Original article

The diagnostic utility of anti-melanoma differentiation-associated gene 5 antibody testing for predicting the prognosis of Japanese patients with dermatomyositis

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Running title: Classification of DM by anti-MDA5 Ab

Abstract

Objective: Interstitial lung disease (ILD), especially rapidly progressive ILD (RPILD), is a major poor prognosis factor in patients with dermatomyositis (DM). We investigated the association of anti-melanoma differentiation-associated gene 5 (MDA5) antibody (Ab) with clinical characteristics and mortality in Japanese patients with DM.

Methods: Seventy-nine DM patients, comprising 58 classic DM and 21 clinically amyopathic DM patients, were enrolled. Serum Abs were screened by immunoprecipitation assays, and an immunosorbent assay (ELISA) was used for MDA5.

The relationships of clinical characteristics and mortality with each Ab were investigated.

Results: Anti-MDA5 Ab was detected in 17 patients. Anti-clinically amyopathic dermatomyositis 140-kDa polypeptide antibodies (anti-CADM140 Abs) were found in 16 out of the 17 anti-MDA5 Ab^+ patients. Skin ulcers, palmar papules, CADM (Clinically amyopathic DM), RPILD, and mediastinal emphysema were highly distributed in anti-MDA5 Ab^+ patients. The mortality at 6 months as well as 5 years was also significantly higher in anti-MDA5 Ab^+ patients than in anti-MDA5 Ab^- patients. In a multivariable Cox regression analysis, mortality was independently associated with anti-MDA5 Ab (relative hazard, 6.33; 95% confidence interval, 1.43-28.0). All of the deaths in anti-MDA5 Ab^+ patients were attributed to respiratory failure of RPILD; however, RPILD did not exacerbate in any of the anti-MDA5 Ab^+ patients who survived the first six months.

Conclusion: The presence of anti-MDA5 Ab identifies the characteristic skin, musculoskeletal, pulmonary and prognostic features in patients with DM. In addition, anti-MDA5 Ab seems to predict a group of patients with CADM complicated fatal RPILD.

Introduction

A number of autoantibodies can be detected in the sera of patients with dermatomyositis (DM), some of which are specific to DM and are known as myositis-specific auto-antibodies (MSAs). Moreover, these auto-antibodies are closely associated with clinical manifestations of DM, such as symptoms, complications, reactivity to therapy and prognosis (1).

In recent years, the autoantibodies found in patients with inflammatory myopathies have been mainly classified into several types by immunoprecipitation assays: anti-aminoacyl-tRNA synthetase antibodies (anti-ARS Abs), antibodies to the signal recognition particle (anti-SRP Abs), anti-Mi2 Abs, PM/Scl-100 Abs and PM/Scl-75 polypeptides Abs (anti-PM-Scl Abs), anti-clinically amyopathic dermatomyositis 140kDa polypeptide Abs (anti-CADM140 Abs), anti-155/140-kDa polypeptide Abs (anti-p155/140 Abs) and autoantibodies to a 142-kDa protein (anti-MJ Abs). These autoantibodies are strongly associated with the clinical presentation (2-6). In this regard, we have reported a high frequency of rapidly progressive interstitial lung disease (RPILD) and CADM (Clinically amyopathic DM) associated with anti-CADM140 Abs (7, 8). Recently, RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) was identified as a major autoantigen in

patients with CADM which is targeted by anti-CADM140 Abs (9, 10).

Gono et al. have also recently reported that anti-MDA5 Ab predicts a fatal outcome in patients with DM combined with RPILD (11); however, the long-term prognosis and other clinical characteristics of anti-MDA5 Ab^+ DM patients remain to be elucidated. In the present study we have tried to investigate the clinical value of anti-MDA5 Ab for DM patients in a single cohort.

