MicroRNA 210 as a Biomarker for Congestive Heart Failure

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MicroRNAs (miRNAs) are endogenous small RNAs that are 18–23 nucleotides long. Recently, plasma miRNAs were reported to be sensitive and specific biomarkers of various pathological conditions. In the present study, we focused on miR-210, which is known to be induced by hypoxia and might therefore be an excellent biomarker for congestive heart failure. Plasma miR-210 levels and expression levels in mononuclear cells and skeletal muscles were elevated in Dahl salt-sensitive rats with heart failure. We also assessed miR-210 expression in patients with heart failure. The miR-210 expression levels in the mononuclear cells of patients with NYHA III and IV heart failure according to the New York Heart Association (NYHA) functional classification system were significantly higher than those with NYHA II heart failure and controls. Although no significant correlation was observed between plasma brain natriuretic peptide (BNP) and plasma miR-210 levels in patients with NYHA II heart failure, patients with an improved BNP profile at the subsequent hospital visit were classified in a subgroup of patients with low plasma miR-210 levels. Plasma miR-210 levels may reflect a mismatch between the pump function of the heart and oxygen demand in the peripheral tissues, and be a new biomarker for chronic heart failure in addition to plasma BNP concentrations.

Key words biomarker; heart failure; microRNA

MicroRNAs (miRNAs) are endogenous small RNAs comprising approximately 18–23 nucleotides, which bind to the 3′-untranslated region of mRNAs of protein-coding genes to downregulate their expression.1,2 miRNAs play an important role in various physiological and pathological processes.3,4 So far, more than 1500 human miRNAs have been identified (http://www.mirbase.org). They are expressed in a tissue- or cell-specific manner.5 Most human protein-coding genes are thought to be targeted by miRNAs,6,7 that appear to function as rheostats to fine tune protein output.8,9

Recently, miRNAs were reported to be present in various body fluids,3,10,11 More than 90 types of miRNAs have been detected in human sera using next-generation sequencing.10 Plasma miRNAs are embedded not only RNA-induced silencing complex (RISC) but also others, exosomes and/or microparticles.12–14 We recently reported that the plasma concentrations of myocardium-specific miRNAs are excellent biomarkers of myocardial infarction.12,14 Other groups also report that plasma miRNAs are sensitive and specific biomarkers of various tissue injuries and pathological conditions.15–18

The present study examined whether circulating miRNAs can be used as biomarkers in patients with heart failure. Recently, Tijen et al. reported that circulating plasma miR-423-5p is most closely related to a clinical diagnosis of heart failure.17 Moreover, we reported that the plasma concentration of miR-126 is negatively correlated with the severity of heart failure.19

In the present study, we determined whether miR-210 is a biomarker for congestive heart failure. Chronic heart failure is characterized by insufficient oxygen supply to the peripheral tissues; miR-210 is highly induced by hypoxia. MiR-210 has already attracted a great deal of attention as a biomarker for various diseases including breast cancer,20 acute cerebral ischemia,21 atherosclerosis obliterans,22 and acute kidney injury.23 Aberrantly accelerated proliferation and metabolism are typical characteristics of cancer cells, which lead to an imbalance between oxygen supply and consumption, causing hypoxia. Moreover, the obliteration of arteries or tissue injury exposes peripheral tissues to hypoxic conditions. In diseases with hypoxia, miR-210 might be a useful auxiliary biomarker (i.e., not for primary diagnosis). It has been established that miR-210 is specifically induced by hypoxia-inducible factor 1α (HIF-1α) during hypoxia. In addition, miR-210 might repress iron–sulfur cluster assembly protein (ISCU), leading to the repression of mitochondrial respiration, reducing oxidative stress, which may protect cells from apoptosis.24

In the present study, miRNA array analysis revealed miR-210 is elevated in the plasma of rats with heart failure. We confirmed that miR-210 is upregulated by hypoxia in rat myocardial cells (H9c2) and tested the hypothesis that the expression level of miR-210 increases in the peripheral tissues of rats with heart failure. Finally, we examined the possibility of miR-210 as a biomarker for heart failure in human patients.

