated using an independent cohort (n = 106). The area under the receiver operating characteristic curve was used to evaluate diagnostic accuracy. In FM patients, hsa-miR-30a, hsa-miR-192, hsa-miR-146a, hsa-miR-155, and hsa-miR-320a were validated as significantly and differentially expressed candidates that could serve as potential markers for diagnosing FM. In addition, the miRNA panel (hsa-miR-155 and hsa-miR-320a) from the multivariate logistic regression model demonstrated high accuracy in the diagnosis of FM and was able to distinguish FM from HCs and NFM. Moreover, the diagnostic value of the miRNA panel was greater than that of CRP and cTn alone or together. The miRNA panel provided the excellent diagnostic capability for FM.

INTRODUCTION

Fulminant myocarditis (FM) is characterized by acute cardiac inflammation that presents suddenly, can lead to cardiogenic shock and electrical instability, and requires inotropic or mechanical circulatory support (MCS). Recent reports demonstrated that patients with FM had a higher rate of cardiac death or need for heart transplantation than did patients with acute nonfulminant myocarditis (NFM)[1,2] presenting with left ventricular systolic dysfunction (LVSD) but who were otherwise hemodynamically stable.[3] Therefore, it is important to identify FM at its earliest stages using the most relevant advanced diagnostic modalities in order to provide treatment as soon as possible.

Although elevated serum cardiac troponin (cTn) is almost always present in FM, an absence of cTn increase does not rule out myocarditis.[4] Cardiac biomarkers in FM can reach levels similar to those in patients with transmural infarctions caused by epicardial coronary occlusions. Experimental and clinical studies suggest that serum cTn can be a useful diagnostic tool early in the course of myocarditis.[5,6] In a registry cohort of patients with myocarditis and preserved left ventricular ejection fraction (LVEF), cTn was increased in all patients, and 99% of patients exhibited abnormal values of C-reactive protein (CRP) or erythrocyte sedimentation rate.[7] Increased levels of biomarkers were similar in patients with and those without adverse cardiac events during follow-up. In a study from Johns Hopkins Hospital, cTn values were associated with giant cell myocarditis, but the researchers did not find a significant correlation between the increase in cTn and patient prognosis.[8] The European Society of Cardiology position statement on the management of acute myocarditis recommends the assessment of serum cTn, erythrocyte sedimentation rate, and CRP to aid in the diagnosis of myocarditis.[9] However, a normal erythrocyte sedimentation rate and CRP level do not exclude myocarditis.[10]

Routine viral serologies are not recommended because of a lack of sensitivity and specificity compared with a viral genome from endomyocardial tissue obtained by biopsy.[9] Endomyocardial biopsy (EMB) represents the gold standard for the diagnosis of FM,[9–11] although its sensitivity may be limited by patchy distribution of the inflammatory infiltrate.[12–15] Considering the above-mentioned reasons, there is an urgent need to develop noninvasive diagnostic biomarkers with ideal sensitivity for early detection of FM.

Table 1. The characteristics of participating patients

	Patients with available data	Acute myocarditis		
		FM	NFM	p value
Overall		99	32	
Demographics				
Age, years	131	40 (26–56)	33 (20–38)	0.359
Age < 15 years	131	5 (5.1)	1 (3.1)	1
Female	131	40 (40.0)	9 (28.1)	0.277
Presenting symptoms				
Dyspnea	131	70 (70.8)	22 (68.8)	0.838
Chest pain	131	68 (68.7)	19 (59.4)	0.42
Syncope	131	34 (34.3)	9 (15.6)	0.032
Prodromal symptoms	131	95 (96)	32 (93.8)	0.549
Fever	131	37 (37.4)	16 (50)	0.219
GI symptoms	131	27 (27.3)	7 (23.3)	0.538
Respiratory symptoms	131	70 (70.7)	27 (84.4)	0.144
ECG at admission				
Normal	131	5 (5.1)	8 (26.7)	0.008
ST-segment elevation	131	51 (51.5)	9 (28.1)	0.024
Other ST-T segment abnormalities	131	44 (44.4)	15 (46.9)	0.723
Life-threatening arrhythmias				
Cardiac arrest	131	26 (26.3)	5 (9.2)	0.012
BBB	131	35 (35.4)	4 (12.5)	0.018
Advanced AV block	131	39 (39.4)	1 (3.1)	< 0.001
Admission laboratory tests				
Increased CRP	131	93 (93.8)	19 (77.8)	0.007
Increased troponin T/I	131	99 (100)	25 (78.1)	< 0.001
Increased CK-MB	131	48 (48.5)	8 (25)	0.023
Echocardiography at admission				
LVEF, %	124	25 (15–35)	36 (29–43)	< 0.0001
LVEDD, mm	99	49 (45–54)	55(50–60)	0.026
Visual dysfunction				
Pericardial effusion	112	24 (28.6)	4 (13.3)	0.013
Coronary angiogram	119	22 (37.3)	11 (37.9)	0.953

Exosomes are 30- to 150-nm extracellular vesicles consisting of nucleotides and proteins that are secreted by specific cell types and are found in various bodily fluids, where they participate in communication between cells.[16] Unlike circulating microRNAs (miRNAs), exosomes are enriched in the circulatory system and are protected from RNase degradation.[17] The identification of exosomal miRNAs in bodily fluids indicates that they have great potential for application in clinical diagnosis or prognosis for cardiovascular diseases.[18–22] The majority of miRNAs isolated from circulation are contained exosomes that are bound to RNA-binding proteins, with few free miRNAs.[17] Through analyzing the change in the quantity of circulating exosomes, it

will be possible to identify miRNAs that can be considered as biomarkers.

