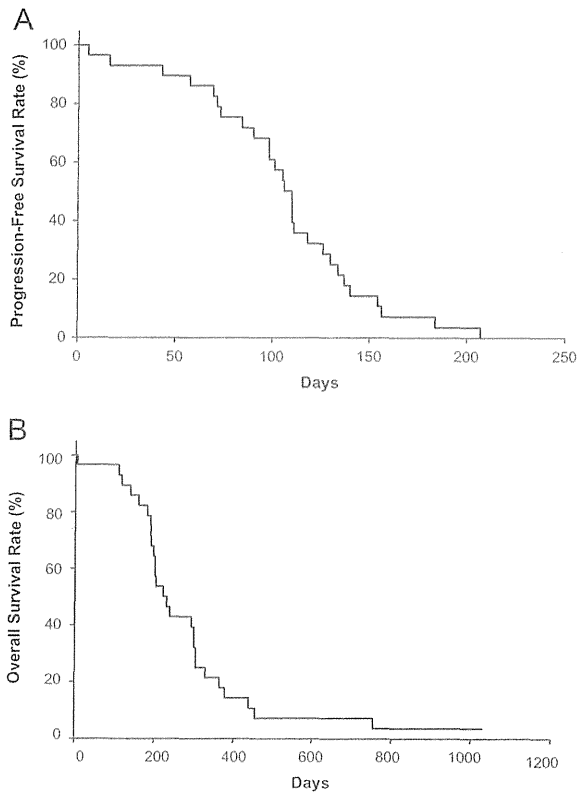

Fig. 1 – (A) Progression-free survival and (B) overall survival.

Table 3 – Toxicity profile.

Toxicity (≥ grade 2)	Grade (CTCAE)			
	Number of patients			
	2	3	4	Grade 3/4 (%)
Hematological				
Neutropenia	0	10	13	23 (79%)
Decreased hemoglobin	11	6	1	7 (24%)
Thrombocytopenia	6	4	3	7 (24%)
Febrile neutropenia	–	1	0	1 (3%)
Non-hematological				
Infection	4	2	0	2 (6%)
Nausea	2	0	0	0
Fatigue	1	0	0	0
Mucositis oral	1	0	0	1 (3%)
Stomach pain	1	0	0	0
Phlebitis	1	0	0	0
Hiccups	1	0	0	0
Pain	1	0	0	0
Interstitial lung disease	0	1	0	1 (3%)
Hyponatremia	0	2	0	2 (6%)
Hypoglycemia	0	0	1	1 (3%)

CTCAE, Common terminology criteria for adverse events.

SCLC, the combination of AMR and CBDCA is worth investigating. Contrary to our expectations, most patients in this study received sufficient cycles of platinum-doublet therapy as first-line chemotherapy. The ORR might have increased if more patients had been treated with insufficient first-line chemotherapy. According to subgroup analysis, this regimen might be suitable for patients treated with CBDCA as first-line chemotherapy. The efficacy of CBDCA plus AMR was not different in patients treated with ETP or CPT as first-line chemotherapy with platinum, which was consistent with our previous result of AMR as second-line chemotherapy [6]. Although the sample size was too small, the above-mentioned results require further validation.

In another Japanese study, even AMR alone demonstrated a quite high response rate (40%) in refractory relapsed SCLC [9], although the result might be biased due to its small sample size ($n=16$), considering the result of a subsequent larger study [7]. Other studies have used combined regimens for relapsed SCLC, some of which suggested high efficacy. However, most of those studies included both sensitive and refractory relapse patterns [4]; thus, their usefulness in refractory relapsed SCLC was unclear.

Toxicity is another important issue for such combination regimens. The above-mentioned previous regimens for relapsed SCLC were generally very toxic. For example, Kubota reported that dose-intensive CODE (CDDP, vincristine, doxorubicin, and ETP) could result in an ORR of approximately 80% in patients with refractory relapsed SCLC; however, that regimen required prophylactic G-CSF support due to severe neutropenia [10]. In contrast, AMR combined with CBDCA showed moderate toxicity in this study, which might be attributable to the dose of CBDCA being AUC 4. We reported this regimen in another study, where toxicity profiles tended to be similar and the efficacy for SCLC was sufficient (ORR was 89% as first-line treatment) [11]. Regarding the AMR dose, the current dose was one level lower than the recommended dose in our phase I and phase II studies of patients with chemotherapy-naïve SCLC because we considered that previously treated patients would be at a higher risk of myelosuppression. Although we believe this combination with the current dosage would be worth investigating in the second-line setting in terms of the risk–benefit balance, there might be scope for increasing the AMR dose to increase its efficacy.

This study has a few limitations. First, the sample size was too small to draw definite conclusions, the efficacy of this combination needs to be confirmed in a future phase III study in which the current regimen could be compared with AMR alone. Second, the drug dose might be insufficient for refractory relapsed cases. Considering that the toxicity of the current dose was moderate, there might be scope to increase the CBDCA or AMR dosage. In addition, the patients that would benefit most from the re-administration of platinum during second-line chemotherapy should be identified.

In conclusion, AMR combined with CBDCA was effective for refractory relapsed SCLC and demonstrated acceptable toxicity. Since treatment options for patients with refractory relapsed SCLC remain limited, further investigation of this regimen is warranted.

Conflict of interest

Akira Inoue received honoraria and research funding from AstraZeneca; Satoshi Oizumi received honoraria from AstraZeneca and research funding from Eli Lilly; Toshihiro Nukiwa received honoraria from Boehringer Ingelheim.

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Clinical Trial Results

Randomized Phase II Trial Comparing Carboplatin Plus Weekly Paclitaxel and Docetaxel Alone in Elderly Patients With Advanced Non-Small Cell Lung Cancer: North Japan Lung Cancer Group Trial 0801

MAKOTO MAEMONDO,[a] AKIRA INOUE,[b] SHUNICHI SUGAWARA,[c] TOSHIYUKI HARADA,[d] YUJI MINEGISHI,[e] KAZUHIRO USUI,[f] KOJI MIWA,[g] NAOTO MORIKAWA,[h] MARIKO KAMBE,[i] KENJI UBE,[j] KANA WATANABE,[a] OSAMU ISHIMOTO,[c] TOMOHIRO SAKAKIBARA,[b] AKIHIKO GEMMA,[e] TOSHIHIRO NUKIWA[k]

[a]Miyagi Cancer Center, Natori, Japan; [b]Tohoku University Hospital, Sendai, Japan; [c]Sendai Kousei Hospital, Sendai, Japan; [d]Hokkaido Social Insurance Hospital, Sapporo, Japan; [e]Nippon Medical School, Tokyo, Japan; [f]NTT Medical Center Tokyo, Tokyo, Japan; [g]Tsuboi Cancer Center Hospital, Kooriyama, Japan; [h]Iwate Medical University School of Medicine, Morioka, Japan; [i]Senseki Hospital, Higashimatsushima, Japan; [j]Iwate Prefectural Central Hospital, Morioka, Japan; [k]South Miyagi Medical Center, Miyagi, Japan

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AUTHOR SUMMARY

ABSTRACT

Background. Standard first-line chemotherapy for elderly non-small cell lung cancer (NSCLC) patients has been monotherapy with vinorelbine or gemcitabine. Docetaxel has also been considered as an alternative option for the elderly population in Japan. We have previously demonstrated the high efficacy of carboplatin plus weekly paclitaxel for elderly NSCLC patients. Consequently, we conducted a randomized phase II study to select the proper regimen for a future phase III trial.

Methods. Eligible patients were aged 70 years or older with newly diagnosed advanced NSCLC. Patients were randomly assigned either to a combination of carboplatin (area under the curve: 6 mg/mL per minute) with weekly paclitaxel (70 mg/m^2) (CP regimen) or to single-agent docetaxel (60 mg/m^2). The primary endpoint of this study was objective response rate. Secondary endpoints were progression-free survival, overall survival, and toxicity profile.

Results. Among 83 eligible patients (41 to CP, 42 to docetaxel), the objective response rates were 54% (95% confidence interval: 39%–69%) and 24% (95% confidence interval: 11%–37%) and median progression-free survival was 6.6 months and 3.5 months in the CP arm and the docetaxel arm, respectively. Severe neutropenia, febrile neutropenia, and nausea were significantly frequent in the docetaxel arm, whereas toxicities in the CP arm were generally moderate. One treatment-related death was observed in the docetaxel arm.

Conclusion. The CP regimen achieved higher activity with less toxicity than single-agent docetaxel. Considering the results of this phase II trial and the IFCT-0501 trial, we have selected the CP regimen for a future phase III trial in elderly patients with advanced NSCLC. ***The Oncologist*** 2014;19:352–353

DISCUSSION

The objective response rate (ORR) of carboplatin (area under the plasma curve: 6 mg/mL per minute) with weekly paclitaxel (70 mg/m^2) (CP regimen) met the primary endpoint of this study, achieving a higher response rate than single-agent docetaxel in this population of elderly patients with non-small cell lung cancer (NSCLC) (Fig. 1). In addition, the CP regimen achieved longer progression-free survival with less toxicity excluding moderate anemia and thrombocytopenia in comparison with docetaxel. Consequently, we have selected the CP regimen as a candidate for a future phase III trial.

