

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- $\gamma$  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- $\gamma$  antibody 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 <sup>a</sup>	Other antibodies
							Pattern	Titre		
1	19	F	Classical DM	–	–	–	Spe.	160	4644	
2	68	F	Cancer-associated DM	–	–	+	Nuc. Dots.	40	3104	
							Diffuse	640		
3	74	F	Classical DM	+	–	–	Ovary	640	1731	ACA
							Spe.	2560		
4	76	F	Classical DM	–	–	–	Dis. spe.	2560	367	
							Diffuse	80		
5	16	F	JDM	–	–	–	Nuc. Dots.	320	326	
							Diffuse	80		
							Nuc. mem.	80		
6	66	F	Classical DM	–	–	–	Mit. spi.	80	255	
							Spe.	160		

<sup>a</sup>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- $\gamma$  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 (microorchidia 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- $\gamma$  [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- $\gamma$  is considered to act as a repressor of TGF- $\beta$  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.

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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- $\gamma$  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

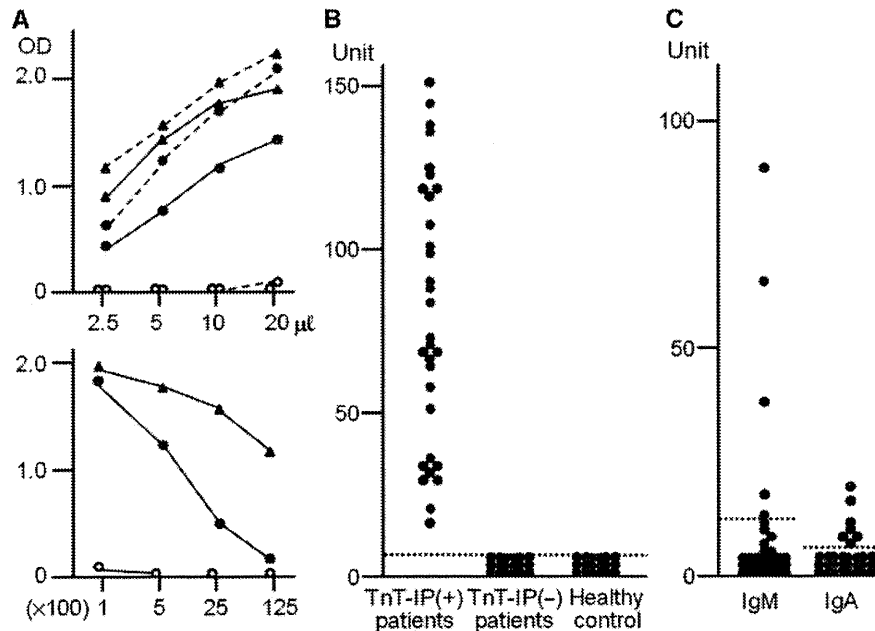
Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3]. Nunc Immobilizer Streptavidin plates (Thermo Scientific Nunc, Roskilde, Denmark) to which streptavidin was covalently coupled via a spacer were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with biotinylated recombinant MDA-5 diluted with T-PBS (50  $\mu$ l/well) and incubated for 1 h at room temperature. After three washes with T-PBS, the wells were blocked with 200  $\mu$ l 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  $\mu$ l/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  $\mu$ l/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  $\mu$ l/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  $\mu$ l/well DT (1  $\mu$ g/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  $\mu$ l/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  $\mu$ l of the final volume per well. In the lower panel, recombinant protein was applied at 10  $\mu$ l diluted with 40  $\mu$ l 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