MATERIALS AND METHODS

Rat Heart Failure Model Dahl salt-sensitive rats fed a high-salt diet for 8 weeks showed a systolic blood pressure (SBP) exceeding 220 mmHg, markedly elevated plasma brain natriuretic peptide (BNP) levels, marked cardiac hypertrophy, and massive proteinuria and were, therefore, considered to have chronic heart failure condition in accordance with previous reports.25–27 Male Dahl salt-sensitive rats (4 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in a temperature-controlled room on a 12-h light/12-h dark cycle and fed low (control group: 0.03%) or

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high (heart failure group: 8%) salt rat diet (Oriental Yeast, Tokyo, Japan) and tap water ad libitum. Body weight and SBP were measured weekly. SBP was measured using the tail-cuff method (BP-98A: Softron, Tokyo, Japan). After 8 weeks of treatment, blood was collected from the inferior vena cava under pentobarbital anesthesia with ethylenediaminetetraacetic acid (EDTA) for RNA measurement and sodium citrate for BNP enzyme-linked immunosorbent assay (ELISA) as an anticoagulant. Plasma was isolated by centrifugation at 16000×g for 15 min at 4°C. Mononuclear cells were isolated by Histopaque-1083 (Sigma-Aldrich, MO, U.S.A.) density gradient centrifugation. The cells were washed 3 times in phosphate-buffered saline (PBS). The heart, kidneys, and skeletal muscles (i.e., the quadriceps femoris) were resected and immediately frozen in liquid nitrogen for transcriptome analysis or Western blot analysis.

The present study was conducted in accordance with the guidelines of the National Cerebral and Cardiovascular Center for the Care and Use of Experimental Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adequate measures were taken to minimize the animals’ pain and discomfort.

**Confirmation of miR-210 as One of the miRNAs Most Markedly Upregulated by Hypoxia** H9c2 cells were obtained from the American Type Culture Collection (ATCC, MD, U.S.A.) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v fetal bovine serum (Gibco BRL, MD, U.S.A.). Cells were exposed to either normoxic conditions (normoxia group: 20% O2, 5% CO2, with N2 balance at 37°C) or hypoxic conditions (hypoxia group: 0–0.1% O2, 5% CO2, with N2 balance at 37°C) for 24 h. The hypoxic culture condition was introduced by using a CulturePal kit provided by Mitsubishi Gas Chemical Company (Tokyo, Japan). The treated H9c2 cells were washed with PBS and collected for transcriptome analysis or Western blot analysis.

**Rat Heart Failure Model and Cell Cultures. Transcriptome Analyses** Plasma RNA was isolated using the mirVana PARIS kit (Ambion, TX, U.S.A.) as described previously. As an internal reference, a known amount of a synthetic artificial miRNA was included in plasma samples as described previously. Total RNA was extracted from H9c2 and mononuclear cells or tissues with TRIzol reagent (Invitrogen, CA, U.S.A.) as described previously.

The expression profiling of 375 miRNAs was performed using the ABI TaqMan Rodent MicroRNA Array kit (Card A: Applied Biosystems, CA, U.S.A.) according to the manufacturer’s instructions. U6 small nuclear RNA included in the TaqMan Rodent MicroRNA Array was used as an endogenous control. No cut-off point was used. The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) was used for amplification and detection. The Ct value was obtained from the amplification plot using SDS software (Applied Biosystems).

The expressions of miR-210 and BNP mRNA were measured using the TaqMan microRNA real-time transcription-polymerase chain reaction (RT-PCR) kit (Applied Biosystems) and the TaqMan gene expression assay kit (Applied Biosystems) as described previously. The 7500 Fast Real-Time PCR System (Applied Biosystems) was used for amplification and detection. The Ct values were obtained from the amplification plot using SDS software.

