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Research article

Diagnostic value of circulating microRNA-21 in chronic lung allograft dysfunction after bilateral cadaveric and living-donor lobar lung transplantation☆

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ABSTRACT

Background: MicroRNAs (miRNAs) involved in the pathogenesis of pulmonary fibrosis have been shown to be associated with the development of chronic lung allograft dysfunction (CLAD) after lung transplantation (LT). We investigated the role of circulating miRNAs in the diagnosis of CLAD after bilateral LT, including cadaveric LT (CLT) and living-donor lobar LT (LDLLT).

Methods: The subjects of this retrospective study were 37 recipients of bilateral CLT (n = 23) and LDLLT (n = 14), and they were divided into a non-CLAD group (n = 24) and a CLAD group (n = 13). The plasma miRNA levels of the two groups were compared, and correlations between their miRNAs levels and percent baseline forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), and total lung capacity (TLC) values were calculated from one year before to one year after the diagnosis of CLAD.

Results: The plasma levels of both miR-21 and miR-155 at the time of the diagnosis of CLAD were significantly higher in the CLAD group than in the non-CLAD group (miR-21, $P = 0.0013$; miR-155, $P = 0.042$). The miR-21 levels were significantly correlated with the percent baseline FEV1, FVC, and TLC value of one year before and at the time of diagnosis of CLAD ($P < 0.05$). A receiver operating characteristic curve analysis of the performance of miR-21 levels in the diagnosis of CLAD yielded an area under the curve of 0.89.

Conclusion: Circulating miR-21 appears to be of potential value in diagnosing CLAD after bilateral LT.

Abbreviations: AR, acute rejection; AUC, area under the curve; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; CLT, cadaveric lung transplantation; CT, computed tomography; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; HLA, human leucocyte antigen; ISHLT, International Society for Heart and Lung Transplantation; LAS, lung allocation score; LDLLT, living-donor lobar lung transplantation; LT, lung transplantation; miRNA, micro-RNA; mRNA, messenger-RNA; PGD, primary graft dysfunction; RAS, restrictive allograft syndrome; ROC, receiver operating characteristic; TGF-β, transforming growth factor-β; TLC, total lung capacity.

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1. Introduction

Lung transplantation (LT), including cadaveric LT (CLT) and living-donor lobar LT (LDLLT), is a definitive therapeutic option for patients with advanced lung disease [1]. However, long-term survival after LT remains poorer than after transplantation other solid organs [2], it is primarily shortened by chronic lung allograft dysfunction (CLAD) [1,3]. CLAD is characterized by fibrotic change in the peripheral small airways or interstitial tissue and pleura, and it develops in approximately 50% of recipients within 5 years after LT [3]. CLAD is diagnosed functionally based on a decrease in forced expiratory volume in 1 s (FEV1) [3]. Since early diagnosis of CLAD prior to the decrease in FEV1 can enable adequate therapeutic interventions, such as enhanced immunosuppression and azithromycin therapy [4], novel biomarkers are needed for early detection of CLAD after LT.

MicroRNAs (miRNAs) are small noncoding RNAs (21–25 nucleotides) that inhibit gene expression by binding to the 3′UTR of target messenger-RNAs (mRNAs) [5], and they are involved in various basic cellular processes, including apoptosis, proliferation, and differentiation [5]. Several miRNAs play a key role in the pathogenesis of fibrosis [6], which is a universal finding in chronic allograft rejection after solid organ transplantation [7]. For example, miR-21 and miR-155 are pro-fibrotic miRNAs that target the transforming growth factor-β (TGF-β) pathway, while miR-29a and miR-200c are anti-fibrotic miRNAs that protect against fibrosis [8,9]. Moreover, circulating miRNAs may be suitable biomarkers for the early detection of CLAD after LT, because their half-lives range from 1.5 h to 13 h, and although rapidly degraded in serum at 37 °C [10], they are stable when stored at 4 °C and below [11]. Circulating miRNAs have recently been shown to be associated with allograft rejection after heart [12], liver [13] and kidney transplantations [14] as well as with CLAD after CLT [15–17], but value of circulating miRNA in the diagnosis of CLAD after LT, especially after LDLLT, has remained unclear. In this study we investigated the relationships between the levels of circulating miRNAs related to pulmonary fibrosis (miR-21, miR-155, miR-29a, and miR-200c) and the development of CLAD after bilateral LT, including after CLT and after LDLLT.

