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Assessment of Plasma miRNAs in Congestive Heart Failure

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Background: MicroRNAs (miRNAs) are endogenous small RNAs that are 21–25 nucleotides in length. Recently, plasma miRNAs have been reported to be sensitive and specific biomarkers of various tissue injuries and pathological conditions. The goal of this study was to assess plasma miRNA profiles and to identify plasma miRNAs that are differentially expressed in patients with heart failure.

Methods and Results: A total of 33 patients with ischemic heart diseases and 17 asymptomatic controls were recruited. In 10 patients with heart failure, miRNAs were assessed at both NYHA Ⅳ and Ⅲ. miRNA array analyses were found to be not appropriate for plasma miRNA profiling. The plasma concentrations of well-characterized miRNAs (miR-126, 122 and 499) were assessed by a real-time reverse transcription-polymerase chain reaction using an artificial small RNA as an internal standard. Plasma concentrations of miR-126 were negatively correlated with age and logBNP. In 10 patients with heart failure, plasma concentrations of miR-126 were up-regulated with improvement of the NYHA class from Ⅳ to Ⅲ.

Conclusions: The plasma concentration of miR-126 was negatively correlated with age and NYHA class, and could be a useful biomarker for heart failure. (*Circ J* 2011; **75:** 336–340)

Key Words: Biomarkers; Endothelium; Heart failure

MicroRNAs (miRNAs) are endogenous small RNAs (21–25 nucleotides in length) that can pair with 3'-UTR sites in the messenger RNAs of protein-coding genes to downregulate their expression.[1] They seem to play important roles in various physiologic and pathologic processes.[2,3] More than 500 human miRNAs have been identified,[4] and it is highly likely that most human protein-coding genes are targeted by these miRNAs.[5,6] miRNAs have been suggested to function as a rheostat to fine-tune protein output.[7,8]

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Recently, the presence of miRNA in various body fluids has been reported.[9–11] Our group and other groups have reported that plasma miRNAs are sensitive and specific biomarkers of various tissue injuries and pathological conditions.[12–15] More recently, Tijsen et al reported that circulating plasma miR-423-5p is most strongly related to the clinical diagnosis of heart failure.[16] In the present study, we explored the hypothesis that plasma miRNA profiling might be useful for evaluating patients with heart failure.

Methods

Study Population

We recruited 33 patients with ischemic heart diseases (myocardial infarction, unstable angina and/or stable angina pectoris) and 17 asymptomatic controls at the National Cardiovascular Center Hospital after obtaining their written informed consent. In 10 patients with heart failure, miRNAs were assessed at both NYHA IV and III. Thus, 60 samples were analyzed in the present study. Patients in cardiogenic shock were not recruited in the present study. This study was approved by the Ethics Committee of the National Cardiovascular Center. The characteristics of the study population are shown in **Table**. The values for fractional shortening (%) and number of diseased coronary vessels (>75%) were those at the closest time point to blood sampling.

Plasma RNA Purification

Blood samples were collected in EDTA-containing tubes (Terumo, Tokyo, Japan) and plasma was isolated by centrifugation at 1,500×g for 15 min at 4°C. Five hundred microliters of plasma was mixed with an equal volume of 2×denaturing solution from the mirVana PARIS Kit (Ambion, Austin, TX, USA) and maintained at –80°C until RNA was

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Table. Patient Characteristics

	Control	NYHA II	NYHA III	NYHA IV	NYHA III (2)
N (F/M)	17 (6/11)	17 (3/14)	6 (1/5)	10 (1/9)	
AP/MI	0/0	4/13	1/5	0/10	
Age (years)	45.2 (16.0)	68.3 (12.9)	73.5 (11.1)	70.2 (11.1)	
Creatinine (mg/dl)	0.81 (0.12)	0.95 (0.30)	1.20 (0.12)	1.28 (0.38)	1.42 (0.48)
AST (IU/L)	23.4 (7.1)	41.9 (57.6)	23.7 (6.1)	28.2 (11.4)	23.8 (6.4)
ALT (IU/L)	23.3 (9.1)	30.6 (25.3)	17.2 (10.4)	16.7 (3.3)	19.9 (6.9)
Log[BNP(pg/ml)]	ND	1.81 (0.08)	2.50 (0.13)	3.01 (0.10)	2.67 (0.10)
Hemoglobin A_{1c} (%)	5.8 (0.4)	5.9 (1.3)	5.9 (1.0)		6.0 (0.8)
DM (%)	11.8 (2/17)	29.4 (5/17)	50 (3/6)		60 (6/10)
N of DV (1/2/3)	ND	8/6/3	2/1/3		1/4/5
FS (%)	ND	35.6 (8.0)	24.1 (12.3)		14.6 (3.2)
Statin (%)	0 (0/17)	35 (6/17)	50 (3/6)	60 (6/10)	60 (6/10)

