Figure 3

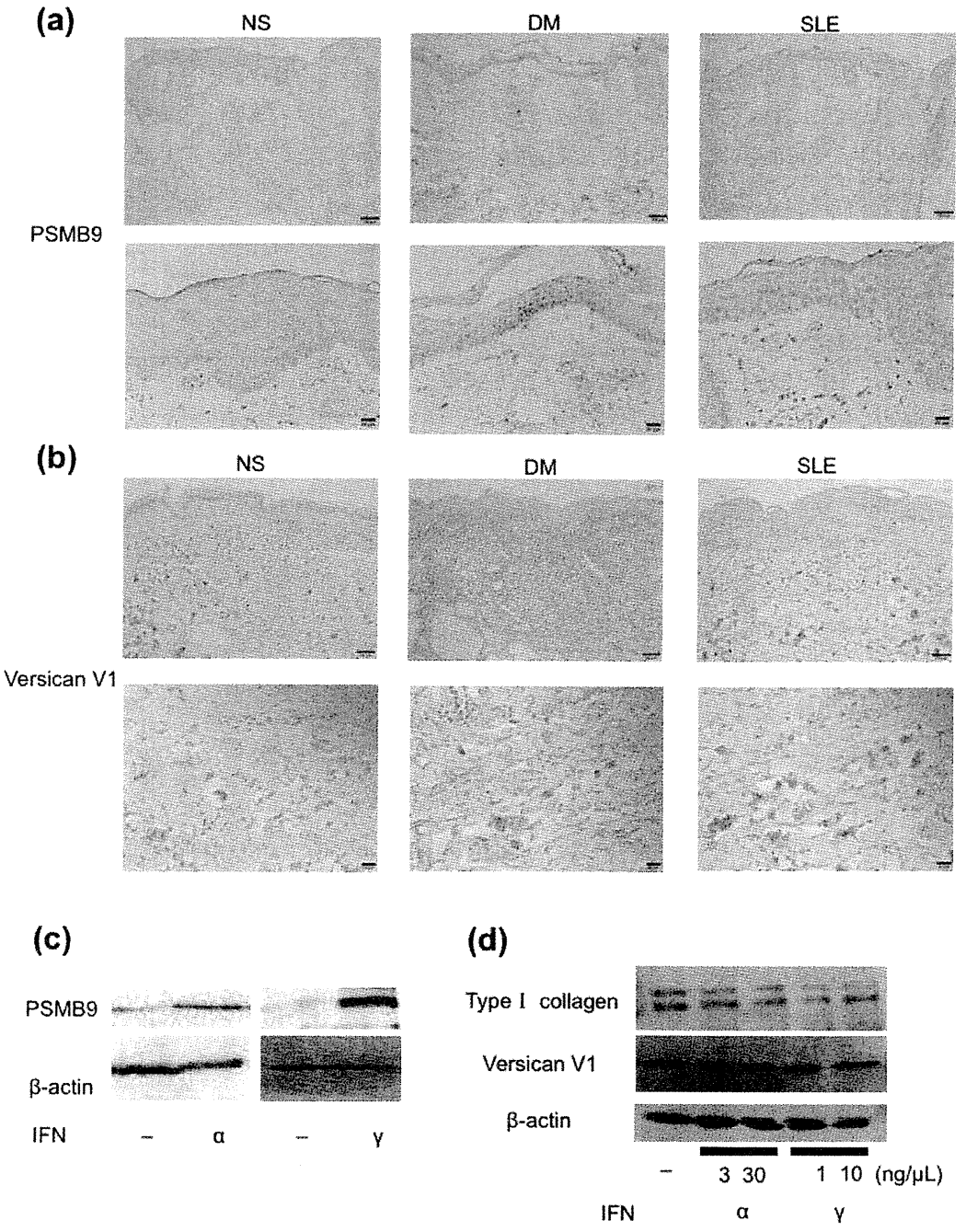


Figure 4

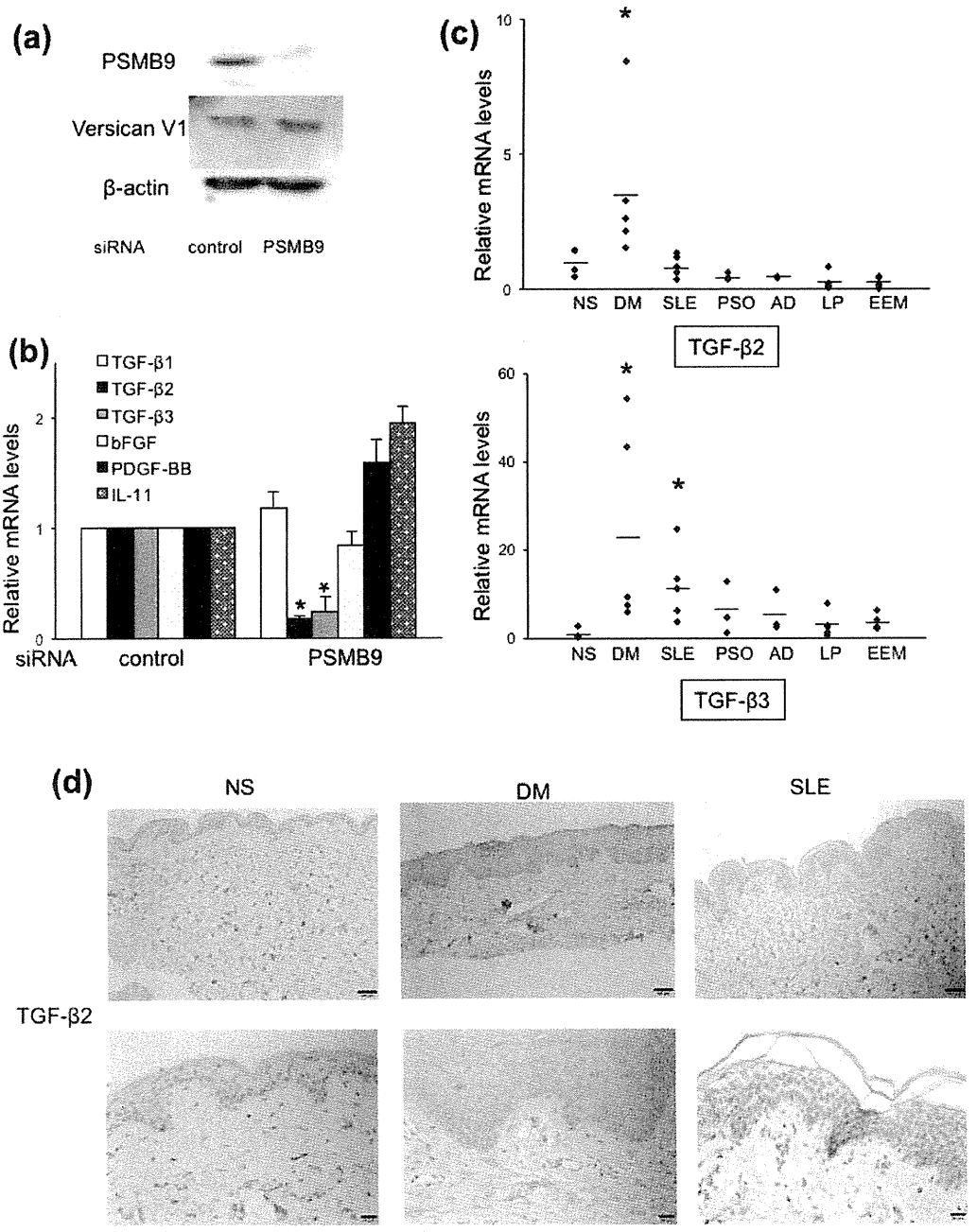


Figure 5

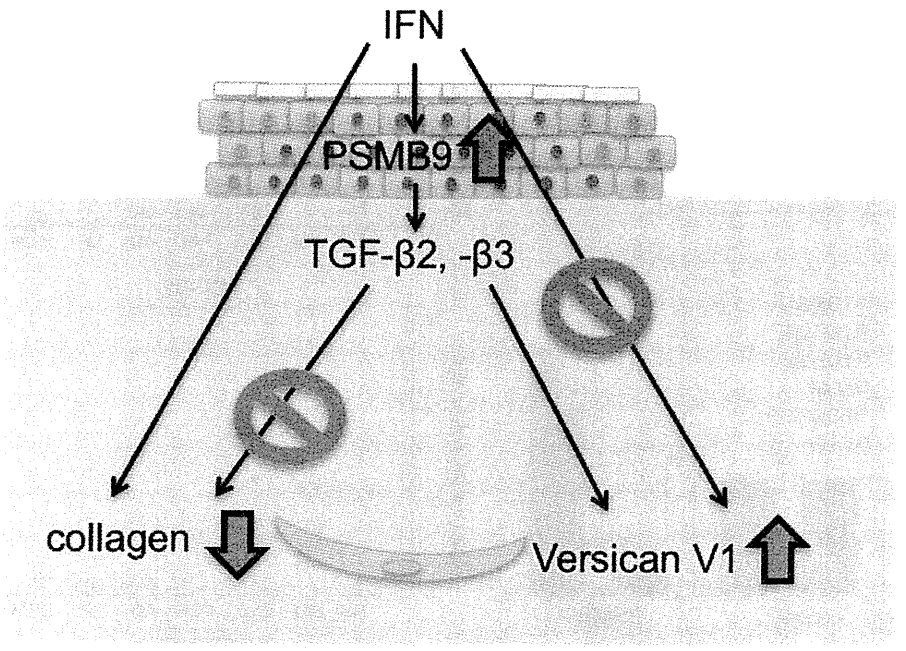
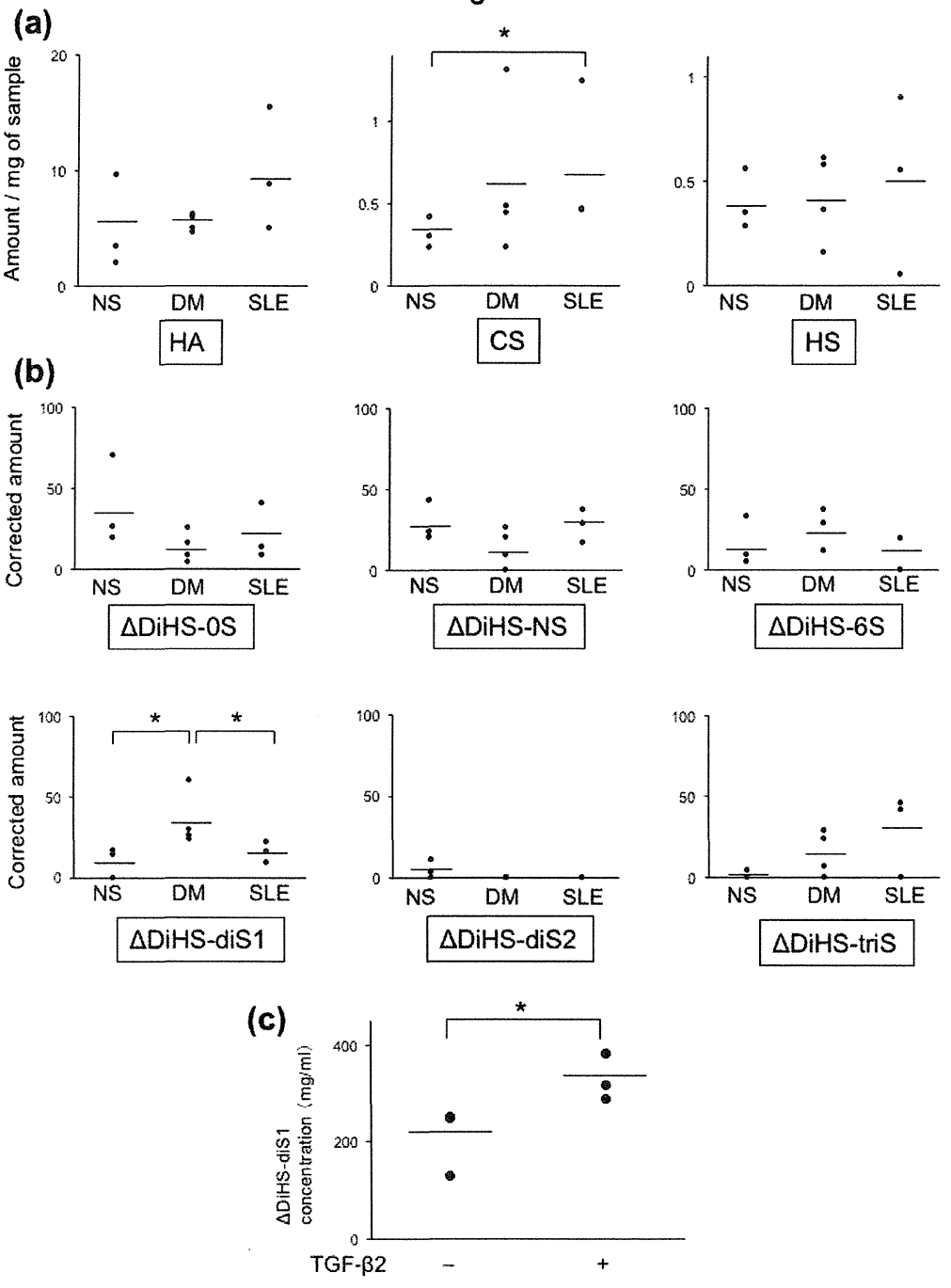


Figure 6



Original Article

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Serum concentrations of Flt-3 ligand in rheumatic diseases

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Summary **Fms-like tyrosine kinase 3 (Flt-3) is a cytokine receptor expressed on the surface of bone-marrow progenitor of hematopoietic cells. Flt-3 ligands are produced by peripheral blood mononuclear cells, and found in various human body fluids. Flt-3 signal is involved in the regulation of vessel formation as well as B cell differentiation, suggesting that Flt-3 signal contributes to the pathogenesis of vascular abnormalities and immune dysregulation in rheumatic diseases. The aim of the present study is to examine serum Flt-3 ligand levels in patients with various rheumatic diseases, and to evaluate the possibility that serum Flt-3 ligand levels can be a useful disease marker. Sera were obtained from 20 dermatomyositis (DM) patients, 36 systemic sclerosis (SSc) patients, 10 systemic lupus erythematosus (SLE) patients, 10 scleroderma spectrum disorder (SSD) patients, 4 mixed connective tissue disease (MCTD) patients, and 12 normal subjects. Flt-3 ligand levels were determined with ELISA. Serum Flt-3 ligand levels were significantly elevated in patients with DM, SSc, SSD and MCTD compared to those in normal subjects. DM patients with elevated Flt-3 ligand levels were accompanied with significantly increased CRP levels and increased frequency of heliotrope rash than those with normal levels. In addition, SSc patients with elevated Flt-3 ligand levels showed significantly reduced frequency of nailfold bleeding. Serum Flt-3 ligand levels can be a marker of cutaneous manifestation in DM and a marker of microangiopathy in SSc. Clarifying the role of Flt-3 ligand in rheumatic diseases may lead to further understanding of these diseases and new therapeutic approaches.**

Keywords: Flt-3, dermatomyositis, systemic sclerosis, systemic lupus erythematosus

1. Introduction

Immune dysfunction and vascular abnormalities are thought to be the common features in rheumatic diseases including rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD) or Sjögren's syndrome (SjS) (*1,2*). For example, autoantibodies, Raynaud's phenomenon, nailfold bleeding (NFB) and skin ulcers are frequently seen in various rheumatic diseases. These vascular abnormalities are characterized by uncontrolled regeneration of the vasculature and vascular losses due to defective maintenance of the vasculature (*3*). However, the mechanism is hardly understood, and needs to be clarified.

