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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

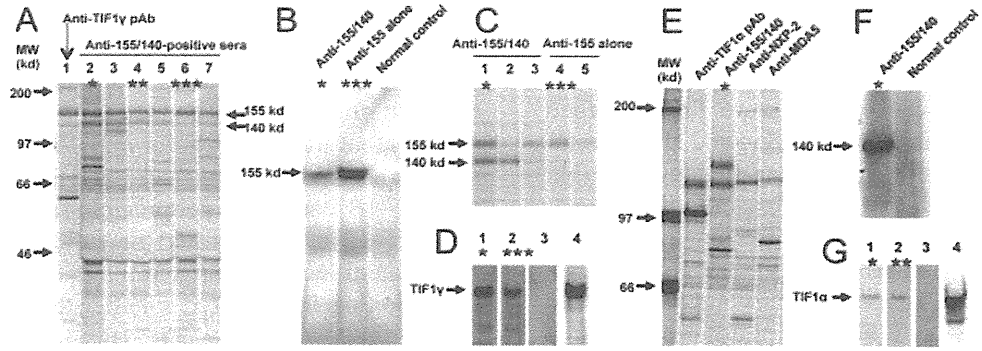

Figure 1. A, Immunoprecipitates with anti-155/140 autoantibodies from ^{35}S-methionine–labeled K562 cell extracts were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography. Lane 1, Anti–transcription intermediary factor 1γ (anti–TIF-1γ) polyclonal antibodies (pAb); lanes 2–7, dermatomyositis (DM) patient sera positive for anti-155/140. B, K562 cell extracts were immunoprecipitated with anti-155/140–positive sera, anti-155–positive sera, or normal control sera and subjected to SDS-PAGE and Western blotting with anti–TIF-1γ polyclonal antibodies. C, TIF-1γ and TIF-1α were immunodepleted from ^{35}S-methionine–labeled cell extracts using anti–TIF-1γ (lanes 2 and 5) or anti–TIF-1α (lane 3) polyclonal antibodies, and immunoprecipitated with anti-155/140–positive sera (lanes 1–3) or anti-p155–positive sera (lanes 4 and 5). Lanes 1 and 4, Control samples immunodepleted with control IgG. D, Recombinant TIF-1γ protein was subjected to Western blotting with anti-155/140–positive sera. Lanes 1 and 2, Anti–TIF-1γ–positive sera; lane 3, control sera; lane 4, anti–TIF-1γ polyclonal antibodies. E, Shown is a comparison of the proteins immunoprecipitated with anti-155/140, anti–NXP-2, or anti–melanoma differentiation–associated protein 5 (anti–MDA-5) antibodies in DM patient sera, or with anti–TIF-1α polyclonal antibodies, analyzed by 7% SDS-PAGE. F, Immunoprecipitates from cell extracts with anti-155/140–positive sera or control sera were probed with anti–TIF-1α polyclonal antibodies. G, Recombinant TIF-1α protein was subjected to Western blotting with anti-155/140–positive sera. Lanes 1 and 2, Anti–TIF-1α–positive sera; lane 3, control sera; lane 4, anti–TIF-1γ polyclonal antibodies. **Asterisks** indicate the same serum samples.

were determined by ELISA. Microtiter plates with 96 wells (Costar) were coated with recombinant TIF-1α or TIF-1γ protein (1 μg/ml) at 4°C overnight. The wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris buffered saline for 1 hour at 37°C. The serum samples that were diluted to 1:100 were first preabsorbed in GST-coated wells and then were added to duplicate wells coated with recombinant TIF-1 proteins for 90 minutes at 20°C. After washing 4 times, the bound antibodies were detected with alkaline phosphatase–conjugated goat anti-human IgG antibodies (Cappel) using *p*-nitrophenyl phosphate (Sigma-Aldrich) as substrate. Absorbance in each well was read using a microplate reader (Bio-Rad) set to 405 nm.

Statistical analysis. Fisher's exact test was employed for comparison of frequencies. The Kolmogorov-Smirnov test was used to determine normality. *P* values less than 0.05 were considered significant.

RESULTS

Confirmation that the 155-kd antigen of anti-155/140 antibodies was TIF-1γ. In IP assays, "anti-p155 antibodies" have been reported to precipitate a 155-kd protein (14), while "anti-155/140 antibodies" have been reported to precipitate 2 proteins—a 155-kd protein and a 140-kd protein (15). If these 2 autoantibodies are the same, the difference in the reacting proteins may be due to the types of the substrate cells (for example, K562 cells versus HeLa cells) or the experimental procedures (for example, degradation of antigen proteins). However, when multiple serum samples were compared simultaneously using the same extract of antigen source, some sera reacted strongly with both proteins, while some reacted strongly with the 155-kd protein but not, or very weakly, with the 140-kd protein (Figure 1A). Therefore, the reactivities for the 155-kd and 140-kd proteins differed among serum samples under the same conditions, suggesting that the antibodies reacted with each protein independently and that the 2 proteins were indeed different. We screened a total of 456 serum samples from DM patients and identified 77 samples that were positive for either the 155-kd or 140-kd protein. Among the 77 samples, 52 were reactive with both the 155-kd and the 140-kd proteins, while 25 were reactive with the 155-kd protein alone. No samples were found to be positive for the 140-kd protein alone.

We sought to confirm that the 155-kd antigen recognized by anti-155/140 antibodies was TIF-1γ, as was preliminarily reported for anti-p155 antibodies (14). K562 cell lysates were incubated with protein A–Sepharose beads preincubated with sera reactive with the 155-kd and 140-kd antigens and those reactive with the 155-kd antigen alone, and precipitated proteins were probed with polyclonal antibodies to TIF-1γ in Western blotting. Polyclonal anti–TIF-1γ antibodies reacted with

both of the immunoprecipitates generated with anti-155/140–positive sera and anti-155–positive sera (Figure 1B). To further confirm that these sera recognized the same antigens, K562 cell extracts were first absorbed using polyclonal antibodies to TIF-1γ, and then immunoprecipitation was performed using patient sera that were reactive with both the 155-kd and 140-kd antigens or with the 155-kd antigen alone. The polyclonal antibodies depleted the 155-kd band (Figure 1C), demonstrating that both anti-p155 antibodies and anti-155/140 antibodies reacted with TIF-1γ. Furthermore, anti-155/140 sera reacted strongly with recombinant TIF-1γ protein in Western blotting (Figure 1D). Therefore, it was formally demonstrated that "anti-p155" and "anti-155/140" antibodies are the same in that they both recognize TIF-1γ as the 155-kd antigen.

The 140-kd antigen is TIF-1α. Next, we sought to identify the 140-kd protein targeted by anti-155/140 antibodies. On 7% polyacrylamide gels, the 140-kd protein appeared different from MDA-5, which is targeted by anti-CADM140 antibodies, and NXP-2 (also known as MORC3), which is targeted by anti-MJ (anti-p140) antibodies (Figure 1E). On the other hand, another TIF-1 family protein, TIF-1α, migrated at a molecular weight identical to the 140-kd antigen precipitated with anti-155/140 antibodies (Figure 1E). Therefore, we examined whether the 140-kd autoantigen targeted by anti-155/140 antibodies was TIF-1α. First, K562 cell lysates were incubated with protein A–Sepharose beads preincubated with anti-155/140–positive sera, and precipitated proteins were probed with polyclonal antibodies to TIF-1α in Western blotting. While control samples from healthy subjects did not show any band, those from anti-155/140–positive sera developed a strong 140-kd band that was recognized by polyclonal anti–TIF-1α antibodies (Figure 1F). Also, in the immunodepletion assay, polyclonal antibodies to TIF-1α depleted the 140-kd band that was recognized by anti-155/140–positive sera (Figure 1C). Moreover, when recombinant GST-tagged full-length human TIF-1α protein was subjected to SDS-PAGE and Western blotting with anti-155/140–positive sera, the sera positive for the antibodies reacted with the recombinant protein (Figure 1G). These sera did not react with GST alone (results not shown). Therefore, the 140-kd antigen of anti-155/140 antibodies was identified as TIF-1α.

A portion of anti-155/140–positive sera react with TIF-1β. TIF-1β also belongs to the TIF-1 family. Therefore, we examined whether TIF-1β was another target recognized by anti-155/140–positive sera. When immunoprecipitated with polyclonal anti–TIF-1β antibodies and subjected to SDS-PAGE, TIF-1β migrated at ~100 kd and appeared as a thick band (Figure 2A). Therefore, we first screened anti-155/140–positive serum samples that also precipitated proteins at ~100 kd. Of 77 serum samples that were positive for anti-155/140 antibodies, 6 samples showed a similar thick band that was identical to the TIF-1β band immunoprecipitated with polyclonal anti–TIF-1β antibodies and that did not match previously identified autoantigens including PL-12 (Figure 2A). We thus investigated whether this immunoprecipitated protein was indeed TIF-1β.

