Elevated Serum APRIL Levels in Patients with Systemic Sclerosis: Distinct Profiles of Systemic Sclerosis Categorized by APRIL and BAFF

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ABSTRACT. ***Objective.*** Elevated serum concentrations of B cell-activating factor belonging to the tumor necrosis factor family (BAFF) are found in systemic sclerosis (SSc) and are associated with the severity of skin sclerosis. We investigated serum levels of a proliferation-inducing ligand (APRIL), a close homolog to BAFF, and its clinical association in patients with SSc as well as its correlation with BAFF.

Methods. Serum APRIL levels from 74 patients with SSc, 25 patients with systemic lupus erythematosus, and 25 healthy subjects were examined by ELISA. Clinical and laboratory measures were compared between SSc patients with elevated serum APRIL levels and those with normal levels. We assessed correlation of serum APRIL and BAFF levels in patients with SSc.

Results. Serum APRIL levels were elevated in SSc patients compared to controls. SSc patients with elevated serum APRIL levels had significantly higher incidences of pulmonary fibrosis than those with normal levels. Serum APRIL levels did not correlate with serum BAFF levels in SSc patients, and there were distinct profiles of SSc categorized by serum APRIL and BAFF levels. High APRIL levels served as a marker for involvement of pulmonary fibrosis and high BAFF levels served as a marker for severe skin sclerosis.

Conclusion. The results suggest that elevated serum APRIL and BAFF levels were differentially associated with disease severity in SSc. (First Release Sept 15 2007; J Rheumatol 2007;34:2056–62)

Key Indexing Terms:
SYSTEMIC SCLEROSIS APRIL BAFF PULMONARY FIBROSIS

Systemic sclerosis (SSc) is a connective tissue disorder characterized by excessive fibrosis in the skin and various internal organs, with an autoimmune background. A variety of immunological abnormalities of T and B cells have been detected in SSc[1]. Autoantibodies are positive in over 90% of patients; autoantibodies associated with SSc include anti-DNA topoisomerase I, anticentromere, anti-RNA polymerase, anti-U3RNP, and anti-Th/To antibodies[2]. Further, hyper-γ-globulinemia and B cell hyperactivity are detected in patients with SSc[3,4]. A recent study demonstrated that SSc patients have distinct abnormalities of blood B cell compartments, characterized by expanded naive B cells and activated memory B cells[5]. In the tight-skin mouse, a genetic model of SSc, chronic B cell activation is critical not only for induction of autoantibodies, but also for the development of skin fibrosis[6]. B cell depletion following treatment with a CD20 monoclonal antibody reduces skin fibrosis of tight-skin mice[7]. Further, we have recently shown that serum levels of B cell-activating factor belonging to the tumor necrosis factor family (BAFF), a potent B cell survival factor, are elevated in SSc[8]. Serum BAFF levels were correlated with skin fibrosis, and SSc B cells were more susceptible to BAFF stimulation *in vitro*[8]. These results suggest that BAFF and its signaling in B cells contribute to B cell abnormalities and disease development in SSc. Although the pathogenesis of SSc remains unknown, B cell abnormalities and dysregulation of BAFF expression are likely to play an important role.

A proliferation-inducing ligand, APRIL, also known as TALL-2 and TRDL-1, is a tumor necrosis factor (TNF) superfamily member (TNFSF13A) with close homology to BAFF and shares many functions in common with BAFF. The gene encoding the APRIL protein is localized to chromosome 17p 13.3[9]. Similarly to BAFF, APRIL is produced by several cell types, including monocytes, macrophages, dendritic cells, and T cells[10-12]. In addition, APRIL and BAFF are expressed by several types of tumors and transformed cell lines[9,13-16]. APRIL expression is upregulated by treatment with interferon-γ (IFN-γ) and IFN-α[10,11]. APRIL shares 2 TNF receptor

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superfamily (TNFRSF) members with BAFF: transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI/TNFRSF13B) and B cell maturation antigen (BCMA/TNFRSF17)[17]. Although it was initially considered that there was no specific receptor for APRIL, recent studies suggest the existence of at least one specific APRIL receptor. Two groups recently identified heparan sulfate proteoglycan on activated T cells as an APRIL-specific receptor[18,19], although the role of this interaction on primary T cells remains unclear. Similarly to BAFF, recombinant APRIL costimulates B cells *in vitro* and *in vivo*[17,20]. In mice overexpressing APRIL, T cell survival and T-independent type II antigen responses are enhanced[21].

