Table I. *Correlation of serum miR-7 levels with clinical and laboratory features in patients with dermatomyositis (DM)*

	DM patients (n=20)	Serum miR-7 Reduced (n=13)	Serum miR-7 Normal (n=7)	p-value
Clinical features				
Gottron's eruption (%)	88.9	100.0	71.4	0.14
Heliotrope coloration (%)	58.8	63.6	50.0	0.48
ILD (%)	38.9	45.5	28.6	0.42
Internal malignancy (%)	25.0	38.5	0.0	0.08
Joint involvement (%)	40.0	42.9	33.3	0.67
Dysphagia (%)	33.3	36.4	25	0.59
Laboratory features				
ANA (%)	45.0	46.2	42.9	0.63
IgG (mg/dl), mean	1,467.8	1,231.3	1,901.3	0.42
CK (IU/l), mean	2,510.8	3,220.8	1,192.1	0.97
Myoglobin (ng/ml), mean	794.8	975.6	484.9	0.93
Aldolase (IU/l), mean	46.0	71.5	10.3	0.32

DM: dermatomyositis; ILD: interstitial lung disease; ANA: antinuclear antibodies; CK: creatinine kinase.

miRNA extraction from tissue and PCR analysis of miRNA expression

Small RNAs were extracted from tissue section using a miRNeasy FFPE kit (Qiagen, Valencia, CA, USA). For PCR array, RNAs were reverse-transcribed into the first strand cDNA using an RT² miRNA First Strand Kit (SABioscience, Frederick, MD, USA). Equal amounts of samples from 3 normal skin or 3 DM skin were pooled and used in a single experiment: the mixed cDNA was added with RT² Real-Time SYBR GREEN/ROX PCR Master Mix (SABioscience) and applied into a 96-well RT2 miRNA PCR Array (SABioscience) that includes primer pairs for 88 human miRNAs (6). PCR was performed on a Takara Thermal Cycler Dice (TP800®) following the manufacturer's protocol. Threshold cycle (Ct) for each miRNA was extracted using Thermal Cycler Dice Real Time System ver2.10B. The raw Ct was normalized using the values of small RNA housekeeping genes.

For quantitative real-time PCR, cDNA was synthesized from total miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio Inc, Shiga, Japan). Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800®) used primers and templates mixed with the SYBR Premix. The sequence of miR-7 primer was designed based on miRBase (http://www.mirbase.org):caaagtgcttacagtgcaggtag. The primer set was prevalidated to generate single amplicons. DNA was amplified for 40 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. Transcript level of miR-7 was normalized to that of U6.

miRNA extraction from serum and PCR analysis of miRNA expression

miRNA isolation from serum samples were performed with miRNeasy RNA isolation kit (Qiagen) following the manufacturer's instructions with minor modification (7, 8). Briefly, 100 µl serum were supplemented with 5 µl of 5 fmol/µl synthetic non-human miRNA (C. elegans miR-39, Takara) as a control providing an internal reference for normalization of technical variations between samples. After Qiazol solution (1 ml) was added and mixed well by vortexing, then samples were incubated at room temperature for 5 min. Aqueous and organic phase separation was achieved by the addition of chloroform. The aqueous phase was applied to RNeasy spin column and RNeasy MinElute spin column. miRNA was eluted from the column with nuclease-free water.

cDNA was synthesized from total miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit. Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800®) used primers and templates mixed with the SYBR Premix. Transcript level of miR-7 was normalized to that of cel-miR-39.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U test for comparison of medians and Fisher's exact probability test for the analysis of frequency. p-values <0.05 were considered significant.

RESULTS

miRNA expression profile in dermatomyositis skin

As an initial experiment, to determine which miRNAs were involved in the pathogenesis of DM, we purified total miRNAs from Gottorn's eruption of 3 DM patients and from normal skins from 3 healthy controls. Equal amount of samples from the 3 normal skin or 3 DM skin were pooled and used in a single experiment; various miRNA expression in normal or DM skin *in vivo* was determined using PCR array, consisting of 88 miRNAs involved in human cell differentiation and development. The miRNA expression profile indicated that 5 miRNAs were overexpressed and 27 miRNAs were suppressed in DM skin compared with normal skin (Table SI; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1459). Among them, we focused on miR-7, one of the miRNAs which are expressed in normal skin but not in DM skin, because miR-7 is thought to be increased in SSc skin fibroblasts and may be involved in the pathogenesis of SSc (9, 10). The array analysis was performed as a single experiment, and statistical significance of the data could not be evaluated. Therefore, we confirmed the array results by real-time PCR using specific primer for miR-7 with increased number of samples (8 DM skin, 6 SSc skin and 7 control skin). Consistent with the array data, the miR-7 levels in DM skin were significantly down-regulated compared with those in normal skin, while they tended to be up-regulated in SSc skin (Fig. 1).

Serum concentrations of miR-7 in dermatomyositis patients

We next tried to determine serum concentration of miR-7 by quantitative real-time PCR and evaluated the possibility that serum miR-7 levels could be a disease marker for DM.