K562 cell lysates were incubated with protein A–Sepharose beads preincubated with serum samples positive for this 100-kd protein, and precipitated proteins were assessed in Western blotting by probing with polyclonal antibodies to TIF-1β. While samples immunoprecipitated with serum from healthy subjects did not show any band, those immunoprecipitated with anti–100-kd–positive sera developed a strong 100-kd band that was recognized by polyclonal anti–TIF-1β antibodies (Figure 2B). This was also confirmed by immunodepletion assay, in which polyclonal antibodies to TIF-1β decreased the 100-kd band (Figure 2C). Moreover, anti–100-kd sera reacted with recombinant GST-tagged human TIF-1β protein, but not with GST alone, in Western blotting (Figure 2D). Therefore, a portion of the patients with anti-155/140 antibodies also possessed autoantibodies that targeted TIF-1β. Taken together, the results show that the TIF-1 family proteins TIF-1α, TIF-1β, and TIF-1γ can be targeted by anti-155/140–positive sera.

To assess the possibility that anti–TIF-1β antibodies appeared independently of anti–TIF-1α or anti–TIF-1γ, we reviewed the results from the 456 patients with DM as well as from the 62 patients with PM, 108 with SLE, and 433 with SSc, and we found an additional serum sample from a 36-year-old woman with clinically amyopathic DM that reacted with the 100-kd protein but not with the 155-kd and 140-kd proteins (Figure 2A). The reactivity with TIF-1β in this sample was also confirmed by the assay using immunoprecipitation and Western blotting with polyclonal anti–TIF-1β antibodies as well as Western blotting using recombinant protein (results not shown). Serum samples from patients with other diseases did not react with the 100-kd protein.

In summary, 78 of 456 DM sera (17%) were positive for at least 1 anti–TIF-1 antibody (anti–TIF-1α, anti–TIF-1β, or anti–TIF-1γ). Among these 78 sera, reactivity with all 3 antibodies was observed in 4 sera (5.1%), reactivity with anti–TIF-1α and anti–TIF-1γ in 48 sera (62%), reactivity with anti–TIF-1β and anti–

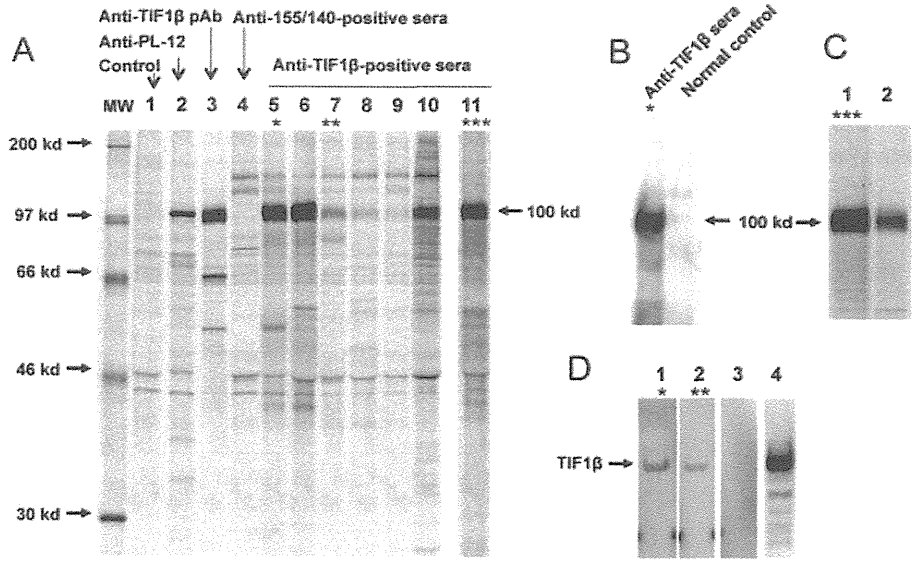

Figure 2. **A,** Immunoprecipitation of the 100-kd autoantigen from ^{35}S-methionine–labeled K562 cell extracts is shown, as described in Figure 1A. Lane 1, Normal human serum; lane 2, prototype serum positive for anti–PL-12 antibodies; lane 3, sample immunoprecipitated with polyclonal antibodies to TIF-1β (the position of the 100-kd TIF-1β antigen is indicated); lane 4, prototype serum positive for anti-155/140 antibodies; lanes 5–11, serum samples from DM patients positive for anti–TIF-1β antibodies (lane 11 shows a serum sample from a 36-year-old woman with clinically amyopathic DM; see Results). **B,** K562 cell extracts were immunoprecipitated with anti–TIF-1β–positive sera or normal control sera and were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and probed with anti–TIF-1β polyclonal antibodies. The molecular weight of TIF-1β (100 kd) is indicated. **C,** Immunodepletion analysis of anti–TIF-1β antibodies is shown. TIF-1β was immunodepleted from ^{35}S-methionine–labeled K562 cell extracts using anti–TIF-1β polyclonal antibodies (lane 2) and then immunoprecipitated with anti–100-kd–positive sera. Control IgG is shown in lane 1. **D,** Recombinant TIF-1β protein was subjected to Western blotting with anti–TIF-1β–positive sera. Lanes 1 and 2, Anti–TIF-1β–positive sera; lane 3, normal control sera; lane 4, anti–TIF-1β polyclonal antibodies. **Asterisks** indicate the same serum samples. See Figure 1 for definitions.

TIF-1γ in 2 sera (2.6%), reactivity with anti–TIF-1γ alone in 23 sera (29%), and reactivity with anti–TIF-1β alone in 1 serum sample (1.3%) (Figure 3A). No sera reacted with anti–TIF-1α alone or with anti–TIF-1α and anti–TIF-1β without reacting with anti–TIF-1γ. Thus, TIF-1γ was the most commonly targeted protein, followed by TIF-1α and TIF-1β. Collectively, these findings demonstrated that anti-155/140–positive sera target all 3 TIF-1 family proteins with varied patterns of reactivity.

Clinical associations. The clinical association of anti–TIF-1α, anti–TIF-1β, and/or anti–TIF-1γ (anti–TIF-1$\alpha/\beta/\gamma$) antibodies was analyzed in the 78 patients. Among them, 74 patients were age >15 years, and 4 patients were age <15 years (Figure 3B). Thus, 17% of adult DM patients (74 of 445) and 36% of juvenile DM patients (4 of 11) were positive for anti–TIF-1$\alpha/\beta/\gamma$ antibodies. This was consistent with reports from the US and Europe that anti-155/140 antibodies are a major serologic subset both in juvenile DM and in adult cancer-associated DM (14,15,22,23). Most of the adult patients were age >45 years at onset, although it was notable that there was another small peak of "young adults" between ages 25 and 39 years. Anti–TIF-1$\alpha/\beta/\gamma$–positive adult DM patients had malignant disease at a rate of 65% (48 of 74), while none of the young adult patients had a history of malignancy. Thus, although this finding was not statistically significant, anti–TIF-1$\alpha/\beta/\gamma$ antibodies may underlie a subset of "young adult DM" that is not associated with malignancy. In contrast, among 64 patients age >40 years, 48 had malignancy (75%). In particular, patients age >60 years had a frequency of malignancy as high as 86% (30 of 35). The cancer sites in 48 patients are shown in Table 1.

DM patients present either the classic DM phenotype (i.e., with muscle involvement) or the clinically amyopathic DM phenotype. In the 78 patients with anti–TIF-1$\alpha/\beta/\gamma$ antibodies, 53 had classic DM (68%), while 25 had clinically amyopathic DM. All patients with juvenile DM were classified as having classic DM. In contrast, among the 8 young adult DM patients positive for anti–TIF-1$\alpha/\beta/\gamma$ antibodies, 6 had no symptoms of muscle involvement and were classified as having clini-

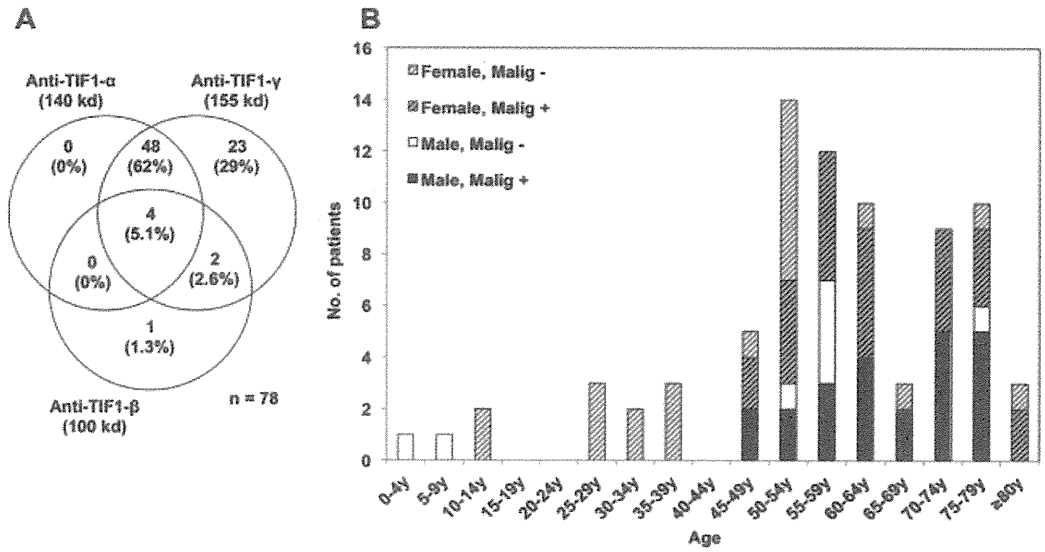

Figure 3. A, Numbers and percent of anti–TIF-1α, anti–TIF-1β, and anti–TIF-1γ antibodies in 78 DM patients. B, Distribution of the age at onset and the presence or absence of malignancy in 78 DM patients positive for anti–TIF-1α, anti–TIF-1β, and anti–TIF-1γ antibodies. Bars show the numbers of female and male patients with or without malignancy (Malig+ and Malig−, respectively). See Figure 1 for other definitions.

cally amyopathic DM. Another young adult patient was initially diagnosed as having clinically amyopathic DM but developed mild myositis 6 months later. The rates of malignancies in patients with adult classic DM and those with clinically amyopathic DM were 69% (34 of 49) and 56% (14 of 25), respectively. Thus, the rate of malignancy was slightly higher in patients with classic DM, although there was no significant difference. Interstitial lung disease was observed in only 3 patients (3.8%).

