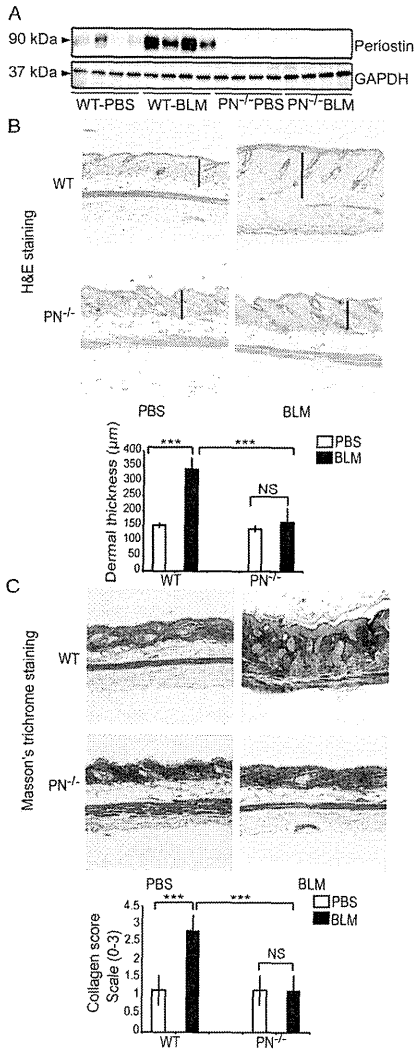


**Figure 2. Periostin gene knockout ($PN^{-/-}$) mice are resistant to BLM-induced cutaneous sclerosis as assessed by dermal thickness and collagen deposition.** A, Western blotting analysis for periostin in skin extracts from WT and $PN^{-/-}$ mice, which were treated with BLM or PBS. B, H&E staining of skin samples from WT and $PN^{-/-}$ mice (original magnification, ×100). Dermal thickness is shown as the black bar in the lower panel and was measured as described in the Materials and Methods. C, Masson's trichrome staining of skin samples from WT and $PN^{-/-}$ mice (original magnification, ×100). Collagen fibers were stained blue. Collagen deposition was scored on a scale of 0–3 as described in the Materials and Methods and is shown in the lower panel. For all assays, 10 mice from each group were analyzed. Values in B and C are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.
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to WT mice [30], suggesting that periostin plays a limited role or is dispensable in certain conditions of fibrosis.

At present, it is unclear whether periostin is upregulated in the fibrotic lesions of scleroderma or plays a role in its pathology. In the present study, we analyzed periostin expression in skin samples from patients with systemic scleroderma, and the role of periostin in this disease, using $PN^{-/-}$ mice in a murine model of bleomycin (BLM)-induced scleroderma that exhibits defined cutaneous sclerosis that mimics human scleroderma [31].

## Results

### Periostin is Overexpressed in Lesional Skin of Patients with Scleroderma

To assess the involvement of periostin in the pathogenesis of scleroderma, we first compared the expression of periostin in sclerotic skin lesions from scleroderma patients and skin from identical areas of healthy donors. Based on western blotting analysis and immunohistochemical staining, periostin expression was markedly elevated in lesional skin from scleroderma patients compared with skin from healthy donors (Figure 1A and 1B). In addition, the distribution pattern of periostin in normal and fibrotic skin tissue appeared to be very different. In normal skin sections, periostin was faintly detectable in the upper dermis. In contrast, in scleroderma lesional skin, more intense staining for periostin was observed in the surrounding ECM throughout the dermis (Figure 1B). Furthermore, we examined periostin expression in the lesional skin from patients with other skin fibrotic diseases (keloid and hypertrophic scar), and found that periostin appeared to be expressed more strongly in lesional skin tissue of scleroderma than in those of keloid and hypertrophic scar (Figure 1B).

### *Periostin Gene Knockout Results in Reduced Symptoms of BLM-induced Cutaneous Sclerosis in Mice*

Given these results above, it was logical to ask whether periostin plays an essential role in the pathophysiology of scleroderma or whether the altered expression of periostin is secondary to the disease process. To resolve this issue, we assessed the role of periostin in BLM-induced murine scleroderma using $PN^{-/-}$ mice [27]. To induce cutaneous sclerosis, we subcutaneously injected mice with BLM or PBS for four consecutive weeks, which has been widely used as an animal model of scleroderma [31]. Skin samples were collected one day after the final injection. To evaluate whether periostin is overexpressed in mice with BLM-induced scleroderma, the proteins extracted from mouse skin were subjected to western blotting analysis (Figure 2A). Indeed, periostin was strongly expressed in BLM-induced sclerotic skin of WT mice compared to skin samples from control PBS-treated mice. Antibody specificity was confirmed by the absence of a corresponding band in samples from $PN^{-/-}$ mice. These results agree with the supposition that elevated expression of periostin is closely linked to the pathogenesis of scleroderma.





Next, histological examinations of mouse skin sections using H&E staining (Figure 2B) were performed. As previously reported in this mouse model [32], a striking increase in dermal thickness and an apparent decrease in the amount of subcutaneous fat tissue (Figure 2B) were observed in WT mice injected with BLM. In contrast, $PN^{-/-}$ mice showed minimal dermal thickening (Figure 2B). WT mice showed a statistically significant increase of 220% ±33% in dermal thickness following BLM treatment ($p<0.01$), whereas, $PN^{-/-}$ mice did not develop apparent dermal thickening (Figure 2B, bar graph, lower panel).

Masson's trichrome staining, which stains collagen fibers blue, was performed to examine the increase of collagen fibers in BLM-treated mice (Figure 2C). WT BLM-treated mice displayed substantial thickening of the dermis with a robust deposition of collagen fibers that replaced the subcutaneous fat. These changes were markedly attenuated in BLM-treated $PN^{-/-}$ mice. Assessment using a four-point (grade 0–3) collagen deposition scoring system confirmed that the difference between $PN^{-/-}$ mice and WT mice was significant (Figure 2C, bar graph, lower panel).

Collectively, these results demonstrate that $PN^{-/-}$ mice display markedly reduced symptoms of BLM-induced cutaneous sclerosis, indicating that periostin is required for the development of BLM-induced cutaneous sclerosis.

### Expression of Fibrogenic Cytokines and ECM Proteins in BLM-treated Mice Skin

Next, we assessed the expression of the main fibrogenic cytokines, TGFβ1 and CCN2 (also called CTGF), by real-time quantitative PCR. The expression of TGFβ1 and CCN2 (CTGF) mRNA after BLM treatment (Figure 3A and 3B) was increased in both WT and $PN^{-/-}$ mice, suggesting that the fibrotic process was initiated similarly in both $PN^{-/-}$ and WT mice. We then assessed the mRNA levels of Col1α1, a major component of dermal collagen fibers in these mice. Col1α1 mRNA levels were increased (536±76%) in WT mice skin after BLM treatment ($p<0.01$), but unexpectedly, not in BLM-treated $PN^{-/-}$ mice (Figure 3C). Thus, while periostin is known to regulate collagen assembly [10], these data suggest that periostin in BLM-induced scleroderma is critical for excessive collagen synthesis.

### Periostin is Required for Myofibroblast Differentiation *in vivo*

It is widely accepted that α-SMA-expressing myofibroblasts, which are induced by fibrogenic cytokines, play key roles in collagen synthesis during the development of scleroderma [33]. To determine whether periostin is required for myofibroblast differentiation in this model, histoimmunohistochemistry for α-SMA (the most widely used myofibroblast marker) was performed on skin derived from WT and $PN^{-/-}$ mice with BLM or after PBS treatment (Figure 4A). α-SMA$^+$ cells were increased in the dermis of skin sections from BLM-treated WT mice compared with skin from PBS-treated WT mice (Figure 4A). In contrast, α-SMA$^+$ cells were not increased in BLM-treated $PN^{-/-}$ mice (Figure 4A). To detect myofibroblasts more specifically, double-labeling histoimmunofluorescence staining for anti-α-SMA and anti-CD34 (a representative vascular endothelial maker) were further performed (Figure 4B). Nonvascular α-SMA-positive CD34-negative spindle-shaped cells (α-SMA$^+$ and CD34$^-$ cells), which indicate myofibroblasts, increased in the dermis of WT mice with statistical significance ($p<0.01$) but not in $PN^{-/-}$ mice (Figure 4C) after BLM treatment.

Supporting these data, western blotting analysis revealed an increase in the expression of α-SMA in skin derived from BLM-treated WT mice, but not $PN^{-/-}$ mice, compared with PBS-treated WT mice (Figure 4D). These results suggest that periostin is required for myofibroblast development in this scleroderma model.

### Periostin is Required for TGFβ1-induced Myofibroblast Differentiation *in vitro*

TGFβ1 is the most potent inducer of myofibroblast differentiation in fibrosis [34]. To investigate the mechanism of action of periostin in myofibroblast generation, we isolated mouse dermal fibroblasts from WT and $PN^{-/-}$ mice and stimulated these cells with TGFβ1 *in vitro*. The induction of α-SMA at 2 hrs after TGFβ1 stimulation appeared similar between WT and $PN^{-/-}$ fibroblasts. However, after longer periods of stimulation (12 hrs, 24 hrs), α-SMA expression levels in $PN^{-/-}$

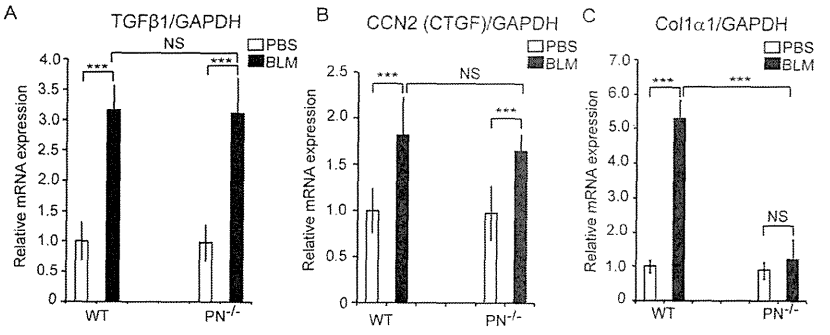


**Figure 3. The expression of fibrogenic cytokines (TGFβ1 and CCN2/CTGF) and collagen type I in BLM-treated mouse skin.** Real-time quantitative PCR analysis was performed to determine mRNA levels of TGFβ1 (A), CCN2 (CTGF) (B), and Col1α1 (C) in mouse skin of WT and $PN^{-/-}$ mice. Values were normalized to GAPDH levels and expressed as relative mRNA levels compared with PBS-treated WT mice. Values are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.
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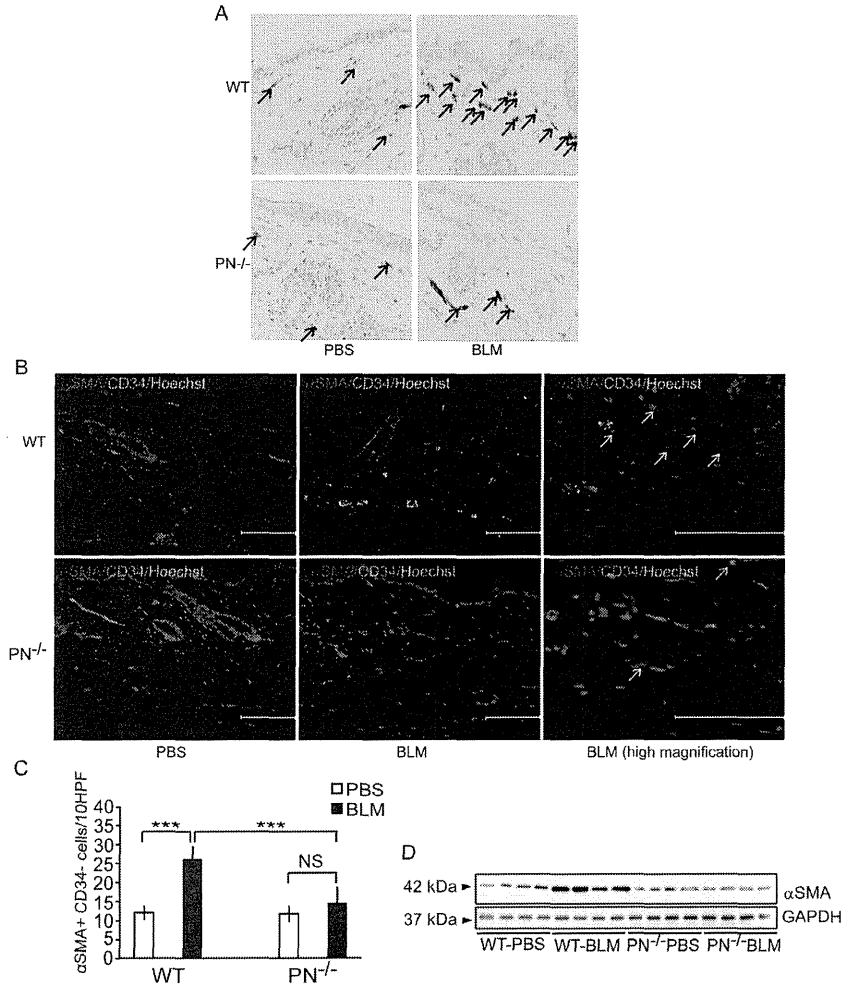


**Figure 4. Periostin is required for dermal myofibroblast development in BLM-treated mice *in vivo*.** A, Representative skin sections from WT and $PN^{-/-}$ mice, stained by immunohistochemistry with anti-α-SMA antibody (original magnification, ×400). α-SMA-positive myofibroblasts are indicated by arrows. B, Representative skin sections from WT and $PN^{-/-}$ mice, doubly stained by immunofluorescence for anti-α-SMA (red) and anti-CD34 (green). α-$SMA^{+}$ $CD34^{-}$ spindle-shaped myofibroblasts are indicated by arrows. Scale bar = 100 μm. Nucleic staining: Hoechst 33342 (blue). C, The number of myofibroblasts per 10 hyper power microscopic fields is shown in the histogram. D, Western blotting analysis of protein extracted from WT and $PN^{-/-}$ mice skin tissues. For all assays, 10 mice from each group were analyzed. Values in C are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.

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$^{-}$ *fibroblasts were significantly lower than those in* WT fibroblasts ($P<0.01$) (Figure 5A). Western blotting analysis, using protein samples extracted from cultured fibroblasts 24 hrs after TGFβ1 stimulation, confirmed that α-SMA protein levels were strongly induced in WT fibroblasts but not in $PN^{-/-}$ fibroblasts (Figure 5B). In addition, WT fibroblasts stimulated with TGFβ1 for more than 12 hrs could upregulate periostin at the protein levels (Figure S2B). These results raise the possibility that periostin protein induced by TGFβ1 may directly or indirectly mediate α-SMA expression in fibroblasts. Therefore, we next stimulated cultured WT dermal fibroblasts with different concentrations of rmPeriostin alone or in combination with TGFβ1 for two hours. While neither α-SMA transcript expression (Figure 5C) nor α-SMA protein expression (Figure 5D) was increased by rmPeriostin stimulation alone, the α-SMA expression level was synergistically enhanced by the combined stimulation of rmPeriostin with TGFβ1, compared to that with TGFβ1 stimulation alone (Figure 5C and 5D). These results suggest that periostin can enhance α-SMA expression in fibroblasts, not by acting alone but by cooperating with TGFβ1.

## Periostin Upregulates Col1α1 Expression via the αv-integrin Mediated Phosphoinositide 3 Kinase (PI3K)/Akt Signaling Pathway *in vitro*

TGFβ1 is also known as a major inducer of collagen synthesis. We therefore investigated Col1α1 transcript levels in WT and $PN^{-/-}$ fibroblasts when they were stimulated with TGFβ1. Similar to the results of α-SMA expression, Col1α1 expression in $PN^{-/-}$ fibroblasts became to be significantly lower than WT *fibroblasts after 12 hours of stimulation* (Figure 6A). This result suggests that periostin may play a role in the Col1α1 expression.

To elucidate whether periostin directly enhances collagen synthesis, the effects of periostin on Col1α1 expression were also examined in cultured WT dermal fibroblasts. Interestingly, Col1α1 *expression was induced two hours after stimulation with* rmPeriostin alone in a dose-dependent manner (Figure 6B). In addition, Col1α1 expression level was further enhanced by the combined stimulation of rmPeriostin and TGFβ1, compared to TGFβ1 or rmPeriostin stimulation alone (Figure 6B), indicating the additive effect of rmPeriostin on TGFβ1-induced collagen induction.

Finally, to further clarify the signaling pathway by which periostin regulates Col1α1 expression, receptor neutralizing and kinase inhibition analyses were performed. After identification the optimal concentration of each inhibitor by a series dilution prior to the initiation of experiments, mouse dermal fibroblasts were pre-treated for two hours with or without various inhibitors at the identified concentrations: a neutralizing antibody against the known periostin receptor of αv-integrin (anti-αv integrin Ab), a tyrosine kinase inhibitor (genistein), a PI3K/Akt kinase inhibitor (LY294002), a mitogen-activated protein (MAP) kinase inhibitor (PD98095), an extracellular signal-related kinase (ERK) inhibitor (U0126), a p38 MAP kinase inhibitor (SB203580), or mammalian target of rapamycin (mTOR) inhibitors (temsirolimus and rapamycin). Fibroblasts were then stimulated with rmPeriostin for two hours to measure Col1α1 mRNA levels by real-time quantitative PCR (Figure 6C). Among these pharmacological inhibitors, only the addition of

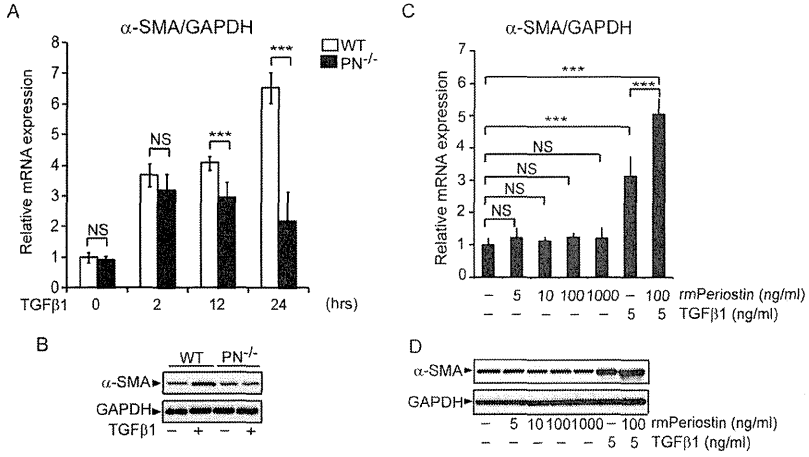


**Figure 5. Periostin is required for TGFβ1-induced myofibroblast differentiation *in vitro*.** A, Real-time quantitative PCR was performed to determine relative mRNA levels of α-SMA in cultured mouse dermal fibroblasts after TGFβ1 stimulation at the indicated times. B, Western blotting analysis for α-SMA with protein extracted from the indicated mouse dermal fibroblasts after TGFβ1 stimulation. C, Relative mRNA levels of α-SMA in cultured WT mouse dermal fibroblasts after the indicated stimulation. D, Western blotting analysis for α-SMA with protein extracted from WT mouse dermal fibroblasts after the indicated stimulation. Values in A and C were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT mice fibroblasts (A) or WT dermal fibroblasts without stimulation (C). Values in A and C are shown as the mean ± SD. NS, no significance; ***, $p<0.01$.

