CLINICAL RESEARCH

Circulating microribonucleic acids miR-1, miR-21 and miR-208a in patients with symptomatic heart failure: Preliminary results

ARN circulant (miR-1, miR-21 et miR-208a) chez les patients ayant une insuffisance cardiaque symptomatique : résultats préliminaires

Grażyna Sygitowicz a,*,1, Mariusz Tomaniak b,1, Olga Błaszczyk c, Łukasz Kołtowski b, Krzysztof J. Filipiak b, Dariusz Sitkiewicz a

a Department of Laboratory Medical Diagnostics, Medical University of Warsaw, Warsaw, Poland
b 1st Chair and Department of Cardiology, Medical University of Warsaw, Warsaw, Poland
c Department of Pharmacogenomics, Medical University of Warsaw, Warsaw, Poland

Received 13 January 2015; received in revised form 8 July 2015; accepted 22 July 2015
Available online 21 October 2015

KEYWORDS
Microribonucleic acid;
Galectin-3;
NT-proBNP;
Cardiovascular disease;
Heart failure

Summary
Background. — Cardiomyocytes produce a wide variety of bioactive molecules that regulate numerous physiological and pathophysiological processes. Recently, it has been recognized that changes in microribonucleic acid (miRNA) expression may lead to cardiac dysfunction.
Aims. — To assess the expression of circulating miRNAs (miR-1, miR-21 and miR-208a) in patients with symptomatic heart failure (HF), and to investigate the relationship between expression of these miRNAs and secretion of N-terminal pro-B-type natriuretic peptide (NT-proBNP) and galectin-3.

Abbreviations: HF, heart failure; LVEF, left ventricular ejection fraction; miRNA or miR, microribonucleic acid; NT-proBNP, N-terminal prohormone of B-type natriuretic peptide; NYHA, New York Heart Association; qRT-PCR, quantitative real-time polymerase chain reaction.
* Corresponding author. Department of Laboratory Medical Diagnostics, Medical University of Warsaw, 1, Banacha Street, 02-097 Warsaw, Poland.
E-mail address: gsygitowicz@poczta.onet.pl (G. Sygitowicz).
1 G. Sygitowicz and M. Tomaniak contributed equally to this article.

http://dx.doi.org/10.1016/j.acvd.2015.07.003
1875-2136/© 2015 Elsevier Masson SAS. All rights reserved.
Background

Micronucleic acids (miRNAs) are recently discovered, small, endogenous, single-stranded, non-coding RNAs comprising 18–25 nucleotides. They are initially transcribed as primary-miRNA with a characteristic stem-loop structure. The primary-miRNA stem-loop structure is cleaved by the enzyme Drosha to ~70 nucleotides in length within the nucleus, and is called precursor-miRNA. Precursor-miRNAs are then exported from the nucleus into the cytoplasm, where the mature miRNA strands regulate gene expression post transcriptionally [1].

More than 600 miRNAs have been discovered; they participate in various physiological processes, including heart development, but are also involved in the pathogenesis of heart failure (HF), myocardial hypertrophy and arrhythmia [2,3]. Cardiac fibroblasts play a key role in the adverse myocardial remodelling that occurs with hypertension,
myocardial infarction and HF. The involvement of miRNAs in these pathological processes has been recently recognized. Indeed, altered miRNA expression during cardiac remodelling has been reported in mice and humans [4]. Aberrant expression of selected miRNAs has been linked with various pathological conditions such as cardiac fibrosis [5]. Recent evidence suggests that miRNAs are differentially expressed in the failing myocardium and play an important role in the progression of HF [6,7]. miRNAs are fundamentally involved in and have an effect on cardiac fibrosis [5,8]. In addition, miRNA controls cardiac fibroblast differentiation [9].