Fms-like tyrosine kinase 3 (Flt-3), also known as CD135, is a cytokine receptor expressed on the surface of bone marrow-derived hematopoietic progenitor cells. Activation of the receptor has been reported to regulate vessel formation as well as the blood cell differentiation. For example, Flt-3 signal is thought to play roles in the angiogenesis of malignant tumors, the process of B cell differentiation, immune response to virus infection, and aging (*4-7*). Furthermore, Flt-3 mutations are found in patients with leukemia (*8*). Thus, there is a possibility that Flt-3 signal is involved in the pathogenesis of vascular abnormalities and immune dysregulation in rheumatic diseases as well as similar dysregulation of immunity and angiogenesis such as malignancy, infection and aging.

On the other hand, Flt-3 ligands, approximately 30 kDa transmembrane glycoproteins, are produced

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by peripheral blood mononuclear cells (*9*), and can be found as soluble homodimeric proteins in various human body fluids including serum. So far, several previous studies have suggested the significance of Flt-3 ligand levels in autoimmune arthritis such as RA and primary Sjogren's syndrome: there was a significant correlation between serum Flt-3 ligand levels and disease activity in RA patients (*10,11*). However, the expression of Flt-3 ligand in other rheumatic diseases has yet to be elucidated. Therefore, in this study, we examined serum Flt-3 ligand levels in patients with various rheumatic diseases, and tried to evaluate the possibility that serum levels of Flt-3 ligand can be a useful disease marker.

2. Materials and Methods

2.1. *Clinical assessment and patient material*

Patients with SSc and SLE fulfilled the criteria proposed by the American College of Rheumatology (ACR) (*12,13*). All SSc patients were grouped into diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy *et al.* (*14*). The concept of scleroderma spectrum disorder (SSD) was originally proposed by Maricq *et al.* to unify typical SSc, early forms of SSc and closely related disorders (*15,16*). After that, Ihn *et al.* redefined SSD as patients that did not fulfill the ACR criteria of SSc but were thought to develop SSc in the future, and proposed a new diagnostic method (*17*). Classical DM patients were diagnosed based on the criteria proposed by Bohan and Peter (*18,19*). Patients with clinically and histopathologically typical cutaneous lesions but without myositis were diagnosed as clinically amyopathic DM (CADM) according to the previous criteria (*20*). MCTD patients had clinical features of SLE, SSc and PM/DM and anti-U1RNP antibodies, but did not satisfy the criteria for these connective tissue diseases (*12,13,18,19*), and were diagnosed according to the criteria of Alarcón-Segovia (*21,22*).

