ESC HEART FAILURE
ESC Heart Failure 2017; **4**: 112–121
Published online 1 December 2016 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/ehf2.12123

ORIGINAL RESEARCH ARTICLE

Dynamic changes of serum microRNA-122-5p through therapeutic courses indicates amelioration of acute liver injury accompanied by acute cardiac decompensation

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Abstract

Aims Recent studies have shown that serum microRNA (miR) abundance is informative for the diagnosis or prognosis of heart failure. However, the dynamics and kinetics of miRs in acute heart failure are largely unknown. Serial measurement and analysis of serum miRs changes in individuals along their therapeutic course could reduce inter-individual variation and should detect potentially important serum miRs related to disease mechanisms. Based on this concept, we profiled serum miR signatures of blood samples that were obtained sequentially on the day of admission and on hospital Day 7.

Methods and results This prospective, observational study included 42 consecutive acute heart failure patients (74 ± 1 years old, 24 male). From admission to Day 7, most of the patients showed clinical improvement. In such a cohort, we detected several fluctuations of serum miRs by two distinct screening methods (quantitative PCR and high-throughput sequencing). One of these fluctuating serum miRs, miR-122-5p, decreased significantly from Day 1 to Day 7 [median arbitrary unit (1st:3rd quantile value); 4.62 [2.39:12.3] to 3.07 [1.67:5.39], $P = 0.007$]. This fluctuation was significantly correlated with changes in serum liver function markers (estimated coefficient and 95% confidence interval; vs change in aspartate aminotransferase 1.69, 0.890–2.484, $P < 0.001$ and $r = 0.560$, vs change in alanine aminotransferase 1.09, 0.406–1.771, $P = 0.007$ and $r = 0.428$).

Conclusions The serum miR signature of patients with acute heart failure might indicate the severity of the disease or patients' response to therapeutic intervention. Notably, serum miR-122-5p levels reflect liver damage in this condition.

Keywords Acute heart failure; Biomarker; Liver injury; MicroRNA; miR-122-5p

Received: 30 May 2016; Revised: 28 September 2016; Accepted: 12 October 2016
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Introduction

Heart failure is increasing in incidence and is having a huge impact on medicine and medical economics. Heart failure includes many pathophysiological conditions and its severity has wide variations from sudden death to a stable state that is observable for years. To manage this complex syndrome effectively, appropriate definition, classification, and standardization of treatments are essential. To accomplish this mission, many researchers have already developed and

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tested numerous biomarkers. Among them, serum protein biomarkers such as brain natriuretic peptide or cardiac troponins have excellent clinical value and are used frequently.[1,2] However, these generalized heart failure biomarkers cannot provide specific information about hemodynamics or organ damage caused by the disease. To meet these demands, the development of new specific biomarkers is urgently required.

Numerous reports have showed that serum microRNAs (miRs) could be specific biomarkers for various diseases. miRs circulate in the blood stream enclosed in small vesicles called exosomes.[3,4] Thus, these circulating miRs are stable in the blood and there are several established methods for their extraction and measurement. There are already many reports, including ours, that show circulating miRs are good biomarkers for cardiovascular diseases.[5–16]

To explore new biomarkers for heart failure, we need to take account of the definition of biomarkers, which are diagnostic, prognosis predictive and responsiveness to disease conditions. In the field of heart failure, many candidate miRs have been reported from the viewpoint of diagnostic properties.[7–12] Some miRs are reported to have prognostic value.[13–16] However, not many reports have elucidated the biological meanings of serum miRs in patients with heart failure. For example, it is unknown whether increased serum miRs in patients compared with healthy controls are pathological or physiological. This is partly because many of these diagnostic or prognosis-related miRs are obtained from case–control studies. Case–control study designs can effectively pick-up disease-related markers. However, inter-individual comparison cannot always give information on causal pathophysiological relationships. Moreover, inter-individual differences, especially between diseased patients and healthy controls, often provide uncontrollable large biases that mask subtle changes in indicators in response to the disease state transition.

Heart failure is a common but unique disorder, in which we can observe a dynamic transition of condition from acute decompensation to its resolution in a relatively a short time period. These characteristics enable us to identify disease-dependent transitions of biomarkers. Characterizing the changes in miRs-signature in response to the severity of heart failure might highlight important disease-related markers and elucidate pathophysiological meanings of serum circulating miRs in heart failure.

Based on such concepts, we designed and conducted this study. Blood samples were collected sequentially from patients with acute cardiac decompensation. The first samples were obtained at the peak of decompensation and the second samples were collected from the same individuals after therapeutic intervention. In such a study design, we observed that several serum miRs changed in response to heart failure therapy. In particular, serum miR-122-5p changed in parallel with liver injury markers in this short time period.

