idiopathic pulmonary alveolar proteinosis have been shown to decrease following whole lung lavage (WLL).[13] We demonstrated for the first time that serum KL-6, SP-A, SP-D, CEA and lactate dehydrogenase levels are elevated and are significantly correlated with disease severity in patients with APAP.[4]

Cytokeratins are the main protein components of the cell cytoskeleton and belong to the family of intermediate filaments. Cytokeratin 19 is a low-molecular weight (40 kD) protein and is expressed in simple epithelia, including bronchial epithelial cells.[14] A fragment of cytokeratin 19, called CYFRA 21-1, can be measured in serum and has been evaluated as a useful tumour marker for non-small cell lung cancer.[14] Moreover, it is also known to be elevated in various types of non-malignant lung disorders,[15] such as bacterial pneumonia, tuberculosis, bronchiectasis and interstitial pneumonia, particularly acute interstitial pneumonia.[16] In addition, increased serum CYFRA 21-1 levels and its decrease after WLL have been reported in pulmonary alveolar proteinosis patients.[17] We have previously reported in a pulmonary alveolar proteinosis patient that high levels of serum CYFRA 21-1 declined following successful GM-CSF inhalation therapy, accompanied by an improvement in arterial blood gas levels and the diffusing capacity of carbon monoxide (DLco).[18] Thus, serum CYFRA 21-1 levels may be related to pulmonary alveolar proteinosis severity.

To assess the value of serum CYFRA 21-1 as a biomarker of APAP reflecting disease severity, we measured serum CYFRA 21-1 levels in APAP patients, and examined the relationship between serum CYFRA 21-1 levels and disease severity using respiratory function tests and arterial blood gas analysis. This is the most comprehensive and largest study to assess the role of serum CYFRA 21-1 as biomarker of APAP.

Table 1 Patient characteristics at the time of diagnosis of APAP

	Mean ± SE or number of cases
Number of patients	48
Age (years)	52.9 ± 1.9
Gender (M/F)	31/17
Smoking history (NS/ES/CS)	17/20/11
Dust exposure (Yes/No)	18/29
MRC (0/1/2/3/4)	9/18/18/3/0
DSS (I/II/III/IV/V)	4/20/8/9/6
PaO_2 (Torr)	66.5 ± 2.0
$AaDO_2$ (Torr)	36.3 ± 2.1
VC, %predicted	87.5 ± 2.9
DLco, %predicted	54.9 ± 2.9
CYFRA 21-1 (ng/mL)	9.03 ± 1.64
KL-6 (U/mL)	7320 ± 1224
SP-D (ng/mL)	224 ± 24
CEA (ng/mL)	10.0 ± 1.3
Anti-GM-CSF autoantibody (ng/mL)	48.5 ± 4.7

Normal serum ranges were CYFRA 21-1 (<2.8 ng/mL), KL-6 (<500 U/mL), SP-D (<110 ng/mL) and CEA (<5.0 ng/mL). The serum CYFRA 21-1 levels of 8 cases out of the total 48 cases were within the normal range. The 8 cases were mild or moderate diseases categorized in DSS 1, 2 and 3, and complaint of mild dyspnoea (MRC 0 and 1).

$AaDO_2$, alveolar-arterial oxygen gradient; APAP, autoimmune pulmonary alveolar proteinosis; CEA, carcinoembryonic antigen; CS, current smoker; DLco, diffusing capacity of carbon monoxide; DSS, disease severity score; ES, ex-smoker; GM-CSF, granulocyte-macrophage colony-stimulating factor; KL-6, Krebs von den Lungen-6; MRC, British Medical Research Council score for shortness of breath upon exertion; NS, non-smoker; PaO_2, partial pressure of oxygen; SP-D, surfactant protein-D; VC, vital capacity.

METHODS

Subjects

Forty-eight consecutive patients diagnosed with APAP at the Kinki-Chuo Chest Medical Center between 2002 and 2010 were enrolled in the study. The diagnosis of APAP was confirmed based on transbronchial lung biopsy, bronchoalveolar lavage, radiological findings and the presence of anti-GM-CSF autoantibody (Table 1). Serum levels of anti-GM-CSF autoantibody were measured at diagnosis by enzyme-linked immunosorbent assay as previously reported with minor modification,[4,19] and its cut-off level to diagnose APAP was 0.5 μg/mL.

Serum CYFRA 21-1 levels of APAP were compared with that of 68 patients with interstitial lung diseases diagnosed at the Kinki-Chuo Chest Medical Center, including idiopathic pulmonary fibrosis ($n = 25$), non-specific interstitial pneumonia ($n = 6$), collagen vascular disease associated interstitial pneumonia ($n = 15$), chronic hypersensitivity pneumonia ($n = 13$) and sarcoidosis ($n = 9$) (Table S1 in the online supporting information).

WLL was performed in 10 patients, and inhaled GM-CSF was administered to 20 patients (Table S3 in the online supporting information). A positive response to the therapy was defined as improvement of alveolar-arterial oxygen gradient ($AaDO_2$) by 10 Torr.

Comprehensive informed consents for measurements of serum biomarkers and comparison with clinical measures were obtained from all subjects. Additional informed consents were obtained on GM-CSF inhalation therapy. The review board of Kinki-Chuo Chest Medical Center approved this prospective study (Approval Number 73, 99).

Procedure of WLL

WLL was performed as previously described by Ramirez,[20] with slight modifications. The patient was in supine position, and a left-sided double-lumen tube was placed, after which one-lung ventilation with an inspiratory oxygen fraction of 1.0 was performed. Saline, warmed to body temperature, was delivered by gravity, and the lavage fluid was also drained by gravity after chest-wall percussion. Lavage

was completed when the appearance of the lavage fluid turned from milky to clear, and the total saline delivered usually reached 15–20 L for a single lung. WLL for the other side was performed about 2 weeks after the first WLL. Response to WLL was evaluated 1 month after the first WLL.

GM-CSF inhalation

Twenty patients were treated with GM-CSF inhalation. Three protocols for GM-CSF inhalation were employed in 15 patients, as described by Tazawa *et al.*[21] One patient from our first pilot study[18] was treated with a daily inhalation of 250 μg of GM-CSF every second week for 24 weeks starting on the first week. Two patients from our second pilot study received 125 μg of GM-CSF inhalation daily during the first 6 weeks, and an additional 125 μg of GM-CSF inhalation daily during the second 6 weeks if the change in $AaDO_2$ was ≥10 Torr, or an additional 250 μg of GM-CSF inhalation daily during the second 6 weeks if the change in $AaDO_2$ was <10 Torr. Twelve patients were from a phase II trial.[21] The remaining five APAP patients were treated with 50 μg of GM-CSF inhalation twice on days 1–8 and no GM-CSF inhalation on days 9–14 for 12 weeks, and 50 μg once on days 1–4 and no GM-CSF inhalation on days 5–14 for the next 12 weeks.

Evaluation of disease severity

Disease severity of the patients were evaluated at the time of diagnosis, and before and after WLL or GM-CSF inhalation using the following parameters: $AaDO_2$, partial pressure of oxygen (PaO_2), percentage of predicted DLco (%DLco), percentage of predicted vital capacity, the British Medical Research Council score for shortness of breath upon exertion and the disease severity score of APAP, as defined by Inoue *et al.*[1] The severity was classified into five grades: grade 1, $PaO_2 \geq 70$ Torr without respiratory symptoms; grade 2, $PaO_2 \geq 70$ Torr with respiratory symptoms; grade 3, 70 Torr > $PaO_2 \geq 60$ Torr; grade 4, 60 Torr > $PaO_2 \geq 50$ Torr; and grade 5, $PaO_2 < 50$ Torr. Arterial blood gas analyses were performed on samples obtained with the patient breathing room air at rest in the supine position for at least 15 min.

Measurement of CYFRA 21-1 and other serum markers

Serum CYFRA 21-1, KL-6, SP-D and CEA levels were measured by commercial enzyme-linked immunosorbent assay kits (CYFRA, Boehringer Mannheim, Tokyo, Japan; KL-6, Eizai, Tokyo, Japan; SP-D, Kyowa Medex, Tokyo, Japan; CEA, Abbott Japan, Tokyo, Japan). These markers were measured at diagnosis, and before and after therapy.

Immunohistochemistry

To detect the location of CYFRA 21-1 in the lung, we performed immunohistochemistry using the modified method of Nakayama *et al.*[15] Immunohistochemistry was performed by the avidin-biotin peroxidase complex method using the VECTASTAIN ABC mouse IgG Kit (Vector Laboratories, Burlingame, CA, USA) and a murine monoclonal anti-cytokeratin-19 antibody, Ks 19.1 (Progen, Biotechnik GmbH, Heidelberg, Germany), which is used for the measurement of serum CYFRA 21-1, at the concentration of 5 μg/mL. As a negative control, mouse IgG was used at the same concentration.

Statistical analysis

Each parameter was presented as mean ± SE. Correlations between serum CYFRA 21-1 and parameters of disease severity were evaluated by Spearman's rank correlation analysis. The differences in serum CYFRA 21-1 levels before and after GM-CSF inhalation or after WLL were determined by the paired *t*-test. Serum CYFRA 21-1 levels of GM-CSF responders and non-responders were compared by the Student *t*-test. Receiver operator characteristic curve analysis was performed to evaluate serum markers as diagnostic tests for APAP. The correlation between the change in serum CYFRA 21-1 and the change in $AaDO_2$ was assessed using the Pearson correlation analysis. A *P*-value of <0.05 was considered statistically significant. All statistical calculations were performed using the JMP version 8.0.2 for Macintosh (SAS Institute Inc., Cary, NC, USA).

