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INTERNAL MEDICINE

□ CASE REPORT □

The Clinical Characteristics of Two Anti-OJ (Anti-Isoleucyl-tRNA Synthetase) Autoantibody-Positive Interstitial Lung Disease Patients with Polymyositis/Dermatomyositis

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Abstract

We herein report the clinical and laboratory characteristics of two anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibody-positive interstitial lung disease patients with polymyositis/dermatomyositis (PM/DM). We compared these characteristics with previously published findings. Previous reports and our present cases show that anti-OJ autoantibody-positive interstitial lung disease (ILD) patients with PM/DM lack the manifestations of Raynaud's phenomenon and sclerodactyly and show good prognoses and responses to glucocorticoid therapy. These results indicate that the presence of anti-OJ autoantibodies may be useful for predicting the prognosis of ILD and its clinical course in PM/DM patients.

Key words: interstitial lung disease, polymyositis/dermatomyositis, anti-aminoacyl-tRNA antibodies

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Introduction

Anti-aminoacyl-tRNA synthetase (anti-ARS) autoantibodies are generally found in patients with polymyositis (PM) and dermatomyositis (DM), particularly in those with associated interstitial lung disease (ILD) (1). Autoantibodies directed against aminoacyl-tRNA synthetases are found in approximately 25% to 35% of patients with PM and DM (2). ARS comprises a family of cytoplasmic enzymes that catalyze the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA and play a crucial role in protein synthesis (3). Eight classes of autoantibodies that react with ARS have been recognized: anti-histidyl (anti-Jo-1), anti-threonyl (anti-PL-7), anti-alanyl (anti-PL-12), anti-glycyl (anti-EJ), anti-isoleucyl (anti-OJ), anti-asparaginyl (anti-KS), anti-phenylalanyl (anti-Zo) and anti-tyrosyl-tRNA (anti-YRS) synthetases (4-6). Anti-Jo-1 antibodies were the first of these antibodies to be discovered and are detectable in approximately 20% to 30% of PM/DM patients (7). Anti-OJ antibodies appear to be associated with clinical manifestations that are generally similar to those of other antisynthetases, including myositis, ILD and Raynaud's phenomenon. In previous studies, anti-OJ antibodies were found in fewer than 2% of all patients with PM/DM (8). Sato et al. (9) reported the clinical characteristics of seven Japanese patients with anti-OJ autoantibodies. These patients lacked any manifestations of Raynaud's phenomenon or sclerodactyly and suffered from ILD associated with PM. This group showed that anti-OJ antibodies are a clinically important

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Table 1. Laboratory and Radiological Data

Patient number	1	2
CRP (0.00-0.20 mg/dL)	0.73	8.65
LDH (120-240 IU/L)	630	267
CK (40-185 IU/L)	874	413
ALD (2.1-6.1 U/L)	20.6	8.2
ANA	160× cytoplasm pattern	640 × cytoplasm pattern
Anti-SS-A Ab	negative	negative
Anti-mitochondrial Ab	negative	negative
Anti-OJ Ab	positive	positive
KL-6 (105-401 U/mL)	2,766	614
SP-D (<110.0 ng/mL)	128	250
SP-A (<43.8 ng/mL)	175	146
%VC	50	78.1
%DLco	53.6	64
electromyogram	myogenic	myogenic
muscle biopsy	not performed	myositis
BALF		
Recovery rate (%)	36.7	38
Total cell counts (/μL)	500	600
Differential cell counts (%)		
Neutrophils	10	2
Lymphocytes	62	41
Macrophages	28	57
HRCT findings	bilateral consolidation ground glass opacities liner opacities	bilateral consolidation ground glass opacities traction bronchiectasis
distribution	lower lobe peribronchovascular	lower lobe peribronchovascular and peripheral

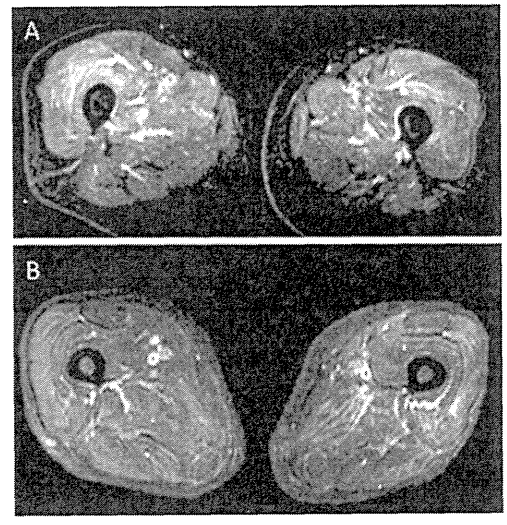

Figure 1. Lower limb muscle MRI in three patients. Fat-suppressed T2-weighted imaging in Case 1 showing (A) diffuse, abnormally high signals in the bilateral quadriceps and in Case 2 showing (B) diffuse, abnormally high signals in the bilateral quadriceps.

marker of a specific subset of anti-ARS syndromes. However, there are still very few reports regarding the clinical characteristics of PM/DM patients with anti-OJ autoantibodies; therefore, collecting cases to identify the clinical features is necessary. We herein present the clinical characteristics of two anti-OJ autoantibody-positive ILD patients with PM/DM.

Case Reports

Case 1

A 67-year-old woman was admitted to our hospital with a 2-month history of dry cough, slight fever and worsening dyspnea on exertion. She also complained of mild myalgia in both thighs. One month earlier, an abnormal chest roentgenogram was found, and she was referred to our hospital for extensive testing. She had a history of diabetes mellitus, which was treated with diet therapy, and carpal tunnel syndrome. She was on no medications. She had never smoked and had no history of alcohol use or pet ownership. She had a family history of diabetes mellitus and liver disease; however, she did not have a family history of connective tissue disease (CTD), rheumatic diseases, collagen vascular diseases or systemic autoimmune diseases. She lived with her husband in an old wooden house that had been standing for 40 years.

A physical examination showed scaly erythema on the dorsum of the hands (Gottron's sign), mechanic's hand and spotty hemorrhages in some nail fold capillaries. Chest auscultation revealed fine crackles in the lower aspects of both lungs. On neurological examination, the patient's Manual Muscle Test (MMT) scores were 4 in the infraspinatus and iliopsoas muscles and 5 in the other muscles. The findings of other neurological assessments were normal, including those of the cranial nerves, sensory function and tendon reflexes. The laboratory findings and results of the pulmonary function tests are shown in Table 1. Magnetic resonance imaging (MRI) of the bilateral thighs showed diffuse, abnormally high T2-weighted imaging (T2WI) signals (Fig. 1A). Findings from needle electromyography of the right quadriceps indicated the presence of myogenic changes with concomitant active denervation. Chest X-ray and high-resolution computed tomography (HRCT) findings of the chest are shown in Figs. 2A, 3A, 3B. HRCT showed bilateral consolidation predominantly distributed along the bronchovascular bundles in both lower lobes and ground glass attenuation. Bronchoalveolar lavage (BAL) evaluation was performed (Table 1), which showed lymphocyte predominance. A Gram stain of the BAL fluid (BALF) was negative. It was difficult to perform a histopathological examination of the muscles or lungs due to deterioration of the patient's respiratory symptoms. These findings led us to diagnose the patient with interstitial pneumonia associated with DM. A fluorescent antinuclear antibody test was positive and showed a cytoplasmic pattern. An anti-Jo-1 antibody test was negative; therefore, we assayed other anti-ARS autoantibodies with an

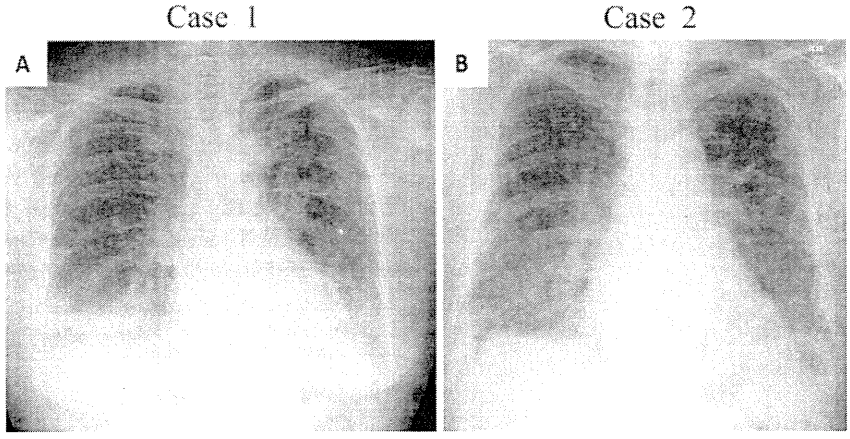

Figure 2. Chest radiographs. Case 1: (A) bilateral consolidation and ground glass attenuation with volume loss in both lower lobes; Case 2: (B) bilateral consolidation and ground glass attenuation with volume loss in both lower lobes.

