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5. Attach File Tool – for inserting large amounts of text or replacement figures.

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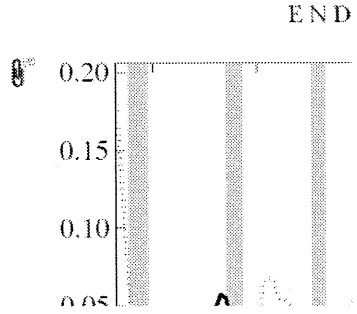

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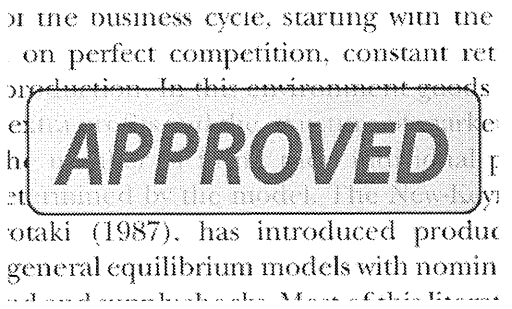

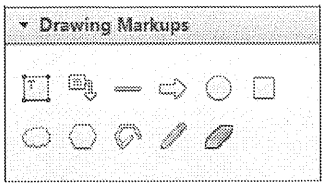

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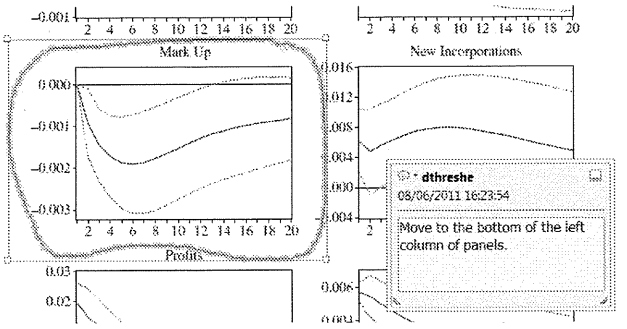

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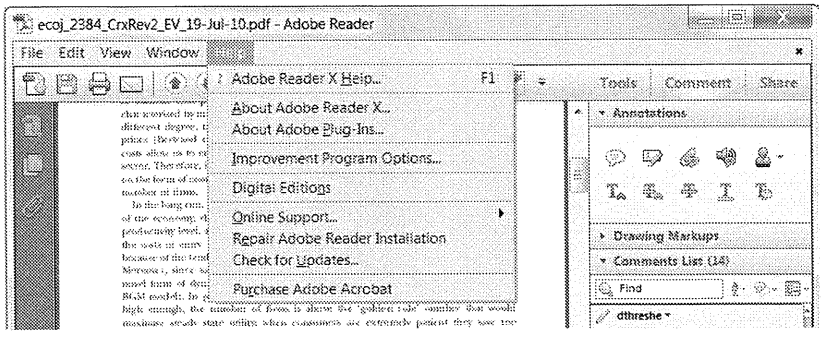

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Development of an ELISA for detection of autoantibodies to nuclear matrix protein 2

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Abstract

Objectives. Autoantibodies characterizing certain forms of inflammatory myopathy, which are myositis-specific autoantibodies, are useful in the diagnosis and prediction of prognosis in DM/PM. This study aimed to identify a subset of DM patients who have anti-nuclear matrix protein 2 (anti-NXP-2) antibodies by using biotinylated recombinant proteins, and to clarify the clinical features of DM patients with these antibodies.

Methods. Sera from 170 Japanese patients with CTDs including 106 with DM, 8 with PM, 21 with SLE, 20 with SSc, 15 with myositis overlap syndrome and 20 healthy controls were screened for anti-NXP-2 antibodies by our novel ELISAs. Positive sera were further examined by immunoprecipitation.

Results. Sera from 6 of the 170 patients with CTDs were confirmed to be positive for anti-NXP-2 antibodies. These six positives were from female patients, with five of the six sera being from adult DM patients and only one of the six being from 1 of the 12 JDM patients. All these patients had myositis. None of the anti-NXP-2-positive patients had interstitial lung disease, but one patient was complicated with ovarian cancer.

Conclusion. Our newly developed ELISA is applicable for the measurement of anti-NXP-2 antibodies. The results show that anti-NXP-2 antibodies, which have been characterized in JDM, exist in adult DM patients. Further studies using large populations are necessary to elucidate the characteristic clinical features and the prognosis of patients with anti-NXP-2 antibodies, especially for adult patients.

Key words: anti-MJ antibody, anti-NXP-2 antibody, dermatomyositis, ELISA, myositis-specific autoantibody.

Introduction

The idiopathic inflammatory myopathies (IIMs) are a group of acquired, systemic autoimmune diseases that include PM, DM and inclusion body myopathies [1, 2]. Several myositis-specific autoantibodies (MSAs) that are associated with certain clinical forms of IIMs have been identified, and they are useful tools for predicting the prognosis of IIMs. For example, anti-melanoma differentiation-associated protein 5 (MDA5)-antibody-positive patients demonstrate rapid progressive interstitial lung disease (ILD) and anti-transcriptional intermediary factor 1 (TIF1)-γ-antibody-positive patients are often complicated with cancer [3]. Recently anti-nuclear matrix protein 2 (anti-NXP-2, also called anti-MJ) antibodies that react to a 140-kDa polypeptide have been described in cohorts of JDM patients in the UK and Argentina [4].

Anti-NXP-2 antibodies were reported to be associated with calcinosis, although the antibodies were investigated only in cohorts of juvenile patients [4–6]. The prevalences of anti-NXP-2 antibodies in adult patients with CTDs have been unknown. We hypothesized that anti-NXP-2 antibodies could be detected not only in JDM patients, but also in adult DM cases, and that these antibodies could be a new serological marker for a certain disease subset of adult DM. To investigate the presence of anti-NXP-2 antibodies in sera from patients with various CTDs, this study used our newly developed ELISAs and immunoprecipitation (IPP) with biotinylated recombinant protein. We clarified the prevalence of the antibodies against NXP-2 in adult DM and examined clinical features associated with anti-NXP-2 antibodies.

