

Longitudinal analysis of serum KL-6 levels in patients with systemic sclerosis: association with the activity of pulmonary fibrosis

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Abstract

Objective

To determine whether changes in serum KL-6 levels reflect the activity of pulmonary fibrosis (PF) in patients with systemic sclerosis (SSc).

Methods

KL-6 levels were determined by ELISA in 39 SSc patients. In a retrospective longitudinal study, 250 serum samples were analyzed during a follow-up period of 0.3-6.1 years.

Results

KL-6 levels at the first visit were higher in patients with SSc, especially with PF, compared with healthy controls. In the longitudinal study, KL-6 levels in 4 patients with anti-topo I Abs increased rapidly, parallel to the progression of PF. Four patients with inactive PF exhibited elevated, but stable levels of KL-6 during the follow-up. The 31 patients with almost normal KL-6 levels during the follow-up exhibited no deterioration or new onset of PF.

Conclusion

Rapidly increased serum KL-6 levels during disease course were associated with new onset or deterioration of PF.

Key words

Systemic sclerosis, KL-6, pulmonary fibrosis, disease activity, longitudinal study.

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Introduction

Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by sclerotic changes in the skin and internal organs. Pulmonary fibrosis (PF) occurred in more than 50% of SSc patients and is the major cause of death in SSc patients (1,2). To evaluate the activity of PF, previous studies have identified several important signs, including patchy areas with a ground-glass or reticular appearance on high resolution computed tomography (HRCT) and neutrophilic alveolitis by bronchoalveolar lavage analysis (1). However, simpler, easier, and non-invasive serological markers would be helpful to closely monitor the activity of PF in SSc.

KL-6, a glycoprotein antigen first described by Kohno *et al.* (3), is expressed mainly on type II pneumocytes in alveoli and respiratory bronchiolar epithelial cells (4). KL-6 is elevated in the sera of patients with interstitial lung diseases, including idiopathic interstitial pneumonia (IIP), hypersensitivity pneumonia, radiation pneumonia, sarcoidosis, and pneumonia related to collagen diseases (4-8). In interstitial lung diseases, serum KL-6 levels have been shown to be significantly higher in patients with active disease than in those with inactive disease (4, 5). These findings indicate that KL-6 is a useful serological marker not only for the clinical diagnosis of interstitial lung diseases, but also for evaluation of disease activity.

Recent studies (9, 10) have shown that serum KL-6 levels are increased in SSc patients with PF compared with those without PF. Furthermore, KL-6 levels have been shown to correlate with the extent of PF in SSc. However, Yamane *et al.* reported that elevated KL-6 levels were associated with PF activity while we did not find this always to be the case. Furthermore, KL-6 levels were analyzed at only one or two time points during the disease course in these studies (9, 10). Therefore, the precise clinical significance of changes in KL-6 levels over the long term has not been fully clarified. In particular, it remains unknown whether and to what extent KL-6 levels change in association with

the deterioration or new onset of PF. This issue is most clinically relevant when monitoring the PF activity by means of serum KL-6 levels in patients with SSc.

In this study, to determine whether changes in serum KL-6 levels are associated with the new onset or worsening of PF, we performed a retrospective longitudinal study of serum KL-6 levels in SSc patients, especially those with anti-topoisomerase I antibodies (anti-topo I Abs), which is frequently related to the presence of PF (1).

Materials and methods

Patients

Serum samples were obtained from 39 Japanese patients with SSc (33 females and 6 males). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology (ACR) (11). These patients were grouped according to the classification system proposed by LeRoy *et al.* (12): 14 patients had limited cutaneous SSc (lSSc) and 25 had diffuse cutaneous SSc (dSSc). Anti-topo I Abs were positive for 25 patients; anticentromere Abs for 11; and anti-RNA polymerase I Abs for 3. These patients were aged 2-72 years (mean age 49). The mean disease duration was 4.3 (range: 0.2-20) years. In patients with anti-topo I Abs, the mean disease duration was 3.9 (0.2-20) years. The duration of the disease was calculated from the time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. At their first visit, 8 patients had been treated with low-dose steroids (prednisolone, 5-20 mg/day) and two with low-dose D-penicillamine (100-500 mg/day). None of SSc patients had received immunosuppressive therapy. Twenty patients with systemic lupus erythematosus (SLE) who fulfilled the ACR criteria (13) were examined as disease controls. Thirty-two healthy age- and sex-matched Japanese individuals were used as healthy controls.