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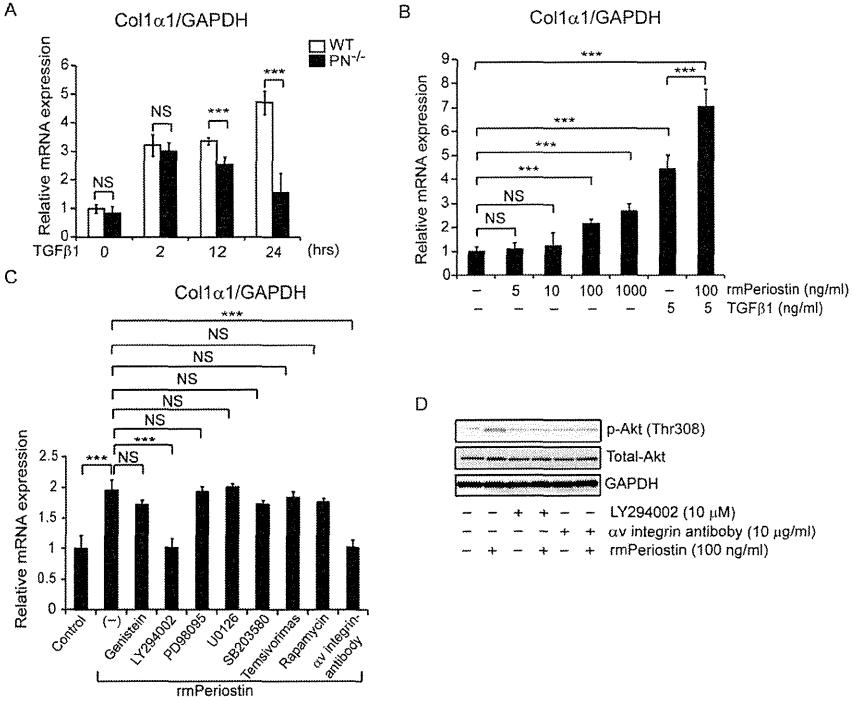


**Figure 6. Periostin upregulates the expression of Col1α1 via αv-integrin mediated-PI3K/Akt signaling pathway *in vitro*.** A, Real-time quantitative PCR was performed to determine relative mRNA levels of Col1α1 in cultured dermal fibroblasts from WT and $PN^{-/-}$ mice after TGFβ1 stimulation at the indicated times. B, Relative mRNA levels of Col1α1 in WT mouse dermal fibroblasts with the indicated stimulation. C, Relative mRNA levels of Col1α1 in cultured WT mouse dermal fibroblasts treated with rmPeriostin in the presence or absence of the indicated neutralizing antibody or kinase inhibitors. D, Phosphorylation of Akt in cultured WT mouse dermal fibroblasts treated with or without rmPeriostin in the presence or absence of LY294002 or anti-αv neutralizing antibody. Values in A, B, and C were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT mice fibroblasts (A) or WT dermal fibroblasts without stimulation (B and C). NS, no significance; ***, $p<0.01$.
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LY294002 (10 μM) and anti-αv integrin Ab (10 μg/ml) abrogated periostin-induced upregulation of Col1α1 expression. In addition, rmPeriostin promptly activated Akt (Thr308) in WT mouse dermal fibroblasts (Figure 6D), implying the direct activation of the PI3K/Akt pathway by rmPeriostin. We also confirmed that the nontoxic concentration of LY294002 and anti-αv integrin Ab efficiently blocked Akt phosphorylation in fibroblasts treated with rmPeriostin (Figure 6D). Thus, periostin appears, at least in part, to directly increase Col1α1 expression in murine scleroderma via the αv integrin-mediated PI3K/Akt pathway.

## Discussion

Matricellular proteins are ECM proteins that modulate cell-matrix interactions as well as cellular functions. They are highly expressed in injured and remodeled tissues and during embryonic development, and have been implicated in the pathophysiology of various fibrotic conditions. Like other matricellular proteins, periostin is thought to play a fundamental role in tissue development and remodeling [10,27,35]. Using $PN^{-/-}$ mice, the importance of periostin in various fibrotic conditions has been uncovered. However, it is still unknown whether periostin is involved in scleroderma. Our study is the first to assess the role of periostin in scleroderma.

As expected, we show herein the enhanced expression of periostin in lesional skin from patients with scleroderma and in BLM-induced sclerotic mouse skin, compared with hypertrophic scar, keloid, normal skin and PBS-treated mouse skin. These observations support the notion that periostin is involved in the process of skin fibrosis.







$PN^{-/-}$ mice were used to examine the contribution of periostin in the pathogenesis of scleroderma. The results of histological analysis showed that before the subcutaneous injection of BLM, there were no significant differences in dermal thickness or collagen production between WT and $PN^{-/-}$ mice. However, in the BLM-induced mouse scleroderma model, a reduced sclerotic response was shown in the skin of $PN^{-/-}$ mice, suggesting that periostin is critically involved in the pathogenesis of scleroderma.

The enhanced generation of α-SMA-positive myofibroblasts is determined to be a hallmark of and an essential process for scleroderma [33]. In the present study, BLM-induced myofibroblast formation was distinctly impaired in $PN^{-/-}$ mice. A similar reduction in the development of α-SMA-positive myofibroblasts has been observed previously in $PN^{-/-}$ mice subjected to various pathogenic conditions such as myocardial infarction [17,36], wound healing [37] and tumor engraftment [27]. These observations collectively indicate the important role of periostin in myofibroblast development *in vivo*.

One possible mechanism by which periostin can increase myofibroblast number is the promotion of myofibroblast recruitment through the αv-integrin pathway [17,21]. It is also well known that myofibroblast differentiation is critically regulated by TGFβ1 and TGFβ1-induced matricellular proteins such as CCN2 and fibronectin [4,38,39]. In the present study, myofibroblast differentiation induced by TGFβ1 *in vitro* was attenuated in $PN^{-/-}$ fibroblasts (Figure 5A and 5B), although we found no impairment of cell viabilities in $PN^{-/-}$ fibroblasts during culture (Figure S1 and Text S1). Moreover, this impairment in $PN^{-/-}$ fibroblasts was rescued by the addition of rmPeriostin *in vitro* (Figure S3A). Interestingly, however, we found that periostin stimulation alone did not induce α-SMA expression in WT fibroblasts, but the TGFβ1-induced α-SMA expression could be enhanced in combination with rmPeriostin. Similar to our findings, a previous study showed that periostin is required for embryonic fibroblasts to respond properly to TGFβ1 [40]. Thus, it appears that periostin likely plays a critical role as a co-factor that augments TGFβ1-induced α-SMA expression. This action of periostin is reminiscent of other matricellular proteins such as CCN2 in facilitating TGFβ1 action [38]. Thus, periostin, in cooperation with other TGFβ1-induced matricellular proteins, may provide integrated extracellular signals for a proper TGFβ1 response. In addition, periostin may also augment TGFβ1 activity *via* the activation of latent TGFβ1, as suggested by a previous study on airway epithelial cells [41].

Our findings also suggest that periostin directly contributes to excessive collagen synthesis in scleroderma. Previously, in various disease models utilizing $PN^{-/-}$ mice, reductions in collagen accumulation, similar to our observations, were reported [17,27–29]. However, it is unknown whether periostin directly regulates collagen synthesis. In this study, both $PN^{-/-}$ mice upon bleomycin injection *in vivo* and $PN^{-/-}$ fibroblasts stimulated with TGFβ1 *in vitro* exhibited reduced Col1α1 mRNA production. Furthermore, rmPeriostin induced Col1α1 mRNA expression in dermal fibroblasts *in vitro*. These effects of periostin are presumably direct and mediated *via* the αv-integrin mediated-PI3K/Akt pathway because 1) rmPeriostin can induce a prompt activation of Akt in fibroblasts and 2) Col1α1 induction was abrogated by αv-integrin neutralization or PI3K inhibition. It is known that periostin can bind to several types of integrins (e.g., αvβ3, αvβ5, and αvβ4), which act as receptors that activate downstream signaling pathways including PI3K/Akt [13]. Our findings also raise the intriguing possibility that TGFβ1-induced Col1α1 expression, unlike α-SMA expression, is mediated by the action of periostin. These observations of periostin differ from those obtained using $CCN2^{-/-}$ fibroblasts, in that Col1α1 production normally increases after TGFβ1 stimulation [4]. It is tempting to speculate that Col1α1 production in $CCN2^{-/-}$ fibroblasts might be compensated by the effects of TGFβ1-induced periostin. Thus, we assume that periostin, upon induction by TGFβ1, not only acts as a co-factor of TGFβ1 activity, but also, at least in part, directly mediates part of the TGFβ1 response.

Our time-course experiments *in vitro* revealed that mRNA levels of α-SMA and Col1α1 were similar between WT and $PN^{-/-}$ fibroblasts at the early phase of TGFβ1 stimulation (0 hrs, 2 hrs), but became prominently lower in $PN^{-/-}$ fibroblasts than that in WT fibroblasts after longer incubation with TGFβ1 (12 hrs, 24 hrs) ($P<0.01$) (Figure 5A and 6A). This difference at late phase can be explained by de novo periostin secretion, which is induced by TGFβ1 in WT fibroblasts. Indeed, as reported previously [19], periostin was strongly induced in fibroblasts by TGFβ1 in a dose-dependent manner (Figure S2A). Moreover, the protein synthesis and secretion of periostin was undetectable at 2 hrs but became detectable after 12 hrs of stimulation (Figure S2B). Notably, TGFβ1-induced expression of α-SMA and Col1α1 in $PN^{-/-}$ fibroblasts could be rescued by addition of rmPeriostin to the culture media (Figure S3A and S3B). Upon these results described above, periostin, induced by TGFβ1 in fibroblasts, is likely involved in fibrosis process of scleroderma, at least in part *via* enhancing α-SMA expression and mediating Col1α1 induction in these cells.

The unexpected data we encountered in the present study was that, in $PN^{-/-}$ fibroblasts, TGFβ1-induced α-SMA and Col1α1 mRNA levels were peaked at 2 hrs and slightly declined thereafter (Figure 5A and 6A). Because it is well known that the fibrotic effect of TGFβ1 is regulated by its negative feedback mechanisms, the absence of periostin may render these feedback mechanisms predominant. Furthermore, our preliminary data suggest that the expression of decorin, which is known as a potent inhibitor of TGFβ1/Smad signaling [42], is increased in $PN^{-/-}$ fibroblasts compared to WT cells (data not shown). Thus, periostin may accelerate the fibrotic action of TGFβ1 not only by increasing α-SMA and Col1α1 mRNA expression but also by counteracting against negative feedback signaling of TGFβ1. Further studies are underway to reveal the role of periostin in regulating negative-feedback signaling molecules such as decorin and Smad7 in TGFβ1 signaling.

It should be noted that periostin is reported to have a number of functions that may related to skin fibrosis. Similar to other matricellular proteins like thrombospondin-2 [41] and SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM-40) [42], periostin is known to be involved in collagen assembly [10]. Moreover, we recently reported that rmPeriostin can promote the proliferation of mouse dermal fibroblasts *in vitro* [21], at least in part *via* periostin-PI3K/Akt pathway. Additionally, according to recent evidence [29,43], periostin may also contribute to scleroderma *via* the regulation of the Notch1 signaling pathway, another important pathway in skin sclerosis [44–46].

It is generally known that fibrotic processes in skin are regulated by a complex network of matricellular proteins. Inhibition of just one matricellular protein can often disrupt the balance of this organized network and lead to exacerbation [43] or attenuation [4] [6,44] of skin fibrosis under pathogenic conditions. The present study is the first to show that periostin is one of these pivotal matricellular proteins that accelerates pathologic fibrosis in both BLM-induced skin sclerosis and human scleroderma. Our findings suggest that periostin promotes disease by enhancing myofibroblast differentiation and collagen synthesis via the augmentation

and mediation (at least in part) of TGFβ1 activity. Periostin may also contribute to the pathogenesis of scleroderma via the proliferation and recruitment of myofibroblasts [17,24], enhancement of Notch1 signaling [29,45], and promotion of collagen assembly [10]. Thus, our observations and those of others collectively indicate that periostin is involved in the multiple steps of skin fibrosis and is an attractive target for the treatment of scleroderma.

We hope that our findings will contribute to both a better understanding of scleroderma pathogenesis and the development of novel therapeutic approaches, including the possible inhibition of periostin function, for the treatment of scleroderma.

## Materials and Methods

### Human Samples

The frozen biopsy tissues and paraffin-embedded tissue sections obtained from lesional skin of well-defined patients with diffuse systemic scleroderma (n = 12; male: female ratio 2:10, mean age 52.4 years [range 24–76 years]), lesional skin of patients with keloid (n = 8; male: female ratio 2:4, mean age 48.5 years [range 21–68 years]), hypertrophic scar (n = 7; male: female ratio 2:5, mean age 50.5 years [range 34–72 years]), and corresponding sites of healthy donors (n = 12; male: female ratio 3:9, mean age 49.2 years [range 26–65 years]) were used in this study. Written informed consent was obtained from all participants prior to study inclusion. The study was approved by the Medical Ethics Committee of Osaka University (Case number 2011-3/17-10193).

### Rearing Management of Animals

WT mice (C57BL/6 strain) were obtained from CLEA Japan, Inc. (Osaka, Japan). Periostin gene knockout ($PN^{-/-}$) mice (C57BL/6 strain) were generated as previously described [27]. All animal care and experimentation were performed in accordance with the institutional guidelines of the National Institute of Biomedical Innovation, Osaka, Japan (NIBIO) (Approval No. DS2147R1).

### BLM-induced Scleroderma Model and Tissue Sample Preparation

BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and sterilized by filtration. BLM or PBS (100 μl) was injected subcutaneously as described by us previously [46]; one day after the final injection, the skin at the injected site was removed and processed for analysis as previously described [47].

### Histopathological Analysis, Assessment of Skin Thickness, and Collagen Synthesis

Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E Fisher Scientific), and dermal thickness was calculated as described previously [48]. To assess dermal collagen deposition, semi-quantitative analysis using Masson's trichrome staining, in which collagen fibers are stained blue, was used. Collagen deposition was graded by examining five randomly chosen fields at 100× magnification in a blinded manner using three observers. The grading criteria were as follows: grade 0 = no collagen fibers; grade 1 = few collagen fibers; grade 2 = moderate amount of collagen fibers; and grade 3 = excessive amount of collagen fibers.

### Immunohistochemistry and Immunofluorescence Staining

Paraffin sections were prepared as referred to above and then subjected to immunohistochemistry and immunofluorescence staining as described previously [47,49]. The primary antibodies used were rabbit anti-periostin (1:3,000 dilution; Abcam, Cambridge, MA), mouse anti α-smooth muscle actin (α-SMA; 1:3,000 dilution; Sigma-Aldrich, St. Louis, MO) and rat anti-CD34 antibody (1:50 dilution; Abcam, Cambridge, MA), followed by the DAKO LSAP+System-AP (DakoCytomation) and Dako ChemMate Envision kit/HRP(DAB), or followed by the secondary antibody (anti-mouse Alexa Fluor 555, anti-rat Alexa Fluor 488, Invitrogen). The slides were visualized using a light microscope or Keyence Biozero confocal microscope. α-SMA-positive spindle cells ($\alpha$-SMA$^+$ cells) or α-SMA-positive and CD34-negative spindle-shaped cells ($\alpha$-SMA$^+$ CD34$^-$ cells) were counted in 10 non-contiguous random grids under high-power magnification fields (400×) by confocal microscope. Results are expressed as the mean ± standard deviation (SD) of positive spindle-shaped fibroblasts per field.

### Cell Culture

Neonatal murine primary dermal fibroblasts were isolated from the skin of 10-day-old WT mice and cultured as previously described [47]. After 24 hours of serum starvation, dermal fibroblasts were treated with TGFβ1 (2–12 ng/ml) or recombinant mouse periostin (rmPeriostin) (5–1,000 ng/ml) for the indicated periods prior to extraction of RNA and protein extraction. Cells were used at passage three. In each experiment, obtained fibroblasts were examined at the same time and under the same culture conditions (e.g., cell density, passage, and days after plating).

### Neutralizing and Kinase Inhibition Assays

Cells were grown on 6-well plates; after extensive washing with PBS to remove all sera, cells were serum-starved for 24 hours. Subsequently, the cells were incubated for 2 hours with the neutralizing antibody against αv-integrin (anti-αv-integrin Ab, Biolegend, San Diego, CA) and kinase inhibitors (Cell Signaling Technology, Beverly, MA) at the indicated concentrations: anti-αv-integrin Ab (10 μg/ml), genistein (10 μM), LY294002 (10 μM), PD98095 (50 μM), U0126 (20 μM), SB203580 (25 μM), temsirolimus (10 μM), and rapamycin (500 nM). Cells were then stimulated for 2 hours with 100 ng/ml rmPeriostin in the same media. After stimulation, total RNA was isolated. To analyze protein phosphorylation, cells were collected after five minutes of periostin stimulation. We performed a serial dilution to identify the optimal concentration of each inhibitor prior to the initiation of experiments by MTT assay and western blotting analysis, the nontoxic and effective concentration was used in neutralizing and kinase inhibition assay.

### RNA Isolation and Real-time Quantitative Polymerase Chain Reaction (PCR)

Total RNA from mouse skin tissues or cultured fibroblast cell pellets was isolated with RNeasy spin columns (Qiagen, Valencia, CA) following the manufacturer's instructions. The integrity of the RNA was verified by gel electrophoresis. Total RNA (100 ng) was reverse-transcribed into first-strand complementary DNA (cDNA) (QuantiTect Reverse Transcription Kit, Qiagen). The primers used for real-time PCR were as follows: TGFβ1, sense 5′-cgaatgtctgacgtattgaagaaca-3′, antisense 5′-ggagcccgaagcggacta-3′; CCN2/CTGF, sense 5′-caaagcagctgcaaatacca-3′, antisense 5′-gacaggcttggcgattttag-3′; α-SMA, sense 5′-tctctatgctaacaacgtcctgtca-3′, antisense 5′-ccaccgatccagacagagtactt-3′; collagen type-I alpha 1 (Col1α1), sense 5′-gagccctcgctccgtactc-3′, antisense 5′-tgttcctactcagccgtctgt-3′; and GAPDH, sense 5′-tgtcatcatacttggcaggtttct-3′, antisense 5′-catggccttccgtgttccta-3′. Each reaction was performed in triplicate. Variation within samples was less than 10%. Statistical analysis was performed with the Student's paired *t* test.