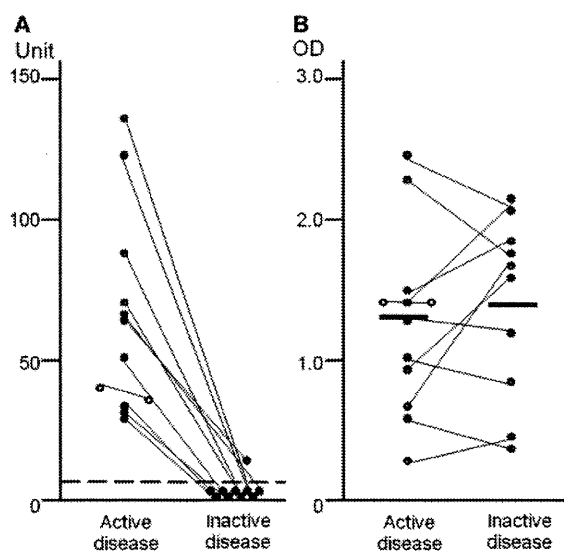
levels were compared between serum samples at active and inactive disease states (Fig. 2A). Except for one patient who still had anti-MDA-5 antibodies but whose titre was dramatically reduced at 5 years from disease onset, in all the sera the anti-MDA-5 antibodies were absent during remission. These 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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## Correspondence

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

Skin involvement in multiple myeloma (MM) is uncommon.<sup>1-3</sup> 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.<sup>4</sup> 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.

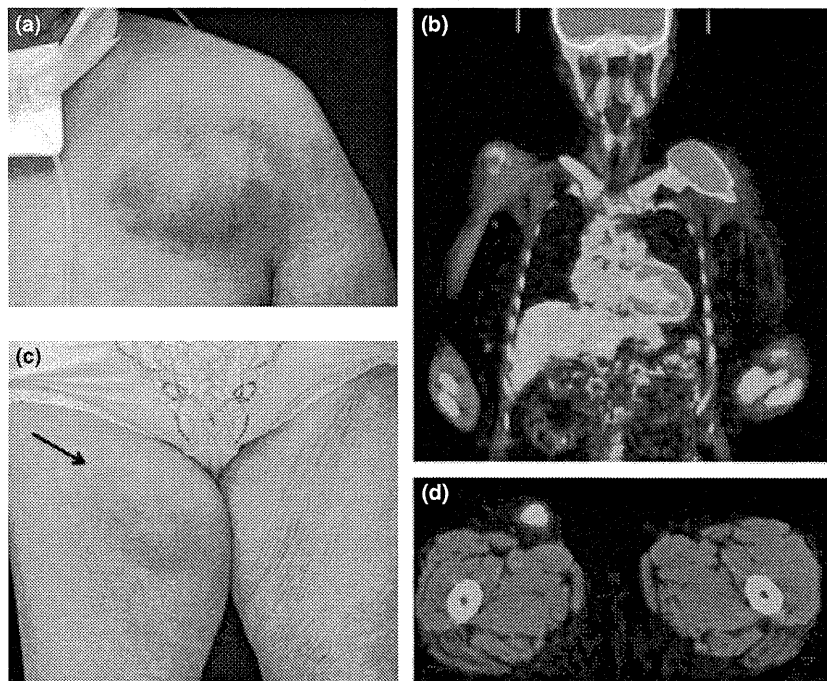
A 68-year-old woman was referred to the outpatient clinic of our dermatology department with two large subcutaneous masses, one on the left shoulder and the other on the right thigh. Both masses were well-demarcated, elastic hard subcutaneous tumors, approximately 10 cm in diameter, and neither adhered 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  $\gamma$ -globulin

$\lambda$ -light chain, although no  $\kappa$ -light chain-positive cell was seen. IgA  $\alpha$  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.