In a retrospective longitudinal study, we analyzed 250 serum samples from 39 SSc patients. The mean follow-up period was 2.9 (0.3-6.1) years with 6.3 (2-12) timepoints. In addition to the 8

patients already treated with low-dose steroids and 2 patients already treated with low-dose D-penicillamine at their first visit, low-dose steroids (prednisolone, 5-20 mg/day) and low-dose D-penicillamine (100-500 mg/day) were started in 15 patients and 9 patients during the follow-up period, respectively. For subacute deterioration of PF, one patient received steroid pulse therapy, followed by 40 mg/day of oral prednisolone and one patient was treated with 750 mg/day of cyclophosphamide pulse therapy, followed by 30 mg/day of oral prednisolone. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C before use.

Clinical assessment

Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at their first visit, with evaluations especially for PF during follow-up visits. The modified Rodnan total skin thickness score was assessed as described elsewhere (14). Organ system involvement was defined as described previously (15). Pulmonary interstitial fibrosis was defined as bibasilar interstitial fibrosis on chest radiogram and HRCT. A pulmonary function test (PFT), including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), was also carried out. When the DLco and VC were $< 75\%$ and $< 80\%$, respectively, of the predicted normal values, they were considered to be abnormal. The PF activity was assessed by changes in serial examinations by HRCT and PFT. We used an HRCT scoring system based on the method of Kazerooni and co-workers (16). Each lobe of the lung was scored for the extent of ground-glass opacity (HRCT-ground glass) and reticular opacities and honeycombing (HRCT-fibrosis) on a scale of 0-5 as follows: score 0 (absent), 1 ($< 5\%$), 2 (5-25%), 3 (25-50%), 4 (50-75%), 5 ($> 75\%$). Mean value for all lobes was incorporated into a fibrotic (HRCT-fibrosis) and ground-glass (HRCT-ground glass) score for each patient and scores were then averaged for the two readers. SSc patients with a smoking habit or other respiratory disorders,

which could have affected %DLco or %VC, were excluded from this study.

Detection of serum KL-6

Serum KL-6 levels were measured with specific ELISA kits (Eitest KL-6, Eisai, Tokyo, Japan), according to the manufacturer's protocol. Briefly, 96-well plates were coated with mouse anti-KL-6 monoclonal Abs, and the serum samples diluted to 1:200 were added to duplicate wells for 2 hours at 20°C . After washing, the bound Abs were detected with peroxidase-conjugated mouse anti-KL-6 monoclonal Abs. In this assay system, the cut-off value was established as 500 U/ml by receiver-operating characteristic analysis, as described elsewhere (17). The sensitivity of the assay was 26 U/ml. The intra and inter-assay coefficients of variation were $< 8\%$ and $< 10\%$, respectively.

Statistical analysis

Comparisons between two experimental groups of data were performed using a Mann-Whitney U-test. Comparisons among three or more experimental groups were performed using a one-way ANOVA followed by a Bonferroni's test. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. P values less than 0.05 were considered statistically significant.

Results

Clinical association of serum KL-6 levels at the first visit

We first assessed clinical correlation of KL-6 levels in patients with SSc at their first visit. In this study, we focused on SSc patients with anti-topo I Abs since this autoantibody is frequently associated with the presence of PF (1). For comparison, SSc patients with anticentromere or anti-RNA polymerase Abs or SLE patients were also included in this study. Elevated KL-6 levels were observed in 15% (6/39) of SSc patients and all of them were positive for anti-topo I Abs (Figs. 1 and 2). Serum KL-6 levels at the first visit were significantly elevated in patients with SSc compared with healthy controls ($p < 0.05$) and patients with SLE

($p < 0.005$). There was no significant difference in KL-6 levels between patients with SLE and healthy controls. Serum KL-6 levels were significantly increased in SSc patients with anti-topo I Abs relative to those found in SSc patients with anticentromere Abs ($p < 0.01$), SLE patients ($p < 0.001$), or normal controls ($p < 0.0001$). By contrast, serum KL-6 levels were similar for SSc patients with anticentromere Abs, those with anti-RNA polymerase Abs, and normal controls.