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Materials and methods

Patients and sera

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 170 Japanese patients with CTDs. The CTD cohort consisted of 106 patients with DM [including 12 with JDM, 38 with clinically amyopathic DM (ADM) and 14 with cancer-associated DM], 8 with PM, 21 with SLE, 20 with SSc and 15 with myositis overlap syndrome (Table 1). The group of patients with myositis overlap syndrome consisted of seven patients with overlapping CTDs and eight with MCTD. Serum samples were collected at the time of diagnosis or before aggressive therapy. Twenty healthy Japanese individuals were also assessed as normal controls. All the DM patients, except those clinically ADM, and all the PM patients fulfilled Bohan and Peter's criteria [7, 8]. All the clinically ADM patients fulfilled Sontheimer's criteria [9]. The clinically ADM group included patients who had developed ILD within 6 months after disease onset. Patients were classified as having JDM if they were <16 years at the onset of DM according to the criteria of Bohan and Peter [10]. Patients were classified as cancer-associated DM if the internal malignancy was diagnosed within 3 years (before or after) of the DM diagnosis based on previous studies [11]. SLE was diagnosed by the ACR criteria for SLE [12]. SSc was diagnosed according to the SSc diagnostic criteria [13] established by the Ministry of Health, Labour and Welfare of Japan, which were modified from the ACR criteria [14]. MCTD was diagnosed according to the MCTD diagnostic criteria [15]. Patients were classified as having myositis in overlap with another CTD if they met published criteria for the CTD. The clinical records were retrospectively reviewed and the following information was recorded: demographic data including gender, age at onset, diagnosis, date of serum sampling and clinical features including the presence of cutaneous signs of DM (heliotrope rash or Gottron's papules), RP or calcinosis. Elevated creatine kinase (CK) was defined by elevation of CK above the normal range in clinical laboratory tests. Patients were diagnosed as having ILD, which was defined by fibrosis on chest radiographs or chest CT scans. Internal malignancy was investigated according to the results of whole-body CT, endoscopy, colonoscopy, gynaecological examination and breast cancer screening. The ages at the time of sera collection and gender ratios of each clinical group are summarized in Table 1. In the juvenile-onset patients with DM, two patients were originally seen at other hospitals far outside our area but then transferred to our hospital. Their intervals between disease onset and serum sampling were 26 and 28 years. All the patients and healthy individuals in the present study gave fully informed consent for participation, including provision of serum samples. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISAs

Specific binding of serum antibodies to recombinant NXP-2 was analysed using direct solid-phase ELISA. This method was based on our previous protocol, which quantitatively measured the antibodies against MDA-5 [16]. Instead of a conventional optical system for ELISA, this study used a microplate luminometer to increase the

TABLE 1 Patient's groups and anti-NXP-2 antibody frequencies

Clinical group	Age[a], range, years	Age[a], mean (S.D.), years	Gender, M:F	Total, *n*	α-NXP-2, *n* (%)
Total DM	3–84	51 (18)	33:73	106	6 (5.7)
Clinically ADM	3–84	48 (19)	9:29	38	0
Cancer-associated DM	48–80	66 (10)	6:8	14	1 (7.1)
Classical DM	16–80	49 (17)	18:36	54	5 (9.3)
Adult DM	19–84	55 (14)	26:68	94	5 (5.3)
Clinically ADM	23–84	54 (13)	5:27	32	0
Cancer-associated DM	48–80	66 (10)	6:8	14	1 (7.1)*
Classical DM	19–80	53 (15)	15:33	48	4 (8.3)*,**
JDM	3–32	18 (9)	7:5	12	1 (8.3)
Clinically ADM	3–32	16 (12)	4:2	6	0
Classical DM	16–27	20 (5)	3:3	6	1 (17)
Total other CTDs	18–75	45 (13)	6:58	64	0
PM	32–70	59 (13)	1:7	8	0
SLE	18–57	38 (11)	3:18	21	0
SSc	26–55	45 (8)	0:20	20	0
Overlap syndrome	24–75	49 (16)	2:13	15	0

[a]Age at the time of sera collection. In JDM, two patients were originally seen at other hospitals far outside our hospital. Their intervals between disease onset and serum sampling were 26 and 28 years. *$P < 0.05$ in classical adult DM and cancer-associated DM (5/62) *vs* other CTDs (0/64) by Fisher's exact test ($P = 0.0265$). **$P < 0.05$ in classical adult DM (4/48) *vs* other CTDs (0/64) by Fisher's exact test ($P = 0.0313$). M: male; F: female.

sensitivity, thereby reducing the required amount of biotinylated recombinant protein for the assays. The full-length NXP-2 cDNA clone was purchased from Kazusa DNA Research Institute (Chiba, Japan) and its DNA sequences were confirmed to be identical to GenBank accession number D50926. Biotinylated recombinant NXP-2 was produced from the cDNA, using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) to which streptavidin was covalently coupled via a spacer were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with biotinylated recombinant NXP-2 diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature with gentle agitation. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Diluted sample sera with blocking buffer (50 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG antibody conjugated with HRP (Dako, Glostrup, Denmark) as a secondary antibody (50 μl/well) at 1:30 000 dilution after five washes. After incubation for 1 h at room temperature, the plates were washed five times and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA) (50 μl/well) as the substrate according to the manufacturer's protocol. Then, the relative luminescence unit (RLU) was determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU subtracted background was used for data analysis. A standard curve was obtained from serial concentrations of a serum sample containing a high titre of the anti-NXP-2 antibody. The cut-off level was set at 17.0 U, based on 5 S.D. above the mean value obtained from 20 healthy control sera.

Detection of anti-NXP-2 antibodies using IPP

IPP was performed using transcription and translation (TnT) products as previously described [17–20], with minor modifications. Briefly, 10 μl of patient sera was mixed and incubated with 20 μl of a 50% slurry of Protein G Sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) and 270 μl IPP buffer (PBS containing 1% Nonidet P-40) at 4°C for 1 h. Sepharose beads were mixed and incubated with 270 μl binding buffer (IPP buffer containing 0.5% BSA) and 10 μl of the TnT products, which was pre-cleared using the sepharose beads, at 4°C for 1 h. The beads were washed five times with IPP buffer, suspended in Laemmli sample buffer and the IgG-bound proteins retained by the sepharose beads were electrophoresed on 7.5% SDS-PAGE gel. The proteins were electrophoretically transferred onto Immobilon-P transfer membranes (Millipore Corporation, Billerica, MA, USA) and the biotinylated proteins were subsequently detected with Western Blue Substrate (Promega).

IIF

IIF was performed by standard methods [21] using HEp-2 cells (Fluoro HEPANA Test; MBL, Nagoya, Japan).

Statistical analysis

The frequency of antibodies between each CTD and control was analysed using Fisher's exact test. The associations between clinical features and antibodies in DM were analysed using Fisher's exact test or unpaired Student's *t*-test. SPSS version 17.0 for Windows (SPSS Japan, Tokyo, Japan) was used to perform the statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Establishment of ELISA with biotinylated recombinant NXP-2

For the screening of anti-NXP-2 antibodies in large numbers of serum samples, we established an ELISA system that uses biotinylated recombinant NXP-2. In the present study, by using a luminometer, we succeeded in decreasing the required amount of TnT product for the assay from 10 μl/well in the previous system to 1 μl/well in our newly developed system, based on the results of serial dilution experiments (data not shown). We screened a total of 170 serum samples obtained from patients with various CTDs. Based on the cut-off level (17.0) at 5 S.D. above the mean value, six patients with DM and one patient with PM were classified into the anti-NXP-2-positive group (Fig. 1). When the cut-off was set at 3 S.D. above the mean value (9.7), one sample from a patient with SLE was just beneath the cut-off (9.2). Subsequently serum samples from these eight patients were used for IPP to confirm whether they were truly positive for the anti-NXP-2 antibodies.

