Table 1. Summary of miRNA expression profiles in the skin of LSc and Keloid by PCR array analyses

micro RNA	LSc	Keloid	Healthy control	micro RNA (continue)	LSc	Keloid	Healthy control
let-7a	2.97	74.9	-0.12	miR-134	9.67	6.36	6.62
let-7b	2.16	1.06	0.05	miR-137	13.5	68.1	10.4
let-7c	6.42	67.9	3.31	miR-141	8.68	5.53	3.74
let-7d	9.43	7.90	7.18	miR-142-3p	5.41	68.0	5.21
let-7e	6.17	74.9	3.13	miR-142-5p	11.1	12.2	10.1
let-7f	11.37	75.0	9.14	miR-146a	5.83	67.9	2.90
let-7g	4.57	67.9	1.84	miR-146b-5p	6.25	3.47	3.20
let-7i	4.55	2.12	3.16	miR-150	3.37	ND	1.35
miR-1	9.17	8.96	-0.89	miR-155	7.47	6.84	6.99
miR-7	10.6	7.87	7.47	miR-181a	7.33	4.39	5.57
miR-9	12.0	7.65	7.75	miR-182	9.05	67.9	4.90
miR-10a	6.83	74.9	3.45	miR-183	10.6	68.1	7.39
miR-10b	6.47	2.26	1.70	miR-185	8.22	5.98	5.61
miR-15a	7.91	4.40	4.56	miR-18a	9.07	67.9	6.80
miR-15b	6.11	3.12	2.40	miR-18b	8.18	9.42	7.94
miR-16	2.96	-0.12	-1.28	miR-192	8.67	7.26	6.90
miR-17	7.20	4.80	3.80	miR-194	8.78	7.15	6.36
miR-20a	6.69	4.26	3.34	miR-195	2.97	-0.67	-1.43
miR-20b	10.3	8.46	7.44	miR-196a	8.00	4.58	3.96
miR-21	0.68	-4.87	-1.77	miR-205	2.48	0.64	-0.69
miR-22	5.06	1.60	2.59	miR-206	11.9	13.8	3.11
miR-23b	3.57	0.87	-0.84	miR-208	14.2	17.0	10.6
miR-24	2.70	0.25	-0.41	miR-210	8.28	7.03	5.97
miR-26a	1.89	74.9	-1.94	miR-214	6.45	2.62	3.51
miR-33a	11.6	7.83	6.54	miR-215	12.0	74.7	9.72
miR-92a	3.03	1.49	-0.56	miR-218	48.5	74.9	ND
miR-93	7.13	5.24	4.95	miR-219-5p	15.7	11.3	9.56
miR-96	10.7	ND	6.34	miR-222	5.84	68.0	4.42
miR-99a	4.94	1.01	0.55	miR-223	4.66	ND	2.20
miR-100	4.96	1.78	0.83	miR-301a	12.5	10.2	9.51
miR-101	9.03	6.42	4.10	miR-302a	9.47	14.6	12.8
miR-103	5.50	3.07	3.15	miR-302c	12.9	14.2	16.2
miR-106b	7.88	67.8	5.07	miR-345	9.39	8.06	8.31
miR-122	5.87	4.20	10.8	miR-370	8.70	8.84	10.3
miR-124	11.4	12.0	9.30	miR-371-3p	15.1	ND	13.3
miR-125a-5p	4.71	1.91	0.01	miR-375	8.91	8.48	5.10
miR-125b	1.47	-2.36	-3.23	miR-378	7.29	5.78	2.51
miR-126	3.26	0.35	-0.55	miR-424	6.27	0.34	4.99
miR-127-5p	12.5	9.32	11.2	miR-452	11.8	9.70	7.94
miR-128a	8.33	6.15	3.85	miR-488	15.3	14.8	14.2
miR-129-5p	11.1	12.9	9.51	miR-498	18.7	20.8	16.4
miR-130a	9.99	7.69	6.22	miR-503	12.0	8.56	ND
miR-132	9.51	7.07	6.51	miR-518b	14.6	16.4	17.0
miR-133b	12.5	14.8	4.57	miR-520g	13.0	12.0	10.2

A mixture of equal amounts of miRNAs from three LSc, three Keloid, and three healthy controls were prepared. The miRNA expression profiles were examined by PCR array. The raw threshold cycle (Ct) was normalized using housekeeping miRNA. The ΔΔCt (the raw Ct of each miRNA – Ct of housekeeping miRNA) is shown. The yellow boxes indicate the decreased miRNAs, which were one tenth lower than healthy controls, and the orange boxes indicate the increased miRNAs, more than tenfold higher. The red-letter indicates the miRNAs decreased in LSc and with no change in keloid patients. ND; not detected.

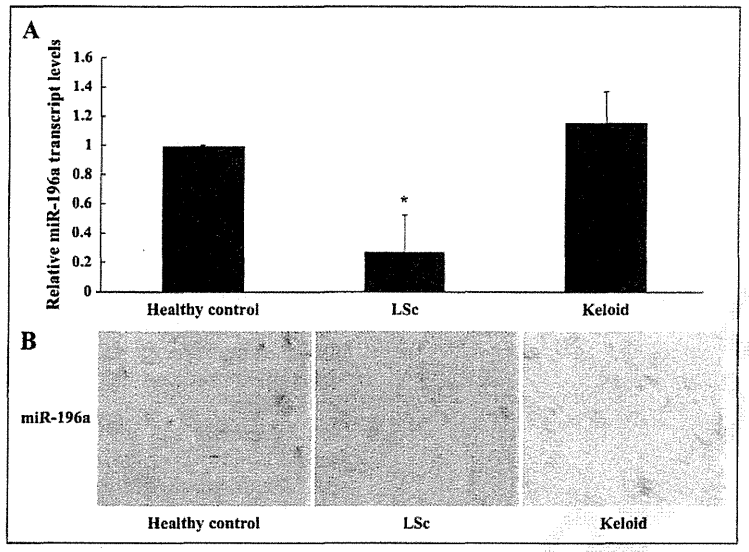

Figure 1. A) Mean expression levels of miR-196a in the skin tissue of 3 healthy control skins, 3 localized scleroderma (LSc) and 3 keloids were measured by real–time PCR using specific primer for miR-196a. Data was presented as mean+S.E. * $p<0.05$ was carried out with Mann-Whitney test as compared with the values of healthy controls (1.0). **B)** *In situ* detection of miR-196a in the paraffin-embedded, formalin-fixed tissues of healthy control skin, localized scleroderma (LSc) and keloid. The nucleus was counterstained with nuclear fast red. The miR-196a stained brown.

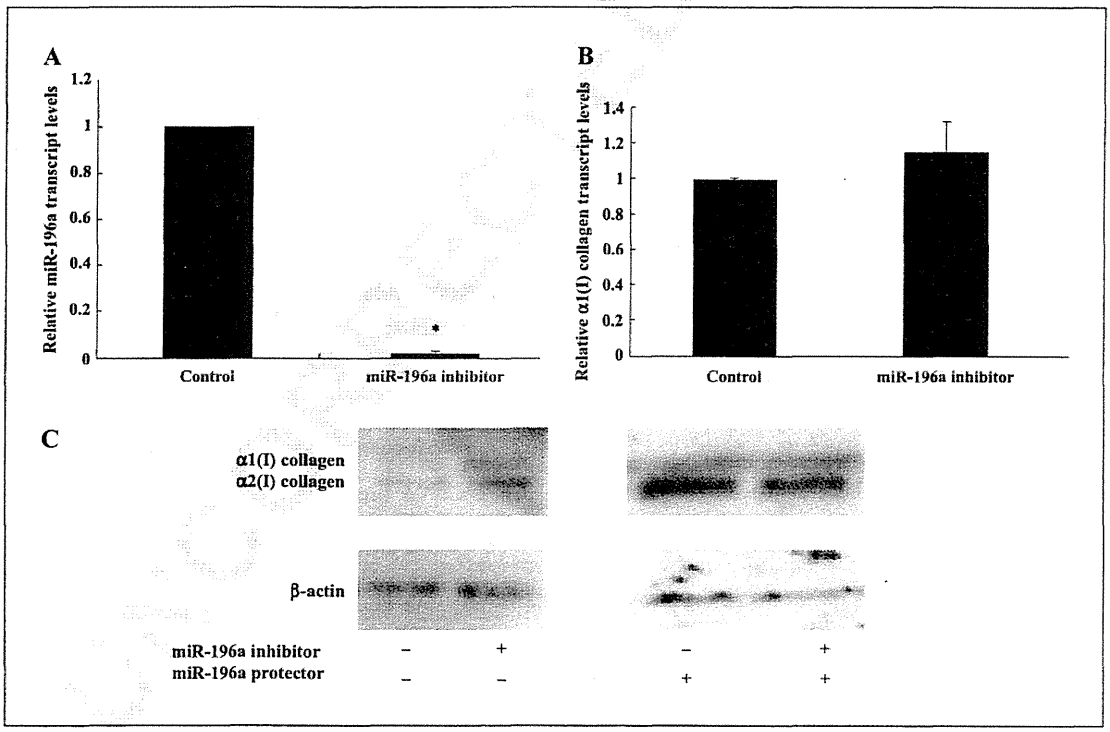

Figure 2. A,B) The expression levels of miR-196a **(A)** and α1(I) collagen mRNA **(B)** in HDFs transfected with control or the specific inhibitor was measured by real-time PCR. The mean value transfected with control was set at 1. * $p<0.05$. **C)** HDFs were transfected with miR-196a inhibitor for 72hours in the presence or absence of miR-196a protector. Control inhibitor or protector was used for the control. Cell lysate were subjected to immunoblotting.
The protein levels of type I collagen with or without miR-196a inhibitor and protector, examined by immunoblotting.

pathogenesis of LSc remains to be elucidated. In the present study, miR-196a may play some role in the pathogenesis of cutaneous fibrosis, including SSc and LSc, but there may be a different etiology in keloids. This result supports the hypothesis that SSc and LSc could very likely represent two ends of a continuous spectrum of disease.

