INTRODUCTION

Cardiorespiratory fitness is not only a measure of physical function but also a key factor closely associated with morbidity and mortality in patients with cardiovascular diseases.\(^1\)\(^-\)\(^3\) Cardiopulmonary exercise testing (CPET) is currently the most reliable examination method to evaluate parameters related to cardiopulmonary fitness such as oxygen consum-
tion and ventilatory efficiency during exercise. However, performing CPET in patients as part of routine clinical care is complicated and presents multiple barriers such as the need for equipment, trained personnel, time, and patient condition and effort. Consequently, there is a need to explore biomarkers that can serve as reliable and practical alternatives to CPET.

MicroRNAs (miRs) are non-protein-coding ribonucleic acid molecules that control posttranscriptional gene expression and downstream cellular function by directly binding to specific sequences in mRNA transcripts. miRs can be secreted as stabilised factors into the blood stream or contained either in microvesicles or as free RNA–protein complexes that later mature to circulating miRs. Circulating concentrations of specific miRs have been used as biomarkers of various disease states including cardiovascular diseases. However, few studies have determined the relation between circulating miRs and cardiorespiratory fitness in patients with cardiovascular diseases.

Against this background, we conducted the present pilot study to investigate the correlations between circulating levels of specific miRs and parameters of CPET in patients after acute myocardial infarction (MI). We focused on the miR-181 family and miR-484 because these miRs are known to be associated with the mitochondrial function of cardiomyocytes. Members of the miR-181 family are multifunctional miRs that were originally identified as specifically expressed in haematopoietic cells and modulate haematopoietic lineage differentiation. A series of experimental studies by Das et al. showed that members of the miR-181 family (miR-181a, b, and c) modulate the myocardial response to oxidative stress in cardiomyocytes. miR-484 has been shown to take part in the process of cell proliferation and apoptosis. In cardiomyocytes, miR-484 suppresses translation of the mitochondrial fission protein Fis1 and inhibits fission and apoptosis.

Identifying a potential link between cardiopulmonary performance and novel biomarkers would provide not only a new diagnostic tool but also new insight into the potential pathophysiology of cardiovascular disease. We hypothesised that circulating miR-181c and miR-484 may be candidates for such biomarkers.

**METHODS**

**Study Patients**

We examined data from 20 patients with acute MI (age, 70.2 ± 9.9 years; 15 men). Patients with prior histories of cardiac surgery, chronic obstructive pulmonary disease, and those on haemodialysis were excluded. All subjects underwent primary percutaneous coronary intervention within 24 h after the onset of MI. The diagnoses of acute MI were based on clinical symptoms, electrocardiographic changes, blood examinations, and coronary angiograms. Circulating levels of creatine kinase (CK), CK-MB, and troponin-I levels were measured until they peaked. Two-dimensional (2D) and Doppler echocardiography were performed using digital echocardiography equipment (TUS-A400, Canon Medical Systems, Tochigi, Japan) before discharge; 2D measurements included the left ventricular (LV) end-diastolic dimension and the left atrial dimension. The LV ejection fraction (LVEF) was calculated using the biplane modified Simpson’s method. Additionally, all patients underwent CPET in the convalescent phase following acute MI.

The study protocol conformed to the guidelines of the Helsinki Declaration for human research and was approved by the institutional review board of Showa University Fujiigaoka Hospital/Fujiigaoka Rehabilitation Hospital (approval number: 2016017). Written informed consent was obtained from all individual participants included in this study.

**Quantification of miR Expressions**

Circulating levels of miR-181 family members and miR-484 were quantified using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) in a similar manner to that previously described. Serum from peripheral blood was collected on days 1 and 7 after admission and stored at −80°C. Serum samples from apparently healthy Japanese subjects (n=5; age, 63.6 ± 4.8 years; 3 men) were purchased from BioIVT (Westbury, NY, USA) and used as the control. Total RNA was extracted from patient serum and reverse-transcribed. The generated cDNA was amplified using labelled TaqMan probe and primer sets for miR-181a-5p, b-5p, and c-5p and miR-484 with a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The fold change was calculated using the 2–ΔΔCT formula, where CT is the threshold cycle. ΔCT was calculated by subtracting the CT values of each miR in a sample from the CT value of the exogenously added cel-miR-39-3p in that sample. To determine the ΔΔCT, these ΔCT values were subsequently compared with the values of each control sample (normalised to a fold change of 1).