### Western Blotting Analysis

Proteins from skin samples and cell pellets were extracted, and 5 μg of extracted protein was used for western blotting analysis as described previously [47]. The primary antibodies were used at the following dilutions: anti-α-SMA (Sigma-Aldrich), 1:500; anti-periostin (R&D Systems, Minneapolis, MN), 1:500; anti-periostin (Abcam, Cambridge, MA), 1:1,000; anti-phospho-Akt (Cell Signaling Technology, Beverly, MA), 1:1,000; anti-Total Akt (Cell Signaling Technology), 1:1,000; and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500. We used anti-GAPDH antibody as a loading control.

### Statistical Analysis

The data were expressed as the mean ± SD. The Student's two-tailed *t*-test (Microsoft Excel software, Redmond, WA) was used for comparison between two groups. When analysis included more than two groups, one-way analysis of variance was used. P-values less than 0.05 were considered statistically significant.

## Supporting Information

**Figure S1 TGFβ1 does not affect cell viability of WT and $PN^{-/-}$ dermal fibroblasts.** Cell viabilities of WT and $PN^{-/-}$ dermal fibroblasts were assessed by MTT assay after treatment with TGFβ1 (5 ng/ml) for 2–24 hours. Data are shown as mean ± SD. NS, no significance.
(TIF)

**Figure S2 Periostin is induced by TGFβ1 in WT dermal fibroblasts in a dose- and time-dependent manner.** A, Real-time quantitative PCR was performed to determine relative mRNA levels of periostin in cultured WT dermal fibroblasts at two hours after TGFβ1 treatment at the indicated concentrations. B, Western blotting analysis for periostin with protein extracted from WT dermal fibroblasts or culture supernatants after TGFβ1 treatment at the indicated times. Values in A were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT dermal fibroblasts without TGFβ1 treatment. Values in A are shown as the mean ± SD. NS, no significance; ***, $p<0.01$. (TIF)

**Figure S3 The effects of TGFβ1 in the induction of α-SMA and Col1α1 were recovered by addition of rmPeriostin to cultured $PN^{-/-}$ fibroblasts.** Real-time quantitative PCR was performed to determine relative mRNA levels of α-SMA (A) and Col1α1 (B) in cultured dermal fibroblasts at 24 hours after TGFβ1 treatment. Values in A and B were normalized to GAPDH levels and expressed as relative mRNA levels compared with WT dermal fibroblasts without TGFβ1 treatment. Values in A and B are shown as the mean ± SD. NS, no significance; ***, $p<0.01$. (Note: Data of WT and $PN^{-/-}$ group shown here and those presented in Figure 5A and 6A are from the same data set.)
(TIF)

**Text S1 Supplementary materials and methods.**
(DOC)

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## Author Contributions

Conceived and designed the experiments: LY SS HM MF AK TN IK. Performed the experiments: LY SS HM MF MT SM YK SK. Analyzed the data: LY SS HM MF MT YK SK. Contributed reagents/materials/analysis tools: AK MT SM YK SK. Wrote the paper: LY SS HM MF.

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TOPICS

## Serum leucine-rich alpha-2 glycoprotein is a disease activity biomarker in ulcerative colitis

*Serada S, Fujimoto M, Terabe F, Iijima H, Shinzaki S, Matsuzaki S, Ohkawara T, Nezu R, Nakajima S, Kobayashi T, Plevy SE, Takehara T, Naka T*

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# 潰瘍性大腸炎の疾患活動性マーカーとしての血清ロイシンリッチアルファ2グリコプロテイン

世良田 聡* 藤本 穣* 仲 哲治*
Satoshi Serada Minoru Fujimoto Tetsuji Naka

*Key words*：炎症性腸疾患，潰瘍性大腸炎，バイオマーカー

## 論文の背景

炎症性腸疾患(inflammatory bowel disease：IBD)は再燃と寛解を繰り返す原因不明の慢性炎症性疾患であり，潰瘍性大腸炎(ulcerative colitis：UC)とクローン病(Crohn's disease：CD)に分類される．近年，IBDに対する免疫抑制薬，調節薬などを用いた治療法に加え，IBDの病態と関係の深いサイトカインの一つであるTNF-αを標的とした抗体医薬など生物学的製剤が使用され始めたことによって劇的な治療効果が発揮されたことにより，寛解を目指した治療が現実になりつつある[1),2)]．IBDの診療においては，内視鏡検査・X線検査などの画像診断，生検組織の病理学的診断といった炎症局所の評価が重要であることは明白であるが，これらの評価法以外にも，バイオマーカーの測定を併用することで炎症性腸疾患の診断や治療が低侵襲的，簡便で客観的，かつ低コストで行える可能性がある．

CDの疾患活動性を把握する活動性マーカーとしてはC-reactive protein(CRP)，赤沈検査(ESR)，白血球数などが知られている．これらの検査マーカーはCDの炎症局所である腸以外の炎症においても高値を示す．また，UCにおいてはこれらのマーカーはCDほど有用ではないといわれている[3)]．さらに，UCの疾患活動性を評価するために内視鏡的な所見は重要な情報であるが，内視鏡による検査は侵襲性が高いことが問題であり，短期間に繰り返して検査することが困難である．そのため，UCの治療に伴う疾患活動性の適切な評価を行うに当たり，侵襲性が少ない新規疾患活動性マーカーが必要とされている．

筆者らはこれまでにインフリキシマブ(TNF-α阻害抗体)治療前と治療後の関節リウマチ同一患者血清に対して定量的プロテオミクス手法を用

*独立行政法人医薬基盤研究所創薬基盤研究部免疫シグナルプロジェクト(〒567-0085 大阪府茨木市彩都あさぎ7-6-8)
*Laboratory for Immune Signal, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki-city, Osaka 567-0085, Japan

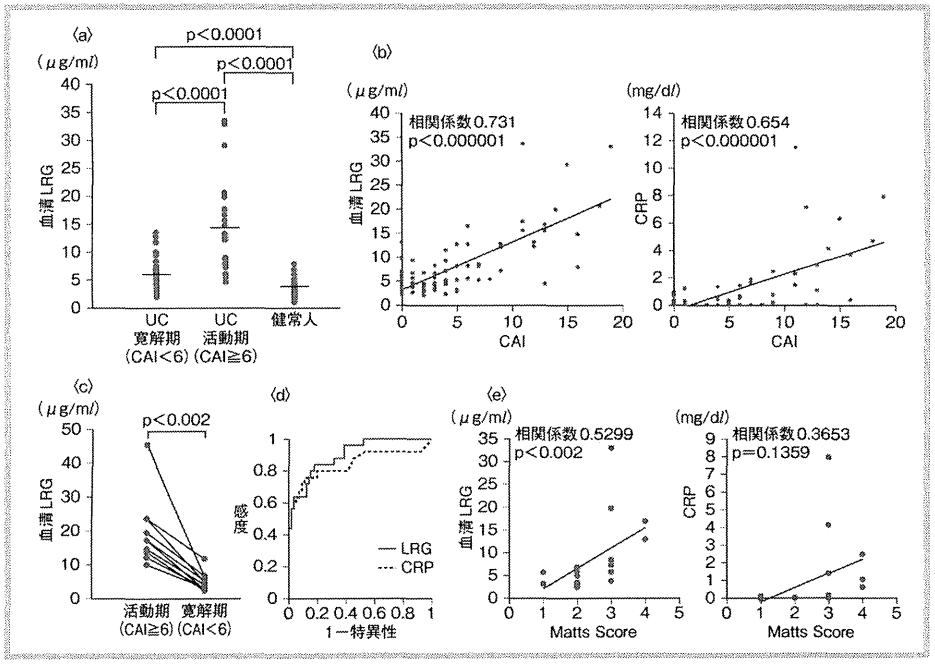


**図　潰瘍性大腸炎の疾患活動性マーカーとしての leucine-rich α-2 glycoprotein (LRG) の有用性**

a：潰瘍性大腸炎患者血清中の LRG 濃度
b：血清 LRG 濃度，および CRP と CAI との相関
c：活動期，寛解期における血清 LRG 濃度の変動
d：活動期と寛解期を区別する血清 LRG 濃度と CRP の ROC 曲線解析
e：血清 LRG 濃度，および CRP と内視鏡スコア (Matts Score) との相関
CAI：clinical activity index, ROC：receiver operating characteristic
〔Serada S, et al：Inflamm Bowel Dis　2012；18：2169-2179 より改変〕

いることで，治療によって発現変動を示す血清タンパク質を網羅的に探索した．その結果，CRP や serum amyloid A (SAA) のような既知の炎症マーカータンパク質のみならず，leucine-rich alpha-2 glycoprotein (LRG) というタンパク質が治療後よりも治療前にて高値を示すことを明らかにした[4]．LRG は 1977 年に発見された血清中に存在する糖タンパク質で，ロイシンリッチリピートと呼ばれる特徴的なドメインを八つ含むタンパク質であるが，生理的機能はまだ明らかにされていない[5]．疾患活動性マーカーとしての血清 LRG の有用性を検討するため，ELISA 法を用いて関節リウマチ患者血清中の LRG を定量した結果，LRG は関節リウマチの優れた疾患活動性マーカーとなりうることを明らかにした．さらに，血清 LRG が CD においても優れた疾患活動性マーカーとなりうることを明らかにしている．本論文は UC の疾患活動性マーカーとしての血清 LRG の有用性について報告している．



## 論文の概要

UC 患者 82 例を対象とし，血清中の LRG 濃度を ELISA 法により定量した．その結果，血清 LRG 濃度は活動期 (25 例) において，寛解期 (57 例)，および健常人 (50 例) の血清 LRG 濃度よりも有意に高値を示した (図 a)．UC の疾患活動性スコアである Clinical Activity Index (CAI) と血清 LRG 濃度との相関を解析した．その結果，血清 LRG 濃度は CRP よりも CAI と強く有意に相関した (図 b)．さらに，寛解期の患者血清中の LRG 濃度は活動期に比較して有意な低下を認めた (図 c)．また，receiver operating characteristic (ROC) 曲線解析から，UC 活動期と寛解期を区別するマーカーとして血清 LRG 濃度は CRP よりも優れていた (図 d)．UC の炎症の状態を把握するうえで内視鏡所見は直接的な指標となる．そこで，血清 LRG 濃度と内視鏡スコア (Matts Score) との相関を解析した．その結果，血清 LRG 濃度は CAI だけでなく内視鏡スコアに対しても CRP よりも強く相関関係を示した (図 e)．また，LRG は炎症局所の腸組織において高発現することが免疫組織化学染色法にて確認された．LRG は正常腸組織からは発現がほとんどみられないものの，UC 患者の炎症局所である腸組織において高発現するため，疾患活動性とより強く相関することが示唆されており，この点は炎症時に肝臓から産生される CRP と異なった特徴と考えられる．

IL-6 欠損マウスを用いて炎症性腸疾患モデルの血清 LRG 濃度を解析した．その結果，野生型マウス，IL-6 欠損マウスのいずれにおいても dextran sulfate sodium (DSS) 誘導性腸炎を誘導することにより血清 LRG 濃度の上昇が野生型マウスと IL-6 欠損マウスの間において同程度で認められた．このことから，LRG の発現には IL-6 が必ずしも必要ではないことが明らかとなり，従来の炎症マーカーとして使用されている CRP とは発現調節機序が異なることが判明した．

以上の結果，血清 LRG は CRP とは異なる UC の新規疾患活動性マーカーとなりうることが明らかとなった．興味深いことに，血清 LRG は IL-6 非依存性の発現調節機序が存在する炎症マーカーとなりうる．このことより，IL-6 阻害療法を受けるため CRP 値が常に陰性化するため，CRP を疾患活動性マーカーや感染症検出マーカーとして使えない関節リウマチなどの自己免疫疾患患者において，LRG が疾患活動性マーカー，あるいは感染症を検出できる有用性の高いマーカーとなることが期待される．

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*Key words*：inflammatory bowel diseases, ulcerative colitis, biomarker

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# Elevated Levels of Pentraxin 3 in Systemic Sclerosis

## Associations With Vascular Manifestations and Defective Vasculogenesis

Yuichiro Shirai,[1] Yuka Okazaki,[2] Yumiko Inoue,[1] Yuichi Tamura,[1] Hidekata Yasuoka,[1] Tsutomu Takeuchi,[1] and Masataka Kuwana[2]

***Objective.*** **To clarify the role of pentraxin 3 (PTX3), a multifunctional pattern recognition protein that can suppress fibroblast growth factor 2 (FGF-2), in systemic sclerosis (SSc)–related vasculopathy.**

***Methods.*** **We assessed 171 SSc patients and 19 age- and sex-matched healthy control subjects. Circulating PTX3 and FGF-2 levels were measured by enzyme immunoassay, and CD34+CD133+CD309+ endothelial progenitor cells (EPCs) were counted by flow cytometry. Correlations between PTX3 and FGF-2 and the presence or future development of vascular manifestations, including digital ulcers and pulmonary arterial hypertension (PAH), were identified by univariate and multivariate analysis. The effect of PTX3 on EPC differentiation was evaluated in proangiogenic cultures of mouse bone marrow cells in combination with colony formation assay.**

***Results.*** **Circulating PTX3 and FGF-2 levels were significantly higher in SSc patients than in healthy control subjects. PTX3 was elevated in SSc patients who had digital ulcers or PAH, while FGF-2 was reduced in SSc patients with PAH. Multivariate analysis identified elevated PTX3 as an independent parameter associated with the presence of digital ulcers and PAH, and PTX3 levels were a useful predictor of future occurrences of digital ulcers. Reduced FGF-2 was independently associated with the presence of PAH. EPC counts were significantly lower in patients with digital ulcers or PAH and correlated negatively with circulating PTX3 concentrations. Finally, PTX3 inhibited EPC differentiation in vitro.**

***Conclusion.*** **In SSc patients, exposure to high concentrations of PTX3 may suppress EPC-mediated vasculogenesis and promote vascular manifestations such as digital ulcers and PAH.**

Systemic sclerosis (SSc) is a multisystem disease characterized by microvascular abnormalities and excessive fibrosis (1). It has been suggested that the pathogenic process of SSc is triggered by endothelial damage and the subsequent activation of immune cells and fibroblasts, resulting in excessive accumulation of extracellular matrix (2). Although several soluble mediators, including growth factors, cytokines, chemokines, and proangiogenic and antiangiogenic factors, are known to play critical roles in the pathogenesis of SSc, the mechanisms regulated by the interactions of these mediators are not clearly understood (2).

Pentraxin 3 (PTX3) is a pattern recognition protein belonging to the pentraxin superfamily (3). C-reactive protein (CRP), a short pentraxin, is primarily produced in the liver in response to interleukin-6 (IL-6). In contrast, PTX3 is produced locally at the inflammation site by macrophages, dendritic cells, endothelial cells (ECs), smooth muscle cells, and fibroblasts (4), and it is induced by Toll-like receptor agonists or proinflammatory cytokines such as *IL-1β* and tumor necrosis factor α, but not IL-6. Recently, several lines of evidence have shown that PTX3 has nonredundant roles in antimicrobial innate immunity, inflammation, extracellular matrix deposition, and neovascularization (4). Specifically, it binds to apoptotic cells and selected pathogens, and it activates and modulates the classical complement pathway by binding to C1q. PTX3 is also a component of the extracellular matrix and contributes to fibrosis in this role. Finally, PTX3 acts as an antiangiogenic factor by binding to fibroblast growth factor 2 (FGF-2) with high affinity and specificity and inhibiting FGF-2–dependent EC proliferation and neovascularization (5).

The pleiotropic effects of PTX3 on inflammation and fibrosis, along with its inhibition of neovascularization, suggest that it is an intriguing candidate as a mediator in the pathogenesis of SSc. In fact, circulating PTX3 levels are elevated in SSc patients (6), and PTX3 is up-regulated in ECs and fibroblasts in affected skin (6–8). In addition, cultured fibroblasts derived from SSc skin constitutively expressed PTX3 in the absence of agonistic stimulation (6,7). The silencing of PTX3 gene expression by small interfering RNA restored the impaired ability of cultured SSc microvascular ECs to form capillary-like tubes and promote vascularization (9). These findings suggest that PTX3, constitutively produced at the affected site, is involved in the pathogenesis of SSc. To test this hypothesis, we examined the roles of PTX3 in SSc pathogenesis by evaluating potential correlations between SSc manifestations and circulating levels of PTX3 and FGF-2, and by investigating the mechanisms underlying these correlations.

## PATIENTS AND METHODS

**Patients and controls.** This study included 171 consecutive patients with SSc (15 men and 156 women) who visited an SSc clinic at Keio University Hospital between 2007 and 2013. All patients met the American College of Rheumatology (ACR)/European League Against Rheumatism 2013 classification criteria for SSc (10), and 137 patients (80%) also met the 1980 ACR preliminary classification criteria (11). Peripheral blood samples were collected from all patients when they entered the study and from a subset of patients 2 years later. Peripheral blood was also collected from 19 healthy control subjects matched for age and sex (4 men and 15 women). The heparinized blood was immediately separated into platelet-poor plasma and mononuclear cells, which were used to count endothelial progenitor cells (EPCs). Serum and plasma samples were stored at −80°C until use. We obtained informed written consent from all subjects prior to collecting samples in accord with the tenets of the Declaration of Helsinki, and all study protocols were approved by the Institutional Review Board of Keio University School of Medicine.

**Clinical assessment.** A complete medical history, physical examination, and laboratory evaluation were performed for each patient at the time of enrollment, and limited evaluations were conducted at each followup visit. Patients were carefully monitored for new-onset digital ulcers and pulmonary arterial hypertension (PAH) through December 2013. We collected the following data for each patient: age, sex, disease subset, disease duration from first appearance of non–Raynaud's phenomenon symptoms, disease duration from first appearance of Raynaud's phenomenon, modified Rodnan skin thickness score (MRSS) (12), and SSc-related organ involvement, including digital ulcers, interstitial lung disease (ILD), PAH, and any heart, upper gastrointestinal (GI), lower GI, or renal involvement. We also recorded treatment profiles and any risk factors for atherosclerosis, including hypertension, dyslipidemia, diabetes mellitus, and whether the patient was a current or former smoker. SSc was classified as diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) according to the system described by Medsger (13).