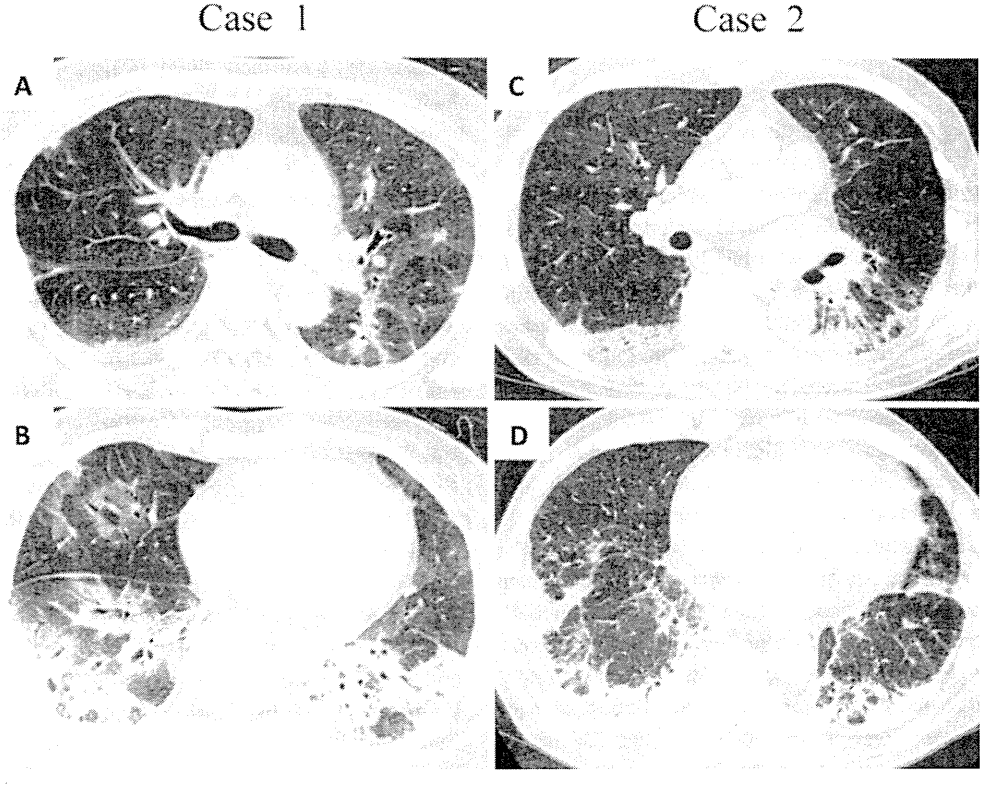

Figure 3. High-resolution chest CT scans. Case 1: (A, B) bilateral consolidation predominantly distributed along the bronchovascular bundles with volume loss in both lower lobes and areas of ground glass attenuation and liner opacities; Case 2: (C, D) bilateral consolidation predominantly distributed in peripheral areas with volume loss in both lower lobes and areas of ground glass attenuation, intralobular reticular opacities and traction bronchiectasis.

RNA immunoprecipitation assay and found that the anti-OJ antibody test was positive. The patient was treated with high-dose methylprednisolone (1 g/day intravenously for three days) followed by oral prednisolone (1 mg/kg/day) and cyclosporine (5 mg/kg/day). Her clinical condition and chest radiography findings showed remarkable improvements after 12 months of treatment.

Case 2

A 74-year-old man was admitted to our hospital with a 1-month history of fever, fatigue and worsening dyspnea on exertion. Two weeks prior to admission, he noticed a pro-

Table 2. Clinical Characteristics

Patient number	1	2
Gender	female	male
Age	67	74
Diagnosis	DM	PM
initial symptoms		
cough	+	+
dyspnea	+	+
arthritis	-	-
weight loss	-	-
muscle weakness	+	-
fever	-	+
myositis	+	+
Rash	+	-
Raynaud's phenomenon	-	-
abnormal capillaries of nail folds		
micro hemorrhage	+	+
enlarged capillary	-	-
sicca	-	-
mechanic's hand	+	-
	Gottron sign (+)	
treatment	PSL pulse→1.0mg/kg	PSL 1.0mg/kg
	CyA	
effect	Improved	Improved

ductive cough and was seen at the nearest clinic. He was diagnosed with bronchitis and treated with levofloxacin and a combination cold remedy. However, his clinical condition did not improve and instead gradually deteriorated. Therefore, he was referred to our hospital for extensive testing. He had a history of atrial fibrillation, mitral regurgitation, mitral stenosis and diabetes mellitus and had been hospitalized seven years earlier for approximately three months with infectious endocarditis caused by Group A *Streptococcus*. His medications included metildigoxin, fulosemide, warfarin, amlodipine, candesartan and glimepiride. He had never smoked and had no history of alcohol use or pet ownership. He had no family history of collagen or muscle disease.

On physical examination, he was found to have cornification, but no erythema, spread in a symmetric fashion over the metacarpophalangeal joints along with spotty hemorrhages in some nail fold capillaries; however, no muscle weakness was observed. Cardiac auscultation revealed a systolic murmur (Levine III/VI), and chest auscultation showed fine crackles in the lower aspects of both lungs. On neurological examination, the MMT scores were full in all muscles. The findings of other neurological assessments were normal. The laboratory findings and results of the pulmonary function tests are shown in Table 1. Although the patient did not show any muscle symptoms, an MRI study of the bilateral thighs showed diffuse, abnormally high T2-WI signals (Fig. 1B). Findings from needle electromyography of the right quadriceps indicated the presence of myogenic changes with concomitant active denervation, and a muscle biopsy of the left quadriceps revealed myositis. The findings of chest X-ray and HRCT of the chest are shown in Figs. 2B, 3C, 3D. HRCT showed bilateral consolidation predominantly distributed along the bronchovascular bundles in both lower lobes and ground glass attenuation with traction bronchiectasis. A BAL analysis was performed (Table 1), which showed lymphocyte predominance. These findings led us to diagnose the patient with interstitial pneumonia associated with PM. Later, an anti-OJ antibody test was shown to be positive. The patient was treated with oral prednisolone (1 mg/kg/day). His clinical condition and chest radiography findings showed remarkable improvements, and no deteriorations were observed over a 3-year treatment period.

Discussion

Previous studies have indicated that ILD is associated with high mortality in PM/DM patients. ILD occurs in as many as 53% of PM/DM patients (10) and may lead to life threatening complications, including ventilator failure and secondary pulmonary hypertension (11). Anti-ARS antibodies are directed against cytoplasmic enzymes that catalyze the formation of the aminoacyl-tRNA complex from an amino acid and its cognate tRNA (3). Marguerie et al. (12) described a series of 29 PM/DM patients with anti-ARS antibodies who were more likely to have fevers, dyspnea, mechanic's hands, arthritis and ILD than those without such antibodies. Yoshifuji et al. (10) stratified 74 PM/DM patients into anti-ARS-positive (including two anti-OJ-positive patients) and -negative groups and found that ILD and mechanic's hand occurred significantly more frequently in the anti-ARS-positive group.

To date, eight antisynthetase antibodies have been identified, and anti-Jo1, anti-PL7 and anti-PL12 antibodies are the strongest markers for ILD; anti-Jo1 antibodies are the most common (13, 14). Anti-OJ autoantibodies are a type of anti-ARS antibodies originally identified after the original sera from "patient OJ" showed significant inhibition of isoleucyl-tRNA synthetase activity (15). To the best of our knowledge, two reports have been published regarding the clinical significance of anti-OJ autoantibodies in ILD patients with PM/DM (9, 16). Additionally, there are seven reports describing the clinical significance of anti-OJ autoantibodies (9, 10, 16-20). Sato et al. (9) screened 1,135 Japanese patients who had or were suspected of having CTDs and detected seven patients positive for anti-OJ autoantibodies (four with PM, three with IIPs), all of whom had ILD. These results suggested that the presence of anti-OJ autoantibodies is more closely associated with ILD than myositis. Targoff et al. (16) reported the cases of six anti-OJ autoantibody-positive PM/DM patients with ILD; however, he did not describe the details of these cases, including the symptoms observed on first visit and the responsiveness to the therapy. The characteristics of our present two patients are summarized in Table 2, and previous reports and our cases are summarized together in Table 3. Yoshifuji et al. (10) reported that, in anti-ARS autoantibody-positive PM/DM patients, the development of ILD often precedes that of myopathy. Our patients experienced respiratory symptoms that preceded the development of other symp-

