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*Corresponding author at:
Department of Histology and Embryology,
Center of Biostructure Research,
Medical University of Warsaw, Chałubińskiego 5,
02-004 Warsaw, Poland.
Tel.: +48 22 6295282; fax: +48 22 6295282
E-mail address: Monika.Oldak@wum.edu.pl (M. Ołdak)

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Letter to the Editor

Circulating miR-29a levels in patients with scleroderma spectrum disorder

Systemic sclerosis (SSc) or scleroderma is an acquired disorder which typically results in fibrosis of the skin and internal organs [1–3]. The pathogenesis of tissue fibrosis may include inflammation, autoimmune attack and vascular damage, resulting in the activation of fibroblasts to produce excessive amounts of various collagens, mainly type I collagen which consists of α1(I) and α2(I) collagen [4]. However, the mechanism underlying the dysregulation of type I collagen in SSc is still unknown.

We focused on microRNA (miRNA) as the regulator of type I collagen expression in SSc. miRNAs, short ribonucleic acid molecules on average only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the three prime untranslated regions (3′ UTRs) of mRNAs, leading to gene silencing. Although recent publications have demonstrated that miRNAs are involved in the pathogenesis of various disorders, the role of miRNA in SSc is not determined. Moreover, serum miRNA has attracted attention for its potential as disease marker [5]. In this study, we try to evaluate the possibility that serum levels of miR-29a can be a useful marker for SSc. MiR-29a can bind to 3′ UTR of both α1(I) collagen and α2(I) collagen based on miRNA target gene predictions using the TargetScan (version 5.1, http://www.targetscan.org/), a leading program in the field [6].

There has been no report demonstrating the expression of hsa-miR-29a in cell-free body fluid. To validate that the miRNA is indeed detectable in human serum, total miRNA was extracted from sera of healthy individual and the level of miR-29a was determined by quantitative real-time PCR using specific primer set. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. MicroRNA isolation from serum samples were performed with miRNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions with minor modification [5]; immediately before isolation, 100 μl of serum were supplemented with 5 μl of 5 fmol/μl synthetic non-human miRNA (*C. elegans* miR-39, Takara Bio Inc., Shiga, Japan) as controls providing an internal reference for normalization of technical variations between samples. cDNA was synthesized from miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara). DNA was amplified for 50 cycles of denaturation for 5 s at 95 °C and annealing for 20 s at 60 °C. As shown in Fig. 1a, the amplification of hsa-miR-29a was observed, and Ct values were increased by the serial dilution of the miRNA. Thus, hsa-miR-29a was thought to be detectable and quantitative in the serum using our method.

Next, serum samples were obtained from 61 patients with SSc (12 men and 49 women; age range, 29–85 years); 23 patients had diffuse cutaneous SSc (dcSSc) and 38 patients had limited cutaneous SSc (lcSSc), as described previously [7]. Twelve patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought to develop SSc in the future based on the criteria proposed by Ihn et al., were also included in this study [8–10]. Although we expected serum miR-29a levels are down-regulated in SSc sera because the expression of type I collagen, the target of the miRNA, is up-regulated in SSc, there was no statistically significant difference between healthy control subjects and SSc patients in hsa-miR-29a transcript levels corrected for the levels of cel-miR-39 in the same samples (Fig. 1b), Also, we could not find a significant difference in the values between dcSSc patients and lcSSc patients. On the other hand, miR-29a levels in SSD patients were dramatically decreased as compared with other groups; healthy controls, lcSSc or dcSSc patients. miR-29a down-regulation only in SSD patients may contribute to the pathogenesis of tissue fibrosis seen in SSc: dysregulation of type I collagens may be triggered by decreased

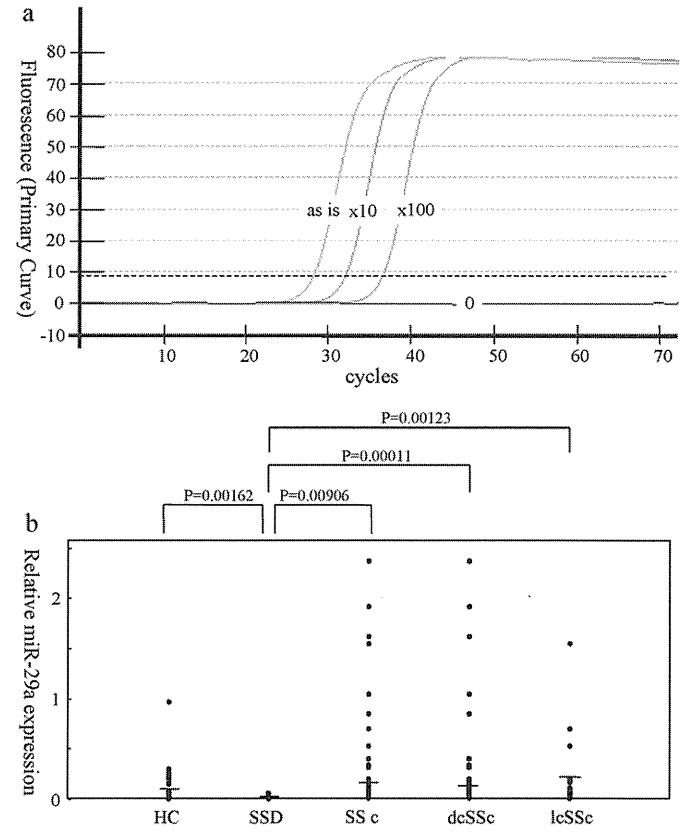

Fig. 1. (a) hsa-miR-29a is present in serum sample. Serial dilution of cDNA (as is, 10-fold dilution, 100-fold dilation and 0) synthesized from serum miRNA was used as template for real-time PCR. Amplification curves of gene-specific transcripts are shown to illustrate the process of exponential increase of fluorescence. Horizontal dotted line indicates the threshold. (b) Serum concentrations of miR-29a in patients with systemic sclerosis (SSc, $n = 61$) or scleroderma spectrum disorder (SSD, $n = 12$) and in healthy control subjects (HC, $n = 20$). MiR-29a levels were measured with real-time PCR. Serum miR-29a concentrations are shown on the ordinate. Bars show means. *P*-values less than 0.05 by Mann–Whitney test were considered significant. dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc.

Table 1a
Correlation of serum miR-29a levels with clinical and serological features in patients with systemic sclerosis (SSc).

	Serum miR-29a		
	Patients with reduced miR-29a levels ($n=30$)	Patients with normal miR-29a levels ($n=31$)	P-value
Age at the serum sampling (mean year)	60.8	57.6	0.54
Age at onset (mean year)	51.7	50.3	0.94
Duration of disease (year) (mean year)	7.5	4.2	0.80
Type (diffuse: limited)	0.5	0.5	0.49
m-TSS (point)	9.9	11.8	0.48
Clinical features			
Pitting scars	57.7	37.5	0.15
Ulcers	29.6	33.3	0.79
Nailfold bleeding	50.0	54.2	0.78
Raynoud's phenomenon	92.6	84.6	0.36
Telangiectagia	40.0	40.0	0.31
Contracture of phalanges	92.3	81.8	0.27
Calcinosis	0	0	1.00
Diffuse pigmentation	40.9	35.7	0.76
Short SF	77.3	70.0	0.59
Sicca symptoms	66.7	52.9	0.43
Organ involvement			
Pulmonary fibrosis	37.0	46.4	0.48
Mean %VC (%)	96.0	99.9	0.49
Mean %DLco (%)	86.2	79.2	0.23
Pulmonary hypertension (mmHg)	33.7	27.4	0.04*
Oesophagus	20.8	24.0	0.79
Heart	44.4	38.5	0.66
Liver	20.0	14.8	0.62
Kidney	6.7	0.0	0.16
Joint	53.3	64.3	0.55
Thyroid	25.9	40.9	0.27
Thrombosis	0	0	1.00
Malignant tumor	0	4.2	0.31
ANA specificity			
Anti-topo I	26.6	22.6	0.71
Anti-centromere	33.3	32.2	0.93
Anti-U1 RNP	13.3	9.7	0.65

Unless indicated, values are percentages.
m-TSS, 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-U1 RNP, anti-U1 ribonucleoprotein antibody.
* $P<0.05$ versus patients with normal miR-29a levels using Fisher's exact probability test.

Table 1b
Clinical and laboratory features of 12 patients with SSD.

Patient no.	Sex	Age	Skin sclerosis	Pulmonary changes	Antinuclear antibodies	NFB	Raynaud phenomenon	Point	Relative miR-29a levels
1	F	68	–	–	U1-RNP	–	Triphasic	6	0.0513
2	F	48	Swollen	–	Others	–	Triphasic	5	0.0023
3	F	62	–	+, VC ≧ 80%	ACA	–	Triphasic	8	0.0226
4	M	47	–	–	Topo-I	–	–	5	0.0019
5	F	75	–	+, VC≧80%	ACA	–	Triphasic	8	0.0334
6	F	57	Swollen	–	Others	One or two fingers	Triphasic	6	0.0522
7	F	44	–	–	Topo-I	–	–	5	0.0219
8	M	46	–	–	Topo-I	–	–	5	0.0555
9	F	80	–	–	ACA	One or two fingers	Triphasic	7	0.0144
10	F	58	Swollen	–	ACA	One or two fingers	Triphasic	8	0.0040
11	F	45	–	–	Topo-I	–	–	5	0.0033
12	F	56	–	–	Topo-I	–	Biphasic	6	0.0116

For the diagnosis of SSD, a total score was obtained as the sum of the following five factors [10]:
(1) Extent of skin sclerosis: 10 points for truncal sclerosis, 5 points for skin sclerosis limited to the extremities and face, 3 points for sclerodactylia alone and 1 point for swollen fingers.
(2) Pulmonary changes: 4 points for pulmonary fibrosis accompanied by decreased vital capacity (<80%) and 2 points for pulmonary fibrosis accompanied by normal vital capacity (≧80%).
(3) Antinuclear antibodies: 5 points for positive anti-topoisomerase I antibody (Topo-I), 3 points for positive anticentromere antibody (ACA) or anti-U1RNP antibody, 2 points for anti-nucleolar antibody and 1 point for other positive ANA.
(4) Pattern of Raynaud's phenomenon: 3 points for triphasic (pale-purple-red), 2 points for biphasic (two of the above colours) and bilateral, and 1 point for biphasic and hemilateral, or monophasic (pale or purple only) and bilateral.
(5) Nailfold bleeding (NFB): 2 points for NFB in three or more fingers, and 1 point for NFB in one or two fingers.
The conditions with 9 or more points are consistent with SSc and those with 5–8 points are considered as SSD.
SSD, scleroderma spectrum disorder; VC, vital capacity; Topo-I, anti-topoisomerase I antibody; ACA, anti-centromere antibody; U1 RNP, anti-U1 ribonucleoprotein antibody; NFB, nailfold bleeding

miR-29a at the SSD stage, but maintained by other factors in SSc patients.