SSc-related organ involvement was defined as described previously (14) with modifications. A digital ulcer was defined as a loss of both epidermis and dermis in an area at least 2 mm in diameter located at the volar surface of the digit distal to the proximal interphalangeal digital crease (15). ILD was defined as bilateral reticulations, ground-glass opacity, and/or honeycombing seen on chest radiograph or high-resolution computed tomographic scan of the lungs. PAH was defined as a mean pulmonary arterial pressure of ≥25 mm Hg and pulmonary vascular resistance of >3 Wood units, measured by right-sided heart catheterization with the patient at rest, after exclusion of pulmonary hypertension due to left-sided heart disease (an end-expiratory pulmonary artery wedge pressure of >15 mm Hg), advanced ILD (forced vital capacity <70% of predicted), or chronic thromboembolism (16). Heart involvement was defined as clinical evidence of symptomatic pericardial effusion, congestive heart failure, or arrhythmia considered to be due to SSc and requiring treatment. Upper GI involvement was defined as distal esophageal hypomotility demonstrated by cine-esophagram or manometry. Lower GI involvement was defined as radiologic evidence of wide-mouthed colonic sacculations or small intestinal dysmotility, malabsorption, or small bowel bacterial overgrowth requiring administration of antibiotics. Renal involvement was defined as acute or subacute development of renal insufficiency often, but not always, associated with accelerated hypertension and/or microangiopathic hemolytic anemia. Extensive ILD was defined as described by Goh et al (17).

**SSc-related autoantibodies.** The following antibodies were identified by indirect immunofluorescence using commercially prepared slides of monolayer HEp-2 cells (MBL) and immunoprecipitation assays: anticentromere, anti–topoisomerase I, anti–RNA polymerase III, anti–U1 RNP, anti–U3 RNP, anti–U11/U12 RNP, anti-Th/To, anti–PM-Scl, anti-Ku, and anti-RuvBL1/2 (18).

**Measurement of PTX3 and FGF-2 in the circulation.** Concentrations of PTX3 in plasma and FGF-2 in serum were measured in duplicate using enzyme immunoassay kits according to the manufacturers' instructions (Perseus Proteomics and R&D Systems, respectively). The lower limits of detection for PTX3 and FGF-2 were 0.1 ng/ml and 3 pg/ml, respectively. The coefficients of variation of 2 values ranged from 0 to 0.25. In some analyses, the ratio of PTX3 (ng/ml) to FGF-2 (ng/ml) was used as an indicator of the anti/proangiogenic activity of FGF-2 signaling.

**Quantification of EPCs.** The absolute number of CD34+CD133+CD309+ EPCs in peripheral blood was deter-

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[1]Yuichiro Shirai, MD, Yumiko Inoue, MD, Yuichi Tamura, MD, PhD, Hidekata Yasuoka, MD, PhD, Tsutomu Takeuchi, MD, PhD: Keio University School of Medicine, Tokyo, Japan; [2]Yuka Okazaki, BS, Masataka Kuwana, MD, PhD: Keio University School of Medicine and Nippon Medical School Graduate School of Medicine, Tokyo, Japan.

Address correspondence to Masataka Kuwana, MD, PhD, Department of Allergy and Rheumatology, Nippon Medical School Graduate School of Medicine, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: kuwanam@nms.ac.jp.

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mined as described previously (19) and is shown as the number per 1 ml of peripheral blood, determined using FlowCount fluorospheres (Beckman Coulter) as an internal calibrator.

**EPC colony formation assay.** The ability of mouse bone marrow stem cells to differentiate into EPCs in the presence of a series of proangiogenic factors, including FGF-2, was assessed by colony formation assay as described previously (20). Briefly, bone marrow mononuclear cells were isolated from the thigh bones of 10–12-week-old male C57BL/6J mice. The cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma-Aldrich) and seeded on Pronectin (Sigma-Aldrich)–coated 60-mm culture dishes. After 24 hours, nonadherent mononuclear cells were collected and cultured on Pronectin-coated UpCell 6-well plates with EGM-2 supplemented with SingleQuots (Lonza), which contains 2% fetal bovine serum and other components (hydrocortisone, insulin-like growth factor 1 [IGF-1], epidermal growth factor [EGF], vascular endothelial growth factor [VEGF], FGF-2, ascorbic acid, and heparin) with no information available on individual concentrations, either with or without recombinant mouse PTX3 (5 or 20 n*M*; R&D Systems).

After 6 days, adherent cells were subjected in duplicate to a colony assay that can be used to evaluate colony-forming unit (CFU)–EC colonies and CFU–granulocyte–erythrocyte–macrophage–megakaryocyte (GEMM) colonies simultaneously. The culture medium used was MethoCult (StemCell Technologies) with stem cell factor (100 ng/ml), IL-3 (20 ng/ml), IGF-1 (50 ng/ml), EGF (50 ng/ml), VEGF (50 ng/ml), FGF-2 (50 ng/ml) (all from R&D Systems), insulin (10 $\mu$g/ml; PromoCell), and transferrin (200 $\mu$g/ml; Sigma-Aldrich). After 14 days, CFU-EC and CFU-GEMM colonies were individually counted using a microscope at 20× magnification. Results are shown as the mean of the 2 measurements.

**Statistical analysis.** All continuous variables are shown as the mean ± SD. Paired and unpaired comparisons of continuous variables were made using Wilcoxon tests or Mann-Whitney U tests, respectively. Categorical variables were compared using Fisher's exact test or a chi-square test when appropriate. The correlation coefficient (r) was calculated using Pearson's regression model. Variables that best explained the risk of digital ulcers and PAH were identified by multivariate logistic regression analysis combining clinical parameters (sex, age at onset and at entry into the study, disease duration from first appearance of non–Raynaud's phenomenon symptoms and from first appearance of Raynaud's phenomenon, disease subset, Raynaud's phenomenon, individual organ involvement, and previous history of digital ulcers), autoantibodies, corticosteroid use, and circulating levels of PTX3 and FGF-2. Results are presented as odds ratios (ORs) with 95% confidence intervals (95% CIs). The Cox proportional hazards regression model was used to determine factors associated with an increased risk of the future development of digital ulcers. Results are presented as hazard ratios (HRs) with 95% CIs. The cutoff value that best discriminated 2 groups was determined by receiver operating characteristic (ROC) curve analysis. Survival analysis was performed using the Kaplan-Meier method, and survival was compared between 2 groups by log rank test. All statistical analyses were performed using SPSS 19.0 statistical software.

## RESULTS

**Levels of circulating PTX3 and FGF-2 in SSc patients.** Demographic and clinical characteristics were recorded and blood samples were obtained from each of the 171 SSc patients as they entered the study (Table 1). Our cohort consisted mainly of patients with lcSSc (74%). The mean ± SD disease durations from the first appearance of non–Raynaud's phenomenon symptoms and from the first appearance of Raynaud's phenomenon were 9.5 ± 7.2 years and 14.0 ± 9.9 years, respectively. Figure 1 shows that in comparison to healthy control subjects, SSc patients had significantly higher

**Table 1.** Demographic and clinical characteristics of the 171 SSc patients at the time of enrollment*

| | |
|---|---|
| Female | 156 (91) |
| Age at SSc onset, mean ± SD years | 51 ± 14 |
| Age when enrolled in study, mean ± SD years | 60 ± 14 |
| Disease duration from first appearance of non–Raynaud's phenomenon symptoms, mean ± SD years | 9.5 ± 7.2 |
| Disease duration from first appearance of Raynaud's phenomenon, mean ± SD years | 14.0 ± 9.9 |
| Diffuse cutaneous SSc | 44 (26) |
| Modified Rodnan skin thickness score, mean ± SD | 6.1 ± 7.5 |
| Raynaud's phenomenon | 165 (96) |
| Previous history of digital ulcer | 16 (9) |
| Organ involvement | |
| Digital ulcer | 17 (10) |
| Interstitial lung disease | 83 (49) |
| Interstitial lung disease, extensive stage | 29 (17) |
| Pulmonary arterial hypertension | 21 (12) |
| Heart involvement | 11 (6) |
| Upper gastrointestinal involvement | 92 (54) |
| Lower gastrointestinal involvement | 8 (5) |
| Renal involvement | 2 (1) |
| Autoantibodies | |
| Anticentromere | 69 (40) |
| Anti–topoisomerase I | 46 (27) |
| Anti–U1 RNP | 29 (17) |
| Anti-Th/To | 8 (5) |
| Anti–RNA polymerase III | 7 (4) |
| Anti–U3 RNP | 2 (1) |
| Anti-Ku | 1 (1) |
| C-reactive protein, mean ± SD mg/dl | 0.25 ± 0.53 |
| Treatment | |
| Corticosteroids (≤10 mg/day prednisolone equivalent) | 23 (13) |
| Immunosuppressants | 9 (5) |
| Phosphodiesterase 5 inhibitors | 12 (7) |
| Endothelin receptor antagonists | 8 (5) |
| Parenteral prostanoids | 3 (2) |
| Risk factors for atherosclerosis | |
| Hypertension | 15 (9) |
| Dyslipidemia | 10 (6) |
| Diabetes mellitus | 3 (2) |
| Current or former smoker | 1 (1) |

* Except where indicated otherwise, values are the number (%). SSc = systemic sclerosis.





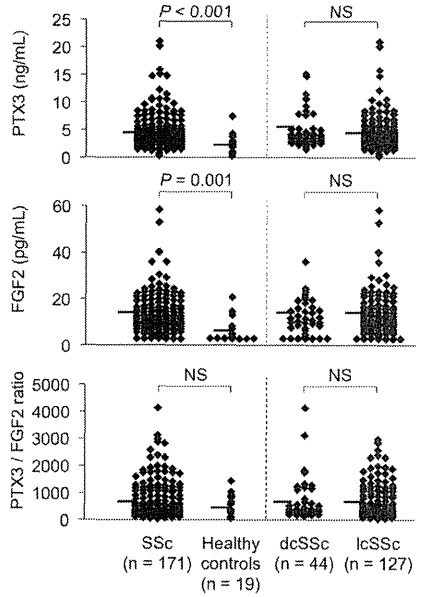


**Figure 1.** Levels of circulating pentraxin 3 (PTX3) and fibroblast growth factor 2 (FGF-2) and the PTX3:FGF-2 ratio in patients with systemic sclerosis (SSc) and in healthy control subjects. SSc patients were classified as having diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc). Symbols represent individual subjects; bars show the mean. *P* values were determined by Mann-Whitney U test. NS = not significant.

levels of circulating PTX3 (mean ± SD 4.8 ± 3.3 ng/ml versus 2.4 ± 1.7 ng/ml) and FGF-2 (12.0 ± 9.4 pg/ml versus 5.8 ± 5.0 pg/ml). There were no differences in PTX3 or FGF-2 levels between SSc patients with dcSSc and those with lcSSc (Figure 1). The PTX3:FGF-2 ratio, which we used as an indicator of the relative anti/proangiogenic activity of FGF signaling, was not different between SSc patients and healthy controls. We did not find correlations between PTX3, FGF-2, and CRP levels in patients with SSc (r < 0.1 for all comparisons).

**Association of organ involvement with circulating levels of PTX3 and FGF-2.** PTX3 and FGF-2 levels were compared between SSc patients grouped by the presence or absence of specific organ involvement (further information is available at http://www.nms-rheum.jp). Statistically significant differences were detected when patients were divided by the presence or absence of digital ulcers or PAH (Figure 2). PTX3 was significantly elevated in patients with digital ulcers compared to those without digital ulcers, while there was no difference in FGF-2 concentrations. The presence of PAH, like the presence of digital ulcers, was associated with increased PTX3. However, in contrast to the similarity in FGF-2 levels between patients with and those

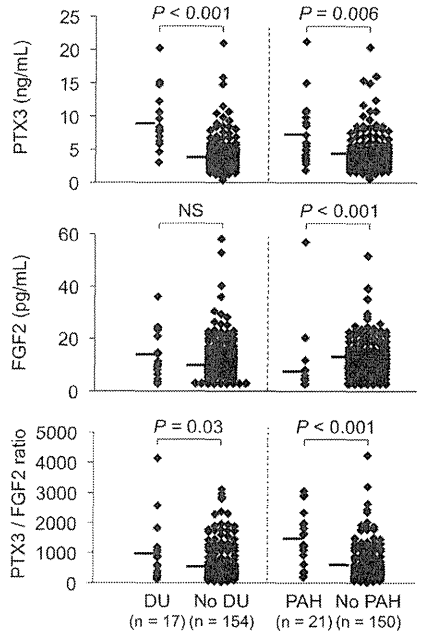


**Figure 2.** Levels of circulating PTX3 and FGF-2 and the PTX3:FGF-2 ratio in SSc patients grouped by the presence or absence of digital ulcers (DU) or pulmonary arterial hypertension (PAH). Symbols represent individual patients; bars show the mean. *P* values were determined by Mann-Whitney U test. See Figure 1 for other definitions.

without digital ulcers, levels of FGF-2 were decreased among patients with PAH compared to those without PAH (Figure 2). The mean ± SD FGF-2 level in SSc patients with PAH (8.1 ± 12.3 pg/ml) was comparable to that in healthy controls ($P = 0.8$). The PTX3:FGF-2 ratio was significantly higher in patients with digital ulcers or PAH than in those without, suggesting that FGF-2 signaling was suppressed. When we assessed potential associations of PTX3 and FGF-2 levels with risk factors for atherosclerosis and with specific treatments, PTX3 levels were found to be significantly higher in SSc patients receiving corticosteroids than in those not receiving them ($P = 0.02$).

As the behavior of FGF-2 appeared to change in association with digital ulcers or PAH, we divided SSc patients into 4 groups based on the presence or absence of digital ulcers and the presence or absence of PAH, and we evaluated the distribution of PTX3 and FGF-2 levels in each group (Figure 3). When PTX3 and FGF-2 levels were divided as high or low relative to the mean (3.88 ng/ml for PTX3 and 9.82 pg/ml for FGF-2), differences in patient distribution in the groups became apparent. All but 1 patient with digital ulcers was included in the high-PTX3 group, independent of the presence or absence of PAH, and the frequency of high PTX3 was significantly greater in patients with digital ulcers than in those without digital ulcers (94% versus 45%; $P < 0.001$). On the other hand, patients with PAH were more frequently included in the low–FGF-2 group than were those without PAH (86% versus 45%; $P < 0.001$). Thus, it is likely that different PTX3 and FGF-2 profiles regulate the development of digital ulcers and PAH. However, the PTX3:FGF-2 ratio was elevated both in patients with digital ulcers and in patients with PAH (Figure 2), indicating the presence of common antiangiogenic properties.

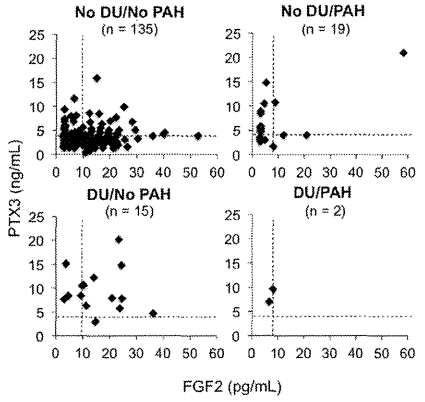


**Figure 3.** Distribution of PTX3 and FGF-2 levels in 4 groups of SSc patients stratified by the presence or absence of digital ulcers (DU) and pulmonary arterial hypertension (PAH). Dashed lines indicate mean PTX3 and FGF-2 levels in SSc patients (3.88 ng/ml and 9.82 pg/ml, respectively). Symbols represent individual patients. See Figure 1 for other definitions.

**PTX3 and FGF-2 as independent parameters associated with digital ulcers or PAH.** Using multivariate analysis, we further determined whether circulating PTX3 and FGF-2 levels were independently associated with the presence of digital ulcers or PAH (further information is available at http://www.nms-rheum.jp). Univariate analyses identified disease duration from the first appearance of non–Raynaud's phenomenon symptoms, dcSSc, a previous history of digital ulcers, upper GI involvement, and anti–topoisomerase I antibody as parameters potentially associated with the presence of digital ulcers ($P < 0.1$). Multivariate logistic regression analysis of these parameters along with PTX3 and FGF-2 levels identified the following as independent parameters positively associated with digital ulcers: PTX3 (OR 1.50 [95% CI 1.22–1.85], $P < 0.001$), anti–topoisomerase I antibody (OR 8.01 [95% CI 1.13–57.1], $P = 0.04$), and a previous history of digital ulcers (OR 61.7 [95% CI 7.09–537], $P < 0.001$).

To identify independent parameters associated with PAH, we conducted multivariate analysis with 5 variables: the absence of anti–topoisomerase I antibody, which was selected by univariate analysis; disease duration from the first appearance of Raynaud's phenomenon and lcSSc, which were previously reported to be associated with PAH (21); and PTX3 and FGF-2 (further information is available at http://www.nms-rheum.jp). PTX3 was the sole parameter positively associated with PAH (OR 1.23 [95% CI 1.08–1.40], $P = 0.002$), while FGF-2 was identified as an independent parameter negatively associated with PAH (OR 0.92 [95% CI 0.85–0.99], $P = 0.02$). When these analyses were repeated using the PTX3:FGF-2 ratio instead of PTX3 and FGF-2 levels, the PTX3:FGF-2 ratio was selected as an independent parameter associated with digital ulcers (OR 1.001 [95% CI 1.000–1.002], $P = 0.04$) and PAH (OR 1.001 [95% CI 1.001–1.002], $P < 0.001$).

**Predictors of a new occurrence of digital ulcers.** Of 171 patients at the initial evaluation, 148 had never developed digital ulcers. These patients were prospectively followed up for a mean ± SD of 3.4 ± 2.5 years, and 18 (12%) developed digital ulcers for the first time during the followup period. ROC curve analysis was used to determine whether circulating levels of PTX3 and FGF-2, as well as the PTX3:FGF-2 ratio, were predictive of new digital ulcer development (Figure 4A). PTX3 levels and the PTX3:FGF-2 ratio were found to be useful in predicting the future onset of digital ulcers, but FGF-2 levels were not. The cutoff values that best discriminated a risk of future digital ulcer development were 4.3 ng/ml for PTX3 and 344 for the PTX3:FGF-2 ratio. When SSc patients were divided by high or low PTX3 levels relative to the cutoff values, the cumulative occurrence rate of new digital ulcers changed significantly (Figure 4B). We were unable to conduct a similar analysis of PAH predictors because only 1 patient developed PAH during the followup period.

Using the Cox proportional hazards regression model, we further assessed whether the circulating PTX3 level was an independent parameter predicting the future development of digital ulcers (further information is available at http://www.nms-rheum.jp). In this analysis, the time origin was the time of enrollment. The variables included were dcSSc and anti–topoisomerase I antibody, which were selected by univariate analysis ($P < 0.1$); disease duration from the first appearance of non–Raynaud's phenomenon symptoms, which was previously shown to be associated with digital ulcers (22,23); and PTX3 and FGF-2. This identified PTX3 as the sole independent predictor for the new development of digital ulcers (HR 1.25 [95% CI 1.09–1.42], $P = 0.001$). When the analysis was repeated using the PTX3:FGF-2 ratio instead of PTX3 and FGF-2, the PTX3:FGF-2 ratio was selected as the sole predictor for the future development of digital ulcers (HR 1.001 [95% CI 1.000–1.001], $P = 0.003$).