**ISCU Western Blot Analysis** A rabbit polyclonal antibody against rat ISCU was obtained from Santa Cruz Biotechnology (CA, U.S.A.). H9c2 cells or tissues of Dahl salt-sensitive rats were homogenized in Triton-based lysis buffer, and the protein concentration was determined using the bicinchoninic acid method (Pierce, IL, U.S.A.). Equal amounts of protein (5 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and transferred to a nylon membrane (GE Healthcare, Buckinghamshire, U.K.). After blocking with 5% bovine serum albumin (BSA), the membranes were incubated with the primary antibody (1:10000 dilution) overnight at 4°C. Membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution for 1h). The expression levels were quantified by densitometry (Luminescent Image Analyzer LAS-1000: FUJIFILM, Tokyo, Japan).

**Plasma BNP ELISA** Plasma BNP concentrations were assayed using the AssayMax Rat BNP-45 ELISA Kit (AssayPro, MO, U.S.A.) according to the manufacturer’s protocol. Absorbance at 450 nm was measured using a Wallac 1420 ARVO MX/Light system (PerkinElmer, MA, U.S.A.). Standard points and samples were determined in duplicate.

**Assessment in Heart Failure Patients. Assessment of miR-210 Levels in Mononuclear Cells** Mononuclear cells were isolated from 13 patients hospitalized for congestive heart failure (8 and 5 patients classified as New York Heart Association (NYHA) II, and NYHA III and IV, respectively, according to the NYHA functional classification system) and 6 healthy control subjects. Plasma miR-210 concentrations were not determined because these samples were derived from samples of a previous study. Total mononuclear cells were isolated by Ficoll-Paque Plus (Pharmacia, NJ, U.S.A.) density gradient centrifugation. The collected cells were washed 3 times with PBS. The total RNAs of mononuclear cells were extracted with TRIzol reagent and analyzed using real-time RT-PCR.

**Assessment of Plasma miR-210 Levels** Thirty-nine patients with heart failure were recruited from our outpatient clinic. Blood samples were collected in tubes containing EDTA as an anticoagulant, plasma was obtained, and total RNA was purified as described above. Plasma BNP

### Table 1. Physiological Data of Dahl Salt-Sensitive Rats Fed the Low- and High-Salt Diets (n=9 and n=13, Respectively)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>SBP (mmHg)</th>
<th>Relative heart ratio (%)</th>
<th>Relative BNP mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks</td>
<td>8 weeks</td>
<td>0 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Low-salt diet</td>
<td>110.6±5.9</td>
<td>323.8±8.2</td>
<td>100.8±4.8</td>
<td>133.7±4.3</td>
</tr>
<tr>
<td>High-salt diet</td>
<td>99.9±6.3</td>
<td>244.4±26.1</td>
<td>97.2±7.4</td>
<td>218.0±21.6</td>
</tr>
</tbody>
</table>

The values represent the mean±S.D. *p<0.01. Body weight decreased in the high-salt diet group. In contrast, SBP, the relative heart ratio (heart weight/bodyweight), and BNP mRNA expression were significantly greater in the high-salt diet group than the low-salt diet group. These data show that the high-salt diet induced heart failure.
concentrations were obtained from chart data. All patients were classified as having NYHA II heart failure caused by a previous myocardial infarction. In 24 out of 39 patients, plasma BNP concentration was reassessed during the subsequent hospital visit approximately 3 months later. The patients were divided into “improved” and “unimproved” subgroups on the basis of the changes (i.e., decrease and increase, respectively) of their plasma BNP levels at the subsequent visit. Then, we evaluated whether plasma miR-210 levels at first visit could be used to predict the change in BNP.

Written informed consent was obtained from all participants. The present study was approved by the Ethics committee of the National Cerebral and Cardiovascular Center and performed in accordance with the Code of Ethics of the World Medical Association.