2. Methods

2.1. Subjects

This was a retrospective cohort study of the 37 of the 41 patients who received bilateral LT for advanced lung disease at Okayama University Hospital between October 1998 and November 2018 who remained after excluding the four patients who underwent lung re-transplantation. Blood samples were collected from the 37 subjects consisting of patients who developed CLAD (CLAD group, n = 14) and patients who did not develop CLAD (non-CLAD group, n = 23) between September 2016 and October 2019 (Fig. 1), and both their pre- and intra-operative characteristics and their post-operative outcomes were assessed. The priority of each patient as an LT recipient was estimated by calculating their lung allocation score (LAS) in November 2018 by using the LAS calculator on the OPTN website (https://optn.transplant.hrsa.gov/resources/allocation-calculators/las-calculator/). It was possible for the maximum total number of human leukocyte antigen (HLA) mismatches in the LDLLT group to equal 12, because two different donors were involved in the procedure for each recipient. CLAD-free survival time was defined as the interval between the LT and the time CLAD was diagnosed. Overall survival time was defined as the interval between the LT and the date of death. The Institutional Review Board of Okayama University Hospital approved the study protocol (No. 1706–043) on June 30, 2017. Written informed consent to the study was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Procedure

Candidates for CLT are required to be registered with the Japan Organ Transplant Network. Since the LAS system has not been adopted in Japan, the allocation of lungs from deceased donors is still based mainly on wait time. Lung donation for transplantation after cardiac death has not been approved in Japan. LDLLT is performed for patients who cannot await CLT, and patients must meet the

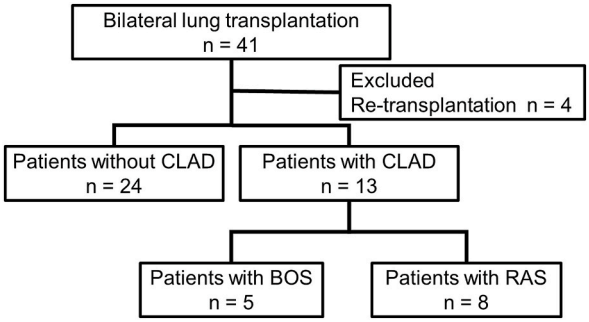

Fig. 1. The 37 patients who underwent bilateral lung transplantation were divided into a group that did not develop chronic lung allograft dysfunction (CLAD) (non-CLAD group, n = 24) and a group that developed CLAD (CLAD group, n = 13). The CLAD group consisted of patients with bronchiolitis obliterans syndrome (BOS; n = 5) and restrictive allograft syndrome (RAS; n = 8).

same criteria as for CLT. Our institution accepts only blood relatives within the third degree or a spouse as living donors [18]. The size-matching protocol and transplant procedures for LDLLT have been reported previously [19]. Graft ischemic time was defined as the ischemic time to the second transplanted lung.

2.3. Postoperative care

The postoperative management of LT recipients, such as immunosuppressive therapy and prophylactic therapy, has been described previously [20,21]. Grades of primary graft dysfunction (PGD) were determined according to the definition of PGD proposed by the International Society for Heart and Lung Transplantation (ISHLT) [22]. Acute rejection (AR) was treated with bolus intravenous glucocorticoids on three consecutive days. Azithromycin was not used for prophylaxis for CLAD. The pulmonary function tests, i.e. the FEV1, forced vital capacity (FVC), and total lung capacity (TLC), were performed at 3, 6, and 12 months and then annually after LT. In accordance with the classification system proposed by the ISHLT [3,23], CLAD consisting of bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS) was functionally diagnosed based on a decrease in FEV1 to <80% of the baseline value, which was calculated as the average of the two best FEV1 values obtained at least 3 weeks apart. The averages of the other pulmonary function test parameters measured at the time of the best FEV1 measurements were adopted as their baseline values. Lung ventilation scintigraphy for delayed washout imaging [24] and lung perfusion scintigraphy to check a perfusion shift to the contralateral functioning lung [25] were performed to detect CLAD after LT, especially after LDLLT, at the same time as the pulmonary function tests were performed. Recipients underwent blood test, chest radiography, computed tomography (CT) of the chest, and an electrocardiogram at the same time to make the differential diagnosis of CLAD [26].