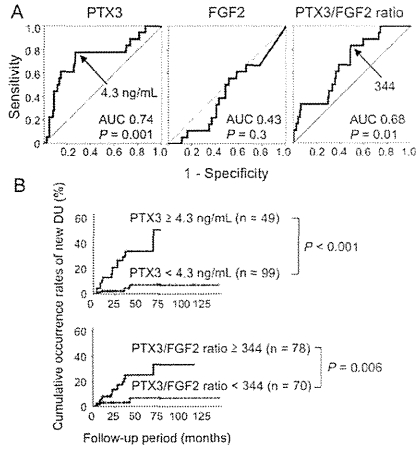


**Figure 4.** Predictors for new digital ulcers (DU) in 148 SSc patients who had never developed digital ulcers. **A,** Receiver operating characteristic curve analysis evaluating optimal cutoff values of PTX3 and FGF-2 levels and the PTX3:FGF-2 ratio for predicting new occurrences of digital ulcers. AUC = area under the curve. **B,** Kaplan-Meier analysis to evaluate cumulative occurrence rates of new digital ulcers in SSc patients stratified by PTX3 levels and the PTX3:FGF-2 ratio. $P$ values were determined by log rank test. See Figure 1 for other definitions.

**Changes in PTX3 and FGF-2 levels after an interval of 2 years.** Followup blood samples obtained 2 years after the initial evaluation were available for 37 SSc patients, 9 of whom developed digital ulcers during this period. The levels of circulating PTX3 and FGF-2 were fairly stable between study enrollment and 2 years later, regardless of whether there was any new onset of digital ulcers (4.7 ± 3.3 ng/ml versus 4.4 ± 3.2 ng/ml and 10.7 ± 10.4 pg/ml versus 10.9 ± 7.1 pg/ml, respectively). Taken together, these results suggest that elevated PTX3 levels and PTX3:FGF-2 ratios are useful biomarkers to predict the future development of digital ulcers in SSc patients, and that high, persistent concentrations of PTX3 in the circulation may lead to the occurrence of digital ulcers.

**Correlations between PTX3 and EPCs in the circulation.** It was recently reported that the number of circulating EPCs is inversely correlated with the presence of digital ulcers, and that a low EPC count in SSc patients is a risk factor for developing digital ulcers (24,25). Thus, we investigated the potential correlation between circulating PTX3 levels and EPC counts. Circulating EPC counts obtained at the same time as PTX3 and FGF-2 levels were available for 70 patients. EPC counts were significantly lower in patients with digital ulcers than in those without digital ulcers and significantly lower in patients with PAH than in those without PAH (Figure 5A). EPC counts were negatively correlated with circulating PTX3 levels and PTX3:FGF-2 ratios, but there was no correlation with FGF-2 levels (Figure 5B). These findings demonstrated an association between reduced EPC counts and high concentrations of

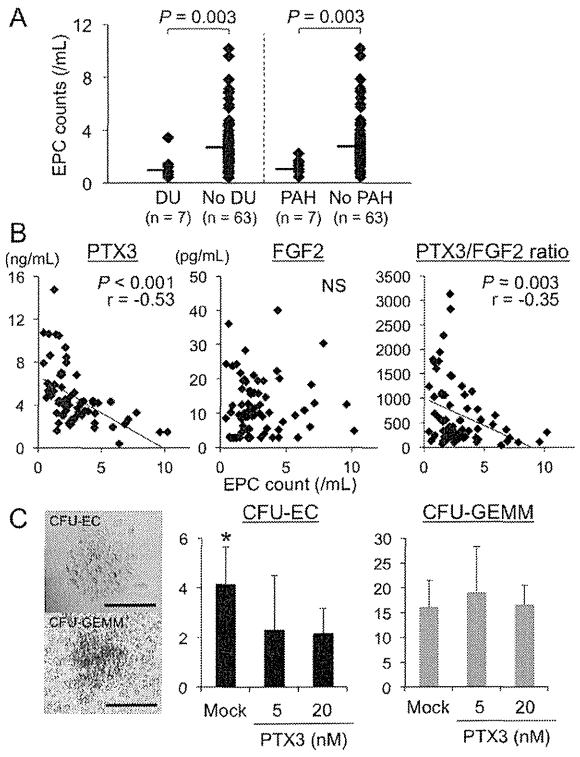


**Figure 5.** Relationship between endothelial progenitor cell (EPC) counts and concentration of circulating PTX3 in SSc patients. **A,** EPC counts in SSc patients stratified by the presence or absence of digital ulcers (DU) or pulmonary arterial hypertension (PAH). Symbols represent individual patients; bars show the mean. *P* values were determined by Mann-Whitney U test. **B,** Correlation between EPC counts and concentrations of PTX3 or FGF-2 in the circulation or between EPC counts and the PTX3:FGF-2 ratio. Symbols represent individual patients. **C,** Effect of PTX3 on in vitro EPC differentiation. Mouse bone marrow mononuclear cells were cultured under proangiogenic conditions in the presence or absence (mock) of PTX3. Left, Images showing typical morphology of colony forming unit–endothelial cell (CFU-EC) colonies and CFU–granulocyte–erythrocyte–macrophage–megakaryocyte (GEMM) colonies. Bars = 0.2 mm. Middle and right, Effect of PTX3 on formation of CFU-EC colonies (middle) and CFU-GEMM colonies (right). Values are the mean ± SD of 10 independent measurements. * = $P = 0.01$ versus 5 n*M* of PTX3 and $P = 0.005$ versus 20 n*M* of PTX3. See Figure 1 for other definitions.

PTX3 in the circulation in the context of the occurrence of digital ulcers or PAH.

**Inhibition of EPC differentiation by PTX3.** To investigate the effect of PTX3 on EPC differentiation, we used an in vitro mouse system in which nonadherent bone marrow mononuclear cells were cultured under EPC differentiation conditions (which included FGF-2) in the presence or absence of PTX3, followed by colony-forming assays to count CFU-EC and CFU-GEMM colonies (Figure 5C). In these cultures, PTX3 reduced the formation of CFU-EC colonies to approximately half, but had no effect on CFU-GEMM colony formation. Further increases in the concentration of PTX3 did not increase the inhibitory effect. This suggests that an FGF-2–independent pathway is also involved in this in vitro EPC differentiation, since PTX3 is known to directly inhibit binding of FGF-2 to its receptor (5). Thus, exposure to high concentrations of PTX3 may suppress EPC differentiation, leading to a negative correlation between PTX3 levels and EPC counts.





## DISCUSSION

In this study, we found that circulating PTX3 was elevated in SSc patients, irrespective of the disease subset. Interestingly, elevated PTX3 levels were associated with vascular manifestations such as digital ulcers and PAH, but not with the fibrotic aspects of the disease. This was confirmed by multivariate analysis, in which PTX3 was identified as an independent parameter for the presence of digital ulcers and PAH. Elevated PTX3 was also identified as a good predictor for the future development of digital ulcers. Together, these findings suggest that PTX3 primarily promotes SSc vasculopathy, which is consistent with previous studies (6,26). However, while Iwata and colleagues also found correlations between elevated PTX3 and various fibrotic aspects of SSc, including dcSSc, high MRSS, pulmonary fibrosis, and heart involvement, they did not find a correlation with PAH (6). Although the exact reasons for this discrepancy are uncertain, it may be due to differences in the methods of measuring PTX3 levels and the definitions for the involvement of various organs, as well as the lower proportion of patients with dcSSc in our study. Since circulating PTX3 levels stayed fairly stable over the course of this study, PTX3 should prove to be a useful biomarker for the future development of digital ulcers in SSc patients.

Since PTX3 exerts its antiangiogenic effects primarily by competing with FGF-2 for binding to its receptor, for which PTX3 has higher affinity (5), the balance between circulating PTX3 and FGF-2 can reasonably be used to assess net anti/proangiogenic activity. Since FGF-2 was also elevated in SSc patients compared to healthy subjects, as other studies have also shown (27,28), the PTX3:FGF-2 ratio remained comparable between SSc patients and healthy controls. However, as with PTX3, our results clearly identified the PTX3:FGF-2 ratio as an independent parameter for the presence of digital ulcers and PAH, as well as a predictor for the future development of digital ulcers.

The roles of FGF-2 in neovascularization are mediated in part by the promotion of angiogenesis, a process in which ECs proliferate and sprout from preexisting vessels (29,30). It has been suggested that chronic tissue ischemia and a lack of compensatory angiogenesis lead to vascular manifestations in SSc patients (31). In this regard, the angiogenic capacity of ECs derived from SSc skin was reduced by FGF-2 and VEGF in vitro (9). On the other hand, we have proposed that vasculogenesis, a vascularization process that involves the recruitment and in situ differentiation of bone marrow–derived EPCs, is also defective in SSc patients (32); this is based on the reduced counts and impaired differentiation potential of EPCs in SSc (19,33). However, the underlying mechanisms of defective vasculogenesis in SSc are not well understood. In our present study, we showed that EPC counts were inversely correlated with the level of circulating PTX3 or the PTX3:FGF-2 ratio, and that PTX3 could inhibit differentiation of stem cells into EPCs in in vitro cultures with FGF-2. Therefore, it is likely that exposure to a high concentration of PTX3 suppresses the FGF-2–mediated processes in both angiogenesis and vasculogenesis, increasing the risk of digital ulcers and PAH, although we did not show direct evidence of an inhibitory effect of PTX3 on FGF-2 in our assay system.

While elevated PTX3 was associated with both digital ulcers and PAH, SSc patients with PAH uniquely exhibited FGF-2 levels similar to those in healthy controls. This suggests that digital ulcers and PAH, in part, have distinct pathogenic processes in SSc. In this regard, investigators studying patients from French and Canadian registries reported that the occurrence of digital ulcers did not necessarily correlate with that of PAH (22,23), but the use of pulmonary vasodilators in patients with PAH may prevent the onset of digital ulcers. On the other hand, the relatively normal FGF-2 levels seen in patients with SSc-associated PAH contrasts sharply with the elevated FGF-2 seen in patients with idiopathic PAH (34), and cultured pulmonary ECs derived from patients with idiopathic PAH also overexpress FGF-2 (35). Furthermore, a series of studies using animal models for medial hypertrophy of the pulmonary arterioles showed that FGF-2 expression is enhanced in pulmonary arterioles, and that knocking down FGF-2 or administering FGF receptor antagonists reverses pulmonary vascular remodeling (35–37).

Differences in FGF-2 behavior in SSc-associated PAH and idiopathic PAH may be responsible for the

distinct pulmonary vascular histologic features seen in these 2 conditions. Specifically, SSc-associated PAH is characterized by intimal fibrosis of the pulmonary arterioles and venules (pulmonary venoocclusive disease–like changes) and the absence of the plexogenic arteriopathy that is typical of idiopathic PAH (38). Comprehensive gene expression profiling of lung samples showed that SPARC and thrombospondin 1 are up-regulated in patients with SSc-associated PAH in comparison with those with idiopathic PAH (39). Interestingly, these molecules are known to suppress autocrine and paracrine FGF-2 production loops (40,41). Therefore, it is likely that levels of FGF-2 signaling in the pathogenic process of PAH modify pulmonary vascular remodeling.

In summary, circulating PTX3 was elevated in SSc patients and was a useful biomarker predicting the presence of digital ulcers and PAH as well as the future development of digital ulcers. In addition, PTX3 may contribute to SSc vasculopathy by inhibiting vasculogenesis-mediated neovascularization through its suppressive effects on FGF-2. Further studies are necessary to elucidate these roles and the complex interactions of anti/proangiogenic factors in the development of vascular manifestations of SSc.



## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kuwana had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Shirai, Tamura, Kuwana.
**Acquisition of data.** Shirai, Okazaki, Inoue, Tamura, Yasuoka, Takeuchi, Kuwana.
**Analysis and interpretation of data.** Shirai, Kuwana.

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# Biologic-free remission of established rheumatoid arthritis after discontinuation of abatacept: a prospective, multicentre, observational study in Japan

**Tsutomu Takeuchi[1], Tsukasa Matsubara[2], Shuji Ohta[3], Masaya Mukai[4], Koichi Amano[5], Shigeto Tohma[6], Yoshiya Tanaka[7], Hisashi Yamanaka[8] and Nobuyuki Miyasaka[9]**

## Abstract

**Objective.** The aim of this study was to determine whether biologic-free remission of RA is possible with discontinuation of abatacept.

**Methods.** Japanese RA patients in 28-joint DAS with CRP (DAS28-CRP) remission (<2.3) after >2 years of abatacept treatment in a phase II study and its long-term extension entered this 52 week, multicentre, non-blinded, prospective, observational study. At enrolment, the patients were offered the option to continue abatacept or not. The primary endpoint was the proportion of patients who remained biologic-free at 52 weeks after discontinuation. Clinical, functional and structural outcomes were compared between those who continued and those who discontinued abatacept.

**Results.** Of 51 patients enrolled, 34 discontinued and 17 continued abatacept treatment. After 52 weeks, 22 of the 34 patients (64.7%) remained biologic-free. Compared with the continuation group, the discontinuation group had a similar remission rate (41.2% *vs* 64.7%, $P = 0.144$) although they had a significantly higher mean DAS28-CRP score at week 52 (2.9 *vs* 2.0, $P = 0.012$). The two groups were also similar with regard to mean HAQ Disability Index (HAQ-DI) score (0.6 for both, $P = 0.920$), mean change in total Sharp score (ΔTSS; 0.80 *vs* 0.32, $P = 0.374$) and proportion of patients in radiographic remission (ΔTSS ⩽ 0.5) at the endpoint (64.3% *vs* 70.6%, $P = 0.752$). Those attaining DAS28-CRP < 2.3 or < 2.7 without abatacept at the endpoint had significantly lower HAQ-DI score and/or CRP at enrolment. Non-serious adverse events occurred in three patients who continued or resumed abatacept.

**Conclusion.** Biologic-free remission of RA is possible in some patients after attaining clinical remission with abatacept. Lower baseline HAQ-DI or CRP may predict maintenance of remission or low disease activity after discontinuation of abatacept.

**Trial registration:** UMIN Clinical Trials Registry, http://www.umin.ac.jp/ctr/ (UMIN000004137).

**Key words:** rheumatoid arthritis, abatacept, biologic-free remission, observational study.

[1]Division of Rheumatology, Department of Internal Medicine, School of Medicine, Keio University, Tokyo, [2]Department of Rheumatology, Matsubara Mayflower Hospital, Kato, [3]Department of Rheumatology, Taga General Hospital, Hitachi, [4]Division of Rheumatology and Clinical Immunology, Department of Medicine, Sapporo City General Hospital, Sapporo, [5]Department of Rheumatology and Clinical Immunology, Saitama Medical Center, Saitama Medical University, Kawagoe, [6]Clinical Research Center for Allergy and Rheumatology, National Hospital Organization Sagamihara National Hospital, Sagamihara, [7]First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health Hospital, Kitakyushu, [8]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo and [9]Tokyo Medical and Dental University, Tokyo, Japan.

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Correspondence to: Tsutomu Takeuchi, Division of Rheumatology, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
E-mail: tsutake@z5.keio.jp













## Introduction

RA is a systemic inflammatory disease characterized by polyarthritis and progressive joint destruction. In RA, synovial monocyte-/macrophage-like cells and dendritic cells serve as antigen-presenting cells (APCs) due to their expression of antigen–MHC class II complexes and co-stimulatory molecules such as CD80 and CD86 [1]. Activated $CD4^+$ T cells expressing CD28 significantly infiltrate into the synovial membrane of affected joints and exacerbate synovitis and joint destruction by secreting inflammatory cytokines and activating synovial cells and osteoclasts [2–4]. The activation of $CD4^+$ T cells is therefore an important stage in the development of rheumatic synovitis, with the CD28-mediated co-stimulatory signal being required for full T cell activation and playing a major role in the immunopathological process of RA.

Abatacept is a genetically engineered humanized fusion protein consisting of the extracellular domain of human cytotoxic T lymphocyte-associated molecule 4 (CTLA-4) connected to a modified Fc region (hinge-CH2-CH3 domain) of human immunoglobulin G-1. Abatacept is a novel anti-rheumatic drug that acts by modulating the activation of naive T cells through the competitive binding of co-stimulation molecules expressed on APCs (CD80 and CD86) and blockade of $CD4^+$ T cell co-stimulation via CD28 [5].

Abatacept has been reported to control disease activity, prevent or delay joint destruction and improve quality of life [6–12]. Further, abatacept exhibits similar efficacy in Japanese MTX-intolerant patients with active RA, achieving clinical remission [28-joint DAS with CRP (DAS28-CRP) <2.6] in 24.6% of patients after 24 weeks [7]. Due to the high cost of biologic DMARDs and concerns regarding their long-term safety, the potential for biologic-free remission has been identified as an issue for further investigation [13, 14]. No previous studies have addressed this potential therapeutic application of abatacept despite evidence of its ability to suppress $CD4^+$ T cell activation in autoimmune diseases such as RA.

Thus we conducted the present study in Japanese RA patients who had completed a phase II study of abatacept [7] and its long-term extension in order to determine whether clinical remission attained with the drug was sustained following its discontinuation.

## Methods

Before enrolment in this study, written informed consent was obtained from each participating patient according to the Declaration of Helsinki (updated 2008). Prior to the start of the study, the institutional review board of each centre reviewed and approved the study.

### Study design and patients

In the previous phase II study [7], 194 Japanese RA patients received double-blind treatment with abatacept or placebo for 24 weeks in addition to prior MTX therapy and 174 of them entered its long-term extension and received open-label abatacept for a mean of 37.7 months (range 3.6–45.1). Those who had completed the phase II study [7] and its long-term extension were eligible for this multicentre, non-blinded, prospective, observational study if they were in clinical remission (DAS28-CRP < 2.3) and not receiving any other biologic therapy at enrolment. Inclusion criteria for the phase II study were age ⩾20 years; fulfilment of the 1987 ACR criteria for the diagnosis of RA with a functional status of class I, II or III; previous treatment with MTX at 6–8 mg/week for at least 12 weeks and one or more of the following: ⩾10 swollen joints (66-joint count), ⩾12 tender joints (68-joint count) or CRP ⩾ 1.0 mg/dl.

### Procedures

At enrolment, patients were offered the option to continue or discontinue abatacept during the study. Those who discontinued abatacept treatment (discontinuation group) were periodically followed up for disease activity. Those who chose to continue abatacept (continuation group) were treated with the drug every 4 weeks at its approved dosage and received similar follow-up. Abatacept could be restarted at a fixed dose of 10 mg/kg in response to a sign of relapse (DAS28-CRP > 2.7 at two consecutive visits) or at the investigator's discretion. If restarted after an interval of ⩽12 weeks, administration was every 4 weeks, whereas if started after an interval of >12 weeks, the first two doses were administered every 2 weeks and subsequent doses every 4 weeks.

During the study, dose modifications of non-biologic DMARDs (e.g. MTX) and glucocorticoids were allowed at the investigator's discretion. Concomitant administration of NSAIDs was permitted, but that of biologic agents was not.

