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*).

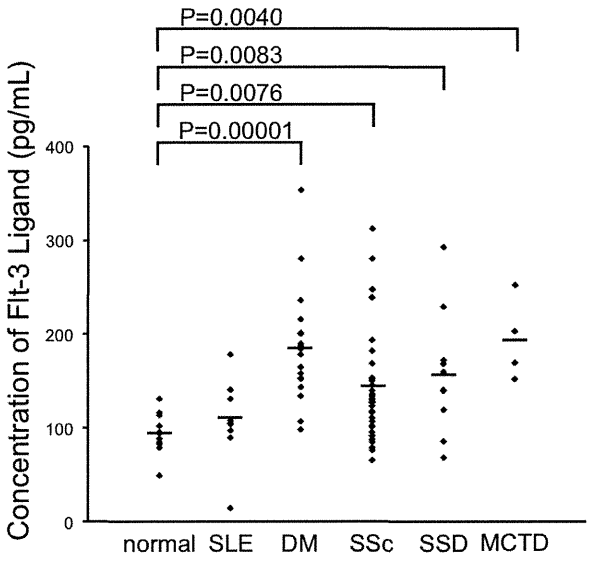

Figure 1. The serum levels of Flt-3 ligand in rheumatic diseases. Levels of Flt-3 ligand in sera of patients with systemic lupus erythematosus (SLE, $n = 10$), dermatomyositis (DM, $n = 20$), systemic sclerosis (SSc, $n = 36$), scleroderma spectrum disorder (SSD, n = 10), mixed connective tissue disease (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.

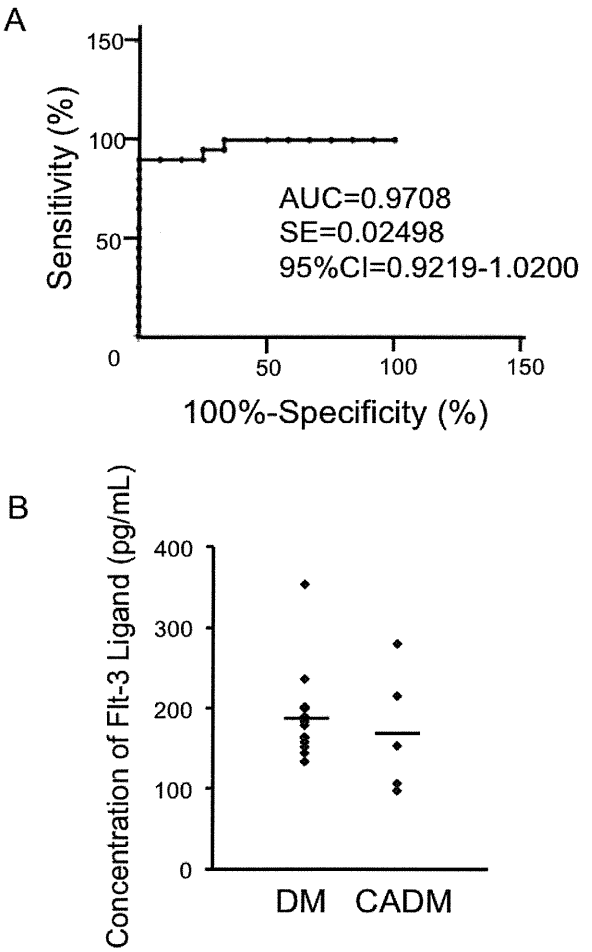

Figure 2. The serum levels of Flt-3 ligand in dermatomyositis. (A) Receiver operating characteristic (ROC) curve for serum Flt-3 ligand to distinguish dermatomyositis (DM) patients from normal subjects. AUC; areas under curves, SE; standard error, CI; confidence interval. **(B)** Levels of Flt-3 ligand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