Table 1a shows the association of serum miR-29a levels with the clinical features in SSc patients. Considering that collagen expression in SSc patients is up-regulated as described above, we regarded reduction of miR-29a levels as the meaningful change. We found that SSc patients with reduced miR-29a levels had significantly higher right ventricular systolic pressure by Doppler echocardiography than those with normal levels. Though the cause of pulmonary hypertension in SSc is still uncertain, our results suggested that miR-29a also takes part in the pathogenesis of pulmonary hypertension. On the other hand, as shown in Table 1b, clinical features of each SSD patient were not correlated with the levels of miR-29a. Taken together, serum miR-29a levels is not likely to be a specific marker for clinical manifestations of SSD, but the miRNA may play an important role in the pathogenesis of this disease.

The concept of SSD has been proposed by Maricq et al. originally to unify typical SSc, early forms of SSc and closely related disorders including mixed connective tissue disease (MCTD) [8,9]. Thereafter, Ihn et al. established a new diagnostic method using a points system to distinguish patients with SSD from those with early SSc [10]. Although the point system has not been accepted in the world wide basis, because progressive fibrosis of SSc is often irreversible, at least clinically, there is an urgent need to develop new strategies to diagnose patients as early as possible and follow-up carefully. For that purpose, the concept of SSD should be further understood and characterized. Our study is the first to examine serum miRNA levels using sera from SSD as well as SSc, and is shedding new light on the definition of SSD. To note, it may be difficult to distinguish early stage SSc from SSD, because skin sclerosis is sometimes not apparent in early SSc, especially in lcSSc. Serum levels of miR-29a levels may be useful for the differentiation of SSc from SSD.

As the limitation of this study, we could not collect large number of SSD patients because of the rarity of this condition. However, our approach may be effective to clarify the property of SSD. Larger studies are needed in the future.

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Yoshio Kawashita
Masatoshi Jinnin*
Takamitsu Makino
Ikko Kajihara
Katunari Makino
Noritoshi Honda
Shinich Masuguchi
Satoshi Fukushima
Yuji Inoue
Hironobu Ihn
Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan

*Corresponding author. Tel.: +81 96 373 5233;
fax: +81 96 373 5235
E-mail address: jinjin1011@hotmail.com
mjin@kumamoto-u.ac.jp

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Letter to the Editor

Differential hyaluronan homeostasis and expression of proteoglycans in juvenile and adult human skin

Cutaneous ageing is a complex biological process affecting all skin components. It consists of two independent, clinically and biologically, distinct processes. The first is intrinsic or innate ageing, which affects the skin in the same pattern as it affects all internal organs. The second is extrinsic ageing, which is the result of exposure to external factors, mainly ultraviolet (UV) irradiation [1].

Extracellular matrix molecules are highly implicated in the ageing process and exhibit specific alterations in extrinsic and intrinsic skin ageing [2]. Among them, hyaluronic acid (HA) is of high importance since it has the unique capacity of binding water, thus providing viscosity and hydration to the dermis. In photo-ageing process, HA homeostasis shows specific alterations since in photo-aged skin HA of reduced size is elevated and exhibits abnormal deposition [3].

In this study, we have tried to elucidate alterations in HA homeostasis and proteoglycan expression associated with intrinsic skin ageing. We employed juvenile skin tissue specimens ($n = 10$, mean age 5 years, 4 mm punch biopsies) collected from foreskin of children undergoing surgery for phimosis. Adult photo-protected skin tissue specimens ($n = 16$, mean age 72 years, 4 mm punch biopsies) were collected from the area behind the ear lobe. Total glycosaminoglycans were isolated and purified from skin tissue specimens, as previously described [3]. Aliquots of total glycosaminoglycans were assayed for HA content by ELISA. Gene

Expression of Matrix Metalloproteinase-13 Is Controlled by IL-13 via PI3K/Akt3 and PKC-δ in Normal Human Dermal Fibroblasts

Chikako Moriya[1], Masatoshi Jinnin[1], Keitaro Yamane[1], Keishi Maruo[1], Faith C. Muchemwa[1], Toshikatsu Igata[1], Takamitsu Makino[1], Satoshi Fukushima[1] and Hironobu Ihn[1]

IL-13, a T helper type 2 cytokine, is reported to be increased in the tissue of patients with atopic dermatitis (AD). In addition, chronic lichenified plaques in AD show thickened epidermis and dermis. We hypothesized that IL-13 is involved in tissue remodeling by altering the expression of matrix metalloproteinases (MMPs). In this study, we examined the MMP-related genes targeted by IL-13 in human dermal fibroblasts using a complementary DNA microarray. We focused on the *MMP-13* gene, which was identified as one of the MMPs suppressed by IL-13. IL-13 downregulated both MMP-13 protein and mRNA expression. IL-13 suppressed MMP-13 expression more effectively in the presence of protein kinase C (PKC)-δ inhibitor, whereas IL-13 upregulated MMP-13 in the presence of inhibitors of phosphoinositide 3-kinase (PI3K)/Akt pathway or *Akt3*-specific small interfering RNA. Our results suggest that MMP-13 expression is negatively controlled by PI3K/Akt3 and positively regulated by PKC-δ in the presence of IL-13. Taken together, these findings indicate that IL-13 may induce the formation of thickened dermis in AD by decreasing collagen degradation. Blockade of IL-13 signaling cascades in AD patients may be a new therapeutic approach.

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INTRODUCTION

T helper type 2 responses are essential for the allergic reaction and host defense against extracellular parasites. IL-4 and IL-13, both of which are major T helper type 2 cytokines, mediate immune responses typically characterized by eosinophilia, basophilia, mastocytosis, enhanced B-cell class switching, antibody production, and plasma accumulation of IgE and IgG1. IL-4 and IL-13 share a common receptor subunit and have overlapping functions (LaPorte *et al.*, 2008). However, IL-13 might be the dominant effector cytokine because IL-13 is much more abundant *in vivo* (Aleksza *et al.*, 2002; Kaminishi K *et al.*, 2002; Terao *et al.*, 2003; Tazawa *et al.*, 2004).

Recent studies have indicated that IL-13 is also associated with the regulation of extracellular matrix (ECM) synthesis and tissue remodeling (Wynn, 2004; Zheng *et al.*, 2009). The amount of ECM in the tissue might be controlled through a balance among ECM production, ECM degradation by matrix metalloproteinases (MMPs), and inhibition of MMPs by tissue inhibitors of metalloproteinases (Visse and Nagase, 2003). MMPs are a family of structurally related zinc-dependent endopeptidases collectively capable of degrading essentially all ECM components. Human MMP gene family can be divided into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs, and novel MMPs based on their structure and substrate specificity (Birkedal-Hansen *et al.*, 1993b; Kähäri and Saarialho-Kere, 1997). Among them, collagenases have a major role in the digestion of type I collagen, the most abundant mammalian ECM in the skin (Birkedal-Hansen, 1993a; Birkedal-Hansen *et al.*, 1993b; Sternlicht and Werb, 2001; Chakraborti *et al.*, 2003). In this study, we identified the MMP-related genes targeted by IL-13 in human dermal fibroblasts using a complementary DNA (cDNA) microarray. We then focused on the MMP-13 gene, which was identified as one of the MMPs downregulated by IL-13 in the microarray analysis. We clarified the mechanisms by which IL-13 regulates MMP-13 expression in dermal fibroblasts.

RESULTS

Effects of IL-13 on MMP-related genes

The technique of differential hybridization of cDNA expression arrays was used to identify differences in the expression pattern of genes between control and IL-13-treated fibroblasts. When the gene expression ratio (IL-13 treated/control) of >1.5 or <0.67 was considered significant, among the MMP-related genes, *MMP-10*, *MMP-13*, and *MMP-16* were

[1]*Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan*

Correspondence: Masatoshi Jinnin, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan. E-mail: mjin@kumamoto-u.ac.jp

Abbreviations: AD, atopic dermatitis; cDNA, complementary DNA; ECM, extracellular matrix; MMP, matrix metalloproteinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA

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Table 1. Effects of IL-13 on the expression profiles of MMP-related genes as measured with the cDNA microarray

Gene name	Gene accession number	Expression ratio
Matrix metalloproteinase-1 (interstitial collagenase)	NM_002421	1.31
Matrix metalloproteinase-2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	NM_004530	0.89
Matrix metalloproteinase-3 (stromelysin 1, progelatinase)	NM_002422	1.33
Matrix metalloproteinase-7 (matrilysin, uterine)	NM_002423	0.98
Matrix metalloproteinase-10 (stromelysin 2)	NM_002425	0.29
Matrix metalloproteinase-11 (stromelysin 3)	NM_005940	1.05
Matrix metalloproteinase-13 (collagenase 3)	NM_002427	0.34
Matrix metalloproteinase-15 (membrane inserted)	NM_002428	1.65
Matrix metalloproteinase-16 (membrane inserted)	NM_005941	0.08
Matrix metalloproteinase-19	NM_022790	0.79
Tissue inhibitor of metalloproteinase-2	NM_003255	1.13
Tissue inhibitor of metalloproteinase-3 (Sorsby fundus dystrophy, pseudoinflammatory)	NM_000362	0.73

Abbreviations: cDNA, complementary DNA; MMP, matrix metalloproteinase.

downregulated and only *MMP-15* was upregulated by IL-13 (Table 1). *MMP-1* and *MMP-3* were slightly induced by IL-13, but not significantly. Expression of tissue inhibitors of metalloproteinases was not significantly affected by IL-13. Thus, IL-13 may decrease the activity of ECM degradation by altering the balance between MMPs and tissue inhibitors of metalloproteinases.

Expression of MMP-13 is downregulated by IL-13 in human dermal fibroblasts

MMP-13 is one of the collagenases that have a major role in the digestion of type I collagen, as described above (Birkedal-Hansen, 1993a; Birkedal-Hansen *et al.*, 1993b; Sternlicht and Werb, 2001; Chakraborti *et al.*, 2003). *MMP-13* mRNA expression was also analyzed by quantitative real-time PCR. The level of *MMP-13* mRNA was reduced by the treatment with IL-13 for 24 hours (Figure 1a). Then we determined whether IL-13-mediated downregulation of *MMP-13* mRNA resulted in a decrease of the protein level. Immunoblotting revealed that IL-13 decreased MMP-13 protein expression in a time-dependent manner (Figure 1b and c). ELISA specific for MMP-13 also showed that MMP-13 concentration in culture supernatant was decreased by the treatment with IL-13 for 48 hours (Figure 1d). These results confirm the microarray data that the stimulation of human dermal fibroblasts with IL-13 downregulates MMP-13 expression.