Methods

Patients inclusion, blood samples, and clinical data collection

Blood samples and clinical data were collected from 42 consecutive patients who required admission to treat new-onset heart failure or acute aggravation of chronic heart failure. Diagnosis of heart failure was made by more than two cardiologists, based on physical examination, electrocardiography, chest radiography, echocardiography, and laboratory tests. Echocardiography was performed on the day of admission. Blood samples were collected within 6 h after admission in serum separator tubes (Neotube, Nipro, Osaka, Japan) and incubated at room temperature at least 30 min. Then, the samples were centrifuged at 3500 rpm for 10 min and serum was dispensed to 2 mL tubes as aliquots and stored at −80°C until analysis. Clinical data were obtained from direct interview or chart review. The study protocol was approved by the clinical ethical board of Hyogo Prefectural Amagasaki Hospital and Kyoto University Hospital. Written informed consent was obtained from all participants.

Screening experiments

Two sets of pooled serum samples (Day 1 and Day 7) obtained from the first 10 patients were screened for fluctuating miRs by two distinct methods; quantitative PCR (qPCR) panel and high-throughput sequencing (HTS). These methods were performed in parallel and the intersection of fluctuating miRs in these two assays was validated in individual samples using qPCR.

MicroRNA profiling by high-throughput sequencing

Exosomes were isolated using ExoQuick polymer precipitation methods (System Biosciences, Mountain View, CA, USA) from pooled serum. Exosome RNA was purified from isolated exosomes using a SeraMir RNA kit (System Biosciences). RNA NGS libraries were constructed using modified Illumina adapter methods with a TailorMix miRNA Sample Preparation Kit (SeqMatic, Inc. Fremont, CA, USA) and indexed with separate bar codes for multiplex sequencing on either a HiSeq 2500 instruments using a 1 × 50 bp single-end run setting. All procedures were performed in accordance with the manufacturer's instructions. The output sequences were filtered by sequence quality, and trimmed of adapter sequences. The remaining sequences were aligned to the human genome build 37. Read counts for each miR were calculated based on genomic feature format file obtained from the miRbase website (http://www.mirbase.org/ftp.shtml). Inter-sample

DOI: 10.1002/ehf2.12123

normalization was performed using R[17] and the DESeq package.[18] Briefly, as a normalization factor, the size factor was obtained as a median of the geometric mean of each miRNA read count. The calibrated read counts were calculated by dividing the raw read count by the size factor.

$$ReadCount_{Normalized} = \frac{ReadCount_{Raw}}{\underset{i}{median}\frac{ReadCount_i}{\left(\prod_{k=1}^{m} ReadCount_{ik}\right)^{\frac{1}{m}}}}$$

MicroRNA profiling by quantitative PCR panel

Exosomes were isolated from 500 μL of pooled serum using a miRCURY Exosome Isolation Kit (Exiqon, Vedbaek, Denmark) and a miReasy kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's instructions with a final volume of 50 μL. Twenty microliter of isolated RNA was used for cDNA synthesis using a Universal cDNA synthesis kit II (Exiqon). The synthesized cDNA was diluted 50 times and applied for qPCR panel analysis (Human miRNome miR PCR panel I+II). miRs with Cq values more than 40 were regarded as invalid. The inter-plate variances were normalized by inter-plate calibrators, which were loaded onto each plate (raw CQ values—mean IPC CQ values). After inter-plate calibration, inter-sample normalization was performed by subtracting prespecified miR Cq values (miR-425-5p, miR-423-5p, miR-103-3p, miR-191-5p, and miR-93-5p). In the previous report, miR-423-5p was shown to be a heart-failure-related miRNA. We normalized miR 423-5p using four inter-sample calibrators, excluding miR-423-5p. However, we did not see significant expression changes between the Day 1 and Day 7 samples (log twofold change = −0.27).

$$Cq_{Calibrated} = Cq_{Raw} - \frac{1}{n}\sum_{i=1}^{n} Cq_{IPC_i}$$

$$Cq_{Normalized} = Cq_{Calibrated} - \frac{1}{m}\sum_{i=1}^{m} Cq_{Reference_i}$$

Validation assay

Serum samples were thawed and centrifuged at 13 000 ***g*** for 10 min and 300 μL of supernatant was collected. Exosomes were isolated from serum using a miRCURY Exosome Isolation Kit (Exiqon) in accordance with the manufacturer's instructions. Briefly, 120 μL of precipitation buffer was added to serum, which was incubated on ice for 60 min to precipitate exosomes and then centrifuged at 1 500 ***g*** for 30 min at 4°C. The pellet was subsequently re-suspended in 160 μL of resuspension buffer. Isolated exosomes were lysed in 750 μL of QIAzol (QIAGEN) detergent that includes 1.5 pmole of Sp6 RNA (Exiqon) and 1 μg MS2 RNA (Roche, Basel, Switzerland). RNA was extracted from this lysate in accordance with the manufacturer's instructions include in the miR easy kit (QIAGEN). Finally, RNA was eluted in 40 μL of nuclease free water and 2 μL of the solution was used for cDNA synthesis. Reverse transcription was performed in 10 μL volume using the Universal cDNA synthesis Kit (Exiqon). One-fortieth of the obtained cDNA was used for individual qPCR assays using a miRCURY micro-RNA assay (Exiqon) and Light cycler LC96 system (Roche). Sp6 RNA level was concomitantly measured and used as an internal control. In this article, the serum concentrations of miR-122-5p in individual assays are expressed as an estimated concentration that was determined using the spike-in SP6 concentration. The coefficient of variation of Cq_{miR122} was 3.8% in our assay protocol.