The patient characteristics are shown according to the patient groups.
The characteristics of 10 patients with NYHA IV heart failure were assessed twice (at NYHA IV and NYHA III (2)).
Values are expressed as mean (standard deviation).
F/M, numbers of females and males; AP/MI, number of patients with angina pectoris/number of patients with demonstrated myocardial infarction; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BNP, plasma brain natriuretic polypeptide (pg/ml); DM, presence of diabetes mellitus; N of DV, number of diseased vessels (>75% as assessed by coronary angiography); FS, fractional shortening as assessed by echocardiography; Statin, prescription of statins.

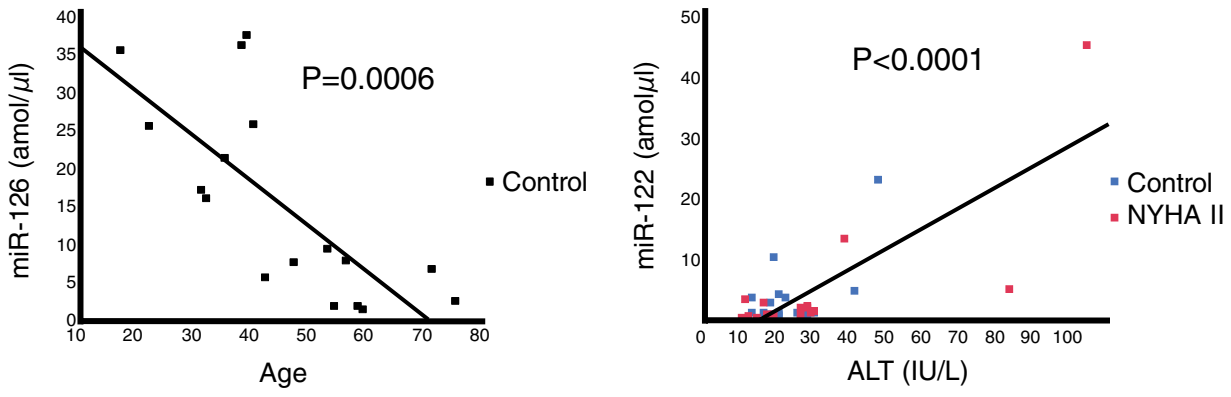

Figure 1. Plasma concentrations of miR-126 and -122 in the study population. (**Left**) Plasma concentrations of miR-126 were highly and negatively correlated with age (r^2=0.52; P=0.0006; N=17) in the asymptomatic control group. (**Right**) Plasma concentrations of miR-122 were significantly correlated with ALT activity (r^2=0.57; P<0.0001; N=34) in the control and NYHA II groups.

isolated. Before RNA isolation, 1 amol of synthetic small RNA (5'-rCrArC rGrUrG rUrGrG rCrArA rUrGrC rArUrU rArCrU rGrArG rUrU-3') was added to the mixture as an internal reference to validate the procedure (RNA purification and real-time reverse transcription-polymerase chain reaction (RT-PCR)). RNA isolated from 500 μl of plasma was finally resuspended in 20 μl of RNase-free water.

Expression Profiling of Plasma miRNA

Expression profiling of miRNA was performed using an ABI TaqMan MicroRNA Array kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. In brief, RNA derived from 100 μl of plasma or 1 μg of tissue RNA was reverse-transcribed using Megaplex RT primers (Applied Biosystems). cDNA was amplified using TaqMan PreAmp Master Mix (Applied Biosystems) according to the manufacturer's protocol and subjected to real-time PCR using a TaqMan Human MicroRNA Array with an ABI 7900HT system. SDS software v2.3 and RQ manager v1.2 (Applied Biosystems) were used to obtain the quantitative cycle (Cq) value. We performed plasma miRNA profiling in 8 patients with heart failure and 3 normal controls. Expression profiles of human heart, liver, vascular smooth muscle and umbilical vein endothelial cells are provided as a **Supplemental File 1**.