RESULTS

Cut-off level of CYFRA 21-1 for diagnosis of APAP

Serum CYFRA 21-1 levels were significantly elevated in APAP (9.03 ± 1.64 ng/mL) (Table 1) as compared with interstitial lung diseases (2.96 ± 0.22 ng/mL) (Table S1). Cut-off level of serum CYFRA 21-1 to diagnose APAP was 3.80 ng/mL by receiver operator characteristic analysis (Fig. 1). Area under receiver operator characteristic curve of CYFRA 21-1 was similar to that of KL-6 (Fig. 1, Table S2 in the online supporting information).

Correlation between serum CYFRA 21-1 levels and other parameters reflecting disease severity

Serum CYFRA 21-1 levels were significantly correlated with British Medical Research Council score, disease severity score, $AaDO_2$ and %DLco, but not with percentage of predicted vital capacity. Serum KL-6 and SP-D were significantly correlated with all five parameters (Table 2).

Change in serum CYFRA 21-1 and $AaDO_2$ levels 1 month after WLL

In all 10 patients treated with WLL, serum CYFRA levels significantly decreased 1 month after WLL (Fig. 2a). $AaDO_2$ before WLL and 1 month after WLL was evaluated in nine patients, except for one patient whose disease was too severe to evaluate $AaDO_2$ under room air conditions. The decrease in $AaDO_2$ in

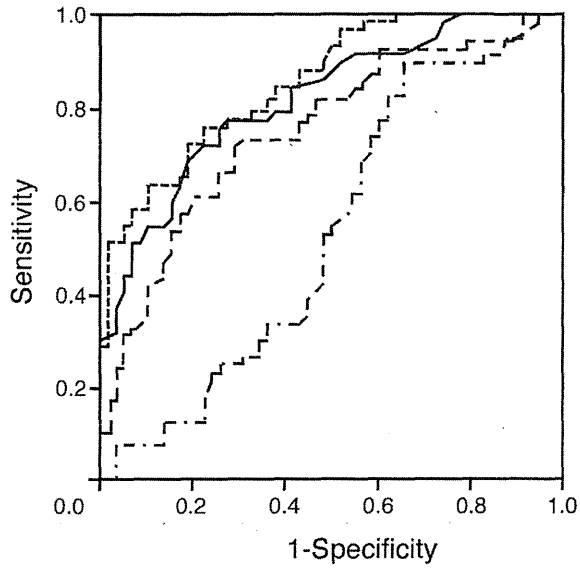

Figure 1 Receiver operator characteristic (ROC) curves showing comparison of serum markers including KL-6, surfactant protein-D (SP-D), carcinoembryonic antigen (CEA) and CYFRA 21-1 as diagnostic test for autoimmune pulmonary alveolar proteinosis (APAP). Levels of serum markers were measured in 68 patients with interstitial lung diseases (ILD), including idiopathic pulmonary fibrosis; 25 patients, non-specific interstitial pneumonia; 6 patients, collagen vascular disease associated interstitial pneumonia; 15 patients, hypersensitivity pneumonia; 13 patients and sarcoidosis; 9 patients, and 48 patients of APAP. Area under ROC curve of CYFRA 21-1, KL-6, CEA and SP-D was 0.8171, 0.8516, 0.7505 and 0.4951, respectively. Cut-off level of serum CYFRA 21-1 to diagnose APAP was 3.80 ng/mL. Details of serum markers in ILD (Table S1) and results of ROC curve analysis (Table S2) were described in the online supporting information. ——, CYFRA 21-1; ····, KL-6; – – –, SP-D; - - -, CEA.

Table 2 Relationship (ρ) between serum markers and disease severity markers in APAP patients

	CYFRA 21-1	KL-6	SP-D	CEA
MRC	0.6127*	0.6113*	0.5117**	0.5909*
DSS	0.6441*	0.6991*	0.5044*	0.5635*
$AaDO_2$	0.7474*	0.7239*	0.6091*	0.5914*
%DLco	−0.6793*	−0.7309*	−0.6878*	−0.4356***
%VC	−0.2326	−0.5097**	−0.5222*	−0.2403

* $P < 0.0001$; ** $P < 0.001$; *** $P < 0.01$.

The relationship between serum markers and disease severity markers was evaluated by ρ using the Spearman rank correlation analysis.

$AaDO_2$, alveolar-arterial oxygen gradient; APAP, autoimmune pulmonary alveolar proteinosis; CEA, carcinoembryonic antigen; DLco, diffusing capacity of carbon monoxide; DSS, disease severity score; KL-6, Krebs von den Lungen-6; MRC, British Medical Research Council score for shortness of breath upon exertion; SP-D, surfactant protein-D; VC, vital capacity.

nine patients following WLL ($\Delta AaDO_2$) was significantly correlated with the decrease in CYFRA 21-1 after WLL (ΔCYFRA 21-1) (Fig. 2b; $n = 9$, $r = 0.7621$, $P = 0.0170$), but not with ΔKL-6 ($n = 9$, $r = 0.4378$, $P = 0.2386$), ΔSP-D ($n = 9$, $r = 0.4686$, $P = 0.2033$) and ΔCEA ($n = 9$, $r = 0.3948$, $P = 0.2930$).

Change in serum CYFRA 21-1 and $AaDO_2$ following GM-CSF inhalation therapy

In GM-CSF-effective cases ($n = 11$), serum CYFRA 21-1 levels diminished significantly after GM-CSF inhalation therapy ($P = 0.002$); however, in GM-CSF-ineffective cases ($n = 9$), serum CYFRA 21-1 levels did not change significantly (Fig. 3). Although the characteristics of responders were not different from that of non-responders, except for age (Table S3), the serum CYFRA 21-1 levels in responders were significantly higher compared with non-responders before GM-CSF inhalation therapy (Fig. 4). There was no significant difference in serum KL-6, SP-D and CEA between responders and non-responders (Table S3). Multivariate logistic regression analysis with a stepwise method to predict effectiveness of GM-CSF inhalation revealed that serum CYFRA level (ng/mL) is a significant predictive factor; however, other factors, including the other serum markers, were insignificant (Table 3, Table S4 in the online supporting information).

Immunohistochemistry for CYFRA 21-1 detection

Immunohistochemical analysis of the transbronchial lung biopsy specimens obtained from one patient was performed. CYFRA 21-1-positivity was detected in the proteinaceous material in the alveolar spaces and in the hyperplastic alveolar epithelial cells (Fig. 5).

DISCUSSION

Receiver operator characteristic curve analysis revealed that CYFRA 21-1 is a diagnostic marker of APAP. CYFRA 21-1 is also a sensitive serum marker for APAP reflecting disease severity that is comparable to KL-6 and CEA, two markers that we have previously described.[4,22] In the present study, serum CYFRA 21-1 levels were significantly correlated with other disease severity parameters. Serum CYFRA 21-1 levels significantly decreased in APAP patients in whom $AaDO_2$ improved following GM-CSF inhalation therapy and WLL. Thus, we propose that CYFRA 21-1 is an important serum marker for diagnosis and disease severity of APAP. No significant relationship of CYFRA 21-1 with percentage of predicted vital capacity was found. However, no significant correlation might be a natural consequence of the percentage of predicted vital capacity decreasing below normal levels only in severe APAP, as previously reported.[4]

Elevation of serum markers in APAP patients is associated with impaired metabolism through the dysfunction of alveolar macrophages and through augmented permeability of each marker observed in interstitial lung diseases.[23,24] Increased permeability is necessary for KL-6, a large molecule with a molecular weight of more than 1000 kD,[25] to flow into the blood.

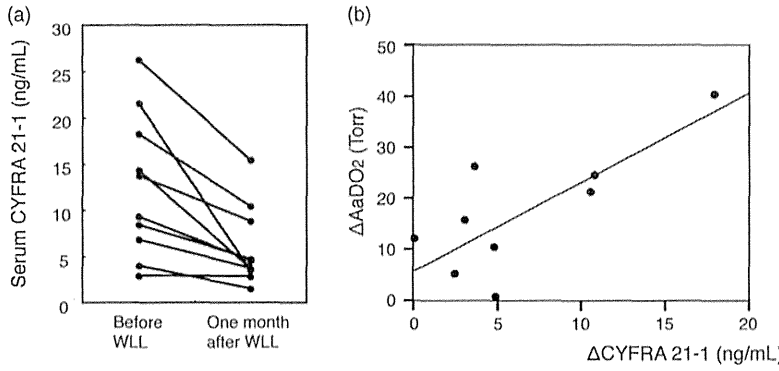

Figure 2 Serum CYFRA 21-1 levels (mean ± SE) in patients treated with whole lung lavage (WLL) ($n = 10$) decreased significantly after the treatment (from 12.53 ± 2.42 ng/mL to 5.90 ± 1.36 ng/mL) ($P = 0.0041$) (a). Alveolar-arterial oxygen gradient ($AaDO_2$) was evaluated before and after the WLL in nine patients. Decrease in CYFRA 21-1 (ΔCYFRA 21-1) significantly correlated with the decrease in $AaDO_2$ ($\Delta AaDO_2$) in nine patients 1 month after WLL ($P = 0.0170$). Statistical analyses were performed by the paired *t*-test (a) and the Pearson correlation analysis (b).