Among the numerous miRNAs reported to influence the process of maladaptive cardiac remodelling, the strongest preclinical and clinical data appear to support the roles of miR-1, miR-21 and miR-208 in this process [10]. miR-1 is one of the most abundant miRNA in the heart, and plays a protective role against cardiac hypertrophy or HF by regulating several hypertrophy-associated genes, which include transcription factors, receptor ligands, apoptosis regulators and ion channels, as well as exacerbating arrhythmogenesis when overexpressed [10–14]. Predominantly expressed in cardiac fibroblasts, miR-21 is one of the most greatly upregulated miRNAs during cardiac hypertrophy; miR-21 induces cardiac fibrosis and protects cardiomyocytes against apoptosis. Interestingly, pharmacological antagonism of miR-21 suppresses cardiac remodelling after pressure overload in the heart [13]. Furthermore, in experimental studies, miR-208a was sufficient to induce cardiac remodelling and modulate the expression of hypertrophy-associated genes, and the systemic delivery of miR-208a inhibitors prevented pathological myosin switching and cardiac remodelling while improving cardiac function and survival [10,12].

The aims of this study were to assess the level of expression of miRNA-1, miRNA-21 and miRNA-208a in the sera of patients with symptomatic HF, and to evaluate the relationship between serum miRNAs and the serum concentrations of galectin-3 and N-terminal prohormone of B-type natriuretic peptide (NT-proBNP) in relation to the severity of decompensated HF.

Methods

Study population

Sixty-one patients hospitalized for symptomatic HF in the 1st Chair and Department of Cardiology, Medical University of Warsaw, between October 2013 and August 2014 were enrolled in this prospective, single-centre registry. Symptomatic HF comprised acute decompensated heart failure — de novo or decompensation of chronic heart failure [15] — as well as symptomatic chronic HF, with New York Heart Association (NYHA) class ≥ II.

Patients with symptomatic HF requiring hospitalization were included within 24 hours of admission. The inclusion criteria also involved clinical or radiological signs of pulmonary congestion and left ventricular ejection fraction (LVEF) <50%, computed according to Simpson’s method [16].

Patients with acute coronary syndromes, active infection, Cushing’s syndrome, primary hyperaldosteronism, Addison’s disease, liver cirrhosis, acute renal failure, paraneoplastic syndromes, subarachnoid haemorrhage, chronic lung diseases, acute and chronic pulmonary embolism, and myopathies were excluded from the study.

Data on time of chronic HF diagnosis, risk factors, cardiovascular conditions, family history and previously used medications were collected. All enrolled patients underwent a physical examination, with special emphasis on clinical features of HF, an electrocardiogram and routine laboratory tests.

The local ethics committee approved the protocol of the study, and all patients provided written informed consent.

Echocardiographic studies

Each patient underwent a complete echocardiographic examination using an iE33 ultrasound system (Philips, Amsterdam, Netherlands). LVEF was calculated according to Simpson’s method [16].

Sample collection

Blood samples were collected during the 24 hours after admission. Blood samples were collected after overnight fasting into plastic tubes with clot activator. Large gauge needles were used to avoid potential platelet activation and miRNA release, as suggested by Witwer et al. [17].

Circulating RNA extraction

Total RNA was extracted from 300 μL of serum using a commercial column-based system (NucleoSpin® miRNA Plasma Kit; Macherey-Nagel, Düren, Germany), according to the manufacturer’s instructions. Before RNA isolation, serum samples were thawed and vortexed. Total RNA was eluted by adding 30 μL of ribonuclease-free water to the membrane of the spin column. RNA was stored at −80°C until further analysis. A fixed volume of 4 μL RNA solution from the 30 μL RNA isolation eluate was put into the reverse transcription reaction.