Quantification of Plasma-Derived miRNAs by Real-Time RT-PCR

Concentrations of miR-126, 122 and 499 in plasma were measured using a TaqMan microRNA real-time RT-PCR kit[17] (Applied Biosystems) according to the manufacturer's protocol with modifications. The concentrations of miR-126

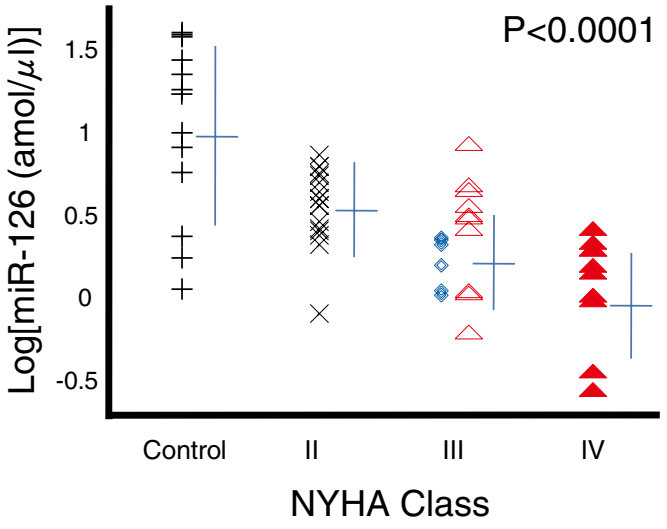

Figure 2. Plasma concentrations of miR-126 according to the NYHA group. Plasma concentrations of miR-126 are shown according to the NYHA group. Ten patients with NYHA IV were assessed twice and are indicated by (▲). Mean values and standard deviations are indicated by horizontal and vertical lines. ANOVA identified significant differences in the plasma concentrations of miR-126 among the 4 groups (P<0.0001). Subsequent Tukey's Honestly Significant Difference (HSD) test indicated significant differences between the following groups: Control (Ct) and NYHA II, Ct and III, Ct and IV, and II and IV (P<0.01).

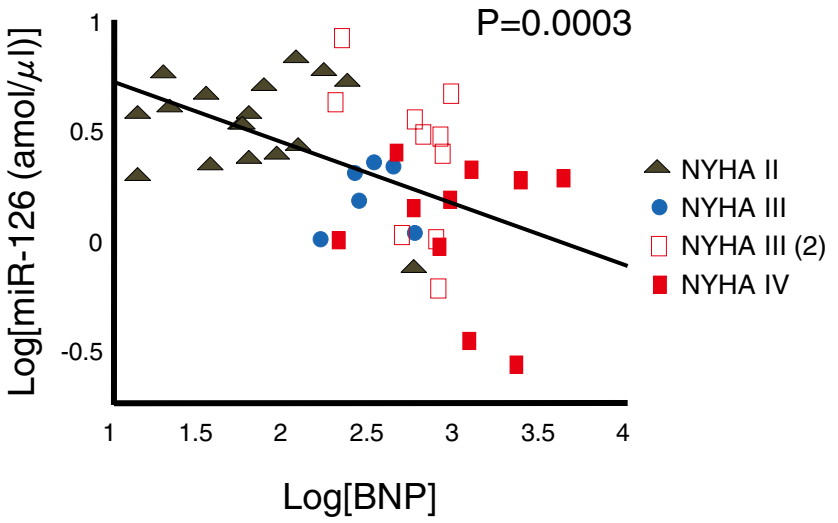

Figure 3. Correlation between brain natriuretic peptide (BNP) and miR-126. Correlations between plasma concentrations of BNP and miR-126 were analyzed in patients with NYHA II, III and IV. A significant negative relationship was observed (r^2=0.25; P=0.0003; N=43). NYHA III (2) indicates the values at NYHA III for the 10 patients who were assessed at both NYHA IV and III.

