

# Elevated Circulating TWEAK Levels in Systemic Sclerosis: Association with Lower Frequency of Pulmonary Fibrosis

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**ABSTRACT. Objective.** To determine serum levels of tumor necrosis factor-related weak inducer of apoptosis (TWEAK) and its clinical associations in patients with systemic sclerosis (SSc).

**Methods.** Serum TWEAK levels from 70 patients with SSc were examined by ELISA. In a retrospective longitudinal study, sera from 23 patients with SSc were analyzed (followup 0.8–7.2 yrs).

**Results.** Serum TWEAK levels were elevated in patients with SSc ( $n = 70$ ) compared with healthy controls ( $n = 31$ ) and patients with systemic lupus erythematosus ( $n = 22$ ). Among patients with SSc, there were no differences in serum TWEAK levels between limited cutaneous SSc and diffuse cutaneous SSc. Patients with SSc who had elevated TWEAK levels less often had pulmonary fibrosis and decreased vital capacity than those with normal TWEAK levels. In the longitudinal study, SSc patients with inactive pulmonary fibrosis or without pulmonary fibrosis consistently exhibited increased TWEAK levels, while those with active pulmonary fibrosis showed decreased TWEAK levels during the followup period.

**Conclusion.** TWEAK levels were increased in patients with SSc, and associated with a lower frequency of pulmonary fibrosis in patients with SSc. TWEAK could be a protective factor against the development of pulmonary fibrosis in this disease and as such would be a possible therapeutic target. (J Rheumatol First Release June 15 2009; doi:10.3899/jrheum.081310)

## Key Indexing Terms:

SYSTEMIC SCLEROSIS      ELISA      CYTOKINE      PULMONARY FIBROSIS  
TUMOR NECROSIS FACTOR-RELATED WEAK INDUCER OF APOPTOSIS

Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by sclerotic changes in the skin and internal organs. In addition, SSc is generally regarded as an autoimmune disorder because of the presence of antinuclear antibodies. Although the pathogenesis of SSc remains unclear, many studies have suggested that some cytokines or growth factors regulate the induction of SSc by stimulating the synthesis of extracellular matrix components, injuring

the endothelial cells, and modulating the function of leukocytes<sup>1,2</sup>. These cytokines or growth factors are produced partly by inflammatory cells infiltrating the affected tissues, such as skin or lungs, of patients with SSc<sup>1-3</sup>.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily originally identified as a weak inducer of apoptosis in certain tumor cell lines<sup>4</sup>. TWEAK is cleaved into a circulating trimeric form that is thought to mediate its biological effects. The receptor for TWEAK, fibroblast growth factor-inducible gene 14, is widely expressed on a variety of tissues<sup>5</sup> and is highly upregulated in the context of tissue injury, regeneration, and inflammatory responses<sup>6,7</sup>. TWEAK induces upregulation of adhesion molecules such as intercellular adhesion molecule-1 and E-selectin on vascular endothelial cells<sup>8</sup>. TWEAK also induces secretion of cytokines and chemokines such as interleukin 6 (IL-6), IL-8, CCL2, CCL5 and CXCL10 from human umbilical vein endothelial cells<sup>8</sup>, synoviocytes, dermal fibroblasts<sup>9</sup>, and keratinocytes<sup>10</sup>. Serum TWEAK levels in patients with rheumatoid arthritis are significantly elevated and reflect disease activity<sup>11</sup>. Further, anti-TWEAK treatment significantly reduces the severity in mouse models of collagen-induced arthritis<sup>12,13</sup>, lupus nephritis<sup>14</sup>, and multiple sclerosis

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Supported by a grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan.

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Accepted for publication March 5, 2009.

sis<sup>15</sup>. Thus, TWEAK may play an important role in the pathogenesis of autoimmunity. We examined serum levels of TWEAK in patients with SSc, and related these results to clinical features. In addition, we undertook a retrospective longitudinal study of TWEAK levels in some of these patients to determine the changes in TWEAK over time.

