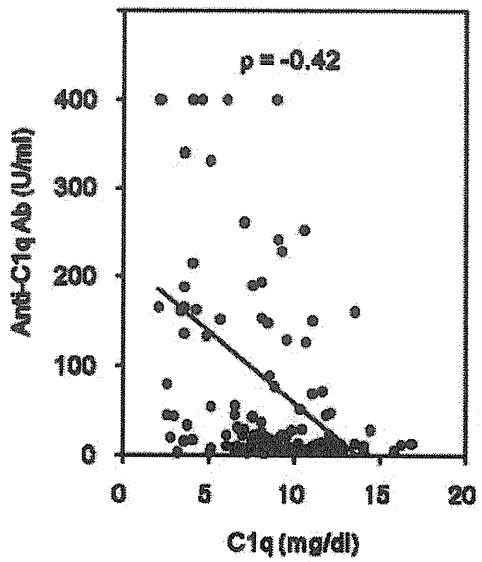

Figure 3. Significant negative correlation ($P < 0.0001$) between anti-C1q antibody (Ab) titers and the levels of C1q measured in 126 consecutive patients with active systemic lupus erythematosus.

[range −147% to 99%; $P = 0.00097$]) (Figure 4). The SLEDAI-2K scores in these 20 patients also decreased (median decrease 66% [range 25–100%], $P < 0.0001$). Four of these 20 patients initially had active lupus nephritis. In the only patient in whom the titer of anti-C1q antibodies increased (from 162 units/ml to 400 units/ml), a new rash had developed, and anti-dsDNA antibody positivity and hypocomplementemia persisted at the time of reevaluation (615 days later), although other SLE-related manifestations had improved. In the only patient in whom anti-C1q antibody titers did not change (400 units/ml before and after treatment), anti-dsDNA antibody positivity and hypocomplementemia persisted, although other SLE-related manifestations improved. In the latter patient, the anti-C1q antibody titers exceeded the upper limit of the assay; thus, there may have been some difference between the actual titers before and after treatment.

DISCUSSION

In our study of a large cohort of 126 consecutive patients with SLE, we observed that anti-C1q antibodies are associated with SLE global activity. However, in contrast to the results of previous, predominantly retrospective studies (4,10,12,16) that frequently involved fewer subjects (9,17), our results demonstrated that anti-C1q antibodies are not specifically associated with active lupus nephritis. We also showed that anti-C1q antibody titers significantly decreased as the patients' condition improved with clinical treatment. To our knowledge, this study is the first to investigate associations between anti-C1q antibodies and clinical and serologic parameters of SLE using a large prospective or consecutive cohort of patients with SLE.

This study demonstrated that the presence of anti-C1q antibodies is significantly associated with SLE. When serum samples from patients with SLE and those from normal healthy control subjects were compared, the specificity of the presence of anti-C1q antibodies for the diagnosis of SLE was as high as 90% and that for the PPV was as high as 98%. The prevalence of these antibodies in patients with RA and patients with SSc was as low as that in normal healthy control subjects. However, because anti-C1q antibodies have been observed to occur in many disease conditions (8), their presence cannot be said to be specific for any one disease (6).

Anti-C1q antibodies were detected in the sera of patients with active SLE who had a variety of manifes-

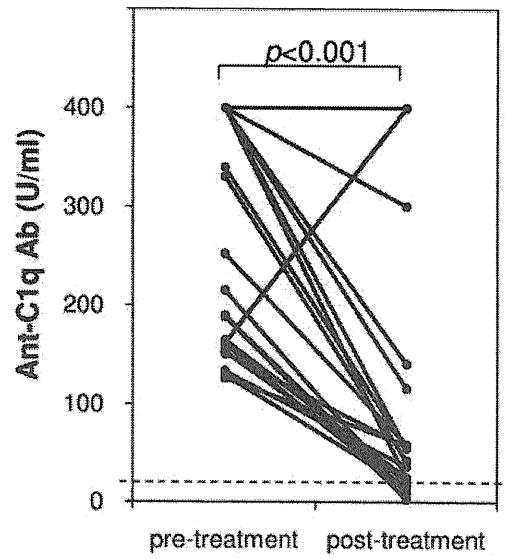

Figure 4. Anti-C1q antibody (Ab) titers before and after treatment in 20 patients with active systemic lupus erythematosus (SLE) who had high titers of anti-C1q antibodies (>125 units/ml) at the initial evaluation. The broken line indicates the cutoff value (15 units/ml) recommended by the manufacturer of the enzyme-linked immunosorbent assay kit. The anti-C1q antibody titers decreased significantly in accordance with clinical amelioration of disease following treatment (median decrease 84% [range −147% to 99%]; $P = 0.00097$).

tations. Leukopenia was shown to be significantly associated with anti-C1q antibodies. Interestingly, Armstrong et al also observed a correlation between the presence of anti-C1q antibodies and hematologic disease as well as renal disease in patients with SLE (29). However, another study demonstrated that the incidence of organ manifestations other than nephritis was the same in patients with and those without high titers of anti-C1q antibodies (12). As in earlier studies (4,17), we observed good correlations between anti-C1q antibodies and recognized markers of SLE activity: the SLEDAI, anti-dsDNA antibodies, C3, C4, CH50, and IC by C1q assay. Our findings indicate that anti-C1q antibodies are associated with SLE global activity but not specifically with active lupus nephritis. Although the main function of C1q is to clear ICs from tissues and self antigens generated during apoptosis (4), C1q has other biologic functions and could play roles as both facilitating and inhibiting/protective factors (30). As one example, Lood et al recently reported a novel function for C1q in the regulation of IC-induced production of interferon-α and other cytokines by plasmacytoid dendritic cells (31). It appears that anti-C1q antibodies may have several different pathogenic roles in SLE (6,32) and may account for various clinical manifestations of the disease.

Unlike previous studies, our study did not reveal a specific association between anti-C1q antibodies and active lupus nephritis. We speculate that the discrepancy between previous findings and our results may arise from differences in the patient populations. Most previous studies were retrospective (not consecutively or randomly sampled) (4,10,12,16) and were frequently small in size (9,17). In addition, most previous studies were led by departments/divisions of nephrology and/or focused on nephritis, usually severe; this could lead to sampling bias, because patients with severe lupus nephritis might be more likely to be included in such studies than in population samples of all SLE patients. Therefore, these earlier studies may not adequately reflect the full spectrum of SLE.

In contrast, our cohort is more likely to accurately represent the population of lupus patients commonly seen at clinics/hospitals specializing in rheumatology. Although many of the previous studies have shown a high NPV of anti-C1q antibodies for the occurrence of severe lupus nephritis, ranging up to 100%, it is worth noting that not all studies demonstrated such a definite result (6,8). Several explanations for the variation among studies have been proposed, including the following: across studies, the timing of anti-C1q antibody testing in relation to the renal flare was not uniform, different definitions of active lupus nephritis and positive test results were used, and assays were not standardized (32). Our study avoided these issues because 1) serum samples were always obtained before initiation or reinforcement of treatment, on admission or at the time of renal biopsies, when performed, 2) active lupus nephritis was defined consistently using the most standardized method, and 3) we used the same commercial kit as was used in previous studies (9,17).

In many previously reported cases, the levels of anti-C1q antibodies increased prior to the exacerbation of lupus nephritis (6). Thus, in some of the patients in our study who did not have active lupus nephritis, overt lupus nephritis may have developed over time if treatment had not been initiated. In fact, the number of patients with clinically overt nephritis in this study was only a small subset of the entire group (21 of 126 patients). Furthermore, some of our SLE patients who did not fulfill the criteria for active lupus nephritis did have renal biopsies, which revealed class II, III, IV, or V lupus nephritis. Our early clinical intervention could partly account for this discrepancy.

The NPV of anti-C1q antibodies for active nephritis was as high as 87% in our study, in accordance with previous reports (4,9,10), while the PPV was only 19%. Because nephritis does not develop in many other diseases involving the presence of anti-C1q antibodies (8), indications are that anti-C1q antibodies are required but not solely sufficient for development of renal inflammation in SLE (11,32). Flierman and Daha proposed a hypothetical model of lupus nephritis pathogenesis that involves 4 steps (11). First, circulating ICs containing nucleosomes deposit in the glomerulus by charge interactions with the glomerular basement membrane. Second, glomerular IC deposits bind C1q. Third, the exposure of neoantigens within the solid-phase C1q molecule serves as a focus, i.e., planted antigen, for circulating anti-C1q antibodies. Last, full activation of the classical pathway of the complement system leads to tissue injury mediated by the membrane attack complex as well as the influx of inflammatory cells, such as neutrophils. In addition, SLE patients who have anti-dsDNA antibodies but not anti-C1q antibodies may exhibit some degree of renal disease that may be only mild and/or effectively regulated via complement regulators.

