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Original Article

Circulating miR-21 and miR-423-5p as biomarkers for heart failure in heart valve disease patients

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Abstract: Objective: The potential diagnostic value of circulating miRNAs in heart failure (HF) due to heart valve disease (VHD) remains elusive. The purpose of this study was to investigate the potential value of serum miR-21 and miR-423-5p for HF diagnosis. Methods: Serum samples from 60 VHD patients with HF were collected for investigation, and 60 healthy subjects' samples were used as the control. The expression level of serum miR-21 and miR-423-5p was measured by QRT-PCR. ROC curve analysis and Pearson correlation analysis were performed to determine the diagnostic value. Results: The expression of miR-21 and miR-423-5p in sera was markedly increased in VHD patients than normal control (both $P < 0.05$). Besides, serum miR-21 and miR-423-5p showed independent and effective diagnostic utilities for HF due to VHD. The AUC of serum miR-423-5p was 0.805 for distinguishing the VHD patients with pulmonary hypertension (PH). Conclusions: Serum miR-21 and miR-423-5p showed potential diagnostic values on HF due to VHD. Moreover, serum miR-423-5p could function as an effective biomarker for diagnosing VHD patients with PH.

Keywords: Circulating microRNA, miR-21, miR-423-5p, valvular heart disease, heart failure, ROC curve

Introduction

As a debilitating chronic disease, heart failure (HF) has become one of the most tough health problems worldwide. The prevalence of HF is expected to accelerate in the next couples of years mainly due to the unhealthy habits established in modern society [1, 2]. HF is often characterized with ventricular filling and decreased ejection fraction [3, 4]. At the late stage or severe state of multiple vascular cardiac diseases, patients were often suffered from HF [5]. In order to diagnose HF more accurately and precisely, significant efforts have been made to indentify a series of biomarkers for HF diagnosis in clinic. The B-type natriuretic peptide (BNP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) were considered as promising biomarkers for HF diagnosis [6]. Also, BNP and NT-proBNP can serve as adjunctive biomarkers for evaluating the severity and defining the progression of HF in congenital cardiac diseases [7, 8]. Although great values were implied for BNP and NT-proBNP in the prediction of HF, there are still some limitations and the results from several studies remains controversial. It was reported that single BNP measurement was not enough to determine the degree of congestion in acute systolic HF [9]. Recent study showed an inverse association between BNP and NT-proBNP levels and body mass index, indicating the limitation in diagnosis of obesity patients with HF [10]. Thus, novel non-invasive biomarkers were required for noninvasive diagnosis on HF.

Valvular heart disease (VHD) has become an increasingly common type in patients who suffered from age-related cardiac disorders [11]. Although the prevalence of VHD gradually decreased, the incidence of VHD was still estimated at approximately 2.5% in industrialized countries [12]. With the progression of VHD, the endocardiosis and cardiac dysfunction would eventually lead to HF [13]. We discovered that after interval treatments on patients with VHD, the accuracy and specificity of NT-proBNP on evaluating the severity of VHD reduced significantly. Moreover, to date, the diagnostic values of biomarkers for VHD patients with pulmonary

hypertension (PH) still remain limited [14, 15]. Hence, we aimed to find alternative biomarkers for VHD diagnosis in the present study.

MicroRNAs (miRNAs), as a group of non-coding RNAs with 21-23 nucleotides in length, have been proven to serve critical roles in the process of various human cardiac diseases, such as myocardial infarction, chronic myocarditis, as well as HF [16-18]. Many reports revealed that aberrant expression patterns of miRNAs were associated with cardiac dysfunction, indicating that miRNAs might be involved in the mechanisms of heart diseases and qualified to be promosing biomarkers for HF [19-22]. Multiple miRNAs in biofluids were recognized as circulating biomarkers for HF [23, 24]. Wong et al. discovered that the specific miRNA panels enjoyed great discrimination power than NT-proBNP in HF with reduced and preserved left ventricular ejection fraction [25]. Moreover, the plasma miR-30d level was identified to be closely associated with response to cardiac resynchronization therapy (CRT) in HF patients with dyssynchrony [26]. The article of Lai et al. also demonstrated circulating miRNAs could be biomarkers in the early stage of HF in Chinese population [27]. The miRNAs in VHD patients were also identified as potential biomarkers for disease progression, while the diagnostic value remained unclear [28].