Reports have described elevated serum APRIL levels in patients with systemic lupus erythematosus (SLE) and with Sjögren's syndrome[22,23], in addition to BAFF[24-27]. Further, inhibition of APRIL and BAFF by TACI-Ig, a soluble decoy receptor for both ligands, was found to be successful in treating a murine model of SLE[28,29]. These lines of evidence suggest that increased serum APRIL may play a role in the pathogenesis of SLE. However, Stohl, *et al* reported that APRIL might serve as a downmodulator of serological and/or clinical autoimmunity in patients with SLE[30]. To date, serum APRIL levels in SSc have not been investigated. We examined serum APRIL levels and their relation to clinical features, and associations between APRIL and BAFF, in patients with SSc.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 74 Japanese patients with SSc (65 women, 9 men). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology (ACR)[31]. Patients' median age was 52 years (range 20–77) and the median disease duration was 2.4 years (range 0.2–30). These patients were grouped according to the classification system proposed by LeRoy, *et al*[32]: 42 patients (34 women, 8 men) had diffuse cutaneous SSc (dcSSc) and 32 (31 women, 1 man) had limited cutaneous SSc (lcSSc). Anti-topoisomerase I antibodies were positive in 36 patients; anticentromere antibodies in 30; and anti-RNA polymerases I/III antibodies in 8. Disease duration of patients with dcSSc and lcSSc was 2.1 (range 0.2–18) and 3.0 (range 0.3–30) years, respectively. At the first visit, 4 patients had been treated with low-dose steroids (prednisolone, 5–20 mg/day) and 7 patients with low-dose D-penicillamine (100–300 mg/day). No SSc patient had received other immunosuppressive therapies. None had a recent history of infection or other inflammatory diseases, or a history of cancer. As a disease control, we also examined serum samples from 25 patients with SLE [21 women, 4 men, age 43 yrs (range 20–58)] who fulfilled the ACR criteria[33]. Twenty-five age- and sex-matched healthy Japanese individuals [21 women, 4 men, age 52 yrs (range 26–72)] were recruited as healthy controls. Fresh venous blood samples were centrifuged shortly after clot formation and 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 limited evaluations during followup visits. Skin score was measured by the scoring technique of the modified Rodnan total skin thickness score (modified Rodnan TSS)[34]. Organ system involvement was defined as described[35,36], as follows: lung involvement: bibasilar fibrosis on chest radiography and high-resolution computed tomography; esophagus: hypomotility shown by barium radiography; joint: inflammatory polyarthralgias or arthritis; heart: pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney: malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle: proximal muscle weakness and elevated serum creatine kinase. Pulmonary fibrosis (PF) was defined as bibasilar interstitial fibrosis on chest high-resolution computed tomography with ground-glass appearance. Pulmonary function testing, including vital capacity (VC) and diffusing capacity for carbon monoxide (DLCO), was also carried out. Patients with SSc who smoked or had respiratory disorders that might have affected the %VC or %DLCO were excluded from the study.

The study protocol was approved by Kanazawa University Graduate School of Medical Science, and informed consent was obtained from all patients.

Enzyme-linked immunosorbent assays (ELISA). ELISA kits were used for measuring serum APRIL (Bender MedSystems, Vienna, Austria) and serum BAFF levels (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. All sera were preabsorbed with protein A (Amersham Biosciences, Piscataway, NJ, USA) to deplete immunoglobulin. Each sample was tested in duplicate. The detection limits of the assays were as follows: APRIL, 0.4 ng/ml, and BAFF, 3.38 pg/ml. The intra and interassay coefficients of variation in the APRIL ELISA kit were 8.1% and 7.1%, respectively.