2.4. miRNA expression analysis

Blood samples were centrifuged at 3500×*g* for 10 min. Plasma samples were transferred to microcentrifuge tubes and centrifuged at 16,000×*g* for 10 min to remove residual cells and then stored at −20 °C. Total RNAs were extracted from 200 μL plasma samples with the mirVana PARIS kit (Ambion) and stored at −80 °C. Complementary DNA (cDNA) was synthesized from 2 μL of total RNA with a Taqman Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was analyzed by using the StepOnePlusTM real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). The PCR amplification conditions were 95 °C for 20 s, 40 cycles at 95 °C for 1 s, and 60 °C for 20 s miRNA expression was calculated by the delta-delta-CT method. The miR-16 (Taqman assay ID: 477,860_mir), miR-21 (Taqman assay ID: 477,975_mir), miR-155 (Taqman assay ID: 483,064_mir), miR-29a (Taqman assay ID: 478,587_mir), and miR-200c (Taqman assay ID: 478,351_mir) primer and probe sets were purchased from Thermo Fisher Scientific and used according to the manufacturer's instructions. miR-16 was used as an endogenous

Table 1
Patient characteristics.

	Non-CLAD group (n = 24)	CLAD group (n = 13)	*P-value*
Preozperative variables			
Age, years	36 (8–64)	27 (10–61)	0.32
Sex, female	14 (61%)	7 (50%)	0.76
BMI (kg/m2)	17.5 (12.2–29.0)	17.7 (10.8–25.3)	0.79
Lung donor			
Living	8 (33%)	6 (46%)	0.50
Cadaveric	16 (67%)	7 (54%)	
Diagnosis			0.50
Interstitial lung disease	8 (33%)	2 (15%)	
Pulmonary hypertension	2 (8.3%)	3 (23%)	
Pulmonary graft-versus-host disease	8 (33%)	3 (23%)	
Lymphangioleiomyomatosis	2 (8.3%)	1 (7.7%)	
Other lung diseases	4 (16%)	4 (30%)	
Lung allocation score	43.7 (31.6–69.2)	45.8 (33.9–91.0)	0.47
CMV mismatch (recipient negative/donor positive)	5 (22%)	2 (15%)	0.98
Total number of HLA-A, HLA-B, and HLA-DR mismatches	5 (3–7)	5 (4–9)	0.49
Intraoperative variables			
Operative time (min)	518 (299–785)	514 (223–669)	0.22
Total ischemic time (min)	517 (74–787)	248 (89–665)	0.32
Cardiopulmonary bypass use, yes	24	13	0.11
Postoperative variables			
Maximum grade of PGD (0–72 h)	2 (0–3)	2 (0–3)	0.41
Acute rejection, yes	5 (22%)	8 (57%)	0.067
Antibody-mediated rejection, yes	1 (4.3%)	1 (7.1%)	1.00
Postoperative GERD	0	1 (7.1%)	0.80
Time between transplantation and most recent follow-up examination (days)	2626 (1570–3711)	2887 (248–7540)	0.47

Notes: Data are presented in the form of numbers (%) or median values (range).
Abbreviations: BMI, body mass index; CMV, cytomegalovirus; GERD, gastroesophageal reflex disease; HLA, human leukocyte antigen; PGD, primary graft dysfunction.

control for the miRNA expression analysis [27]. Because approximately 50% of LT patients develop CLAD within 5 years after LT [1], plasma miRNA levels at 5 years after LT were adopted as the control values in the non-CLAD group. Plasma miRNA levels were also measured using the blood samples from 5 healthy adult volunteers.

2.5. Statistical analysis

The Mann-Whitney *U* test for continuous variables and Fisher's exact test for categorical variables were used to evaluate differences in the patient characteristics of the two groups. The Kruskal-Wallis test of variance followed by the Bonferroni test was used to evaluate differences among three groups. The time-course of the miRNA levels were expressed as means ± standard error of the means. Missing data were not replaced. The Spearman's rank correlation coefficient was calculated between the miRNA levels and the percent baseline values of pulmonary function tests, including FEV1, TLC, FVC. We performed a receiver operating characteristics curve (ROC) analysis to set the optimal cut-off value for the miRNA levels between the CLAD group and the non-CLAD group at the time of the diagnosis of CLAD. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [28], a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R commander that was designed to add statistical functions frequently used in biostatistics.

3. Results

Fig. 1 shows a schematic diagram of the study cohort. Thirteen patients developed CLAD after bilateral LT, and they consisted of 5 patients who developed BOS and 8 patients who developed RAS. Table 1 summarizes the patient characteristics. There were no significant differences between the two groups in terms of risk factors for CLAD, including cytomegalovirus mismatches, HLA mismatches, PGD grades, AR, and gastroesophageal reflux disease [29].

