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Circulating miRNAs as Prospective Biomarker for Progressive Pulmonary Fibrosis in Comparison with Surfactant Protein D in Mice

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ABSTRACT

As for the serum biomarkers clinically available for progressive pulmonary fibrosis including idiopathic pulmonary fibrosis, Surfactant Protein D (SP-D) and Krebs von den Lungen-6 antigen (KL-6) have been used for more than 20 years in Japan not only for supporting diagnosis but detecting the disease progress. However, both of these markers are produced chiefly by alveolar type II epithelial cells, which might induce inconsistent attitude with disease progress. Therefore, we tried to find out novel biomarkers for progressive pulmonary fibrosis from circulating miRNAs in the mouse model of pulmonary fibrosis, in comparison with SP-D using a newly established detecting system for mouse SP-D. The serum levels of mouse SP-D after bleomycin-induced lung injury, revealed positive correlation with lung inflammation by day 7, but not so much with increased fibrosis until day 21. Then we compared the expression patterns of miRNAs in the serum and lung tissues after bleomycin-induced lung injury, and found that miR-30d was increased most toward day 21, followed by miR-122, miR-690, miR-1907, and miR-3096b-5p, though none of which were increased in the fibrotic lung tissues. Only miR-322 and miR-874 were moderately increased in both serum and lung tissues on day 21. These results indicate that the plausible biomarkers for pulmonary fibrosis in circulating miRNAs are in dependent of secretion from fibrotic lung tissue cells, and may suggest promising targets for treatment against progressive pulmonary fibrosis.

INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a specific form of chronic, progressive, fibrosing interstitial pneumonia of unknown cause [1], and its concept is shifting to irreversible pulmonary fibrosis of many entities [2] despite the proven efficacy of novel antifibrotic therapies through large scale of world-wide clinical trials. Although the Forced Vital Capacity (FVC) in pulmonary function test of patients with IPF has been used to assess disease progression and treatment response in these clinical studies, identifying predictive circulating blood biomarkers could help identify specific biologic pathways for treatment [3]. As for the diagnostic and prognostic efficacy of the serum levels of Surfactant protein (SP)-D, and SP-A, and Krebs von den Lungen-6 antigen (KL-6) in patients with IPF, a significant amount of data has been accumulated in Japan for more than twenty years due to their availability in clinical practice [4]. However, these

proteins are produced and secreted chiefly by alveolar type II epithelial cells, and the inconsistency of these biomarkers with the aggravation of clinical symptoms of patients with IPF [3,4]. In this context, we investigate new biomarkers of pulmonary fibrosis by focusing circulating micro-RNAs (miRNAs), which are endogenous 17 to 22 nucleotide non-coding RNA molecules with possibility of a biologically important effect on post-transcriptional gene expression [5]. Consequently, miRNAs regulate several cellular processes like proliferation, apoptosis and differentiation [6], and have been revealed that sufficiently stable miRNAs can be isolated from the serum of cancer and sepsis, acute myocardial infraction patients [7-10]. Although several studies have already reported plausible information on miRNAs in association with lung fibrosis [11-16], little information has revealed on circulating miRNAs. Our final goal is to find out reliable biomarkers for detecting progressive fibrosis in the lungs of IPF patients by profiling the distinct circulating miRNAs in the cell free circulating fluids of IPF patients, but these circulating miRNAs are known to be susceptible to diets and medications according to patients' conditions [17,18]. Therefore, in this current study, we examined to detect miRNAs in the sera, and in the lung tissues as well, in the mouse model of bleomycin-induced lung injury which covers both inflammation and fibrosis after lung injury, in comparison with the serum level of mouse SP-D, which is a standard biomarker for human patients with interstitial pneumonia. We chose novel monoclonal antibodies established against rat SP-D (Yamasa Corporation, Japan), because mice lack KL-6 and because SP-D is more reliable than SP-A for detecting interstitial pneumonia. SP-D, a hydrophilic collagenous glycoprotein, is a member of the collectin surperfamily, is secreted into alveolus chiefly by alveolar type II cells and binds to several microorganisms to regulate host immune defense [19]. Furthermore, it has been recognized that SP-D protects against lung injury and fibrosis to modulate pro-inflammatory cytokines and anti-oxidant enzymatic scavenger systems, and to regulate numbers of macrophages, fibrocytes, and profibrotic cytokines [20,21].

The purpose of our current study is to reveal the defect of serum level of SP-D as a circulating biomarker in the disease process of progressive fibrosis after bleomycin-induced lung injury, and find out prospective biomarkers in circulating miRNAs, which may offer the future studies for excellent biomarkers and novel treatment as well for patients with progressive pulmonary fibrosis, including IPF [22].

MATERIAL AND METHODS

Animals and bleomycin-induced lung injury and fibrosis

We purchased wild-type C57BL/6J mice from Charles River (Japan, Yokohama). Pulmonary fibrosis was induced in the 10- to 12-wk-old male C57BL/6 mice by intra-tracheal instillation of 1.0 mg/kg bleomycin chlorate (Nippon-kayaku, Tokyo, Japan) dissolved in 50μl of saline on day 0. On days 0 (control), 3, 7, 14, and 21, the mice (n=6-8 in each group) were anesthetized and sacrificed. Animal experiments were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and this study was approved by the Institutional Animal Care and Use Committee of Tohoku University.

Bronchoalveolar Lavage (BAL) and cell counts

The lungs were lavaged twice with 0.75 ml of PBS. After centrifugation (3000rpm, 2 min), the supernatants were stored for analyses, and cell pellets were resuspended in 1 ml of PBS for total and differential cell counts. Total BAL cells were counted with a hemocytometer. The BAL differential was determined from ≥200 cells in Cytospin preparations stained with Diff-Quik (Sysmex), and the cells were identified as macrophages, eosinophils, lymphocytes, or neutrophils on the basis of cellular morphology and staining characteristics.

Collagen assay

The right lungs harvested on days 0, 3, 7, 14, and 21 were used for collagen assay. The Sircol collagen assay kit (Biocolor Ltd, Belfast, UK) was performed following the manufacturer's instructions.

ELISA

The Serum and BALF level of SP-D was measured using ELISA kit (Murine SP-D KIT YAMASA EIA).