Our study investigated serum exosomal miRNA profiles in an independent validation cohort, with the intention to identify a panel of miRNAs for the diagnosis of FM. The cohort included healthy controls (HCs) and patients with NFM and FM. The purpose of this study is to identify serum-derived exosomal miRNA biomarkers for use as a noninvasive detection method in order to improve the specificity and sensitivity of FM diagnosis.

RESULTS
Study population

The overall population included 236 patients (FM = 99, NFM = 32, HCs = 105). The primary characteristics and a comparison between patients with FM and NFM are presented in Table 1. The median age of FM patients was 40 years (interquartile range [IQR], 26–56 years) with a female prevalence of 40%. Prodromal symptoms were more commonly observed in FM patients. Compared with those with NFM, at baseline, patients with FM exhibited lower LVEF and higher levels of CRP, cTn, and MB isoenzyme of creatine kinase (CK-MB). Cardiac arrest requiring resuscitation maneuvers, ventricular fibrillation, and sustained ventricular tachycardia occurred more frequently in FM patients.
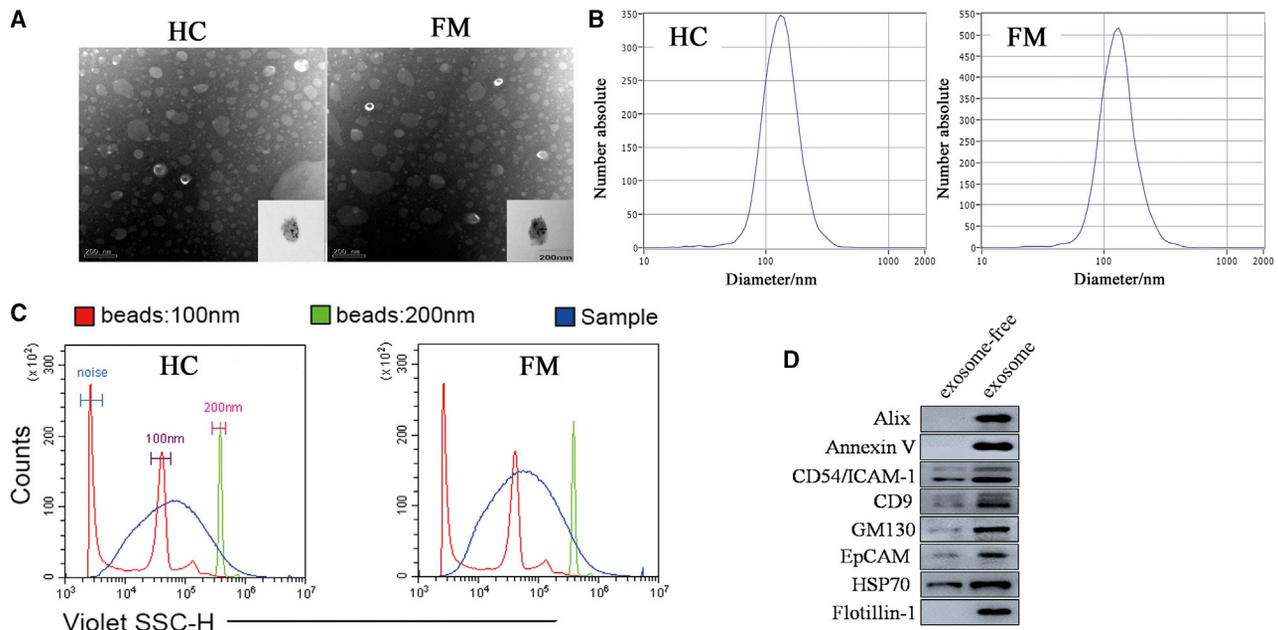
Isolation and characterization of exosomes

Exosomes isolated from serum of HC and FM patients were analyzed by transmission electron microscopy, nanoparticle tracking analysis (NTA), and bead-based flow cytometry. Representative images show that the purified exosomes were mainly round-shaped vesicles ranging from 50 to 200 nm in diameter with a clear membrane structure and exhibited the characteristic cup-shaped morphology (Figures 1A–1C). Western blot analysis validated the expression of the known exosomal biomarkers, such as Alix, CD9, CD54, and HSP70, which was consistent with previously reported characteristics of exosomes (Figure 1D). Both types of exosomes contained abundant mounts of RNA, and the number of particles or RNA in HCs and FM was indistinguishable (Figure S1).

Serum-derived exosome miRNA screening

To screen for candidate serum exosomal miRNAs associated with FM, miRNA high-throughput sequencing was used to evaluate the two groups (FM = 5 and HCs = 5). As shown in Figure 2A, the sequencing results identified 161 miRNAs that were differentially contained in FM relative to HCs (threshold at >2-fold change and p < 0.05), of which 89 miRNAs were upregulated and 72 miRNAs were downregulated.