Statistical analysis. Statistical analysis was performed using Mann-Whitney U test for comparison of values, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test 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. Data are shown as the median (range) unless otherwise indicated.

RESULTS

Elevated serum APRIL levels in SSc. Serum APRIL levels were significantly higher in SSc patients than in healthy controls (Figure 1). Similarly, serum APRIL levels were significantly higher in SLE patients than in healthy controls. As for the SSc subgroups, serum APRIL levels tended to be higher in patients with dcSSc compared with lcSSc, and patients with dcSSc exhibited significantly increased APRIL levels compared to controls ($p < 0.005$). In addition, serum APRIL levels in patients with dcSSc were comparable to those in patients with SLE. Although the variability of APRIL levels in each subset was very high, serum APRIL levels were significantly elevated in patients with dcSSc and SLE compared to controls (Figure 1).

Clinical correlation of serum APRIL levels in SSc. Clinical and laboratory measures obtained at the first evaluation were compared between SSc patients with elevated serum APRIL levels and those with normal levels (Table 1). When values higher than the mean + 2 SD (26.8 ng/ml) of the control serum samples were considered to be elevated, serum APRIL levels were elevated in 27% (20/74) of SSc patients. Patients with dcSSc (15/42, 36%) more frequently had elevated serum APRIL levels than patients with lcSSc (5/32, 16%), although the difference was not statistically significant. SSc patients with elevated APRIL levels exhibited slightly higher modified Rodnan TSS than those with normal levels. However, serum APRIL levels did not correlate with modified Rodnan TSS in SSc patients (Figure 2). As for clinical features, SSc patients with elevated serum APRIL levels had pitting scars more frequently than those with normal levels ($p < 0.05$). In addition, SSc patients with elevated serum APRIL levels had a significantly higher incidence of PF than those with normal levels