## MATERIALS AND METHODS

**Patients.** Serum samples were obtained from 70 Japanese patients with SSc (62 female, 8 male) at the first visit. All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR)<sup>16</sup>. These patients were grouped according to the classification system proposed by LeRoy, *et al*<sup>17</sup>: 26 patients had limited cutaneous SSc (ISSc) and 44 had diffuse cutaneous SSc (dSSc). Anti-topoisomerase I (topo I) antibodies (Ab) were positive for 27 patients; anticentromere Ab for 21; anti-RNA polymerase Ab for 8; anti-U1RNP Ab for 2; anti-U3RNP Ab for 2; and anti-Th/To Ab for 1. The remaining patient showed negative antinuclear Ab. These patients were aged 10–73 years (mean age 49). The mean disease duration was  $5.0 \pm 6.8$  (range 0.2–32) years.

Twenty-two patients with systemic lupus erythematosus (SLE) who fulfilled the ACR criteria<sup>18</sup> acted as disease controls. Thirty-one age- and sex-matched healthy Japanese individuals were used as healthy controls.

In a retrospective longitudinal study, we analyzed serum samples from 23 SSc patients twice (22 female, 1 male; 16 with dSSc and 7 with ISSc). Two samples from each patient were obtained: at the initial visit and the last visit when serum samples were collected. The median followup period was 2.5 (range 0.8–7.2) years with 2 timepoints. The mean disease duration was  $3.7 \pm 4.4$  (range 0.2–13.3) years. Anti-topo I Ab were positive for 13 patients; anticentromere Ab for 7; anti-RNA polymerase I Ab for 2; and anti-U1-RNP Ab for 1. These patients were aged 10–73 years (mean age 49).

Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at  $-70^{\circ}\text{C}$  before use.

**Clinical assessment.** Complete medical histories, examinations, and laboratory tests were conducted for all patients at their first visit, with evaluations especially for pulmonary fibrosis (PF) during the followup visit. Organ system involvement was defined as described<sup>19,20</sup>: lung = bibasilar fibrosis on chest radiography and high-resolution computed tomography (HRCT); esophagus = hypomotility shown by barium radiography; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure with no other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. The modified Rodnan total skin thickness score (TSS) was measured by summing the skin thickness measurements, and determined by palpation on a 0–3 scale in 17 body areas<sup>21</sup>. In particular, PF was defined as bibasilar interstitial fibrosis on chest radiographs, and ground-glass opacities, reticular opacities, or honeycombing on chest HRCT. In addition, pulmonary function testing (PFT), including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO), was also evaluated to examine the severity of PF. When the DLCO and VC were  $< 75\%$  and  $< 80\%$ , respectively, of the predicted normal values, they were considered to be abnormal. SSc patients with a smoking habit or other respiratory disorders that could have affected %DLCO or %VC were excluded from our study. The activity of the PF was initially determined by chest HRCT and PFT. Specifically, PF was considered to be active when the following 2 criteria were met: a ground-glass appearance or reticular pattern on HRCT of the chest<sup>22</sup>, and  $> 10\%$  change in VC or  $> 15\%$  change in the DLCO within 1 year<sup>23</sup>.

The study protocol was approved by the Nagasaki University Graduate School of Biomedical Sciences and Nagasaki University Hospital, and informed consent was obtained from all patients.

**Detection of serum TWEAK.** Serum TWEAK levels were measured with specific enzyme-linked immunosorbent assay (ELISA) kits (Bender Medsystems, Vienna, Austria), according to the manufacturer's protocol.

This ELISA system can detect all circulating TWEAK isoforms. Each sample was tested in duplicate. The detection limit of this assay was 9.7 pg/ml.

**Statistical analysis.** The Mann-Whitney U-test was used to compare TWEAK levels, Fisher's exact probability test to compare frequencies, and Bonferroni's test used for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between 2 continuous variables. *p* values less than 0.05 were considered statistically significant.

## RESULTS

**Serum TWEAK levels were elevated in patients with SSc.** Serum TWEAK levels at the first visit were significantly higher in patients with SSc ( $196.9 \pm 129.7$  pg/ml) than in healthy controls ( $41.5 \pm 33.5$  pg/ml;  $p < 0.001$ ) and patients with SLE ( $43.4 \pm 39.3$  pg/ml;  $p < 0.001$ ; Figure 1). As for subgroups of SSc, TWEAK levels in both dSSc ( $192.6 \pm 128.8$  pg/ml) and ISSc ( $204.1 \pm 133.4$  pg/ml) patients were significantly higher than in healthy controls ( $p < 0.001$  for both) or patients with SLE ( $p < 0.001$  for both). There was no significant difference in serum TWEAK levels between patients with dSSc and ISSc.