Although monotherapy with third-generation agents has been regarded as the preferred treatment option for elderly patients with NSCLC [1–6], Quoix et al. recently reported the results of IFCT-0501, a phase III study comparing a similar CP regimen (carboplatin [area under the plasma curve: 6 mg/mL per minute] plus weekly paclitaxel at 90 mg/m^2) with monotherapy with either vinorelbine or gemcitabine in an elderly population [7]. IFCT-0501 demonstrated significant superiority to the CP regimen in terms of the efficacy (ORR and overall survival); however, severe toxicity in the CP arm, including a treatment-related death (TRD) rate of 4.4%, was of concern. The dose of paclitaxel in the current study was 70 mg/m^2, and this could explain the lower toxicity of CP. No TRDs have been

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Correspondence: Akira Inoue, M.D., Ph.D., Tohoku University Hospital, 1-1, Seiryocho, Aobaku, Sendai, 9808574 Japan. Telephone: 81-22-717-8539; E-Mail: akinoue@idac.tohoku.ac.jp Received December 13, 2013; accepted for publication February 12, 2014; first published online in ***The Oncologist Express*** on March 28, 2014. http://dx.doi.org/10.1634/theoncologist.2013-0411

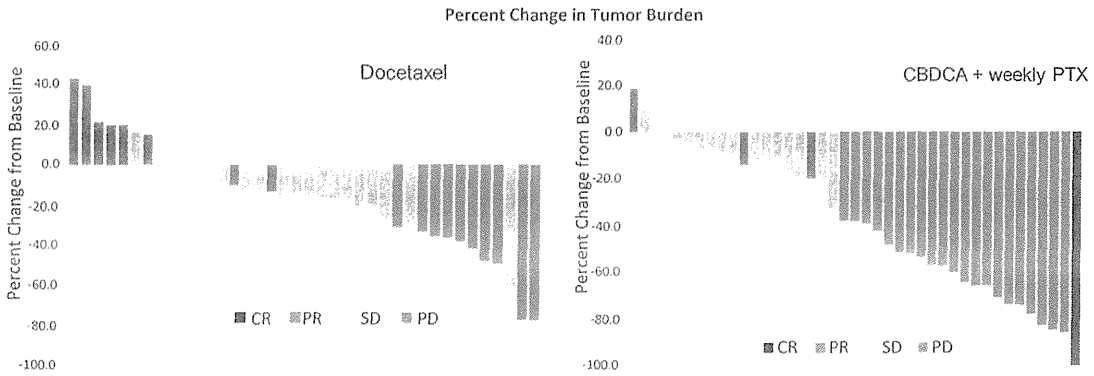

Figure 1. Waterfall plots of the docetaxel arm and the CP arm in this study.
Abbreviations: CBDCA, carboplatin; CP, carboplatin with weekly paclitaxel; CR, complete response, PD, progressive disease; PR, partial response; PTX, paclitaxel; SD, stable disease.

observed in the CP arm of this study or in our previous study using the same regimen.

Regarding the efficacy of CP, the ORR and progression-free survival in this study (54% and 6.6 months) are consistent with results achieved with the same regimen in our previous study (55% and 6.0 months) [8]. Because the evaluation of response in this study was performed by centralized review blinded as to the treatment, we believe the results were not biased. Furthermore, the ORR of the docetaxel arm in this study (24%) was quite consistent with previous results achieved with docetaxel in Japanese phase III trials with elderly NSCLC patients (23% in WJTOG9904 and 25% in JCOG0802) [6, 9]. Importantly, the rate of febrile neutropenia, an independent and poor prognostic factor in elderly NSCLC patients receiving chemotherapy, has been consistently high (>10%) in the docetaxel arm in the current study and in previous Japanese studies. In addition, one TRD was observed in the docetaxel arm in this study. All of these observations suggest that monotherapy with docetaxel might be more toxic than CP for elderly patients.

Author disclosures and references available online.

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Case report

Development of pulmonary alveolar proteinosis following exposure to dust after the Great East Japan Earthquake

Shu Hisata[a,*], *Hiroshi Moriyama*[b], *Ryushi Tazawa*[c], *Shinya Ohkouchi*[a], *Masakazu Ichinose*[a], *Masahito Ebina*[a]

[a]*Department of Respiratory Medicine, Tohoku University Graduate, School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan*

[b]*Division of Respiratory Medicine, Graduate School of Medical and Dental Sciences, Niigata University, 1-757, Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan*

[c]*Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Chuo-ku, Niigata 951-8520, Japan*

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ABSTRACT

We report a unique case of pulmonary alveolar proteinosis that developed 3 weeks after the Great East Japan Earthquake and the subsequent tsunami. The patient had inhaled dust repeatedly while visiting her devastated neighborhood without wearing a protective mask. Five weeks after the earthquake, lung samples taken from the patient showed foreign particle deposition; however, her serum was negative for GM-CSF autoantibody. The patient's clinical symptoms resolved following whole lung lavage. We conclude that inhalation of fine dust particles after natural disasters may cause the onset of pulmonary alveolar proteinosis.

1. Introduction

The Great East Japan Earthquake deeply impacted respiratory health care in the affected areas. During the acute phase of the disaster, in addition to an increase in the number of patients with "tsunami lung" caused by near-drowning, there was also an increase in the number of patients with common respiratory diseases such as pneumonia and acute exacerbations of COPD and bronchial asthma [1]. During the sub-acute phase of the disaster, patients presented with

Abbreviations: PAP, pulmonary alveolar proteinosis; GM-CSF, granulocyte macrophage colony-stimulating factor; COPD, chronic obstructive pulmonary disease; CT, computed tomography; HRCT, high-resolution computed tomography; BALF, bronchoalveolar lavage fluid; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D; CEA, carcinobembryonic antigen; TBLB, transbronchial lung biopsy

*Corresponding author. Tel.: +81 22 717 8534; fax: +81 22 717 8549.
E-mail address: hisata@idac.tohoku.ac.jp (S. Hisata).

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allergic lung inflammation, including hypersensitivity pneumonitis and organizing pneumonia. These respiratory conditions occurred not only in the victims of the tsunami but also in the workers engaged to clean up the debris, which contained rubble from buildings and industrial waste material from the sea [2].

Previous studies have shown that dust exposure may be related to the pathogenesis of PAP. Exposure to dust has been reported as a cause of secondary PAP, in which serum GM-CSF autoantibodies are considered negative, but this has not been fully confirmed [3]. Further, recent studies have raised the hypothesis that an inhaled agent may instead be the trigger of the development of autoimmune PAP, characterized by positive GM-CSF autoantibodies [4]. To our knowledge, no increase in the incidence of PAP after natural disasters or the World Trade Center attacks has been reported [5]. However, specific materials contained in the debris from disasters can induce PAP

Here, we report a case of PAP that developed after exposure to dust following the Great East Japan Earthquake.

2. Case presentation

A 63-year-old Japanese woman was referred to our institute for worsening lung infiltrates, dyspnea, and hypoxia. She had never smoked and had a past history of hypertension.

Although she was not otherwise injured her house was completely destroyed in the large tsunami triggered by the Great East Japan Earthquake on March 11 of 2011. After the earthquake, she repeatedly retrieved personal effects from the rubble without wearing a mask. Since large amounts of sludge and burned embers were scattered throughout the area, she was exposed to various kinds of inhaled dust. Three weeks after the earthquake, she developed dry cough and her chest X-ray showed bilateral reticular shadows (Fig. 1Aa). The computed tomography (CT) of her chest showed diffusely-distributed ground glass opacity in the subpleural area (Fig. 1Ab). At the previous hospital, analysis of her bronchoalveolar lavage fluid (BALF) revealed lymphocytosis (lymphocytes: 89.0%, CD4/CD8: 3.6) without turbidity and a transbronchial lung biopsy (TBLB) did not indicate PAP. However, upon re-evaluation, we detected particles within the lung (Fig. 2A), and an electron probe X-ray microanalysis revealed the deposition of silicon, oxygen, and aluminum, while other specific elements were not detected (data not shown). On clinical suspicion of idiopathic interstitial pneumonias, she was treated with prednisolone, cyclosporin, and methylprednisolone pulse therapy. Eight months later, she was referred to our University Hospital as the treatment was not fully effective.

On admission, her blood pressure was 137/98 mm Hg; pulse, 105 beats/min; and body temperature, 36.8 °C. Chest examination revealed slight bilateral inspiratory crackles, and a chest X-ray showed a significant loss of lung volume (Fig. 1Ba). High-resolution computed tomography (HRCT) of the chest showed diffuse ground-glass opacities with superimposed interlobular septal thickening and intralobular lines (Fig. 1Bb). Five weeks after admission, pulmonary function testing showed that the patient had a severe, restrictive pattern (vital capacity, 1.17L; 52.7% predicted) with reduced

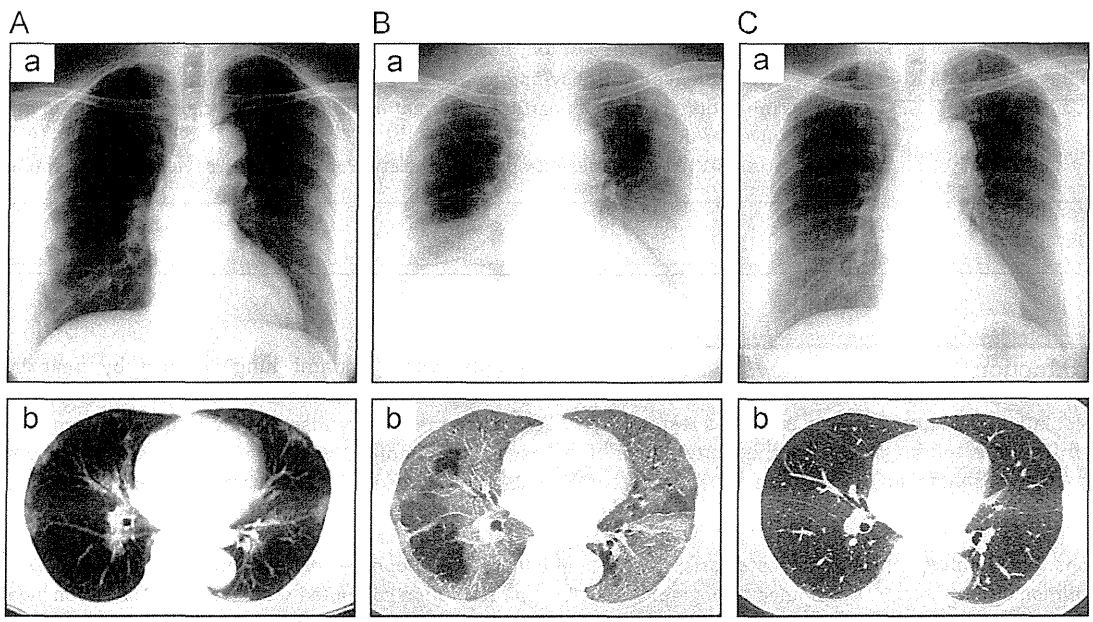

Fig. 1 – X-ray (a) and computed tomography (b) of the chest (A) at the initial visit in the previous hospital (April 2011), showing limited ground glass opacity in the subpleural area; (B) at 3 weeks after admission to our hospital (January 2012), showing significant loss of lung volume and a wide range of ground-glass opacities with superimposed interlobular septal thickening and intralobular lines; (C) at 6 months after the whole lung lavage (August 2012), showing improvements in the lung volume and ground glass opacities.