Table 1. Sites/type of malignancies and autoantibody reactivity to TIF-1 proteins in the 48 dermatomyositis patients with cancer*

	Total (anti–TIF-1γ positive) (n = 48)†	Anti–TIF-1α positive (n = 36)	Anti–TIF-1β positive (n = 2)†
Lung	14	10	0
Stomach	11	8	0
Colon and rectum	4	4	0
Ovary	4	4	2
Breast	4	3	0
Thymus	3	2	0
Gall bladder and bile duct	2	1	0
Uterus	2	1	0
Prostate	1	1	0
Pancreas	1	1	0
Epipharynx	1	1	0
Lymphoma	1	1	0
Renal pelvis	1	1	0
Bladder	1	1	0
Thyroid	1	0	0
Unknown origin	1	0	0

* Values are the number of patients. Patients with cancer at multiple sites are included in the numbers for each involved site.
† All patients having malignancy were positive for anti–transcription intermediary factor 1γ (anti–TIF-1γ) antibodies.
‡ Both patients were also positive for anti–TIF-1α.

When the correlation with antibody reactivity was assessed, patients positive for both anti–TIF-1α and anti–TIF-1γ antibodies were found to have a 73% rate of malignancy (36 of 49), while those positive for anti–TIF-1γ antibodies alone had a 50% rate of malignancy (12 of 24) (Table 2). Thus, the incidence of malignancy was significantly higher in those with anti–TIF-1α and anti–TIF-1γ antibodies than in those with anti–TIF-1γ antibodies alone ($P < 0.05$). There was no specific association between cancer type or site and reactivity with TIF-1α and TIF-1γ. Additionally, among the 7 patients who were positive for anti–TIF-1β antibodies, 2 were diagnosed as having malignancies, both of which were ovarian cancer. Patients positive for both anti–TIF-1α and anti–TIF-1γ antibodies had internal malignancy and truncal erythema more frequently than those positive for anti–TIF-1γ antibodies alone (Table 2).

Longitudinal changes in serum antibody titers were also assessed in 8 patients positive for anti–TIF-1α and anti–TIF-1γ antibodies. After treatment, the titer of anti–TIF-1γ antibodies as measured by ELISA had decreased in all patients (Figure 4), although they remained positive in immunoprecipitation assays (data

Table 2. Demographic, clinical, and laboratory features in the adult dermatomyositis patients with both anti–TIF-1α and anti–TIF-1γ antibodies and in those with anti–TIF-1γ antibodies alone*

	Anti–TIF-1α and anti–TIF-1γ antibodies (n = 49)	Anti–TIF-1γ antibodies alone (n = 24)	*P*
Age at onset, mean (range) years	62 (29–89)	57 (27–75)	NS
No. men/no. women	22/27	9/15	NS
Skin eruptions			
Heliotrope rash	62	67	NS
Gottron's papules	82	83	NS
Perionychia erythema	62	50	NS
Nailfold punctate hemorrhage	38	39	NS
Truncal erythema	77	33	<0.01
Calcinosis	0	6	NS
Ulceration	3	17	NS
Clinical features			
Muscle weakness	75	61	NS
Raynaud's phenomenon	10	0	NS
Arthritis	3	6	NS
Fever	18	11	NS
Organ involvement			
Interstitial lung disease	2	8	NS
Internal malignancy	73	50	<0.05
Laboratory findings			
Elevated CK	69	56	NS
Highest CK level, mean (range) IU/liter	1,456 (55–8,670)	850 (40–2,805)	NS

* Except where indicated otherwise, values are the percent of patients. Anti–TIF-1 = anti–transcription intermediary factor 1; NS = not significant; CK = creatine kinase.

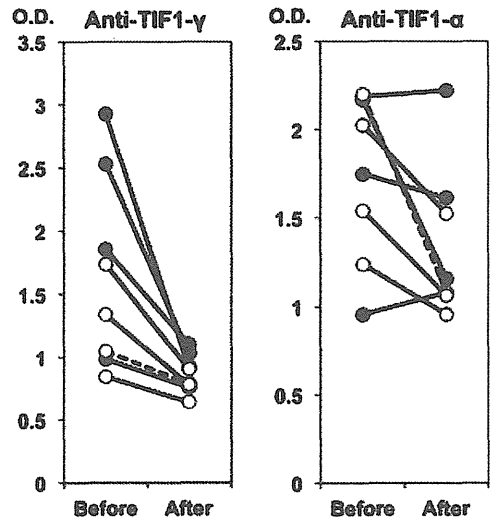

Figure 4. Relative titer of anti–TIF-1γ and anti–TIF-1α antibodies in sera from 8 DM patients before and after treatment. Recombinant TIF-1γ and TIF-1α proteins were coated onto microtiter plates, and antibody binding in the serum samples obtained at the first visit (before) and when patients underwent treatment and the disease was inactive (after) was evaluated by enzyme-linked immunosorbent assay. Solid circles indicate DM patients with malignancy; open circles indicate DM patients without malignancy; solid lines indicate adult patients with DM; dashed line indicates a patient with juvenile DM. OD = optical density (see Figure 1 for other definitions).

not shown). In contrast, the titer of anti–TIF-1α antibodies decreased in 6 patients after treatment, while 2 patients showed a slight increase in antibody titer (Figure 4).

DISCUSSION

In the current study, we have confirmed that the 155-kd protein recognized by anti-155/140 antibodies is TIF-1γ, and we have demonstrated that the 140-kd antigen is TIF-1α. Moreover, a portion of the patients also had autoantibodies directed to TIF-1β. Therefore, the TIF-1 family of proteins is targeted by anti-155/140 antibodies. Also, it was formally confirmed that anti-p155 and anti-155/140 antibodies both react with TIF-1γ and thus are indeed the same. This study also assessed the largest number of anti–TIF-1α/β/γ–positive patients (n = 78) to date and demonstrated that 65% of adult patients had cancer. It is also noteworthy that anti–TIF-1α/β/γ antibodies underlie a distinct subset of "young adult DM" without malignancy, in addition to juvenile DM and adult malignancy-associated DM.

While a few non-DM patients have been documented to have anti–TIF-1α and/or anti–TIF-1γ (anti–TIF-1α/γ) antibodies, including 1 patient with SLE (14)

and 1 patient with PM (24), anti–TIF-1α/γ antibodies are considered highly specific for DM. Anti–TIF-1α/γ antibodies have been detected in 18–23% of the adult DM patients in the US and European populations (14,22,24). In the current study, the prevalence of anti–TIF-1α/γ antibodies was 16%. This was higher than in our previous study, in which we observed anti–TIF-1α/γ positivity to be 7% (25 of 376) (21). This is mainly because our previous study only included patients having autoantibodies reactive with both TIF-1α and TIF-1γ. Nonetheless, the positivity may still be slightly lower than that reported in Caucasian populations. This may be due to ethnic differences since, for example, anti-CADM140 antibodies appear more frequent in Asian populations than in Caucasian populations (11,25,26).

The association of anti–TIF-1α/γ antibodies with cancer has been described in a number of reports. The incidence of cancer in anti–TIF-1α/γ–positive patients with adult DM is 42–75% (14,15,21,22,24,27). On the other hand, positivity for anti–TIF-1α/γ antibodies in cancer-associated DM is 43–75%. Another subset in which anti–TIF-1α/γ antibodies are frequently detected is juvenile DM. The frequency of anti–TIF-1α/γ antibodies in juvenile DM is 23–29% (14,23). The current study also confirmed that anti–TIF-1α/γ antibodies are frequently present in juvenile DM in a Japanese population. Moreover, use of a large population in our study had the merit of detecting a "young adult" population of DM patients who were positive for these antibodies, in addition to patients with juvenile DM, although this finding was not statistically significant due to the small number. These patients were age <40 years, female, and predominantly categorized as having clinically amyopathic DM. None of these patients had a history of malignancy. Nonetheless, further accumulation and followup of cases are needed to clarify whether these patients are at risk of malignancy, and whether this finding is also true across racial groups.