Patients, Material and Methods

Patients

Sera samples were obtained from 79 patients with DM who were undergoing medical treatment at the Graduate School of Biomedical Sciences, Nagasaki University, from September 1999 to August 2010, and were stored at -20˚C until use. Most of the sera samples were obtained at the first visit so the interval from initiation of therapy was minimal. We collected the data from all of the DM patients examined in our department. Twenty-one patients did not fulfil Bohan and Peter's criteria (12, 13) but fulfilled Sontheimer's criteria (CADM) (14, 15) because of the absence of clinical skeletal muscle symptoms and the presence of persistent clinical DM skin features. Clinical manifestations, laboratory data, radiographic data and the presence of internal

malignancies were extracted from medical records and verified by TK, NI and KF. The patients were diagnosed with ILD according to the results of chest X-ray and high-resolution chest computed tomography, reported by Japanese-board-certified radiologists. All of the subjects underwent routine examination of internal malignancies and chest radiography. A subset of patients with RPILD was defined as those presenting with progressive dyspnea and progressive hypoxemia, and a worsening of interstitial change on chest radiography within 1 month from the onset of respiratory symptoms, as described previously (2). A signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University, was obtained from each patient.

Immunoprecipitation and ELISA

MSAs, including anti-CADM140 Abs, anti-ARS Abs and anti-155/140 Abs, were detected by immunoprecipitation assays using extracts of leukemia cell line K562, as described previously (3). Interpretation of the results of immunoprecipitation was undertaken without knowledge of patients' clinical status. An enzyme-linked immunosorbent assay (ELISA) system using recombinant MDA5 as an antigen source was performed as described previously (10). All samples were examined in duplicate, and the antibody units were calculated from the optical density at 450 nm, using a

standard curve obtained from serial concentrations of a serum sample containing a high titer of anti CADM-140 Abs. The cutoff level was set at 8.0 units, based on 10 SDs above the mean value obtained from 32 healthy control sera. Interpretation of the results of ELISA was undertaken without knowledge of the clinical status of the patients and the results of immunoprecipitation assays.

Statistical analysis

Fisher's exact probability test and the Mann-Whitney U-test were used to compare the differences. We also examined the cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years by the multivariate Cox proportional hazard model adjusted for patient age at symptoms onset, gender, with or without corticosteroids and with or without immunosuppresants. A p-value <0.05 was considered significant.

Results

Clinical characteristics of anti-MDA5 Ab$^+$ patients

Table 1 summarizes the 17 DM patients with anti-MDA5 Ab and the 62 DM patients without anti-MDA5 Ab. There were 21 patients with CADM in the present study and we have found that anti-MDA5 Ab is detected in 14 out of 21 patients. In this group,

eleven out of 14 patients complicated RPILD (79%) and 7 patients died (50%). Our present data confirm the recent publications regarding the characteristics of anti-MDA5 Ab^+ patients including the CADM, RPILD, low CK, high ferritin and high mortality found in these patients (11). Since anti-MDA5 Ab is mostly attributed to anti-CADM 140 Abs, a high prevalence of palmar papules and mediastinal emphysema, which has been reported as typical of anti-CADM 140 Abs^+ DM patients by our group (7), was also preferentially found in anti-MDA5 Ab^+ patients. The present finding that skin ulcers are highly prevalent in anti-MDA5 Ab^+ patients is new, however. Muscle biopsy or lung biopsy was not performed. Skin biopsies were taken from 8 patients positive for anti-MDA5 antibodies, and 6 patients were diagnosed pathologically with dermatitis consistent with DM. One patient revealed only mild mucin deposition, and another revealed only hyperpigmentation. Only the small number of patients were taken biopsy might show a limitation of the present study. Electromyography was performed in one anti-MDA5 Ab^+ patient revealing myogenic conversion consistent with myositis. Only one patient was found to have preceding interstitial lung diseases among anti-MDA5 Ab^+ patients. Skin manifestations preceded interstitial lung diseases in the other patients. We showed the typical images about mediastinal emphysema, palmar pustule and regional ulcers in anti-MDA5 Ab^+ patients with CADM (Fig 1). In the frequency of

cancer, anti-MDA5 Ab^+ patients have no malignancy (0/17) whereas 6 out of 62 (10%) patients in anti-MDA5 Ab^- group were complicated malignancies. Anti-155/140 Abs were found in all of the six patients with cancer. We confirmed the profile of autoantibodies regarding the presence or absence of anti-MDA 5 Ab: namely, all DM patients positive for anti-ARS Abs, anti-155/140 Abs and other types of autoantibodies were among the anti-MDA5 Ab^- group. There was no overlap between anti-MDA5 Ab and any other types of autoantibodies. Immunoprecipitation of anti-CADM 140 Abs from patients with anti-MDA5 Ab is shown in Figure 2.