Statistical Analysis Data are presented as mean±S.D. Statistical analysis was performed by analysis of variance (ANOVA), regression analysis, and contingency table analysis using the JMP statistical analysis package (SAS Institute, Cary, NC, U.S.A.).

RESULTS

miRNA Array Analysis in Dahl Salt-Sensitive Rats with and without Heart Failure A high-salt diet for 8 weeks induced markedly high blood pressure and heart failure in Dahl salt-sensitive rats as reported previously. SBP, the relative heart ratio, and BNP mRNA expression levels also increased with feeding of the high-salt diet (Table 1). These data demonstrate that the high-salt diet induced heart failure.

The plasma RNAs of the control and heart failure groups were then subjected to miRNA array analysis (Table 2). Eleven miRNAs including miR-210 increased significantly in the heart failure group.

Confirmation of Hypoxia-Induced miRNAs in H9c2 Cells Because heart failure is characterized by a deficiency in oxygen supply relative to peripheral oxygen demand, we hypothesized that miRNAs involved in hypoxia might be upregulated in heart failure. Therefore, H9c2 cells exposed to normoxic or hypoxic conditions were subjected to miRNA array analysis. The results showed that miR-210 expression levels increased markedly in cells under the hypoxic culture condition, which was validated by real-time RT-PCR (Figs. 1A, B). ISCU is an important target of miR-210 and the induction of miR-210 downregulates its expression. However, ISCU levels in H9c2 cells did not differ between normoxic and hypoxic conditions (Fig. 1C).

Assessment of miR-210 Levels in Dahl Salt-Sensitive Rats with Heart Failure Given the results of the miRNA

<table>
<thead>
<tr>
<th>miRNA</th>
<th>$C_T$ value control</th>
<th>$C_T$ value heart failure</th>
<th>$\Delta C_T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a</td>
<td>25.44</td>
<td>21.59</td>
<td>3.85</td>
</tr>
<tr>
<td>miR-15b</td>
<td>23.74</td>
<td>21.74</td>
<td>2.00</td>
</tr>
<tr>
<td>miR-15c</td>
<td>25.89</td>
<td>22.79</td>
<td>3.10</td>
</tr>
<tr>
<td>miR-16</td>
<td>27.07</td>
<td>24.94</td>
<td>2.13</td>
</tr>
<tr>
<td>miR-19</td>
<td>22.13</td>
<td>18.94</td>
<td>3.19</td>
</tr>
<tr>
<td>miR-20a</td>
<td>25.98</td>
<td>22.79</td>
<td>3.19</td>
</tr>
<tr>
<td>miR-20b</td>
<td>28.60</td>
<td>23.29</td>
<td>5.31</td>
</tr>
<tr>
<td>miR-30b</td>
<td>23.96</td>
<td>20.34</td>
<td>3.62</td>
</tr>
<tr>
<td>miR-31</td>
<td>25.11</td>
<td>20.58</td>
<td>4.53</td>
</tr>
<tr>
<td>miR-34</td>
<td>27.65</td>
<td>23.12</td>
<td>4.53</td>
</tr>
</tbody>
</table>

$\Delta C_T$: $C_T$ value of control, $C_T$ value of heart failure.
array analysis in rats with heart failure and confirmation of miR-210 upregulation in cells cultured under hypoxic conditions, we investigated whether miR-210 is a biomarker for heart failure. Thus, we examined miR-210 expression levels in rats fed the low- and high-salt diets (Control: n=9) and high-salt diets (Heart failure: n=13). Expression levels of miR-210 increased up to 15.0 fold in plasma (Fig. 2A). The miR-210 expression levels of mononuclear cells, the heart, the kidneys, and skeletal muscle were examined in order to clarify the tissues in which miR-210 levels increased. Although miR-210 expression levels were unchanged in the heart and kidneys (Figs. 2C, D), they increased up to 4.5 and 2.1 fold in mononuclear cells and skeletal muscle, respectively (Figs. 2B, E). However, ISCU expression was not downregulated in Dahl salt-sensitive rats with heart failure (Fig. 2F).