This report is the first to describe the presence of autoantibodies against TIF-1β in DM. A protein array analysis revealed that anti–TIF-1β antibodies were detected in 8 of 43 patients with colorectal cancer and 1 of 40 controls without cancer (28). In our study, 7 DM patients were positive for anti–TIF-1β antibodies. Among them, 6 were also positive for anti–TIF-1α/γ antibodies, while 1 was positive for anti–TIF-1β antibodies alone. Two patients were classified as having cancer-associated DM (ovarian cancer), while 2 other patients were classified as having "young adult" DM without cancer. No patients with juvenile DM were positive for anti–TIF-1β antibodies. Nonetheless, this finding may be due to a relatively small number of patients with juvenile DM.

The TIF-1 family, a subgroup of the tripartite motif–containing (TRIM) proteins, consists of at least 3 members: TIF-1α (TRIM24), TIF-1β (KAP1, TRIM28), and TIF-1γ (TRIM33). Additionally, TIF-1δ has been identified in mice (29), while its function remains relatively unknown. Studies have revealed intriguing roles of TIF-1 proteins in carcinogenesis. TIF-1α ubiquitinates the tumor suppressor gene p53 (30) and also activates estrogen-dependent genes associated with cellular proliferation and tumor development (31). The depletion of TIF-1α expression in human breast cancer cells causes spontaneous apoptosis (30), and aberrant overexpression of TIF-1α in breast cancer patients is frequent and correlates with poor survival (31). In contrast, in liver, TIF-1α is shown to act as a functional tumor suppressor gene by inhibiting the retinoic acid pathway in mice (32). TIF-1β has an antiapoptotic effect by inhibiting p53 acetylation and promoting p53 ubiquitination (33), and is overexpressed in gastric cancer (34). TIF-1γ, which appears to contribute to transforming growth factor β signaling, exerts a protective role in pancreatic carcinogenesis in mice by cooperating with $Kras^{G12D}$ (35).

Collectively, these findings demonstrate that TIF-1 proteins play pivotal positive and/or negative roles in carcinogenesis, suggesting the possibility that the autoantibodies to these proteins develop during antitumor immune responses that contribute to the development of cancer-associated DM. Indeed, Casciola-Rosen and colleagues have demonstrated that myositis autoantigen expression is markedly increased in cancers known to be associated with myositis but not in their related normal tissues, and have proposed that autoimmune response directed against cancer cross-reacts with regenerating muscle cells, enabling a feed-forward loop of tissue damage and antigen selection in cancer-associated myositis (36). With regard to the close relationship of TIF-1 proteins with p53, it has been well appreciated that autoantibodies to p53 are detected in patients with a wide variety of cancers (37). The close association of TRIM proteins with interferon (IFN)–mediated immunity is also noteworthy (38,39). Large numbers of TRIM proteins are up-regulated by IFN, and some are also reported to regulate IFN expression in turn. Since IFN is implicated in the pathogenesis of DM, it is intriguing to hypothesize that TIF-1 proteins serve as a bridge between cancer and IFN-mediated immunity.

In summary, the current study revealed that anti-155/140 antibodies that are frequently detected in

patients with cancer-associated DM target the TIF-1 family members TIF-1α, TIF-1β, and TIF-1γ. While TIF-1γ is the most commonly recognized antigen, antibodies to TIF-1α are also frequently detected. A small number of patients exhibited reactivity with TIF-1β. Since these TIF-1 proteins are highly homologous, it is plausible to hypothesize that TIF-1γ is the original target and that other antigens are recognized by cross-reactivity, especially based on the observation that most sera had reactivity with TIF-1γ. In immunoprecipitation assays using the sera that were preabsorbed with recombinant TIF-1γ protein, the reactivity with TIF-1α was substantially reduced in many cases (data not shown), supporting this notion that the autoantibodies predominantly target homologous sequences. In contrast, as shown in Figure 4, the directionality of the change in titer of anti–TIF-1α and anti–TIF-1γ antibodies was sometimes discordant, suggesting that not all antibodies are directed to the homologous sequences. Therefore, precise determination of the epitopes will be needed in the future. The TIF-1 family plays pivotal roles in oncogenesis, including p53 regulation, and overexpression of these proteins in tumor tissues has been reported, suggesting that autoantibodies to TIF-1 proteins may result from a misdirected antitumor response. Different reactivity to TIF-1 proteins in individual patients may be dependent on the tissue and/or types of the tumors.

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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. Fujimoto 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. Fujimoto.

Acquisition of data. Fujimoto, Hamaguchi, Kaji, Matsushita, Ichimura, Kodera, Ishiguro, Ueda-Hayakawa, Asano, Ogawa, Fujikawa, Miyagi, Mabuchi, Hirose, Akimoto, Hatta, Tsutsui, Higashi, Igarashi, Seishima, Hasegawa, Takehara.

Analysis and interpretation of data. Fujimoto, Hamaguchi, Kaji, Matsushita, Hasegawa, Takehara.

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The Multicenter Study of a New Assay for Simultaneous Detection of Multiple Anti-Aminoacyl-tRNA Synthetases in Myositis and Interstitial Pneumonia

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Abstract

Objective: Autoantibodies to aminoacyl-tRNA synthetases (ARSs) are useful in the diagnosis of idiopathic inflammatory myopathy (IIM) with interstitial pneumonia (IP). We developed an enzyme-linked immunosorbent assay (ELISA) system using a mixture of recombinant ARS antigens and tested its utility in a multicenter study. **Methods:** We prepared six recombinant ARSs: GST-Jo-1, His-PL-12, His-EJ and GST-KS expressed in *Escherichia coli*, and His-PL-7 and His-OJ expressed in Hi-5 cells. After confirming their antigenic activity, with the exception of His-OJ, we developed our ELISA system in which the five recombinant ARSs (without His-OJ) were mixed. Efficiency was confirmed using the sera from 526 Japanese patients with connective tissue disease (CTD) (IIM n = 250, systemic lupus erythematosus n = 91, systemic sclerosis n = 70, rheumatoid arthritis n = 75, Sjögren's syndrome n = 27 and other diseases n = 13), 168 with idiopathic interstitial pneumonia (IIP) and 30 healthy controls collected from eight institutes. IIPs were classified into two groups; idiopathic pulmonary fibrosis (IPF) (n = 38) and non-IPF (n = 130). Results were compared with those of RNA immunoprecipitation. **Results:** Sensitivity and specificity of the ELISA were 97.1% and 99.8%, respectively when compared with the RNA immunoprecipitation assay. Anti-ARS antibodies were detected in 30.8% of IIM, 2.5% of non-myositis CTD, and 10.7% of IIP (5.3% of IPF and 12.3% of non-IPF). Anti-ARS-positive non-IPF patients were younger and more frequently treated with glucocorticoids and/or immunosuppressants than anti-ARS-negative patients. **Conclusion:** A newly established ELISA detected anti-ARS antibodies as efficiently as RNA immunoprecipitation. This system will enable easier and wider use in the detection of anti-ARS antibodies in patients with IIM and IIP.

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Introduction

A number of autoantibodies can be detected in sera from patients with idiopathic inflammatory myopathy (IIM), some of which are specific to IIM (known as myositis-specific autoantibodies: MSAs). Detection of these autoantibodies is closely associated with IIM clinical manifestations [1,2].

Among MSAs, autoantibodies against aminoacyl-tRNA synthetases (ARSs) are the most frequently detected in adult IIM patients. To date, eight anti-ARS antibodies have been described. Anti-Jo-1 (histidyl-tRNA synthetase) [3,4] is the most common, occurring in approximately 20% of IIM patients [2,5]. Anti-PL-7 (threonyl) [6], anti-PL-12 (alanyl) [7,8], and anti-EJ (glycyl) [9] occur in ~3–4%, and anti-OJ (isoleucyl) [10] and anti-KS (asparaginyl) [11] occur in < 2% of IIM patients. Anti-tyrosyl- and anti-phenylalanyl-tRNA synthetases were also reported in one case each [12,13]. Patients with anti-ARSs show a spectrum of common clinical manifestations known as anti-synthetase syndrome (ASS), including myositis, interstitial pneumonia (IP), non-erosive arthritis, fever, Raynaud's phenomenon, and mechanic's

hands. Of note, the prevalence of IP in anti-ARS-positive patients is as high as 75–95% and IP sometimes precedes myositis [1,14,15]. Yoshifuji *et al.* reported that anti-ARS-positive patients with IP respond better to initial corticosteroid therapy but suffer from a significantly higher recurrence than anti-ARS-negative patients [1]. Therefore, anti-ARS antibodies are useful not only in diagnosing IIM but also in predicting late-onset myopathy in IP-proceeding patients and the clinical course of IP in myositis.

Currently, anti-ARS antibodies are detected using an enzyme-linked immunosorbent assay (ELISA), immunodiffusion or immunoprecipitation, but all of the antibodies are not routinely detected except for anti-Jo-1. To detect anti-ARS antibodies more readily, we established an ELISA system using a mixture of five recombinant ARS antigens: Jo-1, PL-7, PL-12, EJ, and KS. Our intention was to detect these autoantibodies simultaneously as "multiple anti-ARS antibodies". This ELISA system that we developed could be used to detect not only anti-ARS-positive myositis patients but also anti-ARS-positive idiopathic interstitial pneumonia (IIP) patients.