Circulating miRNA reverse transcription and amplification

Complementary deoxyribonucleic acid (cDNA) synthesis was performed with the Universal cDNA Synthesis kit (Exiqon, Vedbaek, Denmark) according to the manufacturer’s protocol. The reaction volume was 20 μL. Endogenous abundance of miRNA was measured using quantitative real-time polymerase chain reaction (qRT-PCR) on the Viia™ 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The triplicate reactions in 15 μL were carried out using Exi-LIGHT SYBR® Green Master Mix (Exiqon, Vedbaek, Denmark) with the locked nucleic acid (LNA™) primer sets for miRNA-1 (MIMAT0000416), miRNA-21-5p (MIMAT000076) and miRNA-208a (MIMAT000241), plus miRNA-103-3p (MIMAT000101) as an internal control. In each reaction, 6 μL of 10× diluted cDNA was used. Thermal cycling consisted of an initial denaturation followed by 50 cycles of amplification. A melt curve was performed after each cycle to indicate the specificity of the primers. The threshold cycle (Ct) for each reaction was determined, defined as a cycle number at which exponential phase of the amplification plot crosses the threshold, significantly above the background. If the Ct
value was >40, the miRNA concentration was considered undetectable. Relative expression of investigated miRNAs compared with healthy volunteers was calculated using the \( \Delta \Delta Ct \) method, the most usual way to analyse gene expression experiments. For each sample, \( \Delta Ct \) was obtained, which indicates the difference in Ct values between the miRNA of interest and the endogenous control (miR-103-3p) \( (\Delta Ct = C_{miR-x} - C_{miR-103}) \). Then, the mean \( \Delta Ct \) value for the control group was calculated and subtracted from the \( \Delta Ct \) value obtained for the individuals from the experimental group \( (\Delta \Delta Ct = C_{miR-x} - C_{miR-103}) \). To find the relative expression, the fold value was calculated using the \( 2^{-\Delta \Delta Ct} \) formula. A fold value \( (2^{-\Delta \Delta Ct}) \) lower or higher than 1 indicates down- or upregulation, respectively, compared with the healthy controls.

The results are presented as fold change in relation to the miRNA expression in the serum samples of the control subjects, who were 17 age- and sex-matched healthy volunteers, age: 59 ± 12.4 years; 13 (76%) were men. Normalization for variations in RNA input was conducted using miR-103-3p, which, in our samples, varied little between patients, with a coefficient of variation = 7.94%. Moreover, this miRNA was found to be stably expressed in plasma. In addition, we analysed the expression of miR-16, but it occurred to be less favourable, as the coefficient of variation occurred to be higher (11.6%).

Biochemical analysis

Blood samples were collected after overnight fasting into plastic tubes with clot activator. After centrifugation, part of the serum samples were used for determination of the concentrations of creatinine, high-sensitivity C-reactive protein, NT-proBNP, troponin I; the remaining serum was immediately frozen at −70 °C for later measurements of the concentration of galectin-3. Serum galectin-3 concentration was measured using the reagents and instrument from the VIDAS\textsuperscript{®} family (bioMérieux SA, Marcy-l’Étoile, France). The assay principle combines a one-step immunonas assay sandwich method with final fluorescent detection (enzyme-linked fluorescence assay [ELFA]). The concentrations of creatinine, high-sensitivity C-reactive protein, troponin I and NT-proBNP were measured using the Flex\textsuperscript{®} Reagent Cartridge and the Dimension Xpand instrument (Siemens Health Care Diagnostics, Erlangen, Germany).

Statistical analysis

For statistical analyses, the data analysis software system STATISTICA, version 10 (StatSoft, Inc., Tulsa, OK, USA) was used. Continuous variables were tested for normal distribution by the Shapiro-Wilk test. Results for normally distributed continuous variables are expressed as means ± standard deviations, and we used the unpaired Student’s t test to compare mean values. Continuous variables with non-normal distribution are presented as median values and interquartile ranges (range from the 25th to the 75th percentile). Between-group comparisons of distributions were performed using the Mann-Whitney U test and Wilcoxon’s signed-rank sum test. Correlations among continuous variables were assessed using Spearman’s rank correlation coefficient \( r \). Categorical variables are expressed as numbers (percentages) and were compared using Fisher’s exact test. To evaluate the expression levels of miRNAs circulating within the two groups, we decided to use Student’s t test for two independent groups. \( P \)-values < 0.05 were considered statistically significant.

Results

Patients’ characteristics

Sixty-one patients were divided into two subgroups according to NYHA functional class. The first group included 35 patients with cardiac functional classes II and III, with a mean age of 68.8 ± 13.0 years; the second group included 26 patients with cardiac functional class IV, with a mean age of 72.0 ± 10.4 years. There were no statistically significant differences in terms of age and sex between these two groups (Table 1).