A study concerning Dachshunds suggested that miR-21 showed a trend of down-regulation in the mild to moderate VHD-HF group [29]. Moreover, miR-423-5p expression was increased in HF patients than control despite the body mass index, indicating it might be a convincing predictor for HF [30]. Meanwhile, through our clinical validations, we predicted miR-423-5p expression might be associated with VHD failure patients with HF. Therefore, this study intended to confirm the diagnostic value of miR-21 and miR-423-5p in HF due to VHD.

Materials and methods

Subjects

Sixty VHD patients with HF and sixty healthy individuals without significant cardiovascular disease from Sichuan Provincial People's Hospital (Chengdu, China) between August 2014 and April 2016 were recruited in the present study. The disease history of VHD patients was documented in hospitalized medical records. The assistance examinations such as general 12-leads ECG (Electrocardiograph), TTE (transthoracic echocardiography), and detection of expression levels of specific indicators including NT-proBNP, hsCRP (hyper-sensitive C-reactive protein) as well as physical examinations were conducted on participants. The patients who was over 50 years old and suspected to be suffered from coronary diseases were underwent coronary angiography. The state of HF was clarified according to NYHA (New York Heart Association) class [31, 32]. The patients included in this research should meet the following criterion: (1) age over 18 years old; (2) confirmed to be suffered from VHD through the above gold examinations; (3) owned the definite symptoms of HF, which were in accordance with the NYHA II-IV class diagnosis criterion. The exclusion criterion were listed below: (1) patients who had the history of myocardial infarction or underwent acute HF, or coronary artery stenosis area over 75% by coronary angiography, or treated stent implantation before; (2) patients with primary pulmonary diseases; (3) patients with hyperthyroidism, hypothyroidism, and chronic anemia; (4) who suffered from primary or secondary hypertension. The serum samples from 60 healthy subjects were also enrolled as control. Furthermore, patients were diagnosed with PA according to the updated WHO clinical classification [33], inclusion criteria including mean pulmonary artery pressure (mPAP) $\geq$ 25 mmHg, pulmonary wedge pressure $\leq$ 15 mmHg.

For each participant, 5 ml fasting blood was withdrawn in ethylenediaminetetraacetic acid-containing tubes. The whole blood samples were processed for serum extraction immediately, and the serum samples were stored at -80°C until further processing.

The study was approved by the Ethic Committee of Sichuan Provincial People's Hospital and performed in compliance with the Helsinki Declaration. Written informed consent documents were obtained from all the participants prior to their inclusion.

Cardiac ultrasound examination

LVEDd (left ventricular end-diastolic diameter) and LVEF (left ventricular ejection fraction) were measured based on Simpson biplane method [34]. The max reversal flow velocity of tricuspid valve was detected by Doppler ultrasound at apical four chamber view.

Table 1. The sequences of QRT-PCR primers

Gene name	Primer sequence (5' to 3')
miR-21	ACACTCCAGCTGGGTAGCTTATCAGACTGAT ACTGGTGTCGTGGAGTCG
miR-423-5p	ATGGTTCGTGGGTGAGGGGCAGAGA GCGAGAGCAGGGTCCGAGGTATTC
U6	CTCGCTTCGGCAGCACA AACGCTTCACGAATTTGCGT

Table 2. Clinical characteristics of participants enrolled in this study

Characteristics	VHD	Control	*P* value
Case number	60	60	
Age (year)	60.25 ± 12.03	55.92 ± 9.18	0.497
Gender (male/female)	25/35	28/32	0.581
Log (NT-proBNP)	3.89 ± 0.46	1.18 ± 0.51	< 0.001
HsCRP (mg/L)	6.38 ± 2.78	3.19 ± 1.80	< 0.001
LVEDd (mm)	52.14 ± 7.69	50.05 ± 5.21	0.084
LVEF (%)	56.23 ± 11.75	N.A	

NT-proBNP, N-terminal pro-brain natriuretic peptide; HsCRP, High-sensitive C-reactive protein; LVEDd, Left ventricular end-diastolic diameter; LVEF, Left ventricular ejection fraction. N.A = Non-available.