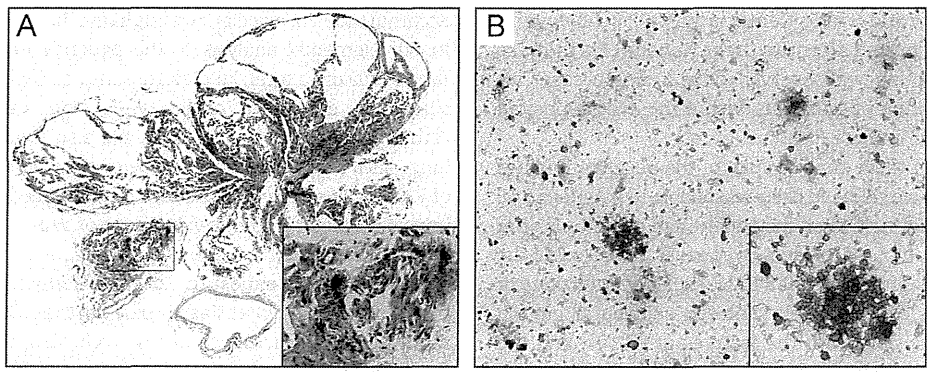

Fig. 2 – Pathological findings. (A) Hematoxylin–Eosin staining of patient's lung tissue obtained in April 2011, by transbronchial lung biopsy at the previous hospital, showing dark brown particles without typical findings as protein alveolar proteinosis (×20); lower right box: partial enlargement of black box (×200). (B) Appearance of bronchoalveolar lavage fluid obtained in January 2012 at our hospital, showing large foamy macrophages and amorphous materials (×100); lower right box: enlargement of a foamy macrophage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

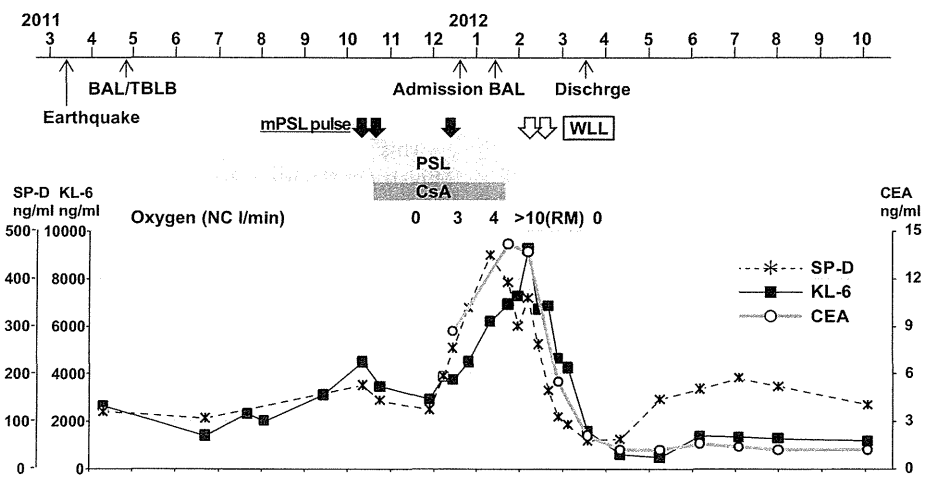

Fig. 3 – The patient's clinical course is outlined and clearly shows improving clinical parameters following admission to our hospital and the whole lung lavage procedure. WLL: whole lung lavage; PSL: prednisolone; mPSL: methylprednisolone; CsA: cyclosporin A; NC: nasal cannula; RM: reservoir face mask. Cut off: SP-D < 109 ng/mL, KL-6 < 435 U/mL, and CEA < 5.0 ng/mL.

carbon monoxide diffusing capacity (38.2% predicted). The patient's serum levels of Krebs von den Lungen-6 (KL-6) and surfactant protein D (SP-D) were elevated to 3752 U/mL and 254 ng/mL, respectively (Supplementary Table S1).

At first, steroid and immunosuppressive therapy had been continued, because an acute exacerbation of interstitial pneumonia could not be excluded. However, the patient was re-evaluated in response to the HRCT imaging as described above. Her BALF showed a milky appearance with large foamy macrophages and amorphous materials (Fig. 2B). TBLB could not be performed owing to severe hypoxia. Her serum was negative for GM-CSF autoantibody.

Under the diagnosis of PAP, the doses of prednisolone and cyclosporine were gradually decreased. Even after the discontinuation of these agents, her hypoxia progressed at a relatively rapid rate, and 5 days before whole lung lavage, her alveolar–arterial oxygen gradient was 71.8 Torr. Under extracorporeal membrane oxygenation, she underwent bilateral whole lung lavage at 2-week intervals (Fig. 3). Subsequently, her hypoxia, infiltration observed on HRCT, and levels of serum biomarkers (KL-6, SP-D, CEA) dramatically improved (Fig. 1Ca, Cb; Fig. 3). After 1 year, despite having slightly elevated levels of serum biomarker, she had no further respiratory symptoms.

3. Discussion

In this report, we describe a case of PAP that developed after the tsunami triggered by the Great East Japan Earthquake. To the best of our knowledge, no cases of PAP have been reported in association with other major tsunami disasters [6]

or following the World Trade Center attacks [5]; however a case of PAP was reported after the Great Hanshin Earthquake in Japan [7]. Although we observed only a single case, we believe that inhalation of materials within the dust deposited by the tsunami can induce PAP.

In this case, we believe that, in spite of the negative findings of bronchoscopy, it would have been reasonable to diagnose PAP at the patient's initial visit. First, the patient had symptoms after exposure to large amounts of dust over several weeks. At the initial visit, analysis of the patient's BALF excluded infectious or malignant diseases, the CT-image showed subpleural ground glass opacities, consistent with the features of PAP [8] and the infiltrates continued to expand despite steroid therapy. Second, 5 weeks after the tsunami her lung-specimen showed plenty of deposits. Since she had never smoked, the presence of these particles suggested that she had been exposed to large amounts of dust. The clinical symptoms resolved almost entirely after whole lung lavage therapy was performed. After admission to our hospital, the sub-acute exacerbation could have been precipitated by the prednisolone or cyclosporin treatment, as has been previously reported [9].

Previous studies have shown that secondary PAP can occur as a consequence of underlying conditions (such as hematologic or autoimmune diseases), infections, or exposure to inhaled dust including silica, titanium, aluminum, cement, and tin [3]. However, the presence of serum GM-CSF autoantibodies was not confirmed in most of the reported cases. In a large cohort of Japanese patients, secondary PAP without GM-CSF autoantibodies was limited to those with hematologic or autoimmune comorbidities [10]. In contrast, 26% of patients with autoimmune PAP had a history of dust exposure [11]. Furthermore, a case of autoimmune PAP associated with exposure to indium-tin oxide has been reported [12]. These studies have raised the hypothesis that an inhaled agent may be the trigger for the development of autoimmune PAP [4]. In the case presented here, the serum level of GM-CSF autoantibodies barely exceeded the cutoff (0.9 μg/mL in December 2012, cut off <0.5 μg/mL) 1 year after the initial visit to our hospital, while autoantibodies could not be detected in the active phase of PAP (Supplementary Table S2). *In vitro* studies revealed that the serum from this patient had an inhibitory effect on GM-CSF signaling (Supplementary Fig. S1). Accordingly, in this case we propose that a low titer of GM-CSF autoantibodies might be associated with the development of PAP. However, there are limitations to this interpretation. First, our *in vitro* studies cannot clarify whether this inhibitory effect on GM-CSF signaling is due to the presence of GM-CSF autoantibodies. We cannot exclude the possibility that other factors in the serum inhibited GM-CSF signaling and thus caused PAP. Second, during the active phase of PAP the patient was negative for GM-CSF autoantibody, and as such there is a discrepancy between the titer of autoantibody and the activity of PAP. The level of serum immunoglobulin G decreased to 383 mg/dL at the initial visit to our hospital (Supplementary Table S2), suggesting that large amounts of steroid and immunosuppressive agents could lower the level of autoantibodies. Furthermore, the effect of immunosuppression on the pathogenesis of PAP has not been determined.

The contribution of element comprising particle deposition within the lung to the development of PAP is not fully understood, although an association between PAP and iron accumulation in alveolar macrophages has been reported [13]. In our elemental analysis of the patient's lung specimen, the ratio of silicon, oxygen, and aluminum was high, which indicates silica and aluminum oxide, while iron was also detected. Although silicon and aluminum are also found in the normal lung, silica and aluminum have been reported to cause the onset of PAP [3]. Therefore, the amount of an inhaled agent may be an important factor in the development of PAP.

In conclusion, we present a unique case of PAP that developed after exposure to dust following the tsunami triggered by the Great East Japan Earthquake. In the future, monitoring the incidence of PAP following disasters, as well as assessing the air for hazardous substances in affected areas, will be required.

Conflict of interest

The authors have no potential conflict of interest related to the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.resinv.2013.04.005.