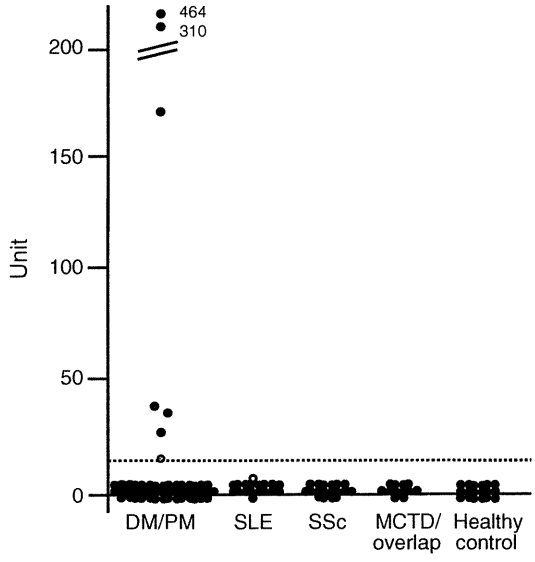
Detection of anti-NXP-2 antibodies using IPP

After the initial screening by ELISA we investigated antibodies against NXP-2 in sera from 7 anti-NXP-2-positive patients, 1 equivocal patient and 24 anti-NXP-2-negative patients for their ability to immunoprecipitate biotinylated recombinant NXP-2. Six of the seven anti-NXP-2-positive sera in ELISA showed a distinct protein band with a molecular weight of 140 kDa in IPP assays (Fig. 2, lanes 1–6). All were from patients with DM, whereas the serum from the one patient with PM did not react to the recombinant (Fig. 2, lane 7). The serum from an SLE patient with an equivocal level of anti-NXP-2 antibodies in ELISA was negative in IPP (Fig. 2, lane 8). Furthermore, anti-NXP-2 antibodies were not detected in normal control sera as well as 24 anti-NXP-2-negative sera in ELISA by IPP assays (representative negative sera, Fig. 2, lane 9).

Clinical and serological features of DM patients with anti-NXP-2 antibodies

Of the 106 DM sera, 6 (5.7%) had anti-NXP-2 antibodies (Table 1). In contrast, anti-NXP-2 antibodies were not detected in any serum from patients with other CTDs.

FIG. 1 ELISA using biotinylated recombinant NXP-2 protein. Measurement of anti-NXP-2 antibodies in 170 serum samples from patients with various CTDs or 20 healthy control subjects. We used the 1 μl/well of TnT mixture and the diluted patient serum samples at 1:1000 for measuring all samples (closed circles). The antibody units were calculated from the RLU using a standard curve obtained from serial concentrations of a serum sample containing a high titre of the anti-NXP-2 antibody. Broken line indicates the cut-off value (17.0 U). Six samples from patients with DM, one sample from a patient with PM (grey circle, just around the cut-off value) and one sample from a patient with SLE (open circle, under the mean values of 20 healthy controls + 3 S.D.) were introduced to IPP assays with biotinylated recombinant proteins (see Fig. 2).



Anti-NXP-2 antibodies were found at a higher frequency in DM patients than in patients with other CTDs (6/106 *vs* 0/64, $P = 0.0844$). Concerning JDM, only 1 (8.3%) of the 12 JDM patients had serum that reacted with NXP-2. The patient with anti-NXP-2 antibodies had no findings associated with calcinosis. As for adult DM, sera from 5 (5.3%) of 94 patients were positive. In adult cancer-associated DM, serum from 1 (7.1%) of 14 patients was positive for anti-NXP-2 antibodies. This patient had ovarian cancer that resulted in a fatal outcome. Adult patients with classical DM had anti-NXP-2 antibodies with a significantly higher frequency than patients with other CTDs (4/48 *vs* 0/64, $P = 0.0313$). Even if adult patients with cancer-associated DM were added to the classical adult DM group, a significant difference would still remain between adult patients with DM and patients with other CTDs (5/62 *vs* 0/64, $P = 0.0265$). According to these results, anti-NXP-2 antibodies were detected not only in JDM, but also in cancer-associated adult DM and classical adult DM. No patients with any other CTD, clinically ADM or healthy individuals had anti-NXP-2 antibodies.

FIG. 2 IPP of biotinylated recombinant NXP-2 with patient's sera. The input lane contains half the dose (5 μl) of biotinylated recombinant NXP-2 protein that was used for the IPP assay. The band in this lane that migrated at around 140 kDa corresponds to NXP-2. Lanes 1–6 contain the NXP-2 immunoprecipitated by the sera of patients that were anti-NXP-2-positive in ELISA. Lane 7 is the serum that was just above the cut-off level in ELISA. Lane 8 is the equivocal serum in ELISA. Lane 9 is serum from a healthy control. We arranged the samples from lanes 1–9 according to the levels of ELISA units. The asterisk denotes biotinylated NXP-2.

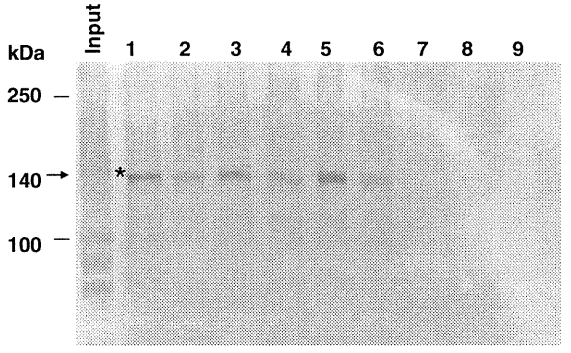


Table 2 compares the clinical features between anti-NXP-2-positive patients and anti-NXP-2-negative patients. There was little significant difference in gender, age at onset, diagnosis or prevalence of DM-specific skin symptoms (heliotrope rash and Gottron's papules or sign) and RP. All six of the anti-NXP-2-positive patients had myopathy, but none of them were complicated with ILD. The frequency of calcinosis was relatively higher in the positive group, although the difference was not significant.

Table 3 summarizes the serological information of the six patients with anti-NXP-2 antibodies in the present study. IIF revealed diverse nuclear staining patterns with various titres in all the anti-NXP-2-positive sera. The IIF titres did not correspond well to the ELISA units.