Survival rate of patients with anti-MDA5$^+$ patients

Ten patients (12%) died within 5 years from the first treatment. The cumulative 6-month survival rates were 57.4 and 98.4% for DM with anti-MDA5 Ab and those without anti-MDA5 Ab, respectively (Fig. 3). The survival rates from first visit to our hospital after adjusting for age, gender, with or without corticosteroids and with or without immunosuppresants were significantly different between each subset (p = 0.0151). The first visit to our hospital was almost identical to the diagnosis of each patient. The presence of anti-MDA5 Ab was independently associated with mortality (relative hazard, 6.33; 95% confidence interval, 1.43-28.0) in a multivariable Cox regression model that included patient age at onset, gender, with or without corticosteroids and with or

without immunosuppresants. We have tried to compare the variables within anti-MDA5 Ab^+ DM patients who is alive or dead and found that the regime of therapy was not different between two groups though PaO2/FiO2 and serum CPK level was higher in the former. The value of anti-MDA5 Ab is significantly lower in the former (Table 2). All the deaths in the anti-MDA5 Ab^+ patients were attributed to respiratory failure of RPILD. However, importantly, there was no acute exacerbation or progressive worsening of ILD by CT images after initial treatments in any of the anti-MDA5 Ab^+ patients. In fact, all of the deaths of anti-MDA5 Ab^+ patients occurred within the first 6 months (Fig. 3). In addition, there was no patient required home oxygen therapy after discharge among anti-MDA5 Ab^+ patients who is alive during the first 6 months. We showed a short case presentation describing a patient with CADM positive for anti-MDA5 Ab. A 60-year-old female developed erythemas on upper eyelids, fingers and elbows in July 2005. Three months later, she developed exertional dyspnea. A CT scan revealed interstitial lung shadow (Fig. 4A). We measured anti-CADM-140 antibody levels and anti-MDA5 antibody levels, which were both positive (anti-140 kDa antibodies were detected by immunoprecipitation assay, and the titer of anti-MDA5 antibodies was 544.109 unit). She has been treated at our outpatient department and in a stable condition (Fig. 4B).

Discussion

Other Japanese groups recently identified the characteristics of anti-MDA5 Ab^+ DM patients (11). Our present data confirmed their findings. Additionally, we have shown some new characteristic of these patients, such as high frequencies of palmar papules, skin ulcers and mediastinal emphysema as well as no overlapping of other types of autoantibodies. These data may help physicians to recognize features of anti-MDA5 Ab^+ patients among DM patients. Since physicians are urged to start intense immunosuppressive therapy early for anti-MDA5 Ab^+ DM patients, this information may be clinically indispensable.

Although the prognosis of anti-MDA5 Ab^+ patients was worse than that of anti-MDA5 Ab^- patients, none of the surviving anti-MDA5 Ab^+ patients experienced acute exacerbation or progressive worsening of ILD after the initial treatment. This is quite different from the anti-MDA5 Ab^- patients since ILD recurred in several of these patients, and death ensued during the long-term follow-up (Fig. 3). One of the characteristics of anti-MDA5 Ab^+ patients is hyperferritinemia (11, 16). There are many reports evaluating hyperferritinemia in patients with autoimmune diseases (17). The highest ferritin levels in autoimmune disorders are typically seen in patients with macrophage activation syndrome (MAS), often associated with adult-onset Still's

