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A possible contribution of lipocalin-2 to the development of dermal fibrosis, pulmonary vascular involvement and renal dysfunction in systemic sclerosis

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Conflicts of interest
None declared.

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Summary

Background Lipocalin-2 is an adipocytokine implicated in apoptosis, innate immunity, angiogenesis, and the development of chronic kidney disease.
Objectives To investigate the role of lipocalin-2 in systemic sclerosis (SSc).
Materials and methods Serum lipocalin-2 levels were determined by enzyme-linked immunosorbent assay in 50 patients with SSc and 19 healthy subjects. Lipocalin-2 expression was evaluated in the skin of patients with SSc and bleomycin (BLM)-treated mice and in Fli1-deficient endothelial cells by reverse transcriptase-real time polymerase chain reaction, immunoblotting and/or immunohistochemistry.
Results Although serum lipocalin-2 levels were comparable between patients with SSc and healthy controls, the prevalence of scleroderma renal crisis was significantly higher in patients with SSc with elevated serum lipocalin-2 levels than in those with normal levels. Furthermore, serum lipocalin-2 levels inversely correlated with estimated glomerular filtration rate in patients with SSc with renal dysfunction. Among patients with SSc with normal renal function, serum lipocalin-2 levels positively correlated with skin score in patients with diffuse cutaneous SSc with disease duration of < 3 years and inversely correlated with estimated right ventricular systolic pressure in total patients with SSc. Importantly, in SSc lesional skin, lipocalin-2 expression was increased in dermal fibroblasts and endothelial cells. In BLM-treated mice, lipocalin-2 was highly expressed in dermal fibroblasts, but not in endothelial cells. On the other hand, the deficiency of transcription factor Fli1, which is implicated in SSc vasculopathy, induced lipocalin-2 expression in cultivated endothelial cells.
Conclusions Lipocalin-2 may be involved in renal dysfunction and dermal fibrosis of SSc. Dysregulated matrix metalloproteinase-9/lipocalin-2-dependent angiogenesis due to Fli1 deficiency may contribute to the development of pulmonary arterial hypertension associated with SSc.

What's already known about this topic?

- Adipokines have been shown to play various important roles in systemic sclerosis (SSc).
- Lipocalin-2 is a member of the adipokines, which are implicated in apoptosis, innate immunity, angiogenesis, and the development of chronic kidney disease.

What does this study add?

- Lipocalin-2 potentially contributes to the development of skin sclerosis, pulmonary arterial hypertension and renal damage in SSc, further supporting the critical roles of adipokines in the pathogenesis of this disease.

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vasculopathy and tissue fibrosis with unknown aetiology.[1] Recently, adipokines have attracted much attention as a new cytokine family associated with this disease.[2–7]

Lipocalin-2, a 25-kDa secretory glycoprotein originally identified in human neutrophil granules,[8] is an adipokine with diversified functions relevant to apoptosis[9] and innate immunity[10] as well as glucose metabolism and insulin sensitivity. Besides neutrophils and adipocytes, lipocalin-2 is also expressed in macrophages[11] and in several tissues such as liver[11–13] and kidney.[14–16] Lipocalin-2 expression is induced by liver injury[13,17] and urinary lipocalin-2 levels reflect the severity of hepatic fibrosis.[18] In acute kidney injury, lipocalin-2 is not only a serum/plasma and urinary marker of its severity but also a central effector of progressive renal tissue damage.[16,19–22] Furthermore, lipocalin-2 is abnormally expressed in some malignant tumour cells and may play an important role in tumorigenesis and metastasis by regulating the stabilization of matrix metalloproteinase (MMP)-9,[23,24] epithelial mesenchymal transition,[25,26] angiogenesis[27–30] and/or apoptosis.[31] Thus, lipocalin-2 has multifaceted functions related to angiogenesis, fibrosis, kidney injury and immunity, all of which are associated with SSc.

Based on these backgrounds, we investigated the potential role of lipocalin-2 in SSc by a series of experiments with clinical samples, human dermal microvascular endothelial cells (HDMECs) and SSc animal models.

Materials and methods

Patients

Serum samples, frozen at −80 °C until assayed, were obtained from 50 patients with SSc [46 women, four men; age, median (25th–75th percentiles): 58·5 years (53–67); disease duration, 3·0 years (1·5–10); body mass index (BMI), 21·1 kg m^{-2} (19·1–23·3)] and 19 healthy individuals [17 women, two men; age, 57 years (44·5–62·5); BMI, 22·4 kg m^{-2} (20·2–24·0)]. Patients being treated with corticosteroids or immunosuppressants were excluded. Patients were grouped by LeRoy's classification system:[32] 28 with diffuse cutaneous SSc (dcSSc) and 22 with limited cutaneous SSc (lcSSc). All patients fulfilled the new classification criteria of SSc.[33,34] The prevalence of each organ involvement was as follows: interstitial lung disease (ILD), 21 of 28 for dcSSc and four of 22 for lcSSc; elevated right ventricular systolic pressure (RVSP), six of 27 for dcSSc and seven of 21 for lcSSc; digital ulcers, six of 27 for dcSSc and two of 22 for lcSSc; scleroderma renal crisis (SRC), two of 28 for dcSSc and zero of 22 for lcSSc. The median and 25th–75th percentiles of modified Rodnan total skin thickness score (MRSS) were 9·0 (6·5–17·0) for dcSSc and 2·0 (1·5–4·5) for lcSSc. The whole study was performed according to the Declaration of Helsinki and approved by the ethical committee of the University of Tokyo Graduate School of Medicine.

The measurement of serum lipocalin-2 levels

Specific enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum lipocalin-2 levels (R&D Systems, Minneapolis, MN, U.S.A.). Briefly, polystyrene 96-well plates coated with anti-lipocalin-2 antibody were incubated with 50 μL of 20-fold diluted serum at 4 °C for 2 h. Then, the wells were washed and incubated at 4 °C for 2 h with anti-lipocalin-2 antibody conjugated with horseradish peroxidase. Next, the wells were washed again, tetramethylbenzidine was added, and the cells were incubated at room temperature for 30 min. Finally, sulphuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum lipocalin-2 levels were calculated using standard curves. This ELISA kit recognizes full-length human lipocalin-2 with an assay range of 0·156–10 ng mL^{-1}. Some samples were diluted a further 10-fold to be quantified within the assay range.

Clinical assessment

The definition of disease onset and disease duration and the details of assessment for organ involvement are described in the table legends and in a previous report.[35]

RNA isolation, reverse transcriptase–real time polymerase chain reaction, immunoblotting and immunohistochemistry in human dermal microvascular endothelial cells and/or skin sections

Gene silencing of Fli1 in HDMECs, the generation of bleomycin (BLM)-induced skin fibrosis in mice, RNA isolation from those cells and skin tissue, reverse transcriptase-real time polymerase chain reaction, immunoblotting and immunohistochemistry were carried out as described previously.[36–38] The sequences of primers were as follows: LCN2-forward 5′-CCCAGCCCCACCTCTGA-3′, LCN2-reverse 5′-CTTCCCCTGGAATTGGTTGTC-3′; Lcn2-forward 5′-TGCCACTCCATCTTTCCTGTT-3′, Lcn2-reverse 5′-GGGAGTGCTGGCCAAATAAG-3′; FLI1-forward 5′-GGATGGCAAGGAACTGTGTAA-3′, FLI1-reverse 5′-GGTTGTATAGGCCAGCAG-3′; 18S rRNA-forward 5′-CGCCGCTAGAGGTGAAATTC-3′, 18S rRNA-reverse 5′-TTGGCAAATGCTTTCGCTC-3′; Gapdh-forward 5′-CGTGTTCCTACCCCCAATGT-3′, Gapdh-reverse 5′-TGTCATCATACTTGGCAGGTTTCT-3′. Antibodies for human and mouse lipocalin-2 were purchased from Abcam (Cambridge, U.K.) and Merck Millipore (Darmstadt, Germany), respectively.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, U.S.A.).[38] Putative Fli1 binding site was predicted by Tfsitescan. The primers were as follows: LCN2/F-437, 5′-ACCCTTCCCTGACCCTTAAA-3′; LCN2/R-205, 5′-ACTCCCTCTGTGCCTTCCTT-3′. The amplified DNA products were resolved by agarose gel electrophoresis.

Statistical analysis

Statistical analysis was carried out with the Mann–Whitney U-test for the comparison of skewed distribution, the two-tailed paired t-test for the comparison of normally distributed paired data, a Kruskal–Wallis test and a Steel–Dwass test for multiple comparison, and a Fisher's exact probability test was used for the analysis of frequency. Correlations with clinical data were assessed by Spearman's rank correlation coefficient. The normal distribution was evaluated by the Shapiro–Wilk test. Data with skewed distribution were shown as median with 25th–75th percentiles, while those with normal distribution were shown as mean ± SD. Statistical significance was defined as a P-value of $< 0{\cdot}05$.

Results

Serum lipocalin-2 levels in systemic sclerosis

There was no significant difference in serum lipocalin-2 levels among dcSSc, lcSSc and healthy controls [5·7 ng mL^{-1} (2·8–16·2), 5·7 ng mL^{-1} (3·4–12·6) and 5·1 ng mL^{-1} (3·1–9·6), respectively; P = 0·88 (multiple comparison); Fig. 1a]. Importantly, there was a subgroup of patients with dcSSc with quite high serum lipocalin-2 levels, while all of the patients with lcSSc had serum lipocalin-2 levels distributed within the range of healthy controls, suggesting that elevation of serum lipocalin-2 levels reflects some aspect of disease process in dcSSc.