As shown in Fig. 2A and B, the plasma levels of miR-21 and miR-155, which are involved in the pathogenesis of fibrosis, were significantly higher at the time of diagnosis of CLAD in the CLAD group than in the non-CLAD group (miR-21, P = 0.0013; miR-155, P = 0.042). By contrast, at the time the diagnosis of CLAD was made in the CLAD group there were no significant differences between the two groups in plasma levels of miR-29a and miR-200c, which are related to anti-fibrosis (miR-29a, P = 0.48; miR-200c, P = 0.21) (Fig. 2C and D). The results for the time-course changes in miRNA levels showed significant difference in the plasma levels of miR-21 and miR-155 at the time of the diagnosis of CLAD, but that there were no significant differences between the two groups a year before or a year after the diagnosis of CLAD (miR-21, P < 0.001; miR-155, P = 0.016) (Fig. 3A–C). Moreover, there were no significant differences between the BOS group and RAS group in plasma levels of miR-21, miR-155, miR-29a, and miR-200c at the time of the diagnosis of CLAD (miR-21, 8.51 (7.51–16.05) vs. 12.01 (7.95–13.43), P = 1.00; miR-155, 6.90 (4.43–14.36) vs. 18.14 (9.80–34.63), P = 0.19; miR-29a, 5.78 (4.41–7.15) vs. 3.39 (1.52–5.10), P = 0.28; miR-200c, 41.29 (32.66–41.54) vs. 21.55 (17.69–50.21), P = 0.50).

It is noteworthy that the plasma miR-21 levels had significant negative correlations with the baseline FEV1, FVC, and TLC values both a year before (FEV1, P = 0.012, r = −0.49; FVC, P = 0.0058, r = −0.52; TLC, P = 0.0079, r = −0.53) and at the time of the

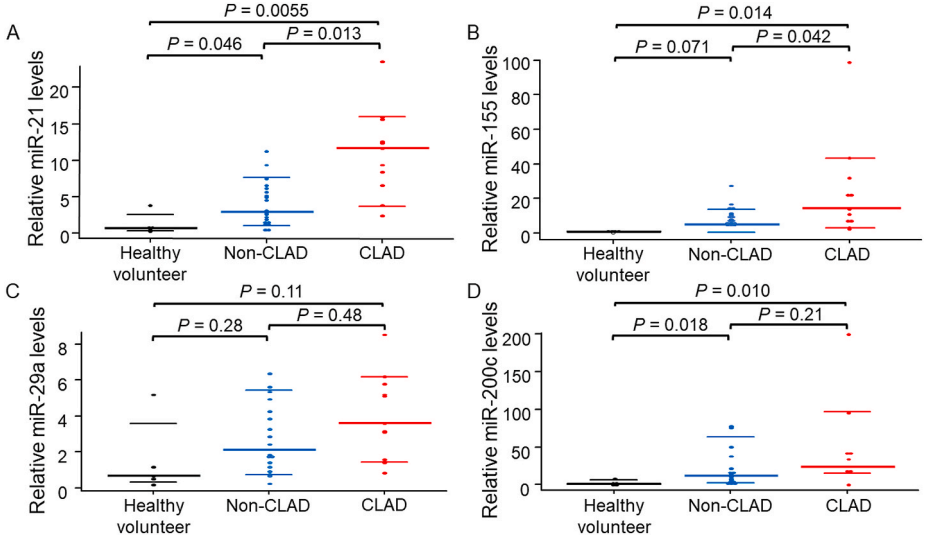

Fig. 2. Plasma miRNA levels at the time of the diagnosis of chronic lung allograft dysfunction (CLAD) are shown as median values (black lines), interquartile ranges (boxes) and 5th and 95th percentiles (whiskers). (A, B) After bilateral lung transplantation the CLAD group had significantly higher plasma levels of the pro-fibrotic miRNAs miR-21 and miR-155 than the non-CLAD group (miR-21, 2.97 (1.41–5.79) vs. 11.73 (7.51–14.15), P = 0.0013; miR-155, 4.90 (1.90–8.72) vs. 14.28 (6.97–26.90), P = 0.042). (C, D) There were no significant differences between the plasma levels of the *anti*-miRNAs miR-29a and miR-200c in the non-CLAD group and the CLAD group (miR-29a, 2.14 (1.23–4.18) vs. 3.62 (2.33–5.48), P = 0.48; miR-200c, 12.50 (4.49–26.74) vs. 24.63 (18.45–41.54), P = 0.21).

