Original Paper

Serum Exosomal MiR-92b-5p as a Potential Biomarker for Acute Heart Failure Caused by Dilated Cardiomyopathy

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Key Words

Exosome • MiR-92b-5p • Biomarker • DCM • AHF

Abstract

Background/Aims: MicroRNAs (miRNAs) can be used as biomarkers for cardiovascular diseases, especially for heart failure. However, there are few reports on serum exosomal miRNA biomarkers in patients with acute heart failure (AHF) due to Dilated cardiomyopathy (DCM). **Methods:** We analyzed 3 different serum exosomal miRNAs (exo-miR-92b-5p, exo-miR-192-5p, and exo-miR-320a) in 43 patients with DCM-AHF and 34 healthy volunteers as a control group (CG) by using exosome separation followed by a quantitative reverse-transcript PCR assay. Exosomes were identified by electron microscopy, NaNO₂S-90, and western blot analyses (CD63 and Hsp70). **Results:** Serum exo-miR-92b-5p expression was increased in DCM-AHF patients compared to the CG (Mann-Whitney *U*-test: $P < 0.001$). Exo-miR-92b-5p was positively related to age and some ultrasound data (Spearman's correlation: exo-miR-92b-5p vs. age, $r = 0.297$, $P = 0.014$; exo-miR-92b-5p vs. left atrial diameter, $r = 0.431$, $P < 0.001$; exo-miR-92b-5p vs. left ventricular diastolic diameter, $r = 0.419$, $P < 0.001$; exo-miR-92b-5p vs. left ventricular systolic diameter, $r = 0.446$, $P < 0.001$). Exo-miR-92b-5p was also negatively related to other ultrasound data (Spearman's correlation: exo-miR-92b-5p vs. left ventricular fraction shortening, $r = -0.497$, $P < 0.001$; exo-miR-92b-5p vs. left ventricular ejection fraction, $r = -0.482$, $P < 0.001$). The discrimination of DCM-AHF patients from the CG by exo-miR-92b-5p was demonstrated by a receiver operating characteristic curve (exo-miR-92b-5p: cutoff value = 0.0023, area under the curve = 0.808, $P < 0.001$, sensitivity = 62.8%, specificity = 85.3%). **Conclusion:** Serum exo-miR-92b-5p is a potential biomarker for the diagnosis of DCM-AHF.

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Published by S. Karger AG, Basel

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Introduction

Dilated cardiomyopathy (DCM) is one of the most common forms of cardiomyopathy and is the main cause of heart failure (HF) [1]. Acute heart failure (AHF) is a serious pathophysiological state of DCM. Presently, the diagnosis of AHF due to DCM (DCM-AHF) is mainly based on ultrasound data and clinical biochemical indices, such as brain natriuretic peptide (BNP). Ultrasound data can be used to determine whether pathogenesis is mainly caused by DCM, whereas the change of BNP levels can help to estimate whether a patient with DCM is in the dangerous state of AHF. However, the data from ultrasound examination and clinical biochemical testing of BNP are neither specific nor cost-effective for the diagnosis of DCM-AHF. Therefore, novel clinical diagnostic biomarkers need to be developed for the benefit of patients with DCM-AHF.

The cardiovascular system has specific expression profiles (including RNAs and proteins) to maintain its normal function. For example, miR-34a reduces the shortening of telomeres, damage of the DNA response, and myocardial cell apoptosis by targeting protein phosphatase 1 regulatory subunit 10 [2], and miR-10a regulates vessel formation by targeting fms-related tyrosine kinase 1 [3]. However, the expression profiles might change during cardiovascular disease (CVD) [4]. For example, miR-29 was decreased after acute myocardial infarction, which targets a cadre of mRNAs encoding proteins involved in fibrosis to promote the fibrotic response [5]. This was confirmed in another report in which miR-29b was shown to target insulin-like growth factor 1 and phosphatidylinositol 3-kinase and contribute to muscle atrophy [6]. Microarray profiling also showed alterations in genes related to ion channels by comparing DCM patients with controls: sodium voltage-gated channel beta subunit 2 was increased, while potassium voltage-gated channel subfamily J member 5/8, chloride intracellular channel 2/3, and calcium voltage-gated channel auxiliary subunit B2/A1C were decreased [7].