Normal human organ expression level

RNAs extracted from normal human organs were purchased from Clontech (Mountain View, CA, USA). Ten nanogram of total RNA was used for reverse transcription reactions as described earlier and one-eightieth of synthesized cDNA was used for qPCR reactions.

Data representation and statistical analysis

Normally distributed continuous variables were summarized as mean ± standard deviation and tested using *t*-test. Continuous variables with skewed distribution were summarized as median and first and third quintiles, and tested using Wilcoxon rank sum test. All comparisons between Day 1 and Day 7 data were tested as paired samples. Correlations were assessed using Pearson correlation coefficients and summarized as estimated coefficients and its 95% confidence interval. Multivariate analysis was performed using multiple linear regression model to test independency of explanatory variables, which showed significant correlations in single variable analysis. All statistical tests were performed using R software version 3.1.2.

Results

Patient characteristics and the therapeutic responses

In this study, 42 acute heart failure patients were enrolled. *Tables 1* and *2* show the baseline characteristics and echocardiographic variables of the study population. The mean age was over 75 years old, and average ejection fraction was around 45%. These data are roughly consistent with a

Table 1 Baseline characteristics

Age (years)	75.7 ± 11.4
BMI	22.0 ± 4.2
Gender, male (%)	45 (19)
Concomitant disorders (%)	
Ischemic heart disease	48 (20)
Atrial fibrillation	45 (19)
With pacemaker	21 (9)
Diabetes	48 (20)
Medication on admission (%)	
RAS inhibitor	40 (17)
Aldosterone blocker	31 (13)
Beta blocker	45 (19)
Loop diuretic	74 (31)
Thiazide diuretic	7 (3)
Antiplatelet	48 (20)
Anticoagulant	38 (16)
Calcium channel blocker	43 (18)
Intravenous drug use (%)	
Diuretics	82 (36)
Inotrope	14 (6)
Nitroglycerin	33 (14)
Natriuretic peptide	33 (14)

BMI, Body mass index; RAS, Renin angiotensin system.
Data is expressed as mean ± standard deviation or percentage (count).

Table 2 Echocardiographic variables

LVDd (mm)	45.9 [40.5:51.7]
LVDs (mm)	33.1 [28.2:43.7]
IVSd (mm)	11.5 ± 2.6
PWTd (mm)	12.6 ± 2.5
LADs (mm)	42.5 [39.0:48.7]
Teichholz-EF (%)	44.6 ± 18.8
Modified Simpson-EF (%)	46.5 ± 18.6

EF, ejection fraction; IVSd, interventricular septum thickness end diastolic; LVDd, left ventricular internal diameter end diastolic; LVDs, left ventricular internal diameter end systolic; LADs, left atrial internal diameter end systolic; PWTd, posterior wall thickness end diastolic.
Data is expressed as mean ± standard deviation or median (1st:3rd quantile value).

previously reported large-scale registry series of acute heart failure.[19,20]

Changes in clinical variables during the therapeutic course are shown in *Tables 3* and *4*. Blood pressure, heart rate, body weight, and oxygen demand were decreased in response to therapeutic intervention. The resolution of laboratory tests was also observed. Abnormalities in liver function tests were improved, and serum natriuretic peptide and troponin levels decreased. However, the indices of renal function worsened, which may be the result of successful aggressive volume reduction.

Screening and validation for fluctuating microRNA and validation

To obtain a precise and reproducible result, we performed two distinct screening methods, qPCR panel and HTS. Fluctuating miRs levels detected in both methods were considered as candidates for further evaluation. Sera from the first 10 patients in our study population were pooled and analyzed by qPCR panel and HTS. A summary of the results of screening experiments with the two assay systems is shown in *Figure 1*. HTS-based and qPCR-based methods detected 187 and 167 miRs at both time points. Among these miRs, 125 were detected in both methods and at both time points. The relative changes detected in these systems showed significant correlations ($r > 0.6$, Supporting Information *Figure S1*). We plotted these detected miRs in *Figure 2*, and highlighted miRs with names that showed consistently increases or decreases by more than twofold in both methods. Some of these differently expressing miRs, such as miR-16-5p, miR-92-3p, miR-486-5p, miR-25-3p, and miR-101-3p, are the members of miRs that are known to exist abundantly in red blood cells[21] and their copy numbers in serum are also known to be increased by hemolysis. Although the pathophysiological significance of hemolysis in heart failure is still unclear, we excluded these miRs from the validation experiment in this study. After excluding these hemolysis-related miRs, miR-122-5p was the most abundant and significantly changed miR. To validate this fluctuation, we measured miR-122-5p levels in each sample by qPCR. As shown in *Figure 3* and *Figure S2*, miR-122-5p levels were significantly decreased from Day 1 to Day 7.