The transfection of a specific inhibitor for miR-196a into HDFs resulted in the increase of type I collagen protein

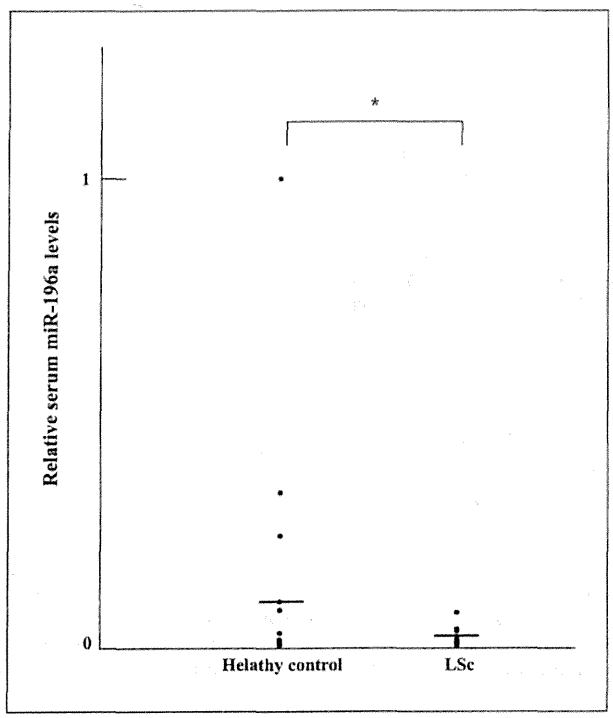

Figure 3. miRNAs were extracted from the sera of 34 LSc patients and 22 healthy controls. The serum miR-196a levels were measured using a specific primer for miR-196a by real-time PCR and corrected by the levels of cell miR-39. The horizontal bars show the mean value in each group. The maximum value in the healthy control was set at 1. * $p<0.05$ as compared with the values of healthy controls.

expression *in vitro*. Actually, according to miRNA target gene predictions using the TargetScan (http://www.targetscan.org/), MiRanda (http://www.microrna.org/), DIANAmicroT (http://diana.cslab.ece.ntua.gr/microT/), and PicTar (http://pictar.mdcberlin.de/), the leading program in the field [35-38], miR-196a is the potent regulator of both α1(I) and α2(I) collagen chains, components of type I collagen protein expression. We also demonstrated the decreased serum concentration of miR-196a in LSc patients. Not only a local decrease of miR-196a in the skin but systemic down-regulation of the miRNA may be involved in the formation of LSc. LSc sometimes poses many diagnostic challenges: Johnson *et al.* described that 63% patients were given the diagnosis more than 6 months after onset. Thus delay in diagnosis and treatment may cause depressed lesions of the face or deformities of the extremities, as described in the introduction [4, 39]. Serum miR-196a levels may be helpful as a diagnostic biomarker for LSc. Although we could not find any significant correlation between serum miR-196a levels and clinical or laboratory findings, this may be because of the small number of patients due to the rarity of this disease. Larger studies should be performed in the future.

We have reported that there were no significant differences in serum miR-196a levels between SSc and healthy controls [29]. The discrepancy between SSc and LSc may be explained by the notion that the fibrosis in lesions tends to be severer in LSc compared with SSc; for example, fibrosis in LSc sometimes extends to the muscular tissue or the bone beneath cutaneous lesions, but not in SSc. Thus, lower miR-196a levels in LSc sera may reflect severer tissue fibrosis.

miRNA-target therapies have been ongoing in human clinical trials for hepatitis C and liver cancer. The strategy of miRNA-target therapies for skin fibrosis is channeled into two categories: increasing the expression of anti-fibrotic miRNAs and decreasing the expression of the profibrotic miRNAs [22]. The fibrosis of the skin, subcutaneous tissue and muscle in LSc is often refractory to current treatments, corticosteroids or immunosuppressive therapies, because the fibrosis is sometimes severer than in SSc. In order to enhance the effect of miRNA-target therapies, many more of the miRNAs involved in the pathogenesis of fibrosis must be revealed.

We considered the miR-196a family as the LSc-specific miRNAs. However, as seen in *table 1*, there were other miRNAs down-regulated in LSc but not in keloids, such as miR-9, 10b, 15a, b, 20a, 23b, 99a, 100 or 126. The relationship between these miRNAs and LSc needs to be examined in the future.

LSc may be a good model of cutaneous fibrotic conditions. Clarifying the regulatory mechanisms of collagen by miRNAs may lead to the development of new serum markers and therapeutic values for LSc, by transfection into involved skin *in vivo*. ■

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Brief Report

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Serum levels of leptin receptor in patients with systemic sclerosis

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Summary **Microvascular damage is one of the primary pathologic components of systemic sclerosis (SSc). Serological abnormalities of angiogenic and angiostatic factors in SSc have previously been described. Like these factors, the plasma levels of leptin were significantly elevated in patients with SSc in comparison to normal controls. However, leptin receptor has not been examined in patients with SSc. The current study used sandwich ELISA to evaluate the serum levels of leptin receptor in patients with SSc. Serum samples were obtained from 36 patients with SSc. Samples were also obtained from 12 healthy control subjects and 10 patients with scleroderma spectrum disorder (SSD) who did not fulfill the criteria for SSc but who had the potential to develop SSc. Mean serum leptin receptor levels were significantly higher in patients with SSD than in patients with SSc (255.7 ng/mL *vs.* 184.6 ng/mL, $p < 0.05$ according to a *Mann-Whitney* test). There were no statistically significant differences between healthy control subjects and patients with SSc. Clinical parameters were evaluated, and the frequency of esophageal reflux was significantly lower in patients with elevated serum leptin receptor levels than in those with reduced levels (6.3% *vs.* 35.3%, $p < 0.05$). In summary, these results suggest that the serum levels of leptin receptor are a clinically useful marker of SSD, and measurement of serum leptin receptor over time in patients with SSD may lead to early detection of SSc.**

Keywords: Leptin receptor, systemic sclerosis, scleroderma spectrum disorder, ELISA

1. Introduction

Systemic sclerosis (SSc), or scleroderma, is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of SSc is still unclear, it includes inflammation, autoimmune attack, and vascular damage. The condition leads to the activation of fibroblasts and abnormal accumulation of extracellular matrix, mainly in the form of type I collagen (*1,2*).

Vascular damage is one of the primary pathologic components of SSc. Raynaud's phenomenon, or aberrant nailfold bleeding, is known to be an early vascular event associated with this disease. Telangiectasias, pitting scars, skin ulcers, impaired wound healing, and pulmonary hypertension are frequently observed in the disease process and can severely affect the quality of life of these patients. Serological abnormalities of angiogenic and angiostatic factors, including vascular endothelial growth factor, angiopoietin-2, and platelet-derived growth factor, in SSc have previously been described; uncontrolled activation of such signaling rather than its inactivation may be the cause of the disturbed vessel morphology in sclerotic skin (*3,4*).

Significantly increased plasma levels of leptin were also reported in patients with SSc in comparison to normal controls (*5*). Leptin, the *ob* gene product consisting of 146 amino acid residues, is known to be secreted by adipocytes (*6*). Leptin helps to regulate body weight by affecting food intake, energy expenditure, and thermogenesis (*7*). Furthermore, leptin is involved in many physiological processes, including angiogenesis, by stimulating endothelial cell proliferation (*8*).

Leptin takes action by binding to its receptor. The leptin receptor usually consists of an extracellular domain and cytoplasmic portion and is restricted to the cell surface. Leptin receptor levels are highest in

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infants, decrease into adolescence, and remain stable throughout adulthood (*9*). The receptor is expressed predominantly in areas of the hypothalamus, indicating that the leptin receptor also plays an important role in regulating body weight (*7*). That said, the receptor is also associated with conditions that negatively affect health. The extracellular domain of the receptor can be secreted into body fluid in soluble form. Soluble leptin receptor is found to be up-regulated in patients with obesity as well as in chronic heart failure, end-stage renal disease, and anorexia (*10*). However, serum leptin receptor levels have not been examined in patients with rheumatic diseases.

The current study posited that leptin signaling contributes to the pathogenesis of vascular damage in SSc and it sought to evaluate the potential for serum levels of leptin receptor to be a useful marker of SSc.