Sera were obtained from randomly chosen 15 classical DM patients (age range: 27-86 years, mean ± SD: 54.1 ± 18.4; disease duration range: 1-24 months, mean ± SD: 7.4 ± 7.5), 5 CADM patients (age range: 33-81 years, mean ± SD: 61.4 ± 19.4; disease duration range: 1-108 months, mean ± SD: 31.2 ± 38.5), 36 patients with SSc (13 dcSSc and 23 lcSSc, age range: 24-85 years, mean ± SD: 62.2 ± 14.6; disease duration range: 1-768 months, mean ± SD: 75.0 ± 151.7), 10 SSD patients (age range: 44-75 years, mean ± SD: 54.9 ± 10.7; disease duration range: 1-120 months, mean ± SD: 27.9 ± 40.1), 10 SLE patients (age range: 23-57 years, mean ± SD: 35.0 ± 11.9; disease duration range: 2-84 months, mean ± SD: 21.3 ± 27.6) and 4 MCTD patients (age range: 44-73 years, mean ± SD: 59.8 ± 12.6; disease duration range: 24-228 months, mean ± SD: 123.0 ± 83.4). Clinical and laboratory data reported in this study were obtained at the time of serum sampling. None of the patients received systemic treatments such as steroid or immunosuppressant at the serum sampling.

Control serum samples were also collected from 12 normal subjects (age range = 32-81 years, mean ± SD: 47.1 ± 16.4). All serum samples were stored at – 80°C prior to use. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki.

2.2. *Nailfold bleeding*

Nailfold bleeding (NFB) was evaluated macroscopically by the presence of splinter hemorrhages of the nailfold capillaries in more than one finger by 2 investigators in a blinded manner.

2.3. *Serum concentrations of Flt-3 ligand*

Levels of serum Flt-3 ligand were measured with a specific ELISA kit (R&D Systems, Minneapolis, MN, USA) (*23*). Briefly, anti-Flt-3 ligand monoclonal antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by the addition of peroxidase-conjugated antibodies to Flt-3 ligand. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase, and the absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 540 nm. The concentration of Flt-3 ligand in each sample was determined by interpolation from a standard curve.

2.4. *Statistical analysis*

Statistical analysis was carried out with Mann-Whitney U test for the comparison of medians, and Fisher's exact probability test for the analysis of frequency. p values less than 0.05 were considered significant. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of Flt-3 ligand levels using GraphPad Prism ver. 5.01 (MDF software, Tokyo, Japan).

3. Results

3.1. *Serum concentrations of Flt-3 ligand in patients with various rheumatic diseases*

The serum Flt-3 ligand levels in patients with various rheumatic diseases are shown in Figure 1. Serum samples were obtained from 20 DM patients (including 5 CADM patients), 36 SSc patients, 10 SSD patients, 10 SLE patients, 4 MCTD patients, and 12 normal

subjects. SSD patients did not fulfill the criteria of SSc but were thought to develop SSc in the future, while CADM patients had typical cutaneous lesions of DM but lacked myositis (*15-17,20*).

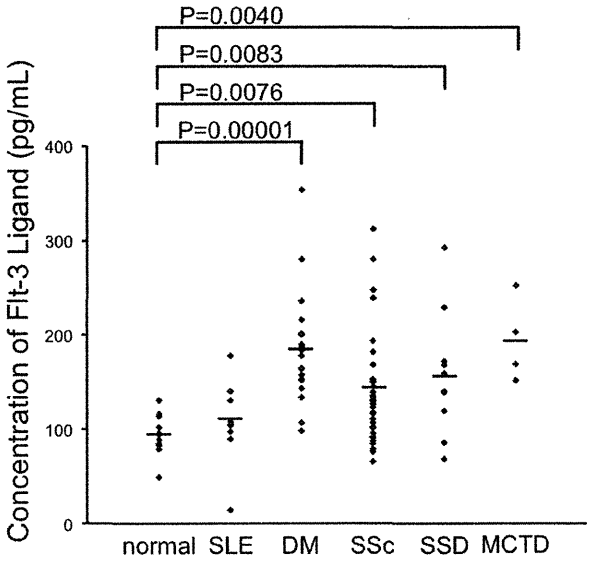

Figure 1. The serum levels of Flt-3 ligand in rheumatic diseases. Levels of Flt-3 ligand in sera of patients with systemic lupus erythematosus (SLE, n = 10), dermatomyositis (DM, n = 20), systemic sclerosis (SSc, n = 36), scleroderma spectrum disorder (SSD, n = 10), mixed connective tissue disease (MCTD, n = 4) and normal subjects (n = 12) were analyzed with ELISA. Bars show the means.

The mean age of normal subjects was 47.1 years, and the ratio of female was 75.0%. Summary of clinical/laboratory features in patients with these rheumatic diseases enrolled in this study are shown in Tables and Supplemental Tables. For example, in DM patients, the mean age at the serum sampling was 56.0 years, and mean CK levels were 2,400.8 IU/L (normal values; 57-284 IU/L for male and 45-176 IU/L for female) (Table 1). Among the rheumatic diseases, DM patients had elevated serum Flt-3 ligand concentration (mean ± SD: 185.1 ± 57.4 pg/mL), and there was a statistically significant difference in the values between DM patients and normal subjects (94.4 ± 21.0 pg/mL, p = 0.000011, Figure 1). According to the manufacturer's data, mean normal serum Flt-3 ligand level was 93.9 pg/mL, which is consistent with our results. In ROC curve analysis for serum Flt-3 ligand levels to distinguish DM patients from normal subjects, the area under the curve (AUC) was 0.9708 (95% confidence interval; 0.9219-1.020, Figure 2A): AUC more than 0.9 means that the level of Flt-3 ligand is useful for diagnosis of DM patients. When the most optical cut-off point was set at 132.8 pg/mL according to the Youden index (0.90), the sensitivity was 90.0% and the specificity was 100.0%. There were no significant differences between patients with classical DM and CADM (189.9 ± 52.4 *vs.* 170.8 ± 55.0 pg/mL, p = 0.51, Figure 2B).

Levels of serum Flt-3 ligand also showed significant elevation in patients with SSc compared to in normal

Table 1. Correlation of serum Flt-3 ligand levels with clinical/serological features in patients with dermatomyositis

Items	All patients (n = 20)	Serum Flt-3 ligand	
		Patients with elevated Flt-3 ligand levels (n = 10)	Patients with normal Flt-3 ligand levels (n = 10)
Age at the time of serum sampling (mean years)	56.0	61.9	50.0
Gender (ratio of female, %)	60.0	50.0	70.0
Duration of disease (mean months)	13.4	6.3	20.3
Type (DM:CADM)	15:5	8:2	7:3
Clinical features			
Gottron's sign (%)	83.3	100.0	70.0
Heliotrope rash (%)	75.0	100.0*	50.0
Laboratory features			
IgG (mg/dL)	1379.2	1365.4	1392.9
CK (IU/L)	2400.8	2155.2	2646.4
Myoglobin (ng/mL)	694.6	602.6	786.7
Aldolase (U/L)	37.0	21.6	50.7
CRP (mg/dL)	0.83	1.48*	0.19
Organ involvement			
Muscle weakness (%)	76.5	87.5	66.7
Interstitial kung disease (%)	35.3	42.6	30.0
Dysphasia (%)	30.8	40.0	20.0
Joint (%)	28.6	50.0	20.0
ANA specificity			
ANA (+)	50.0	70.0	30.0
(levels, index)	48.6	67.3	20.5
Anti-Jo-1 (%)	10.0	10.0	10.0
(levels, IU/mL)	1.68	1.74	1.61

The cut-off vale was set at the mean + 4SD of the value in healthy control subjects. DM, dermatomyositis; CADM, clinicallyamyopathic dermatomyositis; CK, creatin kinase; ANA, antinuclear antibodies; Anti-Jo-1, Anti-Jo-1 antibodies. * $p < 0.05$, versus patients with normal Flt-3 ligand levels using Fisher's exact probability test.

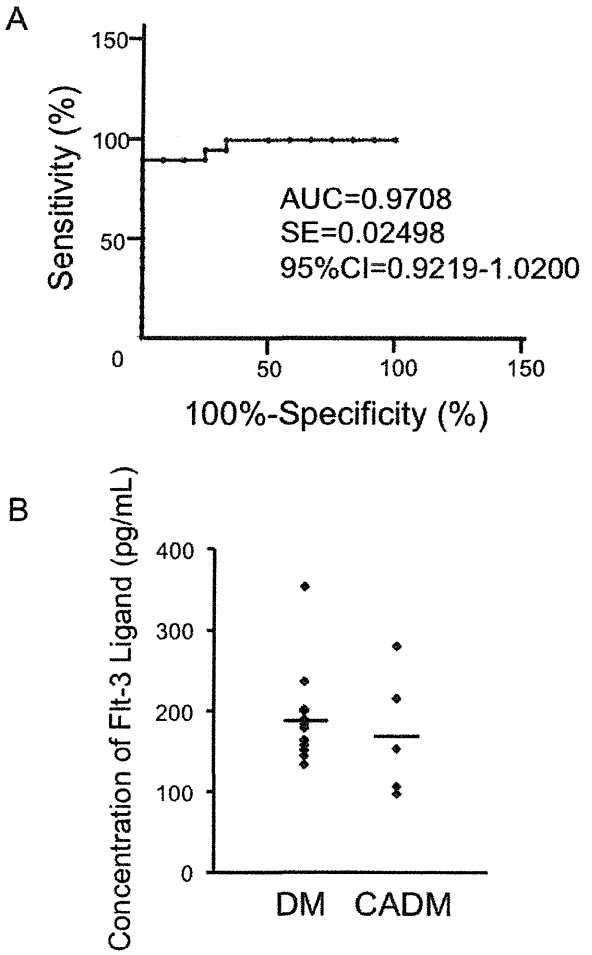

Figure 2. The serum levels of Flt-3 ligand in dermatomyositis. (A) Receiver operating characteristic (ROC) curve for serum Flt-3 ligand to distinguish dermatomyositis (DM) patients from normal subjects. AUC; areas under curves, SE; standard error, CI; confidence interval. **(B)** Levels of Flt-3 ligand in sera of patients with classical DM (n = 15) and clinically amyopathic DM (CADM, n = 5) were analyzed with ELISA. Bars show the means.