Materials and Methods

Patients

Serum samples were obtained from 694 Japanese adult patients with connective tissue disease (CTD) and IIP who had been followed at eight University Hospitals in Japan and 30 healthy volunteers. Patient diagnoses included IIM (n = 250), systemic lupus erythematosus (SLE) (n = 91), systemic sclerosis (SSc) (n = 70), rheumatoid arthritis (RA) (n = 75), SS (n = 27), other diseases (n = 13), and IIP (n = 168). The diagnoses of IIM, SSc, SLE, and RA were made on the basis of corresponding criteria proposed by Bohan and Peter [16] or the American College of Rheumatology [17,18,19]. IIP was defined as IP of unknown cause in which a patient did not fulfill classification criteria for any specific CTD or vasculitis, or whose lung disease was potentially caused by a drug or occupational-environmental exposure [20]. Patients with IIP were classified into two groups; an idiopathic pulmonary fibrosis (IPF) (n = 38; 12 by histological diagnosis) group and a non-IPF (n = 130; according to the typical radiographic patterns of chest high-resolution computed tomography) group.

All patients and healthy volunteers gave their written informed consent to participate in this study prior to sample collection that was performed in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (Approval number: E544) and also by institutional review boards of all participating centers (Table S1).

Immunoprecipitation

The presence of anti-ARS antibodies was determined by RNA immunoprecipitation (RNA-IP) as previously described [21]. The immunoprecipitated RNA was resolved using urea-polyacrylamide gel electrophoresis and visualized using silver staining. Each anti-ARS antibody was identified according to its mobility and tRNA pattern compared with standard serum.

Construction of expression plasmids for ARS-encoding cDNAs

For the expression and purification of recombinant proteins, full-length cDNAs of PL-12, EJ, PL-7, Jo-1, KS, and OJ (GenBank accession Numbers: D32050, U09587, NM_152295, AY995220, and BC001687, respectively) were first amplified using RT-PCR with HeLa total mRNA as a template. CDNAs for PL-12 and EJ were inserted into pET30a(+) (Novagen, Madison, WI, USA) and expressed as C-terminal His-tagged proteins. CDNAs for Jo-1 and KS were subcloned into pGEX4T-1 and pGEX6P-1 (GE Healthcare UK Ltd, Buckinghamshire, England), respectively, and expressed as N-terminal GST fusion proteins. CDNAs for PL-7 and OJ were engineered with a cMyc-epitope tag and His-tag sequence at their 3′ ends, and inserted into the pFastBacDual vector for baculovirus expression (Invitrogen, Carlsbad, CA, USA). Correct construction of plasmids was confirmed using DNA sequencing.

Expression and purification of recombinant ARSs

Expression and purification of His-tagged recombinant proteins: PL-12 and EJ were expressed in *Escherichia coli* BL-21(DE3) codon plus RIL bacteria (Stratagene, La Jolla, CA, USA). Competent cells were transformed with the vectors and the cells were incubated on Luria-Bertani (LB) agar plates containing 50 μg/mL kanamycin for 15 h at 37°C. A single colony was cultured in LB liquid medium containing kanamycin at 37°C. Addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to the medium was used to induce expression of recombinant PL-12 and EJ proteins. After a 2-h incubation, cells were harvested using centrifugation and resuspended in ice-cold phosphate buffered saline (PBS) at pH 7.5. The cells were sonicated and soluble cell lysates containing the His-tagged recombinant proteins were separated using centrifugation.

PL-7 and OJ were expressed in baculovirus-infected Hi-5 cells. Each of the expression vectors was transfected into SF-9 cells using Cellfectin (Invitrogen), and the baculovirus stock was prepared from the transfectant culture supernatant. Hi-5 cells infected with baculovirus were incubated for 72 h at 26°C and were harvested using centrifugation, and soluble cell lysates containing recombinant proteins were prepared as described above.

Soluble His-tagged recombinant ARSs were purified using immobilized metal ion affinity chromatography. Cell extracts were applied to TALON® Metal Affinity Resin columns (Clontech, Palo Alto, CA, USA), and the columns were washed with PBS containing 10 mM imidazole. Purified PL-12, EJ, PL-7, and OJ were eluted with PBS containing 50 mM imidazole.

Expression and purification of recombinant GST-ARS fusion proteins: Jo-1 and KS were also expressed in *E. coli* BL-21(DE3) codon plus RIL bacteria in the presence of ampicillin. Transformation, cultivation, induction, and extraction of soluble cell proteins were performed as described for PL-12 and EJ proteins. Soluble GST-Jo-1 and GST-KS fusion proteins were purified on Glutathione Sepharose 4B columns (GE Healthcare UK Ltd.) and eluted with Tris-HCl (pH 8.0) containing 15 mM GSH.

Immunoblotting of recombinant antigens

Purified recombinant ARS antigens were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane as described by Towbin *et al.* [22] with minor modifications. After blocking with 5% skimmed milk, the membrane was incubated for 60 min with serum diluted 1:100 and then incubated for 60 min with a 1:1000 dilution of goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL, Nagoya, Japan). Immunoreactive bands were detected using the Western Blot Detection System WEST-one (iNtRON Biotechnology, Gyeonggi-do, Korea).

ELISA

For detection of each ARS autoantibody, purified recombinant ARSs were individually coated on 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA). PL-12, EJ, PL-7, and

Jo-1 were diluted in PBS to a final concentration of 2.5 μg/mL, and KS to 5.0 μg/mL. Each diluent was added at 100 μL/well and incubated overnight at 4°C. The plates were washed twice with PBS, and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose overnight at 4°C. Sera from patients and normal healthy donors were diluted 1:100 in PBS containing 0.15% Tween 20 (PBS-T), 1% casein enzymatic hydrolysate, and 0.2 mg/mL *E. coli* extract, and 100 μL was applied to each well. After incubation for 60 min at room temperature (RT), the wells were washed four times with PBS-T. Goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL) was diluted 1:7000 in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid (peroxidase stabilizer), and 100 μL was added to each well. After incubation for 60 min at RT, the wells were washed four times with PBS-T, and 3,3',5,5'-tetramethylbenzidine substrate was then added. After a 30-min incubation at RT, the reaction was stopped by adding 100 μL of 0.25 N sulfuric acid and absorbance was read at 450 nm (A_{450}).

For simultaneous detection of five ARS autoantibodies, purified recombinant ARSs were diluted and mixed together in PBS and coated on plates. The final concentrations of PL-12, EJ, PL-7, Jo-1, and KS were 1.25 μg/mL, 0.63 μg/mL, 1.25 μg/mL, 0.63 μg/mL, and 2.5 μg/mL, respectively. The total protein concentration of the mixture was 6.25 μg/mL. ELISA plate preparation and assays were performed as described above. Conversion from A_{450} to a unit value (U/mL) was calculated using the following formula:

$$Unit\ Value\,(U/mL) = \frac{A_{450} < Sample > - A_{450} < Blank >}{A_{450} < Positive > - A_{450} < Blank >} \times 100$$

A_{450}<Positive> is the absorbance for an anti-Jo-1-positive patient serum that corresponds to a 100 U/mL value. A_{450}<Blank> is the background absorbance of buffer that does not contain serum. A_{450}<Sample> is the absorbance of a tested serum. The cutoff point was defined at 25 U/mL based on the analysis of the receiver operating characteristic curve in this multicenter study.

Statistical analysis

Statistical analyses were performed using StatView version 5.0 software. Clinical information of anti-ARS-negative and positive non-IPF patients was compared using the two-sample t-test or the Fisher's exact test.

Results

Autoantigen preparation

We first prepared six recombinant His-tagged ARS antigens, which were all expressed in *E. coli*. Immunoblot analysis showed that four of them, Jo-1, PL-12, EJ, and KS, were identified by their corresponding autoantibodies as well as by using an ELISA, whereas PL-7 and OJ reacted weakly with their corresponding autoantibodies (data not shown). Because we hypothesized that poor antigenic activity of recombinant PL-7 and OJ was due to a lack of posttranslational modification or proper structural folding, we prepared both fusion proteins expressed in eukaryotic Hi-5 cells using the baculovirus system. We confirmed antigenic activity of the new recombinant PL-7 using an ELISA (Fig.ô 1a) but the activity was lost when examined using immunoblotting (Fig.ô 1c). Recombinant PL-7, denatured using urea or SDS, had weaker antigenic activity than non-denatured PL-7, showing that the 3-dimensional protein structure played an important role in the reaction between the threonyl-tRNA synthetase and the anti-PL-7 antibody (Fig.ô 1a). Because of this antigenic characteristic of PL-7, we decided to prepare other recombinant ARSs, without denaturing reagents, as soluble polypeptides in PBS. Because His-Jo-1 and His-KS were insoluble, they were expressed as GST-recombinant proteins. ELISA revealed that the five newly prepared ARS antigens, His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7, displayed suitable antigenic reactivity. Immunoblotting also showed that four of the five ARS antigens, except for His-PL-7, had sufficient antigenic activity (Fig.ô 1b and c).

The recombinant OJ expressed in Hi-5 cells had weak antigenic activity, as confirmed using both immunoblotting and an ELISA (data not shown), suggesting that it is difficult to prepare a recombinant OJ as a single polypeptide that retains antigenic activity.