The titers of anti-C1q antibodies in SLE patients showing a variety of clinical manifestations significantly decreased and, in some patients, decreased even beyond their cut-off levels (<15 units/ml) as the disease condition improved with treatment, as previously reported (9,16). These findings also support the view that anti-

C1q antibodies are associated with SLE global activity but not specifically with active lupus nephritis. It is possible that anti-C1q antibodies could be useful as a surrogate marker of SLE disease activity in patients positive for this antibody.

When anti-C1q antibody titers were compared with the levels of C1q in patients with SLE, the correlation was significant and negative; when the 2 parameters were measured together, they seemed to have a mirror-like pattern of serum concentrations, as previously reported (17,33). These findings also suggested that the 2 parameters are potential markers of SLE global activity. We speculate that a decrease in the number of C1q antibodies in patients with active SLE is mainly attributable to excessive consumption of C1q by complement activation by anti-C1q antibodies and by the formation and tissue deposition of C1q–C1q antibody complexes (33–35). According to an alternative theory, anti-C1q antibodies are a mere product of this activation itself, which creates neoepitopes in the C1q molecule (17,36).

Although our study was appreciably larger than the majority of previous studies dealing with anti-C1q antibodies and SLE, an insufficient number of patients with SLE still limited our ability to draw definitive conclusions about the significance of anti-C1q antibodies in SLE. Another potential weakness of our study arises from the variation in treatment protocols among the patients, reflecting different clinical presentations. Followup durations also varied among the patients, because reevaluation samples were collected at certain time points cross-sectionally. Because our study was not designed to validate anti-C1q antibodies as a biomarker for predicting lupus flares, as suggested previously (6), we are not able to comment on this issue, except to note that in most of our study patients, lupus flares of any form did not occur during followup. In addition, because almost all of the patients in the present study were Japanese, it is not clear whether anti-C1q antibodies have a different effect on lupus nephritis in patients with different ethnic backgrounds. However, several studies on patients of other ethnicities support our findings (29).

In conclusion, anti-C1q antibodies are associated with SLE global activity but not specifically with active lupus nephritis. The results of this study should caution clinicians against relying too much on anti-C1q antibodies as a diagnostic marker of lupus nephritis. Anti-C1q antibodies may, however, be useful as a surrogate marker for disease activity in patients with SLE in whom this antibody is present. Standardization of the measurement of anti-C1q antibodies and its increased use would be of benefit in the routine clinical diagnosis and treatment of SLE.

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AUTHOR CONTRIBUTIONS

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

Study conception and design. Katsumata, Kawaguchi, Yamanaka.

Acquisition of data. Katsumata, Miyake, Kawaguchi, Okamoto, Kawamoto, Gono, Baba.

Analysis and interpretation of data. Katsumata, Kawaguchi, Hara.

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CASE REPORT

Serum ferritin correlates with activity of anti-MDA5 antibody-associated acute interstitial lung disease as a complication of dermatomyositis

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Abstract Dermatomyositis (DM) is occasionally complicated by interstitial lung disease. Acute/subacute interstitial pneumonia (A/SIP) with DM is intractable and life threatening. Clinically amyopathic dermatomyositis (C-ADM) is also reported to be complicated with A/SIP, especially in those patients with anti-melanoma differentiation-associated gene 5 (MDA5) antibody. In the present cases, we indicate that serum ferritin level correlated with activity of A/SIP with DM. Two patients, a 65-year-old woman and a 30-year-old woman, were diagnosed with anti-MDA5 antibody-associated A/SIP with DM. Serum ferritin was high, 1600 and 770 mg/dl, respectively, on admission. Immunosuppressive therapy ameliorated A/SIP in both cases. Similarly, serum ferritin was also decreasing. However, A/SIP was recurrent and progressive, and serum ferritin was also increasing again in one case. In conclusion, serum ferritin correlates with disease activity of anti-MDA5 antibody-associated A/SIP with DM. Intensity of treatment may be decided according to serum ferritin level.

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Keywords Dermatomyositis · Acute interstitial pneumonia · Ferritin · Melanoma differentiation-associated gene 5 · Macrophage activation

Introduction

Dermatomyositis (DM) is characterized by inflammation of skin and muscle [1], occasionally complicated by interstitial lung disease (ILD), which is classified into two subsets: acute/subacute interstitial pneumonia (A/SIP) and chronic interstitial pneumonia (CIP) [2, 3]. A/SIP is of prime importance in clinical management of patients with DM, because it is an intractable and life-threatening complication [4]. Clinically amyopathic dermatomyositis (C-ADM) has typical skin lesions with amyopathy or hypomyopathy [5] and was recently reported to be complicated by A/SIP, especially in those patients with anti-CADM-140 antibody [6]. Recently, anti-CADM-140 antibodies in patients' sera were reported to react specifically with melanoma differentiation-associated gene 5 (MDA5) protein, which is involved with the innate immune system, confirming the identity of MDA5 as the CADM-140 autoantigen [7, 8]. Fathi et al. [9] reported that patients with myositis plus ILD needed careful evaluation of clinical features as well as pulmonary function tests and radiological features during follow-up, because the course of ILD could not be predicted on first examination. In previous study, we demonstrated that serum ferritin levels predict development and severity of A/SIP as a complication of dermatomyositis [10]. However, it has not been clarified whether serum ferritin levels in patients with C-ADM reflect improvement by treatment with immunosuppressive therapies.

In the present two cases, we indicate that serum ferritin levels correlated with activity of anti-MDA5 antibody-associated A/SIP with DM.

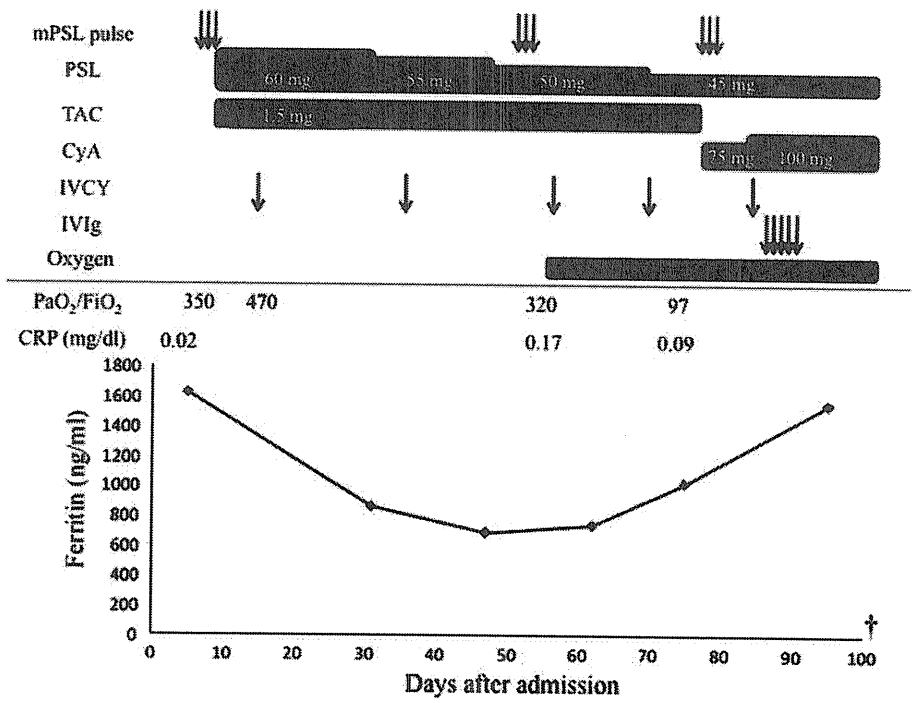

Fig. 1 Serum ferritin and clinical course in case 1. *mPSL pulse* methylprednisolone pulse therapy, *PSL* prednisolone, *TAC* tacrolimus, *CyA* cyclosporine, *IVCY* intravenous cyclophosphamide therapy, *IVIg* intravenous immunoglobulin therapy, *CRP* C-reactive protein