Exosomes are endogenous nano-vesicles (size 30–100 nm) that can carry biological information and may regulate the function of target cells [8, 9]. The contents of exosomes could be proteins and nucleic acids (including DNAs and RNAs). Accordingly, researchers are passionate about identifying and analyzing the information delivered by exosomes.

MicroRNAs (miRNAs) are one of the molecules carried in exosomes [10, 11]. Due to their small size and protection given by exosomes, miRNAs can escape from nuclease degradation in the serum or plasma. In addition, miRNAs have been extensively reported as biomarkers in many human CVDs, including DCM. For example, Gupta et al. demonstrated that the levels of miR-548 family members (miR-548c and miR-548i) in peripheral blood mononuclear cells were decreased in 44 patients with relatively stable chronic HF (CHF) compared with 48 non-failing controls [12]. In another study, miR-21 was found to be increased in patients with DCM, while miR-29 (miR-29a/29b/29c) and miR-133 (miR-133a/133b/133c) family members were decreased [13]. Meanwhile, miRNA biomarkers have also been identified in patients with HF. For example, miR-423-5p, miR-320, miR-22, and miR-92b were increased in patients with CHF [14], and miR-192, miR-194, and miR-34a were elevated in patients with HF caused by acute myocardial infarction (AMI) [15]. Besides, plasma miR-210 expression levels were significantly higher in patients with New York Heart Association class III and IV HF than in those with class II HF and controls [16]. Some reports have identified markers related to acute exhaustive exercise in patients with CHF. For example, Xu et al. reported that serum miR-21, miR-378, and miR-940 levels were significantly up-regulated immediately following acute exercise [17]. Despite these reports on serum or plasma miRNA biomarkers, to the best of our knowledge, there are few reports on miRNA biomarkers in patients with DCM-AHF, especially miRNA biomarkers from serum exosomes. In this study, we compared 3 different serum exosomal miRNAs (exo-miRNAs) between patients with DCM-AHF and an age- and sex-matched control group (CG). We aimed to analyze their potential use as biomarkers for DCM-AHF.

Materials and Methods

Inclusion and exclusion criteria of the patients and healthy volunteers

We prospectively recruited 43 DCM-AHF patients and 34 age- and sex-matched CG volunteers at the Affiliated Hospital of Ningbo University from March 2015 to April 2017. Patients with DCM were diagnosed using the 1995 WHO/ISFC criteria [18]. The diagnosis of AHF was based on the guidelines of the American Heart Association [19, 20]. Eligible patients with DCM-AHF were those with new-onset or acute decompensation CHF as the primary cause of admission. Exclusion criteria for all participants were pregnancy and known or treated malignancies.

The characteristics of the patients with DCM-AHF were as follows: average age 62 years (range, 28–86 years); 32 men and 11 women. Patients with any other diseases such as coronary heart disease, primary valvular heart diseases, congenital heart diseases, rheumatic diseases, and tumors were excluded. CG volunteers had no coronary or valvular diseases or no evidence of cardiomyopathy, as confirmed by echocardiography. The characteristics of the volunteers from the CG were as follows: average age 57 years (range, 21–83 years); 23 men and 11 women.

This study was approved by the Institutional Review Board of the Affiliated Hospital of Ningbo University, and all participants provided written informed consent.

Serum sample collection

Venous blood samples were obtained from the patients with DCM-AHF when they were admitted to hospital and from the volunteers at 07:00 before breakfast.

Serum samples were collected using pro-coagulation tubes, followed with standing for 2 h and centrifuging at 1660×g for 10 min at room temperature. At least 800 µL serum was collected from each sample and stored at -80°C.

Exosome isolation and particle size identification

Serum was centrifuged at 2000 × g for 20 min in order to sediment debris. Subsequently, an exosome isolation reagent (RiboBio, Guangzhou, China) was added to the serum and incubated at 4°C for 30 min. Exosomes were collected by centrifugation at 15000 × g for 2 min at 4°C. The size and concentration of the exosomes were quantified by NaNOZS-90 (Malvern Instruments Ltd., Malvern, UK).

Transmission electron microscopy

The exosomes were resuspended in 30 µL of 1× phosphate-buffered saline, and then 10 µL of the sample was dropped onto a copper grid and left to subside for 1 min. Filter paper was used to absorb the remaining fluid, followed by dropping 10 µL uranyl acetate on the copper grid, which was left to subside for another 1 min, again removing the remaining fluid using filter paper. The sample was air-dried for 10 min at room temperature and examined under a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) at 80 kV.