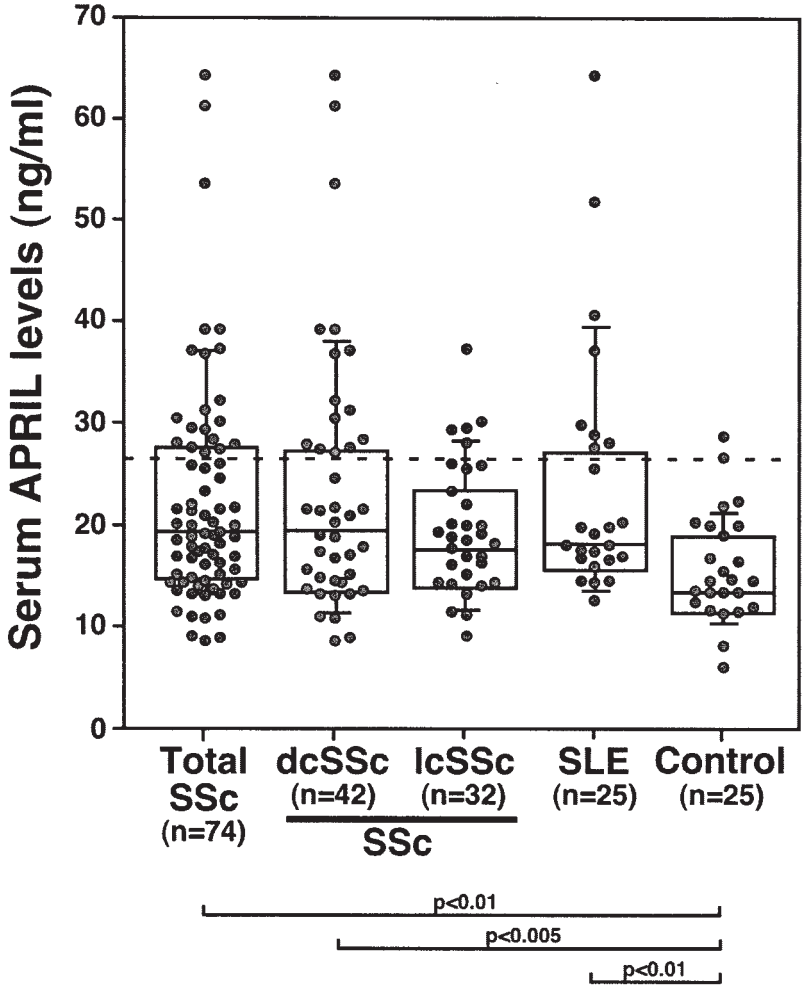

Figure 1. Serum APRIL levels in patients with autoimmune diseases at the first evaluation were determined by specific ELISA. Broken line indicates cutoff values (mean + 2 SD of control samples). Lines inside boxes indicate median; outer borders indicate 25th and 75th percentiles; extending bars indicate 10th and 90th percentiles. dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, SLE: systemic lupus erythematosus.

(65% vs 37%, respectively; $p < 0.05$). Further, %VC in SSc patients with elevated serum APRIL levels was significantly decreased compared to those with normal levels ($p < 0.05$), although %DLCO was comparable between SSc patients with elevated and those with normal APRIL levels. APRIL levels did not correlate with %VC or %DLCO in patients with SSc (Figure 2). There were no significant differences in the frequencies of other organ involvement such as intestinal tract, heart, kidney, joint, and muscle, between SSc patients with elevated APRIL levels and normal levels. On the other hand, serum IgG levels were significantly increased in SSc patients with elevated APRIL levels compared with those with normal levels ($p < 0.05$; Table 1). Levels of IgA and IgM were also higher in SSc patients with elevated APRIL levels than in those with normal levels, but the difference was not statistically significant. APRIL levels in SSc patients correlated positively with serum IgG and IgM concentrations ($p < 0.05$, $p < 0.005$, respectively; Figure 2). However, serum APRIL levels did not correlate significantly with serum levels of anti-topoisomerase I antibody determined by ELISA, anticentromere levels by ELISA, C-reactive protein, or erythrocyte sedimentation rates (Table 1; other data not shown). Collectively, elevated APRIL levels were associated with PF and hyper-γ-globulinemia in patients with SSc.

Distinct profiles of SSc categorized by APRIL and BAFF. APRIL is a close sequence homolog of BAFF, and shares 2 TNF receptor family members with BAFF. Our previous observations[8] revealed that serum BAFF levels were elevated in SSc patients compared to healthy controls, and they correlated positively with the extent of skin fibrosis. Therefore, we assessed the correlation of serum APRIL and BAFF levels in patients with SSc: serum APRIL levels did not correlate with serum BAFF levels in SSc patients (Figure 3). When values higher than the mean + 1 SD of serum samples with SSc

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum APRIL levels. All the clinical and laboratory measures and serum APRIL levels were obtained at the first evaluation.

	SSc	
	Elevated APRIL, n = 20	Normal APRIL, n = 54
Median (range) age at onset, yrs	54 (22–73)	50 (20–77)
Sex (female/male)	17/3	48/6
Median (range) disease duration, yrs	3.0 (0.2–30)	2.0 (0.2–27)
Subtype SSc		
Diffuse cutaneous	75%	50%
Limited cutaneous	25%	50%
Median (range) modified Rodnan TSS	15 (2–42)	9.5 (2–42)
Clinical features, %		
Pitting scars	65*	33
Contracture of phalanges	55	54
Diffuse pigmentation	65	44
Telangiectasia	50	43
Organ involvement, %		
Lung	65*	37
Median (range) % VC	87 (34–132)*	102 (42–134)
Median (range) % DLCO	54 (26–112)	59 (24–91)
Esophagus	70	56
Heart	30	20
Kidney	5	2
Joint	40	24
Muscle	20	13
Laboratory findings, %		
Anti-topoisomerase I	65	61
Anticentromere	30	26
Anti-RNA polymerases I/III	5	13
Median (range) ESR, mm/h	18 (3–113)	14 (1–94)
Median (range) CRP, mg/dl	0.2 (0–3.8)	0.1 (0–3.0)
Median (range) IgG, μg/ml	1543 (1200–3320)*	1480 (722–2302)
Median (range) IgA, μg/ml	326 (127–769)	288 (98–645)
Median (range) IgM, μg/ml	186 (95–829)	152 (59–512)