There has been no report demonstrating the expression of miR-7 in cell-free body fluid such as serum or urine. To validate that the miRNA in human serum is indeed detectable, miRNA was extracted from sera of healthy individual and quantitative real-time PCR was

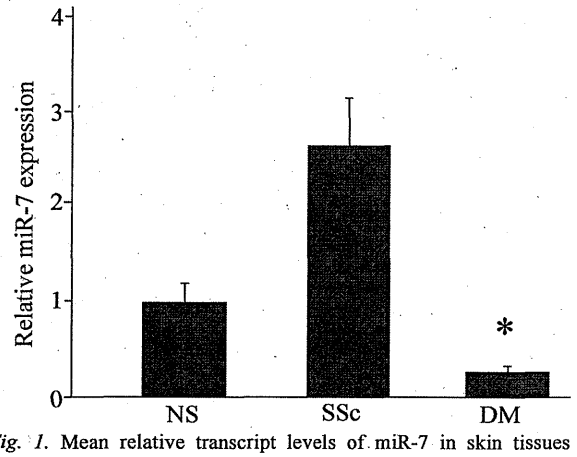

Fig. 1. Mean relative transcript levels of miR-7 in skin tissues of dermatomyositis (DM), systemic sclerosis (SSc) and normal subjects (NS). Error bars represent standard error. *$p<0.05$ compared with the values in NS (1.0).

performed using primer set specific for miR-7, as described in previous publications (7, 8). The amplification of miR-7 was observed, and Ct values were increased by the serial dilution of the miRNA (data not shown). Thus, miR-7 is likely to be not only detectable, but quantitative, in the serum by our method.

The serum miR-7 levels in patients with DM are shown in Fig. 2. Serum samples were obtained from 20 patients with DM, 5 with CADM, and 10 with PM. Seventeen healthy control subjects, 10 SLE patients and 10 SSc patients were also included in this study.

Mean serum miR-7 levels were lower in DM and CADM patients than in healthy control subjects, and there was statistically significant difference in the values between control subjects and DM patients ($p<0.00001$) or CADM patients ($p=0.00036$). In addition, serum

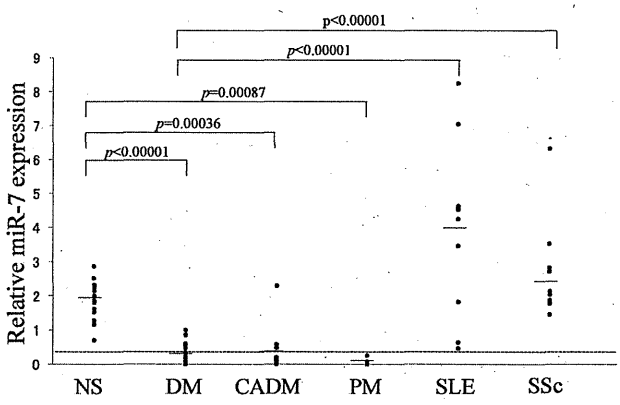

Fig. 2. Serum concentrations of miR-7 in patients with dermatomyositis (DM), clinically amyopathic dermatomyositis (CADM) or polymyositis (PM) and normal subjects (NS). miR-7 levels were measured with real-time PCR, as described in Materials and Methods. The maximum value in patients with DM was set at 1. Serum miR-7 concentrations are shown on the ordinate. Bars show means. The dotted line indicates the cut-off. *p*-values are determined using a Mann–Whitney *U* test. SLE: systemic lupus erythematosus; SSc: systemic sclerosis.

miR-7 levels were also significantly decreased in PM patients compared with healthy controls ($p=0.00087$). On the other hand, miR-7 levels in patients with SLE or SSc were slightly higher than those in healthy controls, but not statistically significant. We also found significant difference in miR-7 levels between DM patients and patients with SLE ($p<0.00001$) or SSc ($p<0.00001$). Taken together, the decrease in serum miR-7 levels was highly specific to the patients with DM, CADM or PM.

When we set the cut-off value at mean –3 SD of serum miR-7 levels in healthy controls subjects, the serum miR-7 levels were decreased in 13 of 20 DM patients (65%), 7 of 10 CADM patients (70%), all of 5 PM patients (100%), and none of 10 SLE and SSc patients. All the values in healthy controls were above the cut-off.

Association of serum miR-7 levels with clinical or laboratory features of dermatomyositis

Finally, we examined the association of serum miR-7 levels with clinical manifestations of DM patients (Table I). Although not statistically significant ($p=0.14$), the frequency of Gottron's eruption tended to be increased in patients with reduced serum levels of miR-7 than those with normal miR-7 levels, which is consistent with the array result that miR-7 expression is reduced in Gottron's eruption *in vivo* (Table SI). In addition, patients with decreased miR-7 levels tended to have higher prevalence of Heliotrope coloration, lung involvement, internal malignancy, joint involvement or dysphagia, as well as higher levels of CK, myoglobin or aldolase, but we could not find statistical significance.

DISCUSSION

In this study, we first identified several overexpressed or suppressed miRNAs in DM skin compared with normal skin by the miRNA PCR array analysis. Among them, miR-7 expression was down-regulated in DM skin. miR-7 expression is reported to be decreased in various cancer cells including glioblastoma and breast cancer (11, 12). On the other hand, there has been little research on miR-7 expression in the skin. We have shown that miR-7 expression is increased in SSc dermal fibroblasts by array analysis (9, 10). This study also showed that miR-7 expression is up-regulated in SSc skin. Thus, the expression of miR-7 may be differently regulated in each autoimmune disease.