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Light chain (κ/λ) ratio of GM-CSF autoantibodies is associated with disease severity in autoimmune pulmonary alveolar proteinosis☆☆

Takahito Nei[a,b], Shinya Urano[a], Yuko Itoh[a], Nobutaka Kitamura[c], Atsushi Hashimoto[a], Takahiro Tanaka[a], Natsuki Motoi[a], Chinatsu Kaneko[a], Ryushi Tazawa[a], Kazuhide Nakagaki[d], Toru Arai[e], Yoshikazu Inoue[e], Koh Nakata[a,*]

[a] *Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata, Japan*
[b] *Department of Respiratory Medicine, Nippon Medical School, Tokyo, Japan*
[c] *Department of Medical Informatics, Niigata University Medical and Dental Hospital, Niigata, Japan*
[d] *Laboratory of Infectious Diseases and Immunology, College of Veterinary Medicine, Nippon Veterinary and Life Science University, Tokyo, Japan*
[e] *Department of Diffuse Lung Diseases and Respiratory Failure, National Hospital Organization Kinki-Chuo Chest Medical Center, Japan*

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Abstract Previous studies demonstrated that antigranulocyte colony-stimulating factor autoantibody (GMAb) was consistently present in patients with autoimmune pulmonary alveolar proteinosis (aPAP), and, thus, represented candidature as a reliable diagnostic marker. However, our large cohort study suggested that the concentration of this antibody was not correlated with disease severity in patients. We found that the κ/λ ratio of GMAb was significantly correlated with the degree of hypoxemia. The proportion of λ-type GMAb per total

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* Corresponding author at: Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Niigata 951-8520, Japan. Fax: +81 25 227 0377.
E-mail addresses: takahitonei@gmail.com (T. Nei), hoshisagashi@yahoo.co.jp (S. Urano), y-itoh@med.niigata-u.ac.jp (Y. Itoh), nktmr@m12.alpha-net.ne.jp (N. Kitamura), noiredge2007@yahoo.co.jp (A. Hashimoto), belltatnk@gmail.com (T. Tanaka), natsumo87@gmail.com (N. Motoi), kanegon0719@gmail.com (C. Kaneko), ryushi@med.niigata-u.ac.jp (R. Tazawa), nakagaki@nvlu.ac.jp (K. Nakagaki), to-arai@kch.hosp.go.jp (T. Arai), GlichiYi@aol.com (Y. Inoue), radical@med.niigata-u.ac.jp (K. Nakata).

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λ-type IgG was significantly higher in severely affected patients than those in mildly affected patients, but the proportion of κ-type was unchanged. The κ/λ ratio was significantly correlated with both KL-6 and SP-D, which have been previously reported as disease severity markers. Thus, the light chain isotype usage of GMAb may not only be associated with the severity of aPAP, but may also represent a useful disease severity marker.

1. Introduction

Detection of granulocyte macrophage-colony stimulating factor (GM-CSF) autoantibody (GMAb) is known to be an excellent test with almost 100% sensitivity and specificity for the serological diagnosis of autoimmune pulmonary alveolar proteinosis (aPAP), which comprises 90% of all acquired PAP cases. This indicates its potential value in the routine clinical diagnosis of the disease; however, the test has neither approval nor commercial availability for clinical use [1]. On the other hand, our previous report demonstrated that serum GMAb levels were not correlated with the degree of hypoxemia [2] (according to disease severity score; partial pressure of oxygen in arterial blood, PaO_2; and alveolar–arterial oxygen difference, $AaDO_2$), but were moderately correlated with serum surfactant protein-A, -D, Krebs von den Lungen (KL)-6, and carcinoembryonic antigen (CEA) levels [1]. Because the autoantibodies were polyclonal, thereby recognizing multiple target epitopes on GM-CSF molecules with variable binding avidity, the loss of GM-CSF bioactivity in the lungs of patients with aPAP was thought to be affected not only by the concentration but also by multiple properties of GMAb such as binding avidity, neutralizing capacity, or targeting epitope. Thus, no characteristic correlation has been demonstrated between the properties of GMAb and the degree of hypoxemia.

Each B lymphocyte expresses only one isotype of light chain, κ or λ, which remains fixed for the life of the B lymphocyte. While immunoglobulin synthesis is matured and continually stimulated, the λ chain immunoglobulin concentration reaches a plateau by one year after birth and is maintained throughout the child's life [3]. On the other hand, the concentration of the κ chain, which increases gradually until 20 years of age, reflects the concentration of immunoglobulins as a whole [3,4]. The κ/λ ratio of immunoglobulin in normal adults ranges from 0.85 to 1.86 [5,6], from which it then becomes divergent in patients with monoclonal gammopathy or some autoimmune diseases. Studies suggest a selective preference for either κ- or λ-light chains in autoantibody formation, such as rheumatoid factor (RF) [7,8], anti-cardiolipin antibodies [9], anti-neutrophil antibodies [10], several thyroid-stimulating antibodies [11,12], anti-lamin B antibody [13], and circulating immune complexes in juvenile idiopathic arthritis [14]. Thus, measuring κ/λ ratios in some autoantibodies may be useful to identify the state of activation of B cells involved in some autoimmune diseases. Studies suggest selective preference for either κ or λ chains in autoantibody formation.

During a previous study on characterization of GMAb in patients with aPAP in comparison to pharmaceutical immunoglobulin (IVIG), which was produced from pooled normal sera of more than 1000 normal subjects, we measured concentrations, binding avidities, and κ/λ ratios of GMAb. We noticed that the κ/λ ratio was much higher for GMAb than for whole IgG in both IVIG and aPAP groups, but it decreased as the disease severity of aPAP increased. The aim of this study was to assess the potential use of the κ/λ ratio as a disease severity marker in aPAP. In addition, we discuss the possibility that a selective preference in light chain isotype usage might be associated with the pathogenesis of aPAP.

2. Materials and methods

2.1. Subjects

Forty-six patients with aPAP were enrolled in this study. All patients were diagnosed with PAP by computed tomography findings and lung biopsy or bronchoalveolar lavage findings, and diagnosis was confirmed by the existence of GM-CSF autoantibodies in sera according to the diagnostic criteria (http://www.pap-guide.jp/en/). The median age, gender, proportion of symptomatic individuals, mean arterial blood oxygen pressure, and mean percent vital capacity were comparable to those in our previous large cohort study [2]. All serum and plasma samples were gathered in our institution to measure the level of GM-CSF autoantibodies after written informed consent to collect samples. All participants provided written informed consent; minors provided consent in accordance with the Declaration of Helsinki. Healthy volunteers were also enrolled into the study as healthy subjects (HS) after agreement with written informed consent. All patients with aPAP were categorized by disease severity score (DSS) at enrollment, as previously described [2], from least severe (DSS-1) to most severe (DSS-5).

2.2. Pharmaceutical immunoglobulin (IVIG)

Eight different batches of pharmaceutically-prepared immunoglobulin, Venoglobulin-IH™, were kindly provided by Mitsubishi Pharma Corporation (Tokyo, Japan).

2.3. Enzyme-linked immunosorbent assay (ELISA)

2.3.1. Whole IgG

The serum concentration of whole IgG was measured by using a human IgG ELISA quantitation kit (Bethyl, Montgomery, TX, USA) according to the manufacturer's instructions.

2.4. GM-CSF autoantibody

Serum and culture medium GM-CSF autoantibody levels were measured using direct ELISA as previously reported [15–17]. In brief, micro-ELISA plates (Maxisorp™ flat-bottom, clear, 96-well plates; Nunc, Roskilde, Denmark) were coated with

recombinant human (rh) GM-CSF produced in *Saccharomyces cerevisiae* (Leukine™, sarglamostim; Genzyme, Boston, MA, USA) at 2 μg/ml with phosphate-buffered saline (PBS) at 4 °C overnight. After washing with PBS containing 0.1% Tween™-T (0.1% PBS-T) (MP Biomedicals, Solon, OH, USA), plates were stabilized with blocking solution (Stabilcoat™, SurModics, Eden Prairie, MN, USA) for 1 h at room temperature. Standard human monoclonal GM-CSF autoantibodies with λ light chain isotypes were kindly provided by Dr. Kenzo Takada (Evec Co. Ltd., Sapporo, Japan). Standard GM-CSF autoantibody was diluted in PBS containing 1% bovine serum albumin (1% BSA/PBS) and was used as a standard for antibody measurement. Serum and plasma samples were diluted into 1% BSA/PBS at a 3000-fold dilution, and a volume of 50 μl of prepared samples and standard was transferred to plates. After keeping plates at room temperature for 1 h and washing with 0.1% PBS-T, autoantibodies captured by rhGM-CSF were detected by peroxidase-labeled anti-human Fcγ, Fcμ, or Fcα antibody (Dako Corporation, Carpinteria, CA, USA). After washing, color was developed using tetramethylbenzidine (TMB) solution, and the absorbance was measured at 450 nm.

2.5. Light chain assay

Based on the same ELISA method as above, peroxidase-conjugated anti-human lambda light chain antibody (Bethyl) was used for antibody detection. As the human monoclonal antibody had λ-chains, we used this as the standard for λ-type GMAb. The κ-type GMAb level was calculated by subtraction of each λ-type GMAb level from the GMAb level.

2.6. Binding avidity

The rhGM-CSF (Leukine®) was dialyzed against PBS (pH 7.4) and biotinylated using the NHS-PEO-biotin kit and monomeric avidin kit (Pierce Biotech, Rockford, IL, USA) according to the manufacturer's instructions. The purity of biotinylated GM-CSF (bGM-CSF) was almost 100%. For estimation of binding avidity, the serum GMAb concentration was fixed at 0.685 nM (100 ng/ml) in every assay and was incubated with various concentrations of bGM-CSF (0–1.25 nM) at 4 °C for 1 h and then transferred into a 96-well plate, which was previously coated with 1 μg/ml of anti-human IgG (Bethyl). The bound bGM-CSF was reacted with streptavidin-peroxidase. Then, the reactant was colored with TMB solution, and the OD (at 450 nm) was measured. Based on Lineweaver–Burk plots of the concentration of bGM-CSF and OD values, the average binding dissociation constant (Kd) was determined from the concentration of bGM-CSF at 50% of the maximal OD value.