**Cardiopulmonary Exercise Testing**

Symptom-limited CPET using a cycle ergometer (StrengthErgo-8, Mitsubishi Electric Engineering, Nagoya,
Japan) was performed at the beginning of participation in the outpatient cardiac rehabilitation program (25 ± 9 days after the onset of acute MI), as previously described. In brief, after warming up, patients performed an incremental exercise test with a ramp protocol (10 W/min) until exhaustion and then recovered for 5 min. Expired gas was collected and analysed continuously using an AE-310S gas analyser (Minato, Osaka, Japan). Peak oxygen consumption (VO$_2$), the anaerobic threshold (AT), and the slope of the relationship between ventilation (VE) and carbon dioxide output (VCO$_2$) were determined. AT was determined by the V-slope method. Peak VO$_2$ was defined as the highest VO$_2$ value achieved during peak exercise. Peak VO$_2$ expressed as a percentage of the age and sex-predicted value (peak VO$_2$-%predict) was calculated with the use of a formula based on Japanese normal subjects, as previously described. The VE versus VCO$_2$ (VEvsVCO$_2$) slope was calculated as the slope of the linear regression line between VE and VCO$_2$ from the start of exercise to immediately before the respiratory compensation point; if the subject did not reach that point, the line from the start of the exercise to the end of the exercise was used. The slope is the ratio of total ventilation required for CO$_2$ excretion. Total ventilation is the sum of dead space ventilation and alveolar ventilation, and pulmonary blood flow is involved in alveolar ventilation. Therefore, increased ventilation/perfusion mismatching is one of the principal mechanisms by which the slope becomes steeper.

**Statistical Analysis**

All data were expressed as means ± standard deviations, unless otherwise indicated. Student’s t-test was used to compare the differences between the groups. Comparisons between three groups were performed using one-way analysis of variance followed by the Tukey–Kramer multiple comparison test. To compare the fold changes, the ΔΔCT value was used for analysis. The correlation coefficients between two parameters were determined using Pearson’s simple linear regression analysis. All statistical analyses were conducted using the JMP Pro 14 software package (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Characteristics of Patients after Acute MI**

Table 1 shows the patients’ characteristics. The mean age was 70 years, and men made up 75% of the patients. Almost all included patients were diagnosed with ST-segment elevation MI. Culprit lesions were localised in the left anterior descending (LAD) and right coronary artery in 80% of cases. All patients underwent primary percutaneous coronary intervention on admission. Medical treatment after admission was performed according to the current guidelines. Echocardiography revealed mildly reduced LVEF after MI.

CPET was performed during the early convalescent phase after acute MI to determine the cardiopulmonary fitness of the patients. CPET analysis showed impaired exercise tolerance and reduced ventilatory efficiency (Table 2). A peak VO$_2$ <85% of that predicted by the patient’s age and sex category is considered an indicator of reduced exercise capacity. A VEvsVCO$_2$ slope >34 is considered the cut-off score for predicting worse prognosis in patients with cardiovascular disease. The present CPET analysis revealed reduced peak VO$_2$-%predictive (mean 74%) and increased VEvsVCO$_2$ slope (mean 34) in patients after acute MI.

**Time Course Changes in Circulating Levels of miR-181s after Acute MI**

We evaluated changes in the circulating mir levels between the 1st and 7th day after acute MI (Fig. 1). The circulating mirs from apparently healthy subjects were used as controls. In a fold-change analysis, the circulating levels of miR-181a on the 1st and 7th days after acute MI were significantly higher than those in the controls. miR-181b levels gradually increased until the 7th day, although the differences were not statistically significant. miR-181c levels on the 7th day were significantly higher than those on the 1st day and in controls. Circulating levels of miR-484 appeared to be unchanged.