2. Materials and Methods

2.1. *Clinical assessment and patient characteristics*

The rheumatic diseases of systemic lupus erythematosus (SLE), dermatomyositis (DM), and SSc as are associated with vasculopathy or dysfunction of endothelial cells were studied. Patients with SSc or SLE fulfilled the criteria proposed by the American College of Rheumatology (ACR) (*11,12*). SSc was categorized as diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy *et al.* (*13*). The concept of scleroderma spectrum disorder (SSD) was originally proposed by Maricq *et al.* to unify typical SSc, early forms of SSc, and closely related disorders, including mixed connective tissue disease (MCTD) (*14,15*). Ihn *et al.* later redefined SSD as patients did not fulfill the criteria for SSc but some later developed SSc, so they suggested a new method of diagnosis using a point system to distinguish SSD from early SSc. A total score was obtained as the sum of the following five factors: *i*) extent of skin sclerosis (maximum, 10 points), *ii*) pulmonary changes (maximum, 4 points), *iii*) antinuclear antibodies (maximum, 5 points), *iv*) pattern of Raynaud's phenomenon (maximum, 3 points), and *v*) nailfold bleeding (maximum, 2 points). A score of 9 or more points is consistent with SSc and a score of 5 to 8 points is consistent with SSD (*16*). Patients diagnosed with SSD who fulfilled the criteria proposed by Ihn *et al.* (*16*) were also included in the current study. Classical DM was diagnosed based on the criteria proposed by Bohan and Peter (*17*). Clinically and histopathologically typical cutaneous lesions without classical myositis were diagnosed as clinically amyopathic DM (CADM) in accordance with previous criteria (*18,19*). Clinical and laboratory data reported in the current study were obtained at the time of serum sampling.

2.2. *Measurement of leptin receptor levels*

Levels of serum leptin receptor were measured with a specific ELISA kit (Human leptin receptor, BioVendor Laboratory Medicine, Czech Republic) (*20*). Briefly, monoclonal anti-human leptin receptor antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies against leptin receptor. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase and absorbance at 450 nm was measured. Wavelength correction was performed based on absorbance at 630 nm. The level of leptin receptor in each sample was determined by interpolation from a standard curve.

2.3. *Statistical analysis*

Statistical analysis was carried out with a *Mann-Whitney* test for the comparison of median, and Fisher's exact probability test for the analysis of frequency. A p less than 0.05 was considered significant.

3. Results and Discussion

3.1. *Serum levels of leptin receptor in patients with SSc*

Serum leptin receptor levels in patients with various rheumatic diseases and in healthy control subjects are shown in Figure 1. Serum samples were obtained from 36 patients with SSc (13 dcSSc and 23 lcSSc). Samples were also obtained from 12 healthy control subjects, 10 patients with SLE, 15 patients with DM, 5 patients with CADM, and 10 patients with SSD who did not fulfill the criteria for SSc but who had the potential to develop SSc. Patients with diabetes, obesity, atherosclerosis, or metabolic syndrome and those who had been treated were excluded.

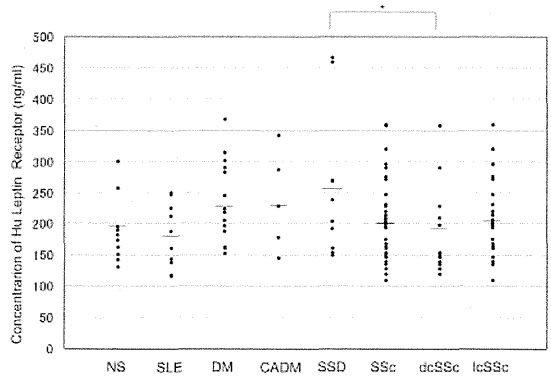

Figure 1. Serum concentrations of leptin receptor in patients with SSc, SSD, SLE, classical DM, CADM, and NS. SSc was classified as dsSSc or lcSSc. Serum leptin receptor levels were measured with an ELISA kit as described in materials and methods. Serum leptin receptor concentrations are shown on the ordinate. Bars indicate means. A p less than 0.05 is considered significant.

Mean serum leptin receptor levels were highest in patients with SSD. Although mean leptin receptor levels were elevated in patients with SSD compared to those in patients with SSc or in healthy control subjects, there were no statistically significant differences among the groups. Mean serum leptin receptor levels were significantly higher in patients with SSD than those in patients with dcSSc (255.7 ng/mL *vs.* 184.6 ng/mL, $p < 0.05$). Serum leptin receptor levels may transiently increase in the SSD stage but normalize in the SSc stage. Because progressive tissue fibrosis caused by SSc is often irreversible, at least clinically, new strategies need to be quickly developed to diagnose patients as early as possible and follow them closely. Accordingly, the concept of SSD should be better understood and characterized. The current findings suggest that elevated serum leptin receptor levels may serve as a useful marker for the differentiation of SSD from SSc. Moreover, patients with SSD frequently have an increased risk of developing SSc in the future. Measurement of serum leptin receptor levels over time in patients with SSD may lead to early detection of SSc. The current study did have one limitation in that it involved a small sample of patients with SSD because SSD is a fairly rare disease. However, the current approach may be a useful way to diagnose SSD. Studies with larger samples are needed in the future.

3.2. *Correlation of serum leptin receptor levels with clinical and serological features of SSc*

Table 1 shows the clinical and laboratory features of SSc in conjunction with elevated or reduced leptin receptor levels. There were no significant differences between these two groups in term of sex, age of onset, or the prevalence of dcSSc. However, esophageal reflux was significantly less prevalent in patients with elevated serum leptin receptor levels than in those with reduced levels (6.3% *vs.* 35.3%, $p < 0.05$). As noted earlier, leptin receptor was thought to possibly be involved in the pathogenesis of vascular abnormalities in patients with SSc, but serum levels were not associated with the prevalence of Raynaud's phenomenon, pitting scars, nailfold bleeding, or pulmonary hypertension. Esophageal reflex is treatable and reversible but cannot be detected by serology. Serum leptin receptor may serve as a clinically useful marker. Since patients with SSD had higher leptin receptor levels than both patients with SSc and control subjects and since patients with SSc and elevated serum levels had less esophageal reflux, leptin receptor levels may increase in the very early stage of this disease and thus act to mask the SSc phenotype. However, the exact role of leptin receptor in the pathogenesis of SSc remains unclear. Further studies are needed to clarify these aspects.

In conclusion, mean serum leptin receptor levels were highest in patients with SSD. These results suggest that elevated serum leptin receptor levels may serve as a useful marker for the differentiation of SSD from SSc and the reduced prevalence of esophageal reflex in SSc.

Table 1. Correlation of serum leptin receptor levels with clinical and serological features in patients with systemic sclerosis (SSc)

Items	Serum leptin receptor levels	
	Elevated ($n = 18$)	Low ($n = 18$)
Age at the time of serum sampling (mean years, interquartile range)	63.7	59.5
Duration of disease (mean years, interquartile range)	102	41.3
Type (diffuse: limited)	6:11	8:09
MRSS (point)	8.4	13.17
Clinical features		
Pitting scars/ulcers	41.2	50
Nailfold bleeding	58.8	66.7
Raynaud's phenomenon	88.2	92.9
Telangiectasia	25	21.4
Contracture of phalanges	85.7	85.7
Calcinosis	0	12.5
Diffuse pigmentation	36.4	20
Short SF	69.2	81.8
Sicca symptoms	53.9	53.9
Organ involvment		
Pulmonary fibrosis	29.4	44.4
Mean % VC (%)	105	97.6
Mean % DLco (%)	89.6	84.2
Pulmonary hypertension	27.9	28.3
Oesophagus	6.3*	35.3
Heart	37.5	17.7
Kidney	0	0
Joint	50	66.7
Thrombosis	0	0
ANA specificity		
Anti-topoI	22.2	33.3
Anti-centromere	50	27.8
Anti-U1 RNP	16.7	16.7

Unless indicated, values are percentages. MRSS: Modified rodnan total skin thickness score; SF: Sublingual frenulum; VC: Vital capacity; DLco: Diffusion capacity for carbon monooxidase; ANA: Antinuclear antibodies; Anti-topo I: Anti-topoisomerase I antibody; Anti-centromere: Anti-centromere antibody. *$p < 0.05$ versus patients with normal leptin receptor levels according to a *Mann-Whitney* test.