disease (AOSD) (18). It is well known that many viruses produce double-stranded (ds) RNA that can be recognized by two major arms of the innate immune system: the toll-like receptors (TLR) and the Rig-I-like receptors (RLR). MDA5 is a member of the RLR family that recognizes dsRNA within the cytosolic compartment and induces the production of inflammatory cytokines and cell surface molecules involved in the antiviral response (19). Considering that MAS could be induced by varying infectious agents (20) and given the critical role of MDA5 in the innate immune defense against viruses, one hypothesis is that the production of anti-MDA5 Ab is an epiphenomenon during virus infection that is associated with the onset of CADM and RPILD; namely, infection of the skin and lung epithelium by certain viruses. In general, innate immune responses do not recur; therefore, we have not found the exacerbation of ILD during the follow-up periods of anti-MDA5 Ab^+ DM patients.

Most patients with ILD complicated in DM appear to be well controlled by corticosteroids and immunosuppresants (21). In contrast, patients with RPILD observed in DM were resistant to a variety of treatments (22, 23). We have introduced corticosteroids, cyclophosphamide and calcineurin inhibitor to the anti-MDA5 Ab^+ patients with RPILD. We could not find the significant difference of therapy between alive and dead patients. PaO2/FiO2, serum CPK level and the value of anti-MDA5 Ab

before treatment were prognostic factor statistically. We showed the duration of preceding symptoms to diagnosis in patients positive for anti-MDA5 antibodies. Although we do not have any definitive evidence, shorter duration from preceding symptoms to the treatment could lead better outcome (Supplemental Table 1). Thus, anti-MDA5 Ab^+ patients who have typical CADM with signs of ILD are recommended to treat promptly with the combination of corticosteroids, cyclophosphamide and calcineurin inhibitor.

In conclusion, the measurement of anti-MDA5 Ab by ELISA enables us predict the prognosis of the patients with CADM complicated fatal RPILD. The characteristics of anti-MDA5 Ab^+ DM patients could be explained by the nature of MDA5 in innate immune responses to viruses. A multicenter, prospective study is warranted to confirm our results.

Key Messages

・ Anti-MDA5 Ab is associated with characteristic pulmonary and skin involvements in patients with DM.

・ Anti-MDA5 Ab predicts the patients with CADM complicated fatal RPILD.

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Disclosure statement: The authors have no conflicts of interest to declare.

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rapidly progressive diffuse alveolar damage. J Dermatol 1998;25:32.6.

Figure Legends

Table 1. Comparison of clinical manifestations between patients with anti-MDA5 antibody and patients without anti-MDA5 antibody. Ages are presented as mean values (S.D.), while laboratory markers are medians (interquartile range). P-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values.

Figure 1. Typical clinical manifestations of patients with anti-MDA5 Ab. The palmar pustules (A) were mainly located near the metacarpophalangeal and proximalinterphalangeal joints (arrows) and multiple ulcer regions (B) were also observed. Chest CT scan (C) shows mediastinal emphysema in the middle of the chest cavity (arrows).

Figure 2. Immunoprecipitation with anti-CADM140 Ab from the 35S-labelled K562 cell extract. Lanes 5-12 and 18-25 show the results with anti-CADM140-positive sera from DM patients with anti-MDA5 Ab^{+} (A-P). The results of the prototype sera of

anti-155/140 Abs and anti-CADM140 Abs are also shown (lanes 3, 16 and 4, 17). One sera of anti-MDA5 Ab^+ patient immunoprecipitated not anti-CADM 140 Abs but anti-U1-ribonucleoprotein (RNP) Ab that was deleted from Fig. 2.

Figure 3. The adjusted cumulative survival rates in the presence or absence of anti-MDA5 Ab. The cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years were examined as described in the Text. Survival rate of anti-MDA5 Ab^+ patients was significantly low as compared with anti-MDA5 Ab- patients. $p = 0.0151$, between the two groups.

Table 2 Comparison of clinical parameters between alive and dead anti-MDA5 Ab^+ patients. Ages are presented as mean values (S.D.), while laboratory markers are medians (interquartile range). P-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values.