KL-6 levels in anti-topo I Ab-positive SSc patients with PF were significantly higher than those without PF ($p < 0.05$; Fig. 2). Furthermore, KL-6 levels correlated inversely with %VC ($r = -0.42$, $p < 0.05$) and %DLco ($r = -0.52$, $p < 0.05$) in SSc patients with anti-topo I Abs (Fig. 3). PF was not detected in any SSc patients with anticentromere or anti-RNA polymerase Abs nor in any SLE patients. Serum KL-6 levels were compared with the presence of organ involvement other than PF in SSc patients with anti-topo I Abs; however, no clinical association was observed (data not shown). In addition, KL-6 levels did not significantly correlate with modified Rodnan total skin thickness score (data not shown). Furthermore, KL-6 levels did not significantly correlate with anti-topoisomerase I antibody levels (data not shown). Thus, the elevated serum KL-6 levels were overall associated with the presence and severity of PF in SSc patients with anti-topo I Abs at their first visit.

Longitudinal study of serum KL-6 levels

To determine whether the changes in serum KL-6 levels correlated with the new onset or deterioration of PF, we retrospectively analyzed 250 serum samples from 39 SSc patients. 25/39 patients had anti-topo I Abs. For comparison, 11 patients with anticentromere Abs and 3 patients with anti-RNA polymerase Abs were also assessed. Based upon changes in KL-6 levels, SSc patients with anti-topo I Abs were classified into the following 3 groups. The first group included 4 patients with anti-topo I Abs who showed a rapid, dramatic increase in KL-6 levels (502-

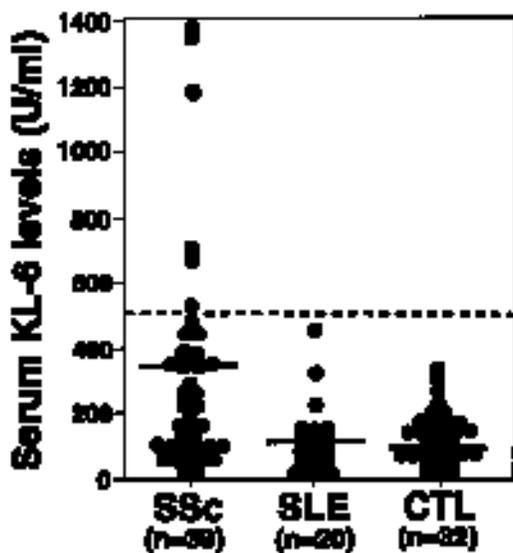


Fig. 1. Serum KL-6 levels in SSc patients with anti-topo I Abs (Topo I), SLE patients and healthy controls (CTL). Serum KL-6 levels were determined by ELISA. Horizontal lines show the median values. The dotted line indicates the cut-off value (500 U/ml).