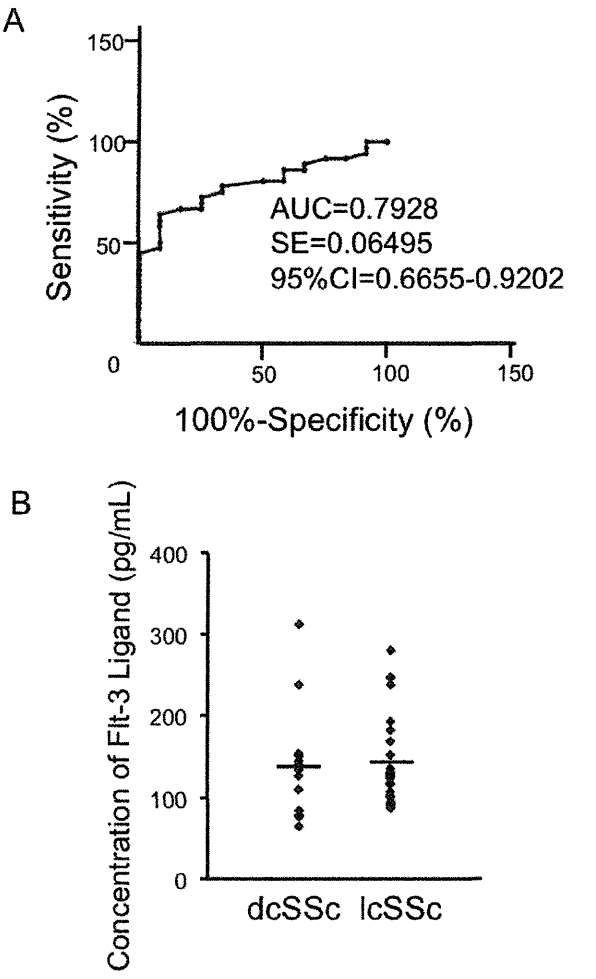

Figure 3. The serum levels of Flt-3 ligand in systemic sclerosis. (A) Receiver operating characteristic (ROC) curve for serum Flt-3 ligand to distinguish systemic sclerosis (SSc) patients from normal subjects. AUC; areas under curves, SE; standard error, CI; confidence interval. **(B)** Levels of Flt-3 ligand in sera of patients with diffuse cutaneous SSc (dcSSc, n = 13) and limited cutaneous SSc (lcSSc, n = 23) were analyzed with ELISA. Bars show the means.

subjects (144.7 ± 60.9 *vs.* 94.4 ± 21.0 pg/mL, p = 0.0026). In ROC curve analysis for serum Flt-3 ligand levels to distinguish SSc patients from normal subjects, AUC was 0.7928 (95% confidence interval; 0.6655-0.9202, Figure 3A): AUC more than 0.7 indicates sufficient diagnostic performance of serum Flt-3 ligand levels. When the most optical cut-off point was set at 114.8 pg/mL by the Youden index (0.56), the sensitivity and specificity were 63.9% and 91.7%, respectively. We could not find any significant differences in Flt-3 ligand levels between dcSSc patients and lcSSc patients (139.8 ± 69.1 *vs.* 147.6 ± 57.3 pg/mL, p = 0.72, Figure 3B).

Patients with SSD and MCTD also showed significantly higher serum Flt-3 ligand levels than normal subjects (157.7 ± 65.9 *vs.* 94.4 ± 21.0 pg/mL, p = 0.0083 and 194.3 ± 44.1 vs. 94.4 ± 21.0 pg/mL, p = 0.0040, respectively) (Supplemental Tables 1 and 2, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=5*). On the other hand, serum Flt-3 ligand levels were slightly higher in patients with SLE compared with normal subjects, but the difference was not statistically significant (111.0 ± 43.3 *vs.* 94.4 ±21.0 pg/mL, p = 0.25) (Supplemental Table 3, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=5*).

Taken together, significant increases of serum Flt-3 ligand levels were found in patients with DM, SSc, SSD, and MCTD. When the cut-off value was set at the mean + 2SD of Flt-3 ligand levels in normal subjects (136.4 pg/mL), all the values in normal subjects were below the cut-off. On the other hand, increased serum Flt-3 ligand levels were found in 17 of the 20 DM patients (85.0%), 14 of the 36 SSc patients (38.9%), 7 of the 10 SSD patients (70.0%), all of the 4 MCTD patients (100.0%) and 3 of the 10 SLE patients (30.0%). Furthermore, when the cut-off was set at mean + 4SD of values in normal subjects (178.5 pg/mL), increased serum Flt-3 ligand levels were seen in 10 of the 20 DM patients (50.0%), 8 of the 36 SSc patients (22.2%), 2 of the 10 SSD patients (20.0%), and 2 of the 4 MCTD patients (50.0%).

Table 2. Correlation of serum Flt-3 ligand levels with clinical/serological features in patients with systemic sclerosis

Items	All patients (n = 36)	Serum Flt-3 ligand	
		Patients with elevated Flt-3 ligand levels (n = 14)	Patients with normal Flt-3 ligand levels (n = 22)
Age at the time of serum sampling (mean years)	62.2	64.9	60.3
Gender (ratio of female, %)	77.7	75.0	77.2
Duration of disease (mean months)	75.0	58.2	86.6
Type (diffuse:limited)	13:23	6:8	7:15
m-TSS	11.0	11.6	10.5
Clinical features			
Pitting scars (%)	43.8	50.0	40.0
Ulcers (%)	32.0	20.0	40.0
Nailfold bleeding (%)	48.7	26.7*	63.6
Raynaud's phenomenon (%)	90.3	100.0	84.2
Telangiectasia (%)	23.1	18.2	26.7
Contracture of phalanges (%)	85.7	100.0	77.8
Calcinosis (%)	5.9	0.0	10.0
Diffuse pigmentation (%)	28.6	42.9	21.4
Short SF (%)	75.0	85.7	70.6
Sicca symptoms (%)	53.9	45.5	60.0
Laboratory features			
CRP (mg/dL)	0.75	0.95	0.57
Organ involvement			
Pulmonary fibrosis (%)	37.1	35.7	38.1
Mean %VC (%)	102.9	100.0	104.9
Mean %DLCO (%)	87.0	85.1	88.4
Pulmonary hypertension (%)	28.1	27.0	29.0
Esophagus (%)	20.6	35.7	10.0
Heart (%)	29.4	28.6	30.0
Kidney (%)	0.0	0.0	0.0
Joint (%)	57.9	71.4	50.0
Thrombosis (%)	0.0	0.0	0.0
ANA specificity			
Anti-topo I (%)	27.8	21.4	31.8
(levels, IU/mL)	36.7	33.8	39.6
Anti-centromere (%)	38.9	50.0	31.8
(levels, IU/mL)	74.5	73.0	76.1
Anti-U1 RNP (%)	16.7	21.4	13.6
(levels, IU/mL)	10.4	12.9	7.9
Anti-SS-A (%)	30.6	57.1	13.6
(levels, IU/mL)	28.0	22.6	32.3

The cut-off vale was set at the mean + 2SD of the value in healthy control subjects. m-TSS, modified Rodnan's total skin thickness score; SF, sublingual frenulum; VC, vital capacity; DLco, diffusion capacity for carbon monooxidase; ANA, antinuclear antibodies; Anti-topo I, anti-topoisomerase I antibody; Anti-centromere, anti-centromere antibodies; Anti-U1 RNP, anti-U1 RNP antibodies; Anti-SS-A, anti-SS-A antibodies. *$p < 0.05$ versus patients with normal Flt-3 ligand levels using Fisher's exact probability test.

3.2. *Correlation of serum Flt-3 ligand levels with clinical manifestations and laboratory data in patients with DM or SSc*

Then we examined correlation of serum Flt-3 ligand levels with clinical and serological features of patients with DM or SSc. As shown in Table 1, when Flt-3 ligand levels > mean + 4SD of the values in normal subjects (178.5 pg/mL) were regarded as elevated, DM patients with elevated Flt-3 ligand levels tended to be accompanied with heliotrope rash at a significantly higher prevalence than those with normal levels (100.0% *vs.* 50.0%, $p = 0.029$). Furthermore, C-reactive protein (CRP) levels (normal values; < 0.3 mg/dL) in DM patients with elevated Flt-3 ligand levels were significantly higher than in those without (1.48 *vs.* 0.19 mg/dL, $p = 0.019$). We could not find any significant differences between patients with and without elevated Flt-3 ligand levels in terms of the frequency of Gottron's sign, the levels of muscle enzymes, or the positive rates and the levels of antinuclear antibody (ANA) and Jo-1 antibody. Duration of disease (between the onset of symptoms and the first hospital visit) was slightly shorter in patients with elevated Flt-3 ligand levels, but not statistically significant.