Table 3. Literature Review and Our Cases

	Sato et al.[9]	Targoff et al.[16]	Friedman et al.[20]	other reports[17-19]	our cases	Total
No patients	7	9	2	3	2	**23**
Age at onset, mean±S.D. years	53±18	52±13	36, 50	54±8	67, 74	**53±11**
No male/no. female	3/4	4/5	1/1	0/3	1/1	**9/14**
Dignosis						
PM/DM with ILD	3/7	6/9	0/2	0/3	2/2	**11/23(47.8%)**
PM,RA with ILD	1/7	0/9	0/2	0/3	0/2	**1/23(4.3%)**
IIPs	3/7	3/9	2/2	3/3	0/2	**11/23(47.8%)**
fever	3/7	NA	0/2	0/3	1/2	**4/14(2.9%)**
ILD	7/7	8/9	2/2	3/3	2/2	**22/23(95.7%)**
arthritis	4/7	6/7*	1/2	2/3	0/2	**13/21(61.9%)**
Raynaud's phenomenon	0/7	1/7*	0/2	1/3	0/2	**2/21(9.5%)**
myositis	4/7	8/9	0/2	1/3	2/2	**15/23(65.2%)**

PM: polymtositis, DM: dermatomyositis, RA: rheumatoid arthritis, IIPs: idiopathic intestitial pneumonias

* a part of data were not available. NA, data not a vailable

The diagnosis of IIPs (idiopathic interstitial pneumonias) was based on consensus classification of IIPs.[10]

toms. On physical examination, neither sclerodactyly, Raynaud's phenomenon nor sicca were found in either patient. Although Raynaud's phenomenon is often associated with the presence of anti-Jo-1 antibodies, it may not occur in patients with anti-OJ antibodies (Table 3).

The response of ILD to glucocorticoid therapy is significantly better in anti-ARS positive PM/DM patients than in anti-ARS negative patients. Three of the four anti-OJ autoantibody-positive ILD patients with PM reported by Sato et al. also responded to glucocorticoid therapy. Both of our patients also showed good clinical responses following treatment with high-dose glucocorticoids without immunosuppressants. There may be a tendency for ILD patients with anti-OJ autoantibodies to show good prognoses and good responses to glucocorticoid monotherapy. Although ILD has a tendency to recur in anti-ARS autoantibody-positive patients (10), in both of the present cases, the ILD remained static since the first treatment.

Performing a histopathological analysis of ILD is often difficult in patients with clinical symptoms due to the risk of deterioration. Although histopathological analyses could not be performed in the present cases, the CT findings suggested a diagnosis of ILD. To the best of our knowledge, there have been few detailed reports regarding the chest CT findings of ILD in PM/DM patients with anti-OJ autoantibodies. Both of our cases showed similar findings on chest HRCT, including bilateral consolidation and ground glass attenuation with volume loss in the lower lobes and peribronchovascular predominance. Koreeda et al. (17) reported the chest HRCT findings of one anti-OJ autoantibody-positive ILD patient without PM/DM, which included bilateral ground glass opacities, consolidations volume loss in the lung bases and peribronchovascular predominance. These reported findings are similar to the HRCT findings observed in our patients. Such findings may be associated with the presence of anti-OJ autoantibodies.

As described above, there are no reports regarding the detailed radiological and histopathological findings of ILD in PM/DM patients with anti-OJ autoantibodies; however, some reports have described the details of radiological and histopathological analyses of idiopathic interstitial pneumonia (IIPs) patients with anti-OJ autoantibodies. The results of a previous histopathological analysis of six IIP patients with anti-OJ autoantibodies (9, 16-18) revealed an NSIP pattern in two of the six patients, a cryptogenic organizing pneumonia pattern in two patients, an UIP pattern in one patient and no diagnosis in one patient. Unfortunately, in our series, we could not perform video-assisted thoracoscopic surgery (VATS) due to a deterioration of respiratory symptoms. However, the chest HRCT findings were not typical of IPF/UIP, as in previous reports. The histopathological analysis described above may indicate good responses to steroid therapy in ILD patients with anti-OJ autoantibodies (21).

Our results and those of previous reports suggest that anti-OJ autoantibody-positive PM/DM patients with ILD exhibit distinct features compared to ILD patients with other anti-ARS antibodies. The course of ILD in such patients may show a good response to glucocorticoid therapy and be associated with a good prognosis, as in other anti-ARS antibody-positive patients (10). Lymphocyte-predominant BAL and HRCT findings of bilateral consolidation predominantly distributed along the bronchovascular bundles in both lower lobes and areas with ground glass attenuation and linear opacities may be clinical features of anti-OJ autoantibody-positive PM/DM patients with ILD. In such patients, ILD has a tendency to follow a subacute pattern and not become a chronic disease. Although anti-OJ autoantibody screening tests are not available, except in some research institutes, testing for anti-OJ autoantibodies is very important clinically because it may distinguish subtypes of both PM/DM and anti-ARS syndromes and predict a patient's prognosis. The clinical properties of ILD patients with PM/DM may be classified according to the presence or absence of anti-OJ autoantibodies. Further accumulation and analysis should be conducted to clarify whether anti-ARS autoantibodies truly affect the prognoses of ILD patients

with PM/DM and distinguish between the differences observed among types of anti-ARS autoantibody-positive ILD.

Author's disclosure of potential Conflicts of Interest (COI).
Ishida T: Honoraria, Abott, Taisyo-Toyama and Daiichi-Sankyo.

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Low Prevalence of Anti-DFS70/LEDGF Antibodies in Patients with Dermatomyositis and Other Systemic Autoimmune Rheumatic Diseases

To the Editor:

We read with interest the article by Mahler, *et al*[1]. They screened a large number of sequential serum samples and sera from patients with various conditions as well as healthy individuals for anti-dense fine speckled 70 autoantibodies (anti-DFS70ab). Their data confirmed the observations that anti-DFS70ab are significantly more prevalent in healthy individuals than in patients with systemic autoimmune rheumatic diseases (SARD) and that, when they are positive, anti-DFS70ab in SARD are usually accompanied by additional SARD-related antibodies. However, their SARD cohort did not include patients with dermatomyositis (DM). We have confirmed that anti-DFS70ab were less prevalent in our DM cohort than in healthy individuals[2]. We also found some anti-DFS70ab-positive patients with DM who exhibited interesting patterns of change in autoantibody titers in longitudinal sera.

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 116 Japanese patients with DM, 108 of which were used in our previous study on anti-TIF1-γ/α (155/140) and anti-Mi-2 autoantibodies[3]. The sera were from 103 patients with adult-onset DM [35 with clinically amyopathic DM, 17 with cancer-associated DM, and 51 with classical DM] and 13 with juvenile DM (6 with clinically amyopathic DM and 7 with classical DM). The definitions of DM, clinically amyopathic DM, cancer-associated DM, and juvenile DM were based on the criteria used in our previous studies[3,4]. The ages at disease onset were 1 to 80 years (mean 47 yrs). Eighty-one patients were female and 35 were male. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and complied with the Declaration of Helsinki guidelines.

Antinuclear antibody testing was performed by indirect immunofluorescence (IIF) on HEp-2 cell substrates (MBL Co. Ltd.)[2], and anti-DFS70ab levels were measured by ELISA (MBL). DM-specific autoantibodies including anti-Mi-2, anti-TIF1-γ/α, anti-MDA5 (CADM-140), and anti-NXP-2 (MJ) antibodies were measured by the ELISA we developed, which uses *in vitro* transcription and translation recombinant protein[3,4,5].

The anti-DFS70ab ELISA found 7 positive patients in our DM cohort (6.4%; Figure 1A). Patients were confirmed to have anti-DFS70ab by immunoblotting analysis with bacterially expressed recombinant protein[2], although 2 of them did not show the characteristic DFS pattern in IIF studies (data not shown). Their clinical features are summarized in Table 1. The findings for the anti-DFS70ab-positive patients did not differ markedly from those for the anti-DFS70ab-negative patients with DM. The frequency of anti-DFS70ab in each DM subset (clinically amyopathic DM, cancer-associated DM, adult classical DM, and juvenile DM) was 9%, 6%, 2%, and 15%, respectively. Five of 7 DM patients with anti-DFS70ab had additional DM-specific autoantibodies. Anti-MDA5 antibodies were found in only 3 anti-DFS70ab-positive patients compared to 28 anti-DFS70ab-negative patients. Anti-TIF1-γ antibodies were present in 1 anti-DFS70ab-positive patient, and anti-NXP-2 antibodies were present in another anti-DFS70ab-positive patient, while they were present in 18 and 5 anti-DFS70ab-negative patients, respectively. Anti-TIF1-α and anti-Mi-2 antibodies were not found. In the other 2 patients with anti-DFS70ab, no DM-specific autoantibodies were detected, although other myositis-specific autoantibodies such as anti-tRNA synthetase antibodies including anti-Jo-1, anti-PL-7, anti-PL-12, anti-EJ, and anti-KS were also investigated. However, serum from one of them revealed many unidentified polypeptides by immunoprecipitation using radiolabeled cell extract (data not shown). These data confirm that, in DM as well, anti-DFS70ab are rarely observed and that, when they are positive, they are usually accompanied by additional DM-specific autoantibodies.