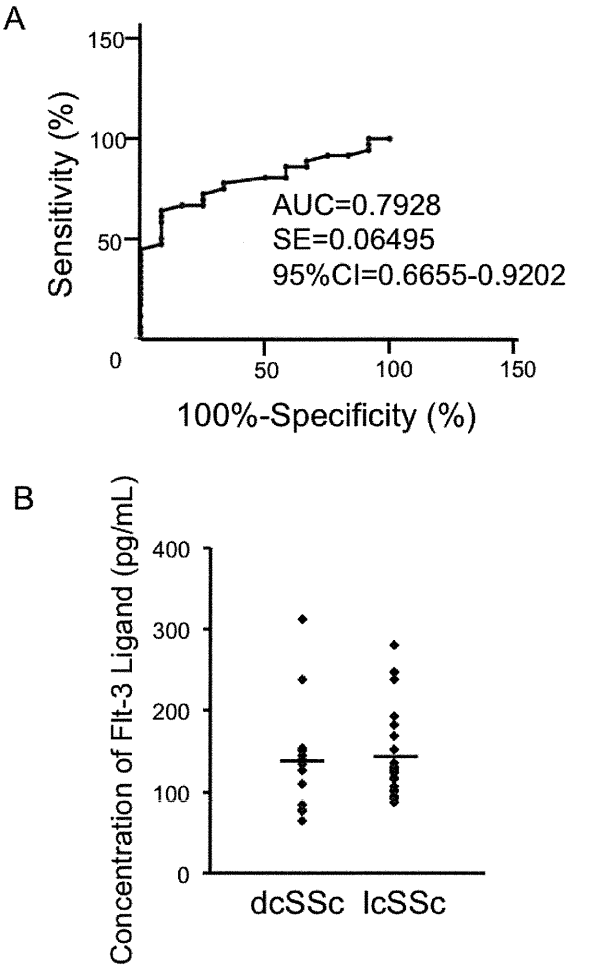

Figure 3. The serum levels of Flt-3 ligand in systemic sclerosis. (A) Receiver operating characteristic (ROC) curve for serum Flt-3 ligand to distinguish systemic sclerosis (SSc) patients from normal subjects. AUC; areas under curves, SE; standard error, CI; confidence interval. **(B)** Levels of Flt-3 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.

Acknowledgements

This study was supported in part by a grant for scientific research from the Japanese Ministry of Education, Science, Sports and Culture, by project research on intractable diseases from the Japanese Ministry of Health, Labour and Welfare, and by Lydia O'leary Memorial Foundation.

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(Received September 17, 2015; Revised October 21, 2015; Accepted October 24, 2015)

International Journal of
Dermatology

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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Conflicts of interest: None.

doi: 10.1111/ijd.12958

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

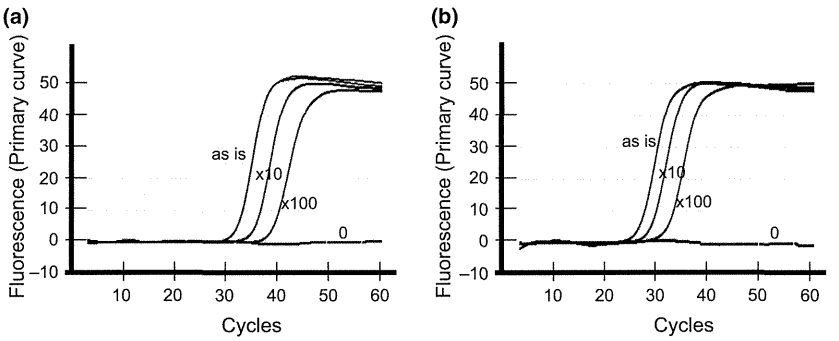

Figure 1 The presence of miR-214 in the hair shaft (a) or hair roots (b) was shown. Serial dilutions of cDNA (as is, 10-fold dilution, 100-fold dilution, and water) synthesized from hair miRNAs were used as templates for real-time polymerase chain reaction. The amplification curves of transcripts are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