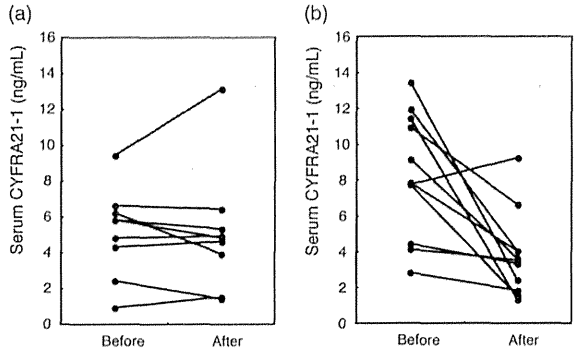

Figure 3 Measurement of serum CYFRA 21-1 levels (mean ± SE) before and after granulocyte-macrophage colony-stimulating factor (GM-CSF) inhalation. In GM-CSF-ineffective cases (non-responders, $n = 9$), there was no significant difference before (5.13 ± 0.82 ng/mL) and after the inhalation therapy (5.10 ± 1.14 ng/mL) (a), and in GM-CSF-effective cases (responders, $n = 11$), the serum CYFRA 21-1 levels significantly decreased from 8.29 ± 1.04 ng/mL to 3.74 ± 0.71 ng/mL after the inhalation therapy ($P = 0.002$) (b). Statistical analysis was performed by the paired *t*-test.

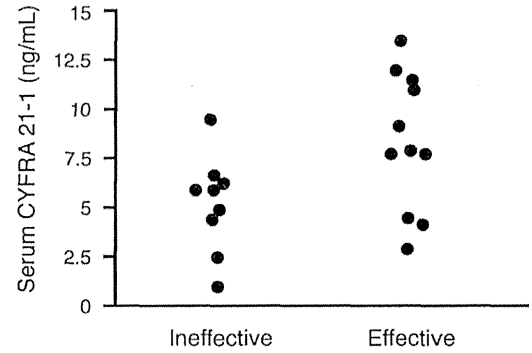

Figure 4 Serum CYFRA 21-1 levels before the inhalation of granulocyte-macrophage colony-stimulating factor (GM-CSF) were significantly higher in GM-CSF-effective cases (responders, $n = 11$, 8.29 ± 1.04 ng/mL) than those in GM-CSF-ineffective cases (non-responders, $n = 9$, 5.13 ± 0.82 ng/mL), as demonstrated by the Student *t*-test ($P = 0.017$). Three GM-CSF-effective cases showed comparatively low levels of serum CYFRA 21-1, although alveolar-arterial oxygen gradient of three patients was more than 40 Torr. Serum levels of Krebs von den Lungen-6, surfactant protein-D and carcinoembryonic antigen were also low in two out of three patients (data not shown) (values were presented as mean ± SE).

Inoue *et al.* reported that the increase in serum KL-6 level was due to increased permeability of the alveolar wall to blood flow in berylliosis.[26] The permeability of the alveolar-airway barrier might be modulated by GM-CSF. GM-CSF increases alveolar epithelial barrier function in vitro[27] and suppresses the apoptosis of alveolar epithelial cells.[28] Thus, it is possible that the deficiency of GM-CSF caused by the presence of anti-GM-CSF autoantibodies in APAP conversely leads to the dysfunction of the alveolar epithelial barrier and increased permeability.

Based on a previous report on non-malignant pulmonary diseases, elevation of serum CYFRA 21-1 is due to epithelial damage and its increased production in the epithelium.[16] CYFRA 21-1 is expressed in hyperplastic type II pneumocytes and metaplastic cells in patients with idiopathic pulmonary fibrosis.[16] Although the pathophysiology is unknown, alveolar epithelial cell hyperplasia is sometimes observed in the lung specimens of APAP patients.[29] In agreement with this finding, we also pointed out the existence of alveolar epithelial cell hyperplasia in the present study. Thus, increased production of CYFRA 21-1 from alveolar epithelial cells may be another reason for the elevation of serum CYFRA 21-1 levels in APAP patients.

Yoshimasu *et al.* calculated the half-life of CYFRA 21-1 to be 1.5 h from the disappearance curve of serum concentration after the resection of lung cancer.[30] The half-lives of SP-D and KL-6 are not known; however, they are predicted to be longer than that of CYFRA 21-1 when considering their higher molecular weights. A short half-life is an important characteristic as a serum marker because levels of a serum marker with a short half-life can change simultaneously with the change in disease activity. To evaluate this point, the disappearance rate of each serum marker 1 month after WLL was examined in the present study; however, there was no significant difference between the markers (data not shown). This is because 1 month is too long an interval to evaluate the disappearance rate of each of the serum markers.

Table 3 Multivariate logistic regression analysis to predict effectiveness of GM-CSF inhalation in APAP

Parameters	Odds ratio	95% CI	*P*-value
CYFRA 21-1 ≥ 6.4 ng/mL	9.333	1.372–94.188	0.0213

Multivariate logistic regression analysis with a stepwise method to predict effectiveness of GM-CSF inhalation was performed, using serum levels of CYFRA 21-1, carcinoembryonic antigen (CEA), Krebs von den Lungen-6 (KL-6), surfactant protein-D (SP-D), %vital capacity (VC), % diffusing capacity of carbon monoxide (DLco), alveolar-arterial oxygen gradient ($AaDO_2$), disease severity score (DSS), gender, age and British Medical Research Council score for shortness of breath upon exertion (MRC score). Each parameter was classified into two groups by median except for gender: CYFRA 21-1 ≥ 6.4 ng/mL, CEA ≥ 7.3 ng/mL, KL-6 ≥ 4090 U/mL, SP-D ≥ 213 ng/mL, %VC ≥ 89%, %DLco ≥ 47.5%, $AaDO_2$ ≥ 44 Torr, DSS ≥ 4, MRC ≥ 3, age ≥ 53. Univariate analysis revealed that serum CYFRA 21-1 level was only a significant factor to predict good response of GM-CSF inhalation to GM-CSF inhalation (Table S4 in the online support information). Using stepwise method, serum CYFRA 21-1 level was also only a significant predictor for the response of GM-CSF inhalation therapy and other serum factors were insignificant.

APAP, autoimmune pulmonary alveolar proteinosis; CI, confidence interval; GM-CSF, granulocyte-macrophage colony-stimulating factor.

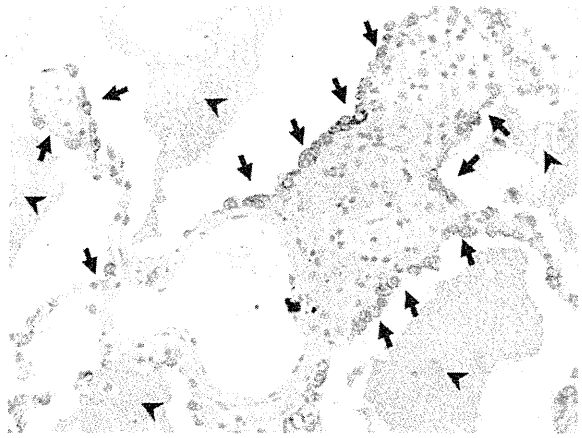

Figure 5 Immunohistochemical analysis of transbronchial lung biopsy specimens obtained from one patient. CYFRA 21-1-positivity was observed in lipoproteinaceous material in the alveolar spaces (arrowhead) and in the hyperplastic alveolar epithelial cells (arrow).

Serum CYFRA 21-1 levels in responders to GM-CSF inhalation were significantly higher compared with non-responders. The same tendency has been reported for serum KL-6;[21] however, there was no difference in the levels of the other serum markers between responders and non-responders in our examination. Serum CYFRA 21-1 was a significant factor to predict effectiveness of GM-CSF inhalation therapy by multivariate logistic regression analysis. Hence, serum CYFRA might be a useful serum marker to predict the effectiveness of GM-CSF inhalation. However, studies of additional APAP patients treated with GM-CSF inhalation are needed to make definite conclusions, and response to GM-CSF itself needs to be judged cautiously considering the possibility of spontaneous regression of some cases of APAP.[4]

It remains unresolved from our investigation which is the best serum marker of APAP. KL-6 is a very good marker, and is highly correlated with both symptoms and pulmonary function tests. However, another important ability requested for a good marker is to predict treatment response and prognosis. We expect that serum CYFRA 21-1 might be able to forecast effectiveness of GM-CSF inhalation shown above and disease severity changes, for example spontaneous regression, due to its short half-life. Future studies are needed to reach definite conclusions.