Discussion

There is increasing evidence that MSAs are associated with clinical subsets of DM/PM. Several MSAs have shown positive polypeptide bands ~140–160 kDa on SDS-PAGE by the traditional assay of IPP with cell extracts, including anti-NXP-2, anti-Mi-2, anti-MDA-5, anti-TIF1-γ and anti-OJ antibodies. It is difficult to differentiate these antibodies by molecular weight. We used biotinylated recombinant proteins and successfully detected anti-MDA-5 and anti-TIF1-γ antibodies in our IPP study [3]. However, there are technical limits to handling large numbers of serum samples efficiently with IPP. Thus we needed to establish an ELISA system. In our previous study, taking advantage of the high affinity between biotin

and avidin, we used streptavidin-coated ELISA plates for simultaneous purification and immobilization of the biotinylated protein for the detection of anti-MDA-5 antibodies [16]. However, improving the sensitivity of the ELISA was required, because this ELISA system was not suitable for measuring large numbers of serum samples. The TnT system cannot produce a practical amount of recombinant proteins compared with the *Escherichia coli* expression system. In this study, we established highly sensitive ELISA with TnT products by using a luminometer.

This study examined the specificity of anti-NXP-2 antibodies in Japanese patients with DM. Anti-NXP-2 antibodies were finally detected in sera of 6 of 170 patients with CTDs by ELISA and IPP. We conclude that only the DM patients who screened positive by ELISA were confirmed to have anti-NXP-2 antibodies. The results of ELISA and IPP in this study with biotinylated recombinant NXP-2 were inconsistent with previously reported findings of anti-NXP-2 antibodies being detected in JDM patients, especially patients with calcinosis in Argentina and the UK [4–6], Espada *et al.* [5] reported that 25% of JDM patients were found to have anti-NXP-2 (anti-MJ) antibodies and that anti-NXP-2-positive juvenile patients demonstrated severe disease characterized by muscle atrophy with contractures. Some of them also had large-joint arthritis, dysphagia, cutaneous vasculitis and calcinosis universalis. Gunawardena *et al.* [6] showed that anti-140-kDa protein (NXP-2) antibodies were significantly associated with the presence of calcinosis when compared with the overall juvenile myositis cohort. In our study, only 1 (8.3%) of 12 JDM patients were anti-NXP-2 positive. The prevalence of other MSAs in our JDM patients was 42%, i.e. five patients with anti-TIF1-γ antibodies (data not shown). The prevalence of anti-NXP-2 antibodies in JDM was low in our study, but this may depend on the fact that some patients had long periods (26 and 28 years) between onset and serum sampling. In contrast to adult DM, in which calcification is relatively uncommon, it is estimated that 20–40% of JDM patients have calcification [22, 23]. In our study, 2 of the 12 JDM patients were complicated with calcinosis. Both patients were anti-TIF1-γantibody positive (data not shown).

Few reports have addressed the correlation between adult-onset DM and calcinosis. In our study, 4 (4.3%) of 94 adult DM patients had calcinosis. Among adult DM patients with calcinosis, only one patient (Patient 3 in Table 3) was detected as having anti-NXP-2 antibodies. She had additional anti-centromere antibodies (ACAs) and multiple cutaneous calcinosis. Although ACAs are known to be associated with calcinosis, it is not clear whether the

TABLE 2 Clinical features of anti-NXP-2-positive and -negative DM patients

Feature	Anti-NXP-2 antibodies	
	Positive ($n=6$)	Negative ($n=100$)
Gender, male:female	0:6	33:67
Age at onset, mean (range), years	50.7 (16–73)	50.9 (1–80)
Age at diagnosis, mean (range), years	51.3 (16–74)	51.5 (3–84)
Type of skin lesion		
Heliotrope rash, *n* (%)	4 (67)	52 (52)
Gottron's papules or sign, *n* (%)	4 (67)	68 (68)
RP, *n* (%)	1 (17)	11 (11)
Calcinosis, *n* (%)	1 (17)	5 (5)
Elevation of CK, *n* (%)	6 (100)	74 (74)
ILD, *n* (%)	0 (0)*	41 (41)
JDM, *n* (%)	1 (17)	11 (11)
Internal malignancy, *n* (%)	1 (17)	13 (13)

*$P=0.0796$ by Fisher's exact test.

TABLE 3 Laboratory data for the anti-NXP-2-positive patients

Patient no.	Age, years	Gender, M/F	Diagnosis	Calcinosis	ILD	Cancer	IIF		ELISA units[a]	Other antibodies
							Pattern	Titre		
1	19	F	Classical DM	–	–	–	Spe. Nuc. Dots.	160 40	4644	
2	68	F	Cancer-associated DM	–	–	+ Ovary	Diffuse Nuc. Dots.	640 640	3104	
3	74	F	Classical DM	+	–	–	Spe. Dis. spe.	2560 2560	1731	ACA
4	76	F	Classical DM	–	–	–	Diffuse Nuc. Dots.	80 320	367	
5	16	F	JDM	–	–	–	Diffuse Nuc. mem. Mit. spi.	80 80 80	326	
6	66	F	Classical DM	–	–	–	Spe.	160	255	

[a]ELISA units of anti-NXP-2 antibodies. Spe.: speckled; Nuc. dots.: nuclear dots; Dis. spe.: discrete speckled; Nuc. mem.: nuclear membrane; Mit. spi.: mitotic spindle.

concomitant presence of the two kinds of antibodies was coincidental and which antibody was more pathogenic of calcinosis. Six patients with anti-NXP-2 antibodies presented no common characteristic clinical features other than myositis. Their myositis was moderate and their CK levels were not elevated by >500 U/l. We noticed that none had ILD, which sometimes occurs in adult DM. One (Patient 2 in Table 3) of the six patients had cancer. She was found to have ovarian cancer at the same time as being diagnosed with DM. Anti-TIF1-γ antibodies, which had been associated with cancer in our previous study [3], were not detected in her serum. The present findings suggest that DM patients who are anti-NXP-2 positive do not always have benign forms of DM, at least not adult patients, even when their myositis is moderate. One of the few published studies on anti-NXP-2 antibodies in adult DM patients is a preliminary report [24]. They reported that the frequency and clinical associations of anti-NXP-2 autoantibodies varied between adults and juveniles.

By IIF, the staining patterns by anti-NXP-2-positive sera were mainly divided into speckled or diffuse. NXP-2, also called MORC3 (microrchidia family CW-type zinc finger 3), is a MORC-family protein that is characterized by three conserved domains consisting of RNA-binding, nuclear matrix-binding and coiled-coil domains [25]. They are structurally separated and they may have important roles in diverse nuclear functions, including regulation of transcription, maintenance of nuclear architecture and RNA metabolism. NXP-2 is a nuclear matrix protein that is shown as speckled-like staining in IIF. The nuclear staining of NXP-2 becomes diffuse after nuclear matrix treatment with RNase [25]. Nuclear domains, which are demonstrated as nuclear dots by anti-MORC3 antibodies in IIF [26], were not clearly detected by patients' sera. The differences among IIF patterns in anti-NXP-2-positive patients might have resulted from other concomitant antibodies. It is also possible that altered conformational structures of NXP-2 affect the accessibility of antibodies to antigenic epitopes. In any case, IIF patterns are not useful for screening anti-NXP-2 antibodies.