**Correlation Between Clinical and CPET Parameters and Circulating miRs on the 7th Day after MI**

Next, we examined the correlations between the miR levels after MI and clinical and CPET parameters to explore the clinical significance of the circulating mirs. The absolute levels of circulating mirs were expressed as –ΔCT. The mir levels on the 1st day of hospitalisation did not correlate significantly with the clinical and CPET parameters. On the 7th day, the levels of the mirs were as follows: miR-181a, −8.4 ± 0.9; miR-181b, −8.2 ± 1.1; miR-181c, −15.2 ± 1.5; and miR-484, −2.9 ± 3.2. The peak levels of CK, CK-MB, and troponin-I in the peripheral circulation reflect the extent of myocardial damage after acute MI. The levels of circulating miR-181 family members and miR-484 did not significantly correlate with age, myocardial damage factors, or values measured by echocardiography (LVEF and end-diastolic...
Table 1. Patient characteristics (n=20)

| Characteristic                              | Value        |
|--------------------------------------------|--------------|
| Age, years                                 | 70.2 ± 9.9   |
| Male sex, n (%)                            | 15 (75)      |
| Diagnosis, n (%)                           |              |
| STEMI                                      | 19 (95)      |
| Non-STEMI                                  | 1 (5)        |
| Risk factors, n (%)                        |              |
| DM                                         | 5 (25)       |
| HT                                         | 9 (45)       |
| DLP                                        | 13 (65)      |
| Current smoking                            | 6 (35)       |
| Culprit vessel, n (%)                      |              |
| LAD                                        | 8 (40)       |
| RCA                                        | 8 (40)       |
| Cx                                         | 4 (20)       |
| Peak levels of myocardial enzymes          |              |
| CK, IU/L                                   | 2691 ± 2045  |
| CK-MB, IU/L                                | 245 ± 157    |
| TnI, ng/L                                  | 148 ± 140    |
| Medication, n (%)                          |              |
| Dual antiplatelet therapy                  | 20 (100)     |
| ACEi/ARB                                   | 13 (65)      |
| β-blocker                                  | 19 (95)      |
| Statin                                     | 18 (90)      |
| Echocardiography                           |              |
| LVEDD, mm                                  | 49.0 ± 12.1  |
| LA dimension, mm                           | 34.4 ± 9.0   |
| LVEF, %                                    | 49.2 ± 14.0  |

ACEi/ARB, angiotensin converting enzyme inhibitor/angiotensin type-I receptor blocker; CK, creatine kinase; Cx, circumflex coronary artery; DLP, dyslipidaemia; DM, diabetes mellitus; HT, hypertension; LA, left atrium; LAD, left anterior descending coronary artery; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; RCA, right coronary artery; STEMI, ST-segment elevation myocardial infarction; TnI, troponin-I.

Table 2. Parameters of cardiopulmonary exercise testing (n=20)

| Parameter                      | At rest | AT         | Peak         |
|--------------------------------|---------|------------|--------------|
| Work rate (W)                  | 0       | 44 ± 13    | 84 ± 23      |
| Heart rate (bpm)               | 68 ± 8  | 93 ± 13    | 116 ± 14     |
| R (gas exchange ratio)         | 0.92 ± 0.08 | 0.93 ± 0.06 | 1.18 ± 0.11 |
| VO2 (ml/min/kg)                | 3.5 ± 0.5 | 11.2 ± 1.9 | 17.3 ± 3.4   |
| Peak VO2-%predict (%)          | –       | –          | 73.7 ± 11.6  |
| VE vs VCO2 slope               | –       | –          | 33.8 ± 4.4   |

AT, anaerobic threshold; VCO2, carbon dioxide output; VE, ventilation; VO2, oxygen consumption

diameter) (Table 3).

Pearson’s simple linear regression analysis demonstrated that circulating levels of miR-181c and miR-484 on the 7th day of hospitalisation had a significant positive correlation with AT, peak VO2, and peak VO2-%predict from the CPET analysis, whereas miR-181a and miR-181b levels did not show any significant correlation with these variables. Moreover, circulating levels of miR-181c were inversely associated with
Distinct Kinetics of Circulating miR-181c and miR-484 Between Patients with High and Low Exercise Capacity

Although the expression patterns after MI were different for circulating miR-181c and miR-484, the levels of both these miRs on the 7th day of hospitalisation were positively associated with exercise capacity in patients with MI. Therefore, we compared the expression levels of these circulating miRs on the 7th day between patients characterised with high or low exercise capacity. The patients were divided into two groups according to the Weber–Janicki classification for exercise tolerance in cardiovascular diseases: the high exercise capacity group (class A and class B (peak VO$_2$ >16 ml/min/kg, n=13)) and the low exercise capacity group (class C (12 < peak VO$_2$ <16 ml/min/kg, n=7)). None of the patients in this study were in class D or E of this classification. miRs from healthy subjects were used as the reference for the fold change analysis.