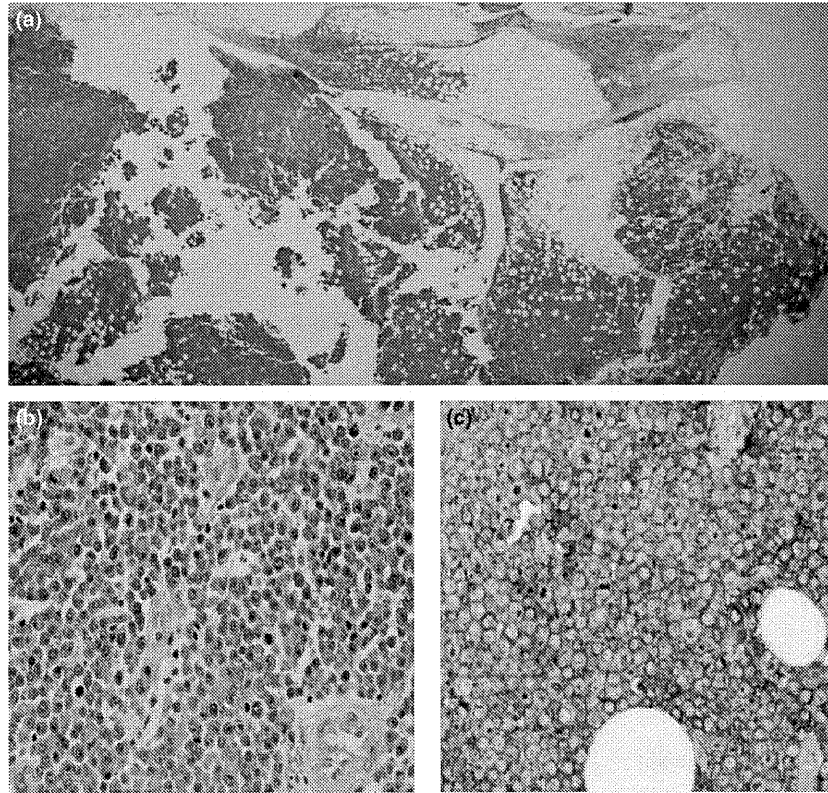
Eight months before the onset of the skin tumors, MM was suspected from high serum  $\gamma$ -globulin levels and an unusual fracture of the left upper arm. Quantitative immunoglobulin tests showed an IgA- $\lambda$  spike. Bone marrow aspiration and biopsy revealed large numbers of atypical plasma cells, 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

LOW RESOLUTION COLOR FIG



**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,  $\times 20$ ). (b) High magnification shows that the infiltrating cells are atypical plasma cells (hematoxylin and eosin, original magnification,  $\times 400$ ). (c) Immunostaining reveals that most of the atypical cells are strongly positive for  $\gamma$ -globulin  $\lambda$ -light chain (original magnification,  $\times 400$ )

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.<sup>2,3</sup> Thus, metastatic skin lesions in MM are very rare. Requena *et al.*<sup>4</sup> 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.<sup>4</sup> 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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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)<sup>1</sup>, also known as drug rash with eosinophilia and systemic symptoms (DRESS)<sup>2</sup>, 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<sup>1</sup>.

Granulysin is a cytotoxic molecule produced against virus-infected cells, tumor cells, transplant cells, bacteria, fungi and parasites<sup>3</sup>. 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)<sup>4</sup>. 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<sup>5</sup>.

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:  $55.4 \pm 19.9$  years) were obtained from multiple institutions. All the patients had actively progressing reactions meeting the criteria for DIHS, as previously defined<sup>1</sup>. 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 maculopapular type and erythema multiforme-type reactions. The granulysin concentrations of the serum samples were measured with an ELISA as previously described<sup>6</sup>. 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%,  $21.9 \pm 12$  ng/ml). In serum samples taken from day 11 to day 20 (n=11), we detected prolonged high serum granulysin levels (63.6%,  $16.1 \pm 14.8$  ng/ml). Serum granulysin levels decreased gradually after day 21 (n=20) (30%,  $7.6 \pm 3.4$ ) (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%,  $1.6 \pm 0.6$  ng/ml) and in 24 patients with ODSRs, elevated granulysin was detected in only one patient (4.16%,  $3.5 \pm 3.4$  ng/ml)<sup>5</sup>.

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, lymphadenopathy, 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<sup>7</sup>, and reactivation of HHV<sup>8</sup>. 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<sup>9</sup>.

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<sup>8</sup>. 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<sup>+</sup> T lymphocytes, which are directed against herpes viruses such as EBV<sup>10</sup>.