Case reports

Case 1

A 65-year-old Japanese woman was admitted with skin eruption of the whole body and myalgia of the extremities. These symptoms had gradually worsened during the preceding month. She complained of no respiratory symptoms. On admission, general examination revealed a heliotrope rash and Gottron's papules of the bilateral metacarpophalangeal, and elbow and knee joints. Neurological findings revealed mild weakness of the proximal upper and lower extremities. Laboratory investigations revealed that levels of serum creatine kinase (CK), aspartate aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), triglyceride, C-reactive protein (CRP), and ferritin were 712 IU/l, 160 IU/l, 77 IU/l, 456 mg/dl, 0.17 mg/dl, and 1620 ng/ml, respectively. Pancytopenia was not revealed. Although both antinuclear antibody and anti-aminoacyl tRNA synthetase (ARS) antibodies, including anti-Jo-1 antibody, were negative, anti-MDA5 antibody was detected by methods described previously [7]. Electromyography and magnetic resonance imaging demonstrated no evidence of myositis. The patient was diagnosed with C-ADM with anti-MDA5 antibody. She complained of dyspnea on exertion 6 days after admission. Blood gas analysis showed that the level of partial pressure of oxygen (pO_2) was lower than that at admission, with a decrease from 98 [PaO_2/FiO_2 490 (normal range, more than 300)] to 70 mmHg (PaO_2/FiO_2 350) at room air. High-resolution computed tomography (HRCT) of the chest demonstrated consolidations of the bilateral upper lobe of the lung and ground-glass opacities of the bilateral lower lobe. The respiratory symptom worsened for 1 week after admission. Infection such as cytomegalovirus and *Pneumocystis jirovecii* was not revealed. White blood cell counts and the level of CRP were within normal limits. Thus, the patient was diagnosed with anti-MDA5 antibody-associated AIP with DM. The clinical course is illustrated in Fig. 1. We selected combination therapy, including intravenous corticosteroid pulse therapy (1 g/day methylprednisolone × 3 days) followed by oral 60 mg/day prednisolone, 1.5 mg/day tacrolimus, and intravenous cyclophosphamide therapy (IVCY). The combination therapy ameliorated the A/SIP. The level of pO_2 was improved, reaching 94 mmHg (PaO_2/FiO_2 470) at room air. The level of serum ferritin also correlated with disease activity of AIP plus DM and was decreasing after treatment. Just before the third IVCY, the patient experienced dyspnea again. Blood gas analysis showed that the level of pO_2 was decreasing, and was measured as 64 mmHg (PaO_2/FiO_2 320) at room air. Oxygen had to be administered by using a nasal cannula and face mask. Although we investigated the possibility of infection such as cytomegalovirus and *Pneumocystis jirovecii*, the complication of infection was not revealed and the value of CRP was within normal limits. Thus, AIP with C-ADM was considered to be recurrent and progressive. IVCY was administrated biweekly. However, hypoxia worsened and ground-glass opacities were extended in the

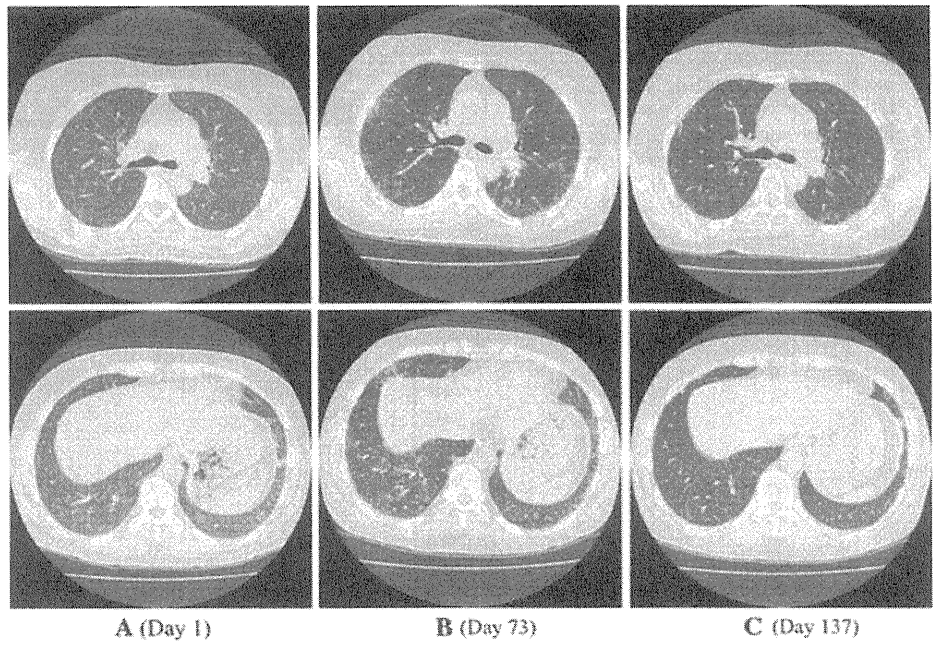

Fig. 2 High-resolution computed tomography of the lung in case 2: on admission (**a**), and at day 73 (**b**) and day 137 (**c**) after admission, respectively

lung. Tacrolimus was switched to cyclosporine. Moreover, intravenous immunoglobulin therapy (0.4 mg/kg body weight) was added to biweekly IVCY. However, the ILD attributed to DM was more progressive, accompanied by elevation of serum ferritin. The patient died from respiratory failure 3 months after admission.

Case 2

A 30-year-old Japanese woman was admitted with skin eruption of the face and hands and myalgia of the extremities. These symptoms had gradually worsened during the preceding 3 months. She complained of no respiratory symptoms. On admission, general examination revealed Gottron's papules of the bilateral metacarpophalangeal, and elbow and knee joints. Neurological findings revealed weakness of the proximal upper and lower extremities. Laboratory investigations revealed that levels of serum CK, ALT, γ-GTP, triglyceride, CRP, and ferritin were 468 IU/l, 71 IU/l, 96 IU/l, 231 mg/dl, 3.79 mg/dl, and 770 ng/ml, respectively. Pancytopenia was not revealed. Although both antinuclear antibody and anti-ARS antibody were negative, anti-MDA5 antibody was positive as in case 1. Electromyography, magnetic resonance imaging, and muscle biopsy demonstrated evidence of myositis. HRCT demonstrated no evidence of ILD on admission (Fig. 2a). The patient was diagnosed with DM with anti-MDA5 antibody. She complained of dyspnea on exertion 3 weeks after admission. Blood gas analysis showed that the level of pO_2 and PaO_2/FiO_2 were decreasing, measured as 69.5 mmHg and 348 at room air, respectively. HRCT demonstrated ground-glass opacities of the bilateral lobe in the lung (Fig. 2b). She was diagnosed with anti-MDA5 antibody-associated AIP with DM. The clinical course is illustrated in Fig. 3. We selected combination therapy, including oral 50 mg/day prednisolone, 150 mg/day cyclosporine, and IVCY (600 mg/body weight). However, the respiratory symptom and further radiological examinations worsened weekly, and oxygen had to be administered. Her AIP with C-ADM was considered progressive. The dose of IVCY was increased from 600 to 750 mg/body weight at the third course. This intensive treatment ameliorated the activity of AIP, which resulted in improvement of the ground-glass opacities of HRCT (Fig. 2c), the level of pO_2, and the dyspnea. The level of serum ferritin also correlated with disease activity of AIP and was decreasing to a normal level after treatment. Additionally, the level of interleukin (IL)-18 correlated with ferritin and disease activity of AIP. Normal range of serum IL-18 has not been determined precisely, but we measured the concentrations of serum IL-18 in healthy donors ($n = 30$). The median (range) concentration was 50.5 pg/ml (18–121 pg/ml). The concentrations of IL-18 in her sera were very high compared with those in healthy donors. She was discharged with a prescription for 35 mg prednisolone daily. There has been no recurrence in her for 2 years after discharge. She has felt fine and began to work.