Figure 4 A chest CT scan before and after treatment. A reticular shadow was revealed in the lower lung field (A) and improved after four years later onset of the disease (B). Arrows indicate the legion which is improved by the treatment.

Supplemental Table 1 The duration of preceding symptoms to diagnosis in patients positive for anti-MDA5 antibodies.

Table 1

Variables	Anti-MDA5 Ab positive (n=17)	negative (n=62)	p value
Age at onset, years	55.5 (13.0)	55.3 (15.0)	0.27
Female, n (%)	15 (88)	37 (60)	0.056
Skeletal muscle and skin features			
Muscle weakness, n (%)	4 (24)	38 (62)	**0.005**
Gottron's sign, n (%)	13 (76)	32 (52)	0.07
Ulcer region, n (%)	10 (59)	7 (12)	**0.00007**
Heliotrope rush, n (%)	8 (47)	23 (39)	0.56
Palmar papules, n (%)	11 (65)	13 (22)	**0.0014**
Periungual erythema, n (%)	10 (59)	24 (41)	0.2
Clinical diagnosis			
CADM, n (%)	14 (82)	7 (11)	**4.2×10^{-9}**
Pulmonary involvement and malignancy			
ILD, n (%)	16 (94)	37 (61)	**0.008**
Rapidly progressive ILD, n (%)	12 (71)	4 (7)	**9.8×10^{-9}**
Mediastinal emphysema, n (%)	6 (35)	1 (2)	**2.1×10^{-5}**
Malignancies, n (%)	0 (0)	6 (10)	0.17
Laboratory data			
CPK, IU/L	173 (53-468)	905 (107-1607)	**0.00024**
KL-6, U/mL	1361 (825-1903)	1040 (345-1510)	0.36
Ferritin, ng/mL	1365 (894-1751)	180 (90-244)	**0.016**
Therapy			
Maximum PSL, mg/day	40 (35-50)	40 (22.5-50)	0.99
Immunosuppressant, n (%)	16 (94)	29 (47)	0.17
Outcome			
Death, n (%)	7 (41)	3 (5)	**6.6×10^{-6}**
MSA profile			
Anti-140Ab positive, n (%)	16 (94)	0 (0)	**3.76×10^{-15}**
Anti-155/140Ab positive, n (%)	0 (0)	7 (11)	0.35
Anti-ARS Ab positive, n (%)	0 (0)	30 (48)	**0.002**
Autoantibodies negative	1 (6)	25 (40)	**0.005**
Anti MDA5 antibody titer	230 (22-448)	1.3 (1.1-1.9)	**1.62×10^{-10}**

Figure 1.