Liver function test abnormalities and miR-122-5p

To clarify the physiological significance of this change, we explored the origin of miR-122-5p in the human body. We measured expression levels of miR-122-5p in normal human organs. As shown in *Figure 4*, miR-122-5p was specifically and abundantly expressed in the liver. This result was consistent with previous reports.[22] Thus, we hypothesized that serum miR-122-5p might reflect liver damage and assessed correlations between miR-122-5p level and standard liver damage markers. As shown in *Figure 5*, miR-122-5p expression levels in serum showed good correlations with serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.

In our cohort, patients showed an increased level of liver damage markers on admission, and these abnormalities improved after therapeutic intervention. We also assessed whether this resolution could be detected using serum miR-122-5p levels. *Figure 6* showed the correlation between changes in serum liver function markers and serum miR-122-5p levels. Serum miR-122-5p levels also decreased consistently with serum liver injury markers. Correlations between miR-122-5p changes and other indices are shown in *Tables 5* and *6*. Pretreatment with diuretics before admission and larger decrease in body weight achieved by therapy

Table 3 Changes of clinical parameters

	Day 1	Day 7	*P*-value
Heart rate (/min)	82.5 [68.2:103.2]	71.5 [60.0:81.0]	<0.001
Systolic blood pressure (mmHg)	139.2 ± 31.6	122.3 ± 18.2	<0.001
Diastolic blood pressure (mmHg)	77.3 ± 23.3	69.1 ± 12.4	0.013
Body weight (kg)	53.6 [47.0:63.6]	50.0 [41.4:60.9]	<0.001
Oxygen requirement	40% (17)	17% (7)	0.030
White blood cell count (10^8/mL)	61.3 ± 17.8	57.1 ± 15.2	0.136
Red blood cell count (10^8/mL)	349 ± 70.9	367 ± 79.9	0.006
Hemoglobin (g/dL)	10.6 ± 2.1	11.1 ± 2.3	0.017
Platelet count (10^{10}/mL)	19.6 ± 6.7	22.0 ± 6.6	<0.001
Albumin (g/dL)	3.4 ± 0.5	3.3 ± 0.6	0.305
AST (IU/L)	23.0 [18.2: 31.0]	18.0 [15.0:21.0]	<0.001
ALT (IU/L)	17.0 [11.2:26.0]	12.5 [9.2:19.5]	<0.001
ALP (IU/L)	241.5 [197:344]	226.0 [172:271]	<0.001
Total bilirubin (mg/dL)	0.60 [0.50:0.98]	0.50 [0.40:0.80]	0.008
Blood urea nitrogen (mg/dL)	23.4 [16.8:34.2]	34.7 [25.1:49.0]	<0.001
eGFR (mL/min/1.73 cm^2)	44.2 ± 26.0	39.1 ± 24.8	0.001
Sodium (mEq/L)	139.9 ± 3.9	140.8 ± 5.5	0.281
Potassium (mEq/L)	3.95 ± 0.57	4.01 ± 0.44	0.518
CPK (IU/L)	91.5 [61.5:210.5]	45.5 [36.2:65.8]	<0.001
CRP (mg/dL)	0.60 [0.18:1.31]	0.51 [0.23:1.53]	0.746
BNP (pg/mL)	586 [385:872]	261 [189:535]	<0.001
Troponin I (ng/mL)	0.050 [0.034:0.162]	0.027 [0.014:0.066]	0.003

ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BNP, Brain natriuretic peptide; CPK, Creatine phosphokinase; CRP, C-reactive protein; eGFR, Estimated glomerular filtration ratio.
Data is expressed as percentage (count), mean ± standard deviation or median (1st:3rd quintile value).

Table 4 Correlations between clinical characteristics or changes in vital signs and serum microRNA (miR)-122-5p change

Explanatory variable	Estimated coefficient [95% CI]	*P*-value
Age (years)	0.024 [−0.028:0.075]	0.357
BMI (kg/cm^2)	0.231 [−2.883:3.345]	0.882
Heart rate (/min)	−0.015 [−0.037:0.008]	0.207
Systolic blood pressure, Day 1 (mmHg)	−0.001 [−0.019:0.018]	0.931
Diastolic blood pressure, Day 1 (mmHg)	−0.016 [−0.041:0.009]	0.192
Δ Heart rate	0.752 [-1.979:3.483]	0.581
Δ Systolic blood pressure	−0.001 [−0.023:0.020]	0.916
Δ Diastolic blood pressure	−0.015 [−0.043:0.013]	0.291
Δ Body weight	10.618 [0.384:20.853]	0.042