Expression of miRNA

miRNA-1 was downregulated in the serum of patients with symptomatic HF. The degree of expression of miRNA-1 decreased with the severity of NYHA class. This effect was greater in patients in NYHA class IV than in those in NYHA class II/III \( (P = 0.007) \) (Fig. 1A). In contrast, miR-21 was overexpressed in patients with symptomatic HF, and the expression of miR-21 was independent of disease severity (Fig. 1B). In both groups of patients (NYHA classes II/III and IV), the concentration of miR-21 was the same \( (2.00 [1.01–4.84] \text{ change fold vs. } 2.092 [1.48–4.46] \text{ change fold}; P = 0.95) \). The qRT-PCR results obtained for miR-208a indicated no amplification or amplification was irreproducible (standard deviation for amplifying replicates >0.5). Moreover, we observed multiple peaks in the melting curves in the reactions with amplification, meaning that the quality of the data was low and inconsistent with specific and reliable detection. Thus, in our study, there is little evidence for the presence of miR-208a in the analysed plasma samples, suggesting no miR-208a leakage from the injured heart into the circulation in the control and symptomatic HF groups.

Relationship between miRNA expression and biomarkers of cardiac function

To examine whether changes in miRNA expression influence cardiac fibrosis and neurohormonal activation in the failing heart, the concentrations of serum galectin-3 and NT-proBNP were determined. We observed significant negative correlation between decreasing miR-1 expression and serum NT-proBNP concentration \( (r = -0.389; P = 0.023) \) in patients in NYHA class II/III (Fig. 2A). However, this correlation was not statistically significant in patients in NYHA class IV \( (r = 0.025; P = 0.90) \). It must also be noted that we did not observe any relationship between changes in miR-1 expression and serum galectin-3 concentration in patients in NYHA class II/III \( (r = -0.299; P = 0.08) \) or NYHA class IV \( (r = -0.109; P = 0.60) \).

Overexpression of miR-21 was significantly correlated with serum galectin-3 concentration in patients in NYHA class IV \( (r = 0.422; P = 0.032; \text{ Fig. 2B}) \). In patients in NYHA
class II/III, however, we did not observe this correlation \( r = -0.092; \, P = 0.60 \). Overexpression of miR-21 did not influence the concentration of NT-proBNP, regardless of disease severity; \( r = -0.075 \) in patients in NYHA class II/III \( (P = 0.67) \); and \( r = -0.182 \) in patients in NYHA class IV \( (P = 0.38) \).

It should be noted that there was a significant positive correlation between serum creatinine and galectin-3 concentrations in patients in NYHA class II/III \( (r = 0.441; \, P = 0.008) \) and NYHA class IV \( (r = 0.640; \, P = 0.0006) \). Interestingly, we found a negative association between creatinine concentrations and miR-1 expression in the NYHA class II/III group \( (r = -0.463; \, P = 0.005) \). There was no statistically significant association between miR-21 expression and renal function variables. Furthermore, we did not observe significant differences between the NYHA class II/III and class IV groups in terms of troponin concentrations \( (0.017 [0.01-1.153] \, \text{ng/mL vs.} \, 0.12 [0.034-0.921] \, \text{ng/mL, respectively}) \).

**Discussion**

The main finding of this study was the observation that in patients with the most exacerbated clinical symptoms of HF...
Microribonucleic acids and heart failure

Figure 1. A. Expression of microribonucleic acid-1 (miR-1) in patients with symptomatic heart failure. B. Expression of microribonucleic acid-21 (miR-21) in patients with symptomatic heart failure. NYHA: New York Heart Association.

(NYHA functional class IV), miR-1 was significantly downregulated. miR-1 is the second most abundant miRNA in the human heart [18]. It has been evaluated mainly in acute myocardial infarction studies, showing notable upregulation and achievement of peak concentrations earlier than cardiac troponins [19]. Evidence of its expression in acute HF has received far less interest so far, and the available data remain controversial [7,14,20–23].