IL-13 reduces MMP-13 expression via phosphoinositide 3-kinase/Akt signaling pathway

Subsequently, we investigated which signaling pathways were involved in the IL-13-mediated MMP-13 downregulation. First, cells were pretreated with phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (Figure 2a) or Akt inhibitor (Figure 2b) for 1 hour before the stimulation with IL-13. IL-13 could not downregulate MMP-13 protein expression in the presence of these PI3K/Akt inhibitors but induced it significantly (Figure 2a and b). Interestingly, the addition of these inhibitors in the absence of IL-13 decreased basal MMP-13 expression. These results suggest that the PI3K/Akt signaling pathway is essential for both basal MMP-13 expression and the reducing effect of IL-13 on MMP-13 expression.

Similar result was obtained by quantitative real-time PCR examining the IL-13 effect on *MMP-13* mRNA expression in the presence of LY294002 or Akt inhibitor. IL-13 induced the *MMP-13* mRNA expression in the presence of these PI3K/Akt inhibitors, rather than reducing it (Figure 2c).

In order to confirm whether IL-13 can activate the Akt signaling pathway, whole-cell lysates were prepared from the cells treated with IL-13, and immunoblotting was performed. The phosphorylation levels of Akt at Thr 308 or Ser 473 were increased after 3–6 hours of IL-13 treatment (Figure 2d). Thus, IL-13 is likely to downregulate MMP-13 expression via activation of the PI3K/Akt pathway.

Isoform-specific effect of Akt on IL-13-mediated MMP-13 downregulation

We then examined which isoform of Akt is associated with the IL-13-mediated downregulation of MMP-13. To inhibit each Akt isoform specifically, we used small interfering RNA (siRNA) specific for *Akt1/2* or *Akt3*. First, the knockdown efficiency of siRNA was evaluated by immunoblotting (Figure 3a and b). Expression of Akt1/2 or Akt3 was downregulated by transfection of siRNA for *Akt1/2* (Figure 3a) or *Akt3* (Figure 3b), respectively, as compared with control siRNA or transfection reagent only.

Then, MMP-13 expression in the cells transfected with these siRNA in the presence or absence of IL-13 was analyzed by immunoblotting. IL-13 significantly induced MMP-13 protein in the presence of *Akt3* siRNA (Figure 3c and d), which is consistent with the above result that IL-13 induced

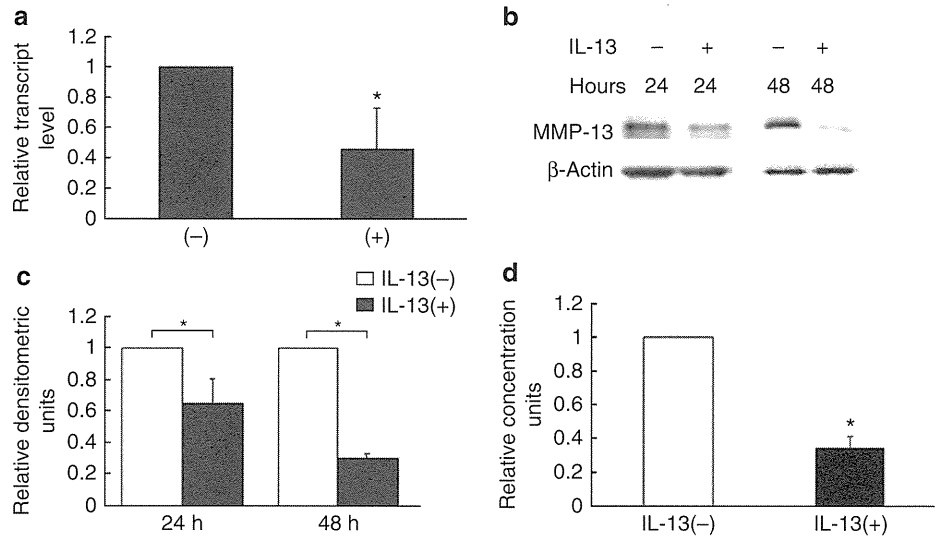

Figure 1. IL-13 downregulates matrix metalloproteinase (MMP)-13 at a transcriptional and post-transcriptional level in human dermal fibroblasts. Cells were incubated in the presence or absence of IL-13 (10 ng ml^{-1}). (**a**) Real-time PCR for *MMP-13* was performed. The value in untreated cells was set at 1. (**b**) Conditioned media and cell lysates were subjected to immunoblotting with MMP-13 and β-actin antibody. (**c**) MMP-13 protein levels quantitated by scanning densitometry and corrected for β-actin are shown relative to the level in untreated cells (1.0). Data are expressed as the mean ± SD of five independent experiments. (**d**) Cells were incubated in serum-free medium for 48 hours in the presence or absence of IL-13 before collection of medium. Specific ELISA for detecting MMP-13 levels was performed as described in "Materials and Methods". *$P<0.05$ as compared with the value in untreated cells.

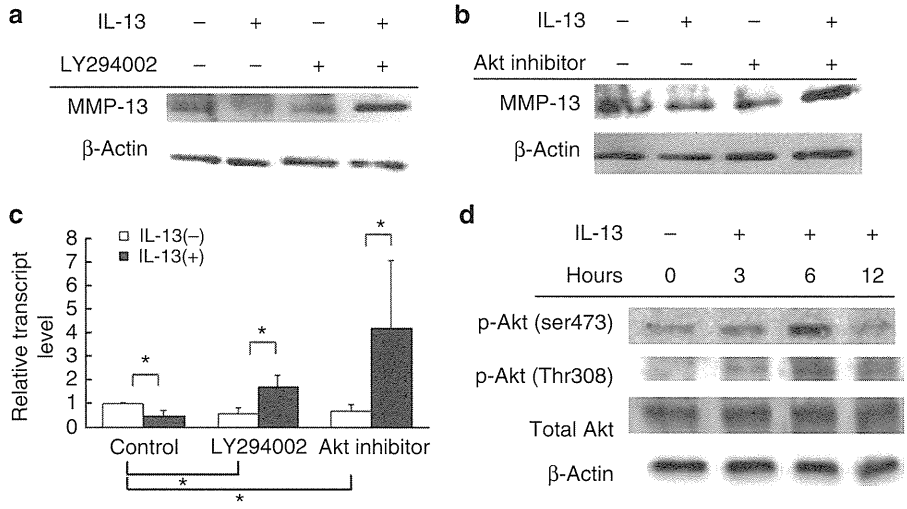

Figure 2. Effect of LY294002 or Akt inhibitor on IL-13-mediated matrix metalloproteinase (MMP)-13 downregulation. (**a**, **b**) Human dermal fibroblasts were treated with 30 mM LY294002 (**a**) or 10 mM Akt inhibitor (**b**) for 1 hour before the addition of IL-13 (10 ng ml^{-1}) for 24 hours. Immunoblotting was performed as described in Figure 1b. One experiment representative of five independent experiments is shown. (**c**) Cells were treated with LY294002 (30 mM) or Akt inhibitor (10 mM) 1 hour before the addition of IL-13 (10 ng ml^{-1}) for 24 hours. *MMP-13* mRNA levels were determined by quantitative real-time PCR as described in Figure 1a. (**d**) Human dermal fibroblasts were treated with IL-13 (10 ng ml^{-1}) for the indicated time. Cell lysates were subjected to immunoblotting with antibodies for phosphorylated Akt (Thr 308 or Ser 473). One experiment representative of three independent experiments is shown. *$P<0.05$ as compared with the value in untreated cells.

MMP-13 expression in the presence of PI3K/Akt inhibitors (Figure 2). On the other hand, in the presence of *Akt1/2* siRNA, IL-13 did not alter MMP-13 expression. Considering that *Akt3* siRNA and PI3K/Akt inhibitors had similar effects on MMP-13 expression, Akt3 may be the main pathway that mediates the IL-13 effect on MMP-13 expression.

PKC-δ inhibitor amplified the reducible effect of IL-13 on MMP-13 expression

As shown above, PI3K/Akt inhibitors downregulated basal MMP-13 expression in the absence of IL-13, whereas PI3K/Akt inhibitors upregulated the expression of MMP-13 in the presence of IL-13 (Figure 2). Conversely, IL-13 decreased

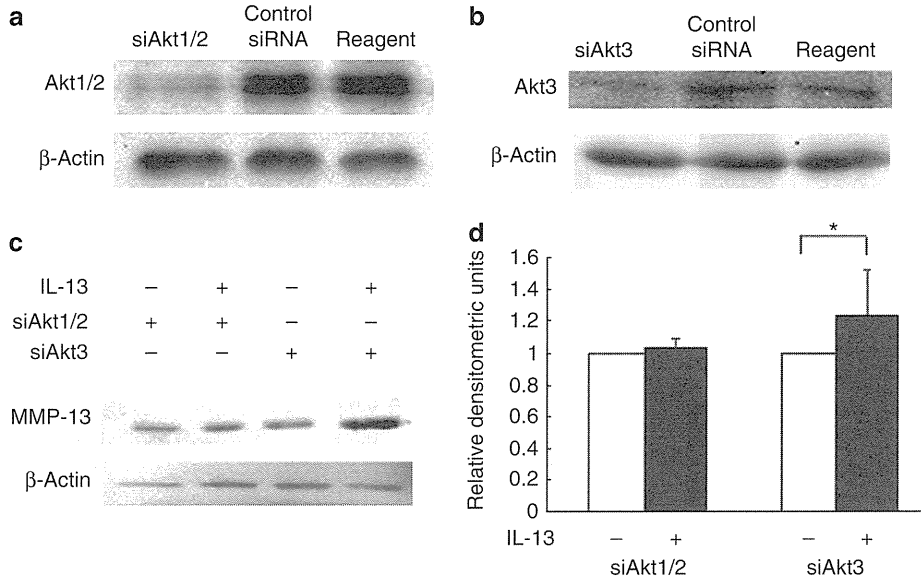

Figure 3. Effect of small interfering RNA (siRNA) specific for each Akt isoform on IL-13-mediated matrix metalloproteinase (MMP)-13 downregulation. (**a**, **b**) Cells were transfected with siRNA specific for *Akt1/2* (siAkt1/2) or *Akt3* (siAkt3). Immunoblotting was performed as described in Figure 1b. The results in the cells treated with control siRNA or transfection reagent only are also shown as controls. (**c**, **d**) Cells were transfected with siRNA specific for Akt isoform as described in Figure 3a and b. Then cells were incubated with or without IL-13 for 24 hours (**c**). The levels of MMP-13 protein quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the level in cells without IL-13 treatment (1.0) (**d**). $^*P<0.05$ as compared with the value in cells without IL-13 treatment.