Serum lipocalin-2 levels correlated with skin score in diffuse cutaneous systemic sclerosis with disease duration of < 3 years

We next evaluated if serum lipocalin-2 levels correlate with fibrotic markers in dcSSc. Regarding MRSS, there was no correlation with serum lipocalin-2 levels in total dcSSc (r = 0·22, P = 0·28). Given that severe organ damage generally occurs within 5–6 years after disease onset, especially the first 3 years, in dcSSc,[39] patients with dcSSc with disease duration of < 6 years (n = 20) or of < 3 years (n = 18) were also assessed. Notably, there was a significant positive correlation in dcSSc with disease duration of < 3 years (r = 0·53, P = 0·024; Fig. 1b), while not in dcSSc with disease duration of < 6 years (r = 0·39, P = 0·085). In contrast, serum lipocalin-2 levels did not correlate with the percentage of predicted vital capacity (%VC) or the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) in any of the above three groups. In lcSSc, serum lipocalin-2 levels correlated with neither skin nor lung fibrotic parameters. Collectively, lipocalin-2 may be associated with dermal fibrosis, but not ILD, in early dcSSc.

Correlation of serum lipocalin-2 levels with vascular clinical features in systemic sclerosis

Because lipocalin-2 potentially regulates angiogenesis,[27–30] we next examined if serum lipocalin-2 levels correlate with

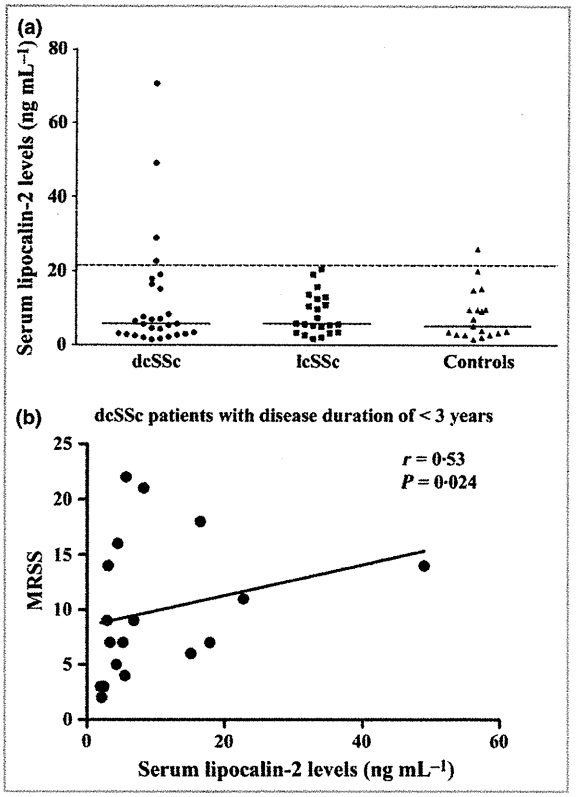

Fig 1. Serum lipocalin-2 levels in patients with SSc and healthy controls. (a) Serum lipocalin-2 levels were determined by a specific ELISA in dcSSc, lcSSc and healthy controls. The dashed line indicates the cut-off value (21·6 ng mL^{-1}; mean + 2 SD of healthy controls). The horizontal bars indicate the median value in each group. Elevated serum levels of lipocalin-2 were found in four of 28 patients with dcSSc and none of 22 patients with lcSSc. (b) Serum lipocalin-2 levels correlated with MRSS in patients with dcSSc with disease duration of < 3 years (r = 0·53, P = 0·024). The solid line represents the regression line. dcSSc, diffuse cutaneous SSc; ELISA, enzyme-linked immunosorbent assay; lcSSc, limited cutaneous SSc; MRSS, modified Rodnan total skin thickness score; SSc, systemic sclerosis.

SSc vasculopathy. To this end, patients with SSc were classified into two groups with the cut-off value of 21·6 ng mL^{-1} (mean + 2 SD of normal controls): patients with elevated serum lipocalin-2 levels (four patients dcSSc as shown above the dotted line in Figure 1a) and those with normal levels. As shown in Table 1, there was no significant difference between these two groups in terms of sex, age of onset, BMI and disease duration. Consistent with the correlation of serum lipocalin-2 levels with MRSS in dcSSc with disease duration of < 3 years, there was a trend towards the elevation of MRSS in patients with SSc with elevated serum lipocalin-2 levels compared with those with normal levels [12·3 ng mL^{-1} (10·5–14·3) vs. 7·7 ng mL^{-1} (2·0–9·0), P = 0·074]. Furthermore, all patients with SSc with elevated serum lipocalin-2 levels had dcSSc with ILD. Regarding cutaneous vascular involvement, the prevalence of pitting scars tended to be higher in patients with SSc with

Table 1 Correlation of serum lipocalin-2 levels with clinical features in SSc

	Patients with elevated serum lipocalin-2 levels (n = 4)	Patients with normal serum lipocalin-2 levels (n = 46)
Patient information		
Sex (male : female)	1 : 3	3 : 43
Age of onset (years)	47·8 (53·5–59·5)	52·3 (47·5–60·8)
Disease duration (years)	5·0 (2·0–7·5)	6·4 (1·5–10·0)
BMI (kg m^{-2})	25·0 (22·4–26·9)	21·1 (19·1–23·1)
Disease subtype (dcSSc : lcSSc)	4 : 0	24 : 22
MRSS	12·3 (10·5–14·3)	7·7 (2·0–9·0)
ILD	100	46
Cutaneous vascular involvement		
Nailfold bleeding	75	67
Pitting scars	75	24
Telangiectasia	75	38
Raynaud phenomenon	75	85
Organ involvement associated with proliferative vasculopathy		
Digital ulcers	25	16
Elevated RVSP	25	28
Scleroderma renal crisis	50*	0

BMI, body mass index; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis. The values of age, disease duration, BMI and modified Rodnan total skin sickness score (MRSS) are presented as median (25th–75th percentiles). In the others, except for sex and disease subtype, values are percentages. Interstitial lung disease (ILD) was defined as bibasilar interstitial fibrosis on chest radiographs or alveolitis on high-resolution computed tomography. The degree of ILD was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) on pulmonary function test. Elevated right ventricular systolic pressure (RVSP) was defined as ≥ 35 mmHg on echocardiogram. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure. Statistical analysis was carried out with Fisher's exact probability test. *$P < 0·05$.

elevated serum lipocalin-2 levels than in those with normal levels (75% vs. 24%, $P = 0·061$), while there were no significant differences in the frequencies of nailfold bleeding, telangiectasia and Raynaud phenomenon. As for organ involvement associated with proliferative obliterative vasculopathy, both of the two patients with SRC had elevated serum lipocalin-2 levels and the prevalence of SRC was significantly higher in patients with SSc with elevated serum lipocalin-2 levels than in those with normal levels (50% vs. 0%, $P = 0·0049$), while the prevalence of digital ulcers and elevated RVSP was comparable between the two groups. We also evaluated the difference in serum lipocalin-2 levels between patients with SSc with and without each clinical symptom, but did not see any significant difference (left columns in Table 2). Collectively, serum lipocalin-2 levels may serve as a useful marker of SRC as well as acute kidney injury caused by other reasons.[19–22]

Correlation of serum lipocalin-2 levels with estimated glomerular filtration rate in patients with systemic sclerosis with renal dysfunction

Because lipocalin-2 is implicated in progressive renal tissue damage,[16] we next evaluated if serum lipocalin-2 levels correlate with estimated glomerular filtration rate (eGFR) in patients with SSc. As various markers reflecting renal dysfunction are often altered when eGFR is below 60 mL min^{-1} 1·73 m^{-2}, we classified patients with SSc into two groups according to eGFR. Expectedly, serum lipocalin-2 levels inversely correlated with eGFR in patients with SSc with eGFR < 60 mL min^{-1} 1·73 m^{-2} ($r = -0·68$, $P = 0·035$; Fig. 2a), while not in those with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2} ($r = 0·04$, $P = 0·79$; Fig. 2b), suggesting that lipocalin-2 contributes to renal dysfunction in SSc.

Correlation of serum lipocalin-2 levels with clinical features in patients with systemic sclerosis with normal renal function

We re-evaluated the clinical correlation of serum lipocalin-2 levels in patients with SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2}. Serum lipocalin-2 levels were comparable among total SSc [5·3 ng mL^{-1} (3·0–9·4)], dcSSc [4·5 ng mL^{-1} (2·7–7·6)], lcSSc [5·6 ng mL^{-1} (3·3–12·7)] and healthy controls [$P = 0·75$ (multiple comparison)]. There was a significant correlation between serum lipocalin-2 levels and MRSS in dcSSc with disease duration of < 3 years ($r = 0·67$, $P = 0·028$), while not in total SSc, total dcSSc, dcSSc with disease duration of < 6 years, and lcSSc. Regarding %VC and %DLco, no correlations with serum lipocalin-2 levels were seen in any of the five subgroups. Furthermore, cutaneous vascular symptoms, including Raynaud phenomenon, telangiectasia, nailfold bleeding and pitting scars, did not affect serum lipocalin-2 levels in patients with SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2} (right columns in Table 2). Regarding organ involvement associated with proliferative obliterative vasculopathy, serum lipocalin-2 levels were significantly decreased by the presence of elevated RVSP, while not by the presence of digital ulcers. Furthermore, serum lipocalin-2 levels inversely correlated with RVSP in patients with SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2} ($r = -0·50$, $P = 0·0019$; Fig. 2c). Collectively, lipocalin-2 may be linked to progressive skin sclerosis and pulmonary vascular involvement leading to pulmonary arterial hypertension (PAH) in SSc.