The previously described DM-specific antigens, Mi-2 [27] and TIF1-γ [28] are nuclear proteins that are also involved in transcriptional regulation. It is notable that antibodies to small ubiquitin-like modifier (SUMO) activating enzyme (SAE), which is involved in a post-transcriptional modification called sumoylation, have been recently described in adult DM [29]. NXP-2 was reported to be a target of SAE and to have a possible role in SUMO-mediated transcriptional repression [30]. Mi-2 is also thought to be directly involved in mediating SUMO-dependent repression [31]. TIF1-γ is considered to act as a repressor of TGF-β superfamily-induced transcription via its E3 ubiquitin ligase activity [32]. The SUMO pathway may play a potential role in the pathogenic mechanisms of DM.

In summary, our newly developed ELISA is applicable for the measurement of anti-NXP-2 antibodies. Our present study revealed that anti-NXP-2 antibodies were observed not only in JDM but also in adult DM. Adult DM patients with anti-NXP-2 antibodies had uniformly moderate myositis without ILD, even though this serological group featured no major demographic differences with respect to the other adult DM patients in our study. To elucidate the characteristic clinical features and the prognosis of patients with anti-NXP-2 antibodies, a much larger cohort is needed, especially for adult patients.

Rheumatology key messages

- A newly established ELISA for biotinylated recombinant proteins is useful for detecting anti-NXP-2 antibodies.
- Anti-NXP-2 antibodies are detected not only in JDM, but also in adult DM.

Acknowledgements

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References

1 Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362:971–82.

2 Mastaglia FL, Phillips BA. Idiopathic inflammatory myopathies: epidemiology, classification, and diagnostic criteria. Rheum Dis Clin North Am 2002;28:723–41.

3 Hoshino K, Muro Y, Sugiura K *et al.* Anti-MDA5 and anti-TIF1-γ antibodies have clinical significance for patients with dermatomyositis. Rheumatology 2010;49:1726–33.

4 Oddis CV, Fertig N, Goel A *et al.* Clinical and serological characterization of the anti-MJ antibody in childhood myositis (abstract). Arthritis Rheum 1997;40:S139.

5 Espada G, Maldonado Cocco JA, Fertig N, Oddis CV. Clinical and serologic characterization of an Argentine pediatric myositis cohort: identification of a novel autoantibody (anti-MJ) to a 142-kDa protein. J Rheumatol 2009;36:2547–51.

6 Gunawardena H, Wedderburn LR, Chinoy H *et al.* Autoantibodies to a 140-kd protein in juvenile dermatomyositis are associated with calcinosis. Arthritis Rheum 2009;60:1807–14.

7 Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N Engl J Med 1975;292:344–7.

8 Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med 1975;292:403–7.

9 Sontheimer RD. Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis sine myositis) as a distinctive subset within the idiopathic

inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol 2002;46:626–36.

10 Bohan A, Peter JB, Bowman RL, Pearson CM. Computer-assisted analysis of 153 patients with polymyositis and dermatomyositis. Medicine 1977;56:255–86.

11 Chinoy H, Fertig N, Oddis CV, Ollier WE, Cooper RG. The diagnostic utility of myositis autoantibody testing for predicting the risk of cancer-associated myositis. Ann Rheum Dis 2007;66:1345–9.

12 Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

13 Takehara K, Sato S. The classification of pathogenesis and the development of therapy in systemic sclerosis: the classification of severity and the guideline for treatment (2004). A research project for systemic sclerosis supported by the Ministry of Health, Labour and Welfare of Japan (Japanese). Tokyo: Ministry of Health, Labour and Welfare, 2004.

14 LeRoy EC, Black C, Fleischmajer R *et al*. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988;15:202–5.

15 Alarcon-Segovia D, Villareal M. Classification and diagnostic criteria for MCTD. In: Kasukawa R, Sharp GC, eds. Mixed Connective Tissue Disease and Antinuclear Antibodies. Amsterdam: Elsevier, 1987:33–40.

16 Muro Y, Sugiura K, Hoshino K, Akiyama M. Disappearance of anti-MDA-5 autoantibodies in clinically amyopathic DM/interstitial lung disease during disease remission. Rheumatology 2011. Advance Access published 30 December 2011, doi: 10.1093/rheumatology/ker408.

17 Ogawa Y, Sugiura K, Watanabe A *et al*. Autoantigenicity of DFS70 is restricted to the conformational epitope of C-terminal alpha-helical domain. J Autoimmun 2004;23:221–31.

18 Okamoto M, Ogawa Y, Watanabe A *et al*. Autoantibodies to DFS70/LEDGF are increased in alopecia areata patients. J Autoimmun 2004;23:257–66.

19 Goto N, Sugiura K, Ogawa Y *et al*. Anti-p80 coilin autoantibodies react with a conserved epitope and are associated with anti-DFS70/LEDGF autoantibodies. J Autoimmun 2006;26:42–51.

20 Tanaka N, Muro Y, Sugiura K, Tomita Y. Anti-SS-A/Ro antibody determination by indirect immunofluorescence and comparison of different methods of anti-nuclear antibody screening. Mod Rheumatol 2008;18:585–92.

21 Watanabe A, Kodera M, Sugiura K *et al*. Anti-DFS70 antibodies in 597 healthy hospital workers. Arthritis Rheum 2004;50:892–900.

22 Pachman LM, Veis A, Stock S *et al*. Composition of calcification in children with juvenile dermatomyositis: association with chronic cutaneous inflammation. Arthritis Rheum 2006;54:3345–50.

23 Rider LG. Calcinosis in juvenile dermatomyositis: pathogenesis and current therapies. Pediatr Rheumatol Online J 2003;1:119–33.

24 Betteridge ZE, Gunawardena H, Chinoy H *et al*. Clinical associations of anti-p140 autoantibodies in adult myositis. Ann Rheum Dis 2010;69(Suppl. 3):127.

25 Kimura Y, Sakai F, Nakano O *et al*. The newly identified human nuclear protein NXP-2 possesses three distinct domains, the nuclear matrix-binding, RNA-binding, and coiled-coil domains. J Biol Chem 2002;277:20611–7.

26 Takahashi K, Yoshida N, Murakami N *et al*. Dynamic regulation of p53 subnuclear localization and senescence by MORC3. Mol Biol Cell 2007;18:1701–9.

27 Wade PA, Gegonne A, Jones PL *et al*. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 1999;23:62–6.

28 Yan KP, Dolle P, Mark M *et al*. Molecular cloning, genomic structure, and expression analysis of the mouse transcriptional intermediary factor 1 gamma gene. Gene 2004;334:3–13.