We focused on 14 candidates with high signal intensities (>300) in the two groups to guarantee stable detection but also considered high differential expression. Figure 2B shows a heatmap of the most differentially expressed and subsequently hierarchically clustered miRNAs. The differential expression of eight selected miRNAs was validated

Figure 1. Characterization of serum exosomes from FM patients and HCs

(A) Transmission electron microscopy (TEM) showing exosome morphology. Scale bar, 200 μm. (B and C) Size distribution of serum exosomes from HCs and FM by NTA analysis and flow cytometry showing that the exosomes are within the expected size range. (D) Serum exosomes from HCs and FM express exosomal markers Alix, TSG101, and CD9, as revealed by western blot analysis.

by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Table S1).

miRNA expression profile for FM versus HCs in the training dataset

To further evaluate the potential of these miRNAs as biomarkers for FM, the changes in the level of the eight individual miRNAs derived from the sequencing dataset were evaluated by qRT-PCR in an independent cohort of FM (n = 60) and HCs (n = 60). Interestingly, when examining the level of these miRNAs, only five miRNAs (hsa-miR-30a, hsa-miR-192, hsa-miR-146a, hsa-miR-155, and hsa-miR-320a) were significantly higher in FM than HCs (p < 0.05) (Figure 2C). To assess the biomarker performance of the above miRNAs, we performed a receiver operating characteristics (ROC) curve analysis. The five miRNAs for FM diagnosis exhibited comparative accuracy in distinguishing FM from HCs (area under the curve [AUC] = 0.834 [95% confidence interal (CI), 0.7518–0.9146], 0.874 [95% CI, 0.8074–0.9401], 0.853 [95% CI, 0.7828–0.9228], 0.901 [95% CI, 0.8594–0.9581], and 0.912 [95% CI, 0.8751–0.9693]) (Figure 3A).

Establishing the predictive miRNA panel

A stepwise logistic regression model to estimate the risk of being diagnosed with FM was applied to the training dataset. Two of the five miRNAs were significant predictors (Table 2). The predicted probability of being diagnosed with FM from the logit model based on the miRNA panel was used to construct the ROC curve (logit (p = FM) = −3.577 + 0.396*hsa-miR-155 + 0.373*hsa-miR-320a). The diagnostic performance for the established miRNA panel was

evaluated using ROC analysis. The miRNA panel was able to distinguish FM from HCs, with an AUC of 0.944 (95% CI, 0.912–0.976) (Figure 3B).

Validating the miRNA panel

The parameters estimated from the training dataset were used to predict the probability of being diagnosed with FM using the independent validation dataset (n = 106). The results showed that there was a higher expression of all five miRNAs in the FM group as compared to the HC group or NFM group (Figure S2). The analysis demonstrated that the miRNA panel had high accuracy in discriminating FM from HCs (AUC = 0.927; 95% CI, 0.8895–0.9646). Furthermore, we subsequently compared the diagnostic value between the miRNA panel, CRP, and cTn. The results showed that the diagnostic value of the miRNA panel was more optimal than that of CRP and cTn alone or together, which had an AUC of 0.927 (95% CI, 0.8895–0.9646), 0.774 (95% CI, 0.6790–0.8692), 0.851 (95% CI, 0.7876–0.9137), and 0.875 (95% CI, 0.8104–0.9399) (Figure 4A). The miRNA panel had the ability to adequately differentiate between FM and NFM (Figure 4B), although the AUC values (AUC = 0.659; 95% CI, 0.5287–0.7893) are low. These data suggested that the miRNA panel from serum-derived exosomes provided the excellent diagnostic capability for FM patients.

DISCUSSION

Early recognition and aggressive management are essential for favorable outcomes of FM. Blood-based tests, such as measurement of circulating exosomal miRNAs, can offer an alternative diagnostic tool for screening and detection of FM. Hence, we investigated the
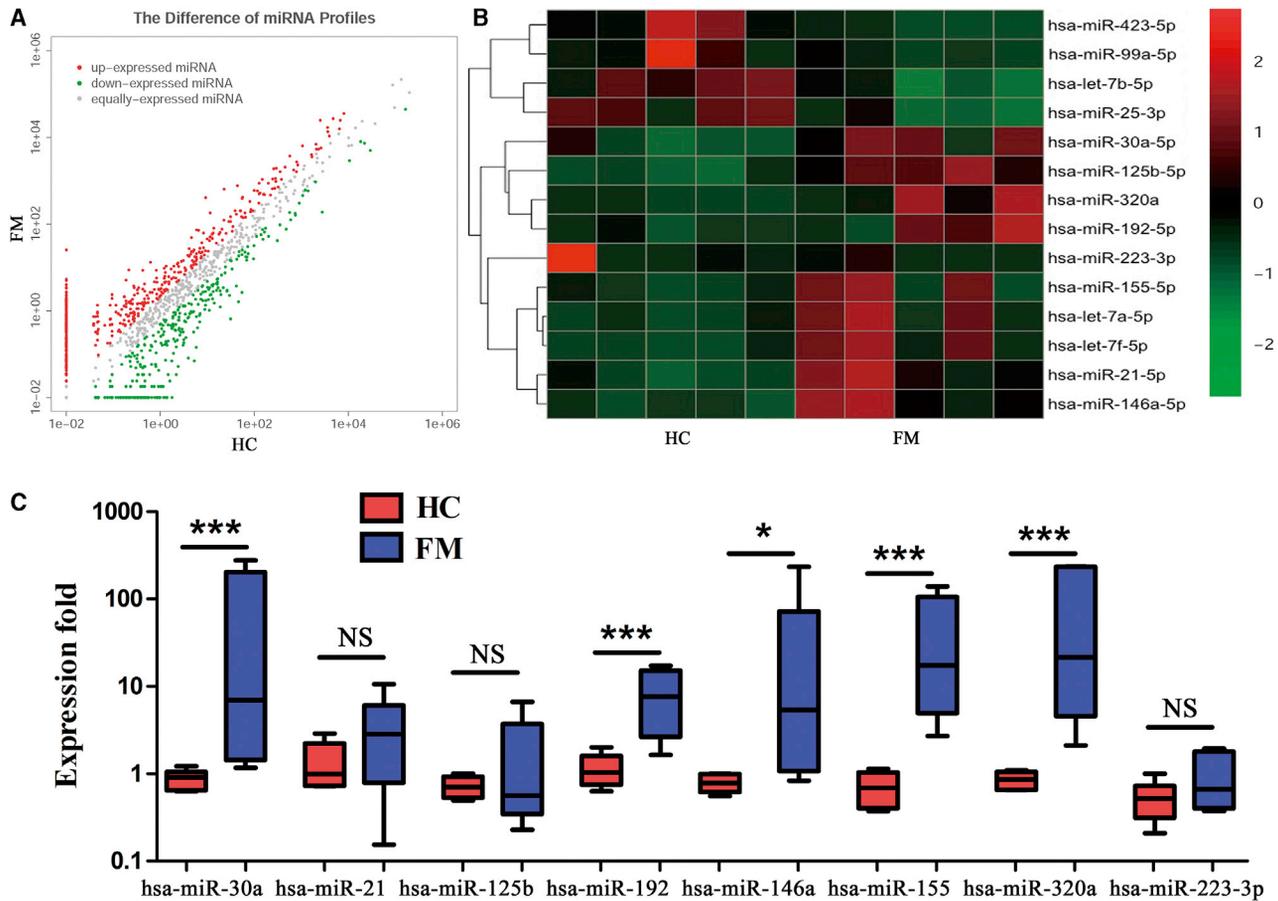
Figure 2. Clustering analysis and confirmation of differential miRNA expression in serum exosomes from HCs and FM