RNA extraction and quantitative reverse transcription-PCR (QRT-PCR)

The RNA from serum samples was isolated using mirVana™ PARIS miRNA isolation kit (Ambion Inc, Austin TX, USA) according to the protocol. RNA sample concentration and purity were assessed via NanoDrop ND-1000 spectrophotometer (Nanodrop, USA). 40 ng of total RNA was reverse transcribed to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). QRT-PCR reactions for miRNAs were performed using the TaqMan MicroRNA PCR Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The sequences of the primers were listed in **Table 1**. The $2^{-\Delta\Delta Ct}$ cycle threshold (Ct) method was used to investigate the relative expression levels of target miRNAs [35, 36], which were normalized to that of U6. Each experiment was repeated triplicate.

Statistical analysis

All statistical analyses were performed with SPSS 21.0 software (Chicago, Illinois, USA). Data were presented in the form of mean ± standard deviation (SD). Receiver-operating characteristic (ROC) curves were plotted and the area under the ROC curve (AUC) was calculated to evaluate the diagnostic value of using serum levels of miRNAs. Student's *t*-test or chi-square test was conducted for the comparison between different groups when appropriate. Pearson correlation analysis was used to estimate the association between two parameters. All statistical tests were 2-tailed and *P* values < 0.05 were considered statistically significant.

Results

Clinical characteristics of participants

60 VHD patients with HF were included in this study, whose average age was 60.25 ± 12.03 years, with 25 male and 35 female. Meanwhile, 60 healthy subjects underwent routine physical examinations were set as control, whose average age of 55.92 ± 9.18 years, with 28 male and 32 female. There was no significant difference of age, gender, and several other factors between VHD group and control group. However, log (NT-proBNP) in VHD group (3.89 ± 0.46) was markedly higher than that in control group (1.18 ± 0.51), as well as the level of HsCRP (6.38 ± 2.78 mg/L versus 3.19 ± 1.80 mg/L). More details about the clinical characteristics of participants were documented in **Table 2**.

Overexpression of serum miR-21 and miR-423 in VHD patients

As shown in **Figure 1A**, serum miR-21 was significantly up-regulated in VHD group rather than that in control group (*P* < 0.05). Similarly, serum miR-423-5p of VHD group expressed significantly higher than that of control group (*P* < 0.05). According to Pearson correlation analysis, we found that the expression levels of serum miR-21 and miR-423-5p was separately positive correlated with HsCRP level in VHD patients, as demonstrated in **Figure 1B**. Besides, serum miR-21 level was also closely correlated to serum miR-423-5p level in VHD group.

The diagnostic value of serum miR-21 and miR-423-5p for HF due to VHD

The ROC curves of serum miR-21, serum miR-423-5p and NT-proBNP were plotted and pre-

