

Circulating microRNAs as novel biomarkers for dilated cardiomyopathy

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Abstract

Background: *Circulating microRNAs (miRNAs) potentially carry disease-specific information. In the current study, we aim to characterize the miRNA signature in plasma from dilated cardiomyopathy (DCM) patients and assess the possible correlation between expression levels of circulating miRNAs and symptom severity in DCM patients.*

Methods: *Using microarray-based miRNA expression profiling, we compared the miRNA expression levels in plasma samples from 4 DCM patients and 3 healthy controls. The expression levels of selected differentially expressed, upregulated miRNAs (miR-3135b, miR-3908 and miR-5571-5p) were validated independently in plasma samples from 19 DCM patients and 20 controls.*

Results: *We observed that plasma miR-3135b ($p < 0.001$), miR-3908 ($p < 0.001$) and miR-5571-5p ($p < 0.001$) were significantly upregulated in DCM patients. The area under receiver operating characteristic (ROC) curves for the 3 miRNAs ranged from 0.83 to 1.00. Moreover, miR-5571-5p levels in plasma were significantly upregulated with severe New York Heart Association (NYHA) classification ($p < 0.05$).*

Conclusions: *The circulating miRNAs (miR-3135b, miR-3908 and miR-5571-5p) have potential as diagnostic biomarkers for DCM. Additionally, miR-5571-5p correlated with NYHA classification. (Cardiol J 2017; 24, 1: 65–73)*

Key words: microRNAs, dilated cardiomyopathy, biomarkers, diagnosis

Introduction

Dilated cardiomyopathy (DCM), which is generally used as an all-encompassing term for a non-ischemic cardiomyopathy with depressed left ventricular (LV) function, is an important cause of sudden cardiac death and heart failure (HF) and also the leading indication for cardiac transplantation in children and adults worldwide [1]. DCM may manifest clinically at a wide range of ages and is often identified when associated with severe

limiting symptoms and disability. The diagnosis of DCM is usually made with echocardiography, which has several disadvantages, including high financial costs, limited availability and high interobserver variability. The use of circulating biomarkers in the diagnostic approach to DCM would offer an attractive alternative. MicroRNAs (miRNAs) are small, highly conserved, non-coding RNAs, which act as endogenous inhibitors of target genes [2–5]. MiRNAs have been shown to be key molecular players in nearly all cellular processes, including

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cardiovascular development and pathophysiology [6, 7]. Gain or loss of function experiments, expression arrays and bioinformatic analysis confirmed that specific miRNAs played essential roles in the biogenesis of DCM [6, 8–13]. This observation laid the theoretical foundation of the diagnostic application of miRNAs to DCM. Subsequently, several studies demonstrated miRNA expression differences in various pathological conditions and characterized the links between specific miRNAs and aspects of pathologies [14, 15]. In addition, circulating miRNAs have many requisite features of good biomarkers, such as accessibility through non-invasive methods; a high degree of specificity and sensitivity; the ability to differentiate pathologies, allowing early detection; sensitivity to relevant changes in the disease; a long half-life within the sample; and the capability for rapid and accurate detection [16]. Therefore, circulating miRNAs have potential as new diagnostic biomarkers for DCM.

In the present study, we aimed to characterize the miRNA signature in plasma from DCM patients compared with healthy controls. We also evaluated the discriminatory power of the significantly upregulated miRNAs and correlated them with symptom severity in DCM.

Methods

Patients and controls cohort

This study has been approved by the Institution Review Board of Beijing Hospital of the Ministry of Health, and all subjects provided written informed consent before the initiation of any study-related procedures. Twenty-three patients with DCM with no identifiable or reversible etiology were recruited from the Department of Cardiology. Subjects that demonstrated persistent (> 3 months) LV systolic dysfunction (LV ejection fraction [LVEF] $\leq 40\%$) as assessed by echocardiography were classified as patients with DCM for the study. Each DCM patient underwent coronary computed tomography or coronary angiography, which revealed that no coronary stenosis was larger than 50%. In addition, a control group, consisting of 23 apparently healthy volunteers with no known heart disease, kidney, lung or hematologic disorders, were recruited; they were age- and gender-matched to the DCM group. Exclusion criteria for all participants were pregnancy, known or treated malignancies, and connective diseases.

