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Title: Autoantibodies to Su/Argonaute 2 in Japanese patients with inflammatory myopathy

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Abstract: Background: Anti-Su antibodies are found in 5-20% of cases of various systemic autoimmune rheumatic diseases and in 5-10% of dermatomyositis (DM)/polymyositis (PM) patients. In 2006, the 100kDa Su antigen was identified as argonaute2 (Ago2), and it was found to play a major role in RNA interference. However, immunoprecipitation (IP) remains the main method for detecting anti-Su and the clinical significance of the antibodies is uncertain.
Methods: Sera from patients with DM/PM (n=224) were screened by an ELISA that uses recombinant biotinylated Ago2 protein. Some serum samples were tested by IP and by indirect immunofluorescence (IIF) analysis.
Results: Seventeen (7.5%, 17/224) sera from DM/PM were positive in ELISA. Of the 33 IP-tested sera (17 ELISA-positive and 16 ELISA-negative with high background), 13 were found to be anti-Ago2/Su-positive in IP and ELISA. Only one IP-positive serum was judged to be ELISA-negative. Among the 13 patients with anti-Su/Ago2, 7 cases also had myositis-specific autoantibodies. Six sera that were positive by both IP and ELISA showed the GW body pattern in IIF. Interestingly, one ELISA-positive serum with an inconclusive result in IP also showed the GW body pattern.
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*Highlights

Highlight

- ● Our study aims to establish an ELISA for anti-Su/Argonate2 (Ago2) antibodies.
- ● Our ELISA was useful for screening anti-Su/Ago2 (sensitivity 93% , specificity 79%).
- ● Of the 224 inflammatory myopathy patients, 13 (5.8%) were anti-Su/Ago2 positive.

*Manuscript

Autoantibodies to Su/Argonaute 2 in Japanese patients with inflammatory myopathy

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antibodies (sensitivity: 93%, specificity: 79%).

Key words

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1. Introduction

Anti-Su antibodies were first detected by double immunodiffusion using calf thymus extract in sera from patients with systemic lupus erythematosus (SLE) (1). MRL/*lpr* mice and pristane-induced SLE model mice also produce anti-Su antibodies (2, 3). In 1994, target antigens of anti-Su antibodies were characterized by immunoprecipitation (IP) as 100/102kDa doublet proteins often accompanied by a 200kDa protein (4). In 2006, the 100kDa component of the Su antigen was identified as Argonaute 2 (Ago2), a key component in RNA interference machinery (5). Ago2 also plays important roles in the regulation of microRNA expression, function, maturation and stability (6). Recently, the roles of microRNAs in the pathogenesis of autoimmune diseases have been studied extensively (7).

In addition to being reported in SLE, anti-Su antibodies have been reported in many other systemic autoimmune rheumatic diseases, including systemic sclerosis (SSc), dermatomyositis/polymyositis (DM/PM), and rheumatoid arthritis (RA). The prevalence is relatively high in SLE (17-24%) and SSc (17-32%), but lower in RA and DM/PM (7-9 %), based on limited studies (1, 4, 8-10). The data on anti-Su in DM/PM patients in the previous studies were from small cohorts; only 17 to 43 serum samples from DM/PM patients were examined (1, 8, 10). The clinical features of anti-Su

antibody-positive DM/PM are not well characterized. One reason for this is because detection has been done by IP or by insensitive, rarely used double immunodiffusion. Thus, in this study, we aimed to establish a simple method for screening anti-Su/Ago2 antibodies and to analyze clinical features associated with DM/PM patients with these antibodies. We have already accumulated data of more than 200 patients with idiopathic inflammatory myopathies in our previous studies (11, 12).

2. Material and Methods

2.1 Patients and Sera

Two hundred twenty-four Japanese patients (68 males, 156 females) were enrolled in the study. Demographic and medical information were collected from chart reviews. One hundred fifty-four of the 224 sera were from patients who had visited the clinic of the Department of Dermatology at Nagoya University Graduate School of Medicine between 1994 and 2015. Sera from 70 other patients were sent to our laboratory from collaborating hospitals to test for myositis-specific autoantibodies. One hundred fifty-nine patients fulfilled the criteria of Bohan and Peter for DM/PM (13), and the remaining 65 met the Sontheimer criteria for clinically amyopathic DM (CADM) (14). Of the 224 patients, 90 patients had classical DM, 65 had clinically amyopathic DM, 25

had cancer-associated DM, 7 had juvenile DM, 20 had PM and 7 had myositis overlap syndrome. The ages at disease onset were 3 to 85 years (mean=52 ± 18).