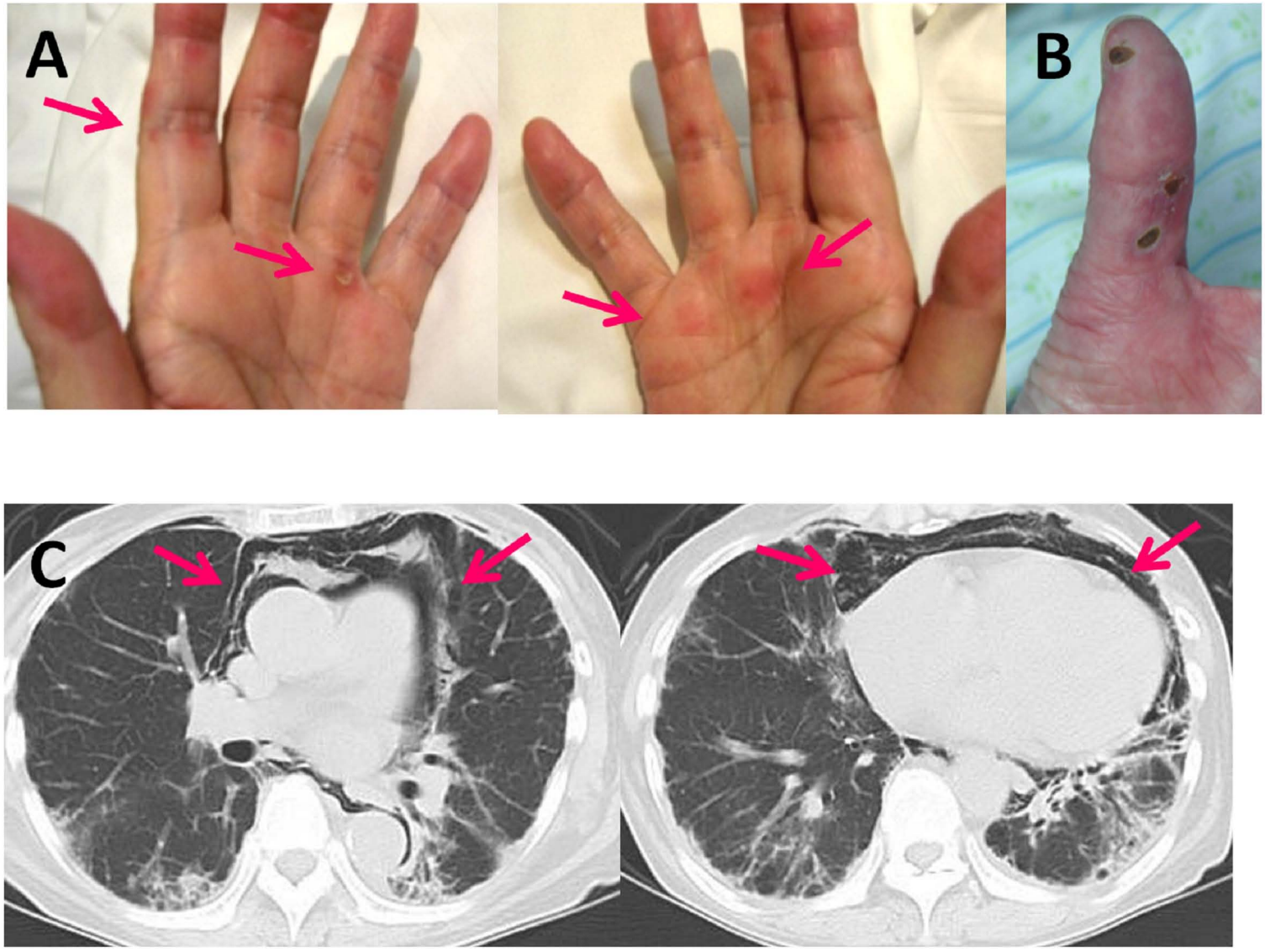