2.7. Statistical analysis

Numerical data were evaluated for a normal distribution using Shapiro–Wilk tests and for equal variance using Levine median tests. Parametric data are presented as means (±SE), and non-parametric data are presented as medians and ranges. Nonparametric data were compared with the use of the Mann–Whitney *U* test (for two groups, non-paired), or Wilcoxon signed-rank test (for two groups, paired). Correlation coefficients were obtained using the Spearman's correlation method. All tests were two-sided, and *P*-values < 0.05 were considered to indicate statistical significance. Data were analyzed by using JMP (8.0.1) software (SAS, Cary, NC). Figures were made by Stat View (v. 5.0) (SAS, Cary, NC) and Microsoft Power Point (Microsoft, Seattle, WA).

3. Results

3.1. Demographic features of the study subjects

Forty-six patients with positive serum GMAb and pathologically-proven PAP were enrolled in this study. The demographic features, including age, gender, symptoms, arterial blood gas analysis, pulmonary function tests (percent vital capacity, %VC; percent forced expiratory volume in one second, $\%FEV_{1.0}$, and, percent diffusing capacity for carbon monoxide, $\%DL_{CO}$), DSS, and serum markers (KL-6 and SP-D) (Table 1) in this group were similar to those of the 223 participants in our previous cross-sectional, large cohort study [2], indicating that patients in the present study had similar backgrounds to the general demographic features of aPAP in Japan. The median PaO_2 was 67.5 mm Hg, and the numbers of patients in each DSS were 11, 6, 18, 8, and 3 for DSS 1 to 5, respectively, with a mean score of 2.7 ± 1.2.

3.2. Association of GMAb concentrations and binding avidities with disease severity

To assess the correlation of GMAb properties with disease severity, we first investigated the concentration and its proportion to whole IgG in the sera of patients with aPAP as compared to IVIG. As shown in Fig. 1A, the concentrations of IgG isotype GMAb were higher than 3.0 μg/ml in all patients, with a mean value of 46.7 ± 34.1 μg/ml, whereas the levels were less than 3.0 μg/ml for IVIG (2.21 ± 0.38 μg/ml, n = 8). The mean percentage of GMAb per total IgG was 21.7-fold higher in aPAP than in IVIG ($P < 0.0001$, Fig. 1B). "Both the concentrations and the percentages did not correlate with the degree of hypoxemia (i.e., PaO_2, $AaDO_2$, and DSS) (Supplemental Table 1).

Thus, we confirmed our previous results in a large cohort study [2] using a different set of samples in this study.

Then, we evaluated the correlation of binding avidity with disease severity. As the concentration of GMAb increased, the avidity to recombinant human GM-CSF was remarkably variable among the patients, with a mean value of 0.48 nM (Fig. 1C). The mean avidity value was higher in IVIG than those in aPAP patients ($P < 0.05$). As the binding avidity of antibodies is generally thought to reflect the frequency and intensity of ligand stimulation, we considered that the avidity of GMAb in aPAP patients might be associated with disease severity. However, the avidity correlated with none of the parameters regarding the degree of hypoxemia (Supplemental Table 1).

Our previous study demonstrated that serum biomarkers KL-6, SP-D, and pulmonary functions correlated with disease severity [2]. Similarly, we confirmed these results using the present data set. Both KL-6 and SP-D were correlated moderately to strongly with the degree of hypoxemia as shown in Supplemental Table 1. Of pulmonary function data, percent vital capacity (%VC) and percent diffusing capacity

Table 1 Demographic data of study subjects.

Characteristics	n	%	Mean ± SD (minimum–maximum)
Number	46		
Age (years)			50.5 ± 14.9 (16–85)
Gender			
Male	29	63	
Female	17	37	
Clinical symptoms			
None	12	26	
Dyspnea on effort	25	54	
Cough	18	39	
Others	3	7	
Arterial blood gas analysis			
PaO_2 (Torr)	46		69.3 ± 13.8 (40.6–99.7)
$PaCO_2$ (Torr)	46		38.4 ± 4.3 (28.6–47.5)
$AaDO_2$ (Torr) [a]	46		33.1 ± 14.7 (2.6–60.2)
Pulmonary function test			
%VC	38		90.4 ± 14.9 (55.6–116.9)
$\%FEV_{1.0}$	35		83.3 ± 10.3 (67.8–126.5)
$\%DL_{CO}$	30		61.2 ± 23.4 (8.4–99.7)
Disease severity score	46		2.5 ± 1.1 (1–5)
Serum markers			
KL-6 (U/ml)	45		4431 ± 4707 (147–23,000)
SP-D (ng/ml)	45		184.4 ± 129.4 (17.3–498)

Definition of abbreviations: $AaDO_2$ = alveolar–arterial oxygen difference; GM-CSF = granulocyte/macrophage-colony stimulating factor; NS = not significant.

P_B; barometric pressure measured by local observatories, P_{H2O}; partial pressure of water vapor in inspired air (assumed to be 47 Torr), F_IO_2; fractional concentration of oxygen in dry gas (assumed to be 0.21), $PaCO_2$; partial pressure of arterial CO_2 measured in arterial blood, R; respiratory quotient (assumed to be 0.8), PaO_2; partial pressure of arterial oxygen measured in arterial blood.

The serum concentration of GM-CSF autoantibody with λ isotype (Igλ-GMAb) was measured by GMAb ELISA(2) using recombinant GM-CSF (Leukine™, Immunex Co., WA) for antigen capture and peroxidase labeled anti-lambda antibody (Bethyl Laboratories Inc., Montgomery, TX) for detecting antibody. The serum concentration of whole GM-CSF autoantibody was measured using peroxidase labeled anti-whole immunoglobulin antibody as the detecting antibody. For the standard, monoclonal human GM-CSF antibody (kindly provided from Dr. Kenzo Takada, Evec Co. Ltd., Sapporo, Japan) with lambda isotype was used. Kappa/lambda ratio was calculated by the following equation.

kappa/lambda ratio = (whole immunoglobulin concentration − Igλ-GMAb) / Igλ-GMAb.

[a] Calculated using the following equation: $AaDO_2 = (P_B - P_{H2O}) \times F_IO_2 / R + \{PaCO_2 \times F_IO_2 \times (1 - R) / R\} - PaO_2$.

of carbon monoxide ($\%DL_{CO}$) demonstrated moderate correlations with the degree of hypoxemia.

3.3. Correlation between κ/λ ratios of GMAb and disease severity

Another important property of GMAb that might affect the blocking capacity of GM-CSF bioactivity is the binding of paratopes, consisting of some parts of variable regions in both light and heavy chains. As the light chain κ/λ ratio is known to change in some autoimmune diseases, we focused on the ratio of GMAb in aPAP patients as compared to IVIG. The mean κ/λ ratio in total IgG was similar between aPAP patients and IVIG with values of 1.23 ± 0.60 and 1.41 ± 0.27, respectively (Fig. 2A), which were comparable to the data in normal subjects reported previously [5,6]. On the other hand, the mean κ/λ ratio of GMAb was higher in IVIG (3.60 ± 0.71) than in aPAP patients (2.12 ± 1.37) ($P < 0.01$, Fig. 2B). The κ/λ ratio of GMAb was strikingly higher in both aPAP patients and IVIG than the κ/λ ratio of the corresponding total IgG (Fig. 2C, $P < 0.001$ and $P < 0.05$, respectively). The κ/λ ratio of GMAb in patients was highly variable, ranging from 0.002 to 5.75 as compared with total IgG (Fig. 2C), suggesting that GMAb-producing B cells or plasma cells are polyclonally activated. The κ/λ ratio of GMAb was significantly higher than the ratio of the total IgG ($P < 0.001$).

When we evaluated the correlation between the κ/λ ratio and the disease severity parameters in aPAP patients, we found that the κ/λ ratio was moderately correlated with PaO_2 (Fig. 3A, $\rho = 0.411$, $P < 0.01$) and inversely and moderately correlated with both $AaDO_2$ (Fig. 3B, $\rho = -0.484$, $P < 0.001$) and DSS (Fig. 3C, $\rho = -0.378$, $P < 0.01$). When the κ-type or λ-type GMAbs were compared between patients with mild and severe aPAP (PaO_2 higher and lower than 67.5 mm Hg, respectively), the κ/λ ratio of GMAb was significantly higher in the former group than the latter (Fig. 4A, $P < 0.05$). Interestingly, the proportion of λ-type GMAb per whole λ-type IgG was significantly higher in the latter group than the former, whereas the proportion of κ-type GMAb per total κ-type IgG was equal between the two groups (Figs. 4B ($P < 0.05$) and 4C). This result suggested that λ-type GMAb predominantly increased in the severe aPAP.

3.4. Correlation between κ/λ ratio of GMAb and other parameters

As both serum markers and pulmonary functions are widely accepted as disease severity parameters, we evaluated whether serum κ/λ ratio correlated with these parameters. The κ/λ ratio weakly to moderately correlated with KL-6 ($\rho = -0.297$, $P < 0.05$) and SP-D ($\rho = -0.360$, $P < 0.05$), but not with %VC and $\%DL_{CO}$ (Supplemental Table 2). Neither the concentrations nor binding avidities of GMAb correlated with the disease severity or the κ/λ ratio of GMAb (Supplemental Table 2). Then, the patients were classified into two groups; group 1: patients with high binding avidity less than 0.24 nM (the mean + 2SD for IVIG) and group 2: patients with low binding avidity over 0.24 nM. In group 1, the serum κ/λ ratio was similar between severe (<67.5 mm Hg in PaO_2) and mild (≧67.5 mm Hg in PaO_2) disease, whereas, in group 2, the κ/λ ratio was higher in mild patients (2.85 + 1.60) than severe (1.43 + 1.08) patients ($P < 0.05$, Fig. 4D). Thus, the association of serum κ/λ ratio with disease severity was obvious in patients with low avidity GMAb but not in patients with high avidity GMAb.