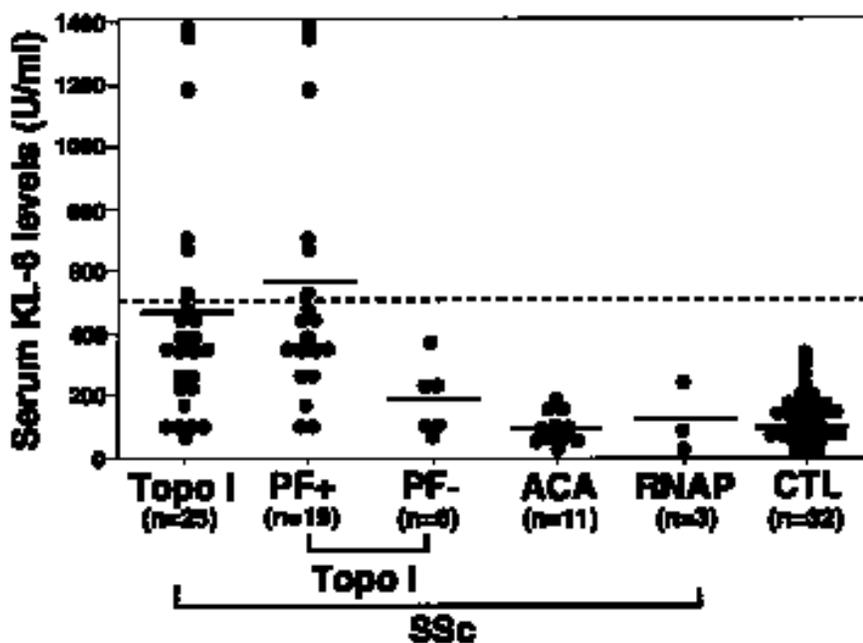


Fig. 2. Serum KL-6 levels in SSc patients with anti-topo I Ab (Topo I), those with anticentromere Ab (ACA), those with anti-RNA polymerase Ab (RNAP), SLE patients, and healthy controls (CTL). In SSc patients with anti-topo I Ab, KL-6 levels in patients with pulmonary fibrosis (PF+) and without PF (PF-) were shown. Serum KL-6 levels were determined by ELISA. Horizontal lines show the median values. The dotted line indicates the cut-off value (500 U/ml).

1514 U/ml increase) during the follow-up period (Fig. 4A). The second group included 4 patients with anti-topo I Abs who exhibited high KL-6 levels (544-1456 U/ml) at their first visit that remained stable during the follow-up period (Fig. 4B). The remaining 17 patients with anti-topo I Abs belonged to the third group, in which KL-6 levels were overall within the normal range during the follow-up period (Fig. 4C).

All patients with anticentromere or anti-RNA polymerase Abs exhibited stable KL-6 levels that remained within normal ranges during the follow-up (Fig. 4D). All 4 patients in the first group whose serum KL-6 levels rapidly and markedly increased during the observation period exhibited subacute deterioration of PF.

The first patient (case 1) showed a slight increase in KL-6 level (554 U/

ml) with mild PF and slightly decreased %DLco (58%) at the first visit (Figs. 4A and 5B and Table I). This patient had been treated with 15 mg/day of oral prednisolone for skin sclerosis and the same dose was continued after the first visit. Four months after the first visit, the KL-6 level suddenly increased to 1,463 U/ml with subacute deterioration of PF, dry cough, and dyspnea. On chest CT, the ground glass appearance and reticular shadow was increased bilaterally in the middle and lower lobes relative to that at the first visit (Fig. 5A). %DLco and %VC decreased by 37% and 59%, respectively. Steroid pulse treatment was started, followed by 40 mg/day of oral prednisolone, but the PF worsened, resulting in the death due to right heart failure 3.2 years after the first visit.

The second patient (case 2) had a normal KL-6 level (55 U/ml) with mild PF and normal PFT at the first visit (Figs. 4A and 5B and Table I). This patient was treated with 20 mg/day of oral prednisolone for skin fibrosis. Six months after the first visit, KL-6 suddenly rose to 956 U/ml and the patient began to have a dry cough. Chest CT revealed a ground glass appearance and reticular shadow bilaterally in the lower lobes (Fig. 5B) with decreased %DLco (39%) and %VC (56%). After 3 years, domiciliary oxygenation was started (KL-6 level: 460 U/ml).

The third patient (case 3) had high KL-6 level (1,191 U/ml) with mild PF, decreased %DLco (44%), and decreased %VC (77%) at the first visit (Fig. 4A and Table I). This patient was treated with 20 mg/day of oral prednisolone. Within 6 months the KL-6 level rose to 1,693 U/ml and PF progressed with decreased %DLco (35%) and %VC (57%). However, 6 months after the first visit, the KL-6 level gradually began to decrease and the progression of lung fibrosis ceased (%DLco, 35% and %VC, 55%).