Immunohistochemistry for SP-D

Mouse lungs were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemistry. The monoclonal antibody against SP-D was provided by YAMASA (Tokyo, Japan).

RNA extraction from lung tissues and sera

Sections (10μm in thickness) were prepared from each FFPE specimen. Paraffin was removed by xylene treatment and tissues were washed with ethanol twice to remove xylene. Tissues

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were then treated with proteinase K at 37°C overnight. Following centrifugation, the supernatant was subjected to RNA purification by silica-based spin column. The degrees of RNA cross-linking and RNA degradation were analyzed by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA in serum was extracted from the using 3D-Gene RNA extraction reagent from liquid sample (Toray, Kamakura, Japan) according to the manufacturer's instructions.

miRNA expression profiling

Extracted total RNA was labeled with Hy5 using the miRCURY LNA Array miR labeling kit (Exiqon, Vedbaek, Denmark). Labeled RNAs were hybridized onto 3D-Gene Mouse miRNA Oligo chips (v.17.0; Toray Industries, Tokyo, Japan). The annotation and oligo nucleotide sequences of the probes were conformed to the miRBase miRNA data base Release 17 (http://microrna.sanger.ac.uk/sequences/). After stringent washes, fluorescent signals were scanned with the 3D-Gene Scanner (Toray Industries) and analyzed using 3D-Gene Extraction software (Toray Industries). The raw data of each spot was normalized by substitution with a mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. The raw data intensities greater than 2 Standard Deviations (SD) of the background signal intensity were considered to be valid. Detected signals for each gene were normalized by global normalization method (the median of the detected signal intensity was adjusted to 25). All the tissue or serum samples examined for miRNA expression profiling were mixed by three mice in each group.

Statistical analysis

Differences were considered to be statistically significant at values of $P \leq 0.05$ by Dunnett test. All data are expressed as the means ± SE. Single, double and triple asterisks indicate statistically significant differences: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. Univariate correlations between variables were compared using Pearson product-moment correlation.

RESULT AND DISCUSSION

Time course of lung injury and fibrosis after bleomycin-induced injury

The mice (n=6-8 in each group) were examined on days 0, 3, 7, 14, and 21 after bleomycin-induced lung injury to investigate the time course of inflammation and fibrosis in this progressive pulmonary fibrosis model. As the parameters of inflammation after lung injury, we estimated the wet lung weight of right lungs (Figure 1A), the concentration of total protein in bronchoalveolar lavage fluid (BALF) (Figure 1B), the serum level of C-Reactive Protein (CRP) (Figure 1C), and the component of cells in BALF (Figure 1D). The results revealed that both the wet lung weight and the total protein level in BALF increased after day 7 and reached to the peak on day 14. The peak of the serum level of C-Reactive Protein (CRP), a marker of inflammation, appeared on day 7, earlier than these parameters after bleomycin-induced lung injury. As for the cell component in BALF, both the macrophages and the lymphocytes peaked on day 14, later than the peak of the neutrophils on day 7, well known chief contributors in lung injury [23].

As the parameters of the degree of pulmonary fibrosis, the total amount of collagen in the lung tissues, measured by collagen assay (Figure 1E) and the Ashcroft scoring system [24] (Figure 1F) estimated by morphologic analysis of lung tissues in elastica-Masson stain (Figure 2A-2D). The amount of collagen in right lungs increased from day 14, and remained in high by day 21 (Figure 1E). The collagen deposition in the lungs as assessed by Ashcroft scores was also increased after day 7 through day 21 (Figure 1F). These results indicated the peak of the inflammation in the mouse lung after bleomycin-induced lung injury was appeared on day 14, but pulmonary fibrosis increased until day 21.