4. Discussion

In this study, we demonstrated that the light-chain ratio (κ/λ ratio) of GMAb in patients with aPAP and IVIG was higher than total IgG, and the κ/λ ratio was significantly correlated with

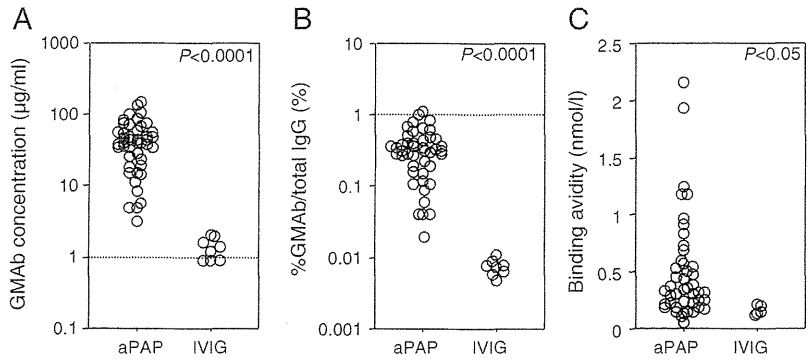

Figure 1 (A) Comparison of GMAb between patients with aPAP (n = 46) and IVIG preparations (n = 8). GMAb concentrations were significantly higher in aPAP patients than IVIG preparations ($P < 0.0001$, Mann–Whitney *U*-test). (B) Comparison of the percent GMAb per total IgG between patients with aPAP (n = 46) and IVIG preparations (n = 8). Percent GMAb per total IgG was significantly higher in the aPAP group than IVIG ($P < 0.0001$, Mann–Whitney *U*-test). (C) Comparison of the binding avidity of GMAb between patients with aPAP and IVIG preparations. The binding avidity of GMAb was significantly higher in IVIG than that of the aPAP group ($P < 0.05$, Mann–Whitney *U*-test).

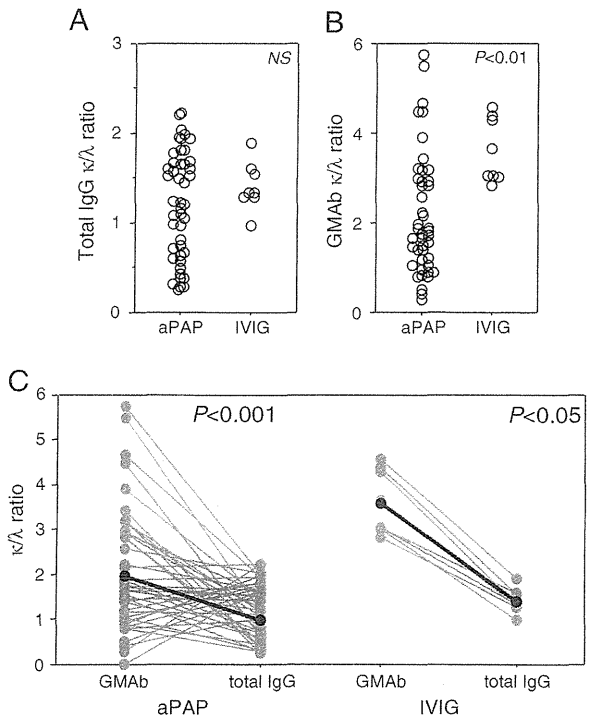

Figure 2 Comparison of the κ/λ ratio of total IgG (A) and GMAb (B) between aPAP patients and IVIG preparations. The mean κ/λ ratio in total IgG was similar between aPAP patients and IVIG preparations, whereas the κ/λ ratio for GMAbs was significantly higher in IVIG as compared to aPAP patients ($P < 0.05$, Mann–Whitney *U*-test). (C) Comparison of the κ/λ ratio as compared between GMAb and total IgG for each individual sample (gray lines) and the average (black line) in aPAP patients (left panel) and IVIG preparations (right panel). For both aPAP patients and IVIG preparations, the κ/λ ratio of GMAb was significantly higher than total IgG ($P < 0.001$ and $P < 0.05$, respectively, Wilcoxon signed-rank test).

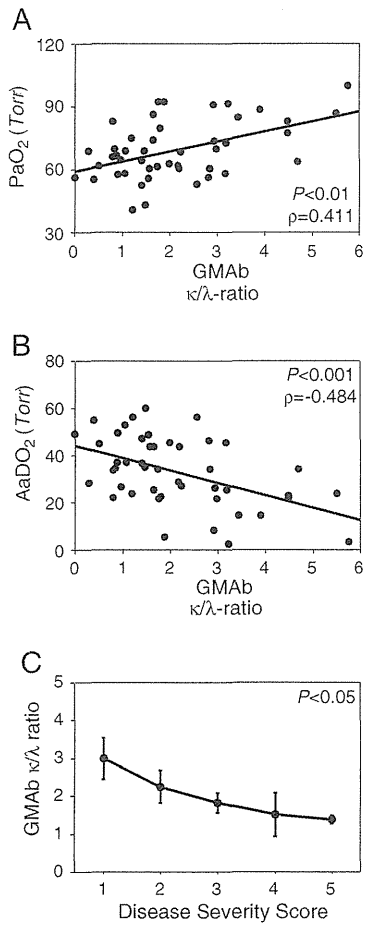

Figure 3 Correlations between the κ/λ ratio of GMAb and clinical parameters: (A) PaO_2 and (B) $AaDO_2$. Each combination was highly correlated ($P < 0.01$, $R = 0.411$; $P < 0.001$, $R = -0.484$, respectively). All data were evaluated by using Spearman's rank correlation coefficient. (C) The κ/λ ratio as a function of DSS. With increasing DSS values, the κ/λ ratio of GMAb gradually trended lower and significant differentiation was observed between the κ/λ ratios and DSS values ($P < 0.05$, Kruskal–Wallis test).

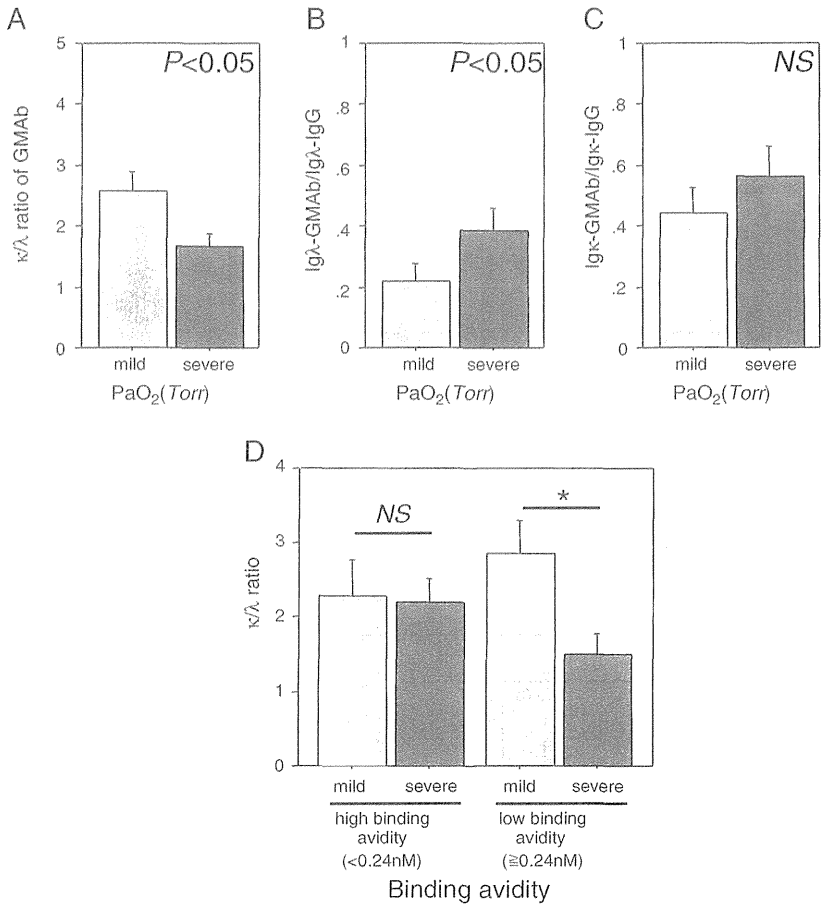

Figure 4 Comparison of the κ/λ ratio of GMAb (A) and the κ- (B) and λ-type (C) GMAb for each light-chain type of IgG between patients with high PaO_2 (mild disease) and low PaO_2 (severe disease), with a median value of 67.5 Torr. The κ/λ ratio of GMAb was significantly higher in the high PaO_2 group as compared to the low PaO_2 group ($P < 0.05$, Mann–Whitney *U*-test). The ratio of λ-type GMAb per whole λ-type IgG was higher in the severe group as compared to the mild group ($P < 0.05$, Mann–Whitney *U*-test); however, the ratio of κ-type GMAb per whole κ-type IgG did not show significant difference between high and low PaO_2 groups. (D) Comparison of the κ/λ ratio in GMAb between two groups segregated by binding avidity of GMAb, patients with high binding avidity less than 0.24 nM (the mean + 2SD for IVIG, n = 16) and patients with low binding avidity over 0.24 nM (n = 30). In the former group, the serum κ/λ ratio of GMAb was similar between severe and mild disease, whereas, in the latter, the κ/λ ratio was higher in the mild disease than that in severe disease (*$P < 0.05$, Mann–Whitney *U*-test).

disease severity (i.e., the degree of hypoxemia). As previous reports and the present data indicated that the serum concentration of GMAb and the binding avidity of GMAb had no correlation with disease severity [2,18,19], the present study is the first to suggest that the light chain isotype usage in GMAb might be associated with disease severity.