Lipocalin-2 expression in systemic sclerosis skin

We next investigated lipocalin-2 expression in the lesional skin of SSc and control subjects by immunohistochemistry (Fig. 3, Table 3). To this end, we selected patients with dcSSc

Table 2 Associations of serum lipocalin-2 levels with clinical features in total patients with SSc and those with SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2}

		Total SSc		SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2}	
Organ involvement	Status	Median (25th–75th percentile)	P-values	Median (25th–75th percentile)	P-values
Raynaud phenomenon	+	5·7 (3·1–12·8)	0·76	5·2 (2·9–10·1)	0·39
	–	6·8 (4·8–10·7)		6·8 (5·3–9·4)	
Nailfold bleeding	+	6·9 (3·2–15·1)	0·51	5·0 (3·0–13·3)	0·99
	–	5·7 (4·7–7·8)		5·6 (5·2–7·6)	
Telangiectasia	+	6·5 (4·9–17·8)	0·28	5·4 (3·5–13·5)	0·75
	–	5·6 (3·3–11·7)		5·6 (3·3–11·1)	
Pitting scars	+	5·7 (3·3–16·9)	0·79	3·3 (2·0–5·9)	0·17
	–	5·7 (3·2–12·6)		5·6 (3·2–12·5)	
Digital ulcers	+	6·3 (4·7–14·5)	0·72	5·6 (2·2–7·0)	0·53
	–	5·7 (3·2–12·5)		5·3 (3·2–11·1)	
Elevated RVSP	+	3·4 (3·0–8·1)	0·25	3·1 (2·8–3·3)	0·01
	–	6·5 (3·9–13·3)		6·1 (3·6–12·8)	

eGFR, estimated glomerular filtration rate; RVSP, right ventricular systolic pressure; SSc, systemic sclerosis.

with disease duration of ≤ 1 year because the most remarkable molecular changes generally occur in the early phase of the diffuse subtype. In patients with dcSSc, lipocalin-2 was detectable to variable degrees in dermal fibroblasts and endothelial cells. In contrast, in healthy controls lipocalin-2 was slightly or not detected in dermal fibroblasts and endothelial cells. Regarding perivascular inflammatory cells, lipocalin-2 expression was variable in each individual cell and slightly increased in patients with dcSSc compared with healthy controls, but the accurate comparison was difficult due to the small number of inflammatory cells in the control skin. These results suggest that lipocalin-2 is mainly expressed in dermal fibroblasts and endothelial cells, especially at high levels in endothelial cells, in patients with dcSSc with disease duration of ≤ 1 year.

Lipocalin-2 expression in bleomycin-treated mice

We further evaluated lipocalin-2 expression in BLM-treated mice. Expectedly, BLM-treated skin exhibited lipocalin-2 mRNA levels significantly higher than phosphate-buffered saline-treated skin (4·2 ± 0·22 vs. 1·0 ± 0·33, P = 0·012; Fig. 4a). However, in contrast to SSc lesional skin, lipocalin-2 expression was elevated in dermal fibroblasts, but not in endothelial cells in BLM-treated fibrotic skin (Fig. 4b). Given that the BLM-treated mouse is an animal model of SSc dermal fibrosis, but not SSc vasculopathy, these results suggest that the upregulated expression of lipocalin-2 in dermal fibroblasts may contribute to the activation of those cells in the fibrotic condition.

Increased expression of lipocalin-2 in Fli1 deficient human dermal microvascular endothelial cells

Considering a potent pro-angiogenic property of lipocalin-2, the inverse correlation of serum lipocalin-2 levels with RVSP suggests the potential role of endothelial lipocalin-2 in pulmonary vascular involvement leading to SSc-PAH. To assess this hypothesis, we focused on the role of transcription factor Fli1 in the pathogenesis of SSc vasculopathy. It is generally accepted that SSc is caused by a complex interplay between genetic factors and environmental influences.[40] Fli1 has been thought to be a potential predisposing factor of SSc because FLI1 gene expression is epigenetically suppressed in SSc skin.[41] Supporting this idea, endothelial cell-specific Fli1 knockout mice spontaneously develop the histological and functional abnormalities characteristic of SSc vasculopathy such as stenosis of arterioles, dilation of capillaries and increased vascular permeability, suggesting that endothelial Fli1 deficiency is a key feature of SSc vasculopathy.[42] Importantly, Fli1 deficiency markedly increases the expression of endothelial MMP-9, which is a key proteolytic enzyme regulating angiogenesis.[42] Given that lipocalin-2 makes a complex with MMP-9 and protects it from autodegradation,[23,43] which promotes angiogenesis under physiological and pathological conditions, we hypothesized that Fli1 deficiency increases the expression of lipocalin-2 as well as MMP-9 in endothelial cells and contributes to the development of aberrant angiogenesis in SSc. Therefore, we examined the effect of Fli1 gene silencing on lipocalin-2 expression in HDMECs. As shown in Figure 5a, gene silencing of Fli1 resulted in the significant increase in lipocalin-2 mRNA levels in HDMECs (1·89 ± 0·50 fold increase, P = 0·021). This result was also confirmed at protein levels by immunoblotting (Fig. 5b). Furthermore, ChIP analysis revealed Fli1 occupancy of the lipocalin-2 promoter (Fig. 5c). These results suggest that Fli1 regulates MMP-9/lipocalin-2-dependent angiogenesis by coordinately modulating the expression of both genes at the transcriptional level in endothelial cells.

Discussion

In this study, a series of clinical and experimental data suggest the potential contribution of lipocalin-2 to the development of

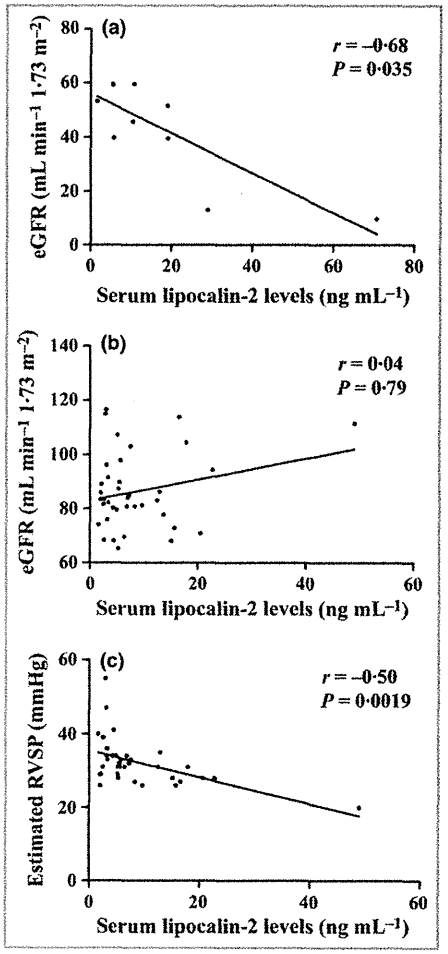

Fig 2. The clinical correlation of serum lipocalin-2 levels in patients with SSc. (a, b) Patients with SSc were classified into two groups: patients with renal dysfunction (a: eGFR < 60 mL min^{-1} 1·73 m^{-2}) and those retaining normal renal function (b: eGFR ≥ 60 mL min^{-1} 1·73 m^{-2}). The correlation of serum lipocalin-2 levels with eGFR was evaluated in these two groups. The solid line represents the regression line. (c) In patients with SSc with eGFR ≥ 60 mL min^{-1} 1·73 m^{-2}, the correlation of serum lipocalin-2 levels with estimated RVSP was evaluated by Spearman's rank correlation coefficient. The solid line represents the regression line. eGFR, estimated glomerular filtration rate; RVSP, right ventricular systolic pressure; SSc, systemic sclerosis.

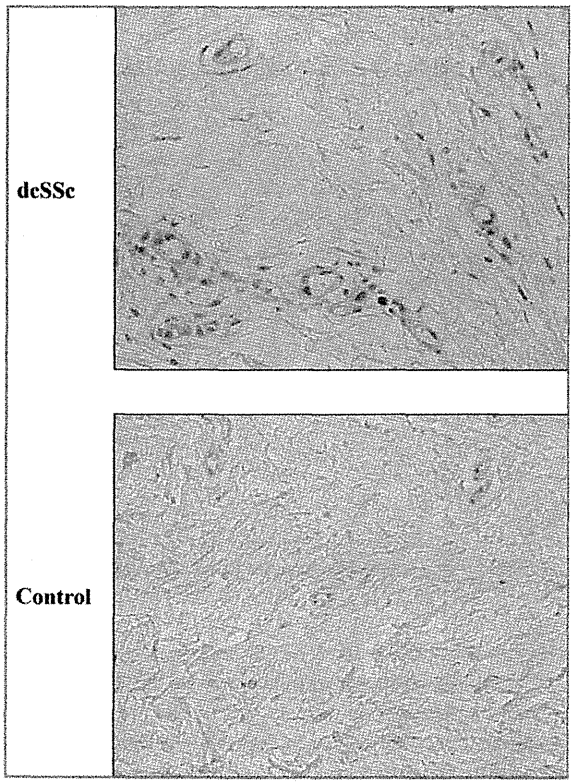

Fig 3. Lipocalin-2 expression in the skin of patients with SSc and healthy controls. Lipocalin-2 expression was evaluated by immunohistochemistry in the skin sections from patients with dcSSc with disease duration of ≤ 1 year and healthy controls. Representative results are shown (original magnification ×200). Horseradish peroxidase activity was detected by 3,3′-diaminobenzidine. Counterstaining was carried out with methyl green. dcSSc, diffuse cutaneous SSc; SSc, systemic sclerosis.

renal dysfunction, progressive skin sclerosis and pulmonary vascular injury leading to PAH in SSc.