Autoantibodies against MDA5 are a serological marker for DM, especially for clinically amyopathic DM complicated with rapidly progressive interstitial lung disease (ILD), often resulting in poor

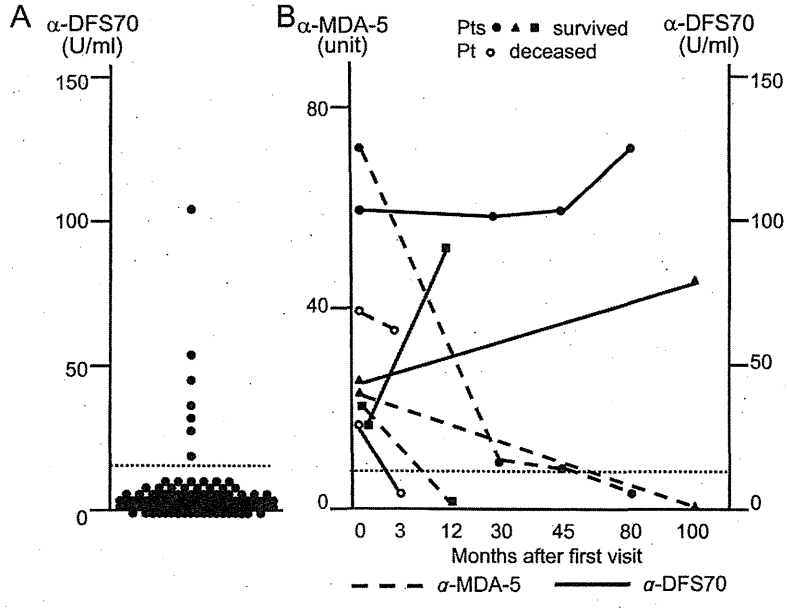

Figure 1. A. Anti-DFS70 antibodies in patients with dermatomyositis, as determined by ELISA. B. Titer changes of anti-DFS70 and anti-MDA5 antibodies in 4 patients with dermatomyositis; 3 survived and 1 died of rapidly progressive interstitial lung disease. Anti-DFS70ab increased in the 3 surviving patients but decreased in the deceased patient. Dotted line represents cutoff values of anti-DFS70 antibodies (15 U/ml) and anti-MDA5 antibodies (6.5 units). Anti-DFS70ab: anti-dense fine speckled 70 autoantibodies.

Table 1. Clinical characteristics of dermatomyositis (DM) patients with anti-DFS70 antibodies in our cohort.

Patient	Sex/Age, yrs	Diagnosis	Complication	Prognosis	Skin Symptoms H	G	C	Elevation of CK*	Anti-DFS70 Titer, U/ml	DM Marker Autoantibodies	Other Autoantibodies
1	F 25	CADM	—	Surviving	+	+	–	–	101.4	MDA5	p80-coilin[8]
2	F 23	CADM	ILD	Surviving	+	+	–	+/–	43.2	MDA5	—
3	F 46	CADM	ILD	Dead	+	+	–	+/–	29.7	MDA5	—
4	F 68	Cancer-associated DM	Ovarian carcinoma	Dead	+	+	–	+	28.0	NXP-2	—
5	M 1	Juvenile DM	—	Surviving	–	+	+	+	50.4	TIF1-γ	—
6	F 6	Juvenile DM	Atopic dermatitis	Surviving	+	+	–	–	30.2	Unidentified	—
7	F 46	Classical DM	—	Surviving	+	–	–	+	18.9	—	—

* Maximum elevation of creatine kinase (CK) is shown; +: more than 1.5-fold of upper limit; +/–: within 1.5-fold of upper limit; –: within upper limit. H: heliotrope rash; G: Gottron's sign; C: calcinosis; CADM: clinically amyopathic dermatomyositis; ILD: interstitial lung disease.

prognosis[6]. Recently, we showed that anti-MDA5ab disappeared in clinically amyopathic DM complicated with ILD during disease remission[5]. In our DM cohort, we had 3 patients with both anti-DFS70ab and anti-MDA5ab. In addition, we recently encountered another patient with juvenile DM complicated with ILD who had both autoantibodies. Although 1 patient died from rapidly progressive ILD, the other 3 patients (1 juvenile DM patient with rapidly progressive ILD, 1 with chronic ILD, and 1 with amyopathic DM without ILD) have survived. In the deceased patient, the anti-MDA5ab level did not change during the therapy, whereas anti-DFS70ab changed to become negative (Figure 1B). In contrast, in the 3 surviving patients, anti-MDA5ab disappeared upon remission of ILD and/or ADM, whereas anti-DFS70ab levels increased. The hypothesis from the Mahler group that anti-DFS70ab serve as "protective autoantibodies"[7] is very attractive. The patterns of change in anti-DFSab titers in the present 4 anti-MDA5-positive patients with DM (1 deceased, 3 surviving) may support the idea of a protective nature of anti-DFS70ab. The higher prevalence of anti-DFS70ab in healthy individuals than in patients with SARD[1,2] may support this hypothesis. Such a possibility regarding anti-DFS70ab should be explored by future study.

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Concise report

Disappearance of anti-MDA-5 autoantibodies in clinically amyopathic DM/interstitial lung disease during disease remission

Yoshinao Muro[1], Kazumitsu Sugiura[1], Kei Hoshino[1] and Masashi Akiyama[1]

Abstract

Objective. Autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) are one of the serological markers for DM. Anti-MDA-5 antibodies are especially associated with rapidly progressive interstitial lung disease (ILD) in amyopathic DM (ADM). It is known that the antibody status of anti-ENAs does not generally change significantly with disease course. For anti-MDA-5 antibodies, however, few longitudinal studies have investigated such changes. This study aimed to establish a quantitative assay for anti-MDA-5 antibodies towards assessing the long-term outcome of ADM patients who had anti-MDA-5 antibodies.

Methods. We established ELISA for measuring anti-MDA-5 antibody levels using *in vitro* transcription and translation recombinant protein. The antibody levels were measured at different time points in 11 clinically ADM patients who tested positive for the anti-MDA-5 antibody on their first visit (range of follow-up 3 months to 16 years).

Results. At the stage of clinical remission, six patients received no medication and the four others received low-dose CS. ELISA showed that anti-MDA-5 antibodies disappeared in nine of the patients and fell to just above the cut-off in one patient; in the patient who died, the antibodies remained.

Conclusion. Our results suggest that anti-MDA-5 antibodies may be useful as a marker for monitoring disease activity in ILD complicated with ADM. Serial monitoring at short intervals is required to evaluate whether anti-MDA-5 antibody levels correlate with ADM disease activity.

Key words: amyopathic dermatomyositis, anti-MDA-5 antibody, interstitial lung disease, prognosis.

Introduction

Myositis-specific autoantibodies are useful for diagnosing PM/DM. DM-specific autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) and transcriptional intermediary factor 1-γ are particularly important, because they are closely associated with life-threatening complications such as rapidly progressive interstitial lung disease (ILD) and internal malignancies, respectively [1–4]. A subgroup of DM patients is known to have typical skin manifestations of DM but with little evidence of myositis, a condition known as clinically amyopathic DM (C-ADM). Initially, anti-MDA-5 antibodies were reported to be serological markers of clinically ADM with rapidly progressive ILD, especially in East Asia [5]; more recently they were found in Caucasian patients with ADM complicated with ILD [6]. Although it has been suggested that patients with anti-MDA-5 antibodies have a poor prognosis, few reports have tracked the long-term outcome of these patients [4, 7].

SLE is also an autoimmune rheumatic disease that is characterized by a fluctuating disease course and a variety of autoantibodies. Many autoantibody specificities (SSA/Ro, SSB/La, Sm, U1-RNP) in lupus patients remain constant over time, whereas reactivity to dsDNA may fluctuate with disease activity, although the pattern of change differs with autoantibody specificity [8, 9]. We have little information on an association between DM-specific

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autoantibodies and the long-term outcome of DM patients [10]. We established a quantitative assay of antibody levels and monitored anti-MDA-5 autoantibodies during long-term follow-up periods in order to assess the long-term outcome of ADM patients with anti-MDA-5 antibodies.