Western blot assay

Serum exosomes were lysed with RIPA lysis buffer and protein concentration was determined. The proteins (20 µg) were electrophoresed by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked for 2 h at room temperature with 5% non-fat milk. The membrane was incubated with primary antibodies (1:1000, anti-CD63 antibody [TS63], ab59479, Abcam, Cambridge, UK; 1:1000, anti-Hsp70 antibody, #4876, CST, Danvers, MA, USA) overnight at 4°C, followed by a secondary antibody (1:2000, goat anti-mouse IgG, HRP-conjugated for CD63 and 1:3000, goat anti-rabbit IgG, HRP-conjugated for Hsp70) for another 1 h. Protein expression was assessed using a LI-COR model 3600 (LI-COR, Durham, NC, USA).

Quantitative reverse transcription-polymerase chain reaction

miRNAs were extracted from blood (serum) using an miRNA Quick Extraction Kit (Biotek, Beijing, China) according to the manufacturer's protocol. Exo-miRNAs were dissolved in 30 µL RNase-free water, followed by an evaluation of their concentration and purity with an ultraviolet spectrophotometer. The expression levels of exo-miR-92b-5p, exo-miR-192-5p, exo-miR-320a were determined using a standard polyA quantitative reverse transcription PCR (RT-PCR) assay.

The sequences of the reverse transcription adaptor primer, forward primers for miR-92b-5p, miR-192-5p, miR-320a, internal reference gene forward primer for miR-451b [21], and universal inverse primer in

the regular polyA RT-PCR assay were as follows: reverse transcription adaptor primer, 5'-gcgag cacag aatta atacg actca ctata gggtt tttt n-3'; miR-92b-5p PF, 5'-aggga cggga cgcgg tgcag tg-3'; miR-192-5p, 5'-atgac ctatg aattg acagc c-3'; miR-320a, 5'-aaaag ctggg ttgag agggc ga-3'; miR-451b PF, 5'-tagca agaga accat tacca tt-3'; and universal reverse primer, 5'-gcgag cacag aatta atacg ac-3'.

The polyA reverse transcription conditions were 37°C for 1 h and 95°C for 5 min to tail polyA at the end of mature miRNAs, which was followed by the reverse transcription reaction: 42°C for 5 min and 85°C for 5 min. The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 62°C for 35 s, and the melt curve stage of the ABI Q5 standard SYBR Green procedure.

Statistical analysis

The SPSS 18.0 Mann-Whitney *U*-test was used to analyze the expression of serum exosome miRNAs between DCM-AHF patients and the CG. Spearman's correlation test was used to analyze the relationship between the expression level of exo-miR-92b-5p and the ultrasound data. Fisher's exact test, Pearson's chi-square test, Mann-Whitney *U*-test, and Student's *t*-test were used to analyze the characteristic features of the DCM-AHF patients and the CG. A receiver operating characteristic (ROC) curve was generated using the expression level of exo-miR-92b-5p to separate DCM-AHF patients from CG volunteers. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Exosome sample identification

The characteristics of exosomes were determined by particle-size analysis, transmission electron microscopy, and western blot analysis. Exosomes in the serum exhibited a double-layer membrane and ranged in size from 30 to 150 nm (Fig. 1A and 1B). In addition, CD63 and Hsp70, which are protein markers of exosomes, were used to confirm the evaluation of exosomes by western blot assay (Fig. 1C).

Comparison of the clinical characteristics between the DCM-AHF patients and CG

The clinical characteristics of the DCM-AHF patients and CG are shown in Table 1. The two groups were similar regarding sex, age, body mass index (BMI), smoking and drinking habits, and hypertension status. Predicted and trivial significant changes, reflecting the different nature of DCM-AHF versus CG were echocardiographic indexes (Mann-Whitney *U*-test, $P < 0.001$), including left ventricular diastolic diameter (LVDD), left ventricular systolic diameter (LVSD), left atrial diameter (LAD), left ventricular ejection fraction (LVEF), and left ventricular fraction shortening (LVFS). However, some confounding factors, such

Fig. 1. Characterization of exosomes extracted from serum. A. The particle sizes of extracted exosomes were confirmed by NaNOZS-90. B. Serum exosomes were analyzed by microscopy, which were highlighted using black arrows. Scale bar = 100 nm. C. The exosome biomarkers CD63 and Hsp70 were identified by western blot assay. E, exosomes; EDS: exosome-depleted supernatant.

as diabetes mellitus (Fisher's exact test, $P = 0.001$) and renal failure (Fisher's exact test, $P = 0.009$), also indicated significant differences between DCM-AHF and CG, which we analyzed further in the following sections.