Although the exact role of lipocalin-2 in tissue fibrosis still remains unknown, lipocalin-2 has been implicated in the development of fibrotic liver diseases in animal models. For instance, experimental chronic liver injury results in rapid and well-sustained induction of lipocalin-2 expression mainly from injured hepatocytes. Furthermore, lipocalin-2 is strongly induced in both primary hepatocytes and immortalized hepatocellular carcinoma cell line HepG2 by the pro-inflammatory cytokine interleukin-1β.[13] Similar to these observations, lipocalin-2 was highly expressed mainly in dermal fibroblasts of BLM-induced fibrotic skin, suggesting that lipocalin-2 is induced by pro-inflammatory cytokines in fibrotic conditions. Consistent with this idea, lipocalin-2 was highly expressed in dermal fibroblasts of dcSSc with disease duration of ≤ 1 year and serum lipocalin-2 levels positively correlated with MRSS in dcSSc with disease duration of < 3 years in which the perivascular infiltration of mononuclear cells in lesional skin and the increase in serum levels of various pro-inflammatory cytokines are characteristic features.[44–46] It currently remains unclear whether lipocalin-2 is directly associated with the mechanism of progressive skin sclerosis or just an epiphenomenon in early dcSSc. Further studies are required to clarify this point in the future.

In acute kidney injury, serum/plasma and urinary lipocalin-2 levels serve as useful diagnostic and prognostic markers.[19–22] Notably, lipocalin-2 is not simply a marker, but a central effector of progressive renal tissue damage.[16] In animal models of chronic kidney disease, lipocalin-2 is the most markedly upregulated gene and renal expression levels and urinary excretion of lipocalin-2 highly reflect tubular damage, while a homozy-

Table 3 Summary of immunohistochemistry for lipocalin-2 in the skin of SSc and healthy control subjects

Subjects	Age, Sex	Disease duration	ECs	Fibroblasts	Infiltrating cells
HC 1	60, F		+/−	+/−	+/−
HC 2	50, F		−	−	+
HC 3	42, F		−	−	+/−
HC 4	37, F		−	−	−
dcSSc 1	68, F	6 months	+++	+	+
dcSSc 2	55, F	1 year	++	+	+
dcSSc 3	21, F	8 months	++	+/−	+/−
dcSSc 4	48, F	1 year	++	+	+

dcSSc, diffuse cutaneous SSc; ECs, endothelial cells; HC, healthy control; SSc, systemic sclerosis; −, no staining; +, slight staining; ++, moderate staining; +++, strong staining. Disease duration means the interval between the onset defined as the first clinical event of SSc other than Raynaud phenomenon and the time skin biopsy was performed.

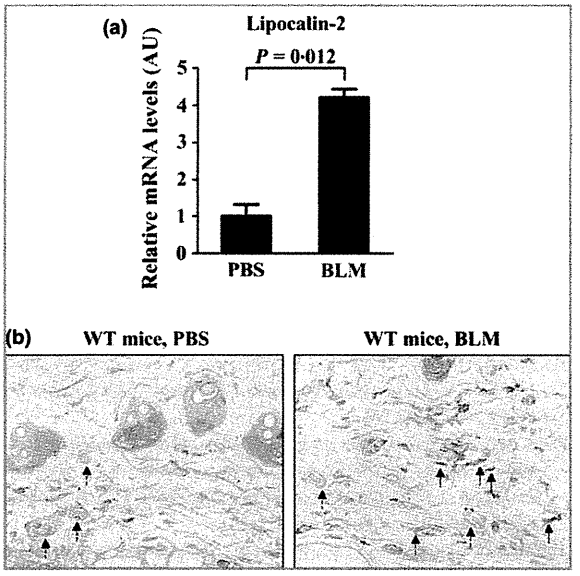

Fig 4. Lipocalin-2 expression levels were increased in the lesional dermal fibroblasts of BLM-treated mice. (a) Wild-type C57BL/6 mice were treated with BLM or PBS for 3 weeks and mRNA levels of lipocalin-2 gene were determined in the lesional skin of these mice by reverse transcriptase-real time polymerase chain reaction (n = 4 for each group). (b) Lipocalin-2 expression was examined by immunohistochemistry in the skin of wild-type C57BL/6 mice treated with BLM or PBS for 3 weeks. Representative results are shown (original magnification ×200). Horseradish peroxidase activity was detected by 3,3′-diaminobenzidine. Counterstaining was carried out with methyl green. Regular and dotted arrows represent lipocalin-2-positive dermal fibroblasts and lipocalin-2-negative endothelial cells, respectively. For (a), results of controls or relative value compared with the controls are expressed as means ± SD. Statistical analysis was carried out with the Mann–Whitney U-test. AU, arbitrary unit; BLM, bleomycin; PBS, phosphate-buffered saline; WT, wild type.

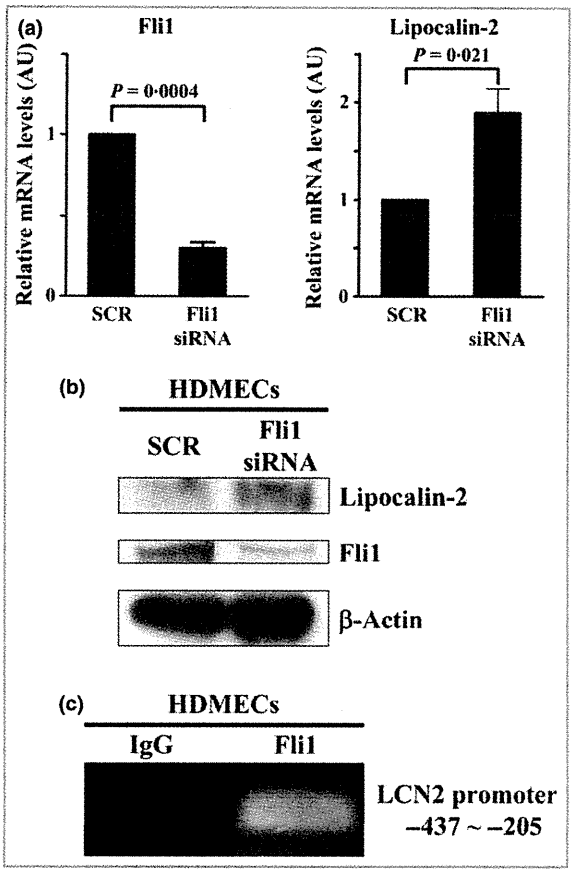

Fig 5. The contribution of Fli1 deficiency to the upregulated expression of lipocalin-2 in endothelial cells. (a) mRNA levels of Fli1 and lipocalin-2 genes in HDMECs transfected with Fli1 siRNA or nonsilencing scrambled RNA (SCR) were examined by reverse transcriptase-real time polymerase chain reaction (PCR) and normalized to the levels of the human 18S rRNA gene. Four independent experiments were carried out. (b) Under the same conditions, the protein levels of Fli1 and lipocalin-2 were evaluated by immunoblotting. Equal loading was confirmed by immunoblotting for β-actin. The representative results of four independent experiments are shown. (c) Chromatin was isolated from HDMECs and immunoprecipitated using rabbit anti-Fli1 antibody or rabbit immunoglobulin G. After isolation of bound DNA, PCR amplification was carried out using lipocalin-2 promoter-specific primers. One representative of three independent experiments is shown. For (a), results of controls or relative value compared with the controls are expressed as means ± SD. Statistical analysis was carried out with a two-tailed paired t-test. AU, arbitrary unit; HDMECs, human dermal microvascular endothelial cells.

gous disruption of the lipocalin-2 gene prevents the development of chronic renal lesions and preserves kidney function.[16] Given that serum lipocalin-2 levels inversely correlated with eGFR in patients with SSc with renal dysfunction and were extremely elevated in patients with SRC, lipocalin-2 may contribute to chronic kidney disease in SSc. Further analyses regarding whether serum and urinary lipocalin-2 levels serve as a predictor of SRC are under way in our laboratory.