Materials and methods

Patients

The patients were seen or consulted in the Department of Dermatology, Nagoya University Graduate School of Medicine from 1994 to 2011. From our department serum bank, we used sera from 51 patients with DM, including 30 with C-ADM and 1 with C-ADM overlapping with scleroderma. These patients were diagnosed as having DM or C-ADM based on the criteria of Bohan *et al.* [11] and of Sontheimer [12], respectively. In general, C-ADM presents as typical skin lesions and amyopathy or hypomyopathy for >6 months. The ADM group included patients who developed fatal ILD within 6 months after disease onset. Of these 51 patients, 41 were characterized in our previous study [3]: 21 were anti-MDA-5 positive and 20 were negative. This study also included additional serum samples from 10 other DM patients with anti-MDA-5 antibodies, who were seen after our previous study [3] and defined by our immunoprecipitation assays with recombinant MDA-5. The anti-MDA-5-positive serum samples totalled 31 (male : female = 5 : 26). The mean age was 48.9 (range 11–80) years. One patient with JDM was included. Twenty sera were collected from healthy blood donors and used as normal controls.

In the 31 patients with anti-MDA-5 antibodies, sera from 10 patients with ADM were taken both at their first visit and at inactive disease periods after therapy. Serum from one other patient with ADM (female, aged 46 years) was taken at her first visit and just before death from ILD 3 months later. All the patients except one were female, and their ages ranged from 23 to 60 years. They were non-smokers and had no evidence of cancer. Ten of the patients developed ILD within 6 months after disease onset, whereas one patient had no lung involvement during the course. The first sera samples from all the patients were characterized as having had anti-MDA-5 antibodies previously [3]. The range of follow-up was 5–16 years, except for the patient who died. All the patients and healthy individuals in the present study gave fully informed consent for participation, including provision of sera samples. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISA

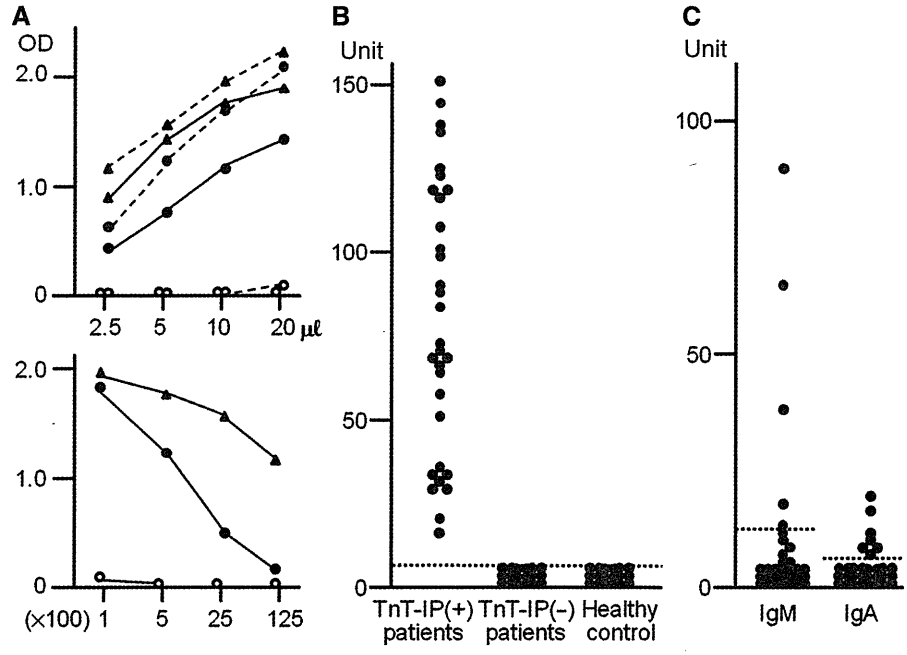
Specific binding of serum autoantibodies to recombinant MDA-5 was analysed using direct solid-phase ELISA. Biotinylated recombinant MDA-5 was produced from full-length MDA-5 cDNA using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3]. Nunc Immobilizer Streptavidin plates (Thermo Scientific Nunc, Roskilde, Denmark) to which streptavidin was covalently coupled via a spacer were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with biotinylated recombinant MDA-5 diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Diluted sample sera with blocking buffer (75 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG, IgM or IgA antibody conjugated with HRP (Dako, Glostrup, Denmark) as a secondary antibody (75 μl/well) at 1 : 30 000 dilution after five washes. After incubation for 1 h at room temperature, the plates were washed five times and incubated with Ultra TMB (Pierce, Rockford, IL, USA) (75 μl/well) as the substrate, according to the manufacturer's protocol. Then, optical density (OD) at 450 nm was determined using Multiskan FC (Thermo Scientific, Waltham, MA, USA). Each serum sample was tested in duplicate, and the mean OD subtracted background was used for data analysis. An in-house ELISA was used for measuring anti-diphtheria toxoid (DT). In brief, plates (Medisorp, Thermo Scientific Nunc) were coated with 50 μl/well DT (1 μg/ml in PBS) (List Biological Laboratories, Campbell, CA, USA) and blocked with 3% BSA in T-PBS. The sera samples were diluted 1 : 100 in 3% BSA in T-PBS. Anti-human IgG antibody conjugated with HRP and a substrate was used in the manner described above.

Results

ELISA with biotinylated recombinant MDA-5

To measure anti-MDA-5 antibodies in sera quantitatively, we tried to establish an ELISA that uses biotinylated recombinant MDA-5. Based on the results of two different anti-MDA-5-positive sera (Fig. 1A), we decided to use the 10 μl/well of TnT mixture and the diluted patient serum samples at 1 : 500 for measuring all samples. The unit of each sample was calculated as that sample's OD divided by the OD of the standard positive serum #1251 and then multiplied by 100. With the cut-off value determined as the mean value of 20 control sera + 3 S.D., 31 serum samples that had been identified as positive for anti-MDA-5 antibodies by immunoprecipitation also tested positive in these ELISA, and 20 serum samples from patients that were identified as being without anti-MDA-5 antibodies by immunoprecipitation also tested negative (Fig. 1B). We also measured IgM- and IgA-class antibodies using these assays as a positive control for the IgG anti-MDA-5 antibody level of #1251 (Fig. 1C). Both immunoglobulin classes of anti-MDA-5 antibodies were present, but in minor populations.

Fig. 1 ELISA using biotinylated recombinant MDA-5 protein. (**A**) Serial dilution of biotinylated protein (upper panel) or sera (lower panel) for ELISA. Closed circle and triangle: anti-MDA-5-positive sera in immunoprecipitation analysis. Open circle: healthy individual serum. In the upper panel, broken and solid lines denote sera diluted to 1 : 100 and 1 : 500, respectively. Recombinant protein was diluted with T-PBS to 50 μl of the final volume per well. In the lower panel, recombinant protein was applied at 10 μl diluted with 40 μl of T-PBS per well. Serum dilution was 1 : 100–1 : 12 500. (**B**) Measurement of anti-MDA-5 antibodies in 71 serum samples. All samples were classified as positive or negative for anti-MDA-5 antibodies, as determined by immunoprecipitation assay with biotinylated proteins. Broken line indicates the cut-off value (6.5 U), calculated from the mean OD values of 20 healthy controls + 3 S.D. (**C**) Isotype analysis of anti-MDA-5 antibodies. Thirty-one IgG anti-MDA-5-positive serum samples were also analysed for IgM and IgA class antibodies. Broken lines indicate the cut-off value (9.4 U for IgM and 6.0 U for IgA) calculated from the mean values of 20 healthy controls + 3 S.D.