Fig. 1. Fold changes in circulating miR-181a/b/c and miR-484 levels in patients after acute myocardial infarction compared with those in controls. The time course changes in expression levels were distinct among the circulating miRs. Circulating levels of miR-181c on the 7th day were prominently elevated in patients after acute myocardial infarction compared with those in controls. Con, healthy control subjects (n=5); MI, patients after acute myocardial infarction (n=20); d1, d7, the 1st and 7th days after admission. *P <0.01 vs. Con; #P <0.05 vs. MI d1.

The analysis of miR-181c found that the expression levels in the two groups were higher than the reference values (Fig. 2). The expression levels in patients with class A or B exercise tolerance were 14-fold higher than the reference and significantly higher than those in patients with class C exercise tolerance. The expression pattern of miR-484 with respect to the reference group was different from that of miR-181c. The levels of miR-484 in the class A and B groups were marginally higher than the reference, whereas the miR levels in the class C group were markedly lower than the reference. The expression levels of miR-484 in patients from class A and B groups were significantly higher than those in patients with class C exercise tolerance.

Circulating Levels of miR-181c and miR-484 in Patients with LAD and Non-LAD Lesions

LAD lesions are recognised as an important factor in risk stratification for coronary artery diseases. Consequently,
we also divided the patients into two groups according to the culprit vessels in acute MI: the LAD lesion group (n=8) and the non-LAD lesion group (n=12). We then compared the CPET parameters and the expression levels of circulating miR-181c and miR-484 between the two groups. From CPET analysis, the VEvsVCO\textsubscript{2} slope was significantly higher in the LAD lesion group than in the non-LAD lesion group (37.5 ± 3.4 vs. 33.1 ± 4.2, P <0.05), whereas indices for exercise capacity were not significantly different between the two groups. The LAD lesion group showed relatively lower expression levels of miR-181c and miR-484 than the non-LAD lesion group (–ΔCT: −15.7 ± 1.2 vs. −14.9 ± 1.7 for miR-181c and −3.4 ± 2.8 vs. −2.5 ± 3.5 for miR-484), although the differences were not statistically significant.

### Association of the Circulating miR Levels with Changes in Cardiopulmonary Fitness after Cardiac Rehabilitation

Fifteen of the 20 patients participated in and completed a 5-month outpatient cardiac rehabilitation program. The program significantly improved peak VO\textsubscript{2} (16.2 ± 2.5 to 18.4 ± 3.5 ml/min/kg, P <0.05) and VEvsVCO\textsubscript{2} slope (35.8 ± 4.4 to 33.3 ± 4.9, P <0.05) in these participants. The changes in exercise capacity and ventilatory inefficiency were not associated with circulating levels of miR-181c or miR-484 on day 7 after acute MI.