GMAb has been widely accepted as the causative pathogen of aPAP [1,20] because GMAb showed consistently high concentrations in both the lungs and blood with strong binding avidities to GM-CSF, and its injection is known to reproduce PAP in nonhuman primates [21]. In the late 90s, Svenson et al. demonstrated that a high avidity but low concentration of GMAb was ubiquitously present in every batch of pharmaceutically-prepared immunoglobulin (IVIG), and such antibodies neutralized GM-CSF bioactivity [22]. This suggested the presence of GMAb in the sera of normal subjects because IVIG is the product from pooled sera of normal donors. More recently, we confirmed that a low concentration of GMAb was ubiquitously present in the sera of normal subjects, showing similar biological properties in aPAP [23]. In this regard, we first report the difference in properties of GMAb between IVIG and aPAP patients: the κ/λ ratio of GMAb is significantly higher in IVIG than that in aPAP patients.

Considering that GMAbs consistently, but not exceedingly, exist in the sera of healthy subjects, membrane GMAb$^+$ B cell clones seemed to be stimulated by intrinsic GM-CSF in healthy subjects. As κ-chain was predominant in GMAbs in IVIG, it is likely that some membrane GMAb$^+$ B cell clones in healthy subjects underwent class switching without undergoing apoptosis or receptor editing. On the other hand, increased λ-type GMAb in severely affected aPAP patients might be due to the selective expansion of λ-type membrane GMAb$^+$ B clones in the peripheral lymphatic tissues.

Recently, Wang et al. investigated 19 monoclonal antibodies against GM-CSF established from six patients with aPAP

and demonstrated that the affinity of each antibody correlated with the neutralizing capacity [24]. In this regard, it is plausible that the binding avidity might be associated with the κ/λ ratio, and thus, correlated with disease severity through the neutralizing capacity. However, our present data excluded this possibility (i.e., the binding avidity correlated with neither the κ/λ ratio nor the disease severity).

In our previous reports, the correlation coefficients between the disease severity and the serum markers of LDH, CEA, SP-A, KL-6, and SP-D were 0.29–0.53 [2]. The correlation coefficient of the κ/λ ratio with the disease severity ($\rho = -0.378$) was comparable with the coefficients described above. It is of interest for future studies to assess how closely the κ/λ ratio of GMAb correlates with the longitudinal change of disease severity and how precisely the κ/λ ratio predicts the prognosis of aPAP.

In patients with aPAP, the κ/λ ratio of GMAb was remarkably variable compared with total IgG. The variability may be partially due to the frequency of antigen stimulation, receptor editing, or clonal expansion in the secondary lymphatic organs. As λ chain usage was shown to be correlated with disease severity in this study, it is plausible to consider that some critical sequences for binding with GM-CSF are coded in the λ chain of GMAb. Moreover, as a light chain binds antigens with a corresponding heavy chain, such critical sequences may be also coded in heavy chains, especially in CDR3 regions, which are known to be major paratopes. These molecular issues remain to be investigated in future studies.

5. Conclusion

Investigation of the light-chain isotype ratio of GMAb in patients with aPAP and healthy subjects revealed that the κ chain is predominant in general, but the proportion of the λ chain increases as disease severity increases in patients with aPAP. This study may provide an important clue as to the mechanism for the production of GMAb. For future study, it is intriguing to investigate whether the disease progression is associated with the serum κ/λ ratio retrospectively and prospectively.

Conflict of interest statement

None of the authors have a financial relationship with a commercial entity with an interest in the subject of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2013.10.002.

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Serum VEGF-D concentration as a biomarker of lymphangioleiomyomatosis severity and treatment response: a prospective analysis of the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial

Lisa R Young, Hye-Seung Lee, Yoshikazu Inoue, Joel Moss, Lianne G Singer, Charlie Strange, Koh Nakata, Alan F Barker, Jeffrey T Chapman, Mark L Brantly, James M Stocks, Kevin K Brown, Joseph P Lynch 3rd, Hilary J Goldberg, Gregory P Downey, Jeffrey J Swigris, Angelo M Taveira-DaSilva, Jeffrey P Krischer, Bruce C Trapnell, Francis X McCormack, for the MILES Trial Group

Summary

Background VEGF-D is a lymphangiogenic growth factor that has a key role in tumour metastasis. Serum VEGF-D concentrations are increased in most patients with lymphangioleiomyomatosis, a rare neoplasm associated with mTOR-activating tuberous sclerosis gene mutations, lymphadenopathy, metastatic spread, and pulmonary cyst formation. We used data from the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial to assess the usefulness of serum VEGF-D concentration as a marker of severity and therapeutic response to sirolimus in patients with lymphangioleiomyomatosis.

Methods In the MILES trial, patients with lymphangioleiomyomatosis who had forced expiratory volume in 1 second (FEV_1) of 70% or less of predicted were randomly assigned (1:1) to 12 months masked treatment with sirolimus or placebo. Serum VEGF-D concentrations were measured at baseline, 6 months, and 12 months. We used a linear regression model to assess associations of baseline VEGF-D concentrations with markers of disease severity, and a linear mixed effects model to assess the associations of VEGF-D concentrations with between-group differences in clinical, physiological, and patient-reported outcomes.

Findings We included 42 patients from the placebo group and 45 from the sirolimus group in our analysis. Baseline VEGF-D concentrations in individual patients varied from 0·34 ng/mL to 16·7 ng/mL. Baseline VEGF-D concentrations were higher in patients who needed supplemental oxygen than in those who did not need supplemental oxygen (1·7 ng/mL [IQR 0·99–3·36] *vs* 0·84 ng/mL [0·52–1·39]; p<0·0001) and in those who had a bronchodilator response than in those who did not (2·01 ng/mL [0·99–2·86] *vs* 1·00 ng/mL [0·61–2·15]; 0·0273). Median serum VEGF-D concentrations were similar at baseline in the sirolimus and placebo groups, and fell from baseline at 6 and 12 months in the sirolimus group but remained roughly stable in the placebo group. Each one-unit increase in baseline log(VEGF-D) was associated with a between-group difference in baseline-to-12-month FEV_1 change of 134 mL (p=0·0007). In the sirolimus group, improvement in baseline-to-12-month FEV_1 occurred in 15 of 23 (65%) VEGF-D responders (ie, those in whom baseline-to-12-month VEGF-D concentrations decreased by more than they did in any patients in the placebo group) and four of 15 (27%) VEGF-D non-responders (p=0·0448).

Interpretation Serum VEGF-D is a biologically plausible and useful biomarker in lymphangioleiomyomatosis that correlates with disease severity and treatment response. Measurement of serum VEGF-D concentrations could inform the risk–benefit analysis of sirolimus therapy in patients with lymphangioleiomyomatosis and reduce the numbers of patients needed for clinical trials.

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Introduction

Lymphangioleiomyomatosis is a rare neoplasm that results in progressive cystic lung disease and respiratory failure,[1] and affects women almost exclusively. Disease can occur in two forms: sporadic lymphangioleiomyomatosis or as tuberous-sclerosis-complex-associated lymphangioleiomyomatosis.[2] In addition to round, uniform, thin-walled cysts distributed randomly throughout the lungs, patients with lymphangioleiomyomatosis can have renal angiomyolipomas and complications resulting in lymphatic infiltration and obstruction, including lymphadenopathy, cystic lymphangioleiomyomas, and chylous fluid collections in the abdomen and chest.[3] Lymphangioleiomyomatosis is deemed a low-grade metastasising neoplasm,[1,4] and spread through lymphatic channels and recurrence after lung transplantation have been noted.[5] Inappropriate lymphangiogenesis and abundant lymphatic channel formation in lymphangioleiomyomatosis lung lesions seem to be driven by expression of VEGF-C, VEGF-D, and their cognate receptor, VEGFR3.[6,7]

VEGF-D is a lymphangiogenic growth factor that has a key role in tumour metastasis.[8] Serum VEGF-D

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Divisions of Pediatric Pulmonary Medicine and Allergy, Pulmonary and Critical Care, Vanderbilt University School of Medicine, Nashville, TN, USA (L R Young MD); Pediatrics Epidemiology Center, Department of Pediatrics, University of South Florida, Tampa, FL, USA (H-S Lee PhD, Prof J P Krischer PhD); Department of Diffuse Lung Diseases and Respiratory Failure, Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Center, Osaka, Japan (Y Inoue MD); National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA (J Moss MD, A M Taveira-DaSilva MD); Division of Respirology, Department of Medicine, University of Toronto, Toronto, ON, Canada (L G Singer MD); Division of Pulmonary and Critical Care Medicine, Medical University of South Carolina, Charleston, SC, USA (Prof C Strange MD); Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata, Japan (Prof K Nakata MD); Division of Pulmonary and Critical Care, Oregon Health and Science University, Portland, OR, USA (Prof A F Barker MD); Respiratory Institute, Cleveland Clinic Foundation, Cleveland, OH, USA (J T Chapman MD); Division of Pulmonary and Critical Care, University of Florida, Gainesville, FL, USA (Prof M L Brantly MD);

University of Texas Health Science Center at Tyler, Tyler, TX, USA (Prof J M Stocks MD); Department of Medicine, National Jewish Health, Denver, CO, USA (Prof K K Brown MD, Prof G P Downey MD, J J Swigris DO); Division of Pulmonary and Critical Care Medicine, University of California, Los Angeles, Los Angeles, CA, USA (Prof J P Lynch 3rd MD); Division of Pulmonary and Critical Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA (H J Goldberg MD); Division of Pulmonary Biology and Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA (Prof B C Trapnell MD); and Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA (Prof B C Trapnell, Prof F X McCormack MD)

Correspondence to:
Dr Lisa R Young, Division of Pulmonary Medicine, Vanderbilt University School of Medicine, 2200 Children's Way, Nashville, TN 37232-9500, USA
lisa.young@vanderbilt.edu

or

Prof Francis X McCormack, Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0564, USA
frank.mccormack@uc.edu