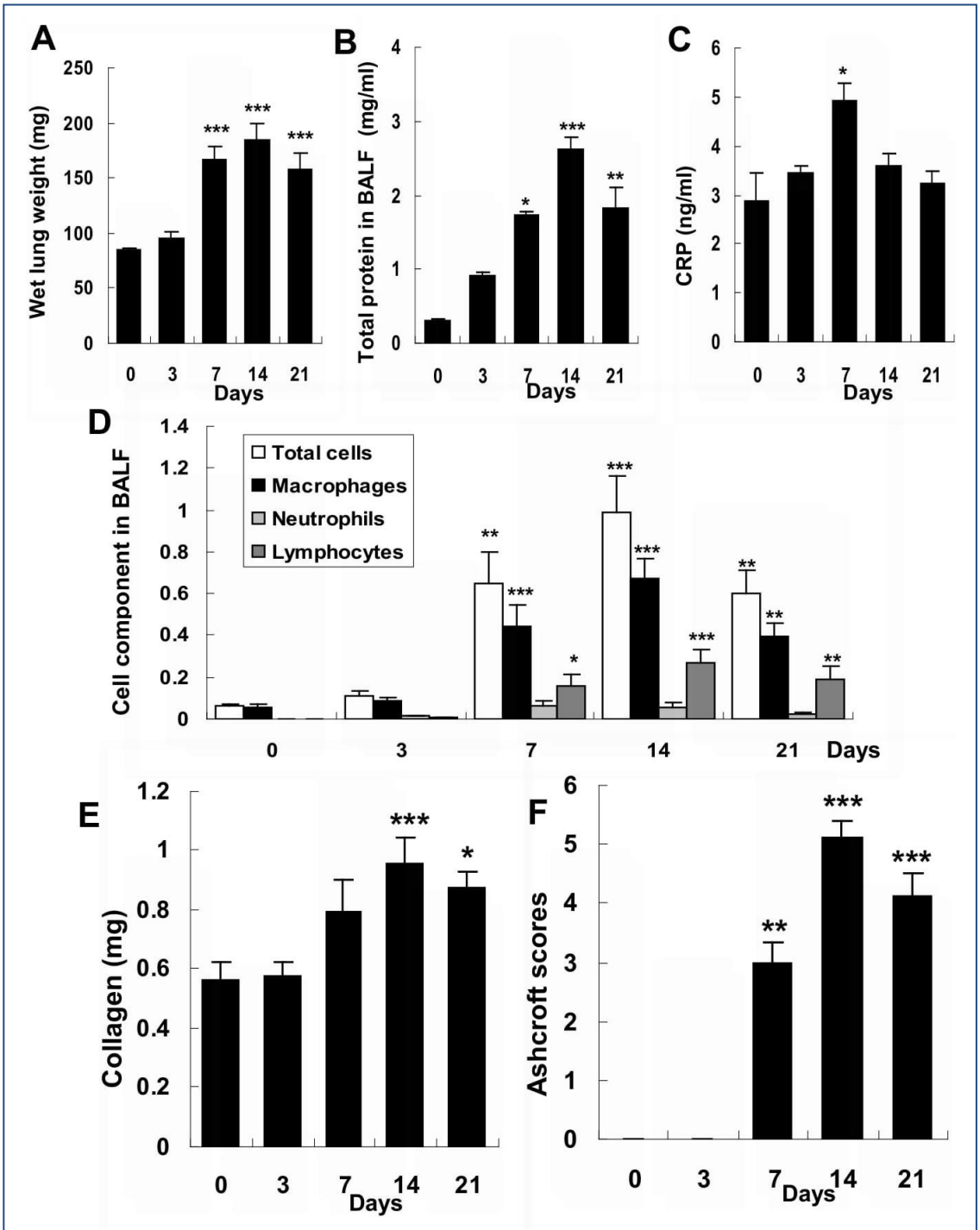

Figure 1: Characteristic alterations of mouse lung after bleomycin-induced lung injury.

Alterations of the wet lung weight (mg) of right lungs (A), the concentration of total protein (mg/ml) in the BALF (B), CRP in the serum (C), the cell component in BALF (D) on days 3, 7, 14, and 21 after leomycin-induced lung injury. The amount of collagen (mg) in the right lungs (E) and Ashcroft score of the left lungs (F) of each group. Data bars show means ± SE (n= 6-8 in each group), compared with control (day 0, n=3). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

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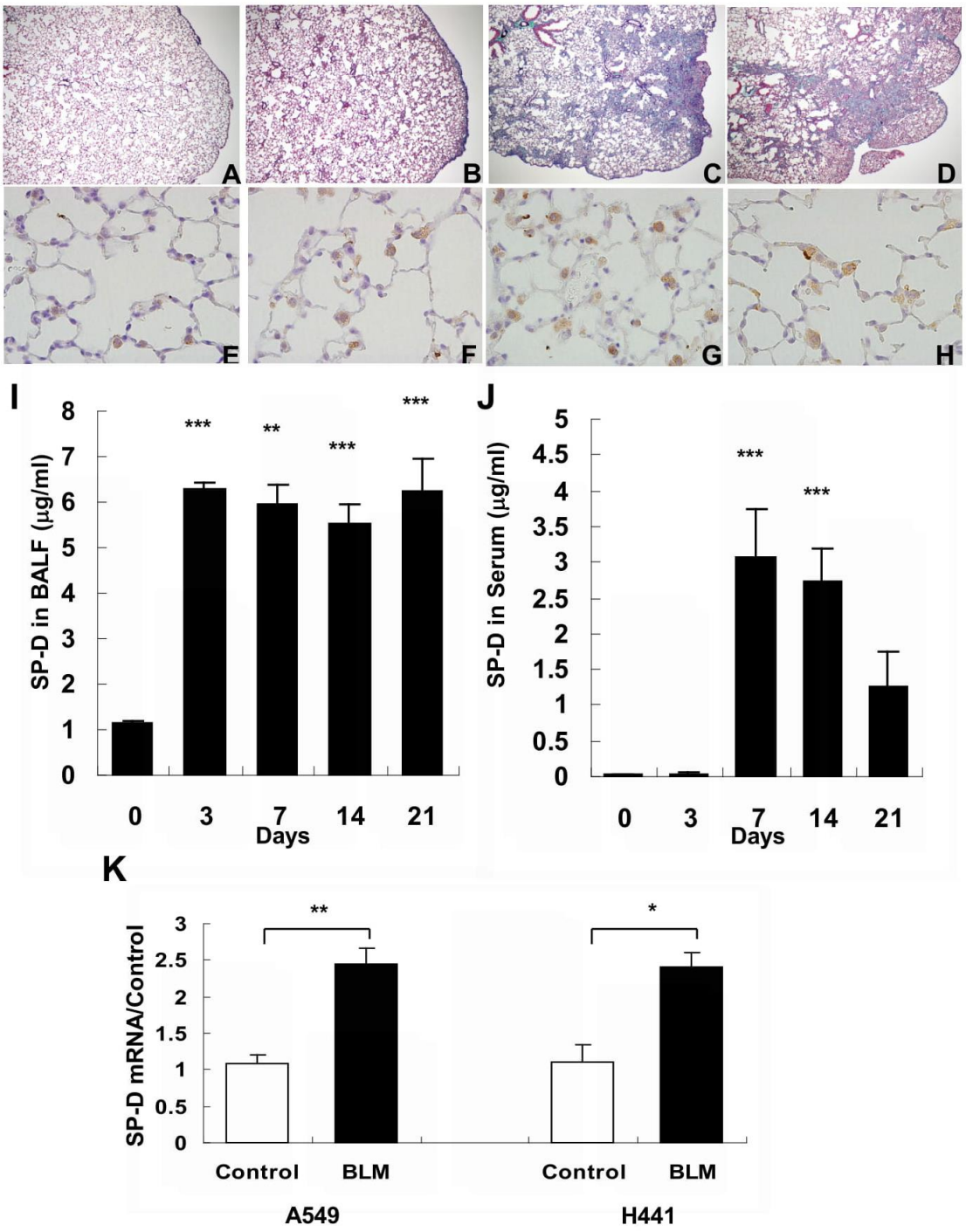

Figure 2: Evaluation of pulmonary fibrosis and distribution of SP-D expression after bleomycin-induced lung injury.

A-D: Photomicrographs of lung tissues on day 0, without treatment (A), and on day 7 (B), day 14 (C), and day 21 (D) after bleomycin injury (elastica-Masson staining at original magnification x40)

E-H: Immunohistochemical staining for SP-D in the lung tissues before treatment of bleomycin. day 0 (E), day 7 (F), day 14 (G), and day 21 (H) after bleomycin injury (counterstaining by hematoxylin, original magnification at x1,000).