All of the clinically relevant data were collected for the DCM group and control group: clinical, echocardiographic, electrocardiographic,

and baseline laboratory, as well as the relevant demographic data.

Sample acquisition and handling

An 8 mL aliquot of blood was obtained from all participants directly into sodium citrate tubes. The whole blood was allowed to stand for ~ 3 h at -4°C before centrifuging at 1,500 g for 10 min at room temperature. The resultant plasma was aliquoted into Eppendorf tubes and stored at -70°C .

Clinical biochemical markers

Peripheral venous blood samples were tested for the levels of hemoglobin (HGB), creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) using standard protocols in the core clinical laboratory at Peking University Fifth Hospital. Plasma N-terminal-pro-B type natriuretic peptide (NT-proBNP) was measured by electrochemiluminescence immunoassay (Elecys proBNP II assay) on an automated Cobas e601 analyzer according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, German). The quality controls were measured within 2-standard deviation values. The range of detection for NT-proBNP was 5–3,500 pg/mL.

Echocardiography

Doppler echocardiographic assessment was performed by one of 2 blinded operators in accordance with the European Society of Echocardiography recommendations. All data represent the mean of 3 measurements on sequential cardiac cycles. LVEF was calculated by the Simpson's method.

RNA extraction

Total RNA was isolated using TRIzol (Invitrogen) and purified with RNeasy mini kit (QIAGEN) according to manufacturer's instructions. RNA quality and quantity was measured by using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies), and RNA Integrity was determined by gel electrophoresis.

Microarray processing and analysis

RNA labeling and array hybridization was performed according to Exiqon's manual. The samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on the miRCURY™ LNA Array (v.18.0) (Exiqon) according to the manufacturer's guidelines. Following hybridization, the slides were obtained and washed several times using Wash buffer kit (Exiqon). Then, the slides were

scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥ 30 in all samples were chosen for calculating the normalization factor. Expression data were normalized using the median normalization. After normalization, the miRNAs were considered to be significantly differentially expressed only when they exhibited a mean change > 2 -fold relative to the controls and had a q-value < 0.05 between the DCM patients and control groups.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

To confirm the findings obtained by analyzing the miRNA profiling, we measured the expression of upregulated miRNAs using TaqMan qRT-PCR. All reactions were carried out using Gene Amp PCR System 9700 (Applied Biosystems), according to the manufacturer's recommendations. In brief, the reverse transcription reaction was performed in 20 μL mixture consisting of 0.3 μL of 10 μM RT primer, and 100 ng RNA sample, 2 μL of 2.5 mM dNTPs, 0.2 μL MMLV reverse transcriptase, 2 μL of 10 \times reverse transcription buffer, 0.3 μL RNase inhibitor, and nuclease-free water. The reaction mixture was incubated for 30 min in 16 $^{\circ}\text{C}$, 40 min in 42 $^{\circ}\text{C}$, and 5 min in 85 $^{\circ}\text{C}$, and then kept in -20 $^{\circ}\text{C}$. Next, qPCR was performed in a final volume of 10 μL containing 1 μL PCR probe, 2 μL product from the RT reaction, 5 μL 2X PCR Master Mix, and 2 μL nuclease-free water. The thermal cycle started with 10 min at 95 $^{\circ}\text{C}$, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 10 s and 60 $^{\circ}\text{C}$ for 1 min. MiR-191-5p was selected as the internal control miRNA to normalize the cDNA levels of different samples because the concentration of miR-191-5p was approximately the same in various samples (detailed data can be found in Table 1). The threshold cycle (Ct) values obtained for each miRNA were then normalized to the respective miR-191-5p Ct values to obtain the ΔCt values and eventually used to plot relative expression values.

Data analysis and statistics

Data are presented as mean \pm standard deviation unless indicated otherwise, and plasma miRNA levels are presented as fold change relative to controls. The miRNA expression fold changes were calculated using the arithmetic formula $2^{-\Delta\Delta\text{Ct}}$.