The fourth patient (case 4) had a normal KL-6 level (368 U/ml) with no PF and slightly decreased %DLco (64%; Figs. 4A and 5C and Table I). This patient was treated with 20 mg/day of oral prednisolone for skin fibrosis. Two months after the first visit, the KL-6

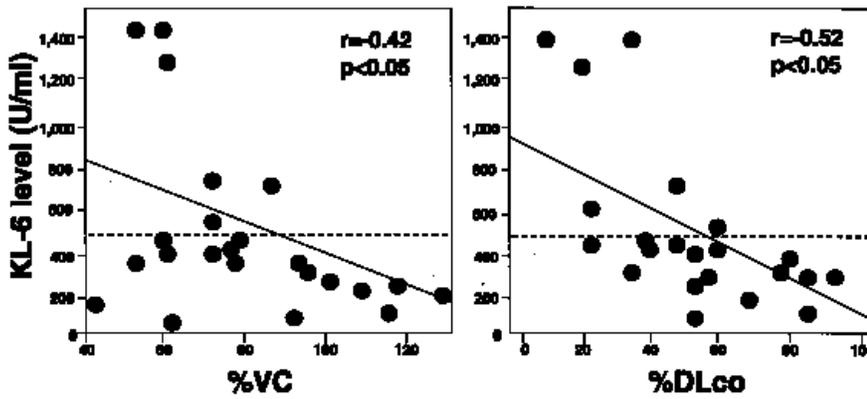


Fig. 3. The correlation of serum KL-6 levels against %VC or %DLco in SSc patients with anti-topo I Abs. Serum KL-6 levels were determined by ELISA. The dotted line indicates the cut-off value (500 U/ml).

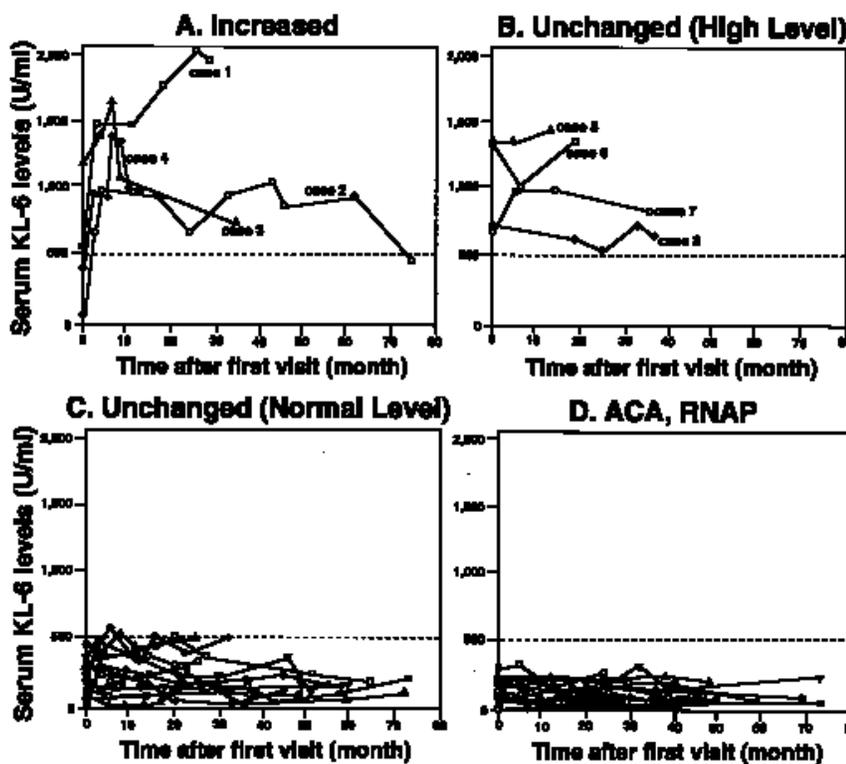


Fig. 4. Serial changes in serum KL-6 levels during the follow-up period in patients with SSc. Patients with SSc were classified into the following 4 groups: SSc patients with anti-topo I Abs who showed dramatic and rapid increases in serum KL-6 levels (A), those who had high KL-6 levels at their first visit that remained stable during the follow-up (B), those who had almost normal and stable KL-6 levels during the follow-up (C), and SSc patients with anticentromere Abs (ACA) and anti-RNA polymerase Abs (RNAP; D). Serum KL-6 levels were determined by ELISA. The dotted line indicates the cut-off value (500 U/ml).