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A simple and effective reconstructive technique for repairing defects of the upper lip using a hatchet flap

Dear Editor,

The upper lip is important in both an aesthetic and functional sense.[1,2] Various methods of reconstructing defects after surgical resection for upper lip tumors have been reported.[3–5] The upper lip is divided into medial and lateral aesthetic subunits, and we believe that it is important for the scars to match the aesthetic units or subunits.[2] Most flaps used for upper

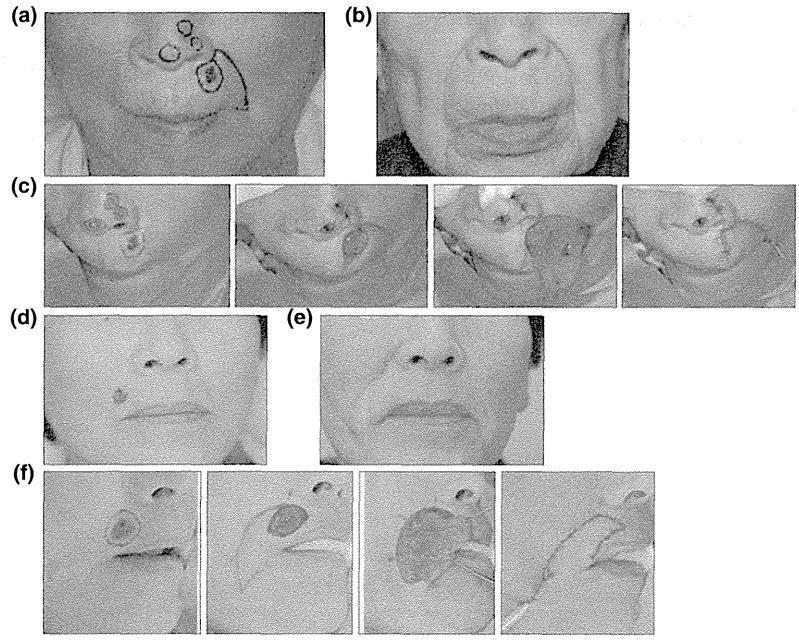

Figure 1. Case 1 (a–c). (a) Basal cell carcinoma located of the left side of the upper lip in an 88-year old male treated with a hatchet flap. (b) Six-month postoperative photograph of the patient. (c) Excision of the basal cell carcinoma of the upper lip (left end). The design of the hatchet flap after excision (second from the left end). The hatchet flap was elevated (third from the left end). The hatchet flap was meshed (the right end). Case 2 (d–f). (d) Basal cell carcinoma of the left side of the upper lip in a 79-year old female. (e) Six-month postoperative photograph of the patient. (f) Excision of the basal cell carcinoma of the upper lip (left end). The design of the hatchet flap after excision (second from the left end). The hatchet flap was elevated (third from the left end). The hatchet flap was meshed (the right end).

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lip reconstruction are beyond the aesthetic the unit or subunit. We herein report two cases reconstructed with hatchet flap.

CASE 1

An 88-year-old Japanese male presented with a 4-year history of a nodule on the left upper lip (Fig. 1a), which was diagnosed as basal cell carcinoma based on the histological findings. He subsequently underwent resection (surgical margin, 5 mm) with reconstruction of the 13 × 14 mm defect using a hatchet flap (Fig. 1c).[1] We confirmed the complete resection histologically. After 6 months, the patient was satisfied with the results of the surgery (Fig. 1b).

CASE 2

A 79-year-old Japanese female presented with a 3-year history of a nodule on the right upper lip (Fig. 1d) and was histologically diagnosed as basal cell carcinoma. We resected the tumor completely and reconstructed the 15 × 16 mm defect as in Case 1 (Fig. 1f). After 6 months, she was satisfied with the result of the surgery (Fig. 1e).

The patients were followed up for 14 (Case 1) and 25 (Case 2) months with no severe postoperative complications. Both flaps survived with a good match for color and texture. The flaps in the present two cases were dissected from the orbicularis oris muscle in the area of the upper lip. Hatchet flaps are superior to other flaps because they leave no conspicuous distortion of the mouth.[1] In addition, most hatchet flaps can be made without spreading out the lateral subunit of the upper lip.

In Case 2, the flap was beyond the nasolabial fold, and the scar had thus become slightly displaced from the nasolabial fold, however, a new nasolabial fold was created, and the symmetry of the patient's face was therefore relatively well preserved. Designing a large flap in the lateral subunit of the upper lip, as in Case 1, can help to avoid such issues.

Hatchet flaps have some disadvantages. For example, a trap door deformity can develop if a hatchet flap is used for a large defect near the center of the philtrum.[1] This flap is also not suitable for reconstruction for highly invasive tumors.

Hatchet flaps are useful and convenient for performing lateral part of upper lip reconstruction. These flaps leave minimal scars in inconspicuous areas, as the majority of scars match the lateral subunits, as shown in Case 1. In the setting of upper lip reconstruction, hatchet flaps are adaptable for defects measuring up to 20 mm in diameter without causing conspicuous distortion of the mouth.[1] We recommend this easy technique as the one of the best options with good patient acceptance.

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

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Aesthetic reconstruction of defects in the lateral side wall of the nose using a local V-Y flap

Dear Editor,

The nose is a prominent feature of the face; therefore, nasal reconstruction requires a good aesthetic outcome. The aesthetic units of the face have previously been described, with the nose being divided into subunits of the roof, dorsum, lateral side wall, tip, alar lobule and columella.[1] We believe that it is important for the scar to match the aesthetic unit or subunit.[2,3] Although various methods of reconstruction for defects after surgical resection of cancer in the lateral side wall of the nose have been reported, most of these reports have proposed methods of reconstruction beyond the aesthetic unit or subunit.[2,3] We herein report three cases of V-Y flap reconstruction

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CONCISE COMMUNICATION

A case of anaplastic large cell lymphoma of skeletal muscle

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ABSTRACT

Anaplastic large cell lymphoma (ALCL) is a high grade non-Hodgkin lymphoma (NHL) that is comprised of the malignant proliferation of large lymphoid cells, which express CD30. Primary ALCL of the skeletal muscle is extremely uncommon. A 51-year-old Japanese female presented at our hospital with a 2-month history of severe pain and swelling of the right leg. A gallium-67 SPECT/CT scan showed a large mass involving the skeletal muscles from the gluteus to femoris. A biopsy of the mass demonstrated diffuse infiltration of medium and large neoplastic cells with round or lobulated hyperchromatic pleomorphic nuclei. A subset of Reed-Sternberg-like cells was also identified. Immunohistochemically, the neoplastic cells were strongly positive for CD4 and CD30, but negative for CD3, CD8, anaplastic lymphoma kinase (ALK), CD20, CD79α, CD21 and CD23. Based on the histological examination, this patient was diagnosed to have ALK-negative ALCL of the skeletal muscle. Further studies are needed to clarify the biological behavior of primary skeletal muscle ALCL.

Key words: ALK, CD30, extranodal involvement, non-Hodgkin lymphoma, T cell lymphoma.

INTRODUCTION

Anaplastic large cell lymphoma (ALCL) is a high grade non-Hodgkin lymphoma (NHL) that is comprised of the malignant proliferation of large lymphoid cells, which express CD30.[1] Most of the cases of ALCL occur in nodal sites, but a few cases develop in extranodal sites. The extranodal sites of ALCL include the skin, bone, soft tissue, and gastrointestinal tract.[2] Primary skeletal muscle ALCL is extremely uncommon and few cases have been described in the literature.[3]

We herein, report the case of a Japanese female diagnosed with anaplastic lymphoma kinase (ALK)-negative ALCL of the skeletal muscle.

CASE REPORT

A 51-year-old Japanese female presented with a 2-month history of severe pain and swelling of the right leg.

She had a history of atopic dermatitis from childhood. Since about 5 years prior to this episode, the condition of her dermatitis had often been exacerbated, she had sometimes developed erythroderma (Fig. 1a). Every time her dermatitis got worse, we treated her and tried to clarify the cause of her dermatitis aggravation, and considered the potential existence of a malignant tumor and malignant lymphoma. During exacerbations, she had increased serum IgE levels (1039–24 090 IU/mL) with eosinophilia (400–6209/μL). A histological examination revealed spongiosis and infiltration of lymphocytes and eosinophils. (Fig. 1b, c). We had therefore thought that the cause of her dermatitis was atopic dermatitis.

The patient was admitted to our university hospital for further evaluation after she had experienced a fever and night sweats for a week. A physical examination showed multiple hypopigmented plaques and macules present in the skin all over her body. Her right thigh was swollen (Fig. 2a), but there was no sign of lymphadenopathy or hepatosplenomegaly. As before, a histological examination of the skin revealed spongiosis and the infiltration of lymphocytes and eosinophils. Clonal T-cell receptor beta gene rearrangement was not detected in the skin by Southern blotting. The laboratory blood tests including the blood cell count and, renal functional parameters were within the normal range. However, the serum levels of lactate dehydrogenase (1384 U/L), creatine kinase (528 U/L), myoglobin (271.4 ng/mL), aldolase (67.3 U/L), soluble-interleukin 2 receptor (12 564 U/mL), and C-reactive protein (27.38 mg/dL) were markedly increased. A gallium-67 SPECT/CT scan showed a large mass involving the right gluteus maximus muscle, iliopsoas muscle, obturatorius internus muscle, external obturator muscle, biceps femoris muscle, semimembranosus muscle and semitendinosus muscle (Fig. 2b). Based on imaging findings, we suspected malignant lymphoma of the skeletal muscle. We performed CT-guided needle biopsy of the