Continuous clinical variables were compared between the two groups by a 2-sided unpaired

Table 1. MiR-191-5p expression of all samples. Coefficient of variation: 1.1%. Circulating miR-191-5p is stably expressed across all samples and thus is a suitable reference gene for normalizing reverse-transcription polymerase chain reaction data in this study.

Sample	(miR-191-5p) threshold cycle
Control1	22.071
Control2	22.469
Control3	22.278
Control4	21.988
Control5	22.113
Control6	21.469
Control7	22.111
Control8	21.609
Control9	21.933
Control10	21.678
Control11	21.711
Control12	21.849
Control13	22.078
Control14	21.906
Control15	21.893
Control16	22.060
Control17	21.548
Control18	21.946
Control19	21.895
Control20	21.585
Dilated1	21.821
Dilated2	21.977
Dilated3	22.007
Dilated4	21.603
Dilated5	21.714
Dilated6	21.644
Dilated7	22.083
Dilated8	21.887
Dilated9	21.988
Dilated10	22.237
Dilated11	22.042
Dilated12	21.677
Dilated13	21.491
Dilated14	21.465
Dilated15	21.536
Dilated16	22.153
Dilated17	22.372
Dilated18	22.075
Dilated19	21.943

t-test for Gaussian data or Mann-Whitney tests for non-Gaussian data. Chi-square tests were used to compare categorical clinical variables.

Table 2. Characteristics of the dilated cardiomyopathy (DCM) group and the control group.

Variables	Cohort 1 (screening by microarray)		Cohort 2 (validation by qRT-PCR)	
	Controls (n = 3)	DCM (n = 4)	Controls (n = 20)	DCM (n = 19)
Age [years]	49.3 ± 8.4	53.7 ± 7.6	56.7 ± 9.1	59.2 ± 13.1
Male	2 (66.7%)	4 (100%)	16 (80%)	16 (84.2%)
Body mass index [kg/m ²]	27.2 ± 0.7	25.4 ± 2.7	25.4 ± 2.1	27.0 ± 5.0
Diabetes	0 (0%)	0 (0%)	0 (0%)	2 (10.5%)
Hypertension	0 (0%)	2 (50%)	0 (0%)	12 (63.2%) [^]
Coronary artery disease	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Atrial fibrillation	0 (0%)	0 (0%)	0 (0%)	9 (47.4%) [^]
NT-proBNP [pg/mL]	29.7 (5.9–51.4)	1352.8 (772.0–18050.0) [#]	39.2 (13.4–62.1)	1991.9 (851.5–3706.0) [^]
LVEF [%]	68.3 ± 2.1	20.5 ± 3.1 [^]	65.8 ± 4.9	27.5 ± 7.7 [^]
LAD [mm]	31.0 ± 2.6	47.3 ± 5.7 [^]	34.8 ± 4.1	45.4 ± 6.5 [^]
LVEDD [mm]	44.3 ± 2.3	71.0 ± 9.1 [^]	45.1 ± 3.3	61.4 ± 7.1 [^]
NYHA grade:				
Class I/II		2 (50%)		6 (31.5%)
Class III/IV		2 (50%)		13 (68.5%)
Hemoglobin [g/L]	153.7 ± 27.5	152.8 ± 14.1	153.3 ± 17.2	149.7 ± 19.1
eGFR [mL/min]	116.9 ± 28.5	80.7 ± 11.4	105.5 ± 22.2	91.1 ± 34.9
ALT [U/L]	14.3 ± 1.2	22.0 ± 13.0	29.2 ± 29.2	19.9 ± 9.3
AST [U/L]	19.0 ± 5.6	25.8 ± 7.2	28.3 ± 11.0	23.8 ± 4.6
Beta-blockers	0 (0%)	4 (100%) [#]	0 (0%)	18 (94.7%) [^]
ACEI/ARB	0 (0%)	4 (100%) [#]	0 (0%)	18 (94.7%) [^]
Spirolactone	0 (0%)	4 (100%) [#]	0 (0%)	17 (89.5%) [^]
Digitalis	0 (0%)	3 (75%)	0 (0%)	4 (21.1%) [#]
Antiplatelet	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Anticoagulation	0 (0%)	0 (0%)	0 (0%)	9 (47.4%) [^]
ICD/CRT	0 (0%)	1 (25%)	0 (0%)	13 (68.4%) [^]