Decline in anti-MDA-5 antibodies during remission

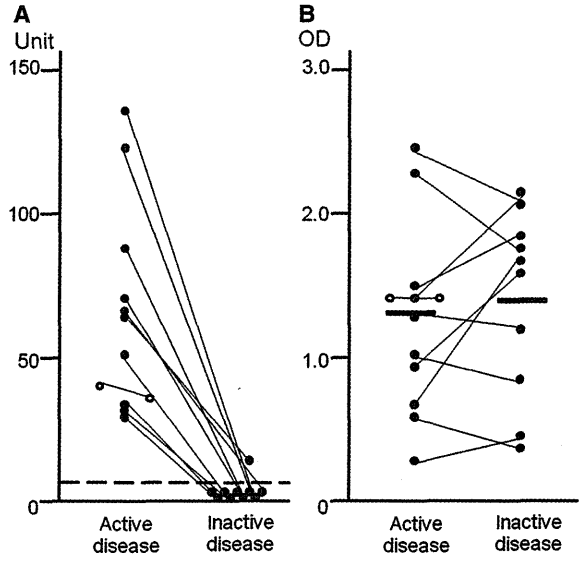
From 31 patients whose initial serum samples had anti-MDA-5 antibodies, sera were retaken during remission periods from the 10 patients with C-ADM. As a treatment for ILD in nine of these patients, methylprednisolone pulse therapy and immunosuppressive drugs were administered to eight patients and seven patients, respectively. The following immunosuppressive drugs were administered: ciclosporin to two patients, the combination of ciclosporin and i.v. CYC to two patients; ciclosporin, AZA and i.v. CYC to two patients and AZA and i.v. CYC to one patient. After initial therapy, 6 of the 10 patients were in clinical remission, which was defined as no evidence of active skin rash, myositis and lung involvement for >6 months without drug therapy. The remaining four patients also entered clinical remission, but with therapy of low-dose CS (prednisolone <7.5 mg/day). None of the 10 patients showed aggravated interstitial findings in their chest radiograph examinations for >5 years. The sampling of sera during remission ranged from 5 to 15 years after the first sampling. IgG-class anti-MDA-5 antibody levels were compared between serum samples at active and inactive disease states (Fig. 2A). Except for one patient who still had anti-MDA-5 antibodies but whose titre was dramatically reduced at 5 years from disease onset, in all the sera the anti-MDA-5 antibodies were absent during remission. These were also confirmed to be negative in the same ELISA plate with 20 sera samples from healthy individuals, and also by immunoprecipitation using this biotinylated protein (data not shown).

We also measured anti-DT antibodies in the same serum samples because we wondered whether the disappearance of anti-MDA-5 antibodies related to general immunosuppression. ELISA results showed that antibodies against DT remained at similar levels (Fig. 2B).

Discussion

In a Japanese multicentre study, 5-year survival in patients with anti-MDA-5 antibodies was 56% [4]. However, the long-term outcome of ADM patients has been seldom reported in terms of longitudinal serological findings. Since we recently examined >10 ADM patients

Fig. 2 Decrease of anti-MDA-5 antibody levels in remission. (**A**) Anti-MDA-5 antibody levels in 10 patients with ADM who were positive for serum anti-MDA-5 antibodies at their first visit decreased during inactive disease periods under the cut-off level (6.5 U), which is shown by the broken line. The open circle indicates the patient who died 3 months after disease onset. (**B**) Titres of anti-DT antibodies in ELISA. The same serum samples as used in (**A**) were measured. Horizontal bars show the mean values of OD for the sera group in the active stage (except for the patient indicated by the open circle) and for the sera group in the inactive stage.



with anti-MDA-5 antibodies who experienced clinical remission for >5 years, we investigated anti-MDA-5 antibodies in these surviving patients. Our results showed that all but one patient lost anti-MDA-5 antibodies in sera and went into remission.

Kuwana *et al.* [13] examined serial changes in anti-topo I antibody levels in patients with SSc and found that, in some patients with a favourable outcome, loss of anti-topo I antibodies occurred within 10 years after the first visit. Kinetic studies of *in vitro* T-cell proliferation indicated that the disappearance of anti-topo I antibodies was due to loss of activation of topo I-reactive T cells. Expressions of cryptic epitopes by protein cleavage are probably important for the autoantibody response. MDA-5, which plays important roles in the innate immune system during RNA viral infections, is degraded in cells infected with different picornaviruses [14]. Whether such cleavage might lead to autoimmune responses against MDA-5 needs further investigation.

In summary, we have identified the disappearance of anti-MDA-5 antibodies in ADM remission. The precise factors or mechanisms that define positive/negative immune response to MDA-5 among ADM patients remain unknown. Future studies should address whether anti-MDA-5 antibody levels are useful as indicators for response to therapy. To confirm anti-MDA-5 antibodies as a marker for increased disease activity, future studies would need to determine whether anti-MDA-5 antibodies reappear during disease activity.

Rheumatology key messages

- Anti-MDA-5 antibodies could be an important serological marker for ILD in ADM patients.
- The tracking of anti-MDA-5 antibodies could be useful for monitoring disease activity in ILD complicated with ADM.

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Concise report

Clinical features of anti-TIF1-α antibody-positive dermatomyositis patients are closely associated with coexistent dermatomyositis-specific autoantibodies and anti-TIF1-γ or anti-Mi-2 autoantibodies

Yoshinao Muro[1], Asuka Ishikawa[1], Kazumitsu Sugiura[1] and Masashi Akiyama[1]

Abstract

Objective. Myositis-specific autoantibodies (MSAs), which characterize certain forms of inflammatory myopathy, are useful in the diagnosis and prediction of prognosis in DM/PM. Anti-transcriptional intermediary factor 1-α (TIF1-α) antibodies were recently reported to be associated with cancer-associated DM in conjunction with anti-TIF1-γ antibodies. This study aimed to identify a subset of DM patients who have anti-TIF1-α antibodies by using biotinylated recombinant proteins and to clarify the clinical and other serological features of DM patients with these antibodies.

Methods. Sera from 202 Japanese patients with CTDs, including 108 with DM and 20 healthy controls, were screened for anti-TIF1-α antibodies by our novel ELISAs. Positive sera were further examined by immunoprecipitation and also investigated for the detection of anti-TIF1-γ and anti-Mi-2 antibodies.

Results. Sera from 12 patients with DM were confirmed to be positive for anti-TIF1-α antibodies. None of the patients with other CTDs and none of the healthy controls had the antibodies. Seven anti-TIF1-α-positive patients simultaneously had anti-TIF1-γ antibodies and the other five had anti-Mi-2 antibodies, both of which are well known to be MSAs. These double-positive patients with anti-TIF1-α and anti-γ antibodies included three JDM and two cancer-associated adult DM patients, whereas all the double-positive patients with anti-TIF1-α and anti-Mi-2 antibodies were classical adult DM.

Conclusion. Although MSAs have been regarded as mutually exclusive, anti-Mi-2 antibody-positive patients simultaneously have anti-TIF1-α antibodies. Anti-Mi-2 antibody-positive patients are associated with classical DM without cancer even with the simultaneous presence of anti-TIF1-α antibodies.

Key words: autoantibodies, dermatomyositis, Mi-2, TIF1-α, TIF1-γ.

Introduction

The idiopathic inflammatory myopathies (IIMs) are a group of systemic autoimmune diseases that include PM, DM and inclusion body myopathies [1]. Several myositis-specific autoantibodies (MSAs) are associated with certain clinical forms of IIMs, and they are useful tools for predicting the prognosis. For example, anti-MDA5-antibody-positive patients demonstrate rapid progressive interstitial lung disease (ILD) and anti-transcriptional intermediary factor 1-γ (TIF1-γ) antibody-positive patients are often complicated with cancer [2]. Very recently, anti-p155/140 antibodies, which are serological markers of cancer-associated DM [3, 4], were analysed by Fujimoto *et al.* [5]. They determined that p140 is identical to TIF1-α, whereas p155 is known as TIF1-γ [6]. Their study showed that TIF1-β was also targeted in DM patients, but infrequently, although anti-TIF1-γ antibodies alone were frequently detected. Interestingly, anti-TIF1-α antibodies were always associated with anti-TIF1-γ antibodies.

MSAs have been regarded as mutually exclusive [1, 7]. Fujimoto *et al.* [5] showed that autoantibodies against the

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TIF1 protein family occurred in various combinations, often in patients with cancer-associated DM. However, they concentrated on analysing anti-p155/140-positive sera in a large cohort to find autoantibodies against the TIF1 protein family. In this study, we investigated sera from patients with various CTDs for the presence of anti-TIF1-α antibodies by using our developed ELISAs and immunoprecipitation (IPP) with biotinylated recombinant protein. We confirmed that anti-TIF1-α antibodies are specific to DM and are often associated with anti-TIF1-γ antibodies. Surprisingly, the other anti-TIF1-α antibody-positive sera also had anti-Mi-2 antibodies.