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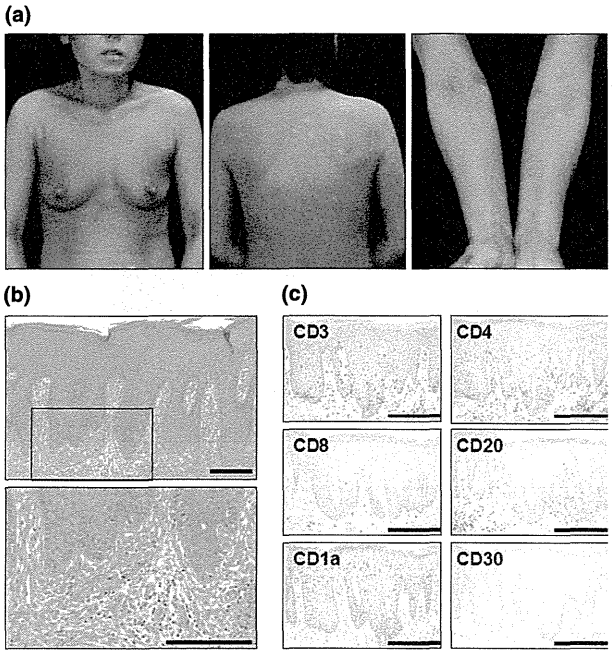

Figure 1. A skin lesion at the initial visit (4 years before the diagnosis). (a) Her skin was generally dry and slightly scaly. Multiple hypopigmented plaques and macules were present in the skin all over the body. (b, c) A biopsy specimen from the patient. H&E staining revealed compact hyperkeratosis and irregular acanthosis. Mild spongiosis was seen throughout the epidermis, and there was lymphocyte and eosinophil infiltration in the upper dermis (b). Immunohistochemical staining showed that the infiltrating cells were reactive for CD3, CD4, CD20 and slightly for CD8, but were negative for CD1a and CD30. Scale bars: 500 μm (b), 20 μm (c).

mass to obtain a diagnosis. The biopsy of the mass demonstrated a diffuse infiltration of medium and large neoplastic cells with round or lobulated hyperchromatic pleomorphic nuclei. A subset of Reed-Sternberg-like cells was also identified (Fig. 3a). Immunohistochemically, the neoplastic cells were strongly positive for CD4 and CD30, but negative for CD3, CD8, ALK, CD20, CD79α, CD21 and CD23 (Fig. 3b). Epstein Barr virus (EBV) was not detected. The findings of bone marrow aspiration were also normal. Based on the histological and immunophenotypic findings, she was diagnosed to have CD30-positive ALK-negative ALCL. Therefore, we planned to give the patient systemic chemotherapy. However, her general condition rapidly deteriorated. She died of multiple organ failure 2 months after the admission to our hospital. An autopsy was not performed.

DISCUSSION

Anaplastic large cell lymphoma is comprised of two clinically distinct subtypes referred to as primary systemic ALCL and primary cutaneous ALCL.[4] Additionally, according to the fourth edition of the World Health Organization (WHO) classification, there are two types of systemic ALCL: ALK-positive ALCL, and

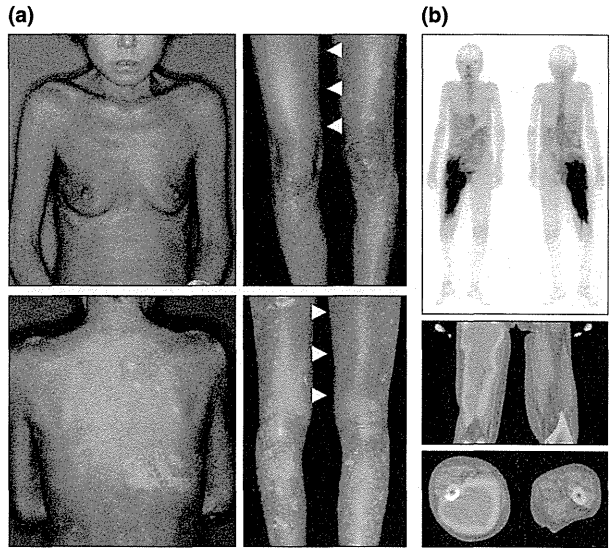

Figure 2. The skin and imaging findings during the admission. (a) Her skin was scaly, brownish red, hot and dry, and multiple hypopigmented plaques and macules were present in the skin all over her body. The right thigh was swollen (arrow heads). (b) A gallium-67 SPECT/CT scan showed the involvement of the right gluteus maximus muscle, iliopsoas muscle, obturatorius internus muscle, external obturator muscle, biceps femoris muscle, semimembranosus muscle and semitendinosus muscle. Based on imaging findings, we suspected malignant lymphoma.

ALK-negative ALCL.[4] The presence of the ALK protein is important because of the positive linkage between the presence of ALK and the prognosis of the patient.[5] Our case had ALK-negative systemic ALCL.

The age of patients with ALK-positive ALCL is approximately two decades younger than that of patients with ALK-negative ALCL.[5] Patients with ALK-positive ALCL have a more favorable prognosis compared with ALK-negative patients (5-year overall survival: 70–80% and 33–49%, respectively).[5,6] This prognostic advantage is present in both children and younger adults with ALCL.[7]

Distinguishing ALCL from large-cell transformation of mycosis fungoides is often challenging.[8] If the patient's skin lesions that we diagnosed as atopic dermatitis were CTCL, the mass involving the muscles could be large cell transformation of CTCL. Although we examined T-cell receptor beta chain gene rearrangement using Southern blotting to detect the clonality of the skin lesion, the results could be false-negative. Furthermore, it is also possible that the skin lesion might be non-specific rash associated with ALCL as is sometimes seen in all forms of leukemia/lymphoma. Therefore, it is important to carefully follow patients with erythroderma in consideration of the potential existence of a malignant tumor or malignant lymphoma, even if the patient was diagnosed to have atopic dermatitis.

The most common extranodal sites of ALCL are the skin, subcutaneous tissue, bone marrow, bone, lung and gastrointestinal organs. Lesions in the skeletal muscles are extremely rare in extranodal ALCL.[9] Some cases may pres-

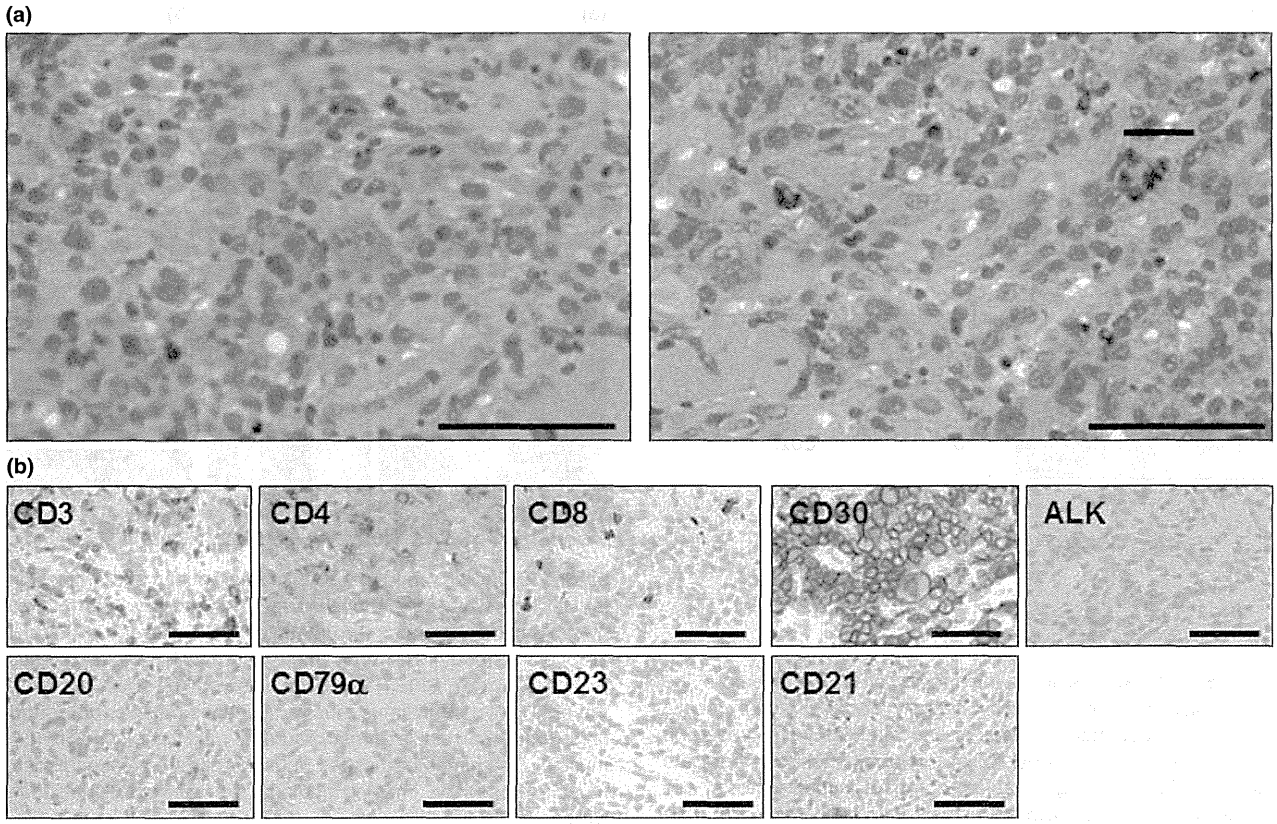

Figure 3. A CT-guided needle biopsy of the mass revealed ALK negative anaplastic large cell lymphoma. (a) H&E staining showed that there were isolated Reed-Sternberg-like cells (arrow in the left panel) and dense infiltration of pleomorphic cells with prominent nucleoli and mitotic figures (in the right panel). (b) Immunohistochemical staining demonstrated that the tumor cells were reactive for CD4 and CD30, but were negative for CD3, CD8, ALK, CD20, CD79α, CD21 and CD23. Scale bars, 200 μm.

ent with rhabdomyolysis, and less frequently with compartment syndrome.[10,11] Our patient presented with severe pain and B-symptoms (fever and night sweats), and with primary skeletal muscle CD30-positive, ALK-negative ALCL. The patient died 2 months after her admission, and similar survival of affected cases has been reported previously.[5,12] However, some reports indicated that patients with primary skeletal muscle ALCL responded to systemic chemotherapy. Most of these patients had the aforementioned favorable prognostic factors (ALK-positive, young age).[13,14]

Further studies are needed to clarify the biological behavior of primary skeletal muscle ALCL, and to identify effective treatments.