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concentrations are increased in most patients with lymphangioleiomyomatosis, but use of these concentrations as a marker of disease severity, progression, and response to treatment has not been established.[9–12] Serum VEGF-D concentrations are normal in women with other cystic lung diseases, such as pulmonary Langerhan's cell histiocytosis, emphysema, follicular bronchiolitis, lymphoid interstitial pneumonia, and Birt-Hogg-Dubé syndrome.[10,13] We have previously reported that serum concentrations of 0·8 ng/mL distinguished lymphangioleiomyomatosis from mimics that present as thin-walled cysts visible on high-resolution CT (sensitivity 73%, specificity 100%); at a cutoff of 0·6 ng/mL, diagnostic sensitivity of serum VEGF-D was 84% and specificity was 98%.[10] VEGF-D concentrations are increased in women with tuberous-sclerosis-complex-associated disease to an extent greater than that in women with sporadic lymphangioleiomyomatosis.[10] Furthermore, in a cross-sectional study of women with tuberous sclerosis complex, a serum concentration of 0·8 ng/mL effectively discriminated between patients with and without cystic changes on chest CT.[10]

The mTOR signalling pathway is constitutively activated in lymphangioleiomyomatosis because of loss of *TSC1* or *TSC2* function, resulting in dysregulated cell growth, motility, and survival.[1] Activation of the mTOR pathway is blocked by sirolimus, and several studies have shown the efficacy of mTOR inhibition in the treatment of manifestations of tuberous sclerosis complex, including angiomyolipomas,[14–16] subependymal giant cell astrocytomas,[17] and, most recently, lymphangioleiomyomatosis.[18–20] The Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial[18] was an international, randomised, double-blind, placebo-controlled trial, which showed that sirolimus stabilised lung function and was associated with improvements in quality of life and functional performance. In the trial, mean serum concentrations of VEGF-D at baseline were similar in the placebo and sirolimus groups; concentrations fell during 12 months in the sirolimus group but remained stable in the placebo group.[18] We analysed data from the MILES trial to establish the usefulness of serum VEGF-D concentration as a marker of severity and response to treatment in lymphangioleiomyomatosis. Some of our results have been previously reported in abstract form.[21]

Methods

Background and study population

Our study population comprised participants in the MILES trial (who were enrolled at one site in Canada, two sites in Japan, and ten sites in the USA) who provided more than one serum sample drawn during the trial (ClinicalTrials.gov number, NCT00414648).[18] Briefly, inclusion criteria for the MILES Trial were forced expiratory volume in 1 s (FEV_1) of 70% or less of predicted (ie, moderate-to-severe lung disease based on FEV_1), a definite diagnosis of lymphangioleiomyomatosis based on compatible cystic changes on chest CT and either biopsy confirmation or a history of angiomyolipomas, tuberous sclerosis complex, or chylothorax. Late in the enrolment period, a serum VEGF-D concentration of 0·8 ng/mL or greater was added as an inclusion criterion. Participants were randomly assigned to receive dose-adjusted, double-blinded sirolimus or placebo for 1 year. At that point, sirolimus or placebo was discontinued and patients were followed up for an additional year. Only data from the first year were included in our analysis. Pulmonary function testing and patient-reported outcomes were gathered serially. The MILES trial was approved by the institutional review boards at the University of Cincinnati, Cincinnati Children's Hospital Medical Center, and all participating sites. Analysis of VEGF-D as a biomarker was prespecified in the MILES trial protocol, and VEGF-D measurements were submitted to the data coordinating centre before reporting of the primary outcome in the MILES trial.

Procedures

Serum VEGF-D concentrations were measured at baseline, 6 months, and 12 months. VEGF-D testing was done in a lab that met College of American Pathologists and Clinical Laboratory Improvement Amendments standards, by technicians masked to treatment assignment and clinical data. Reagents included in the Quantikine Human VEGF-D Immunoassay (R&D Systems, Minneapolis, MN, USA) were used in measurements. Additional methods and performance characteristics of the test are provided in the appendix. Pulmonary function testing methodology from the MILES trial has been previously reported.[18]

Statistical analysis

We used a linear regression model to assess the relation between baseline VEGF-D concentrations and results from pulmonary function tests, 6 min walk tests, and patient-reported outcomes. The Wilcoxon rank-sum test was used to test for differences in baseline serum VEGF-D concentrations between subgroups defined by the historical presence or absence of complicating disease features (eg, angiomyolipoma, pneumothorax). We used Fisher's exact test to test for differences in proportions.

We used linear models to assess whether or not the association between serum VEGF-D concentrations and pulmonary measures differed between treatment groups. Although patients were randomly assigned to either placebo or sirolimus in the MILES trial, we adjusted for age and height in the model to account for differences between participants at the North American and Japanese sites. We examined the interaction between serum VEGF-D concentrations at baseline and treatment assignment on pulmonary function tests, 6 min walk tests, or patient-reported outcomes. General linear models were used to assess absolute changes from baseline to

12 months, and linear mixed-effects models were used to assess changes with time. In the linear mixed-effects model analysis, we used the Kenward-Roger correction to adjust the degree of freedom to improve performance when data were missing.[22] Log-transformed VEGF-D concentrations were used to better fit the linear models. We deemed p values less than 0·05 to be significant. All reported p values are two-sided and unadjusted for multiple testing. We did all statistical analyses in SAS, version 9.2.

Role of the funding source

The funding sources had no role in study design, data analysis, the decision to publish, or writing of this Article. Raw data were electronically submitted to the data management coordinating centre and accessed by H-SL and JPK. The corresponding author had full access to all the data and the final responsibility to submit for publication.

Results

At baseline, serum samples were available for 87 of the 89 women with lymphangioleiomyomatosis who participated in the MILES trial (figure 1). The clinical characteristics of the trial population have been previously reported.[18] 23 (26%) participants had a baseline FEV_1 of greater than 60% of predicted, 17 (20%) had measurements between 51% and 60% of predicted, 23 (26%) had measurements between 41% and 50% of predicted, 15 (17%) had measurements between 31% and 40% of predicted, and nine (10%) had measurements less than or equal to 30% of predicted. Withdrawal during the treatment period did not differ significantly between the placebo (seven of 42 patients) and sirolimus (five of 45 patients) groups (p=0·54).

Baseline VEGF-D concentrations ranged from 0·34 ng/mL to 16·7 ng/mL. Age of participants and serum concentrations of VEGF-D were not significantly related (appendix). Of the 79 patients with sporadic lymphangioleiomyomatosis, 54 (68%) had serum VEGF-D concentrations greater than or equal to 0·8 ng/mL, 58 (73%) had concentrations of 0·7 ng/mL or greater, and 63 (80%) had concentrations of 0·6 ng/mL or greater. Baseline concentrations were higher than 0·8 ng/mL in seven of the eight patients with tuberous-sclerosis-complex-associated lymphangioleiomyomatosis. Median baseline serum concentrations of VEGF-D did not differ significantly between patients with tuberous-sclerosis-complex-associated disease (1·95 ng/mL [IQR 0·96–5·39]) and those with the sporadic form of the disease (1·27 ng/mL [0·66–2·59]; p=0·27).

Serum VEGF-D concentrations at baseline were significantly higher in participants who used supplemental oxygen than in those who did not use supplemental oxygen (1·7 ng/mL [IQR 0·99–3·36] *vs* 0·84 ng/mL [0·52–1·39]; p<0·0001) and in those who had a positive bronchodilator response than in those who did not (2·01 ng/mL [0·99–2·86] *vs* 1·00 ng/mL [0·61–2·15]; 0·0273; figure 2). Patients with a history of pneumothorax had significantly lower concentrations than did those without such a history (p=0·0015; figure 2). Baseline concentrations of serum VEGF-D were not significantly associated with menopausal status or historical presence or absence of angiomyolipoma (figure 2).

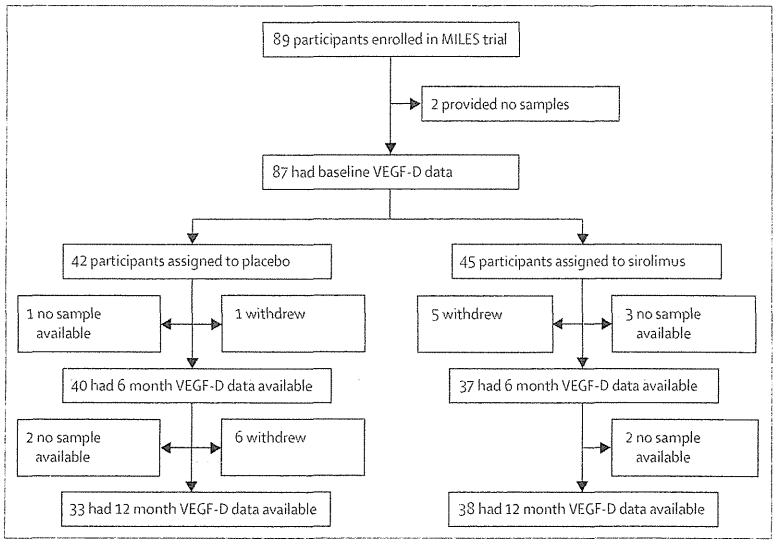

Figure 1: **Study population**
MILES=Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus.

Baseline VEGF-D concentrations were associated with markers of airflow obstruction and hyperinflation. Increased VEGF-D concentrations were positively associated with forced vital capacity (FVC), total lung capacity, residual volume, functional residual capacity, and several patient-reported outcomes, including scores on the functional performance inventory and the St George's respiratory questionnaire (table 1), which has recently been validated as a longitudinal measure in lymphangioleiomyomatosis.[23] The associations remained significant when we did sensitivity analyses that excluded either participants with tuberous-sclerosis-complex-associated disease or those who were enrolled in the MILES trial on the basis of increased VEGF-D concentrations (n=12) (data not shown).

Serum VEGF-D concentrations were similar at baseline in the placebo and sirolimus groups.[18] VEGF-D concentrations remained roughly stable with time in the placebo group but fell significantly with time in the sirolimus group (p<0·0001; appendix). Between-group differences in concentration were significant at 6 months (p=0·0123) and 12 months (0·0047). Baseline log(VEGF-D) correlated negatively (Spearman correlation −0·413; p=0·0168) with percentage change in FEV_1 during 12 months in individual patients in the placebo group and positively (0·409; 0·0087) with percentage change in