(A) Scatterplot of the RNA sequencing expression data from serum exosomes from HCs and FM. (B) Heatmap of 14 differentially expressed miRNAs in serum exosomes from HCs (n = 5) and FM (n = 5). Intensity plot shows relative upregulation (red) and downregulation (green). (C) Relative expression fold of selected miRNAs was determined by quantitative real-time PCR. Median (horizontal line), 25th, and 75th percentiles (box), and 5th and 95th percentiles (whiskers) of miRNAs are shown. Wilcoxon's rank-sum test comparing the miRNA levels between HCs (n = 60) and FM (n = 60). ***p < 0.001; *p < 0.05; NS, no significance. Data are represented as mean ± SD.

diagnostic potential of exosomal miRNAs by analyzing circulating exosomal miRNA profiles of FM patients and HCs. To our knowledge, this is the first demonstration of changes in circulating exosomal miRNA expression in a FM cohort.

In this study, 161 miRNAs were significantly and differentially expressed in the samples, of which, 89 miRNAs were upregulated, whereas 72 miRNAs were downregulated in FM patients, compared with HCs. Furthermore, hsa-miR-30a, hsa-miR-192, hsa-miR-146a, hsa-miR-155, and hsa-miR-320a were identified as candidates in FM patients and could serve as potential circulating markers for diagnosing FM. The miRNA panel with hsa-miR-155 and hsa-miR-320a, which are miRNAs from the multivariate logistic regression model, demonstrated high accuracy in the diagnosis of FM, and the AUC value was markedly greater than that of individual miRNA, CRP, or cTn.

Studies of diagnosis and evaluation of patients presenting with suspected FM can be considered in several categories, which include im-

aging, histology, and biomarkers. Both cardiac magnetic resonance (CMR) and echocardiography are useful for identifying FM. CMR can provide increased imaging quality for detecting FM in patients with relatively mild cases of myocarditis, and the method is especially important in institutions without the capacity for EMB. However, CMR may not be considered early in the diagnostic algorithm of FM because of patient instability (> 10 days later than in NFM), and patients with FM more often display diffuse late gadolinium enhancement (LGE) relative to patients without FM.[1]