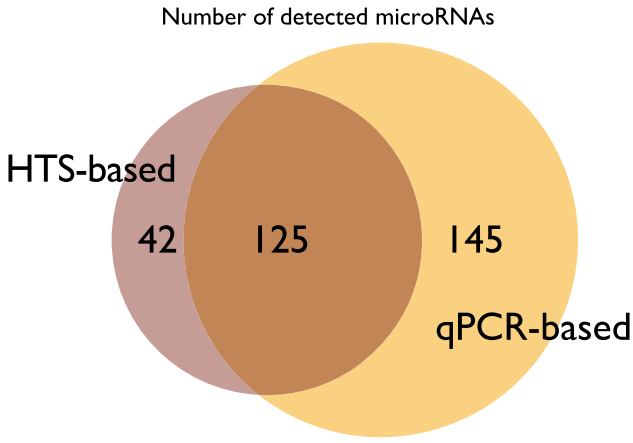
Estimated Pearson's correlation coefficients and its 95% confidence interval (CI) are shown.

showed positive relationships with miR-122-5p levels. Even after correction for these factors, the fluctuation in liver injury markers was the strongest determinant of miR-122-5p changes (*Table 7*).

Discussion

In this study, we successfully observed dynamic changes in serum miR-122-5p abundance through therapeutic courses for acute heart failure using two distinct screening methods, qPCR panel and HTS. Quantification of miR-122-5p levels in individual samples revealed a substantial correlation

Figure 1 Screening schema and its consistency. (A) The number of microRNAs (miRs) detected by quantitative PCR (qPCR) or high-throughput sequencing (HTS) in both Day 1 and Day 7 samples shown in a Venn diagram. (B) Consistency of screening result about each miRs. 'Decreased' indicates the number of miRs whose abundance at Day 7 was decreased from Day 1. 'Increased' indicates the abundance at Day 7 was increased from Day 1.



MicroRNA expression changes from Day 1 to Day 7

qPCR panel	High throughput sequence: Decreased	Increased	
Decreased	65	15	80
Increased	34	11	45
	99	26	125

Figure 2 Changes in the serum microRNA signature. Screening result from high-throughput sequencing (HTS) (A) and quantitative PCR (qPCR) (B). Horizontal axis indicates relative abundance in each method. Vertical axis indicates relative change as log 2-fold change.

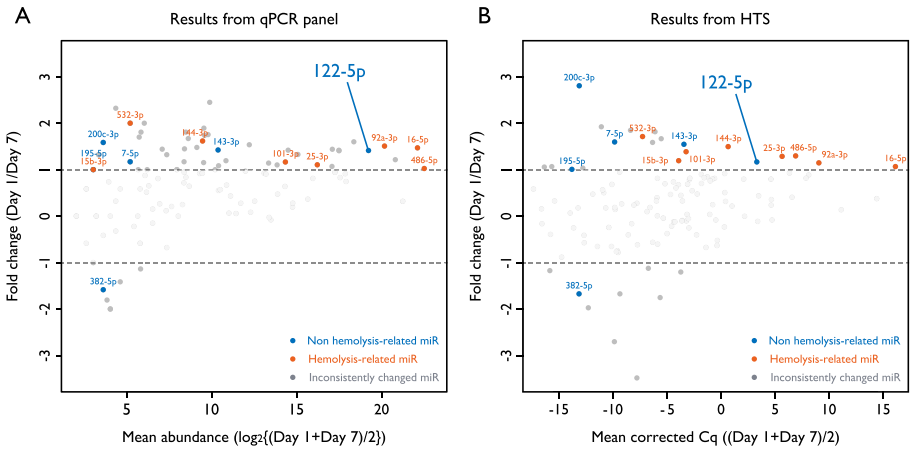


Figure 3 Fluctuation of serum microRNA (miR)-122-5p level in individual samples. Two dots connected with a line indicated the samples from one patient. Significance was tested using a paired Wilcoxon rank sum test.

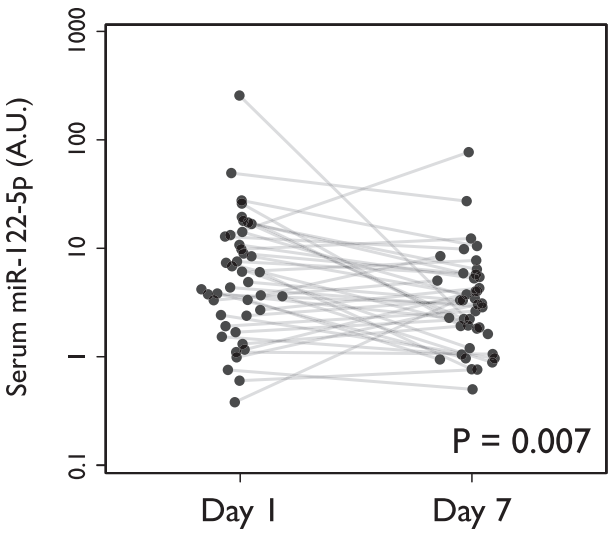
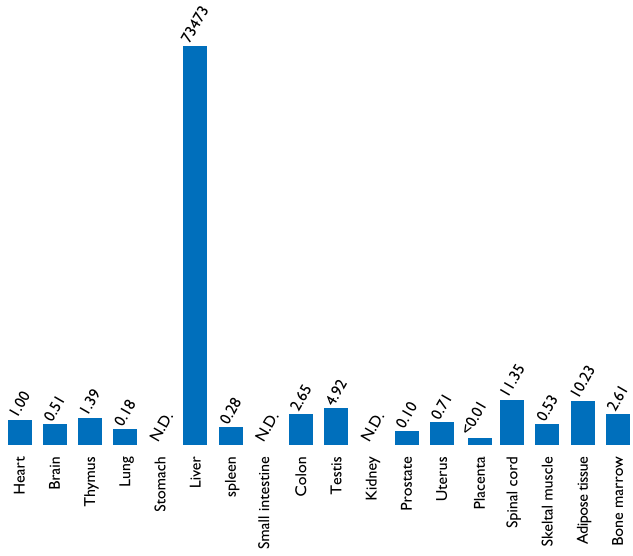