MMP-13 expression in the absence of PI3K/Akt inhibitors, whereas IL-13 upregulated it in the presence of the inhibitors. To explain the paradoxical effects of IL-13 and PI3K/Akt inhibitors, we expected the presence of another downstream target of IL-13, which competes with PI3K/Akt. Pretreatment of cells with the p38 inhibitor SB203580 did not affect the IL-13-mediated downregulation of *MMP-13* mRNA (data not shown). On the other hand, in the presence of Rottlerin, a selective protein kinase C (PKC)-δ inhibitor, IL-13 decreased MMP-13 expression more effectively than in the absence of Rottlerin (Figure 4a). In the absence of IL-13, basal MMP-13 expression was not significantly affected by Rottlerin. Thus, PKC-δ is thought to have an inducible effect under the stimulation with IL-13.

It is generally accepted that inactive PKC isoforms are located in the nucleus and translocated to the plasma membrane and/or cytoskeleton after activation (Mochly-Rosen *et al.*, 1990; Tourkina *et al.*, 2001). To examine the activation of PKC-δ by IL-13, immunofluorescence microscopy using anti-PKC-δ antibody was performed. The result revealed that PKC-δ was translocated to the plasma membrane in IL-13-treated fibroblasts (Figure 4b).

Taken together, our results suggest that IL-13 activates both PI3K/Akt and PKC-δ. The effect of PKC-δ on MMP-13 expression was opposite to that of PI3K/Akt3, probably competing with it. Considering that IL-13 stimulation results in MMP-13 downregulation, PI3K/Akt3 may be dominant to PKC-δ (Figure 5). Based on this hypothetical model, in the presence of PI3K/Akt inhibitors, the addition of IL-13 can increase MMP-13 expression because PKC-δ is still activated, and the reducing effect of IL-13 on MMP-13 expression is amplified in the presence of Rottlerin. On the other hand, in the absence of IL-13, PI3K/Akt inhibitors decrease basal MMP-13 expression because PI3K/Akt is essential for basal MMP-13 expression.

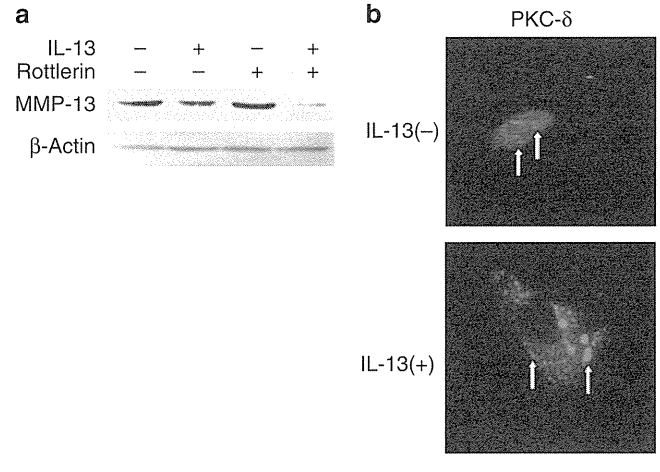

Figure 4. Activation of protein kinase C (PKC)-δ by IL-13 in human dermal fibroblasts. (**a**) Human dermal fibroblasts were treated with 3 μM Rottlerin for 1 hour before the addition of IL-13 (10 ng ml^{-1}) for 24 hours. Conditioned media or cell lysates were subjected to immunoblotting with antibody for matrix metalloproteinase (MMP)-13 or β-actin, respectively. (**b**) The subcellular translocalization of endogenous PKC-δ by IL-13 was visualized by immunofluorescence microscopy. Human dermal fibroblasts were serum-starved for 24 hours and incubated in the presence or absence of IL-13 (10 ng ml^{-1}) for 1 hour. Punctate stainings of PKC-δ are indicated by arrows.

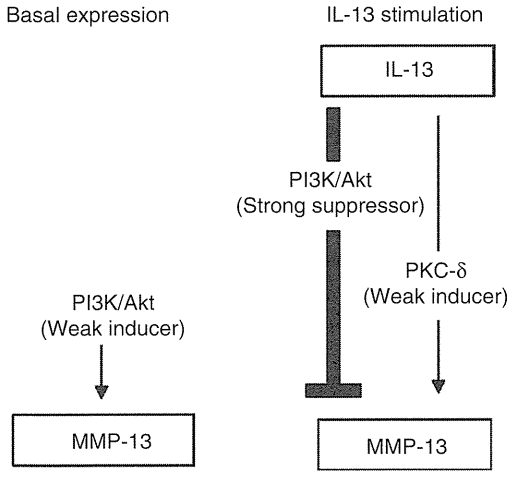

Figure 5. Diagram summarizing major pathways that are involved in matrix metalloproteinase (MMP)-13 expression. In the absence of IL-13 (left), basal expression of MMP-13 is positively controlled by PI3K/Akt. Thus, PI3K/Akt inhibitors decrease basal MMP-13 expression. In the presence of IL-13 (right), PI3K/Akt and PKC-δ are activated. PI3K/Akt3 has a reducing effect and PKC-δ has an inducing effect on MMP-13 expression. Because PI3K/Akt3 is dominant over PKC-δ, IL-13 stimulation results in downregulation of MMP-13. Addition of PI3K/Akt inhibitors increases MMP-13 expression because PKC-δ is still activated, whereas Rottlerin causes a further decrease of MMP-13.

DISCUSSION

In this article, we have presented several major findings. First, IL-13 can alter the expression of MMP-related genes by cDNA microarray analysis. Second, expression of MMP-13 is downregulated by IL-13 in human dermal fibroblasts. Third, inhibitors of the PI3K/Akt pathway decreased basal MMP-13 expression. Finally, IL-13 upregulated MMP-13 expression in the presence of PI3K/Akt inhibitors or Akt3-specific siRNA, whereas IL-13 suppressed MMP-13 more effectively in the presence of Rottlerin.

Akt is present in all eukaryotes and is involved in a wide variety of cellular functions, including proliferation, cell survival, and differentiation (Cho *et al.*, 2001; Bernal-Mizrachi *et al.*, 2004). In mammals, three isoforms—Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ—share a high degree of amino-acid identity and are activated by similar pathways in a PI3K-dependent manner (Brazil *et al.*, 2004). However, each isoform may have specific functions. For example, Akt3, but not Akt1, mediates MMP-13 upregulation induced by IL-1 or oncostatin-M in cultured human chondrocytes (Litherland *et al.*, 2008). Our study suggests that Akt3 may be the key molecule in downregulation of MMP-13 as well as upregulation.

The PKC family of proteins comprises at least 10 isozymes with diverse functions that are involved in numerous important cellular processes (Jimenez *et al.*, 2001). Studies have established that the activation of PKC is necessary for the stimulation of gene expression in response to various cytokines and that several cellular responses to IL-13 are regulated by a PKC-dependent signal-transduction pathway. For example, PKC-δ and -ζ mediate IL-4/IL-13-induced germline ε-transcription in human B cells (Ikizawa *et al.*, 2001).

Our hypothetical scheme is shown in Figure 5. In the presence of IL-13, the effect of PKC-δ on MMP-13 expression was opposite to that of PI3K/Akt3. We previously reported that IL-13 stimulates the transcription of tenascin-C and α2(I) collagen genes via the PI3K/Akt and PKC signaling pathway (Jinnin *et al.*, 2004, 2006). Interestingly, in the regulatory mechanism of tenascin-C by IL-13, both PI3K/Akt and PKC had positive effects on tenascin-C expression. PI3K/Akt and PKC signaling activated by IL-13 may have a major role in both ECM production and degradation, cooperating or competing with each other. On the other hand, MMP-13 expression was unchanged by inhibitors of p38 mitogen-activated protein kinase in human dermal fibroblasts in the presence of IL-13 (data not shown), although the activity of p38 mitogen-activated protein kinase was reported to be essential for MMP-13 induction by transforming growth factor-β in gingival fibroblasts cultured in collagen gel (Ravanti *et al.*, 1999). This discrepancy may be explained by the speculation that p38 mitogen-activated protein kinase activation is essential for MMP-13 upregulation, whereas downregulation of MMP-13 is mediated by PI3K/Akt and PKC.

In atopic dermatitis (AD), chronic lichenified plaques show thickened epidermis and dermis. In the thickened dermis, deposition of ECM such as type I collagen or tenascin-C is found (Toda *et al.*, 2003). Therefore, fibrosis occurs in chronic AD skin lesions by increased ECM deposition, including type I collagen or tenascin-C, and the fibrosis may have some role in the pathogenesis of AD. However, the mechanisms of the fibrosis in AD are still unknown. In addition, in AD patients, higher numbers of IL-13-positive cells are seen in skin lesions, reflecting T helper type 2 inflammation of this disease (Hamid *et al.*, 1996). In IL-13 transgenic mice, the skin showed thickening of the epidermal and dermal layers (Zheng *et al.*, 2009), which is similar to AD skin. IL-13 signaling at sites of T helper type 2 inflammation of AD skin may be the key participant that stimulates ECM production, such as type I collagen or tenascin-C via PI3K/Akt or PKC, as described above. Also, IL-13-mediated MMP-13 downregulation may accelerate ECM deposition in AD skin by reducing ECM degradation activity. Our study contributes to a better understanding of the mechanisms of fibrosis in AD skin. Controlling MMP-13 expression via the balance of PI3K/Akt3 and PKC-δ is a new therapeutic approach to chronic and untreatable AD.

There were some limitations of our study. First, LY294002 or Akt inhibitor increased MMP-13 expression more than two-fold in the presence of IL-13 (Figure 2c), whereas Akt3-specific siRNA increased MMP-13 only up to 20% (Figure 3d). This discrepancy may be explained by the differences in the method (e.g., immunoblotting or real-time PCR) and reagent (kinase-specific inhibitor or siRNA) used in each experiment. Otherwise, this may indicate the synergistic effect of Akt1/2 and Akt3 to downregulated MMP-13 expression. Furthermore, although our results indicated that the PI3K/Akt3 pathway mediates strong suppression of MMP-13 expression by IL-13 and that inhibition of the pathway leads to increased MMP-13 expression that exceeds the

amplitude of the "native" inhibitory action of the cytokine, there may be a more complex mechanism than that illustrated in Figure 5. For example, PI3K/Akt3 may interact with PKC-δ or other signaling pathways directly or indirectly to mediate the effect of IL-13. Further studies are needed to clarify these points.