(122 or 499) and the small RNA as an internal reference were determined simultaneously in the same well of the detection plate by following the FAM and VIC signals, respectively, using the ABI Prism 7500 system (Applied Biosystems). To determine the absolute copy numbers of miR-126 (122 or 499), the real-time RT-PCR assay was conducted with known amounts of synthetic human miR-126 (122 or 499) (Integrated DNA Technologies). All samples were determined in duplicate. The mean copy number (concentration) obtained from the two Cq values was assigned to each sample's value.

Statistical Analysis

Unless otherwise indicated, values are expressed as the mean±standard deviation (SD). Values were $\log_{10}$-transformed when appropriate. Results were analyzed by ANOVA followed by Tukey's Honestly Significant Difference (HSD) test, a paired t-test, or single/multiple regression analyses. Statistical analysis was performed using the JMP statistical package v7.0 (SAS Institute, Cary, NC, USA).

Results

First, we performed expression profiling of plasma miRNAs using the ABI TaqMan MicroRNA Array kit in patients with heart failure. Although the miRNA array system appears to be useful for identifying tissue- or cell-specific miRNAs, the reliability of this system for assessing several-fold differences was low, as described in detail in the **Supplemental File 2**. We were unable to identify plasma miRNAs that were differentially expressed in heart failure. Indeed, we were unable to reconfirm the report by Tijsen et al that circulating plasma miR-423-5p was most strongly related to the clinical diagnosis of heart failure.[16] One possible intriguing observation in our array analyses was the relatively high global Cq values in participants with CHF compared with those in healthy participants.

Next, we changed our strategy and evaluated the concen-

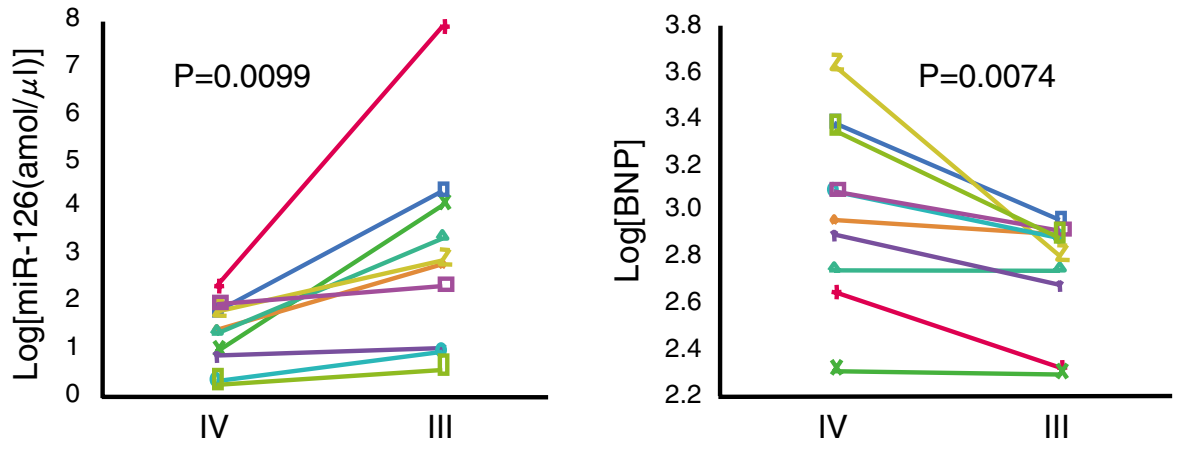

Figure 4. Changes in plasma miR-126 according to clinical improvement. In 10 patients with heart failure, plasma miR-126 concentrations were assessed at both NYHA IV and III. As the clinical condition improved, plasma BNP levels were significantly reduced (P=0.0074) and plasma concentrations of miR-126 were significantly increased (P=0.0099).

trations of well-characterized miRNA species (miR-126, miR-122 and miR-499) in plasma samples of the study population using a recently established reliable assessment method.[15] miR-122, miR-126 and miR-499 are almost specifically expressed in the liver, endothelial cells and the ventricle of the heart, respectively (**Supplemental Files 1**,**2**). Thus, it is likely that we can identify the pathophysiological significance of the up- or down-regulation of these miRNAs in plasma. Moreover, our examination using synthetic miRNAs for miR-122, -126 and -499 re-confirmed that real-time RT-PCR for these 2 miRNAs was highly reliable.