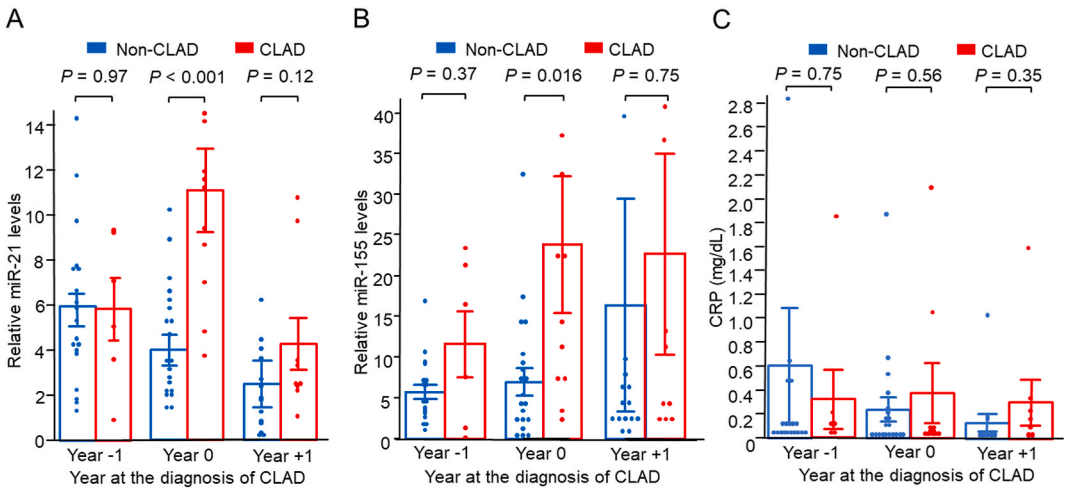

Fig. 3. Plasma miR-21 and miR-155 levels were measured from a year before the diagnosis of chronic lung allograft dysfunction (CLAD) (year −1) to a year after the diagnosis of CLAD (year +1). The plasma miR-21 (A) and miR-155 (B) levels in the CLAD group at the time of the diagnosis of CLAD were significantly higher than in the non-CLAD group (miR-21, 3.98 ± 0.68 vs. 11.11 ± 1.83, P < 0.001; miR-155, 6.93 ± 1.68 vs. 23.76 ± 8.42, P = 0.016). There were no significant differences between the plasma levels of miR-21 and miR-155 in the two groups at year −1 and year +1. The serum levels of *C*-reactive protein did not differ in the two groups from year −1 to year +1 (C).

diagnosis of CLAD (FEV1, P = 0.054, r = −0.49; FVC, P = 0.0089, r = −0.47; TLC, P = 0.0052, r = −0.49) as well as with the baseline FVC and TLC values a year after the diagnosis of CLAD (FVC, P = 0.045, r = −0.42; TLC, P = 0.0088, r = −0.53) (Fig. 4A). By contrast, the plasma miR-155 levels had significant negative correlations with the baseline TLC values from a year before to a year after the diagnosis of CLAD (Year −1, P = 0.019, r = −0.47; Year 0, P = 0.014, r = −0.55; Year +1, P = 0.0015, r = −0.55) (Fig. 4B).

An ROC analysis demonstrated good performance of the plasma miR-21 levels for diagnosing CLAD after bilateral LT at the time of the diagnosis of CLAD, with an area under the curve (AUC) of 0.89 (sensitivity = 75%; specificity = 95%) (Fig. 5A), and the analysis of miR-155 levels yielded an AUC of 0.79 (sensitivity = 67%; specificity = 84%) (Fig. 5B).

In addition, the plasma miR-21 and miR-155 levels at the time of the diagnosis of CLAD after bilateral LDLLT in the CLAD group were significantly higher than in the non-CLAD group (miR-21, 2.97 (1.41–5.79) vs. 9.39 (5.07–12.51), P = 0.016; miR-155, 4.90 (1.89–8.72) vs. 14.28 (6.97–26.81), P = 0.031). Similarly, the plasma miR-21 levels at the time of the diagnosis of CLAD after bilateral cadaveric LT in the CLAD group were significantly higher than in the non-CLAD group (2.97 (1.41–5.79) vs. 13.66 (10.92–15.98), P < 0.001). Moreover, the plasma miR-21 levels of the CLAD group after bilateral LDLLT were similar to the levels after bilateral CLT (9.39 (5.07–12.51) vs. 13.66 (10.92–15.69), P = 0.41).