We have presented results indicating that downregulation of the expression of miRNA-1 is correlated with the increase of serum NT-proBNP concentration in patients with symptomatic HF in NYHA class II/III. As there were no patients with acute myocardial infarction included in this study, the miR-1 expression pattern was not influenced by the presence of acute ischaemia or myocardial necrosis, which could potentially increase the expression of this miRNA type [11].

NT-proBNP is a well-established biomarker recommended for acute HF diagnosis in patients presenting with acute dyspnoea, and for stratification of risk in subjects with chronic HF [15]. To date, two types of miRNAs have appeared to correlate positively with NT-proBNP: miR-499 and miR-423-5p [24,25]. Recently, the concentrations of four other miRNAs, which were increased in sera of patients with HF, namely miR-423-5p, miR 320a, miR-22 and miR-92b, were combined by Goren et al. into a single ‘miRNA score’, which was also significantly associated with elevated serum BNP concentrations [26]. Another miRNA type investigated as a potential biomarker of congestive HF was miR-126, which was significantly downregulated in patients with ischaemic heart disease and congestive HF, being negatively correlated with BNP [27].

We observed significant miR-1 downregulation in NYHA class IV patients and its reverse correlation with NT-proBNP, a validated prognostic variable. To the best of our knowledge, these data demonstrate for the first time that circulating miR-1 may be a novel biomarker for predicting the exacerbation of HF.

Our findings correspond with observations confirming the inhibitory effect of miR-1 on cardiomyocyte number. miRNA-133
is another muscle-specific miRNA, which forms a bicistronic cluster with miRNA-1. The regulation of cardiomyocyte apoptosis by miR-1 and miRNA-133 has also been suggested by Xu et al. [30]. miR-1 and miR-133 produced opposing effects on apoptosis, induced by oxidative stress in a rat embryonic ventricular cell line or in neonatal rat ventricular myocytes. These experiments provide evidence of miR-1 being pro-apoptotic and miR-133 being antiapoptotic.

According to preclinical studies, downregulation of the muscle-specific miR-1 mediates the induction of pathological cardiac hypertrophy [31]. Some authors have suggested that its downregulation may also induce ventricular tachyarrhythmias by altering the expression and function of connexin 43 located in gap junctions [20]. Some reports have suggested that the concentrations of miR-1 are lower in acute HF, which is in line with our results [32]. However, other observations were noted in the study by Zhang et al., who found that elevated plasma miR-1 concentrations may predict the onset of HF, and are negatively correlated with LVEF (P = 0.0001) [14]. In our study, we did not find any relationship between miR-1 concentrations and LVEF. However, Zhang et al. [14] selectively enrolled patients after acute myocardial infarction, in whom the miR-1 concentration might have been altered by the ischaemia, blurring the cause–effect connection.

Another important finding of this study was that the concentrations of galectin-3, a novel, promising biomarker of adverse cardiac remodelling, were higher in the NYHA class IV group than in the NYHA class II/III group. This soluble β-galactoside-binding protein released by activated macrophages is involved in numerous pathological processes, including inflammation, tumour growth and fibrosis [33,34]. Of note, we found a significant correlation between galectin-3 and NT-proBNP concentrations, consistent with some previous reports [35,36].

Interestingly, in the group of chronic HF patients with the most pronounced clinical symptoms of the disease (NYHA functional class IV), we observed a significant association between galectin-3 and miR-21 serum concentrations. miR-21 is one of the most highly expressed miRNAs during the process of cardiac remodelling, but the exact role of miR-21 in the cardiac stress response remains unclear. We did not find any significant correlation between NYHA functional class and miR-21 concentrations in our study. However, according to Thum et al. [13], miR-21 was highly expressed not in cardiomyocytes, but in cardiac fibroblasts, and its expression was higher with the severity of HF exacerbation. This miRNA molecule was reported to inhibit the apoptosis of cardiac fibroblasts and to enhance signal transduction of the mitogen-activated protein kinase signalling pathway, leading to cardiac hypertrophy and myocardial fibrosis, which are known as important pathological factors implicated in the development of HF [37]. In preclinical studies, silencing of miR-21 function by a specific antagonist in the pressure overload-induced HF model was able to reverse the above-mentioned pathological processes and attenuate cardiac dysfunction [37]. These observations suggest that miRNA therapeutic interventions might be feasible in the chronic HF setting. Of note, the strong positive correlation between the serum concentrations of miR-21 and galectin-3 demonstrated in this study confirms the postulated role of miR-21 in fibrosis, and constitutes a further validation of this molecule as an attractive disease target in the management of HF.