### Efficacy outcomes

The primary outcome measure of this study was the proportion of patients who remained biologic-free at 52 weeks after discontinuation of abatacept. Secondary and tertiary outcomes were efficacy and safety, respectively.

RA disease activity was assessed in terms of DAS28-CRP and DAS28-ESR at weeks 0, 4, 12, 24, 36 and 52. If a patient resumed abatacept treatment, this assessment was made at the time of resumption as well as after 12 and 24 weeks.

In accordance with DAS28-CRP scores, disease activity was classified as remission (<2.3), low (⩽2.3 to <2.7), moderate (⩽2.7 to <4.1) or high (⩾4.1) [15]. The proportion of patients in each disease activity class at each specified time and the proportion of patients in DAS28-CRP remission (<2.3) at week 52 were calculated.

Similarly, disease activity was classified by DAS28-ESR as remission (<2.6), low (LDA; ⩽2.6 to <3.2), medium (MDA; ⩽3.2 to <5.1) or high (HAD; ⩾5.1) [15]. To assess disease impact on a patient's level of functional ability, the HAQ Disability Index (HAQ-DI) was determined at weeks 0, 4, 12, 24, 36 and 52.

Radiographic progression of joint destruction was assessed in terms of van der Heijde-modified total Sharp score (mTSS) [16, 17] at weeks 0 and 52 or at the time of withdrawal from the study, where possible. Changes from baseline in TSS (ΔTSS), joint erosion (ΔJE) score and joint space narrowing (ΔJSN) score at week 52 were determined. The proportion of patients with no (ΔTSS $\leqslant$ 0), little (ΔTSS $\leqslant$ 0.5; defined as radiographic remission) and rapid radiographic progression (RRP; ΔTSS $\geqslant$5) [18] was calculated.

### Time to abatacept treatment resumption

The mean time to resumption of abatacept treatment was determined in the discontinuation group.

### Safety

Patients remaining on abatacept were monitored for adverse events (AEs) throughout the study period. In the discontinuation group, AE monitoring was done only if and after abatacept was resumed following relapse. To investigate the relationship between the immunogenicity of abatacept and its tolerability, the anti-abatacept antibody titre in blood was measured at the time of discontinuation, time of resumption and 24 weeks after resumption of abatacept, if applicable.

### Statistical analysis

Missing data were imputed by linear extrapolation (radiographic assessments) or last observation carried forward (LOCF) (other efficacy variables). Continuous metric data were summarized in terms of descriptive statistics and were expressed as the mean (S.D.). Data between the two groups were compared using Wilcoxon's rank sum test (demographic and baseline characteristics, DAS28, HAQ-DI, ΔTSS, ΔJE and ΔJSN) or Fisher's exact test (proportion of patients in DAS28-CRP remission at week 52 and the proportions of patients with ΔTSS $\leqslant$0, $\leqslant$0.5 and $\geqslant$5).

## Results

### Patient disposition and baseline characteristics

Fifty-one consenting patients were enrolled and chose to either discontinue ($n=34$) or continue ($n=17$) abatacept. Nine of the 34 patients from the discontinuation group restarted abatacept at the investigator's discretion ($n=8$) or due to relapse ($n=1$). Six patients from the discontinuation group (with an additional patient withdrawn after resumption) and two from the continuation group dropped out of the study, leaving a total of 28 and 15 patients, respectively. Nineteen patients from the discontinuation group remained biologic-free at week 52 (Fig. 1). The demographic and baseline characteristics of the 51 patients enrolled are summarized in Table 1. The two groups had comparable baseline characteristics, except for significantly shorter disease duration and significantly less joint damage in terms of JSN and TSS in those who discontinued abatacept at enrolment ($P<0.05$ for all comparisons).

### Efficacy outcomes

Of the 34 patients who discontinued abatacept at enrolment, 22 patients from an intention-to-treat (ITT) analysis (64.7%) remained biologic-free after 52 weeks. While the mean DAS28-CRP score remained constant in the continuation group, it gradually increased over time in the discontinuation group, leading to a significant difference between the groups at week 52 (2.9 *vs* 2.0, $P=0.012$).

This was also true when the subgroup of discontinuing patients who remained in the study and never restarted abatacept ($n=19$) were compared with the continuing patients remaining in the study ($n=15$; 2.8 *vs* 2.1, $P=0.036$).

Fig. 2 shows the proportion of patients in each RA disease activity class at specified times. In the discontinuation group there was a tendency towards a decrease in the proportion of patients in DAS28-CRP remission and an increase in the proportion of those with HDA as follow-up progressed. At week 52 (LOCF), the proportion of patients in remission was 41.2% in the discontinuation group compared with 64.7% in the continuation group ($P=0.144$). Sixteen of the 17 continuing patients (94.1%) experienced no disease flare (DAS28-CRP < 2.7), while 20 of the 34 discontinuing patients (58.8%) were in remission or maintained LDA. Compared with the 14 patients who failed to do so, these 20 patients had significantly lower baseline HAQ-DI scores and CRP ($P=0.036$ and $P=0.048$, respectively). Of the 19 patients who went without abatacept for 52 weeks, 7 were in remission at the endpoint and 12 were not. These two subgroups had comparable baseline characteristics, except that more patients in remission than not in remission at the endpoint were in functional remission (HAQ-DI $\leqslant$ 0.5) at enrolment (100% *vs* 41.7%, $P=0.016$). The mean time-averaged DAS28-CRP (TA-DAS28-CRP) [19, 20] was 1.9 (S.D. 0.4) for those who maintained LDA compared with 3.0 (S.D. 0.7) for those who failed to do so ($P<0.0001$).

In contrast to consistently low (<2.6) scores in the continuation group, the mean DAS28-ESR score in the discontinuation group increased slightly, from 2.4 at baseline to 2.7 at week 4, 3.1 at week 12, 3.3 at week 24, 3.5 at week 36 and 3.6 at week 52. According to the endpoint DAS28-ESR scores, 24.2% of the discontinuing *vs* 47.1% of the continuing patients were in remission, 30.3% *vs* 35.3% had LDA, 27.3% *vs* 17.6% had MDA and 18.2% *vs* 0% had HDA. The mean HAQ-DI scores for the two groups followed similar time courses and were 0.6 for both groups at week 52 ($P=0.920$; Fig. 3).

The TSS at weeks 0 and 52 was similar in the discontinuation and continuation groups, but the baseline TSS was higher for the continuation group (Fig. 4A). Mean ΔTSS (0.80 *vs* 0.32, $P=0.374$) and ΔJE (−0.02 *vs* 0.32, $P=0.466$) were similar for the two groups, while mean ΔJSN was significantly greater in the discontinuation group (0.82 *vs* 0, $P=0.035$; Fig. 4B). After correction by linear extrapolation, the proportion of patients in radiographic remission (ΔTSS $\leqslant$ 0.5) was 64.3% in the discontinuation group compared with 70.6% in the continuation group ($P=0.752$; Fig. 4C). No radiographic progression was seen in 42.9% and 47.1% of patients, while RRP was seen in 14.3% and 0% of patients in the discontinuation and continuation groups, respectively (Fig. 4C). The four patients who showed RRP after discontinuation had significantly higher CRP at enrolment in this study and lower RF in the previous phase III study compared with the 24 patients who did not show RRP in this group ($P=0.034$ and $P=0.020$, respectively).

Fig. 1 Patient disposition

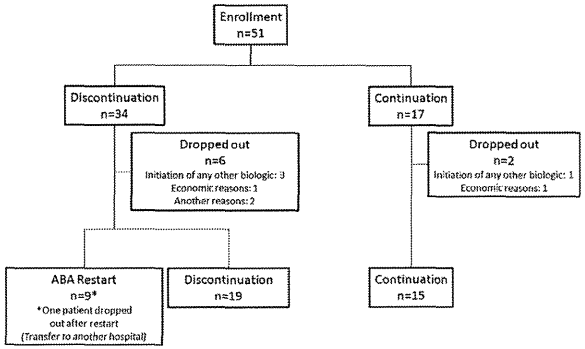



Table 1 Patient characteristics

| | Discontinuation ($n=34$) | Continuation ($n=17$) | *P*-value |
|---|---|---|---|
| Age, mean (S.D.), years | 56.9 (11.4) | 60.9 (9.5) | 0.195[a] |
| Male, *n* (%) | 5 (14.7) | 4 (23.5) | 0.443[b] |
| Female, *n* (%) | 29 (85.3) | 13 (76.5) | |
| RA disease duration, mean (S.D.), years | 9.6 (5.2) | 15.3 (10.5) | 0.018[a] |
| DAS28-CRP, mean (S.D.) | 1.8 (0.4) | 1.7 (0.5) | 0.803[a] |
| Tender joint count (0–28), mean (S.D.) | 0.3 (0.6) | 0.1 (0.5) | 0.788[a] |
| Swollen joint count (0–28), mean (S.D.) | 0.5 (0.8) | 0.6 (0.9) | 0.429[a] |
| HAQ-DI, mean (S.D.) | 0.5 (0.5) | 0.5 (0.5) | 0.356[a] |
| CRP, mean (S.D.), mg/dl | 0.3 (0.5) | 0.2 (0.2) | 0.285[a] |
| ESR, mean (S.D.), mm/h | 18.7 (9.5) | 17.6 (8.5) | 0.790[a] |
| DAS28-ESR, mean (S.D.) | 2.4 (0.5) | 2.3 (0.6) | 0.705[a] |
| MMP-3, mean (S.D.), ng/ml | 79.5 (63.3)[c] | 75.3 (46.3)[d] | 0.707[a] |
| RF, mean (S.D.), IU/ml | 72.8 (128.5)[c] | 50.7 (76.1)[e] | 0.822[a] |
| RF positive, *n* (%) | 14 (48.3)[c] | 6 (60.0)[e] | 0.394[b] |
| PGA (0–100 mm VAS), mean (S.D.) | 12.7 (10.7) | 17.4 (15.2) | 0.363[a] |
| Erosion, mean (S.D.) | 29.9 (37.9)[f] | 62.0 (58.4) | 0.015[a] |
| Joint space narrowing, mean (S.D.) | 28.6 (27.2)[f] | 55.5 (41.2) | 0.020[a] |
| TSS (0–448), mean (S.D.) | 58.5 (64.1)[f] | 117.5 (97.7) | 0.016[a] |
| Concomitant use of MTX, *n* (%) | 19 (55.9) | 12 (70.6) | 1.000[b] |
| MTX dose, mean (S.D.), mg/week | 6.7 (2.2)[g] | 8.7 (2.3)[h] | 0.211[a] |
| Concomitant use of PSL, *n* (%) | 12 (35.3) | 8 (47.1) | 0.372[a] |
| PSL dose, mean (S.D.), mg/day | 4.0 (2.8)[i] | 3.9 (2.8)[j] | 0.538[a] |

PGA: patient's global assessment of disease activity; VAS: visual analogue scale; RF: rheumatoid factor; TSS: total Sharp score; PSL: prednisolone. [a]Wilcoxon's rank sum test; [b]Fisher's exact test; [c]$n=29$; [d]$n=14$; [e]$n=10$; [f]$n=28$; [g]$n=17$; [h]$n=12$; [i]$n=9$; [j]$n=8$.

FIG. 2 Proportion of disease activity

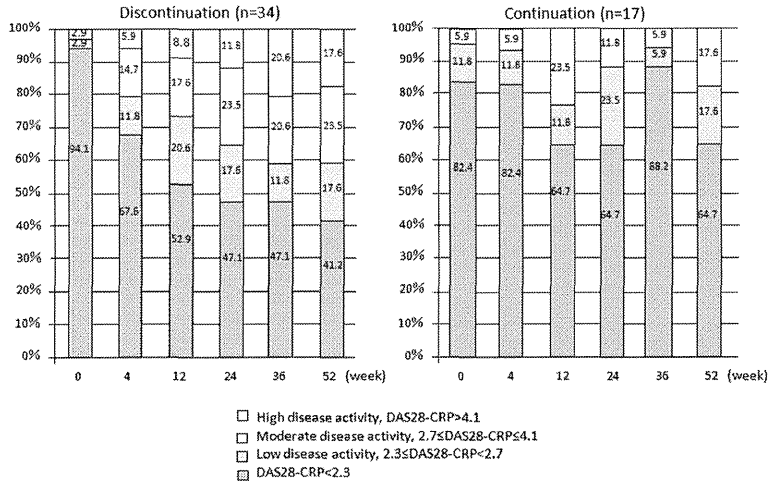
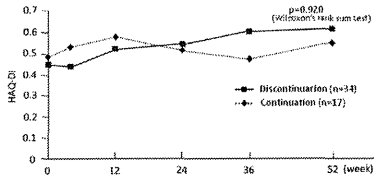



FIG. 3 Transition diagram of HAQ-DI




DI: Disability Index.

In the discontinuation group, 10 of the 14 patients in DAS28-CRP remission at week 52 were evaluable for ΔTSS, of whom 7 (70%) were in radiographic remission. In the continuation group, all 11 patients in DAS28-CRP remission at week 52 were evaluable for ΔTSS and 7 (63.6%) were in radiographic remission.

### Resumption of abatacept treatment

Nine patients resumed abatacept treatment after a mean interval of 149.6 days (S.D. 34.5). After resumption, the mean DAS28-CRP score steadily decreased, from 5.0 (S.D. 1.1) to 3.7 (S.D. 1.6) at 12 weeks and to 3.7 (S.D. 1.7) at 24 weeks, as was observed in the previous phase II/III study [from 4.8 (S.D. 0.8) at baseline to 3.0 (S.D. 0.9) at week 12 and to 2.8 (S.D. 0.9) at week 24; not significant by Wilcoxon's rank sum test].

In the previous study, time to remission in those who resumed ($n = 9$) and did not resume ($n = 25$) abatacept was similar ($P = 0.643$; log rank test); clinical remission was achieved in 2 of 9 (22.2%) *vs* 13 of 25 (52.0%) patients at week 24 and in 88.9% *vs* 96.0% of patients at the endpoint, respectively. The two populations also had comparable demographic and baseline characteristics.

### Safety

Non-serious AEs occurred in one patient who resumed abatacept (acute upper respiratory tract infection) and two patients who continued the drug (acute bronchitis in one and low back pain, cystitis, constipation, common cold and left scapulohumeral periarthritis in the second). No serious AEs were reported. Anti-abatacept antibody titre was measured in 26 of the 34 patients upon discontinuation of abatacept, as well as in 7 of 9 and 6 of 9 patients immediately and at 24 weeks after resumption. Positive titres were recorded in four patients (15.4%) upon discontinuation, in two patients (28.6%) immediately after resumption and in no patients at 24 weeks after resumption. Two of the four patients with positive titres upon discontinuation restarted abatacept. Both patients had positive titres again upon resumption, but not after 24 weeks. None of the patients with positive anti-abatacept antibody titre developed AEs or responded poorly to abatacept.

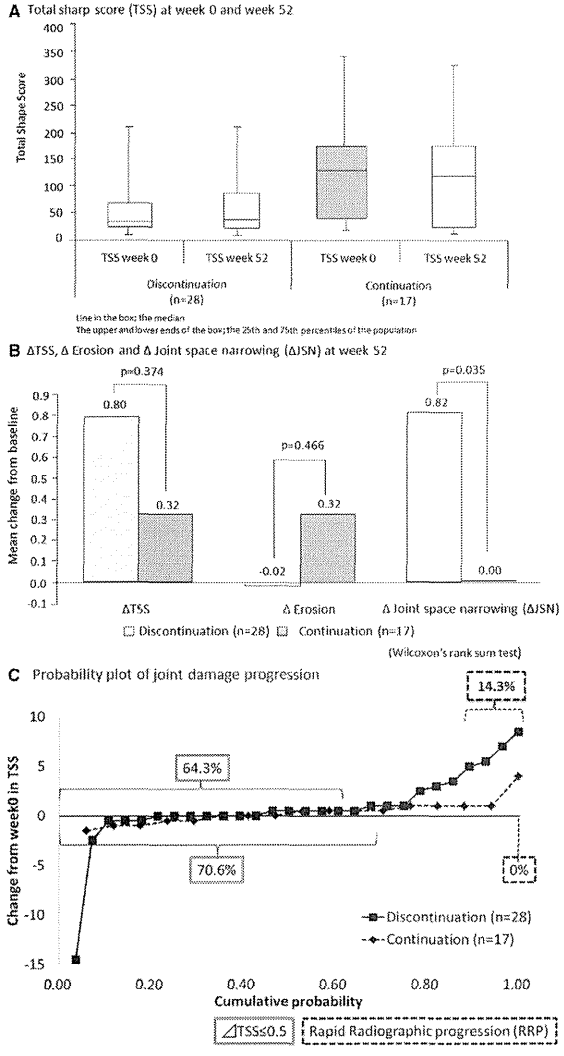








FIG. 4 Total Sharp score



## Discussion

Accumulating evidence suggests that $CD4^+$ T cells play a key role in RA-associated inflammation [21–23], although the extent to which they contribute to this disease is not fully understood. Abatacept, which blocks a T cell co-stimulation pathway, has been shown to have favourable efficacy and tolerability profiles in Japanese and non-Japanese MTX-intolerant, TNFinhibitor-intolerant or MTX-naive [early (<2 years)] RA patients [7–12].

The ACR and European League Against Rheumatism treatment recommendations propose that remission or LDA should be the primary target for treatment of RA [24]. Combined therapy with currently available biologic and non-biologic DMARDs can help attain current treatment targets in the majority of RA patients. Nonetheless, the high costs of biologic agents have encouraged ongoing efforts to reduce the economic burden upon patients, including trials to discontinue biologic therapy in patients in sustained clinical remission. While existing data support the potential for biologic-free remission following intensive treatment with TNFinhibitors [25–28], definitive evidence for this potential following discontinuation of abatacept is limited. One study suggested that there was no further radiographic or MRI progression of joint destruction after discontinuation of abatacept in patients with undifferentiated inflammatory arthritis or very early RA [29]. Here we determined the potential of abatacept in promoting biologic-free remission in RA patients already in clinical remission.

At week 52, 64.7% of the patients who discontinued abatacept in an ITT population remained biologic-free (primary endpoint). In a drug-free follow-up of 102 RA patients (mean disease duration 5.9 years) who attained LDA with infliximab [25], 55% of the patients maintained LDA and 39 of the 83 patients (47%) who had achieved remission (DAS28 < 2.6) at enrolment remained in remission for 1 year. In a similar study for adalimumab [28], 14 of 22 patients (64%) maintained LDA (DAS28-CRP < 2.7) without the drug for 1 year. On comparison with these TNF inhibitors, abatacept seems to have a similar potential in the induction of biologic-free remission.