4. Discussion

The results of study showed that the plasma levels of the pro-fibrotic miRNAs miR-21 and miR-155 at the time of the diagnosis of CLAD after bilateral LT, including LDLLT and CLT, were significantly higher in the CLAD group than in the non-CLAD group. Moreover, with the exception of the baseline FEV1 values a year after the diagnosis of CLAD the plasma miR-21 levels were significantly correlated with the baseline FEV1, FVC, and TLC values from a year before to a year after the diagnosis of CLAD. Based on the results of the ROC analyses, plasma miR-21 levels may be a diagnostic marker for CLAD after both bilateral CLT and LDLLT at the time of the diagnosis of CLAD. This is the first study to assess the diagnostic value of plasma miRNAs levels in CLAD and the relationship between plasma miRNA levels and baseline FEV1, FVC, and TLC values after LT.

The results of this study showed that the levels of the pro-fibrotic miRNAs miR-21 and miR-155 at the time of the diagnosis of CLAD were significantly higher than in the non-CLAD group, whereas the levels of the anti-fibrotic miRNAs miR-29 and miR-200c were not significantly lower in the CLAD group. MiR-21 has been shown to target Smad 7 and thereby enhance the TGF-signaling pathway and promote fibrotic lung diseases, including idiopathic pulmonary fibrosis [30]. Consistent with the results of the present study, a recent study showed elevated serum miR-21 levels in CLAD patients compared to non-CLAD patients [17]. Moreover, miR-155 has been shown to be increased in exosomes in the serum of the CLAD patients [31] and to induce immune responses that lead to BOS in a mouse model [32]. The similar levels of pro-fibrotic miRNAs a year after the diagnosis of CLAD might indicate that the target miRNAs could increase especially in the progression of lung fibrosis and decrease once the lung fibrosis reached a plateau in CLAD. By contrast, the similar levels of the anti-fibrotic miRNAs miR-29a and miR-200c in the CLAD group and non-CLAD group in our study may reflect the fact that some TGF-β-dependent anti-fibrotic miRNAs, such as miR-29a [33] and miR-200c [34], are feedback regulators of TGF-β signaling. The circulating miRNAs analyzed in the present study included miRNAs contained in exosomes, apoptotic bodies that bind to proteins or lipids, and miRNAs that leaked out as a result of cell destruction in the plasma [14,35,36]. Since exosomes can function as cargo for cell-to-cell communication [14,36], the measurement of exosomal miRNAs might offer further evidence of diagnostic value