I&J: Concentration of SP-D in BALF (I) and in serum (J) on days 0, 3, 7, 14, and 21. Data bars show means ± SE (n= 6-8 in each group), compared with control (day 0, n=3). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Alteration of SP-D in the bleomycin model

The distribution of the pulmonary cells producing SP-D were observed by immunohistochemical staining using the same monoclonal antibody used for ELISA in this study (Figure 2E-2H). We confirmed the main producing cells of SP-D are the alveolar type II epithelial cells by showing intercellular immunoreactivity with SP-D, and also increased its intensity after bleomycin-induced lung injury (Figure 2E-2H). The time course of the concentration of SP-D in BALF detected by ELISA revealed the augmented level of SP-D on day 3 were remained until day 21 (Figure 2I). Interestingly, the serum level of SP-D was augmented on day 7, but gradually decreased by day 21 (Figure 2J). In addition, we examined the cell cultures, A549 and H441, both of which sustain the nature of alveolar epithelial cells, to estimate the direct effects of bleomycin on the production of SP-D by these epithelial cells, and observed both of these cell lines augmented production of SP-D after incubation with 10μM bleomycin for 48 hours (Figure 2K).

The decrease of the serum level of SP-D after day 7 suggests the SP-D is the circulating biomarker of lung inflammation, which does not show the fibrotic degree after lung injury. We previously showed the damage of superficial lymphatic capillaries in the subpleural and interlobular septa lesions by extensive fibrosis in the lungs of patient with IPF [25]. Considering the main function of these lymphatic capillaries is alveolar clearance as superficial lymphatics, the decrease of the serum level of SP-D in the fibrotic stage, in spite of augmented levels in BALF, in this mouse model of progressive pulmonary fibrosis, may suggest the poor response of serum level of SP-D in the terminal stage of extensive pulmonary fibrosis in patients with IPF. As for another plausible mechanism for the inconsistency in the concentration of SP-D between in serum and in BALF, we presented before the disruption of tight junctions between epithelial and endothelial cells by upregulated TGF-β in the mouse models of progressive pulmonary fibrosis after bleomycin-induced lung injury [26], which may deteriorate the absorption of SP-D across the alveolar-capillary membrane.

Expression patterns of circulating miRNAs after bleomycin-induced injury

To find out the prospective biomarkers in circulating miRNA in this mouse model of progressive pulmonary fibrosis, we estimated the alteration of miRNA expression after bleomycin-induced lung injury using miRNA Oligo chip arrays (TORAY, Japan). Because our mouse model revealed that there was a peak on day 7 in inflammation in the lung while fibrosis increased by day 21, we considered day 7 after bleomycin-induced lung injury as a lung inflammation phase and day 21 as fibrotic phases.

Data were clustered according to the Pearson correlation coefficients using hierarchical cluster analysis (Figure 3). In total, 896 circulating miRNAs were expressed above background in the serum, and 143 circulating miRNAs in the serum on day 21 showed above two-fold changes or under one-half changes compared with control (day 0) (Figure 3).

We compared the circulating miRNAs modulated for both lung injury phase on day 7 and fibrosis phase on day 21 (Figure 4). Five miRNAs (miR-30d, miR-122, miR-690, miR-1907, miR-3096b-5p) were upregulated above two folds on both day 7 and day 21 (Figure 5A). Three miRNAs (miR-501-3p, miR-15a, miR-20b) were downregulated under one-half change on day 7 and upregulated above two-fold changes on day 21 (Figure 5B). There was only one miRNA (miR-3095-3p) recognized to be upregulated on day 7 and downregulated on day 21 in turn (Figure 5C). Two miRNAs (miR-504, miR-500*) were similarly downregulated under one half change in both phase (Figure 5D).