Discussion

We have demonstrated that serum ferritin level correlates with activity of AIP with C-ADM in presence of anti-MDA5

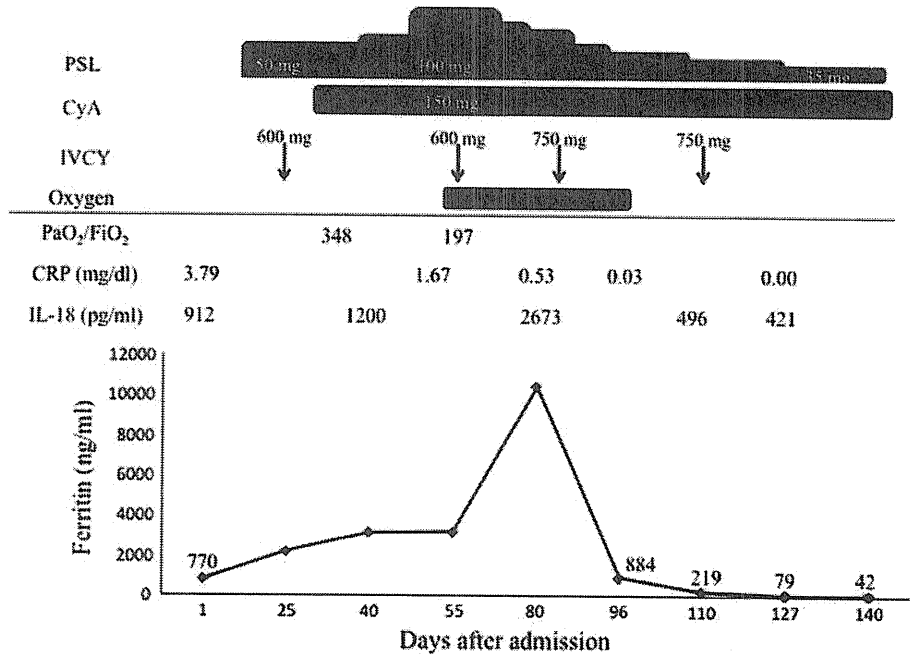

Fig. 3 Serum ferritin and clinical course in case 2. *PSL* prednisolone, *CyA* cyclosporine, *IVCY* intravenous cyclophosphamide therapy

antibody, suggesting that serum ferritin may be a valuable biomarker for AIP with C-ADM, although ferritin levels alone are not sufficient to predict real survival of each patient. Nakashima et al. reported that anti-MDA5-positive patients frequently showed hyperferritinemia and acute progressive ILD with poor prognosis. Additionally, the worse the ILD of the anti-MDA5-positive patients became, the higher their serum ferritin concentrations were [8]. Because the levels of serum ferritin correlated well with activity of AIP, it is possible that overproduction of ferritin reflects the pathogenesis of AIP with DM involved in anti-MDA5 antibody. Ferritin is the major molecule for iron storage, and it plays a crucial role in sequestration of potentially harmful molecules of reactive iron [11]. Ferritin can be secreted by the liver, T lymphocytes, and macrophages. Very high serum levels of ferritin have been reported in systemic-onset juvenile idiopathic arthritis (SoJIA), adult-onset Still disease (AOSD), and hemophagocytic syndrome related to connective tissue diseases (e.g., SoJIA, AOSD, DM, systemic lupus erythematosus), which is in the spectrum of secondary hemophagocytic lymphohistiocytosis (HLH) [12, 13]. "Macrophage activation syndrome" (MAS) is now considered a special term to refer to a form of secondary HLH seen in the context of rheumatic disorders [13]. The pathophysiology of MAS involves lack of regulation of T lymphocytes and excessive production of cytokines, such as tumor necrosis factor α (TNF-α), IL-1β, IL-6, and IL-18, resulting in activation of macrophages [12]. High levels of serum ferritin can reflect aberrant production of ferritin by activating macrophages. IL-18 levels were investigated only in the present case 2. IL-18 level correlated with ferritin and disease activity of AIP. The levels of hepatobiliary enzymes and triglyceride were increasing on admission in both case 1 and case 2. Pancytopenia was revealed and worsening at the same time as AIP was progressive. The pathogenesis of anti-MDA5 antibody-associated A/SIP with DM may be equivalent to MAS with respect to excessive alveolar macrophage activation in the lung.

MDA5 is concerned with the innate immune system. MDA5 initially recognizes picornaviruses such as coxsackievirus and evokes antiviral responses by producing type I interferons and TNF-α [14]. Previously, coxsackievirus infection was reported to be one of the contributing factors in the pathogenesis of juvenile DM [15]. Anti-MDA5 antibody-associated A/SIP may be one of the cytokine storms that contribute to viral infections such as coxsackievirus. On the other hand, alveolar macrophages, which are activated by some antigens, microbes or autoimmune stimuli, are induced to produce leukotriene B4 and IL-8 [16]. These mediators stimulate neutrophils to induce the fibrosis process in the lung. Thus, alveolar macrophage may be activated by viral infection and injury of the lung in anti-MDA5 antibody-associated A/SIP with DM. Regulation of T cells and macrophages, such as the HLH-2004 protocol [13], may be immediately necessary to improve A/SIP before lung fibrosis becomes irreversible.

In conclusion, serum ferritin correlates with disease activity of anti-MDA5 antibody-associated A/SIP with DM, suggesting that elevated levels of serum ferritin can be related to severity of A/SIP. A strong and combined

immunosuppressant therapy may be chosen according to serum ferritin level.

Conflict of interest None.

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Circulating miR-142-3p levels in patients with systemic sclerosis

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Summary

Background. Recently, increased evidence has shown that serum micro (mi)RNA levels are a useful biomarker for the diagnosis, prognosis and therapeutic value of various diseases. However, serum miRNA has not been investigated in patients with systemic sclerosis (SSc), to our knowledge.

Aim. To investigate the possibility that serum levels of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p), one of the miRNAs regulating the expression of integrin αV, could be a specific disease marker for SSc.

Methods. Serum samples were obtained from 61 patients with SSc and 20 healthy controls. Patients with systemic lupus erythematosus (SLE), dermatomyositis (DM) and scleroderma spectrum disorder (SSD), who did not fulfil American College of Rheumatology criteria for SSc but might develop SSc in the future, were included as disease controls in this study. miRNAs were purified from serum, and miR-142-3p levels were measured with a quantitative real-time PCR assay.

Results. Serum miR-142-3p levels in patients with SSc were significantly higher than in patients with SSD, SLE or DM, and healthy control groups. Patients with increased miR-142-3p levels tended to have a short sublingual frenulum.

Conclusions. Our data indicate that serum levels of miR-142-3p may be elevated specifically in patients with SSc, correlating with the severity of this disease, and may be useful diagnostic markers for the presence of SSc and for the differentiation of SSc from SSD.

Introduction

Systemic sclerosis (SSc) or scleroderma is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack and vascular damage, leading to the activation of fibroblasts and disturbed interactions with different components of the extracellular matrix (ECM).[1,2] Thus, abnormal SSc fibroblasts, which are responsible for the fibrosis, may develop from a subset of cells that have escaped from normal control mechanisms.[3,4]

Although the mechanism of activation of dermal fibroblasts in SSc is presently unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by transforming growth factor (TGF)-β1.[5] The principal effect of TGF-β1 on mesenchymal cells is its stimulation of ECM deposition. Fibroblasts from affected SSc skin cultured *in vitro* produce excessive amounts of various collagens, mainly types I and III,[6,7] and display increased transcription of corresponding genes,[8] suggesting that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF-β1 signalling.

TGF-β1 is normally secreted as a latent precursor complex, including the bioactive peptide of TGF-β1 and latency-associated peptide (LAP)-β1. TGF-β1 forms a noncovalent complex with LAP-β1, called the small

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latent complex (SLC). In this configuration, TGF-β1 is inactive because it cannot bind to its receptors. TGF-β1 activation is a complex process, involving conformational changes induced by either cleavage of LAP-β1 by various proteases such as plasmin, or by physical interactions of LAP-β1 with other proteins such as integrins and thrombospondin1, leading to the release of bioactive and mature TGF-β1.[9–12]

We previously reported that total (active plus latent) and active TGF-β1 levels in culture media are as high for SSc fibroblasts as for normal fibroblasts.[13] Thus, the activation of dermal fibroblasts in SSc may be a result of stimulation by autocrine TGF-β signalling, without increasing the concentration of active TGF-β1. To explain how the endogenous latent TGF-β1 is activated in SSc fibroblasts, we recently reported overexpression of αVβ5 and αVβ3 integrin in these cells. These integrins may recruit and activate the SLC on the cell surface of SSc fibroblasts. Recruitment and/or activation of latent TGF-β1 in the pericellular region may enhance the incidence of interaction between active TGF-β1 and its receptors, leading to the self-activation of SSc fibroblasts without increasing levels of TGF-β1.[11,14] Therefore, integrin overexpression is thought to be the most upstream event of TGF-β1 activation and collagen upregulation in SSc fibroblasts. However, the mechanism of integrin overexpression in SSc is still unknown.