Several studies have indicated that serum miRNAs are stable: miRNAs in serum are thought to be encapsulated in microvesicles shed from cell plasma membrane (13–17). Such microvesicles including miRNAs as well as RNA or DNA can be incorporated into adjacent cells, resulting in the alteration of gene expression in the recipient cell (17). Accordingly, serum miRNA may not merely come from apoptotic cells, but have some

functional roles (18). Although there has been no report determining serum miRNA in DM patients or determining serum miR-7 levels in human, we expected that miR-7 expression may also be decreased in serum of DM patients, and can be correlated with the disease activity of DM. Our results indicate that serum miR-7 levels are specifically decreased in DM patients as well as in patients with PM or CADM, but not decreased in those with other autoimmune diseases including SSc. Thus there is a possibility that serum miR-7 levels can be used as a diagnostic marker for PM/DM. Although we could not find significant association of serum miR-7 levels with clinical and laboratory findings of DM patients, this may be because of the small number of patients. In addition, we could not address the cellular source of miR-7 in the skin, the function of the miRNA, the detailed mechanism(s) by which miR-7 expression is decreased in the skin and sera of DM patients, and the role of decreased miR-7 in the pathogenesis of DM in this study. miR-7 has been reported to be expressed in several cell types including lymphocytes and fibroblasts (9, 19), and may target inflammatory molecules including fibroblast growth factor (FGF) 11 or CC chemokine ligand (CCL) 16 according to TargetScan (http://www.targetscan.org/). Our speculation is that, for example, miR-7 expression is decreased in the infiltrated lymphocyte or fibroblasts of DM skin, which may result in increased production of such inflammatory molecules, leading to the inflammation of skin. In addition, decreased serum miR-7 may indicate systemic down-regulation of miR-7 expression (e.g. in muscles or lung as well as skin). Larger studies are needed to clarify these points in the future.

Nakasa et al. (20) have reported that intravenous injection of miR-146a in collagen-induced arthritis model mice could prevent joint destruction. Clarifying the up- or down-stream events of down-regulated miR-7 in DM patients may lead to further understanding of the disease and a new therapeutic approach.

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Serum miR-21 levels in patients with dermatomyositis

Sirs,

There are only a few reports about the relationships between microRNAs (miRNAs) and polymyositis/dermatomyositis (PM/DM): Eisenberg *et al.* found that expression of several miRNAs are up- or down-regulated in the muscle tissues of PM/DM (1). First, we also determined the miRNA levels in muscle tissues of DM patients. Total miRNA were obtained from muscle samples of 4 DM patients and 4 healthy control subjects (2). Consistent with the previous report (1), miR-21 expression in the muscle tissues was significantly elevated in DM patients (Fig. 1a).

Accordingly, we tried to evaluate the possibility that serum levels of miR-21 can be a useful marker for the diagnosis and the evaluation of disease activity of DM. Serum samples were obtained from 30 DM patients (12 men and 18 women; mean age, 54.8 years). Control serum samples were also collected from 20 healthy volunteers, 10 clinically amyopathic DM (CADM) patients, 5 PM patients, 20 systemic lupus erythematosus (SLE) patients, and 10 systemic sclerosis (SSc) patients. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study. By the real-time PCR using hsa-miR-21 primer and total miRNA purified from serum of healthy volunteer (2), the amplification curve of miR-21 was observed, and Ct values were increased by the serial dilution of the miRNA (Fig. 1b). Thus, using our method, hsa-miR-21 was likely to be detectable and quantitative in the serum.

Then, before miRNA isolation, serum was supplemented with synthetic non-human miRNA (C. elegans miR-39, Takara) as controls providing internal reference for normalisation of technical variations between samples (3, 4). Mean serum levels of miR-21 corrected for C. elegans miR-39 levels in the same samples were significantly higher only in DM patients than those in normal subjects (Fig. 1c, p=0.041). It has been reported that serum miR-21 levels are increased in various human neoplastic disorders (5-7). Our results indicate miR-21 expression is also increased in the muscles and sera of DM patients, and may be useful for the diagnosis of DM. miR-21 expression has been previously described to be up-regulated in T lymphocytes of SLE patients (8-10), whereas serum miR-21 levels in SLE patients were higher than those in normal subjects, but not statistically significant in our study. This discrepancy may be due to the small number of patients or the different expression pattern of miR-21 between lymphocytes and serum.

When the cut-off value was set at mean value+2SD of the healthy controls, there was no significant difference between patients with elevated serum miR-21 levels (n=12) and those with normal levels (n=18) in terms of the prevalence of Gottron's sign (80.0 *vs.* 86.7%), Heliotrope rush (83.3 *vs.* 66.7%), lung involvement (50.0 *vs.* 40.0%), or internal malignancies (33.3 *vs.* 18.8%). Serum creatine kinase levels were not significantly different between patients with and without elevated miR-21 levels (1130.3 *vs.* 2861.1 U/l). However, we found mean serum IgG levels were significantly higher in patients with elevated miR-21 levels than those without (1837.4 *vs.* 1283.6 mg/dl, p=0.0041 by Mann-Whitney U-test). Serum IgG level is thought to reflect the abnormal activation of immune system and regarded as one of the disease markers in DM. Thus, serum miR-21 may also be correlated with the disease activity of DM and be involved in the pathogenesis of this disease. Another possibility is that increased miR-21 expression in DM is linked to the muscle reconstruction. Clarifying the role of miRNAs in this disease may lead to further understanding of the disease and new therapeutic approaches. Larger studies are needed by measuring serum levels of miR-21 as well as other miRNAs in increased number of patients in the future.