Another important observation in this study was the inverse correlation of serum lipocalin-2 levels with RVSP in patients with SSc with normal renal function. Evidence suggests that dysregulated angiogenesis largely contributes to SSc-PAH. For instance, gene expression profiling of peripheral blood mononuclear cells, which is strikingly similar between SSc-PAH and idiopathic PAH, exhibits significant upregulation of vascular endothelial cell growth factor and MMP-9 in mild PAH compared with severe PAH and healthy controls.[47] Furthermore, serum MMP-9 levels are decreased in patients with SSc with class II or III PAH compared with those without PAH and inversely correlate with the severity of PAH in patients with SSc-PAH.[48] Given that MMP-9 expression and gelatinolytic activity in isolated pulmonary arteries are increased in monocrotaline-induced animal models of PAH which resemble the initiation phase of PAH,[49] MMP-9 expression in the pulmonary artery is likely to be increased during the initiation phase but starts decreasing afterwards along with the progression of pulmonary arterial involvement in SSc-PAH. Because Fli1, whose deficiency is deeply associated with SSc vasculopathy,[42] directly regulates lipocalin-2 and MMP-9 gene expression, it is plausible that, similar to MMP-9, serum lipocalin-2 levels inversely correlate with the severity of pulmonary vascular involvement leading to PAH in SSc. Importantly, lipocalin-2 stabilizes MMP-9 by forming a complex[8,43] and thereby prevents its autodegradation.[28,43,50] Therefore, dysregulated MMP-9/lipocalin-2-dependent angiogenesis may contribute to the developmental process of SSc-PAH.

Recently, the role of lipocalin-2 in atherosclerosis and endothelial function has been reported in the context of obesity. Obesity promotes the accumulation of lipocalin-2 in blood and arteries and induces endothelial dysfunction and atherosclerosis.[51–53] However, unlike in the general population,[12] serum lipocalin-2 levels did not correlate with BMI in patients with SSc ($r = 0{\cdot}02$, $P = 0{\cdot}88$), suggesting that lipocalin-2 may be associated with the development of SSc vasculopathy through a unique mechanism different from obesity-mediated vascular complications. Further studies are required to clarify this point.

In summary, this is the first study regarding the potential role of lipocalin-2 in SSc. Consistent with previous reports, serum lipocalin-2 levels also serve as a useful marker of renal damage in SSc. Furthermore, lipocalin-2 may be involved in the mechanism of progressive skin sclerosis and pulmonary vascular injury leading to PAH in SSc. Although further studies with a larger number of patients are required to confirm the current preliminary conclusion drawn from the small number of patients, these results definitely provide us with a new clue to further understand the role of adipokines in the pathogenesis of SSc.

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ORIGINAL ARTICLE

Serum omentin levels: A possible contribution to vascular involvement in patients with systemic sclerosis

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ABSTRACT

Adipokines have been shown to be potentially involved in various pathological processes of systemic sclerosis (SSc), including inflammation, vasculopathy and fibrosis, through their pleiotropic effects. Omentin is a member of the adipokines, and has a protective effect against vascular inflammation and pathological remodeling leading to atherosclerosis as well as a vasodilatory effect. To assess the potential role of omentin in the development of SSc, we determined serum omentin levels by enzyme-linked immunosorbent assay in 66 SSc and 21 control subjects and evaluated their clinical correlation. Serum omentin levels were significantly decreased in diffuse cutaneous SSc patients compared with limited cutaneous SSc patients, while comparable between total SSc patients and healthy controls. In diffuse cutaneous (dc)SSc, patients with a disease duration of 5 years or less had serum omentin levels significantly lower than those with a disease duration of more than 5 years. In total SSc, serum omentin levels were significantly higher in patients with elevated right ventricular systolic pressure than in the others, while serum omentin levels did not correlate with fibrotic and systemic inflammatory parameters. These results suggest that a loss of omentin-dependent protection against vascular inflammation and remodeling may be related to pathological vascular events of early dcSSc. The elevation of serum omentin levels may serve as a marker of vascular involvement leading to pulmonary arterial hypertension in SSc, which is possibly due to the compensatory induction of omentin against the increased pulmonary vascular tone.

Key words: omentin, pulmonary arterial hypertension, systemic sclerosis, vascular inflammation, vasodilation.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem chronic disease characterized by vasculopathy and fibrosis of the skin and certain internal organs with an autoimmune background. Although tissue fibrosis is a prominent clinical feature of SSc, autoimmune attacks and vascular injuries prior to the onset of fibroblast activation appear to play critical roles in the pathogenesis of this complicated disorder.[1,2] Recently, a large attention has been paid to adipokines as a member of cytokine families contributing to the pathological activation of various cell types in SSc.[3–10]

Omentin is a member of the adipokines, and was identified in a human omental fat cDNA library and initially described as intelectin in murine small intestinal Paneth cells.[11,12] Omentin has been reported to be preferentially expressed and secreted by visceral adipose tissues, especially adipose tissue stromal vascular cells, but not adipocytes.[13] Adipokines are generally related to impaired glucose tolerance and a lack of omentin contributes to the development of insulin resistance and type 2 diabetes.[13,14] As well as other adipokines, omentin has various effects on endothelial cells (EC) and vascular smooth muscle cells (vSMC), linking metabolic syndrome with vascular complications, such as hypertension and atherosclerosis. In the isolated aorta of rat, omentin induces a phosphorylation of endothelial nitric oxide synthase (eNOS) at serine 1177, leading to nitric oxide (NO) production and subsequent vasodilation.[15] In humans, consistently, circulating omentin concentration positively correlates with endothelium-dependent vasodilation,[16] which is primarily mediated by NO.[17] Importantly, circulating omentin concentration increases in parallel with blood pressure,[18] suggesting that omentin has a compensatory mechanism in the regulation of vascular tone. Omentin also has anti-inflammatory effects on EC and vSMC. In human umbilical vein EC (HUVEC), omentin suppresses tumor necrosis factor (TNF)-α-induced cyclooxygenase-2 expression through activation of eNOS pathway. In vSMC, omentin inhibits TNF-α-induced expression of vascular cell adhesion molecule 1 and platelet-derived growth factor BB-induced migration through antioxidant mechanism.[19,20] Thus, omentin is an anti-inflammatory adipokine with a potent vasodilatory effect.

Given that vascular inflammation, remodeling and impaired endothelial function are involved in the development of

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SSc,[21–23] the multifaceted roles of omentin in these vascular events encouraged us to investigate the contribution of this adipokine to the pathological process of this disease. As an initial step to address this issue, we investigated serum omentin levels and their clinical association in SSc.

METHODS

Patients

Serum samples, frozen at −80°C until assayed, were obtained from 66 SSc patients (64 women, two men; age, median [25–75 percentile], 59 years [47.8–68.3]; disease duration, 6 years [2–19.5]; body mass index, 20.7 kg/m^2 [19.0–23.0]; systolic blood pressure, 110 mmHg [100–127]; diastolic blood pressure, 64 mmHg [60–72]) and 21 healthy individuals (18 women, three men; age, 53 years [44.5–66]; body mass index, 22.1 kg/m^2 [19.8–23.8]; systolic blood pressure, 118 mmHg [108–128]; diastolic blood pressure, 68 mmHg [62–72]) after obtaining informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Patients treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by LeRoy's classification system:[24] 35 patients with limited cutaneous SSc (lcSSc) (34 women, one man; age, 62 years [54–73]; disease duration, 7 years [2–20]; body mass index, 20.7 kg/m^2 [18.9–23.0]; systolic blood pressure, 118 mmHg [105–130]; diastolic blood pressure, 61 mmHg [59–73]) and 31 with diffuse cutaneous SSc (dcSSc) (30 women, one man; age, 51 years [46–63]; disease duration, 5 years [2–18]; body mass index, 20.6 kg/m^2 [18.9–22.7]; systolic blood pressure, 105 mmHg [98–124]; diastolic blood pressure, 64 mmHg [60–70]). All patients fulfilled the new classification criteria of SSc.[25]

Measurement of serum omentin levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum omentin levels (BioVendor Laboratory Medicine, Brno, Czech Republic). Briefly, 96-well polystyrene plates coated with antihuman omentin antibody were incubated with 40-fold diluted serum at 37°C for 2 h. After washing the wells, biotin-labeled polyclonal antihuman omentin antibody was added and incubated at 37°C for 30 min. Then, the wells were washed and incubated at 37°C for 30 min with horseradish peroxidase-conjugated streptavidin. Next, the wells were washed again, tetramethyl-benzidine added and incubated at room temperature for 10 min. Finally, sulfuric acid was added to terminate the reaction and absorbance at 450 nm was measured. Serum omentin levels were calculated using a standard curve.

Clinical assessments

Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time of blood sampling. The clinical and laboratory data were obtained when the blood samples were drawn. Skin score was measured using modified Rodnan total skin thickness score (MRSS).[26] The degree of interstitial lung disease (ILD) was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) on pulmonary function test and by ground-glass opacity score and fibrosis score.[27] Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more on echocardiogram. Scleroderma renal crisis (SRC) was defined as malignant hypertension and/or rapidly progressive renal failure. Estimated glomerular filtration rate (eGFR) was calculated from routine creatinine measurements using the Modification of Diet in Renal Disease equation.[28]

Statistical analysis

Statistical analysis was carried out with the Mann–Whitney *U*-test to compare the distributions of two unmatched groups, with one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, Fisher's exact probability test for the analysis of frequency, and Spearman's rank correlation coefficient to evaluate the correlation with clinical data. Statistical significance was set at $P < 0.05$.