29 Betteridge Z, Gunawardena H, North J, McHugh N. Identification of a novel autoantibody directed against small ubiquitin-like modifier activating enzyme in dermatomyositis. Arthritis Rheum 2007;56:3132–7.

30 Rosendorff A, Sakakibara S, Lu S *et al*. NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression. Proc Natl Acad Sci USA 2006;103:5308–13.

31 Stielow B, Sapetschnig A, Krüger I *et al*. Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. Mol Cell 2008;29:742–54.

32 Agricola E, Randall RA, Gaarenstroom T, Dupont S, Hill CS. Recruitment of TIF1γ to chromatin via its PHD finger-bromodomain activates its ubiquitin ligase and transcriptional repressor activities. Mol Cell 2011;43:85–96.

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

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Abstract

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

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

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

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

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

Introduction

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

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

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

Materials and methods

Patients

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

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

ELISA

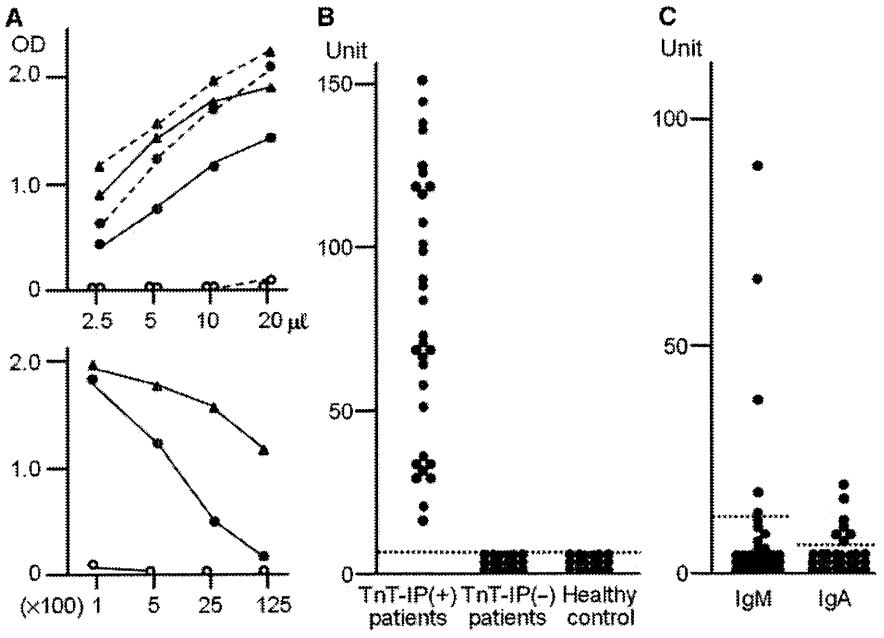
Specific binding of serum autoantibodies to recombinant MDA-5 was analysed using direct solid-phase ELISA. Biotinylated recombinant MDA-5 was produced from full-length MDA-5 cDNA using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3].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

Results

ELISA with biotinylated recombinant MDA-5

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

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



Decline in anti-MDA-5 antibodies during remission

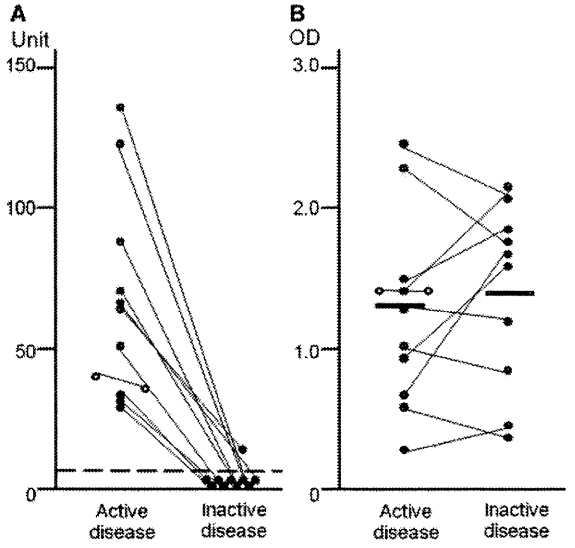
From 31 patients whose initial serum samples had anti-MDA-5 antibodies, sera were retaken during remission periods from the 10 patients with C-ADM. As a treatment for ILD in nine of these patients, methylprednisolone pulse therapy and immunosuppressive drugs were administered to eight patients and seven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

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

Discussion

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

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



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

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

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

Rheumatology key messages

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

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References

1 Sato S, Hoshino K, Satoh T *et al.* RNA helicase encoded by melanoma differentiation-associated gene 5 is a major autoantigen in patients with clinically amyopathic dermatomyositis: association with rapidly progressive interstitial lung disease. Arthritis Rheum 2009;60:2193–200.

2 Kaji K, Fujimoto M, Hasegawa M *et al.* Identification of a novel autoantibody reactive with 155 and 140kDa nuclear proteins in patients with dermatomyositis: an association with malignancy. Rheumatology 2007;46:25–28.

3 Hoshino K, Muro Y, Sugiura K *et al.* Anti-MDA5 and anti-TIF1-γ antibodies have clinical significance for patients with dermatomyositis. Rheumatology 2010;49:1726–33.

4 Hamaguchi Y, Kuwana M, Hoshino K *et al.* Clinical correlations with dermatomyositis-specific autoantibodies in adult Japanese patients with dermatomyositis: a multicenter cross-sectional study. Arch Dermatol 2011;147:391–8.

5 Sato S, Kuwana M. Clinically amyopathic dermatomyositis. Curr Opin Rheumatol 2010;22:639–43.

6 Fiorentino D, Chung L, Zwerner J *et al.* The mucocutaneous and systemic phenotype of dermatomyositis patients with antibodies to MDA5 (CADM-140): a retrospective study. J Am Acad Dermatol 2011;65:25–34.

7 Gono T, Kawaguchi Y, Satoh T *et al.* Clinical manifestation and prognostic factor in anti-melanoma differentiation-associated gene 5 antibody-associated interstitial lung disease as a complication of dermatomyositis. Rheumatology 2010;49:1713–9.

8 Tench CM, Isenberg DA. The variation in anti-ENA characteristics between different ethnic populations with systemic lupus erythematosus over a 10-year period. Lupus 2000;9:374–6.

9 Faria AC, Barcellos KSA, Andrade LEC. Longitudinal fluctuation of antibodies to extractable nuclear antigens in systemic lupus erythematosus. J Rheumatol 2005;32: 1267–72.

10 Marie I, Lahaxa L, Benveniste O *et al*. Long-term outcome of patients with polymyositis/dermatomyositis and anti-PM-Scl antibody. Br J Dermatol 2010;162:337–44.

11 Bohan A, Peter JB, Bowman RL *et al*. A computer-assisted analysis of 153 patients with polymyositis and dermatomyositis. Medicine 1977;56:255–86.

12 Sontheimer RD. Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis siné myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol 2002;46:626–36.