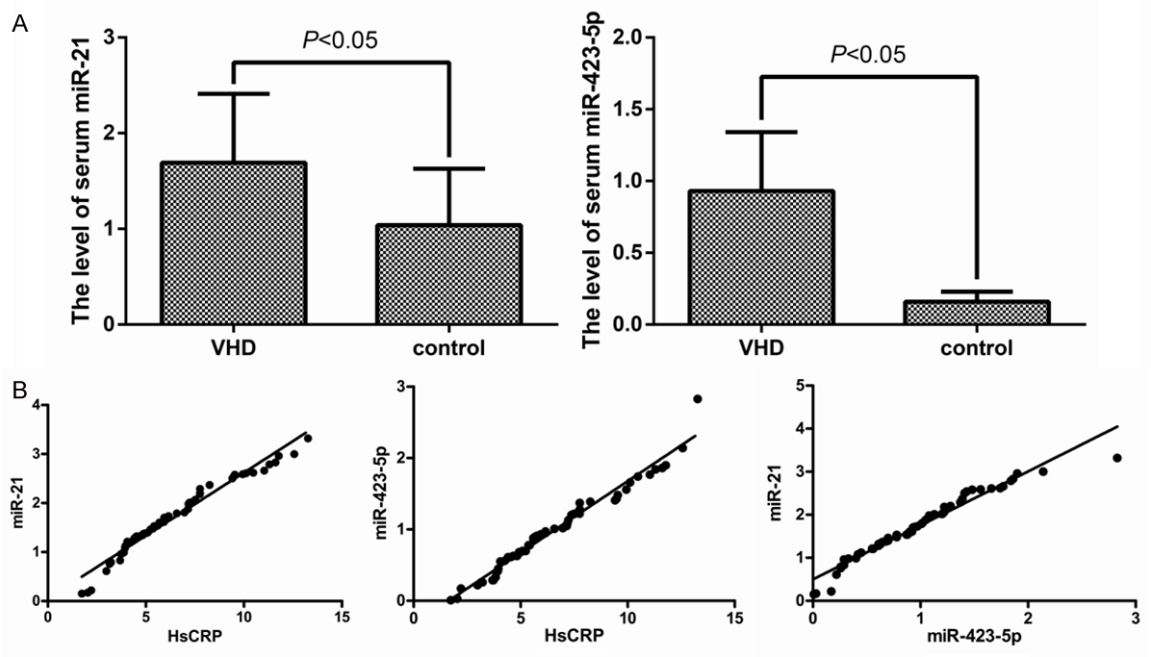

Figure 1. Relative expression of serum miR-21 and miR-423-5p detected by QRT-PCR. A. The significant higher expression level of miR-21 and miR-423-5p in VHD group rather than control group (all $P < 0.05$). B. The positive correlation among miR-21, miR-423-5p, and HsCRP in VHD group.

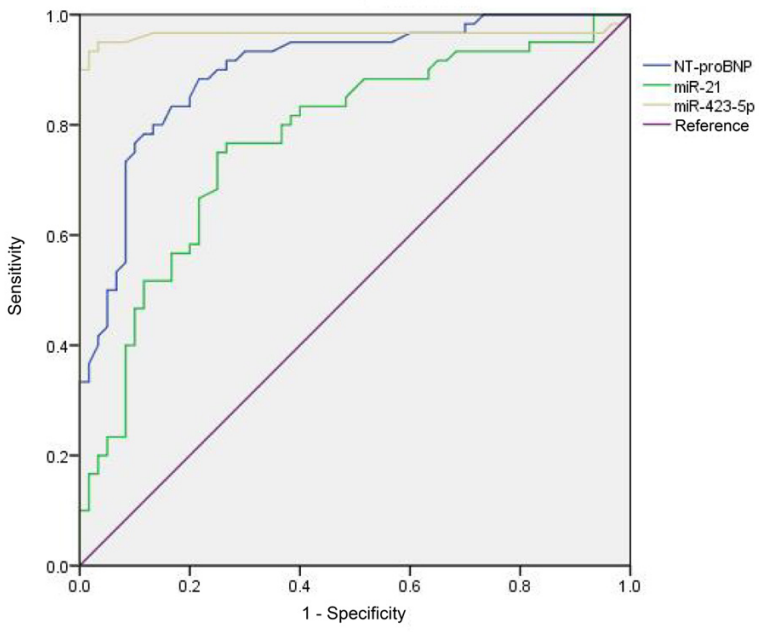

Figure 2. ROC curves of miR-21, miR-423-5p and NT-proBNP.

sented in **Figure 2**. With the AUC of 0.772, it showed that serum miR-21 had the relative promising diagnosis utility for HF due to VHD, which might function as a supplementary biomarker for HF diagnosis in clinic. Moreover, the AUC of serum miR-423-5p was 0.965, even higher than NT-proBNP (with the AUC of 0.900), indicating it could serve as an accurate diagnosis biomarker for HF in VHD patients.

Serum miR-423-5p could determine VHD with PH

Interestingly, as shown in **Figure 3A**, we observed that serum miR-423-5p had probable higher levels in VHD patients with PH (pulmonary hypertension). Hence, the VHD patients were allocated into two groups based on whether suffered from PH. The clinical characteristics of VHD with PH or non-PH patients were presented in **Table 3**. It turned out that serum miR-423-5p level in VHD with PH group was significantly higher than that in VHD with non-PH group ($P < 0.05$), while no significant difference was found on serum miR-21 and NT-proBNP (which did not show in