Exo-miR-92b-5p expression is increased in patients with DCM-AHF

In this study, we pretested 9 serum exo-miRNAs in small groups and found that 3 different serum exo-miRNAs (i.e., exo-miR-92b-5p, exo-miR-192-5p, and exo-miR-320a) were relatively stably expressed (Table 2). Further formal tests showed that exo-miR-92b-5p was increased in DCM-AHF patients compared with the CG (Mann-Whitney U -test: $P < 0.001$; median [25–75%]: DCM-AHF 0.0062 [0.0010–0.0119] vs. CG 0.0007 [0.0002–0.0016], Fig. 2A). The expression of exo-miR-192-5p and exo-miR-320a was not significantly different between DCM-AHF and CG (Mann-Whitney U -test and median [25–75%]: exo-miR-192-5p, $P = 0.454$, DCM-AHF 0.0028 [0.0010–0.0178] vs. CG 0.0035 [0.0010–0.0066], Fig. 2B; exo-miR-320a, $P = 0.351$, DCM-AHF 0.0146 [0.0009–0.1398] vs. CG 0.0055 [0.0006–0.0705], Fig. 2C).

In order to characterize the main expression form of exo-miR-92b-5p in serum, we compared its expression between exosomes and exosome-depleted supernatant (Fig. 3), and found that miR-92b-5p was mainly expressed in exosomes.

In order to distinguish whether exo-miR-92b-5p expression was affected by diabetes mellitus and renal failure, as mentioned above (Table 1), we further compared the expression of exo-miR-92b-5p between DCM-AHF patients with and without diabetes mellitus as well as DCM-AHF patients with and without renal failure. There was no significant difference in exo-miR-92b-5p expression between DCM-AHF patients with diabetes mellitus and those without diabetes mellitus as well between DCM-AHF patients with renal failure and those without renal failure (Fig. 4).

Table 1. Characteristics of the DCM-AHF and CG subjects ^a, Student t -test ; ^b, Pearson's Chi-square test; ^c, Fisher's exact test; ^d, Mann-Whitney U -test

Variable	CG (n = 34)	DCM (n = 43)	P-value
Age (years)	57±2	62±2	0.107 ^a
Sex (male)	23 (67.6%)	32 (74.4)	0.514 ^b
Smoking	12 (35.3%)	20 (46.5%)	0.321 ^b
Drinking	8 (23.5%)	19 (44.2%)	0.059 ^b
Diabetes mellitus	4 (11.8%)	20 (46.5%)	0.001 ^c
Hypertension	22 (64.7%)	22 (51.2%)	0.233 ^b
Renal failure	1 (2.9%)	11 (25.6%)	0.009 ^c
BMI (kg/m ²)	23.5±0.5	24±0.6	0.427 ^a
BNP (ng/L)	NA	927.8 [637.4–1470.8]	NA
LAD (mm)	33.5[31.0–37.0]	47.0 [42.0–52.0]	<0.001 ^d
LVDD (mm)	48.5[45.8–51.0]	65.0 [59.0–73.0]	<0.001 ^d
LVSD (mm)	29.9±0.6	52.3±1.1	<0.001 ^a
LVEF(%)	68.9±0.8	39.1±1.4	<0.001 ^a
LVFS(%)	38.8±0.6	20.3±0.8	<0.001 ^a

Fig. 2. Comparison of the expression levels of serum exo-miRNAs between DCM-AHF patients and the CG. A. Exo-miR-92b-5p (Mann-Whitney U -test: DCM-AHF vs. CG, $P < 0.001$). B. Exo-miR-192-5p (Mann-Whitney U -test: DCM-AHF vs. CG, $P = 0.454$). C. Exo-miR-320a (Mann-Whitney U -test: DCM-AHF vs. CG, $P = 0.351$).