Echocardiography is important for bedside assessment of cardiac anatomy and function as well as for differentiating acute myocarditis from inflammatory cardiomyopathies and other cardiac conditions with overlapping clinical presentations. However, there is no specific criteria defined by a set of standardized findings for myocarditis, and therefore, echocardiography has a limited capacity to predict a fulminant disease course.[8,23] The gold standard for diagnosis of FM is histologic confirmation of myocarditis by EMB. Because this is an invasive procedure and
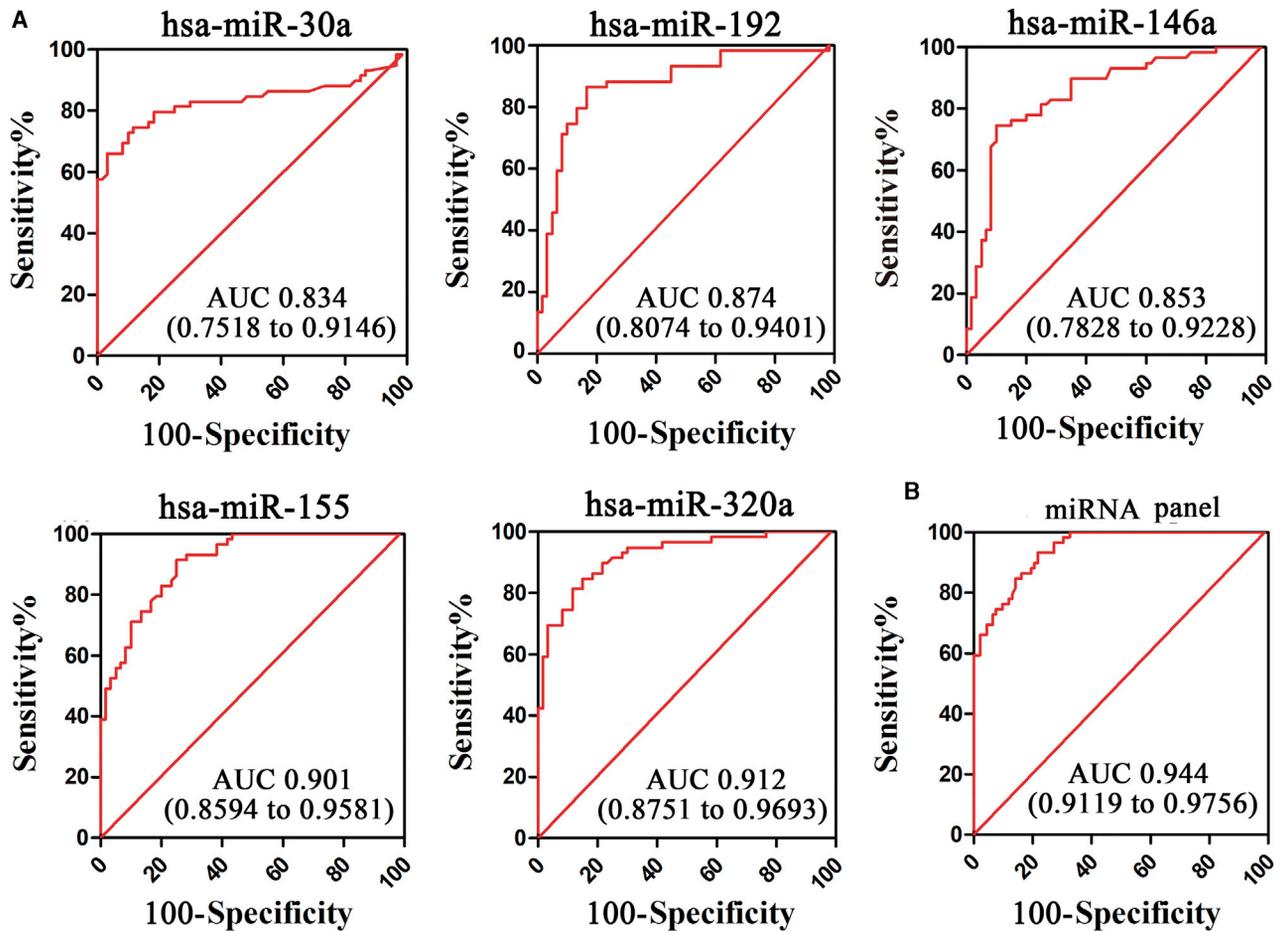
Figure 3. ROC curve analysis for five differentially expressed miRNAs in the diagnosis of FM

(A) AUC estimation for the five differentially expressed miRNAs in HCs and FM. The cohort includes 60 HC and 60 FM patients. (B) ROC curve analysis for the miRNA panel in discriminating the FM (n = 60) from the HC group (n = 60) in the training dataset.

not widely offered, the added diagnostic value of EMB for myocarditis has been challenged over time.[12,24] Consequently, there is an urgent need to develop new and noninvasive diagnostic biomarkers for the detection of FM. A number of biomarkers can assist both in diagnosing FM and estimating the extent of the disease. Markedly elevated plasma concentrations of cTn, CK-MB, and CRP, as well as urea nitrogen, creatinine, and liver transaminases, are nonspecific, indirect markers of myocardial dysfunction.[5–9] It has been reported that FM was associated with significantly higher concentrations of CRP and CK-MB than NFM.[6] However, these biomarkers have diagnostic overlap with myocardial infarction and other causes of heart failure[4,10] and, thus, there is not yet a robust biomarker specific to myocarditis.

miRNAs are the elements most heavily scrutinized in exosomes for their role as new biomarkers in cardiovascular diseases. Focusing on myocarditis, the number of studies using blood-based biomarkers of myocarditis has been fewer, although there have been many studies showing miRNA dysregulation in myocarditis. Corsten et al.[25] inves-

tigated 14 acute myocarditis plasma samples for several myomiRNAs and inflammation-related miRNAs (miR-223, miR-146a, miR-146b, and miR-155) and found modest but significant elevation in miR-208b and miR-499. Goldberg et al.[26] found that the level of cardiac- and inflammatory-associated miR-21 and miR-208a was significantly increased during the acute phase of pediatric viral myocarditis compared to the resolution/chronic phase. More recently, Wang et al.[27] compared miR-1, miR-146b, and other cardiovascular biomarkers between 119 children with myocarditis and 120 age-matched controls and found that these miRNAs were inferior to cTn, interleukin (IL)-18, and tumor necrosis factor alpha (TNF-α). Fan et al.[28] detected the expression of 10 candidate miRNAs in the serum exosomes in 23 patients with viral myocarditis and found that upregulated miR-30a and miR-181d may have the potential to be used as biomarkers for viral myocarditis diagnosis.