MATERIALS AND METHODS

Regents

Recombinant human IL-13 was obtained from R&D Systems (Minneapolis, MN). The antibody for MMP-13 was purchased from Lab Vision (Fremont, CA). Primary antibodies against actin, Akt1/2, Akt3, phosphorylated Akt (Thr308 and Ser473), and PKC-δ were from Santa Cruz Biotechnology (Santa Cruz, CA). Akt inhibitor was from Calbiochem (La Jolla, CA). Rottlerin was from Merck (Darmstadt, Germany). Lipofectamine RNAiMAX transfection regent was purchased from Invitrogen Corporation (Carlsbad, CA).

Cell cultures

Human dermal fibroblasts were obtained by skin biopsy from five healthy donors as described previously (Ihn *et al.*, 1996, 1997). All biopsies were obtained with institutional review board approval and written informed consent according to the Declaration of Helsinki principles. Primary explant cultures were established in 25 cm^2 culture flasks in modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin, streptomycin, and amphotericin B (Gibco, Carlsbad, CA). Monolayer cultures were maintained at 37 °C in 5% CO_2 in air. Fibroblasts between the third and seventh subpassages were used for experiments.

Microarray

The cDNA microarray hybridization experiments were performed using Intelligene Human Cancer CHIP, version 2.1 (Takara, Tokyo, Japan) according to the manufacturer's directions. The protocol used and a complete listing of the nearly 800 genes related to cancer on the Human Cancer CHIP are available on the Web. An ~300-bp cDNA region of each gene, which has minimal homology with other genes registered in the cDNA database, is spotted on this DNA chip. Poly $(A)^+$ mRNA was isolated and used to prepare cDNAs that were fluorescently labeled with either Cy3 (IL-13 untreated) or Cy5 (IL-13 treated) fluorescent dye. Samples were mixed and applied to DNA microarrays. The arrays were scanned using ScanArray (GSI Group Japan Corporation, Tokyo, Japan), and the data were analyzed using Quant Array (BM BIO, Tokyo, Japan) (Hishikawa *et al.*, 2001). A gene expression ratio (Cy5/Cy3) of >1.5 or <0.67 was considered significant.

Western blotting analysis

Human dermal fibroblasts were cultured until they were subconfluent. Cells were serum starved, and then the medium was collected. The medium was concentrated 10 times using Amicon Ultra (Millipore, Billerica, MA). The remaining cells were washed with cold phosphate-buffered saline twice and lysed in lysis buffer (Denaturing Cell Extraction Buffer, Biosource, Camarillo, CA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma, St Louis, MO). Cell lysates were quantitated using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. Equal amounts of protein were loaded and separated on 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Nonspecific binding to the membrane was blocked for 1 hour at room temperature with 5% nonfat milk or 1% fetal albumin. Membranes were incubated overnight at 4 °C with the primary antibodies diluted as follows: MMP-13 (1:200), Akt1/2 (1:200), Akt3 (1:200), phosphorylated Akt at Thy308 (1:500), and phosphorylated Akt at Ser473 (1:500). Then membranes were incubated with secondary antibody for 1 hour at room temperature. The immunoblots were developed using the enhanced chemiluminescence detection system from Amersham Biotech (Piscataway, NJ). Image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

RNA isolation and reverse transcription

Total RNA was extracted from cultured human dermal fibroblasts using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using the PrimeScript RT reagent Kit (Takara) with both oligo-dT primer and random hexamers.

Quantitative real-time PCR

Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800) was performed using primers and templates mixed with the SYBR Premix Ex Taq Kit (Takara). Primer sets for MMP-13 and 18S ribosomal (r18S) RNA were purchased from Takara. Primer sets were prevalidated to generate single amplicons. Two microliters of first-strand cDNA product was used for amplification in duplicate in a 25 μl reaction solution containing 12.5 μl of SYBR Green/Fluorescein PCR master mix and 0.5 μl of each primer. DNA was amplified for 45 cycles of denaturation for 5 seconds at 95 °C and annealing for 30 seconds at 66 °C. Data generated from each PCR reaction were analyzed using the Thermal Cycler Dice Real Time System, version 2.10B (Takara). Specificity of reactions was determined by melting curve analysis. Data were analyzed using the comparative C_t method. The r18S RNA was used as a normalizer.

The transient transfection

siRNAs against *Akt1/2* and *Akt3* were purchased from Santa Cruz Biotechnology. Human dermal fibroblasts were plated in six-well plates. At 20% confluence, cells were transfected with siRNA for *Akt1/2*, *Akt3*, or non-targeting control RNA mixed with Lipofectamine RNAiMAX transfection reagent and incubated without media change for 48 hours.

Immunofluorescence microscopy

Dermal fibroblasts were grown in four-well LAK TEK chambers (Nunc, Naperville, IL) to subconfluence as described above. After 24 hours of serum starvation, cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline, and blocked with 10% fetal calf serum in 0.5% Triton X-100 in phosphate-buffered saline. Cells were stained with anti-PKC-δ antibody as the primary antibody, washed, and incubated with FITC-conjugated secondary antibody. To visualize the antibody staining, a fluorescence microscope (Carl Zeiss Meditec, Dublin, CA) was used (Asano *et al.*, 2004).

Statistical analysis

Data presented as bar graphs are the means + SD of at least three independent experiments. Statistical analysis was performed using the Mann–Whitney *U*-test. *P*-values <0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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ORIGINAL ARTICLE

Efficacy and safety of intravenous cyclophosphamide pulse therapy with oral prednisolone in the treatment of interstitial lung disease with systemic sclerosis: 4-year follow-up

Akiko Tochimoto · Yasushi Kawaguchi · Masako Hara · Mutsuto Tateishi · Chikako Fukasawa · Kae Takagi · Emi Nishimagi · Yuko Ota · Yasuhiro Katsumata · Takahisa Gono · Eiichi Tanaka · Hisashi Yamanaka

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Abstract Interstitial lung disease (ILD) is a noteworthy condition in the treatment of systemic sclerosis (SSc) because of its associated mortality and morbidity; however, the efficacy of various treatments for ILD has been controversial in previous reports. In this study, we examined the efficacy and safety of intravenous cyclophosphamide (IVCY) pulse therapy with prednisolone (PSL) for the treatment of ILD with SSc. A total of 121 patients with SSc were screened and evaluated for ILD, using high-resolution computed tomography of the chest, pulmonary function testing, and bronchoalveolar lavage. Thirteen patients with active ILD were enrolled in this study. The treatment protocol for ILD was 0.4 g/m^2 of body surface area of IVCY monthly plus 0.8 mg/kg of body weight of PSL daily. Two to six doses of IVCY were administered, depending on the remission of ILD. Initial PSL doses were maintained for a month and then gradually tapered to 10 mg daily. An activity index of ILD showed improvements in all patients in the 12 months after the initial intervention; however, four patients experienced recurrence of ILD after 24 months, and one additional patient had recurrence of ILD after 36 months. Seven patients reached the 48-month point with no recurrence of ILD. This long observational study for 48 months showed the efficacy of IVCY with PSL for active alveolitis in the first year. However, because five patients had recurrence of ILD more than 1 year after the treatment, it would be necessary to consider maintenance therapy for ILD beyond 1 year.

A. Tochimoto · Y. Kawaguchi (✉) · M. Hara · M. Tateishi · C. Fukasawa · K. Takagi · E. Nishimagi · Y. Ota · Y. Katsumata · T. Gono · E. Tanaka · H. Yamanaka
Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-Ku, Tokyo 162-0054, Japan
e-mail: y-kawa@ior.twmu.ac.jp

Keywords Systemic sclerosis · Cyclophosphamide · Prednisolone · Interstitial lung disease

Introduction

Systemic sclerosis (SSc) is a chronic multisystem autoimmune disease characterized by tissue fibrosis of unknown etiology. Pulmonary fibrosis and infection is one of the most frequent causes of mortality in SSc, along with pulmonary arterial hypertension. In most patients, alveolitis is an initiating event during the pulmonary fibrotic process, developing into pulmonary fibrosis with honeycombing [1]. It is generally believed that pulmonary fibrosis is an irreversible and untreatable complication; however, several reports suggest that the early pulmonary involvement of alveolitis can be controlled by immunosuppressants [i.e., cyclophosphamide, prednisolone (PSL)] [2–7]. Various agents have been evaluated to treat interstitial lung disease (ILD) in SSc [8, 9]. In previous uncontrolled studies, oral or intravenous cyclophosphamide (IVCY) has variably improved lung function parameters, including forced vital capacity (FVC) levels and/or computed tomography (CT) results [10–12]. Three prospective randomized placebo-controlled trials of cyclophosphamide (CY) were published in 2006 [13–15]. In one of these, the group treated with oral CY showed a significant increase of FVC at 1 year [13]. This trial did not allow the use of any corticosteroids. By contrast, the other two studies [14, 15] selected combination therapy with both CY and corticosteroids. The results showed that lung functional parameters did not change significantly after treatment; however, there was a trend toward statistical significance for the difference between the CY-treated group and the control group. Both reports demonstrated that CY could be a promising

disease-modifying medication, improving lung function for up to a year. Based on those studies, the European League Against Rheumatism recommended that CY be considered for the treatment of SSc-related ILD [16]; however, the long-term effectiveness of CY remains controversial. In the present study, we investigated the efficacy of IVCY therapy with oral PSL in Japanese patients with SSc-related ILD during treatment and for the 4 years following the beginning of the treatment.