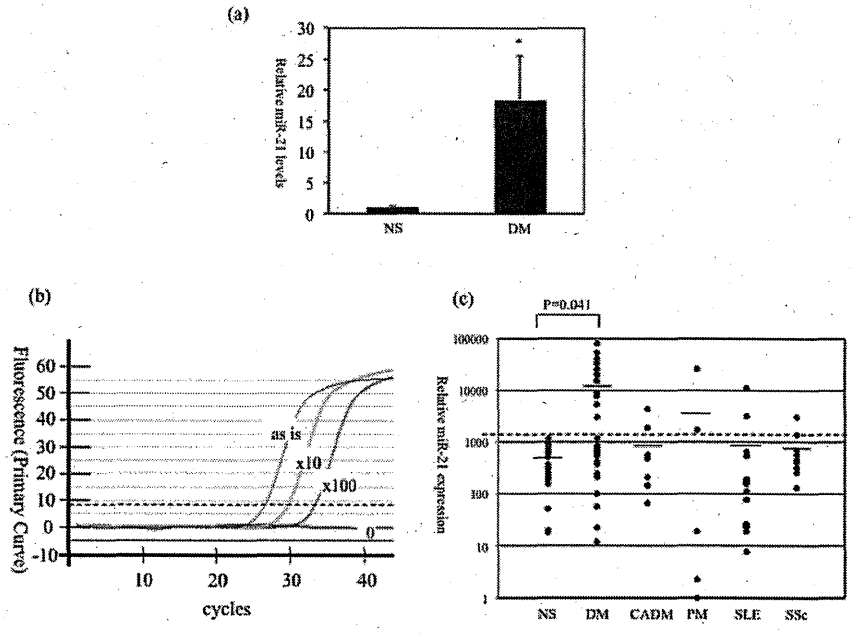

Fig. 1. (a) Mean relative transcript levels of miR-21 in muscle tissues from 4 normal subjects (NS) and 4 DM patients were determined by real-time quantitative PCR. *P<0.05 as compared with the value in samples from NS (1.0).
(b) miR-21 is present in serum sample. Serial dilution of cDNA (as is, 10-fold dilution, 100-fold dilation and 0) synthesised from serum miRNA was used as template for real-time PCR. Amplification curves of gene-specific transcripts are shown to illustrate the process of exponential increase of fluorescence. Horizontal dotted line indicates the threshold.
(c) Serum concentrations of miR-21 in patients with dermatomyositis (DM), clinically amyopathic dermatomyositis (CADM), polymyositis (PM), systemic lupus erythematosus (SLE), or systemic sclerosis (SSc) and in normal control subjects (NS). Serum was added with synthetic non-human miRNA (C. elegans miR-39) as a control providing an internal reference for normalisation of technical variations between samples. Then, total miRNA was purified. miR-21 levels were measured with real-time PCR, and corrected for the levels of C. elegans miR-39 in the same samples. miR-21 concentrations are shown on the ordinate. The horizontal dotted line indicates the cut-off level (mean+2SD of the values in NS). Bars show means. The minimum value was set at 1.

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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

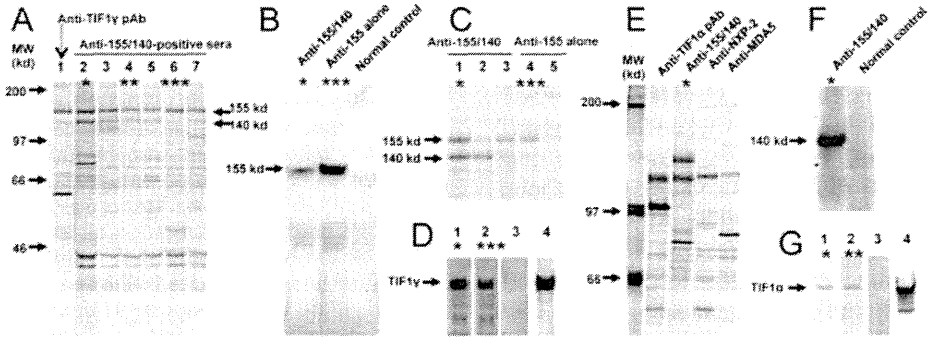

Figure 1. A, Immunoprecipitates with anti-155/140 autoantibodies from ^{35}S-methionine–labeled K562 cell extracts were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography. Lane 1, Anti–transcription intermediary factor 1γ (anti–TIF-1γ) 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–

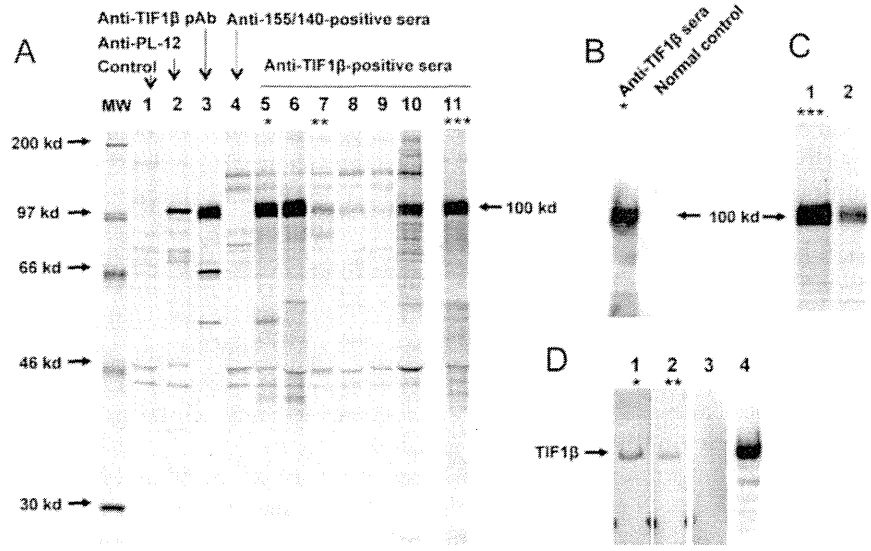

Figure 2. **A,** Immunoprecipitation of the 100-kd autoantigen from ^{35}S-methionine–labeled K562 cell extracts is shown, as described in Figure 1A. Lane 1, Normal human serum; lane 2, prototype serum positive for anti–PL-12 antibodies; lane 3, sample immunoprecipitated with polyclonal antibodies to 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-