Establishing an ELISA system for simultaneous detection of five ARS antibodies

To detect multiple ARS antibodies simultaneously, we developed an ELISA system using a mixture of the five recombinant ARSs except for OJ. We tested a variety of antigen mixtures to estimate the most appropriate ratio and concentration to use, and we found that anti-ARS-positive sera showed reactivity with all five different ARSs with the highest sensitivity and specificity occurring at antigen concentrations of 0.63, 1.25, 1.25, 0.63, and 2.5 μg/mL (6.25 μg/mL in total) for histidyl-, threonyl-, alanyl-, glycyl-, and asparaginyl-tRNA synthetases, respectively. To assess potential cross-reactivity, we compared the absorbance values (A_{450}) obtained using an ELISA on every single recombinant ARS with those obtained with the new ELISA using the ARS mixture. When tested using a single-peptide-ELISA, each of the five anti-ARS antibodies showed reactivity with only its corresponding autoantigen. Samples positive for anti-PL-7, PL-12, or KS antibodies showed higher A_{450} values with the new mixed-peptide-ELISA than with the single-peptide ELISA, whereas the samples positive for anti-Jo-1 or EJ antibodies showed no significant difference in A_{450} values obtained with the two ELISAs. Such differences in A_{450} values may be due to different peptide-coating efficiencies because the total peptide concentration was higher in the mixed-peptide-ELISA than in the single-peptide ELISA (data not shown).

Clinical significance of anti-ARS ELISA in CTD

To confirm the efficiency of this newly established ELISA, we screened a total of 694 serum samples from patients with various CTDs and IIP, and 30 healthy controls. The results were compared between the ELISA and the RNA-IP assay (Fig.ô 2). A total of 102 samples were positive for anti-ARS antibodies using the ELISA and all of them, except for one, were identified to have any anti-ARS, other than anti-OJ, using the RNA-IP assay (Tableô 1). The sensitivity and specificity of the new ELISA in the detection of anti-ARS antibodies (including anti-OJ) compared with the RNA-IP technique were 97.1% and 99.8%, respectively (Tableô 1). Anti-ARS antibodies were detected in 30.8% (77/250) of IIM and 2.5% (7/276) of other CTDs (Tableô 2). None of the healthy controls were positive (Fig.ô 2). In IIM, 30.8% (33/107) of polymyositis (PM), 35.5% (33/93) of dermatomyositis (DM), 13.0% (3/23) of amyopathic DM, and 33.3% (1/3) of overlap myositis were positive for anti-ARS antibodies (Tableô 3). Among the 95 anti-ARS-positive IIM patients, 85 (89.4%) had IP, 54 (56.8%) arthralgia/arthritis, 24 (25.3%) had mechanic's hand, 37 (38.9%) had high fever, and 31 (32.6%) had Raynaud's phenomenon, which were consistent with previous reports [15]. The prevalence of these ASS symptoms was significantly higher in the anti-ARS-positive patients than in the negative patients (data

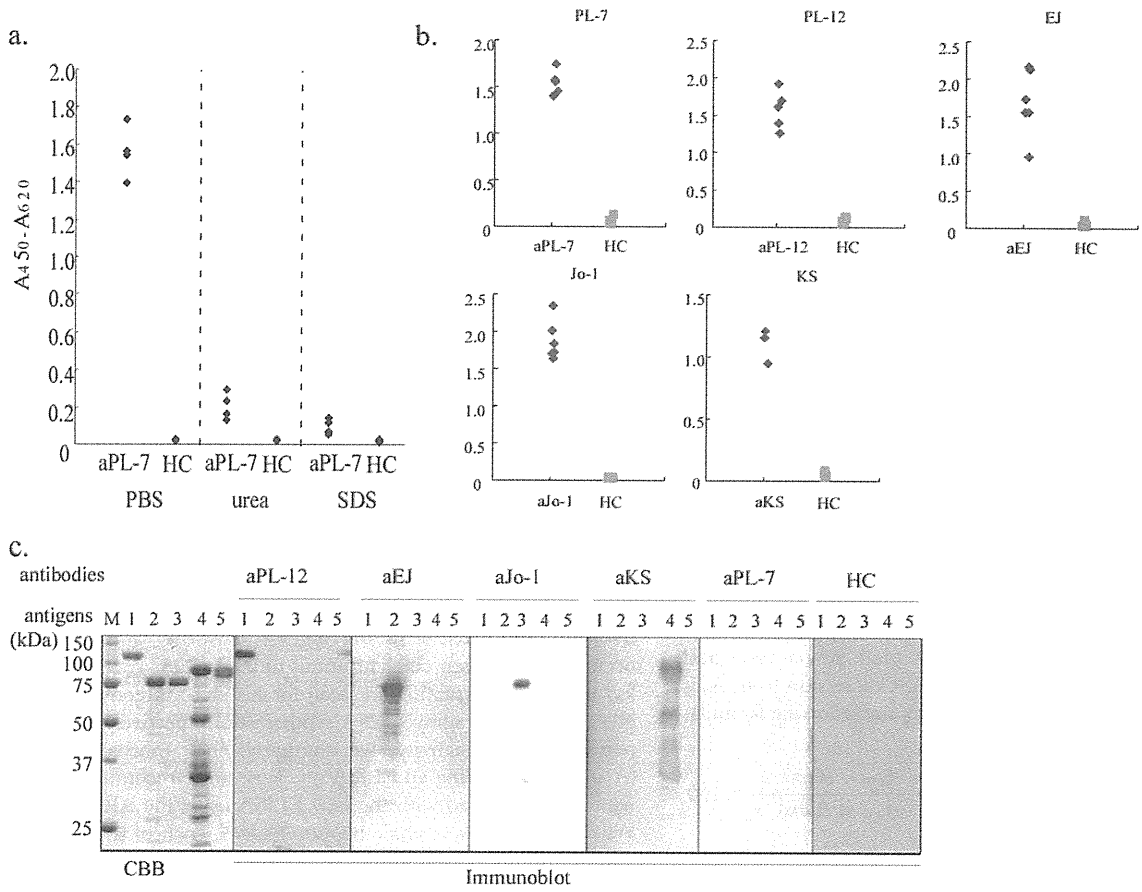

Figure 1. Antigenic activity of recombinant autoantigens a. Antigenic activity of PL-7 in various conditions. Left, purified recombinant PL-7 was eluted and diluted in PBS and coated on ELISA plates. Middle and Right, purified recombinant PL-7 was eluted in PBS and diluted in 8M urea and 2 × SDS sample buffer, respectively, and then coated onto ELISA plates. b. Five recombinant ARS antigens (His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7) were prepared as soluble polypeptides in PBS and their antigenic activity was tested in an ELISA using sera from five patients each containing corresponding autoantibodies (only GST-KS was tested using sera from three patients). Six healthy controls were used in each ELISA. c. Purified recombinant ARS antigens were electrophoresed on SDS-PAGE and transferred to a PVDF membrane followed by immunoblot analysis. CBB; Coomassie Brilliant Blue staining of gels, M; molecular weight marker, HC; healthy control, Lane 1; His-PL-12, Lane 2; His-EJ, Lane 3; GST-Jo-1, Lane 4; GST-KS and Lane 5; His-PL-7.
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not shown). There were seven anti-ARS-positive patients with other CTDs; two SSc patients were positive for anti-PL-12, two SLE patients were positive for anti-KS or anti-PL-12, and three RA patients were positive for anti-KS, anti-OJ or anti-PL-12.

Table 1 Comparison of the results between the new ELISA system and RNA-IP.

		RNA-IP		
		+	-	
anti-ARS ELISA	+	101*	1*	102
	-	0* (3)†	622* (619)†	622
	total	101* (104)†	623* (620)†	724

*The results detecting the five anti-ARS antibodies (anti-Jo-1, PL-12, EJ, KS, and PL-7) are described (sensitivity: 100%, specificity: 99.8%).
†Numbers in parenthesis are the results detecting all anti-ARS antibodies (including anti-OJ) (sensitivity: 97.1%, specificity: 99.8%).
doi:10.1371/journal.pone.0085062.t001

Clinical significance of anti-ARS ELISA in IIP

Anti-ARS antibodies were positive in 10.7% (18/168) of IIP patients. Only two patients (5.6%) with IPF were positive for anti-ARS; conversely, 16 patients (12.1%) with non-IPF were positive for anti-ARS antibodies (Tableô 2). To investigate whether the anti-ARS-positive IIP were clinically distinct from anti-ARS-negative IIP patients, we compared clinical backgrounds and treatments between anti-ARS-positive and negative non-IPF patients (Tableô 4). The anti-ARS-positive patients were significantly younger and a higher proportion was female ($p<0.01$), and they were treated more frequently with glucocorticoids (GC) or the combination of GC and immunosuppressants ($p<0.05$ and $p<0.01$, respectively).