Figure 4 MicroRNA (miR)-122-5p expression in normal human organs. Relative expression levels are expressed as relative values to miR-122-5p in the heart.



between the changes in miR-122-5p and serum liver injury markers. This is the first report that shows a serum miR pattern reflects organ damage caused by acute heart failure and these changes occurred within a clinically relevant period. These properties support the application of miRs as point-of-care biomarkers.

At present, the mechanism of the serum miR-122-5p increase remains to be elucidated. However, miR-122-5p is specifically expressed in the liver in human[22] and data from patients with hepatitis and *in vitro* data suggest that miR-122-5p released from the liver is based on the destruction of hepatocytes.[23] Therefore, it is reasonable to suppose that the fluctuation in serum miR-122-5p levels could reflect liver damage caused by acute heart failure. This is consistent with the observation that heart failure patients are prone to liver damage based on hemodynamic abnormalities. In line with our results, Nikolaou *et al.* showed serum liver function markers were elevated on admission for acute heart failure, and these abnormalities improved in parallel with clinical resolution.[24] They also showed significant relationships between baseline liver function tests and prognosis. Indeed, patients who needed therapy with intravenous inotropic agent did not show tendencies for a decrease in miR-122-5p from Day 1 to Day 7, which may indicate the sustained liver damage still occurs in such severe cases.

Figure 5 Correlation between serum microRNA (miR)-122-5p and AST or ALT activities. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity are expressed after log 2 transformation. miR-122-5p was expressed in arbitrary units (AUs). The red line indicates the estimated regression line. Dotted lines show 95% confidence intervals of the estimated regression line.

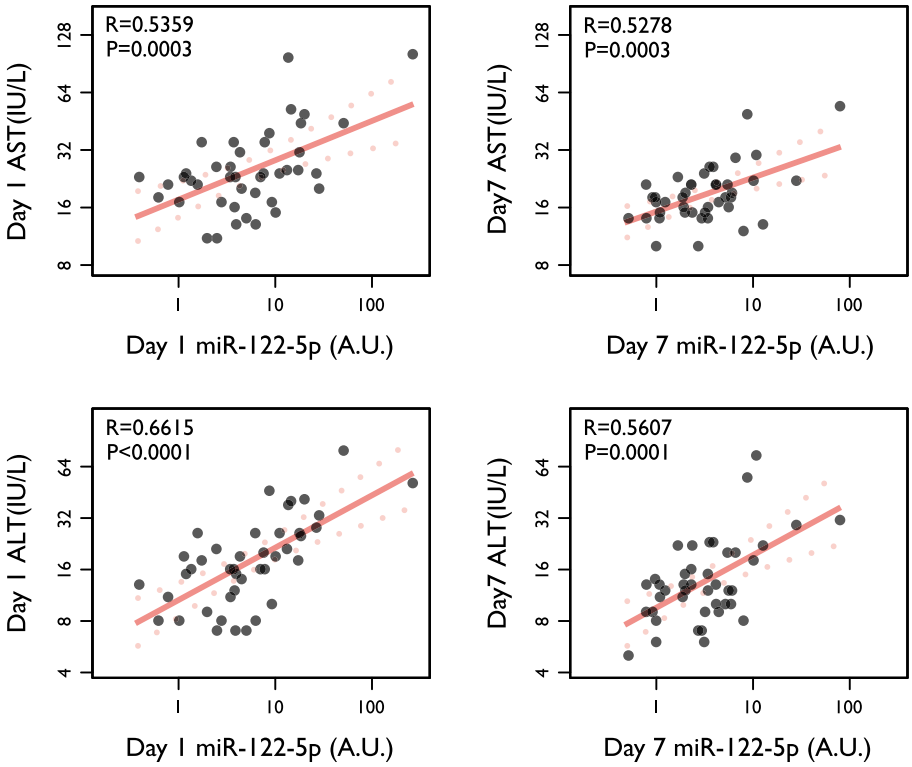
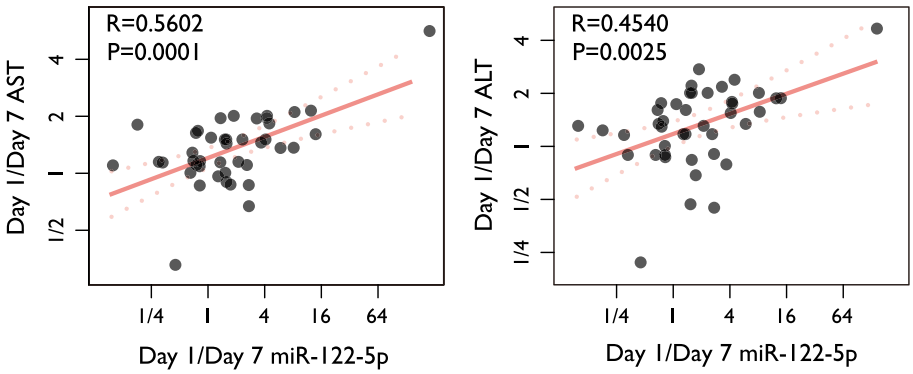