Plasma concentrations of miR-126 were highly and negatively correlated with age (**Figure 1 Left**; r^2=0.52; P=0.0006; N=17) in the control group. Plasma concentrations of miR-126 were significantly different among the Control and NYHA II-IV groups (**Figure 2**). Mean values of age were not significantly different among the NYHA II, III and IV groups (P=0.73). Plasma concentrations of miR-126 were not correlated with the prescription of statin, hemoglobin A_{1c}, or creatinine (data not shown). Plasma concentrations of miR-126 were negatively correlated with brain natriuretic peptide (BNP) (r^2=0.25; P=0.0003; **Figure 3**; N=43) in the NYHA II-IV groups.

Next, we assessed the plasma concentrations of miR-126 twice in 10 patients with heart failure when they were at NYHA IV and later when they improved to NYHA III. **Figure 4** shows that plasma concentrations of miR-126 were up-regulated with improvement of the NYHA class from IV to III. Thus, the heart failure condition, as well as age, significantly influenced plasma concentrations of miR-126.

Plasma concentrations of miR-122 were significantly correlated with ALT activity (**Figure 1 Right**; r^2=0.57; P<0.0001; N=34) in the control and NYHA II groups. Further studies are required to determine whether the assessment of plasma concentrations of miR-122 may be a new biomarker of liver dysfunction. The plasma concentrations of miR-499 were close to or below the limit of detection in the control subjects and below the limit of detection in patients at NYHA II-IV as previously reported.[15] Again, further studies are needed to determine whether this implies an absence of myocardial necrosis even in patients with heart failure at NYHA IV, or whether this assay method is less sensitive than highly sensitive troponin-based assays.

Discussion

The present study confirmed for the first time that plasma concentrations of miR-126 decreased with age and were down-regulated by poor conditions related to congestive heart failure (CHF). Recently, Fichtlscherer et al reported that plasma concentrations of miR-126 were down-regulated in patients with coronary artery disease.[18] Moreover, Zampetaki et al reported that plasma concentrations of miR-126 were down-regulated in patients with type 2 diabetes.[19] As shown in **Table**, patients with heart failure at NYHA III or IV were more likely to have diabetes and advanced atherosclerosis. The down-regulation of plasma concentrations of miR-126 in patients with heart failure at NYHA IV may partly be ascribed to these 2 factors.

The next question is whether assessment of the plasma concentration of miR-126 or other miRNAs has any clinical significance for the assessment of and therapeutic options for CHF. Plasma BNP and cardiac troponins are often assessed as biomarkers of heart failure and are both products of cardiomyocytes. miR-126 is highly enriched in endothelial cells and is expected to reflect some conditions of vascular endothelial cells. The reduced plasma concentrations of miR-126 in patients with CHF based on atherosclerosis were somewhat surprising. As Fichlscherer et al speculated,[18] we would expect that endothelial activation, since it occurs in patients with atherosclerosis, diabetes, or CHF, induces the release of microparticles and remnants of apoptotic cells, and would thereby increase the concentration of miR-126. It is possible that a perfusion defect and/or cellular aging may reduce the metabolic activity and/or renewal of endothelial cells, which could lead to a reduction in the release of miRNAs, including miR-126. It is also possible that endothelial activation may activate the degradation of circulating miRNAs and thereby reduce plasma concentrations of miRNAs. Further studies are necessary to determine whether plasma

miR-126 is really promising as a new biomarker for various vascular diseases including CHF.

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Supplemental Files

Supplemental File 1

Data S1. miRNA Array Analysis of Human Liver, Umblical Endothelial Cell, and Heart

Supplemental File 2

Limitations of MicroRNA (miRNA) Array Analysis

Figure S1. Identification of tissue-specific miRNA.

Figure S2. Poor reliability of miRNA array system.

Figure S3. Poor correlation between miRNA array and individual real-time RT-PCR assessments.

Figure S4. A typical example of miRNA array analysis.

Please find supplemental file(s);
http://dx.doi.org/10.1253/circj.CJ-10-0457