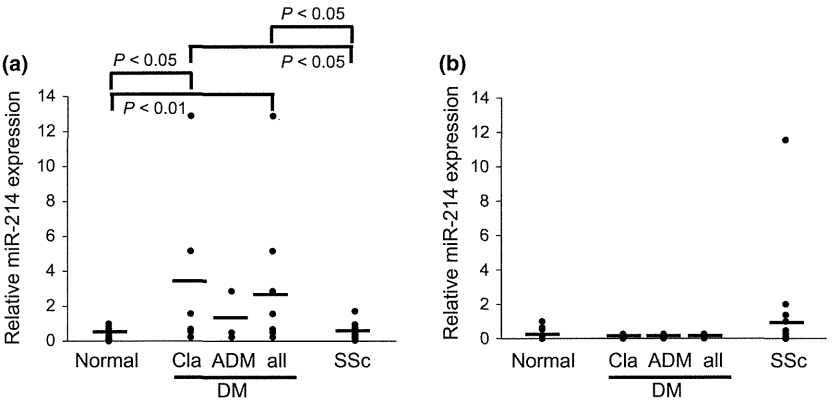

Figure 2 miR-214 levels measured by quantitative real-time polymerase chain reaction are plotted along the ordinate. miRNAs were obtained from hair shafts (a) or hair roots (b) of normal subjects (normal), patients with dermatomyositis (DM) and scleroderma (SSc). Bars show means. The maximum value in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

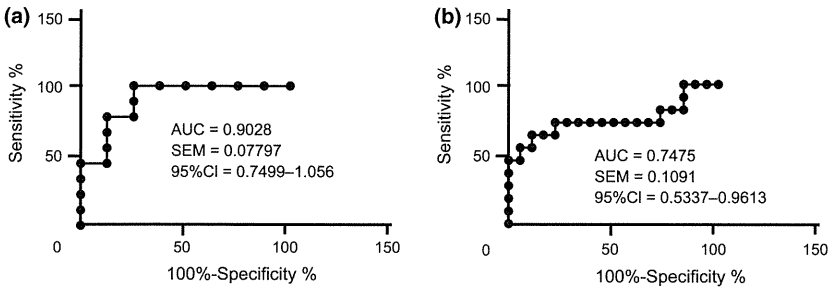

Figure 3 Receiver operating characteristic curve for miR-214 in the hair shafts (a) or hair roots (b) to distinguish patients with dermatomyositis from normal subjects. AUC, areas under curves; CI, confidence interval; SEM, standard error of the mean

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.

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MORH-D-15-00190 Received: Mar-24-15; Accepted: Aug-11-15

Original article

Abnormal distribution of AQP5 in labial salivary glands is associated with poor saliva secretion in patients with Sjögren's syndrome including neuromyelitis optica complicated patients

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Running title: AQP in NMO with SS

ABSTRACT

Objective: To investigate whether aquaporins (AQPs) are involved in salivary gland dysfunction in patients with neuromyelitis optica (NMO) complicated with Sjögren's syndrome (SS).

Methods: Eight primary SS (pSS) patients, four NMO spectrum disorder (NMOsd) patients complicated with SS (NMOsd-SS), and three control subjects were enrolled. Immunohistochemistry of labial salivary glands (LSGs) was performed to determine the expressions of AQP4, AQP5 and tumor necrosis factor-alpha (TNF-α). In vitro expression of AQP5 was examined by Western blotting in cultured primary salivary gland epithelial cells (SGECs).

Results: No expression of AQP4 was shown in all LSGs. AQP5 was clearly expressed in the all acini, but the predominant localization of AQP5 in the apical side was diminished in the patients with pSS or NMOsd-SS compared to the controls and tended to be even lower in NMOsd-SS than pSS. The abnormal localization of AQP5 was associated with poor saliva secretion. No difference was found in TNF-α expression in the LSGs between patients with pSS and NMOsd-SS. AQP5 expression of SGECs in vitro was not changed by TNF-α or interleukin-10.