13 Kuwana M, Kaburaki J, Mimori T *et al*. Longitudinal analysis of autoantibody response to topoisomerase I in systemic sclerosis. Arthritis Rheum 2000;43:1074–84.

14 Barral PM, Morrison JM, Drahos J *et al*. MDA-5 is cleaved in poliovirus-infected cells. J Virol 2007;81:3677–84.

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Metastatic skin lesions of multiple myeloma presenting as two extraordinarily large subcutaneous tumors

Skin involvement in multiple myeloma (MM) is uncommon.[1–3] It usually occurs at the late stage of MM as a result of direct skin invasion from underlying bony osteolytic lesions of MM, and metastatic skin lesions without underlying bony involvement are extremely rare.[4] Here we report a female patient with MM showing two extraordinarily large subcutaneous tumors as metastatic skin lesions of MM, without any other extramedullary involvement.

A 68-year-old woman was referred to the outpatient clinic of our dermatology department with two large subcutaneous masses, one on the left shoulder and the other on the right thigh. Both masses were well-demarcated, elastic hard subcutaneous tumors, approximately 10 cm in diameter, and neither adhered to the underlying tissue (Fig. 1). Skin biopsy specimens of the masses showed that the tumor consisted of proliferated atypical plasma cells (Fig. 2). Immunohistochemical staining revealed that all the plasma cells involving the subcutis expressed γ-globulin λ-light chain, although no κ-light chain-positive cell was seen. IgA α chain expression was not observed in the infiltrating plasma cells. X-ray images showed no apparent abnormality in bones of the left shoulder or the right thigh. We diagnosed the tumors as skin metastatic lesions of MM.

Eight months before the onset of the skin tumors, MM was suspected from high serum γ-globulin levels and an unusual fracture of the left upper arm. Quantitative immunoglobulin tests showed an IgA-λ spike. Bone marrow aspiration and biopsy revealed large numbers of atypical plasma cells, and the patient was diagnosed with MM. By the time of her initial visit to our dermatology clinic, three cycles of chemotherapy with melphalan, prednisolone, and bortezomib had been performed and subsequent treatment with lenalidomide and dexamethasone had been tentatively ceased.

We diagnosed the present case as hematogenous or lymphogenous metastasis, because the masses were well-demarcated from the underlying tissue and computed tomography and X-ray images of the left shoulder and

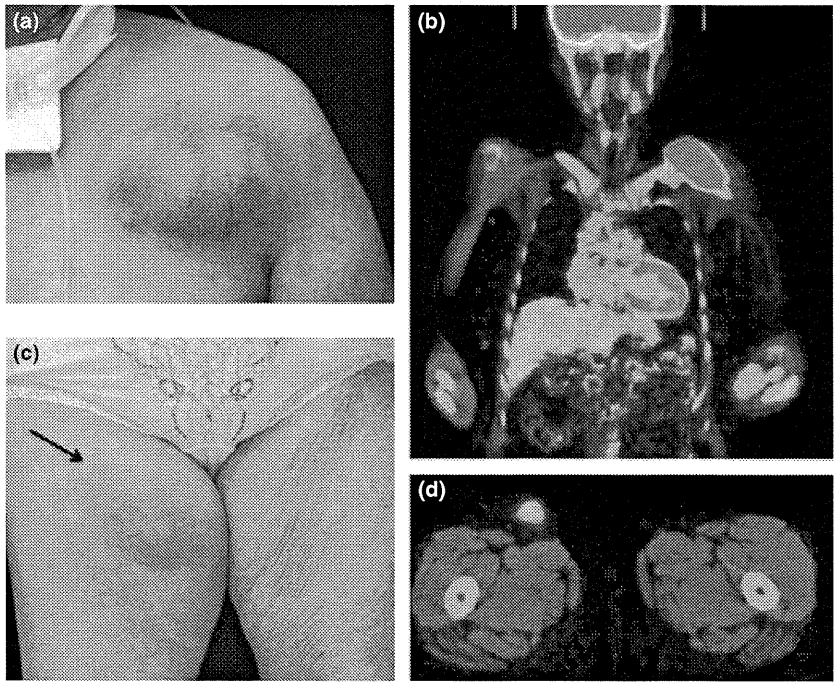

Figure 1 (a,c) Large subcutaneous masses are seen on the left shoulder (a) and right thigh (c, arrow). (b,d) F-18 FDG PET/computed tomography shows markedly high uptake on the left shoulder (b) and right thigh (d), locations corresponding to the subcutaneous tumors

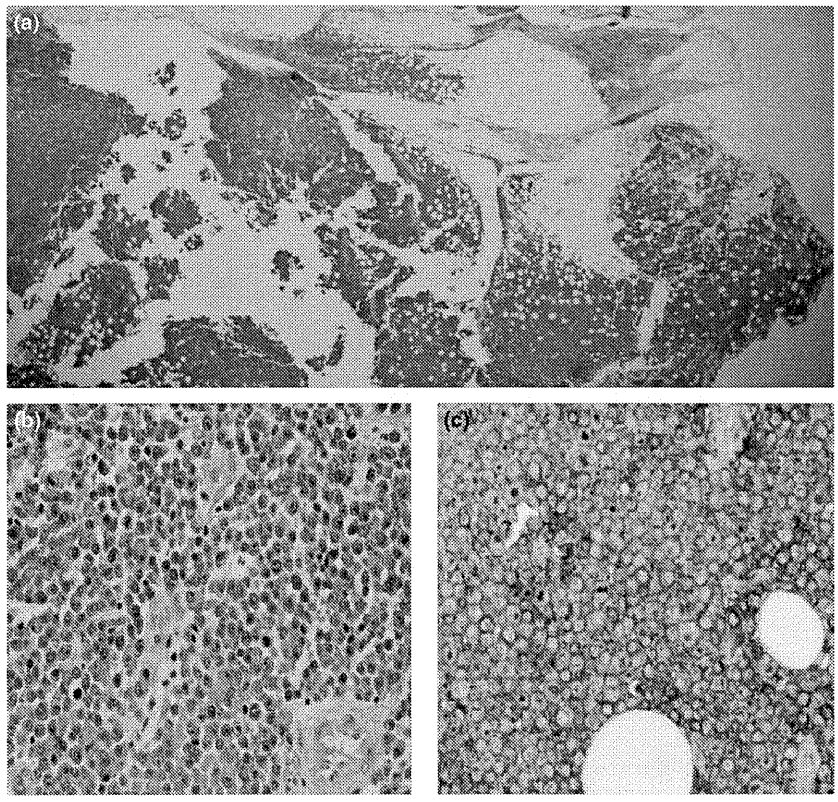

Figure 2 Histopathological features of the skin biopsy specimen from the left shoulder. (a) Low magnification shows diffuse, dense infiltration of mononuclear cells in the subcutaneous fat (hematoxylin and eosin, original magnification, ×20). (b) High magnification shows that the infiltrating cells are atypical plasma cells (hematoxylin and eosin, original magnification, ×400). (c) Immunostaining reveals that most of the atypical cells are strongly positive for γ-globulin λ-light chain (original magnification, ×400)

right thigh bones showed no abnormal changes suggestive of MM.