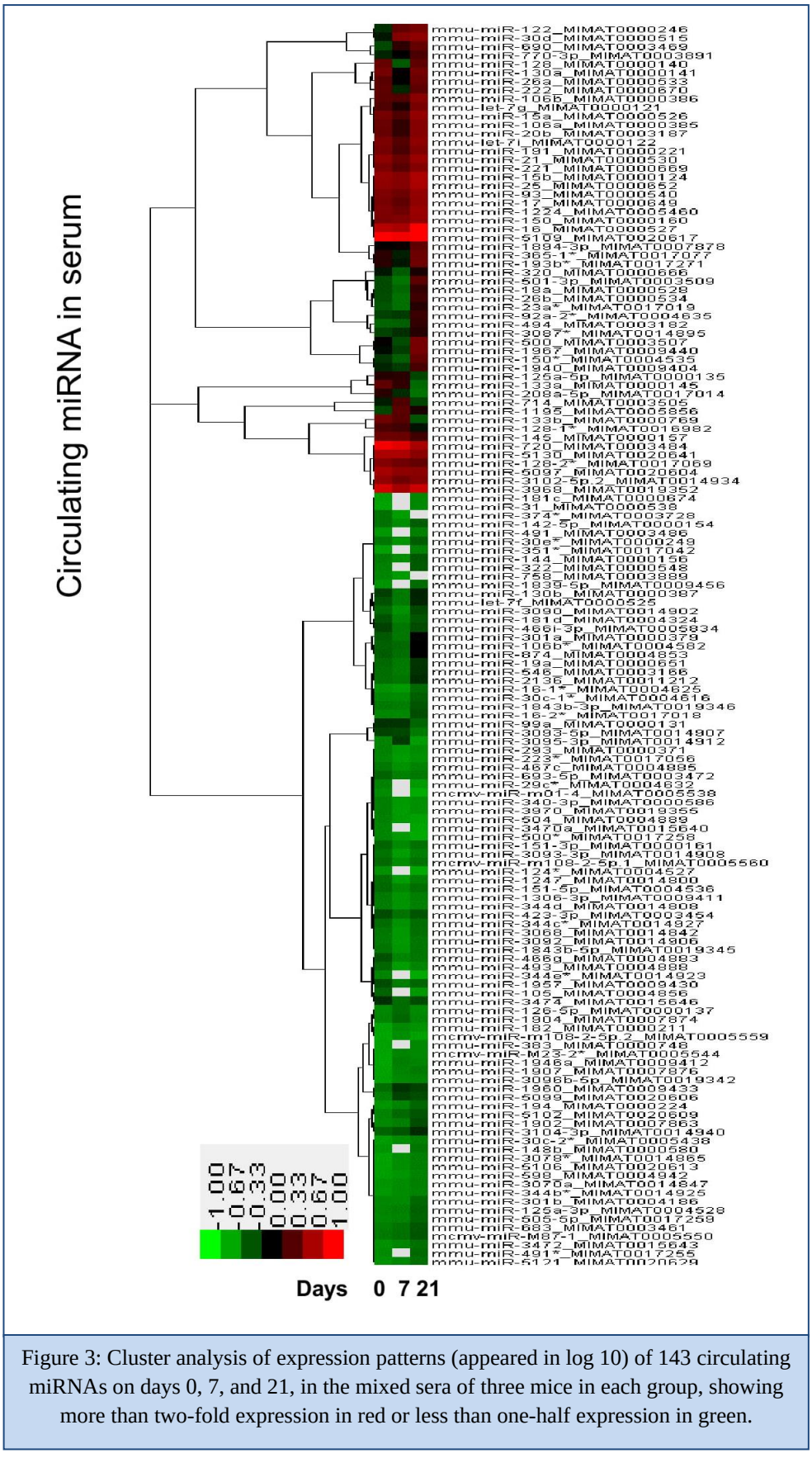

Figure 3: Cluster analysis of expression patterns (appeared in log 10) of 143 circulating miRNAs on days 0, 7, and 21, in the mixed sera of three mice in each group, showing more than two-fold expression in red or less than one-half expression in green.

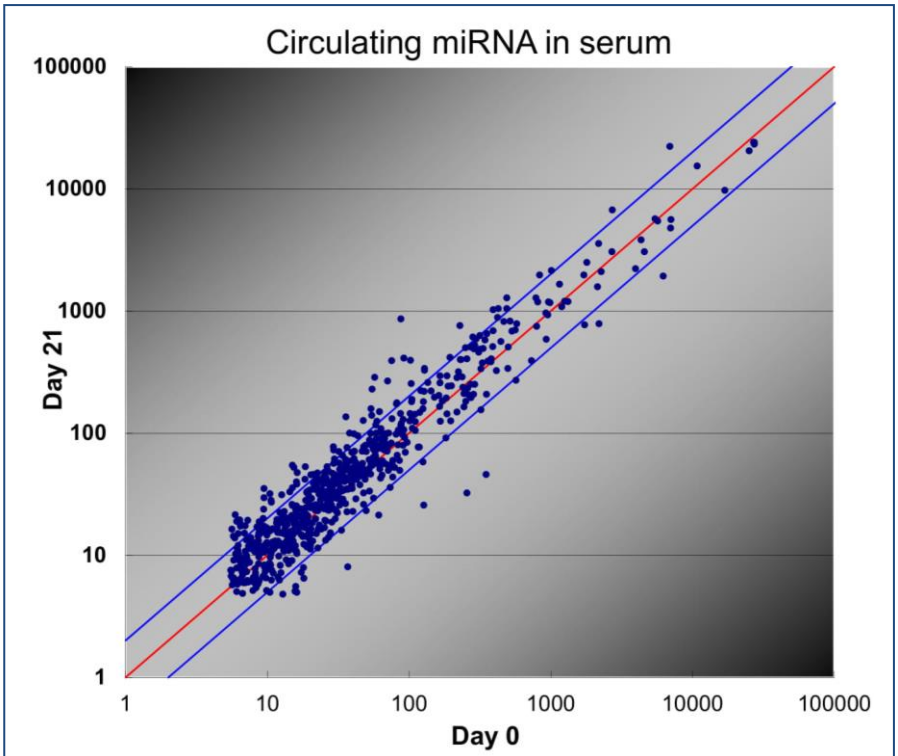

Figure 4: Regression line of expression patterns (appeared in log 10) of 896 circulating miRNAs in comparison between day 0 and day 21.

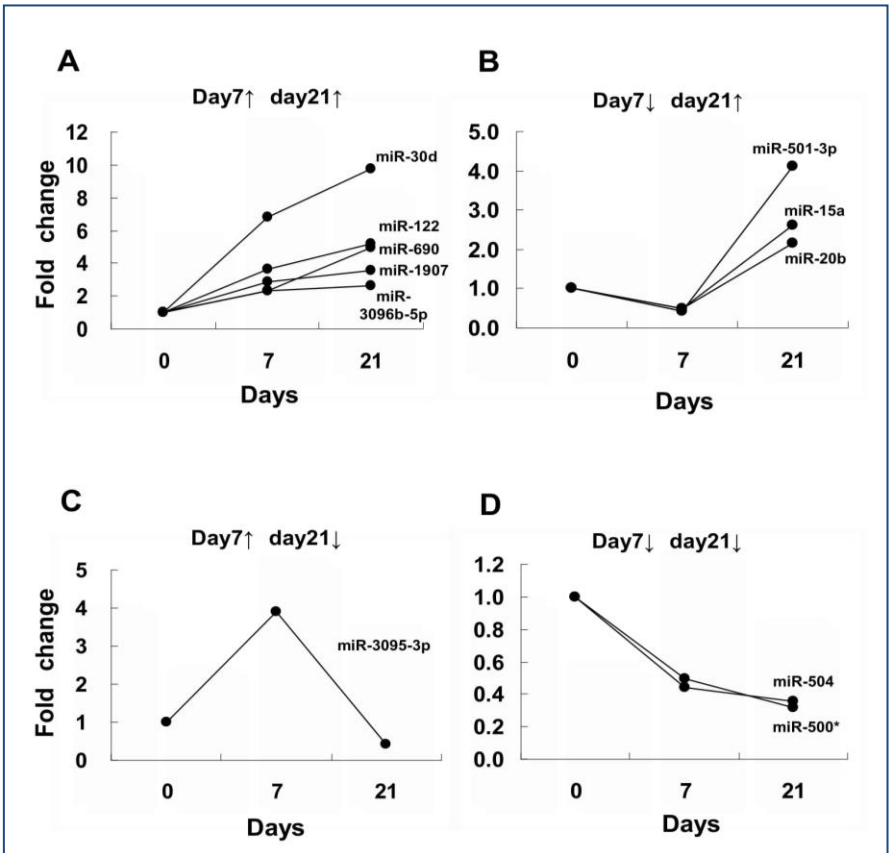

Figure 5: Alteration pattern of circulating miRNAs after bleomycin injury.