Conclusion: Our results suggest that AQP5 but not AQP4 contributes to salivary secretion in patients with SS including those with NMO complicated with SS.

Key words: Neuromyelitis optica, Sjögren's syndrome, aquaporin, labial salivary glands.

Abbreviations

AQP; aquaporin, MNCs; mononuclear cells, NMO; neuromyelitis optica, SS; Sjögren's syndrome, NMOsd: NMO spectrum disorder, NMOsd-SS: NMOsd patient with SS; pSS, primary SS; MS; multiple sclerosis, SGECs; salivary gland epithelial cells, LSG; labial salivary gland, FS; focus score

Introduction

Primary Sjögren's syndrome (pSS) is characterized by dryness of the mouth, eyes, and other mucous membranes, and it is sometimes accompanied by extraglandular manifestations,[1,2,3] including both peripheral and central neurological disorders. As central neurological complications in pSS, meningoencephalitis, diffuse non-focal symptoms, cerebellar ataxia and also multiple sclerosis-like manifestations including acute transverse or progressive myelopathy, sensory deficits and optic neuropathy are reported.[4]

Neuromyelitis optica (NMO) develops in middle-aged women, and the main symptoms of NMO are loss of vision and acute myelitis extending over three or more vertebral segments.[5–7] These symptoms worsen while exacerbation and remission repeat.[8,9] An Attack in NMO is often more severe than that in multiple sclerosis (MS)[9–12], and tends to give a serious disability. In 1992, Preston et al. identified aquaporin (AQP) 1 as a functional unit of membrane water channels.[13] At present, 13 members of the AQP gene family have been identified (AQP0–12) in the human genome.[14] AQP4 is expressed in the central nervous system and is involved in the transport of water in the central nervous system.[15]

Anti-AQP4 antibody was identified and detected in the serum of patients with NMO.[16,17] Since MS and NMO have different serological profiles such as presence of anti-AQP4 antibody, they are thought to be distinct entities.[18,19] Although the pathogenesis of SS remains to be clarified, changes in the distribution of AQP5 expression in salivary glands of SS patients have been reported in pathology studies.[20]

The infiltration of mononuclear cells (MNCs) was observed in the labial salivary glands (LSGs) of patients with myelitis over several segments.[21] Pathological similarity might therefore exist between NMO and SS, but research to determine this has not been performed, to the best of our knowledge. It should be shown whether or not AQP4 detected in NMO appears in LSGs of NMO patients with SS by reason of complication of these disorders. Another point is if the distribution of AQP5 found in LSGs of patients with SS is affected by the presence of NMO. Additionally, we here show if TNF-□ that is reported to inhibit expression of AQP5 in a kind of salivary gland epithelial cells actually inhibits expression of AQP5 in primary cultured salivary gland epithelial cells (SGECs) derived from the patients with SS. Moreover, we note how interleukin 10 (IL-10) that is a representative Th2 cytokine and a counterpart of Th1 cytokine such as TNF-□ acts on expression of AQP5 in SGECs *in vitro*. In the present study, we focused on the expression and localization of AQP4 and AQP5 in LSGs of patients with neuromyelitis optica spectrum disorder (NMOsd) or pSS.

Patients and Methods

Patients

Eight patients with primary SS (pSS), four NMOsd patients complicated with SS (NMOsd-SS), and three control subjects were enrolled. The diagnosis of NMOsd was based on the 2007 Wingerchuk criteria.[4] AQP4 antibody was measured at Tohoku University and Cosmic Corporation. AQP4 antibodies were positive in three of the four NMOsd patients.