Materials and methods

Patients and sera

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 202 Japanese patients with CTDs. They consisted of 108 patients with DM [including 13 with JDM, 38 with clinically amyopathic DM (ADM) and 15 with cancer-associated DM], 9 with PM, 24 with SLE, 20 with SSc, 26 with SS and 15 with myositis overlap syndrome (Table 1). Sera from 21 cancer patients that were used in our previous study [2] were also analysed. Twenty healthy individuals were assessed as normal controls. All the DM patients except those with clinical ADM and all the PM patients fulfilled Bohan and Peter's criteria [8, 9]. All the clinically ADM patients fulfilled Sontheimer's criteria [10]. The clinically ADM group included patients who had developed ILD within 6 months after disease onset. Patients were classified as having JDM if they were aged <16 years at the onset of DM [11] and as cancer-associated DM if internal malignancy was diagnosed within 3 years (before or after) of the DM diagnosis [12]. The criteria of other CTDs were based on the established criteria for these diseases used in our previous studies [2, 13]. The ages at disease onset and gender ratios of each clinical group are summarized in Table 1. All the patients and healthy individuals gave fully informed consent for participation. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISAs

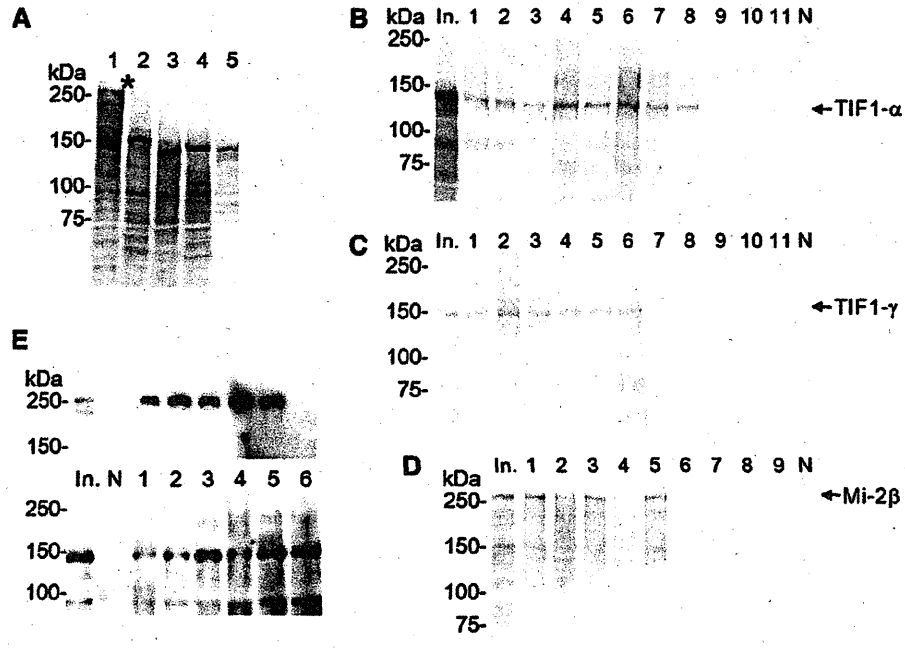
Specific binding of serum antibodies to recombinant TIF1-α or Mi-2β was analysed using our recently established sensitive ELISA [14]. This method was based on our previous protocol, which quantitatively measured the antibodies against MDA-5 [15]. Instead of a conventional optical system, this ELISA uses a microplate luminometer for increased sensitivity, thereby reducing the amount of recombinant protein required for the assays. The full-length TIF1-α cDNA clone was purchased from Kazusa DNA Research Institute (Chiba, Japan). The full-length Mi-2β cDNA clone [16] was a kind gift

Table 1 Patient groups and anti-TIF1-α antibody frequencies

Clinical group	Age at onset, mean (range), years	Gender, M:F	Total, *n*	Anti-TIF1-α (*n* = 12)		Anti-TIF1-α ELISA unit[a], mean (range)
				Anti-TIF1-γ	Anti-Mi-2	
Total DM	47 (1-80)	33:75	108	7	5	41.9 (−12.8 to 472.5)
Clinically ADM	45 (1-73)	9:29	38	5	5	42.1 (−4.3 to 366.4)
Cancer-associated DM	65 (48-80)	6:9	15	2	0	45.8 (−6.3 to 279.0)
Classical DM	44 (1-80)	18:37	55	0	0	40.7 (−12.8 to 472.5)
Adult DM	52 (19-80)	26:69	95	4	5	38.3 (−12.8 to 472.5)
Clinically ADM	51 (20-73)	5:27	32	2	0	23.7 (−4.3 to 268.4)
Cancer-associated DM	65 (48-80)	6:9	15	2	0	45.8 (−6.3 to 279.0)
Classical DM	49 (19-80)	15:33	48	0	5	45.8 (−12.8 to 472.5)
Juvenile DM	9 (1-16)	7:6	13	3	0	68.0 (13.8 to 366.4)
Clinically ADM	6 (1-11)	4:2	6	3	0	140.0 (13.8 to 366.4)
Classical DM	11 (1-16)	3:4	7	0	0	6.2 (−6.4 to 16.3)
Total of other CTDs	42 (15-81)	11:83	94	0		15.2 (−30.5 to 63.2)
PM	52 (32-67)	1:8	9	0		27.9 (13.1 to 63.2)
SLE	30 (15-51)	4:20	24	0		11.1 (−30.5 to 43.7)
SSc	40 (22-55)	0:20	20	0		16.1 (−23.2 to 58.2)
Overlap syndrome	49 (23-69)	2:13	15	0		16.9 (7.8 to 62.5)
SS	47 (21-81)	4:22	26	0		13.0 (10.2 to 39.5)
Cancer	58 (48-78)[b]	5:15[b]	21	0[c]		Not done

[a]The cut-off level is 121.3 and 83.0 U based on 5 S.D.s and 3 S.D.s above the mean value obtained from 20 healthy control sera, respectively. No patient had an ELISA in the range of 83.0-121.3 U. M:F = male:female. [b]Information on the age and gender of one patient was not available. [c]All sera from cancer patients were examined for anti-TIF1-α antibodies by IPP.

FIG. 1 Anti-TIF1-α antibodies coexist with anti-TIF1-γ or anti-Mi-2 antibodies.



(**A**) Biotinylated recombinant proteins used in our laboratory were subjected to 7.5% SDS-PAGE and analysed by immunoblotting. Lane 1: Mi-2β; lane 2: TIF1-γ; lane 3: TIF1-α; lane 4: NXP-2; lane 5: MDA-5. Mi-2β was shown at the larger size of 250 kDa, probably due to the addition of N-terminal haemagglutinin tag and C-terminal V6His tag indicated by an asterisk [16]. (**B**) IPP of recombinant TIF1-α. In.: the input was a full dose (10 μl) of biotinylated TIF1-α protein that was used for the IPP assay. Lanes 1–8 contain the TIF1-α immunoprecipitated by the sera of different DM patients. In lanes 9–11, anti-TIF1-γ-positive sera from DM patients, which immunoprecipitated TIF1-γ, did not immunoprecipitate TIF1-α. N: normal control. (**C**) IPP of recombinant TIF1-γ. In.: the input was half the dose (5 μl) of the biotinylated TIF1-γ protein used for the IPP assay. Lanes 1–6 contain the TIF1-γ immunoprecipitated by the sera of different DM patients. In lanes 7–11, anti-TIF1-α-positive and anti-Mi-2-positive sera from DM patients, which immunoprecipitated TIF1-α and Mi-2β, did not immunoprecipitate TIF1-γ. N: normal control. (**D**) IPP of recombinant Mi-2β. In.: the input was half the dose (5 μl) of the biotinylated Mi-2β protein used for the IPP assay. Lanes 1–5 contain the Mi-2β immunoprecipitated by the sera of different DM patients. In lanes 6–9, anti-TIF1-α/γ-positive sera from DM patients, which immunoprecipitated TIF1-α and TIF1-γ, did not immunoprecipitate Mi-2β. N: normal control. (**E**) Immunoprecipitates from cell extracts with anti-TIF1-α-positive sera were probed with anti-Mi-2α MoAb (upper panel) and with anti-TIF1-α PolyAb (lower panel). Lane In. contains a half dose of the input of K562 cell extracts. Lane N: normal control; lanes 1–5: anti-TIF1-α/Mi-2-positive sera; lane 6, anti-TIF1-α/TIF1-γ-positive serum.

from Drs Kato and Takahashi at Nagoya University. Biotinylated recombinant protein was produced from the cDNA, using the transcription and translation (TnT) T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol (Fig. 1A) [2, 15]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with 1 μl of TnT product diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Sample sera diluted with blocking buffer (50 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG antibody conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) (50 μl/well) at 1:30 000 dilution. After incubation for 1 h at room temperature, the plates were washed and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA) (50 μl/well) as the substrate. Relative luminescence unit (RLU) number was then determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU subtracted background was used for data analysis.

A standard curve was obtained from serial concentrations of a serum sample containing a high titre of the autoantibody against each antigen.