Patients and methods

Patients

We prospectively and consecutively enrolled Japanese patients with SSc who were admitted to Aoyama Hospital, Tokyo Women's Medical University, between May 2000 and November 2004. To be eligible for the present study, SSc patients had to fulfill the American College of Rheumatology classification criteria for SSc [17]. Skin thickness was assessed using the modified Rodnan total skin thickness score (maximum possible score 51) [18]. ILD was defined as high-resolution computed tomography (HRCT) of the chest that included one or more of the following features: isolated ground-glass opacities, honeycombing and the concurrent presence of ground-glass attenuation, and traction bronchiectasis and/or bronchiolectasis. The HRCT scans were evaluated by two rheumatologists and a radiologist. Active ILD was defined when patients had both an increase in the reticular/linear pattern or a new appearance of ground-glass opacity and worsening of clinical symptoms such as dyspnea or cough. Furthermore, patients had to meet at least one of the following criteria: (1) elevation of serum KL-6 levels; (2) FVC decrease of more than 10%; (3) PaO_2 decrease of more than 5 mmHg; or (4) increase in the percentage of lymphocytes, neutrophils, and eosinophils in bronchoalveolar lavage (BAL) fluid. Exclusion criteria were any immunosuppressive treatment or previous high dose of PSL. We also excluded patients with other collagen diseases (i.e., systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, polymyositis, dermatomyositis) and patients with isolated pulmonary arterial hypertension (PAH), defined as right ventricular systolic pressure (RVSP) at rest >40 mmHg detected by echocardiography and confirmed by right-heart catheterization. This protocol was approved by our institutional ethics committee, and informed consent was obtained from each patient. All patients were tested for the presence of significant antibodies. Anti-nuclear antibodies were detected using indirect immunofluorescence. Anti-topoisomerase I antibody, anti-U1-RNP antibody, anti-Sm antibody, anti-centromere antibody, anti-SS-A antibody, and anti-SS-B antibody levels were examined using direct immunodiffusion method. Anti-double-stranded (ds) DNA antibody was examined by radioimmunoassay.

Therapeutic regimen

All patients in whom active ILD was determined received 0.4 g/m^2 body surface area of IVCY per month for 2–6 months and oral PSL daily (0.8 mg/kg body weight). The number of IVCY treatments varied because IVCY was discontinued when the patient no longer fulfilled the criteria for active ILD described above. A month after the first IVCY treatment, we began to reduce the PSL dose, first to 2.5 mg daily for 2 weeks, and the dose was then increased to 10 mg/day as a maintenance dose. Patients whose ILD severity index scores (described below) improved after the second IVCY treatment did not receive further IVCY.

Evaluation of outcome

The response to this therapy was analyzed by examining clinical symptoms, HRCT, the pulmonary function test (PFT), and the serum KL-6 level. These tests were performed at baseline and at 6, 12, 24, 36, and 48 months. To assess these values, we created indices for each finding according to its severity, as shown in Table 1. We calculated the total number of points by adding the point values for each index. The primary endpoint was the change in the total number of points over the 48-month period. The secondary endpoints were progression of ILD due to ineffectiveness of therapy; recurrence of ILD, as defined by the entry criteria for this study; and death.

Statistics

We calculated each patient's dyspnea score, HRCT score, %FVC score, serum KL-6 score, and the total score. Between-group differences were estimated using the Mann–Whitney *U*-test. A P value of <0.05 was considered as significant.

Results

Clinical and immunological characteristics

As outlined in Fig. 1, we evaluated 121 patients with SSc who were consecutively admitted to our hospital. Thirteen patients (11 women, 2 men; median age 55 years) presented with active ILD and were enrolled in the study. Table 2 summarizes the baseline characteristics and clinical manifestations of the patients. Eleven patients in this study had diffuse cutaneous SSc and 2 had limited

Table 1 Severity scoring of ILD

	Score
Dyspnea score	
Unable to carry out any physical activity without discomfort	5
Unable to walk >100 m due to dyspnea	4
Dyspnea with walking on a level surface	3
Dyspnea with walking up stairs or up a slope	2
Dry cough	1
No dyspnea	0
HRCT score[a]	
Interstitial shadow in the upper, middle, and lower zones	4
Interstitial shadow in the middle and the lower zones	3
Interstitial shadow in the lower zone	2
Interstitial shadow in the lung base alone	1
Without interstitial shadow	0
With ground-glass opacity	+1
%FVC score	
<50	5
50–60	4
60–70	3
70–80	2
80–90	1
90	0
KL-6 score	
>2500 (U/ml)	5
2000–2500	4
1500–2000	3
1000–1500	2
500–1000	1
<500	0

ILD interstitial lung disease, *HRCT* high-resolution computed tomography, *FVC* forced vital capacity

[a] Upper zone, lung apex to aortic arch; middle zone, aortic arch to inferior pulmonary veins; lower zone, inferior pulmonary veins to lung base

cutaneous SSc. The median modified Rodnan total skin-thickness score (mRTSS) was 30 (range 5–44). The median duration from the diagnosis of SSc, defined as the onset of SSc-related manifestations except for Raynaud's phenomenon, was 10 months (range 3–108 months). Five patients had anti-topoisomerase I antibody and 3 patients had anti-U1-RNP antibody. All patients had anti-nuclear antibody. Seven patients had had BAL; 4 of these had abnormal findings. The mean FVC was 81%, and the mean DLco was 83%.

Response to therapy

The duration of IVCY therapy ranged from 2 to 6 months; 1 patient was withdrawn from the study and 2 patients

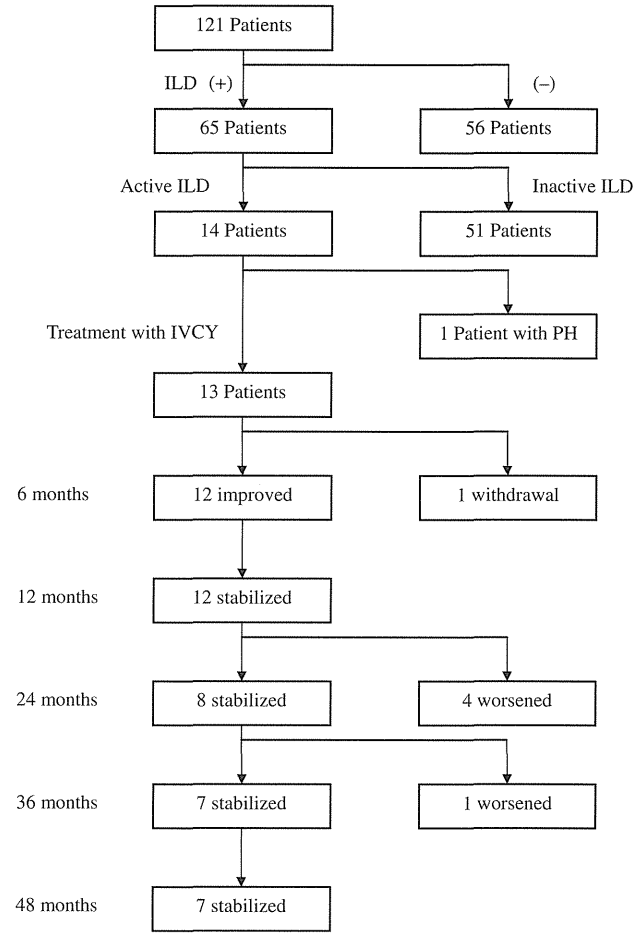

Fig. 1 Patients enrolled in the present 4-year follow-up study, *ILD* Interstitial lung disease, *IVCY* intravenous cyclophosphamide, *PH* pulmonary hypertension

completed 2 months, 3 patients completed 3 months, 1 patient completed 4 months, 3 patients completed 5 months and 3 patients completed 6 months of therapy. By 6 months after the treatment started, ILD had improved in all patients, as indicated by changes in their ILD severity index scores (Fig. 2). After 12 months, all patients were stabilized. After 24 months, 8 patients were stabilized, 3 patients had worsened, and 1 patient had died. Of the 8 patients who were stable after 24 months, 7 patients were stabilized at 36 and 48 months and 1 patient had worsened at 36 months. As noted above, skin thickening was evaluated using mRTSS during this study, and the mean mRTSS was significantly reduced at 6, 12, 24, 36, and 48 months.

Evaluation of ILD variables

As shown in Table 3, there were significant differences in the dyspnea score and total score between baseline and 48 months ($P = 0.001$ and $P = 0.01$, respectively). The dyspnea score had improved significantly at 3 months, improved further from 3 to 12 months, and then remained

Table 2 Characteristics of 13 patients with systemic sclerosis (SSc)

Patient no.	Sex	Age (years)	Disease type	mRTSS	Disease duration (months)	BALF			Autoantibodies except for ANA	Outcome
						L (%)	*N* (%)	*E* (%)		
1	F	61	Dc	30	8	11	–	–	ND	Withdrawal
2	F	64	Dc	30	96	NT			Topo-I	Improved
3	F	47	Dc	39	7	NT			Topo-I	Improved
4	F	52	Dc	20	10	NT			U1-RNP	Improved
5	F	47	Lc	5	7	12	–	–	U1-RNP	Worsened
6	F	73	Dc	30	14	61	5	–	ND	Worsened
7	M	55	Lc	14	36	41	–	–	SS-A, SS-B	Improved
8	F	55	Dc	27	108	40	5	5	Topo-I, CENP	Worsened
9	M	64	Dc	33	12	12	–	9	Topo-I	Worsened
10	M	55	Dc	15	3	NT			U1-RNP, SS-A	Worsened
11	F	50	Dc	23	12	7	–	1	Topo-I	Improved
12	F	67	Dc	44	10	NT			ND	Improved
13	F	43	Dc	34	8	NT			ND	Improved

Dc diffuse cutaneous SSc, *Lc* limited cutaneous SSc, *mRTSS* modified Rodnan total skin-thickness score, *BALF* bronchoalveolar lavage fluid, *L* lymphocyte, *N* neutrophil, *E* eosinophil, *ANA* anti-nuclear antibody, *Topo-I* anti-topoisomerase I antibody, *CENP* anti-centromere antibody, *U1-RNP* anti-U1-snRNP antibody, *SS-A* anti-SS-A antibody, *SS-B* anti-SS-B antibody, *NT* not tested, *ND* not detected