After discontinuation of abatacept, the mean DAS28-CRP score gradually increased and reached a level significantly higher than in the continuation group at week 52. This was also true when the mean endpoint DAS28-CRP score was compared between the 19 patients who went without abatacept and the 15 patients who continued the drug for 52 weeks. In the discontinuation group, the number of patients in DAS28-CRP remission decreased and the number of patients with HDA increased. HAQ-DI and CRP are two baseline parameters that were significantly different between those with ($n = 20$) and without ($n = 14$) LDA at week 52. In addition, HAQ-DI is the only baseline parameter that was significantly different between those in remission ($n = 7$) and those not in remission ($n = 12$) without abatacept at week 52. These findings suggest that the HAQ-DI or CRP immediately before discontinuation of abatacept may predict the probability of subsequent maintenance of remission or LDA. According to TA-DAS28-CRP data, those with LDA at the endpoint maintained LDA throughout the period of follow-up. Comparison between the discontinuation and continuation groups showed similar proportions of patients in clinical remission at week 52 and similar changes in the HAQ-DI over time, indicating that the effects of abatacept on clinical and functional outcomes are durable even after discontinuation.

In RA, joint destruction progresses over time, causing significant disability, which imposes an enormous social burden. Although the recently introduced biologic agents, including abatacept, can prevent or delay joint destruction in a proportion of patients, it is not known if they prevent disease relapse following discontinuation. In the present study, radiographic assessment of joint destruction showed no significant difference between those who discontinued and those who continued abatacept with regard to mean ΔTSS or the percentage of patients with ΔTSS $\leqslant 0$, $\leqslant 0.5$ or $\geqslant 5$. These data confirm that abatacept exerts a sustainable effect in preventing or delaying joint damage and thus keeps patients in radiographic remission even after discontinuation. These radiographic benefits of abatacept appear to be comparable to those of infliximab and adalimumab (in early RA), as evidenced by 67% [25] and 81% [27] of patients with LDA remaining in radiographic remission after discontinuation of those drugs.

As a proportion of RA patients have to suspend their biologic therapy for economic or other reasons, we also assessed the efficacy and safety of re-treatment with abatacept after relapse. Re-treatment with abatacept was effective in controlling disease activity but may be less effective than the initial treatment with abatacept, which was evaluated in the previous phase II study [7].

Abatacept was well tolerated after resumption and during extended use, with only non-serious AEs being reported in three patients. Regarding the immunogenicity of abatacept, two of the limited number of patients assessed were positive for anti-abatacept antibody at the resumption of treatment but were negative after 24 weeks. The disappearance of anti-abatacept antibody after resumption of abatacept treatment may reflect the immunomodulatory effect of the drug.

The present study has several limitations. First, this was an exploratory study about the possibility of biologic-free remission after attaining clinical remission with abatacept. This study had no hypothesis to be tested because no data were available about this possibility with any other biologic DMARDs when we planned this study. Second, this was a small, non-randomized, observational study. Only Japanese RA patients who had completed a phase II study of abatacept [7] and its long-term extension and were in DAS28-CRP remission (<2.3) were enrolled, and for ethical reasons they were offered the option to continue abatacept or not at enrolment. As an expected consequence, the two groups were not well matched at baseline; those who chose to discontinue the drug were at an earlier stage of RA and had less progressive joint damage. Therefore data comparing the two groups should be interpreted cautiously. Third, we imputed missing data for non-radiographic efficacy variables using LOCF, a less favoured method than multiple imputation. This might introduce uncertainly about the reliability of the disease activity data and compromise their interpretation. Despite these limitations, the results are informative, as they indicate that the clinical remission achieved after abatacept treatment is potentially maintained following discontinuation of the drug in some of the patients, particularly in those who have also achieved a low HAQ-DI score and/or low CRP after the treatment. Given that the decision to continue or discontinue abatacept after attaining clinical remission was made by individual patients and their physicians, this finding will also be helpful for implementing the treat-to-target principle in RA practice.

**Rheumatology key messages**

- The effects of abatacept on clinical, functional and structural outcomes in RA continue after its discontinuation.
- Biologic-free remission of RA can be maintained after attaining sustained clinical remission with abatacept.
- Lower HAQ DI or CRP may predict maintenance of RA remission or low disease activity after discontinuation of abatacept.

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ORIGINAL ARTICLE

# Obstacles to the implementation of the treat-to-target strategy for rheumatoid arthritis in clinical practice in Japan

Yuko Kaneko[1], Takao Koike[2], Hiromi Oda[3], Kazuhiko Yamamoto[4], Nobuyuki Miyasaka[5], Masayoshi Harigai[6], Hisashi Yamanaka[7], Naoki Ishiguro[8], Yoshiya Tanaka[9], and Tsutomu Takeuchi[1]

[1]*Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan,* [2]*Sapporo Medical Center NTT East Corporation, Sapporo, Hokkaido, Japan,* [3]*Department of Orthopaedic Surgery, Saitama Medical University, Iruma District, Saitama, Japan,* [4]*Department of Allergy and Rheumatology, Graduate School of Medicine, the University of Tokyo, Bunkyo, Tokyo, Japan,* [5]*Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo, Tokyo, Japan,* [6]*Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo, Tokyo, Japan,* [7]*Institute of Rheumatology, Tokyo Women's Medical University, Shinjuku-ku, Tokyo, Japan,* [8]*Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan, and* [9]*The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka, Japan*

**Abstract**

*Objective.* To clarify the obstacles preventing the implementation of the treat-to-target (T2T) strategy for rheumatoid arthritis (RA) in clinical practice.
*Methods.* A total of 301 rheumatologists in Japan completed a questionnaire. In the first section, participants were indirectly questioned on the implementation of basic components of T2T, and in the second section, participants were directly questioned on their level of agreement and application.
*Results.* Although nearly all participants set treatment targets for the majority of RA patients with moderate to high disease activity, the proportion who set clinical remission as their target was 59%, with only 45% of these using composite measures. The proportion of participants who monitored X-rays and Health Assessment Questionnaires for all their patients was 44% and 14%, respectively. The proportion of participants who did not discuss treatment strategies was 44%, with approximately half of these reasoning that this was due to a proportion of patients having a lack of understanding of the treatment strategy or inability to make decisions. When participants were directly questioned, there was a high level of agreement with the T2T recommendations.
*Conclusion.* Although there was a high level of agreement with the T2T recommendations, major obstacles preventing its full implementation still remain.

**Keywords**

Implementation, Obstacle, Rheumatoid arthritis, Treat-to-target

**History**

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## Introduction

Recent insights into rheumatoid arthritis (RA) are leading to a new era in which the pursuit of remission and the prevention of irreversible joint destruction and physical functional impairment is the primary goal of treatment [1,2]. In parallel, there is evidence that treating RA to a target value via composite measures of disease activity results in a significantly improved clinical outcome [3,4]. To help facilitate this future goal the Treat-to-Target (T2T) strategy is an international initiative aimed at proposing global standard procedures in the management of RA. The T2T steering committee has developed 4 overarching principles and 10 recommendations based on clinical evidence through systematic literature review and expert opinion refined from a Delphi-like process [5].

In 2010 an anonymous worldwide survey was conducted on the proposed T2T recommendations for implementation that involved over 1901 rheumatologists. Results showed a very high level of agreement with scores of more than 8.5 for all of the recommendations [6]. However, the study also revealed that in some of the items approximately 10% of participants did not apply the recommendations in clinical practice, and 20–45% of these participants indicated that they would not change this practice [6,7]. In Japan, we have observed similar results of high agreement, but approximately 10% of non-application as a part of the study. Further, a year later, preliminary interviews performed in Japan with 30 rheumatologists and rheumatology nurses suggested that while they agreed with the concept of the T2T recommendations, they were not yet ready to implement them in routine clinical practice. We therefore aimed to identify the obstacles preventing the implementation of the T2T recommendations by rheumatologists at present, with a view to encourage their implementation in the future.

Here, we quantitatively surveyed the current implementation of the T2T recommendations and investigated the obstructions to their implementation.

Correspondence to: Tsutomu Takeuchi, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan. Tel: +81-3-5363-3786. Fax: +81-3-5379-5037. E-mail: tsutake@z5.keio.jp

## Subjects and methods

### Participants of the survey

Rheumatologists registered on a major website for Japanese physicians were contacted nationwide through e-mail and screened

to select those who were using the disease activity score (DAS) 28 to evaluate disease activity in RA patients and had started administering biological drugs to more than five RA patients in the previous year. The selected rheumatologists were asked to anonymously complete a web-based questionnaire in November 2011. The sample size was defined in advance as 300, and the survey ended when the number of subjects reached over 300 after a period of 10 days.

### Questionnaire

The participants were indirectly questioned about their implementation of basic components of the T2T strategy in a multiple-choice questionnaire. For example, when questioned on the application of Statement 1 ("The primary target for treatment of rheumatoid arthritis should be a state of clinical remission."), the question was not phrased as "Do you apply Statement 1?" In contrast, the participants were first asked "Do you set any 'treatment targets' when treating RA patients with moderate to high disease activity?" Participants were then asked "What are your 'treatment targets' when treating RA patients with moderate to high disease activity? Please choose one or more corresponding answers from the following: 1. Resolution/relief of joint pain, 2. Resolution/relief of joint swelling, 3. Reduction of inflammatory markers (e.g., C-reactive protein [CRP], erythrocyte sedimentation rate [ESR]), 4. Inhibition of progression of joint destruction, 5. Maintenance/improvement of patients' daily life activities, 6. Achievement of low disease activity, 7. Achievement of clinical remission, or 8. Others." Participants who selected "Yes" for the first question and "7. Achievement of clinical remission" for the second were regarded as properly implementing Statement 1. The questionnaire can be viewed in the Supplementary Questionnaire available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2014.926607. To avoid any bias, we did not disclose the fact that these questions regarded the T2T recommendations until the final section. The questionnaire was developed by T.T. by reference to the preliminary interviews performed with 30 rheumatologists and rheumatology nurses and approved by the other investigators.

In the last section, the participants were directly questioned on their level of agreement with each of the 10 recommendations on a 10-point Likert scale (1 = fully disagree to 10 = fully agree) and the degree to which each recommendation was being applied in clinical practice on a 4-point Likert scale (never, not very often, very often, or always). This methodology was consistent with the international T2T perception survey that was conducted in 2010 [6].

We used descriptive statistics to present proportions of participants pertinent to each issue.

## Results

### Demographic data of participants

Of the 301 participants, 277 (92.0%) were between 30 and 60 years of age. The number of participants working in each type of health care facility was as follows: university hospitals, 101 (33.6%); general hospitals, 139 (46.2%); and private clinics, 61 (21.3%). The number of participants with a certificate from the Japan College of Rheumatology for each type of membership was as follows: instructor, 92 (30.6%); specialist, 134 (44.5%); and general member, 60 (19.9%). The average number of RA patients seen by each rheumatologist per month was 149.9.

### Implementation of treating RA to a primary target of remission using validated composite measures

Of the 301 participants, 228 (75.7%) set treatment targets for nearly all of their RA patients with a moderate to high disease activity, and 72 (23.9%) for a proportion of their RA patients.

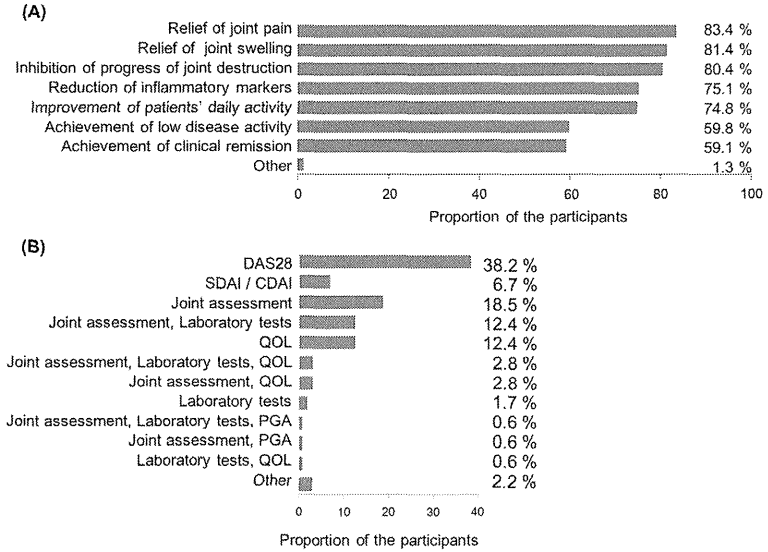


Figure 1. (A) Targets set by rheumatologists in clinical practice. (B) Definitions of clinical remission as treatment targets. The upper two answers included composite measures, and the others included only individual variables. DAS28, disease activity score 28; SDAI, simplified disease activity score; CDAI, clinical disease activity score; Joint, joint assessment; Lab test, laboratory test; PGA, patient global assessment; QOL, quality of life.

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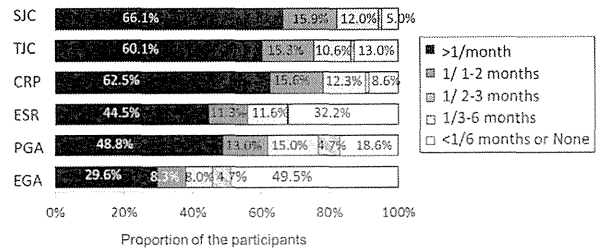
Figure 2. The proportion of rheumatologists who monitored each variable at the indicated intervals. SJC, swollen joint count; TJC, tender joint count; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PGA, patient global assessment; EGA, estimator global assessment.




However, only 178 (59.1%) participants set clinical remission as a target (Figure 1A), and of those only 80 (44.9%) used composite measures that included joint assessments (Figure 1B). Of the 301 participants, 26.6% were using remission defined using validated composite measures of disease activity as targets of treatment in routine clinical practice as recommended.

### Frequency of disease activity monitoring

The frequencies of monitoring variables pertinent to disease activity in RA patients with moderate to high disease activity are shown in Figure 2. The most frequently monitored variables were swollen joint count (SJC) and tender joint count (TJC), and the least monitored was evaluator global assessment (EGA). The number of participants who monitored each variable at least every month was a follows: SJC, 199 (66.1%); TJC, 181 (60.1%); C-reactive protein, 188 (62.5%); erythrocyte sedimentation rate, 134 (44.5%); patient global assessment, 147 (48.8%); and EGA, 89 (29.6%).

### Assessment of structural changes and functional impairment

Regarding the assessment of structural changes 264 participants (87.7%) monitored the X-rays of RA patients with moderate to high disease activity. However, only 177 participants (58.8%) were performing X-ray assessment in more than 80% of their patients (Figure 3). In terms of functional impairment only 131 rheumatologists (43.5%) were routinely monitoring the Health Assessment Questionnaire (HAQ), and this number fell to 67 (22.2%) when limiting to those who performed HAQ assessment in more than 80% of RA patients (Figure 3).

### *Communication between physicians and patients*

When the participants were questioned on communication with patients regarding their treatment strategy and regimen, 131 (43.5%) answered that they did not discuss with all their patients (Figure 4A). Of these participants, 63 (48.1%) answered that this was due to a proportion of patients being unable to understand the treatment strategy and regimen, and 77 (58.8%) that it was due to a proportion being unable to make a decision on the treatment (Figure 4B). When asked whether they calculated DAS28 scores and informed the patients of this during consultations, 176 (58.5%) did not do so at all, and only 64 (20.9%) informed more than 80% of their patients (Figure 4C). One hundred and fourteen (37.9%) participants did not calculate DAS28 during the consultations (i.e., calculate after the patient has left the office or at other available time), with the majority (95, 83.3%) of these citing the lack of time to calculate DAS28 during consultations as a reason (Figure 4D).

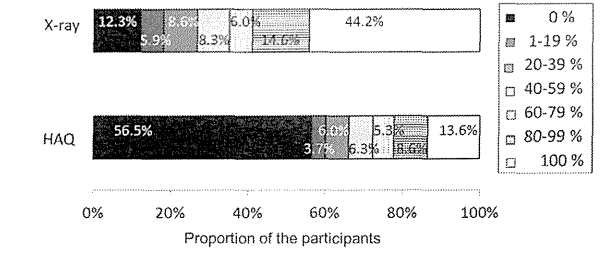
### Support system for implementation of T2T recommendations

As the implementation of T2T requires joint assessment, calculating composite measures of disease activity, and the education of and communication with RA patients, we investigated the proportion of rheumatologists who conducted these multiple processes in clinical practice. Joint assessment was conducted by 286 (95.0%) participants (Figure 5A). Two hundreds seventy-nine (92.7%) calculated DAS28 themselves while 22 (7.3%) were helped by nurses or other medical staffs (Figure 5B). Although nearly all participants explained the disease, the laboratory tests for RA, and treatment of RA to their patients, only 14.0–15.9% of participants received assistance from other healthcare providers, such as nurses or pharmacists (Figure 5C).

### Agreement with and application of T2T recommendations from questions in a direct manner

In the last section of the questionnaire, participants were directly questioned on their level of agreement with the T2T recommendations and the extent to which they applied them in clinical practice. There was a high level of agreement, with each of the 10 recommendations receiving a score of greater than 8.0 (Figure 6A). The highest scores were for Recommendations 1 (9.1) and 9 (9.0), and the lowest for Recommendations 8 (8.0) and 10 (8.1). In terms of application into clinical practice, the

Figure 3. The proportion of rheumatologists who routinely monitored X-rays or Health Assessment Questionnaires (HAQ) for the indicated percentages of RA patients with moderate to high disease activity. HAQ, health assessment questionnaire.



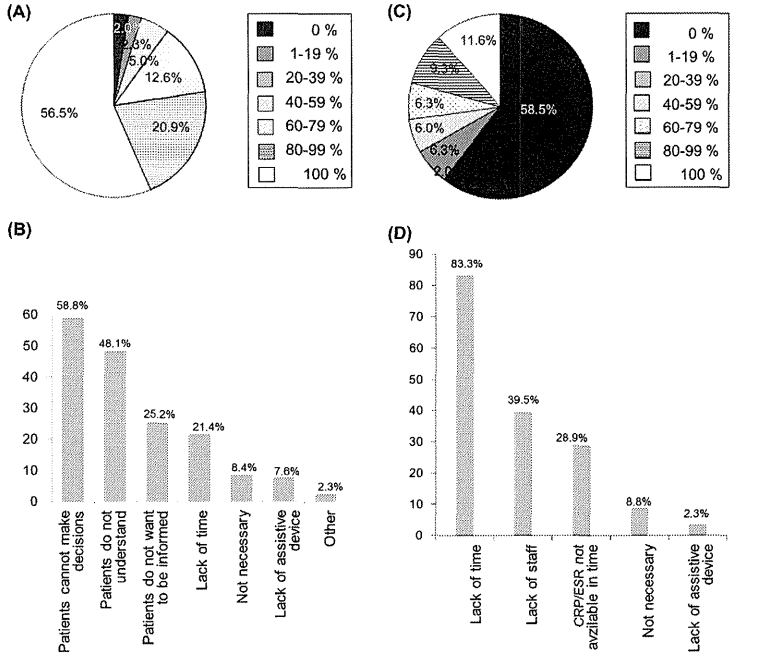



Figure 4. (A) The proportion of RA patients with moderate to high disease activity with whom the rheumatologists were discussing their treatment strategy and (B) the reasons for not discussing with all their patients ($n = 131$). Multiple answers are allowed. (C) The proportion of patients the rheumatologists informed of their DAS28 score during consultations and (D) the reasons for not calculating DAS28 during consultations ($n = 114$). Multiple answers are allowed.