Values are shown as mean ± standard deviation, n (%), or median (interquartile range); [^]p < 0.05; [#]p < 0.01; qRT-PCR — quantitative reverse-transcription polymerase chain reaction; NT-proBNP — N-terminal-pro-B type natriuretic peptide; LVEF — left ventricular ejection fraction; LAD — left atrial dimension; LVEDD — left ventricular end-diastolic dimension; NYHA — New York Heart Association; eGFR — estimated glomerular filtration rate; ALT — alanine aminotransferase; AST — aspartate aminotransferase; ACEI — angiotensin-converting enzyme inhibitor; ARB — angiotensin receptor blockers; ICD — implantable cardioverter-defibrillator; CRT — cardiac resynchronization therapy

Receiver operating characteristic (ROC) analysis was carried out for each miRNA validated by qRT-PCR, as well as NT-proBNP, and the area under the ROC curve (AUC) was calculated to evaluate its predictive power for DCM.

Associations between the New York Heart Association (NYHA) classification and miRNAs were evaluated as follows: a 2-sided unpaired t-test was used to compare the miRNAs of samples with NYHA classifications of class I–II with the samples with NYHA classifications of class III–IV.

Data analysis was performed using the Statistical Package for the Social Sciences (version 19.0; SPSS Inc., Chicago, IL, USA). The probability level for statistical significance was set at α = 0.05.

Results

Patient characteristics

To select candidate plasma miRNAs for DCM detection, we performed an initial genome-wide microarray screening of Cohort 1: samples derived from 4 DCM patients and 3 matched controls. Then, we studied Cohort 2, which was composed of 19 DCM patients and 20 controls for independent validation. The clinical characteristics of the DCM patients and controls are shown in Table 2. In both cohorts, the two groups were similar regarding age, gender, body mass index, HGB, liver function (ALT, AST) and renal function (estimated glomerular filtration rate [eGFR]). Predicted

significant changes, reflecting the differences between DCM patients vs. controls were in LVEF, left atrial dimension, LV end-diastolic dimension and NT-proBNP. In Cohort 2, DCM patients had significantly higher levels of NT-proBNP: ~1,991.9 pg/mL compared with controls: ~39.2 pg/mL. The LVEF in DCM patients was ~27.5% compared with controls with a LVEF of ~65.8%.

MiRNAs microarray of plasma in DCM

To determine the differential miRNA expression in DCM, we performed plasma miRNA microarray analysis with 4 DCM patients and 3 controls. The microarray covered all human, mouse and rat miRNAs annotated in miRBase 18.0, as well as all viral miRNAs related to these species. The expression profiles of altered miRNAs following the genome-wide miRNA microarray showed distinctive differences between control and DCM patient sample. Of 3,100 miRNAs detected on the microarray, 47 miRNAs were found to be differentially expressed in DCM patients compared with controls with a fold change > 2 and $q < 0.05$, among which 30 miRNAs were increased. Especially miR-3135b, miR-3908, and miR-5571-5p had the most significantly increased level.

In some stances, studies have reported that the peripheral blood mononuclear cells had similar expression of certain gene expression compared to pathological tissues. To test whether differentially expressed miRNAs of plasma from DCM patients mimic the pattern of cardiac miRNAs in end-stage HF because of DCM, 9 miRNAs representing the cardiac miRNA signature were assessed [10]. Surprisingly, we observed that 3 miRNAs (miR-1, miR-342, and miR-145) were barely detectable in the microarray and that none of the 6 remaining miRNAs significantly mirror the cardiac miRNA expression pattern (Fig. 1). This indicates that circulating miRNA in plasma may be different and might have a unique miRNA profile.