Then, rats with and without heart failure were prepared...
Six patients were improved; their plasma miR-210 levels were lower (0.42 ± 0.25, n = 6) than those of not improved patients (0.65 ± 0.25, n = 18, p < 0.05). As a result, no patients with higher plasma miR-210 level had a tendency to improve (Fig. 3C).

**DISCUSSION**

Based on the result of increased miR-210 by hypoxia in the in vitro experiment, we showed that plasma miR-210 may be available to know the condition of heart failure. Additionally, we found that the information obtained from the measurement of plasma miR-210 levels is different from plasma BNP concentration.

miRNA array analysis of the plasma of Dahl salt-sensitive rats with heart failure revealed various candidate biomarker miRNAs, including miR-15a, miR-15b, miR-20a, miR-103, miR-130a, miR-130b, miR-195, miR-210, miR-301b, miR-451, and miR-494 (Table 2). The miR-15 family (i.e., miR-15a, miR-15b, and miR-195) has been reported to regulate the postnatal mitotic arrest of cardiomyocytes. Meanwhile, the miR-130 family (i.e., miR-130a and miR-130b) has been reported to enhance HIF-1α translation, while miR-494 has been reported to activate the Akt pathway, which confers protective effects against ischemia/reperfusion-induced cardiac injury. MiR-20a has been reported to modulate the translation of E2F transcription factors that regulate cell proliferation and apoptosis. However, the physiological functions of miR-103 and miR-301b are still incompletely understood. Therefore, these may be worth investigating in further detail in future studies.

The miRNA array data of the rats were compared with those of the human sample obtained from a previous study. The results show that rats and humans with severe heart failure exhibited upregulated plasma miR-210 and miR-451 levels. MiR-451 is highly expressed in erythroid cells, hemolysis, which is frequently observed in congestive heart failure, appears to be the reason for the upregulation of plasma miR-451 in heart failure. Therefore, we focused on miR-210 as a possible biomarker for congestive heart failure.

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**Table 3. Characteristics of Patients with NYHA II Heart Failure**

(n = 5 and n = 5, respectively) in order to assess the correlation between plasma BNP and miR-210 levels. The plasma BNP concentrations of rats with heart failure (228.1 ± 147.8 pg/mL) were significantly higher than those without heart failure (18.8 ± 3.5 pg/mL, p < 0.03). Moreover, plasma miR-210 levels were strongly correlated (r² = 0.7531) with plasma BNP, a conventional marker of heart failure (Fig. 2G).

**Assessment of Plasma miR-210 Levels in Patients with Heart Failure** We hypothesized that miR-210 is upregulated in patients with severe heart failure. Indeed, the miR-210 expression levels in mononuclear cells were significantly higher in patients with NYHA III and IV heart failure than those with NYHA II or healthy controls (Fig. 3A, p < 0.05). However, the miR-210 expression levels in mononuclear cells were not significantly different between healthy controls and patients with NYHA II heart failure.

Accordingly, 39 patients with NYHA II heart failure were recruited from our outpatient clinic. Their characteristics are summarized in Table 3. The correlation between plasma BNP and miR-210 levels was examined; no significant correlation was observed in these patients (Fig. 3B). However, plasma BNP concentrations were reassessed at the subsequent hospital visit approximately 3 months later; consequently, plasma miR-210 levels of all the improved patients were in the lower range (0.42 ± 0.10, n = 6) than those of not improved patients (0.65 ± 0.25, n = 18, p < 0.05). As a result, no patients with higher plasma miR-210 level had a tendency to improve (Fig. 3C).
miR-210 is well known to be upregulated by hypoxia.\textsuperscript{35,36} It is also reported that miR-210 is regulated via both HIF-dependent\textsuperscript{37,38} and HIF-independent mechanisms.\textsuperscript{39} and that miR-210 is associated with angiogenesis.\textsuperscript{40} Indeed, the present results confirm the induction of miR-210 by hypoxia. Chronic heart failure is characterized by a deficiency in oxygen supply relative to the demand of the peripheral tissues. Thus, from this perspective, miR-210 might be the promising candidate biomarker for heart failure.