**Clinical correlation of serum TWEAK levels.** Values higher than the mean + 2 SD (108.5 U/ml) of the control serum samples were considered to be elevated in this study. Elevated TWEAK levels were observed in 74% (52/70) of total patients with SSc, 73% (32/44) of patients with dSSc,

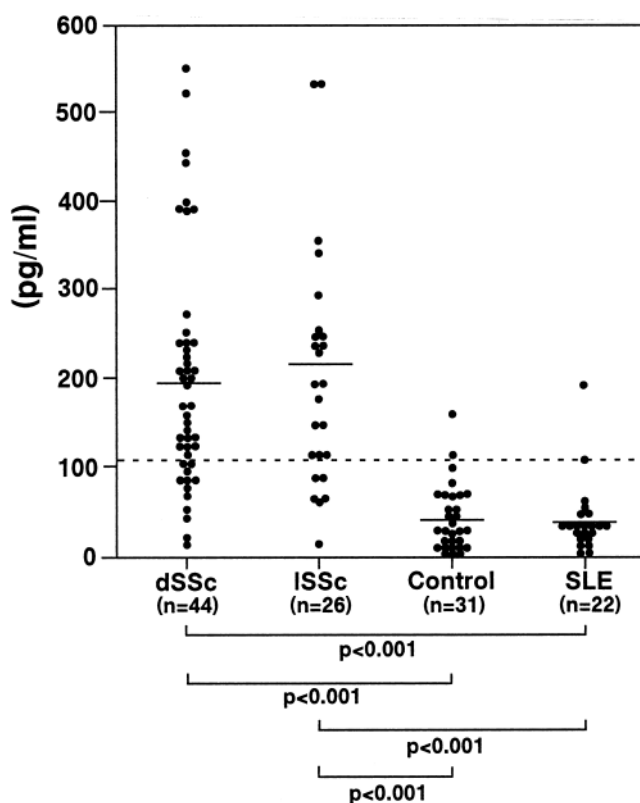


Figure 1. Serum levels of TWEAK in patients with dSSc, ISSc, SLE, and healthy controls at first visit. Serum TWEAK levels were determined by a specific ELISA. Bars indicate mean value in each group. Broken line indicates cutoff value (mean + 2 SD of the normal control samples).

and 77% (20/26) of patients with ISSc. The frequency of PF and decreased %VC in SSc patients with elevated TWEAK levels was lower than in those with normal TWEAK levels (42% vs 78%;  $p < 0.01$ ; and 21% vs 50%;  $p < 0.05$ , respectively; Table 1). Consistent with these findings, TWEAK levels were significantly lower in SSc patients with PF and decreased %VC (Figure 2). In addition, patients with elevated TWEAK levels less often had raised C-reactive protein (CRP) levels than those with normal TWEAK levels (10% vs 33%;  $p < 0.05$ ). Thus, raised TWEAK levels were associated with a lower frequency and severity of PF and increased CRP.

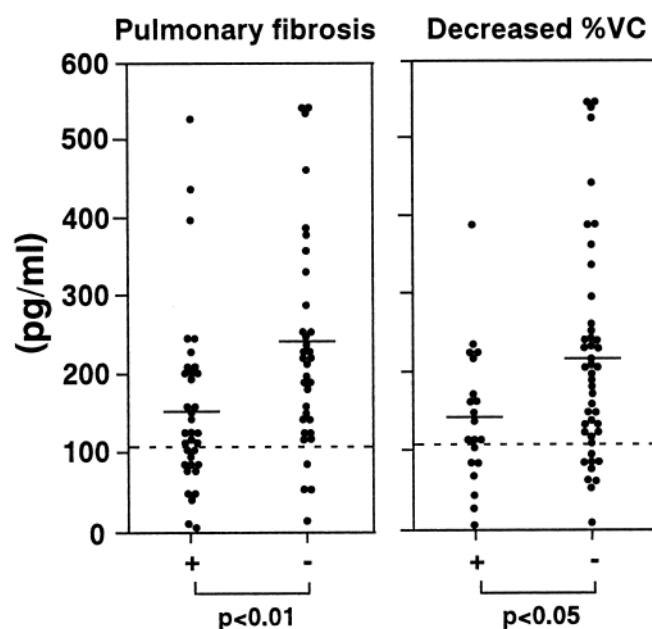
**Longitudinal study of serum TWEAK levels.** To assess changes in serum TWEAK levels over time, serum samples from 23 patients with SSc were analyzed twice during an interval of 0.8–7.2 years (mean). The patients were divided into 3 groups: a group of 6 patients who had active PF (Figure 3A), one of 5 patients who had inactive PF (Figure 3B), and one of 12 patients who did not have PF (Figure 3C).