Quantitative RT-PCR analysis of plasma miRNAs in DCM

Within Cohort 1, miR-3135b, miR-3908, and miR-5571-5p had been shown to have significantly upregulated expression. To verify the differential expression patterns of these miRNAs, we further examined these 3 miRNAs in independent Cohort 2, which consisted of 19 DCM patients and 20 controls, by qRT-PCR. Consistent with the aforementioned results, miR-3135b ($p < 0.001$), miR-3908 ($p < 0.001$), and miR-5571-5p ($p < 0.001$) were significantly upregulated in DCM patients com-

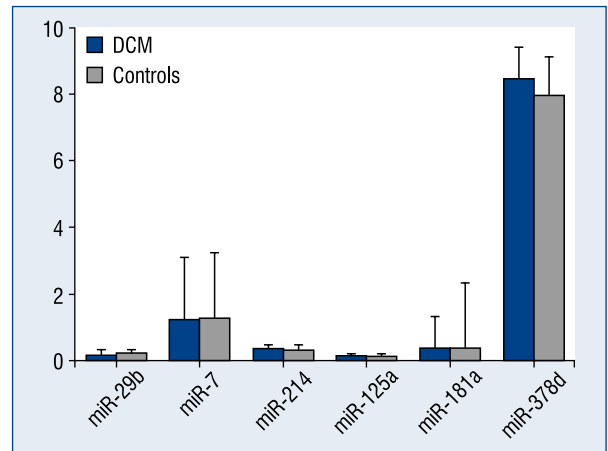


Figure 1. MiRNA microarray analysis showed that 6 miRNAs representing the cardiac miRNA signature of dilated cardiomyopathy (DCM) patients were not significantly differentially expressed in plasma from DCM patients compared with plasma from controls. Data presented as the mean \pm standard deviation.

pared with controls (Fig. 2). Furthermore, our studies revealed that the miR-3135b, miR-3908, and miR-5571-5p expression levels were even altered in plasma from patients who had just been classified into NYHA class I/II, suggesting that plasma miRNAs changes could be observed even in patients with mild symptoms.

Furthermore, ROC analysis showed miR-3135b, miR-3908, and miR-5571-5p with significant AUC ranged from 0.83 to 1.00, implying that they had the discriminatory power to distinguish DCM patients from controls (Fig. 3). For comparison with the conventional biomarker of HF, we used NT-proBNP measurements from these patients and NT-proBNP exhibited an AUC value of 0.99. Thus, miR-3135b and miR-5571-5p had a similar discriminative power for DCM with NT-proBNP.

Because miRNAs are normally excreted by the kidney [17], it is possible that miRNA levels in the plasma are affected by renal function. To assess whether such an effect may have caused a bias in our study, we compared the miRNA levels between DCM patients whose values for the eGFR were above the median and the samples with values that were below the median. There was no significant ($p < 0.05$) difference for each miRNA.

Correlation between miRNAs and symptom severity in DCM

The 3 miRNAs were tested for a possible association with symptom severity in DCM. NYHA