On the other hand, when SSc patients were divided into two groups according to the cut-off value (136.4 pg/dL, mean + 2SD of normal subjects), patients with elevated Flt-3 ligand levels showed a significantly reduced prevalence of NFB as compared to those with normal levels (26.7 *vs.* 63.6%, $p = 0.027$, Table 2). There were no significant differences between patients with elevated Flt-3 ligand levels and those with normal levels in the frequency of major organ involvements

or other vascular involvements including Raynaud's phenomenon, thrombosis, pitting scar or telangiectasia. Furthermore, CRP levels were not significantly different between the two groups. The positive rates and the levels of antibodies against topoisomerase I, centromere, U1 RNP or SS-A were not significantly different between patients with and without elevated Flt-3 ligand levels. Duration of disease was shorter in patients with elevated Flt-3 ligand levels, albeit insignificant.

4. Discussion

We demonstrated several novel findings in this study. First, levels of serum Flt-3 ligand were significantly elevated in patients with DM, SSc, SSD, and MCTD compared to normal subjects. Second, SSc patients with elevated Flt-3 ligand levels showed significantly reduced prevalence of NFB compared to those with normal levels. And lastly, in DM patients, those with elevated Flt-3 ligand levels were accompanied with significantly increased CRP levels and increased frequency of heliotrope rash than those with normal levels.

NFB is one of the earliest vascular events in SSc, and the main factor of the diagnostic criteria for SSD. Although we also performed capillaroscopy on several SSc patients with or without increased serum Flt-3 ligand levels, we could not find any significant differences in the frequency of capillaroscopic findings including bleeding, dilated loops and giant loops between the two groups, probably because the number of patients determined was limited and insufficient to allow statistically based conclusions. Considering the significant elevation of Flt-3 ligand levels in SSD patients and the reduced frequency of NFB in SSc patients with elevated Flt-3 ligand levels, there is a possibility that Flt-3 ligand may start to elevate at the prodromal stage of SSc, and have suppressive effect on NFB. Increased Flt-3 ligand levels may activate Flt-3 signal, and may stimulate vascular formation to repair vascular abnormalities of NFB in these patients.

Although patients in this study were randomly chosen, DM patients showed high CK levels (2,400.8 IU/L) and high CRP levels (0.83 mg/dL) on average. Thus, one of the reasons why DM patients showed the elevated serum Flt-3 ligand values is possibly due to their high disease activities at the sampling point. Higher CRP levels and a higher frequency of heliotrope rash in DM patients with elevated Flt-3 ligand levels indicated that an abnormal increase of Flt-3 ligand may be correlated with the disease activity and the formation of the skin lesions. Crowson *et al.* previously reported that skin lesions of DM showed more severe endothelial injury, telangiectasia, and deposition of fibrin or C5b-9 on vessels than those of lupus erythematosus, whereas the density of superficial vascular plexus was lower in lesions of DM compared to lupus erythematosus (*24*). Several investigators also have demonstrated a marked reduction of capillary number in the involved muscles of DM (*25,26*). These results indicate that vascular abnormalities are present in DM, and play a role in the pathogenesis of skin lesions. Given that Flt-3 signals may be mediated in the vessel formation process, and that uncontrolled activation of angiogenic factors rather than their inactivation is thought to cause vascular abnormalities in rheumatic diseases (*27,28*). The excessive expression of Flt-3 ligands in DM may also induce dysregulation of the vessel formation, resulting in the formation of heliotrope rash. Consistently, MCTD patients included in this study were accompanied with heliotrope rash, and showed the highest Flt-3 ligand levels among rheumatic diseases, although we could not determine ROC curve analysis or the association with clinical/laboratory findings due to the small number of the patients. On the other hand, although SLE patients are sometimes accompanied with a variety of microangiopathy phenotypes, weaker changes of vessels in SLE than in DM as described above may explain the lower serum Flt-3 ligand levels in SLE.

Taken together, our results suggested that Flt-3 signal may play a different role in each rheumatic disease. Telangiectasia and endothelial injury are commonly observed both in skin lesions of DM including heliotrope rash and in the microangiopathy such as NFB (*24,29*), which may be associated with increased Flt-3 ligand levels in patients with DM or SSc. However, there are also several differences between these two abnormalities (*e.g.* distribution or the presence of bleeding). Further researches on the similarities and the dissimilarities are needed in the future.

On the other hand, we had expected that the association of serum Flt-3 ligand levels and immunodysfunction in patients with DM or SSc, but the positive rates and the levels of ANA or other antibodies were not significantly different between patients with and without elevated serum Flt-3 ligand levels.

As the limitation of this study, because the onset ages of SSc, DM, SLE, and MCTD were usually different (*30,31*), the patients were not age-matched. There was no correlation between serum Flt-3 ligand levels and the age of individuals included in this study ($r = 0.399$). Furthermore, Kinn *et al.* have reported that the expression levels of Flt-3 ligand in healthy skin did not show any significant correlation with aging (*7*), and Metcalf *et al.* have suggested that Flt-3 ligand levels in peripheral blood mononuclear cells were similar between adult people and old people (*32*). Thus, the direct correlation between aging and serum Flt-3 ligand levels is not obvious. However, the mean ages of both SSc and DM patients were higher than that of SLE patients, and the levels of Flt-3 ligand in SSc and DM

were higher than those in SLE. Additionally, the mean age of DM or SSc patients with elevated Flt-3 ligand levels tended to be older than the counterpart. Because normal aging is usually associated with minimal/ chronic inflammation coined by inflammaging, there is a possibility that the difference of Flt-3 ligand levels can be explained by aging factor. Future studies to measure circulating levels of Flt-3 or Flt-3 ligand in normal aging as well as malignancy or viral infection are needed. On the other hand, albeit insignificant, disease duration of the elevated Flt-3 ligand groups in both SSc and DM patients was shorter than the counterpart. This result suggests that these patients may have more severe symptoms. These points should also be clarified in the future by larger studies.

In conclusion, serum Flt-3 ligand levels may be a marker of cutaneous manifestations in DM patients and a marker of microangiopathy in SSc patients. Clarifying the role of Flt-3 ligand in rheumatic disease by systematic approach is required for further understanding of the diseases and new therapeutic approaches.

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ORIGINAL ARTICLE

Enhanced CCR9 expression levels in psoriatic skin are associated with poor clinical outcome to infliximab treatment

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ABSTRACT

Infliximab is an anti-tumor necrosis factor (TNF)-α antibody drug that suppresses TNF-α and its associated inflammatory responses. Although infliximab therapy generally results in a 75% or greater improvement in the Psoriasis Area and Severity Index from baseline in psoriasis patients, there is the heterogeneity of therapeutic efficacy in psoriasis patients among patients of a similar PASI baseline score. However, there are few published reports about the predictors of the clinical response among psoriasis patients who undergo biologic therapies. We thus evaluated the possible existence of biologic markers that would indicate poor prognosis of infliximab using skin biopsy specimens. This was because we assumed that the inhibitors for upregulated chemokine/chemokine receptors in non-responders may have the ability to reduce the occurrence of psoriatic eruptions. PCR array analyses identified that the levels of various chemokines and chemokine receptors were increased in non-responders in comparison to responders. Immunohistochemical analyses revealed that upregulation of the CCR9 protein levels was not associated with the pretherapeutic PASI score, but with poor response to infliximab. Our results indicated that the expression levels of CCR9 in lesional skin may be a useful biologic marker of the clinical efficacy of infliximab therapy in psoriasis patients.

Key words: CCR9, chemokine, clinical outcome, infliximab, psoriasis.

INTRODUCTION

The clinical manifestation of psoriasis is sharply marginated erythema with silver scaling. These symptoms reflect epidermal hyperplasia and the infiltration of inflammatory cells. The pathogenesis of psoriasis involves the upregulation of several cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-17 and IL-22 in inflammatory cells.[1] TNF-α targeted therapy suppresses multiple pro-inflammatory pathways including those involving TNF-α and T-helper (Th)17 responses.[2]

Chemokines constitute a family of small cytokines and signaling proteins that direct the movement of circulating leukocytes.[3] Several chemokines have attracted attention with respect to their potential roles in the pathogenesis of psoriasis.[4] For example, CC chemokine ligand (CCL)5, chemokine (C-X-C motif) ligand (CXCL)9, CXCL10 and CXCL11 are upregulated in psoriatic skin lesions,[5–8] while CC chemokine receptor (CCR)6 and CCL20 induce IL-23-mediated psoriasiform dermatitis in mice.[9,10]

Treatments with biologic therapies often achieve dramatic improvement in the Psoriasis Area and Severity Index (PASI) in patients with psoriasis. Approximately 70% patients treated with biologic therapies experience at least 75% improvement from their baseline PASI (PASI75).[11,12] However, the beneficial clinical effects are not achieved in all patients and the severity of PASI does not predict a lack of response; that is to say there is heterogeneity in the clinical responses to biologic therapies. Furthermore, biologic therapies are expensive and occasionally cause severe adverse events.[13,14] Thus, there exists a need to investigate the markers that are useful for predicting the effect of biologic therapies in psoriasis patients.