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<sup>3</sup>.

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<sup>3</sup>. 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<sup>4</sup>, and we revealed that serum granulysin increased in early stage of SJS/TEN<sup>5</sup>. We speculated that granulysin is involved in SJS/TEN pathogenesis, inducing keratinocyte death in the early stage of

these diseases, whereas serum granulysin in DIHS might be released against virus-infected cells. This speculation is consistent with the present data that show the duration of DIHS manifestation to coincide with the timing of elevated serum granulysin levels. Recently we developed a rapid immunochromatographic test to detect high serum granulysin level in 15 minutes<sup>6</sup>. We expect that monitoring of serum granulysin by the rapid test might contribute to the early diagnosis of DIHS as well as of SJS/TEN. In conclusion, serum granulysin might help early diagnosis and predict disease prognosis.

#### Figure Legend

Fig 1. Granulysin levels of healthy controls, and of ODSRs patients and DIHS patients at different stages of the diseases.

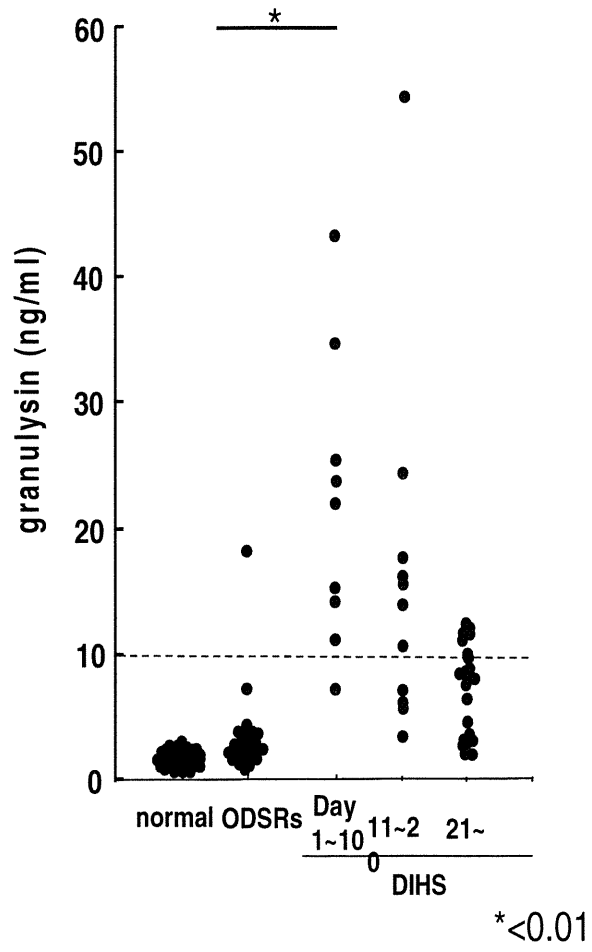
In DIHS patients, we examined the concentration of granulysin for three terms: day 1 to 10, day 11 to 20, and after day 21. The granulysin level was elevated from day 1 to 20, compared to those for ODSRs and normal controls (\*:p<0.01).



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Fig 1



## CORRESPONDENCE

**Intraepidermal neutrophilic IgA pemphigus successfully treated with dapsone**

A 25-year-old woman presented with a 2-month history of erythematous, intensely itchy macules and vesicles on the extremities and trunk. Before onset, she was in good health and took no medication. Physical examination revealed pinkish or reddish, edematous, well-demarcated erythema (figure 1A). The lesions tended to coalesce, forming annular patterns, some of which had vesicles around the margins, forming a sunflower-like configuration. The oral cavity and genital area were unaffected. Histopathological findings of a pustule revealed intraepidermal blisters with neutrophil infiltrates without prominent acantholysis (figure 1B). Laboratory examinations, including serum immunoglobulins, and ELISA for anti-desmoglein 1 and 3 were within normal ranges. Chest X-ray, electrocardiogram, and blood tests revealed no other related diseases and monoclonal gammopathy. DIF of the erythematous lesion revealed IgA deposition in the intercellular space throughout the epidermis (figure 1C). IIF revealed circulating IgA autoantibodies binding to the cell surfaces of the entire epidermis of normal human skin (titer: 64×). Immunoblot analysis using epidermal extracts from normal human skin and recombinant desmocolin 3 showed no specific bands for either IgA or IgG antibodies. These findings led to the diagnosis of IEN-type IgA pemphigus. Treatment was initiated with topical corticosteroids, achieving only a slight effect; dapsone (50 mg per day) was therefore started. The