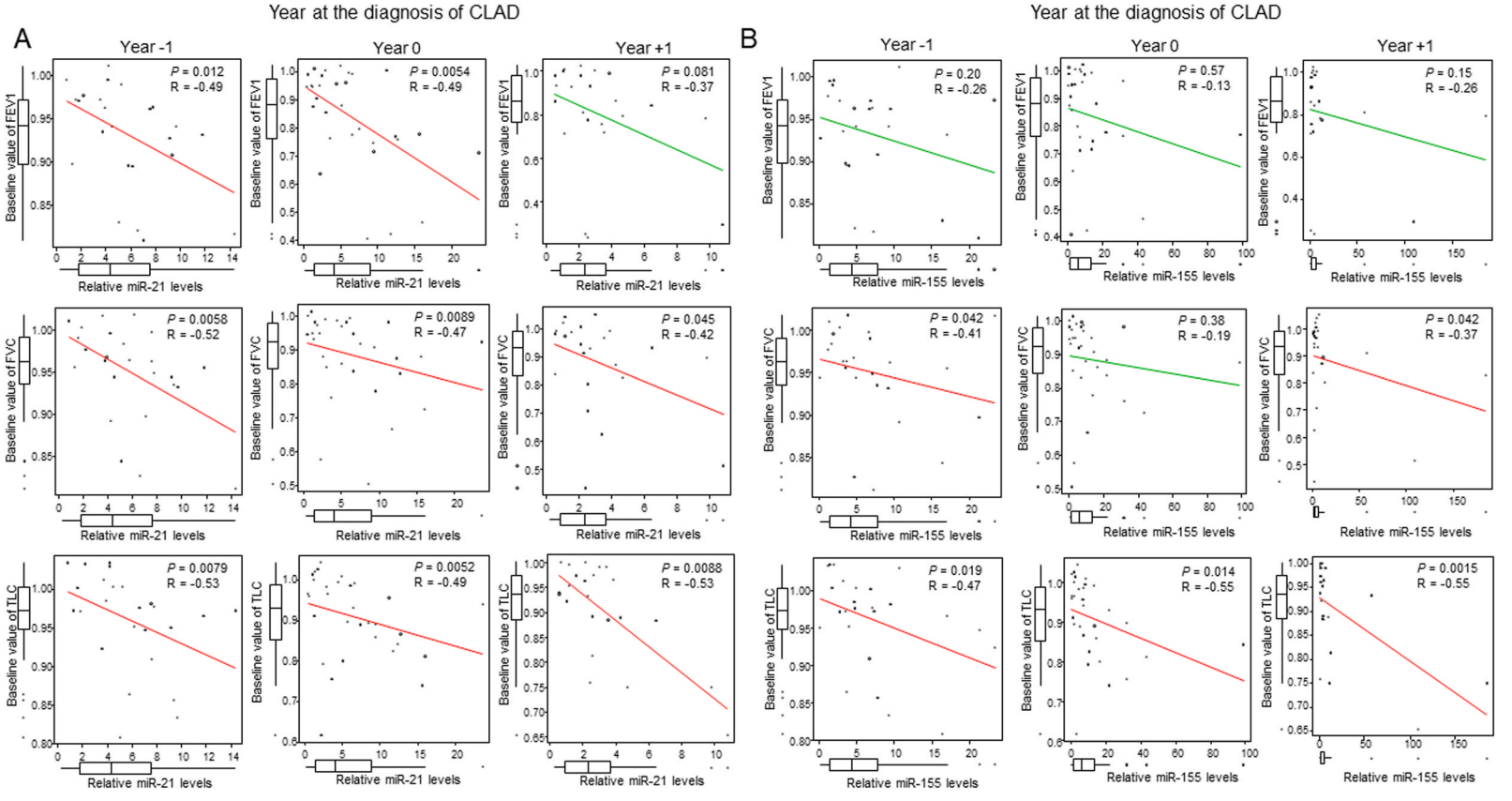

Fig. 4. Correlations between plasma microRNA levels and baseline forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), and total lung capacity (TLC) values. (A) With the exception of the baseline FEV1 value at year +1, the miR-21 levels had significant negative correlations with the baseline FEV1, FVC, and TLC values from a year before the diagnosis of chronic lung allograft dysfunction (CLAD) (year −1) to a year after the diagnosis of CLAD (year +1). (B) The miR-155 levels had significant negative correlations with the baseline TLC values from year −1 to year +1 and with the baseline FVC values at year −1 and year +1, but the miR-155 levels did not have significant correlations with the baseline FEV1 values.

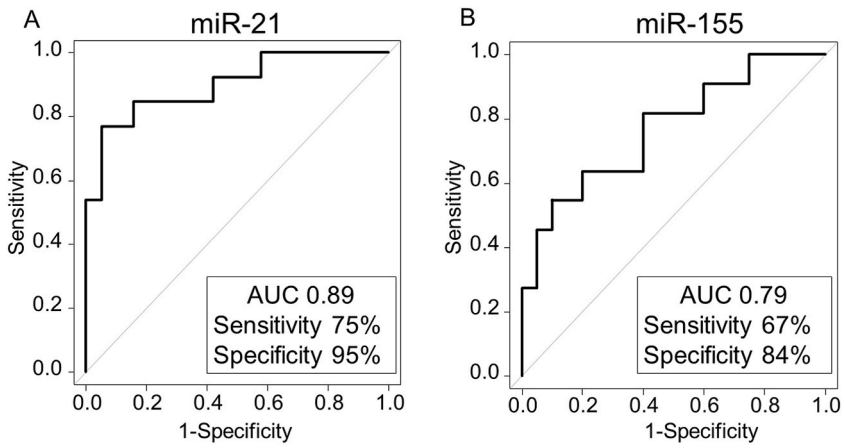

Fig. 5. Receiver operating characteristic curve (ROC) analyses to determine the performance of plasma levels of miRNAs for the diagnosis of chronic lung allograft dysfunction (CLAD) at the time of the diagnosis of CLAD. In the ROC analysis of miR-21, an area under the curve (AUC) was 0.89 (sensitivity = 75%; specificity = 95%) (A), and analysis of miR-155 yielded an AUC of 0.79 (sensitivity = 67%; specificity = 84%) (B).

of miRNAs in the future.