level suddenly increased to 886 U/ml and chest CT revealed a ground glass shadow bilaterally in the lower lobes compared to that at the first visit. Eight months after the first visit, the KL-6 level rose to 1,311 U/ml. On chest CT, the ground glass shadow in the lower

lobes was increased bilaterally (Fig. 5C). %DLco and %VC were decreased to 49% and 79%, respectively. Cyclophosphamide pulse therapy (750 mg/day) was started, followed by 30 mg/day of oral prednisolone. After the first cyclophosphamide pulse treatment, the

KL-6 level decreased to 996 U/ml and the progression of lung fibrosis appeared to cease.

Thus, rapid and dramatic increases in KL-6 levels were associated with the new onset or deterioration of PF in SSc. In the 4 patients from the second group, KL-6 levels were elevated at their first visit, but remained high during the follow-up period (Fig. 4B). Two patients (cases 5 and 6) had relatively high KL-6 levels (>1,000 U/ml). These two patients, whose disease duration was more than 6 years, had moderate but inactive PF with a honeycomb pattern on chest CT at their first visit and unchanged PFT during the follow-up (Table I). The remaining 2 patients (cases 7 and 8) had KL-6 levels of 500-1,000 U/ml and exhibited only mild and inactive PF during the follow-up (Table I). The disease duration of these 2 patients was 0.4 (case 8) and 5.0 (case 7) years.

The 17 patients of the third group had almost normal levels of KL-6 during the follow-up. Eleven patients had mild and inactive PF and 6 patients had no PF (Fig. 4C). There was no significant change in HRCT grade, %DLco, or %VC between the first and final evaluation (Table I). Those patients with anti-centromere or anti-RNA polymerase Abs who had normal, stable KL-6 levels did not exhibit new onset of PF during the follow-up (Fig. 4D and Table I). In all the patients examined in this study, treatment with low-dose steroids and low dose D-penicillamine did not affect KL-6 levels during the observation period.

Discussion

In the current study, serum KL-6 levels were elevated in SSc patients, especially those with anti-topo I Abs (Figs. 1 and 2) at the first visit. Increased KL-6 levels were associated with the presence of PF and decreased %DLco and %VC in SSc patients with anti-topo I Abs (Figs. 2 and 3). KL-6 levels did not correlate with organ involvement other than PF. These results confirm previous results from cross-sectional analysis showing that serum KL-6 levels reflect the presence and extent of PF in patients with SSc.