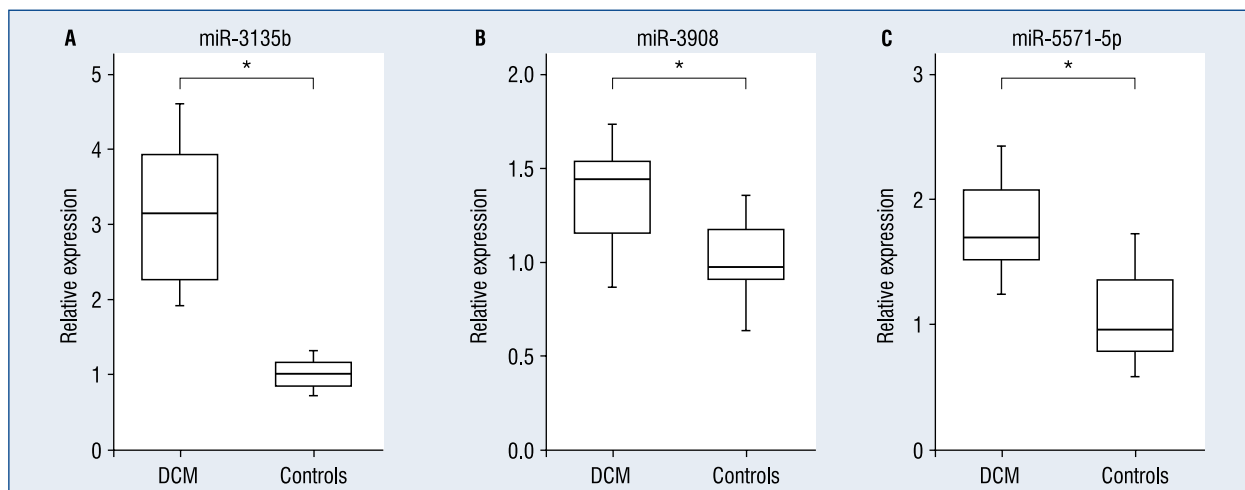


Figure 2. Levels of miR-3135b (A), miR-3908 (B), and miR-5571-5p (C) in dilated cardiomyopathy (DCM) patients compared with controls by reverse-transcription polymerase chain reaction. Data are presented as fold over internal control -miR-191-5p; *p < 0.05.