Multiple myeloma is an uncommon disease, and only 2.2–3.5% of MM cases were reported to have hematogenous or lymphogenous skin metastasis.[2,3] Thus, metastatic skin lesions in MM are very rare. Requena *et al.*[4] reported that most skin lesions of secondary cutaneous plasmacytoma from MM appear in the form of papules or subcutaneous nodules 1–5 cm in diameter.[4] The present patient is noteworthy for having had two extraordinarily large tumors, 10 cm in diameter, without other apparent MM lesions being found anywhere on her body.

The patient had been treated successfully with combination chemotherapy, and no other apparent extramedullary lesions were detected when the two skin tumors were found. The present case suggests that skin metastasis of MM can occur in the form of large subcutaneous tumors, even in patients who are successfully treated and have no other extramedullary lesions. In light of this, we have to keep skin metastasis of MM in mind for differential diagnosis of large skin tumors during treatment and follow-up of patients with MM.

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References

1 Bluefarb SM. Cutaneous manifestations of multiple myeloma. *Arch Dermatol* 1955; 72: 506–522.

2 Hayes DW, Bennett WA, Heck FJ. Review of literature and pathologic studies: extramedullary lesions in multiple myeloma. *Arch Pathol* 1952; 53: 262–272.
3 Wuepper KD, MacKenzie MR. Cutaneous extramedullary plasmacytomas. *Arch Dermatol* 1969; 100: 155–164.
4 Requena L, Kutzner H, Palmedo G, *et al.* Cutaneous involvement in multiple myeloma: a clinicopathologic, immunohistochemical, and cytogenetic study of 8 cases. *Arch Dermatol* 2003; 139: 475–486.
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Prolonged elevation of serum granulysin in drug-induced hypersensitivity syndrome

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Key words

Drug-induced hypersensitivity syndrome (DIHS), Eosinophilia and systemic symptoms (DRESS), Granulysin

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Drug-induced hypersensitivity syndrome (DIHS)[1], also known as drug rash with eosinophilia and systemic symptoms (DRESS) [2], has been established as a clinical entity in severe cutaneous adverse drug reactions. DIHS is characterized by the limited number of causative drugs, late onset, clinical similarity to infectious mononucleosis-like syndrome and prolonged clinical course due to relapse[1].

Granulysin is a cytotoxic molecule produced against virus-infected cells, tumor cells, transplant cells, bacteria, fungi and parasites[3]. It plays an important role in the host defense against pathogens. A recent paper reported that granulysin is highly expressed in blisters of two other severe cutaneous adverse drug reactions: Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN)[4]. In addition, we found that serum granulysin is more elevated in patients with early-stage SJS/TEN than in those with ordinary drug-induced skin reactions[5].

This paper investigates the serum granulysin level of DIHS patients. We assembled serum samples of patients with DIHS and analyzed the correlation between granulysin concentrations with clinical manifestations and disease courses.

Sera of 15 patients with DIHS (10 men and 5 women; average age: 55.4 ±19.9 years) were obtained from multiple institutions. All the patients had actively progressing reactions meeting the criteria for DIHS, as previously defined[1]. The disease onset (day 1) was defined as when the skin eruption appeared. Sera of patients with ordinary drug skin reactions (ODSRs) (n=24) and healthy controls

(n=31) were also obtained. ODSRs included maculopapulartype and erythema multiforme-type reactions. The granulysin concentrations of the serum samples were measured with an ELISA as previously described[6]. In brief, serum samples were incubated on plates coated with RB1 antibody (MBL) and then reacted with biotinylated RC8 antibody (MBL). We performed assays in blind of the clinical features.

In serum samples taken from day 1 to day 10 (n=9), 8 samples showed elevated serum granulysin levels over 10 ng/ml (88.9%, 21.9 ±12 ng/ml). In serum samples taken from day 11 to day 20 (n=11), we detected prolonged high serum granulysin levels (63.6%, 16.1±14.8ng/ml). Serum granulysin levels decreased gradually after day 21 (n=20) (30%, 7.6 ± 3.4) (Figure 1). By day 20, the skin eruptions of all the DIHS patients had disappeared. As we reported previously, in 31 healthy control subjects, no increase of granulysin level was detected (0%, 1.6±0.6 ng/ml) and in 24 patients with ODSRs, elevated granulysin was detected in only one patient (4.16%,3.5±3.4 ng/ml) [5].

To distinguish DIHS from ODSRs, the following clinical information is helpful: limited causative drugs, late onset after medication, manifestations similar to infectious mononucleosis such as fever, lymphoadenopathy, hepatitis and hematological abnormalities. However, because of the diversity of ODSRs and similarity to viral exanthema, DIHS sometimes poses a diagnostic challenge. In addition, some cases suffer from multiple organ failure. Therefore, early diagnosis and appropriate treatment is essential.

Unique mechanisms have been implicated in DIHS development, including detoxification defects leading to reactive metabolite formation and subsequent

immunological reactions[7], and reactivation of HHV[8]. In addition, it is increasingly apparent that there is a genetic predisposition to adverse drug reactions. Human leucocyte antigen-related genes have been identified as predictors of DIHS[9].

In particular, the observation that HHV reactivation occurs during the acute phase of DIHS has led to suggestions of a pathogenic link. Shiohara *et al.* identified early reactivation of HHV6 and EB virus, with later involvement of HHV7 and CMV[8]. The resulting expansion of virus-specific T cells might mediate the clinical disease. Recent paper showed that cutaneous and visceral symptoms of DIHS/DRESS are mediated by activated $CD8^+$ T lymphocytes, which are directed against herpes viruses such as EBV[10].

Granulysin exhibits potent cytotoxicity against a broad panel of microbial targets, including tumor cells, transplant cells, bacteria, fungi and parasites, damaging negatively charged cell membranes because of its positive charge[3]. Granulysin plays important roles in the host defense against pathogens and induces apoptosis of the target cells in a mechanism involving caspases and other pathways [3]. In the present study, we showed that granulysin levels of sera were significantly elevated in DIHS patients compared to those of ODSRs. It is suggested that, in DIHS, activation of virus-specific cytotoxic T cells resulted in granulysin release in circulated blood. In contrast, granulysin was identified as the most highly expressed cytotoxic molecule in blisters of SJS/TEN resulting massive keratinocyte apoptosis[4], and we revealed that serum granulysin increased in early stage of SJS/TEN[5]. We speculated that granulysin is involved in SJS/TEN pathogenesis, inducing keratinocyte death in the early stage of