Interestingly, our study confirmed that five miRNAs (hsa-miR-30a, hsa-miR-192, hsa-miR-146a, hsa-miR-155, and hsa-miR-320a)

Table 2. miRNA profile and diagnostic performance of the training dataset

miRNAs	B (Beta)	S.E. (Standard Error)	Wald	df (degrees of fredom)	Sig. (Significance)	Exp(B)	95% CI for EXP (B)	
							Lower	Upper
Hsa-miR-30a	−0.007	0.007	0.962	1	0.327	0.993	0.664	0.839
Hsa-miR-192	0.000	0.024	0.000	1	0.984	1.000	0.747	0.886
Hsa-miR-146a	0.000	0.091	0.000	1	1.000	1.000	0.743	0.882
Hsa-miR-155	0.396	0.129	9.482	1	0.002	1.486	0.821	0.927
Hsa-miR-320a	0.373	0.083	20.247	1	0.000	1.452	0.897	0.969

MicroRNA panel, AUC = 0.944 (95% CI, 0.912–0.976). Logit (p = FM) = −3.577 + 0.396 * hsa-miR-155 + 0.373 * hsa-miR-320a.

showed differential expression in the comparison of FM versus NFM or HCs. The similarities between our results and others were few, and there may be some reasons for this phenomenon. Many circulating miRNAs are passively released from apoptotic and necrotic cells,[29,30] and therefore, they may not precisely reflect the biological changes that occur in these vascular lesions. In contrast, exosomes are actively secreted into the peripheral blood by different cell types, and they play a role in cardiovascular protection and repair through miRNA transfer. This indicates that exosomes carry specific miRNAs as well as their own miRNA biogenesis machinery. Therefore, exosomal miRNAs may truly represent specific molecular biomarkers compared with cell-free miRNAs.

Moreover, compared with the studies describing the diagnosis of myocarditis using circulating miRNAs, our study is unique for the following reasons. First, we screened a large number of serum miRNAs via sequencing, which enabled a greater likelihood of identifying potential diagnostic markers. Second, Cel-miR-39 was added as a normalizer for serum exosomal miRNA quantification in our study. In fact, RNU6B is not native to plasma or serum, being released only after the death of cells. Thus, it should not be used as a normalizing control for this type of sample,[31,32] and studies based on RNU6B normalization should be questioned. Furthermore, we included not only the FM and HC groups, but a NFM group as well.

FM patients had worse outcomes than patients with NFM, which necessitated the research for markers that distinguished FM from NFM. Using a stepwise logistic regression model, it was determined that hsa-miR-155 and hsa-miR-320a were significant predictors. The miRNA panel had the ability to distinguish FM from HCs and NFM, with an AUC of 0.927 and 0.659, respectively. Furthermore, the diagnostic value of the miRNA panel was more robust than that of CRP and cTn alone or together, which had an AUC of 0.927, 0.774, 0.851, and 0.875, respectively. These data suggested that the miRNA panel from serum-derived exosomes provides the excellent diagnostic capability for FM patients.

Despite these advantages, our study has several limitations. First, it is a rather small, single-center study. Second, current guidelines do not support the routine use of EMB for the diagnosis of myocarditis, and therefore, such a biopsy and analysis were not performed. Further-more, considering that serum exosome populations are heterogeneous and can be derived from all types of cells, especially blood cells, future research should determine the origin of exosomes present in the peripheral blood and whether the level of miRNAs in circulating exosomes correlates with specific cell miRNA components. In addition, further functional studies of dysregulated circulating miRNAs are required to confirm the role of hsa-miR-155 and hsa-miR-320a in FM.

This study investigated the exosome-derived miRNA profiles in serum from patients with FM using direct miRNA sequencing and qRTPCR. In particular, the miRNA panel for FM diagnosis exhibited high accuracy in distinguishing FM from HCs and NFM. Our results support the hypothesis that circulating exosomal miRNA profiles can serve as noninvasive biomarkers of FM. However, multicenter large prospective studies would be necessary to confirm these findings.

MATERIALS AND METHODS
Study approval
This study was approved by the Ethics Committee of Yijishan Hospital under approval number 2013110017. All participants provided written informed consent.

Sample collection
Peripheral blood samples from 99 FM patients, 32 NFM patients, and 105 HCs who had no history of basic or chronic diseases were collected from the Yijishan Hospital of Wannan Medical College. The study was conducted according to the Declaration of Helsinki and the principles of good clinical practice. All consecutive patients with a diagnosis of acute myocarditis were included from January 1, 2014, to March 31, 2019. The characteristics of the participating patients are described in Table 1.

The inclusion criteria for patients with FM were according to the 2013 European Society of Cardiology position statement, the 2017 Chinese Society of Cardiology expert consensus statement, and the 2009 International Consensus Group on Cardiovascular Magnetic Resonance in Myocarditis statement.[9,33,34]

To be specific, the diagnostic criteria for FM were as follows: (1) acute presentation, defined by the onset of cardiac symptoms within
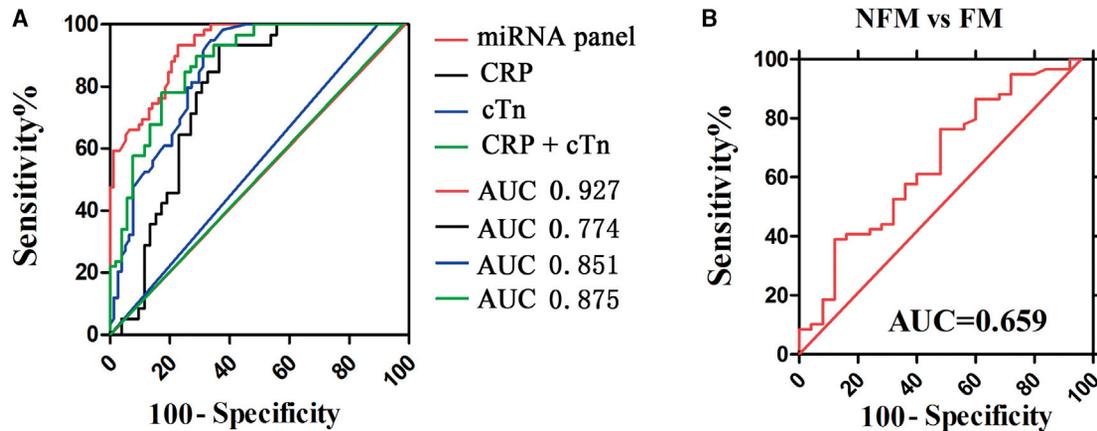
Figure 4. Comparative analysis of the ROC curve for the miRNA panel, CRP, and cTn alone or together in the diagnosis of FM
(A) Comparative analysis of the ROC curve for the miRNA panel, CRP, and cTn alone or together in the validation dataset. ROC plots for the miRNA panel (red), CRP (black), cTn (blue), and CRP and cTn (green) in discriminating the FM (n = 34) from the HC group (n = 40). (B) ROC plots for the miRNA panel in discriminating the FM (n = 34) from the NFM group (n = 32).