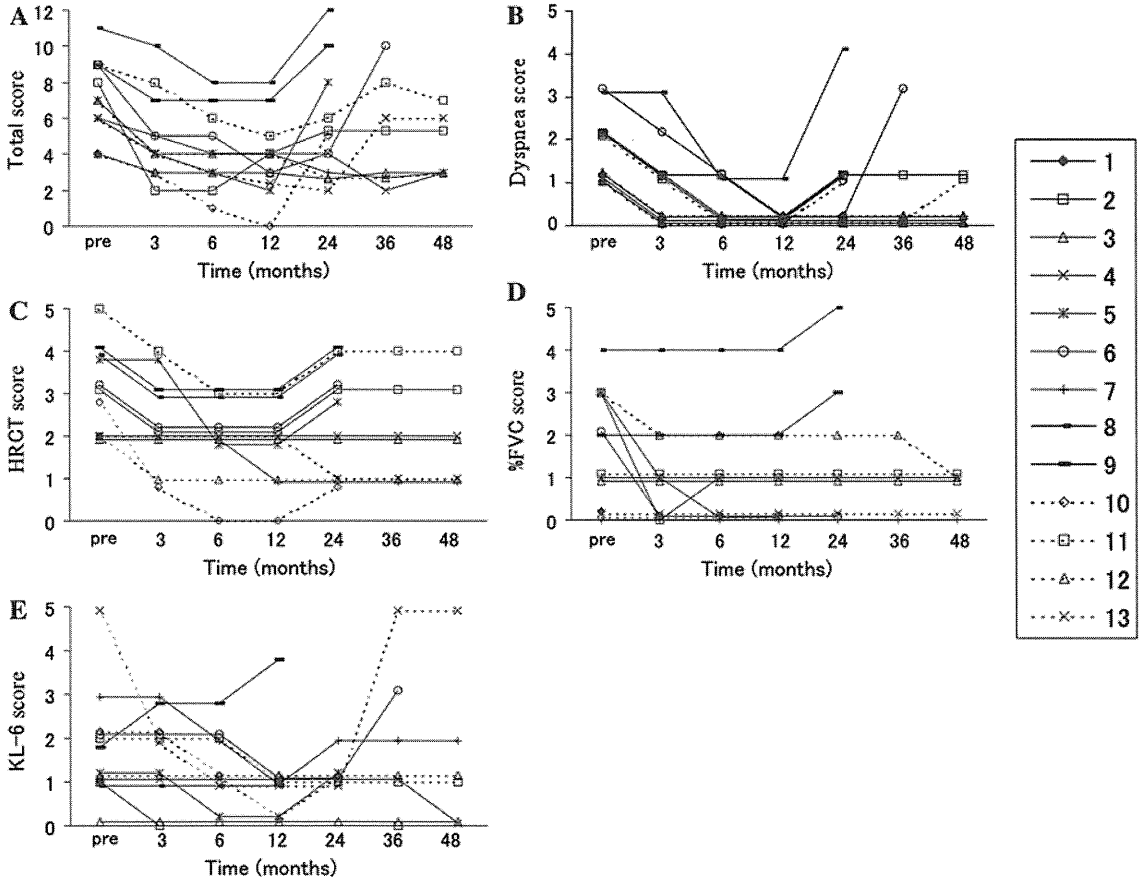

Fig. 2 Severity scoring during follow-up. **a** Total severity score; **b** dyspnea score; **c** high-resolution computed tomography (*HRCT*) score; **d** %forced vital capacity (*%FVC*) score; **e** *KL-6* score

Table 3 Comparison of the evolution of the dyspnea score, CT score, FVC score, KL-6 score, and total number of points

	Baseline	3 months	6 months	12 months	24 months	36 months	48 months
Dyspnea	1 (1–3)	0.5 (0–3)*	0 (0–1)***	0 (0–1)***	0 (0–1)***	0 (0–1)***	0 (0–1)**
HRCT	3 (2–5)	2 (1–4)	2 (0–3)*	2 (0–3)*	2 (1–4)	2 (1–4)	2 (1–4)
%FVC	1 (0–4)	1 (0–4)	1 (0–4)	1 (0–4)	1 (0–2)	1 (0–2)	1 (0–1)
KL-6	1 (0–5)	1.5 (0–3)	1 (0–3)	1 (0–4)	1 (0–2)	1 (0–5)	1 (0–5)
Total	7 (4–11)	4 (2–10)*	4 (1–8)**	4 (0–8)**	3.5 (2–6)**	3 (2–8)*	3 (3–7)*

Results are expressed as means (ranges), and the differences were estimated by the paired Wilcoxon's test. P values were determined in comparison with baseline. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4 Comparison of the evolution of the FVC and KL-6

	Baseline	3 months	6 months	12 months	24 months	36 months	48 months
%FVC	82 ± 15	86 ± 13	89 ± 9	91 ± 13	87 ± 10	87 ± 11	87 ± 11
KL-6 (U/ml)	1229 ± 1211	935 ± 513	919 ± 482	773 ± 505	788 ± 307	1195 ± 1559	1361 ± 1995

Results are expressed as means ± SD

at the same level until the end of the study at 48 months. The HRCT score had improved significantly at 6 and 12 months compared with the baseline. The %FVC and KL-6 scores showed trends for improvement between baseline and 3–24 months. The raw data for %FVC and KL-6 are shown in Table 4. The total score also showed significant improvement at 3 months, improved further from 3 to 24 months, and then remained at the same level until the end of the study at 48 months.

Adverse effects

The therapy was well tolerated overall. One patient developed viral infectious myocarditis after the first IVCY and was withdrawn from the study. This infectious complication was not associated with neutropenia or lymphopenia. No severe bone marrow suppression or hemorrhagic cystitis was observed.

Discussion

We assessed the efficacy and safety of IVCY pulse therapy combined with PSL in Japanese patients with SSc-related ILD. The therapy was well tolerated and improved or stabilized ILD variables for up to 12 months after treatment began. However, after 12 months, 4 patients (33%) showed deteriorated HRCT and/or increased %FVC. All 4 were treated again using the same dose of IVCY. Three patients improved with the additional IVCY therapy, but one patient died of severe bacterial pneumonia. After 24 months, one patient redeveloped active ILD but improved with additional IVCY therapy. The regimen of IVCY pulse therapy combined with PSL was effective and safe for the treatment of active ILD with SSc for 12 months, but the therapeutic effects were not sustained in all patients beyond the first year.

The North American Scleroderma Lung Study group evaluated the efficacy of 12 months' oral CY therapy [13]. Patients treated with oral CY had significantly less change in %FVC compared with placebo-treated patients. The treatment with CY also had the beneficial effect of improving dyspnea, functional ability, health-related quality of life, and skin thickness. However, at 2 years, overall differences between the treatment groups were small, especially for the PFT results (evolution rate of FVC −2.0% for CY vs. −2.3% for placebo), with no significant beneficial effect demonstrated [19]. In the present study, 5 of 12 patients (42%) had worsened more than 12 months after the first IVCY treatment. These results suggest the need to combine a maintenance therapy, such as azathioprine, mycophenolate mofetil, or calcineurin inhibitors, with IVCY plus PSL as an induction immunosuppressive therapy.

The results of 2 studies of CY followed by oral azathioprine were recently reported. In the UK Fibrosing Alveolitis in Scleroderma Trial, 45 patients with SSc-related ILD were randomized to receive PSL (20 mg/day) and 6 CY infusions (0.6 g/m^2 monthly) followed by oral azathioprine or placebo. Estimation of the relative treatment effect (active treatment vs. placebo) adjusted for baseline FVC and treatment center revealed a favorable FVC outcome of 4.19%. This between-group difference showed a trend toward statistical significance ($P = 0.08$) [15]. A retrospective multicenter open-label study of French patients with worsening ILD examined 27 patients who received 6 IVCY treatments followed by 18 months of immunosuppressive therapy [14]. That study indicated that

IVCY followed by oral maintenance immunosuppressive therapy for worsening ILD stabilized 51.8% of the patients with progressive ILD at 2 years.

The doses of IVCY in our study were lower than those used in previous trials in the United States and Europe. This factor may explain why only one of our patients (8%) experienced an adverse reaction. The therapeutic regimen used in our study is safe and tolerable for Japanese patients.

In conclusion, the combination of IVCY and oral PSL improved dyspnea, the HRCT score, and %FVC in all patients, and the improved therapeutic effects were seen for a year after treatment began. More than 1 year post-therapy, however, we observed recurrence of ILD in about half of the patients, a finding which indicates the importance of adding maintenance therapy after IVCY-plus-corticosteroid therapy for the treatment of ILD complicated with SSc.

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Conflict of interest None.

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Association of Hepatocyte Growth Factor Promoter Polymorphism With Severity of Interstitial Lung Disease in Japanese Patients With Systemic Sclerosis

Kana Hoshino,[1] Takashi Satoh,[1] Yasushi Kawaguchi,[2] and Masataka Kuwana[1]

Objective. **To examine associations of single-nucleotide polymorphisms (SNPs) within genes for hepatocyte growth factor (HGF) and its receptor c-*met* with disease susceptibility and organ involvement in Japanese patients with systemic sclerosis (SSc).**

Methods. **Four SNPs (*HGF* –1652 C/T, +44222 C/T, and +63555 G/T, and c-*met* –980 T/A) were analyzed in 159 SSc patients and 103 healthy control subjects with the use of a polymerase chain reaction–based assay. The influence of the *HGF* –1652 SNP on transcription activity was evaluated with a luciferase reporter assay and an electrophoretic mobility shift assay (EMSA).**

Results. **There was no difference in the distribution of *HGF*/c-*met* SNPs between SSc patients and controls. *HGF* –1652 TT was found much more frequently in SSc patients with end-stage lung disease (ESLD) than in those without (41% versus 8%; $P = 0.0004$). This association was confirmed by a replication study involving a separate cohort of 155 SSc patients. Kaplan-Meyer analysis revealed that *HGF* –1652 TT carriers had a higher probability of developing ESLD than did CT or CC carriers. The *HGF* promoter carrying the *HGF* –1652 T allele had lower transcription activity than did the promoter carrying the C allele. EMSA showed the presence of a potential negative transcription regulator that binds specifically to the *HGF* promoter carrying a T allele at position –1652. Finally, TT carriers had a relative inability to increase circulating HGF levels even in the presence of advanced interstitial lung disease.**

Conclusion. **A SNP in the *HGF* promoter region may modulate the severity of interstitial lung disease by controlling the transcriptional efficiency of the *HGF* gene.**

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis of the skin and internal organs, microvasculopathy, and the presence of circulating antinuclear antibodies (ANAs). The cause of the disease remains unclear, but both genetic and environmental factors contribute to its development (1). Recent findings indicate that a series of growth factors and cytokines are involved in the pathogenesis of SSc (2). For example, transforming growth factor β (TGFβ) is a profibrotic factor that stimulates fibroblast collagen production and promotes extracellular matrix deposits. Other profibrotic factors include connective tissue growth factor (CTGF) and platelet-derived growth factor.

In contrast, hepatocyte growth factor (HGF), a ligand for the c-*met* receptor tyrosine kinase, is an antifibrotic factor that helps to regenerate damaged tissue by inhibiting fibrosis and promoting angiogenesis (3). A series of studies have shown that HGF suppresses fibrosis by inhibiting collagen production, inducing myofibroblast apoptosis, and degrading the extracellular matrix (4,5). Since HGF counteracts many of the profibrotic actions of TGFβ, it has been proposed that a balance between HGF and TGFβ may play a decisive role in the pathogenesis of fibrosis (6,7). However, patients with SSc have increased circulating levels of HGF as compared to those in healthy controls (8). This level of HGF may not be sufficient to inhibit the ongoing fibrotic process.