* $p < 0.05$ vs SSc patients with normal APRIL levels.

patients (APRIL, 33.0 ng/ml; BAFF, 2.66 ng/ml) were considered as high levels, SSc patients were classified into 3 distinct populations (Figure 3): the "APRIL-high" group with high APRIL levels and normal BAFF levels (Figure 3, lower right), the "BAFF-high" group with high BAFF levels and normal APRIL levels (upper left), and the normal group, with normal APRIL and BAFF levels (lower left). Interestingly, no patient had high levels of both APRIL and BAFF; thus increased APRIL and BAFF levels were mutually exclusive. The APRIL-high group consisted of 7 patients with dcSSc and a patient with lcSSc, while the BAFF-high group consisted of 9 patients with dcSSc. The remaining normal group consisted of 26 patients with dcSSc and 31 with lcSSc (Table 2). SSc patients in the BAFF-high group exhibited significantly higher modified Rodnan TSS than those in APRIL-high and normal groups ($p < 0.05$; Table 2). By contrast, patients in the APRIL-high group had significantly higher incidences of PF than those in the normal group (75% vs 35%; $p < 0.05$). In addition, %VC and %DLCO in SSc patients in the APRIL-high group were significantly decreased compared to those in the normal group ($p < 0.05$). Although patients in the APRIL-high and BAFF-high groups were virtually all dcSSc patients, who generally have severe skin sclerosis and PF, high APRIL levels and high BAFF levels differentially served as a marker for involvement of PF and severe skin sclerosis, respectively. Thus, the APRIL-high group and the BAFF-high group represent distinct populations.

DISCUSSION

While elevated serum BAFF levels were observed in patients with SSc and have been associated with the severity of skin sclerosis[8], serum levels of APRIL, a BAFF homolog, had not been assessed in SSc. In our study, serum APRIL levels were found to be significantly elevated in patients with SSc relative to healthy controls (Figure 1). SSc patients with elevated serum APRIL levels had a significantly higher incidence of PF and decreased %VC than those with normal levels (Table 1), while serum APRIL levels did not correlate with the extent of skin fibrosis (Table 1 and Figure 2). In addition, there were distinct profiles of SSc patients categorized by serum APRIL