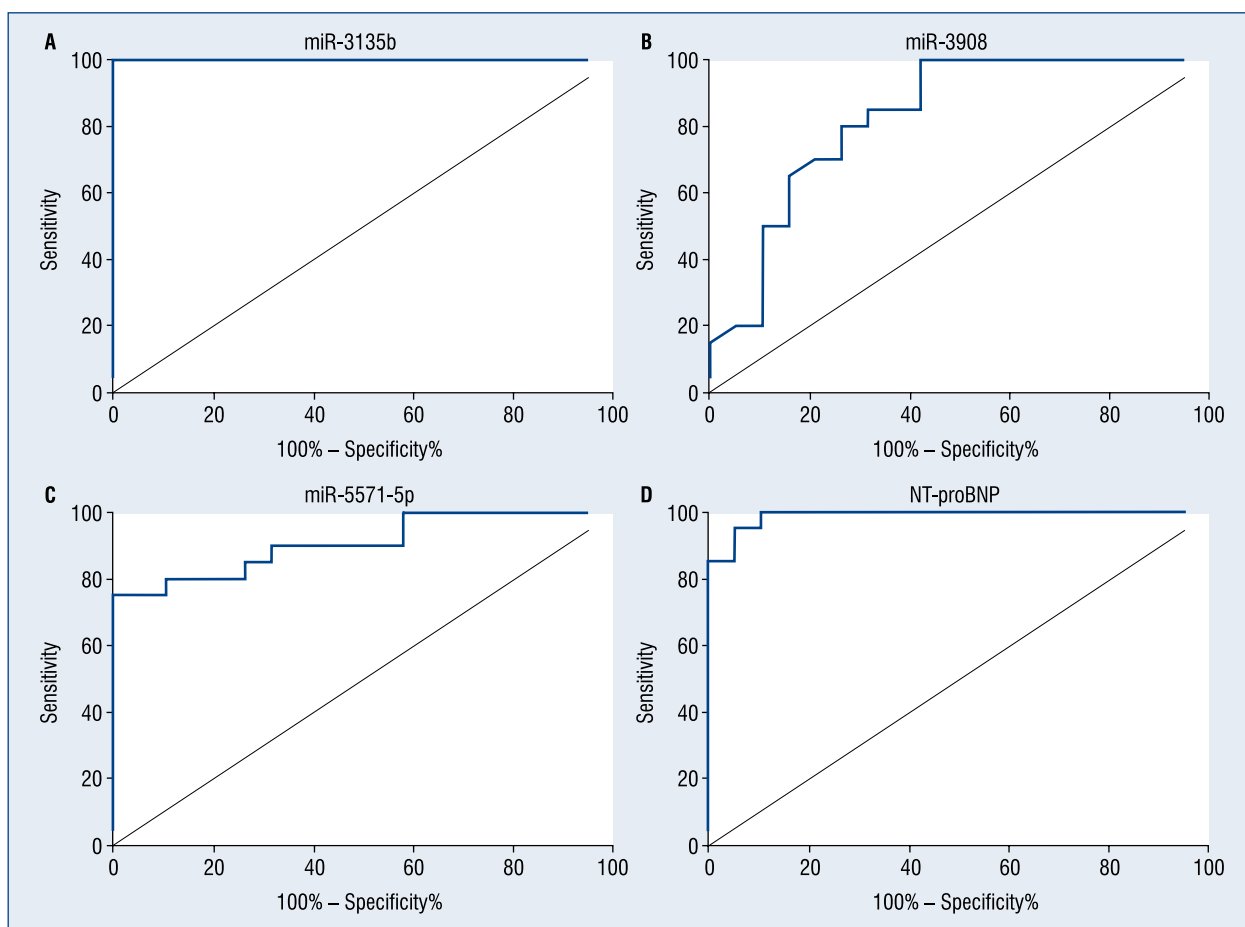


Figure 3. Receiver operating characteristic (ROC) analysis. ROC curves for miR-3135b (A), miR-3908 (B), and miR-5571-5p (C) showed the area under the ROC curve (AUC) to be 1.00, 0.83 (95% CI 0.70–0.96), and 0.91 (95% CI 0.82–1.00), respectively. Additionally, N-terminal-pro-B type natriuretic peptide (NT-proBNP) values were used for ROC analysis, and the AUC was 0.99 (95% CI 0.97–1.00) (D).

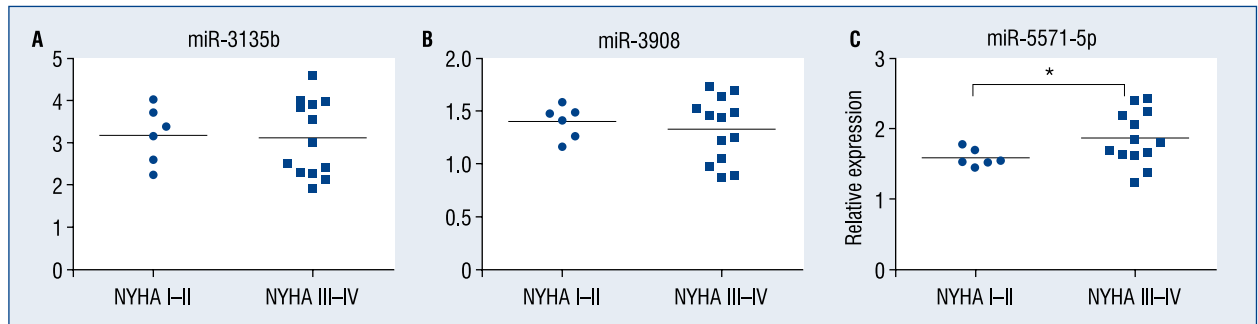


Figure 4. Significant associations between symptom severity and individual microRNA. There are no significant associations between New York Heart Association (NYHA) and miR-3135b (A) or miR-3908 (B). Additionally, more severe NYHA functional classification is associated with higher miR-5571-5p levels (C); * $p < 0.05$.

classification was based on the severity of symptoms and physical activity. We compared the miRNAs between NYHA I-II ($n = 6$) and NYHA III-IV ($n = 13$). Interestingly, the elevated miR-5571-5p ($p < 0.05$) had a significant association with more severe NYHA classification (Fig. 4). In addition, miR-3135b and miR-3908 had no significant difference between NYHA I-II and NYHA III-IV.

Discussion

As the roles of miRNAs in cardiovascular diseases are increasingly recognized, we aimed to investigate the potential of circulating miRNAs as biomarkers in DCM. Our study identified a novel cluster of plasma miRNAs (miR-3135b, miR-3908, and miR-5571-5p) that was significantly upregulated in DCM patients. In addition, miR-3135b and miR-3908 had the discriminatory power to differentiate between healthy controls and DCM patients nearly similar to the classical NT-proBNP, and miR-5571-5p levels had significant correlation with symptom severity.