Age-appropriate cancer screening and computed tomography of the chest for the evaluation of interstitial lung disease (ILD) were performed. Patients were classified as having cancer-associated DM if a diagnosis of a malignancy was made within 3 years prior to the development of the DM-associated symptoms or after the onset of the first DM symptoms. This study was approved by the ethics committees of the Nagoya University Graduate School of Medicine and University Hospital.

2.2 Laboratory tests and serologic assays

Serum samples were screened for antibodies against SS-A/Ro60 and U1-RNP using commercial enzyme-linked immunosorbent assay (ELISA) kits (MBL®, Nagoya, Japan). In addition, anti-Mi-2, anti-TIF1γ, anti-MDA-5, anti-NXP-2, anti-HMG-CoA, anti-Ku70/80, anti-SRP54, anti-PM/Scl-75/100 and anti-SAE1/2 were tested by ELISA using biotinylated recombinant proteins (15). When the results obtained by anti-aminoacyl-transfer RNA synthetase (anti-ARS) ELISA kit (MBL®) were positive, the individual anti-ARS of EJ, Jo-1, KS, PL-7 and PL-12 were tested by the in-house ELISA (15).

2.3 Recombinant protein

The full-length cDNA clone of human Ago2 in pDEST17 vector was a gift from Dr. Edward Chan (University of Florida) (16). Biotinylated recombinant protein was produced from the cDNA using the T7 Quick Coupled Transcription/Translation System (Promega®, Madison, WI, USA) (15).

2.4 Enzyme-linked immunosorbent assay (ELISA)

All sera were tested by ELISA for antibodies to Ago2, following our previously published protocols (15). A 96-well Immobilizer Streptavidin plate (Thermo Scientific Nunc®, Roskilde, Denmark) was incubated with 1 µl of biotinylated recombinant Ago2 protein/50 µl PBS containing 0.05% Tween20/well. The wells were then incubated with diluted patient sera (1:1,000 dilution), followed by incubation with horse radish peroxidase-conjugated goat anti-human IgG antibody (1:30,000 dilution; Dako®, Grostrup, Denmark). Anti-Ago2 reactivity was determined by incubation with substrate, and relative luminescence units (RLU) were read. The RLU of the samples was converted into units using a standard curve created with a serially diluted prototype positive serum.

2.5 Immunoprecipitation (IP)

Autoantibodies in sera were tested by IP of ^{35}S-methionine radiolabeled K562 cell extract, SDS-PAGE, and autoradiography. Anti-Su antibodies were identified by the presence of the characteristic 100/102-kDa doublet Su antigens (4).

2.6 Indirect immunofluorescence (IIF)

Sera were tested by IIF with two different commercial kits using HEp-2 cells (Fluoro HEPANA Test: MBL®, and Premmune HEp20-10: EUROIMMUN®, Luebeck, Germany) according to their protocols.

2.7 Statistical analysis

The results were analyzed by Fisher's exact test, Mann-Whitney U test, or log rank test, as appropriate, using SPSS version 22 (IBM, Armonk, NY, USA). P values less than 0.05 were considered significant.

3. Results

3.1 Detection of anti-Su/Ago2 antibodies by ELISA and immunoprecipitation

An in-house ELISA was used to detect anti-Ago2 antibodies in sera. The cutoff value was defined as a mean plus 5 standard deviations of the ELISA unit (=2.7 units) as obtained from the 40 healthy controls. All healthy control sera were anti-Ago2-antibody-negative in our ELISA. Of the 224 DM/PM patients' sera, 17 (7.6 %) were ELISA-positive.

Next, 33 sera (including 17 ELISA-positive sera and 16 ELISA-negative sera that had units close to the cutoff value) were tested by IP for the presence of 100/102 kDa Su proteins. Anti-Su/Ago2 was confirmed by IP in 13 of 17 ELISA-positive sera. One of the 17 ELISA-positive sera strongly immunoprecipitated a protein that migrated close to the 100/102kD Su antigen, and whether the serum had anti-Su or not was inconclusive (Figure 1a, lane 15). In contrast, only one of 16 ELISA-negative sera that showed relatively high reactivity but was below the cutoff in the anti-Ago2 ELISA was positive for anti-Su/Ago2 by IP (Figure 1a, lane 14). From these data, our ELISA showed a sensitivity of 93% (13/14) and a specificity of 79% (15/19), compared to anti-Su/Ago2 antibody detection by IP as the gold standard.