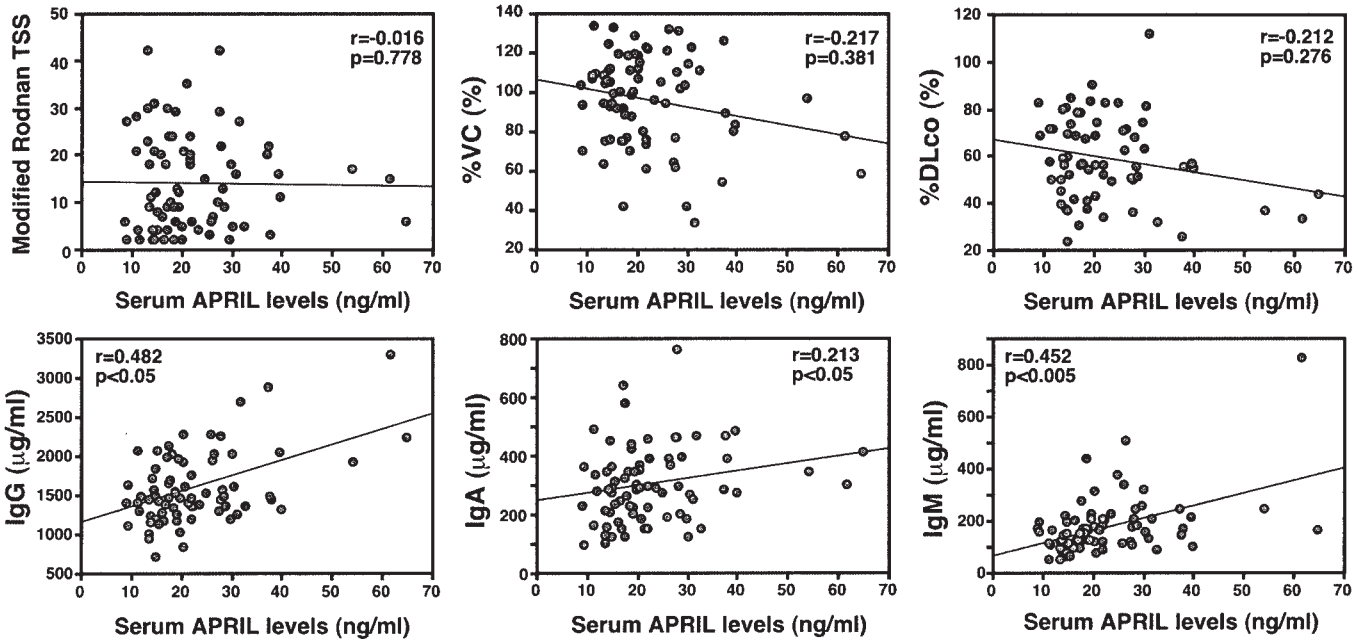

Figure 2. Correlation of serum APRIL levels against the modified Rodnan total skin thickness scores (TSS), %VC, %DLCO, and serum levels of IgG, IgA and IgM in patients with SSc at the first evaluation. Serum APRIL levels were determined by a specific ELISA.

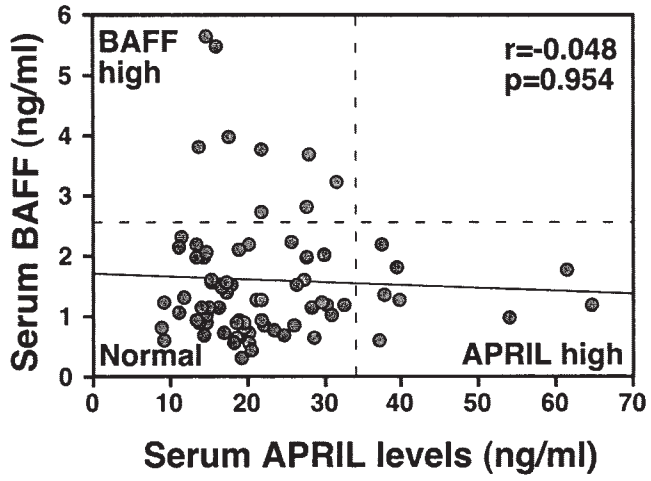

Figure 3. Correlation of serum APRIL levels against serum BAFF levels in patients with SSc at the first evaluation. Serum APRIL and BAFF levels were determined by specific ELISA. Broken lines indicate cutoff value of APRIL or BAFF (mean + SD of SSc patient samples).

and BAFF levels (Figure 3): high APRIL levels served as a marker for involvement of PF, and high BAFF levels served as a marker for severe skin sclerosis (Table 2). Our results suggest that elevated serum APRIL levels are associated closely with the presence and severity of PF in SSc.

BAFF and APRIL are essential components of B cell homeostasis and potent B cell survival factors associated with systemic autoimmune disease in animals[28,37,38]. Mice overexpressing BAFF exhibit elevated B cells in the spleen and lymph nodes and characteristics of autoimmune diseases, including spontaneous autoantibody production, immunoglobulin deposits in the kidneys, and glomerulonephritis, showing an autoimmune phenotype similar to patients with SLE[28,37-39]. Mice overexpressing APRIL show elevated T cell survival and proliferation[21]. BAFF and APRIL also have an important role in T cell activation and survival[40,41]. Further, BAFF and APRIL share the ability to induce IgG and IgA class-switching[11,15,16]. In our study, serum APRIL levels in SSc patients correlated positively with immunoglobulin levels (Figure 2). However, dysregulated expression of APRIL is not specific for SSc — elevated APRIL levels have also been reported in SLE and Sjögren's syndrome[22,23]. Nonetheless, by enhancing class-switching, BAFF and APRIL may contribute not only to the genesis of hyper-γ-globulinemia, but also to immunocomplex-induced inflammation in SSc.