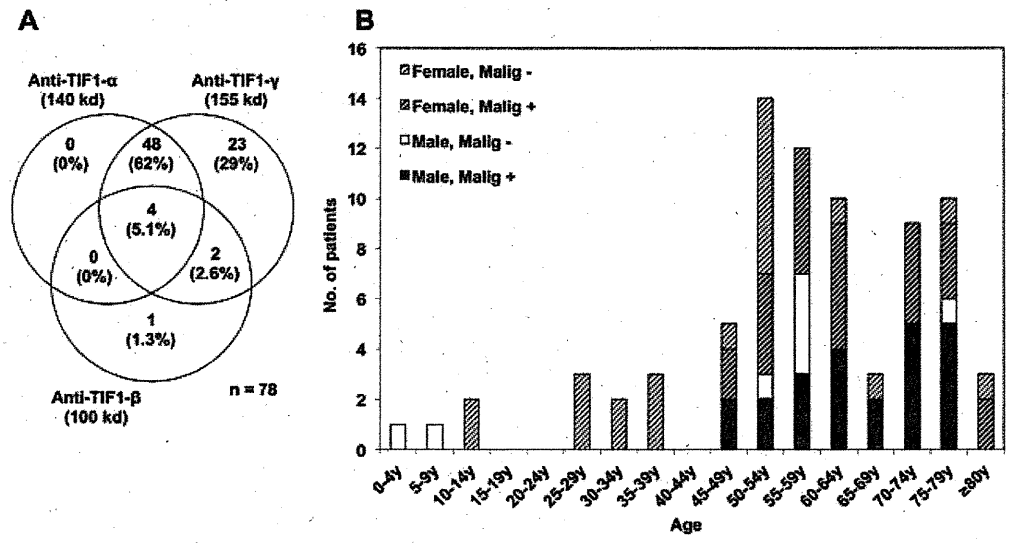

Figure 3. **A,** Numbers and percent of anti–TIF-1α, anti–TIF-1β, and anti–TIF-1γ antibodies in 78 DM patients. **B,** Distribution of the age at onset and the presence or absence of malignancy in 78 DM patients positive for anti–TIF-1α, anti–TIF-1β, and anti–TIF-1γ antibodies. Bars show the numbers 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.

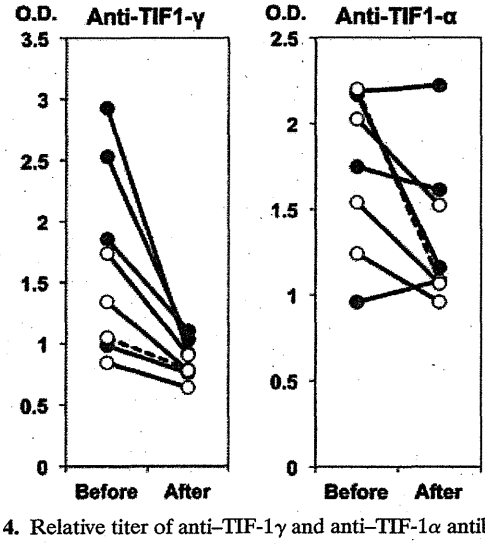

Figure 4. Relative titer of anti–TIF-1γ and anti–TIF-1α antibodies in sera from 8 DM patients before and after treatment. Recombinant TIF-1γ and TIF-1α proteins were coated onto microtiter plates, and antibody binding in the serum samples obtained at the first visit (before) and when patients underwent 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.

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CONCISE REPORT

Anti-NXP2 autoantibodies in adult patients with idiopathic inflammatory myopathies: possible association with malignancy

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ABSTRACT

Objectives Myositis-specific autoantibodies (MSAs) are useful tools for identifying clinically homogeneous subsets and predicting prognosis of patients with idiopathic inflammatory myopathies (IIM) including polymyositis (PM) and dermatomyositis (DM). Recent studies have shown that anti-NXP2 antibody (Ab) is a major MSA in juvenile dermatomyositis (JDM). In this study the frequencies and clinical associations of anti-NXP2 Ab were evaluated in adult patients with IIM.

Methods Clinical data and serum samples were collected from 507 adult Japanese patients with IIM (445 with DM and 62 with PM). Eleven patients with JDM, 108 with systemic lupus erythematosus, 433 with systemic sclerosis and 124 with idiopathic pulmonary fibrosis were assessed as disease controls. Serum was examined for anti-NXP2 Ab by immunoprecipitation and western blotting using polyclonal anti-NXP2 Ab.

Results Seven patients (1.6%) with adult DM and one (1.6%) with adult PM were positive for anti-NXP2 Ab. Except for two patients with JDM, none of the disease controls were positive for this autoantibody. Among eight adult patients with IIM, three had internal malignancies within 3 years of diagnosis of IIM. Another patient with DM also had a metastatic cancer at the diagnosis. All of the carcinomas were at an advanced stage (stage IIIb–IV).

Conclusions While less common than in juvenile IIM, anti-NXP2 Ab was found in adult IIM. Anti-NXP2 Ab may be associated with adult IIM with malignancy.