We previously discovered that the mRNA levels of CCL22, CCL24, CCRL2, GPR17 and CCR7 were upregulated in the responders (with a fold change of >100) using a polymerase chain reaction (PCR) array.[15] We also revealed that increased levels of CCL22 expression in lesional skin predict good prognosis.[15] No reports have indicated an association between chemokines or chemokine receptors and poor response to biologic therapies in psoriasis patients. Moreover, if upregulated chemokines and chemokine receptors were found in non-responders, then their inhibitors may have the ability to improve psoriatic eruptions in patients with infliximab resistance. The aim of this study was to evaluate the correlation between the expression levels of chemokines/chemokine receptors and the treatment efficacy of infliximab.

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METHODS

Patients

The present retrospective study recruited consecutive patients with psoriasis vulgaris or psoriatic arthritis who received infliximab therapy at Kumamoto University Hospital between March 2010 and November 2014. The eligible patients met all of the following criteria: a histological diagnosis of psoriasis; a PASI of 8 or more; no organ disorder; and the availability of sufficient tissue in paraffin blocks for assessment by PCR array and immunohistochemistry. Although the rule of tens is known as an inception criterion in biologic therapy,[16] patients with a PASI of 8 or more (rather than ≥10) were assessed to provide the investigation with a greater number of specimens. Institutional review board approval and written informed consent for this study were obtained according to the Declaration of Helsinki. Skin tissue samples were obtained from 27 patients with psoriasis before infliximab treatment. Infliximab (5 mg/kg) was i.v. administrated at weeks 0, 2, 6 and every 8 weeks thereafter. Immediately after removal, the skin samples were fixed using formalin and embedded in paraffin.

RNA isolation from tissue, and the PCR array

RNA was extracted from the paraffin slices of the skin samples using an RNeasy FFPE kit (Qiagen, Valencia, CA, USA) and reverse transcribed into first-strand cDNA using an RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA). For the PCR array, the cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix, and the mixture was added to 96-well plates with the Chemokines & Receptors PCR array (PAHS-022Z; SABiosciences). PCR was performed on a Takara Thermal Cycler Dice instrument (TP800®; Takara, Kyoto, Japan).

Immunohistochemical analysis

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigens were retrieved via incubation with citrate buffer solution (pH 6) for 20 min at 95°C. Immunohistochemistry was performed with CCR9 antibodies (1:200) (Abcam, Cambridge, UK), as previously described.[17]

Statistical analysis

The statistical analyses were performed using the Mann–Whitney *U*-test for comparisons of medians and Fisher's exact probability test for analyses of frequency. Correlations were assessed according to Pearson's correlation coefficient. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Patient characteristics

A total of 27 patients fulfilled the above eligibility criteria. Most patients (81.5%) were male. The median patient age was 51.2 years (range, 27–83) at baseline. We next evaluated the therapeutic response to treatment at 22 weeks from baseline. Psoriasis patients were categorized as either responders, in whom PASI75 was achieved, or non-responders, in whom PASI75 was not achieved (Table 1). Among the 27 patients who underwent infliximab therapy, five patients (18.5%) were non-responders. There were no statistically significant differences in the clinical findings (sex, age, disease duration, pretherapeutic PASI, presence of arthritis, bodyweight or body mass index) between responders and non-responders.

Upregulated chemokines and chemokine receptors in the non-responders

At first, to determine the RNA that can be used as predictors of the refractory psoriasis, we performed PCR array analysis of chemokine/chemokine receptor. A mixture was prepared from equal amounts of cDNA obtained from the sections of three responders and three non-responders, and the cDNA expression profile of each group was evaluated using a PCR array. Several RNA were found to be upregulated in the non-responders in comparison to the responders (Table 2). Among these markers, CCR9 mRNA drew our attention because it was the most overexpressed gene in the non-responders in comparison to responders (13.19-cycle difference; 9345.14-fold change according to the ΔΔCt method). There are no reports about the role of CCR9 in dermatological diseases including psoriasis. However, CCR9 receives a lot of attention in regard to the pathogenesis of autoimmune diseases including Crohn's disease (CD)[18,19] and rheumatoid arthritis (RA).[20,21] Furthermore, clinical trials of CCR9 inhibitors have been initiated in patients with CD.[22]

Enhanced expression levels of CCR9 protein in psoriatic skin correlate with a poor response to infliximab

In order to investigate the expression of CCR9 protein, paraffin-embedded sections, which were obtained from the skin of both responders and non-responders, were stained for anti-CCR9 antibodies. The typical immunostaining intensity of CCR9-stained dermal tissues is shown in Figure 1. The CCR9-stained

Table 1. Correlations between the clinical manifestations of the patients with psoriasis treated with infliximab (5 mg/kg)

	Responder (n = 22)	Non-responder (n = 5)	P
Sex (male/female)	17:5	4:1	0.894
Age (years), mean ± SD	50.9 ± 14.1	52.4 ± 9.4	0.596
Disease duration (years), mean ± SD	9.4 ± 6.5	9.3 ± 12.7	0.382
PASI before infliximab, mean ± SD	22.1 ± 12.0	21.0 ± 12.5	0.157
Arthritis (%)	36.4	40.0	0.879

A responder was defined as a patient who achieved at least a 75% improvement in Psoriasis Area and Severity Index (PASI) at 22 weeks from baseline. SD, standard deviation.

tissues were categorized according to their immunoreactivity ([−]–[+++]).

We examined the strength of the correlation between protein immunoreactivity and therapeutic efficacy. The CCR9 protein expression levels in psoriatic skin were negatively correlated with PASI improvement ($R = -0.571$, $P = 0.002$) (Fig. 2a). This means that the overexpression of CCR9 predicted the resistance to infliximab therapy in the psoriasis patients. Furthermore, we measured the relationship between the immunostaining intensity of the CCR9 protein and the pretreatment PASI scores and found no statistically significant association ($R = 0.070$, $P = 0.732$) (Fig. 2b). Based on these findings, we found that high expression of the CCR9 protein is associated with poor response to treatment, but not PASI score before infliximab therapy.

Table 2. Summary of upregulated RNA (with a fold change >5) in the non-responder skin samples according to the PCR array analysis

Gene name	Ct value	Fold change
CCR9	13.19	9345.14
CMKLR1	12.49	5752.61
CCL3	4.61	24.42
CXCL13	4.20	18.38
CXCL7	4.05	16.56
CXCR4	3.97	15.67
CXCL4L1	3.43	10.78
CXCR3	3.31	9.92
CCL28	3.29	9.78
CCR1	3.25	9.51
CCL15	3.22	9.32
CXCL5	2.43	5.39
CXCL10	2.33	5.03

A mixture was prepared from equal amounts of cDNA obtained from the sections of involved skin in three responders and three non-responders, and the cDNA expression profile of each group was evaluated using a polymerase chain reaction (PCR) array. The ΔΔCt (raw Ct value of each mRNA − Ct value of the RNA housekeeping gene) was calculated. CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CMKLR, chemokine-like receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor.

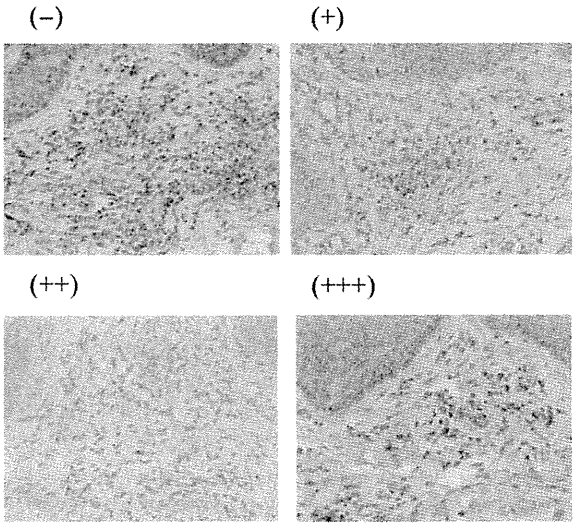

Figure 1. Representative images of semiquantitative scoring using the immunohistochemical staining of CCR9 ([−], [+], [++], [+++]) (original magnification ×200).

DISCUSSION

In this study, we initially investigated the relationship between the mRNA expression levels of chemokine/chemokine receptor mRNA and poor treatment outcomes in psoriasis patients treated with infliximab using a PCR array. We identified several chemokine/chemokine receptor mRNA that were overexpressed in non-responders. Among them, we focused on CCR9, the expression of which was found to be remarkably upregulated in the non-responders. An immunohistochemical analysis revealed that the CCR9 protein expression levels were negatively correlated with the PASI score improvement rate, while there were no statistically significant differences between relative CCR9 protein levels and pretreatment PASI scores.