Figure 6 Correlation between changes of serum microRNA (miR)-122-5p and changes of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) activities. Relative changes in miR-122-5p, AST and ALT activity are shown after log 2 transformation. The red line indicates the estimated regression line. Dotted lines show the 95% confidence interval of the estimated regression.



Some reports have examined the relationship between miR-122-5p and heart failure. Corsten *et al.* showed that serum miR-122-5p was increased in patients with acute heart failure.[25] On the contrary, Fukushima *et al.* reported no relationship between miR-122-5p and New York Heart Association (NYHA) functional status in patients with chronic, stable heart failure.[26] This discrepancy might partly be explained by the fluctuation that was observed in our study. That is, heart failure patients showed increased miR-122-5p in serum in the acute phase with liver injury, but they did not show any elevation of miR-122-5p in serum during the stable phase. This finding was also consistent with the observation that patients with cirrhosis but without ongoing liver damage do not show elevated serum miR-122-5p level.[23]

Table 5 Relationships between clinical characteristics and serum microRNA (miR)-122-5p change

Explanatory variable (number of yes)	No	Yes	*P*-value
Male (23)	1.51 [1.14:3.36]	1.52 [0.74:3.29]	0.783
Ischemic heart disease (20)	2.14 [0.86:3.45]	1.38 [0.70:2.35]	0.225
Atrial fibrillation (19)	1.52 [0.76 :3.09]	1.46 [0.77:3.56]	0.745
Diabetes (20)	1.49 [0.70:2.53]	1.92 [0.79:4.38]	0.288
Viral hepatitis (2)	1.49 [0.75:3.27]	3.35 [2.98:3.71]	0.281
RAS inhibitor (17)	1.52 [0.80:2.63]	1.46 [0.73:3.94]	0.960
Aldosterone blocker (13)	1.52 [0.80:2.63]	1.46 [0.63:4.07]	0.591
Beta blocker (19)	1.47 [0.78:2.62]	1.68 [0.76:4.14]	0.671
Loop diuretics (31)	2.63 [1.36:5.98]	1.46 [0.70:2.56]	0.041
Statin (14)	1.49 [0.79:2.86]	1.52 [0.68:3.75]	0.927
Antiplatelet (20)	1.52 [0.86:2.60]	1.49 [0.71:3.98]	0.832
Anticoagulant (16)	1.49 [0.79:3.31]	1.67 [0.70:3.37]	0.828
Calcium channel blocker (18)	1.35 [0.67:3.67]	1.67 [1.31:3.04]	0.413
Oxygen requirement, Day 1 (17)	1.68 [0.79:3.18]	1.24 [0.73:3.54]	0.309
Oxygen requirement, Day 7 (7)	1.52 [0.77:2.91]	1.47 [0.88:3.81]	0.974
Inotrope use (6)	1.60 [0.79:3.64]	1.02 [0.69:1.42]	0.159
Natriuretic peptide use (15)	1.51 [0.70:2.62]	1.52 [1.14:4.01]	0.482
Nitroglycerin use (13)	1.52 [0.75:3.98]	1.47 [0.79:2.63]	0.839

RAS, Renin angiotensin system.
Data are presented as median of Day1/Day7 miR-122-5p (AU) and [1st:3rd quantile value]. Statistical significances were tested using Wilcoxon-rank sum tests.

Table 6 Correlations between serum laboratory measurements and microRNA (miR)-122-5p changes

Explanatory variable	Estimated coefficient [95% CI]	*P*-value
Δ White blood cell count	0.023 [−0.009:0.055]	0.157
Δ Red blood cell count	−0.001 [−0.015:0.014]	0.923
Δ Hemoglobin	−0.014 [−0.521:0.493]	0.955
Δ Platelet count	0.044 [−0.089:0.177]	0.509
Δ Albumin	1.247 [−0.168:2.662]	0.083
Δ AST	2.434 [1.284:3.583]	<0.001
Δ ALT	1.570 [0.585:2.554]	0.003
Δ ALP	4.479 [1.927:7.031]	0.001
Δ Total bilirubin	0.215 [−1.331:1.761]	0.780
Δ Blood urea nitrogen	0.069 [−1.375:1.512]	0.924
Δ GFR	0.007 [−0.055:0.069]	0.821
Δ Sodium	−0.046 [−0.155:0.063]	0.400
Δ Potassium	0.886 [−0.070:1.842]	0.069
Δ Creatine kinase	0.958 [−0.013:1.929]	0.053
Δ CRP	−0.046 [−0.509:0.418]	0.844
Δ BNP	0.628 [−0.304:1.560]	0.180
Δ Troponin-I	0.362 [-0.273:0.998]	0.255

ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BNP, Brain natriuretic peptide; CPK, Creatine phosphokinase; CRP, C-reactive protein; eGFR, Estimated glomerular filtration ratio; NT-pro BNP, N-terminal pro BNP.
Estimated Pearson's correlation coefficients and its 95% confidence interval (CI) are shown.

On the other hand, we did not detect a significant fluctuation of known heart failure-related miRs, such as miR-423-5p, miR-22, miR-320a. miR-92b, miR-208, and miR-499, in this study. This result was consistent with the previous report by Seronde *et al.*[27] They measured several heart-failure-specific serum miRs (miR-1, miR-21, miR-126, and miR-423-5p) on admission and the fifth admission day and did not see any significant fluctuations. It seems that these markers were relatively stable in patients with heart failure irrespective its severity or specific characteristics.

The physiological significance of the presence of miRs in the serum of heart failure patients has been largely unclear. Our result advocates a concept that some of the fluctuating serum miRs in patients with acute heart failure could reflect the organ damage caused by hemodynamic abnormality. Additionally, short-term resolution of organ damage could also be observed as a change in serum miRs. Based on these findings, measurement of organ-specific miRs might afford effective ways to monitor organ damage, which is caused by acute heart failure or other etiologies. Moreover, measurement of serum miRs on other settings or specific subtypes of heart failure, could identify a variety of miRs that relate to specific conditions or heart failure subtypes.

Circulating miRNAs in the blood now offer a new form of biomarker for disease diagnosis. Recent studies have proposed the use exosomal miRNAs as diagnostic markers in human diseases,[28] because the quantity of miRNAs in exosomes exhibited greater differences between healthy individuals and cancer patients than observed in sera.[29] It was also shown that more reads of miRNAs are detected in exosomal samples compared with those detected in serum or plasma.[30] Therefore, we utilized exosome-derived miRNA for the analysis. Traditional isolation of exosomes from body fluids is based on ultracentrifugation in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes away from other vesicles and particles. These protocols can take up to 30 h and are not suitable for assaying many samples. Recently, several polymers have been developed for the isolation of exosomes. By tying up water molecules, the polymer forces less-soluble components, such as vesicles, out of solution, which allows them to be collected easily using a short, low-speed centrifugation step. Thus, many

Table 7 Single and multivariate analysis of significant determinants of serum miR-122-5p

Explanatory variable	Single-variable analysis			Multi-variable analysis		
	Estimated coefficient	95% CI	*P*-value	Estimated coefficient	95% CI	*P*-value
Loop diuretic on admission	−0.363	−0.661:−0.066	0.018	−0.289	−0.543:−0.034	0.027
Δ Body weight	0.319	0.012:0.626	0.042	0.175	−0.087:0.437	0.183
Δ AST	0.560	0.296:0.825	<0.001	0.407	0.149:0.666	0.003
Loop diuretics on admission	−0.363	−0.661:−0.066	0.018	−0.319	−0.577:−0.060	0.017
Δ Body weight	0.319	0.012:0.626	0.042	0.200	−0.066: 0.466	0.136
Δ ALT	0.454	0.169:0.739	0.003	0.382	0.109: 0.655	0.008

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.

reports on exosomes-derived RNA have already been published by use of this method without any internal control.[31–36]

In conclusion, we found a dynamic miR signature transition in a timely fashion during the therapeutic course for acute heart failure. Among these miRs, serum miR-122-5p levels reflected liver damage in this condition. These findings warrant the importance of a larger study about the relationships between serial measurement of miRs and clinical indices or prognosis of acute heart failure.

Conflicts of interest

None declared.

Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers, 26116716, 26293186, and 25670383 to K. Ono, 26860556 to Y. Kuwabara, 20565577 to T. Horie, and 25293182 to T. Kimura. This work was also supported by grants from Japan Foundation for Aging and Health to H. Arai.

Supporting information

Supporting information may be found in the online version of this article:

Figure S1. Correlation between two screening experiments. Abundance determined by high-throughput sequencing (HTS) is shown on the horizontal axis and abundance determined by quantitative PCR (qPCR) is shown on the vertical axis. Only microRNAs (miRs) that were detected by both methods are shown. Pearson's correlation coefficient between experiments is shown.

Figure S2. Distribution of serum microRNA (miR)-122-5p abundance and change. Distribution of miR-122-5p abundance at each time point is shown in Figure S2A. Changes in miR-122-5p abundance are also shown in Figure S2B. The number of patients is shown on the horizontal axis. Relative abundances or relative changes are shown on the vertical axis.

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