In Figure 3A, one patient was already treated with low-

**Table 1.** Clinical and laboratory findings in patients with SSc showing elevated serum TWEAK levels at the initial visit.

	Elevated TWEAK, n = 52	Normal TWEAK, n = 18
Age at onset, yrs, mean $\pm$ SD	43 $\pm$ 15	49 $\pm$ 15
Male: Female	5:47	3:15
Disease duration, yrs, mean $\pm$ SD	5.1 $\pm$ 6.5	4.8 $\pm$ 7.4
Clinical features		
dSSc	62	67
ISSc	38	33
TSS points, mean $\pm$ SD	13.0 $\pm$ 8.7	15.1 $\pm$ 12.0
Pitting scars	48*	17
Contracture of phalanges	47	37
Diffuse pigmentation	56	88
Telangiectasia	46	33
Calcinosis	10	6
Organ involvement		
Pulmonary fibrosis	42**	78
Decreased %VC	21*	50
Decreased %DLCO	60	89
Pulmonary hypertension	14	19
Esophagus	62	67
Heart	16	22
Kidney	2	0
Joint	19	17
Muscle	12	17
Laboratory findings		
Anti-topoisomerase I antibody	37	50
Anticentromere antibody	33	22
Increased IgG	31	39
Elevated ESR	38	47
Elevated CRP	10*	33

Unless noted otherwise, values are percentages. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs SSc patients with normal serum TWEAK levels. TWEAK: tumor necrosis factor-related weak inducer of apoptosis; SD: standard deviation; dSSc: diffuse cutaneous systemic sclerosis; ISSc: limited cutaneous SSc; VC: vital capacity; TSS: Rodnan total skin thickness score.



**Figure 2.** Serum TWEAK levels at first visit in presence and absence of PF, and decrease in %VC. Bars indicate mean value in each group. Broken lines indicate cutoff value.

dose steroids at the first visit. During the followup period, low-dose steroids (prednisolone 5–20 mg/day) were started in 2 patients and low-dose D-penicillamine in one (100–500 mg/day). During the followup period, no patient with SSc received immunosuppressive therapy. Among the 6 patients in this group, 3 showed elevated TWEAK levels at the first visit, and in all of these the levels decreased to the normal range. In the remaining 3 patients in this group, TWEAK levels remained normal during the followup period. In this group, Case 1 showed normal TWEAK concentration (95 pg/ml) with slightly decreased DLCO (58%) at the first visit. Ground-glass shadow was observed in bilateral lower lobes on chest HRCT. This patient had been treated with 15 mg/day oral prednisolone for skin sclerosis and the same dose was continued after the first visit. Four months later, ground-glass and reticular shadow was increased in bilateral middle and lower lobes on chest HRCT relative to that observed at the first visit. In addition, 12 months after the first visit, serum TWEAK level was decreased to 23 pg/ml with decreased DLCO (39%) and VC (56%). Although steroid pulse treatment was started, followed by 40 mg/day oral prednisolone, PF continued to deteriorate; 2.7 years after the first visit, this patient died of right heart failure due to PF.

Figure 3B shows data for one patient already treated with low-dose D-penicillamine at the first visit. During the followup period, low-dose steroids (prednisolone 5–20 mg/day) were started in 2 patients and low-dose D-penicillamine in one (100–500 mg/day). During the followup period, no patient with SSc received immunosuppressive therapy. All 5 patients in this group showed elevated TWEAK