### Table 3. Correlations between circulating miR levels on the 7th day after MI and clinical and CPET parameters

| Clinical parameters | miR-181a (–ΔC\textsubscript{T}) | Correlation coefficient | P | miR-181b (–ΔC\textsubscript{T}) | Correlation coefficient | P | miR-181c (–ΔC\textsubscript{T}) | Correlation coefficient | P | miR-484 (–ΔC\textsubscript{T}) | Correlation coefficient | P |
|---------------------|---------------------------------|-------------------------|---|---------------------------------|-------------------------|---|---------------------------------|-------------------------|---|---------------------------------|-------------------------|---|
| Age                 | –0.347                          | 0.134                   |   | –0.173                          | 0.467                   |   | –0.415                          | 0.077                   |   | –0.044                          | 0.853                   |   |
| Peak CK             | 0.033                           | 0.889                   |   | 0.085                           | 0.723                   |   | –0.131                          | 0.592                   |   | –0.259                          | 0.271                   |   |
| Peak CK-MB          | 0.047                           | 0.846                   |   | 0.156                           | 0.512                   |   | –0.043                          | 0.862                   |   | 0.047                           | 0.844                   |   |
| Peak TnI            | 0.070                           | 0.770                   |   | 0.018                           | 0.939                   |   | –0.018                          | 0.940                   |   | –0.238                          | 0.313                   |   |
| LVEF                | –0.308                          | 0.187                   |   | –0.118                          | 0.620                   |   | –0.049                          | 0.842                   |   | 0.245                           | 0.297                   |   |
| LV EDD              | –0.070                          | 0.770                   |   | –0.015                          | 0.950                   |   | –0.234                          | 0.336                   |   | –0.057                          | 0.811                   |   |
| CPET parameters     |                                 |                         |   |                                 |                         |   |                                 |                         |   |                                 |                         |   |
| AT                  | 0.263                           | 0.263                   |   | 0.205                           | 0.386                   |   | 0.478                           | 0.038*                  |   | 0.555                           | 0.011*                  |   |
| Peak VO\textsubscript{2} | 0.390                         | 0.089                   |   | 0.319                           | 0.171                   |   | 0.649                           | 0.003*                  |   | 0.513                           | 0.021*                  |   |
| Peak VO\textsubscript{2}-%predict | 0.294                  | 0.208                   |   | 0.317                           | 0.174                   |   | 0.565                           | 0.012*                  |   | 0.553                           | 0.012*                  |   |
| VEvsVCO\textsubscript{2} slope | –0.165                  | 0.486                   |   | 0.008                           | 0.974                   |   | –0.525                          | 0.021*                  |   | –0.112                          | 0.639                   |   |

*Statistically significant (P<0.05)

**DISCUSSION**

The results of the present study demonstrated that circulating levels of miR-181c and miR-484 on the 7th day after acute MI were positively associated with peak VO\textsubscript{2} determined by CPET in patients in the early convalescent phase after MI. miR levels could allow the discrimination of patients with high and low exercise capacity according to the Weber–Janicki classification. Circulating miR-181c also showed an inverse correlation with ventilatory inefficiency. However, circulating miR levels were not correlated with the location of the culprit lesions, peak levels of myocardial enzymes, or LVEF. These findings suggest that circulating miR levels may be regulated by not only cardiac condition but also a more complex pathophysiology, including functional impairment of the skeletal muscle. Moreover, circulating levels of miR-181c and miR-484 did not reflect changes in cardiopulmonary fitness after a 5-month outpatient cardiac rehabilitation program.

Among patients with heart failure, plasma concentrations of norepinephrine and brain natriuretic peptide (BNP) are known to be biomarkers associated with adverse clinical outcomes.\textsuperscript{23,24} Peak VO\textsubscript{2} and the VEvsVCO\textsubscript{2} slope as measured by CPET are indices of cardiorespiratory fitness and are likely more powerful predictors of morbidity and mortality in patients with cardiovascular diseases.\textsuperscript{1,3,22–24} However, performing CPET continues to be impractical for routine clinical care in many countries. Furthermore, BNP was found not to be an alternative biomarker to CPET.\textsuperscript{26}
In the field of exercise physiology, there has been great interest in exploring circulating miRs as potential biomarkers. Recently, several studies have shown changes in some circulating miRs in response to different types of exercise. Combining the results of exercise testing with the levels of selected circulating miRs allowed accurate discrimination between patients with coronary artery disease and healthy controls. In contrast, Hortman et al. focused specifically on the exercise-induced changes in specific miRs, but did not find any clinical value related to the diagnosis of coronary artery disease. However, few reports have demonstrated a direct association between circulating miRs and exercise capacity. One report showed that circulating miR-181a levels at baseline were negatively associated with gait speed changes after aerobic exercise training in obese older adults. Against this background, the present study is the first to show that certain types of circulating miRs, i.e., miR-181c and miR-484, are associated with cardiorespiratory fitness evaluated accurately by CPET in patients with cardiovascular disease.