30 days before admission; (2) LVSD at admission, defined as LVEF < 50% at first echocardiogram; (3) use of MCSs in the early phase (day 1 or 2 of admission); (4) severe hemodynamic compromise requiring high doses of vasopressors ($\geq$ 5 mg/kg/min of dopamine or dobutamine); and (5) presence of myocarditis confirmed by CMR performed before discharge. Only CMR-confirmed patients were enrolled. The exclusion criteria were as follows: (1) patients with possible acute coronary syndrome but unable to undergo coronary angiography to distinguish the two conditions; (2) patients with myocardial injury caused by sepsis, chemotherapeutical agents, or poison; (3) patients with unstable hemodynamics or shock caused by hypovolemia. FM was defined in accordance with previously published reports[1,3,23] as a low cardiac output syndrome requiring inotropes and/or MCS, whereas NFM was defined by hemodynamic stability without the need for inotropes or MCS.

Study design
Our blood samples were allocated to three phases (Figure 5), which are described as follows.

Discovery phase
Ten samples were screened with an exosome small RNA sequencing platform (RiBoBio, Guangzhou, PR China). A Mann-Whitney U test was performed to discover differentially expressed miRNAs in the two groups: FM versus HCs. From the differentially expressed miRNAs, eight candidate miRNAs discovered via small RNA sequencing with p <0.05 and fold expression change >2 were selected for further testing by qRTPCR.

Training phase
The eight discovered miRNAs were first tested with quantitative real-time PCR in a training cohort of serum samples from 120 participants. Five miRNAs were differentially expressed between the FM and HC groups. These participants were used to construct the diag-

nostic miRNA panel based on the logistic regression model for the differentiation between FM and HCs.

Validation phase
The parameters of the logistic model from the training phase were applied to an independent cohort of 106 samples to validate the diagnostic performance of the selected miRNA panel.

Serum exosome isolation
Blood samples were centrifuged at 1,500 × g for 15 min at 4°C. The collected supernatants (2.5 mL serum diluted 5× with PBS) were centrifuged at 1,000 × g for 10 min at 4°C. The supernatant fraction was further centrifuged at 12,000 × g for 30 min at 4°C. This supernatant was sterile filtered through a 0.22-μm filter. Exosomes were pelleted from this filtrate at 120,000 × g for 2 h at 4°C. The exosomes pellets were resuspended in 0.5 mL phosphate-buffered saline (PBS), PBS, and protein was quantified using a Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Characterization and quantification of exosomes
Exosome protein extraction and western blot analysis were performed according to the described protocol. For NTA, Zetaview equipment (PMX, Germany) was used for real-time tracking and analysis of the exosomes. All samples were diluted with PBS (1:300) before NTA analysis, and then, 100 μL of the sample was loaded into the exosome analysis chamber. For western blot analysis, exosomes were homogenized in lysis buffer with proteinase inhibitors, resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and then incubated with primary antibodies (Abcam, Cambridge, UK) at 4°C overnight. The membranes were then rinsed four times with Tris-buffered saline containing 0.5%
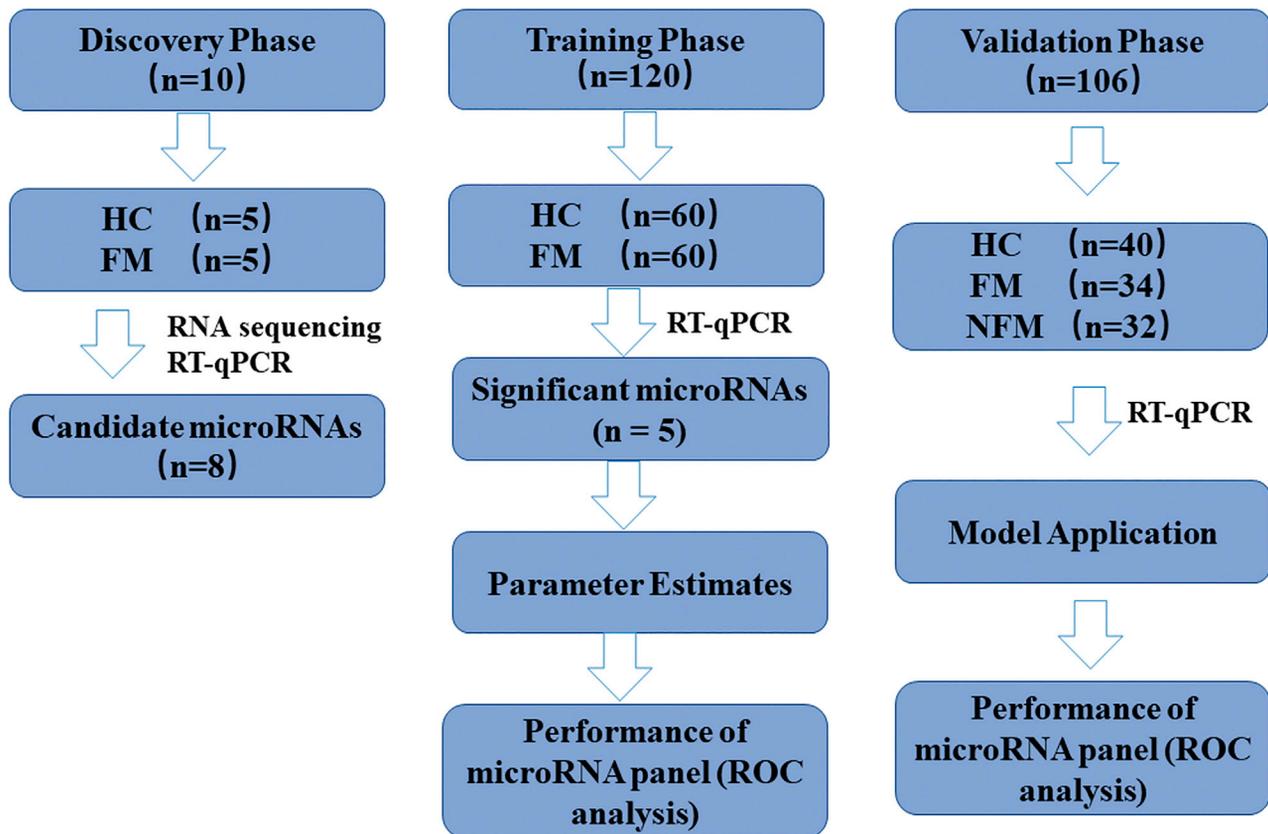
Figure 5. Study design
FM, fulminant myocarditis; NFM, nonfulminant myocarditis; HCs, healthy controls; ROC, receiver operating characteristics; qRT-PCR, quantitative reverse transcriptase PCR.