Fig. 3. Comparison of the expression levels of miR-92b-5p between exosomes and exosome-depleted supernatant. A. Relative expression levels of miR-92b-5p in exosomes and supernatant (exosome-depleted supernatant). B. Ratio of miR-92b-5p expression in exosomes and supernatant (exosome-depleted supernatant).

Fig. 4. Comparison of the expression levels of serum exo-miR-92b-5p with clinical characteristics. A. Patients with or without diabetes mellitus. B. Patients with or without renal failure.

Relationship between the expression of exo-miR-92b-5p and other clinical characteristics

We further analyzed the relationship between exo-miR-92b-5p and other clinical characteristics, and found that exo-miR-92b-5p was significantly related to age and echocardiographic indexes (Fig. 5). Exo-miR-92b-5p expression was positively related to LAD (Fig. 5A, Spearman's correlation, $r = 0.431$, $P < 0.001$), LVDD (Fig. 5C, Spearman's correlation, $r = 0.419$, $P < 0.001$), LVSD (Fig. 5D, Spearman's correlation, $r = 0.446$, $P < 0.001$), and age (Fig. 5F, Spearman's correlation, $r = 0.297$, $P = 0.014$), whereas it was negatively related to LVEF (Fig. 5B Spearman's correlation, $r = -0.482$, $P < 0.001$) and LVFS (Fig. 5E, Spearman's correlation, $r = -0.497$, $P < 0.001$).

Table 2. Choice of candidate exo-miRNAs and references. Note: "-" = unrecorded

Candidate Targets		Pretest non-detection rate	Range of CT
miRNAs	miR-1-2	7/15(47%)	-
	miR-210	3/5(60%)	-
	miR-499a	4/5(80%)	-
	miR-499b	4/5(80%)	-
	miR-208b	13/15(87%)	-
	miR-423	11/15(73%)	-
	miR-320a	0/10(0)	-
	miR-192-5p	0/10(0)	-
	miR-92b-5p	0/10(0)	-
References	U6	1/9(11%)	28-31
	miR-484	0/6(0)	25-34
	miR-16	7/12(58%)	26-39
	miR-642-3p	0/6(0)	28-32
	miR-451b-5p	0/6(0)	22-24

Exo-miR-92b-5p is a potential diagnostic biomarker for DCM-AHF

The discrimination of DCM-AHF patients from CG volunteers by using exo-miR-92b-5p was demonstrated by a ROC curve with an area under the curve (AUC) of 0.808 (95% confidence interval, 0.712–0.903, $P < 0.001$) (Fig. 6). When the expression value of exo-miR-92b-5p was equal to or greater than 0.0023, which predicts that patients have DCM-AHF, a sensitivity of 62.8% and specificity of 85.3% were generated for the identification of DCM-AHF.

Discussion

Circulating miRNAs are potential biomarkers for diverse CVDs [22, 23]. In the present study, we aimed to identify potential serum exo-miRNAs as diagnostic biomarkers for DCM-AHF. We found that serum exo-miR-92b-5p was significantly increased in patients with DCM-AHF. Further analysis also indicated that serum exo-miR-92b-5p is a potential biomarker for DCM-AHF. However, some issues require further discussion.

It is known that miRNAs are potential biomarkers for various diseases, because of their stability in human plasma or serum. Mitchell et al. first reported such a phenomenon in human prostate cancer [24]. However, as further research has been conducted, the stability of miRNAs has been found not only to be attributed to their small size but also to their protection in exosomes. Circulating miRNAs exist as 2 forms, namely, free miRNAs and exo-miRNAs. Gall et al. confirmed that there are differences between serum free nucleic acids and exosome-contained nucleic acids [25]. They reported that the majority of miRNAs were contained in exosomes in serum [25]. miRNAs, which are extracted from isolated exosomes, can be tested more sensitively than those in whole serum [25]. Some reports have supported this view. For example, Jansen et al. compared the expression of miRNAs between plasma and circulating microvesicles in 181 patients with stable coronary artery diseases, and found that miR-126 and miR-199a, which were contained in microvesicles, were related to cardiovascular events, but not the free nucleic acids circulating in the plasma [26]. Certainly, there has been some debate over this issue. For example, Goren et al. compared the expression of circulating miRNAs between