While the role of CCR9 has already been investigated in relation to various inflammatory disorders. To our knowledge,

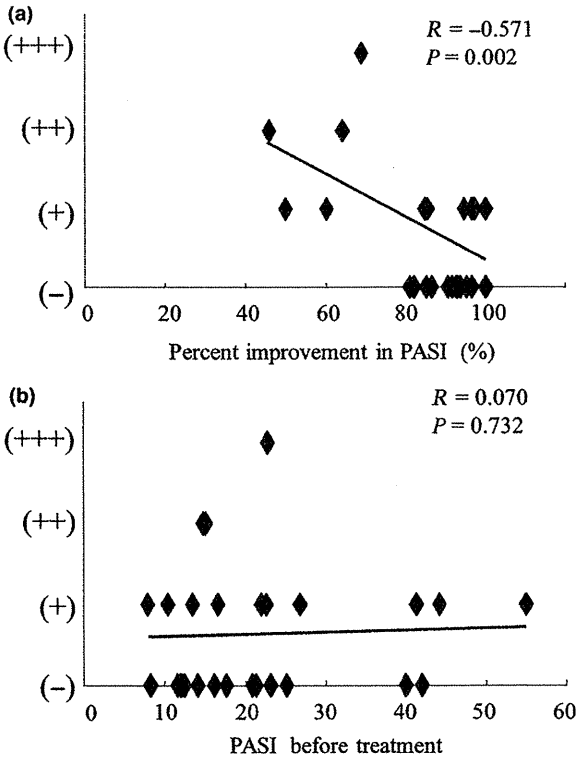

Figure 2. (a) Correlation between the semiquantitative evaluation of the CCR9 protein and the percentage of Psoriasis Area and Severity Index (PASI) improvement. (b) Correlation between the semiquantitative evaluation of the CCR9 protein and the PASI score before infliximab treatment.

our study is the first to indicate that CCR9 expressions may be involved in the pathogenesis of psoriasis.

CCR9 is a receptor on T/B lymphocytes and dendritic cells.[23] It is involved in lymphocyte migration.[24] As stated previously, CCR9 plays an important role in inflammatory diseases including CD[18,19] and RA.[20,21] Although anti TNF-α antibodies have proven to be highly effective, as in the case of psoriasis, not all patients respond to treatment with TNF-α inhibitors. Recent reports have indicated that a CCR9 inhibitor improves the disease progress in experimental mouse models of CD[19] and RA.[21] Moreover, anti-CCR9 therapies have recently been developed in new clinical trials for CD. The effectiveness of CCX282-B, which is an inhibitor of CCR9, has been demonstrated in a phase II study[25] and a multicenter/double-blind study.[22]

The TNF-α and Th17 pathway plays a key role in the pathogenesis of RA, CD and psoriasis,[26] which indicates that the pathogenesis of psoriasis is generally similar to RA and CD. Clinical trials of the use of CCR9 inhibitor in the treatment of CD patients are ongoing. Moreover, in our results, increased CCR9 expression levels in psoriatic skin implied a poor response to infliximab treatment. Taken together, these results suggest that in the future (as with CD), additional therapy using CCR9 inhibitors may be effective in treating psoriasis which is resistant to existing biologic agents.

In conclusion, our findings demonstrated that CCR9 expression levels may regulate the clinical response to biologic therapies. However, our results were obtained from a small study population and further studies of these biomarkers are needed to verify the results in a large series of patients. Clarifying the connection between the pathogenesis of psoriasis and the upregulation of CCR9 may lead to a greater understanding of psoriasis and the development of new therapeutic approaches.

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CONFLICT OF INTEREST: None declared.

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Report

microRNA level is raised in the hair shafts of patients with dematomyositis in comparison with normal subjects and patients with scleroderma

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Abstract

Background The diagnosis of dermatomyositis is sometimes difficult. We tried to evaluate the possibility that levels of *Homo sapiens* microRNA-214 (hsa-miR-214) in hair roots or hair shafts can be a useful marker of the disease.

Methods A single hair root and five pieces of hair shafts were obtained from nine patients with dermatomyositis, 15 normal subjects, and 18 patients with scleroderma before treatment. RNAs were purified from the hair roots and hair shafts using commercially available kits. cDNA was synthesized from the RNA, and miR-214 levels were measured with quantitative real-time polymerase chain reaction. Receiver operating characteristic curve analysis was performed to evaluate the diagnostic performance of hair microRNA levels.

Results The levels of miR-214 in hair shafts of patients with dermatomyositis were significantly higher than those of normal subjects and patients with scleroderma. By receiver operator curve analysis of hair shaft miR-214 levels to distinguish patients with dermatomyositis from normal subjects, the area under the curve was 0.90. The duration between symptom onset and the first visit to the hospital was significantly shorter in patients with elevated hair shafts miR-214 levels, suggesting that they have more severe subjective symptoms. On the other hand, we could not find significant differences in hair root miR-214 levels among normal subjects and patients with dermatomyositis and scleroderma.

Conclusions Hair shaft miR-214 levels are useful for diagnosis and evaluating the disease severity of dermatomyositis. Hair microRNA levels may have potential to be a novel and less invasive biomarker.

Introduction

Dermatomyositis is one of the autoimmune disorders characterized by myositis and cutaneous eruptions. In addition, patients are sometimes accompanied with interstitial pneumonia and/or internal malignancy.

The diagnosis of dermatomyositis is not difficult when patients have muscle or lung involvement as well as cutaneous manifestation. However, for example, amyopathic dermatomyositis lacks myositis and needs to be diagnosed only by cutaneous manifestations. Gottron's sign and heliotrope rash are frequent and characteristic, but they are clinically similar to other diseases such as knuckle pads or angioedema, respectively;[1–4] other various disorders were also reported to mimic dermatomyositis skin and were initially misdiagnosed.[5–7] In addition, patients with dermatomyositis sometimes have atypical skin manifestations, which makes the diagnosis more challenging for dermatologists. The histopathological findings, including basal layer liquefaction degeneration, mild lymphocyte infiltration, or dermal mucin deposition, are characteristic of dermatomyositis but not specific. Therefore, the development of new diagnostic markers of this disease will be beneficial for patients.

microRNAs (miRNAs), small non-coding RNAs on average only 22 nucleotides long, have the ability to regulate the expression of other genes. By binding to the 3′-untranslated regions of target mRNAs, miRNAs can

inhibit the translation of the mRNAs.[8] A lot of research has shown the involvement of miRNAs in the pathogenesis of various diseases, such as skin diseases, cancers, infections, and lifestyle-related diseases.

Increasing evidence has indicated that extracellular miRNA levels are useful as the disease markers. Considering that RNAs, including miRNA, usually respond to any stimuli earlier than proteins, RNA levels may be more sensitive biomarkers. We have investigated whether miRNAs in serum can be the novel disease markers of dermatomyositis and previously reported that serum levels of miR-21 were significantly elevated in the patients.[9] Furthermore, we recently showed the potential of hair miRNAs as the independent disease markers, because hair miRNA levels were not significantly correlated with serum miRNA levels in each individual.[10] Eisenberg *et al.* reported that expression of several miRNAs is up- or downregulated in the muscle tissues of dermatomyositis compared with that of normal control muscles.[11] Among them, we focused on *Homo sapiens* miRNA-214 (hsa-miR-214), because its role in autoimmune disorders has not been investigated. Although miR-214 was not detected in the human sera by our method,[12] the miRNA may be detectable in the hair roots or hair shafts. In this study, we tried to evaluate the possibility that levels of the unique miRNA in hair roots and hair shafts become an effective biomarker for the diagnosis and the examination of disease activity in dermatomyositis.

Materials and methods

Collection of patients' materials

A single hair root and five pieces of hair shafts were obtained from nine patients with dermatomyositis (three men and six women; age range, 24–79 years) before treatments. These patients were classified into six classical dermatomyositis or three amyopathic dermatomyositis categories based on the criteria proposed by Bohan and Peter or by Sontheimer, respectively.[13–15] These patients did not have internal malignancies or skin cancers. Hair samples were also obtained from 15 normal subjects and 18 patients with systemic sclerosis. All hair samples were preserved at −80 °C before use. Institutional review board approval and written informed consent were obtained before patients and normal subjects were entered into this study according to the Declaration of Helsinki.

microRNA extraction from a hair root and hair shafts

Hair roots and hair shafts were washed in water and ethanol. A single hair root was incubated with Isogen (Nippon Gene, Toyama, Japan) overnight at room temperature. On the other hand, five hair shafts were dissolved by incubation in extraction buffer supplemented with enzyme solution and lysis solution of Isohair (Nippon Gene) for 90 min at 55 °C. Samples were then supplemented with Isogen.

After chloroform was added, each sample was mixed well by vortexing and was incubated for 5 min at 4 °C. The aqueous and organic phases were separated by centrifuge. RNA was precipitated from the aqueous phase by adding isopropanol and ethanol as described previously.[10,16]

Quantitative real-time polymerase chain reaction

To synthesize cDNA from the total RNA, including small RNA derived from hair roots and hair shafts, we used Mir-X miRNA First Strand Synthesis and SYBR quantitative real-time polymerase chain reaction (PCR) Kit (Takara Bio, Shiga, Japan).[10] Quantitative real-time PCR with Takara Thermal Cycler Dice (TP800) used primers and templates mixed with SYBR Premix. The sequence of hsa-miR-214 primer was designed based on miRBase (http://www.mirbase.org). DNA amplification was performed by 50 cycles of denaturation for 5 s at 95 °C and annealing for 20 s at 60 °C.[17,18]

Statistical analysis

Differences between two groups were compared using Mann–Whitney's *U*-test. The analysis of frequency was performed by Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

Results

Because there has been no previous research that proves the presence of hsa-miR-214 in human hairs, we first tried to determine whether miR-214 is detectable in hair roots or hair shafts. Total miRNA was extracted and purified from hair shafts and hair roots of healthy individuals as described previously,[10] and the levels of miR-214 were evaluated by real-time quantitative PCR using primer set specific for miR-214 (Fig. 1a,b). The amplification curve of hsa-miR-214 was observed by PCR using miRNAs obtained from hair shafts (Fig. 1a) or hair roots (Fig. 1b), and C_t values were increased by the serial dilution of the miRNAs. Accordingly, we confirmed that hsa-msiR-214 is detectable and quantitative in both hair roots and hair shafts.