In the present study, we focused on micro (mi)RNA as the regulator of integrin expression. miRNAs, short ribonucleic acid molecules, on average only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the 3′ untranslated regions (UTRs) of mRNAs, leading to gene silencing. We investigated serum levels of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) in patients with SSc. miR-142-3p can bind to the 3′ UTR of the integrin αV subunit, based on miRNA target gene predictions using TargetScan (version 5.1; http://www.targetscan.org/), a leading software program in the field.[15] Recently, there has been increasing evidence showing that serum miRNA levels are a useful biomarker for diagnosis, prognosis and therapeutic value, especially for various malignant tumours. Thus, we investigated the possibility that serum levels of miR-142-3p might be a disease marker for SSc, reflecting the overexpression of integrin αV levels.

Methods

The study was carried out in accordance with the Declaration of Helsinki. Institutional review board approval and written informed consent were obtained before participants were entered into the study.

Clinical assessment and patient material

Serum samples were obtained from 61 patients with SSc (12 men, 49 women; mean ± SD age 60.4 ± 13.2, range 29–85). All patients with SSc were grouped according to the classification system proposed by LeRoy *et al.*[16] Based on previous descriptions,[17] 23 patients had diffuse cutaneous (dc)SSc and 38 patients had limited cutaneous (lc)SSc. In addition, 12 patients diagnosed as having scleroderma spectrum disorder (SSD; 1 man, 11 women; mean ± SD age 55.4 ± 11.9, range 44–80), who did not fulfil the American College of Rheumatology criteria for SSc but might go on to develop SSc in the future based on the criteria proposed by Ihn *et al.*, were also included.[18–20]

Control serum samples were also collected from 20 healthy volunteers matched for age and gender (6 men, 14 women; mean ± SD age 44.0 ± 13.8, range 28–83). We also included serum samples from other connective-tissue disorders as disease controls; eight patients with systemic lupus erythematosus (SLE; two men, six women; mean ± SD age 38.9 ± 15.3, range 22–65) and eight patients with dermatomyositis (DM; three men, five women; mean ± SD age 64.9 ± 17.6, range 31–86).

Diagnostic method of SSD using the points system

For the diagnosis of SSD, a total score was obtained as the sum of the following five factors:[18–20] (i) extent of skin sclerosis: 10 points for truncal sclerosis, 5 points for skin sclerosis limited to the extremities and face, 3 points for sclerodactylia alone and 1 point for swollen fingers; (ii) pulmonary changes: 4 points for pulmonary fibrosis accompanied by decreased vital capacity (≤ 80%) and 2 points for pulmonary fibrosis accompanied by normal vital capacity (≥ 80%); (iii) antinuclear antibody (ANA): 5 points for positive antitopoisomerase I antibody, 3 points for positive anticentromere antibody or anti-U1 ribonucleoprotein antibody, 2 points for antinucleolar antibody and 1 point for other positive ANA; (iv) pattern of Raynaud phenomenon: 3 points for triphasic (pale purple-red), 2 points for biphasic (two of the above colours) and bilateral, and 1 point for biphasic and hemilateral, or monophasic (pale or purple only) and bilateral; and (v) nailfold bleeding (NFB): 2 points for NFB in ≥ 3 fingers, and 1 point for NFB in one or two fingers.

Conditions with ≥ 9 are consistent with SSc, and those with 5–8 points consistent with SSD.

Extraction of microRNA and quantitative real-time PCR

There has been no report demonstrating the expression of hsa-miR-142-3p in cell-free body fluid, to our knowledge. To validate that the miRNA is indeed detectable in human serum, miRNA was extracted from sera of healthy individuals, and the levels of miR-142-3p were determined by quantitative real-time PCR using a primer set specific for miR-142-3p.

Isolation of miRNA from serum samples was performed with a commercial kit (miRNeasy RNA Isolation kit; Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions with minor modification.[21] Briefly, 100 μL of serum were supplemented with 5 μL of 5 fmol/μL synthetic non-human miRNA [*Caenorhabditis elegans* miR-54 (cel-miR-54), Takara Bio Inc., Shiga, Japan] as controls, providing an internal reference for normalization of technical variations between samples. Lysis reagent (1 mL; Qiazol solution; Qiagen Inc.) was added and mixed well by vortex, then samples were incubated at room temperature for 5 min. The aqueous and organic phases were separated by the addition of chloroform. The aqueous phase was then added to columns (RNeasy and RNeasy MinElute spin columns; Qiagen Inc.), and miRNA eluted with nuclease-free water.

Complementary (c)DNA was synthesized from miRNA (Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR kit; Takara Bio Inc). Quantitative real-time PCR was carried out on a thermal cycler (Thermal Cycler Dice (TP800)®; Takara Inc.), and primers and templates were mixed with the SYBR Premix. The sequence of the hsa-miR-142-3p primer was designed based on the miRBase sequence (http://www.mirbase.org): TGTAGTGTTTCCTACTTTATGGA. DNA was amplified using 50 cycles of denaturation for 5 s at 95 °C and annealing for 20 s at 60 °C. The relative fold changes of gene expression between hsa-miR-142-3p and cel-miR-54 were calculated using standard curves.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney test for the comparison of serum miR-142-3p levels, the Student *t*-test for the comparison of means in clinical and serological features, and the Fisher exact probability test for the analysis of frequency in clinical and serological features. The Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and Statcel 97 (OMS, Tokorozawa, Japan) were used to evaluation statistical significance. $P < 0.05$ was considered significant.

Results

hsa-miR-142-3p was present in the serum

We found detectable and quantitative levels of has-miR-142-3p in the serum of healthy controls (Fig. 1). Amplification of hsa-miR-142-3p was observed, and Ct values were increased by serial dilution of the miRNA.

Serum concentrations of hsa-miR-142-3p

Serum miR-142-3p levels in patients with SSc are shown in Fig. 2. Serum miR-142-3p levels in patients with SSc were significantly higher than those in healthy control subjects or in patients with SSD, SLE or DM. When patients with SSc were classified as lcSSc or dcSSc as described in Methods, there was no significant difference between the two groups. In addition, there was no significant difference between patients with SSD and healthy controls. When the cut-off value was set as the mean + 2SD of the healthy controls, the values for all patients with SSD were below the cut-off line, whereas increased serum concentrations of miR-142-3p were still present in 32 of the 61 patients (52.5%) with SSc.

Correlation of serum miR-142-3p levels with clinical and laboratory results for patients with SSc

Table 1 shows the association of serum 142-3p levels with the clinical features in patients with SSc. Although there was no significant difference ($P = 0.08$), patients

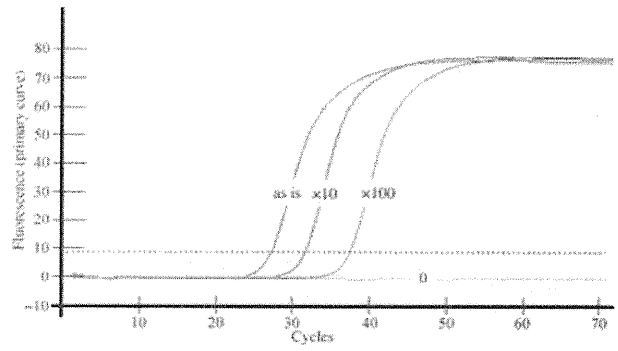

Figure 1 *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) was found in serum samples from healthy controls. Serial dilutions of cDNA (10-fold dilution, 100-fold dilation and undiluted) synthesized from serum micro RNA were used as template for real-time PCR. Amplification curves of gene-specific transcripts are shown to illustrate the process of exponential increase of fluorescence. The horizontal dotted line indicates the threshold.