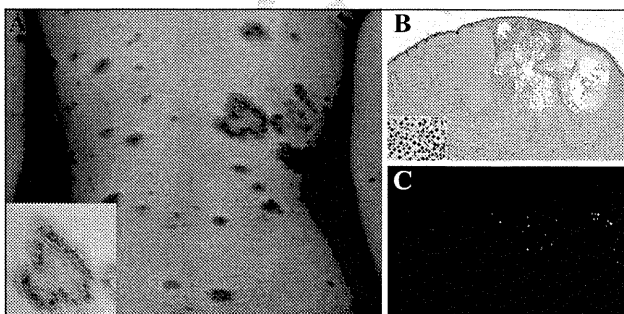
pruritus and lesions improved but the symptoms recurred after four weeks. For that reason the dose was raised to 75 mg dapsone and the itchiness subsided within a few days. Two weeks later, only pigmented macules with no active lesions were observed. The titer of IIF also decreased from 64× to 16×.

IgA pemphigus is a distinct group of auto-immune intraepidermal blistering diseases that present with vesiculopustular eruption, neutrophil infiltration with or without acantholysis. IgA autoantibodies that target keratinocyte cell surfaces and desmosomal components in the epidermis have been detected in DIF and IIF [1]. IgA pemphigus is divided into two major subtypes: the IEN type, and the SPD type. While SPD-type IgA pemphigus shows subcorneal pustules, the IEN type is characterized by pustule formation, mainly in the middle or lower epidermis.

In DIF, SPD-type IgA pemphigus involves cell surface IgA binding only in the upper epidermis, whereas IEN-type IgA pemphigus shows binding throughout the epidermis [2]. Desmocolin 1 has been identified as an autoantigen in SPD-type IgA pemphigus, suggesting that it plays an important role in the pathogenesis of this disease subtype [3]. Although autoantibodies against desmogleins [4] and desmocolins [5] have been reported in some cases of IEN-type IgA pemphigus, the specific autoantigen remains unidentified. In our case, we were also unable to detect specific autoantibodies using immunoblot analysis. Interestingly, a case with clinical and histological features compatible with SPD-type IgA pemphigus, but for which anti-desmocolins antibodies were not detected, was diagnosed as IEN-type IgA pemphigus [6]. That report suggested that the subtypes of IgA pemphigus might be considered to be divided by autoantigens.

In contrast to the common types of pemphigus, like pemphigus vulgaris, treatment for some cases of IgA pemphigus does not require corticosteroid or other immunosuppressive therapy. These cases of IgA pemphigus are well controlled using only anti-inflammatory treatments, such as dapsone, colchicine or isotretinoin [1]. Dapsone may be useful in treating IgA pemphigus due to its effect in suppressing neutrophilic infiltration. However refractory cases require plasmapheresis or cyclophosphamide. In the present case, oral administration of dapsone quickly caused the symptoms to subside. In IgA pemphigus, it is important to make the correct diagnosis and to choose a suitable therapy to avoid the side effects by the prolonged use of systemic corticosteroids. ■

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**Figure 1.** A) Pinkish and reddish edematous erythema with vesicles around the margins are scattered on the trunk. B) Histopathological findings of a pustule reveal intraepidermal blisters with neutrophil infiltrates. C) Direct immunofluorescence of the perilesional skin biopsy specimen reveals IgA deposits on the keratinocyte cell surfaces.

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