Given the function of miRNAs, the circulating miRNAs could be a more sensitive diagnostic marker as compared to the conventional diagnostic methods of CLAD, including pulmonary function test and imaging. Although we found significant differences in the miRNAs at the time of the diagnosis of CLAD, there were no significant differences in the miRNAs one year before the diagnosis of CLAD in the present study. Our results suggest that the annual measurement of serum miRNAs might not be enough for early detection of CLAD. For further analysis, more frequent measurement of miRNAs, such as semiannual or triannual measurement, before the diagnosis of CLAD might be useful to evaluate potential of early detection of CLAD. By contrast, the change of the circulating miRNAs related to fibrosis might be affected by the development of other fibrotic diseases.

The results of our study showed that, with the exception of the baseline FEV1 values a year after the diagnosis of CLAD, the circulating miR-21 levels of the CLAD group were significantly negatively correlated with their baseline FEV1, FVC, and TLC values from a year before to a year after the diagnosis of CLAD. In addition, the circulating miR-21 and miR-155 levels from a year before to a year after the diagnosis of CLAD were significantly negatively correlated with the baseline TLC values in the CLAD group, even though TLC might be affected by the hyperinflation of affected lungs with BOS. Although there were no significant differences in the levels of the four miRNAs between the BOS group and the RAS group in this study, these findings suggest that circulating miR-21 and miR-155 levels may reflect the development of CLAD, especially RAS, after bilateral LT. In fact, circulating miR-21 and miR-155 levels have been shown to be associated with FVC in patients with idiopathic pulmonary fibrosis [37]. Although the numbers of patients with BOS and the RAS in our study were too small to demonstrate any significant differences in their circulating miRNA levels, miRNA measurements may contribute to the differential diagnosis of clinical subtypes of CLAD in the future. In view of the results of the ROC analyses in our study, miR-21 may be a more sensitive and minimally invasive diagnostic marker than miR-155 for CLAD after bilateral LT.

The features of CLAD after bilateral LDLLT differ from the features of CLAD after bilateral CLT. CLAD after bilateral LDLLT usually develops unilaterally because of the different immunological features of the blood-relative donors of the two lungs [25,38,39]. Moreover, CLAD develops later after bilateral LDLLT than after CLT because of the size mismatch between the donor and the recipient [39] and delayed generation of donor-specific antibody [40]. Despite these differences between CLAD after CLT and after LDLLT, we observed similar circulating miR-21 and miR-155 levels in the CLT group and LDLLT group in the present study. Our data indicated that the circulating miRNA levels may depend on the severity of the fibrosis in CLAD, not on the laterality or the time of the diagnosis of CLAD.

The present study had several limitations. First, it was a retrospective study conducted at a single lung transplant center, and the number of LT subjects was small. Second, because the number of measurement of miRNAs was limited, earlier and more frequent measurement of miRNAs prior to the diagnosis of CLAD will be needed to validate miRNAs as diagnostic markers of CLAD. Third, the circulating miRNAs consisted of miRNAs contained in exosomes and apoptotic bodies that bind to proteins or lipids, and miRNAs that leaked out as a result of cell destruction in the plasma, whether they were functional or not. Fourth, our study focused on a limited number of miRNAs due to cost-effectiveness considerations, and, obviously, a comprehensive analysis of miRNAs would be ideal. However, because LDLLT has been performed exclusively in Japan, our findings in this study provide valuable information that circulating miRNAs, especially miR-21, may serve as a diagnostic marker for CLAD both after bilateral CLT and LDLLT.

5. Conclusion

The CLAD group had significant higher plasma miR-21 and miR-155 levels at the time of the diagnosis of CLAD than the non-CLAD group had after bilateral LT, including CLT and LDLLT. In particular, the plasma miR-21 levels were significantly correlated with the

decreases in FEV1, FVC, and TLC values a year before and at the time of the diagnosis of CLAD after bilateral CLT and LDLLT. In view of the results of ROC analyses, the circulating miR-21 levels may make it possible to diagnose CLAD after bilateral CLT and LDLLT.

Author contribution statement

Toshio Shiotani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Seiichiro Sugimoto and Shinichi Toyooka: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Yasuaki Tomioka, Haruchika Yamamoto, Shin Tanaka and Kentaroh Miyoshi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Ken Suzawa, Kazuhiko Shien, Hiromasa Yamamoto, and Mikio Okazaki: Contributed reagents, materials, analysis tools or data.

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Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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