RESULTS

Serum omentin levels in SSc

Because serum omentin levels are markedly elevated and positively correlate with serum creatinine levels in patients with end-stage renal disease undergoing chronic hemodialysis,[29] we first evaluated the correlation of serum omentin levels with eGFR in SSc patients with renal dysfunction (eGFR, <60 min/mL per 1.73 m^2). In five SSc patients with renal dysfunction, serum omentin levels did not correlate with eGFR ($r = 0.37$, $P = 0.50$). Furthermore, an SSc patient with SRC showed serum omentin levels (579.2 ng/mL) within the range of healthy controls (as shown in Fig. 1). Thus, renal dysfunction appeared not to affect serum omentin levels in SSc patients.

We next compared serum omentin levels between SSc patients and healthy controls. Although there was no significant difference in serum omentin levels between total SSc patients and healthy controls (761.4 [627.6–1029] vs 849.5 ng/mL [690.0–1040], $P = 0.69$), dcSSc patients had serum omentin levels significantly lower than lcSSc patients (715.4 [609.0–922.8] vs 980.2 ng/mL [693.5–1138], $P = 0.013$ [one-way ANOVA], $P < 0.05$ [Tukey's multiple comparison test]; Fig. 1). There was also a trend toward a decrease in serum omentin levels in dcSSc patients compared with healthy controls, but it did not reach a statistical significance ($P = 0.058$ by Mann–Whitney *U*-test). These results suggest that serum omentin levels may be related to disease process associated with dcSSc.

Serum omentin levels were decreased in dcSSc with disease duration of 5 years or less

Because dcSSc is characterized by extensive fibrotic response, especially in the skin and lung, a significant decrease of serum omentin levels in dcSSc suggests the potential contribution of omentin to the fibrotic process of this disease. Therefore, we examined the correlation of serum omentin levels with dermal and pulmonary fibrotic markers, such as MRSS, %VC and %DLco in total SSc, but failed to

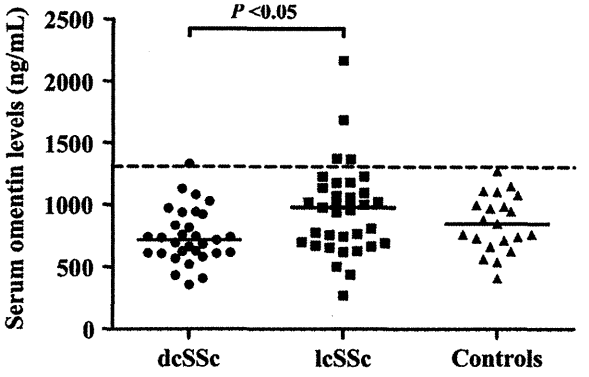

Figure 1. Serum levels of omentin in systemic sclerosis (SSc) patients and healthy individuals. Serum omentin levels were determined by a specific enzyme-linked immunoassay in healthy controls and diffuse cutaneous (dc)SSc and limited cutaneous (lc)SSc patients. The dashed line indicates the cut-off value (1307 ng/mL; mean ± 2 standard deviations of healthy controls). The horizontal bars indicate the median value in each group. Elevated serum levels of omentin were found in one of 31 dcSSc patients and four of 35 lcSSc patients. Statistical analysis was carried out with one-way ANOVA followed by Tukey's post-hoc test.

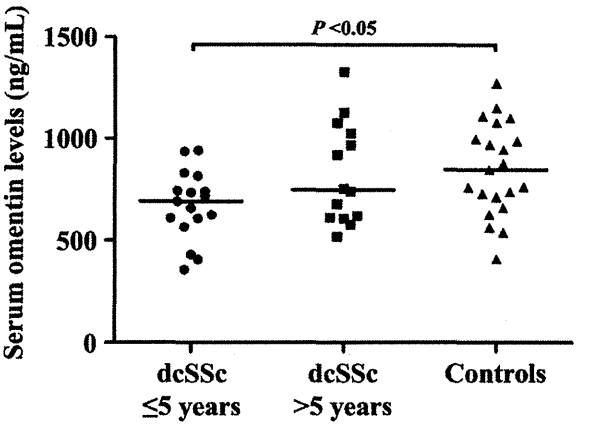

Figure 2. Serum omentin levels in diffuse cutaneous systemic sclerosis (dcSSc) patients further classified into subgroups based on the disease duration. dcSSc patients were divided into two subgroups: dcSSc patients with disease duration of 5 years or less and those with disease duration of more than 5 years. Serum omentin levels were determined by a specific enzyme-linked immunoassay. The horizontal bars indicate the median value in each group. Statistical analysis was carried out with one-way ANOVA followed by Tukey's post-hoc test.

detect significant correlations (data not shown). We also evaluated the correlation of serum omentin levels with ground-glass opacity score and fibrosis score in SSc patients with ILD, but did not observe any correlations (data not shown). Collectively, these results suggest that omentin is poorly involved in the fibrotic process of SSc.

Because we have demonstrated that serum levels of adipokines alter along with disease duration in dcSSc and a remarkable change is mostly seen in its early stage,[3–5,8] we next compared serum omentin levels between dcSSc with disease duration of 5 years or less and those with disease duration of more than 5 years. As shown in Figure 2, serum omentin levels were significantly decreased in dcSSc patients with a disease duration of 5 years or less (693.5 ng/mL [588.2–780.4]) as compared with healthy controls ($P = 0.035$ [one-way ANOVA], $P < 0.05$ [Tukey's multiple comparison test]), while there was no difference between the other pairs of groups. Taken together with the limited contribution of omentin to the fibrotic process, these results suggest that omentin may be involved in aberrant inflammation and/or vascular remodeling of SSc, which are extensively active in early dcSSc.

Clinical correlation of serum omentin levels in SSc

We next investigated the association of serum omentin levels with clinical features related to SSc vasculopathy. As shown in Figures 1 and 2, a decrease of serum omentin levels in the active stage is characteristic of dcSSc, but some of the SSc patients showed markedly elevated serum omentin levels, especially in lcSSc. In order to determine the clinical correlation of elevated serum omentin levels, we set the cut-off value at 1307 ng/mL (mean ± 2 standard deviations) based on the data of normal controls, which are normally distributed, and classified SSc patients into two groups: SSc patients with elevated serum omentin levels and those with normal levels. According to this criterion, 8% of SSc patients were assigned to the patient group with elevated serum omentin levels (1/31 dcSSc and 4/35 lcSSc). Patient information and the prevalence of clinical features associated with SSc vasculopathy in these two groups are shown in Table 1. There was no significant difference between these two groups in terms of sex, age, body mass index, disease duration and the frequency of dcSSc, while systolic and diastolic blood pressure levels were significantly higher in SSc patients with elevated serum omentin levels than in those with normal serum omentin levels, which is consistent with a previous report.[18] Regarding the frequency of cutaneous vascular manifestations, including Raynaud's phenomenon, nailfold bleeding, telangiectasia and pitting scars, there was no significant difference between two groups. As for organ involvement associated with proliferative obliterative vasculopathy, including digital ulcers, elevated RVSP and SRC, the frequency of elevated RVSP was significantly greater in patients with increased serum omentin levels than in those with normal levels (60% vs 13%, $P = 0.029$), while the frequencies of digital ulcers and SRC were comparable between two groups. We also compared serum omentin levels between SSc patients with each clinical symptom and those without, and confirmed that elevated RVSP is the only clinical symptom which affects serum omentin levels (Table 2). Viewed together, these results indicate that the elevation of serum omentin levels may be associated with pulmonary vascular involvement leading to pulmonary arterial hypertension (PAH).

We finally assessed the involvement of omentin in systemic inflammation of SSc. To this end, we evaluated the association

Table 1. Correlation of serum omentin levels with clinical symptoms in systemic sclerosis (SSc) patients

	Patients with elevated omentin levels (n = 5)	Patients with normal omentin levels (n = 61)
Sex, men : women	0:5	2:59
Age (years)	70.0 (54.5–75.0)	59.0 (47.5–67.5)
Body mass index	18.1 (15.6–23.0)	20.7 (19.2–23.0)
Systolic blood pressure (mmHg)	138 (123–143)*	109 (98–124)
Diastolic blood pressure (mmHg)	72 (67–85)**	60 (58–72)
Disease duration (years)	8.0 (4.0–30.5)	6.0 (2.0–20.0)
dcSSc : lcSSc	1:4	30:31
Cutaneous vascular symptoms		
Raynaud's phenomenon	100 (5/5)	93 (57/61)
Nailfold bleeding	75 (3/4)	69 (42/61)
Telangiectasia	50 (2/4)	55 (30/55)
Pitting scars	40 (2/5)	38 (23/61)
Organ involvements associated with proliferative vasculopathy		
Digital ulcers	40 (2/5)	30 (18/61)
Elevated RVSP	60 (3/5)***	13 (8/61)
Scleroderma renal crisis	0 (0/5)	3 (2/61)

*P = 0.0088 (Mann–Whitney U-test), **P = 0.028 (Mann–Whitney U-test), ***P = 0.029 (Fisher's exact probability test). dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; RVSP, right ventricular systolic pressure.