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

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lip reconstruction are beyond the aesthetic the unit or subunit. We herein report two cases reconstructed with hatchet flap.

CASE 1

An 88-year-old Japanese male presented with a 4-year history of a nodule on the left upper lip (Fig. 1a), which was diagnosed as basal cell carcinoma based on the histological findings. He subsequently underwent resection (surgical margin, 5 mm) with reconstruction of the 13 × 14 mm defect using a hatchet flap (Fig. 1c).[1] We confirmed the complete resection histologically. After 6 months, the patient was satisfied with the results of the surgery (Fig. 1b).

CASE 2

A 79-year-old Japanese female presented with a 3-year history of a nodule on the right upper lip (Fig. 1d) and was histologically diagnosed as basal cell carcinoma. We resected the tumor completely and reconstructed the 15 × 16 mm defect as in Case 1 (Fig. 1f). After 6 months, she was satisfied with the result of the surgery (Fig. 1e).

The patients were followed up for 14 (Case 1) and 25 (Case 2) months with no severe postoperative complications. Both flaps survived with a good match for color and texture. The flaps in the present two cases were dissected from the orbicularis oris muscle in the area of the upper lip. Hatchet flaps are superior to other flaps because they leave no conspicuous distortion of the mouth.[1] In addition, most hatchet flaps can be made without spreading out the lateral subunit of the upper lip.

In Case 2, the flap was beyond the nasolabial fold, and the scar had thus become slightly displaced from the nasolabial fold, however, a new nasolabial fold was created, and the symmetry of the patient's face was therefore relatively well preserved. Designing a large flap in the lateral subunit of the upper lip, as in Case 1, can help to avoid such issues.

Hatchet flaps have some disadvantages. For example, a trap door deformity can develop if a hatchet flap is used for a large defect near the center of the philtrum.[1] This flap is also not suitable for reconstruction for highly invasive tumors.

Hatchet flaps are useful and convenient for performing lateral part of upper lip reconstruction. These flaps leave minimal scars in inconspicuous areas, as the majority of scars match the lateral subunits, as shown in Case 1. In the setting of upper lip reconstruction, hatchet flaps are adaptable for defects measuring up to 20 mm in diameter without causing conspicuous distortion of the mouth.[1] We recommend this easy technique as the one of the best options with good patient acceptance.

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Aesthetic reconstruction of defects in the lateral side wall of the nose using a local V-Y flap

Dear Editor,

The nose is a prominent feature of the face; therefore, nasal reconstruction requires a good aesthetic outcome. The aesthetic units of the face have previously been described, with the nose being divided into subunits of the roof, dorsum, lateral side wall, tip, alar lobule and columella.[1] We believe that it is important for the scar to match the aesthetic unit or subunit.[2,3] Although various methods of reconstruction for defects after surgical resection of cancer in the lateral side wall of the nose have been reported, most of these reports have proposed methods of reconstruction beyond the aesthetic unit or subunit.[2,3] We herein report three cases of V-Y flap reconstruction

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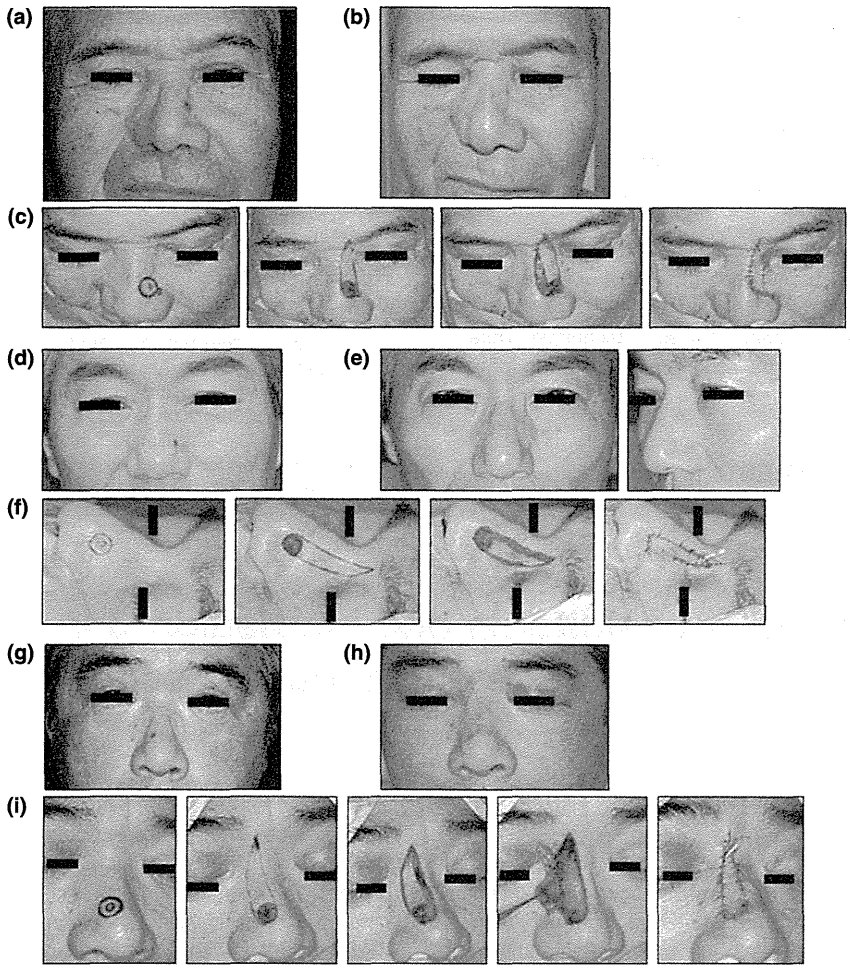

Figure 1. Case 1 (a–c). (a) Basal cell carcinoma in the left lateral wall of the nose of a 75-year-old male. (b) 6-month postoperative photograph of the patient. (c) The design for excision of the basal cell carcinoma of the upper lip (left end). The design of the V-Y flap after excision (second from the left end). The flap was elevated (third from the left end). The flap was meshed (right end). Case 2 (d–f). (d) Basal cell carcinoma in the left lateral wall of the nose of a 54-year-old male. (e) 6-month postoperative photograph of the patient. (f) The design for excision of the basal cell carcinoma of the upper lip (left end). The design of the V-Y flap after excision (second from the left end). The flap was elevated (third from the left end). The flap was meshed (right end). Case 3 (g–i). (d) Basal cell carcinoma in the right lateral wall of the nose of a 61-year-old male. (e) 1-week postoperative photograph of the patient. (f) The design for excision of the basal cell carcinoma of the upper lip (left end). The design of the V-Y flap after excision (second from the left end). The flap was elevated (third and fourth from the left end). The flap was meshed (right end).

in the lateral wall of the nose that was performed in consideration of the aesthetic units of the face.

Case 1: A 75-year-old Japanese male presented with a 5-month history of a black nodule on the left lateral side wall of the nose (Fig. 1a). He was diagnosed with basal cell carcinoma (BCC) based on the histological findings, and subsequently underwent complete resection of the tumor, with reconstruction of a 12 × 13 mm defect using a V-Y flap (Fig. 1c). After 6 months, he was satisfied with the results of the surgery (Fig. 1b).

Case 2: A 54-year-old Japanese male presented with a 1-year history of a gray-black nodule on the left lateral side wall of the nose (Fig. 1d), and was histologically diagnosed with BCC. We resected the tumor completely and reconstructed the 13 × 13 mm defect using a V-Y flap (Fig. 1f). After 6 months, he was satisfied with the results of the surgery (Fig. 1e).

Case 3: A 61-year-old Japanese male presented with a 5-year history of a black nodule on the right lateral side wall of the nose (Fig. 1g) and was histologically diagnosed with BCC. We resected the tumor completely and reconstructed the 14 × 13 mm defect using a V-Y flap (Fig. 1i). After 5 days, he was satisfied with the results of the surgery (Fig. 1h).