The plasma miR-210 levels were increased in Dahl salt-sensitive rats with heart failure induced by 8 weeks of high-salt diet feeding. miR-210 expression levels of mononuclear cells, the heart, the kidneys, and skeletal muscle were examined in order to elucidate the tissues in which miR-210 was elevated. The expression levels of miR-210 did not change in the heart or kidneys. This is probably because the heart and kidneys were not exposed to hypoxic conditions, because the blood flow to these organs might be preferentially preserved.

In contrast, miR-210 expression levels in mononuclear cells and skeletal muscles increased significantly. The skeletal muscles may be the first target of reduced blood supply in congestive heart failure.\textsuperscript{41} The increased plasma miR-210 levels might be attributable to increased miR-210 levels in the skeletal muscle.

Moreover, we assessed the correlation between plasma BNP and miR-210 levels. The results show that plasma miR-210 levels were strongly correlated with plasma BNP, a conventional marker of heart failure. Therefore, the results suggest that plasma miR-210 levels are a prognostic biomarker for chronic heart failure.

Next, we assessed miR-210 expression in human patients with heart failure. The expression levels of miR-210 in the mononuclear cells of patients with NYHA III and IV heart failure were significantly higher than those with NYHA II heart failure and healthy controls. However, miR-210 expression levels in mononuclear cells did not differ between healthy controls and patients with NYHA II heart failure. Furthermore, no significant correlation was observed between BNP and miR-210 plasma levels in patients with NYHA II heart failure. Thus, it is conceivable that the results of the present animal experiment are close to those observed in severe heart failure (NYHA III and IV) patients before medical treatment. Furthermore, the results suggest that the lower correlation in human samples could be attributed to medical treatments. However, none of the patients with higher plasma miR-210 levels showed a tendency toward improved BNP levels. Therefore, plasma miR-210 level is an auxiliary prognostic biomarker for chronic heart failure.

In the aging population, deaths and medical costs attributable to heart failure are increasing rapidly. Managing patients with heart failure is one of the greatest challenges our aging society faces. Therefore, the development of accurate prognostic biomarkers of heart failure is important. Plasma BNP level is an excellent biomarker for assessing patients with heart failure. Plasma BNP levels mainly reflect the degree of ventricular overload,\textsuperscript{42} and are, therefore, an excellent prognostic biomarker for heart failure.\textsuperscript{43} Because miR-210 is significantly induced by hypoxia, miR-210 levels may reflect a mismatch between the pump function of the heart and oxygen demand in the peripheral tissues. In this sense, plasma miR-210 is a potential prognostic biomarker for heart failure in addition to plasma BNP level. However, since the number of samples in the present study was insufficient to determine a suitable plasma miR-210 cut-off point and the assessment of severe patients was not carried out, a larger clinical study is required to confirm this hypothesis. In addition, although mononuclear cells might be suitable for diagnosis, this would be impractical because isolating these cells is inconvenient.

The biological significance of miR-210 induction by hypoxia remains unclear. It has been reported that ISCU is one of the direct targets of miR-210 and is downregulated by hypoxia-induced miR-210 in cancer cell lines.\textsuperscript{38} However, miR-210 induction did not suppress ISCU protein levels under hypoxic conditions in our experiments using rat myocardial cells. Moreover, miR-210–knockout mice are reported to exhibit no gross phenotype.\textsuperscript{44} Therefore, further studies are required to fully clarify the biological functions of miR-210.

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