Table 2. Associations of serum omentin levels with clinical features in systemic sclerosis patients

	Serum omentin levels (ng/mL)		
Clinical symptoms	Patients with symptoms	Patients without symptoms	P
Raynaud's phenomenon	761.4 (627.6–1038), (n = 62)	816.7 (598.9–986.8), (n = 4)	0.69
Nailfold bleeding	877.6 (627.1–1038), (n = 46)	740.4 (626.8–816.4), (n = 19)	0.22
Pitting scars	756.4 (619.9–1044), (n = 25)	766.4 (641.7–1028), (n = 41)	0.61
Telangiectasia	792.4 (627.1–1025), (n = 32)	756.4 (611.0–1031), (n = 27)	0.73
Digital ulcers	765.9 (634.0–1074), (n = 20)	761.4 (625.6–1025), (n = 46)	0.61
Elevated RVSP	1029 (756.4–1331), (n = 11)	743.4 (618.9–1002), (n = 55)	0.011
Scleroderma renal crisis	636.4 (579.2–693.5), (n = 2)	770.9 (634.8–1030), (n = 64)	NA

Median (25–75 percentiles) is shown for each group. NA, not applicable; RVSP, right ventricular systolic pressure.

of serum omentin levels with high-sensitivity C-reactive protein and erythrocyte sedimentation rate in SSc patients, but these inflammatory parameters did not correlate with serum omentin levels (r = −0.003 and −0.07, respectively). These results suggest that omentin has little to do with the induction of systemic inflammatory status in SSc patients.

DISCUSSION

Several lines of evidence have demonstrated the critical contribution of adipokines to the pathogenesis of SSc;[3–10] therefore, the effects of omentin on vascular inflammation, remodeling and endothelial function encouraged us to investigate the potential role of this adipokine in the pathological process of this disease. A significant decrease of serum omentin levels in dcSSc as compared with lcSSc suggests the involvement of omentin in fibrotic response of this disease, but several parameters reflecting dermal and pulmonary fibrosis did not correlate with serum omentin levels. Of note, similar to adiponectin,[3] serum levels of omentin were significantly decreased in dcSSc patients with a disease duration of 5 years or less as compared with those with a disease duration of more than 5 years. As for clinical correlation, elevated RVSP was much more frequently seen in SSc patients with elevated serum omentin levels than in the other patients, and SSc patients with elevated RVSP had serum omentin levels significantly higher than those with normal RVSP. Given that early dcSSc is characterized by the extensive vascular activation leading to the development of tissue fibrosis and vasculopathy, these results suggest that omentin may be involved in the pathological vascular events related to the initiation of disease process as well as the development of PAH in SSc.

Systemic sclerosis vasculopathy is characterized by aberrant vascular remodeling, especially in small arteries, arterioles and capillaries.[1] Increasing evidence suggests that anti-EC antibodies and γδ T cells are involved in triggering initial vascular injury prior to vascular remodeling in SSc.[30–35] Of note, a recent study by Wolf *et al.*[36] revealed that antibodies against intercellular adhesion molecule 1 in patients' sera promotes the production of reactive oxygen species (ROS) in HUVEC, suggesting that SSc sera potentially induce ROS-dependent EC apoptosis and vSMC migration and proliferation, leading to the loss of small vessels and the fibro-proliferative remodeling of small arteries and arterioles characteristically seen in SSc.

These vascular changes induce the constitutive activation of fibroblasts partly through tissue hypoxia. Importantly, omentin activates the eNOS signaling pathway and inhibits oxidative stress, leading to the promotion of cell survival and differentiation in HUVEC.[15,37] In line with these findings, systemic delivery of an adenovirus expressing omentin rescues ischemic limbs in wild-type mice by enhancing blood flow recovery and capillary density, while not in eNOS-deficient mice, indicating that omentin promotes revascularization *in vivo* through the activation of the eNOS signaling pathway.[37] Therefore, a decrease of serum omentin levels may contribute to loss of neovascularization and aberrant vascular remodeling following vascular injuries in early dcSSc, leading to the development of tissue fibrosis and vasculopathy.

Despite a protective effect of omentin against vascular inflammation and remodeling, the elevation of serum omentin levels was associated with elevated RVSP in SSc patients. This finding is counterintuitive, but it is plausible when the altered vascular phenotype of SSc is taken into account. SSc is believed to develop as a result of the complex interaction between genetic factors and environmental influences.[38] Epigenetic analyses have identified many candidate genes which link environmental influences to the development of SSc.[39,40] As for SSc EC, the expression of the *NOS3* gene, which encodes eNOS, is constitutively suppressed through the hypermethylation of the CpG island in its promoter region.[41] Therefore, SSc EC are likely to be resistant to the preventive effects of omentin on vascular inflammation and remodeling because the activation of the eNOS signaling pathway is required for these effects. In healthy humans, serum omentin levels increase in parallel with blood pressure, suggesting that omentin has a compensatory mechanism in the regulation of vascular tone through its vasodilatory effect.[15,16,18] Although there is no data regarding the role of omentin in pulmonary vascular tone, the elevation of serum omentin levels in patients with elevated RVSP may reflect the compensatory effect of omentin on pulmonary arterial pressure. However, the pulmonary arteries of SSc patients may be less responsive to the vasodilatory effect of omentin due to epigenetic suppression of eNOS. Viewed together, serum omentin levels may serve as a serum marker reflecting the severity of pulmonary vascular involvement leading to PAH as a result of its compensatory response to increased pulmonary arterial pressure.

Adipokines have attracted much attention as a new cytokine family involved in the pathogenesis of SSc. Importantly, the expression of a certain subset of adipokines seems to be regulated as a result of SSc-related pathological changes rather than metabolic conditions.[3–8] For example, serum levels of adiponectin are markedly decreased in dcSSc patients with a disease duration of 5 years or less when the most prominent molecular changes occur during the whole course of this disease.[3] This notion is further supported by a decrease of serum omentin levels in dcSSc patients with a disease duration of 5 years or less as shown in the present study. To clarify this point, additional studies focusing on the role of adipose tissue in SSc is required in the future.

In summary, this is the first study reporting a possible contribution of omentin to the development of SSc vasculopathy. Relevant to its protective effect on vascular inflammation and remodeling, serum omentin levels were decreased in the early stage of dcSSc. Furthermore, omentin may be increased as a compensatory effect in SSc patients with pulmonary vascular involvement leading to PAH. The present data further support the emerging idea that adipokines are a member of cytokine families involved in the developmental process of SSc.

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CONFLICT OF INTEREST: The authors declare no conflicts of interest.

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CONCISE COMMUNICATION

Association of anti-RNA polymerase III antibody and malignancy in Japanese patients with systemic sclerosis

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ABSTRACT

Patients with systemic sclerosis (SSc) have an increased risk of malignancy compared with the general population. Recently, SSc patients with anti-RNA polymerase III antibody have been reported to have an increased risk of malignancy as compared with those with other disease-specific autoantibodies in US, European and Australian populations. Therefore, we studied the relationship between disease-specific autoantibodies and malignancy in 261 Japanese SSc patients. The prevalence of malignancy was significantly higher in patients with anti-RNA polymerase III antibody (7/22, 31.8%) than in those with anti-topoisomerase I antibody (2/82, 2.4%) and in those with anticentromere antibody (8/137, 5.8%). Importantly, among seven patients with anti-RNA polymerase III antibody and malignancy, three patients (42.9%) developed malignancy from 6 months before to 12 months after SSc onset. Thus, malignancy complication in Japanese SSc patients with anti-RNA polymerase III antibody is as high as that in other races, suggesting that SSc patients with anti-RNA polymerase III antibody share the same pathological process among different ethnic groups.

Key words: anti-RNA polymerase III antibody, Japanese, malignancy, synchronous onset, systemic sclerosis.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune inflammatory vascular disease characterized by extensive tissue fibrosis with unknown etiology.[1] Recently, the contribution of tumor immunity has attracted much attention in the pathogenesis of a certain SSc subset, namely, SSc patients with anti-RNA polymerase III (RNAP III) antibody. According to reports on US, European and Australian populations, SSc patients with anti-RNAP III antibody develop malignant tumors more frequently than those with anti-topoisomerase I (Topo-I) antibody or anticentromere antibody (ACA). More importantly, malignant tumors concomitantly occur in approximately 50% of SSc patients with anti-RNAP III antibody.[2–5] However, there has been no report regarding the close association of this antibody with malignancy in Japanese SSc patients. Therefore, we investigated the relationship between disease-specific autoantibodies and malignancy in our institution.