Intracellular, but not circulating, miR-181c and miR-484 have been shown to regulate mitochondrial function in cardiomyocytes. miR-181c was originally documented as having roles in the inflammatory response, such as CD4+ T-cell activation. Later, miR-181c was found to be increased in cardiac tissues of patients with congenital ventricular septal defects. Cardiac tissue miR-181c likely plays an important role in the diseased myocardium. Experimental studies revealed that miR-181c is translocated from the nucleus to the mitochondria in rat cardiomyocytes to regulate mitochondrial gene expression and function. miR-181c worsened the damage caused by the overproduction of reactive oxygen species via targeting mt-COX1 in the mitochondrial compartment of cardiomyocytes, whereas miR-181a/b had protective effects by targeting PTEN in the cytosol. Moreover, miR-181 family members are involved in the process of skeletal muscle differentiation. Therefore, an intracellular increase of miR-181c in a myoblast may induce skeletal muscle dysfunction.

miR-484 is closely related to the diagnosis and recurrence of various tumours, such as non-small-cell lung cancer. Doxorubicin induced the elevation of miR-484 expression and apoptosis in murine cardiomyocytes. Foxo3a has been shown to transactivate miR-484 expression in cardiomyocytes. The Foxo3a/miR-484 axis attenuated Fis1 upregulation by binding to the amino acid coding sequence of Fis1 and inhibiting its translation, which in turn inhibited mitochondrial fission and myocyte death. miR-484 likely plays a protective role in damaged cardiomyocytes by regulating mitochondrial function.

We found that the expression kinetics of circulating miR-484 after MI was distinct from that of circulating miR-181c, although both were positively associated with the exercise capacity of these patients. In a sub-analysis, miR-484 levels in patients with low exercise capacity after MI were lower than those in healthy controls and lower than those
in patients with high exercise capacity. In a community-based cohort, lower levels of circulating miR-484 than the median value were associated with the occurrence of atrial fibrillation. Therefore, decreased miR-484 expressions in cardiomyocytes and peripheral blood might reflect impaired cardiopulmonary condition. Increases in intracellular miR-181c potentially lead to myocardial injury and heart failure, whereas increases in miR-484 do not. However, circulating miR-181c in the peripheral blood was significantly lower in patients with heart failure than in healthy subjects. In the present study, circulating levels of miR-181c were lower in post-MI patients with impaired exercise capacity than in those with high exercise capacity. Consequently, the mechanisms and significance of increased miR-181c expression appear to be different between cardiac tissues and the blood stream.

Circulating miR-181c increased up to 7 days after acute MI in the present study. Acute MI induces a generalised inflammatory response 1–3 days after the acute event; subsequently, factors related to tissue repair and/or maintenance of homeostasis are mobilised in response. Circulating miRs are involved in this process. We speculate that miR-181c might gradually increase in cardiac and skeletal muscle tissues as a result of stimulating signals such as acute oxidative stress caused by hypoperfusion. Therefore, to maintain tissue homeostasis, it would be necessary for miR-181c to be washed out from the muscle tissues. We further surmise that impaired exercise tolerance in patients with cardiovascular diseases might be conferred in part by decreased excretion of miR-181c into the peripheral circulation because of the increased needs of cardiomyocytes and skeletal myoblasts.

There are several limitations to this study. Given the relatively low number participants in this pilot study, we cannot rule out the possibility that some of the observed differences and associations were statistically significant simply due to chance. Larger scale prospective studies are needed to further clarify the association of cardiopulmonary fitness with circulating levels of miR-181c and miR-484 in various cardiovascular diseases. Additionally, the underlying mechanisms of linkage between exercise tolerance and circulating miRs remain uncertain. Consequently, further experimental study will be needed to obtain mechanistic insights.

CONCLUSIONS

miR-181c and miR-484 appear to play roles in the pathophysiology of cardiovascular diseases. Circulating levels of miR-181c and miR-484 after acute MI are likely to be predictive of exercise capacity and ventilatory inefficiency in patients in the early convalescent phase. Measurements of circulating miRs may be useful not only for the early detection of cardiopulmonary fitness in patients after MI but also for decision making regarding cardiac rehabilitation.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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