INTRODUCTION

Idiopathic inflammatory myopathies (IIM), including polymyositis (PM) and dermatomyositis (DM), are characterised by chronic inflammation of skeletal muscles and/or skin eruptions.[1] Although the aetiology remains unclear, they are considered as autoimmune diseases. The presence of disease-specific autoantibodies (autoAbs), known as myositis-specific autoAbs (MSAs), is a prominent feature. Moreover, MSAs are strongly associated with distinct clinical phenotypes and thus classify patients into groups with more homogeneous clinical features.[2–4] These MSAs include antibodies (Abs) to aminoacyl-tRNA synthetases, the Mi-2 protein, the signal-recognition particle, transcriptional intermediary factor-1 (TIF1; anti-155/140 Ab)[5–7] and melanoma differentiation-associated gene-5 (MDA5; anti-CADM140 Ab).[8]

Oddis and colleagues first described anti-MJ Ab in a US cohort of juvenile DM (JDM),[9] and Targoff *et al* subsequently identified that the antigen of anti-MJ Ab is nuclear matrix protein NXP2 (MORC3).[10] Gunawardena *et al* and Espada *et al* have demonstrated that anti-NXP2 Ab is among the most common MSAs in JDM.[11 12] In this study we evaluated the frequencies and clinical associations of anti-NXP2 Ab in adult patients with IIM.

METHODS

Patients

Serum samples were obtained from 507 consecutive Japanese adult patients with IIM, 445 with DM and 62 with PM, who had been followed up in the Department of Dermatology, Kanazawa University Hospital and collaborating medical centres from 2003 to 2010. All patients with PM and 365 patients with DM fulfilled Bohan and Peter's criteria.[13 14] The remaining 80 did not, but fulfilled Sontheimer's criteria of clinically amyopathic DM (CADM).[15] Among the patients with DM, as disease controls, 11 with JDM, 108 with systemic lupus erythematosus, 433 with systemic sclerosis and 124 with idiopathic pulmonary fibrosis were assessed. Clinical information was collected retrospectively by reviewing their clinical medical charts.

Immunoprecipitation and western blotting

Serum (10 μl) was incubated with 2 mg protein A-Sepharose beads (Amersham Biosciences, Piscataway, New Jersey, USA) in immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% Nonidet P-40) for 2 h. Beads were then mixed with ^{35}S-labelled or unlabelled K562 cell extracts derived from 10^7 cells and rotated at 4°C for 2 h. After five washes, precipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography or western blotting.

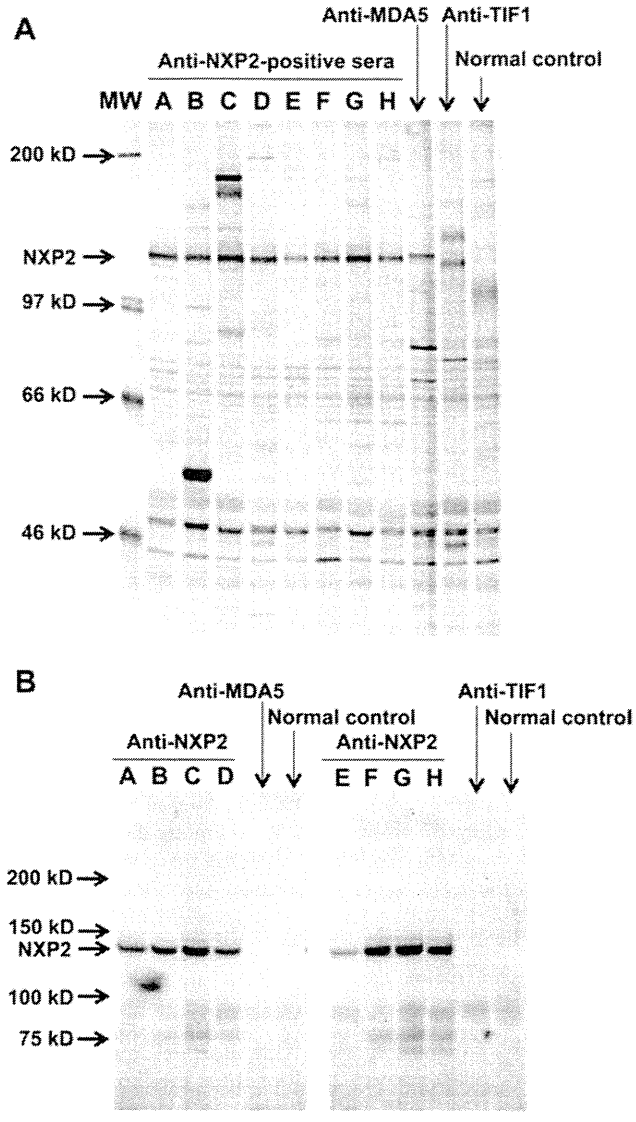

Figure 1 Detection of anti-NXP2 Ab. (A) Immunoprecipitant from ^{35}S-labelled K562 cell extract using serum samples were subjected to 7% SDS-PAGE. Lanes A–H correspond to anti-NXP2-positive patients shown in table 1. As controls, the prototype serum samples positive for anti-TIF1 Ab and anti-MDA5 Ab and normal control serum were also examined. Molecular weights (MW) are shown on the left. The position of the 140 kDa NXP2 protein is indicated by an arrow. (B) K562 cell extracts were immunoprecipitated with anti-NXP2-positive serum (patients A–H), anti-MDA5-positive serum, anti-TIF1-positive serum or normal control serum and subjected to SDS-PAGE, electro-transferred onto nitrocellulose membranes and probed with polyclonal anti-NXP2 Ab. The MW of NXP2 (140 kDa) is indicated by an arrow.