Discussion

Among MSAs/myositis-associated autoantibodies (MAAs), anti-ARSs are the most frequently detected (28–37% [1,23,24]) in adult IIM patients, and anti-ARS-positive patients develop common characteristic symptoms known as ASS. Not only IIM but also apparent IIP patients can be positive for anti-ARS antibodies because IP often precedes myositis [1,14,20,25]. Both myopathy

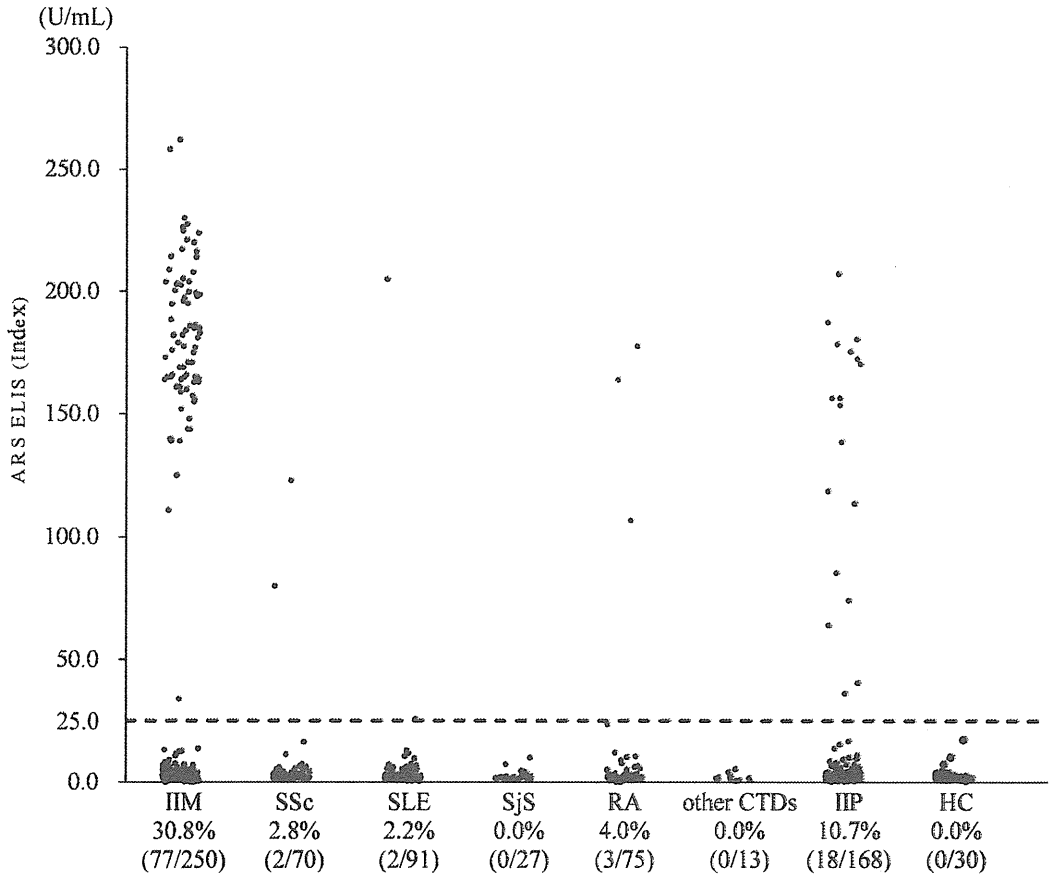

Figure 2. Confirmation of the efficiency of the ELISA system. Using the ELISA system, ARS antibodies were measured in 694 serum samples from patients with various CTDs and IIP, and 30 serum samples from healthy controls. The cutoff value (25 U/mL) is indicated by a horizontal dotted line.
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and IP anti-ARS-positive patients showed a better response to initial GC therapy but it can exacerbate the condition more often in anti-ARS-positive than in anti-ARS-negative patients [1,26]. Therefore, anti-ARS antibodies are useful not only in diagnosis, predicting the clinical course and therapy decisions in IIM, but also in classifying IP patients and predicting late-onset myopathy in IP-preceding patients.

An immunoprecipitation assay has been used to detect each anti-ARS antibody but to date, it can only be performed in a limited number of laboratories. To detect them more easily and routinely, we aimed to establish an ELISA system using the six recombinant ARS antigens to simultaneously detect anti-Jo-1, PL-7, PL-12, EJ, OJ, and KS antibodies. We did not include anti-tyrosyl or phenylalanyl synthetase because they have been reported only in one case each. However, some differences in clinical manifestations and prognoses among patients expressing different ARS antibodies, especially between anti-Jo-1 and non-anti-Jo-1 patients, have been observed [14,15]. However, different treatments for patients expressing different anti-ARSs have not been established. Currently, we treat anti-ARS-positive patients

Table 2 The frequency of each anti-ARS antibody in IIM, other CTD and IIP.

		RNA-IP(%)					
	ARS ELISA	Jo-1	PL-7	PL-12	EJ	KS	OJ
IIM	30.8% (77/250)	13.6	13.2	2.0	6.0	0.0	0.8
other CTDs	2.5% (7/276)	0.0	0.0	1.4	0.0	0.7	0.4
IIP	10.7% (18/168)	3.6	2.4	0.6	1.2	2.4	0.0
IPF	5.3% (2/38)	0.0	0.0	2.6	0.0	2.6	0.0
non-IPF	12.3% (16/130)	4.6	3.1	0.0	1.5	2.3	0.0

doi:10.1371/journal.pone.0085062.t002

Table 3 The frequency of each anti-ARS antibody in subsets of IIM.

IIM classification	Total	Jo-1	PL-7	PL-12	EJ	KS	n (%)
I polymyositis	107	18	7	3	5	0	33 (30.8)
II dermatomyositis	93	13	10	1	9	0	33 (35.5)
III amyopathic dermatomyositis	23	0	2	0	1	0	3 (13.0)
IV malignancy-associated myositis	7	0	1	0	0	0	1 (14.3)
V juvenile myositis	1	0	0	0	0	0	0 (0)
VI overlap myositis	3	1	0	0	0	0	1 (33.3)
VII unclassified	6	2	3	1	0	0	6(37.5)

doi:10.1371/journal.pone.0085062.t003

Table 4 Comparison of clinical backgrounds between anti-ARS (+) and (−) non-IPF patients.

	non-IPF n = 130		
	anti-ARS		
	(−) n = 114	(+) n = 16	p-value
age at the onset of the disease (yr) mean	69.6±9.5	56.9±14.5	<0.01
female (n; (ratio%))	39(34.2)	12(75.0)	<0.01
chronic (n; (ratio%))	104(91.2)	13(81.3)	N.S
subacute + acute (n; (ratio%))	5(4.4)	1(6.3)	N.S
acute (n; (ratio%))	2(1.8)	1(6.3)	N.S
glucocorticoids(GC) (n;(%))	49(43)	11(68.8)	<0.05
GC + immunosuppressants(IS) (n;(%))	19(16.7)	8(50.0)	<0.01
only drugs other than IS (n;(%))	8(7.0)	2(12.5)	N.S
PaO_2 at rest (Torr) mean	75.9±14.9	86.5±37.4	N.S
SpO_2 at rest (%) mean	95.7±2.4	97.1±2.1	<0.05
SpO_2 after 6 min walk test (Torr) mean	88.6±5.5	86.9±6.0	N.S
%VC (%) mean	87.7±22.5	77.9±17.4	<0.05
%DLCO (%) mean	51.0±19.5	58.0±23.1	N.S
KL-6 (U/mL) mean	1132±949	1287±693	N.S
SP-D (ng/mL) mean	207±180	180±136	N.S

%VC: % vital capacity, %DLCO: % diffusing capacity of carbon monoxide.
doi:10.1371/journal.pone.0085062.t004

with expectation of a standard clinical course in which the disease can recur with tapering of GC and in which exacerbation of IP is associated with a poor prognosis [1,14]. Therefore, presently, we are focusing on determining whether a patient with IIM or IIP is anti-ARS positive or not for the first screening when we begin treatment. This is why we decided to use a mixture of ARS antigens and not just single antigens to detect 'multiple anti-ARS antibodies' simultaneously.

We first prepared recombinant ARSs in *E. coli*, but recombinant PL-7 and OJ did not react well with their corresponding autoantibodies either using immunoblotting or an ELISA. For PL-7, structural conformation was important for antigenic activity because the recombinant PL-7 showed good reactivity only when it was expressed in a eukaryotic Hi-5 cell and was not denatured prior to being measured in the ELISA. Conversely, when recombinant PL-7 was denatured with urea or SDS, it was weakly detected with the PL-7 antibody, although its antigenicity was not completely lost. Such antigenic characteristics have also been reported previously by others [27]. This suggests that the synthetase epitope recognized by the anti-PL-7 antibody is in its native tertiary conformation.

In contrast, recombinant OJ (isoleucyl-tRNA synthetase) was not well detected even when it was expressed in Hi-5 cells and analyzed under non-denaturing conditions. This may be due to the unique feature of this isoleucyl-tRNA synthetase, which is a component of the multi-enzyme complex containing nine ARSs with three nonenzymatic factors [28,29]. In screening tests, positivity of anti-OJ in patients' sera was determined by the pattern of immunoprecipitation using HeLa cell extracts as originally described by Targoff *et al.* [28]. But there is a possibility that some 'anti-OJ antibodies' may recognize other components of the multi-enzyme complex rather than isoleucyl-tRNA synthetase itself, or alternatively the structural conformation of the complex may be important for recognition by anti-OJ, as was previously suggested by Targoff *et al.* [10]. They examined 11 patient sera with anti-OJ for evidence of reaction with other components of the complex. Ten out of 11 sera significantly inhibited enzyme activity of isoleucyl-tRNA synthetase, but some of them also significantly inhibited other ARSs such as leucyl-, lysyl-, or arginyl-tRNA synthetases. Moreover, immunoblot analysis of anti-OJ revealed that the majority of the sera could not identify a shared band and only a few sera recognized isoleucyl-tRNA synthetase. These results suggest that most 'anti-OJ sera' may react with multiple synthetases of the multi-enzyme complex or react with conformational epitopes of the complex. For this reason, we considered that it would be difficult to prepare the immunoreactive OJ antigen as a single molecule; therefore, we developed an ELISA system using the other five recombinant ARSs. This may not significantly affect the sensitivity of the ELISA because the prevalence of anti-OJ antibodies in patients is very low among the six anti-ARS antibodies.