The diagnosis of pSS was based on the revised criteria for the diagnosis of pSS as proposed by the American-European Consensus group.[22] In the three control cases, an LSGs biopsy was performed for further examination of dry mouth, and neither abnormality in LSGs nor positive anti-SS-A/Ro, SS-B/La antibodies was detected. Although rheumatoid factor was positive in these subjects, they did not fulfil the AECG classification criteria without positive pathology and autoantibodies. The clinical characteristics of the three groups (control, NMOsd-SS and pSS group) are shown in Table 1.

Antibodies and reagents

Anti-AQP4 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal rabbit anti-AQP5 antibody was purchased from Abcam (Tokyo). Secondary antibodies including donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Hoechst dye 33258 was purchased from Sigma (St. Louis, MO). Tumor necrosis factor-alpha (TNF-α) and mouse anti-human monoclonal antibody was purchased from LSBio (Seattle, WA).

LSGs biopsy

An LSGs biopsy was performed for all patients. For the evaluation of each LSGs biopsy specimen obtained from the lower lip under local anesthesia, we used the grading determined by Chisholm and Mason[23] and the focus score (FS) determined by Greenspan et al.[24] Informed consent for the use of samples obtained by the biopsy was obtained from all of the participating patients, and the study was approved by the human ethics

committee of our institution. For the statistical analysis, the parametric *t*-test was used. P-values <0.05 were considered significant.

Immunohistochemical examination of LSGs

Formalin-fixed, paraffin-embedded sections (3 μm thick) from the patients' and controls' LSGs were mounted on glass slides precoated with aminopropyltriethoxysilane. The sections were then stained using the labeled streptavidin-biotin method as described.[25] In our pre-experiment, microwave epitope retrieval was required for the detection of AQP4/5 and TNF-□. Briefly, endogenous peroxidase was inactivated in a 3% H_2O_2 solution following deparaffinization. Following blocking with 10% goat serum, the sections were incubated with rabbit anti-AQP4/5 polyclonal antibodies (R&D Systems, Minneapolis, MN) in a humid chamber for 60 min at room temperature. Then, all sections were treated with the Histofine Simple Stain Rabbit kit (Nichirei, Tokyo) for 30 min prior to washing and incubation with peroxidase-conjugated streptavidin. The color was developed by soaking the sections in 3.3'-diaminobenzidine and H_2O_2 for 10 min, followed by counterstaining the sections with hematoxylin. Negative control sections were treated with normal rabbit serum. After staining, the degree of staining was evaluated by measuring the brightness with WinROOF software (Mitani Corp., Fukui, Japan).

The LSGs tissue were also double-labeled using mouse anti-TNF-□ antibody (LifeSpan BioSciences, Seattle, WA) with fluorescein isothiocyanate (FITC)-conjugated secondary antibody and rabbit anti-AQP5 antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody. The status of the nucleus was observed by Hoechst staining. The sections were then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and the immunofluorescence signal was scanned by fluorescence microscopy (BIOREVO BZ-9000, Keyence, Osaka, Japan).

Culture of primary salivary epithelial cells

Primary salivary gland epithelial cells (SGECs) from the LSGs were cultured immediately after biopsy following the method reported by Ogawa et al.[26] Briefly, tissues were rinsed with cold sterile phosphate-buffered saline (PBS) containing 100 units/mL of penicillin and 100 μg/mL of streptomycin prior to being minced into fragments. One piece of the tissue was placed in a six-well plate coated with type I collagen (ATG

Techniglass, Aichi, Japan). The culture medium was a defined keratinocyte–serum-free medium (SFM) (Invitrogen Life Technologies, Carlsbad, CA) with the attached supplement, 0.4 μg/mL of hydrocortisone (Sigma) and 25 μg/mL of bovine pituitary extract (Kurabo, Osaka, Japan). Outgrowth of the cells was observed within 1 wk, and when the cells reached confluence, they were subcultured into a 100-mm^2 plate coated with type I collagen.