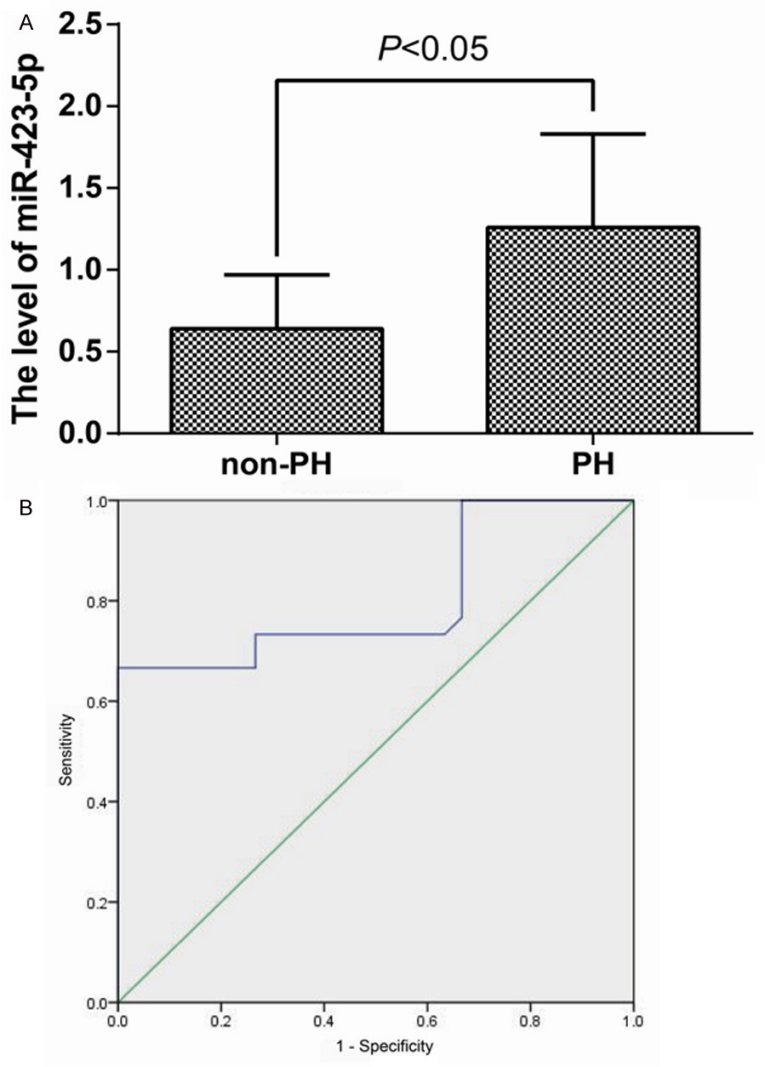

Figure 3. Relationship between miR-423-5p and VHD patients with PH. A. The level of miR-423-5p in VHD patients with PH was significantly higher than that in VHD with non-PH group, $P < 0.05$; B. The ROC curve of miR-423-5p diagnosing VHD with PH.

the article). Next, the ROC curve of serum miR-423-5p on determining VHD with PH was performed. The AUC of serum miR-423-5p on VHD with PH was 0.805 (**Figure 3B**); indicating serum miR-423-5p could determine VHD with or without PH.

Discussion

MiRNAs, as a set of conserved non-coding small RNAs, could regulate targets genes post-transcription involved in the process of cell proliferation, differentiation, apoptosis, and angiogenesis by directly targeting specific mRNAs. After miRNA expression in serum was discovered, multiple studies clarified miRNA could be transferred through cell in various ways, and manage to avoid the degradation of RNase by interacting with other medium, ending with stably exiting in extracellular fluid, including serum, plasma, urine, salvia, sweat and even tear [37, 38]. These findings provided sufficient evidences for circulating miRNAs detection in clinical application. Compared with miRNAs from tissue samples, the circulating miRNAs are easily to acquire, convenient to be measured repeatedly, as well as have the advantages of great stability and excellent sensitivity [39]. Recently, the circulating miRNAs have been reported as important biomarkers for various cardiac diseases [40]. The potential diagnostic utility of circulating miRNAs (miR-125a-5p, -190a, -550a-5p, and -638) was identified for discriminating HF with reduced left ventricular ejection fraction [25]. Besides, the diagnostic and prognostic value of circulating miRNAs in HF was detected through different ways [41, 42]. However, the diagnostic value of miRNAs in HF with VHD was poorly investigated.