No severe postoperative complications were noted in any of the cases. In addition, the flaps survived with a good color and

texture match, and the donor and recipient sites functioned well without severe deformities. We designed the V-Y flap without spreading out the lateral side wall subunit of the nose. The flap was dissected from the perichondrium in the lateral side wall of the nose, and the pedicle included the transverse portion of the nasalis muscle. The flap is adaptable for defects measuring up to 15 mm in diameter without causing a conspicuous scar.

As with open treatment, skin grafting and other local flaps, the application of a V-Y flap is one of the most useful and convenient procedures for reconstructing the lateral wall of the nose. We therefore recommend this easy technique since it has been demonstrated to be associated with good patient acceptance.

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

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Value of serum procalcitonin and interleukin-6 in patients with bullous impetigo and staphylococcal scalded skin syndrome

Dear Editor,

Bullous impetigo (BI) and its generalized form, staphylococcal scalded skin syndrome (SSSS), which are caused by infection with strains of *Staphylococcus aureus*, are highly contagious and blistering skin diseases that often affect children.[1] Although serum levels of procalcitonin (PCT) and interleukin-6 (IL-6) have been shown to correlate with the extent and severity of bacterial infection,[2,3] few studies have measured their serum levels in patients with BI and SSSS. The current study compared the performance of serum white blood cell count (WBC), C-reactive protein (CRP), IL-6 and PCT in distinguishing children with BI and SSSS from healthy controls.

The study included 31 patients with SSSS and 30 patients with BI, along with 28 matched healthy volunteers. The institutional review board of Anhui Medical University approved the study (no. 20140146), informed consent was obtained from a guardian of each child. Serum PCT and IL-6 were determined using an electrochemiluminescence immunoassay method (Elecsys BRAHMS PCT/IL-6 Reagents; Roche Diagnostics, Shanghai, China). Serum CRP was determined using an immune turbidimetric method (DiaSys Diagnostic, Shanghai, China) and WBC using an XE2100 automated blood cell counter (Sysmex, Kobe, Japan). All statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered significant.

As demonstrated in Table 1, mean serum values of WBC, CRP, IL-6 and PCT in BI and SSSS patients were significantly higher than in healthy controls, and PCT and WBC in the SSSS group were significantly higher than in the BI group. Compared with individuals with early stage of the disease, serum PCT and IL-6 values were significantly lower in individuals in whom diseases were in remission. Of all biomarkers, PCT had the highest area under the receiver–operator curve (AUC), with cut-off values of 0.08 or 0.06 ng/mL for the diagnosis of SSSS or BI, respectively.

While IL-6 or PCT are reportedly reliable rule-in or rule-out diagnostic tools in neonatal sepsis, CRP and WBC are of limited potential in this regard.[4] Here, PCT and WBC were biomarkers which differed significantly between SSSS, BI and controls, and PCT had a higher diagnostic AUC than other biomarkers. Compared to the potential diagnostic PCT, due to the lower AUC, negative predictive and accuracy values, WBC, CRP and IL-6 were not suitable for being used as a diagnostic biomarker. Serum levels of both PCT and IL-6 were increased in patients and decreased when diseases were in remission, suggesting that these indices may potentially be used to monitor the efficiency of treatment of these conditions. PCT of 0.1 ng/mL or less has been used as a cut-off to signify the end-point of bacterial infection and discontinuation of antibiotic therapy,[5] and sustained low serum levels of IL-6 have been

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The expression profile of the toll-like receptor family in scleroderma dermal fibroblasts

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Competing interests: none declared.

ABSTRACT

Objectives. *The toll-like receptor (TLR) family is thought to be expressed in many cell types in the skin and play a role in various diseases. The expression pattern and role of TLRs in systemic sclerosis (SSc) is to be clarified. We investigated the expression profiles of TLR-related genes in SSc fibroblasts, and tried to clarify their roles in the pathogenesis of this disease.*

Methods. *The expression profile of TLR-related genes was assessed by gene array. Real-time PCR was used to confirm the array result. The protein expression of TLRs and type I collagen was determined by immunoblotting and immunohistochemistry.*

Results. *PCR array revealed that several genes were up- or down-regulated in SSc fibroblasts compared to normal cells. Among them, both mRNA and protein levels of TLR5 and TLR10 were up-regulated in SSc fibroblasts. The transfection of Smad3 siRNA into SSc fibroblasts resulted in the down-regulation of TLR proteins. There was no significant difference in mRNA half-lives of TLR5 and TLR10 between normal and SSc fibroblasts. Immunohistochemical staining revealed that TLRs expression was strongly detected in SSc fibroblasts in vivo. The stimulation of TLR5 signal with flagellin reduced the expression of type I collagen in SSc fibroblasts, but not in normal fibroblasts.*

Conclusion. *TLR5 and TLR10 expression is increased in SSc fibroblasts in vitro and in vivo, probably at transcript level via the TGF-β/Smad3 activation. Furthermore, TLR5 itself may have suppressive effects on collagen expression, and its overexpression in SSc fibroblasts may be the negative feedback against tissue fibrosis.*

Introduction

Systemic sclerosis (SSc) is an autoimmune disorder characterised by tissue fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, recent techniques such as genome-wide associated study suggested the involvement of various genes in the pathogenesis of SSc (1). Inflammation, autoimmune attack, and vascular damage may lead to the activation of fibroblasts and abnormal accumulation of extracellular matrix (ECM), mainly collagen (2, 3). Thus, abnormal SSc fibroblasts responsible for the fibrosis may develop from cells that have escaped from normal control mechanisms (4, 5).

Although the mechanism of fibroblast activation in SSc is unknown, many of the characteristics found in SSc fibroblasts resemble those in healthy fibroblasts stimulated by transforming growth factor (TGF)-β1 (6, 7). The principal effect of TGF-β1 on fibroblasts is induction of ECM deposition. Fibroblasts from affected SSc skin cultured *in vitro* can produce excessive amounts of collagens (8, 9), indicating that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF-β1 signalling. This notion is supported by the findings that the phosphorylated levels and DNA-binding activity of Smad3, an intracellular signal molecule of TGF-β1, are constitutively up-regulated in SSc fibroblasts (10). TGF-β1 may also contribute to the activation of Abelson kinase (c-Abl) in SSc fibroblasts: the inhibition of c-Abl by imatinib mesylate treatment is reported to improve the skin sclerosis of SSc (11).

There may be other factors that mediate fibroblast activation. The toll-like receptor (TLR) family is thought to play

a key role in the innate immune system. Mammalians are known to have more than 10 types of TLRs on dendritic cells, monocyte/macrophages and B cells which control immune responses by detecting common molecular motifs, including RNA ligands by TLR3, TLR7 and TLR8, DNA ligands by TLR9 and bacterial cell surface proteins by TLR4 (12). In the skin, TLRs also express on many of cell types including keratinocytes and Langerhans cells in the epidermis, or lymphocytes, macrophages, and fibroblasts in the dermis (13).

The expression levels of TLRs are reported to be up- or down-regulated in various diseases, probably contributing to the pathogenesis. Recently, the up-regulation of TLR2, TLR3, and TLR4 in SSc dermal fibroblasts is reported (14-17), suggesting the possibility that immune dysregulation may contribute to fibroblast activation. In this study, we performed PCR array for TLR-related genes using RNA from SSc dermal fibroblasts, and found that the expression of TLR5 and TLR10 is also up-regulated. Therefore, we tried to clarify the mechanism of the up-regulation of the TLRs in these cell types and their roles in the pathogenesis of this disease.

Patients and methods

Cell culture

Human dermal fibroblasts were obtained by skin biopsy of the affected areas (dorsal forearm) from patients with SSc who had <2 years of skin thickening. Control fibroblasts were obtained by skin biopsies from healthy donors. Primary explant cultures were established in 75-cm^2 culture flasks in modified Eagle's medium (MEM; Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal calf serum (FCS) and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA) as described previously (18,19). Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in 95% air, 5% CO_2, and studied between the third and sixth subpassages. Before experiments, cells were serum-starved for 24 hours.

Ethics

Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were recruited into this study according to the Declaration of Helsinki.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan), following the protocol provided by the manufacturer. Then, RNAs were reverse-transcribed into the first strand cDNA using an RT^2 First Strand Kit (SABioscience, Frederick, MD). For RT^2 Profiler PCR Array, the cDNA was mixed with RT^2 Real-Time SYBR GREEN/ROX PCR Master Mix (SABioscience) and the mixture was added into a 96-well RT^2 Profiler PCR Array (Human Toll-Like Receptor Signalling Pathway; SABioscience) that included primer pairs for 84 TLR-related genes. PCR was performed on Takara Thermal Cycler Dice (TP800)® following the manufacturer's protocol. The threshold cycle (Ct) for each gene was extracted using Thermal Cycler Dice Real Time System ver2.10B (Takara Bio Inc., Shiga, Japan). The raw Ct was normalised using the values of housekeeping genes. For quantitative real-time PCR, primers and templates were mixed with the SYBR Premix Ex TaqII (Takara Bio Inc.). Primer sets for 13 TLR-related genes including TLR5, TLR10 as well as GAPDH were purchased from Takara. DNA was amplified for 50 cycles of denaturation for 15s at 95°C, annealing for 35s at 60°C and extension for 30s at 72°C. Transcript levels of each gene of interest were normalised to GAPDH.