Hair miRNA levels were determined in nine patients with dermatomyositis, 18 patients with systemic sclerosis, and 15 normal subjects. miR-214 levels in hair shafts of patients with scleroderma were slightly higher than those of normal subjects but not statistically significant (Fig. 2a). On the other hand, hair shaft miR-214 levels in patients with dermatomyositis were significantly higher than in normal subjects ($P = 0.0056$) and in patients with scleroderma ($P = 0.016$). Although miR-214 was reported as increased in pancreatic cancers or malignant melanoma,[19,20] patients with dermatomyositis in this study

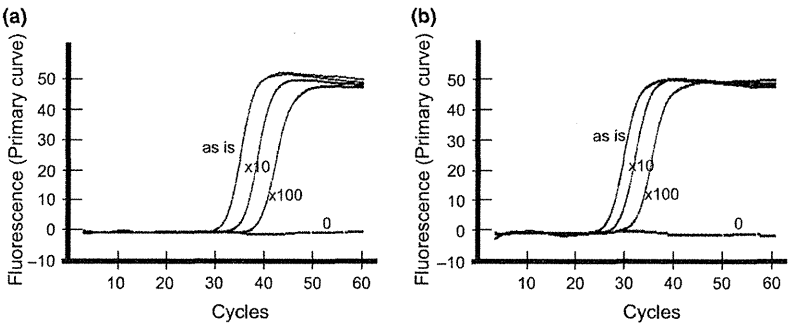

Figure 1 The presence of miR-214 in the hair shaft (a) or hair roots (b) was shown. Serial dilutions of cDNA (as is, 10-fold dilution, 100-fold dilution, and water) synthesized from hair miRNAs were used as templates for real-time polymerase chain reaction. The amplification curves of transcripts are shown to illustrate the process of exponential increase of fluorescence. Horizontal line indicates the threshold

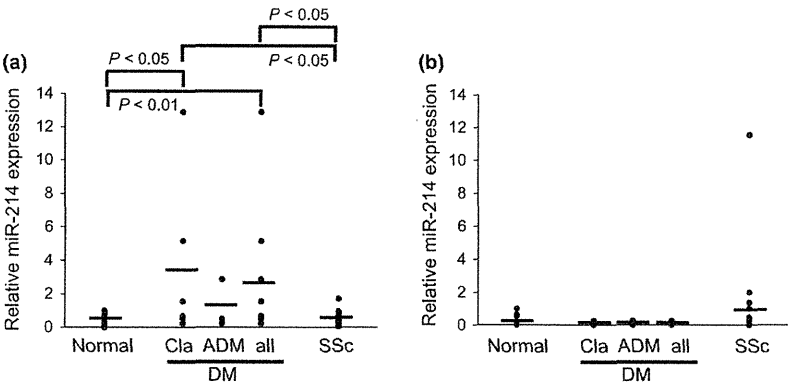

Figure 2 miR-214 levels measured by quantitative real-time polymerase chain reaction are plotted along the ordinate. miRNAs were obtained from hair shafts (a) or hair roots (b) of normal subjects (normal), patients with dermatomyositis (DM) and scleroderma (SSc). Bars show means. The maximum value in normal subjects was set at 1. Patients with DM (all) were classified into classical DM (Cla) and amyopathic DM (ADM)

did not have internal malignancies or skin cancers. When patients with dermatomyositis were classified into classical dermatomyositis and amyopathic dermatomyositis, as described in Materials and Methods, there was a significant difference in miR-214 levels between patients with classical dermatomyositis and normal subjects ($P = 0.010$) or patients with scleroderma ($P = 0.020$). miR-214 levels in amyopathic dermatomyositis were also increased compared to normal subjects or patients with scleroderma but not statistically significant ($P = 0.11$ or 0.23, respectively). We could not find significant differences in hair root miR-214 levels among normal subjects and patients with dermatomyositis and scleroderma (Fig. 2b).

In receiver operator curve analysis for hair shaft miR-214 to distinguish patients with dermatomyositis from normal subjects, the area under the curve (AUC) was 0.90 (95% confidence interval; 0.75–1.06) (Fig. 3a). An AUC more than 0.8 indicates that the level of miR-214 in hair shafts is useful for the diagnosis of patients with dermatomyositis. On the other hand, AUC of hair root miR-214 was 0.75 (95% confidence interval; 0.53–0.96) (Fig. 3b), suggesting that hair root miR-214 levels are less effective than hair shaft miR-214.

We also determined the difference in clinical and serological features between patients with dermatomyositis with normal and elevated hair shaft miR-214 levels (Table 1). Duration of the disease (between symptom onset and the first visit to the hospital) was significantly shorter in patients with elevated miR-214 levels than in those with normal levels (1.5 vs. 9.8 months, $P < 0.05$), suggesting that patients with elevated miR-214 have more severe subjective symptoms. In addition, although not statistically significant, patients with dermatomyositis with elevated miR-214 levels also showed a higher frequency of various findings, including Gottron's sign, heliotrope

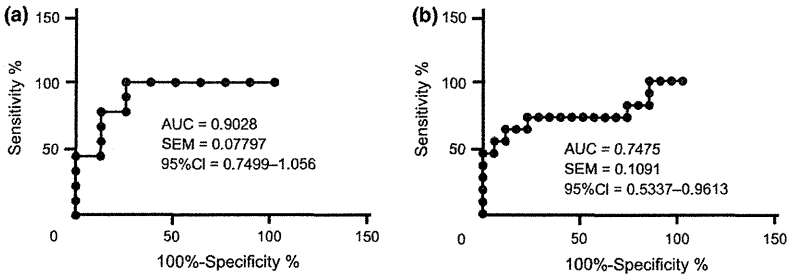

Figure 3 Receiver operating characteristic curve for miR-214 in the hair shafts (a) or hair roots (b) to distinguish patients with dermatomyositis from normal subjects. AUC, areas under curves; CI, confidence interval; SEM, standard error of the mean

Table 1 Clinical and serological features of patients with dermatomyositis with normal or elevated hair shaft miR-214 levels

	Elevated miR-214 (n = 4)	Normal miR-214 (n = 5)
Age (mean years)	49.3	65.2
Duration (mean month)	1.5*	9.8
Gottron's sign	100.0	60.0
Heliotrope rash	50.0	0.0
Lung involvement	25.0	60.0
Cancer	0.0	0.0
Joint pain	75.0	20.0
Dysphagia	50.0	40.0
IgG (mg/dl)	1494.8	1472.6
Creatine kinase (U/l)	1340.3	841.6
Myoglobin (ng/ml)	770.6	246.8
Aldolase (U/l)	20.0	13.2

Unless indicated, values are percentages of patients. Cut-off value was set at mean + 2SD of hair shaft miR-214 levels in normal subjects.
*$P < 0.05$ vs. patients with normal miR-214 levels.

rash, joint pain, and dysphagia, or had higher levels of creatine kinase, myoglobin, or aldolase than those with normal levels. These results suggest that patients with elevated hair shaft miR-214 may also be accompanied by more severe objective symptoms.

Discussion

In the present study, the levels of miR-214 in hair shafts of patients with dermatomyositis showed a significant increase compared to those of normal subjects or patients with scleroderma. According to receiver operator curve analysis, hair shaft miR-214 levels are effective as a diagnostic marker. miR-214 levels in hair shafts were also inversely correlated with disease duration, suggesting that the miR-214 levels can reflect the subjective severity of patients. In addition, although not statistically significant, miR-214 levels are associated with objective severity such as the prevalence of heliotrope rash or levels of creatine kinase. Accordingly, hair shaft miR-214 levels may be clinically useful for the diagnostic and disease activity marker of this disease. Although the reason why miR-214 exists only in hair shafts and hair roots but not in serum remains to be clarified, our previous study revealed that hair miRNA levels are independent from serum miRNA levels.[10] Given that hairs are much easier to obtain than blood samples, hair miRNA levels may have the potential to be novel and less invasive disease markers.

miR-214 has been reported to be associated with the pathogenesis of various tumors, and its expression was found elevated in several cancer cells. For example, increased miR-214 in pancreatic cancers correlates with the resistance to chemotherapy.[19] In malignant melanoma, miR-214 is thought to promote metastasis.[20] Because patients with dermatomyositis, including in this study, did not have internal malignancies, increased miR-214 in the hair was not likely because of the presence of cancer. Further studies should be performed to determine whether miR-214 is not only a disease marker but also involved in the pathogenesis of dermatomyositis. In addition, targets of miR-214 in this disease also need to be addressed.

The diagnosis of patients with amyopathic dermatomyositis is more difficult than that of classical cases. Thus, measurement of hair miR-214 levels will be more meaningful or applicable in diagnosing them. miR-214 levels in amyopathic dermatomyositis were slightly increased compared to normal subjects or patients with scleroderma; however, we could not find significant differences. This may be because of a small number of patients. As a limitation of this study, we could not collect a large number of typical patients due to the rarity of this disease. Because this is the pilot study, larger studies with an increased number of patients are needed to evaluate the usefulness of hair miRNA levels for the diagnosis of amyopathic dermatomyositis in the future.