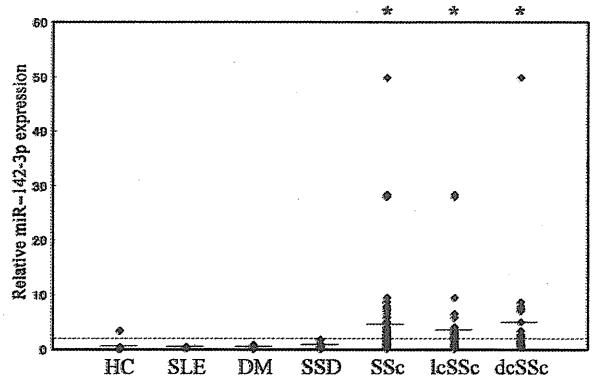

Figure 2 Serum concentrations of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) in patients with systemic sclerosis (SSc), systemic lupus erythematosus (SLE), dermatomyositis (DM) or scleroderma spectrum disorder (SSD), and in healthy control subjects (HC) measured by real-time PCR. miR-142-3p concentrations are shown on the ordinate. The horizontal dotted line indicates the cut-off levels, and bars show means. dcSSc, Diffuse cutaneous SSc; lcSSc, limited cutaneous SSc. *$P < 0.001$ compared with the values in HC, SLE, DM and SSD.

with elevated miR-142-3p levels tended to have a short sublingual frenulum. We could not find significant differences in any other clinical or laboratory features between patients with and without elevated miR-142-3p levels. Considering that a short sublingual frenulum is thought to be caused by fibrosis of the lingual frenulum, serum miR-142-3p levels may be correlated with the activity of tissue fibrosis in this disease.

Discussion

We expected that serum miRNA might be a marker in collagen disease, and that serum miR-142-3p levels would be downregulated in SSc sera because αV integrin, the target of the miRNA, is upregulated in SSc. However, contrary to our expectation, we found that serum miR-142-3p levels were significantly higher in patients with SSc than in healthy control subjects or in patients with SSD, SLE or DM. The increased serum miR-142-3p in patients with SSc may be due to negative feedback against the increased integrin αV expression in dermal fibroblasts. Alternatively, the increased miR-142-3p may contribute to the pathogenesis of this disease by downregulating other targets. This finding needs to be clarified in future studies.

To our knowledge, this study is the first to examine serum miRNA levels using both SSD and SSc sera. The concept of SSD was originally proposed by Maricq *et al.*[18] to unify the conditions of typical SSc, early forms of SSc, and closely related disorders including mixed connective-tissue disease. Later, Ihn *et al.*[19] defined patients with SSD as those who did not fulfil the criteria of SSc but might develop SSc in the future, and established a new diagnostic method using the points system described above to distinguish patients with SSD from those with early SSc. It can be difficult to distinguish early SSc from SSD, because skin sclerosis is sometimes not apparent in early SSc. Progressive fibrosis of SSc is often irreversible, at least clinically,

Table 1 Correlation of serum miR-142-3p levels with clinical and serological features in patients with systemic sclerosis (SSc).

	Patients with		*P*
	Normal 142-3p levels (*n* = 29)	Elevated 142-3p levels (*n* = 32)	
Mean age at serum sampling, years	60.8	67.4	0.29
Mean duration of disease, years	6.3	6.6	0.96
Type, diffuse/limited	12/17	11/21	0.38
m-TSS, score	11.0	11.5	0.86
Clinical features			
Pitting scars/ulcers	41.4	40.6	0.63
Nailfold bleeding	44.8	40.6	0.47
Raynaud phenomenon	79.3	78.1	0.58
Telangiectasia	24.1	21.9	0.54
Contracture of phalanges	72.4	65.6	0.39
Calcinosis	0	0	
Diffuse pigmentation	27.6	15.6	0.20
Short SF	62.1	40.6	0.08
Sicca symptoms	37.9	28.1	0.29
Organ involvement			
Pulmonary fibrosis	34.5	37.5	0.51
Mean %VC	97.7	99.2	0.79
Mean % DL_{co}	73.1	75.5	0.66
Pulmonary hypertension	13.8	15.6	0.57
Oesophagus	20.7	18.8	0.55
Heart	41.4	28.1	0.21
Kidney	0	0	–
Joints	24.1	31.2	0.37
Thrombosis	0	0	–
ANA specificity			
Topo I	17.2	25.0	0.34
ACA	34.5	43.8	0.32
U1 RNP	13.8	0.1	0.44

ACA, anticentromere antibody; ANA, antinuclear antibody; DL_{co}, diffusion capacity for carbon monooxide; m-TSS, modified Rodnan Total Skin Thickness Score; RNP, ribonucleoprotein; SF, sublingual frenulum; Topo I, anti-topoisomerase I antibody; VC, vital capacity. Unless indicated, values are percentages. *P*-values were calculated using the Fisher exact probability test or Student *t*-test.

thus, there is an urgent need to develop new strategies to diagnose patients as early as possible and allow careful monitoring. To allow this, the concept of SSD needs to be further understood and characterized. In this study, we found a significant difference in miRNA levels between patients with SSD and patients with SSc, hence, serum levels of miR-142-3p levels may be useful as the diagnostic marker for the differentiation of SSc from SSD. Moreover, we frequently encounter patients with SSD who are at with increased risk of future development of SSc. Serial time-course measurement of miR-142-3p concentration in patients with SSD might lead to early detection of developing SSc.

Although specific elevation of miR-142-3p levels in patients with SSc indicate that this miRNA plays an important role in the pathogenesis of fibrosis seen in SSc, and that serum miR-142-3p levels are correlated with disease activity, we could not find a significant correlation between serum miR-142-3p levels and the clinical features of SSc. This may be because of the small number of patients, thus larger studies are needed.

Conclusion

Our data indicate that serum levels of miR-142-3p may be elevated specifically in patients with SSc, correlating with the severity of this disease, and may be useful diagnostic markers for presence of SSc and for the differentiation of SSc from SSD.

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Adiponectin expression is decreased in the involved skin and sera of diffuse cutaneous scleroderma patients

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Abstract: In this study, we determined the adiponectin expression in the serum and lesional skin of patients with scleroderma (SSc). Serum adiponectin concentrations were measured in 32 patients with SSc, 10 patients with SLE, 12 patients with dermatomyositis patients and 13 healthy subjects with specific enzyme-linked immunosorbent assays. Adiponectin mRNA was determined in skin tissues of five patients with diffuse cutaneous SSc (dcSSc), seven patients with limited cutaneous SSc (lcSSc) and seven healthy subjects with real-time polymerase chain reaction. There was a significant reduction in serum adiponectin levels in patients with dcSSc. SSc patients with decreased serum adiponectin levels had higher total skin thickness score and higher incidence of pulmonary fibrosis. Adiponectin mRNA levels in skin tissues from patients with dcSSc were also reduced. Serum adiponectin levels may be a useful biomarker for fibrotic condition in patients with SSc. Clarifying the role of adiponectin in collagen diseases may lead to further understanding of the pathogenesis and new therapeutic approach.

Key words: dermatomyositis – scleroderma – systemic lupus erythematosus

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Background

Adiponectin, protein hormone produced by visceral and/or subcutaneous fat cells and secreted into the bloodstream, is thought to regulate metabolic processes of glucose and fatty acid through its receptor type 1 or 2 (1–3). In addition, many studies indicated adiponectin plays some roles in the inflammation, angiogenesis or tissue remodelling; adiponectin induces the production of the anti-inflammatory mediators, blood vessel growth or cardioprotective actions against pressure overload and ischaemia–reperfusion injury (4–8).

Recently, adiponectin has also attracted attention for its involvement in the pathogenesis of collagen diseases. De Sanctis et al. (9) reported that serum adiponectin levels are increased in patients with systemic lupus erythematosus (SLE); this may be associated with cardiovascular involvement of this disease. Furthermore, most recent report showed adiponectin levels are elevated in patients with rheumatoid arthritis (RA), reflecting the disease activity (10). However, no link between adiponectin and other collagen diseases such as systemic sclerosis (SSc) or dermatomyositis (DM) has been established.

Questions addressed

We tried to evaluate the possibility that serum levels of adiponectin can be a useful marker for SSc or DM.

Experimental design

Clinical assessment and patient material

Patients with SSc were grouped into diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy et al. (11,12). Patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfil the ACR criteria of SSc but were thought to develop SSc in the future, and patients with clinically amyopathic DM (CADM), who have clinically and histopathologically typical cutaneous lesions but not myositis, were also included in this study (13–16). Patients with diabetes, obesity, atherosclerosis or metabolic syndrome and those who had received treatments were excluded.

Sera were obtained from 32 patients with SSc (13 dcSSc and 19 lcSSc; seven men and 25 women; age range, 23–85 years), 10 patients with SSD (10 women; age range, 38–73 years), 10 patients with SLE (1 men and 9 women; age range, 22–68 years, SLEDAI range, 12–18), 12 patients with DM (five men and seven women; age range, 26–86 years) and five patients with CADM (one men and four women; age range, 25–78 year). Ten healthy control subjects (four men and six women; age range, 30–55 years) were also corrected. Skin samples were obtained from seven patients with dcSSc, five patients with lcSSc and seven healthy controls.