Fig. 5. The relationship between exo-miR-92b-5p and other clinical characteristics. A. exo-miR-92b-5p vs. LAD (Spearman's correlation: $r = 0.431$, $P < 0.001$). B. Exo-miR-92b-5p vs. LVEF (Spearman's correlation: $r = -0.482$, $P < 0.001$). C. Exo-miR-92b-5p vs. LVDD (Spearman's correlation: $r = 0.419$, $P < 0.001$). D. Exo-miR-92b-5p vs. LVSD (Spearman's correlation: $r = 0.446$, $P < 0.001$). E. Exo-miR-92b-5p vs. LVFS (Spearman's correlation: $r = -0.497$, $P < 0.001$). F. Exo-miR-92b-5p vs. age (Spearman's correlation: $r = 0.297$, $P = 0.014$).

Fig. 6. ROC curve for discriminating DCM-AHF patients from the CG by exo-miR-92b-5p. AUC: 0.859; cutoff value: 0.0023; sensitivity: 62.8%; specificity: 85.3%.

exosomes and unfractionated serum in 10 stable chronic systolic HF patients and 10 controls [14]. They concluded that there was no difference between their levels [14]. Another report showed that extracellular miRNAs were predominantly exosome/microvesicle-free and were associated with Ago proteins [27]. In the present research, we compared the expression of miR-92b-5p between exosomes and exosome-depleted supernatant (Fig. 3), and found that miR-92b-5p was mainly expressed in exosomes, which provides support for the report of Gallo et al. [25].

Before we discuss the main results of this study, we should first discuss one issue that cannot be avoided. What kind of reference should we choose to adjust the results, an internal or external reference? We firstly added cel-miR-39 as an external reference (data not shown) to adjust the final result. We found that although we unified the amount of cel-miR-39, cel-miR-39 expression showed significant differences in different experiments. We then compared some internal references according to previous reports, such as miR-16 [28-32], U6 [32-34], and miR-451 [21]. We found that miR-451b was stably expressed in serum exosomes without any significant difference between patients with DCM-AHF and the CG (Table 2). Finally, we chose miR-451b as our internal reference for serum exo-miRNAs.

Due to the similarity of its basic sequence, miR-92 can be divided into miR-92a and miR-92b. miR-92a contains 2 members (miR-92a-1 and miR-92a-2) with the same sequence, which are located separately in different miRNA gene clusters. Many changes in the expression of miR-92a have been reported in CVDs. For example, Jiang et al. reported that miR-92a expression was decreased in atherosclerosis [35], whereas it presented with the highest expression level in 95 patients with coronary atherosclerosis [36]. Compared to miR-92a, there are much fewer reports on miR-92b in CVDs. One report indicated that miR-92b was expressed higher in CHF patients than in a CG [14]. However, so far as we know, there is no report on the expression of miR-92b in DCM-AHF, and especially no report on serum exo-miR-92b levels in DCM-AHF. Our present study showed that exo-miR-92b-5p was increased in patients with DCM-AHF. Given the sequence similarity between miR-92a and miR-92b, we could predict the function of miR-92b by using reports on miR-92a. Animal experiments showed that miR-92a regulated angiogenesis and functional recovery of ischemic tissues in mice [37]. Therefore, patients with DCM-AHF might be in the ischemic tissue condition, which might lead to the increased expression of serum exo-miR-92b-5p. In spite of such an assumption, basic research on the function of exo-miR-92b-5p in patients with DCM-AHF should be conducted in the near future.

miR-192-5p expression has also been reported in many CVDs. It was reported as a p53-responsive miRNA [38]. The expression of miR-192 was elevated in patients who developed HF within 1 year after AMI compared with 65 matched controls without a subsequent cardiovascular event after discharge [15]. Fang et al. screened miRNA expression using a human plasma/serum microarray and found that miR-192 was up-regulated in hypertrophic cardiomyopathy [39]. To the best of our knowledge, there is no report on the expression of serum exo-miR-192-5p in DCM-AHF. Our present study showed that there was no significant difference between patients with DCM-AHF and the CG for the expression of exo-miR-192-5p (Fig. 2B). We may not be able to compare our results with those from other studies for the following reasons. Firstly, different internal references were used. The reports discussed above used snRNA U6 as an exosomal internal miRNA. However, in the present study, we did not test the stability or expression of snRNA U6, but used exo-miR-451b instead. A different control system might not achieve the same result. Secondly, we focused on a different etiological cause of CVD compared to the previous studies. Reports on HF caused by AMI and hypertrophic cardiomyopathy, as well as DCM-AHF in our present study, might produce different information on the circulatory system. Thirdly, because of a limitation in the number of patients enrolled, we might not have generated a definitive conclusion. If possible, a large-scale, multi-center study should be conducted in the near future.