MiR-21 could promote cardiac fibrosis by regulating Bcl-2, resulting in the development of HF [43]. On one hand, studies showed miR-21 was overexpressed in cardiac myofibroblasts and deficient myocardial cells [44, 45]. On the other hand, Sayed et al. discovered miR-21 as a downstream effector of Akt mediating the suppression of myocaidial cells apoptosis, which means the overexpression of miR-21 may also alleviate HF [46]. The role of miR-21 in HF seems contradictory, which might be explained that the expression of miR-21 was associated with the progression of human cardiac diseases. Other study suggested that miR-21 repressed PDCD4 to enhance valve cell migra-

Table 3. Clinical characteristics of VHD patients with PH and with non-PH

Characteristics	PH	non-PH	*P* value
Case number	25	35	
Age (year)	60.17 ± 12.17	60.33 ± 12.11	0.984
Gender (male/female)	11/14	14/21	0.757
Log (NT-proBNP)	3.83 ± 0.45	3.95 ± 0.47	0.339
HsCRP (mg/L)	6.09 ± 2.75	6.67 ± 2.77	0.441
LVEDd (mm)	52.14 ± 7.69	50.05 ± 5.21	0.237
LVEF (%)	55.60 ± 12.15	56.85 ± 11.51	0.697
Serum miR-21	1.66 ± 0.58	1.72 ± 0.84	0.766
Serum miR-423-5p	1.26 ± 0.57	0.64 ± 0.33	< 0.001

NT-proBNP, N-terminal pro-brain natriuretic peptide; HsCRP, High-sensitive C-reactive protein; LVEDd, Left ventricular end-diastolic diameter; LVEF, Left ventricular ejection fraction.

tion during cardiac valvulogenesis, indicating the potential association between miR-21 and VHD [47]. In the present study, the expression level of serum miR-21 in VHD group was significantly higher than that of control group. It revealed that serum miR-21 might have a potential diagnostic value for HF in VHD patients. Although the predicting efficacy of serum miR-21 was not very sufficient, it could serve as an effective combined indicator with serum miR-423-5p.

The role of miR-423-5p was also investigated in multiple studies. Although Bauter et al. found circulating level of miR-423-5p was not a useful biomarker for indicating left ventricular remodeling after myocardial infarction, other article showed miR-423-5p in plasma owned great prognostic value of acute HF [48, 49]. While miR-423-5p was enriched in pericardial fluid, whose expression pattern was obviously different and independent compared to others circulating microRNAs such as miR-133a, miR-126, and miR-92a, indicating miR-423-5p may serve as a good biomarker for HF due to VHD [50]. Our study revealed that serum miR-423 in VHD group expressed markedly higher than that in control group. Interestingly, we discovered that miR-423-5p was significantly associated with pulmonary hypertension in VHD patients. As the primary lung diseases patients were excluded from this study, the high pressure in pulmonary artery of participants was caused by VHD, which characterized as PH-LHD (pulmonary hypertension due to left heart disease). According to recent reports, about 2/3 of chronic HF patients were suffered from LHD-PH [51]. It was believed that the severe chronic HF was the common reason leading to LHD-PH, and the incidence of diastolic HF with LHD-PH was higher than systolic HF with LHD-PH [52, 53]. In our research, the VHD patients were divided into two groups based on whether suffered from pulmonary hypertension (PH). The data showed that miR-423-5p in serum had greater sensitivity than NT-proBNP in diagnosis of VHD with PH. Accordingly, serum miR-423-5p could be considered as a promising prognostic biomarker for evaluating VHD with PH.

Some intriguing relationships were also observed in this study. The expression levels of serum miR-21 and miR-423-5p in VHD patients were positively associated with HsCRP, indicating these two miRNAs might be involved in the mechanism of inflammation on HF. Studies manifested that miR-21 may activate NF-kB or target PDCD4, one of the stimuli inflammation proteins, and then regulate the inflammation process [47, 54]. However, the role of miR-423-5p in inflammation process of cardiac diseases needed to be further investigated in future. The positive association between circulating levels of miR-21 and miR-423-5p in VHD patients may also need to be explained. It is speculated that miR-21 and miR-423-5p could probably regulate the cardiac function in cooperation, while it needed further functional study for validation.

In conclusion, we discovered that serum miR-21 and miR-423-5p were up-regulated in VHD patients and could serve as potential and independent biomarkers for HF due to VHD diagnosis. Moreover, miR-423-5p showed great sensitivity for evaluating severity of VHD patients with PH and owned excellent diagnostic utility for VHD with PH prognosis. It is expected more studies would be performed to confirm the results of our research, and the mechanisms of miR-423-5p and miR-21 remained to be clarified in the near future.

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Disclosure of conflict of interest

None.

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