We conclude that serum CYFRA 21-1 is a sensitive and useful serum marker for diagnosis and evaluation of disease severity of APAP. CYFRA 21-1 levels might predict the response to GM-CSF inhalation.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Patient characteristics of interstitial lung diseases

Table S2 Details of ROC curve analysis of serum markers

Table S3 Patient characteristics before therapy

Table S4 Univariate logistic regression analysis to predict effectiveness of GM-CSF inhalation in APAP

CHEST

Original Research

DIFFUSE LUNG DISEASE

Duration of Benefit in Patients With Autoimmune Pulmonary Alveolar Proteinosis After Inhaled Granulocyte-Macrophage Colony-Stimulating Factor Therapy

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Background: **Treatment of autoimmune pulmonary alveolar proteinosis (aPAP) by subcutaneous injection or inhaled therapy of granulocyte-macrophage colony-stimulating factor (GM-CSF) has been demonstrated to be safe and efficacious in several reports. However, some reports of subcutaneous injection described transient benefit in most instances. The durability of response to inhaled GM-CSF therapy is not well characterized.**

Methods: **To elucidate the risk factors for recurrence of aPAP after GM-CSF inhalation, 35 patients were followed up, monitoring for the use of any additional PAP therapies and disease severity score every 6 months. Physiologic, serologic, and radiologic features of the patients were analyzed for the findings of 30-month observation after the end of inhalation therapy.**

Results: **During the observation, 23 patients remained free from additional treatments, and twelve patients required additional treatments. There were no significant differences in age, sex, symptoms, oxygenation indexes, or anti-GM-CSF antibody levels at the beginning of treatment between the two groups. Baseline vital capacity (% predicted, %VC) were higher among those who required additional treatment ($P < .01$). Those patients not requiring additional treatment maintained the improved disease severity score initially achieved. A significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group was seen by a Kaplan-Meier analysis and a log-rank test ($P < .0005$).**

Conclusions: **These results demonstrate that inhaled GM-CSF therapy sustained remission of aPAP in more than one-half of cases, and baseline %VC might be a prognostic factor for disease recurrence.**

Trial registry: **ISRCTN Register and JMACCT Clinical Trial Registry; No.: ISRCTN18931678 and JMAIIA00013; URL: http://www.isrctn.org and http://www.jmacct.med.or.jp**

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Abbreviations: A-aDO_2 = alveolar-arterial oxygen difference; Ab = antibody; aPAP = autoimmune pulmonary alveolar proteinosis; AT = additional treatment; BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; DSS = disease severity score; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; ROC = receiver operating characteristics curve; SP = surfactant protein; VC = vital capacity; WLL = whole-lung lavage

Autoimmune pulmonary alveolar proteinosis (aPAP) is a rare lung disease characterized by the accumulation of surfactant protein (SP), which causes progressive respiratory insufficiency.[1-3] The pathogenesis has been attributed to the excessive production of a neutralizing autoantibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) that impairs GM-CSF-dependent surfactant clearance mediated by

alveolar macrophages.[4-8] On pulmonary function testing, the most common pattern seen is that of a restrictive defect, with a disproportionate reduction in diffusing capacity of the lung for carbon monoxide (DLCO) relative to a modest impairment of vital capacity (VC).[2] The disease is usually treated by whole-lung lavage (WLL), which remains the standard therapy to date.

The first patient successfully treated with subcutaneously administered GM-CSF was reported in 1996.[9] In an international multicenter phase 2 trial study, 14 patients were treated with GM-CSF by subcutaneous injection in escalating doses over a 3-month period, with an overall response rate of 43%.[10,11] A subsequent single-center study of 21 patients with aPAP treated with GM-CSF by subcutaneous administration in escalating doses for 6 to 12 months reported an overall response rate of 48%.[12] Several single cases of subcutaneous GM-CSF therapy have reported similar outcomes.[13,14] However, local reaction at sites of injection and other minor toxicities occurred in 85% of patients receiving subcutaneous GM-CSF.[2]

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GM-CSF inhalation is a promising alternative therapy for aPAP that has been demonstrated to lead to functional, biologic, and radiologic improvement.[15-18] Our national, multicenter phase 2 study revealed that the therapy reduced alveolar-arterial oxygen difference ($A\text{-}aDO_2$) by 12.3 mm Hg in 35 patients who completed the therapy, resulting in 24 responders. No treatment-related side effects were noted. Of importance, our previous phase 2 study showed that there was no significant difference in serologic, physiologic, and CT scan testing, except for serum Krebs von den Lungen-6 (KL-6) levels, between the responders and the nonresponders.[18]

There is limited information regarding the duration of benefit after various treatments of aPAP. In the literature analysis of 55 cases with a therapeutic response to WLL, the median duration of clinical benefit from lavage was 15 months.[2] A phase 2 study of subcutaneous GM-CSF administration demonstrated that 45% of patients required WLL during follow-up observation of 39 ± 17.3 months.[12] In a retrospective analysis of inhaled GM-CSF therapy (250 μg bid), five of 12 patients manifest progressive disease during observation.[17] As the disease progresses very slowly and can fluctuate in some cases, it is necessary to evaluate the prognosis by monitoring prospectively at the same time points after the treatment and by disease severity score as well as the need for additional treatment. The aim of this study was to define the duration of benefit among patients who underwent GM-CSF inhalation therapy.

Materials and Methods

Patients and Protocols

The present study prospectively observed patients who participated in a multicenter phase 2 trial (35 patients, registered as ISRCTN18931678 and JMAIIA00013) of GM-CSF inhalation therapy described previously. In brief, patients who had lung biopsy or cytologic findings diagnostic for pulmonary alveolar proteinosis (PAP), including elevated serum anti-GM-CSF antibody (Ab) levels and no improvement during a 12-week observation period, entered the treatment phase. Recombinant human GM-CSF dissolved in 2 mL of sterile saline was inhaled using an LC-PLUS nebulizer (PARI International). The treatment consisted of high-dose GM-CSF administration (125 μg bid on days 1-8, none on days 9-14; sargramostim) for six repetitions of 2-week cycles, then low-dose administration (125 μg once daily on days 1-4, none on days 5-14) for six repetitions of 2-week cycles (for a total dose of 15 mg). The clinical information including physiologic, serologic, and radiologic features obtained[18] was compared with the results of the following 30-month observation.

Patients were regularly evaluated by their physicians at the network hospitals after the GM-CSF inhalation therapy. The worsening dyspnea was evaluated with pulse oximetry, arterial blood gas analysis, or both in outpatient settings. Disease severity in patients was evaluated using PAP disease severity score (DSS) described previously.[19] Patients underwent additional treatments based on

either of the following criteria: (1) DSS is 3 or 4 and symptoms are worsening or (2) DSS 5, as shown in Figure 1. The consortium office of Niigata University contacted the network hospitals every 6 months with a questionnaire regarding additional treatment and disease severity score of the patient. The follow-up clinical information obtained at each network hospital was entered into a database to be compared with the results of the baseline clinical evaluation of each patient. The data were collected from nine clinical research centers in Japan (Hokkaido University, Tohoku University, Chiba University, Kitasato University, Niigata University, Aichi Medical University, National Hospital Organization Kinki-Chuo Chest Medical Center, National Hospital Organization Yamaguchi-Ube Medical Center, and Nagasaki University Institute of Tropical Medicine).

The study was approved by institutional review board of Niigata University (approval No. NH17-006) and the institutional review boards at all participating centers. Informed consent was obtained from all control subjects. The clinical information obtained by the clinical studies was entered into a database to be compared with the results of the 30-month observation. The study was designed and monitored for data quality and safety by a steering committee composed of the principal investigator at each participating site. The steering committee held a conference twice a year, where the findings of the observation were monitored.

BAL Procedures and GM-CSF Autoantibodies

The steering committee edited a standard operational procedure for BAL, which was followed by all participating institutes and described previously.[18,20] The concentration of GM-CSF autoantibodies in BAL fluid (BALF) or in serum were measured using a sandwich enzyme-linked immunosorbent assay as described previously.[4,21]

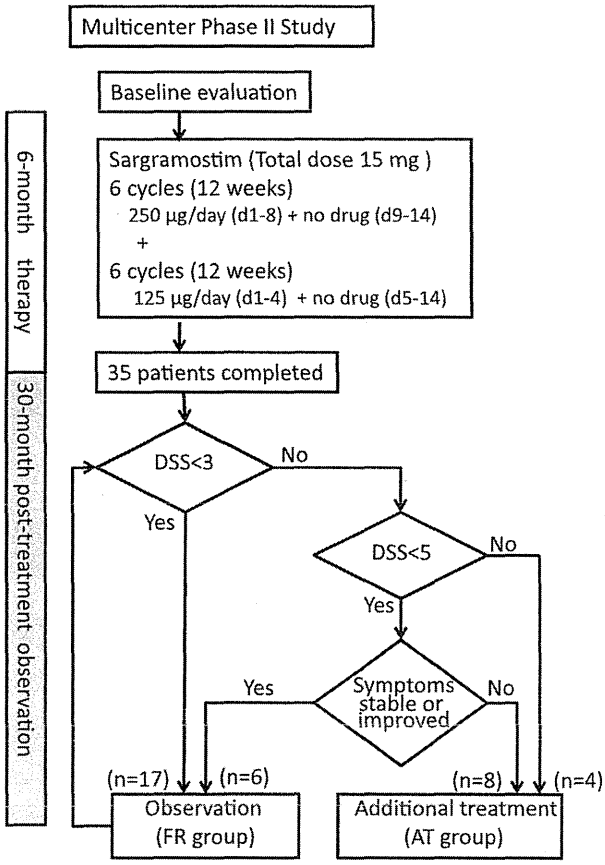

FIGURE 1. Profile of the study cohort. AT = additional treatment; DSS = disease severity score; FR = free from additional treatment.

Statistical Analysis

Numerical results are presented as the mean ± SE or the median and interquartile range (IQR). The χ^2 test was used to evaluate proportions for variables between high and low responders. The paired *t* test was used for comparisons between normally distributed data and the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon signed-rank test. For group comparisons, unpaired *t* tests and Wilcoxon rank-sum tests were used. All *P* values were reported as two-sided. Analysis was performed using JMP software, version 8.0.2 (SAS Institute Inc).