For western blotting, proteins were transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with mouse polyclonal anti-human NXP2 Ab (Abcam, Cambridge, UK) and then with horseradish peroxidase-conjugated goat antimouse IgG Ab (Thermo Scientific, Rockford, Illinois, USA). The membrane was developed using an enhanced chemiluminescence kit (Thermo Scientific).

Additional information regarding methods is available in the online supplement.

Statistical analysis

Fisher exact probability tests were used for comparison of frequencies. The Bonferroni test was examined for multiple comparisons of values following normal distribution. Values that were not normally distributed were evaluated by the Mann–Whitney U test. p Values <0.05 were considered significant.

RESULTS

Detection of anti-NXP2 Abs

Serum samples from 507 adult Japanese patients with IIM were screened for anti-NXP2 Ab. From K562 cell extracts, eight serum samples precipitated a 140 kDa protein which was different from other known autoantigens (figure 1A). Additionally, two patients with JDM were positive for this 140 kDa protein. No serum from other diseases precipitated this 140 kDa protein. On SDS-PAGE, the 140 kDa band was slightly higher than the 140 kDa band of anti-TIF1 Ab and was not accompanied by a 155 kDa band (figure 1A). While this 140 kDa protein migrated closely to MDA5, these serum samples were negative for anti-MDA5 Ab by an ELISA using recombinant MDA5 protein as antigen (data not shown). These serum samples were confirmed to react with NXP2 since precipitated proteins were recognised by polyclonal Abs to NXP2 in western blotting (figure 1B). They also reacted with recombinant human NXP2 protein by western blotting (see figure S1 in online supplement). These serum samples were negative for other MSAs and did not have other known autoAbs, except for patient B who was positive for anti-SS-A/Ro and SS-B/La Abs. In indirect immunofluorescence, five showed negative staining while three stained nuclei in speckled pattern at the maximum dilution of ×80.

Clinical and laboratory profiles of patients with IIM with anti-NXP2 Abs

Among 507 adult patients with IIM, eight were positive for anti-NXP2 Ab: seven (1.6%) in 445 adults with DM and one (1.6%) in 62 adults with PM. All patients with anti-NXP2 Ab showed strong muscle weakness and high elevation of serum creatine kinase levels. Remarkably, internal malignancies found within 3 years of the diagnosis of IIM were present in 37.5% (3/8) (table 2). All these patients had advanced disease (stage IIIb–IV). Additionally, in one patient (C), prostate cancer was found 42 months previously and it was metastatic when the diagnosis of DM was made. However, he was excluded from the statistical analysis since he did not meet the above criteria. Interstitial lung disease (ILD) was not found.

During the follow-up period which varyied from 2 to 61 months, seven of eight patients (all except patient H) were treated with systemic corticosteroid therapy. Patient A also received methotrexate and patient D also underwent intravenous immunoglobulin therapy. While the response to the treatment was favourable in all patients, two (patients C and G) died of malignancy.

Comparison with other MSAs

In addition to seven patients positive for anti-NXP2 Ab, adult patients with DM in this study included 74 patients with anti-TIF1 Ab and 15 with anti-Mi-2 Ab. They also included 51 with anti-MDA5 Ab, 26 with anti-PL-7 Ab, 18 with anti-Jo-1 Ab and 8 with anti-PL-12 Ab. Since anti-TIF1 Ab and anti-Mi-2 Ab are specifically associated with DM, the clinical features of patients with anti-NXP2 Ab were compared with those with anti-TIF1 Ab and anti-Mi-2 Ab (table 2).

Anti-NXP2 Ab was predominantly found in men. Although Gottron's sign was slightly less frequent, there was no significant difference in the frequency of each cutaneous manifestation. The frequency of fever was significantly higher than in patients

Table 1 Clinical and laboratory profile of adult patients with DM or PM positive for anti-NXP2 Ab

Patient	A	B	C	D	E	F	G	H
Age (years)	23	34	54	57	57	61	62	68
Sex	Male	Female	Male	Male	Male	Male	Memale	Male
Diagnosis	DM	DM	DM	DM	DM	DM	PM	DM
Duration (months)	11	21	10	8	23	4	2	7
Heliotrope rash	−	+	+	+	+	−	−	+
Gottron's sign	+	−	+	+	−	−	−	+
Perionychia erythema	−	−	+	+	+	+	−	−
Nailfold punctuated haemorrhage	−	−	+	−	+	+	−	+
V sign	−	−	+	+	+	−	−	−
Shawl sign	−	−	+	+	+	−	−	−
Scratch dermatitis	−	+	−	+	−	+	−	−
Erythema of extensor extremities	−	+	+	+	+	+	−	−
Calcinosis	−	−	−	−	−	−	−	−
Blistering	−	−	−	−	−	−	−	−
Ulceration	−	−	−	−	+	−	−	−
Muscle weakness	+	+	+	+	+	+	+	+
Raynaud's phenomenon	−	−	−	−	−	−	−	−
Fever	−	−	+	−	+	+	+	+
Arthralgia	−	+	−	−	−	−	−	−
Elevation of CK	+	+	+	+	+	+	+	+
Highest CK level (IU/l)	3857	1877	4927	1539	17140	26685	3713	3722
IIF titre	<40	80	<40	40	<40	<40	80	<40
IIF staining pattern	−	Sp	−	Sp	−	−	Sp	−
ILD	−	−	−	−	−	−	−	−
Malignancy	−	−	+	+	−	−	+	+
Origin	−	−	Prostate	Pancreas	−	−	Gallbladder	Lung
Histology	−	−	AC	ND	−	−	AC	SCC
Stage	−	−	IV	IVb	−	−	IVb	IIIb
Period of diagnosis*	−	−	42 months before	6 months after	−	−	Simultaneous	3 months before