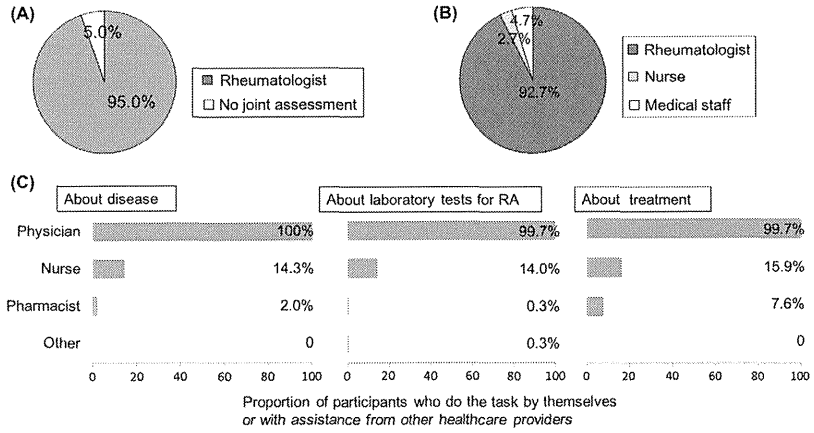



Figure 5. Proportion of participants who (A) assess joints, (B) calculate DAS28 scores, and (C) educate patients with RA in clinical practice by themselves or with assistance from other healthcare providers.





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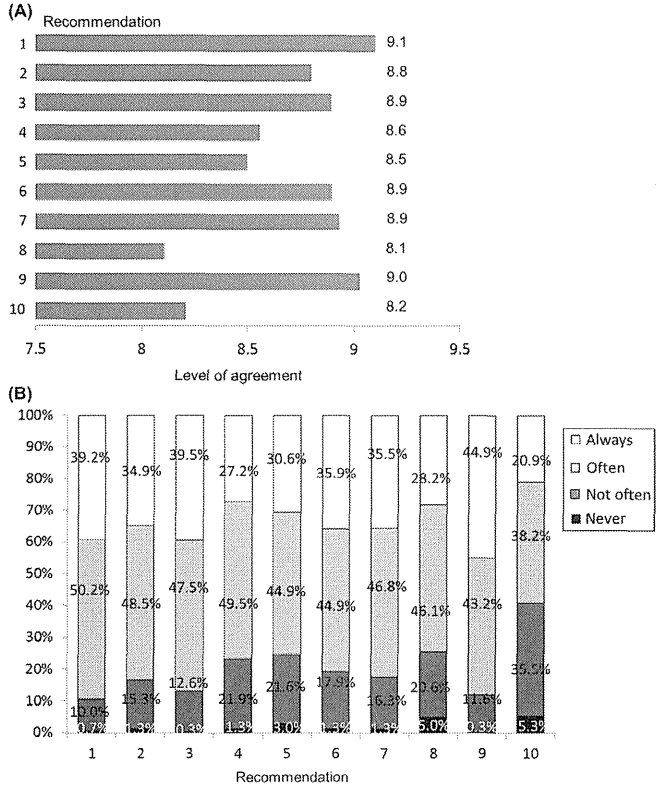
Figure 6. (A) The average level of agreement with and (B) application of the 10 individual T2T recommendations in clinical practice by rheumatologists.




lowest number of responses for "always" and "very often" were for Recommendation 10 (59.1%; Figure 6B). The lowest number of responses for "not very often" and "never" were for Recommendations 1 (10.7%), 3 (12.9%), and 9 (11.9%). There were differences in the results based on indirect questions and those on direct questions on application into clinical practice. The responses for "always" and "very often" regarding Recommendation 1 (clinical remission as a primary target) were 89.4% of participants when directly questioned, which was much higher than the 59.1% when indirectly questioned. A similar trend was observed for Recommendation 7 (consideration of structural damages and functional impairment), that is 82.3% when directly questioned compared to 44.2% (X-ray) or 13.6% (HAQ) when *indirectly questioned*.

## Discussion

Despite the majority of rheumatologists in Japan agreeing with the T2T recommendations for RA, not all implement them properly in clinical practice. This can be attributed to an insufficient understanding of the T2T recommendations by both rheumatologists and patients, a lack of time and support from other healthcare providers during consultations, and patients' passive participation in decision making.

In a similar manner, while the majority rheumatologists agree that they should aim for clinical remission as a primary target of treatment with consideration of structural damages and functional impairment, only 27% of rheumatologists use composite measures appropriately for patients with moderate to high disease activity, and 59% and 22% assess X-ray and HAQ for more than 80% of their patients, respectively. RA cannot be assessed using simple gold standard measures, such as the value of blood pressure when caring for hypertension patients [8], and the complexity of the signs and symptoms requires the application of composite scores [9–11]. However, our results show that physicians tend to interpret the individual variables rather than comprehensive composite measures in clinical practice. This might be partly due to physicians not understanding the T2T recommendations well enough to implement them properly, which could be improved by better educating them on the T2T recommendations. This study also revealed the huge burden on rheumatologists of assessing joints, calculating composite measures, and explaining these aspects of RA to patients. In Japan, patients are free to select rheumatologists or hospitals without any reference, and to see one specified doctor on a regular basis (usually every 1–3 months). Consequently, physicians can only allocate a limited consultation period to each patient and have no time to calculate or explain composite measures. This lack of time is one of the major obstacles in

the implementation of T2T recommendations, and new ways to support physicians should therefore be devised.

Another important finding of this study is the insufficient level of communication between rheumatologists and patients. Nearly half of the rheumatologists involved in this survey answered that a proportion of their patients had a lack of understanding or inability to make decisions regarding their treatment. Further, these rates were much higher than the rheumatologists who referred to the lack of time as the reason why they did not discuss the treatment strategy with all their patients. As a shared decision between patients and rheumatologists is one of the overarching principles of T2T recommendations in RA [5] the next step will be to increase the involvement of patients in the decision making process. The T2T committee has recently launched a worldwide T2T CONNECT project, which is intended to encourage better communication with patients by facilitating intrinsic motivation within patients via Motivational Interviewing method [12] and engaging them in treating RA to target. In order to compensate the shortness of time and to have patients develop understandings, innovative tools that are one of critical elements in the T2T CONNECT project are considered to be helpful. Although some tools such as illustrative leaflets and DAS calculators are now available, more tools should be invented. Moreover, to urge more T2T strategies implementation, we rheumatologists should prove substantial evidence of the benefit of T2T strategies in Japanese patients with RA.

We should note the discrepancy between the results based on indirect questionnaires and the answers to direct questions on components in T2T strategy. It is apparent that not all physicians who answered that they apply T2T recommendations into clinical practice implement them appropriately. Conducting a survey by indirectly questioning the components in T2T strategy may therefore provide a more accurate representation of the actual situation in clinical practice.

There are limitations to consider when interpreting the results of this study. First, as this survey was conducted in Japan the circumstances in clinics are therefore hectic, as mentioned above, and these results may not be representative of other counties. For example, a study from the Netherlands that retrospectively surveyed 100 patients with RA participating in a cohort reported a high availability of DAS28 and successful implementation of the T2T recommendations [13]. A multinational survey with a higher number of participants is therefore warranted. Second, the participants who were contacted through e-mail may not necessarily represent the average rheumatologist. However, we believe from participants' demographic data that they are justifiable enough for analysis. Third, some rheumatologists might agree with T2T strategies but use different evaluative criteria rather than DAS28, SDI, CDAI, or HAQ. These issues are different from an insufficient understanding of T2T strategies, and we could not view them exactly through the questionnaire we used.

In conclusion, while the majority of rheumatologists in Japan agree with the T2T recommendations, there is still a room for improvement in implementation in clinical practice by ensuring that rheumatologists have a sufficient understanding, that support systems allow rheumatologists to implement them more easily, and that patients have better understandings on RA and its treatment strategy and take a greater participation in the decision-making process.

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## Conflict of interest

YK and HO have no competing interest. TK has received research grants from Abbott (Abbvie), Astellas Pharma Inc., Bristol Myers Squibb, Chugai Pharmaceutical Co. Ltd., Eisai Co. Ltd., Mitsubishi Tanabe Pharma, MSD KK, Pfizer Inc. and Takeda Pharmaceutical Co.Ltd., KY has received research grants from Astellas Pharmaceutical, Chugai Pharmaceutical, Eizai Pharmaceutical, Immunofuture Inc, Mitsubishi Tanabe Pharma Corporation, Santen Pharmaceutical and Wyeth. NM has received research grants from Abbott, Astellas Pharmaceutical, Banyu Pharmaceutical, Chugai Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical and Teijin Pharmaceutical. MH has received research grants from Abbott, Astellas Pharma, Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical and UCB Japan. HY has received honorarium for the lecture from AbbVie, Chugai, Daiichi Sankyo, Eisai, Mitsubishi Tanabe, Pfizer, Takeda, Teijin Pharma, and has received research grant from AbbVie, Asahikasei Pharma, Astellasm, Bristol-Myers Squibb, Chugai, Daiichi Sankyo, Eisai, GlaxoSmithKline, Janssen, Mitsubishi Tanabe, MSD, Nippon Kayaku, Pfizer, Santen,Taishotoyama, Takeda, Teijin Pharma. NI has received research grants from Astellas Pharmaceutical, Chugai Pharmaceutical, Eizai Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharma Corporation, Kaken Pharma, Abbott, and Bristol Myers Squibb. YT has received consulting fees, speaking fees, and/or honoraria from Mitsubishi-Tanabe, Eisai, Chugai, Abbott Japan, Astellas, Daiichi-Sankyo, Abbvie, Janssen, Pfizer, Takeda, Astra-Zeneca, Eli Lilly Japan, GlaxoSmithKline, Quintiles, MSD, Asahi-Kasei and has received research grants from Bristol-Myers, Mitsubishi-Tanabe, Abbvie, MSD, Chugai, Astellas, Daiichi-Sankyo. TT has received research grants from Abbott, Astra Zeneca, Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Novartis, Takeda Pharmaceutical and Wyeth.

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## Supplementary material available online

Supplementary Questionnaire.

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CASE REPORT

# Polychondritis presenting with oculomotor and abducens nerve palsies as the initial manifestation

Mitsuhiro Akiyama, Yuko Kaneko, Hironari Hanaoka, Masataka Kuwana and Tsutomu Takeuchi

*Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan*

**Abstract**

We treated a patient with relapsing polychondritis (RP) who presented with intermittent oculomotor and abducens nerve palsies as the first manifestation. Ear swelling and laryngeal edema emerged 7 months later, which led us to diagnose him with RP. Moderate doses of glucocorticoid resolved all symptoms. Our experience with RP accompanied by oculomotor nerve palsy suggests that RP should be considered in patients with cranial nerve palsies so that they may be promptly diagnosed and treated.

**Keywords**

Oculomotor nerve palsy, Abducens nerve palsy, Cranial nerve palsy, Extraocular muscle palsy, Relapsing polychondritis

**History**

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## Introduction

Relapsing polychondritis (RP) is a rare autoimmune disorder in which cartilaginous tissues are the primary targets of destruction, but the immune damage can spread to involve noncartilaginous tissues such as the kidneys, blood vessels, and skin [1]. Although RP is known to present with diverse acute and subacute nervous system complications that may sometimes precede systemic manifestations, cranial neuropathy is an extremely uncommon manifestation [2]. Of the cranial neuropathies, optic neuropathy is reported to occur most frequently, and more than 20 cases have been described [3]. There have also been a few descriptions of patients with abducens, facial, and vestibulocochlear cranial nerve palsies [4]. Moreover, there is a description of a case of trigeminal neuralgia related to RP [5].

We report our experience with a patient who presented with oculomotor and abducens nerve palsies as the first and chief manifestations of RP. Because this was the first case of RP with an oculomotor nerve palsy that we had seen, and it was almost 7 months from the first onset of oculomotor and abducens nerve palsies to the diagnosis of RP, we would like to emphasize that it is important to recognize that RP may cause such manifestations so that rheumatologists can make a timely diagnosis of RP.

## Case report

On 13 February 2013, an 80-year-old man suddenly developed diplopia and ptosis of the left eye without any history of ocular trauma or head injury. He visited an ophthalmologist in our hospital. Neurological examinations showed almost complete third and sixth nerve palsies of the left eye (Figure 1). Pupils were equally round and reactive to light with normal visual acuity. The other cranial nerves were normal. Contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) scans of the patient's brain did not show orbital tumors or meningoencephalitis. Although the cause of these manifestations remained unknown, they resolved completely and spontaneously 3 days later. On 2 March 2013, several painful nodular erythemas appeared on his both arms; a few days later, these lesions had almost completely disappeared. On 17 September 2013, the patient again had diplopia, ptosis of the left eye, and nodular erythema on his arms and legs, but this time he also had a high fever. The patient's right ankle joint was tender and swollen and, at around this time, he found both of his ears to be reddish and painful. He was admitted to another hospital and treated with antibiotics; however, this treatment was not effective. On 30 September 2013, the patient developed laryngeal pain with hoarseness. On 15 October 2013, he was admitted to our hospital.

Physical examination revealed that his temperature was 38.3°C. Neurological examinations showed almost complete third and sixth nerve palsies of the left eye, the same as when he was first seen in February. Both external ears were reddish, swollen, and tender, but the lobes of both ears appeared to have been spared from these manifestations (Figure 2). Several erythematous nodular subcutaneous lesions were on both arms and both legs. His right ankle joint was swollen and tender.

The results of blood and urine tests and a spinal fluid test were tabulated in Table 1. The main results were: 5.400 white blood cells/mm$^3$, 9.6 g/dl hemoglobin, and $304 \times 10^4$ platelets/l. The C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) were elevated, at 140.3 mg/l and 135 mm/h, respectively. Other blood tests, including liver enzyme levels, renal function, blood glucose levels, and total cholesterol, were within the normal ranges. Antinuclear antibody (ANA), rheumatoid factor, anticyclic citrullinated peptide antibody, PR3-ANCA, and MPO-ANCA tests were all negative. Blood cultures did not identify any pathogens. β-D glucan and the Quanti-FERON TB-2G test (QFT) were negative. Urinalysis showed no abnormal findings. The cerebrospinal fluid test was normal.

CT scans revealed thickening and enhancement of the epiglottis and the pyriform fossae (Figure 3a). Indirect laryngoscopy revealed edema and swelling of the supraglottic larynx and epiglottis, consistent with supraglottic laryngitis and epiglottitis (Figure 3b). Skin biopsy of an erythematous nodule on his left leg revealed full-thickness subcutaneous fat infiltration with lymphocytic white blood cells, indicative of panniculitis. A biopsy specimen of the left auricle revealed degenerative changes in the cartilage, but did not show infiltration of plasma cells and lymphocytes into the perichondral area, because the tissue at the border zone between cartilage and connective tissue had been lost during the biopsy process, presumably due to technical issues.

Based on the presence of the typical clinical picture of bilateral auricular chondritis, supraglottic laryngitis, epiglottitis, and recurrent systemic inflammation, as well as on the exclusion of differential diagnoses, such as tuberculosis, bacterial, and fungal infections, we suspected that the patient had RP. He was treated with 40 mg/day prednisolone (0.6 mg/kg/day), and all manifestations improved rapidly. CRP and ESR decreased to normal levels within several days. The prednisolone dosage was gradually reduced without relapse.

## Discussion

Our patient with RP presented with complete oculomotor and abducens nerve palsies that occurred intermittently. Our findings suggest that RP may be an important differential diagnosis for cranial nerve palsies of undetermined origin.

Correspondence to: Mitsuhiro Akiyama, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan. Tel: +81-3-5363-3786. Fax: +81-3-5379-5037. E-mail: hhhirooo@z6.keio.jp







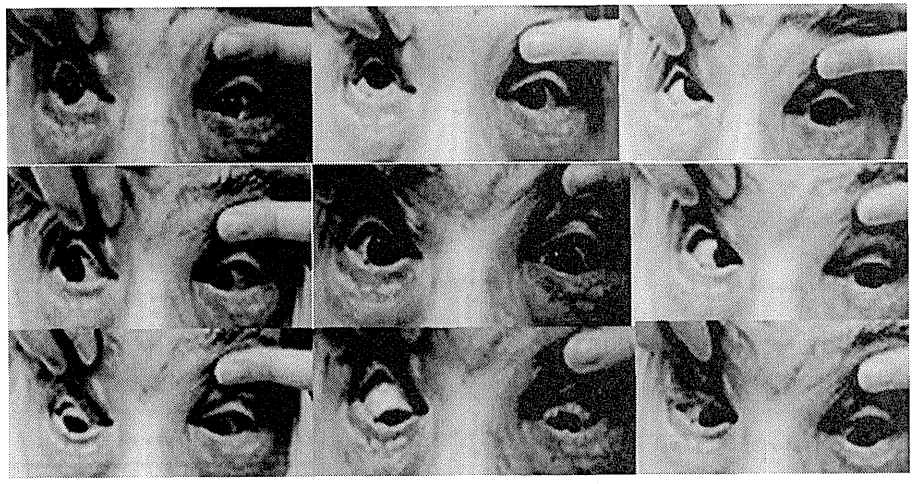

Figure 1. Extraocular movements of the left eye were completely disturbed in all directions.

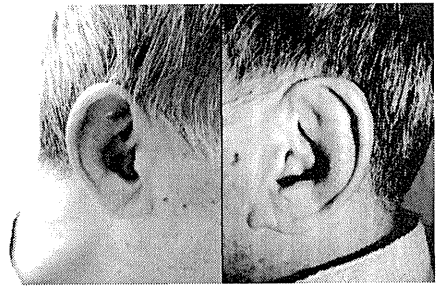

Figure 2. Both ears were reddish and swollen, but the inferior soft lobule, which lacks cartilage, was spared.

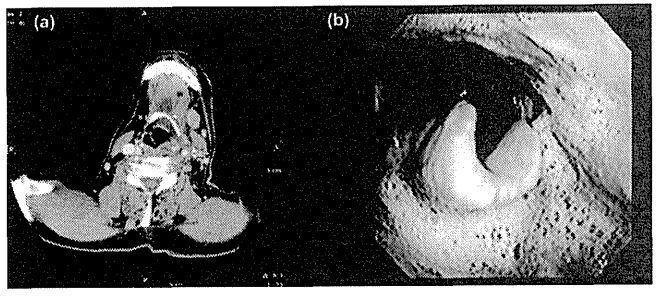


Figure 3. (a) CT scan at the supraglottic level revealed soft tissue thickening and enhancement of the left epiglottic folds and pyriform sinuses (green arrow). (b) Laryngoscope revealed that glottic, laryngeal, and subglottic soft tissues were inflamed.