Measurement of serum adiponectin concentrations

Levels of serum adiponectin (sAdipo) were measured with a specific ELISA kit (R&D Systems) (17). Other methodologies are described in the Data S1.

Results

Serum concentrations of adiponectin

The serum adiponectin (sAdipo) levels in patients with various collagen diseases are shown in Fig. 1. The values were decreased in patients with SSc, but we could not find significant difference

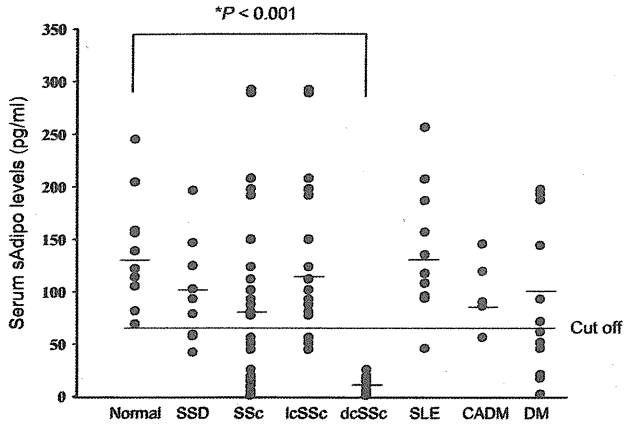

Figure 1. Serum concentrations of adiponectin in patients with collagen diseases. Serum concentrations of adiponectin (sAdipo) determined by ELISA are shown on the ordinate; the horizontal bars show the mean value in each group. The dashed line indicates cut-off, the lowest value in healthy controls. SSD, scleroderma spectrum disorder; SSc, systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; SLE, systemic lupus erythematosus; DM, dermatomyositis; CADM; clinically amyopathic dermatomyositis. *$P < 0.001$ as compared with the values in samples from normal controls.

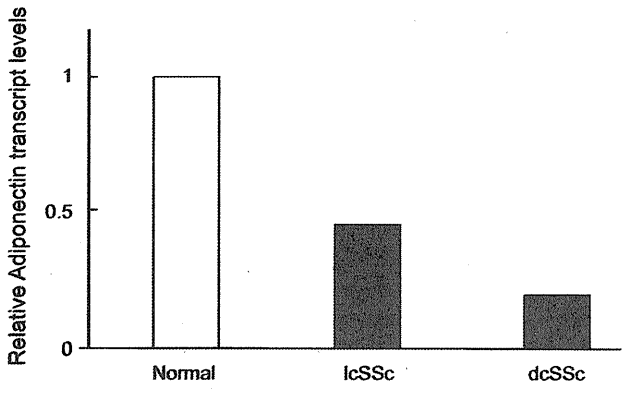

Figure 2. The mRNA expression of adiponectin in involved skin of scleroderma. Mean relative transcript levels of adiponectin in the skin tissues from seven healthy control subjects (Normal), seven patients with diffuse cutaneous scleroderma (dcSSc) and five patients with limited cutaneous scleroderma (lcSSc) were determined by real-time quantitative PCR. The mean transcript level in samples from healthy control subjects was set at 1.

between control subjects and patients with SSc (139.7 ± 54.2 vs 80.1 ± 82.7 pg/ml). However, patients with dcSSc showed significant and dramatic decrease in serum adiponectin levels compared with controls (139.7 ± 54.2 vs 11.2 ± 8.0 pg/ml, $P < 0.001$). To note, mean level in patients with SSD was intermediate between that in patients with SSc and normal controls (100.4 ± 49.3 pg/ml).

On the other hand, the increase in serum adiponectin in patients with SLE was slight compared with those in normal controls, probably due to small number of patients. Patients with DM had decreased serum adiponectin levels, although there was no significant difference compared with normal controls (90.9 ± 72.0 vs 139.7 ± 54.2 pg/ml). The mean value in patients with CADM was also intermediate between that in patients with classical DM and normal controls (100.0 ± 34.0 pg/ml).

Mean relative transcript levels of adiponectin in skin tissues from patients with dcSSc were reduced compared with the value in those from normal controls and patients with lcSSc (Fig. 2), which is consistent with the decreased serum adiponectin levels in patients with dcSSc (Fig. 1). Our results suggested that adiponectin expression is decreased at the mRNA level in the involved skin as well as in sera of patients with dcSSc.

Decreased serum adiponectin was correlated with tissue fibrosis in patients with SSc or DM

When the cut-off value was set at 69.4 pg/ml (the lowest value in healthy controls), as shown in Table S1, there were significant differences in the duration of disease (between symptom onset and first visit to the hospital) between SSc patients with reduced serum adiponectin levels and those without. Also, SSc patients with reduced value had significantly higher Rodnan total skin thickness score (m-TSS), higher prevalence of pitting scars, diffuse pigmentation, pulmonary fibrosis or anti-topoisomerase I antibody, and lower prevalence of anti-centromere antibody. There was no statistically significant difference in the incidence of other clinical or laboratory features.

On the other hand, in Table S2, DM patients with decreased serum adiponectin levels were accompanied with pulmonary fibrosis more frequently compared with those with normal levels.

Conclusions

Our study suggests the serum adiponectin levels are decreased in patients with SSc, mainly correlating with tissue fibrosis (e.g. severe skin sclerosis and pulmonary fibrosis as shown in Table S1). Decreased adiponectin mRNA in involved skin of patients with dcSSc by real-time PCR supports our hypothesis that adiponectin levels are down-regulated in fibrotic tissue. Although the hormone is well implicated in the development of type 2 diabetes, obesity, atherosclerosis or metabolic syndrome (18,19), this is the first study that correlates adiponectin with tissue fibrosis.

Furthermore, although we could not find significant difference in serum adiponectin levels between normal controls and patients with DM, the adiponectin levels were also correlated with fibrotic condition in DM; patients with decreased serum adiponectin levels were accompanied with pulmonary fibrosis. In patients with DM, adiponectin may also play a role in tissue fibrosis.

Taken together, reduced serum adiponectin levels may be a useful biomarker of fibrotic condition in collagen diseases. In addition, considering that SSD/CADM, the incomplete form of SSc/DM, showed intermediate serum value between healthy controls and classical SSc/DM, serial time-course measurement of serum adiponectin concentration in patients with SSD/CADM may lead to early detection of developing SSc/DM. Clarifying the role of adiponectin in the fibrotic condition of collagen diseases may lead to further understanding of the pathogenesis of these diseases and new therapeutic approach.

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Labour and Welfare, and by Lydia O'leary Memorial Foundation. HA performed the research, analysed the data and wrote the paper, MJ analysed the data and wrote the paper, FCM, TM, IK, KM, NH and KS contributed to correct patient samples and analysed the data, SF analysed the data, and HI designed the research study. This research was approved by the Ethics Committee in Kumamoto University (No.177).

Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Correlation of serum adiponectin (sAdipo) levels with clinical and serological features in patients with systemic sclerosis (SSc).

Table S2. Clinical and serological features of dermatomyositis patients with normal or decreased serum adiponectin (sAdipo) concentration.

Data S1. Supplemental Methods.

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Letter to the Editor

No evidence of viral genomes in whole-transcriptome sequencing of three melanoma metastases

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Abstract: Several viruses are known to cause cancer, such as human herpes virus 8 in Kaposi sarcoma and human papilloma viruses in cervical cancer. Recently, Merkel cell polyoma virus (MCPyV) has been described in 80% of Merkel cell carcinomas (MCC). Similarly to MCC and Kaposi sarcoma, melanoma incidence is increased in immunosuppressed patients. We asked whether infection by known or yet unknown viruses may play a role in melanoma development as well. To detect viral sequences expressed in melanoma cells, we analysed three melanoma metastases by whole-transcriptome sequencing and digital transcriptome subtraction. None of the samples investigated harboured viral sequences. In contrast, artificial viral sequences and MCPyV transcripts used as a positive control for the bioinformatics analysis were detected. This renders it less likely that viruses are frequently involved in melanoma induction. A larger number of melanoma transcriptome sequencings are required to rule out viruses as a relevant pathogen.