Line graphs of differentially expressed circulating microRNAs (miRNAs) on day 0, 7, 21. Data are fold changes normalized to expression.

(A) miRNAs with consistent increase pattern (fold ≧ 2) after day 7 (fold ≧ 2) than control.

(B) miRNAs with a pattern of decrease on day 7 (fold ≦ 0.5) but increase on day 21 (fold ≧ 2) .

(C) miRNAs with a pattern of increase on Day 7 (fold ≧ 2) but decrease on day 21 (fold ≦ 0.5).

(D) miRNAs with consistent decrease pattern (fold ≦ 0.5).

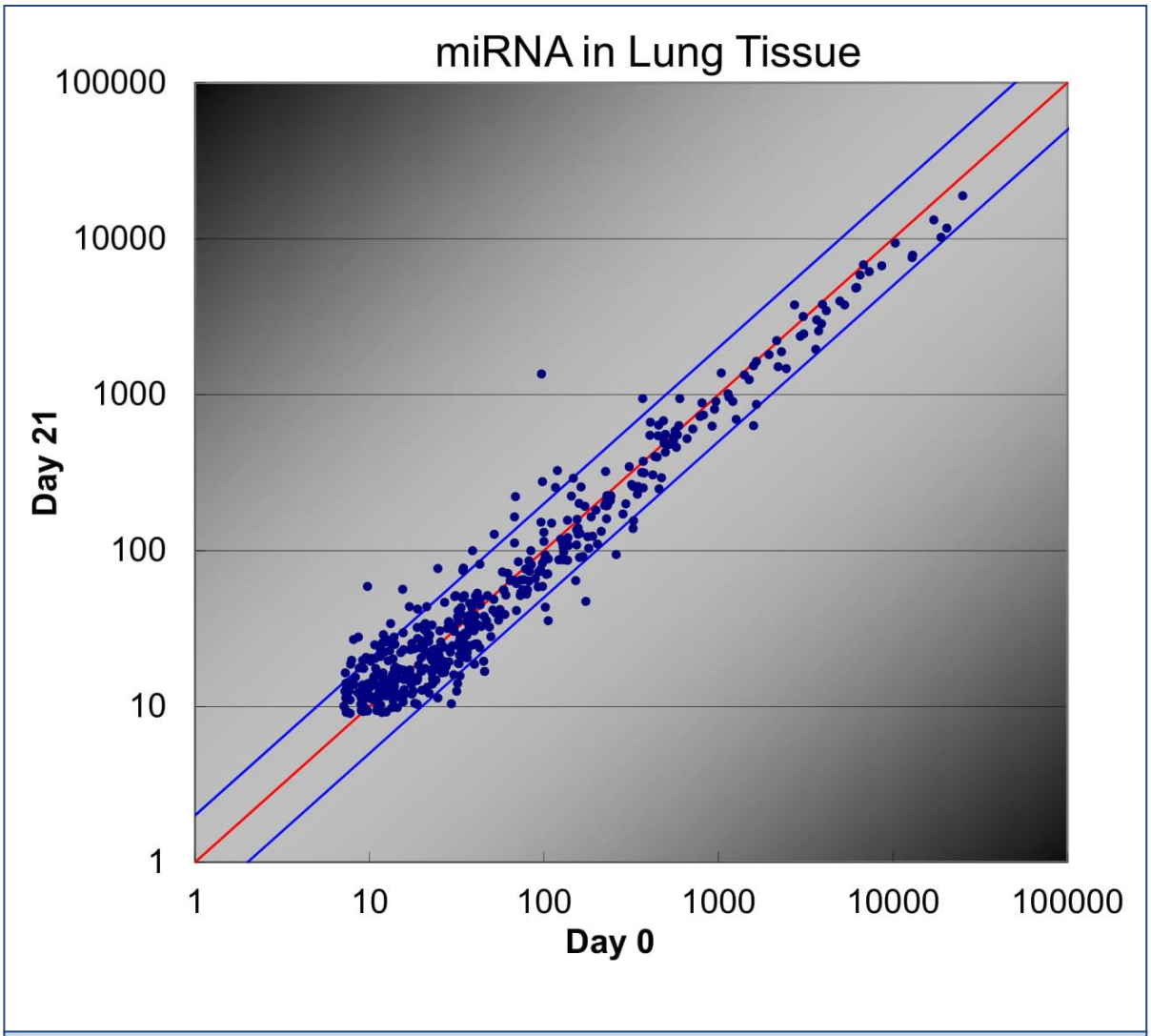

Figure 6: Regression line of expression patterns (appeared in log 10) of 663 miRNAs in lung tissues in comparison between day 0 and day 21.