3.2 Clinical and laboratory profiles of patients with anti-Su/Ago2 antibodies

There was no statistically significant difference in prevalence of myositis subsets, age,

sex or coexisting autoantibodies between the anti-Su/Ago2 antibody-positive group and the anti-Su/Ago2 antibody-negative groups (Table I). Six of the 8 patients who were anti-Su/Ago2 antibody-positive had ILD. None of the anti-Su/Ago2 antibody-positive patients had internal malignancy. In our study, 177 of 224 patients with myositis were available for the evaluation of ILD. Six of 8 (75%) anti-Su-positive patients had ILD, whereas 104 of 177 (61%) anti-Su-negative myositis patients had ILD. The difference was not statistically significant (P=0.7). Moreover, malignancy in anti-Su-positive and -negative patients was 0 of 13 (0%) and 11 of 211 (25%) respectively; no statistical difference was found (Table I).

3.3 IIF analysis of anti-Su/Ago2-positive sera

Twenty-seven sera (17 anti-Ago2 ELISA-positive and 10 below the cutoff value described above) were tested by IIF using 2 different IIF kits to evaluate characteristic GW body (GWB) staining (cytoplasmic discrete foci pattern) (5) for anti-Su (Figures 1b, c). Seven sera showed the GWB pattern by both kits, 6 of which were positive by both IP and ELISA (Table II). The remaining one serum was ELISA-positive, but the IP result was inconclusive because of protein bands that migrate close to the 100/102kD Su antigen (Figure 1a, lane 15). Ten ELISA-negative sera showed no GWB staining.

We compared the clinical characteristics of GW body positive-staining vs. negative-staining anti-Su patients (Table III). Levels of anti-Ago2 determined by ELISA appear higher in the GW body positive-staining group, but the difference is not statistically significant.

4. Discussion

To our knowledge, this is the first study on anti-Su/Ago2 antibodies using anti-Ago2 ELISA and it is the largest myositis cohort. The IP and ELISA results showed very good agreement, and we proved that our ELISA system is effective in screening anti-Su/Ago2 antibodies. In previous studies, anti-Su antibodies were associated with anti-Ku antibodies in overlap syndrome (4), lower levels of IgG anti-β2 glycoprotein I antibodies in primary anti-phospholipid syndromes (17), or the absence of other SLE-related antibodies (1). In our DM/PM cohort, no specific clinical features associated with anti-Su/Ago2 antibodies were found.

Anti-Su/Ago2 antibodies are known to show a unique staining pattern in IIF. Even though Ago2 localizes to GWB, the GWB pattern in IIF is positive for only ~1/3 cases with anti-Su/Ago2 antibodies (5). There are several hypotheses to explain the inconsistent IIF results. It is possible that exposed Ago2 epitopes are affected by post-translational modification or proteins interacting with Ago2. Similarly, it is likely

that Ago2 epitopes in relatively native liquid-phase molecules in IP differ from solid-phase epitopes in IIF slide or recombinant proteins used in ELISA. In the present study, 6 sera that were positive for anti-Su/Ago2 by both IP and ELISA showed a GWB pattern by the 2 different IIF kits, and the densities of GWB seemed similar in both kits. We obtained good concordance in the IIF results using two different HEp-2 kits.

Case 14 showed 1.8 units in ELISA, which was higher than the mean + 2SD of healthy controls but did not reach the ELISA cutoff value. The serum from this patient showed typical IP patterns of anti-Su antibody (Figure line 14, Table II). This might have been the result of differences in antibody affinities to the cell extract (used in IP) and recombinant proteins (used in ELISA) due to the Ago2-epitope divergences discussed above.

There was one anti-Ago2 ELISA-positive serum that showed a GWB pattern in IIF. The serum from this patient immunoprecipitated a strong protein band around 100kDa that comigrates with the characteristic Su/Ago2 bands in IP (Table I); thus, it was not possible to conclude whether this serum had anti-Su/Ago2. Su/Ago2 is a component of cytoplasmic GWB; however, there are many other autoantigens that are components of GWB. Thus, GWB staining alone is not enough to classify this serum as anti-Su/Ago2-positive. Although IP is considered the gold standard for detecting anti-Su

antibodies, our ELISA could sometimes be useful for detecting anti-Su/Ago2 in cases like this. Our newly developed ELISA can be used to detect anti-Su/Ago2 antibodies. To elucidate the clinical features of anti-Su/Ago2 antibody-positive patients with various diseases, future studies in larger cohorts will be needed.