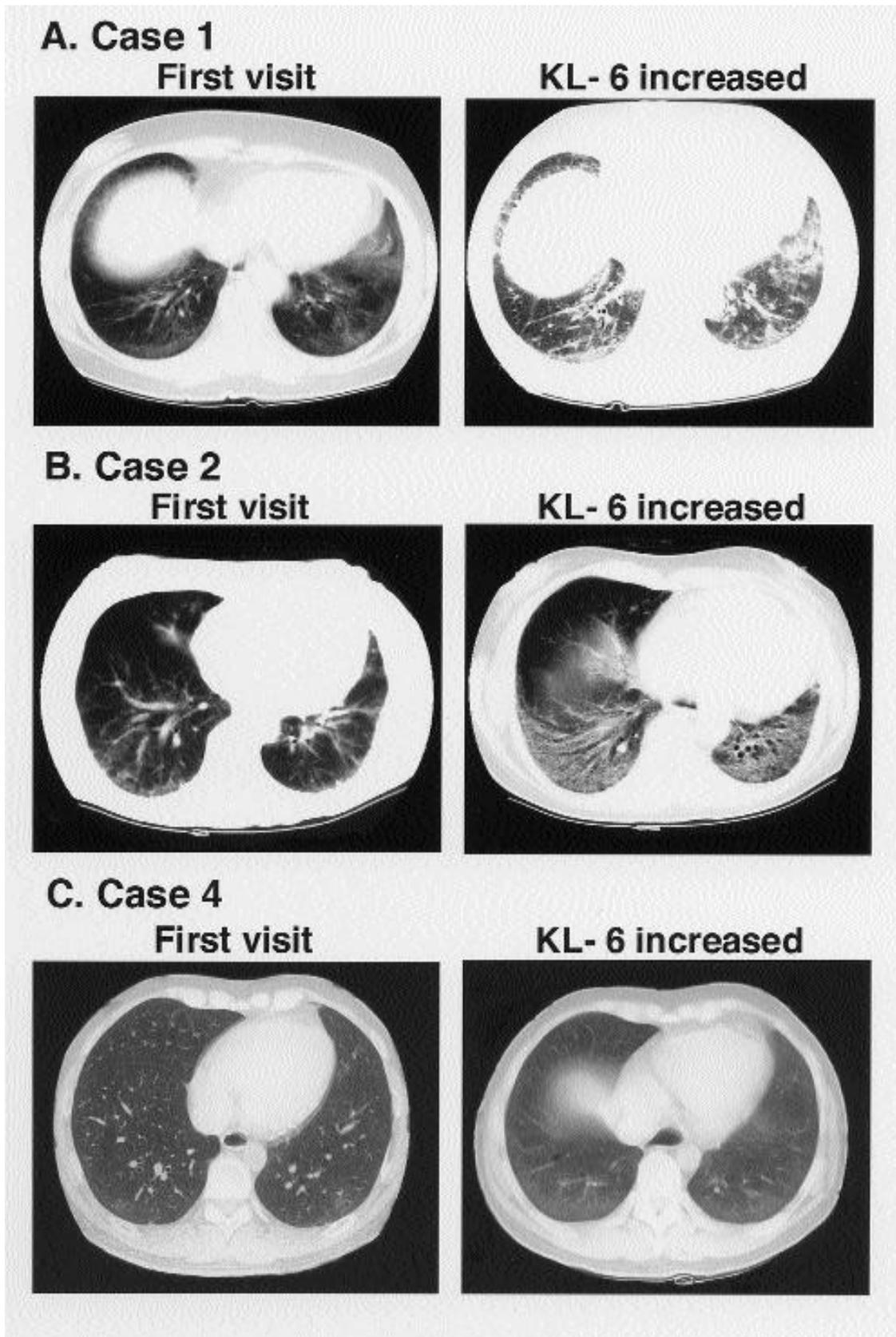


Fig. 5. Chest HRCT at the first visit and at time points when serum KL-6 levels were elevated during the follow-up in patients with SSc positive for anti-topo I Abs. These SSc patients exhibited dramatic and rapid increases in KL-6 levels during the follow-up. The case number was the same as that in Fig. 4A. The time points when KL-6 levels increased were as follows: 24 months after the first visit in case 1 (A); 11 months after the first visit in case 2 (B); and 8 months after the first visit in case 4 (C).

Table I. Chest HRCT gradings and pulmonary function test results at the first visit (Initial), at the time points when KL-6 levels reached their peak (Peak), and at the final visit (Final).

Patient*	HRCT-fibrosis score**			HRCT-ground glass score***			%DLco			%VC		
	Initial	Peak	Final	Initial	Peak	Final	Initial	Peak	Final	Initial	Peak	Final
Group A												
Case 1	0.2	2.2	3.4	1.1	3.2	3.9	58	37	35	81	59	54
Case 2	0.2	2.1	2.8	0.8	2.6	1.1	79	39	38	86	56	51
Case 3	0.5	1.5	2.3	1.4	2.3	1.5	44	35	35	77	57	55
Case 4	0	0	0.3	0	1.1	0.7	64	49	50	104	79	79
Group B												
Case 5	2.3		2.4	0.3		0.2	19		18	67		69
Case 6	1.9		1.9	0.1		0.1	57		56	78		78
Case 7	1.1		1.3	0.2		0.1	57		56	76		78
Case 8	0.7		0.6	0		0	58		54	96		94
Group C												
Median (range)	0.4 (0.2-0.8)		0.5 (0.3-0.8)	0.1 (0-0.4)		0.1 (0-0.4)	71 (58-89)		68 (56-88)	95 (75-106)		93 (73-103)
Group D												
Median (range)	0 (0-0)		0 (0-0)	0 (0-0)		0 (0-0)	74 (71-85)		70 (71-83)	92 (78-108)		91 (78-105)
Control												
Median (range)	0 (0-0)		0 (0-0)	0 (0-0)		0 (0-0)	77 (67-88)		76 (69-87)	98 (76-112)		99 (88-110)