RESULTS

Patient Characteristics and Requirements for Additional Treatments as an Indicator of Recurrence

Demographic data of patients are shown in Table 1. During the 30 months of observation after the end of GM-CSF inhalation, the need for treatments was monitored as an indicator of disease recurrence in each patient. Twenty-three patients were free from additional treatments during 30 months of observation and were designated as FR (free from additional treatment). Twelve patients who required additional treatments, including six patients with recurrence described in our previous study,[18] were designated as AT (additional treatment). Of those, two patients maintained most severe disease (DSS 5) even after the GM-CSF treatment and underwent subsequent WLL. One patient who had dyspnea, cough, and sputum production did not respond to the GM-CSF treatment and underwent subsequent WLL. One patient with cough and dyspnea showed worsening in PaO_2 and cough and had WLL 12 months after the GM-CSF inhalation. The other eight patients with dyspnea showed worsening in PaO_2/oxygen saturation by pulse oximetry (two patients worsened to DSS 5) and underwent additional therapy (e-Fig 1); five underwent additional GM-CSF inhalation treatments, two had WLL, and one patient, a nonresponder, declined WLL and underwent acetylcysteine inhalation, showing much improvement in hypoxia. Median time to additional treatment of the 12 patients was 50.5 weeks, with a range of 8.5 to 117.5 weeks. There was no significant difference in age, sex, symptoms, smoking status, history of dust exposure, arterial blood gas analysis, numbers of responders to GM-CSF inhalation, history of previous lung lavage, and anti-GM-CSF-Ab titer between the FR and AT groups (Table 1). There was no significant difference in disease markers, including baseline levels of PaO_2, A-aDO_2, %VC, %DLCO, CT scan scores, lactate dehydrogenase (LDH), and KL-6 between the patients who underwent WLL (n = 6, AT-WLL group) and those treated with GM-CSF inhalation (n = 5, AT-GM group)

Table 1—*Baseline Clinical Characteristics of Patients Free From Additional Treatment and Those Who Required Additional Treatment After GM-CSF Inhalation*

Characteristic	FR (n = 23)			AT (n = 12)			*P* Value
	No.	%	Median (IQR) or Mean ± SE	No.	%	Median (IQR) or Mean ± SE	
Age, y	23	...	52.5 (48-61)	12	...	52.5 (41.75-58)	.33[a]
Sex			...			...	.54[b]
Female	9	39		6	50		
Male	14	61		6	50		
Responders	17	74	...	7	58	...	.35[b]
Duration of symptoms, mo	23	...	20 (11-61)	12	...	18 (7.75-72)	.78[a]
Symptoms			...			...	
Dyspnea	22	96		12	100		.36[b]
Cough	10	43		7	58		.65[b]
Sputum	8	35		4	33		.71[b]
Smoking status			...			...	.39[b]
Current smoker	8	35		2	17		
Ex-smoker	5	22		2	17		
Never smoked	10	43		8	67		
Dust exposure	22	...	...	11	...	...	.27[b]
Yes	8	36		3	18		
No	14	64		8	82		
Arterial blood gas analysis							
$PaCO_2$, Torr[c]	23	...	38.0 ± 0.7	12	...	39.0 ± 0.9	.40[d]
PaO_2, Torr[c]	23	...	60.6 ± 2.1	12	...	56.3 ± 3.0	.25[d]
A-aDO_2, Torr[c]	23	...	43.5 ± 2.4	12	...	46.2 ± 3.3	.51[d]
Disease severity score	23	...	3 (3-4)	12	...	3.5 (3-5)	.58[a]
GM-CSF autoantibody, μg/mL	23	...	22.8 (8.5-33.2)	12	...	23.1 (16.9-34.2)	.94[a]
Previous lung lavage (>6 mo *prior to study*)			...			...	.22[b]
Yes	5	22		5	42		
No	18	78		7	58		

Thirty-five patients completed both the high-dose and low-dose period of GM-CSF inhalation therapy. A-aDO_2 = alveolar-arterial oxygen difference; AT = additional treatment; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range (range from the 25th to the 75th percentiles of the distribution).

[a]Calculated using the Wilcoxon rank sum test.

[b]Calculated using the χ^2 test.

[c]Measured with patient in a supine position and breathing room air.

[d]Calculated using Student *t* test.

[e]Calculated using the following equation: A-aDO_2 = (PB − PH_2O) × FIO_2 − $PaCO_2$/R + {$PaCO_2$ × FIO_2 × (1 − R)/R} − PaO_2, where PB = barometric pressure measured by local observatories; PH_2O = partial pressure of water vapor in inspired air (assumed to be 47 mm Hg); FIO_2 = fractional concentration of oxygen in dry gas (assumed to be 0.21); and R = respiratory quotient (assumed to be 0.8).

(e-Table 1). However, changes in A-aDO_2 during the GM-CSF treatment were significantly higher in the AT-GM group.

Association of Clinical Parameters With Requirement for Additional Treatment

There was no significant difference in baseline findings in terms of PaO_2, $PaCO_2$, FEV_1, and DLCO between AT and FR groups. Both %VC (% predicted value) and %FVC were higher in the FR group ($P < .01$) (Fig 2A, Table 2, e-Fig 2). There was no correlation between baseline %VC and age ($P = .97$), sex ($P = .41$), baseline PaO_2 ($P = .18$), or baseline %DLCO ($P = .34$). There was no significant difference in high-resolution CT scan scores and serum markers, including LDH, KL-6, carcinoembryonic antigen (CEA), SP-A, and SP-D (Table 2).

As for differential blood cell counts, no significant difference was observed between FR and AT groups, except for numbers of basophils and platelets. The cell density of macrophages in BALF was lower in the FR group than those in the AT group ($P < .05$), whereas lymphocytes were lower in the AT group as compared with the FR group.

Next, clinical parameters at the end of treatment were evaluated. The %DLCO was lower in the AT group than that in the FR group, and serum markers (eg, LDH, KL-6, CEA, SP-D, SP-A) and CT scan scores were higher in the AT group than those in the FR group at the end of treatment ($P < .05$). However, there was no significant difference in A-aDO_2, blood cell counts, and cell differentials in BALF (Table 3). The patients free from additional treatment maintained the improved disease severity score initially achieved (e-Fig 3).

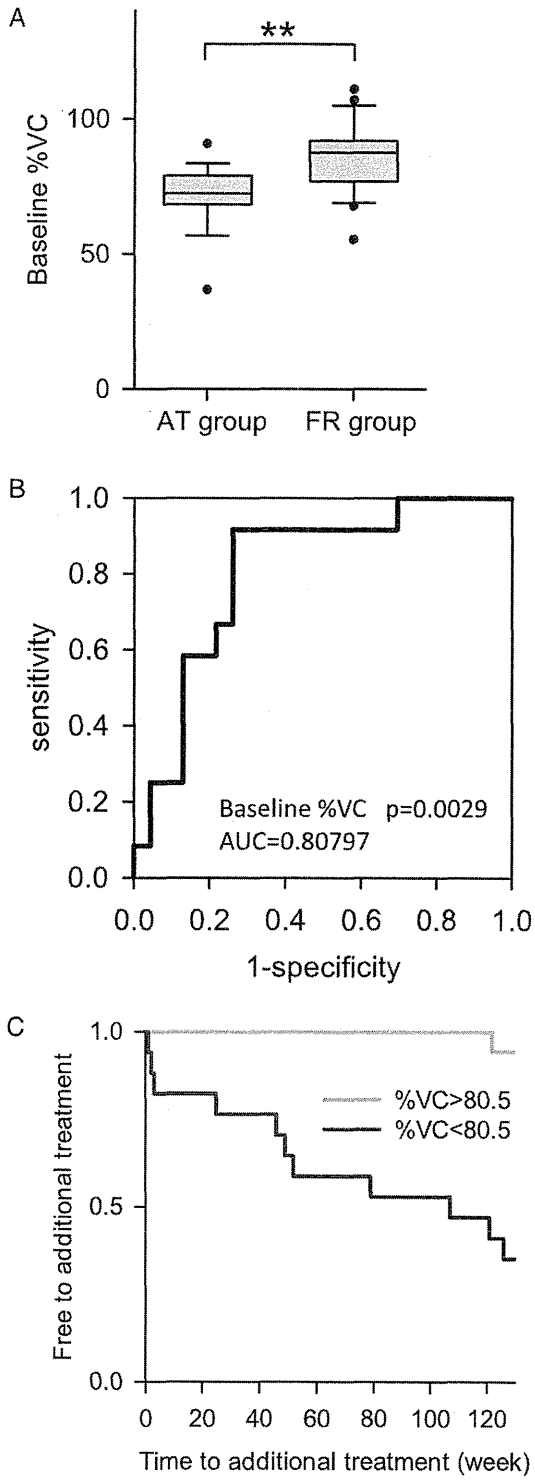

FIGURE 2. The association between VC (% predicted, %VC) and additional treatments during the 30-mo observation period (**$P < .01$). A, Baseline levels of %VC in FR and AT patient groups. B, Receiver operating curve of %VC. C, Kaplan-Meier plot showing patients of the high %VC group (%VC ≥ 80.5) and those of the low %VC group (%VC < 80.5). AUC = area under the receiver operating curve; VC = vital capacity. See Figure 1 legend for expansion of other abbreviations.