*Period of diagnosis indicates when the diagnosis of malignancy was made before or after the onset of idiopathic inflammatory myopathies.
AC, adenocarcinoma; CK, creatine kinase; DM, dermatomyositis; IIF, indirect immunofluorescence; ILD, interstitial lung disease; ND, not done; PM, polymyositis; Sp, speckled; SSC, squamous cell carcinoma.

with anti-TIF1-positive DM. The highest creatine kinase level was similar to that in patients with anti-Mi-2 Ab-positive DM and significantly higher than in patients with anti-TIF1 Ab. The frequency of internal malignancy in those with anti-NXP2 Ab was lower than in those with anti-TIF1 Ab and higher than in patients with anti-Mi-2 Ab, although the differences were not statistically significant.

DISCUSSION

Two studies have recently detected anti-NXP2 Ab in 23% and 25% of patients with JDM in the UK and Argentina, respectively.[11 12] While the number of patients was small, anti-NXP2 Ab was detected in 18% in the control JDM population in this study. Anti-NXP2 Ab is therefore likely to be a major MSA in JDM across racial groups as well as anti-TIF1 Ab, which is detected in 23–29% of patients with JDM.[16] In addition to JDM, Espada *et al* reported that two (28%) of seven patients with juvenile PM were also positive for anti-NXP2 Ab.[12] In this study we identified eight anti-NXP2 Ab-positive adult patients in a Japanese cohort of IIM. The frequencies were 1.6% in both adult DM and adult PM. Therefore, while the population sizes of DM and PM are substantially different, anti-NXP2 Ab has been found at similar frequencies both in juvenile and adult IIM.[12] In contrast, in a preliminary report in a UK cohort, Betteridge *et al* detected anti-NXP2 Ab in 13 (5%) patients with DM but not in patients with PM.[17]

Intriguingly, 37.5% of adult IIM patients positive for anti-NXP2 Ab had malignancy which was found within 3 years, in addition to a patient with metastatic prostate cancer found 42 months previously. Moreover, all of these carcinomas were at an advanced stage. Anti-TIF1 Ab also has a strong association with internal malignancy in adult DM, as it is found in 50–70% of patients with cancer-associated DM.[5 6 18] Therefore, while anti-NXP2 Ab may not be restricted to DM, anti-TIF1 Ab and anti-NXP2 Ab may have a shared property in that they represent two clinical subsets of cancer-associated adult DM and JDM. However, in contrast to our study, Betteridge *et al* reported that anti-NXP2 Ab was not associated with malignancy but with ILD.[17] This may be due to ethnical differences. It may also have resulted from a different distribution of patients between dermatology and rheumatology clinics. Since both studies had relatively small numbers of patients, more studies are needed to evaluate more precisely the clinical relevance of anti-NXP2 Ab in patients with IIM.

Among adult patients with DM, anti-NXP2 Ab appeared to have strong muscle involvement while we could not find any particular cutaneous manifestations related to anti-NXP2 Ab. Unlike anti-NXP2-positive JDM,[11] no adults with DM with anti-NXP2 Ab had cutaneous calcinosis. This may be due to the relatively low incidence of calcinosis in adult DM compared with JDM.

In summary, this study shows that anti-NXP2 Ab occurs in adult patients with IIM and suggests that anti-NXP2 Ab may be correlated with cancer-associated myositis. NXP2 is involved in the activation and localisation of a tumour suppressor gene,

Table 2 Comparison of clinical and laboratory profile of adult patients with dermatomyositis with anti-NXP2 Ab, anti-TIF1 Ab and anti-Mi-2 Ab*

				p Values	
	Anti-NXP2-positive	Anti-TIF1-positive	Anti-Mi-2-positive	vs Anti-TIF1	vs Anti-Mi-2
Number	7	74	15		
Age at onset, mean (range)	57 (23–68)	59 (27–89)	50 (16–67)	NS	NS
Sex (male:female)	6:1	24:34	8:7	0.033	NS
Skin eruptions					
Heliotrope rash	71	62	60	NS	NS
Gottron's sign	57	81	87	NS	NS
Perionychia erythema	57	57	60	NS	NS
Nailfold punctuated haemorrhage	57	40	73	NS	NS
Trunk erythema	57	62	53	NS	NS
Calcinosis	0	2	7	NS	NS
Ulceration	14	7	0	NS	NS
Clinical features					
Muscle weakness	100	69	93	NS	NS
Raynaud's phenomenon	0	9	0	NS	NS
Arthritis	14	3	7	NS	NS
Fever	57	16	20	0.025	NS
Organ involvement					
Interstitial lung disease	0	13	13	NS	NS
Internal malignancy within 3 years	29	66	7	NS	NS
Laboratory findings					
Elevated CK, %	100	63	100	NS	NS
Highest CK level, IU/l, mean (range)	3857 (1539–26685)	425 (40–8670)	3934 (401–10000)	<0.001	NS

*Unless noted otherwise, values are percentages.
CK, creatine kinase.

p53.[19] TIF1 proteins also have a functional relationship with p53.[20] Since these two autoAbs may share similar clinical characteristics, especially the association with cancer, they may develop during antitumour immune responses.

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