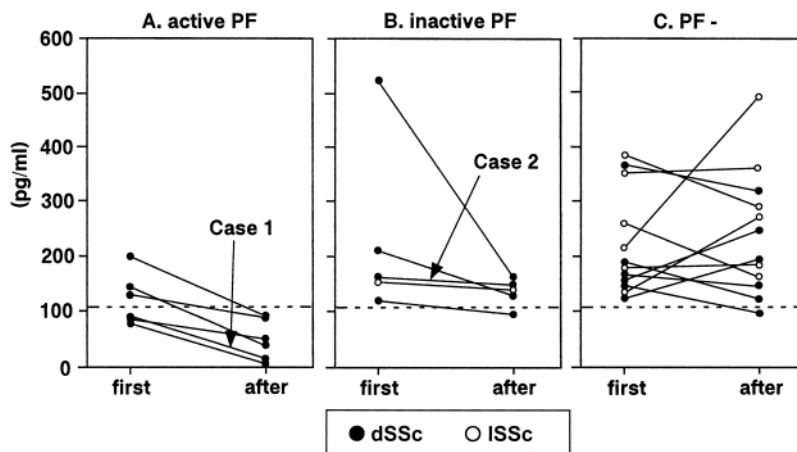


Figure 3. Changes in serum TWEAK levels during followup period in SSc patients with active PF (A), those with inactive PF (B), and those without PF (C). Black symbols represent patients with dSSc, white symbols patients with ISSc. Broken lines indicate cutoff value.

levels at their first visit and in one of these the levels decreased to the normal range. In the remaining 4 patients in this group, TWEAK levels remained elevated during the followup period. In this group, Case 2 showed increased TWEAK concentration (161 pg/ml) with normal DLCO (78%) and VC (86%) at the first visit. Although mild honeycombing shadow was observed in bilateral lower lobes on chest HRCT, no significant change was observed at 3 years after the initial visit; serum TWEAK levels remained high at that time.

As shown in Figure 3C, one patient was already treated with low-dose steroids and 2 received low-dose D-penicillamine at the first visit. After the first visit, 4 patients received low-dose steroid and 2 had low-dose D-penicillamine. During the followup period, no patient with SSc received immunosuppressive therapy. All 12 patients in this group had raised TWEAK levels at their first visit and levels remained high during the followup period.

We also divided these 23 patients into 3 groups as follows: a group of 5 patients who had elevated TWEAK levels at the first visit but decreasing levels during the followup period (Figure 4A), one of 15 patients who had elevated TWEAK levels at the first visit and stable levels during the followup period (Figure 4B), and one of 3 patients who had normal TWEAK levels at the first visit (Figure 4C). In Figure 4A, mean %VC and %DLCO tended to decrease during the followup period, although these changes were not significant. In Figure 4B, mean %VC and %DLCO did not exhibit any significant change during the followup. Notably, mean %VC and %DLCO were significantly decreased (Figure 4C).

Since steroids and D-penicillamine were started in 12 patients after the first visit, the changes in TWEAK levels might have been an effect of these agents. Nevertheless, SSc patients with inactive PF or without PF had a tendency to

have increased TWEAK levels during the disease course. In contrast, TWEAK levels in SSc patients with active PF tended to decrease or remain in the normal range during the followup period.

## DISCUSSION

This is the first report of elevated serum TWEAK levels in patients with SSc. We assessed patients with SSc and SLE, but the TWEAK levels were increased only in the patients with SSc (Figure 1). The TWEAK levels were raised not only in dSSc, but also in ISSc. Further, elevated TWEAK levels were associated with a lower prevalence of PF and better pulmonary function (Figure 2 and Table 1). In our longitudinal study, SSc patients with inactive PF or without PF tended to have elevated TWEAK levels during the followup period. In contrast, SSc patients with active PF tended to show decreased TWEAK levels during the followup period (Figures 3 and 4). Thus, elevated TWEAK levels may be protective against the development of PF in SSc.

TWEAK is largely produced by monocytes/macrophages and induces multiple cytokine/chemokine production including IL-6, IL-8, CCL2, and CXCL10<sup>8,9</sup>. CCL2 has been particularly known as an important chemotactic mediator of monocytes/macrophages<sup>24</sup>. Serum CCL2 levels are significantly increased in patients with SSc<sup>25</sup>, while augmented skin CCL2 expression is observed in the early phase of SSc<sup>25</sup>. TWEAK also upregulates cell-surface expression of adhesion molecules<sup>8</sup>. Further, histological analysis of the initial stage of SSc revealed the presence of perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts<sup>26</sup>. Therefore, TWEAK production from infiltrated monocytes/macrophages may enhance further monocyte/macrophage recruitment by augmenting the expression of CCL2 and/or adhesion molecules, leading to the initiation