Predictive Value of VC for Prognosis After GM-CSF Inhalation

Because only %VC and %FVC differed between FR and AT groups among treatment-related pretreatment factors, the predictive value of parameters for recurrence after GM-CSF inhalation was evaluated using receiver operating characteristics curve (ROC) analysis and Kaplan-Meier analysis of time to additional treatment.

For ROC analysis, the area under the ROC curve was calculated nonparametrically, as proposed by Hanley and McNeil.[22] An additional therapy was defined as a positive indicator for disease recurrence. When the cutoff level of 80.5% was set for %VC, the baseline %VC predicted the additional therapy with a sensitivity of 92% and a specificity of 74% (Fig 2B).

For Kaplan-Meier analysis of time to additional treatment, we divided the patients into two groups, namely the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5). A significant difference in the time to additional treatment between the two groups was seen when the whole period of follow-up was compared ($P = .0001$) (Fig 2C). In the univariate Cox proportional analysis of baseline markers, %VC < 80.5% (hazard ratio, 18.42; 95% CI, 3.55-337.68; $P < .0001$) was associated with additional treatment, whereas no correlations were found between additional treatment and age, sex, baseline PaO_2, changes in A-aDO_2, and baseline levels of LDH, KL-6, SP-A, CEA, and anti-GM-CSF-Ab.

Subgroup Analysis: To test whether VC is an independent predictive factor for the time to additional therapy, we did subgroup analyses because of the small number of the AT patients. The patients were divided into two groups of an upper one-half and a lower one-half regarding age; sex; baseline PaO_2; change in A-aDO_2; baseline levels of LDH, KL-6, SP-A, CEA; and anti-GM-CSF-Ab. In these subgroups, a significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5) was still evident, suggesting that VC might be an independent factor predicting the time to additional therapy (e-Fig 4).

Time Course of Autoantibody Levels: In our previous reports, serum levels of anti-GM-CSF-Ab levels did not change during treatment.[16] To study longitudinal changes of serum levels of anti-GM-CSF-Ab after the inhaled GM-CSF therapy, serum samples were collected for anti-GM-CSF-Ab testing as an optional evaluation after the 30-month observation period. The serum levels were unchanged during the observation period except for three cases (e-Fig 5). In two cases, the serum levels increased by > 100 μg/mL, and one case required an additional treatment, whereas

Table 2—*Baseline Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups Before GM-CSF Inhalation Treatment*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	85.9 ± 2.7	12	71.6 ± 3.8	.0045[a]
FVC, % predicted	23	85.3 ± 2.8	12	71.4 ± 3.9	.0064[a]
FEV_1/FVC	23	87.1 ± 2.0	12	84.9 ± 2.7	.51[a]
DLCO, % predicted	23	57.0 ± 3.4	10	46.0 ± 5.1	.082[a]
HRCT scan scores[b]					
Upper lung region	23	3 (2-5)	12	4.5 (2-5)	.12[c]
Middle lung region	23	4 (3-5)	11	4 (3-5)	.38[c]
Lower lung region	23	4 (3-5)	12	5 (4-5)	.36[c]
Serum biomarkers of PAP					
LDH, IU/L	23	287 ± 19	12	325 ± 26	.26[a]
CEA, ng/mL	23	6.2 ± 1.0	12	8.0 ± 1.4	.30[a]
KL-6, U/L	23	10,038 ± 1,531	12	9,434 ± 2,120	.81[a]
SP-A, ng/mL	23	127 ± 15	12	153 ± 20	.29[a]
SP-D, ng/mL	23	227 ± 25	12	290 ± 34	.14[a]
Hematologic indexes					
WBC count, cells/μL	23	5,608 ± 267	12	6,358 ± 370	.11[a]
Neutrophils, cells/μL	22	3,428 ± 200	12	3,596 ± 271	.62[a]
Monocytes, cells/μL	22	344 ± 21	12	396 ± 28	.15[a]
Lymphocytes, cells/μL	22	1,730 ± 147	12	2,122 ± 198	.12[a]
Eosinophils, cells/μL	22	107 ± 28	12	199 ± 38	.058[a]
Basophils, cells/μL	22	18.3 ± 4.3	12	45.3 ± 5.9	.0008[a]
Hemoglobin, g/dL	23	15.4 ± 0.3	12	14.4 ± 0.4	.058[a]
Platelets, $\times 10^3$ cells/μL	23	224 ± 9.1	11	271 ± 13	.0046[a]
BALF cell classification, %					
Alveolar macrophages	17	63 ± 3.6	5	38 ± 6.7	.0036[a]
Neutrophils	17	5.2 ± 1.5	5	10.8 ± 2.7	.082[a]
Eosinophils	17	0.84 ± 0.32	5	0.40 ± 0.60	.52[a]
Lymphocytes	17	31.2 ± 3.8	5	50.4 ± 7.1	.027[a]

BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; HRCT = high-resolution CT; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; SP = surfactant protein; VC = vital capacity. See Table 1 legend for expansion of other abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously,[18] left lung.

[c]Calculated using the Wilcoxon rank sum test.

the others did not. In the third case, the serum levels decreased to 0.47 μg/mL, and additional treatments were not required.

Discussion

In the present study we have prospectively analyzed, for the time to our knowledge, the requirements of additional therapy and disease severity scores in 35 patients who completed GM-CSF inhalation therapy. The results demonstrate that 23 patients were free from administration of additional treatment during the 30-month observation period, indicating the enduring nature of the therapy. VC could be a useful predictive parameter for the recurrence of disease after GM-CSF therapy. This study contributes to the promotion of GM-CSF inhalation for initial therapy of aPAP.

WLL remains the standard of care today. A retrospective analysis of 231 cases found clinically significant improvement in Pao_2, FEV_1, VC, and DLCO and reported that the median duration of clinical benefit from lavage was 15 months.[2] In a report of 21 patients with PAP who underwent WLL in an experienced center, > 70% of patients remained free from recurrent PAP during 7-year observation.[23] In our study, the median time to application of additional therapy was 30 months after GM-CSF therapy, suggesting the effects of GM-CSF inhalation may be comparable to those of WLL. Notably, the difference in changes in $A\text{-}aDO_2$ during the GM-CSF treatment between the AT-WLL group patients and the AT-GM group patients suggests that nonresponders to the first GM-CSF treatment might be likely to undergo WLL when disease recurred.

In a single-center, phase 2 study for subcutaneous administration of GM-CSF for PAP, Venkateshiah et al[12]

Table 3—*Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups at the End of GM-CSF Inhalation Treatment and Before the 30-Mo Observation*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	93.4 ± 3.0	12	74.2 ± 4.2	.0007[a]
FVC, % predicted	23	80.5 ± 3.3	12	72.2 ± 4.5	.0025[a]
FEV_1/FVC	23	85.6 ± 1.6	12	84.7 ± 2.2	.73[a]
DLCO, % predicted	23	68.4 ± 3.4	11	46.8 ± 4.7	.0006[a]
HRCT scan scores[b]					
Upper lung region	23	2 (2-3)	12	3.5 (2-4)	.036[c]
Middle lung region	23	3 (2-3)	12	4 (2.25-4.75)	.023[c]
Lower lung region	23	2 (2-3)	12	4 (2.25-5)	.0039[c]
Serum biomarkers of PAP					
LDH, IU/L	23	242 ± 13	12	308 ± 18	.0064[a]
CEA, ng/mL	23	2.7 ± 0.6	12	5.7 ± 0.8	.0075[a]
KL-6, U/L	23	3,675 ± 735	12	6,565 ± 1,017	.028[a]
SP-A, ng/mL	23	80 ± 12	12	131 ± 16	.015[a]
SP-D, ng/mL	23	170 ± 34	12	304 ± 47	.027[a]
Hematologic indexes					
WBC count, cells/μL	23	5,213 ± 306	12	5,797 ± 424	.27[a]
Neutrophils, cells/μL	22	2,961 ± 205	12	3,026 ± 277	.85[a]
Monocytes, cells/μL	22	320 ± 30	12	338 ± 41	.74[a]
Lymphocytes, cells/μL	22	1,755 ± 131	12	2,153 ± 177	.080[a]
Eosinophils, cells/μL	22	145 ± 40	12	233 ± 55	.20[a]
Basophils, cells/μL	22	27.4 ± 5.9	12	43.7 ± 8.4	.12[a]
Hemoglobin, g/dL	23	14.8 ± 1.3	12	14.4 ± 1.4	.52[a]
Platelets, × 10^3 cells/μL	23	214 ± 9.0	12	235 ± 12	.17[a]
BALF cell classification, %					
Alveolar macrophages	13	67 ± 4.1	5	58 ± 6.7	.28[a]
Neutrophils	13	6.6 ± 2.2	5	7.4 ± 3.5	.86[a]
Eosinophils	13	0.90 ± 0.46	5	0.82 ± 0.75	.93[a]
Lymphocytes	13	25.6 ± 4.8	5	33.2 ± 7.7	.41[a]

See Table 1 and 2 legends for expansion of abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously,[18] left lung.

[c]Calculated using the Wilcoxon's rank sum test.

reported that nine of 21 patients (43%) required WLL. In a retrospective study of 12 patients who underwent aerosolized GM-CSF therapy, Wylam et al[17] reported that five of 11 responders had recurrence of disease. In four of five patients, the mean time to relapse was 6.3 months and ranged from 5.5 to 12 months.[15] It is notable that the dose of GM-CSF used in their study was twice that used in our study, although the prognosis of our cases was comparable to that of their study.