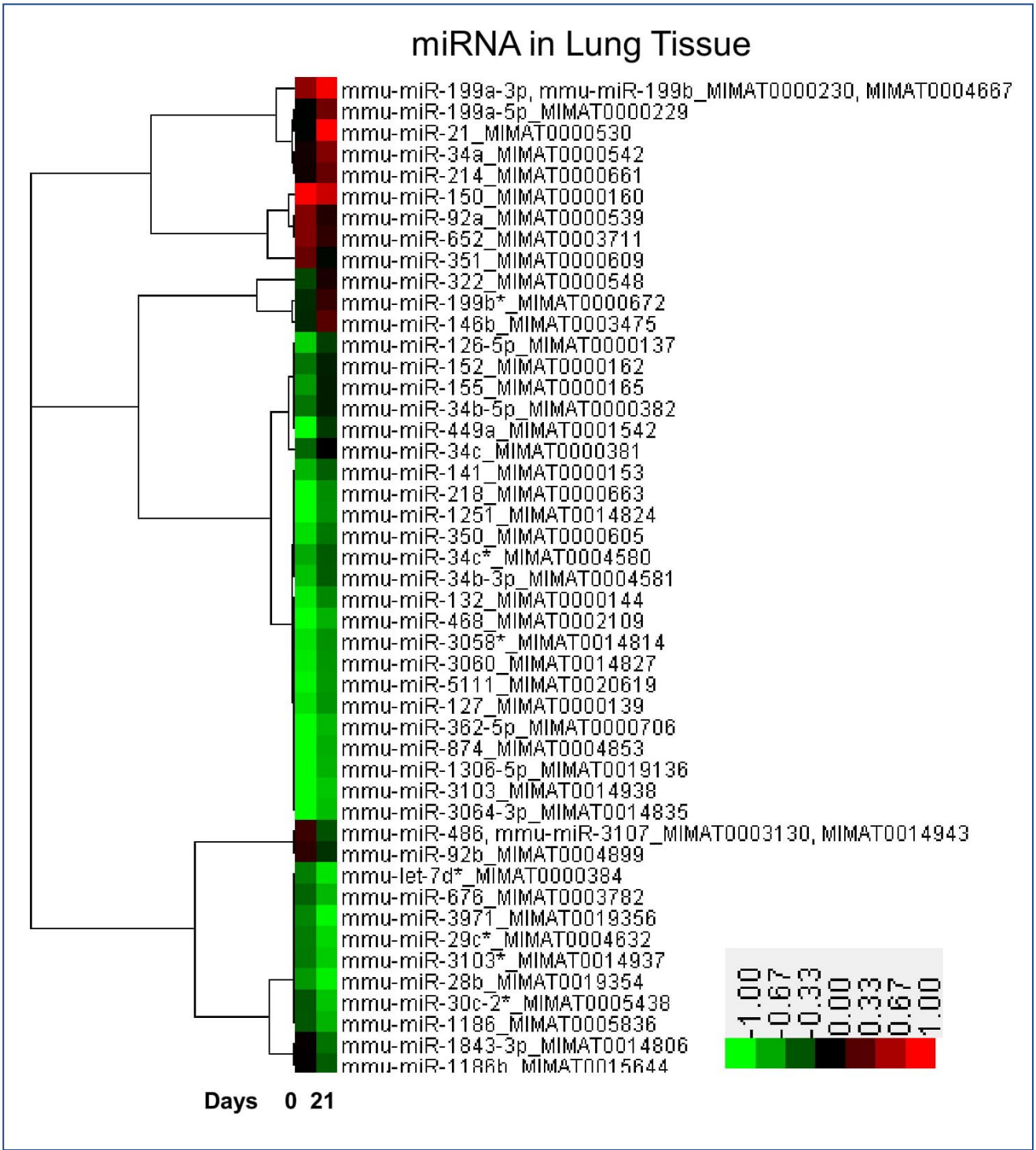

Figure 7: Cluster analysis of expression patterns (appeared in log 10) of 46 miRNAs on days 0 and 21, in the mixed sera of three mice in each group, showing more than two-fold expression in red or less than one-half expression in green.

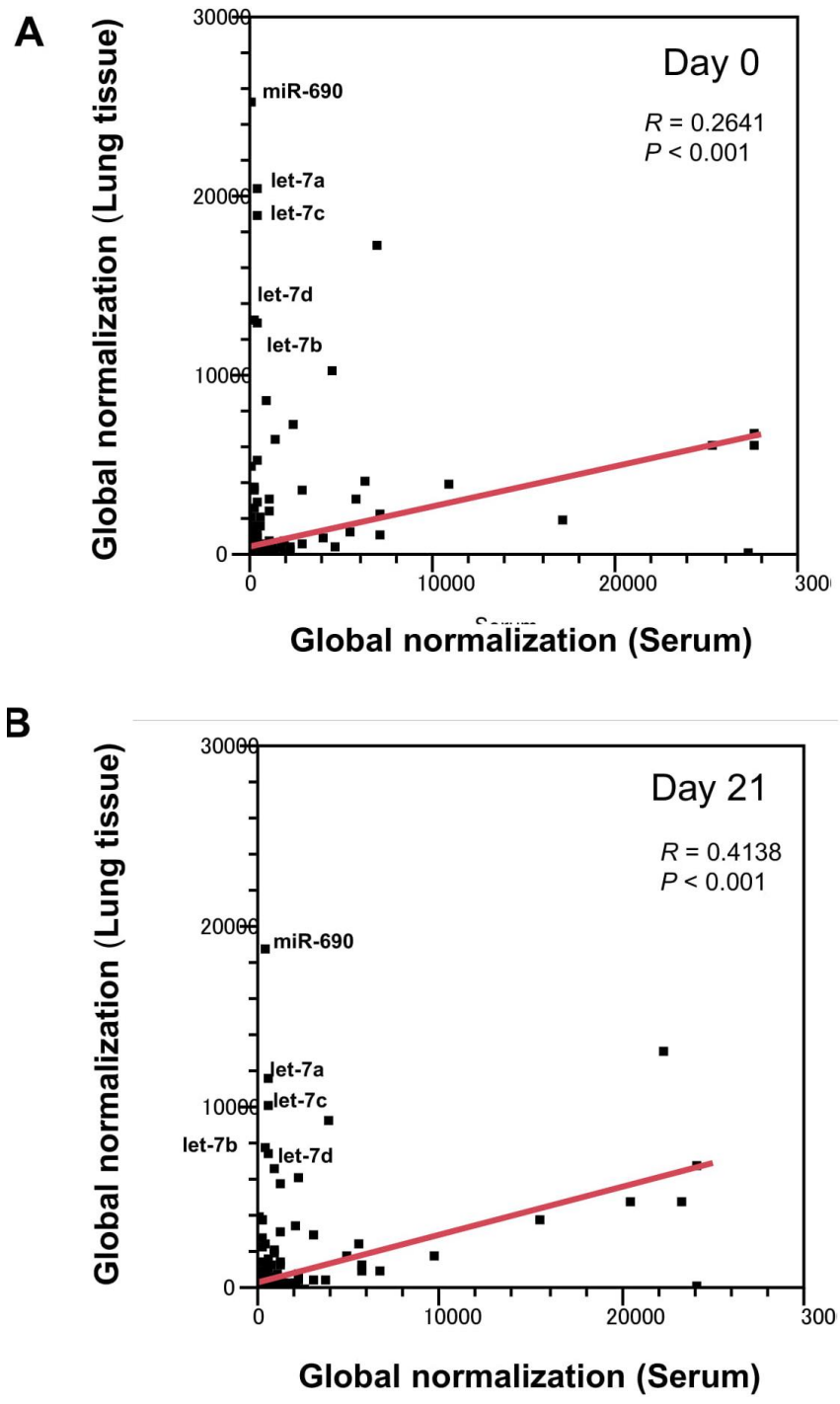

Figure 8: Correlation between circulating miRNA in sera and miRNA in lung tissues.