For the Western blot analysis, we cultured SGECs on 60-mm^2 cover slips precoated with type I collagen. During the cells' on 60-mm^2 culture plates, we stimulated the cells with an assigned concentration of TNF-α or IL-10 (R&D Systems). After 24 h, the expression of AQP5 was analyzed by Western blotting.

Western blot analysis

After stimulation, the cells was collected and lysed by the addition of lysis buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, and 1 mM polymethylsulfonylfluoride [PMSF]) for 25 min on ice. After centrifugation at 14,000 rpm, 15 min, 4°C, the supernatants were collected and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY). Equivalent amounts of protein were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Pagel (Bio-Rad, Tokyo).

The proteins were transferred to a polyvinylidene fluoride (PVDF) filter, which was blocked for 1 h using 5% non-fat dried milk in Tris-buffered saline (TBS, 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, then washed with TBS and incubated at room temperature for 1 h in a 1:1,000 dilution of each antibody. The filter was washed with TBS and incubated with a 1:1,000 dilution of donkey anti-mouse IgG or rabbit IgG, coupled with horseradish peroxidase. An enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) was used for detection.

Results

Clinical features of the pSS patients and NMOsd-SS patients

The clinical features of the patients are shown in Table 1. There was no significant difference between the pSS and NMOsd-SS patients with regard to the frequency of dry eyes and dry mouth. The patients in both groups had decreased salivary secretion as determined by Saxon test. However, the serum IgG concentrations as well as the grade of mononuclear cell (MNC) infiltration in LSGs were significantly higher in the pSS group compared to the NMOsd-SS group (Table 1).

Immunohistochemical evaluation of AQP4 in LSGs

In normal human brain as a positive control for anti-AQP4 antibody, clear staining from the brain surface to cortex was observed (**Fig. 1**). In the LSGs from the control subjects, pSS patients and NMOsd-SS patients, no staining for AQP4 was observed.

Immunohistochemical evaluation of AQP5 in LSGs

In the submandibular gland tissue (positive control) and LSGs of the control subjects, the expression of AQP5 was more intense on the glandular apical side of the acini than the basement membrane side (**Fig. 2**). Although AQP5 staining was also observed in the LSGs of the pSS and NMOsd-SS patients, the intensity of the staining of the apical side was diminished compared to the basement membrane side. In all sections, AQP5 staining was observed only within the glandular structures or acini and was not observed in the aggregation of mononuclear cell infiltration. Data of immunohistochemistry for AQP4 and AQP5 are shown in Table2.

Quantification of AQP5 staining in LSGs

We measured the ratio of the brightness of the apical side and the basement membrane side in order to determine any differences in the AQP5 expression pattern among the pSS, NMOsd-SS and control groups (**Fig. 3a**).

Compared to the brightness ratio of the control group, that of the other two groups was significantly higher. The brightness ratio tended to be higher in the NMOsd-SS group compared to the pSS group ($p=0.14$). When all of the subjects were divided into two groups based on whether they scored $\geq$ 1g/2min or $<$ 1g/2min in

the Saxon test (a measure of saliva secretion), the brightness ratio was higher in the group with less saliva secretion (p=0.05, **Fig. 3b**).

Expression of TNF-□ and AQP5 in LSGs and SGECs

The association between TNF-α and the expression of AQP5 was examined. There was no expression of TNF-α in the salivary glands of the control subjects (**Fig. 4a**). In contrast, mononuclear cells infiltrating around the $AQP5^+$ glandular structures expressed TNF-α in both the pSS and NMOsd-SS salivary glands.

We also examined the expression level of AQP5 in SGECs stimulated with TNF-α or IL-10 by Western blotting. In either group, there was no significant difference in the expression level of AQP5 compared to the control group. AQP5 expression was clearly found in cultured SGECs obtained from pSS patients, and its expression was not changed by TNF-□. In addition, the AQP5 expression in SGECs was not altered by IL-10 **(Fig. 4b).**