Cell lysis and Immunoblotting

Fibroblasts were washed with cold phosphate-buffered saline twice and lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA). Aliquots of cell lysates (normalised for protein concentrations) were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to PVDF membranes, which were blocked in blocking One P buffer (Nacalai Tesque, Kyoto, Japan) for 1 hour and incubated overnight at 4°C with primary antibody for TLR5 (Imgenex corporation, San Diego, CA), TLR10 (Novus Biologicals, Littleton, CO), type I collagen (Southern Biotech, Birminghan, AL) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed with Tris-buffered saline and 0.1%Tween 20 (TBS-T), probed with HRP-conjugated secondary antibody for 1 hour, and then washed with TBS-T again. The detection was performed using ECL system (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's recommendations.

Transient transfection

SiRNA against Smad3 was purchased from Santa Cruz Biotechnology. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used as a transfection reagent. For reverse transfection, control siRNA or Smad3 siRNA (30nM) mixed with transfection reagent were added when fibroblasts were plated, followed by the incubation for 96 hours at 37°C in 5% CO_2.

Immunohistochemical staining

Skin specimens were obtained from 5 SSc patients and 5 control subjects. Control and patient samples were collected and processed immediately after resection in parallel.

Deparaffinised sections were retrieved by incubation with citrate buffer pH6 for 5 minutes with autoclave treatment. Endogenous peroxidase activity was inhibited, after which sections were blocked with 5% normal goat serum for 60 min and then reacted with the antibodies for TLR5 or TLR10 (1:100) overnight at 4°C. After excess antibody was washed out with PBS, samples were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (Nichirei, Tokyo, Japan) for 30 minutes. The reaction was visualised by the diaminobenzidine substrate system (Dojin, Kumamoto, Japan). Slides were lightly counterstained with Mayer's haematoxylin, and examined under a light microscope (BX50, OLYMPUS, Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out with the Mann-Whitney test for the comparison of medians, and Fisher's exact

probability test for the analysis of frequency. P-values less than 0.05 were considered significant.

Results

Expression profile of TLR-related genes in normal and SSc fibroblasts

As an initial experiment, to determine which genes involved in human TLR signalling pathway were up- or down-regulated in SSc, we performed PCR array analysis, consisting of 84 TLR-related genes (Table I). The gene expression profile in 5 dermal fibroblasts derived from the involved skin of SSc patients was compared with that of normal fibroblasts. When 2-cycle difference (=4-fold difference in ΔΔCT method) was considered meaningful, several genes were up- or down-regulated in SSc fibroblasts than normal cells. Among them, 13 genes (BTK, CLEC4E, CSF3, CD180, LY86, NFRKB, PELI1, SIGIRR, ECSIT, TNF, TLR5, TLR9 and TRL10) have not previously investigated in SSc. Real-time PCR using specific primer for each gene with increased number of samples (n=8) revealed that only the expression of TLR5 and TLR10 were still significantly higher in SSc fibroblasts than in normal fibroblasts (Fig. 1a and b). The difference in the expression of other genes became insignificant by increasing sample number; for example, although SIGGER expression was increased in SSc fibroblasts by the array, but it was down-regulated in SSc fibroblasts by real-time PCR albeit insignificant, probably due to the variation among samples.

We also found that the expression of TLR5 in the array were up-regulated in TGF-β-treated normal fibroblasts (7.01-fold difference compared with untreated normal fibroblasts), as well as in SSc fibroblasts (47.2-fold difference) (Table I). Also, TLR10 was increased in both TGF-β-treated normal fibroblasts and SSc fibroblasts. Because many characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β as described above, we expected that genes up- or down-regulated both in normal fibroblasts treated with TGF-β and in SSc fibroblasts play a role in the pathogenesis of SSc. Thus, we focused on TLR5 and TLR10 in the following experiments.

Table I. Summary of TLR-related gene expression in normal and SSc fibroblasts by PCR analysis.

Gene name	NS	NS+TGFβ	SSc	Gene name	NS	NS+TGFβ	SSc
BTK	1.00	0.58	8.46	MAP3K1	1.00	0.55	1.48
CASP8	1.00	0.45	0.26	MAP3K7	1.00	0.66	0.66
CCL2	1.00	3.18	0.80	MAP3K7IP1	1.00	6.02	1.42
CD14	1.00	58.89	224.41	MAP4K4	1.00	0.88	0.87
CD80	1.00	0.00	10.70	MAPK8	1.00	1.34	0.98
CD86	0.00	1.00	0.00	MAPK8IP3	1.00	0.74	0.69
CHUK	1.00	0.99	0.66	MYD88	1.00	1.64	0.62
CLEC4E	0.00	1.00	5.78	NFKB1	1.00	1.45	1.23
CSF2	1.00	1.31	3.10	NFKB2	1.00	0.24	0.00
CSF3	1.00	7.26	162.02	NFKBIA	1.00	0.46	0.74
CXCL10	0.00	1.00	4.72	NFKBIL1	1.00	1.69	2.55
EIF2AK2	1.00	0.72	0.59	NFRKB	1.00	1.77	0.25
ELK1	1.00	0.80	1.30	NR2C2	1.00	0.55	2.00
FADD	1.00	1.29	0.65	PELI1	1.00	9.51	7.78
FOS	1.00	2.01	0.75	PPARA	1.00	0.57	0.75
HMGB1	1.00	0.57	0.24	PRKRA	1.00	1.25	0.97
HRAS	1.00	1.28	1.21	PTGS2	1.00	3.32	0.47
HSPA1A	1.00	1.42	1.72	REL	1.00	3.20	0.33
HSPD1	1.00	2.46	1.97	RELA	1.00	1.45	1.34
IFNA1	1.00	6.50	73.52	RIPK2	1.00	0.78	0.64
IFNB1	0.00	1.00	0.00	SARM1	1.00	0.88	0.97
IFNG	0.00	1.00	0.19	SIGIRR	0.00	0.00	1.00
IKBKB	1.00	1.42	0.63	ECSIT	1.00	31.12	3743.05
IL10	1.00	0.62	10.06	TBK1	1.00	0.50	0.33
IL12A	1.00	1.66	1.32	TICAM2	1.00	0.57	0.47
IL1A	1.00	0.59	0.67	TIRAP	1.00	1.52	1.67
IL1B	1.00	4.29	23.10	TLR1	1.00	1.34	3.05
IL2	0.00	1.00	1.17	TLR10	0.00	1.00	0.03
IL6	1.00	1.93	1.25	TLR2	1.00	0.00	1.11
IL8	1.00	2.27	0.36	TLR3	1.00	0.84	0.34
IRAK1	1.00	2.35	0.64	TLR4	1.00	2.13	47.18
IRAK2	1.00	0.75	0.52	TLR5	1.00	7.01	47.18
IRF1	1.00	0.20	0.40	TLR6	1.00	2.31	0.98
IRF3	1.00	1.23	0.87	TLR7	0.00	1.00	0.00
JUN	1.00	1.16	0.76	TLR8	0.00	1.00	0.00
LTA	0.00	1.00	7.21	TRL9	1.00	36.76	0.00
CD180	0.00	0.00	1.00	TNF	1.00	1.61	1.08
LY86	0.00	0.00	1.00	TNFRSF1A	1.00	0.87	0.67
LY96	1.00	0.55	0.74	TOLLIP	1.00	4.59	2.75
MAP2K3	1.00	0.67	1.64	TRAF6	1.00	1.40	1.57
MAP3K4	1.00	0.90	0.97	TICAM1	1.00	1.06	0.59

A mixture of equal amounts of total RNAs from 5 normal fibroblasts (NS), 5 normal fibroblasts stimulated with TGF-β for 12 hours (NS+TGFβ) or 5 SSc fibroblasts was prepared, and TLR-related gene expression profile in each cell group was evaluated using PCR Array. The fold-change was calculated as 1/2 (raw Ct of each miRNA – mean Ct of small RNA housekeeping genes). The mean fold-change of each miRNA is shown. The values in the normal skin samples were set at 1. When the value of normal skin was 0, the mean value of another sample was set at 1.

TLR5/10 expression is increased at the transcriptional level in SSc dermal fibroblasts

We then determined whether the protein expression of TLR5 and TLR10 was also up-regulated both in normal fibroblasts treated with TGF-β and in SSc fibroblasts. As expected, the protein expression of TLR5 and TLR10 in normal fibroblasts was induced by the ectopic stimulation of TGF-β (Fig. 2a). In addition, we found constitutive up-regulation of TLR5 and TLR10 in SSc fibroblasts compared to normal fibroblasts (Fig. 2b). Therefore, the increase of TLR5 and TLR10 mRNA expression in normal fibroblasts treated with TGF-β and in SSc fibroblasts, which was shown by the PCR array, was thought to result in the overexpression of the TLR5 and TLR10 proteins.

Thus, to examine the possibility that