PAP is often described as a lung disorder with restrictive physiology. In the present study, 18 of 35 patients were in the normal range (≤ 80) in %FVC, whereas the other 17 patients were mildly to moderately restricted, which was comparable to previous studies.[24] Seymour et al[25] investigated 14 patients who underwent subcutaneous GM-CSF administration and suggested that higher VC before treatment was one marker to define responsiveness to GM-CSF therapy. In the present study, VC did not correlate with responsiveness to GM-CSF therapy, but it showed significant association with the requirement for additional treatment. Although limited by the small number of cases, the subgroup analyses suggested that VC is an independent factor from age, sex, baseline Pao_2, change in A-aDO_2, and baseline levels of serum markers, including anti-GM-CSF-Ab. However, there is a possibility that some clinical variables might be intrinsically related to VC. The physicians' decision for retreatment might be influenced by such clinical markers. Notably, a recent study of a series of patients with PAP followed in a reference center reported that the need for lavage was significantly associated with FVC.[26]

Reduction of VC might be due to two different factors: accumulation of surfactant-derived materials in the alveolar space and fibrotic changes of lung tissue. In a study of a quantitative CT scan analysis of patients with PAP who underwent WLL and showed improvements in %DLCO and %FVC, Perez et al[27] demonstrated that there was a reduction in lung weight

following lavage, which correlated with the dry weight of the lavage effluent. The study demonstrated a shift in the regional lung inflation toward more inflated lung with a corresponding increase in the mean lung inflation. Surfactant accumulation might be associated with an elevated ventilation-perfusion mismatch and disproportionately impaired DLCO in patients with aPAP.[2] Seymour et al[25] demonstrated serum levels of SP-A correlated with VC in 14 patients at baseline. The present study also showed that serum levels of SP-A correlated with VC at baseline as well as after treatment. However, requirement of additional therapy was not significantly associated with SP-A at baseline. Surfactant materials might be easily redistributed in alveolar spaces and may not be related to the impairment of lung tissue that might lead to additional treatment.

The other factor, fibrotic changes of lung tissue, might be maintained even after GM-CSF therapy or WLL. Pulmonary fibrosis has been reported to be associated with PAP, and exposure to oxygen or repeated WLL have been suggested as potential contributors to fibrosis. Although irreversible scarring of the lung is rarely associated with PAP, a small fraction of patients with PAP demonstrated substantially impaired %VC and rather poor prognosis. To investigate this possibility, two radiologists reevaluated baseline CT scans of 32 of the 35 participants for findings other than PAP without knowing the study results regarding responsiveness and prognosis of the GM-CSF inhalation. They only pointed out traction bronchiectasis in one patient (responder, FR), bronchiectasis in one patient (responder, FR), and multiple bullae in one patient (responder, AT). Thus, we failed to find any significant association between fibrotic change in CT scan and requirement of additional treatments. In the present study, the mean %VC levels of patients in the FR group improved from 85.9% to 93.4%, whereas those of patients in the AT group changed from 71.6% to 74.2%. The difference in improvement between the groups might be associated with the balance of surfactant accumulation and lung fibrosis in the lungs of patients.

For future studies, it would be useful to explore novel treatment regimens for patients with moderately impaired VC. As shown in this study, inhaled GM-CSF therapy did not change serum levels of anti-GM-CSF-Ab. However, the BALF titers of anti-GM-CSF-Ab were reduced in responders, which was likely due to the improved clearance in alveolar spaces. The future treatments might include a combination of GM-CSF inhalation with WLL to improve the environment of airway/alveolar spaces or with administration of rituximab to reduce the systemic production of anti-GM-CSF-Ab.

In conclusion, this study demonstrated that VC might be clinically useful in predicting the need for additional therapy in patients with aPAP who were treated with inhaled GM-CSF therapy. We believe this study contributes to improving the quality of life and treatments for patients with aPAP.

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Additional information: The e-Figures and e-Table can be found in the "Supplemental Materials" area of the online article.

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稀少肺難病との闘い—リンパ脈管筋腫症の国際共同臨床試験

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Fight Against Rare Lung Diseases
Multi - Center International Sirolimus Trial for Lymphoangioliomyomatosis

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要　旨

リンパ脈管筋腫症（Lymphangioleiomyomatosis ； LAM）は若年女性が罹り，予後不良の難病である．ラパマイシン標的蛋白質（mTOR）阻害薬のシロリムスが有効であることが，医師主導の国際的な多施設共同臨床試験（Multicenter International LAM Efficacy of Sirolimus Trial ； MILES試験）で検証され，2011年3月16日のThe New England Journal of Medicineに報告された．生命科学医療センターと第二内科呼吸器グループは，厚生労働科学研究費「臨床試験推進研究事業」費を得て，近畿中央胸部疾患センターとともに同試験に参加し，試験の成功に貢献した．参加までの経緯と結果について述べたい．

Abstract

Lymphangioleiomyomatosis （LAM） is an uncommon lung disease that affects mostly female. LAM has an insidious onset, and is typically slowly progressive. Until recently, there was no proven therapies for LAM except for lung transplantation. To assess the effect of sirolimus, a mTOR inhibitor, on biological and clinical markers of lung function and to assess the safety, a trial was designed as a multi - center international, phase III, randomized, double - blind, placebo - controlled intention to treat （MILES trial）. Respiratory disease group in The Second Department of Medicine and Bioscience Medical Research Center, Niigata University Hospital participated in the trial with Kinki Chuo Chest Medical Center and enrolled total 24 patients in Japan. On one year of oral sirolimus, patients with LAM and moderate respiratory impairment had significant but modest

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benefits on lung function and measures of quality of life or functional performance. The safety profile of the drug was acceptable and consistent with the known toxicities. Thus, Japanese research group contributed to the success of MILES trial.

Key words: Lymphangioliomyomatosis, clinical trial, FEV1.0, FVC

MILES試験に至る経緯

事の始まりは，2003年にシンシナテイ小児病院のブルーストラップネル教授とシンシナティ大学のフランクマッコーマック教授らが呼びかけて，米国保健研究所の予算で，稀少肺疾患コンソーシアムが結成されたことにある．そのメンバーに近畿中央胸部疾患センターの井上部長とともに選ばれ，肺胞蛋白症，リンパ脈管筋腫症（LAM），α1アンチトリプシン欠損症，嚢胞性線維症，家族性肺線維症の国際共同研究を進めていくこととなった（**図1**）．稀少であるが故に一国のみでは，データベースを作成したり，病因を解明したり，臨床試験を実施するのが難しくても，国際共同で患者を登録することで，集学的な研究が進むことが期待された．

LAMは若年女性に好発し，肺や腎，リンパ管などで起源不明の平滑筋様細（LAM細胞）が異常に増殖する稀少難病である．通常，肺に転移し，多発性嚢胞を形成し，徐々に進行し，呼吸不全に至る．LAMには，常染色体優性遺伝性疾患である結節性硬化症（TSC）に合併するTSC-LAMと非遺伝性の孤発性LAMがある．2003，2006年度に行われた全国疫学調査[1]では，LAM患者264例（うち女性262例）の15年の予後は76％であったが，気胸の既往がある患者が69％，現時点で在宅酸素療法（HOT）を受けている患者は36％を占め，QOLの低さが際立っている．このため，

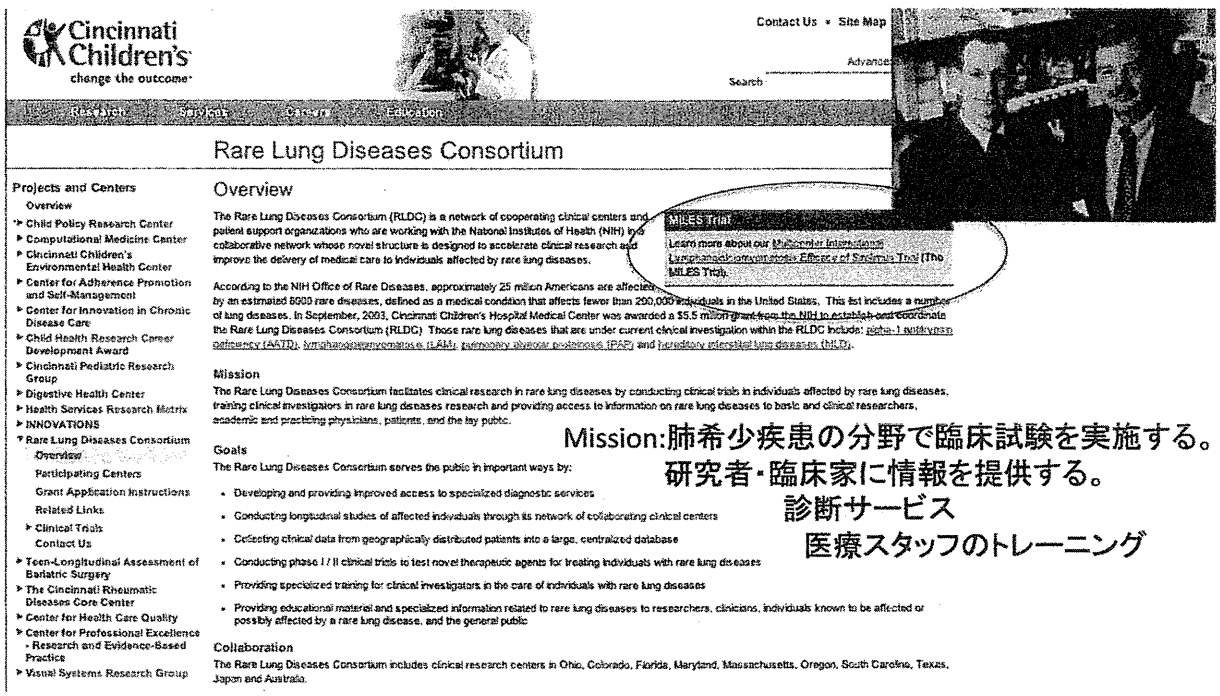

図1　2003年9月　Rare Ling Disease Consortium結成

長年患者団体が中心となり，国の助成を要望してきたが，2009年10月に特定疾患治療研究事業の対象疾患に認定され，支払った医療費に対し公費による払い戻しが受けられるようになった．しかし，有効な治療があってこそ，こうした国の助成が生かされるというものである．