Detection of autoantibodies using IPP

IPP was performed using TnT products of TIF1-α, Mi-2β or TIF1-γ as previously described [2]. Briefly, 10 μl of patient sera was mixed and incubated with 20 μl of a 50% slurry of Protein G Sepharose (GE Healthcare, Buckinghamshire, UK) and 270 μl IPP buffer (PBS containing 1% Nonidet P-40) at 4°C for 1 h. Sepharose beads were mixed and incubated with 270 μl binding buffer (IPP buffer containing 0.5% BSA) and 10 μl of the TnT products, which was pre-cleared using the sepharose beads, at 4°C for 1 h. The beads were washed five times with IPP buffer and suspended in Laemmli sample buffer, and the IgG-bound proteins were electrophoresed on 7.5% SDS–PAGE gel. Immunoblot was performed as previously described [2].

IPP–western blotting

IPP–western blotting was performed using K562 cell extracts without chemical cross-linking [17]. Monoclonal anti-Mi-2α and polyclonal anti-TIF1-α antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA) and MBL (Nagoya, Japan), respectively. HRP-conjugated anti-mouse immunoglobulin and anti-rabbit IgG antibodies were purchased from DAKO (Glostrup, Denmark).

Statistical analysis

The frequency of antibodies between disease groups was analysed using chi-square test with Yates' correction. $P < 0.05$ was considered statistically significant.

Results

ELISA and IPP with biotinylated recombinant TIF1-α

For the screening of anti-TIF1-α antibodies in a large number of serum samples we used an ELISA system with biotinylated recombinant TIF1-α. We examined a total of 202 serum samples from patients with various CTDs. When the cut-off level was set at 121.3 U, based on 5 S.D.s above the mean value obtained from 20 healthy control sera, the 12 sera from patients with DM were positive for anti-TIF1-α antibodies, whereas none of the sera from patients with other CTDs was positive for those antibodies (12 positive sera/108 total DM sera *vs* 0/94 total CTD sera, $P = 0.0012$) (Table 1). Even based on the cut-off level (83.0) at 3 S.D.s above the mean value, the results were not different.

After the initial screening by ELISA, we investigated antibodies against TIF1-α in sera from 12 anti-TIF1-α ELISA-positive patients and several anti-TIF1-α ELISA-negative patients in order to confirm their ability to immunoprecipitate biotinylated recombinant TIF1-α. All 12 anti-TIF1-α-positive sera in ELISA showed a distinct protein band with a molecular weight of 140 kDa in IPP (Fig. 1B). Twelve anti-TIF1-α-negative sera in ELISA and six healthy control sera did not immunoprecipitate the recombinant. Moreover, none of the 21 serum samples from cancer patients immunoprecipitated (data not shown).

Coexistence of anti-TIF1-α and anti-TIF1-γ antibodies

According to the previous report [5], anti-TIF1-α antibodies always coexist with anti-TIF1-γ antibodies. Our anti-TIF1-α-positive sera were examined for anti-TIF1-γ antibodies by IPP (Fig. 1C). Unexpectedly, only 7 of the 12 sera were also positive for anti-TIF1-γ antibodies. Since IPP for anti-TIF1-γ antibodies had been performed on 81 sera from DM patients in our previous study [2], we examined the antibodies by IPP for the remaining 27 sera. We found a total of 16 sera with anti-TIF1-γ antibodies among the 108 patients with DM. Summarizing the results, in the present DM cohort of 108 patients, 12 and 16 patients had anti-TIF1-α antibodies and anti-TIF1-γ antibodies, respectively. Only 7 patients had both anti-TIF1-α antibodies and anti-TIF1-γ antibodies (anti-TIF1-α/γ-positive), and 87 patients had neither anti-TIF1-α antibodies nor anti-TIF1-γ antibodies.

Coexistence of anti-TIF1-α and anti-Mi-2 antibodies

As mentioned above, we found five anti-TIF1-α-positive, anti-TIF1-γ-negative sera. Previously, two of them had been examined and identified as positive for anti-Mi-2 antibodies by IPP with cell extract (data not shown). Thus, in the present study, we investigated anti-Mi-2 antibodies by IPP with the recombinant Mi-2β in the 12 anti-TIF1-α-positive sera (Fig. 1D). All five of the anti-TIF1-α-positive, anti-TIF1-γ-negative sera were positive for anti-Mi-2 antibodies, whereas none of the seven anti-TIF1-α/γ-positive sera was positive for anti-Mi-2 antibodies. In addition, we investigated anti-Mi-2 antibodies for all the sera from the present DM cohort by ELISA with recombinant Mi-2β, although we detected no additional anti-Mi-2-positive sera except for the five sera described above (data not shown). The IPP–western blotting results showed that each protein precipitated by the five sera reacted to mAb against Mi-2α (Fig. 1E, upper panel) and to polyclonal antibody against TIF1-α (Fig. 1E, lower panel). These sera were confirmed to have anti-Mi-2 antibodies, which, in general, react to both Mi-2α and Mi-2β isoforms [7] and to have anti-TIF1-α antibodies.

Clinical features of DM patients with anti-TIF1-α antibodies

Of the 12 anti-TIF1-α-positive patients, 5 patients with anti-Mi-2 antibodies were classical DM without cancer/ILD, a subset of DM that is associated with anti-Mi-2 antibodies [1, 7]. In contrast, the seven patients with anti-TIF1-α/γ antibodies consisted of three patients with JDM, two with adult ADM and two with cancer-associated DM (Table 1). Demographic data of nine adult patients with anti-TIF1-α antibodies, including gender (male: female = 2 : 7) and age at onset of adult DM [mean (S.D.) = 60.0 (13.5)], and clinical features, including the presence of cutaneous signs of DM (heliotrope rash or Gottron's

papules), RP and elevated creatine kinase, were not statistically different compared with anti-TIF1-α-negative patients (data not shown). However, the presence of ILD in anti-TIF1-α-positive patients was significantly less than that for anti-TIF1-α-negative patients (0/9 *vs* 40/86, $P = 0.0098$).

We investigated other MSA or myositis-associated autoantibodies, e.g. anti-MDA5, -MJ -PL-7, -PL-12, -EJ and -KS (by IPP with TnT product), -Jo-1, -SS-A and -U1-RNP (by commercial ELISA kits) in the 12 anti-TIF1-α antibody-positive patients. Only two patients had concomitant anti-SS-A antibodies, and no other autoantibodies were found.

Discussion

Recently, Fujimoto *et al.* [5] reported that the TIF1 protein family of TIF1-α, -β and -γ is an autoimmune target in DM, especially in cancer-associated DM and JDM patients. Our study clarified that anti-TIF1-α antibodies were not specific markers of a certain subset of DM and that clinical features in the antibody-positive patients were influenced by the other coexistent antibodies against TIF1-γ or Mi-2. The most surprising result in our study is that all classical DM patients with anti-Mi-2 antibodies simultaneously carried anti-TIF1-α antibodies in our cohort, because MSAs have been generally regarded as mutually exclusive [1, 7]. Another new finding in this study was that anti-TIF1-α antibodies were found only in patients with DM and not in those with other CTDs or cancer. The previous report [5] investigated the prevalence of the autoantibodies only in DM patients.

The different result in our study from the previous study is that anti-TIF1-α/γ-positive sera (7/108) are seen less than anti-TIF1-γ-positive, anti-TIF1-α-negative sera (9/108). Fujimoto *et al.* [5] showed that anti-TIF1-α/γ-positive sera ($n = 52$) were more numerous than anti-TIF1-γ-positive, anti-TIFI-α-negative sera ($n = 25$). This discrepancy might be caused by the difference of experimental methods. They evaluated anti-TIF1-α/γ-positive sera using conventional IPP assays, whereas our assays use original recombinant proteins that we have developed. The stringency of our IPP buffer was much higher than that of their methods (the present methods, 1% NP-40 and 150 mM NaCl *vs* their methods, 0.1% NP-40 and 50 mM NaCl). They noted the possibility that TIF1-γ IPP would be caused by anti-TIF1-α antibodies that have cross-reactivity to a TIF1-γ sequence. Our assay may have failed to find such cross-reactive antibodies with low avidity.

Some sera from myositis patients have shown several positive polypeptide bands around 140–160 kDa on SDS–PAGE by IPP, corresponding to anti-NXP-2, -MDA5, -TIF1-γ and -OJ antibodies [18]. Although anti-Mi-2 antibodies mainly target Mi-2α/β of ~240 kDa, bands immunoprecipitated by the antibodies included polypeptides ~140 kDa [18, 19]. The Mi-2/NuRD complex is involved in multiple transcriptional regulatory processes and contains many components [20]. Although TIF1-β was reported to interact with Mi-2α [21], there have been no reports of the NuRD complex containing TIF1-α.

Rheumatology key messages

- Anti-TIF1-α antibodies are detected in DM patients with anti-TIF1-γ or anti-Mi-2 antibodies.
- Anti-Mi-2 antibodies are associated with classical DM even when coexistent with anti-TIF1-α antibodies.

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