Recent studies have demonstrated that, in addition to immune cells, cutaneous and respiratory epithelial cells produce considerable amounts of BAFF and APRIL[42,43]. We have reported that BAFF mRNA expression was upregulated in the early affected skin lesions from patients with dcSSc[8], while APRIL expression was not detected (data not shown). Although expressions of BAFF and APRIL have not been investigated in the lung of SSc patients, APRIL expression may be upregulated in lung lesions. If this is the case, this may result in local activation of B and/or T cells, which can account for the distinct roles of APRIL and BAFF in the involvement of lung and skin, respectively. It was notable that increased levels of APRIL and BAFF were mutually exclusive in SSc patients. A possible explanation may be that a different profile of cytokines and/or soluble factors in each subset induced a differential expression pattern of APRIL/BAFF. In SLE and RA, dissociations between serum APRIL and BAFF

Table 2. Clinical and laboratory data of patients with SSc showing APRIL-high or BAFF-high findings. All clinical and laboratory measures, serum APRIL, and serum BAFF levels were obtained at the first evaluation.

	APRIL-high, n = 8	SSc Normal, n = 57	BAFF-high, n = 9
Subtype SSc, %			
Diffuse cutaneous SSc	88*	46	100*
Limited cutaneous SSc	12*	54	0*
Median (range) modified Rodnan TSS	16 (3–32)	9 (2–42)	24 (18–42)**
Organ involvement, %			
Lung	75*	35	55
Median (range) % VC	77 (34–110)*	106 (43–134)	86 (55–127)
Median (range) % DLCO	44 (27–58)*	63 (31–112)	55 (24–69)
Esophagus	75	54	77
Heart	25	21	33
Kidney	13	2	0
Joint	50	18	44
Muscle	25	12	22
Laboratory findings, %			
Anti-topoisomerase I Ab	75	42	77
Anticentromere Ab	13	49	11
Anti-RNA polymerases I/III Ab	13	9	22

* $p < 0.05$ vs SSc patients in normal group. ** $p < 0.05$ vs SSc patients in APRIL-high group and normal group.

levels after treatment with corticosteroids[30] and rituximab[44] have been described, suggesting that production of BAFF and APRIL depends on differential regulation in autoimmune diseases. Our study indicates the importance of APRIL in the development of lung fibrosis in SSc. Since the important role of T cells has been demonstrated in lung fibrosis in SSc[45-47], APRIL may facilitate local T cell activation and survival in the lung. Recently, we reported that BAFF antagonist attenuates the development of skin fibrosis in the tight-skin mouse[48]. While our current study suggests that the blockade of APRIL offers some promise in treating lung disease in SSc, further investigation of the precise mechanism of APRIL involvement in PF is required.

Since there are few established basic therapies for skin sclerosis and lung fibrosis in SSc, new therapeutic agents have been pursued. Serum levels of BAFF and APRIL are elevated in autoimmune diseases[22-27], and blockade of BAFF and APRIL using soluble fusion proteins of BAFF receptors was successful in treating disease models in animals[28,29,49-51]. Moreover, treatment with BAFF antagonists such as humanized anti-BAFF monoclonal antibody has been started in SLE, with reports of safety and efficacy[52,53]. However, a recent report indicated that there are differences between blockade of BAFF alone and blockade of both BAFF and APRIL in a murine model of SLE[51]. Our findings suggest that inhibition of both APRIL and BAFF could be a more effective therapeutic strategy for SSc than inhibition of BAFF alone, since elevated serum APRIL and BAFF levels were differentially associated with the disease severity in SSc.

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