The efficiency of this newly established ELISA system was acceptable because the sensitivity and specificity of the system compared with RNA immunoprecipitation were 97.1% and 99.8%, respectively, even if anti-OJ-positive sera was not excluded. The prevalence of anti-ARS in our IIM cohort was comparable with previous reports [1,2]. It was noteworthy that 10.7% of IIP patients, and in particular, 12.1% of non-IPF patients were positive for anti-ARS antibodies and there were some differences between anti-ARS-positive and negative IIP patients in their clinical backgrounds and treatments. Anti-ARS-positive patients were treated significantly more frequently with GC or the combination of GC and immunosuppressants. However, we are not yet ready to recommend immunosuppressive therapy for anti-ARS-positive IIP patients because we have not yet collected enough data on their clinical response and prognosis. Although some of these anti-ARS-positive IIP patients might develop myopathy later, it suggests that the measurement of anti-ARS antibodies may be useful in stratifying patients into disease subsets, which may help in predicting their clinical course.

A line-blot assay for the detection of multiple MSAs/MAAs (EUROLINE Myositis Profile 3) has been used in which anti-Jo-1, PL-7, PL-12, EJ, and OJ are included. This system can detect and discriminate MSAs/MAAs without further anti-ARS tests, but it does not include anti-KS, which has a stronger association with IIP than myositis [30]. To address this point, our system can more efficiently detect anti-ARS and therefore, is the preferred assay to use for IIP patients than the line-blot assay, although our ELISA does not aim to discriminate specificity for each anti-ARS antibody.

In conclusion, our ELISA system using a mixture of five recombinant ARSs shows similar efficiency to RNA immunoprecipitation and makes it possible to more readily detect anti-ARS antibodies in patients with PM/DM and IIP, and can be widely applied in daily practice.

Supporting Information

Table S1 The list of approval by institutional review boards of all participating centers.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TM RN. Performed the experiments: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Analyzed the data: RN YI. Contributed reagents/materials/analysis tools: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Wrote the paper: RN.

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RESEARCH ARTICLE Open Access

Epidemiologic study of clinically amyopathic dermatomyositis and anti-melanoma differentiation-associated gene 5 antibodies in central Japan

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Abstract

Introduction: Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association. We recently detected autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) in more than 20% of patients with dermatomyositis. Anti-MDA-5 antibodies were associated with the presence of rapidly progressive interstitial lung disease in clinically amyopathic dermatomyositis (CADM). The present study aims to assess the growing prevalence of CADM and the geographical incidence of anti-MDA-5-positive patients.

Methods: We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 patients, including 36 CADM patients. Sera were obtained from 1994 through 2011. Statistical analyses were performed to assess whether CADM development and the presence of anti-MDA-5 antibodies were associated with various parameters, including age at disease onset, season of onset, annual positivity, and population of resident city.

Results: Tertiles based on the year when the sera were collected showed increasing tendencies of CADM and anti-MDA-5-positive patients among all of the dermatomyositis patients. From 1994 to 2010, the relative prevalence of CADM and anti-MDA-5 antibody-positive patients significantly increased. Interestingly, the presence of anti-MDA-5 antibodies in 26 patients was inversely associated with the population of their city of residence.

Conclusions: This is the first study to examine the distribution of anti-MDA-5-positive dermatomyositis phenotypes in Japan. Regional differences in the incidences of these phenotypes would suggest that environmental factors contribute to the production of antibodies against MDA-5, which triggers innate antiviral responses.

Introduction

Idiopathic inflammatory myopathies are a heterogeneous group of autoimmune disorders that target the skeletal muscle and skin. Disease-related death is generally associated with malignancy and interstitial lung disease. The most frequent forms, polymyositis and dermatomyositis (DM), are thought to result from environmental exposure that leads to immune activation in genetically susceptible individuals. Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association [1-5].

A subgroup of DM patients who have typical skin manifestations of DM but little evidence of myositis has been recognized as clinically amyopathic dermatomyositis (CADM) [6]. Although it is still undetermined whether CADM is a distinct clinical entity or just an early phase of classic DM, rapidly progressive interstitial lung disease (ILD) can occur in CADM patients, especially in East Asia [7]. This patient subset with CADM and rapidly progressive ILD has been shown to have specific autoantibodies, originally called anti-CADM-140 antibodies [8]. The target autoantigen is melanoma differentiation-associated gene 5 (MDA-5) [9-11], which

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plays important roles in the innate immune system during RNA virus infections [12].

To better understand this subset of patients, it is important to examine the epidemiologic characteristics of CADM patients with anti-MDA-5 antibodies, whose outcome is often fatal. According to our clinical experiences, we have recently noticed that the prevalence of CADM patients with anti-MDA-5 antibodies seems to be growing, particularly in rural areas. We therefore examined the epidemiologic features of CADM and anti-MDA-5 antibodies in a single cohort of DM patients.

Materials and methods

Patients

We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 Japanese patients (one of them a half-Japanese, half-Filipino boy) with DM, including 36 patients with CADM, 15 patients with cancer-associated DM and 44 patients with classical DM, who were seen by or consulted the Department of Dermatology at Nagoya University Graduate School of Medicine from 1994 to 2011. These patients were diagnosed with DM or CADM based on the criteria of Bohan *et al.* [13] or Sontheimer [6], respectively. In general, CADM presents as typical skin lesions and amyopathy or hypomyopathy that lasts for more than 6 months. The CADM group included patients who developed fatal ILD within the first 6 months after disease onset. Since juvenile DM with rapidly progressive ILD and/or anti-MDA-5 antibodies has been reported in Japan [7,11,14], patients who manifested the disease at < 18 years of age were also included. Patients who were originally seen at other hospitals far outside our area and who then transferred to our hospital were excluded from the present study. Serum samples were obtained from all of the patients between 1 October 1994, the date when we began to build a serum bank of autoimmune rheumatic disease patients, and 30 June 2011. The population data on city of residence in 2010 were obtained from web data published by public offices in 25 cities, eight counties and one village.

The present study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. This study meets and is in compliance with all ethical standards in medicine. Informed consent including that for publication of the study was obtained from all patients according to the Declaration of Helsinki.

Immunoprecipitation

Anti-MDA-5 antibodies were screened by an immunoprecipitation assay using biotinylated recombinant MDA-5 produced from full-length MDA-5 cDNA using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) and the Transcend™ Colorimetric Non-Radioactive Translation Detection System (Promega), according to our published protocol [11]. This method was confirmed to produce consistent results based on a standard immunoprecipitation assay using ^{35}S-methionine-labeled cell extracts [11]. Serum samples from 82 patients were already characterized in our previous report [11]. All serum samples were stored at -70°C until the experiments.

Statistical analysis

The subjects were divided into tertiles based on year the sera were collected, age at collection, age at onset, or population of the city of residence, separately, to examine the associations between each of these factors and the development of CADM and the presence of anti-MDA-5 antibodies. The differences and linear trends across the tertiles were assessed using the chi-square test and the Cochran-Armitage trend test, respectively. SPSS version 17.0 for Windows (SPSS Japan Inc., Tokyo, Japan) was used to perform the statistical analysis. $P < 0.05$ was considered significant.

Results

Patient population

Between 1 October 1994 and 30 June 2011, sera from 95 patients with DM were collected. During 1994 sera were drawn from 24 patients, two-thirds of whom had been diagnosed with DM and treated by our department. The mean age at onset was 46.9 years (range: 1 to 80 years) and that at the time of sera collection was 50.2 years (range: 3 to 84 years). There were 67 (70.5%) female patients. Ten patients developed the disease under 18 years of age.

A review of the medical records indicated that 36 patients (28/36, 77.8% female; 5/36, 13.9% juvenile) had CADM. For these 36 patients, the mean age at onset was 44.9 years (range: 1 to 73 years) and that at the time of sera collection was 48.2 years (range: 3 to 84 years). Based on the immunoprecipitation assays, 26 patients (21/26, 80.8% female; 1/26, 3.8% juvenile) had anti-MDA-5 antibodies. For these 26 patients, the mean age at onset was 46.8 years (range: 11 to 66 years) and that at the time of sera collection was 48.2 years (range: 11 to 71 years). Twenty-five patients with anti-MDA-5 antibodies were diagnosed as CADM, and the remaining patient met the criteria for classical DM. All but one of our patients with anti-MDA-5 antibodies had ILD.

To grasp the overall trend, tertile analysis was conducted based on the number of cases for all patients with DM as well as for patients with CADM and those with anti-MDA-5 antibodies (Table 1). The mean ages at onset and at the time of sera collection did not significantly differ among the tertiles (data not shown), but