METHODS

Patients

We enrolled 261 patients (20 men and 241 women) clinically diagnosed as having SSc with each of three isolated SSc-related autoantibodies, including anti-Topo-I antibody, ACA and anti-RNAP III antibody, who presented to our institution between 2010 and 2014. All patients fulfilled the new classification criteria of SSc.[6] The presence of these three autoantibodies was determined by enzyme-linked immunoassays (ELISA) specific for each autoantibody (an ELISA kit for anti-Topo-I antibody from Fujirebio Diagnostic [Tokyo, Japan]; ELISA kits for ACA and anti-RNAP III antibody from Medical & Biological Laboratories [Tokyo, Japan]). Anti-Topo-I antibody and ACA were evaluated in all patients. Anti-RNAP III antibody was determined in all patients who first visited our department after May 2010, when an ELISA specific for anti-RNAP III antibody was approved by the Japanese Ministry of Health, Labor and Welfare. Regarding SSc patients who first visited our department before May 2010, anti-RNAP III antibody was evaluated at their first visit after May 2010 if patients had speckled antinuclear antibodies detected by indirect immunofluorescence on Hep-2 cells. For each patient, the data of SSc onset, which was defined as the first clinical event of SSc other than Raynaud's phenomenon, disease-specific autoantibodies, modified Rodnan total skin thickness score, complication of malignancy, interstitial lung disease, estimated right ventricular systolic pressure on echocardiography and scleroderma renal crisis, were obtained. SSc patients with anti-RNAP III antibody who did not have malignancy at the first

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Table 1. Neoplastic history in SSc patients with each of disease-specific autoantibodies

Characteristics	Anti-RNAP III (n = 22)	Anti-Topo-I (n = 82)	ACA (n = 137)
Total incidence of cancer	7 (31.8%)	2 (2.4%)	8 (5.8%)
Cancer preceding SSc onset	1 (4.5%)	0 (0%)	0 (0%)
Cancer synchronous to SSc onset	3 (13.6%)	0 (0%)	1 (0.7%)
Cancer succeeding SSc onset	3 (13.6%)	2 (2.4%)	7 (5.1%)

Preceding onset was defined as the diagnosis of cancer more than 6 months before the onset of SSc. Synchronous onset was defined as the diagnosis of cancer from 6 months before to 12 months after the onset of SSc. Succeeding onset was defined as the diagnosis of cancer more than 1 year after the onset of SSc. Two SSc patients with anti-Topo-I antibody had lung carcinoma and follicular carcinoma of thyroid, respectively. In SSc patients with ACA, there were eight cases with malignancy including one lung, one breast, one esophageal, one ureteral, one uterine, one cervical and two colorectal carcinomas. ACA, anticentromere antibody; RNAP III, RNA polymerase III; SSc, systemic sclerosis; Topo-I, topoisomerase I.

screening were followed up at least 1 year after the diagnosis of SSc. Institutional approval was obtained from the University of Tokyo Graduate School of Medicine.

Statistical analysis

Fisher's exact probability test was used for the analysis of frequency. Statistical significance was defined as a P value of less than 0.05.

RESULTS

Among SSc patients enrolled in this study, isolated anti-RNAP III antibody, anti-Topo-I antibody and ACA were found in 22, 82 and 137 patients, respectively. As shown in Table 1, a history of malignancy was present in 17 (7.1%) out of 261 SSc patients. The frequency of malignancy was significantly higher in the anti-RNAP III antibody group (7/22 [31.8%]) than in the anti-Topo-I antibody group (2/82 [2.4%] $P < 0.0005$) and in the ACA group (8/137 [5.8%], $P < 0.005$), while there was no correlation between the types of autoantibodies and malignancy. In addition, the frequency of malignancy diagnosed synchronously with SSc onset (from 6 months before to 12 months after, as previously described)[3] was significantly higher in the anti-RNAP III antibody group (3/22 [13.6%]) as compared with the anti-Topo-I antibody group (0/82 [0%], $P < 0.01$) and the ACA group (1/137 [0.7%], $P < 0.01$). As for the frequency of malignancy which developed preceding SSc onset (more than 6 months before), there were no statistically significant differences between the anti-RNAP III antibody group and the other two groups ($P = 0.21$ for anti-RNAP III antibody versus anti-Topo-I antibody, $P = 0.13$ for anti-RNAP III antibody versus ACA), and as for the frequency of malignancy which developed succeeding SSc onset (more than 1 year after), there were also no statistically significant differences between the anti-RNAP III antibody group and the other two groups ($P = 0.062$ for anti-RNAP III antibody versus anti-Topo-I antibody, $P = 0.14$ for anti-RNAP III antibody versus ACA). The characteristics of the seven patients with anti-RNAP III antibody are presented in Table 2. These seven patients did not show any specific clinical manifestation compared with the other 15 SSc patients with anti-RNAP III antibody (data not shown). Most importantly, 42.9% of malignancy occurred synchronously with SSc onset in the anti-RNAP III antibody group, which was consistent with the results of previous reports (Table 3).[3–5]

DISCUSSION

An increased risk of cancer among SSc patients had been demonstrated by nationwide population-based studies from Europe.[7,8] Possible explanations for the association of the two diseases are as follows: (i) common risk factors, including therapeutic exposure to immunosuppressive drugs and occupational exposure to solvents or silica dust; (ii) effects of chronic inflammation; and (iii) possible genetic predispositions to both diseases. According to these studies, cancer risk is particularly high in male patients, and, for lung cancer, in patients with interstitial lung disease. In addition to these findings, a new novel insight into the contribution of malignancy to autoimmunity in SSc has drawn much attention in the recent half

Table 2. Characteristics of SSc patients with anti-RNAP III antibody and malignancy

Patient no.	Sex	Age	Cancer, staging	Interval	Therapy and/or outcome	MRSS	SSc symptoms
1	F	72	Breast, stage I	5 months	Excision and hormone therapy	25	–
2	F	68	Colorectal, stage IV	1 month	Excision and chemotherapy	14	–
3	F	66	Ureteral, stage IV	−6 months	Relapsed after excision and chemotherapy; died from SRC 18 months after cancer diagnosis	37	SRC ILD
4	F	62	Gastric, stage I	7 years	Excision	23	ILD
5	M	62	Gastric, stage I	10 years	Excision	9	SRC, ILD
6	F	55	Colon, stage I	15 years	Excision	ND	–
7	F	46	Cervical, stage I	−10 years	Excision	31	–

Age, the age at the onset of SSc; ILD, interstitial lung disease; interval, the interval between the onset of SSc and the diagnosis of malignancy (minus means that malignancy is diagnosed prior to the onset of SSc); MRSS, the maximum modified Rodnan total skin thickness score throughout disease course; ND, not determined; SRC, scleroderma renal crisis; SSc, systemic sclerosis.

Table 3. Comparison of the present data with other reports

First author, reference	Reported year	Country or region	Malignancy complication rates in SSc with anti-RNAP III antibody	Synchronous onset
Airo *et al.*[3]	2011	Italy	43.8% (7/16)	42.9% (3/7)
Nikpour *et al.*[4]	2011	Australia	20.3% (14/69)	42.9% (6/14)
Moinzadeh *et al.*[5]	2014	Europe	14.2% (41/288)	55.3% (21/38)
Present study		Japan	31.8% (7/22)	42.9% (3/7)

RNAP III, RNA polymerase III; SSc, systemic sclerosis.

decade. In 2010, Shah *et al.*[2] first reported a possible association of anti-RNAP III antibody with a concomitantly developing SSc malignancy in the small number of US patients. Then, after the specific ELISA kit for anti-RNAP III antibody became commercially available, this notion has been validated in the independent SSc patient groups of European and Australian populations.[3–5] Under such conditions, we further confirmed this notion in Japanese SSc patients. The major findings were as follows: (i) the frequency of malignancy cases in SSc patients with anti-RNAP III antibody is significantly higher than in those with anti-Topo I antibody or ACA; (ii) SSc patients with anti-RNAP III antibody are closely concerned with synchronous malignancy complication; and (iii) the percentage of SSc patients with metachronous malignancy is not significantly different among the three groups of patients with each autoantibody. Importantly, all of these data were similar to those reported in the previous reports (Table 3).[3–5] Therefore, this notion is applicable to worldwide SSc patients, irrespective of their ethnicity.

Recently, a series of excellent studies demonstrated a possible mechanism underlying the close association of anti-RNAP III antibody with malignancy in SSc patients. Joseph *et al.*[9] found genetic alterations of the polymerase III polypeptide A locus in tumor cells in six of eight SSc patients with anti-RNAP III antibody, but in none of eight SSc patients with anti-Topo-I antibody or ACA. Analyses of peripheral blood lymphocytes and serum suggested that these mutations trigger cellular immunity and cross-reactive humoral immune responses. In addition, Shah *et al.*[2] showed that tumor cells from SSc patients with anti-RNAP III antibody express RNAP III protein at high level, while RNAP III expression is under detectable level in tumor cells from SSc patients without anti-RNAP III antibody. Given that RNAP III regulates the transcription of ribosomal RNA and transfer RNA and that the expression levels of RNAP III protein proportionally reflect the growth potential of tumors,[10] the cancer immuno-editing theory may contribute to the development of SSc associated with anti-RNAP III antibody and malignancy. Cancer immuno-editing is a unifying concept explaining the dual impact of immune system on host defense and tumor progression.[11,12] In the initial stage of tumor immunity, tumor growth is suppressed through the destruction of tumor cells and the inhibition of their outgrowth (i.e. elimination phase). However, when the interaction between cancers and immune response is prolonged due to cancer–immune equilibrium (i.e. equilibrium phase), tumor immunity then promotes tumor progression by selecting tumor cells with non-immunogenic phenotype (i.e. escape phase). During the equilibrium phase, generally lasting over a period of many years, tumor cells gain various genetic and epigenetic changes causing their phenotypical alteration. Taking this notion into account, in SSc patients with anti-RNAP III antibody, highly progressive tumor cells escaping from immune system express mutant RNAP III proteins at high level and trigger cellular immunity and cross-reactive humoral immune responses, leading to the development of SSc. A short cancer–autoimmune disease interval has also been described in patients with other autoimmune diseases, such as myositis, vasculitis and systemic lupus erythematosus, to which similar mechanisms could be applicable.[13–15]

In summary, we first confirmed the high prevalence and synchronous onset of malignancy in Japanese SSc patients with anti-RNAP III antibody. These results suggest that SSc patients with anti-RNAP III antibody share the same pathological process among different ethnic groups, even though the racial difference is present in the clinical features of SSc patients.

CONFLICT OF INTEREST: None.

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