Numerous studies have focused on alterations in the levels and compositions of circulating miRNAs in different cardiovascular conditions and pathologies [18, 19]. Upon that, we demonstrated that plasma miR-3135b, miR-3908 and miR-5571-5p might be candidate biomarkers for DCM for the first time. Fan et al. [20] identified miR-423-5p as a biomarker for HF caused by DCM. However, we did not find miR-423-5p levels in DCM to differ from those in the controls. The stage of disease (acute, chronic, treated or untreated), co-morbidity, selected controls and internal references may underlie some of the lack of concordance between these reports. Furthermore, the ROC analysis showed that miR-3135b and miR-5571-5p have

similar diagnostic powers for DCM as NT-proBNP. Thus, miRNA signatures in plasma can potentially be developed into biomarkers for HF in the future and could act as complements to NT-proBNP in some special situations.

The stability of circulating miRNAs raises the intriguing speculation that they are taken up by distant cells to regulate their gene expression. Currently, the potential function of extracellular miRNAs is being studied intensively, and some studies have confirmed that circulating miRNAs may indeed function in cell-to-cell communication [21, 22]. Therefore, the differential expression of miRNAs in plasma in DCM might have its pathophysiological effect and might be the new therapeutic target. Moreover, we discovered that plasma miRNA signature was not analogous to the cardiac miRNA expression pattern, providing new sight into the source and release method of circulating miRNAs.

Improvement in symptoms of HF patients caused by DCM is one of the two major goals of treatment. The symptomatic severity was evaluated by questionnaire on health-related quality of life, 6-min walk test, NYHA classification, NT-proBNP concentration, and other criteria. However, some of the aforementioned methods could be hampered by many factors, including educational background, depression, and co-morbidity (such as chronic obstructive pulmonary disease or osteoarthritis), or could have some limitations in particular situations, including unstable angina pectoris and acute myocardial infarction. NT-proBNP was associated with a wide variety of cardiac and non-cardiac causes [23–26], and some patients with advanced HF had normal NT-proBNP levels or had falsely low NT-proBNP levels. Our study showed the expression levels of miR-5571-5p were in line with

the NYHA classification. Thus, miR-5571-5p might be an objective biomarker for symptomatic severity and might be complementary to pre-existing evaluation methods. Furthermore, the clinically guided up-titration strategy was reported to be suboptimal in achieving target doses of pharmacological agents in HF, which limited the therapeutic effects of the evidence-based HF medications [27–29]. In addition, some meta-analyses concluded that NT-proBNP-guided therapy reduced all-cause mortality in patients with HF compared with usual clinical care [30–32]. Thus, plasma miR-5571-5p might be a candidate biomarker to monitor the disease progression and guide reasonable therapy.

To investigate the physiological functions of the dysregulated miRNAs in response to DCM, the potential targets of the 3 miRNAs were predicted, sequentially, with the commonly used miRNA target prediction software program — TargetScan (<http://www.targetscan.org/>). We found 322 putative targets for miR-3135b and 314 putative targets for miR-3908; miR-5571-5p was not in the TargetScan database. The downregulated genes (targets of the upregulated miRNAs) were successfully annotated with gene ontology analysis (<http://geneontology.org/>). It was easily observed that target genes of miR-3135b and miR-3908 were associated with HF-related biological processes, especially the regulation of cardiac muscle contraction, regulation of calcium ion transport, cardiac cell differentiation, negative regulation of cellular process, regulation of metabolic process, regulation of body fluid levels and cardiac myofibril assembly. All of these imply that the 3 miRNAs play important roles in HF. Such assessment could increase the confidence in the potential use of these circulating miRNAs as potential biomarkers for DCM. Moreover, it is also a step toward further functional studies of these miRNAs.

Limitations of the study

There are several limitations to this study. One of the major limitations of this study is the sample size, as the results need to be confirmed in a larger population. Secondly, we recruited clinically stable patients who had not received acute treatment for some period of time, and we cannot be certain regarding possible interference of chronic medication. Finally, the precise sources of circulating miRNAs remain largely unknown.

Conclusions

In summary, we reported that the expression levels of miR-3135b, miR-3908, and miR-5571-5p

in plasma might be used as biomarkers for DCM, and increased miR-5571-5p expression levels were found to be associated with a poor NYHA classification. Our work demonstrates that they are promising candidates for this challenge.

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Conflict of interest: None declared

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