In this research, we did not observe the expression of miR-208a in the plasma of control subjects or in patients, irrespective of their disease severity. The existing evidence on the expression of this miRNA type is conflicting. Some studies have shown that miR-208a has a positive correlation with cardiac fibrosis in human dilated cardiomyopathy [24]. In contrast, Prado-Uribe et al. [38] demonstrated that expression of miR-208a was dramatically decreased, but cardiac fibrosis was markedly increased, in five of six nephrectomized rats. Some studies have also suggested that miR-208a is required for stress-induced cardiac pathophysiological responses [39]. In our previous paper, we observed significant release of miR-208a in the early phase of myocardial infarction [40]. On the other hand, Zile et al. [41] demonstrated late release of miR-208, 5 days after acute myocardial infarction, which remained up to 90 days. This late release is probably caused by metabolic changes, leading to left ventricular structural remodelling. These conflicting results may suggest that different mechanisms are responsible for the induction of fibrosis in human HF. Whether miRNA-208a can prevent or promote cardiac fibrosis is still to be determined. Further evidence is needed to identify the role of miR-208a in cardiac fibrosis.

Future studies

In preclinical studies, restoration of miR-1 concentrations allowed for a reversal of pressure overload-induced left ventricular hypertrophy and attenuation of maladaptive cardiac remodelling [42]. Hence, miR-1 might also serve as a target for pharmacotherapy. Nevertheless, the diagnostic accuracy and the role of miR-1 in the pathogenesis of symptomatic HF need to be determined in larger cohorts of patients.

Study limitations

The main limitation of this study was its small sample size. Secondly, we could not assess the dynamics of the expression of the assessed miRNAs during the course of therapy, as only a single measurement was taken on admission. Another limitation was the fact that the association between the analysed miRNA and left ventricular hypertrophy was not evaluated. It should be also noted that neither healthy volunteers nor subjects with non-cardiac dyspnoea were included in this work and, as a consequence, we could not verify whether miRNA evaluation might allow for the accurate differentiation between cardiac and non-cardiac causes of dyspnoea. Nevertheless, on the basis of the currently available results of miRNA studies in HF patients, which are controversial because of their high variability and a relatively unfavourable signal-to-noise ratio, one may suggest that the most probable future application of these particles in HF diagnostics will not be associated with the identification of a one disease-specific miRNA type. Instead, following the concept of Goren et al. [26], it appears that further studies aimed at determination of the miRNA ‘set’ or ‘score’, consisting of the most downregulated (such as miR-1) and upregulated miRNAs, are necessary to develop a diagnostic tool that would add valuable information to the biomarkers used currently in HF.
Conclusions

We have, for the first time, demonstrated that circulating miR-1 correlates with the clinical symptoms and biochemical signs of symptomatic HF. Based on these findings, miR-1 may become a biomarker for predicting the exacerbation of HF. miR-1, in combination with other biomarkers of HF, may allow an adequate time window for implementing appropriate therapy and, as a consequence, preventing or attenuating the impending onset of acute HF. The observed correlation between miR-21 expression and galectin-3 confirms its role in HF progression and cardiac remodelling. Nevertheless, further studies are necessary to evaluate the diagnostic efficacy of these molecules.

Acknowledgements

The authors would like to thank bioMérieux SA (Poland) for providing the reagent.

Sources of funding: the research reported in this article was supported by grant No. 4987/B/PO1/2011/40 from the National Research Centre, Poland.

Disclosure of interest

The authors declare that they have no competing interest.

References

[15] McMurray JJ, Adamopoulos S, Anker SD, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012;33:1787–847.