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Figure legend

Figure 1. Immunoprecipitation and indirect immunofluorescence analysis of anti-Su/Ago2-positive sera.

A. Immunoprecipitation for anti-Su/Ago2 antibodies. Lanes 1-13: Anti-Su/Ago2-positive sera in ELISA/IP analysis, lane 14: anti-Su-positive serum in IP analysis, lane 15: anti-Ago2-positive serum in ELISA analysis. Lanes PC and NC: positive control and negative control sera. MWM: molecular weight marker. Lane numbers correspond to the case numbers in Table 2.

B and C. GW body staining pattern by an anti-Ago2/Su antibody-positive serum from a patient with clinically amyopathic dermatomyositis using two indirect immunofluorescence (IIF) kits. B: IIF using EUROIMMUN® kit, C: IIF using MBL® kit.

Table I. Clinical and serological feature of the anti-Su/Ago2-positive and -negative idiopathic inflammatory myopathy patients.

	anti-Su positive % (N=13)	anti-Su negative % (N=211)	P value
Male:female	3:10	65:146	0.75
mean age (mean±SD)	48.0±14.6	52.2±18.1	0.5
Myositis-specific autoantibodies			
MDA5	23 (3)	23 (50)	1
TIF1γ	7 (1)	17 (36)	0.69
ARS	23 (3)	15 (32)	1
negative	46 (6)	33 (70)	1
Clinical diagnosis			
classical DM	30 (4)	40 (86)	0.4
JDM	7 (1)	9 (19)	1
CADM	38 (5)	28 (60)	0.5
Cancer-associated DM	0 (0)	11(25)	0.8
PM	15 (2)	8 (18)	0.3
Overlap	7 (1)	7 (16)	1

ARS: aminoacyl tRNA synthetases, CADM: clinically amyopathic dermatomyositis, DM: dermatomyositis, JDM: juvenile dermatomyositis, MDA5: melanoma differentiation associated gene 5, PM: polymyositis, TIF1γ: transcriptional intermediary factor 1γ

Table II. Clinical and serological characteristics of anti-Su/Ago2 antibody-positive PM/DM patients

Case	Age	Sex	Diagnosis	Concomitant antibodies	ELISA[+] (units)	GWB IIF pattern	CK max (IU/L)	Malignancy	Interstitial lung disease
1	47	M	DM	EJ	4.9	+	582	-	-
2	54	F	DM	PL-7	8.0	+	NA	-	NA
3	50	F	DM	-	25	-	500	-	-
4	50	M	DM	-	17	-	28	-	NA
5	73	M	CADM	ACA	80	+	NA	-	NA
6	57	F	CADM	MDA5	92	+	42	-	+
7	49	F	CADM	MDA5	3.2	-	30	-	+
8	46	F	CADM	MDA5	9.8	+	165	-	+
9	23	F	CADM	-	6.3	-	165	-	+
10	15	F	JDM	TIF1γ, SS-A	4.4	-	24	-	NA
11	54	F	DM+RA	Jo-1, SS-A	40	-	1915	-	+
12	64	F	PM	-	31	-	1076	-	+
13	45	F	PM	U1RNP	313	+	6101	-	NA
14	51	F	Cancer-a. DM	-	1.8	-	NA	+	-
15	74	M	PM	-	4.8	+	1670	+	+

Cases 1-13; Anti-Su/Ago2-positive cases by both immunoprecipitation and ELISA. Case 14; Anti-Su-positive case by

immunoprecipitation. Case 15; Anti-Ago2-positive case by ELISA.

ACA: anti-centromere antibody, CADM: clinically amyopathic dermatomyositis, Cancer-a.: cancer-associated, CK: creatine kinase, DM: dermatomyositis, GWB: GW bodies, IIF: indirect immunofluorescence, JDM: juvenile dermatomyositis, MDA5: melanoma differentiation associated gene 5, NA: not available, RA: rheumatoid arthritis, RLU: relative luminescence units, -: negative, +: positive.

[+]Cut-off value of ELISA: 2.7 unit.

Table III. Clinical and serological features of GW body positive-staining and negative-staining PM/DM patients.

	GW body staining (+) N=6	GW body staining (-) N=7	P value
Age	53.7 ± 10.6	43.57 ± 17.7	0.67
Anti-Ago2 ELISA (unit)	84.6±118	18.13±14.4	0.277
Creatine kinase (IU/L)	1722.5±2928	534±719	0.41
Interstitial lung disease (%)	2 (33%)	4 (57%)	0.59

Figure(s)
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