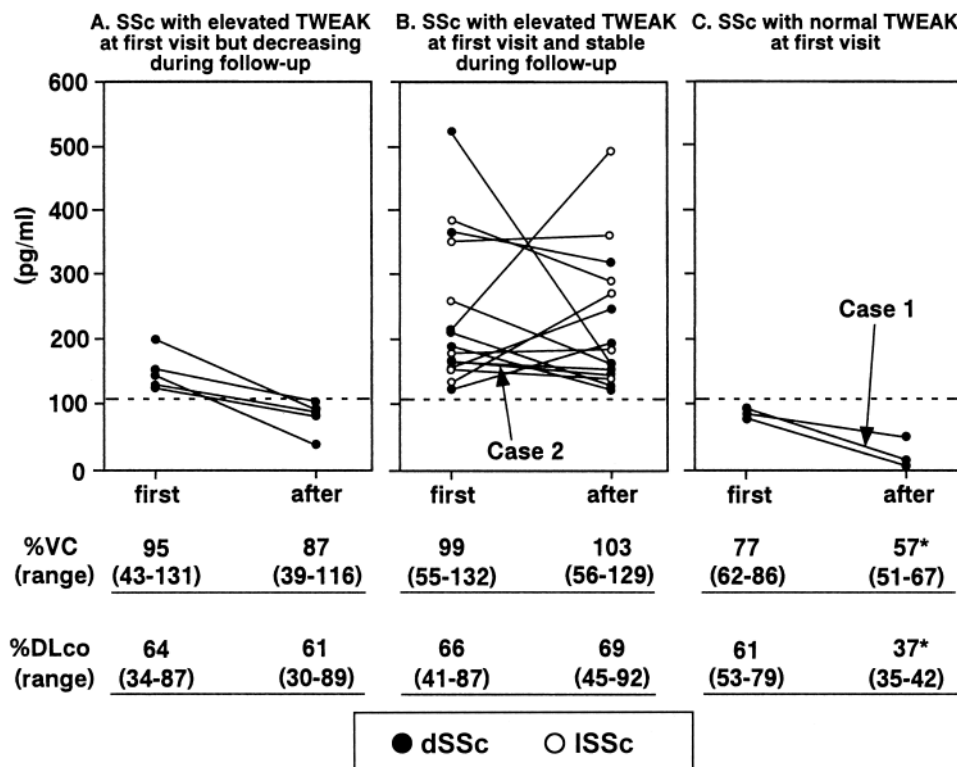


Figure 4. Changes in serum TWEAK levels, mean %VC, and %DLCO during followup period in SSc patients with elevated TWEAK levels at the first visit but decreasing during followup (A), those with elevated TWEAK levels at first visit and stable during followup (B), and those with normal TWEAK levels at first visit (C). Black symbols represent patients with dSSc, white symbols patients with ISSc. Broken lines indicate cutoff value. \* $p < 0.05$  vs %VC or %DLCO at first visit.

of skin sclerosis. In addition, serum levels of IL-6, IL-8, and CXCL10 are also elevated in patients with SSc<sup>27-29</sup>. Therefore, the increase in TWEAK production could be crucial to the onset of SSc in order to induce these cytokines and chemokines. These results also suggest that the measurement of TWEAK in patients who are suspected of SSc may offer an important approach in the diagnosis.

TWEAK enhances CCL5 secretion from dermal fibroblast and keratinocytes *in vitro*<sup>9,10</sup>. Consistently, keratinocytes in patients with SSc strongly express CCL5<sup>30</sup>. Because CCL5 is a powerful chemotactic protein that elicits the infiltration of monocytes/macrophages, T lymphocytes, eosinophils, natural killer cells, and mast cells<sup>31,32</sup>, TWEAK could also be involved in the initiation of skin sclerosis through CCL5. Interestingly, CCL5 levels are also elevated in the bronchoalveolar lavage fluid of patients with SSc, whereas the levels are significantly higher in SSc patients without PF than in those with PF<sup>33</sup>, indicating a regulatory role to prevent PF in SSc. Further, SSc patients with inactive PF or without PF consistently had increased TWEAK levels, while those with active PF had decreased TWEAK levels during the followup period (Figure 3). Therefore, persistent elevation of TWEAK may inhibit the development of PF by enhancing CCL5 production in the lung. Further studies examining the

contribution of TWEAK to the development of each organ involvement in SSc are needed. Nonetheless, the results of our study suggest that persistent administration of TWEAK may be a potential therapy in SSc patients with PF.

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