Tween-20 (TBST), incubated with secondary antibody for 2 h at room temperature, and visualized using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

RNA sequencing
Small RNA libraries were constructed using a NEB Next Ultra Small RNA Sample Library Prep Kit for Illumina according to the manufacturer's instructions. Reverse transcription primer was hybridized after 3′ adaptor ligation of 10 ng RNA per sample, following 5′ adaptor ligation. Twelve cycles of PCR were performed using Illumina feasible barcode primers after first-strand cDNA synthesis. The prepared libraries were resolved on a native 7% polyacrylamide gel. DNA fragments corresponding to 160–180 bp (including 3′ and 5′ adaptors) were recovered in 10 μL of DNase- and RNase-free water.

Libraries were quantified by the Agilent 2100 bioanalyzer using DNA 1000 chips. A total of 36 sequencing libraries were pooled into a single sequencing lane and sequenced using an Illumina HiSeq4000 analyzer (Illumina, USA).The miRNA sequencing data were quantified, and sequences with a length of more than 18 nt were aligned against miRBase (release 21). The miRNA profiling was normalized using reads per million (RPM) mappable miRNA sequences. Analysis of the differentially expressed miRNAs between the two groups was performed using edgeR software. The Gene Expression Omnibus (GEO) database accession number for the miRNA profile data reported in this study is GEO: GSE147517.

Quantitative real-time PCR
The miRNAs were extracted from serum-isolated exosomes using the mirVana miRNA isolation kit per the manufacturer's protocol (Thermo Fisher, MA, USA). The quality, yield, and distribution of miRNAs were analyzed using the Agilent 2100 bioanalyzer with small RNA chips. cDNA was produced using an iScript cDNA synthesis kit (QIAGEN, MD, USA). miRNA levels were measured using a commercial loop miRNA qPCR primer set (RiBoBio, Guangzhou, PR China) according to the manufacturer's instruction. In brief, 2 μg RNA was used as a template and then reverse transcribed using a miRNA-specific reverse transcription (RT) primer. The resulting cDNA was further amplified with a universal reverse primer and a specific forward primer. The cycle parameters were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 65°C for 5 s. Calculations for miRNA expression levels were

performed using the comparative Cq ($^{\Delta\Delta}$Cq) method. Cel-miR-39 was used as an invariant control for serum exosomal miRNA quantification. All reactions were run in triplicate.

Statistics

The baseline characteristics of the population were tabulated using standard descriptors of central tendency and variability (mean ± SD or median [IQR], as appropriate). For sequencing analysis, the Mann-Whitney unpaired test was used for comparison of FM versus HCs. For the data obtained by quantitative real-time PCR, Wilcoxon's rank-sum test was used for the comparison between two groups, and the data are shown as median (IQR). A stepwise logistic regression model was used to select diagnostic miRNA markers based on the training dataset. The predicted probability of being diagnosed with FM was used as a surrogate marker to construct a ROC curve. The AUC was used as an accuracy index for evaluating the diagnostic performance of the miRNA panel. Statistical analysis was performed with IBM SPSS statistics 20.0 and GraphPad Prism version 5.0 software. A p value <0.05 was considered statistically significant.

AUTHOR CONTRIBUTIONS

Y.Z. and K.L. designed the study and analyzed data; X.L., D.W., X.J., and M.Z. conducted experiments and acquired data; Y.Z. and K.L. wrote the manuscript, and all the authors provided critical edits.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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