一方，前世紀の終わりにLAMの病因が解明された．細胞のがん抑制遺伝子TSC－1またはTSC－2に点変異が生じることにより，下流にあるmTORの抑制が効かなくなり，その細胞が異常増殖するために発症することが明らかにされた．mTOR阻害薬のシロリムスが治療薬の候補に挙がり，シンシナティの腎臓病グループが中心となり，2003～04年に第Ⅰ・Ⅱ相試験（CAST試験）が行われた．後腹膜血管筋脂肪腫の大きさの減少を主要評価項目として，患者25例にシロリムスを1年間投与した結果，血管筋脂肪腫の大きさがMRI上で平均53.2％縮小した．驚きだったのは，期待していなかった肺一秒量や努力性肺活量の改善が見られたことである[2]．そこで，同大学のフランクマッコーマック教授らが呼びかけ，肺稀少疾患コンソーシアムのメンバーが動いて，シロリムスのLAMへの有効性と安全性を検証するためのMILES試験が計画された．

国際共同臨床試験の困難な道のり

稀少疾患でも何カ国もが協力すれば，困難な二重盲検試験が可能になる—言うは易く行うは難しで，我々には様々な困難が立ちはだかっていた．日本で稀少疾患に対する新薬の実用化を目指すには，医師主導の治験と未承認薬・適応外検討会議を通す方法があるが，どちらも数年以上を要し，治験には莫大な費用もかかる．また，治験届を申請すると医薬品医療機器総合機構からプロトコールの変更を求められるのは目に見えていた．日本と米国・カナダでは医療制度があまりに異なるからである．マッコーマック教授と協議し，日本では2施設の自主臨床研究としてMILES試験に参加し，米国では日本の臨床試験の主任研究者をマッコーマック教授が務める医師主導の治験として米食品医薬品局（FDA）に提出するという変則的な形を取ることとした．同試験で有効性と安全性を証明し，その後日本で薬事承認を目指すこととなった．

2006年にスタートしてから，患者登録まで，準備期間には約2年間を要した．プロトコルを和訳し，それをさらにバックトランスレートして米国試験本部に送り審査してもらい，米国の倫理委員会での再承認，試験スタッフの倫理教育，プロトコルトレーニングなどの過程と患者向けのMILES試験説明会を行った．2007年11月に米国試験本部からプロジェクトマネージャーが来日し，近畿と新潟の施設を監査し，スタッフに教育をした．その後，試験本部と研究費の契約交渉が開始され，2008年2月に成立した．薬剤の無償供与をしてくれるワイス社との交渉，成立を経て，2008年4月，近畿中央胸部疾患センターと新潟大学医歯学総合病院がサイトオープンした．

研究費も決して潤沢ではなかった．2007年4月－2010年3月までは，幸い厚生労働科学研究費補助金の臨床試験推進事業で外部業者とも委託契約ができたが，期限終了後は，米国の患者支援団体—LAM財団に泣きついてなんとか試験終了にこぎ着けた．LAM財団は，試験中，日本人のLAM患者が新潟と近畿に来院するときの交通費も負担してくれていた．

試験の概要

MILES試験では，18歳以上の女性のLAM患者で，中等～重症患者を対象とした．2006年12月～10年9月に111例が登録され，89例が適格とされた．シロリムスを1日2mg投与する群（46例）とプラセボを投与する群（43例）にランダムに割り付け，投与期間と観察期間に各1年間を予定した．主要評価項目は肺一秒量の1ヵ月間の変化量，副次的評価項目は，努力性肺活量の改善や肺気量，精密肺機能検査，6分間歩行距離，VEGF－D値，QOLのスコアなどの1年間の変化とした．

全例が1年間の服薬を終了する2010年9月初

旬まで試験は続行されたが，予算不足から観察期間は打ち切られ，最終解析が2010年11月に行われた．

シロリムスの効果に改めて驚く

MILES試験中から新潟に来院される患者さんの様子から，シロリムスが効いているのではないかと期待を持っていたが，一昨年の暮れにフランクマッコーマック教授からその結果を聞いたときは震えた．投与期間中の1ヵ月間のFEV1.0の変化量は，プラセボ群の−12±2mLに対し，シロリムス群は1±2mLで有意差が認められた（P＜0.001，図2）．投与期間1年間のFEV1.0の変化量は，プラセボ群に対しシロリムス群で153mL少なかった．投与期間のFVCの変化量は，プラセボ群の−129±233mLに対し，シロリムス群は97±260mLとなった（P＝0.001，図2）．ただし，肺機能が改善したのはシロリムス群の約半数にとどまった．さらに，VEGF-D値，包括的な健康関連のQOLのスコアなども，投与期間にシロリムス群で有意に改善した．控えめに評価しても，シロリムスはLAM患者の一部で肺機能の低下を遅らせる効果があるといえる．またVEGF-D値については，治療効果のバイオマーカーとなる可能性がある．

試験のもう一つの目的はシロリムスの安全性の確認だったが，皮疹，口内炎，下痢，脂質異常症などの有害事象は，プラセボ群と比べてシロリムス群で頻度が高かったが，多くは軽症であった．入院を要する重症の有害事象の頻度は，両群で有意差はなかった．ただし，海外でシロリムス群の患者1例に心膜炎が発生し，心タンポナーデに進行した（ICUで治療後回復した）．

薬事承認までのシナリオ

3月にMILES試験の結果が報道され，患者の中には，シロリムスを個人輸入する人も現れている．危惧されるのは，医師に伝えないことである．LAMではシロリムスの長期服用が必要と考えられるが，長期的な安全性のデータはなく，今後の課題である．また，MILES試験では除外された患者でも，効く可能性もある．試験とは別にシロリムスを用いたところ，劇的に改善したケースがあった．この患者は乳糜胸水の貯留で試験に参加できなかった．呼吸困難がHugh-Jones分類のV度となり，入院と胸水ドレナージ，HOTを要した．患者の強い希望でシロリムスの服用を続行すると，投与開始後約3ヵ月で胸水が徐々に減少し，呼吸困難が軽減．約8ヵ月でHOTから完全に離脱，約1年で社会復帰した．

製造販売元は，ワイス社からファイザー社へと移ったが，2011年6月，ファイザー社は日本での

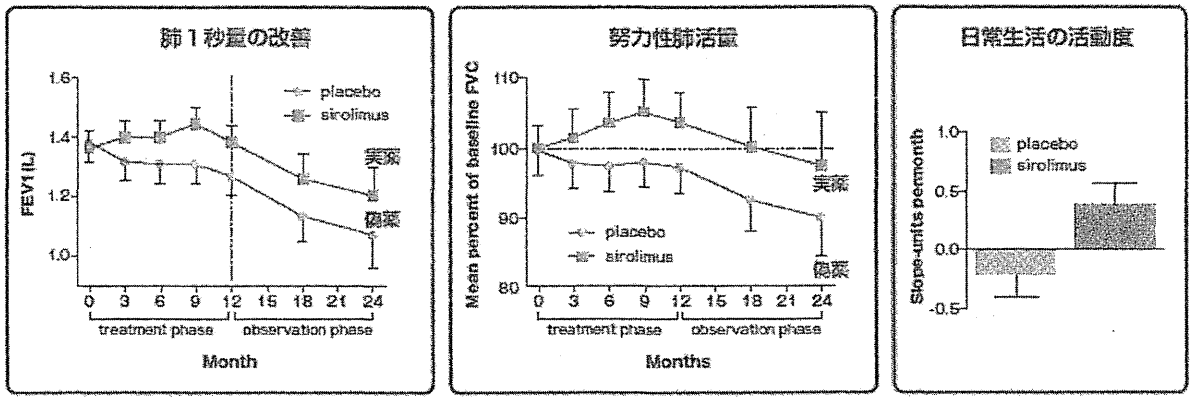

図2　MILES試験の有効性：左：肺1秒量の経時的変化　中：努力性肺活量の経時的変化
右：日常生活の活動量（QOL）の一年間の改善度　■実薬　□偽薬

薬事承認申請はしないことを決めた．その代わり，日本ではシロリムスを他社へライセンスアウトする方針を決め，ノーベルファーマ社が販売権を得た．一方，日本で多くのLAM患者が安全にシロリムスを服用できるようにするため，生命科学医療センターと第二内科呼吸器グループは医師主導治験を企画した．この企画は，2012年4月厚生労働科学研究費難治性疾患等克服研究事業に採択された．6月29日医薬品医療機器総合機構に治験届を提出し，9月5日より全国9施設共同医師主導治験が開始された．順調に進めば2013年6月に薬事承認申請を行ない，2014年3月に承認の見込みである．

文　献

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