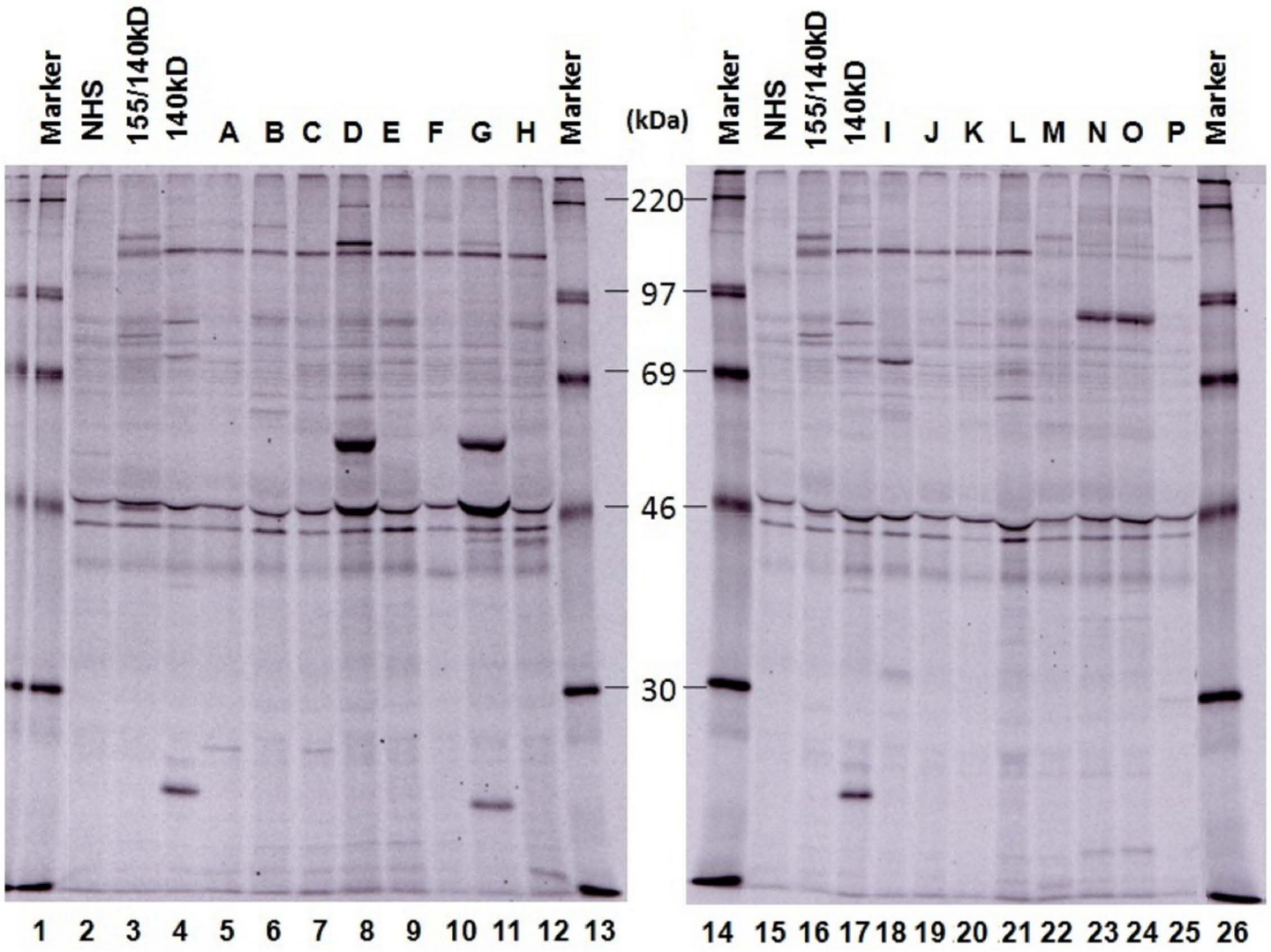