*Patients were divided into the following 4 groups:SSc patients with anti-topo I Abs who showed dramatic and rapid increases in serum KL-6 levels during the follow-up (group A),those who had high KL-6 levels at their first visit that remained stable during the follow-up (group B),those who had almost normal and stable KL-6 levels (group C),and SSc patients with anticentromere and anti-RNA polymerase Abs who had normal stable KL-6 levels (group D). The case numbers are the same as those in Fig. 4A.

**HRCT-fibrosis score on a scale of 0-5, as described in Materials and Methods.

***HRCT-ground glass score on a scale of 0-5, as described in Materials and Methods.

In our longitudinal study, 4 of 25 patients with anti-topo I Abs exhibited rapid and dramatic increases in serum KL-6 levels (> 500 U/ml increase within 6 months of the first visit) during the follow-up period and these changes were associated with the deterioration or new onset of PF (Figs. 4A and 5 and Table I). Furthermore, this was relatively early in the disease course in all 4 patients since their disease durations were less than 2 years (mean 1.4 years). These results suggest that dramatic and rapid increases in KL-6 levels are accompanied by the development of PF in patients with early SSc positive for anti-topo I Abs.

The association of serum KL-6 levels with the PF activity is explained by the finding that KL-6 levels directly reflect alveolar damage and inflammation (4). KL-6 is expressed on regenerating and proliferating type II pneumocytes in pulmonary interstitial diseases more strongly than normal type II pneumo-

cytes. KL-6 levels in bronchoalveolar lavage fluid are also increased and correlated with serum KL-6 levels (4, 6). With an increase of epithelial and vascular permeability, KL-6 may flow into blood vessels, and the soluble form of KL-6 is released into the circulation (18). Despite the association of KL-6 levels with direct alveolar damage and inflammation, treatment with steroid did not influence serum KL-6 levels; especially in case 1, KL-6 levels continued to increase with deterioration of PF despite steroid pulse treatment (Fig. 4A). Consistently, many reports have shown that steroid treatment alone is not effective for PF (2, 19, 20). Several studies have suggested that cyclophosphamide in combination with steroid has a beneficial effect on early PF associated with SSc (21-24). In case 4, after treatment with cyclophosphamide plus steroid, the KL-6 level was decreased with stable HRCT and PFT (Figs. 4A and Table I). Although in this study the

effect of cyclophosphamide treatment on KL-6 levels was assessed in only one patient, it is possible that treatment with cyclophosphamide plus steroid may decrease KL-6 levels, with the stabilization or amelioration of PF in SSc. The 4 patients who exhibited high KL-6 levels at their first visit which remained stable during the follow-up period had inactive PF (Fig. 4B and Table I). Three of the 4 patients had relatively long disease duration (> 5 years) at their first visit (cases 5-7). This indicates that serum KL-6 levels are persistently elevated even in the absence of the PF activity. Since the hyperplasia of type II pneumocytes, which are the major source of KL-6, is observed in established PF (25), the persistent increase in KL-6 levels may reflect the long-term remodeling process of PF. In addition, elevated but stable KL-6 levels were associated with inactive PF during the follow-up period of 3.2 years even in early SSc (case 8, disease dura-

tion 0.4 years at the first visit). Thus, an elevated KL-6 level at one time point, even if it is high, does not always indicate the activity of PF in SSc. Instead, changes over time in KL-6 levels are important to evaluate PF activity. Stable high KL-6 levels during the disease course may reflect the extent of PF rather than the activity and inflammation of PF.

The results of this study suggest the possibility of KL-6 as a simple, easy, and useful serological marker for monitoring the activity of PF. However, it should be noted that serum KL-6 levels must be interpreted in combination with other laboratory tests and radiological findings in order to precisely evaluate the activity or severity of PF. Furthermore, studies with larger numbers of SSc patients will be needed to confirm our findings.

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