Key words: digital transcriptome subtraction – melanoma – RNA – sequencing – virus

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Background

During recent years, viruses have been found to cause cancer in different types of tissues. A vast majority of cervical carcinomas are induced by papilloma viruses (1), and a recent study was able to identify a novel polyoma virus in Merkel cell carcinoma by digital transcriptome subtraction (DTS) (2). The sequencing of four tumors sufficed to identify this novel virus, later named Merkel cell polyoma virus (MCPyV). Subsequent studies screening larger

*These authors contributed equally to the study.

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Myositis-Specific Anti-155/140 Autoantibodies Target Transcription Intermediary Factor 1 Family Proteins

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Objective. **To identify the 140-kd autoantigen recognized by anti-155/140 autoantibodies that are associated with adult cancer-associated dermatomyositis (DM) and juvenile DM and to determine the clinical relevance of anti-155/140 antibodies in a large cohort.**

Methods. **Sera from 456 DM patients were assessed for the presence of anti-155/140 antibodies by immunoprecipitation using K562 cell extracts as substrate. Using immunoprecipitation and Western blotting, we then examined whether anti-155/140–positive sera recognized transcription intermediary factor 1α (TIF-1α), TIF-1β, and TIF-1γ. The clinical associations of antigen reactivity were also evaluated.**

Results. **Anti-155/140–positive sera reacted with 140-kd TIF-1α in addition to 155-kd TIF-1γ. Among sera from 456 DM patients, 52 were reactive with both TIF-1α and TIF-1γ, while another 25 were reactive with TIF-1γ alone. Additionally, 7 were reactive with TIF-1β. Malignancy was more frequently found in adult patients with both anti–TIF-1α and anti–TIF-1γ antibodies than in those with anti–TIF-1γ antibodies alone (73% versus 50%; $P < 0.05$). In addition to juvenile DM patients and middle-aged and older DM patients with high percentages of malignancy, 8 "young adult" DM patients without malignancy had these autoantibodies.**

Conclusion. **Anti-155/140 antibodies target TIF-1 family proteins, TIF-1α and TIF-1β, in addition to TIF-1γ. Since TIF-1 proteins have significant roles in oncogenesis, these antibodies may be produced during misdirected antitumor immunity.**

Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory disorders that mainly affect the muscle and/or skin (1). Clinical manifestations of PM/DM are heterogeneous, with varying degrees of myositis, skin rash, and accompanying symptoms such as interstitial lung disease and internal malignancy. The association of malignancy with PM/DM, which is termed cancer-associated myositis, is well appreciated, particularly in patients with DM (2–6). Since malignant disease is one of the main causes of mortality in these patients, diagnosing occult cancer in them is important and challenging for clinicians.

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Drs. Fujimoto and Takehara have a patent application pending in Japan for diagnostic tools for measuring anti–transcription intermediary factor 1α antibodies.

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PM and DM are considered to be autoimmune diseases. Patients with PM/DM frequently have autoantibodies that target nuclear and/or cytoplasmic antigens. Intriguingly, these autoantibodies are highly specific to PM/DM, appear mutually exclusively, and are closely associated with specific clinical phenotypes within the disease (7–10). Therefore, these myositis-specific autoantibodies (MSAs) are useful tools to define more homogeneous clinical subsets in PM/DM. These include autoantibodies to aminoacyl–transfer RNA synthetases, anti–Mi-2 antibodies, and anti–signal recognition particle antibodies (7–10). In the past several years, new MSAs have been described and characterized, such as anti-CADM140 (anti–melanoma differentiation–associated protein 5 [anti–MDA-5]) antibodies (11,12) and anti–NXP-2 (anti-MJ; anti-p140) antibodies (13). Furthermore, Targoff et al and we have reported anti-p155 antibodies and anti-155/140 antibodies, respectively, which are linked to cancer-associated DM (14,15).

Targoff and colleagues reported that anti-p155 antibodies react with a 155-kd nuclear protein, transcription intermediary factor 1γ (TIF-1γ) (16). While "anti-155/140 antibodies" are assumed to be identical with "anti-p155 antibodies," the 140-kd antigen has not yet been determined. In this study, we have confirmed that "anti-155/140 antibodies" and "anti-p155 antibodies" are the same, since anti-155/140 antibodies also reacted with TIF-1γ. Furthermore, we have demonstrated that the 140-kd antigen is TIF-1α. In addition, a portion of the sera positive for anti-155/140 antibodies were also directed at another TIF-1 family protein, TIF-1β. Thus, anti-155/140 autoantibodies target TIF-1 family proteins that have significant roles in oncogenesis. We also clarified the clinical correlation in a large cohort of patients.

PATIENTS AND METHODS

Patients. Serum samples were obtained from 456 Japanese patients with DM who were consecutively followed up at the Department of Dermatology at Kanazawa University Hospital and collaborating medical centers between 2003 and 2010. Among the 456 DM patients, 373 fulfilled the criteria of Bohan and Peter (17,18); the remaining 83 patients did not, but instead fulfilled Sontheimer's criteria (19) because of the absence of clinical muscle symptoms and the presence of typical DM skin symptoms. These 83 patients were therefore diagnosed as having clinically amyopathic DM. Patients classified as having clinically amyopathic DM included patients with amyopathic DM and patients with hypomyopathic DM. Patients with hypomyopathic DM had DM rashes and subclinical evidence of myositis on electrophysiologic, radiographic, and/or laboratory evaluation (20). Eleven patients were categorized as having juvenile DM, and the other 445 were categorized as having adult DM. Twenty-five patients with anti-155/140 antibodies who were reported previously (21) were included in this study. We assessed 62 patients with PM, 108 with systemic lupus erythematosus (SLE), and 433 with systemic sclerosis (SSc) as disease controls. Sequential serum samples were obtained from 8 DM patients positive for anti-155/140 antibodies (1 with juvenile DM and 7 with adult DM; 4 of the 7 adults had cancer-associated DM).

Clinical information on all patients was collected retrospectively by reviewing their clinical charts. Internal and hematologic malignancies in DM patients were defined using criteria described previously (7). Malignancy was recorded when it was diagnosed within 3 years of the diagnosis of DM. The protocol was approved by Kanazawa University Graduate School of Medical Sciences and Kanazawa University Hospital.

Reagents. Rabbit anti-human TIF-1α (TRIM24), goat anti-human TIF-1β (KAP1, TRIM28), and rabbit anti-human TIF-1γ (TRIM33) polyclonal antibodies were purchased from Abcam. Recombinant proteins used in this study were human full-length TIF-1α protein with glutathione S-transferase (GST) tag (Abnova), human full-length TIF-1β protein with GST tag (Abnova), and human full-length TIF-1γ protein (Origene).

Immunoprecipitation (IP). IP assays were performed using extracts of the leukemia cell line K562 (21). A total of 10 μl of the patient's serum was bound to 2 mg of protein A–Sepharose beads (Amersham Biosciences) in 500 μl of IP buffer (10 m*M* Tris HCl [pH 8.0], 50 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, followed by washing 5 times with IP buffer. Antibody-coated Sepharose beads were mixed with 100 μl ^{35}S-methionine–labeled or unlabeled K562 cell extracts derived from 10^6 cells and rotated at 4°C for 2 hours. After 5 washes, the beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer, and samples were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) followed by autoradiography or Western blotting.

Immunodepletion. Polyclonal antibodies to TIF-1α, TIF-1β, and TIF-1γ (3 μg) were conjugated with protein A–Sepharose beads by incubating for 2 hours at 4°C. These polyclonal antibody–conjugated Sepharose beads were then mixed with ^{35}S-labeled K562 cell extracts and rotated at 4°C for 2 hours. The supernatant was then further incubated with Sepharose beads conjugated with serum autoantibodies that recognized TIF-1 protein(s), as described above. After 5 washes, immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

Western blotting. Proteins immunoprecipitated from K562 extracts or 1 μg of recombinant TIF-1α, TIF-1β, and TIF-1γ proteins were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membrane. After blocking, membranes were incubated with serum samples diluted to 1:100 or with polyclonal antibodies, followed by incubation with horseradish peroxidase–conjugated anti-rabbit IgG (Thermo Scientific), anti-goat IgG (Santa Cruz Biotechnology), or anti-human IgG (MP Biomedicals) antibodies. The membranes were developed using an enhanced chemiluminescence kit (Thermo Scientific).

Enzyme-linked immunosorbent assay (ELISA). Relative levels of serum autoantibodies to TIF-1α and TIF-1γ