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Kana Hoshino, MSc, Takashi Satoh, PhD, Masataka Kuwana, MD, PhD: Keio University School of Medicine, Tokyo, Japan; Yasushi Kawaguchi, MD, PhD: Tokyo Women's Medical University, Tokyo, Japan.

Address correspondence to Masataka Kuwana, MD, PhD, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kuwanam@z5.keio.jp.

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The prevalence and severity of interstitial lung disease (ILD) in SSc patients varies among ethnic groups. ILD is more common and more progressive in African American than in Caucasian patients (9,10). Even in a relatively homogeneous group of SSc patients with serum anti–topoisomerase I (anti–topo I) antibody, Japanese and African American patients were found to have more-prominent deterioration of lung function and lower survival rates than Caucasian patients (11). Together, these findings suggest that ethnicity influences the onset and progression of SSc-associated ILD, likely through differences in both genetic and environmental backgrounds. A recent study of SSc patients showed that increased HGF levels were observed in bronchoalveolar lavage fluid and plasma samples from Caucasian patients but not African American patients (12), suggesting that ethnic differences in susceptibility to pathologic fibrosis in the lungs may be explained by genetic control of HGF expression.

In this study, we selected single-nucleotide polymorphisms (SNPs) within genes for the *HGF* and c-*met* loci in Japanese patients with SSc and examined their associations with disease susceptibility, organ involvement, and autoantibody profiles. We also examined the underlying mechanism of an association between an *HGF* SNP and ILD outcomes.

PATIENTS AND METHODS

Patients and controls. We studied 159 Japanese SSc patients whose cases were followed between 1988 and 2008 at Keio University Hospital (cohort 1). An additional 155 Japanese patients from Tokyo Women's Medical University Hospital were used as a replication cohort (cohort 2). All patients met the American College of Rheumatology preliminary classification criteria for SSc (13). SSc patients were classified as having diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) according to the classification described by Medsger (14). Controls included 103 healthy Japanese volunteers residing in the Tokyo metropolitan area.

All samples were obtained after the patients and control subjects had given their written informed consent. The study protocol was approved by the Institutional Review Boards of Keio University and Tokyo Women's Medical University.

Assessment of clinical features. Records of clinical and laboratory findings were kept prospectively for all patients. A complete medical history, physical examination, and laboratory analyses were performed at each patient's first visit, and more-limited evaluations were performed during followup visits (at least once every 3 months). Forced vital capacity (FVC) measured within 3 months before and 3 months after blood collection was used to examine its association with circulating concentrations of HGF. For all patients, any SSc-related major organ involvement was documented, including that of the peripheral vasculature (digital ulcer), esophagus, heart, kidney, lung parenchyma (ILD), and lung vasculature (pulmonary arterial hypertension).

The criteria used to define individual organ involvement have been described elsewhere (15). ILD was defined as the presence of bibasilar reticulation and/or fibrosis on chest radiograph. End-stage lung disease (ESLD) was defined as the presence of at least 1 of the following 3 features: <50% FVC, required oxygen supplementation in the absence of pulmonary arterial hypertension, or death due to ILD-related causes (16).

Identification of SSc-related ANAs. Serum samples from all SSc patients were analyzed for anticentromere, anti–topo I, and anti–U1 small nuclear RNP (anti–U1 snRNP) antibodies, which are major SSc-related ANAs in the Japanese population (15), using indirect immunofluorescence and immunoprecipitation assays (15).

Genotyping of *HGF* and c-*met* SNPs. Based on heterogeneous allelic distribution in the Japanese population (17,18), we selected for analysis 3 SNPs within the *HGF* gene, namely, –1652 (rs3735520) C/T, +44222 (rs2887069) C/T, and +63555 G/T, as well as 1 SNP within the c-*met* gene, –980 (rs38839) T/A. Genomic DNA was isolated from peripheral blood mononuclear cells using a QIAamp DNA Mini kit (Qiagen). Individual SNPs were determined by amplifying genes of interest by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP). Table 1

Table 1. RFLP conditions and primers used for analysis of the 4 single-nucleotide polymorphisms within the *HGF* and c-*met* genes*

Gene location	Region	Primer sequence (5′→3′)	Annealing temperature (°C)	Restriction enzyme used for RFLP
HGF –1652 C/T (rs3735520)	Promoter	Sense: CAGACAGAGGCTGACAAATG Antisense: CACTTTCAGGGAAAAACAACCTGC	66.5	*Bsl* I
HGF +44222 C/T (rs2887069)	Intron 9	Sense: GCTGACATTCTGCAGGGTGGGC Antisense: CTCCAGGGGGTCAGATAGCTTAG	68.5	*Alu*I
HGF +63555 G/T	Exon 15	Sense: CCTTCCTGCTTCTCAGCAAGGTCAC Antisense: CGAACTGCCACACAGCTGAAG	66.5	*Ssp* I
c-*met* –980 T/A (rs38839)	Promoter	Sense: CAAGTTGGTATGAGAGCCGGAACG Antisense: GAGGAGTTTAACCCTGAGGAGACT	59.7	*Dra*I

* RFLP = restriction fragment length polymorphism.

shows the primer sequences and annealing temperatures used for PCR, as well as the restriction enzymes used for RFLP. PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C, annealing at optimal temperature, and extension at 72°C for 30 seconds each cycle, and a final extension step at 72°C for an additional 5 minutes. The PCR products were digested with the corresponding restriction enzymes, separated on a 12% polyacrylamide gel, and stained with ethidium bromide.

Assessment of *HGF* promoter activity. A series of DNA fragments encoding the promoter region of the *HGF* gene were amplified by PCR using genomic DNA samples known to be homozygous for *HGF* –1652 CC or TT as a template. The 5 fragments generated included nucleotides –460 to –1, –831 to –1, –1313 to –1, and –1756 to –1 with a C allele at –1652, and –1756 to –1 with a T allele at –1652. The sense primer sequences were 5′-GGATCCTGGGGACACCAGAC-3′ (−1756 to −1), 5′-ACCAGAGCATCCACCTCTGGG-3′ (−1313 to −1), 5′-GCTGCCTGCTCTGAGCCCAT-3′ (−831 to −1), and 5′-GAATTGGTCCCCTGCCTGTGCCT-3′ (−460 to −1). The amplification antisense primer sequence was 5′-GGTGCTGCTGGACGGGCTGGC-3′. The genotype was verified by sequencing on an ABI Prism 3100 genetic analyzer using a BigDye Terminator Cycle Sequence Ready Reaction kit (both from Applied Biosystems).

HGF promoter activity was measured with a Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's protocol. Lung squamous cell carcinoma cell line EBC-1 cells were cotransfected with a firefly luciferase plasmid harboring the HGF promoter region and a *Renilla* luciferase reporter plasmid, using Lipofectamine 2000 (Invitrogen). The cells were lysed, and firefly and *Renilla* luciferase activity were each measured with a Micro Lumat Plus LB96V luminometer (Berthold Technologies). The relative *HGF* promoter activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity as an internal reference. The *HGF* promoter region covering nucleotides −460 to −1 was defined as a basic transcription activation domain (19).

Electrophoretic mobility shift assay (EMSA). The interaction of nuclear factors with the DNA covering the polymorphic *HGF* −1652 SNP was evaluated by EMSA, using a LightShift Chemiluminescent EMSA kit (Pierce), according to the manufacturer's directions (20). We used duplex DNA probes composed of complementary oligonucleotides labeled with biotin at the 3′ end: 5′-GATCC/TCTCAAAAGGAATTCT-3′ and 5′-CTAGG/AGAGTTTTCCTTAAGA-3′, with either C/G or T/A at position −1652 (underlined). Nuclear extracts were prepared from primary skin fibroblast cultures, which were derived from the backs of 2 healthy control subjects. The extracts were incubated with biotin-labeled DNA and separated on 4% native polyacrylamide gels. The protein-DNA complex was detected by measuring a chemiluminescent signal. Competition assays were performed using a 400-fold excess of unlabeled duplex DNA as a competitor.

Measurement of serum HGF concentrations. Levels of HGF in serum samples were measured using specific enzyme-linked immunosorbent assay kits (Quantikine; R&D Systems) according to the manufacturer's instructions.

Statistical analysis. The genotype frequency distribution was assessed for Hardy-Weinberg equilibrium. Phenotype frequencies were tested for statistical significance using Pearson's chi-square test for a 2 × 3 contingency table. Corrected P (P_{corr}) values were obtained by multiplying by the number of alleles. Significant differences (overall $P_{corr} < 0.05$) were further analyzed by pairwise comparisons using 2 × 2 chi-square tests. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for statistically significant differences. Continuous variables are reported as the mean ± SD; they were compared using the Mann-Whitney U test. The correlation coefficient (r) was determined using a single regression model. Cumulative survival rates were calculated using the Kaplan-Meier method, and differences between 2 groups were analyzed by the log rank test. All statistical analyses were performed with SPSS statistical software for Windows version 15.0J.

RESULTS

Clinical characteristics of the SSc patients. Demographic and clinical features of the SSc patients who were enrolled in the present study are summarized in Table 2. There were statistically significant differences in the age at onset of SSc, the disease duration, and the proportions of patients with dcSSc and with anti-U1

Table 2. Demographic and clinical characteristics of the 2 cohorts of SSc patients enrolled in the study*

Feature	SSc cohort 1, Keio University (n = 159)	SSc cohort 2, Tokyo Women's Medical University (n = 155)
% female	85	91
Age at onset of SSc, mean ± SD years	46.9 ± 13.7	42.8 ± 12.1†
Disease duration, mean ± SD months	185 ± 111	116 ± 59‡
Clinical features of SSc, % of patients		
Diffuse cutaneous SSc	41	64§
Digital ulcer	30	25
Esophageal involvement	65	53
Heart involvement	7	5
Kidney involvement	3	3
Interstitial lung disease	53	51
Pulmonary arterial hypertension	9	8
End-stage lung disease	11	9
Anti–topo I antibody	37	37
Anticentromere antibody	19	20
Anti–U1 snRNP antibody	18	5¶

* SSc = systemic sclerosis; anti–topo I = anti–topoisomerase I; anti–U1 snRNP = anti–U1 small nuclear RNP.
† $P = 0.005$ versus cohort 1.
‡ $P < 0.0001$ versus cohort 1.
§ $P = 0.0005$ versus cohort 1.
¶ $P = 0.0002$ versus cohort 1.