miR-320a is a controversial miRNA in animal models of CVDs. It was reported as a protective miRNA during the process of myocardial ischemia-reperfusion injury in a rat model [40]. It was increased during myocardial ischemia-reperfusion injury, which may

prevent left ventricular remodeling [40]. While Ren et al. reported that over-expression of miR-320 enhanced cardiomyocyte death and apoptosis in mice undergoing simulated ischemia/reperfusion [41]. They also found that Hsp20, a known cardioprotective protein, was an important candidate target of miR-320 [41]. However, our present study showed that there was no significant difference in miR-320a expression between patients with DCM-AHF and the CG. However, these differences were explained according to our analysis in the above section.

The data shown in Table 1 indicated that serum exo-miR-92b-5p might be affected by other 2 risk factors, namely, diabetes mellitus and renal failure. Therefore, we further analyzed patients with or without the above diseases, and we found that neither diabetes mellitus nor renal failure affected the expression of serum exo-miR-92b-5p. Thus, the differential expression of serum exo-miR-92b-5p might be specific to patients with DCM-AHF.

We also analyzed the relationship between exo-miR-92b-5p expression and other ultrasound indices (Fig. 2), because ultrasound indices might be one of the most effective diagnostic factors for DCM patients. We found that exo-miR-92b-5p was positively related to LAD, LVDD, and LVSD, whereas it was negatively related to LVEF and LVFS. It is easy to understand such a relationship. LAD, LVDD, and LVSD represent the enlargement of the cardiac chambers, whereas LVEF and LVFS indicate decreased cardiac function. Both the positive relationship between exo-miR-92b-5p and the enlargement of the cardiac chambers (LAD, LVDD, and LVSD), as well as the negative relationship between exo-miR-92b-5p and the factors representing cardiac functional decline, indicated that exo-miR-92b-5p was a risk factor for patients with DCM-AHF. Considering the cost-effectiveness of diagnosis by ultrasound indices, it is a preferable strategy to use exo-miR-92b as an alternative diagnostic factor for patients with DCM-AHF.

Until now, BNP or N-terminal pro-BNP has been the most useful biomarker for the diagnostic evaluation and risk stratification of patients with AHF [42]. In spite of its widespread use in clinical practice, it is affected by other factors and is not considered a specific biomarker for DCM-AHF. It is better to compare the relationship between exo-miR-92b-5p and BNP by regression analysis as well as the diagnostic value of BNP by using a ROC curve. However, we could not acquire BNP data from the CG volunteers, because it was beyond our budget to test for BNP and they had no indications for such a test to be performed.

We analyzed the sensitivity and specificity of exo-miR-92b-5p for the diagnosis of DCM-AHF by ROC curve analysis (Fig. 6). According to this analysis, although the sensitivity for exo-miR-92b-5p was not sufficiently high, a high specificity was achieved, which might compensate for the low specificity of BNP.

Still, some limitations of this study should be taken into account. Firstly, we only tested 3 different exo-miRNAs as candidate biomarkers, which might not be considered a comprehensive approach. If possible, we should select typical cases to be screened using a microarray, and identify more candidate exo-miRNAs for further sample validation. Secondly, the number of patients enrolled was not large enough. If possible, we should recruit additional patients for a second cohort to validate our findings. Thirdly, the abnormal expression of exo-miRNAs needs to be examined by basic studies *in vitro* and *in vivo*. Therefore, if possible, a cellular assay and an animal model should be considered in the near future. Fourthly, it is better to verify the specificity of exo-miR-92b-5p for DCM-AHF by comparing its expression in different tissues, but we were unable to obtain those tissues from the patients with DCM-AHF because it required invasive procedures and was ethically restricted.

Conclusion

Serum exo-miR-92b-5p is a putative specific diagnostic biomarker for patients with DCM-AHF.

Acknowledgements

This study was supported by the Ningbo University Science Foundation (Grant Numbers: XYY15016 and XYY16008) Ningbo, Zhejiang, China.

Disclosure Statement

The authors declare no conflicts of interest.

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