Figure 2.


Marker
NHS
155/140kD
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A B C D E F G H
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(kDa)
220
97
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1 2 3 4 5 6 7 8 9 10 11 12 13
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Figure 3.

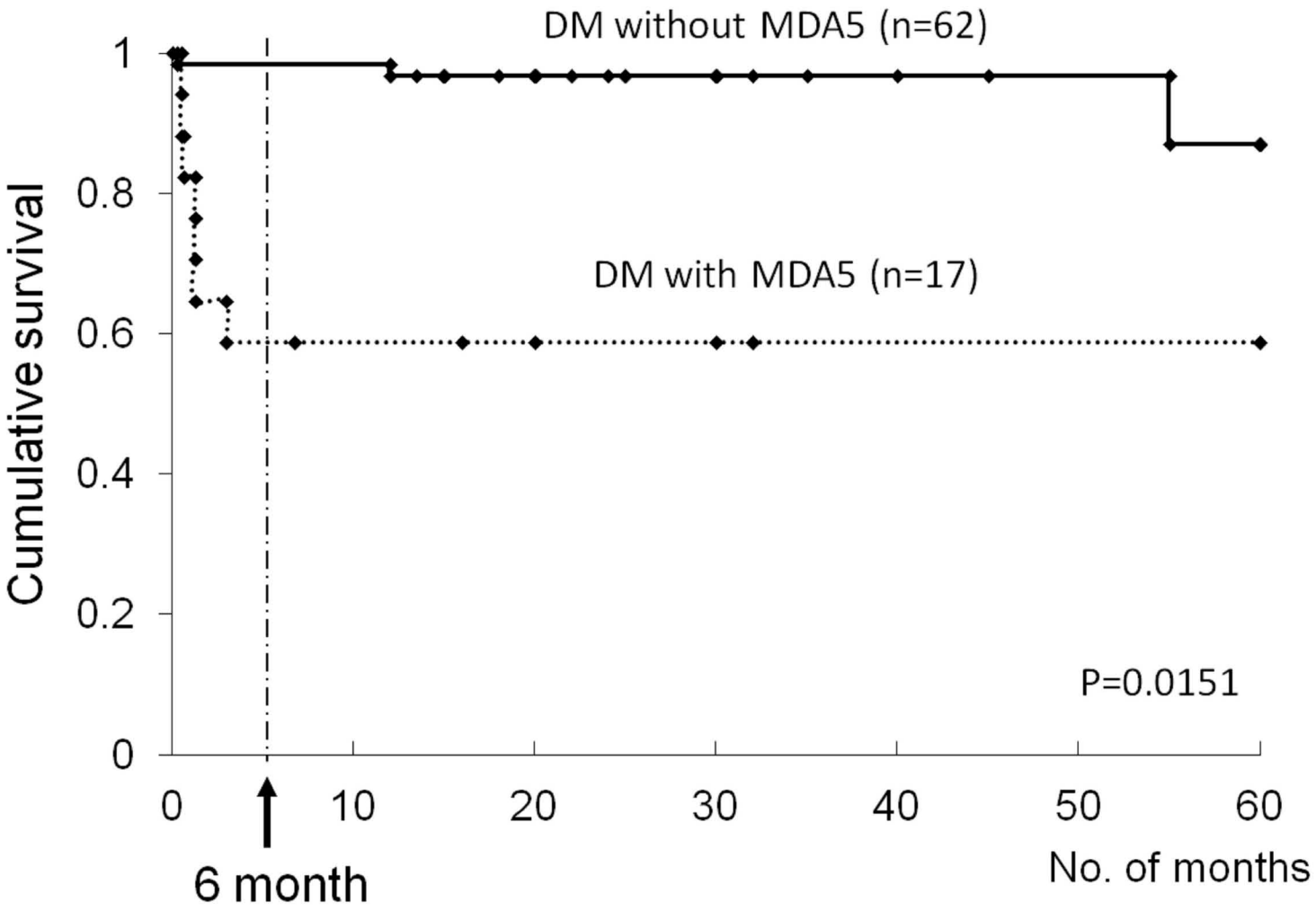
DM without MDA5 (n=62)
DM with MDA5 (n=17)
Cumulative survival
1
0.8
0.6
0.4
0.2
0
P=0.0151
0
10
20
30
40
50
60
6 month
No. of months

Table 2

Variables	Anti-MDA5 Ab positive (n=17)		
	Alive (n=10)	Dead (n=7)	p valure
Age at onset, years	52 (42-58.5)	59 (53-70)	0.051
Female, n (%)	9 (90)	6 (86)	1.00
ulcer region, n (%)	5 (50)	5 (71)	0.70
palmar papules, n (%)	7 (70)	5 (71)	1.00
CPK, IU/L	208 (90.3-864)	169 (33.5-359)	**0.014**
Anti MDA5 antibody titer	168 (16.3-436)	230 (76.0-478)	**0.032**
PaO2/FiO2 before treatment, mmHg	395 (370-462)	203 (114-240)	**0.027**
Therapy			
Steroid pulse therapy, n (%)	5 (50)	7 (100)	0.09
Cyclophosphamide, n (%)	4 (40)	4 (57)	0.84
oral calcinurin inhibitor, n (%)	6 (60)	7 (100)	0.18
intravenous calcinurin inhibitor, n (%)	1 (10)	3 (43)	0.32

Figure 4.