(A) Statistical correlation of miRNA between lung tissue and serum in control.

(B) Statistical correlation of miRNA between lung tissue and serum on 21 days after bleomycin.

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miRNA expression patterns in serum and lung tissue after bleomycin-induced lung injury

To clarify the problem whether these altered levels of circulating miRNAs, upregulated or down regulated after bleomycin-induced lung injury, were secreted from the pulmonary cells in the damaged lung, like circulating SP-D, or delivered from extra-pulmonary organs, we compared miRNA expression patterns between serum and lung tissues. According to a previous study comparing the miRNA expression patterns using total extracted from matched samples of Formalin-Fixed Paraffin-Embedded (FFPE) cells and snap frozen cells revealed that small RNA molecules were less affected and the quantity of miRNA extracted from FFPE cells were equivalent quantities than their snap frozen counterparts [27].

Totally 663 miRNAs were expressed above background in lung tissue (Figure 6), and 46 miRNAs were identified in the lung tissues that showed above two-fold changes or under one-half changes on day 21 after bleomycin-induced injury in comparison with control (day 0) (Figure 7). There were 31 miRNAs found to be upregulated and 15 miRNAs downregulated within the lung tissues on day 21 in comparison with control lungs. The highest 10 miRNAs (Table 1), and the lowest 10 miRNAs (Table 2), in contrast, recognized in the fibrotic lung tissues on day 21 after lung injury, were listed.

Finally, the correlations of secreted miRNAs were compared between serum and lung tissue on day 0 and day 21 after lung injury (Figure 8). As a whole, the global expressions of miRNAs were statistically in poor correlation between the serum and the lung tissues. We recognized only two miRNAs, miR-322 and miR-874, which were augmented both in the serum and in lung tissues on day 21 after bleomycin-induced lung injury (Table 3).

We also found various circulating miRNA were altered in expression, but the correlation ratios between circulating miRNAs and tissue miRNAs were less than 0.5 though there found to be statistic difference. These results suggest there should be circulating miRNA, not only as biomarkers but as targets for treatment of pulmonary fibrosis, including idiopathic pulmonary fibrosis with poor prognosis.

Microrna-21 was revealed to contribute to myocardial disease by stimulating MAP kinase signaling in fibroblasts [28], and also mediate fibrogenic activation of pulmonary fibroblasts and lung fibrosis [15]. Expression of miRNA-21 was found to be increased to ten-fold in fibrotic lung tissue on day 21 after lung injury in our current study, though no alteration of circulating miRNA-21 was observed in serum. MiRNA-155 was shown to regulate lung fibrosis by targeting keratinocyte growth factor [29]. In our study, expression of miR-155 in lung tissue increased to three-fold on day 21 in this study, but again, no expression of circulating miR-155 on days 0 and 7 in serum with only little expression was detected by microarray, which suggest miRNA-155 may be a poor biomarker for lung fibrosis in mouse model.

Table 1: Top 10 of circulating miRNAs increased on day 21 after bleomycin injury.

Name	ID	Ratio: day21/day0
mmu-miR-21	MIMAT0000530	14
mmu-miR-449a	MIMAT0001542	6
mmu-miR-126-5p	MIMAT0000137	3.6
mmu-miR-1251	MIMAT0014824	3.3
mmu-miR-218	MIMAT0000663	3.2
mmu-miR-146b	MIMAT0003475	3.2
mmu-miR-155	MIMAT0000165	3.1
mmu-miR-199a-5p	MIMAT0000229	2.8
mmu-miR-34a	MIMAT0000542	2.7

Table 2: Top 10 of the circulating miRNAs decreased on day 21 after bleomycin injury.

Name	ID	Ratio: day21/day0
mmu-miR-486,	MIMAT0003130,	
mmu-miR-3107	MIMAT0014943	0.27
mmu-miR-1843-3p	MIMAT0014806	0.33
mmu-miR-3971	MIMAT0019356	0.35
mmu-miR-351	MIMAT0000609	0.36
mmu-miR-30c-2*	MIMAT0005438	0.36
mmu-let-7d*	MIMAT0000384	0.39
mmu-miR-150	MIMAT0000160	0.39
mmu-miR-92b	MIMAT0004899	0.42
mmu-miR-1186b	MIMAT0015644	0.42
mmu-miR-92a	MIMAT0000539	0.43

Table 3: The miRNAs increased more than two-fold than control both in sera and lung tissues.

Name	ID	Ratio in serum	Ratio in lung tissue
mmu-miR-322	MIMAT0000548	2.3	2.4
mmu-miR-874	MIMAT0004853	2.4	2.2

CONCLUSION

Our results indicate that the serum level of SP-D is a reliable biomarker for diagnosing inflammation, but not for progressive fibrosis after bleomycin-induced lung injury in mice. Among various amounts of miRNAs in the serum and also fibrotic lung tissues, only two miRNAs, miR-322 and miR-874, are found to be significantly increased in both on day 21 after lung injury. Considering recent information on the possibility that circulating miRNAs may deliver information to affect the functioning cells in the distant organs, highly elevated circulating miRNAs just after lung injury, such as miRNAs, including MiR-30d, 122, 690, 1907, and 3096b, or miRNAs with latent elevation after day 7, such as MiR-501-3p, 15a, 20b, may be not only plausible circulating biomarkers but therapeutic targets providing novel treatment for progressive pulmonary fibrosis in this model mouse and hopefully for patients in future.

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